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ORIGINAL ARTICLES

Hormonal and Nutritional Changes in the Flavedo Regulating Rind Color Development in Sweet Orange [*Citrus sinensis* (L.) Osb.]

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Abstract The objective of this research was to determine the changes in the levels of endogenous gibberellins GA_1 and GA_4, abscisic acid (ABA), and ethylene during fruit coloring of on-tree fruits of sweet orange. The time course of carbohydrates and nitrogen content in the flavedo prior to fruit color break and during peel ripening were also studied. To identify nutritional and hormonal changes in the fruit, 45 days before fruit color break the peduncles of 15–30 fruits per tree of 'Washington' navel, 'Navelate,' and 'Valencia Delta Seedless' sweet orange, located in single-fruited shoots, were girdled to intercept phloem transport. A set of 15–30 fruits per tree remained intact on the peduncle for control. Girdling significantly delayed fruit coloration for more than 2 months; the delay paralleled higher GA_1 and GA_4 concentrations in the flavedo and retarded the rise of ABA concentration prior to color break. Girdling also reduced carbohydrate concentrations and increased N concentrations in the flavedo compared to control fruits; no ethylene production was detected. Therefore, in sweet orange, fruit changes color by reducing active gibberellin concentrations in the flavedo, which are involved in regulating sugars and ABA accumulation and in reducing N fraction concentration as rind color develops. This was demonstrated in vivo without removing the fruit from the tree. Comparable results were obtained with experiments carried out over four consecutive years in two countries (Spain and Uruguay).

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Introduction

Citrus fruit is classified as nonclimacteric fruit (Aharoni 1968; Eaks 1970). However, exogenous ethylene stimulates changes in fruit color (Pons and others 1992) by increasing chlorophyllase *de novo* synthesis (Trebitsh and others 1993; Fujii and others 2007) and enhancing carotenoid biosynthesis pathway genes (Rodrigo and others 2006; Fujii and others 2007; Rodrigo and Zacarias 2007). Moreover, the ethylene antagonist 1-MCP (Serek and others 1995) inhibits chlorophyll loss from green harvested orange fruit (Porat and others 1999).

Fruit color development is under the regulation of various factors, including plant hormones (El-Otmani and others 1995). GA-like activity has been detected up to the onset of chlorophyll loss (García-Luis and others 1985), and the lowest GA-like activity is reached at ripening (Kuraoka and others 1977), but no information about endogenous GA concentration in relation to rind coloration has been reported. Nevertheless, GA_1 and GA_4 have been reported as biologically active gibberellins in developing fruits of *Citrus* (El-Otmani and others 1995), especially in seedless cultivars of sweet orange (Talón and others 1990). Besides that, exogenous gibberellic acid (GA_3) applied prior to color break postpones color development by delaying chlorophyll degradation, reducing carotenoid concentration, and modifying carotenoid composition (Lewis and Coggins 1964; García-Luis and others 1985, 1986).

During ripening, *Citrus* fruit peels accumulate large amounts of colored oxygenated carotenoids (Aung and others 1991; Rodrigo and others 2003; Agustí and others

2007), and transcripts of the CsNCED enzyme, which controls ABA biosynthesis, are expressed at much higher levels in colored than in green fruit (Rodrigo and others 2006; Agustí and others 2007).

In *Citrus*, total sugar concentrations in the flavedo and peel color are positively related, both in vivo and in vitro (Huff 1983, 1984; Holland and others 1999; Iglesias and others 2001; Fidelibus and others 2008). Furthermore, sucrose supplementation to the tree accelerates fruit coloration (Iglesias and others 2001). In in vitro experiments, there is not a significant relationship between fruit color and endogenous nitrogen compounds such as proteins (Lewis and others 1967) or amino acids (Huff 1984). In in vivo experiments, however, nitrate supplementation to the tree delays fruit color break (Alós and others 2006). Besides that, nitrogen compounds added to GA_3 reinforced the effect of GA_3 by delaying peel coloration when applied prior to fruit color break (Agustí and others 1988). Hence, the mechanisms regulating citrus fruit peel ripening remain largely unclear.

To determine nutritional and hormonal changes in the fruit, 1.5 months before fruit color break, peduncles of fruits located in single-fruited shoots were girdled to intercept phloem transport and thus reduce photoassimilate content; concurrently, girdling might intercept gibberellin flows out of fruit, and all together delay fruit coloration. It is important to note that the experiments were carried out in Spain and Uruguay, two countries far away from each other and located in different hemispheres.

The objective of this research was to determine the changes in the levels of endogenous GA_1 and GA_4 biologically active gibberellins, ABA, and ethylene during fruit coloring of 'Washington' navel, 'Navelate,' and 'Valencia Delta Seedless' sweet orange. In addition, the time course of carbohydrate and nitrogen concentrations in the flavedo, total and proteinaceous nitrogen, ammonia nitrogen ($N–NH_4^+$), and nitrate–nitrite nitrogen ($N–NO_2^- + N–NO_3^-$) fractions, prior to fruit color break and during peel ripening, was also studied.

Materials and Methods

Plant Material and Experimental Layout

Experiments were carried out over four consecutive years (2005–2008) in commercial orchards of 'Washington' navel, 'Navelate,' and 'Valencia Delta Seedless' sweet orange [*Citrus sinensis* (L.) Osb.] located at Montevideo (Uruguay), and at Huelva and Valencia (Spain). The trees were 20–25 years old and had been budded onto *Poncirus trifoliata* (L.) Raf. ('Washington' navel) in Uruguay, and onto Carrizo citrange rootstock (*P. trifoliate* × *C. sinensis*) ('Navelate' and 'Valencia Delta Seedless') in Spain. The trees were planted 6 m × 4–5 m apart and grown in a loamy–silty clay soil, pH 6 (Uruguay), loamy-sand soil, pH 6.3 (Huelva, Spain) and loamy-clay soil, pH 8.0 (Valencia, Spain), with drip irrigation. Fertilization, pruning, and pest management were in accordance with optimum commercial practice. A randomized complete block design with single-tree plots and three to four replications was used in the experiments.

Stems of 15–30 single-fruited leafy shoots per tree, randomly selected, were tagged and girdled approximately 45 days before fruit color break to intercept phloem transport. Girdling was performed by removing a 2-mm ring of bark from the peduncle 1.0 cm away from the calyx. Fruits from shoots that were not girdled served as controls.

Samplings and Measurements

Fruits, shoots, and leaves were sampled at the girdling date and at 60 days (2006) and 30 and 45 days (2008) after girdling. Flavedo of ten fruits of 'Washington' navel and 'Valencia Delta Seedless' per treatment and replicate was removed with a scalpel, then frozen immediately with liquid N_2 and stored at −80°C until abscisic acid (ABA), gibberellins (GA_1 and GA_4), carbohydrate (CHO), and nitrogen analyses were performed. A portion of bark close to the calyx of controls and proximal and distal to fruit of girdled stems was sampled, frozen, and stored at −80°C to analyze GA_1 and GA_4 ('Washington' navel, Uruguay) and ABA and CHO ('Washington' navel, Uruguay, and 'Valencia Delta Seedless', Valencia, Spain). Leaves from control and stem-girdled shoots were sampled, frozen, and stored at −80°C to analyze CHO and N fractions. All tissues were lyophilized and then ground to a fine powder before analysis. At the onset of rind color break of control fruits, five fruits per treatment and replicate were sampled to measure ethylene production.

The level of peel color development of all tagged fruit was determined using a Minolta Chromameter CR-300 (Tokyo, Japan) by taking three measurements per fruit in the equatorial zone of the fruit. Measurements were taken every 7 days from girdling date to rind color break. The results are given as *a* and *a/b* ratio of Hunter coordinates. Color readings of *a* denote green when negative and red when positive, and color readings of *b* denote blue when negative (nonexistent for citrus) and yellow when positive. Thus, the *a/b* ratio indicates greenness when negative and redness when positive.

For ethylene production, five fruits of 'Washington' navel (Uruguay) and 'Valencia Delta Seedless' (Valencia, Spain) were incubated in 1.7-L jars. After 4 h of incubation at 20°C, an air sample (2 ml) from the jar headspace was

withdrawn with a hypodermic syringe and injected into a gas chromatograph equipped with a flame ionization detector and a Porapak Q column (2 m in length and 2 mm internal diameter). Temperatures for the injector, the column, and the detector were 130, 80, and 110°C, respectively. Nitrogen at a 45-ml min^{-1} flow rate was used as the carrier gas. Results are expressed as μg g^{-1} FW (fresh weight).

Quantification of ABA was performed by indirect enzyme-linked immunosorbent assay as reported by Zacarías and others (1995) and revised by Lafuente and others (1997). The samples (200 mg) were extracted overnight at 4°C with acetone 80% containing citric acid (0.5 g l^{-1}) and butylated hydroxytoluene (BHT; 100 ml l^{-1}). The extracts were centrifuged and 5 ml of the supernatant was used for ELISA assay following the procedure proposed by Walker-Simmons (1987). Four replicates per sample were incubated with 500 μl of monoclonal antibody (MAb) and 480 μl of Tris-buffered saline (TBS, pH 7.8) (one tablet and 0.2 g $Cl_2Mg{\cdot}6H_2O$ dissolved in 15 ml double-distilled water) at 4°C overnight. Plate wells were incubated at 4°C overnight with 200 μl of ABA-4′ bovine serum albumin (BSA) conjugate, prepared according to Weiler (1979). Wells were washed three times with 200 μl TBS–Tween (1 L TBS with 0.5 ml Tween-20) and 0.2 g BSA. Aliquots (200 μl) of sample incubated with MAb were pipetted into the wells and then plates were kept for 2 h at room temperature. After washing three times with 200 μl of TBS–Tween, each well was filled with 200 μl of the rabbit antimouse alkaline phosphatase conjugate (20 ml TBS containing 20 μl IgG). Plates were incubated at room temperature for 2 h. Wells were washed three times with TBS–Tween, and then 200 μl nitrophenyl phosphate solution (20 ml 0.05 M $NaHCO_3$ containing 20 mg 4-nitrophenyl phosphate disodium salt hexahydrate) was added to each well. Plates were incubated for around 30 min in a forced-draft oven (35°C) until the absorbance at 405 nm of the control sample containing no ABA was approximately 1.0. Replicate ABA standards (ranging from 15 to 250 pg 100 μl^{-1} TBS) were assayed and a linear regression analysis was computed. The amount of ABA in extract samples was calculated based on the coefficient of the ABA standard curve for each plate. (+)-ABA standards, TBS, BSA, antimouse IgG, and 4′-nitrophenyl phosphate disodium salt hexahydrate were purchased from Sigma-Aldrich (Madrid, Spain). MAb was obtained from Idetek, Inc. (San Bruno, CA, USA). BHT, citric acid, acetone, Tween-20, and $NaHCO_3$ were purchased from Scharlab (Barcelona, Spain). Immunoplates (F96 MaxiSorp™) were obtained from Nunc A/S (Roskilde, Denmark) and showed a better ABA-4′-BSA conjugate binding than other plates tested. Results are expressed as μg g^{-1} DW (dry weight).

Gibberellins GA_1 and GA_4 were determined by liquid chromatography and mass spectrometry (LC–MS/MS) as reported by Chiwocha and others (2003), with slight modifications. Extraction with 5 ml of MeOH:H_2O:HOAc (80:19:1, pH 1–3) from 200 mg of lyophilized sample was performed. Deuterium standards ($^2H_2$–GA_1 and $^2H_2$–GA_4) (100 ng) were added, and the mixture was shaken for 12 h at 4°C. Samples were centrifuged at 290 g for 10 min, and the supernatant was removed from the pellet to evaporate the aqueous phase. Each sample was partitioned two times with ethyl acetate ($C_4H_8O_2$), acidified with HOAc 1%, and then it was evaporated to dryness. After resuspending the dry portion in methanol, purification with a C_{18} column (Sep-Pak, Waters-Millipore, Barcelona, Spain) was done. The purified extract was dried in a speed vacuum and reconstituted with 100 μl MeOH (100%) to start quantification using LC–MS/MS. A 10-μl aliquot was injected into a liquid chromatograph (Alliance 2695, Waters, Milford, MA, USA) equipped with a Restek C_{18} column (2.1 × 100 mm, 5 μm) at 25°C. A gradient with a binary solvent system (40% $MeOH^{-1}$:60% H_2O:HOAc 0.2%), which started at a flow rate of 0.2 ml min^{-1}, was used. Samples were analyzed in a double–quadrupole tandem mass spectrometer (Quatro Ultima™ *PT*, Micromass UK Ltd, Stevenage, UK), equipped with an electrospray ion source (ESI–MS/MS) in a negative ion mode. GA_1 and GA_4 were identified by comparing the retention time with pure standards, and monitoring ions by multiple reactions (MRM). Molecular and transitional masses were the following: GA_1 and $^2H_2$–GA_1: 348 > 242 and 350 > 244, and GA_4 and $^2H_2$–GA_4: 332 > 244 and 334 > 246. Quantization was performed by endogenous surface:deuterium surface rates, extrapolated with calibration curves produced with known concentrations.

Soluble carbohydrates were extracted and purified as described by Rivas and others (2006). In brief, they were extracted from 100 mg of sample with ethanol (80%, v/v) at 85°C, with 0.1 ml fucose (60 mg ml^{-1}) as an internal standard. After centrifugation at 15,000 × *g*, the supernatant was evaporated to 0.5 ml in vacuum. Purification was performed through a sequential cation column (Dowex® 1 × 4-100 50-100 Mesh), pH 4.5, prepared with 2 M HCl, and an anion column (Dowex® 1 × 4-100 50-100 Mesh), pH 7.5, prepared with 1 M Na_2CO_3 (Sigma Chemicals, St. Louis, MO, USA), a nylon filter (0.45-μm membrane, Waters-Millipore), and a C_{18} cartridge (Sep-Pak, Waters-Millipore). Purified samples were dried in vacuum and dissolved in 60 μl double-distilled water. Two replicates of 20 μl were injected in the high-performance liquid chromatography (HPLC) Spectra HPLC System® (Spectra, San Jose, CA, USA) equipped with an APS-2 Hypersil, 250 × 4.6-mm column attached to an ion guard precolumn (20 × 0.65 mm) connected to a differential refractometer (Spectra RI150), a vacuum pump (Spectra P2000), and ChromQuest® software system for data processing

(Thermo Quest Inc., San Jose, CA, USA). The solvent was acetonitrile:water (8:2, v/v) applied at a flow rate of 1.5 ml min^{-1} for a 15-min run. Sucrose, glucose, and fructose were identified by their retention times compared with pure standards and were quantified by extrapolation with a calibration curve made with known concentrations of each sugar. A correction factor dependent on fucose recovery was used.

Total and soluble nitrogen fractions were also analyzed. Proteinaceous nitrogen (N-Prot), ammonium nitrogen (NH_4^+–N), and nitrate nitrogen (NO_3^-–N, measured as the combined $NO_3^- + NO_2^-$ content) were determined according to AOAC (2005), Raigón and others (1992), and Beljaars and others (1994). Powdered samples (500 mg DW) were homogenized in 10 ml 5% (w/v) trichloroacetic acid (TCA) at 4°C using a magnetic shaker (RO5; IKA-WERKE GmbH, Staufen, Germany) for 15 min. The test tube was rinsed with 30 ml 5% (w/v) cold TCA, which was added to the homogenate. The homogenate was stored at 4°C for 15 min, then filtered through 90-mm Schleider & Shuell filter paper (Albet, Barcelona, Spain). The residue, containing N-Prot, was rinsed three times with 10 ml 5% (w/v) cold TCA, which was added to the filtered solution. The filtered solution was made up to 100 ml with mQ water and stored at 4°C until NH_4^+–N analysis. For N-Prot analysis, both the solid residue and the filter paper were digested by the micro-Kjeldahl method with 10 ml 96% H_2SO_4, 10 ml H_2O_2, and 3 g catalyst mixture [K_2SO_4:$CuSO_4$:Se (10:1:0.1)], at 450°C for 30 min. The digested sample was then distilled using Foss Kjeltec 2200 Auto Distillation® (FOSS, Höganäs, Sweden) with 40% (w/v) NaOH and 2% (w/v) boric acid and titrated with 0.1 M HCl. Results were expressed as mg N-Prot g^{-1} DW. NH_4^+–N was determined by means of a FIAstar 5000 Analyzer® (Flow Injection System, Höganäs, Sweden) equipped with an ammonium cassette, including a gas diffusion membrane, and a 5027 auto-sampler. Water (mQ) was used as a carrier, 0.5 M NaOH was reagent one, and the acid–base indicator solution was reagent two. Forty microliters of the filtered solution containing NH_4^+–N was injected into the carrier stream as it merged with the NaOH stream. The color shift was measured at 590 and 720 nm. Results were expressed as μg NH_4^+–N g^{-1} DW. For NO_3^-–N fraction analysis, powdered samples (500 mg DW) were homogenized in 50 ml mQ water using a magnetic shaker for 30 min. The homogenized sample was filtered through 90-mm Schleider & Shuell filter paper, and 200 μl of the filtered solution was injected into the FIAstar 5000 Analyzer equipped with a nitrate–nitrite cassette, dialysis membrane, and cadmium-reducing column. The color shift was measured at 540 and 720 nm. Results were expressed as μg NO_3^-–N g^{-1} DW.

Analysis of variance was performed on the data using general linear models and Tukey's test for means separation. The experimental data were analyzed with the MIXED procedure of SAS v9.1.3 software (SAS Institute, Cary, NC, USA).

Results

Stem girdling prior to fruit color break delayed fruit coloration of 'Washington' navel sweet orange, the effect being statistically significant from 30 days after girdling onward (Fig. 1). Nevertheless, stem-girdled fruit became colored because the wound eventually healed and restored the phloem flow, but delayed color break approximately 18 days, and differences with the control fruits were almost constant up to harvest date. Two months after girdling, *a* and *b* values of flavedo color were significantly lower in stem-girdled fruit (1.8 and 53.5, respectively) than in control fruit (22.4 and 58.9, respectively), and the *a*/*b* ratio, as an indicator of fruit ripening, was also significantly reduced (0.03) compared to controls (0.39). Similar trends were found for all orange cultivars studied, regardless of the year or the location (Table 1).

Two months after girdling, once stem-girdled fruit started to change color, the ABA concentration of flavedo of 'Washington' navel was significantly higher for

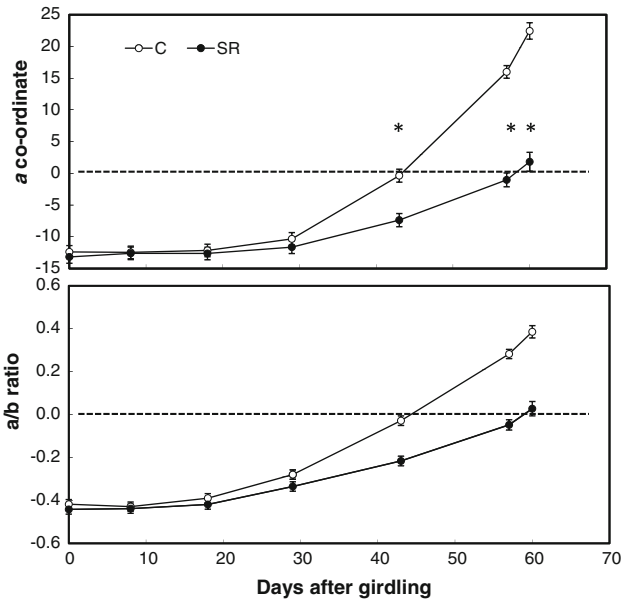

Fig. 1 Effect of stem girdling on the time course of *a* and *a*/*b* Hunter coordinates of the flavedo of fruits of 'Washington' navel sweet orange (Montevideo, Uruguay). *Horizontal line* indicates fruit color break ($a = 0$). Girdling was performed 45 days before fruit color break by removing 2 mm of bark 1.0 cm away from the calyx. Values for 2006. Each value is the average of three replicates of 15 fruits each. Standard errors (SE) are given as *vertical bars*. *Significant differences ($P \leq 0.05$) for a given date. *C* control fruit; *SR* stem-girdled fruit

Table 1 Effect of stem girdling on fruit color of sweet orange at harvest. Influence of cultivar, year, and growing area

Year	Cultivar	Location	*a*		*a/b*	
			Control	Girdled	Control	Girdled
2005	Valencia Delta	Huelva, Spain	20.2 a	17.5 b	0.53 a	0.47 b
	Navelate	Huelva, Spain	19.9 a	15.4 b	0.51 a	0.41 b
2006	Valencia Delta	Huelva, Spain	16.4 a	8.4 b	0.45 a	0.20 b
	Valencia Delta	Valencia, Spain	18.1 a	9.3 b	0.49 a	0.25 b
	Washington navel	Montevideo, Uruguay	16.0 a	6.6 b	0.28 a	0.12 b
2007	Valencia Delta	Valencia, Spain	17.6 a	13.3 b	0.48 a	0.36 b
2008	Valencia Delta	Valencia, Spain	17.4 a	11.8 b	0.47 a	0.31 b
	Washington navel	Montevideo, Uruguay	23.4 a	9.6 b	0.63 a	0.26 b

Different letters in the same line for a given coordinate indicate significant differences ($P \leq 0.05$). Fruit color was established by determining the *a* and *b* Hunter coordinates. Girdling was carried out approximately 45 days before fruit color break by removing 2 mm of bark 1.0 cm away from the calyx. Each value is the average of 3–4 replicates of 15 fruits each

Table 2 Effect of stem girdling on ethylene production of fruit and ABA concentration in the flavedo and the stem-girdled bark proximal and distal to fruit of 'Washington' navel sweet orange (Montevideo, Uruguay, 2006)

	ABA (μg g^{-1} DW) Flavedo	Ethylene (nl g^{-1} FW h^{-1}) Stem-girdled bark	
Control	1.21 ± 0.02 a	0.40 ± 0.02 a	Nil
Girdling	2.31 ± 0.05 b		Nil
Proximal to fruit		1.24 ± 0.10 b	
Distal to fruit		0.58 ± 0.07 a	

Different letters within columns indicate significant differences ($P \leq 0.05$). Values at fruit color break of stem-girdled fruit (60 days after girdling). Girdling was performed 45 days before control fruit color break by removing 2 mm of bark 1.0 cm away from the calyx. Each value is the mean of three replicates of ten fruits or ten stems each

stem-girdled fruit than for control fruit (2.31 vs. 1.21 μg g^{-1} DW, respectively). In addition, the ABA concentration of stem-girdled bark proximal to fruit was significantly increased by girdling (1.24 μg g^{-1} DW) compared to control (0.40 μg g^{-1} DW) (Table 2). Similar results were found for 'Valencia Delta Seedless' (data not shown).

No ethylene production was detected from either the control or the stem-girdled fruit during fruit color development of 'Washington' navel (Table 2), 'Navelate,' and 'Valencia Delta Seedless' sweet orange.

The delay in peel coloration of stem-girdled fruit paralleled higher concentrations of GA_1 and GA_4 in the flavedo. At the girdling date, GA_1 and GA_4 concentrations in the flavedo of control fruit (2.0 and 6.4 ng g^{-1} DW, respectively) were identical to those of stem-girdled fruit (1.9 and 6.5 ng g^{-1} DW, respectively) (Fig. 2). Thirty days later, both GA_1 and GA_4 concentrations were significantly increased in control fruits compared to the values at the date of girdling (4.1 and 51.0 ng g^{-1} DW, respectively),

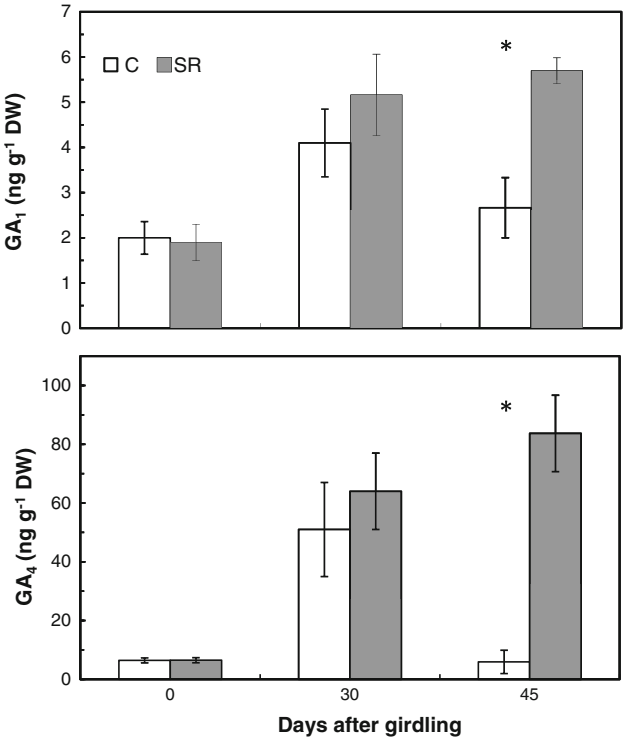

Fig. 2 Effect of stem girdling on GA_1 and GA_4 concentration in the flavedo of 'Washington' navel sweet orange (Montevideo, Uruguay) at fruit-ripening stage. Girdling was performed 45 days before fruit color break by removing 2 mm of bark 1.0 cm away from the calyx. Values for 2008. Each value is the average of three replicates of 10 fruits each. Standard errors (SE) are given as vertical bars. *Significant differences ($p \leq 0.05$) for a given date. C, control; SR, stem-girdled fruit

but girdling did not modify these concentrations significantly (5.2 and 64.0 ng g^{-1} DW). Yet 15 days later, when control fruit changed color, that is, 45 days after girdling (see Fig. 1), GA_1 and GA_4 concentrations dropped significantly in the flavedo of control fruit (2.7 and 5.9 ng g^{-1} DW, respectively), whereas GA_1 remained almost constant

(5.7 ng g^{-1} DW) and GA_4 increased, but not significantly (83.7 ng g^{-1} DW) in the flavedo of stem-girdled fruit. Consequently, when control fruits changed color, the GA_1 concentration in the flavedo of stem-girdled fruit was significantly higher than that of control fruit (5.7 vs. 2.7 ng g^{-1} DW, respectively), and so was the case for GA_4 (83.7 vs. 5.9 ng g^{-1} DW, respectively) (Fig. 2). It is worth emphasizing that the GA_4 concentration was higher than that of GA_1 for all dates and treatments, reaching up to 15 times higher at 45 days after girdling (Fig. 2).

At fruit color break, GA_1 concentration in the stem bark of the control fruits slightly increased (5.0 ng g^{-1} DW) from the girdling date (3.1 ng g^{-1} DW), whereas GA_4 increased by sixfold over the same period (from 2.0 to 11.6 ng g^{-1} DW) (Fig. 3). However, GA_1 concentration in the stem bark of stem-girdled fruits significantly increased (from 3.0 ng g^{-1} DW at girdling date to 13.8 ng g^{-1} DW at fruit color break), differing significantly from that of control fruits at fruit color break (5.0 ng g^{-1} DW) (Fig. 3). GA_4 concentration in the stem bark of stem-girdled fruits remained stable from the girdling date to the onset of color change (from 2.1 to 2.9 ng g^{-1} DW) (Fig. 3).

Total soluble carbohydrate concentration in the flavedo of 'Washington' navel control fruit rose from 77 to

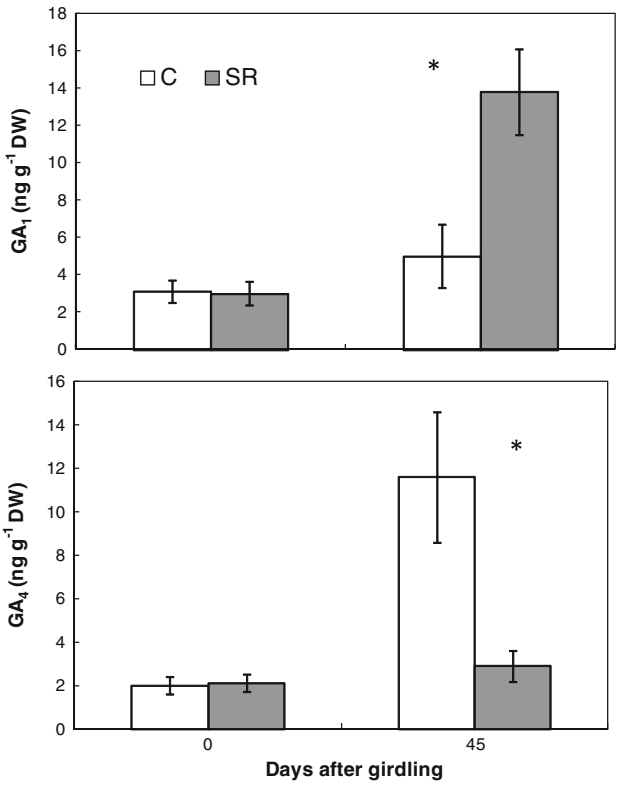

Fig. 3 Effect of stem girdling on GA_1 and GA_4 concentration in the stem bark of 'Washington navel' sweet orange (Montevideo, Uruguay) at fruit-ripening stage. Girdling was performed 45 days before fruit color break by removing 2 mm of bark at 1.0 cm away from the calyx. Values for 2008. Each value is the average of three replicates of 10 stems each. Standard errors (SE) are given as vertical bars. *Significant differences ($p \leq 0.05$) for a given date. C, control; SR, stem girdled bark

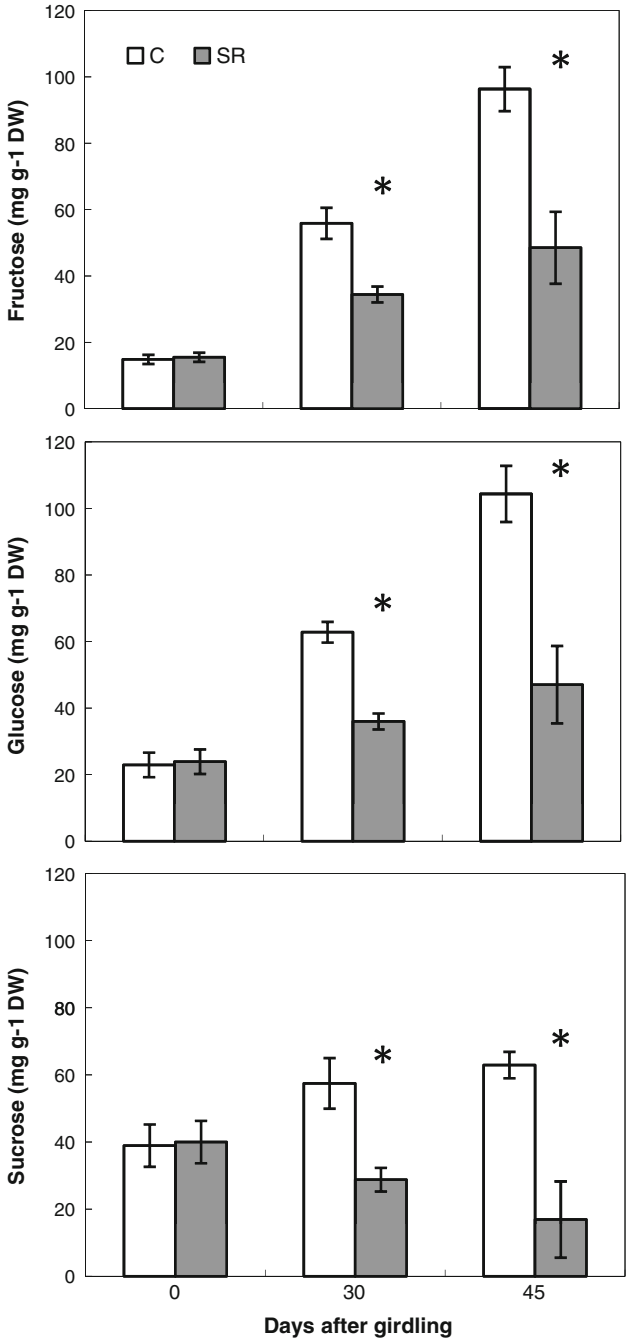

Fig. 4 Fructose, glucose, and sucrose concentration in the flavedo of 'Washington' navel sweet orange (Montevideo, Uruguay) as affected by stem girdling. Girdling was performed 45 days before fruit color break by removing 2 mm of bark at 1.0 cm away from the calyx. Values for 2008. Each value is the average of three replicates of ten fruits each. Standard errors (SE) are given as *vertical bars*. *Significant differences ($P \leq 0.05$) for a given date. *C* control; *SR* stem-girdled fruit

264 mg g^{-1} DW during the 45 days before color break, mainly due to the increase in fructose (15–96 mg g^{-1} DW) and glucose (23–104 mg g^{-1} DW) concentrations (Fig. 4). The sucrose concentration also rose, but in a lower proportion (39–63 mg g^{-1} DW); fructose and glucose concentrations rose continuously up to day 45, and sucrose increased up to day 30, remaining almost constant up to day 45 (Fig. 4). In the flavedo of the stem-girdled fruit, values were always significantly lower at 30 and 45 days. Fructose and glucose concentrations increased continuously up to 45 days after girdling (from 16 to 49 mg g^{-1} DW and from 24 to 47 mg g^{-1} DW, respectively), but in a significantly lower proportion than in control fruit (Fig. 4). By contrast, sucrose concentrations dropped significantly up to day 45 (from 40 to 17 mg g^{-1} DW). Concentrations of all sugars were significantly higher ($P \leq 0.05$) 30 and 45 days after girdling in the flavedo of control compared to stem-girdled fruit.

Total sugar concentration of stem-girdled bark proximal to fruit was significantly lower (68.6 mg g^{-1} DW) 60 days after girdling than in control stem bark (91.8 mg g^{-1} DW) of 'Washington' navel. This difference was due mainly to the lower sucrose concentration, because fructose and glucose concentrations did not differ significantly between treatments. Similar results were found 45 days after girdling for 'Valencia Delta Seedless,' the concentration of all carbohydrates being significantly lower proximal to fruit (Table 3). Consequently, girdling significantly reduced fruit size (data not shown) but did not alter fructose, glucose, and sucrose concentrations in leaves (data not shown).

Total N dropped continuously and significantly in the flavedo of control fruit during fruit ripening (from 11.9 to 7.8 mg g^{-1} DW) (Fig. 5a). However, in the flavedo of stem-girdled fruit, total N concentration fell significantly until 30 days after girdling (from 11.8 to 9.5 mg g^{-1} DW) and remained almost constant until day 45 (9.9 mg g^{-1} DW) (Fig. 5a). This time course of total N concentration parallels that of the N-Prot fraction (Fig. 5b), which accounts for more than 90% of total N. Changes in the concentration of the $NO_3^- + NO_2^-$–N fraction were nil in the flavedo of control and stem-girdled fruit. The concentration of the NH_4^+–N fraction follows a trend similar to that of N-Prot for both types of fruit (data not shown). No significant differences in N-fraction concentrations were found in leaves due to girdling (data not shown).

Table 3 Fructose, glucose, and sucrose concentration in the stem-girdled bark proximal and distal to fruit of sweet orange cv 'Valencia Delta Seedless' (Valencia, Spain, 2006)

Treatment	Fructose	Glucose	Sucrose
Control	12.8 b	14.3 b	26.0 b
Proximal to fruit	4.1 a	4.2 a	9.1 a
Distal to fruit	13.1 b	15.3 b	30.3 b

Different letters within columns indicate significant differences ($P \leq 0.05$). Values at fruit color break. Girdling was carried out 45 days before fruit color break by removing 2 mm of bark 1.0 cm away from the calyx. Values expressed as mg g^{-1} DW. Each value is the mean of three replicates of ten stems each

Discussion

In *Citrus* fruit, peel color development is a complex process involving endogenous and exogenous factors. In our experiments, fructose, glucose, and sucrose concentrations increased in the flavedo as fruit color developed, which corroborates previously reported data (Huff 1984; Sala and

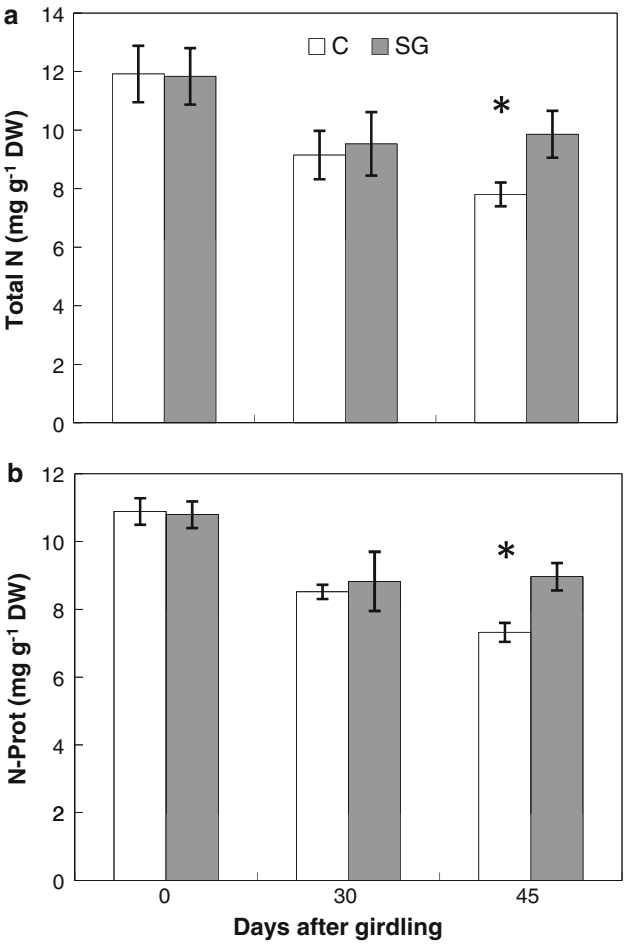

Fig. 5 Total nitrogen (**a**) and N-proteinaceous fraction (N-Prot) (**b**) concentration in the flavedo of control and stem-girdled fruit of 'Washington' navel sweet orange (Montevideo, Uruguay). Girdling was performed 45 days before fruit color break by removing 2 mm of bark at 1.0 cm away from the calyx. Values for 2008. Each value is the average of three replicates of ten fruit each. Standard errors (SE) are given as *vertical bars*. *Significant differences ($P \leq 0.05$) for a given date. **c** control; *SR* stem-girdled fruit

others 1992; Holland and others 1999). Moreover, stem girdling kept the fruit green as total soluble carbohydrates decreased compared to the control. Phloem flux interruption diminished flavedo sucrose concentration during this period, whereas fructose and glucose increased slightly but remained lower than in control fruit. Our results confirm the reducing-sugars requirement to start color break proposed by Huff (1984) and Fidelibus and others (2008). Previous research also reported that sucrose supplementation in vitro (Huff 1983) or in vivo (Iglesias and others 2001) promoted flavedo degreening. In addition, application of ethylene, which promotes color break, upregulates a sucrose transporter and acidic invertase genes (Fujii and others 2007), an effect that is counteracted by GA_3 application (Fujii and others 2008), which delays color development. In maturing fruit of 'Fortune' mandarin, sucrose translocation rather than sucrose synthesis was reported to play a major role in maintaining flavedo sucrose levels (Holland and others 1999). Lower sugar concentrations in the stem-girdled bark proximal to fruit support this hypothesis and partially explain delayed rind color break due to girdling.

Total N concentration and the proteinaceous fraction of the flavedo diminished during color development, in agreement with reports by Iglesias and others (2001). However, other studies found no change in flavedo proteinaceous concentration (Lewis and others 1967) or total N concentration (Win and others 2006) during ripening. In stem-girdled fruit, N decreased until 30 days after treatment and then remained unchanged, in accordance with the delay in fruit color break. Two months after girdling, stem-girdled fruit, which remained green, had a higher N concentration in the flavedo compared to control fruit, which reached orange color. Our results indicate that endogenous N is involved in the process, which is in agreement with results obtained for the exogenous application of nitrate salts in vitro (Huff 1983) or in vivo (Jones and Embleton 1959; Lee and Chapman 1988; Sala and others 1992; Quiñones and others 2004; Alós and others 2006). Application of potassium nitrate and calcium nitrate reduced the rate of chlorophyll degradation, extended the carotenoid composition of green fruit, and reduced β-β-xanthophyll accumulation (Alós and others 2006).

Stem girdling significantly affected the time course of the flavedo GA_1 and GA_4 concentration. In control fruits, gibberellin concentration dropped as rind coloration intensified, whereas in stem-girdled fruits, GA_1 and GA_4 concentrations remained constant. This effect contrasts with that for the bark of girdled stems, particularly in the concentration of GA_4, which fell significantly compared to controls but accumulated in the flavedo of stem-girdled fruit; GA_1, however, had a higher concentration compared to controls, but also accumulated in the flavedo. Accordingly, delayed rind coloration by stem girdling paralleled gibberellin levels (GA_1 and GA_4, in our experiments) in the flavedo.

It is important to note that 2 months after girdling, when the control fruit was fully colored and stem-girdled fruit started to change color, control fruit had significantly lower flavedo ABA concentrations than stem-girdled fruit, that is, girdling delays the rise of ABA concentration of the flavedo prior to fruit color change. This result agrees with those reported for different *Citrus* species for which the onset of fruit degreening has been associated with a marked increase in ABA concentration (Aung and others 1991; Richardson and Cowan 1995; Lafuente and others 1997; Rodrigo and others 2003; Agustí and others 2007), and also with those reported by Richardson and Cowan (1995) who stated that full development of color occurs concomitantly with a decline in ABA concentration.

To our knowledge, no information about endogenous concentrations of GA_1 and GA_4 at the end of stage II and throughout stage III of *Citrus* fruit development has been reported because previous studies were based on GA-like activity. An increase in GA-like activity was reported from anthesis up to the onset of chlorophyll loss, and fruit ripening coincided with a sudden decline in GA-like activity (Kuraoka and others 1977; García-Luis and others 1985). An increased GA-like activity of the flavedo preceded an increase in chlorophyll content in regreening 'Valencia' oranges (Rasmussen 1973). Our results show that for 'Washington' navel sweet orange, endogenous gibberellin concentrations fell in the flavedo before fruit color break, particularly for GA_4. Furthermore, stable concentrations of gibberellin markedly delayed the onset of fruit color change. Therefore, the presence of gibberellins in the flavedo prevents fruit color change, thus providing an explanation as to how gibberellic acid delays fruit coloration of sweet orange, lemon, and grapefruit (Coggins 1981), as well as Satsuma and Clementine mandarins (Kuraoka and others 1977; García-Luis and others 1985; Agustí and others 1988), when applied approximately 2 weeks prior to fruit color change. El-Otmani and others (1995, 2000) reviewed the factors regulating endogenous gibberellin levels in *Citrus* tissues and the use of GA_3 for delaying rind color development of *Citrus* fruit to prolong storage of fruit and delay its rind senescence.

Our results, based on studies with fruits from stem-girdled and ungirdled single-fruited shoots, show that active gibberellin concentrations must be diminished in the flavedo to allow color break, and increased ABA concentration precedes the onset of fruit color change. Considering that *Citrus* fruit produce tiny amounts of ethylene during ripening (Katz and others 2004), our results contribute to the knowledge that gibberellins might indeed be responsible for the endogenous regulation of color development.

In conclusion, 'Washington' navel, 'Navelate,' and 'Valencia Delta Seedless' sweet orange fruit change color as a result of a reduction in active gibberellin concentrations in the flavedo. Our results suggest that gibberellins are involved in regulating sugar (sucrose) translocation, ABA accumulation, and N retention in the flavedo of citrus fruit in relation with color development, but not in regulating ethylene production. We demonstrated it in vivo without removing the fruit from the tree by intercepting phloem transport by girdling the stem of fruited shoots.

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References

Agustí M, Almela V, Guardiola JL (1988) Aplicaciones de ácido giberélico para el control de alteraciones de la corteza de las mandarinas asociadas a la maduración. Invest Agr Prod Prot Veg 3:125–137

Agustí J, Zapater M, Iglesias DJ, Cercós M, Tadeo FM, Talón M (2007) Differential expression of putative 9-cis-epoxycarotene dioxygenases and abscisic acid accumulation in water stressed vegetative and reproductive tissues of citrus. Plant Sci 172: 85–94

Aharoni Y (1968) Respiration of oranges and grapefruit harvested at different stages of development. Plant Physiol 43:99–102

Alós E, Cercós M, Rodrigo MJ, Zacarías L, Talón M (2006) Regulation of color break in *Citrus* fruits. Changes in pigment profiling and gene expression induced by gibberellins and nitrate, two ripening retardants. J Agric Food Chem 54:4888–4895

AOAC (2005) Official methods of analysis of the association of official analytical chemists, 14th edn. AOAC, Arlington, pp 611–613

Aung LH, Houck LG, Norman SM (1991) The abscisic acid content of *Citrus* with special reference to lemons. J Exp Bot 42: 1083–1088

Beljaars PR, van Dijk R, van Der Horst GM (1994) Determination of nitrate in vegetables by continuous flow: interlaboratory study. J AOAC Int 77:1522–1529

Chiwocha SDS, Abrams SR, Ambrose SJ, Cutler AJ, Loewen M, Ross ARS, Kermode AR (2003) A method for profiling classes for plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormones regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. Plant J 35:405–417

Coggins CW Jr (1981) The influence of exogenous growth regulators on rind quality and internal quality of *Citrus* fruits. Proc Int Soc Citriculture 1:214–216

Eaks IL (1970) Respiratory response, ethylene production, and response to ethylene of citrus fruit during ontogeny. Plant Physiol 45:334–338

El-Otmani M, Lovatt CJ, Coggins CW Jr, Agusti M (1995) Plant growth regulators in citriculture: factors regulating endogenous levels in citrus tissues. Crit Rev Plant Sci 14:367–412

El-Otmani M, Coggins CW Jr, Agusti M, Lovatt C (2000) Plant growth regulators in citriculture: world current uses. Crit Rev Plant Sci 19:395–447

Fidelibus MW, Koch KE, Davies FS (2008) Gibberellic acid alters sucrose, hexoses, and their gradients in peel tissues during color break delay in 'Hamlin' orange. J Am Soc Hortic Sci 133: 760–767

Fujii H, Shimada T, Sugiyama A, Nishikawa F, Endo T, Nakano M, Ikoma Y, Shimizu T, Omura M (2007) Profiling ethylene-responsive genes in mature mandarin fruit using a citrus 22 K oligoarray. Plant Sci 173:340–348

Fujii H, Shimada T, Sugiyama A, Endo T, Nishikawa F, Nakano M, Ikoma Y, Shimizu T, Omura M (2008) Profiling gibberellin (GA_3)-responsive genes in mature mandarin fruit using a citrus 22 K oligoarray. Sci Hortic 116:291–298

García-Luis A, Agustí M, Almela V, Romero V, Guardiola JL (1985) Effect of gibberellic acid on ripening and peel puffing in *Satsuma mandarin*. Sci Hortic 27:75–86

García-Luis A, Fornés F, Guardiola JL (1986) Effects of gibberellin A_3 and cytokinins on natural and post-harvest, ethylene-induced pigmentation of *Satsuma mandarin* peel. Physiol Plant 68: 271–274

Holland N, Sala JM, Menezes HC, Lafuente MT (1999) Carbohydrate content and metabolism as related to maturity and chilling sensitivity of cv. *Fortune mandarins*. J Agric Food Chem 47: 2513–2518

Huff A (1983) Nutritional control of regreening and degreening in *Citrus* peel segments. Plant Physiol 73:243–249

Huff A (1984) Sugar regulation of plastid interconversions in the epicarp of *Citrus* fruit. Plant Physiol 76:307–312

Iglesias DJ, Tadeo FR, Legaz F, Primo-Millo E, Talón M (2001) In vivo sucrose stimulation of color change in citrus fruit epicarps: interactions between nutritional and hormonal signals. Physiol Plant 112:244–250

Jones WW, Embleton TW (1959) The visual effect of nitrogen nutrition on fruit quality of 'Valencia' oranges. Proc Am Soc Hortic Sci 73:234–236

Katz E, Martínez-Lagunes P, Riov J, Weiss D, Goldschmidt EE (2004) Molecular and physiological evidence suggests the existence of a system II-like pathway of ethylene production in non-climacteric *Citrus* fruit. Planta 219:243–252

Kuraoka T, Iwasaki K, Ishii T (1977) Effects of GA_3 on puffing and levels of GA_3-like substances and ABA in the peel of *Satsuma mandarin* (*Citrus unshiu* Marc.). J Am Soc Hortic Sci 102: 651–654

Lafuente MT, Martínez TM, Zacarías L (1997) Abscisic acid in the response of *Fortune mandarins* to chilling. Effect of maturity and high-temperature conditioning. J Sci Food and Agric 73: 494–502

Lee LS, Chapman JC (1988) Yield and fruit quality responses of *Ellendale mandarins* to different nitrogen and potassium fertiliser rates. Aust J Exp Agric 28:143–148

Lewis LN, Coggins CW Jr (1964) The inhibition of carotenoid accumulation in navel oranges by gibberellin A_3, as measured by thin layer chromatography. Plant Cell Physiol 5:457–463

Lewis LN, Coggins CW Jr, Labanauskas CK, Dugger WM Jr (1967) Biochemical changes associated with natural and gibberellin A_3 delayed senescence in the navel orange rind. Plant Cell Physiol 8:151–160

Pons J, Almela V, Juan M, Agustí M (1992) Use of ethephon to promote color development in early ripening clementine cultivars. Proc Int Soc Citriculture 1:459–462

Porat R, Weiss B, Cohen L, Daus A, Goren R, Droby S (1999) Effects of ethylene and 1-methylcyclopropene on the postharvest qualities of 'Shamouti' oranges. Postharvest Biol Technol 15: 155–163

Quiñones A, González MC, Montaña C, Primo-Millo E, Legaz F (2004) Fate and uptake efficiency of ^{15}N applied with different

seasonal distributions in *Citrus* trees. Proc Int Soc Citriculture 2:587–592

Raigón MD, Pérez-García M, Maquieira A, Puchades R (1992) Determination of available nitrogen (nitric and ammoniacal) in soils by flow injection analysis. Analysis 20:483–487

Rasmussen GK (1973) The effect of growth regulators on degreening and regreening of citrus fruit. Acta Hortic 34:473–478

Richardson GR, Cowan AK (1995) Abscisic acid content of *Citrus* flavedo in relation to color development. J Hortic Sci 70:769–773

Rivas F, Erner Y, Alós E, Juan M, Almela V, Agustí M (2006) Girdling increases carbohydrate availability and fruit-set in citrus cultivars irrespective of parthenocarpic ability. J Hortic Sci Biotechnol 81:289–295

Rodrigo MJ, Zacarias L (2007) Effect of postharvest ethylene treatment on carotenoid accumulation and the expression of carotenoid biosynthetic genes in the flavedo of orange (*Citrus sinensis* L. Osbeck) fruit. Postharvest Biol Technol 43(1):14–22

Rodrigo MJ, Marcos JF, Alférez F, Mallent MD, Zacarias L (2003) Characterization of pinalate, a novel *Citrus sinensis* mutant with a fruit-specific alteration that results in yellow pigmentation and decreased ABA content. J Exp Bot 54:727–738

Rodrigo MJ, Alquezar B, Zacarias L (2006) Cloning and characterization of two 9-cis-epoxycarotene dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). J Exp Bot 57:633–643

Sala JM, Cuñat P, Collado M, Moncholi V (1992) Effect of nitrogenous fertilization (quantity and nitrogen form) in precocity of color change of 'Navelina' oranges. Proc Int Soc Citriculture 1:598–602

Serek M, Tamari G, Sisler EC, Borochov A (1995) Inhibition of ethylene-induced cellular senescence symptoms by 1-methylcyclopropene, a new inhibitor of ethylene action. Physiol Plant 94:229–232

Talón M, Hedden P, Primo-Millo E (1990) Gibberellins in *Citrus sinensis*: a comparison between seeded and seedless varieties. J Plant Growth Regul 9:201–206

Trebitsh T, Goldschmidt EE, Riov J (1993) Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme, in *Citrus* fruit peel. Proc Nat Acad Sci U S A 90:9441–9445

Walker-Simmons M (1987) ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Physiol 84:61–66

Weiler EW (1979) Use of immunoassay in plant science 7. Radioimmuoassay for the determination of free and conjugated abscisic-acid. Planta 144:255–263

Win TO, Srilaong V, Kyu KL, Poomputsa K, Kanlayanarat S (2006) Biochemical and physiological changes during chlorophyll degradation in lime (*Citrus aurantifolia* Swingle cv. 'Paan'). J Hortic Sci Biotechnol 81:471–477

Zacarías L, Talón M, Ben CW, Lafuente MT, Primo-Millo E (1995) Abscisic acid increases in non-growing and paclobutrazol-treated fruits of seedless mandarins. Physiol Plant 95:613–619