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Biochemical changes in terminal buds of three different walnut (*Juglans regia* L.) genotypes during dormancy break

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ABSTRACT

To the better management of frostbite problem in fruit crops, evaluation of biochemical changes in buds during dormancy break is a key factor. In this study biochemical changes in terminal buds of three different walnut genotypes (early-season, mid-season, and late-season), was investigated during winter dormancy release time. Terminal bud samples were taken from the genotypes in two different times in winter (1st and 20th of March). Variables such as; bud soluble sugars, starch, proline, chlorophyll, total phenolic and protein content as well as catalase (CAT) and guaiacol peroxidase (GPO) enzymes activities were assessed. Analysis of variance ANOVA) revealed significant differences ($p \leq 0.05$) for the measured variables and sampling times among the genotypes. The results indicated that the late growth genotype had the highest content of soluble sugars and proline, and the lowest amount of starch and protein ($p \leq 0.05$) in comparison with early and mid-season growth genotypes. Different enzymes activities were observed in terminal buds among the genotypes. As sampling time delaying, the content of chlorophyll, CAT and GPO enzymes activity increased, while a decrease in proline and total phenol was observed. The results of this study can be considered to a better understanding of the primary mechanisms of bud dormancy break in walnut to reduce the late spring frost damage problem as well as for using in breeding programs.

1. Introduction

Walnut (*Juglans regia* L.) is one of the most important nut trees in the world and has a wide distribution and diversity in Iran and Middle East country (McGranahan et al., 1988). Late spring frost damage is one of the major factors which lead to losing the most walnut production shoot (Xin and Browse, 2000; Sanghera et al., 2011). It is well established that several factors play a key role in resistance of walnut trees to late spring frost, such as genotype, growth stage, the formation of ice crystals, humidity and nutritional status (Rodrigo, 2000; Miranda et al., 2005). Chilling damage severity is different among plant organs (Mahmodzadeh and Imani, 2011) and the flowers are the most sensitive parts (Charrier et al., 2013). It is proven that flower buds are more resistant to cold during deep rest stage, whereas their cold sensitivity increases during bud swelling stage (Ashworth and Wisniewski, 1991).

Dormancy is an occurrence that caused a temporary hold up of observable growth of any plant parts, which categorized as para-dormancy, wherever internal physiological factors inhibit growth, defined as endo-dormancy, and where environmental factors inhibit growth called as eco-dormancy (Lang et al., 1987). To overcome dormancy period plants set some special mechanisms such as changes in their metabolism and growth regulators (carbohydrates, proteins, lipids, tissue water, antioxidant enzymes, organic acids, amino acids, and nucleic acids). They use these mechanisms at the beginning and finishing of the rest period (during winter and early spring) to handle the extreme cold weather condition (Westwood, 1993; Sung et al., 2003). It has been reported that among photosynthesis components, carbohydrate metabolism is the most sensitive to low temperature (Fernandez et al., 2012). There is a decrease in starch content following hydrolysis, therefore during low-temperature exposure, an increase in free saccharides is considerable in plants (Pollock and Lloyd, 1987; Bohnert and Sheveleva, 1998). Saccharides play multiple roles at low-temperature tolerance, such as prevent ice nucleation in the

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apoplast with reducing water availability and preserve water within plant cells as typical compatible osmolytes (Uemura and Steponkus, 1999; Ruelland et al., 2009). Moreover, carbohydrates have a special role in membrane stability and act as reactive oxygen scavenger (Bradford, 1976). Proline is also another protective agent, which plays an important role in plant tolerant to stresses. Proline may act as a mediator of osmotic adjustment, the stabilizer of proteins and membranes, an inducer of osmotic stress-related genes and ROS scavenger (Verbruggen and Hermans, 2008; Szabados and Savoure, 2010).

It is interesting to note that direct relationship has been found between antioxidant enzymes activity and low temperatures resistance (Chen et al., 2006). De la Rosa et al. (2000) stated that during cold stress, sugar content in buds showed an enhancement, and this increment may be due to sugar translocation from shoots and bark to the buds. Accumulation of sugars, saccharides, proteins and osmotic regulators (such as proline) improved cold tolerance in pistachio (Mansouri Dehshoaibi et al., 2011) and tobacco (Konstantinova et al., 2002). Increasing concentration of saccharide and proline, as well as decreasing content of starch at dormancy time in buds, has been reported by Patton et al. (2007). Similar results were reported by Charrier et al. (2013) on walnut trees. Findings on pistachio cultivars revealed that bud dormancy and its resistance to cold stress associated with soluble saccharides, proline and intercellular water content (Nobari et al., 2012). There are many native landraces of walnut in the world that they are sensitive to spring chilling injuries. The purpose of the present study was to illustrate the biochemical changes not such as but rather of soluble sugars, starch, proline, chlorophyll, total phenolic and protein content as well as catalase (CAT) and guaiacol peroxidase (GPO) enzymes activities in walnut terminal buds at dormancy release time, that occur in early, mid, and late seasons walnut genotypes.

2. Materials and methods

2.1. Sample preparation

Terminal buds of three native walnut (*Juglans regia*) genotypes (early, mid and late based on flowering time) were sampled at two times (first and 20th March 2014). Samples were transported in liquid nitrogen to the lab and stored at −80 °C. All trees were received similar standard cultural practices adopted in the orchard. The studied genotypes were selected from Garehasanlo village (37° 32′59.8″ N, 45° 10′35.1″ E, and 1294.1 m above sea level), Urmia County, East Azerbaijan Province, Iran. This region has a cold semi-aired climate with an annual average precipitation of 427.7 mm.

2.2. Determination of soluble sugars

Soluble sugars were determined according to the method of Irigoyen et al. (1992). 0.5 g of freeze-dried buds was homogenized with 5 ml of 95% ethanol. 0.1 ml of alcoholic extract mixed with 3 ml anthrone (150 mg anthrone, 100 ml sulphuric acid 72%). The samples were placed in water bath for 10 min. The absorbance was measured spectrophotometrically at 625 nm using a PD-303 model spectrophotometer (Shimadzu spectrophotometer 2550 UV/Vis, Japan). The content of soluble sugar was determined using a different concentration of glucose as standard in the spectrophotometer.

2.3. Determination of starch

Starch content was determined using the perchloric acid method described by Rose et al. (1991). The left pellet after sugar extraction was further extracted three times with 5 ml of 35% (v/v) perchloric acid with continuous shaking at low speed for 15 min. The extracts were pooled and centrifuged for 5 min at 10000 × g. The supernatants were collected in graduated tubes, then diluted to 20 ml with distilled water. For colorimetric determination, a 1 ml aliquot of the extract was mixed with 5 ml of anthrone reagent (0.175%, w/v, in 75% cold sulfuric acid) in a test tube and mixed gently. The mixture was placed in a water bath for 12min and then set on ice. The mixture's absorbance was read at 620 nm using a spectrophotometer.

2.4. Determination of proline

One ml of alcoholic extract was diluted with 10 ml of distilled water, and 5 ml of ninhydrin (0.125 g ninhydrin, 2 ml of 6 mM NH_3PO_4, 3 ml of glacial acetic acid) and 5 ml of glacial acetic acid was added, and then placed in water bath for 45 min at 100 °C. After cooling samples by placing the test tubes in cold water, the samples were vigorously mixed with 10 ml benzene. Standard solutions of proline were prepared, and finally, absorption of standard solutions and samples at 515 nm was read with the spectrophotometer (Irigoyen et al., 1992).

2.5. Determination of catalase (CAT) enzyme activity

Catalase activity was evaluated by recording the rate of H_2O_2 disappearance at 240 nm using the spectrophotometric method of Aebi (1984). Reaction mixture consisted of 2.5 ml phosphate buffer (50 mM, pH 7.0), 0.2 mL H_2O_2 (1%) and 0.3 mL of enzyme extract. The decomposition of H_2O_2 was determined at 240 nm by extinction coefficient 43.6 $mMcm^{-1}$.

2.6. Determination of guaiacol peroxidase (GPO) enzyme activity

Guaiacol peroxidase activity was measured using the spectrophotometric method described by Aebi (1984). Reaction mixture consisted of 2.5 mL phosphate buffer (50 mM, pH 7.0), 1 mL guaiacol (1%) and 1 mL of enzyme extract. The increase in absorbance at 420 nm was followed for 1 min by extinction coefficient 26.6 $mMcm^{-1}$.

2.7. Determination of chlorophyll (CHL)

The extraction of chlorophyll *a* and *b*, and total chlorophyll was assessed according to the Gross (1991). 0.25 g of the samples were homogenized with 80% acetone. The optical density (O.D.) of the extracted chlorophyll was measured at 645 and 663 nm using a spectrophotometer. Chlorophyll (CHL) a, b and total chlorophyll were calculated by the following formulae (Gross, 1991).

$$CHL_a = (0.0127 \times OD663) - (0.00269 \times OD645),$$

$$CHL_b = (0.0229 \times OD645) - (0.00468 \times OD663)$$

$$TC = (0.0202 \times OD645) + (0.00802 \times OD663)$$

2.8. Determination of total phenol

To extract the phenol content, 0.5 g of terminal buds were homogenized with 10 ml of 85% methanol. The extracts were centrifuged for 15 min at 10,000 × g at 4 °C. Total phenol in the methanol extracts was determined colorimetrically using Folin–Ciocalteu reagent, as described by Slinkard and Singleton (1977). Gallic acid (GAE, Merck, KGaA, Germany) was used as standard and results