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Artigo/Article

Effects of temperature and phytohormones treatments on anthocyanins accumulation in *Citrus sinensis* cv. Sanguinelli

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SUMMARY

Recently is accepted that flavonoids present in citrus fruits are relevant by their potential health-promoting effects, as suggested by the available experimental and epidemiological evidence. The beneficial biological effects of these bioactives compounds may be driven by their antioxidant activity, and they may regulate different signalling pathways involved in cell survival, growth and differentiation. Blood oranges are characterized by the presence of higher amounts of these health promoting substances as flavanones, flavones and anthocyanins. In present study, the evolution of anthocyanin content in juice of cv Sanguinelli during maturation phase are analyzed. This cultivar is one of the citrus fruits that present greater colour in the fruit, due to the presence of the anthocyanins cyanidin 3-glucoside, cyanidin 3-(6´´malonyl glucoside) and peonidin 3-(6´´malonyl glucoside), compounds that accumulate mainly in the pulp. To deepen the knowledge about the regulation of the biosynthesis of these phenolic compounds in citrus fruits, it has been determined, on the one hand, how the environmental temperature affects the accumulation of total anthocyanin in immature fruits harvested. On the other hand, it has been analyzed the effect of the application of different phytoregulators on the biosynthesis of anthocyanins in these fruits. The results reveal that kinetin increases the accumulation of these secondary metabolites, whereas methyl jasmonate and abscisic acid inhibit the total anthocyanin content. These results can define the future strategies in order to regulate anthocyanin biosynthesis and to increase the content of these bioactive compounds in the fruits.

Index terms: cyanidin 3-glicoside, cianidin 3-(6´´malonyl glucoside), peonidin 3-(6´´malonyl glucoside), kinetin.

Efeitos da temperatura e fitohormônios na acumulação de antocianinas em *Citrus sinensis* cv. Sanguinelli

RESUMO

Recentemente, aceita-se que os flavonoides presentes nas frutas cítricas são relevantes por seus possíveis efeitos favoráveis à saúde, conforme sugerido pela evidência experimental e epidemiológica disponível. Os efeitos biológicos benéficos destes compostos bioativos podem ser

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conduzidos pela sua atividade antioxidante e podem regular diferentes vias de sinalização envolvidas na sobrevivência, crescimento e diferenciação celular. As laranjas sanguíneas são caracterizadas pela presença de maiores quantidades dessas substâncias favoráveis à saúde como flavononas, flavonas e antocianinas (Fallico et al, 2017). No presente estudo, analisou-se a evolução do teor de antocianinas no suco da laranja Sanguinelli durante a fase de maturação. Esta cultivar é uma das frutas cítricas que apresentam maior cor na fruta, devido à presença de antocianinas cyanidin 3-glucoside, cyanidin 3-(6´´malonyl glucoside) e peonidin 3-(6´´malonyl glucoside), compostos que se acumulam principalmente na polpa. Para aprofundar o conhecimento sobre a regulação da biossíntese desses compostos fenólicos em cítricos, foi determinado, por um lado, como a temperatura ambiental afeta o acúmulo de antocianina total em frutos colhidos imaturos. Por outro lado, analisou-se o efeito da aplicação de diferentes fitorreguladores na biossíntese de antocianinas nestas frutas. Os resultados revelam que a cinetina aumenta a acumulação desses metabólitos secundários, enquanto que o jasmonato de metila e o ácido abscisico inibem o conteúdo total de antocianinas. Esses resultados podem definir as estratégias futuras para regular a biossíntese de antocianinas e aumentar o conteúdo desses compostos bioativos nos frutos.

Termos de indexação: cyanidin 3-glicoside, cianidin 3-(6´´malonyl glucoside), peonidin 3-(6´´malonyl glucoside), cinetina.

INTRODUCTION

The genus Citrus is characterized by accumulating phenolic compounds with proven beneficial effects for human health, which have been recognized in innumerable studies. Within the sweet oranges [*Citrus sinensis* (L.) Osbeck], we find different varieties called red or sanguine oranges, whose name is due to the accumulation in their peel and pulp of reddish pigments water soluble called anthocyanins. Also contains elevated quantities of various compounds including, hydroxycinnamic acids, and ascorbic acid, carotenoids, and different flavonoids as flavanones, flavones and polymetoxiflavones. Anthocyanins are a class of flavonoids responsible for the pigmentation of many red fruits and including blood oranges. It is worth mentioning its enormous activity as antioxidants, recognized in innumerable studies, in addition they are free radical scavengers, and they present antilipoperoxidantes activity, inhibitors of carcinogenesis process, as well as anti-inflammatory and antiatherogenic properties (Barreca et al., 2011; Barreca et al., 2013; Chaudhary et al., 2012; Del Río et al., 2004; Di Donna et al., 2013; Nogata et al., 2006).

The investigation on chemical composition of blood oranges has grown in recent decades; studies have been carried out concerning the profile and level of anthocyanins (Maccarone et al, 1983; Maccarone et al., 1985), vitamin C (Rapisarda et al., 2001), hydroxycinnamic acids (Fallico et al., 1996; Rapisarda et al., 1998). The major anthocyanins of the blood orange juice have been characterized as cyanidin 3-glucoside, cyanidin 3-(6´´- malonyl glucoside) and several minor anthocyanins (Hillebrand et al., 2004).

In plants, the anthocyanin biosynthesis is probably the most studied plant secondary metabolite pathway. Genes have been isolated for almost every biosynthetic step and a considerable body of knowledge is available on the mechanisms that regulate their expression within the plant cell (Schwinn & Davies, 2004; Andersen & Jordhein, 2006; Davies & Schwinn, 2006). In Citrus, the anthocyanin biosynthetic pathway is relatively well characterized and the expression pattern of structural genes has been investigated in Citrus fruits (Lo Piero et al., 2005; Licciardello et al., 2008; Cultrone et al., 2010; Wang et al., 2016).

Anthocyanins can be modulated by a variety of environmental stimuli, including developmental signals, plant hormones, and environmental stresses such as light, temperature and irrigation (Boss & Davies, 2009). In some plants, the anthocyanin accumulation is enhanced by a plant hormone, ABA (Kataoka et al., 1982; Ban et al., 2003) and suppressed by synthetic auxins (Ban et al., 2003; Davies et al., 1997; Wang et al., 2015). Temperature is another important environmental factor that influences anthocyanin synthesis. Generally, low temperatures, such as 25 °C, increase the anthocyanin biosynthesis, whereas high temperatures, such as 35 °C are associated with anthocyanin degradation and inhibition of anthocyanin accumulation (He et al., 2010).

This work presents a study of the development of the anthocyanin contents in fruit pulp of Sanguinelli orange; Characterization of the different anthocyanins present in fruits; Influence of environmental temperature on the accumulation of anthocyanins in immature fruits harvested and the effect of the application of different phytoregulators on the total content of anthocyanins in juice. The objective is to establish new cultivation programs that allow the commercialization of citrus fruits with higher contents in these bioactive compounds beneficial to human health.

MATERIAL AND METHODS

Plant material

To perform this study, fruits of *C. sinensis* cv. Sanguinelli were used. The cultivar Sanguinelli was originated in Spain by spontaneous mutation of Doble Fina orange. For the realization of the present study, 5 adult trees of about 20 years of age were used. All of them are grafted on Cleopatra mandarin (*C. reshni* Hort. Ex Tanaka), to a planting frame of 5 x 4 m. All trial trees are located on the experimental farm of the Institute of Agricultural and Food Research (IMIDA), in La Alberca (Murcia, Spain). Monthly samples of the five trees were taken, each tree being considered as a sampling unit, during a natural year, to determine the changes in anthocyanins during fruit development. Other cultivars analyzed were cv. Tarocco Messina (TM); cv. Entrefina (E); cv. Murtera (MU); cv. Sangre Oval (SO); cv. Tarocco (T); cv. Maltaise demi Sanguine (MD); cv. Moro Catania (M); cv. Maltaise Blonde (MB); cv. Navel Sangre (NS); cv. Tarocco Rosso (TR), located on the experimental farm IVIA, Valencia, Spain.

Extraction, identification and quantification of anthocyanins

For the extraction of anthocyanins, 8 fruits were squeezed and the juice was homogenized. Then, it was centrifuged for 30 minutes at 1500 rpm and filtered through glass wool. Once the juice has been centrifuged and filtered, an aliquot of 500 μl of juice is taken and mixed with 9.5 ml of methanol with HCl (1% v/v). To quantify total anthocyanins, the absorbance of the filtrate was measured with a UNICAM UV500 spectrophotometer at 530 nm, corresponding to its maximum absorption (Rapisarda et al., 2000). To quantify the amount of anthocyanins present in samples, a standard straight line was made with different concentrations of known sample and we checked the response obtained. The standard used was cyanidin 3-glucoside. In this way the corresponding conversion coefficients were obtained to determine the amount of anthocyanins present in each sample.

Major anthocyanins were identified by reverse phase high pressure liquid chromatography (HPLC). This time, the elution method was different: A solution A, formed by H_2O at pH 2 (reduced with formic acid) and solution B, composed by acetonitrile (ACN) was used as the mobile phase. The different anthocyanins were separated by gradient elution, with an initial composition of 100% (A) for 3 min, increasing the gradient to 70% (A) and 30% (B) during the next 30 minutes. Then, it is increased to 90% (B) for 5 min, to return to the initial conditions. The flow rate was 1 ml min^{-1}. The changes in absorbance at 280 nm and at 520 nm were detected by the diode array detector. Concentrations of the metabolites were determined by the area given the integrator using the response factor of the different standards. The identification of the different compounds was based on the retention time, UV/Vis absorption spectra, rupture pattern (HPLC-MS / MS) and exact mass (HPLC-DAD-TOF).

Effect of environmental temperature on the accumulation of anthocyanins

For study the effect of temperature on anthocyanin biosynthesis in immature fruits, three samples of 45 fruits were harvested in mid-November and each sample was kept in the chamber at one of the following temperatures: 22, 11 and 4 ° C. Each week the total anthocyanin content in 9 fruits of each sample was determined.

Effect of plant growth regulator on anthocyanins content in juice

In order to study how some plant hormones affect anthocyanin biosynthesis in Sanguinelli, in the middle of December, 10 fruits were harvested per treatment. Each sample was treated with the following growth regulators: kinetin, methyl jasmonate and abscisic acid at a concentration of 100 μM. Each phytoregulator was infiltrated in vacuum for 3 hours, dried with paper and stored at room temperature. Seven days after treatment, the total content of anthocyanins in the juice was determined.

RESULTS AND DISCUSSION

Anthocyanins content in juice of different cultivar of blood orange

The study of total anthocyanin content in juice from the major cultivars of blood-grown oranges cultivated in Europe has been carried out. Figure 1 shows the contents of total anthocyanins in different cultivars harvested in mid-November. Of the 11 cultivars analyzed, two of them, stand out over the others, these are cv. Moro Catania and the cv. Sanguinelli, with total anthocyanin concentrations of 31.6 and 30.4 ppm respectively, followed very far by cv. Tarocco Rosso, with 6.9 ppm. Similar results have been described by other authors indicating that anthocyanin biosynthesis depends on the genotype (Fabroni et al., 2016) Sanguinelli is the blood type orange most cultivated in Spain, and these results support the development of the cultivation of this citrus, as an important source of these compounds that bring so many benefits to human health.

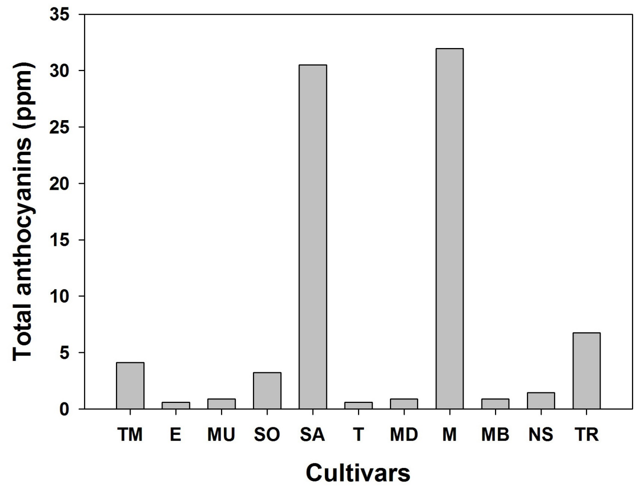

Figure 1. Contents in total anthocyanins of the juices coming from 11 cultivars of sanguine oranges [*C. sinensis* (L.) Osbeck]: cv. Tarocco Messina (TM); cv. Entrefina (E); cv. Murtera (MU); cv. Sangre Oval (SO); cv. Sanguinelli (SA); cv. Tarocco (T); cv. Maltaise demi Sanguine (MD); cv. Moro Catania (M); cv. Maltaise Blonde (MB); cv. Navel Sangre (NS); cv. Tarocco Rosso (TR) harvested in mid-November.

Evolution of the anthocyanins content in juice of Sanguinelli during the development and maturation phases

In order to select the optimum time of harvesting of fruits of this cultivar, the total anthocyanins content were determined from July to April of the following year. The results are shown in Figure 2. The synthesis of anthocyanins begins in mid-November, peaking at the end of February, the anthocyanin slowly decreasing to reach 129.31 ppm in early May. The maximum level of anthocyanins detected (153.33 ppm) are higher than those obtained by other authors for other blood orange varieties. In this sense, values ranging from 80 to 102 ppm for the cv. Moro and from 42 to 37 ppm for the cv. Tarocco has been described, obtaining the highest values at the beginning of March and reducing later (Rapisarda et al., 2000). This decrease observed from February coincides with the increase of the diurnal temperatures stimulates the anthocyanic degradation processes in the fruits (He et al., 2010; Cao et al., 2011).

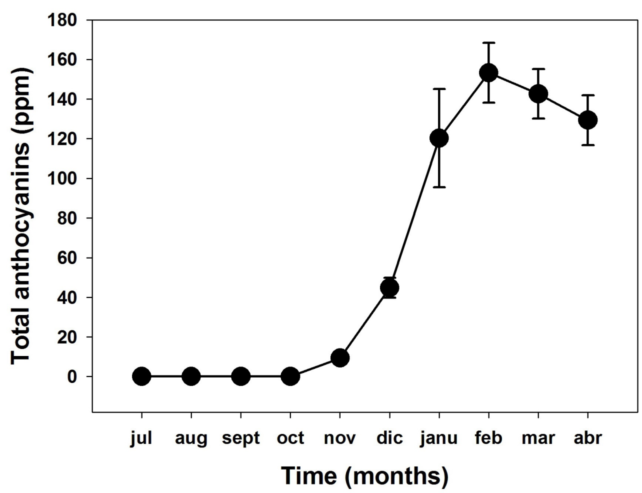

Figure 2. Evolution of the anthocyanins content in juice of the cultivars Sanguinelli during the development and maturation phases.

It has been described that anthocyanin content in fruits is depending on several abiotic conditions, month of harvest and cultivar (Dannehl & Josuttis, 2014; Gómez-Caravaca et al., 2014). In particular, all blood orange varieties require strong day-night thermal climes for intense colour formation in fruit flesh, and varieties such as Moro, with the potential for high pigmentation, are strongly dependent on the prevailing climatic conditions during fruit ripening for full colour development (Butelli et al., 2012).

Identification and distribution of principal anthocyanins present in different part of Sanguinelli fruits

The chromatogram obtained after the HPLC separation of a sample of Sanguinelli juice, using the chromatographic conditions described in Materials and Methods, is shown in Figure 3. In the chromatographic profile, three major peaks, coincident with the commercial standards of cyanidin 3-glycoside (1), cyanidin 3- (6´´-malonyl glucoside) (2) and peonidin 3- (6´´-malonyl glucoside) (3). The identity was confirmed by the coincidences in the rupture patterns when checking its mass spectrum obtained by HPLC-MS/MS. In other citrus fruits has been described that the cyanidin 3- (6´´-malonyl glucoside) is present in a concentration rather high in freshly squeezed juices, but it easily hydrolyses to cyanidin 3-glycoside in processed and stored juices (Scordino et al., 2015).

When analyze the different parts of fruits collected in February (Table 1), we observed that the highest accumulation of anthocyanins occurs in the pulp, followed by central column, albedo and flavedo. These results demonstrate that the edible part of the fruit contains the highest concentration of anthocyanins, which will impact on consumer health.

Effect of environmental temperature on anthocyanin accumulation

From the study of accumulation of anthocyanins with the development of the fruit (Figure 2) it follows that the biosynthesis of these secondary metabolites begins in the middle of November period in which the environmental temperatures decrease. In this section we study the effect of different environmental temperatures on the capacity to accumulate anthocyanins in the pulp of fruits harvested during a period of 28 days.

Figure 4 shows the progress curves of anthocyanins accumulation in immature fruits harvested in November. It is observed that the rate of biosynthesis decreases as the ambient temperature falls, within the range tested. It has been reported that the temperature is important environmental factor that influences anthocyanin synthesis (Tarara et al., 2008). Generally, low temperatures, such as 25 °C, favour the anthocyanin biosynthesis, whereas high temperatures, such as 35 °C are associated with anthocyanin degradation and inhibition of anthocyanin accumulation. In grapes, high night temperatures inhibit genes expressions that are associated with biosynthesis of anthocyanins (Mori et al., 2005; Yamane et al., 2006). In citrus, the anthocyanin content in the fruit juice of

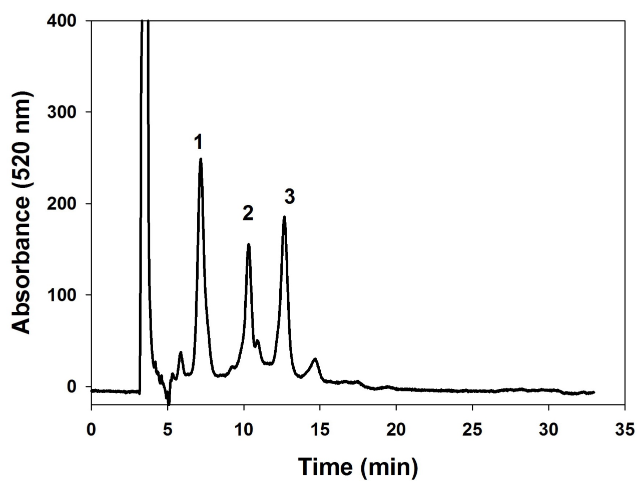

Figure 3. Chromatographic profile of anthocyanins present in fruit juice of *Citrus sinensis* cv. Sanguinelli. (1) cyanidin 3-glycoside, (2) cyanidin 3- (6´´-malonyl glucoside) and (3) peonidin 3- (6´´-malonyl glucoside).

Table 1. Concentrations of the main anthocyanins in different parts of fruit of *Citrus sinensis* cv. Sanguinelli harvested in February

Fruits part	**Cyanidin 3-glucoside** (ppm)	**Cyanidin 3-(6´´malonyl glucoside)** (ppm)	**Peonidin 3-(6´´malonyl glucoside)** (ppm)
Pulp	65.5 ± 5.3	36.1 ± 3.8	44.9 ± 4.7
Central column	28.1 ± 2.9	16.8 ± 3.0	20.3 ± 3.7
Albedo	11.2 ± 2.1	6.1 ± 1.8	7.3 ± 1.3
Flavedo	3.2 ± 0.9	1.8 ± 0.3	3.6 ± 0.9

Data show mean ± SE; n = 3.

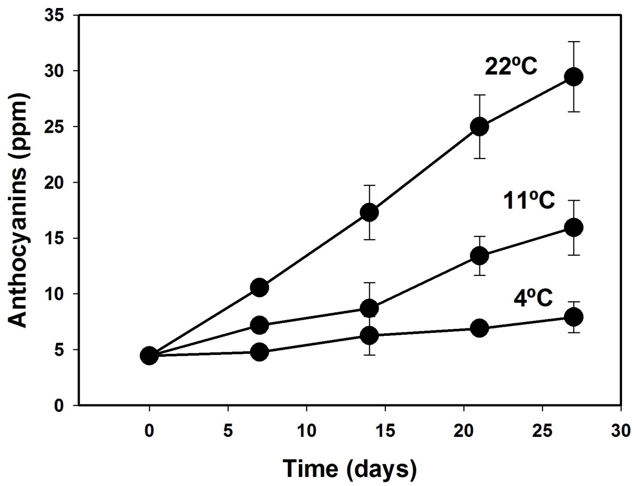

Figure 4. Effect of enviromental temperature on anthocyanin accumulation in juice of fruits of C. *sinensis* cv. Sanguinelli harvested in november.

Figure 5. Effect of plant growth regulator (100 μM) on anthocyanins content in juice of fruits of C. *sinensis* cv. Sanguinelli harvested in november. MJ: methyl jasmonate; K: kinetin; ABA: abscisic acid.

Tarocco was increased rapidly during cold storage at 10 °C for 70 days (Lo Piero et al., 2005; Wang et al., 2016).

Effect of plant growth regulator on anthocyanins content in juice

There are numerous scientific contributions that indicate that the biosynthesis of anthocyanins in different plant materials can be regulated by plant hormones . However, the effect of growth regulators on anthocyanin expression has not been described in citrus fruits. This experiment describes the results obtained in the treatments with three hormones: kinetin, abscisic acid and methyl jasmonate on the total content of anthocyanins in juice of Sanguinelli. Fruits harvested in mid-December were treated with 100 μM of the three hormones described, as described in Materials and Methods. Of all the compounds tested, the one that has shown better results in the accumulation of anthocyanins in pulp in fruits harvested in December, has been the kinetin, phytohormone closely related to the cellular division, increasing about 5% the total content of anthocyanins (Figure 5). In other species it has also been described that cytokinin promotes anthocyanin accumulation in plants (Deikman & Hammer, 1995; Das et al., 2012), and similar results have been observed in the accumulation of other flavonoids (Margna & Vainjärv, 1983).

From these results we can conclude that the cv. Sanguinelli is one of the oranges with the highest anthocyanin content. Its concentration can be modified by controlling the ambient temperature and by treatments with certain phytoregulators such as kinetin. The consumption of citrus fruits with high anthocyanin content, in addition to other flavonoids present in this fruit, can provide numerous benefits for the health of the consumer, preventing a large number of cardiovascular diseases and the onset of certain types of cancer.

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