Murcott seedless: influence of gamma irradiation on citrus production and fruit quality

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Abstract

A Seedlessness is an important economic trait relating to fruit quality, and gamma irradiation is a common technique used to obtain seedless citrus fruits. Herein, we report a study of new seedless 'Murcott' mandarin clones obtained by bud irradiation from the self-compatible not parthenocarpic 'Murcott' mandarin. All irradiated clones examined presented lower seed numbers (from 0.23 to 2.47 seeds per fruit) and reduced pollen germination (from 1.40% to 8.55%) whereas the wild-type 'Murcott' showed an average number of 9.03 seeds per fruit and a pollen germination value of 47.15%. Fruit quality and nutritional bio-components were affected differently; some clones presented no changes compared to the control 'Murcott' mandarin, while other clones showed significant differences. High-performance liquid chromatographic methods were used to identify and quantify of these compounds, using photodiode array, mass and refractive index detectors. Our results indicated high contents in natural antioxidants as vitamin C (from 20.13 to 25.73 mg/100 mL) and phenolic compounds, as flavonoids, in these citrus varieties cultived under the Mediterranean climate. Some of these clones, which ripen late in the season and whose fruit quality is maintained or improved, are in the process of registration. In conclusion, budwood irradiation is a suitable technique to improve cultivars, produce seedless cultivars, adjust ripening time or raise the content of health-promoting compounds. Also this study investigates the influence of temperature during flowering on the number of seeds formed. Findings indicate that low temperatures during flower formation and seed number.

Additional key words: citrus clones; mandarin; nutritional bio-components; pollen germination; temperature.

Resumen

Murcott sin semillas: influencia de la irradiación gamma sobre la producción de cítricos y la calidad de la fruta

El carácter "sin semillas" es un rasgo importante relacionado con la calidad de la fruta, y la inducción de mutaciones mediante irradiaciones gamma es una técnica común utilizada para obtener frutos sin semillas de cítricos. Presentamos aquí el estudio realizado en los nuevos clones que hemos obtenido mediante irradiación, a partir de la variedad autocompatible y no partenocárpica 'Murcott'. Todos los clones irradiados examinados presentaron menor número de semillas (de 0,23 a 2,47 semillas por fruto) y reducción de la germinación del polen (de 1,40% a 8,55%), mientras que la mandarina 'Murcott' control mostró 9,03 semillas por fruto y 47,15% de germinación del polen. La calidad de la fruta y el contenido en compuestos bioactivos y nutricionales se vieron afectados de manera diferente, algunos clones no presentaron cambios con respecto al control, mientras que otros clones sí mostraron diferencias significativas. Nuestros resultados indican un alto contenido en antioxidantes naturales como la vitamina C (20,13 a 25,73 mg/100 mL) y compuestos fenólicos, como flavonoides, en estas variedades de cítricos cultivados bajo el clima mediterráneo. Algunos de estos clones, que maduran a finales de la temporada y cuya calidad de la fruta se mantiene y/o mejora, están en proceso de registro. Además, este estudio examina la influencia de la temperatura durante la floración en el número de semillas formadas. Los hallazgos indican que las bajas temperaturas durante la formación de flores disminuyen el número de semillas.

Palabras clave adicionales: clones de cítricos; compuestos bioactivos y nutricionales; germinación del polen; mandarina; temperatura.

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Introduction

Seedlessness is important in relation to fruit quality, and the production of fruits with few or no seeds has steadily increased (Ye et al., 2009). Seedlessness in citrus mandarin cultivars can be induced by many factors, and many seedless citrus cultivars have been originated from the bud mutation of seedy cultivars (Yamamoto et al., 1995; Shen, 1997; Vardi et al., 2008). Great achievements have been made over the past few decades in seedless breeding techniques with fruits such as citrus, grape (Vitis vinifera L.), litchi (Litchi chinensis Sonn.), loquat (Eriobotrya japonica Lindl. cv. Jiefangzhong), mango (Mangifera indica Linn.) and wampee (Clausena lansium Lour.) (Yamamoto et al., 1995; Raza et al., 2003; Ye et al., 2009). Among citrus cultivars, mutation breeding has been used to improve sweet orange (Citrus sinensis (L.) Osb.), grapefruit (C. paradisi Macf.) and mandarin (C. reticulata Blanco) (Anderson, 2000; Deng, 2000). Most recently we have obtained different clones from irradiated budwood of the seedy 'Moncada' mandarin, self-incompatible parthenocarpic cultivar, assessing the quality characteristics of the fruit, chemical composition and seed number (Bermejo et al., 2011a). Qualities of the control cultivar have to be kept or improved in the new varieties. Fruit weight, size, acidity, maturity index, harvest time, chemical and nutritional composition are important quality traits for fresh citrus consumption and acceptance by the citrus industry. An increase in the consumption of fruits and vegetables is associated with a decrease in the incidence of cardiovascular disease and reduce risks of certain cancers. Citrus fruits and derived products have a beneficial effect on the human health. Thus, citrus fruits have received much attention because of its nutritional and antioxidant properties and nowdays prevention of health problems through nutrition is promoted intensively, due mainly to the contribution of antioxidant compounds including vitamin C, phenolics compounds and carotenoids (Dhuique-Mayer et al., 2005; Cano et al., 2008; Bermejo et al., 2011b). In citrus, the major part of the total antioxidant activity is due to hydropohyllic compound, and some authors have stressed the main role of hesperidin in the total antioxidant capacity of orange juices (Cano et al., 2002; Del Caro et al., 2004; Wu et al., 2007). Some authors have reported that irradiation not only affects seed formation in the fruit, but also lowers acidity and increases lycopene carotenoid (Hensz, 1977) or favours early ripening (Hearn, 1986), while other have reported no changes in seedless cultivars of grapefruits, mandarins and oranges obtained by γ -irradiation of buds (Froneman *et al.*, 1996). In our case, some of the clones obtained showed clear differences in certain fruit quality traits.

Herein, we report a study of new seedless 'Murcott' mandarin clones obtained by gamma irradiation from the seedy self-compatible not parthenocarpic 'Murcott' mandarin. The actual origin of the 'Murcott' cultivar (with an average of 17 seeds per fruit in an open pollinated field) is unknown but is most likely a tangor which is a cross between a tangerine and a sweet orange (Lupo et al., 1991; Jackson & Davies, 1999). Different clones of the self-compatible not parthenocarpic 'Murcott' mandarin, obtained by gamma irradiation, were studied to assess seedlessness, pollen germination, fruit characteristics and quality attributes. Also this study investigates the influence of temperature during flowering, on the number of seeds formed. Findings indicate that low temperatures during flower formation decreased pollen germination and seed number.

Material and methods

Instruments

Polytrom PT3100 homogenizer (Kinematica AG, Switzerland) and an Eppendorf 5810R centrifuge (Eppendorf Iberica, Madrid, Spain) were used for sample treatment. Analysis was made using an Alliance liquid chromatographic system (Waters, Barcelona, Spain) equipped with a 2695 separation module, coupled to a 2996 photodiode array detector and a ZQ2000 mass detector. A thermostat column oven, a reverse-phase column C₁₈ Tracer Excel 5 μ m 120 OSDB (250 mm \times 4.6 mm) (Teknokroma, Barcelona, Spain), a reversephase column C₃₀ YMC S-5 μ m (250 mm × 4.6 mm), a guard column YMC30 S-5 μ m (10 mm × 4.0 mm) (YMC Europe GmbH, Germany), a ICSep ICE-COREGEL 87H3 column (Transgenomic), a ICSep ICE-COREGEL 87H guard kit, and an automatic injector were used for chromatographic separation. Empower 2 software was used for data acquisition. Sample temperature was 5°C, column temperature was 25 or 35°C, and the UV-Vis spectra were recorded from 280 to 400 nm. An HPLC system equipped with a Waters 515 HPLC pump, a Waters 2414 refractive index detector and a 20 µL loop Rheodyne injector were used for sugar analysis. Empower 2 software (Waters, Spain) was used for data processing.

Plant material and sampling

In this research we have used a low-seeded mutation from Brazil, namely clone J (with an average of 9 seeds per fruit), hereafter 'Murcott' mandarin control or wildtype 'Murcott'. Original 'Murcott' budwoods were taken from healthy adult trees of 'Murcott' mandarin control of the Field Collection of Citrus Germoplasm Bank held at Instituto Valenciano de Investigaciones Agrarias (IVIA) located at Moncada (Valencia, Spain). This cultivar demonstrates excellent fruit size, internal quality and good rind colour, and its ripen period is from 15 February to 30 April. Gamma irradiation of 3,000 'Murcott' budwoods was carried out in the year 2000; using a cobalt (⁶⁰Co) source at $50 \pm 10\%$ Gys. Budwoods were then grafted onto one-year 'Carrizo' citrange (C. sinensis (L.) Osb. × Poncirus trifoliata (L.) Raf.) rootstock, held at IVIA, latitude 39°35' N, longitude 0°23' W and altitude 50 m. The following year, around 10,000 buds were selected from the obtained shoots (first generation) and grafted onto 'Carrizo' citrange rootstock, placing 2 buds tree⁻¹, at different heights and orientation, in order to have two sources of irradiated material (second generation). These new plants were planted in containers (3.5 L) in the greenhouse until the spring of 2002, after which they were moved to an experimental field at the IVIA. In total, around of 4,650 plants were transplanted, carrying around 7,725 irradiated buds from 'Murcott' mandarin variety.

The experiments involved 3 adult trees cultivar⁻¹. For this study we used trees of each clone (11 clones) and trees of 'Murcott' mandarin as control. The clones were selected based on fruit seed number. All cultivars shared the same environmental, cultural and soil conditions, thus the differences among cultivars were not influenced by climatic factors or crop techniques.

Moreover, we studied in an independent experiment if the low temperatures slow the activity of the plant. We chose two clones, 'Murcott' mandarin control (clone J) and the clone N306 obtained by gamma irradiation from the self-compatible 'Murcott' mandarin, located at IVIA. The average minimum temperature to which they had been under the flowers during the flowering period and the number of seeds that had formed naturally in the fruit was recorded for seven years, between 2003 and 2009. To determine the temperatures to which flowers were exposed during the process, four Testostor 175 Loggers were placed in the branches of selected genotypes, recording the temperature every 15 min. After full fruit development and maturation, all fruits were counted and harvested and seeds per fruit were counted.

Seed numbers and fruit quality

After full fruit development and maturation, at least 30 fruits were counted and harvested during two seasons (data of seed number are mean of 2008/2009, 2009/2010 and 2010/2011 seasons). Weight, height and diameter were measured for each fruit, and seeds per fruit were counted. Juice was obtained from representative samples, other 30 fruits cultivar⁻¹, collected on four different dates during two years (01/02/2009, 17/02/2009, 01/03/2010 and 15/03/2010), using a Zumonat machine (Somatic-AMD, Spain) and analyzed for °Brix with a refractometer (Atago Co. Ltd., Japan), acidity by titration with 0.1 N NaOH using phenol-phthalein as indicator, maturity index and juice percentage (Bermejo *et al.*, 2011a).

Pollen germination

Twenty flowers per clone were harvested and the anthers collected and dried for 24 h at room temperature in the dark. The pollen grains were hydrated and sown on two glass slide-trays with Brewbaker & Kwack (1963) medium. Ten visual fields were selected from each slide-tray, and the pollen germination percentage recorded.

General procedure for extraction and analysis

Determination of total ascorbic acid. Total vitamin C (ascorbic acid and dehydroascorbic acid) were determined by HPLC-DAD. The procedure used was the reduction of dehydroascorbic acid to ascorbic acid, using DL-dithiothreitol (DTT) as reducing reagent, according to a similar procedure described by Dhuique-Mayer *et al.* (2005) with some modifications to adapt the method to a microliter format (Rojas-Argudo *et al.*, 2012). A reverse-phase C₁₈ column was used with an isocratic mobile phase of methanol:0.6% acetic acid (5:95). The total run time was 10 min at 1 mL min⁻¹, and injection volume was 5 µL. Quantification of ascor-

bic acid was performed at 245 nm by external standard calibration. L-ascorbic acid and DTT were obtained from Sigma (Sigma Co., Barcelona, Spain) and Fluka (Sigma Co., Barcelona, Spain), respectively. All solvents used were of HPLC-grade and ultrapure water (Milli-Q) was used.

Determination of organic acids. Extraction method was the same as the procedure described previously, and organic acids were analysed by HPLC-DAD and HPLC-MS in electrospray ion negative conditions (Bermejo *et al.*, 2011a). An ICSep ICE-COREGEL 87H3 column was used with an isocratic mobile phase of 0.1% H₂SO₄ solution. The total run time was 20 min at 0.6 mL min⁻¹, and injection volume was 5 µL. Compounds were indentified on the basis of comparing their retention times, UV-Vis spectra and mass spectrum data with corresponding authentic standards. All solvents were of HPLC-grade and ultrapure water (Milli-Q) was used. Standards were obtained from Sigma (Sigma Co., Barcelona, Spain).

Determination of flavonoids. Flavonoids were extracted as described earlier (Bermejo et al., 2011a), and analysed by HPLC-DAD and HPLC-MS in electrospray ion positive conditions using a reverse-phase column C₁₈. A gradient mobile phase consisting in acetonitrile (solvent A) and 0.6% acetic acid (solvent B) were used. The flow rate was 1 mL min⁻¹ and injection volume was $10 \ \mu$ L. The conditions were as follow: initial condition of 10% A for 2 min, reaching 75% A in the following 28 min, and then back to initial condition and held for 5 min (total run time 35 min). Compounds were identified on the basis of comparing their retention times, UV-Vis spectra and mass spectrum data with corresponding authentic standards, and concentrations were determined using an external calibration curve. Narirutin was purchased from Extrasynthesis (Extrasynthesis, Genay, France), diosmin and hesperidin were obtained from Sigma Co. (Barcelona, Spain), and didymin, nobiletin and tangeretin were purchased from ChromaDex (Irvine, CA, USA). All solvents used were of HPLCgrade and ultrapure water (Milli-Q) was employed.

Determination of carbohydrates. The extraction method employed was the same as the procedure described previously (Bermejo *et al.*, 2011a), and carbohydrates were analysed by HPLC using a column Tracer Carbohydr 250 mm × 4.5 mm, 5 μ m (Teknokroma, Barcelona, Spain) and a mobile phase composed by acetonitrile:water (75:25) at a flow rate of 1 mL min⁻¹. Fructose, glucose and sucrose sugars were identified comparing their retention time with a standard and quantified using an external calibration curve.

Determination of carotenoids. Carotenoid extraction was carried out according to the method described previously (Bermejo et al., 2011a), and compounds were analysed by HPLC-DAD using a C₃₀ column. The flow rate was 1 mL min⁻¹ and injection volume was 20 µL. A ternary mobile phase of water (A): methanol (B): methyl-tert-butyl ether (C) was used. Initial conditions were 4% A, 86% B and 10% C held for 10 min, then changed to 4% A, 10% B and 90% C for the next 40 min, and back to initial conditions and conditioning column for 10 min, total run time 60 min. Compounds were identified on the basis of comparing their retention times and absorption spectrum characteristics. β -cryptoxanthin was obtained from Extrasynthese (Extrasynthesis, Genay, France), and β -carotene was purchased from Biochemica (Sigma Co., Barcelona, Spain).

Data analysis

Data were expressed as means. One-way ANOVA analyses were carried out with the Statgraphics Plus package, and the Duncan test method (p < 0.05) was applied to experimental data and to estimate significant differences amongst data. For Fig. 1, statistics was generated using Microsoft Office Excel Graphics for Window software.

Results and discussion

Fruit seeds number and pollen germination

The clones were selected based on fruit seed number. The selection process began in 2004 and ended in 2009. Seed number was determined by cutting the fruit transversely in the field. After full fruit development and maturation, all fruits were counted and harvested, and seeds from each fruit were counted. All clones studied presented a lower and significantly different seed number compared to the control 'Murcott' mandarin. The lowest value corresponded to clone M615, with an average number of 0.23 seeds per fruit, followed by clones N235, B418 and G603 (0.47, 0.50 and 0.67 seeds, respectively), whereas the wild-type 'Murcott' showed an average number of 9.03 seeds. None of the new clones had an average seeds number above 2.47 seeds per fruit (Table 1). Moreover, all clones showed reduced pollen germination compared to the control 'Murcott' mandarin, with significantly different

values. Control 'Murcott' mandarin gave a pollen germination value of 47.15%, while clones M615 and N235 presented the lowest pollen germination (2.15% and 1.40%, respectively). All clones had pollen germination values below 8.55% (Table 1).

In view of the above results, we observed that irradiation is a valuable tool to obtain seedless cultivars from seedy ones. Mutation also affects pollen viability. Female and male sterility seem to be directly related, so clones with a lower seed number also present lower pollen viability, and the impairing chromosomes during meiosis activated by irradiation are responsible, in some cases, for sterility (Gmitter *et al.*, 1992; Pardo *et al.*, 2007, 2010).

Fruit quality: nutritional quality

Different fruit quality features have been evaluated in the irradiated 'Murcott' clones under study (see Table 2). Fruit quality characteristics were obtained from representative samples from the whole fruits harvested over two seasons. For the wild-type 'Murcott' fruit, weight, height and diameter values were 94.00 g, 59.00 cm and 45.75 cm, respectively. Weight, height and diameter data differed in some of the clones assayed, but only several clones presented values that were significantly lower than the control (Table 2). °Brix, acidity and maturity index indicate the maturity status of the fruit. Only two clones (K718 and B418) showed significantly different °Brix values than the control mandarin; two clones (B418 and G603) had significantly different acidity values than the control, and three clones (B303, K718 and G603) pre-

Table 1. Seed number and pollen germination (%) in several 'Murcott' clones. Data are expressed as mean \pm standard deviation: data of seed number are mean (30 fruits per year) of 2008/2009, 2009/2010 and 2010/2011 seasons, and data of pollen germination are mean of 2009/2010 and 2010/2011 seasons

Clone	Seed number	Pollen germination (%)
B303	2.13 ± 0.85	4.40 ± 1.70
B418	0.50 ± 0.26	3.20 ± 1.56
C508	1.77 ± 0.68	6.85 ± 1.77
D517	2.47 ± 0.38	7.30 ± 1.27
1208	2.13 ± 0.67	5.05 ± 1.06
K115	1.86 ± 0.50	4.55 ± 1.91
K718	2.00 ± 0.66	8.55 ± 1.77
M615	0.23 ± 0.15	2.15 ± 0.21
N306	2.03 ± 0.81	6.00 ± 0.85
N235	0.47 ± 0.25	1.40 ± 0.14
G603	0.67 ± 0.23	2.45 ± 0.07
Control ^x	9.03 ± 1.46	47.15 ± 6.86

x: 'Murcott' mandarin control.

sented significantly different maturity index values compared to the control. The juice percentage was not influenced by irradiation. The wild-type 'Murcott' had a juice percentage of 51.00%, and the clones assayed gave values of between 45.75% and 54.75% (Table 2).

Vitamin C and organic acid content. Our study revealed few differences in the content of bio-components analyzed in the selected clones (Table 3). The content of vitamin C and other organic acids in fruits and vegetables can be influenced by various factors such as genotypic differences, climatic conditions and

Table 2. Fruit quality data for the different irradiated 'Murcott' clones during 2008/2009 and 2009/2010 seasons^x

Clone	Weight (g)	Diameter (cm)	Height (cm)	°Brix	Acidity (g L ⁻¹)	Maturity index	Juice (%)
B303	98.50 ± 15.61	59.25 ± 3.30	46.50 ± 3.70	16.28 ± 1.20	15.45 ± 0.77	$10.58 \pm 1.03*$	45.75 ± 5.12
B418	$78.25 \pm 10.40*$	$54.50 \pm 3.11*$	$43.50 \pm 2.65*$	$14.18 \pm 1.41*$	$14.60 \pm 1.06*$	9.70 ± 0.80	54.00 ± 2.16
C508	$78.75 \pm 14.15*$	56.75 ± 2.75	$42.50 \pm 1.91*$	16.63 ± 1.42	18.40 ± 2.08	9.05 ± 0.53	49.50 ± 3.11
D517	$78.25 \pm 5.80*$	56.75 ± 2.99	44.75 ± 2.22	17.45 ± 0.79	17.43 ± 1.25	10.08 ± 0.93	54.25 ± 1.89
I208	86.00 ± 4.83	55.50 ± 2.65	$43.50 \pm 1.73*$	16.95 ± 0.62	18.33 ± 1.95	9.30 ± 0.66	52.25 ± 6.60
K115	92.75 ± 5.91	59.50 ± 1.29	46.00 ± 0.82	16.65 ± 0.44	18.30 ± 1.40	9.08 ± 0.76	50.25 ± 4.57
K718	97.25 ± 9.03	57.50 ± 0.58	46.50 ± 1.00	$18.15 \pm 0.83*$	17.05 ± 1.21	$10.65 \pm 0.37*$	54.50 ± 4.12
M615	98.00 ± 10.10	58.50 ± 2.08	46.75 ± 1.50	15.45 ± 0.70	16.05 ± 1.98	9.68 ± 0.78	50.75 ± 2.22
N306	91.00 ± 7.96	59.00 ± 2.45	46.00 ± 2.94	17.38 ± 0.54	16.83 ± 0.67	10.35 ± 0.41	54.75 ± 2.63
N235	$75.75 \pm 4.27*$	$53.50 \pm 2.38*$	$42.50 \pm 1.73*$	16.03 ± 0.53	17.33 ± 0.94	9.28 ± 0.60	54.75 ± 3.10
G603	88.25 ± 7.27	59.00 ± 4.08	45.25 ± 2.87	14.60 ± 1.85	19.55 ± 2.52*	$7.50 \pm 0.83*$	48.50 ± 6.14
Control ^y	94.00 ± 5.29	59.00 ± 1.41	45.75 ± 1.89	15.95 ± 0.77	17.13 ± 1.32	9.35 ± 0.75	51.00 ± 1.41

*: Data are expressed as mean $(n = 4) \pm$ standard deviation; *: Clones with significantly different values compared to the control. y: 'Murcott' mandarin control. cultural practices; furthermore, their nature and concentration largely affect taste characteristics and organoleptic quality (Lee & Kader, 2000; Albertini et al., 2006; Kelebek et al., 2009). The results are in agreement with our previously reported citrus study of several mandarin and orange varieties (Cano et al., 2008; Bermejo et al., 2011a). Ascorbic acid changed significantly in five clones, three of which (G603, I208, D517) showed a higher ascorbic acid content than 'Murcott' mandarin control, which had 22.69 mg/100 mL of juice. Moreover, three organic acids were separated and identified in all clones: citric, malic and succinic acid. As indicated by previous researchers (Kelebek et al., 2009), citric acid was the major organic acid found in all clones (20.89-11.61 g L⁻¹), while malic and succinic acids were present in minor quantities. Thus, the content of organic acids followed a similar trend in all the clones studied, although several clones gave acid values that differed significantly from the 'Murcott' mandarin control (Table 3).

Flavonoid content. In the present study, the most abundant flavanone glycoside identified was narirutin, detected in the highest concentrations, followed by hesperidin, didymin, nobiletin and tangeretin. Narirutin, hesperidin and didymin, differed significantly in several clones and all of them presented higher concentrations than the wild-type 'Murcott' mandarin, whereas in nobiletin and tangeretin we did not find significant differences (Table 4). Flavonoids are widely distributed in fruits, and the highest concentrations found in *Citrus* sp.

correspond to flavanone glycosides, followed by flavones, flavonols and the fully polymethoxylated flavones (PMFs) (Ruiz *et al.*, 2005; Nogata *et al.*, 2006; Weber *et al.*, 2006; Mata-Bilbao *et al.*, 2007). Our results were comparable with several latest reports in mandarin and oranges varieties, and our previous studies on the pulp and rind of orange and mandarin species indicated that the Satsume group present the highest amounts of flavanone glycosides, hesperidin and narirutin, compared to the other varieties studied (Cano *et al.*, 2008; Bermejo *et al.*, 2011a,b; Rojas-Argudo *et al.*, 2012).

Carbohydrate content. The main portions of carbohydrates in citrus fruits are three simple sugars: fructose, glucose and sucrose, they represent the largest percentage of total soluble solids of citrus juice, and the ratios of fructose:glucose:sucrose are generally about 1:1:2 (Kelebek *et al.*, 2009). This ratio was similar for the irradiated 'Murcott' clones under study, and sucrose was present in the largest amounts for all clones (about 96.13 and 127.56 g L⁻¹), although fructose, glucose and sucrose differed significantly in several clones compared to the control (Table 5).

Carotenoid content. All clones presented similar β cryptoxanthin and β -carotene concentrations than the wild-type 'Murcott', with significantly different values for several clones (Table 5). Our results were comparable with several reports. The most abundant carotenoids reported in *Citrus* sp. are β -carotene, β -cryptoxanthin, lutein and zeaxanthin, all of them with antioxidant activity (Pupin *et al.*, 1999; Dhuique-Mayer *et al.*, 2005).

Table 3. Total ascorbic acid (mg/100 mL juice) and organic acids (g L^{-1} juice) content in severa
Murcott' clones. Data are expressed as mean ± standard deviation: data of organic acids are
mean $(n = 4)$ of 2009/2010 season and data of ascorbic acid are mean $(n = 8)$ of 2008/2009 and
2009/2010 seasons

Clone	Ascorbic acid	Citric acid	Malic acid	Succinic acid
B303	24.00 ± 1.15	14.06 ± 1.85*	$4.05 \pm 0.40*$	2.61 ± 0.10
B418	$20.13 \pm 0.98*$	$16.38 \pm 0.40 *$	4.31 ± 0.17	2.51 ± 0.05
C508	24.77 ± 1.33	18.27 ± 0.95	$4.11 \pm 0.40*$	$2.40 \pm 0.04*$
D517	$24.90 \pm 3.37*$	$12.94 \pm 0.44*$	4.27 ± 0.14	$2.31 \pm 0.03*$
I208	25.36 ± 3.58*	20.89 ± 1.90	4.68 ± 0.33	2.61 ± 0.08
K115	24.67 ± 2.72	$14.94 \pm 0.17*$	$3.49 \pm 1.06*$	$2.42 \pm 0.05*$
K718	24.70 ± 2.62	$14.67 \pm 0.42*$	4.84 ± 0.23	2.51 ± 0.04
M615	20.58 ± 1.20	$11.61 \pm 0.66*$	$3.61 \pm 0.17*$	$2.44 \pm 0.05*$
N306	24.65 ± 2.36	$12.84 \pm 0.46 *$	4.38 ± 0.44	$2.43 \pm 0.02*$
N235	$20.53 \pm 1.89*$	19.09 ± 1.12	4.42 ± 0.28	2.55 ± 0.07
G603	$25.73 \pm 0.78*$	$16.39 \pm 0.33*$	$3.37 \pm 1.49*$	2.49 ± 0.03
Control ^x	22.69 ± 1.63	19.44 ± 1.55	4.96 ± 0.09	2.57 ± 0.12

*: Clones with significantly different values compared to the control. ^x: 'Murcott' mandarin control.

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Narirutin	Hesperidin	Didymin	Nobiletin	Tangeretin
18.97 ± 3.78*	12.10 ± 1.64*	3.18 ± 0.74	0.84 ± 0.46	0.56 ± 0.20
15.30 ± 0.73	9.17 ± 2.64	2.98 ± 0.30	0.88 ± 0.58	0.65 ± 0.31
$21.42 \pm 1.82*$	$12.89 \pm 3.48*$	$4.10 \pm 0.22*$	0.81 ± 0.56	0.68 ± 0.53
14.40 ± 1.87	7.61 ± 1.71	2.58 ± 0.45	0.70 ± 0.44	0.61 ± 0.35
$21.87 \pm 5.49*$	$11.97 \pm 1.53*$	$3.61 \pm 1.04*$	0.96 ± 0.40	0.70 ± 0.21
$19.92 \pm 7.81*$	$12.31 \pm 2.80*$	$3.59 \pm 1.46*$	0.99 ± 0.51	0.61 ± 0.25
15.28 ± 1.35	9.20 ± 1.94	2.57 ± 0.33	0.70 ± 0.58	0.56 ± 0.36
$19.00 \pm 0.35*$	9.26 ± 2.51	$3.92\pm0.26*$	1.30 ± 0.59	0.80 ± 0.34
16.01 ± 2.17	8.89 ± 2.97	2.81 ± 0.20	0.69 ± 0.32	0.59 ± 0.26
$17.38 \pm 2.16*$	$13.77 \pm 2.43*$	$3.77 \pm 0.32*$	0.65 ± 0.25	0.54 ± 0.17
$16.17 \pm 5.72*$	7.58 ± 1.49	$3.25 \pm 1.02*$	1.16 ± 0.60	0.80 ± 0.40
12.28 ± 1.02	7.59 ± 1.36	2.51 ± 0.12	0.79 ± 0.46	0.68 ± 0.32
	Narirutin $18.97 \pm 3.78^*$ 15.30 ± 0.73 $21.42 \pm 1.82^*$ 14.40 ± 1.87 $21.87 \pm 5.49^*$ $19.92 \pm 7.81^*$ 15.28 ± 1.35 $19.00 \pm 0.35^*$ 16.01 ± 2.17 $17.38 \pm 2.16^*$ $16.17 \pm 5.72^*$ 12.28 ± 1.02	NarirutinHesperidin $18.97 \pm 3.78^*$ $12.10 \pm 1.64^*$ 15.30 ± 0.73 9.17 ± 2.64 $21.42 \pm 1.82^*$ $12.89 \pm 3.48^*$ 14.40 ± 1.87 7.61 ± 1.71 $21.87 \pm 5.49^*$ $11.97 \pm 1.53^*$ $19.92 \pm 7.81^*$ $12.31 \pm 2.80^*$ 15.28 ± 1.35 9.20 ± 1.94 $19.00 \pm 0.35^*$ 9.26 ± 2.51 16.01 ± 2.17 8.89 ± 2.97 $17.38 \pm 2.16^*$ $13.77 \pm 2.43^*$ $16.17 \pm 5.72^*$ 7.58 ± 1.49 12.28 ± 1.02 7.59 ± 1.36	NarirutinHesperidinDidymin $18.97 \pm 3.78^*$ $12.10 \pm 1.64^*$ 3.18 ± 0.74 15.30 ± 0.73 9.17 ± 2.64 2.98 ± 0.30 $21.42 \pm 1.82^*$ $12.89 \pm 3.48^*$ $4.10 \pm 0.22^*$ 14.40 ± 1.87 7.61 ± 1.71 2.58 ± 0.45 $21.87 \pm 5.49^*$ $11.97 \pm 1.53^*$ $3.61 \pm 1.04^*$ $19.92 \pm 7.81^*$ $12.31 \pm 2.80^*$ $3.59 \pm 1.46^*$ 15.28 ± 1.35 9.20 ± 1.94 2.57 ± 0.33 $19.00 \pm 0.35^*$ 9.26 ± 2.51 $3.92 \pm 0.26^*$ 16.01 ± 2.17 8.89 ± 2.97 2.81 ± 0.20 $17.38 \pm 2.16^*$ $13.77 \pm 2.43^*$ $3.77 \pm 0.32^*$ $16.17 \pm 5.72^*$ 7.58 ± 1.49 $3.25 \pm 1.02^*$ 12.28 ± 1.02 7.59 ± 1.36 2.51 ± 0.12	NarirutinHesperidinDidyminNobiletin $18.97 \pm 3.78^*$ $12.10 \pm 1.64^*$ 3.18 ± 0.74 0.84 ± 0.46 15.30 ± 0.73 9.17 ± 2.64 2.98 ± 0.30 0.88 ± 0.58 $21.42 \pm 1.82^*$ $12.89 \pm 3.48^*$ $4.10 \pm 0.22^*$ 0.81 ± 0.56 14.40 ± 1.87 7.61 ± 1.71 2.58 ± 0.45 0.70 ± 0.44 $21.87 \pm 5.49^*$ $11.97 \pm 1.53^*$ $3.61 \pm 1.04^*$ 0.96 ± 0.40 $19.92 \pm 7.81^*$ $12.31 \pm 2.80^*$ $3.59 \pm 1.46^*$ 0.99 ± 0.51 15.28 ± 1.35 9.20 ± 1.94 2.57 ± 0.33 0.70 ± 0.58 $19.00 \pm 0.35^*$ 9.26 ± 2.51 $3.92 \pm 0.26^*$ 1.30 ± 0.59 16.01 ± 2.17 8.89 ± 2.97 2.81 ± 0.20 0.69 ± 0.32 $17.38 \pm 2.16^*$ $13.77 \pm 2.43^*$ $3.77 \pm 0.32^*$ 0.65 ± 0.25 $16.17 \pm 5.72^*$ 7.58 ± 1.49 $3.25 \pm 1.02^*$ 1.16 ± 0.60 12.28 ± 1.02 7.59 ± 1.36 2.51 ± 0.12 0.79 ± 0.46

Table 4. Flavonoids content (mg/100 mL juice) in several 'Murcott' clones of 2008/2009 and 2009/2010 seasons. Data are expressed as mean \pm standard deviation (n = 7)

^x: Clones with significantly different values compared to the control. ^x: 'Murcott' mandarin control.

Extracts of citrus fruit peel and pulp are an important and complex source of carotenoids, which are natural pigments, in considerable amounts and in all species. β -cryptoxanthin and β -carotene were identified on the basis of comparing their retention times and absorption spectrum characteristics with corresponding authentic standards (Cortés *et al.*, 2004; Bermejo *et al.*, 2011b).

Influence of the temperature on seedless citrus production

This study investigates the influence of temperature during flowering, on the number of seeds formed.

Findings indicate that low temperatures during flower formation decreased pollen germination and seed number. Prevailing ambient temperature during the reproductive phase is an important factor affecting seed and fruit set in different plant species. Under low temperature conditions, the percentage of pollen grain germination is reduced as the result of a drop in starch and sugar concentrations accumulated in pollen grains at the last stages of development (Shaked *et al.*, 2004). Previously we had observed that low temperatures during flower formation decreased in vitro pollen germination and seed number. In particular, pollen viability is more closely related to temperatures during the

Table 5. Content of carbohydrates (g L⁻¹ juice) and carotenoids (mg L⁻¹ juice) in several 'Murcott' clones. Data of sugar composition are mean \pm standard deviation (n = 7) of 2008/2009 and 2009/2010 seasons, and data of carotenoids are mean (n = 4) of 2009/2010 season

Clone	Fructose	Glucose	Sucrose	β-cryptoxanthin	β-carotene
B303	38.28 ± 2.44	32.93 ± 2.17	117.89 ± 7.68	9.11 ± 1.92*	0.35 ± 0.09
B418	39.69 ± 6.16	37.69 ± 8.96	107.10 ± 17.80	10.23 ± 1.79	0.37 ± 0.07
C508	37.98 ± 2.52	34.31 ± 1.78	127.56 ± 8,73*	10.05 ± 1.86	0.41 ± 0.07
D517	$44.98 \pm 5.29*$	$41.70 \pm 5.85*$	119.35 ± 11.09	$7.71 \pm 0.24*$	$0.25 \pm 0.03*$
I208	$41.54 \pm 2.67*$	40.13 ± 4.46	111.42 ± 10.30	$8.42 \pm 0.74*$	$0.28\pm0.02*$
K115	33.33 ± 5.10	29.83 ± 4.07	$96.13 \pm 4.64*$	$7.76 \pm 0.20*$	$0.25 \pm 0.03*$
K718	$42.49 \pm 3.98*$	$41.43 \pm 5.26*$	$101.93 \pm 10.29*$	10.64 ± 0.28	0.40 ± 0.10
M615	38.96 ± 7.34	32.72 ± 7.46	110.87 ± 18.67	10.63 ± 0.26	0.34 ± 0.10
N306	45.17 ± 3.54*	$42.62 \pm 3.50*$	116.48 ± 18.26	9.15 ± 1.83*	0.33 ± 0.08
N235	37.78 ± 3.99	34.10 ± 3.86	101.20 ± 11.95	11.64 ± 0.71	0.39 ± 0.02
G603	33.14 ± 5.08	30.83 ± 5.30	98.57 ± 17.02	$8.43 \pm 0.73^{*}$	$0.28\pm0.02*$
Control ^x	36.29 ± 3.97	34.61 ± 6.51	112.31 ± 8.69	11.50 ± 0.53	0.39 ± 0.02

*: Clones with significantly different values compared to the control. x: 'Murcott' mandarin control.

		Seed number		
lowering period	Temperature (°C) –	Clone J	Clone N306	
ch to 15 May 2003	9.4	9.0	_	
l to 20 May 2004	7.7	6.8	0.9	
ch to 10 May 2005	10.6	10.5	3.3	
ch to 30 April 2006	11.1	9.6	2.9	
ch to 25 April 2007	8.5	8.1	2.5	
ch to 25 April 2008	9.0	9.2	2.8	
ch to 05 May 2009	8.2	7.2	1.2	
ch to 25 April 2007 ch to 25 April 2008 ch to 05 May 2009	8.5 9.0 8.2	8.1 9.2 7.2		

Table 6. Minimum temperatures (average) recorded during the flowering period of each year and seed number during 2003 to 2009 years. Data of seed number are mean of 20-30 fruits per year

green and white button stages (stages BBCH 55 and 56 on the Citrus BBCH phenological scale (Agustí *et al.*, 1997)) than temperatures recorded during the flower formation process itself. The number of ovules potentially developing into seeds was also affected by low temperatures and decreased when temperatures were remarkably low during flowering (Pardo *et al.*, 2007, 2010).

For 7 years, minimum temperatures (average) were recorded during the flowering period of each year (see Table 6). After full fruit development and maturation, all fruits of the 'Murcott' mandarin control and clone N306 (3 adult trees cultivar⁻¹) were harvested and the seeds per fruit were counted. Fig. 1 shows a high correlation between minimum temperatures recorded during the flowering period and seed number. Thus, the low temperatures affect negatively the number of seeds formed.

As conclusions, all irradiated clones examined presented lower seed numbers and reduced pollen germination compared to the control. Fruit quality and nutritional bio-components were affected differently; some clones presented no changes compared to the control 'Murcott' mandarin, while other clones



Figure 1. Correlation between the minimum average temperatures during the flowering period of seven consecutive years and the number of seeds in two clones of Murcott

showed significant differences. One clone (G603), which demonstrate excellent traits regarding seed formation and fruit quality, including fruit size, internal quality, good rind colour and easy peeling, is in the process of being registered. Clone G603 is similar to control in weight and size but its maturity index is lower than the 'Murcott' mandarin control throughout the ripening period. Regarding nutritional quality, clone G603 is particularly different from 'Murcott' mandarin, showing higher ascorbic acid and flavonoid contents. This clone fulfilled the main requirements of our breeding program: low seed number and late maturing, as well as maintaining or improving fruit quality. Some clones with few or no seeds are still the subject of further study. In conclusion, budwood irradiation is a suitable technique to improve cultivars, produce seedless cultivars, adjust ripening time or raise the content of health-promoting compounds.

Also this study investigates the influence of temperature during flowering on the number of seeds formed. Although further experiments with other genotypes, possibly under greenhouse and controlled conditions, are required, it can be concluded that, in general, cold springs cause a decrease in seed number.

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