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Full Length Research Paper

Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.)

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The aim of this study is to determine total phenol amount of the explants and to expose relation between explant age, total phenols and regeneration response for tissue culture and gene transfer systems of cotton (*Gossypium hirsutum* L.). In this study, total phenol amount of 7, 14, 21 and 28 day old cotton (*G. hirsutum* L) var. Nazilli 84S explants and excreted total phenols into culture media were determined during the germination period. Explants were originated from roots, hypocotyls, cotyledons and leaves. After germination, they were cultured on Murashige and Skoog (MS) media, supplemented with 0.1 mg/L kinetin (KIN) and grown at 25°C under fluorescent light (7500 lux), 16 h light and 8 h dark for 3 weeks. Different regeneration ratios were obtained relative to explant age and total phenol amounts. Regenerated shoots were rooted on woody plant medium (WPM) supplemented with 1 mg/L indole-3-butyric acid (IBA).

Key words: *Gossypium hirsutum* L., tissue culture, germination, total phenol, browning.

INTRODUCTION

Malvaceae family member cotton (*Gossypium hirsutum* L.) is one of the most commercially important fiber crops in the world. In addition to textile manufacturing, it produces seeds with a potential multiproduct base such as hulls, oil, linters and food for animals (Mishra et al., 2003; Aragao et al., 2005). For this reason, there is a considerable interest in the development of tissue culture and gene transfer technology for this species. However, cotton has proven to be a relatively difficult species for *in vitro* culture. Genetic differences in tissue culture competence are still one of the limiting factors for the application of biotechnological methods for the study of cotton (Kumria et al., 2003; Sanjaya et al., 2005).

Although regeneration efficiency has been improved via somatic embryogenesis or organogenesis, genotype dependent regeneration, a prolonged culture period, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, lack of shoot elongation, difficulties of rooting and browning which causes death of tissues or explants are the problems associated with cotton tissue culture systems (Kumria et al., 2003). Some of these problems are related to the plant material such as explant age or genotype and others to the culture conditions such as hormones, medium composition, or other physical culture conditions (Ikram-Ul-Haq, 2004).

Phenolic acids are intermediates of phenylpropanoid metabolism (Cvikrová et al., 1996) and precursors of lignin (Lewis and Yamamato, 1990) and phenylpropanoid phytoalexins (Kessmann et al., 1990). Their deposition in cell walls is an important defense mechanism after pathogen infection (Bolwell et al., 1985). When they are excreted from plant root system, they exert inhibitory growth function within adjacent rhizosphere and they affect bacterial flora of the soil (Kefeli et al., 2003). Being an important group of secondary metabolites, phenolics may act as modulators of plant development by regulating indole acetic acid (IAA) catabolism (Arnaldos et al., 2001). They are effective in plant growth regulation, cell differention and organogenesis (Mato et al., 1988). There are two opinions on interactions between phenolics and plant growth and development. One indicates that phenolics are negatively related with plant *in vitro* proliferation while others mention the opposite (Lorenzo

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et al., 2001). In tissue culture studies, phenolic substances, especially oxidized phenolics generally effect *in vitro* proliferation negatively (Arnaldos et al., 2001).

Tissue browning and blackening are also one of the major problems for *in vitro* culturing many economically important plants. When cells are damaged, the contents of cytoplasm and vacuoles are mixed and phenolic compounds can readily become oxidized by air. Oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants (Laukkanen et al., 1999; Compton et al., 1986). Liquid media can be used to reduce phenolic oxidation. In addition frequent subculturing, some antioxidants such as citric acid and ascorbic acid, PVP (polyvinyl pyrolidone) and activated carbon, which are added into medium, can also reduce phenolic oxidation and contribute to regeneration from explants (Toth et al., 1994).

Phenolic concentration is frequently affected by several internal and external factors (Zapprometov et al., 1989). Light appears to induce flavonol synthesis in the chloroplasts and cytoplasm (Kefeli et al., 2003). Some nutrieents, especially carbohydrate supplies influence the phenolic composition (Lux-Endrich et al., 2000). Some stress factors like drought, water, radiation, and pathogen infection from injured surfaces effect concentrations of the phenolics in plants (Kefeli et al., 2003; Zapprometov et al., 1989). Phenol concentrations can also be increased and decreased in different stages of germination (Thomas and Ravindra, 1999). Therefore, determination of the lowest phenol concentration phase during germination and isolation and culturing of explants in this phase will increase regeneration response and success in tissue culture studies.

In this study we determined total phenol amount of 7,14,21 and 28 day old cotton (*G. hirsutum* L) var. Nazilli 84S explants which originated from roots, hypocotyls, cotyledons and leaves and we determined excreted total phenols into MS media spectrophotometrically with Singleton-Rossi method based Folin-Ciocaelteu reagent to find out the best explant age for culturing. We also cultured the cotyledonary nodes, which include hypocotyl pieces as explant on Murashige and Skoog (MS) media, supplemented with 0.1 mg/L kinetin (KIN).

MATERIALS AND METHODS

Plant material, surface sterilization and germination conditions

Seeds of cotton (*G. hirsutum* L.) var. Nazilli 84S were obtained from Nazilli Cotton Research Institute, Aydın-Turkey. This variety is one of the most common planting varieties in western Turkey and it is originated from Nazilli 84. Nazilli 84 is a hybrid form of Coker, Caroline Queen and 153 F genotypes.

Before surface sterilization, cottonseeds were kept under flowing fountain water for 1 h. They were surface sterilized by immersion in 70% ethanol (Sigma Chemical Co.) for 3 min, followed by stirring in 20% commercial bleach (ACE Lever Co.) for 20 min. The surface sterilized seeds were rinsed 3 times with sterile distilled water for 5 min 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 and Skoog) medium.

The medium contained 1 mL MS vitamin solution (Sigma Chemical Co.), 30 g sucrose (Sigma Chemical Co.) and 2.2 g phytagel (Sigma Chemical Co.). The pH of the media was adjusted to 5.7 with 1 M NaOH (Merck) before autoclaving. After autoclaving, 4.3 g basal salt mixture (Sigma Chemical Co.) that sterilized by micro filter was added into MS media. 20 mL MS media was poured into Magenta vessels (Sigma Chemical Co.) and 5 seeds were germinated in each Magenta vessel. Seeds were kept at growth chamber with photoperiod of 16 h light (7500 lux) and 8 h dark, at 25^{o}C and 70% humidity.

Total phenols of Nazilli 84S explants (root, hypocotyl, cotyledon and leaf) and excreted total phenols into MS media were spectrophotometrically determined at 7, 14, 21 and 28th days of the germination period.

Chemical analyses

Total phenol amounts of 7, 14, 21 and 28 day old cotton (*G. hirsutum* L) var. Nazilli 84S were analyzed according to Folin-Ciocalteu method (Singleton et al., 1965; Singleton et al., 1999; Chandler et al., 1983) by using gallic acid as the standard and the results were given as gallic acid equivalents (GAE) (Waterman et al., 1994).

Sample preparation for determination of total phenols

In this study totally 100 germinated plants (25 plants for each week and treatment) were used. For each measurement 0.1 g of plant pieces (totally 2.5 g of roots, hypocotyls, cotyledons and leaves) were dissected out from the plant, mixed and homogenized. After homogenization, approximately 500 mg of samples were placed in 25 mL of 95% ethanol (Sigma Chemical Co.) and kept at 0^{o}C in fridge for 48 h. 5 ml samples were taken and centrifuged at 13000 rpm for 15 min, 1 mL of supernatant was transferred to a test tube and orderly added 1 mL of 95% ethanol and 5 mL double distilled water. 0.5 mL (50 %) of Folin-Ciocalteu solution reagent was added to each sample. After 5 min, 1 mL of 5% Na_2CO_3 (Merck) was added, the samples were mixed and allowed to stand for 1 h in darkness and absorbencies were measured at 760 nm using Shimadzu 240 UV double-beam spectrophotometer in 1.0 cm quartz cells against 95% ethanol as blank. For MS media's total phenol determination, all media were mixed after plants had been removed and the same procedure above was used. Total phenol contents were standardized against gallic acid and expressed as micrograms phenol/g sample gallic acid equivalents (GAE). The linearity range for this assay was determined as 5-500 µg/L GAE (R^2=0.9997).

Establishment of tissue culture systems

Surface sterilized seeds were cultured on hormone free MS media for germination. After germination, 7, 14, 21 and 28 day old cotyledonary nodes, which include hypocotyl pieces cut into 0.5 cm long pieces, then cultured on MS media supplemented with 0.1 mg/L kinetin. They were grown under the same culture conditions of germination. Different regeneration ratios were obtained relating to explants age and total phenol amounts. Regenerated shoots were rooted on WPM (woody plant medium) supplemented with 1 mg/L indole-3-butyric acid. Rooted plants became ready to be transferred to the soil after 15 days.

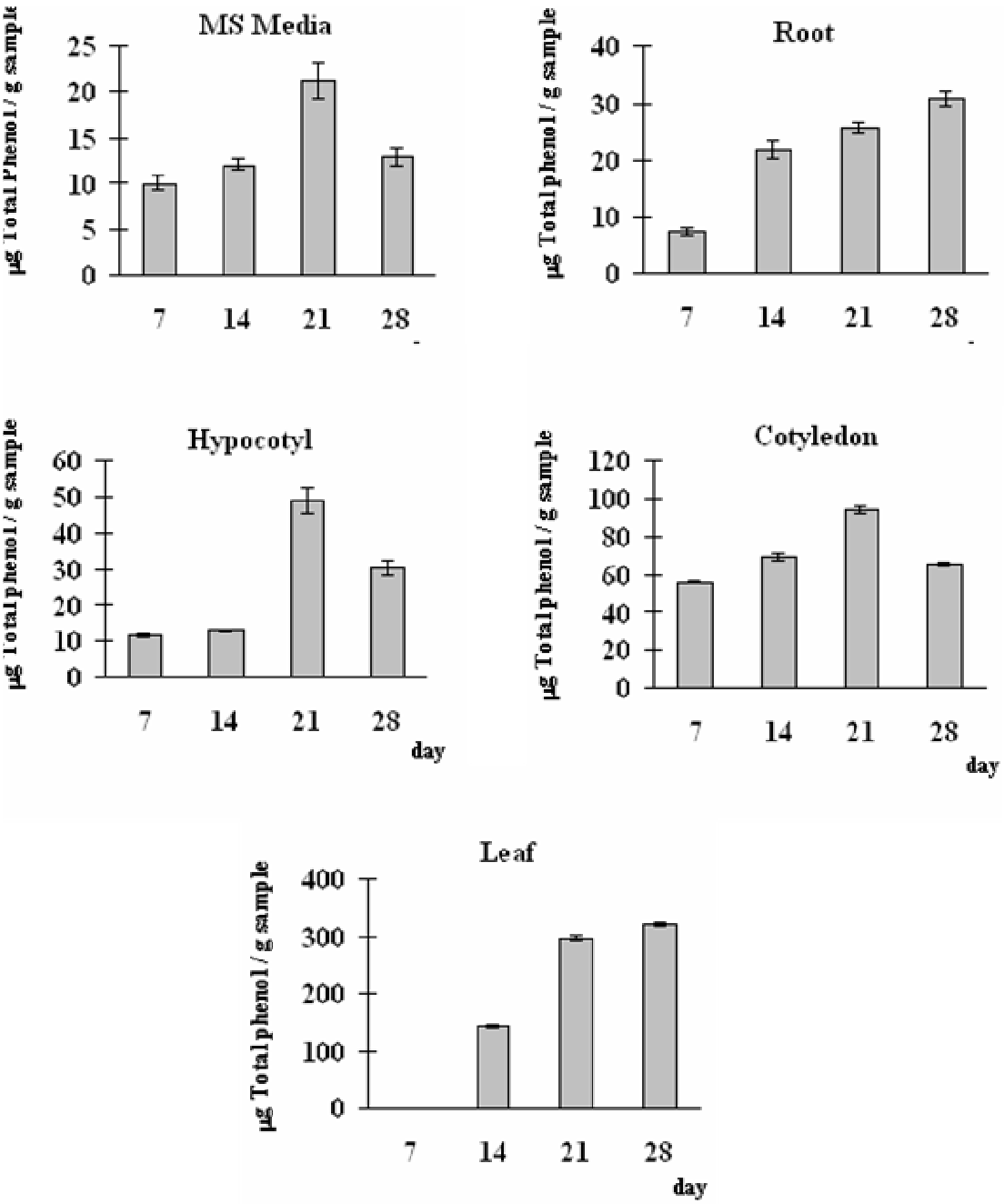

Figure 1. Total phenol amount of root, hypocotyl, cotyledon and leaf explants of cotton (*Gossypium hirsutum* L.) and MS media in 7th, 14th, 21st and 28th day of germination. Each assay is a mean of five replicates ± standard deviation. Total phenols were calculated as Gallic acid equivalents (GAE).

RESULTS AND DISCUSSION

In cotton tissue culture studies hypocotyls, cotyledons and meristematic shoots were generally used as explants. The results obtained correlated to the plant material such as explant age or genotype, the culture conditions such as hormones, medium composition or other physical culture conditions. In this study, the lowest amount of total phenols was found in the first 7 days of germination (Figure 1). Total phenols continuously increased in roots between 7-28th day and leaves between 14-28th day, but in hypocotyls, cotyledons and MS media they increased between 7-21st day and decreased after the 21st day. Increasing rate was the highest between 14-21st day for all materials excluding roots. Total phenol amounts of 7 and 14 day old hypocotyls were close to each other.

There are some reports, which show total phenols could increase or decrease, in different stages of germi-

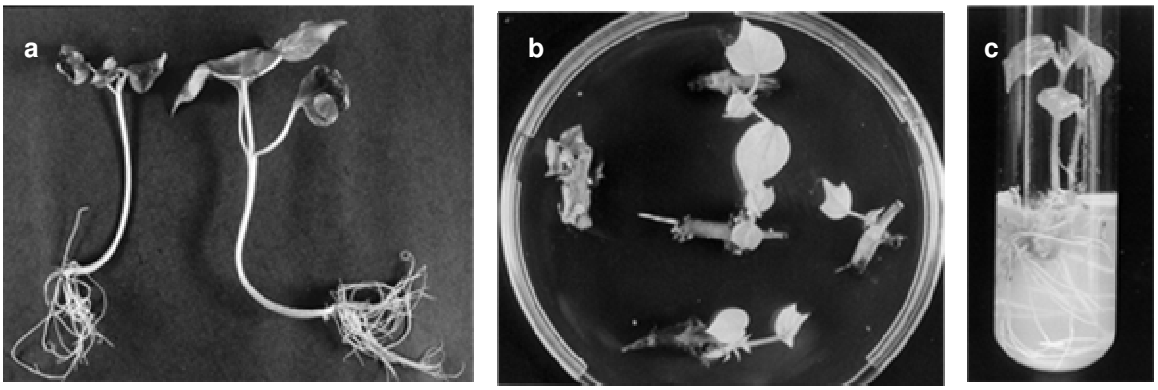

Figure 2. 1 and 2-week old seedlings, which germinated on hormone, free MS media (a). Direct plant regeneration from cotylodanory nodes which include hypocotyl pieces on MS + 0.1 mg/L Kinetin (b). Root induction on MS + 1 mg/L indole-3-butyric acid (c).

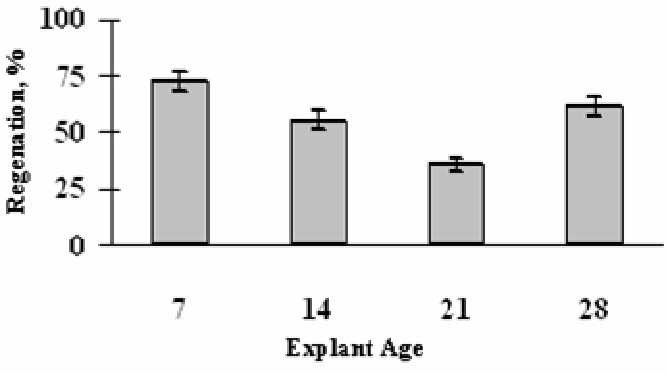

Figure 3. Effect of explant age on regeneration response and direct plant regeneration from cotyledonary nodes which include hypocotyl pieces on MS + 0.1 mg/L Kinetin in 7th, 14th, 21st and 28th days of germination. Each assay is a mean of five replicates ± standard deviation.

nation. Several authors have also described the phenolics are negatively related to seed germination and *in vitro* proliferation and their results are apparently similar with ours (White, 1994; Prasad, 1999). Another group of authors pointed out the positive correlation between phenolics and totipotency (Thomas and Ravindra 1999). They stated that phenolics affect germination and plant tissue culture response (shoot induction) positively. Muscolo et al. (2001) showed that phenolics affect respiratory enzymes in seed germination and total phenols amount change in different stages of germinating *Fagus sylvatica* L. and *Pinus laricio*. They also mentioned that the phenolic compounds bioassayed inhibited seed germination. Weinberger and Vladut (1981) determined some phenolics (especially nonyl phenols) reduced the germination of both jack pine (*Pinus banksiana* Lamb.) and white birch (*Betula papyrifera* March). One treatment with high concentrations of nonyl phenol ethoxylate with 5-ethylene oxide units caused a delay in bud break and up to 50% bud kill in germinating apples. Peaches and grapes have the same symptoms (White, 1994). Nonyl phenol at concentrations below 1 ppm inhibited growth and caused loss of chlorophyll in duck-weed (*Lemna minor*). In the aquatic fern *Salvinia molesta*, concentrations of 2.5 ppm reduced frond production and caused death. In both of these, plants chloroplasts were damaged by the phenolics (Prasad, 1989).

In tissue culture study, 7, 14, 21 and 28 day old cotyledonary nodes, which include hypocotyl pieces were cultured on MS media supplemented with 0.1 mg/L kinetin and different regeneration ratios obtained as followed (Figure 2). The regeneration responses of our explants were supported by the results of total phenol contents of explants (Figure 3). Based on the results of this study, we recommend culturing explants in first seven days of germination and related to reduction of total phenols after 3rd week, explants can also be cultured after 28 days for cotton tissue culture studies.

There are many reports on explant age and regeneration response of cotton (*G. hirsutum* L.). In a study, 3 and 5 day old meristematic shoots of *G. hirsutum* and *G. barbadense* were cultured and the best regeneration response was obtained from 5 day old explants (Gould et al., 1991). In another study, 5, 6, 7, 8, 9 and 10 day old cotyledonary nodes of 10 different genotypes were cultured and 6 day old explants gave the best regeneration response (Gupta et al., 2000). In a similar study, 7, 14, 21, 28, 34 and 45 day old cotyledonary nodes containing hypocotyl pieces were cultured and the best regeneration response was obtained from 14 and 35 day old explants (Luo et al., 2000). In addition, 14 day of leaf, epicotyl, hypocotyl, and cotyledon pieces were cultured and although callus induction was obtained, regeneration was not observed (Hemphill et al., 1998). Another study indicates that when 5 and 7 day old hypocotyls and cotyledonary nodes of *G. hirsutum* L. var.

Coker were cultured, callus and then somatic embryos could be obtained (Kumria et al., 2003).

As explained above, in cotton tissue culture studies, scientists were more successful by using explants in the 1st week or after the 4th week of germination. All these results showed that, total phenol amounts of explants together with genotype, culture conditions and the age of explant could affect regeneration efficiency. Although phenolics affect negatively in cotton tissue culture, in other species phenolics can affect germination and plant tissue culture response (shoot induction) negatively or positively. Cvikrová et al. (1996) used alfalfa leaves for callus induction and showed that phenolic contents increased in three days of culture and then decreased. The most intensive phenolic production and increase of the total phenols preceeded the most intensive cell division period. Lorenzo (2001) cultured sugarcane and determined total phenols in different stages of tissue culture. Their experiment demonstrated a close relationship between phenolic production and *in vitro* sugarcane shoot formation. There are some other studies about the relationship between proliferation and phenolics carried out. Herman (1991) mentioned that high production of shikimic acid was positively correlated with *Pinus sylvestris*.

Other groups of researchers have also pointed out negative correlation between phenolics and *in vitro* proliferation. Thomas and Ravindra (1999) studied with four age groups of shoots (one week old, 2-4 weeks old, 2-4 months old and 1 year old) and they observed maximum phenol exudation, medium discoloration and rapid explant browning with one week old shoots in mango. Laukkanen (1999) cultured calli from shoot tips of mature Scots pine and obtained brownish green (14 day old), greenish brown (28 day old) and totally brown (42 day old) calli and examined peroxidase (POD) and polyphenol oxidase (PPO) activity in those explants. They found that PPO activity increased rapidly during culture but the increase slowed down after 28 day of culture. Cox (1996) also mentioned that nonyl phenols stops or slows growth in sugar beets and tomato tissue cultures.

Literature indicates that the role of a phenolic depends on its chemical structure, plant species, the biological process in study (organogenesis or somatic embryogenesis) and its developmental step. We believe that metabolized phenols affect tissue culture systems positively with auxin metabolism (rapid cell division and synthesis of the cell wall and other related components). But oxidized phenols turn into highly toxic quinones and polymerized material causing discoloration of the medium and death of the explants. It will be very useful to find out the least phenol synthesizing germination age for some problematic species (in tissue culture studies) like cotton, mango, coconut etc. This study will help in the establishment of tissue culture and gene transfer systems of problematic species or genotypes, which have tissue browning and blackening problems and also lower regeneration efficiency.

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