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Biochemical Changes in Green and Etiolated Stems of MM106 Apple Rootstock

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Abstract: In the present study, total chlorophyll a, chlorophyll b, carotenoids, anthocyanin, carbohydrate, phenolic constituents, antioxidant activity and root formation percentage of MM106 apple rootstock in etiolated and green stem cuttings were investigated. The rooting studies were carried out climatic room in dark and daily period at 25°C. Chlorophyll a, chlorophyll b, carotenoids and anthocyanin contents of etiolated stems were significantly lower than green stems. Similarly, total phenolic and carbohydrate contents and antioxidant activity were found lower in etiolated stems but root formation percentage increased significantly with etiolation.

Key words: Antioxidant activity, carbohydrates, etiolation, MM106 rootstock, phenolic compounds, pigments, rooting

INTRODUCTION

Much research has been done to explain the action of various treatment methods and it has been reported that etiolation of stems causes anatomical and physiological changes. Lack of chlorophyll, increase of internode length, increased succulence and decreased mechanical strength of stem tissues that generate roots are some of the etiolation effects (Maynard and Bassuk, 1988). Etiolation promotes adventitious root formation on stem cuttings of many wood species (Anderson, 1981; Bassuk et al., 1984). It is well known that the environmental factors which influence the rooting of cuttings include light quality, intensity and duration (Andersen, 1986). Cuttings from etiolated stems usually root better than those obtained from green ones (Magdalene, 1998; Xuebo and Brewbaker, 2006).

Light influences almost every stage of plant development. Young seedlings grown in the dark exhibit etiolated growth; light triggers de-etiolation, a suite of developmental changes that allow optimal photosynthetic growth (Maloof et al., 2000). In another study, Quercus petraea and Quercus robur seedlings raised under diminishing light showed a greater total height, leaf area, leaf area ratio and chlorophyll content, but lower leaf thickness, root weight, shoot/root ratio, net assimilation rate and relative growth rate (Vera, 2000).

Anthocyanin accumulation is associated with greening and etiolation (Krause et al., 1995; Hoch et al., 2001). Anthocyanins acts as anti oxidants and the association between anthocyanins and oxidative stress appears to be related to the ability of anthocyanins to reduce excitation pressure and hence, the potential for oxidative damage (Steyn et al., 2002).

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu et al., 1998). Some studies show that antioxidant activity and phenolics are influenced by light and dark treatment in plants (Arezki et al., 2001; Shohael et al., 2006; Wu et al., 2006). It was reported that higher irradiance caused a decrease in phenolic rooting inhibitors (Druart, 1982).

Carbohydrates serve as an energy source and yield the carbon needed for the production of new tissues. So, if cuttings derived from stock plants depleted of carbohydrates are rooted under conditions where net photosynthesis cannot occur, the energy charge will be too low to support rooting (Veierskov, 1988). The total carbohydrate content of the shoots was affected by etiolation treatment (Elamrani et al., 1994; Darbelley et al., 1997).

Many studies have been published concerning the propagation MM106 apple rootstock related to various factors (Wilkinson and Withnall, 1970; Welander and Snygg, 1986; Maynard and Bassuk, 1988; Noiton et al., 1992). However, there are no reports on how the antioxidant activity, total phenolic, anthocyanin, chlorophyll a (chl a), chlorophyll b (chl b), carotenoids and carbohydrate content of the etiolated and green stem

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cuttings of MM106 apple rootstocks. Determination of these constituents in MM106 apple rootstock is important for evaluation of metabolic changes related to light and etiolation. Therefore, the aim of this study was to investigate the effects of light and etiolation on antioxidant activity, total phenolic, anthocyanin, chl a, chl b, carotenoids and carbohydrate contents of the stem cuttings of MM106 apple rootstocks.

MATERIALS AND METHODS

Plant material: Rooting studies of MM106 apple rootstocks were carried out with the stem cuttings collected in 2003. Stem cuttings of MM106 apple rootstocks measuring 20 to 25 cm in length and 5 to 6 mm in diameter were used. Three groups of replicates each consisting of 15 cuttings were then planted immediately in pots filled with sand. The rooting studies were carried out climatic room in dark and daily period at 25°C. Observations on morphological characters such as root number were recorded 60 days later after plantation. For each analysis 15 cuttings were taken and split into 3 sets of 5 replicates and stem cuttings were sampled.

Antioxidant activity-DPPH assay: Fresh stem cuttings of etiolated and green of MM106 apple rootstock were extracted using MeOH (3 h with constant shaking). The methanol extract was separated and evaporated under vacuum until dryness. Then corresponding extracts were tested for their free radical scavenging ability using assay given below. Stable free radical 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) has a characteristic purple color in methanol. This stable free radical is scavenged in the presence of antioxidant components of the extract (by free radical scavengers) and purple color is bleached. Bleaching is measured by spectrophotometer at 517 nm and bleaching capacity of the extract is evaluated as inhibition percentage.

The DPPH assay was carried out following the same method as reported elsewhere (Gulluce *et al.*, 2003). Fifty micro liters of various concentrations of the MM106 extracts dissolved in methanol was added to 5 mL of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition percent (1%) of $DPPH^{\cdot}$ was calculated in the following way:

$$1\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Inhibition is concentration dependent and extract concentration

providing 50% inhibition (IC_{50}) is calculated form the graph-plotted inhibition percentage against extract concentration. IC_{50} values of the etiolated and green plant is compared and lower IC_{50} value is defined as higher activity.

Assay for total phenolics: Total phenolic constituents fresh stem cuttings of etiolated and green of MM106 apple rootstocks were performed employing the literature methods involving Folin-Ciocalteu reagent and gallic acid as standard (Chandler and Dodds, 1983; Slinkard and Singleton, 1977). Briefly, 50 mg of fresh stems of MM106 was homogenized in 2.5 mL ethanol and flask was kept in a water bath at 25°C for 24 h with continuous shaking. After filtration 1 mL of supernatant solution was taken in a volumetric flask, 1 mL ethanol and 5 mL distilled water and 1 mL Folin-Ciocalteu reagent were added and flask was shaken thoroughly. After 3 min, 3 mL of solution 2% (w/v) Na_2CO_3 was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated to all standard gallic acid solutions (0-1000 µg/0.1 mL) and standard curve was obtained. Total phenolic compounds of the sample were calculated using the calibration curve by using gallic acid standard. The assay was carried out in triplicate.

Assay for total anthocyanins: Fresh samples etiolated and green were extracted with 12 mL of 1% (w/v) HCl in methanol for 2 days at 3 to 5°C with continuous shaking. The extracts were filtered. The assay was carried out in triplicate. Following this absorbance of the samples were measured at 530 and 657 nm and anthocyanin concentrations were calculated using following equation (Mancinelli *et al.*, 1975).

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

Assay for total chlorophylls: Fresh samples etiolated and green were extracted by following the method given elsewhere (De Kok and Graham, 1989). The absorbance of the supernatant was measured at 662, 645 for total chlorophylls and 470 nm for carotenoids. Concentrations of these pigments were calculated as described by Lichtenthaler and Wellburn (1983).

Assay for total carbohydrates
Extraction and analysis of soluble sugar: Fresh etiolated and green stem samples were dried and grounded. Dried stem samples were extracted by following the method given elsewhere (Ebell, 1970). Absorption of the samples was recorded at 625 nm. Total sugar concentrations of the samples were calculated from the calibration graph drown from glucose standard solutions (Ebell, 1970).

Extraction and analysis of starch: Five milliliter cold water and 6.5 mL of perchloric acid (52%) was added to the residue material used for sugar analysis and mixed for 15 min. Following the method given elsewhere (McCready *et al.*, 1950) absorption of the samples was recorded at 630 nm. Starch concentrations of the samples were calculated from the calibration graph drawn from glucose standard solutions by multiplying 0.92 (McCready *et al.*, 1950).

Statistical analysis: Data from three replications of all treatments were subjected to analysis of variance using SPSS 8.0 for Windows for all statistical analysis. Differences between means at 5% ($p<0.05$) level were considered as significant.

RESULTS AND DISCUSSION

In etiolated stems MM106 apple rootstocks root formation were found higher than green MM106 stems ($p<0.05$). Root formation of etiolated stems was 96 and 87% in green stems. Xuebo and Brewbaker (2006) reported that etiolation treatments increased rooting ability significantly for two difficult-to-root of *Leucaena* hybrids. In a study carried out with *Artocarpus heterophyllus* Lam. stems, etiolation and IBA treatment was reported promoting the root formation (Mukherjee and Chatterjee, 1979). Similarly, *Malus domestica, Rhododendron* and *Kalmia latifolia* stems were kept 14 h in dark and 14 h in dark. It was reported that best root formation had been observed with etiolated stems and this promoting effect directly related to temperature (Hansen and Potter, 1997).

In etiolated stems chlorophyll a, chlorophyll b, carotenoids and anthocyanin contents are significantly lower than green MM106 stems ($p<0.05$) (Table 1). Etiolation or light reduces production of chemicals that responsible from pigment production. In a study with *Arabidopsis thaliana,* pigment levels were found to be affected from the light intensity and pigment levels were found lower in dark (De Kok and Graham, 1989). Wu *et al.* (2006) reported that chlorophyll and β-carotene induction stimulated by light and light quality. In another study, the reaction of *Araucaria angustifolia* stems exposed to low light intensity had been investigated (Duarte and Dillenburg, 2000) and chlorophyll constituent of those stems had been found unchanged and the ratio between chlorophyll a and chlorophyll b remained at the same value. However, anthocyanin biosynthesis and flavoring had been promoted with red light in American cranberry (Zhou and Singh, 2002). Anthocyanin accumulation requires light and generally coincides with periods of

Table 1: Chl a, chl b, carotenoids and anthocyanin levels of MM106 apple rootstocks stems etiolated and green (60 days later). Each value indicates the mean±standard deviation

Parameters	Etiolated stems	Green stems
Chl a[a]	1.18±0.02	3.50±0.20
Chl b[a]	0.93±0.06	1.89±0.07
Carotenoids[a]	0.97±0.05	3.21±0.07
Anthocyanin[b]	0.13±0.03	0.41±0.01

[a]mg g^{-1} fresh weight; [b]Absorbance at 530 nm

Table 2: Antioxidant activity, total phenolic, total sugar and starch levels of MM106 apple rootstocks stems etiolated and green (60 days later). Each value indicates the mean±standard deviation

Parameters	Etiolated stems	Green stems
Antioxidant activity, IC_{50}[a]	64.45±2.89	58.00±1.97
Total phenolic[b]	34.00±2.82	44.50±0.70
Sugar[c]	74.75±0.81	101.44±9.00
Starch[c]	6.68±0.50	9.58±0.91

[a]µg mL^{-1} fresh weight; [b]µg mg^{-1} fresh weight; [c]mg g^{-1} dry weight

high excitation pressure and increased potential for photooxidative damage due to an imbalance between light capture, CO_2 assimilation and carbohydrate utilization (Steyn *et al.*, 2002). Present finding show that chlorophyll a, chlorophyll b, carotenoids and anthocyanin contents lower in etiolated stems than green stems, indicating that light may play important role on pigment levels.

All investigated parameters showed significant decrease in etiolated stems (Table 2). Total phenolic constituent decreased to 34.0 µg mg^{-1} while it was 44.5 µg mg^{-1} in green stems. It was previously reported (Goupy *et al.*, 1990) that phenolic constituent of etiolated *Cichorium endivia* leaves decreased and plant produced more flavanol rather than phenols. In another study showed that phenolic contents changed by light and light quality. Content of total phenolic on matured somatic embryos of *Eleutherococcus senticosus* found high fluorescent light than dark (Shohael *et al.*, 2006). Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids. Phenolic acids have been repeatedly implicated as natural antioxidants in fruits, vegetables and other plants. Different phenolic compounds have differing amounts antioxidant activity (Sakihama *et al.*, 2002; Zheng and Wang, 2001). Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu *et al.*, 1998). In the present study, antioxidant activity of etiolated MM106 stems were also found lower than green stems (Table 2) ($p<0.05$). IC_{50} value of etiolated MM106 stems increased to 64.4 µg mL^{-1} since lower IC_{50} means higher activity.

In the case of sugar and starch contents of MM106 stems both dramatically decreased by etiolation (Table 2). Carbohydrates serve as an energy source and yield the

carbon needed for the production of new tissues. Energy reserves are accumulated in storage organs (stem, crown and/or roots) during cold acclimation of temperate (Levitt, 1980). Some studies revealed no relationship between etiolation and carbohydrate reserves (Stout, 1984; Mckenzie *et al.*, 1988). In contrast to above study sugar and starch concentration decreased in etiolated stem cuttings. Darbelley *et al.* (1997) reported that the changes in α-amylase activity and in starch and free sugar content were investigated in correlation with lipid mobilization in *Helianthus annuus* in discontinuous light and in darkness. In light-grown cotyledons, photosynthesis contributes to increase the carbohydrate levels. It has been reported that etiolated shoots of *Oryza sativa* L. were not photosynthetic, only precursors of the photosynthetic proteins were identified (Komatsu *et al.*, 1999). In etiolated MM106 stems total carbohydrate contents were found lower in present study since etiolated MM106 stems were not possibly photosynthetic.

CONCLUSIONS

The present study indicated that the antioxidant activity, total phenolic, anthocyanin, chl a, chl b, carotenoids, carbohydrate contents are found quite different in etiolated and green stems of MM106. Etiolation reduces total phenolic, anthocyanin, chl a, chl b, carotenoids, carbohydrate contents and antioxidant activity but enhances root formation. Etiolation may be used as effective pretreatment step to improve root formation.

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