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Determination of Physiological Changes in Related to Effects of Exogenous Indole-Butyric-Acid and Callus Formation in Some Kinds of Apple (*Malus sylvestris* Miller) Stem Cuttings

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Abstract: In this study**,** the effect of exogenous indole-butyric-acid and callus formation on the total chlorophyll *a*, chlorophyll *b*, carotenoids, anthocyanin and carbohydrate contents in cuttings stems of three apple (*Malus sylvestris* Miller) kinds ('Golden delicious', 'Starkrimson delicious' and 'Misket delicious') were investigated. The callus formation percentage increased with indole-butyric-acid treatment of three apple kinds but it was decreased at certain concentrations of indole-butyric-acid (3000 mg/L) in some apple kinds ('Golden delicious' and 'Starkrimson delicious'). For all of the three apple kinds, total chlorophyll *a*, chlorophyll *b*, carotenoids contents of the callus stems were lower than non-callus stems, however anthocyanin content was high in callus stems of three apple kinds. In all indole-butyric-acid treated (2000 and 3000 mg/L) apple kinds, it was showed an increase in chlorophyll *a*, chlorophyll *b*, carotenoids and anthocyanin contents of the callus compared to control. Although carbohyrate contents were decreased by callus formation, there were no significant differences between callus treated with indole-butyric-acid (2000 and 3000 mg/L) and control callus in three apple kinds.
Key Words: Apple (*Malus sylvestris* Miller), Callus, Carbohydrate, Indole-butyric acid, Pigments

Bazı Elma (*Malus sylvestris* Miller) Çeşitlerinin Gövde Çeliklerinde Eksojen İndol-Butirik-Asit ve Kallus Oluşumunun Etkileri İle İlgili Fizyolojik Değişimlerin Belirlenmesi

Özet: Bu çalışmada**,** üç elma (*Malus sylvestris* Miller) çeşidinin ('Golden', 'Starkrimson' ve 'Misket') gövde çeliklerinde, eksojen indol-butirik-asit ve kallus oluşumunun toplam klorofil *a*, klorofil *b*, karotenoid ve karbohidrat içeriği üzerine olan etkileri incelenmiştir. Üç elma çeşidinin kallus oluşum yüzdesi, indol-butirik-asit uygulaması ile artmış fakat bazı elma çeşitlerinde (Golden ve Starkrimson) indol-butirik-asit'in belirli konsantrasyonlarında (3000 mg/L) azalmıştır. Üç elma çeşidinin hepsinde, kalluslu gövdelerdeki toplam klorofil *a*, klorofil *b*, karotenoid içerikleri kallussuz gövdedekilerden daha düşüktü, bununla birlikte üç elma çeşidinin kalluslu gövdelerinde antosiyanin içeriği yüksekti. İndol-butirik-asit uygulanan (2000 ve 3000 mg/L) bütün elma çeşitlerinde, klorofil *a*, klorofil *b*, karotenoidler ve antosiyanin içerikleri, kallus-kontrolüne göre artma göstermiştir. Üç elma çeşidinde, kallus oluşumu sonucu karbohidrat içeriği azaldığı halde, indol-butirik-asit uygulanan kallus ile kontrol kallusu arasında önemli bir farklılık yoktu.

Anahtar Kelimeler: Elma (*Malus sylvestris* Miller), Kallus, Karbohidrat, İndol-butirik-asit, Pigmentler

Introduction

Apples are conventionally propagated by cutting, grafting and tissue culture techniques [1, 2]. The propagation of apples from cuttings is difficult, because most of apple cultivars and rootstocks do not form roots readily under conventional nursery conditions [1]. IBA is the most used auxin to stimulate the rooting process in cuttings because of: 1) its high ability to promote root initiation [3, 4] and 2) its weak toxicity and great stability in comparison to naphthalene acetic acid and indole-3-acetic acid [5, 6]. The rooting studies with grapevine stem cuttings have shown that the number of roots is increased after the IBA treatment and this is related to total phenol and protein contents, which was also increased [7].

Chlorophylls (Chl) and carotenoids occur in many tissues, in addition to leaf blades, including petioles, buds, cotyledons, cortical parenchyma of young twigs, and the phelloderm of older stems of some species [8]. Carotenoids are essential components of the photosynthetic apparatus in plants, algae, and cyanobacteria, in which is they protected against photooxidative damage and contribute to light harvesting for photosynthesis [9]. Anthocyanins, a large group of water-soluble pigments responsible for red to purple and red to blue colours of fruits and vegetables, are commonly used in

acidic solutions as a red pigment in soft drinks, jams, confectionery and bakery products [10]. Anthocyanins may act as antioxidants, and the relation between anthocyanins and oxidative stress seems to be interested in the ability of anthocyanins to reduce excitation pressure and, hence, the potential for oxidative damage [11].

Carbohydrates are the direct products of photosynthetic activity and constitute a source of energy and metabolites as well as structural building blocks. They represent to up to three quarters of the dry weight of ligneous plants [8]. They serve as an energy source and provide the carbon which is necessary for the production of new tissues. Energy reserves are accumulated in storage organs (stem, crown and/or roots) during cold acclimation [12, 13].

The propagation of apples from cuttings is difficult. Therefore, determination of pigments (chl *a*, chl *b*, carotenoids and anthocyanin) and carbohydrate contents in three apple kinds is important in explaining the physiological changes during the formation of callus and the effect of exogenous IBA treatment. For this reason, we aimed to investigate total chlorophylls, carotenoids, anthocyanin and carbohydrate contents related to callus formation and IBA treatment in three apple kinds (‘Golden delicious’, ‘Starkrimson delicious’ and ‘Misket delicious’).

Materials And Methods

Plant Material

Rooting studies of three apple (*Malus sylvestris* Miller) kinds (Golden delicious, Starkrimson delicious and Misket delicious) were carried out with the stem cuttings collected in February 2000-2002. Stem parts were collected at altitude 623m in Tokat-Turkey. Stem cuttings of three apple kinds measuring 15 to 20 cm in lenght and 7 to 8 mm in diameter were used. Different concentrations of the IBA (2000 mg/L and 3000 mg/L) were used for the treatments. The bases of the stem cuttings were soaked for 5 second in IBA solution at different concentration and cuttings soaked in distilled water served as control. Three groups of replicates each consisting of 15 cuttings were then planted immediately in pots filled with sand-perlite. The rooting studies were incubated at 17-24 °C (12 h light and 12 h dark). Observations on morphological characters such as callus number were recorded after planting (Table 1). For each analysis 15 cuttings

were taken and split into 3 sets of 5 replicates and basal parts in stem cuttings were sampled.

Assay for total chlorophylls and carotenoids

Fresh stem samples were extracted by following the method of De Kok and Graham [14]. The absorbance of the supernatants was measured at 662, 645 for chlorophylls and 470 nm for carotenoids. Concentrations of these compounds were calculated as described by [15]. All the experiments were performed in triplicate.

Assay for total anthocyanins

One gram of fresh stem samples were extracted with 12 ml of 1% (w/v) HCl in methanol for 2 days at 3 to 5 ^{o}C with continuous shaking. The extracts were filtered and centrifuged at 5000 *g* [16, 17]. The assay was carried out in triplicate. The absorbance of samples were measured at 530 and 657 nm and anthocyanin concentrations were calculated using following equation [17].

$A = (A_{530} - A_{657}/3)$

Assay for total Carbohydrates

Extraction and analysis of soluble sugars

Fresh stem samples were dried and grounded. Dried stem samples were extracted by following the method given elsewhere [18]. Absorbance of the samples was recorded at 625 nm. Total sugar concentrations of the samples were calculated using the calibration curve by using glucose standard [19].

Extraction and analysis of starch

Five mililitres of cold water and 6.5 mL of perchloric acid (52%) were added onto the residual material used for sugar analysis and mixed for 15 minutes. Twenty mililitres of water were then added. The samples were centrifuged and supernatant was separated. The same procedure was repeated with residue and obtained supernatants were combined and left for 30 minutes at 0 ^{o}C. After filtration the volumes of supernatants were adjusted to 100 mL with water [18]. Two and a half mililitres of supernatant were aliquoted into test tubes and 10 mL of cold anthrone solution (2%)

were added onto them. The samples were then heated at 100 °C for 7.5 minutes. Tubes were immediately transferred into an ice bath and cooled down to room temperature. Absorbance of samples were recorded at 630 nm. Starch concentrations were calculated using the calibration curve by using glucose standard and by multiplying the results with 0.92 [18].

Statistical analysis

Data from three replications of all treatments were subjected to analysis of variance using SPSS 8.0 for Windows (SPSS, Chicago, IL, USA) for all statistical analysis. $P < 0.05$ was considered statistically significant.

Results

Influence of IBA concentration on callus formation

Callus formation percentage of three apple kinds responded differently to IBA concentration (Table 1). The callus formation percentage increased with IBA treatment of three apple kinds but it is decreased at certain concentrations of IBA (3000 mg/L) in 'Golden delicious' and 'Starkrimson delicious'. Callus formation percentage of 'Misket delicious' was higher than other apple kinds (Golden delicious and Starkrimson delicious) (Table 1). The callus formation percentage in control group was 35% in 'Golden delicious', 37% in 'Starkrimson delicious' and 42% in 'Misket delicious'.

Table 1. Callus formation (%) in 'Golden delicious', 'Misket delicious' and 'Starkrimson delicious'. Data are the mean ± *SD*.

Apple kinds	Control	IBA treatment	
		2000 mg/L	3000 mg/L
Golden delicious	35±6	46±4	31±6
Misket delicious	42±5	62±6	76±4
Starkrimson delicious	37±6	43±5	34±5

Total chlorophyll *a*, chlorophyll *b*

Total chlorophyll contents of three apple kinds are shown in Figure 1. The chlorophyll contents in 'Golden delicious' (3.2 µg g^{-1}) were higher than other apple kinds (Starkrimson delicious and Misket delicious) in non-callus stem cuttings, whereas the total chlorophyll contents were significantly decreased with callus formation in all apple kinds (Figure 1; $P<0.05$). The total chlorophyll contents of the callus control group in all apple kinds were found to be lower than that of with treatment IBA (2000 and 3000 mg/L) (Figure 1). Although chl *a* and chl *b* contents were decreased with callus formation, its increased in IBA treated (2000 and 3000 mg/L) three apple kinds (Figure 1).

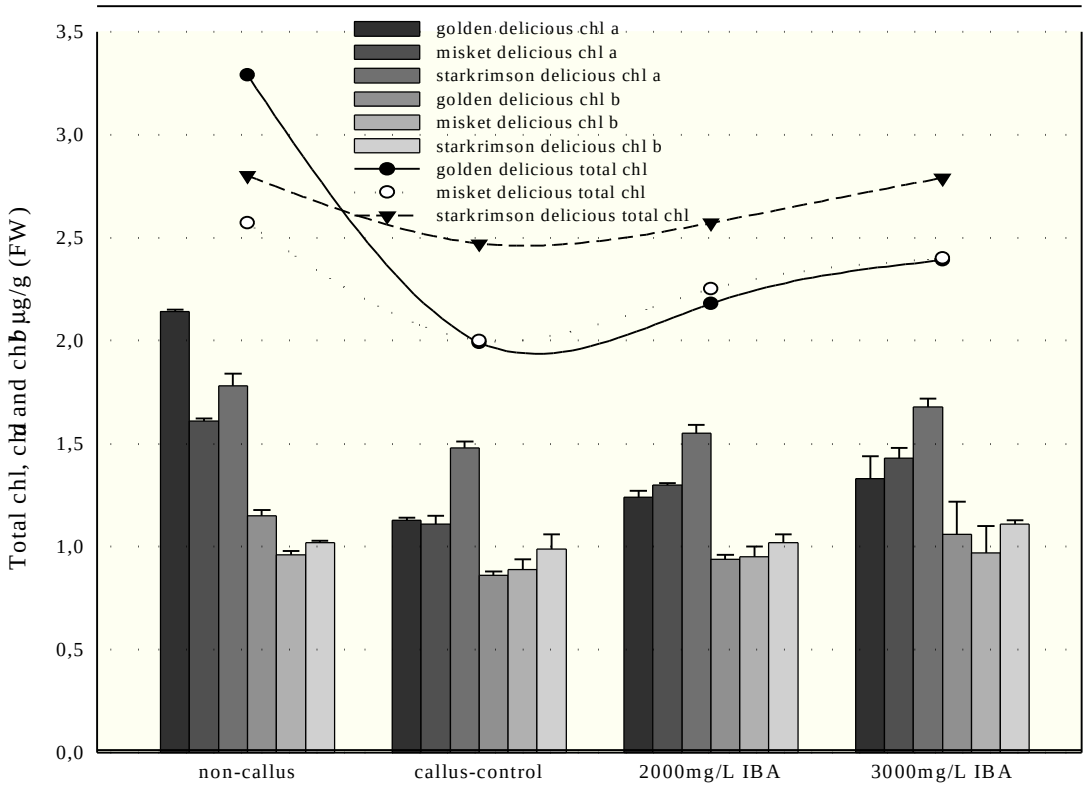

Figure 1. Total chlorophyll, chlorophyll *a* and chlorophyll *b* contents of 'Golden delicious', 'Starkrimson delicious' and 'Misket delicious' related to IBA treatment (non-callus: stem cuttings without callus formation, callus-control: callus formed stem cuttings, not IBA treated) (chl, chlorophyll) (FW, Fresh weight)

Total carotenoids

Carotenoids were significantly decreased with callus formation in all apple kinds compared to non-callus cutting (Figure 2; $P<0.05$). However, the concentrations of carotenoids in all the apple kinds were significantly increased after treatment with 2000 and 3000 mg/L IBA to callus control (Figure 2). The carotenoids contents in Golden delicious (2.1 μg g^{-1}) are higher than other apple kinds (Starkrimson delicious and Misket delicious) in non-callus stem cuttings (Figure 2).

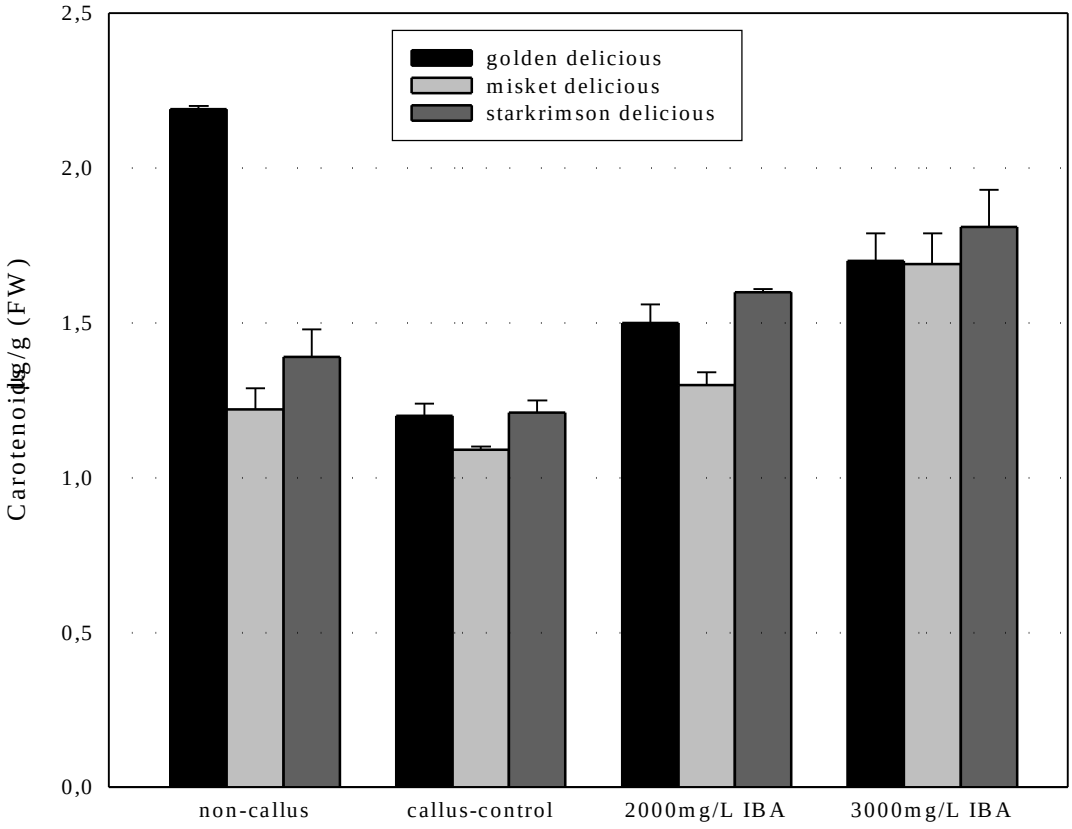

Figure 2. The carotenoids contents of 'Golden delicious', 'Starkrimson delicious' and 'Misket delicious' related to IBA treatment (non-callus: stem cuttings without callus formation, callus-control: callus formed stem cuttings, not IBA treated) (FW, Fresh weight)

Total anthocyanin concentrations

Figure 3 shows the total anthocyanin concentrations in three apple kinds of stem cuttings. The anthocyanin contents of three apple kinds were increased with callus formation and following IBA treatment ($P<0.05$). The highest anthocyanin

concentration was found following the treatment of 3000 mg/L IBA (0.87 at A_{530}) in 'Starkrimson delicious'.

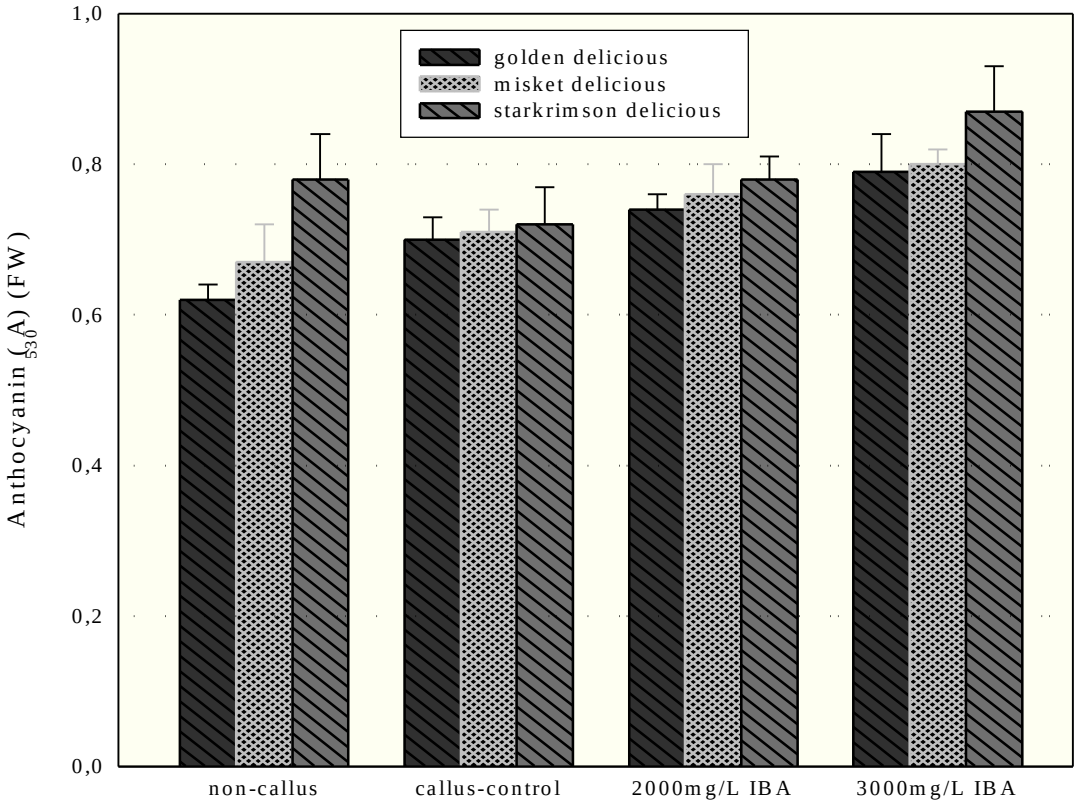

Figure 3. The anthocyanin contents of 'Golden delicious', 'Starkrimson delicious' and 'Misket delicious' related to IBA treatment (non-callus: stem cuttings without callus formation, callus-control: callus formed stem cuttings, not IBA treated) (FW, Fresh weight)

Total carbohydrates

The soluble sugars and starch levels in stem tissues of all apple kinds were significantly decreased with callus formation (Figure 4). The soluble sugars in callus control were 14 mg g^{-1} in 'Golden delicious', 24 mg g^{-1} in 'Starkrimson delicious' and 15 mg g^{-1} in 'Misket delicious'. The decline in callus formation in stem tissues of Golden delicious were nearly five times lower than non-callus (62 mg g^{-1} in non-callus and 14 mg g^{-1} in callus control). However, the levels of soluble sugars and starch of all control callus stems of all the apple kinds were similar in IBA treatment (2000 and 3000 mg/L). Total carbohydrate contents in stem tissues of all apple kinds were declined with callus formation and IBA treatment. Total carbohydrate contents in 'Golden delicious'

were found higher than other apple kinds (‘Starkrimson delicious’ and ‘Misket delicious’) (Figure 4; $P < 0.05$).

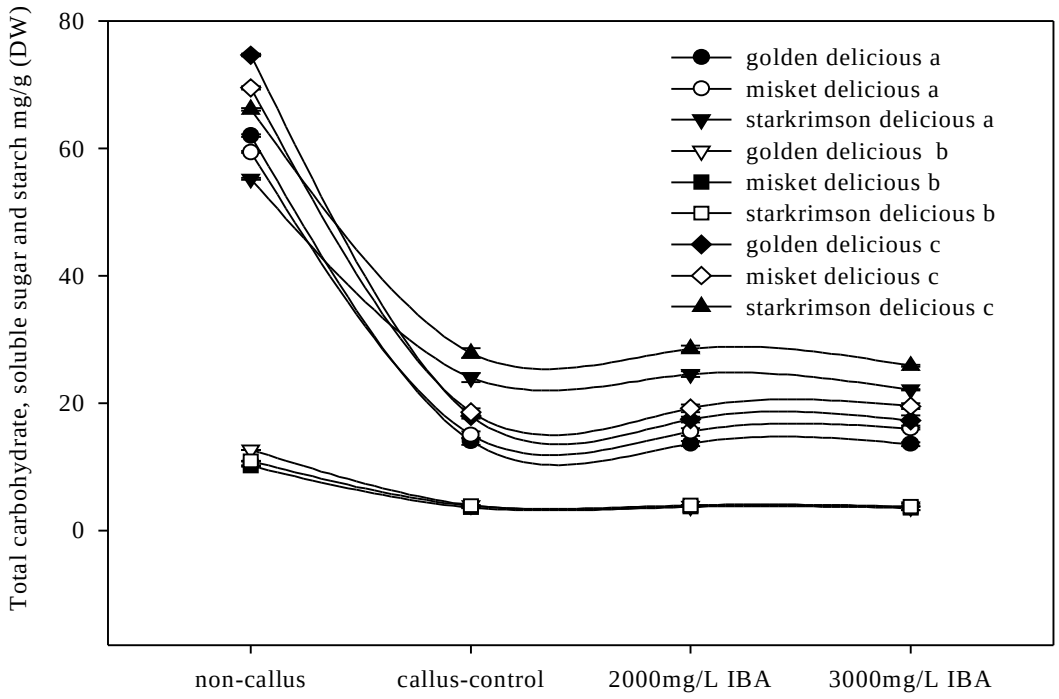

Figure 4. Total carbohydrates, soluble sugars and starch contents of ‘Golden delicious’, ‘Starkrimson delicious’ and ‘Misket delicious’ related to IBA treatment (a, soluble sugars; b, starchs; c, total carbohydrates) (non-callus: stem cuttings without callus formation, callus-control: callus formed stem cuttings, not IBA treated) (DW, Dry weight)

Discussion

Qaddoury and Amssa [20] reported that the root formation in date palm offshoots was significantly improved by IBA treatment and that phenolic contents increased immediately after IBA treatment and then decreased. In another study it was reported that rooting percentage and average number of roots in olive cuttings affected by cultivar, collection season and IBA concentration [21]. According to Azimi and Bisgrove [22], rooting percentage is increased by IBA treatment in *Rosa* species and decreased at certain concentrations of IBA in some *Rosa* species. The results of our study have indicated that the callus formation increased by IBA treatment of three apple kinds and decreased at certain concentrations of IBA (3000 mg/L) in Golden delicious

and Starkrimson delicious. Callus formation percentage have been changed depending on apple kinds (Table 1).

Wiesman and Lavee [23], indicated that photosynthesis in the cuttings olive during of rooting is very low and has almost no effect on the carbohydrate content. In the present study, before rooting chl *a*, chl *b* and carotenoids contents were high but, callus formation caused a decrease in pigment contents and an increase in anthocyanin contents of the three apple kinds (Figure 1, 2, 3). The decrease at the pigment level (chl *a* chl *b* and carotenoids) during callus formation could be explained by basal part of cuttings could not take light in pots and in consequences of this, the pigment levels had been decreased in relation to light in three apple kinds. In the another study, it is reported that anthocyanin production of adventitious roots grown in the dark was lower than that of the branched roots of intact plants cultivated in the field and IBA treatment caused of enhancement of the anthocyanin contents [24]. Callus tissues, derived from seedlings of roselle (*Hibiscus sabdariffa* L.), were shown to produce two cyanidin glycosides as major anthocyanin pigments. Both callus growth and anthocyanin synthesis were remarkably stimulated by 2,4-dichlorophenoxyacetic acid [25]. Kaur et al. [7] have shown that chl *a*, chl *b* and total chl contents in leaves of grapevine stem cuttings is enhanced after IBA treatment. In the present study, the chl *a*, chl *b* carotenoids and anthocyanin contents in all the apple kinds were significantly increased by the following treatment of 2000 and 3000 mg/L IBA to callus control (Figure 1, 2, 3; $P < 0.05$).

Rapaka et al. [26] reported that adventitious root formation were related with initial carbohydrate reserves and current photosynthesis in *Pelargonium*. Haissig [27] reported that rooting ability of cuttings has been in relation to carbohydrate content. The another study showed that mother plant sugar availability could affect the adventitious rooting response of *Eucalyptus* cuttings [28]. In the present study, the soluble sugars and starch levels in stem tissues of all apple kinds were significantly decreased with callus formation and IBA treatment (Figure 4). While total carbohydrate contents were found higher in 'Golden delicious', its callus formation was lower than other apple kinds (Figure 4; Table 1). The decrease in carbohydrate contents during callus formation could be explained by using it as an energy source for callus formation. This

phenomenon suggests high carbon and energy costs for the production of new organs during growth and development.

Consequently, the total chlorophylls, carotenoids, anthocyanin and carbohydrate contents significantly changed by callus formation and IBA treatment in stem cuttings of three apple kinds. Herein, we also have reported that initial carbohydrate level and photosynthesis is important to callus formation. The production of new organs are related to high carbon and energy source. Exogenous plant growth regulator (IBA) may be effective for callus formation and enhances total chlorophylls, carotenoids and anthocyanin compounds in stem cuttings.

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