Effect of Benzylaminopurine on the Flavanones Hesperidin, Hesperetin 7-*O*-Glucoside, and Prunin in Tangelo Nova Fruits

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A study of the flavonoid composition of the fruit of tangelo Nova by high-performance liquid chromatography and nuclear magnetic resonance revealed the presence of hesperidin, as the major flavanone, together with hesperetin 7-*O*-glucoside and prunin. The maximum levels of these flavanones are associated with the logarithmic phase of fruit growth. Treatment of recently set fruit with 20 ppm of benzylaminopurine delayed the fall of hesperidin concentration and accelerated this process in hesperetin 7-*O*-glucoside and prunin, compared with the evolution of these compounds in control fruit. The possible effect of benzylaminopurine on the biosynthetic pathway of these flavanones and/or on their transport in this plant material is discussed.

Keywords: *Cell growth; citrics; cytokinins; flavonoid evolution; flavonoid regulators; growth regulators*

INTRODUCTION

In previous papers we showed that the degree of flavonoid expression in various *Citrus* species is related to certain stages of cell growth, the highest levels of these secondary metabolites being detected in very young tissues both in the fruit (Castillo et al., 1992, 1993; Benavente-García et al., 1993; Del Río and Ortuño, 1994; Ortuño et al., 1995) and in callus cultures (Del Río et al., 1992). These results agreed with those of other authors (Hasegawa and Maier, 1981; Jourdan et al., 1985; Vandercook and Tisserat, 1989; Berhow and Vandercook, 1991).

It is known that the developmental process in plants is regulated by the action and balance of the different groups of growth regulators, which may act as promotors or inhibitors of these processes. Cytokinins, for example, play an important role in the juvenile stages of fruit growth in *Citrus* (García-Martínez and García-Papí, 1979; Stewart and Barthe, 1984; Hernández Miñana et al., 1989). However, there is little available information concerning the possible involvement of these compounds in the secondary metabolism of plants (Coggins et al., 1969; Cho et al., 1988; Hinderer et al., 1984; Wilson et al., 1990; Shaw et al., 1991; Berhow and Vandercook, 1992; Ortuño et al., 1993; Cho and Harper, 1993; García Puig et al., 1993, 1995).

In light of the above, and as part of a wider study to characterize the biosynthethic pathway of these secondary compounds and their possible regulation in *Citrus*, we analyzed the effect of a cytokinin, benzylaminopurine, on flavonoid expression in the treated fruit of tangelo Nova.

MATERIALS AND METHODS

Plant Material and Hormonal Treatments. Tangelo Nova, a mandarin hybrid (*Citrus reticulata* B) × tangelo orlando (*C. reticulata* × *Citrus paradisi* Macf.), trees, located in the experimental plantation of the Centro Regional de Investigaciones Agrarias (Murcia) in Alhama (Murcia), were used. In May 1991, when 90% of the flower petals had fallen (coinciding with fruit set), 14 trees were selected and sprayed with an aqueous solution of 20 ppm of benzylaminopurine using 5 L/tree. The wetting agent used was polyethylene glycol at 0.1%. The same treatment was applied to other groups of 14 trees at 15, 43, and 73 days after anthesis. The corresponding controls were treated with a 0.1% aqueous solution of polyethylene glycol alone. In May 1992, following the procedures mentioned above, another 14 trees were selected and treated with successive applications of 20 ppm of benzylaminopurine. The first treatment involved the recently set fruit and the second the whole trees 15 days later. The corresponding controls were treated with a 0.1% aqueous solution of polyethylene glycol only. Of the 14 trees selected, 4 were used to obtain data concerning fruit diameter, while the rest were used to measure growth and cell parameters.

Measurement of Growth and Cellular Parameters. The equatorial diameters of the fruits left on the trees (50 per tree) were measured for four trees in each assay with a digimatic caliper (Mitutoyo, Tokyo) at different ages, and the mean values at each age were used to express the development of growth in the fruit. Twenty fruits were collected at each age from the remaining 10 trees (2/tree) in each assay and then mixed before being divided into two lots of 10 fruit for the fresh and dry weight measurements. Different cell parameters (cell diameter and cell density) of the flavedo tissue corresponding to control and treated fruits were analyzed by light microscopy. The conditions and procedures followed for the processing of the tissues to obtain the corresponding semithin sections were similar to those described in previous papers (Ortuño et al., 1990; Del Río et al., 1991, 1992). Cell size and cellular density were measured in these semithin sections using an ocular micrometer and an integration plate, respectively, coupled to a Photomicroscope II (Carl Zeiss, Oberkochem, Germany). In all cases, Student's *t*-test was used to evaluate the significance of differences in the mean values. Prior to the *t*-test, the data were transformed into a logarithmic function.

Isolation, Chromatographic Analysis, and Identification of Flavonoids. Three fruits per tree for each experiment (control and treated) were collected, mixed, and divided into three lots of 10 fruit before being dried at 50 °C to constant weight. The dried fruits were ground and shaken with dimethyl sulfoxide (DMSO) (6 mg of dry weight/mL) for 30 min for extraction. The corresponding extracts were filtered

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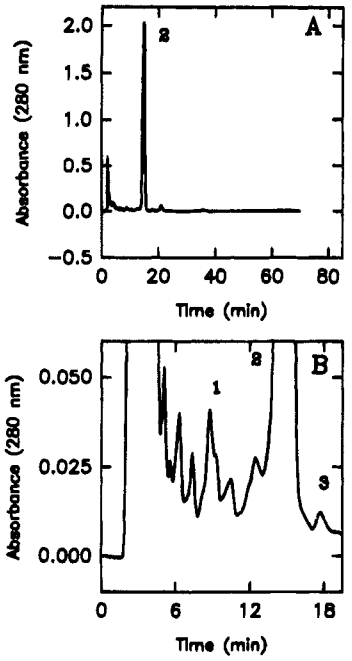

Figure 1. (A) HPLC elution profile of a dimethyl sulfoxide extract of immature tangelo Nova fruits. (B) Amplified profile. The column was eluted with water–methanol–acetonitrile–acetic acid (15:2:2:1 by volume) with a flow rate of 1 mL/min, at 35 °C. Eluent was monitored at 280 nm. Identification: 1, prunin; 2, hesperidin; 3, hesperetin-7-*O*-glucoside.

through a 0.45 μm nylon membrane before analysis by HPLC with a Beckman liquid chromatograph, a Model 110 B solvent delivery module, and a System Gold Module 168 diode array detector (Beckman Instruments, San Ramon, CA). Reversed phase chromatography was performed with a C_{18} analytical 7μ-Bondapak column (Waters Associates, Milford, MA) with an average particle size of 5 μm using isocratic elution with a water–methanol–acetonitrile–acetic acid (15:2:2:1 by volume) solution at a flow rate of 1 mL/min at 35 °C. The change in absorbance was monitored at 280 nm with a V/UV diode array detector. In these conditions the prunin, hesperidin, and hesperetin 7-*O*-glucoside standards showed retention times of 10.4, 14.7, and 17.7 min, respectively. For the isolation and purification of hesperidin the following procedure was used: 50 g of dried, ground fruit was extracted by shaking for 1 h with 500 mL of water (pH 11) with 3 N NaOH. The supernatant was filtered and the hesperidin precipitated after the pH was adjusted to 4 with 3 N HCl. The precipitate was crystallized in methanol and then analyzed by NMR (Mabry et al., 1970). The procedure described by Castillo et al. (1993) was followed in the isolation and purification of prunin and hesperetin 7-*O*-glucoside.

Chemicals. The reagents used were benzylaminopurine from Sigma; polyethylene glycol (commercial Mojasaf) from Safor, S.A., Spain; and prunin, hesperidin, and hesperetin 7-*O*-glucoside from Zoster, S.A., Spain.

RESULTS AND DISCUSSION

Identification of the Flavanones Present in Tangelo Nova Fruit. The flavonoid composition of tangelo Nova fruit has not been described. Figure 1A shows a typical chromatogram in which the presence of a major compound (compound **2**) is revealed with a retention time coinciding with that of the flavanone rutinoside hesperidin (R_t = 14.7 min). Two other minor compounds (compounds **1** and **3**, Figure 1B) are revealed with retention times of 10.4 and 17.7 min, coinciding with those of prunin and hesperetin 7-*O*-glucoside, respectively.

The absorption spectra of these compounds, obtained by means of a V/UV diode array detector, have two

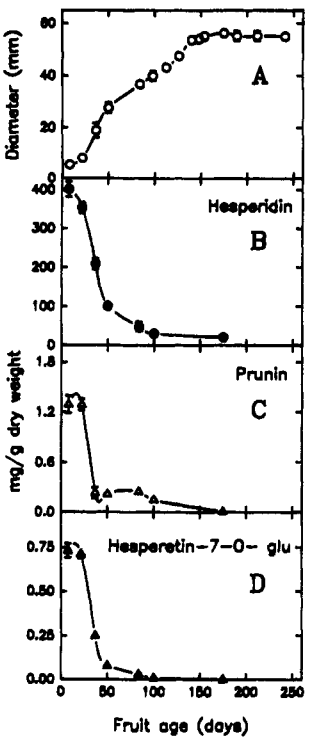

Figure 2. Evolution of equatorial diameter and flavanone content during the growth of tangelo Nova fruits. Growth data (A) were obtained from 100 representative fruits and the ±SE is indicated. The mean values of hesperidin (B), prunin (C) and hesperetin 7-*O*-glucoside (D) (mg/g of dry weight) are represented, and the vertical bars denote ±SE (n = 3), when larger than symbols.

maxima when eluted in the same solvent as used in Figure 1: at 283 and 329 nm for compound **1**, at 283 and 320 nm for compound **2**, and at 284 and 327 nm for compound **3**. These data are consistent with the compounds having flavanone skeletons identical to those of prunin, hesperidin and hesperetin 7-*O*-glucoside.

Compounds **1**–**3** were isolated according to the procedure described under Materials and Methods. The ^{1}H NMR and ^{13}C NMR spectra of compounds **1** and **3** were identical to those obtained by us in previous papers (Castillo et al., 1993) for prunin and hesperetin 7-*O*-glucoside, respectively. The corresponding spectra for compound **2** were similar to those obtained for the hesperidin standard.

Changes in the Levels of Hesperidin, Prunin, and Hesperetin 7-*O*-Glucoside during the Development of Tangelo Nova Fruit. Tangelo Nova fruit present a sigmoid growth curve in which three phases can be detected. The logarithmic phase includes the first 50 days following anthesis, while the linear phase extends from 50 to 150 days following anthesis, after which time the maturation phase begins (Figure 2A).

Hesperidin, the major flavanone of tangelo Nova fruit, reaches its highest levels during the exponential phase of fruit growth (400 mg/g of dry weight, Figure 2B) and then decreases during the linear phase of growth. These results agree with those obtained by us and other authors in other species of *Citrus* (Hasegawa and Maier, 1981; Jourdan et al., 1985; Vandercook and Tisserat, 1989; Berhow and Vandercook, 1991; Castillo et al., 1992, 1993; Del Río et al., 1992; Benavente-García et al., 1993; Del Río and Ortuño, 1994; Ortuño et al., 1995).

A similar evolution was noted for the other two flavanones detected in these fruit, prunin and hesperetin 7-*O*-glucoside (Figure 2C,D), whose levels repre-

Table 1. Cellular Characteristics of Exocarp of Tangelo Nova Fruits[a]

age of fruit (days)	cell size					
	A		B		C	
	I	II	I	II	I	II
0	9.9 ± 1.2	9.3 ± 1.5				
15	11.2 ± 0.9	9.6 ± 1.5	10.0 ± 0.8	9.6 ± 0.6		
43	9.4 ± 1.4	9.5 ± 1.4	11.2 ± 1.7	11.3 ± 1.4	11.4 ± 1.9	10.3 ± 2.3

age of fruit (days)	cell density		
	A	B	C
0	73.7 ± 7.1		
15	97.7 ● 9.9	96.7 ± 10.9	
43	86.7 ± 7.8	73.7 ± 4.8	90.6 ± 7.0

[a] Control (A) fruits were treated with 20 ppm of benzylaminopurine at 0 days (mean diameter of fruit 5.6 ± 1.0 mm) (B); these latter were treated again with 20 ppm of benzylaminopurine 15 days after the first treatment (C). Data correspond to the cellular size of the three cell layers nearest the epidermis (I and II indicate equatorial and polar cell diameters, respectively, in μm), and the cell density (number of cells /10^4 μm^2) for these same three cell layers. In all cases, the data represent the mean values of at least 40 measurements and the standard error is indicated.

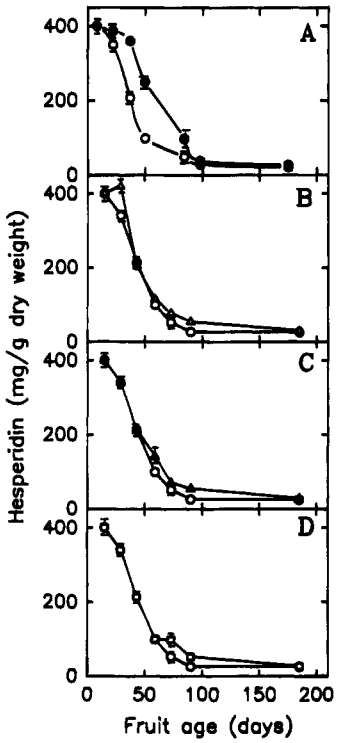

Figure 3. Effect of benzylaminopurine on the hesperidin levels present in tangelo Nova fruits. Data represent the mean values of hesperidin (mg/g of dry weight), and the vertical bars denote ±SE ($n = 3$), when larger than symbols. The treatments with 20 ppm of benzylaminopurine were carried out on the recently set fruit (A, ●), 15 (B, △), 43(C, ▲) and 73 (D, □) days after anthesis. Control (○).

sented no more than 0.6 and 0.2%, respectively, of the total flavonoid content.

Effect of Benzylaminopurine Treatments on the Flavanone Content in Tangelo Nova Fruits. The application of 20 ppm of benzylaminopurine to recently set fruit delays the fall in hesperidin concentration by approximately 40 days (Figure 3A), although it then decreases at a rate similar to that of the control fruit. When the same concentration of benzylaminopurine is applied to fruit at 15, 43, and 73 days, only slight increases with respect to the hesperidin levels of the control are noted between 8 and 13 days after application (parts B, C, and D of Figure 3, respectively). It is concluded, therefore, that the most effective application is that to the recently set fruit since no additional delays in the fall of hesperidin levels are caused if the same fruit are treated again with 20 ppm of benzyalminopurine (data not shown).

Although evidence exists to show that cytokinins are involved in fruit growth, particularly in the control of the cell division which is known to occur in young developing fruits (Wareing and Phillips, 1978), in the conditions assayed here treatment with benzylaminopurine does not stimulate cell division since optical microscopy shows no significant differences in cell size or packing compared with the control fruit (Table 1). Furthermore, macroscopic determination of growth shows no significant differences in fruit diameter or fresh and dry weight (data not shown).

We therefore consider that the influence on hesperidin synthesis might be linked to an increase in the enzymatic activities which take part in the biosynthetic pathway of this flavanone and/or to an increase in the quantity transported.

In support of the first hypothesis, other authors have also suggested that another growth regulator, gibberellic acid, might affect the activities of some of the enzymes involved in flavonoid biosynthesis (Hinderer et al., 1984). It has been suggested that cytokinins may act as direct regulators of enzyme activity rather than of enzyme synthesis (Wareing and Phillips, 1978), and they have also been found to affect the accumulation of phenolic compounds in fruits (Shulman and Lavee, 1971, 1973). Indeed, when the evolution of the other two flavanones in the extracts (prunin and hesperetin 7-*O*-glucoside) was analyzed, a different behavior was seen. Thus, while the application of benzylaminopurine favors the accumulation of hesperidin (Figure 3A), the concentration of the glucosyl flavanones falls after this cytokinin is applied to trees, as can be seen from Figures 4 and 5, in which the evolution of prunin and hesperetin 7-*O*-glucoside (milligrams per gram of dry weight), respectively, in fruits developed on control trees and on trees treated close to fruit set is depicted. The application of 20 ppm of benzylaminopurine almost halves the levels of prunin (Figure 4) during the exponential growth phase of the fruit (see Figure 2A). Similar results were obtained for the other flavanone, hesperetin 7-*O*-glucoside (Figure 5).

These results suggest that benzylaminopurine in a concentration of 20 ppm activates some of the enzymes which take part in the biosynthetic pathway of hesperidin. The fact that the levels of prunin fall might indicate an increase in hydroxylase activity (catalyzing the hydroxylation of naringin to give eriodictiol) and/or methyltransferase activity (catalyzing the methylation

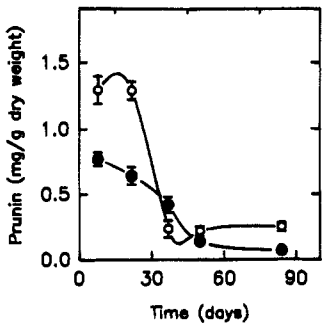

Figure 4. Effect of benzylaminopurine on the prunin levels present in tangelo Nova fruits. Data represent the mean values of prunin (mg/g of dry weight), and the vertical bars denote ±SE (n = 3), when larger than symbols. The treatment with 20 ppm of benzylaminopurine was carried out on the recently set fruit (●). Control (○).

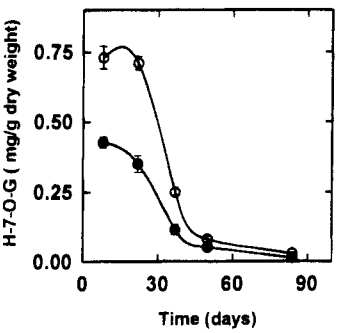

Figure 5. Effect of benzylaminopurine on the hesperetin 7-*O*-glucoside levels present in tangelo Nova fruits. Data represent the mean values of hesperetin 7-*O*-glucoside (mg/g of dry weight), and the vertical bars denote ±SE (n = 3), when larger than symbols. The treatment with 20 ppm of benzylaminopurine was carried out on the recently set fruit (●). Control (○).

of eriodictiol to give hesperidin). Furthermore, the fact that the levels of hesperetin 7-*O*-glucoside fall suggests that benzylaminopurine activates rhamnosyltransferase, an enzyme which catalyzes the transfer of rhamnose to hesperetin 7-*O*-glucoside to give hesperidin.

With regard to the second hypothesis mentioned above, it is known that cytokinins affect source–sink relations (Brenner, 1988), so there might be a possible effect on the transport of these compounds toward the fruit (the site of benzylaminopurine application), bearing in mind that the mobilization of these flavanones has already been described in various *Citrus* species both at plant level (Berhow and Vandercook, 1991; Castillo et al., 1992) and in cell and callus cultures (Gavish et al., 1989; Lewinsohn et al., 1989; Mansell and McIntosh, 1991; Del Río and Ortuño, 1994). Furthermore, there may be a specific transport of hesperidin into the fruit of this plant material when the other flavanones (prunin and hesperetin 7-*O*-glucoside) decrease, since, as we have observed in *Citrus aurantium*, rhamnoglucoside transport is favored in relation to the transport of monoglucosides and aglycons (data not shown).

In light of the above, we think that benzylaminopurine has a possible double effect on hesperidin expression in tangelo Nova, at the synthesis level (by modulation of the enzymatic activities involved) and/or in the modulation of the transport processes since, as we have ascertained in other *Citrus* species (data not shown), the flavonoid level in fruit not only depends on *in situ* synthesis but also on polarized transport processes.

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