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Effect of 2,4-D on fruit sugar accumulation and invertase activity in sweet orange cv. Salustiana

Alexander Rebolledo Roa*[1], Amparo García-Luís[2], José Luís Guardiola Barcena[2(R.I.P)], Consuelo Monerri Huguet[2]

[1]Corporación Colombiana de Investigación Agropecuaria – CORPOICA, C.I. Palmira Unidad de Fisiología, Colombia
[2]Universidad Politécnica de Valencia. España. Departamento de Producción Vegetal, Spain

***Corresponding author: arebolledor@corpoica.org.co**

Abstract

Small fruit size is a common problem in Salustiana oranges. Many reports exist on the use of synthetic auxins to enhance citrus fruit size. Few of these studies delve on the effect of auxins on carbohydrate metabolism and its relationship with enzyme activity. Sugar balance is an important trait for fruit quality and depends on the enzymatic metabolism at different tissue levels. The effect of 2,4-Dichlorophenoxyacetic acid (2,4-D) applications on fruit growth, sugar content, and invertase activity was studied on sweet orange fruit on 20-year-old trees of Citrus sinensis (L.) Osbeck cv. Salustiana. Carbohydrates were quantified via High-Performance Liquid Chromatography (HPLC) and the sugars released by the invertase activity with the Somogy–Nelson method. Then, the growth variables of the fruit were characterized (diameter and fresh and dry weight). The results showed that application of 2,4-D (20 mgL^{-1}, 3.6 L per tree) increased fruit size at maturity (5 mm). Application of 2,4-D increased sugar levels in the fruit and influenced the activity of the different invertase isoforms. The increased activity of the acid invertase isoform suggests a direct effect of 2,4-D on the increased fruit sink strength; it is also related to increased consumption of sugars during the initial fruit development stage.

Keywords: *Citrus sinensis* (L.) Osbeck, enzymatic activity, fruit growth, plant growth regulators, reducing sugars, sucrose, total sugars.
Abbreviations: 2,4-D _ 2,4-Dichlorophenoxyacetic acid; DAA _ days after anthesis; DW _ dry weight; FW _ fresh weight; HPLC _ high-performance liquid chromatography; RS _ reducing sugars; TS _ total sugars.

Introduction

Carbohydrate synthesis, as well as the capacity to transport sugars to non-photosynthetic tissue, is a key characteristic of plants. Carbohydrate supply to citrus fruit growth can be supported by that reserved during the initial bud sprouting and flowering stages. The following growth stages including fruit set, vegetative and fruit development are mainly supported by actual photosynthetic rates (Syvertsen and Lloyd, 1994; Nebauer et al., 2011; Zhang et al., 2013). After phloem unloading in sinks, sucrose is degraded into hexoses for diverse uses by invertase (INV) that hydrolyses sucrose into glucose and fructose. In fact, the enzymatic activity responsible for the first metabolic reaction of sucrose can be a possible key link between the production of photoassimilates in the source tissue and the growing capacity in sink organs (Farrar, 1996; Ruan 2012).
The Indole-3-acetic acid (IAA) concentration at fruit level has been found noticeably higher during postanthesis and sharply decreasing soon thereafter. The maximum IAA levels coincide with differentiation and cellular division processes during the fruit's slow growth phase (Laskowski et al., 2008; Pattison et al., 2014). Fruit sink strength in citrus increases with exogenous auxin applications (Guardiola and García-Luís, 2000); faster fruit growth until ripening is the main result (Guardiola and Lázaro, 1987; Ortola et al., 1988; Agusti et al., 1994; Rebolledo et al., 2012). Guardiola et al., (1993) found reduced sensitivity of citrus fruit tissues to growth regulators; with such being higher during early fruit development. The function of invertases (INV) has been deduced from a correlation between activity and development of physiological processes like tissue growth or sugar accumulation in sink organs (Lowell et al., 1989; Ruan et al., 2010; Ruan 2012). High acid invertase activity (apoplasmic or cell-wall and vacuolar isoforms) has been found in tissues with faster growth (Ricardo and Ap Rees, 1970; Lowell et al., 1989; Ruan et al., 2010; Dai et al., 2011). These invertases hydrolyze sucrose under conditions of high hexose requirement. The role of acid invertase is also known to regulate sucrose concentration in several plant tissues (Hubbard et al., 1989; Ross and Davis, 1992). High activity of these enzymes was found during early fruit development of Satsuma mandarin (Kubo et al., 2001) and Ponkan tangerine (Jiang et al., 2014). Acid invertase activity declines rapidly during fruit development and disappears during early maturity stages, whereas alkaline invertase shows an opposite trend before finally declining. During the fruit's early stage, high activity of acid invertase, sucrose synthase (SUS), and alkaline invertase (Cytoplasmic isoforms) were found involved in sucrose catabolism (Echeverria, 1990; Jiang et al., 2014). High alkaline invertase activity in the vesicles of grapefruit was found during the linear phase of fruit growth characterized by cell expansion (Lowell et al., 1989).
Invertases can also be involved in long-distance transport by creating a concentration gradient between the loading and unloading sites of the phloem (Ruan et al., 2010; Ruan 2012).

Hydrolysis products considerably increased cell osmotic pressure with possible participation in cellular expansion and plant growth (Gibeaut et al., 1990; Ruan 2012). Reduction of alkaline invertase was found from the beginning of the rapid fruit growth period in vesicles of Ponkan tangerine (Jiang et al., 2014). Sugar balance is an important trait for fruit quality and depends on enzymatic metabolism at different tissue levels. Many reports exist on the use of synthetic auxins to enhance citrus fruit size, but few of these delve on its effect on carbohydrate metabolism and its relationship with enzyme activity. The objective of this study was to evaluate the effect of 2,4-D application on fruit growth rate, sugar content, and invertase activity in sweet oranges of the Salustiana cultivar.

Results

Effect of 2,4-D application on fruit growth pattern

Application of 2,4-D increased fruit size. The effect on growth variables was observed immediately after anthesis. The differences between untreated and treated trees increased as the fruit development stage advanced. At time of harvest, differences in diameter were of 5 mm in fruit of a single-flowered leafy inflorescences and 4 mm in multiple-flowered leafy inflorescences (Fig. 1a). There was a higher effect on fruit from single-flowered leafy inflorescences than from multiple-flowered leafy inflorescences. Increased fresh weight (FW) was low throughout early fruit growth until 43 days after anthesis (DAA). From this day, a lineal increase was observed until 240 DAA, along with increased diameter. At the time of harvest, the differences in FW were of 35.5 g in fruit from single-flowered leafy inflorescences and 24 g in multiple-flowered leafy inflorescences, being higher in fruit treated with 2,4-D (Fig. 1b). Like the fresh fruit weight, dry matter accumulation was low during the early fruit growth period until 43 DAA. Differences during this time were kept close to zero. In fruit from single-flowered leafy inflorescences there was a first period of slow accumulation of dry matter between 53 and 133 DAA. In fruit from multiple-flowered leafy inflorescences, this period was extended to 148 DAA. From this day, there was an increase, stabilized at 198 DAA in the first inflorescence type (single-flowered leafy) and 40 DAA later in the other inflorescence type (Fig. 1c).

Effect of 2,4-D application on sugar content

Application of 2,4-D increased total sugars (TS) levels on the pericarp, remaining constant from anthesis to 28 DAA with 0.3% of the FW. A slow increase in TS content was obtained from 43 DAA with the same trend on both 2,4-D-treated and untreated trees with the highest levels in 2,4-D-treated trees. At 150 DAA, the TS concentration in the pericarp was 1.3% of FW in 2,4-D-treated and 0.8% in untreated trees (Fig. 2A). Sucrose content in the pericarp showed a similar variation pattern on both 2,4-D-treated and untreated trees. Decreased sucrose concentration was observed after anthesis, but then a slow recovery was presented until 76 DAA – representing 0.4% of the FW. Sucrose content showed reduction at 93 DAA, being 0.13% higher in 2,4-D-treated compared to untreated trees (Fig. 2B).

The 2,4-D application increased reducing sugar (RS) content in the pericarp without modifying the variation pattern and showed the lowest differences for the treatments assayed

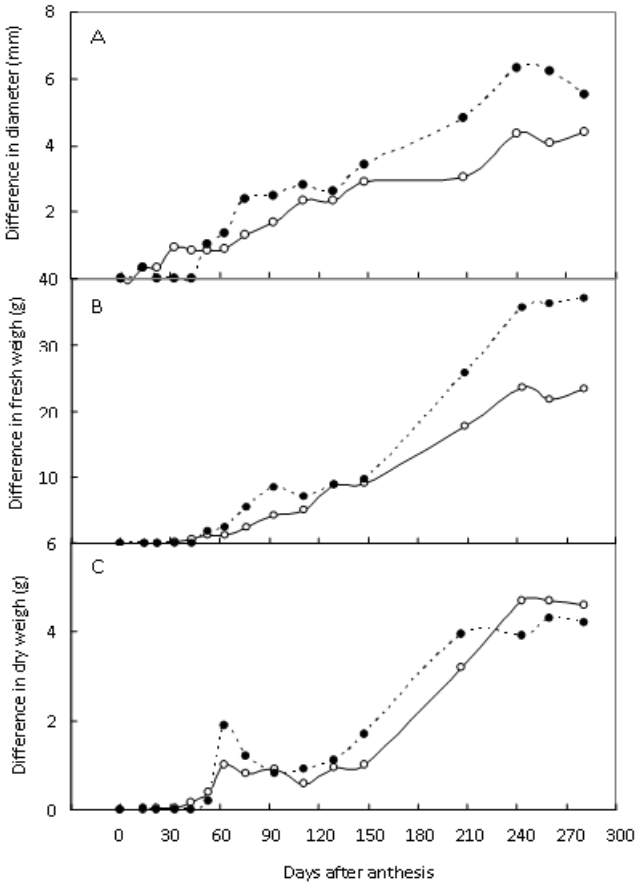

Fig 1. Difference in diameter (A), fresh (B) and dry (C) weight between 2,4-D-treated and untreated 'Salustiana' trees in single- (- - -●- - -) and multiple- (—○—) flowered leafy inflorescence

between 43 and 110 DAA (0.1% in absolute terms), increasing at 150 DAA with 0.4% of the FW (Fig. 2C). TS content in the vesicles was higher on 2,4-D-treated fruits. At the beginning of the study, a 0.3% difference was found between treatments; higher in 2,4-D-treated fruits (0.6% of the FW). Differences increased with progress in the development stage until reaching 0.5% (Fig. 2D). Sucrose content was also higher in the 2,4-D treatment. At 110 DAA, the difference reached 0.35% and then remained unchanged until the end of the study period (Fig. 2E). The RS content was higher in 2,4-D-treated fruits, representing 1.5% of the FW with 0.5% difference in relation to untreated fruits (Fig. 2F). Fructose content in 2,4-D-treated fruit was higher than in untreated fruits. At 150 DAA, differences of 0.2% were noted between treatments (Fig. 3A). Until 43 DAA, low glucose content was found in the pericarp and then the glucose content was higher in the 2,4-D-treated fruits than in untreated fruits; at 150 DAA, glucose content reached 0.6% in the former and 0.4% in the latter (Fig. 3B). Fructose and glucose content showed similar behavior until 110 DAA. At the beginning of the study period, the fructose content on untreated fruits was undetectable, while in 2,4-D-treated fruits 0.1% of the FW was obtained. At 150 DAA, fructose content was higher in 2,4-D-treated tress than in untreated trees, representing 0.6% of the FW and being 0.2% higher (Fig. 3C). Glucose was found in 2,4-D-treated trees at 55

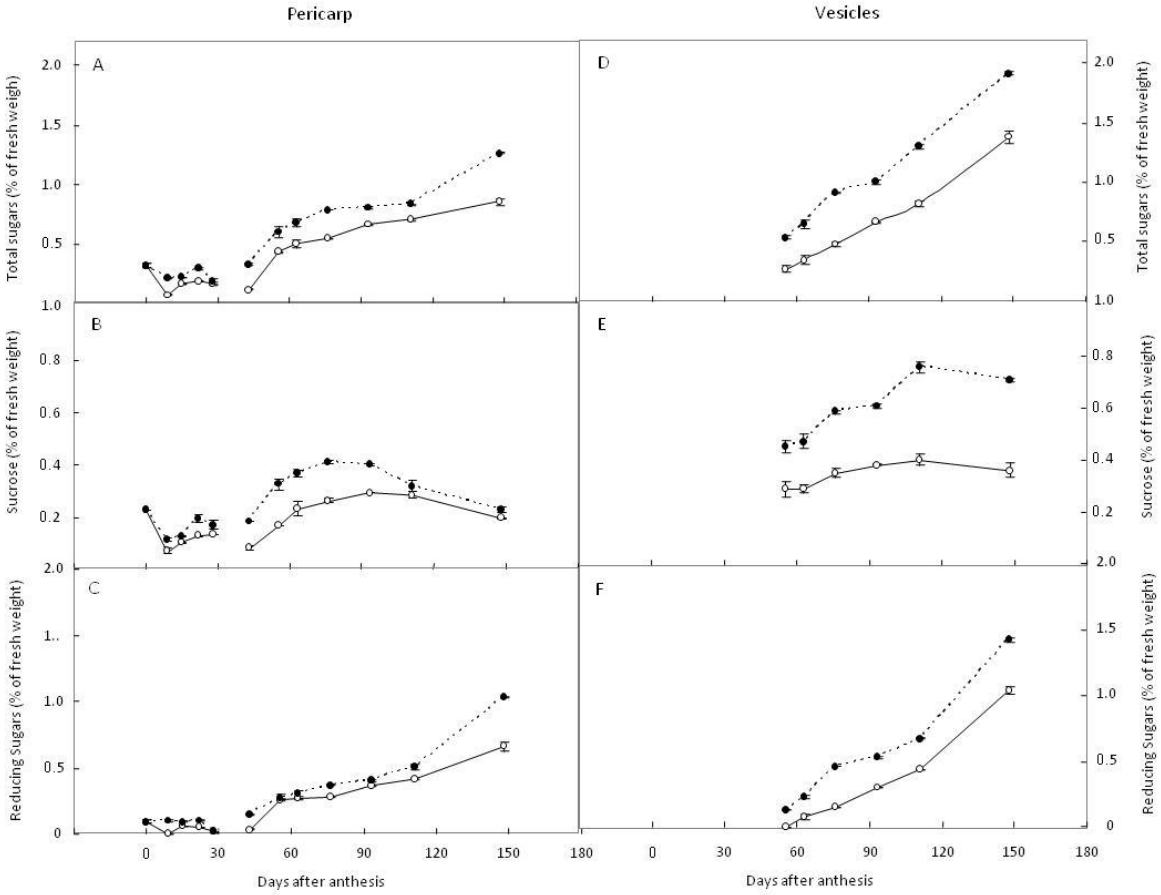

Fig 2. Changes in total sugar (A, D), sucrose (B, E), and reducing sugar (C, F) concentration on fruit pericarp and vesicles of multiple-flowered leafy inflorescence treated with 2,4-D (··●··) and untreated (—○—). Bars represent standard error of two independent samples

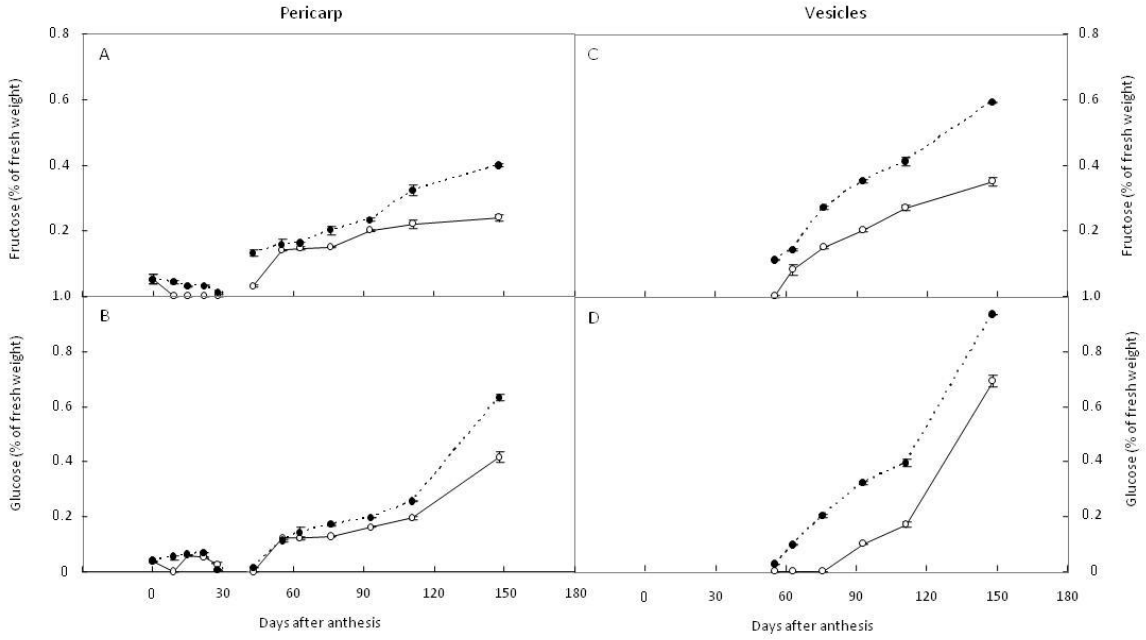

Fig 3. Changes in fructose (A, B) and glucose (C, D) concentrations infruit pericarp and vesicles of multiple-flowered leafy inflorescence treated with 2,4-D (··●··) and untreated (—○—). Bars represent standard error of two independent samples.

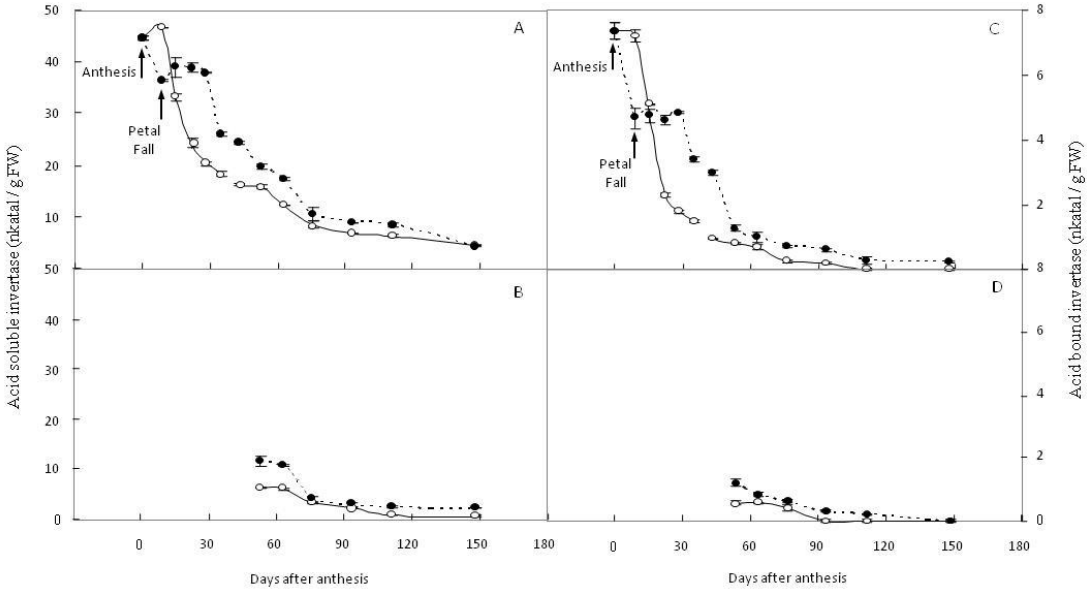

Fig 4. Acid soluble and bound invertase activity on pericarp (A, C) and vesicles (B, D) of multiple-flowered leafy inflorescence treated with 2,4-D (- - ● - -) and untreated (—○—). Bars represent standard error of two independent samples.

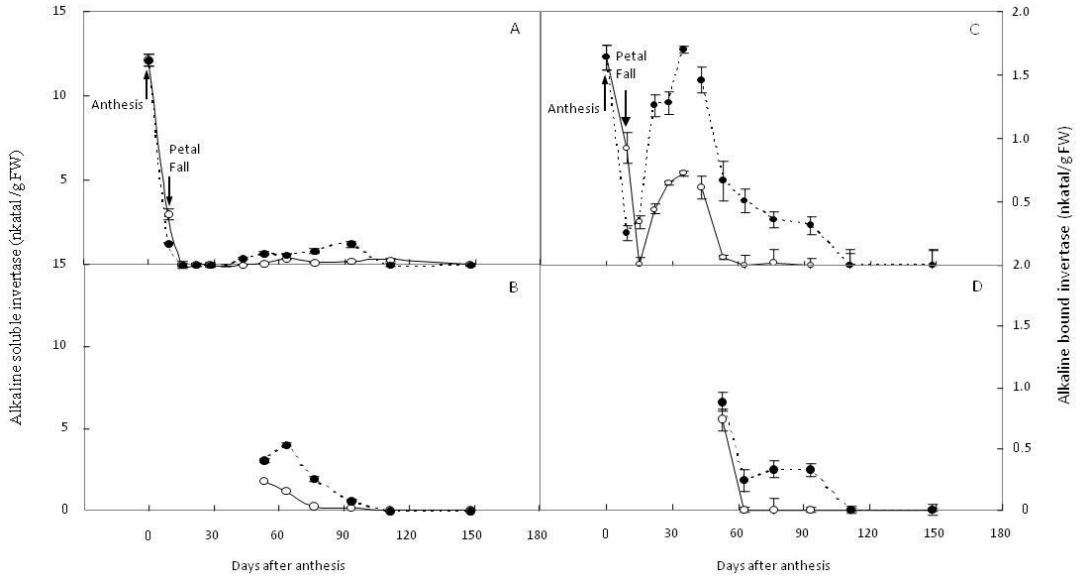

Fig 5. Alkaline soluble and bound invertase activity on pericarp (A, C) and vesicles (B, D) of multiple-flowered leafy inflorescence treated with 2,4-D (- - ● - -) and untreated (—○—). Bars represent standard error of two independent samples.

DAA, while in untreated trees it was found at 93 DAA. Glucose represented 0.9% of the FW at 150 DAA (Fig. 3D).

Effect of 2,4-D application on invertase activity

The pericarp of 2,4-D-treated fruits showed reduction of the acid soluble invertase after anthesis with constant activity from 9 to 28 DAA; the invertase activity was higher on treated trees between 14 and 120 DAA. Both 2,4-D-treated and untreated trees showed faster reduction until 76 DAA. At 150 DAA, the fruits in both treatments showed similar invertase activity (Fig. 4A). This isoform had higher activity on vesicles of 2,4-D-treated trees throughout the study period. From 63 DAA, the activity was low in both treatments, being 2.2 nkatal/g FW in 2,4-D-treated and 0.6 nkatal/g FW in untreated trees (Fig. 4B). The effect of 2,4-D application on the acid-bound invertase showed the same trend as acid-soluble invertase, but in anthesis the activity was lower (7 nkatal/g FW FW) (Fig. 4C). Acid-bound invertase showed low activity in vesicles. At 53 DAA, fruits of 2,4-D-treated trees reached 1.2 nkatal/g FW. Activity decreased slowly until becoming undetectable at 150 DAA (Fig. 4D). At the beginning of the fruit growth period, no difference was perceived on the activity of alkaline-soluble

invertase in the pericarp between untreated and 2,4-D-treated fruits. In both cases, the activity diminished abruptly after anthesis and was undetectable at 15 DAA. From 28 DAA, the activity of this isoform was slightly higher in the 2,4-D treatment, but lower than 1 nkatal/g FW (5A). Vesicles showed high activity with 6.4 nkatal/g FW on 2,4-D-treated fruits, undetectable at 110 DAA (Fig. 5B). Alkaline-bound invertase showed low values on the pericarp; activity lower than 2 nkatal/g FW. After anthesis, activity decreases and was slower on untreated fruits than on 2,4-D-treated trees (Fig. 5C). In both untreated and 2,4-D-treated trees, the alkaline-bound invertase showed minor activity of 1 nkatal/g FW on vesicles. From 53 to 93 DAA, the activity was higher on 2,4-D-treated trees and then it was undetectable in both treatments (Fig. 5D).

Discussion

Plant hormones play an integral role in controlling plant growth, differentiation, and development regulating fruit sink strength (Guardiola and García Luís, 2000; Iglesias et al., 2007; Ruan et al., 2012; Pattison et al., 2014), carbohydrate partition (Brenner and Cheikh, 1995), and phloem unloading (Tanner, 1980; Iglesias et al., 2007; Ruan et al., 2012). Also, evidence was available on the regulatory role of several ph ytohormones on the extracellular invertase activity, as well as increased carbohydrate requirement by the fruit (Roitsch et al., 2003). Increased fruit sink strength due to auxin application led to faster growth until ripening (Guardiola and Lázaro, 1987; Agustí et al., 1994; Chao and Lovatt, 2010; Rebolledo et al., 2012). In this study, applications of 2,4-D increased fruit size in sweet orange Salustiana cultivars. This effect was evident immediately after anthesis and at time of harvest there was a 4.5-mm difference in fruit size. This behavior was previously reported in Clementine Esbal mandarin with the same 2,4-D concentrations used in this study (17 and 20 mgL^{-1}) (Duarte and Guardiola., 1996). Applying 15 mg l^{-1} 3,5,6-TPA to Clementine mandarin trees during the fruit cell division stage significantly increased fruitlet abscission and reduced fruitlet growth rate through reduced photosynthate accumulation in the fruit (Mesejo et al., 2012). Most studies have focused on the characterization of the response of several mandarin cultivars to hormonal treatments before June drop (Guardiola and Lázaro, 1987; Ortolá et al., 1991; Agustí et al., 1994; Georgiu, 1998; Roussos and Tassis, 2011; Yıldırım et al., 2012). In Valencia orange cultivars and Shamouti, application of auxin in flowering was not effective (Erner et al., 1993). The 2,4-D application, herein, increased sugar levels and affected the activity of the invertase isoforms. There was an increase in sucrose and RS during the fruit's early development stage, as well as on the acid invertase activity. This behavior suggests a direct effect of 2,4-D on increased fruit sink strength. It seems that auxin plays a key role in the regulating mechanism of the extracellular invertase activity (Glasziou, 1969; Weil and Rauch, 1990; Roitsch et al., 2003; Ruan et al., 2012). The function of invertases has been deduced from a correlation between activity and development of physiological processes like tissue growth or sugar accumulation in sink organs (Lowell et al., 1989; Ruan et al., 2010; Ruan 2012; Jiang et al., 2014). Tissue with faster growth and low sucrose content have shown high activity of acid invertase (Ricardo and Ap Rees, 1970; Lowell et al., 1989; Ruan et al., 2010; Dai et al., 2011; Jiang et al., 2014). Thus, the role of the acid invertase on plant tissue was to hydrolyze sucrose under conditions of high demand for hexoses. Sucrose hydrolysis has been explained in Marsh grapefruit (Lowell et al., 1989) and Satsuma mandarin (Kato and Kubota, 1978; Kubo et al., 2001) by the action of the acid invertase in the post-phloem path. This activity was present only during early development, whereas, the enzyme is inactivated with the advancement of the fruit's development stage. Moreover, some authors indicate that acid-soluble invertase activity with the production of hexoses results in the formation of a sucrose concentration gradient, which keeps constant transport of sugars into the fruit (Echeverria et al., 1997; Jiang et al., 2014). Application of 2,4-D increased sugar content in vesicles and increased the activity of soluble alkaline invertase, which rapidly decreased to undetectable levels by day 110. In the treated fruits, sucrose represented 45% of total sugars (TS). Increased fruit size due to auxin treatment is associated with increased absolute juice content (El-Otmani et al., 1993). In Satsuma mandarin, cv. 'Okitsu', sucrose was accumulated continuously during the cell enlargement period, but 3,5,6-TPA only increased its concentration temporarily because differences between control and treated fruits were significant only from 113 to 141 DAA (Agusti et al., 2002). In citrus trees, photoassimilates are transported in the form of sucrose (Koch and Avigne, 1990; Koch, 2004), but glucose and fructose are also found in various concentrations in ripe fruit vesicles. Lowell et al., (1989) indicated high activity of alkaline invertases in the vesicles during the fruit's second development stage. Furthermore, they proposed a joint degradation activity with sucrose synthase. On the other hand, Jiang et al. (2014) found high activity of neural invertase in vesicles of Ponkan tangerine, reducing during the advance of the fruit's development stage.

Materials and Methods

Plant material and treatments

The study was carried out in Valencia (Spain) and conducted on 20-year-old Salustiana sweet orange (Citrus sinensis (L.) Osbeck) trees grafted on rootstock citrange Troyer (Citrus sinensis (L.) Osbeck x Poncirus trifoliata (L.) Raf.). In the orchard during the same year, trees with different flowering intensities were often present. Trees with high flowering intensity were selected. The 2,4-D application (Viriman, 10% (p/v) solution of isopropyl ester) was carried out on 11 trees and the same number was selected as untreated trees. The 2,4-D was applied once the trees showed 50% of flowers in anthesis on single- and multi-flowered leafy inflorescences. The concentration was 20 mgL^{-1} and 3.6 L/tree. The application was made on the entire foliar surface of the tree. A non-ionic wetting agent, alkyl polyglycol ether, at a final concentration of 20% (p/v) was added to all solutions.

Fruit growth variables

Fruit growth studies were conducted on single- and multiple-flowered leafy inflorescences. One hundred inflorescences from each type were tagged. The multiple-flowered leafy inflorescences had five leaves and flowers, respectively. It was taken into consideration that the final flower bud from each inflorescence was about to open. The fruit variables were fresh and dry weight, respectively, and diameter. Measurements were made every 10 days, when data were recorded. Fruit diameter was determined by measuring the equatorial region of the apical fruit in each inflorescence. Twenty fruits were collected to determine FW and DW and take into reference the fruit diameter of the tagged inflorescences.

Carbohydrate analysis

Carbohydrates were quantified via HPLC method on whole fruit and separated tissue (vesicles and pericarp). Samples were collected every 10 days, when data were recorded. Fruit was dried to constant weight at 70 °C for 72 h. Ground dried tissue (0.4 g) was added to 15 mL 80% ethanol and incubated in a water bath at 80 °C for 10 min to extract the soluble sugars. The extract was centrifuged for 10 min at 7500 rpm, the ethanol was evaporated, and the tissues were re-extracted three more times as above. The extract was centrifuged at 15,000 rpm for 15 min and then passed through a C_{18} Sep-Pak cartridge balanced with 10 mL of methanol and 10 mL of 0.1 M acetic acid. The extract obtained was filtered through Millipore Millex-HV hydrophilic PVDF 0.45-µm filter.

Sugars were analyzed with a Perkin-Elmer HPLC System, detected by using a 410 Refractive Index Detector (Waters Associates), and quantified by peak area comparison using standard curves for sucrose, glucose, and fructose. Results were calculated by using the chromatographic Millennium 32 processing software (Water Associates). Sugars were eluted through Tracer Carbohidrat Column (25 x 0.46 cm) of 5-µ particle size packed with Tekcnokcroma pre-column using water as the mobile phase at a flow rate of 1.5 mL min^{-1}.

Enzyme Extraction and Assays

Invertase activity was evaluated on whole fruit and separated tissue (vesicles and pericarp) of 2,4-D-treated and untreated trees. Samples were collected every 10 days, when data were recorded. Enzymes were extracted from 1 g of fresh frozen tissue by grinding in a chilled mortar with quartz sand and 10 mM citrate-phosphate tampon (pH 7.5: extraction tampon). The homogenate was washed three times with 10 mL of extraction tampon and then filtered through 4 layers of gauze. Each filtrate was centrifuged at 12,000 rpm for 20 min. The supernatant was gauged in a 50 mL volumetric flask with extraction tampon and used to determine soluble invertase activity. The precipitate was resuspended with 50 mL of extraction tampon to determine insoluble invertase (bound to cell-wall components). The complete process was carried out at 4 ºC to ensure the enzymes remain inactive. One mL of each extract and 1 mL of 0.2 M sucrose were poured into the following tampons for incubation:

1.50 mM Citrate – phosphate, pH 4.5: to determine acid invertases

2.10 mM Citrate – phosphate, pH 7.5: to determine alkaline invertases

Because of acid invertase activity at this pH, a third incubation was necessary to define the alkaline invertase activity: 3. 10 mM TRIS, pH 7.2: inhibits 100% the alkaline invertase activity without affecting the acid invertase (Kato and Kubota, 1978). Alkaline invertase activity was obtained for subtraction with the value obtained at point 2. The samples and targets were incubated in a double boiler at 30 ºC for 30 min. The sugar released from sucrose hydrolysis was determined with the Somogy-Nelson method (Nelson, 1944). Once the incubation process ended, 2 mL of Somogy reagent was poured into the samples and targets to stop the enzymatic reaction. All samples and targets were agitated and then boiled in a double boiler for 15 min. The samples were cooled and then poured into 2 mL of Nelson reagent plus 4 mL of distilled water. After 10 min the samples were filtered through Whatman filter paper Nº 2, agitating and reading at optic density of 500 nM. The standard curve considers 1 mL of extraction tampon, three dissolutions of glucose between 0 and 20 ppm, and 1 mL of each tampon. The standard curve was processed in parallel with the samples processed after adding the Somogy reagent. The results were expressed in nkatal/g FW.

Statistical analyses

Differences among treatments and control were compared by using one-way ANOVA and means were separated by Duncan´s test at 95% confidence limit. All statistical evaluations were performed by using IBM SPSS Statistics 19 for Windows (SPSS Inc., an IBM Company, Chicago, IL, USA).

Conclusions

High metabolic activity during early fruit development is related to increased consumption of carbohydrates and, in fact, to low sugar concentration values detected in this phase. Sugar accumulation occurs with the cell expansion onset in the vesicles. Distribution of sugars in fruit tissues is related to invertase activity. The acid invertase isoforms are more involved during early fruit growth, being higher at anthesis and decreasing with the advance of the fruit development stage. Sugar accumulation during cell expansion of the vesicles is related to an increase of the alkaline invertase isoform, which later decreases rapidly. The 2,4-D application increased fruit size immediately after anthesis with a final 4.5-mm difference at harvest in relation to untreated fruits. Increased fruit size was accompanied by increased sugar levels and influenced by the different invertase isoforms. The increase of acid invertase activity suggests a positive direct effect of 2,4-D on fruit sink strength during early fruit development. Participation of alkaline invertase in the sugar accumulation process that takes place in the vesicles is herein verified.

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