Journal of Plant Physiology 174 (2015) 124–130

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Biochemistry

Different *Citrus* rootstocks present high dissimilarities in their antioxidant activity and vitamins content according to the ripening stage

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ARTICLE INFO

Article history:
Received 19 October 2014
Received in revised form 29 October 2014
Accepted 29 October 2014
Available online 6 November 2014

Keywords:
Antioxidant activity
Ascorbic acid
α-Tocopherol
Citrus sinensis
Ripening stage rootstock

ABSTRACT

"Lane Late" sweet orange grafted on six different citrus rootstocks and grown in the Guadalquivir valley (Seville, Spain) were picked at different ripening stages in two consecutive seasons to characterize their antioxidant activity (free radicals scavenging activity, reducing power and lipid peroxidation inhibition) and quantify their main antioxidant compounds (vitamin E and vitamin C). Linear discriminant analysis and 2-way ANOVA were applied to compare the effects induced by citrus rootstock and ripening stage. The results showed that differences in antioxidant activity and related compounds are mainly dependent on the citrus rootstock, despite ripening stage had also some particular effects. Changes observed in 2012 showed less marked differences among the citrus rootstock. Nevertheless, Cleopatra rootstock showed the highest antioxidant activity in both years, indicating that an increase in its cultivation might be a good solution to sweet orange farmers. Concerning the ripening stage, samples collected in January presented higher vitamin contents, while those collected in April showed higher antioxidant activity. This result allows deciding the harvesting period according to the desired effect.

Introduction

Citrus cultivation is the second most important crop in Andalusia (Spain). Sweet orange is the most important product, representing more than 70% of the total Andalusian citrus production surface. Moreover, Seville is the Spanish region with the highest production of sweet orange, contributing with more than 40% of the total production (Consejeria de Agricultura, 2010).

However, the sweet orange production in this region has been directed on a single pattern, "Carrizo" citrange (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.) without taking into account important aspects such as the diversity in agroclimate conditions at Andalusia, the high concentration of supply in the market and the increasing concern about the fruit quality and a healthier lifestyle (Dauchet et al., 2008).

The introduction of new citrus rootstocks in sweet orange production is a good choice to adapt the crop to different requirements. But the kind of pattern used can influence the chemical composition and the antioxidant activity of the fruit (Sanchez-Rodriguez et al., 2012). On the other hand, the ripening stage might also exert important effects over the chemical composition and the antioxidant activity (Nuncio-Jauregui et al., 2014; Zhang et al., 2010).

With regard to chemical composition, orange is typically considered as one of the products with high contents of antioxidants and vitamins (Del Caro et al., 2004; Dhuique-Mayer et al., 2005). It is well known that the intake of antioxidant compounds is associated with the prevention of various human oxidative stress-related diseases such as neurodegenerative and cardiovascular diseases, stroke, cataractogenesis, diabetes and cancer (Halliwell, 2011; Mateos et al., 2005; Schreckinger et al., 2010). The principal reason is because the oxidative stress-related diseases are produced when the normal balance between the production of free radicals and their neutralization by antioxidant defences tends to the overproduction of free radicals in the organism. In particular, the antioxidants present in orange such as tocopherols

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http://dx.doi.org/10.1016/j.jplph.2014.10.013
0176-1617/

(vitamin E) and ascorbic acid (vitamin C) are believed to prevent us against degenerative malfunctions due to their role as scavengers of overproduced free radicals. Moreover, the intake of ascorbic acid (vitamin C) is critical because of the human inability to synthesize it.

In this context, the present work evaluates the quality of "Lane Late" sweet orange grafted on six different citrus rootstocks and grown in the Guadalquivir valley (Seville) according to the ripening stage (harvested at the beginning and end of the season) in two consecutive seasons (2011 and 2012). Besides characterizing the antioxidant activity in these different conditions, the main antioxidant compounds (which represent the most valued attributes in this fruit) were also quantified.

Materials and methods

Samples

Seven-years-old trees of Lane Late variety were grafted on three new citrus rootstocks, Forner-Alcaide n°5 (FA5), Forner-Alcaide n°13 (FA13) and Forner-Alcaide n°41 (FA41) (hybrids of *Cleopatra mandarin* × *Poncirus trifoliata*), and three traditional rootstocks, Carrizo citrange (CA) (*Citrus sinensis* × *P. trifoliata*), *Citrus macrophylla* (MP) and *C. mandarin* (*Citrus reshni*) (CL) to evaluate the effect of the citrus rootstocks on bioactive compounds and antioxidant activity. Moreover, samples were harvested in different ripening stages, at the beginning and end of the season (January and April) during two consecutive years (2011 and 2012). The production yield of each tree presented similar values (≈5 kg/m^3) for all rootstocks studied. After being collected and subsequently separation of the peel (eight by treatment and replication), the pulp was lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh), mixed to obtain homogenous samples and stored in a desiccator, protected from light, until further analysis.

All samples reached the minimum requirements for quality standards imposed by regulation (color index >6; maturity index >6.5; equatorial diameter >53 mm and over 33% of juice) (Regulation (CE) N°1221/2008. European Commission, 5th December, Official Journal of the European Union, 1–80).

Standards and reagents

Ethyl acetate 99.8% and n-hexane 95% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), α-tocopherol and ascorbic acid standards were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Methanol and all other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Antioxidant activity evaluation

Each lyophilized sample (0.5 g) was extracted by stirring with 20 mL of methanol/water (80:20, v:v) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 20 mL of methanol/water (80:20, v:v) for 1 h. The combined hydromethanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness and re-dissolved in methanol/water (80:20, v:v) for antioxidant activity assays (40 mg/mL). Successive dilutions were made from the stock solution and submitted to the *in vitro* assays already described by Martins et al. (2014), to evaluate the antioxidant activity of the samples. The sample concentrations (mg/mL) providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (ferricyanide/Prussian blue assay) against sample concentrations (Pinela et al., 2012). Trolox was used as a positive control.

Reducing power by ferricyanide/Prussian blue assay

The extract solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, the same with deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in ELX800Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).

DPPH radical-scavenging activity assay

This methodology was performed using the Microplate Reader mentioned above. The reaction mixture on 96 well plate consisted of a solution by the well of the extract solutions with different concentrations (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

Inhibition of β-carotene bleaching or β-carotene/linoleate assay

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing extract solutions with different concentrations (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (absorbance after 2 h of assay/initial absorbance) × 100.

Thiobarbituric acid reactive substances (TBARS) assay

Porcine brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000 × *g* for 10 min. An aliquot (100 μL) of the supernatant was incubated with the different concentrations of the sample solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 μL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000 × *g* for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = $[(A - B)/A] \times 100\%$, where *A* and *B* were the absorbance of the control and the sample solution, respectively.