Naringin and Neohesperidin Levels during Development of Leaves, Flower Buds, and Fruits of Citrus aurantium

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ABSTRACT

The distribution of the flavonones naringin and neohesperidin has been analyzed during the development of the leaves, flower buds, and fruits of Citrus aurantium. These flavonoids are at maximum concentration in the organs studied during the logarithmic phase of growth, gradually decreasing until the organs reach maximum development. However, this decrease in the naringin and neohesperidin concentration in leaves, flower buds, and fruits is due to a dilution of the flavonoids caused by cell growth, because total content per organ continues to increase. The levels of neohesperidin are always greater than those of naringin, although the ratio between the relative concentrations is different in the three organs studied. Leaves have the highest ratios, varying between 8.83 and 5.18, followed by flowers (3.15–1.85), and fruits (2.23–1.02). These observations suggest different relationships between the respective enzymic activities in their biosynthetic pathway.

The flavonoids are an important class of plant secondary metabolites and have been the subject of intense biochemical studies in recent years (7). Citrus species are of much interest because they accumulate large amounts of flavonane glycosides, whose aglycons are early intermediates in the flavonoid biosynthetic pathway (9). Naringin, the 7-β-neohesperidosidic of naringenin (4'-5,7,3-trihydroxy flavanone) and neohesperidin, the 7-β-neohesperidosidic of hesperetin (3',5,7-trihydroxy-4'-methoxy flavanone) are the most abundant flavonoids in Seville orange (Citrus aurantium) (12, 16, 24).

The interest in flavonoids in Citrus is due to their potential physiological and biochemical activity (10), as well as to their organoleptic properties. Flavanone-7-neohesperidosides, particular naringin (11), are extremely bitter, whereas the isomeric flavanone-7-rutinosides, narirutin and hesperidin, are tasteless (27). In addition, flavanone distribution within different Citrus species can be quite distinctive (1). Therefore, naringin and neohesperidin concentrations can be used to differentiate grapefruit, sour orange, and K-early (tangerine × grapefruit hybrid) juice (28). Neohesperidin is a natural source of an intense sweetening agent, neohesperidin dihydrochalcone (3).

Little information is available concerning the individual reactions of the biosynthesis of these compounds in Citrus. The enzymes that take part in the biosynthesis of naringenin, as well as its subsequent glycosylation to naringin, have been characterized (4, 8, 19, 20, 22, 23, 26). However, the biosynthetic pathways of 4'-methoxylated glycosyl flavanones have not been fully described.

As part of a continuous study of the biosynthesis of glycoside flavonoids in Citrus, we have studied the distribution of naringin and neohesperidin during the development of leaves, flower buds, and fruits of Seville orange. This study of naringin and neohesperidin distribution will permit us to know in which stages of the development their levels are highest, to isolate and characterize in the future the key enzymes of the biosynthetic pathways of both flavonoids.

MATERIALS AND METHODS

Plant Materials

Leaves, flower buds, and fruits were obtained from 5-year-old Citrus aurantium cv Seillanoro trees, grown in greenhouses of the University of Murcia. The phloem fluid from the receptacles of decapitated flower buds were collected with a capillary inserted in the ovary hole. The capillaries (1.5 mm i.d.) were left in the plant for 48 h, and their content was weighed and immediately dissolved in DMSO for analysis.

Chemicals

Naringin, neohesperidin, naringenin, and hesperidin were obtained from Zoster S.A., Murcia, Spain.

Extraction, Chromatographic Analysis, Isolation, and Hydrolysis Conditions of Flavonoids

Ten leaves and five flower buds and fruits were collected, immediately dried at 50°C (13), and ground; the flavonoids were extracted with DMSO in the ratio of 2 mg/mL for analytical chromatography. These measurements were repeated for four trees, and the mean values obtained at each age were used to express the distribution of flavonoids in leaves, buds, and fruits. For the isolation of compounds 4 and 7 (Fig. 1), 15 g of leaves (11–20 d old) and 15 g of fruits (3–20 mm diameter) were extracted with DMSO in the ratio 200 mg/mL. The solutions were filtered through a 0.45-μm nylon membrane.

For the location of naringin and neohesperidin in the young leaf, flower bud, and fruit extracts of Citrus aurantium, we used a μBondapak C18 (250 × 4 mm i.d.) analytical column with an average particle size of 5 μm and several solvents: (a) isocratic H2O-Me'OH-acetonitrile-HO acetyl (15:2:2:1); (b) 1 Abbreviation: Me, methyl.
isocratic MeOH-0.01 M H$_3$PO$_4$ (1:2); and (c) gradient solvent A (MeOH) and B (0.01 M H$_2$PO$_4$), the linear gradient profile being 30 to 70% of A in 20 min. In all solvent systems, the flow rate was 1 mL/min at room temperature. The HPLC analysis was performed using a Beckman liquid chromatograph with a model 110B solvent delivery module and a System Gold Module 168 diode array detector (Beckman Instruments, San Ramon, CA). The absorbance change was monitored at 280 nm. Naringin and compound 4 showed, in the different solvent systems, the following retention times: 20.33 min (solvent 1), 24.72 min (solvent 2), and 15.34 min (solvent 3). Neohesperidin and compound 7 showed the following retention times: 30.34 min (solvent 1), 37.11 (solvent 2), and 16.61 min (solvent 3).

For the isolation of compounds 4 (naringin) and 7 (neohesperidin), the following semipreparative column was used: Nucleosil C$_{18}$, 5 μm (250 × 10 mm i.d.), eluted with H$_2$O-MeOH-acetonitrile-HO acetyl (15:2:2:1) and a flow rate of 3 mL/min. For the identification and isolation of the sugars of both compounds (rhamnose and glucose), an IR detector (Shimadzu Co., Kyoto, Japan) and the following semipreparative column was used: Nucleosil-NH$_2$ 7 μm (250 × 10 mm i.d.) eluted with acetonitrile-H$_2$O (85:15) and a flow rate of 3 mL/min at 30°C. Products 4 and 7 and the respective sugars of the hydrolysis were isolated by several chromatographs in their respective semipreparative columns, and the fractions were collected with a Pharmacia FRAC 100 (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Product 4 (1 g), from several semipreparative chromatographs of plant material extracts, was hydrolyzed in 20 mL of 2N sulfuric acid under reflux for 2 h, and a white precipitate was obtained. The cooled suspension was neutralized with NaOH and the precipitate filtered and washed with distilled water. A white precipitate (0.4 g) was obtained (compound IV). Product 7 (1.5 g), from several semipreparative chromatographs of plant material extracts, was hydrolyzed in 250 mL of 2N sulfuric acid under reflux for 6 h, and a white-beige precipitate was obtained. The cooled suspension was neutralized with NaOH and the precipitate filtered and washed with distilled water. A white-beige precipitate (0.6 g) was obtained (compound VII). Compounds 4, 7, IV, and VII were identified by their melting points (Gallenkamp, Loughborough, England), mass spectra (electron impact MS) (Hewlett-Packard Co., Palo Alto, CA), and the $^1$H NMR (200 MHz) and $^{13}$C NMR (50 MHz) spectra (Bruker, Bremen, Germany) in hexadeutero-DMSO, which was capable of dissolving the products. The sugars were identified by their mass spectra (electron impact MS) (Hewlett-Packard).

**RESULTS**

**Identification of Naringin and Neohesperidin in Leaves, Flower Buds, and Fruits of *C. aurantium***

The analysis by HPLC of DMSO extracts of leaves, flower buds, and fruits during their development shows the presence of many flavonoid structures. Figure 1 shows a characteristic chromatogram of an extract from *C. aurantium* fruits (5–6 mm diameter). The chromatograms of leaf and bud extracts showed the same peaks, although with different relative proportions. Peaks whose spectra are similar to or identical with flavanone-, flavone-, or flavonol-type structures are numbered (1–9). In this chromatogram, compounds 4 and 7 are of note because they present a retention time identical with that of naringin and neohesperidin and coincide with those shown by the other chromatographic systems (see “Materials and Methods”). Despite this similarity, these compounds were isolated to confirm their identity.

The absorption spectra of these compounds show two maxima (in the elution solvent of Fig. 1), at 283 and 326 nm for compound 4 and 284 and 327 for compound 7. These data are consistent with the compounds having flavanone skeletons identical with those of naringin and neohesperidin. These compounds were isolated with a semipreparative column (see “Materials and Methods”), and two white products were obtained, with melting points of 171 and 244°C, respectively.

Hydrolysis of compounds 4 and 7 in 2N sulfuric acid gave a white (compound IV) and white-beige (compound VII) precipitate, respectively. Compounds IV and VII had UV spectra, melting points, mass, $^1$H NMR and $^{13}$C NMR spectra identical with those of the flavonones, naringenin and hesperitin, respectively. The sugars resulting from hydrolysis were isolated by semipreparative chromatography (see “Materials and Methods”) and identified as rhamnose and glucose by comparing their mass spectra with those of authentic sugars. Both $^1$H and $^{13}$C NMR spectra for compounds 4 and 7 corresponded to the flavanone structure (21) with glycosylation at position 7, which is characteristic of a neohesperidoside structure (18). This led us to believe that the compounds in question referred to naringin and neohesperidin, respectively (18, 21).

**Changes in the Levels of Naringin and Neohesperidin during Development of *C. aurantium* Leaves**

Both flavanones are at their highest concentration in the logarithmic phase of development (Fig. 2). Neohesperidin...
always more concentrated than naringin and is 8.83 times greater in 11-d-old leaves. However, these concentrations are not constant and gradually decrease as leaves grow. These decreases in concentration take place at different rates, and when the leaf is fully developed, the neohesperidin concentration is only 5.18 times greater than that of naringin.

Both naringin and neohesperidin increase on a per leaf basis as the leaf grows (Fig. 2). Furthermore, the rate of neohesperidin synthesis and/or accumulation is greater than that for naringin. These results confirm that the decreases in concentration of both flavonoids in Figure 2 are possibly due to a dilution effect (15). However, the decrease in the relative proportions of the concentrations of neohesperidin and naringin seems to indicate the presence of a different turnover for each.

Changes in the Levels of Naringin and Neohesperidin during Development of C. aurantium Flower Buds

Buds reach their maximum development after 27 to 30 d, and the fresh weight decreases drastically as the petals drop (Fig. 3). Both flavonoids show an initial decrease in concentration as the bud swells. No differentiated structures appear at this stage. Old buds (9 to 11 d) show an increase in naringin concentration from 6.49 to 11.37 μmol/g fresh weight by 19 d. The neohesperidin concentration increases after 16 d and reaches 25.52 μmol/g fresh weight at 18 d. These increases coincide with the formation of the different organs of the flower (ovary, petals, stigma, filaments). Subsequently, during bud enlargement, there is a second decline in concentration before it increases again, coinciding with pollination and the fall of petals and stamens.

The observed decrease in the concentration of both flavonoids seem to be linked to dilution resulting from cell growth in the buds, similarly to that described in the leaves, rather than to a degradative process. The first increases detected in naringin and neohesperidin are observed during the division and differentiation stages of the cells. However, the subsequent increase after pollination (31-d-old buds) is principally due to the fall of petals. The petals represent a large proportion of total flower weight and dilute the total concentration of the flavonoids. This can be confirmed by analyzing naringin and neohesperidin levels in the different parts of the flower. Table I shows the concentration (μmol/g fresh weight) and percentage of each of these flavonoids contributed by the different parts of the flower and the neohesperidin to naringin ratio in each one of these parts. The ovary and stigma show the highest concentrations of both flavonoids, followed by filaments, receptacles, petals, and anthers, in decreasing order of concentration. When the contribution of each of the structures to the total content per flower of these flavonoids is analyzed, we find that the petals are responsible for 25.09 and 15.61% of neohesperidin and naringin, respectively, because, although their concentrations are low (4.57 and 5.41 μmol/g fresh weight, respectively), they represent a high percentage of the total weight of the flower (42.34%). The unequal distribution of both flavonoids within the flower is shown in Table I. In the receptacle and petals, they are present in almost equal proportions, whereas in the stigma and anthers neohesperidin is, respectively, 3.83 and 3.41 times more concentrated than naringin and is more than double in the ovary (2.42). These different ratios between the different bud struc-

<table>
<thead>
<tr>
<th>Structure</th>
<th>Concentration</th>
<th>% of Total</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptacle</td>
<td>8.10</td>
<td>7.93</td>
<td>18.17</td>
</tr>
<tr>
<td>Ovary</td>
<td>59.82</td>
<td>144.81</td>
<td>26.28</td>
</tr>
<tr>
<td>Stigma</td>
<td>16.91</td>
<td>64.86</td>
<td>11.37</td>
</tr>
<tr>
<td>Petals</td>
<td>4.57</td>
<td>5.41</td>
<td>25.09</td>
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<tr>
<td>Filaments</td>
<td>6.79</td>
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<td>18.50</td>
</tr>
<tr>
<td>Anthers</td>
<td>1.22</td>
<td>4.16</td>
<td>0.59</td>
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tures suggest the existence of different turnovers for each flavonoid.

The overall levels of naringin and neohesperidin in buds show a similar pattern to those in leaves, and their development is accompanied by an increase of both flavonoids (Fig. 4). Maximum values are reached at full development of the bud, and the final decrease in both values is due to petal loss because these are responsible for approximately 25% of overall flavonoid content, as mentioned above. Neohesperidin levels are greater than those of naringin throughout development, as in leaves.

Changes in the Levels of Naringin and Neohesperidin during Development of *C. aurantium* Fruits

Growth of the fruit of bitter orange, as measured by fruit diameter, is sigmoidal (Fig. 5), reaching its maximum size (61 mm diameter) at approximately 200 d. From this time, the processes of maturation begin, with no appreciable change in diameter. Neohesperidin levels are higher in the first stages of development, coinciding with the "lag," and reach their highest concentration in fruit of 5 to 6 mm diameter. When the linear phase begins, a marked decrease in neohesperidin is observed. Naringin concentration remains constant in fruits up to 15 mm diameter, at which stage it decreases to reach the levels of neohesperidin in the senescence phase. With regard to the overall content of both flavanones per fruit (Fig. 6), the increase in synthesis and/or accumulation rates in the day following anthesis is similar to the pattern found in leaves and buds. The neohesperidin synthesis and/or accumulation rate exceeds that of naringin (Fig. 6, inset), although after fruits reach 10 mm diameter these rates even out. This contrasts with that which occurs in leaves, in which neohesperidin rates remain higher than those for naringin throughout development. This suggests that during fruit development both flavonoids experience a change in their metabolism and reach maximum levels in fruit of 30 to 35 mm diameter.

![Figure 5](https://academic.oup.com/plphys/article-lookup/doi/10.1104/pp.99.1.67)

**Figure 5.** Accumulation of mean fruit diameter (○) and naringin (●) and neohesperidin (▲) concentration according to age in *C. aurantium*. Vertical bars, ± SE when larger than symbols.

**Changes in the Levels of Naringin and Neohesperidin during Development of *C. aurantium* Fruits**

**Relationships between Neohesperidin and Naringin in the Development of Leaves, Flower Buds, and Fruit of *C. aurantium***

The ratio between neohesperidin and naringin concentrations during the development of the leaves decreases from 8.83 for those 11 d old to 5.18 when they reach their maximum size (43 d) (Fig. 2). In buds and fruits, the values of this ratio are always smaller than those described for leaves. In buds, they are higher than 3 during the first phase of the logarithmic stage of growth (1–10 d) and decrease sharply to values slightly greater than 2 during the stages in which the different parts of the buds are differentiated. There is then another slight decrease to 1.61 while the bud finishes developing. The bud then opens, is fecundated, and loses petals and stamens; this process increases again slightly to almost 2 (Fig. 3).

In fruits, this ratio varies between 3.15 and 1.02, and three distinct zones are discernible where these values oscillate. It increases from 2.23 to its maximum of 3.15 as fruits grow from 3 to 7 mm in diameter. The ratio then decreases sharply to 1.13 in fruits of 35 mm diameter. At this size, the fruit of *C. aurantium* accumulates maximum overall levels of both.

![Figure 4](https://academic.oup.com/plphys/article-lookup/doi/10.1104/pp.99.1.67)

**Figure 4.** Changes in the mean total content per flower bud of naringin (○) and neohesperidin (▲) according to age in *C. aurantium*. Vertical bars, ± SE when larger than symbols.

![Figure 6](https://academic.oup.com/plphys/article-lookup/doi/10.1104/pp.99.1.67)

**Figure 6.** Changes in mean total content per fruit of naringin (○) and neohesperidin (▲) according to age in *C. aurantium*. Vertical bars, ± SE when larger than symbols.
flavonoids. From this stage onward and until maximum size is reached, the ratio decreases gradually to reach a value of 1.02. At this stage, the fruit completes its development, and the period of maturation begins.

Relationships of Neohesperidin and Naringin Levels among Leaves, Flower Buds, and Fruits during the First Stages of the Reproductive Cycle in *C. aurantium*

Table II shows the distribution of these flavanones in the different organs of *C. aurantium* during the initial stages of the reproductive cycle. The respective concentrations of neohesperidin and naringin and the relationship between them are shown in leaves before anthesis, during the final stage of flowering, and during the logarithmic and linear phase of fruit growth; in 30-d-old buds; in the stems that attach these buds to the tree; in the phloematic fluids of the receptacles of these buds; and finally, in fruits of 10 and 25 mm diameter. Neohesperidin and naringin concentrations are higher in leaves than in the buds and the stems of these buds. However, even higher concentrations are found in the phloematic fluids, especially in the case of naringin. The highest ratio between the two is found in leaves, followed by stems and fluids; the lowest ratio is in buds at 30 d.

As indicated before, the synthesis and/or accumulation rate of neohesperidin in fruits up to 10 mm diameter is greater than that of naringin, and the values tend to even out when the fruits reach this size (Fig. 6, inset). When the fruits reach 25 mm in diameter, the flavanones begin to reach their highest levels with regard to quantity per fruit (Fig. 6). Fruits of 10 mm diameter show high quantities of neohesperidin and naringin, which is much higher than those of leaves, and the ratio between both concentrations is almost three times greater.

This tendency remains the same when the fruits reach 25 mm diameter, although the concentration of both flavanoids decreases in leaves and fruits. The greatest decrease in concentration is shown by neohesperidin, which is approximately 50% in fruits and 40% in leaves. Naringin decreases much less drastically in both organs. This means that in leaves the neohesperidin to naringin ratio remains practically constant at approximately 1, whereas in fruits it decreases from 2.83 to 1.56.

**DISCUSSION**

In this paper, we have used a profile protocol to examine the distribution of the flavanones, naringin and neohesperidin, in the leaves, buds, and fruits of *C. aurantium* during their development. By means of semipreparative chromatography and structural studies of the aglycons and sugars present in the molecules, we confirmed the presence of neohesperidin and naringin in the above-mentioned organs. The main aim of this paper is to describe naringin and neohesperidin levels during the development of the three organs. In this way, we hope to obtain the necessary information to improve isolation and characterization of the specific enzymes involved in the biosynthetic pathways of these flavonoids in *C. aurantium* in the future.

The quantitative studies described here concentrate on three particular aspects in the three organs analyzed: the measurement of the concentrations and overall quantities of both flavanones in each organ and the calculation of the neohesperidin to naringin ratio. From these data it is possible to reach a series of conclusions regarding the metabolism of these flavonoids in *C. aurantium*. Some have already been noted for each particular organ; an overall summary is given below.

The relative levels of these molecules are highly characteristic of a given organ and are markedly affected by the age of the developing organ. Our results confirm previous findings concerning flavanone-glycoside accumulation in *Citrus paradisi* and *Citrus limon* (1, 15, 29), which demonstrated that the concentration is greater in young tissues and that there is a decrease in all tissues during maturation (2). The high levels of these flavanoids during the lag of development might be connected with protection against both insects and pathogens.

However, it must be pointed out that in both leaves and fruits the decrease in concentration observed, as stated in "Results," is due to dilution of enlarging cells, because during the development of these organs, before they enter their respective maturation periods, the overall quantities of naringin and neohesperidin increase. It is evident that the slopes observed for the decrease in concentration and increases in overall flavonoid quantities are different for both organs, suggesting the presence of different metabolisms for both flavanoids in leaves and fruits of *C. aurantium*. On the other hand, different synthesis and/or accumulation profiles exist for naringin and neohesperidin in the same organs, but this does not imply that the flavonoids are derived from different precursors. It is more likely that the variations observed are a result of the change in activity of certain late enzymes involved specifically in the formation of the two related aglycons.

In *C. aurantium* buds, as has been described in the "Results," no constant pattern is observed for the changes in the concentration of either flavonoid, because the cellular differentiation stages and subsequent growth of the bud organs, which produced respective increases and decreases in these
concentrations, do not occur simultaneously. Therefore, the delay in neohesperidin concentration increase, when compared with that of naringin, might indicate the precursor role of naringenin in neohesperidin synthesis (20).

The study of the neohesperidin to naringin ratio during leaf, bud, and fruit development casts some light on the biosynthetic pathways of these flavanones. This ratio generally decreases during the development of the organs studied. However, the rates of the decline and the absolute values of the neohesperidin to naringin ratio are not the same in the different organs. The high ratios detected in leaves suggest that there is high activity of the enzymes involved in the synthesis of neohesperidin from naringenin (20). This is particularly true of methyltransferase activity (5, 17) in the lag of leaf development, although there is a subsequent diminution of this activity as the leaf grows. Our previous experiments showed the presence of a high 4'-O-methyltransferase activity in C. aurantium leaves of <20 mm in length (data not shown), which seems to confirm this suggestion. The decrease in activity might be partly due to a diminution in S-adenosylmethionine levels, given its active participation as a methylating agent in different components of the cell wall, which are actively synthesized during plant cell growth.

Buds and fruits present lower neohesperidin to naringin ratios. During bud development, the ratio between both flavanoids oscillates and is related with the stages of cell division and the subsequent development of different parts of the flower. These results are in accordance with those of other studies that suggest that these flavanoids are rapidly synthesized in tissues during the cell division phase and that synthesis slows down in the cell enlargement phase (1).

The data in Table I confirm that the flower organs that present the highest mitotic activity (ovary and stigma) have the highest concentrations of these flavanoids and the highest neohesperidin to naringin ratios, similarly to that observed in leaves during their cellular division stage (20 mm in length).

A study of all the values described in Table II concerning the first stages of the reproductive cycle of the tree (concentrations of neohesperidin and naringin and their relationship) provides valuable information regarding the presence or absence of synthesis of these flavanones in the different organs.

An analysis of the phloematic fluids in the receptacle (Table II) of these buds suggests that both flavanoids may be transported to the different organs in the tree. Given that the neohesperidin to naringin ratio in leaves during this period (4.35) is much higher than that in the fluids, it can be deduced that naringin is more readily transported through the phloem. It can also be seen that naringin concentration is higher in buds than in leaves, the neohesperidin to naringin ratio being noticeably lower (1.67). The fact that this ratio is also lower than that in the stems and fluids examined seems to indicate that in this period the buds have a greater capacity for synthesizing naringin than neohesperidin. These data seem to confirm that this capacity for naringin synthesis is the cause of the decrease in the ratio between both flavanoids in buds at the conclusion of their swelling stage (1–10 d) and at the moment that the differentiation of the flower organs begins (10–19 d). Thus, the variations observed in neohesperidin to naringin ratios in the different organs of the buds may be due to a differential exogenous contribution of both flavanoids to the tissue and/or to changes in the activity of enzymes involved in the synthesis of these compounds.

The higher concentrations of neohesperidin and naringin in fruits of 10 mm diameter than in leaves (Table II) seem to indicate that the fruits are important sites of synthesis and/or accumulation of these flavanones. Although in fruit of 25 mm diameter the concentration of neohesperidin and naringin and their ratio remain higher than the respective values in leaves (Table II), the decrease in these concentrations and ratios points to a diminution in the activity of flavonane synthase enzyme and of the enzymes involved in the subsequent synthesis of neohesperidin during the development of the fruits (20).

The final conclusion that we can reach is that all the organs analyzed of the C. aurantium tree are capable of synthesizing and/or accumulating these flavanones. It is clear that there is a process of flavonone transport as is shown by the levels of neohesperidin and naringin in phloematic fluids and by our preliminary results in the study of this phenomenon (data not shown). It can also be concluded that in all organs studies the biosynthesis of these flavanoids is more important during the stages of cell differentiation and less intensive in periods of growth and subsequent maturation and that their biosynthetic pathway is regulated differently in each organ.

LITERATURE CITED

NARINGIN AND NEOHESPERIDIN LEVELS IN CITRUS AURANTIUM