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Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips

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Browning and subsequent death of the cultured explants are major problems for many tissue culture non/hard-adaptive species and usually depended on the phenolic compounds and the quantity of total phenols. Many studies, using shoot tips as explant source indicated various problems such as phenolic exudation, media discoloration, rooting deficiencies and explant browning and death. The phenols are synthesized by the plants and in many tissue culture studies, excreted and then oxidized phenols effect *in vitro* proliferation negatively. In addition, amount of phenolics can be more or less in different stages of organogenesis due to metabolic actions. Therefore determination of phenolics and calculating the amounts of phenols may be another research area for many tissue culture studies. In this study, total phenol amounts of shoot tip cultures were evaluated during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.). Cotton *var*. Nazilli 84S was used as explant source and total phenols of young leaves, shoots and the MS (Murashige and Skoog) media (for excreted phenols from explants to medium) were calculated in 7-14-21 and 28 days of culturing period. Seeds were germinated in hormone free MS media in 7 days. After germination, 7 day old meristematic shoot tips were dissected out from seedlings and cultured on MS media, supplemented with 0.1 mg/L Kinetin (KIN). They were grown at $25^{o}C$ under fluorescent light (7500 lx) 16 h light and 8 h dark for 3 weeks. Singleton-Rossi method based Folin-Ciocaelteu reactive was used for determination of total phenol amount of the young plantlets and it was observed that different parts of the plantlets synthesized more or less amounts of phenolics in different stages of organogenesis.

Key words: Cotton, tissue culture, phenolics, browning, organogenesis, shoot tip.

INTRODUCTION

Phenolic compounds occur as secondary metabolites in all plant species and they are generally characterized by a benzene ring and one hydroxyl group (Antolovich et al., 2000; Kefeli et al., 2003). They are also extremely diverse compounds, for example carnosol and rosmanol, which are diterpenes were identified in herbs and spices while the main phenolics are isoflavone glycosides and several phenolic acids like ferulic, caffeic and chlorogenic acids which are present in soybean (Robards et al., 1999). Plant phenols are classified into major groupings distinguished by the number of constitutive carbon atoms in conjunction with the structure of basic phenolic skeleton (Robards et al., 1999; Antolovich et al., 2000). In Table 1, the most important classes of phenolic compounds in plants were shown (Harborne, 1980).

Shikimate is the starting product for the biosynthesis of most phenolic compounds. They are also acidic sub-

stances, due to the dissociability of their -OH groups. Many phenols are rather reactive compounds and as long as no steric inhibition due to additional side chains occurs, they form hydrogen bonds. The composition and synthesis of phenolics in plant tissues may determine by genetic and environmental conditions like oxidative reactions during culturing, processing and storage (Lux-Endrich et al., 2000).

Plant phenolics are modulators of indole acetic acid (IAA) catabolism. Some monophenols like synaptic acid and ferulic acid, at low concentrations, inhibit enzymatic oxidation of IAA and this results in cell elongation and cell division and subsequent plant growth and development (Volpert et al., 1995; Arnaldos et al., 2001). Plant Phenolics increase the rigidity of plant cell walls acting as molecular bridges between cell wall components (Fry, 1986). They are precursors of lignin (Lewis and Yamama-

Table 1. The most important classes of phenolic compounds in plants according to J. B. Harborne (1980).

Number of C-atoms	Basic skeleton	Class
6	C_6	Simple phenols, benzoquinones
7	C_6-C_1	Phenolic acids
8	C_6 - C_2	Acetophenone, phenylacetic acid
9	C_6 - C_3	Hydroxycinnamic acid, polypropene, coumarin, isocoumarin
10	C_6 - C_4	Naphtoquinone
13	C_6-C_1-C_6	Xanthone
14	C_6-C_2 - C_6	Stilbene, anthrachinone
15	C_6-C_3 - C_6	Flavonoids, isoflavonoids
18	$(C_6 - C_3)_2$	Lignans, neolignans
30	$(C_6 - C_3 - C_6)_2$	Biflavonoids
N	$(C_6 - C_3)_n$ $(C_6)_n$ $(C_6 - C_3 - C_6)_n$	Lignins Catecholmelanine (condensed tannins)

to, 1990) and phenylpropanoid phytoalexins (Kessmann et al., 1990). They have also an important role on plant defense mechanism. 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). Another important feature is their ability to form chelate complexes with metals. Also, they are easily oxidized, and so form polymers (dark aggregates). The darkening of cut or dying plant parts is caused by this reaction. They have inhibiting or stimulating effects on plant growth which are variable from any species to one (Ozyigit et al., 2007).

Cotton (*Gossypium hirsutum* L.) is the world's leading natural fiber and second largest oilseed crop. In addition to textile manufacturing, cotton and cotton-by products are the sources of wealth of consumer-based products, livestock feed, fertilizer, foodstuffs and paper (Mishra et al., 2003; Ozyigit et al., 2006). Due to the economic importance of producing cotton based goods through genetic engineering, a great deal of effort has been made to develop systems to recover fertile transgenic cotton plants (Aragaro et al., 2005). Thus, 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. Initial efforts to transform cotton, using *Agrobacterium tumefaciens*, were applicable to only a few varieties (Aragaro et al., 2005; Ozyigit et al., 2007). Although several investigators have worked extensively on plant regeneration through somatic embryogenesis from *vars.* Coker and Sicala, Siokara and Acala, this genotype depended organogenesis, prolonged culture period, low conversion rate of somatic embryos into plantlets, lack of shoot elongation and difficulties of rooting and browning which causes death of tissues are major problems of cotton tissue culture and gene transfer technology (Kumria et al., 2003; Ul-Haq 2004; Ozyigit et al, 2007)

In tissue culture studies, explants are dissected out and cultured on any media. When explants are cut, the con-

tents of the cytoplasm and vacuoles mixes and comes out from the explant and phenolic compounds can readily become oxidize by air. Oxidized phenolic compounds inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants or causes rooting deficiencies (Arnaldos et al., 2001; Ozyigit et al., 2007). Residual phenolics are also important on browning and thus the phenolics which synthesized by the explants in any periods of organogenesis have important role on browning and rooting problems.

In this study, total phenols of young leaves, shoots and the MS (Murashige and Skoog) media (for excreted phenols from explants to medium) were calculated in 7, 14, 21 and 28 days of culturing period and it was observed that different parts of the plantlets synthesized more or less amounts of phenolics at different stages of organogenesis.

MATERIAL AND METHODS

Seeds of cotton *var*. Nazilli 84S, which is one of the most common planting varieties in Western Turkey, were obtained from Nazilli Cotton Research Institute, Aydın-Turkey. Before surface sterilization, cottonseeds were kept under flowing tap water for 1 h and they were surface sterilized by immersion in 70% ethanol for 3 min, followed by stirring in 20% commercial bleach 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 which contained 4.3 g basal salt mixture, 30 g sucrose and 2.2 g phytagel. The pH of the media was adjusted to 5.7 with 1 M NaOH before autoclaving. After autoclaving, 1 mL MS vitamin solution, that sterilized by micro filter was added into MS media. 20 mL MS media was poured into Magenta vessels and 5 seeds were germinated in each Magenta vessel. Seeds were kept at growth chamber with photoperiod of 16 h light (7500 lx) and 8 h dark, at 25°C and 70% humidity. After germination, 7 day old meristematic shoot tips were cut 1 - 2 mm long and then cultured on MS media supplemented with 0.1 mg/L kinetin. They were grown under the same culture conditions of germination. No sub-culturing was made during regeneration period.
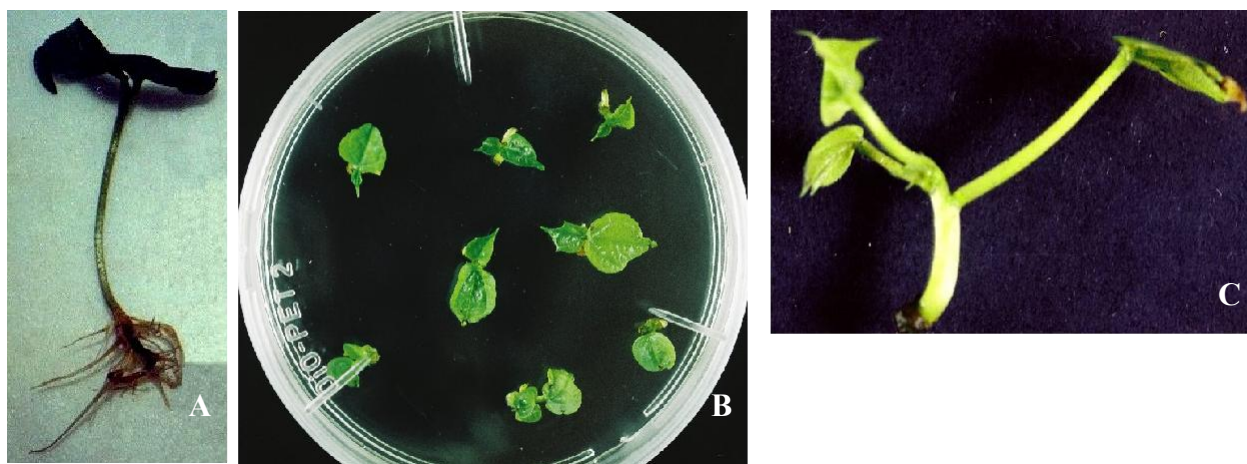
Figure 1. A. One week old germinated seedlings on MS supplemented with no hormones. **B-C.** 2 and 4 week old regenerated plantlets on MS supplemented with 1 mg/l KIN.

Total phenols of young leaves, shoots and the MS (Murashige and Skoog) media (for excreted phenols from explants to medium) were spectrophotometrically determined in 7, 14, 21 and 28th day of organogenesis. The 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).

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 and kept at $0^{o}C$ in fridge for 48 h. 5 ml samples were taken and centrifuged at 13000 rpm for 15 min. Then 1 mL of supernatant was transferred to a test tube and 1 mL of 95% ethanol and 5 mL double distilled water were added. 0.5 mL (50%) of Folin-Ciocalteu solution reagent was added to each sample. After 5 min, 1 mL of 5% Na_2CO_3 was added, the samples were mixed and allowed to stand for 1 h in darkness and measured absorbencies at 760 nm were measured using 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 ∝g/L GAE (R^2 = 0.9997).

RESULTS AND DISCUSSION

Previous studies on tissue culture and total phenolics were generally made with callus cultures (Nishikawa et al., 1999; Arnaldos et al., 2001; Angelova et al., 2001; Islam et al., 2003; Tang and Newton, 2004). In this study, direct regeneration response, total phenol amounts of regenerated plant parts (leaf, shoot) and the medium in 4 weeks of *in vitro* regeneration period (Figure 1) was investigated. As it is known, phenolics are synthesized in

leaves and then carried to other tissues and organs. Therefore, amounts of total phenolics in leaves are more than the other tissues and organs of the plants. In this study, total phenols were least in MS media while the highest were in leaves and the increasing and decreasing levels were similar for the 3 types of samples. In the first 2 weeks total phenols were less, after the second week an increase of total phenolics were observed, and in the third week the amounts of total phenolics peaked (Figure 2). Phenol levels of leaves less decreased between the third and forth weeks while the medium and shoot's phenolics were less increased.

There are some reports which show the parallel results with this study. In a similar study, dark and light periods were performed to the cultured plantlets and it was observed that phenolic contents were increased in 8 weeks of culturing periods in transformed root cultures of *Scutellaria baicalensis* Georgi. The increasing period was started after the fifth weeks of light and third weeks of dark conditions for clone C and in second week in light and in third week in dark with clone W (Nishikawa et al., 1999). This report also showed that genotype and light are also have effects on the synthesis of plant phenolics. In another study, Islam et al. (2003) studied with sweet potato cell suspension cultures and observed increase of total phenolics during 2 weeks and then less decrease of total phenol contents after 2 weeks (Islam et al., 2003). Like this study, meristematic tissues of sugarcane were cultured in liquid medium and it was observed that plant phenolics were increased during the 20 days of culture (gallic acid represented 82% of total phenolics) and decreased during the last 10 days (31 - 40 days of culture). The most intensive period of phenolic excretion (11 - 20 days) was preceded the most intensive period of shoot formation (21 - 30 days) (Lorenzo et al., 2001).

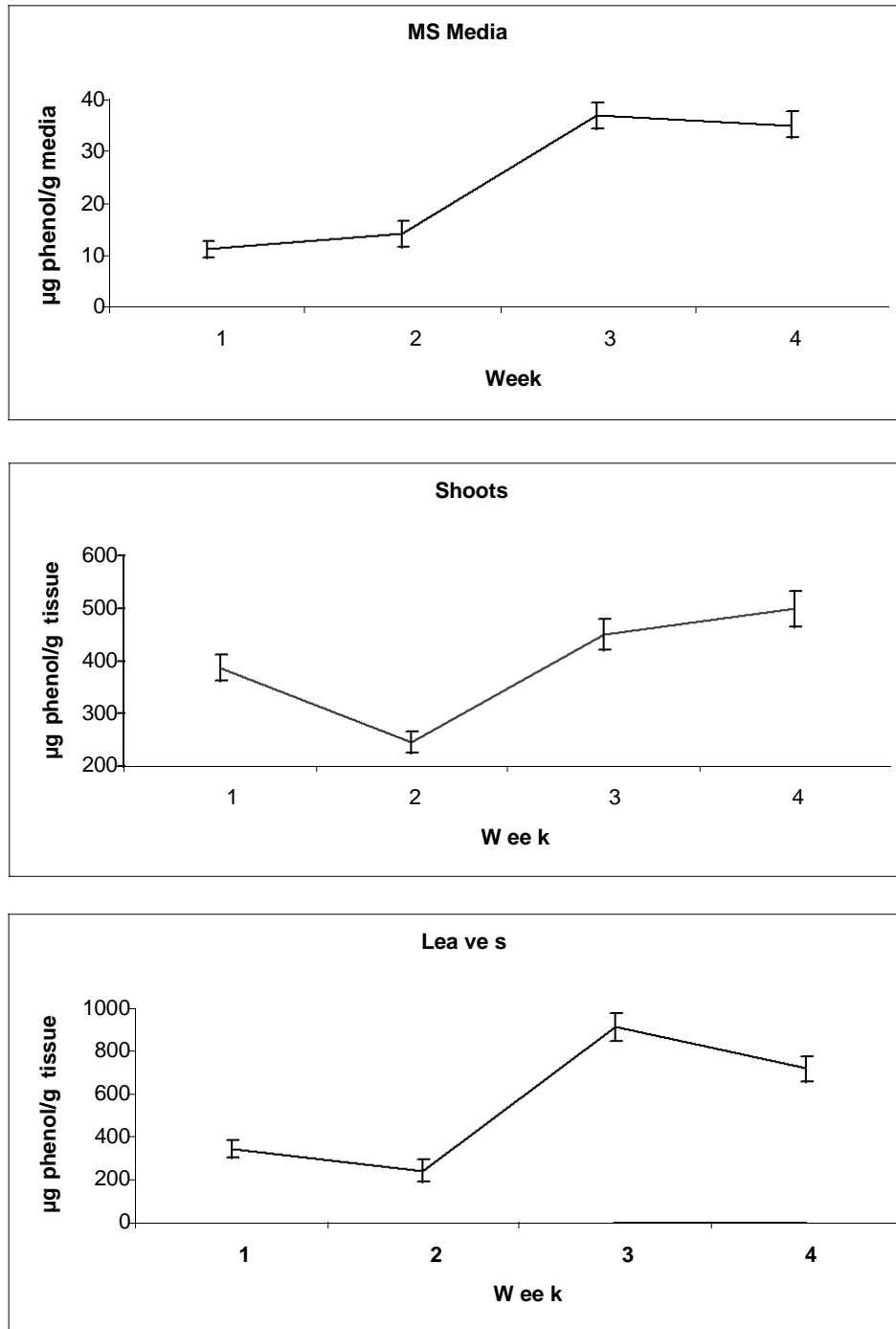There are two opinions about total phenolics and *in*

Figure 2. Total phenol amounts of MS media, shoots and leaves of cotton (*Gossypium hirsutum* L.) in 7th, 14th, 21st and 28th days of *in vitro* organogenesis. Each assay is a mean of five replicates± standard deviation. Total phenols were calculated as gallic acid equivalents (GAE).

vitro proliferation. While some authors described the phenolics being positively related to *in vitro* proliferation other say the negative. The negative effect is resulted with enzymatic discoloration. Tang and Newton (2004)

studied with Virginia pine's browned and non-browned callus tissues and they detected a slight enhanced level of polyphenol oxydase activity (PPO) at the first 3 weeks and the level reached a maximum at third week for

browning and non-browning callus cultures. In non-browning callus cultures, PPO activity subsequently declined while continued to increase in browning callus cultures. PPO is a nuclear encoded copper-containing enzyme which widely distributed in the plant species and catalyzes the oxidation of phenols to quinones (Kim et al., 2001). PPO is localized in the plastids, while its phenolic substrates are mainly present in the vacuoles (Lobreaux et al., 1995). PPO-mediated browning reaction occurs only after a loss of this sub-cellular compartmentation like wounding (Kim et al., 2001). PPO plays role pigment formation, oxygen scavenging and pseudocyclic phosphorylation in chloroplast, and defense mechanism against insects and plant pathogens (Tang and Newton, 2004; Kefeli et al., 2003; Yoruk and Marshall, 2003). Ozyigit et al. (2007) mentioned 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 (PPO activity) affect negatively as turned into highly toxic quinones and polymerized material causing discoloration of the medium and death of the cultured explants (Ozyigit et al., 2007).

In addition, especially in direct regeneration studies, the cut (scarred) surfaces of explants are important for phenolic exudation and oxidation. Although the phenols being metabolized, affect both regeneration and growth positively, if the surfaces are scarred, then exudation and oxidation (PPO activity) take place in these surface's cells, especially rooting parts of explants or young plantlet's phenolics affected negatively and no rooting was obtained while high regeneration responses observed. Oxidation of hydroxycinnamoyl causes the formation of phenolic bridges between polysaccharide chain, polysaccharide and lignins and also between polysaccharide and structural proteins. The consequence phenolic bridges are a loss of cell wall extensibility, thus leading to the cessation of the cell wall (Kroon and Williamson, 1999).

Conclusion

Whether the medium (liquid or solid), regeneration (direct or indirect) and explant types (meristematic tissues, hypocotyls, cotyledons or leaves) are different, it was similarly observed that total phenolics of *in vitro* cultured explants were less at the beginning of *in vitro* proliferation and then increased and less decreased after weeks for many tissue cultured plants. The important factor is oxidizing or metabolizing reactions for the success of *in vitro* organogenesis. If the POD activity is high, the explants show browning, less regeneration responses or death. Although the phenolic contents are high, if POD activity is less, phenols metabolize and play positive role on *in vitro* proliferation as lignin precursors and IAA catabolism modulators. By the way, phenolic oxidations of the scarred areas of direct regenerated explants are important features on rooting success.

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