Influence of Ethylene and Ethephon on the Sesquiterpene Nootkatone Production in *Citrus paradisi*

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The effect of ethylene and ethephon treatments on nootkatone accumulation in the rind of grapefruit was investigated. Considerable increases in the levels of this sesquiterpene were observed in the picked and unpicked grapefruits treated. The changes induced in the maturation–senescence stage of grapefruit by these treatments consisted of an accelerated carotenogenesis process in the rind, along with morphological changes in the exocarp and ultrastructural changes in the plastids. These results suggest that ethylene regulates nootkatone biosynthesis by accelerating the maturation–senescence processes in grapefruit rind.

INTRODUCTION

The synthesis of the sesquiterpene nootkatone has been correlated with the development of morphological differentiation in the cells of *Citrus paradisi* calli (Del Río et al., 1991) and the maturation–senescence process in the *C. paradisi* fruits (Del Río et al., 1992a). In addition, a high increase in nootkatone level was observed in this species after picking, probably due to an acceleration of the maturation–senescence process involved in the expression of this secondary metabolite (Del Río et al., 1992a).

It is known that maturation and senescence, like any other developmental process in plants, is regulated by the action and balance of the different groups of growth regulators, which may act as promoters or inhibitors of these processes (Rhodes, 1980; Frenkel, 1986). However, there is little available information on the possible involvement of these compounds in the secondary metabolism of plants (Coggins et al., 1969; Cho et al., 1988; Wilson et al., 1990; Shaw et al., 1991; Berhow and Vandercook, 1992).

As part of a wider study to characterize the biosynthesis of this terpenic compound and its possible regulation, the effect of different treatments with ethylene and ethylene-releasing compounds, such as ethephon [(2-chloroethyl)-phosphonic acid], on nootkatone production was studied along with possible changes induced by these treatments in the maturation–senescence stage of grapefruit.

MATERIALS AND METHODS

Plant Material and Hormonal Treatments. Forty grapefruit (*C. paradisi* L. cv. Star Ruby) from 8-year-old trees on *Citrus aurantium* L. rootstocks, located in the experimental plantation of the Centro Regional de Investigaciones Agrarias (Murcia) in Orihuela (Alicante), were used.

Twenty-eight weeks after anthesis, an age when nootkatone synthesis has still not started in grapefruit of this variety (Del Río et al., 1992a), 10 trees were sprayed with an aqueous solution of 200 ppm of ethephon using 5 L/tree. The wetting agent used was poly(ethylene glycol) at 0.1%. Another 10 trees acted as control and were treated only with an aqueous solution of poly-(ethylene glycol) at 0.1%. In other assays, 360 fruits from the remaining 20 trees in the plantation (18 fruits/tree) were picked at the same age as indicated; 90 were treated with 100 ppm of ethylene atmosphere for 48 h (with 90 control fruits left untreated), and 90 were treated with an aqueous solution of 480 ppm of ethephon for 30 min and maintained at room temperature. In this last case, the wetting agent was Triton X-100 at 0.1%, and the corresponding 90 control fruit were treated only with an aqueous solution of Triton X-100 at 0.1%.

Extraction and Measurement of Nootkatone. Samples were taken from the unpicked fruits 0 (immediately after treatment), 14, 28, 70, 98, and 126 days after the corresponding hormonal treatments. Harvested fruit samples were analyzed 4, 25, 35, 55, and 70 days after hormonal treatment. At each of the indicated ages, the rinds (flavedo plus albedo) of five grapefruit (picked and unpicked) were assayed. The samples were chopped into 0.5-cm pieces and mixed. Four grams of fresh weight (FW) of this mixture was used in each case for the analysis of the nootkatone by GLC–MS (Hewlett-Packard Model 5993) according to the procedure described previously (Del Río et al., 1992a).

Measurement of Ripening and Carotenoid Determination. Twenty unpicked grapefruit (2 per tree) corresponding to 2, 4, 10, and 14 weeks after treatment were assayed to measure total acidity (TA), as citric acid (grams per liter), and the index of ripeness, expressed as the ratio between total soluble solids (TSS, percent at 20 °C) and TA, both measured from the juice of the corresponding fruits according to the procedure described by Ortiz et al. (1987).

The carotenoids present in the rind of these fruits were isolated according to the method described by Ting and Rouseff (1986), using 30 g of tissue per assay chosen at random for subsequent spectrophotometric determination.

Microscopic Study. At different ages, rind segments (2 × 2 × 1 mm) corresponding to the grapefruits of the different assays were used to obtain the transverse (only exocarp) sections. The conditions and procedures followed for the processing of the tissues to obtain the corresponding semithin and ultrathin sections suitable for light and electron microscopy, respectively, were similar to those described in previous papers (Ortuño et al., 1990; Del Río et al., 1991). Oil cavity diameter and cell size were measured in semithin sections using an ocular micrometer coupled to a photomicroscope II (Carl Zeiss). The ultrathin sections were viewed with a Zeiss EM10 electron microscope (Carl Zeiss).

Chemicals. The reagents used were as follows: Nootkatone from Extrasynthèse, S. A. (Genay, France); ethephon [commercial Ethrel, 48% (2-chloroethyl)phosphonic acid] from Etisa (Barcelona); ethylene from Abello, Oxigeno-Linde, S. A. (Barcelona); Triton X-100 from Merck (FRG); poly(ethylene glycol) (commercial Mojasaf) from Safor, S. A. (Valencia, Spain).

RESULTS

Effect of Ethylene and Ethephon Treatments on the Accumulation of Nootkatone in Grapefruit.

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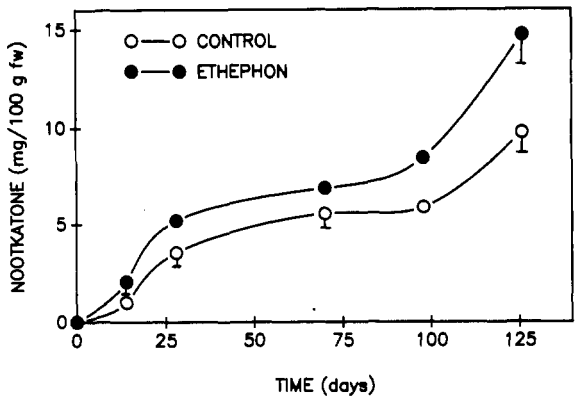

Figure 1. Nootkatone levels in the fruit of *C. paradisi* L. (var. Star Ruby) control and treated with 200 ppm of ethephon. The time refers to number of days since treatment. Vertical bars denote ± SE (n = 3), when larger than symbols.

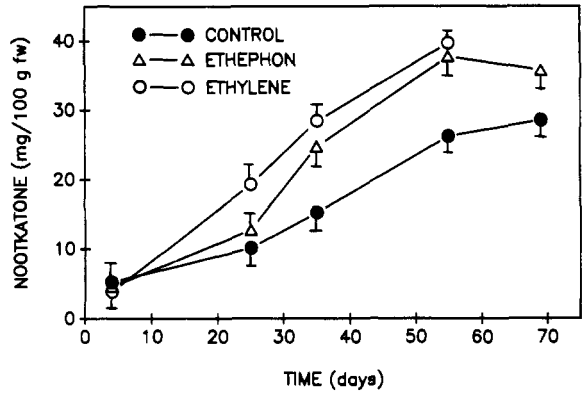

Figure 2. Nootkatone levels in picked fruits treated with 100 ppm of ethylene or 480 ppm of ethephon. The time refers to number of days since treatment. Data represent mean values ± SE (n = 3).

Figure 1 shows the nootkatone found in the rind of grapefruit (*C. paradis* L. var. Star Ruby) fruits left on the tree at different times after application of ethephon and the levels of nootkatone in the control fruits. The ethephon-treated trees (200 ppm) show higher levels of nootkatone at all ages, reaching increases of 25 and 59% above those of the control at 70 and 126 days, respectively.

Similarly, in fruits which had been picked, treatment with ethylene or ethephon also raised nootkatone levels above those of the control (Figure 2). Increases of 50 and 40%, respectively, were observed 55 days after treatment with 100 ppm of ethylene and 480 ppm of ethephon.

Furthermore, it can be seen that the act of picking itself produced substantial increases in nootkatone (Figures 1 and 2) as described previously (Del Río et al., 1992a).

Effect of Ethephon on Several Parameters Related with the Pulp and Rind Maturation Processes in Grapefruit. Table I shows the changes observed at different times in the total soluble solids, the total acidity, and the index of ripeness of the juice from the control fruits and from those treated with 200 ppm of ethephon which were left on the tree. In both cases, total acidity decreased and the index of ripeness increased as the fruit matured; the percentage of soluble solids remained practically unchanged (12.8–14.2%). No modifications in internal maturation were observed up to the age of fruit assayed. These results agree with those obtained by other researchers in different species of *Citrus* (Fishler and Monselise, 1971; Agustí et al., 1981; Coggins, 1981).

The external maturity of grapefruit is initially characterized by a loss of chlorophyll followed by no or a slight

Table I. Evolution of Some Indicators of Internal Maturity in Grapefruit (*C. paradisi* L. Var. Star Ruby)[a]

weeks after treatment	TSS, %		TA, g/L		index (TSS/TA)	
	A	B	A	B	A	B
2	14.2 ± 0.3	13.6 ± 0.5	29.4 ± 0.8	34.1 ± 5.4	4.8	4.0
4	12.8 ± 0.5	13.5 ± 0.7	29.5 ± 2.0	29.1 ± 1.9	4.3	4.6
10	14.2 ± 0.6	14.2 ± 0.7	27.2 ± 2.7	27.4 ± 0.8	5.2	5.2
14	13.1 ± 0.1	13.6 ± 0.4	24.6 ± 1.2	26.3 ● 2.0	5.3	5.2

[a] Control (A) and fruit treated with 200 ppm of ethephon 28 weeks after anthesis (B) were used at 2, 4, 10, and 14 weeks after treatment, and the following parameters were determined: total soluble solids (TSS), total acidity (TA), and index of ripeness (TSS/TA), as described under Materials and Methods. Data represent mean values ± SE (n = 2).

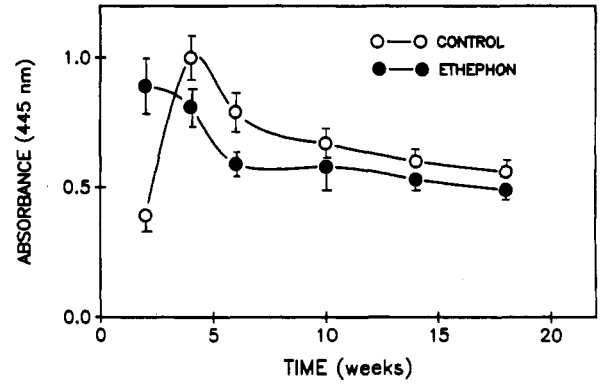

Figure 3. Evolution of absorbance values (445 nm) for rind extracts from the fruit of *C. paradisi* L. (var. Star Ruby) control and treated with 200 ppm of ethephon. The time indicates weeks since treatment. Data represent mean values ± SE (n = 2).

Table II. Effect of Ethephon on the Morphological Parameters in the Exocarp of Unpicked Grapefruit[a]

age of fruits	treatment	oil cavity diameter, μm	cellular size, μm	
			a	*b*
32	control	198 ± 74	56 ± 12	24 ± 3
32	ethephon	245 ± 63	70 ± 17	20 ± 4
42	control	240 ± 48	64 ± 13	22 ± 4
42	ethephon	253 ± 46	77 ± 10	26 ± 5

[a] Fruits treated with 200 ppm of ethephon 28 weeks after anthesis and untreated (control fruits) were used. Age refers to number of weeks after anthesis. Data correspond to the oil cavity diameter (μm) and cellular size in the first cellular layer near this cavity (*a* and *b* indicate the polar and equatorial cellular diameters, respectively, in μm). In all cases, the data represent the mean values of at least 40 measurements and the standard error is indicated.

increase in carotenoids, whose levels decrease later as maturity progresses (Coggins and Jones, 1977; Gross et al., 1983). Our results using ethephon (Figure 3) show an acceleration in the process, the maximum values and subsequent decreases in absorbance at 445 nm, which corresponds to the maximum absorption of β-carotene, being reached earlier than in the control.

Cellular Changes in the Exocarp of Unpicked Grapefruit Treated with Ethephon. The changes of several morphological parameters in the exocarp of unpicked fruits treated with ethephon (200 ppm) and untreated ones (control) are represented in Table II. These results revealed that the ethephon treatment accelerated the processes which led to increases in the oil cavity diameter and in the size of nearby cells.

In addition, ultrastructural studies revealed that morphological changes were observed in the cells near the oil cavity when the fruits were treated with ethephon (Figure 4). Thus, 30 weeks after anthesis in the plastids of the exocarp of control fruits (Figure 4A), the plastoglobules

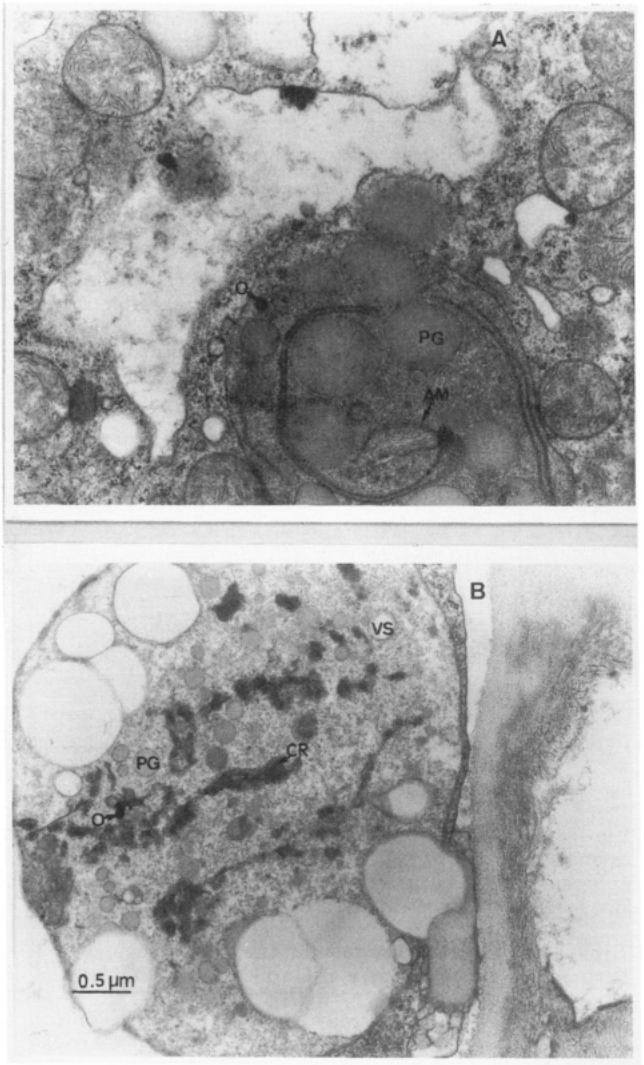

Figure 4. Exocarp chromoplast of unpicked grapefruit 30 weeks after anthesis: untreated (A) and treated with 200 ppm of ethephon 28 weeks after anthesis (B). AM, achlorophyllous membranes; CR, pigment crystalloids; O, osmiophilic structures; PG, plastoglobules; VS, membrane remnants.

increased in volume to become the predominant structural elements in which the lipophilic carotenoids accumulated and contained achlorophyllous membranes arranged in circular groups, similar to those observed in other species (Gross et al., 1983). At the same age (30 weeks after anthesis and 4 weeks after treatment with ethephon), there is a considerable reduction in the volume of the plastoglobules found in the plastids of the exocarp, which is in agreement with the acceleration of the carotenogenesis processes already described for ethephon in this plant material (Figure 3). The development of pigment crystalloids was also observed (Figure 4B).

DISCUSSION

These results show that treatment with ethylene or ethylene-releasing agents (ethephon) caused considerable increases in nootkatone levels (Figures 1 and 2). Bearing in mind that ethylene is considered a growth regulator capable of accelerating the maturation–senescence processes in citris (Scott and Leopold, 1967; Goldschmidt and Galily, 1974; Iwahori, 1978), the results described here would agree with those obtained in previous studies, in which it was demonstrated that the processes which can accelerate maturation–senescence in grapefruit, such as harvesting (Del Río et al., 1992a) or degreening (Del Río et al., 1992b), lead to increases in the levels of nootkatone. These results agree with those of other authors who state that treatment with gibberellic acid, a known growth regulator, capable of delaying senescence in *Citrus* (Monselise and Goren, 1965; Lewis et al., 1967; Coggins et al., 1969; Goldschmidt et al., 1977; Guardiola et al., 1981; McDonald et al., 1987), produced a decrease in the sesquiterpene valencene levels in navel orange (Coggins et al., 1969) and in the levels of nootkatone in marsh grapefruit (Wilson et al., 1990).

The ethylene released after ethephon treatment seemed to accelerate the processes of maturation–senescence in grapefruit, as is shown by the acceleration in carotenogenesis (Figure 3), the morphological changes in the exocarp (Table II), and the ultrastructural changes in the plastids (Figure 4). This treatment with ethephon did not seem to affect the internal maturity of the fruit (Table I), due probably to the lack of vascular connections between the rind and pulp (Monselise, 1977).

Some authors have suggested that plastids are the intracellular sites of essential oil production and of the mechanisms which control terpenoids transport toward the exterior (Bosabalidis and Tsekos, 1982a,b; Cockburn and Wellburn, 1974). Our results suggest that the increase in nootkatone levels in grapefruit produced by the action of ethylene might be associated with the action of this growth regulator in the ultrastructural changes observed in the corresponding plastids (compare parts A and B of Figure 4), in which are included those relative to the processes of carotenogenesis (Figure 3).

However, other processes might be governed by this regulator of fruit development, some of which might be those shown here (see Table II) and which might in turn influence the expression of this secondary metabolite.

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