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Changes in Organic Acids and Sugars during Early Stages of Development of Acidic and Acidless Citrus Fruit

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Most of the studies on organic acids and sugars in citrus were performed during fruit maturation, and less is known before this stage of development. The aim of our study was to investigate acids and sugars in lemon, lime, and orange from fruit-set toward development. We chose to compare organic acid and sugar accumulation among acidic and acidless varieties within three species. We estimated the acidity by titrimetry and quantified the concentrations of seven organic acids and three sugars by reverse HPLC. During the first 50 days of development, quinic acid was the major organic acid whatever the variety. Afterward, citric acid predominated in acidic varieties, while in acidless, malic acid exceeded it. Fructose substituted citric acid in acidless and could be synthesized either from citric acid or directly from glucose. Our results provided the first complete report on sugar and organic acid accumulation during the early stages of fruit development in several citrus varieties.

KEYWORDS: Acidity; citric acid; fructose; fruit development; lemon; lime; orange

INTRODUCTION

Organic acids and sugars are among the major compounds of citrus fruit pulp. Their nature and concentration largely affect taste characteristics and organoleptic quality (*1*). Organic acids and sugars vary according to species, varieties, and also environmental and horticultural conditions such as climate, rootstock, and irrigation (*2*−*3*).

Most of the studies on organic acid and sugar content were performed during fruit maturation of species such as orange, grapefruit, mandarin, and lemon (*4*−*6*). They showed that acidity, mainly due to citric acid, decreases during maturation of orange, grapefruit, and mandarin, whereas it remained constant in lemon. The total sugars increase throughout whatever the species. In contrast, only a small amount data were obtained before maturation. Acidity increases and becomes constant during the second stage of development, defined by Bain (*7*), while the total sugars increase (*8*−*9*). It is worth noting that there is very little information available on individual organic acids and sugars and their respective pattern of accumulation during fruit development (*10*−*13*). However, all citrus fruit does not follow this common behavior. In fact, some acidless varieties resulting from spontaneous mutations are characterized by a very low acidity and a lack of citric acid (*14*−*17*). But, it has not been reported yet if this modification in citrus fruit metabolism might have influences on sugar content in the juice vesicles.

The aim of the present work was to investigate the changes in organic acid and sugar content in three species (lime, lemon, and orange) during the early stages of fruit development of acid and acidless varieties. Our results showed that the differences in acidity are reflected by variations in organic acids and sugars, and we also showed the relationship between organic acid and sugar content under those contrasted situations.

MATERIALS AND METHODS

Samples. Fruit were collected from the citrus arboretum located at the Station de Recherches Agronomiques INRA-CIRAD of San Giuliano, Corsica, France. The acidic varieties investigated were orange (*Citrus sinensis* L. cv. Salustiana) grafted onto trifoliate orange (*Poncirus trifoliata* (L.) Raf.), lemon (*C. limon* (L.) Burm cv. Villafranca), and lime (*C. latifolia* Tan. cv. Tahiti) both grafted onto sour orange (*C. aurantium* (L.)). The acidless varieties were orange (*C. sinensis* L. cv. Iaffaoui) grafted onto trifoliate orange, lemon (*C. limon* (L.) Burm cv. Sweet Lemon), and lime (*C. limettioïdes* Tan. cv. Brazil Sweet Lime) both grafted onto sour orange. Soon after flowering, a large number of very young fruit were identified, on each of the three trees per variety, to have fruit reaching the same development when sampling. Harvesting was performed from June (2 weeks after fruit-set) to November 2002 (15 samplings). Four fruits per variety were collected at each sampling. Each fruit was peeled, and its endocarp was lyophilized, ground in liquid nitrogen, and stored at −20 °C until analysis.

Titratable Acidity. Fifty milligrams of powdered sample was suspended in 5 mL of bidistilled water. Acidity was measured (titration to pH 8.1) using an automatic titrator Mettler DL25 (Mettler-Toledo, France) according to the AOAC method (*18*). Each sample was analyzed in duplicate. Acidity was expressed as milliequivalent per gram of fresh pulp (meq g^{-1} f.p.).

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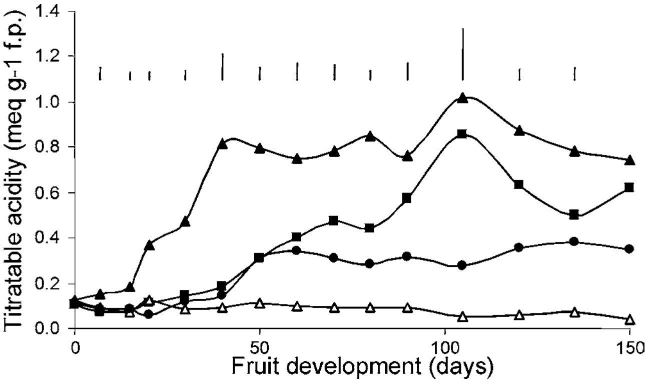

Figure 1. Change in titratable acidity in citrus fruit pulp during fruit development of Tahiti lime (▲), Villafranca lemon (■), Salustiana orange (●), and Brazil sweet lime (△). Titratable acidity of acidless varieties of lemon and orange were similar to those of Brazil sweet lime. Values are means ($n = 4$), and vertical lines represent the least significant difference (lsd) evaluated by a *t*-test.

HPLC Chemicals. Acetonitrile (HPLC grade); potassium dihydrogen phosphate (KH_2PO_4); citric, malic, oxalic, ascorbic, quinic, tartaric, and succinic acids; fructose; glucose; and sucrose were purchased from Sigma (St. Louis, MO).

HPLC Analysis. One hundred milligrams of powdered samples was suspended in 5 mL of water and centrifuged at 160*g* for 20 min. The supernatant was filtered through a 0.45 μm acetate cellulose membrane filter (Sartorius, Goettingen, Germany). The separation of organic acids and sugars was achieved using an analytical HPLC unit (Perkin-Elmer, Series 200, France).

Organic acid separation was performed using an ion-exclusion column (Spheri-5 RP-18, 220 mm × 4.6 mm, 5 μm) thermostated at 20 °C. Elution was carried out with a mobile phase made of 25 mM KH_2PO_4 solution, adjusted to pH 2.4 with H_3PO_4. The flow rate of the mobile phase was 1.0 mL min^{-1}. Detection was performed with an UV detector set at 210 nm. Organic acid quantification was achieved by plotting the absorbance of each organic acid on a standard curve.

Sugar separation was performed with a NH_2 bound silica column (Waters, 4.6 mm × 250 mm, 4 μm) at 35 °C. Elution was carried out isocratically with a mobile phase made of acetonitrile/water (70:30, v/v) at a flow rate of 1.0 mL min^{-1}. Detection was performed with a refractometer index detector. Sugar quantification was achieved according to their refractive index recorded to external standards.

Data acquisition was achieved using TotalChrom software for Windows version 6.2 (Perkin-Elmer Instruments, Boston, MA). Each sample was analyzed in triplicate. Organic acid and sugar concentrations were expressed as milligram per gram of fresh pulp (mg g^{-1} f.p.).

Statistics. Data were subjected to a one-way analysis of variance (ANOVA) using the GLM procedure of SAS (SAS Institute Inc.), and the least significant difference (lsd) between means was assessed using a *t*-test ($P < 0.05$).

RESULTS

Changes in Titratable Acidity and Organic Acids During Fruit Development. Titratable acidity of the three acidic varieties increased early in fruit development (20 days for lime and 50 days for lemon and orange), while it remained constant for the acidless varieties (**Figure 1**). Lime and lemon were the most acidic fruit, reaching maximum values of 1.0 and 0.8 meq g^{-1} f.p., respectively, about 100 days after fruit-set. Titratable acidity of acidic orange was 0.3 meq g^{-1} f.p. throughout development. The lowest titratable acidity was measured in the three acidless varieties ($\leq$0.1 meq g^{-1} f.p). Thus, on the basis of the titratable acidity, we could identify three different profiles between the six varieties used: acidic (acidic lime and lemon), intermediate (acidic orange), and acidless (acidless lime, lemon, and orange).

We clearly identified seven organic acids involved in citrus fruit pulp acidity: citric acid, malic acid, quinic acid, tartaric acid, succinic acid, oxalic acid, and ascorbic acid. Three of them changed during fruit development: oxalic, quinic, and citric acids. Quinic and oxalic acids peaked during the first 50 days of development in all varieties (**Figure 2**). Quinic acid was the major organic acid during this period, and for instance, it accounted for 47−64% of the total organic acids, while citric acid accounted for less than 14% of the total organic acids (**Figure 2** and **Table 1**). Citric acid increased early in the three acidic varieties (15 days for acidic lime or 30 days for acidic lemon and orange). It was the major organic acid of maturing acidic fruit (48, 39, and 25 mg g^{-1} f.p for lime, lemon, and orange, respectively) even if its concentration decreased, at 120 days of orange development (12 mg g^{-1} f.p.). In contrast, the sweet lime was characterized by a lack of citric acid increase and by the lowest citric acid concentration (2 mg g^{-1} f.p.) along development, and the same behavior and values were observed for the two other acidless varieties. At the end of the studied fruit development, citric acid accounted for 71, 79, and 45% of the total organic acids (respectively, for lime, lemon, and orange), while in acidless varieties it only accounted for less than 17% (**Figure 2** and **Table 1**). The four other organic acids (malic, tartaric, succinic, and ascorbic acid) remained relatively constant during the whole development, and no difference could be noticed between acid and acidless fruit (**Table 1**). However, in acidless varieties, the malic acid concentration exceeded the citric acid concentration (1.2-fold) and was significantly different between acidic and acidless limes (**Figure 2**). In fact, citric acid was the only organic acid for which a significant difference could be observed between all acidic and acidless varieties.

Changes in Sugar Content During Fruit Development. Citrus fruit pulp contained three major sugars: fructose, glucose, and sucrose (**Figure 3**). Acidic orange contained the highest concentration of glucose (44 mg g^{-1} f.p.) as compared to acidic lime and lemon (on average, 11 mg g^{-1} f.p. for each) and acidless orange, lime, and lemon (respectively, 10, 15, and 2 mg g^{-1} f.p.). The glucose concentration was significantly higher in the acidic varieties, except for the limes that showed nonsignificant differences. Acidic lemon contained the highest concentration of sucrose (19 mg g^{-1} f.p.), which was significantly different from acidic lime and orange (respectively, 7 and 5 mg g^{-1} f.p.) or acidless lemon, lime, and orange (respectively, 4, 4, and 7 mg g^{-1} f.p.). Fructose was the only sugar for which a significant difference could be observed between all acidic and acidless varieties. The acidless fruit had the highest fructose concentration with maximal values of 45, 50, and 55 mg g^{-1} f.p. for lime, lemon, and orange, respectively. The acidic fruit were divided into two groups, the most acidic fruit (lemon and lime) that had the lowest fructose concentration (6 and 3 mg g^{-1} f.p., respectively) and the acidic orange with a maximal value of 36 mg g^{-1} f.p. at 150 days of fruit development.

Highest Fructose Concentration for Acidless Varieties. We have plotted all citric acid values after 50 days of fruit development against glucose, sucrose, and fructose. We did not observe any correlation with glucose and sucrose (data not shown). By contrast, with fructose, we observed three distinct groups (**Figure 4**). The first group (G1) was characterized by high concentrations in citric acid and low concentrations in fructose and corresponded to acidic lemon and lime. The second group (G2) was characterized by high concentrations in fructose

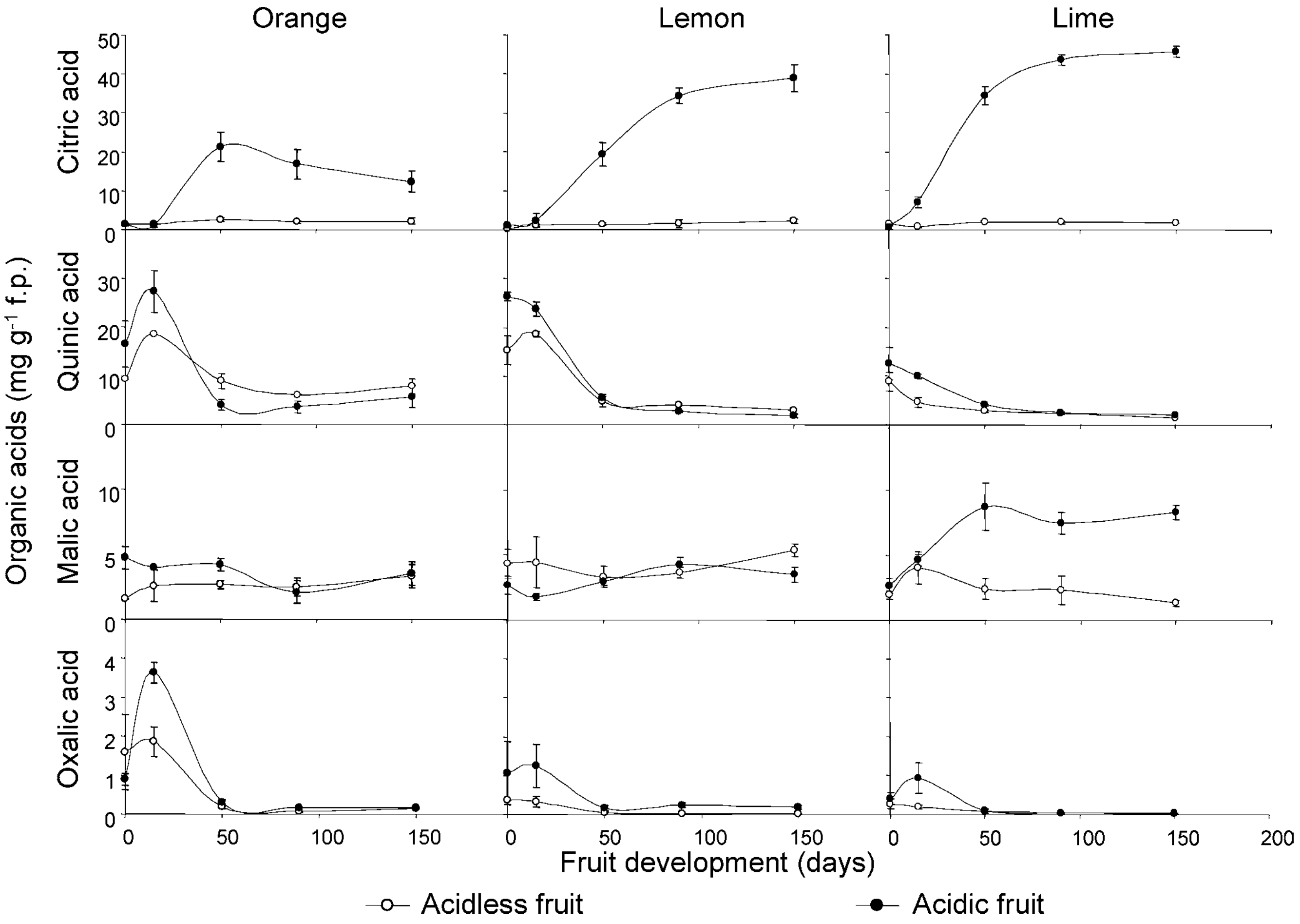

Figure 2. Change in four organic acid concentrations in citrus fruit pulp during fruit development of acidic (●) and acidless (○) varieties. Each point on the graph shows the mean and standard deviation of three measurements done on each of the four samples.

Table 1. Other Organic Acids[a] of Pulp from Citrus Fruit

	organic acids (mg g^{-1} f.p.)					
	ascorbic acid		succinic acid		tartaric acid	
days	acidic	acidless	acidic	acidless	acidic	acidless
			Orange			
0	0.3 ± 0.1	0.0 ± 0.0	1.5 ± 0.6	1.2 ± 0.2	5.8 ± 0.5	4.3 ± 0.0
15	0.3 ± 0.0	0.1 ± 0.0	2.7 ± 1.7	1.8 ± 0.5	7.1 ± 0.7	4.9 ± 0.0
50	0.1 ± 0.0	0.1 ± 0.1	2.9 ± 1.1	3.0 ± 0.8	7.7 ± 0.0	3.4 ± 0.0
90	0.1 ± 0.0	0.2 ± 0.1	3.1 ± 0.0	2.3 ± 1.0	8.9 ± 0.9	3.6 ± 1.0
150	0.2 ± 0.1	0.2 ± 0.1	3.1 ± 1.1	3.7 ± 0.2	5.2 ± 0.8	4.1 ± 1.2
			Lemon			
0	0.3 ± 0.1	0.3 ± 0.1	2.3 ± 0.9	1.1 ± 0.4	6.1 ± 1.2	7.3 ± 1.1
15	0.2 ± 0.1	0.3 ± 0.1	2.8 ± 0.1	1.7 ± 0.2	6.3 ± 0.2	7.2 ± 1.6
50	0.2 ± 0.0	0.4 ± 0.1	4.0 ± 0.6	3.9 ± 1.0	5.6 ± 1.0	7.3 ± 1.7
90	0.1 ± 0.0	0.3 ± 0.1	1.7 ± 0.3	2.2 ± 0.4	3.9 ± 0.8	6.5 ± 1.0
150	0.3 ± 0.0	0.3 ± 0.1	1.4 ± 0.2	1.8 ± 0.6	4.7 ± 0.6	6.4 ± 1.5
			Lime			
0	0.3 ± 0.0	0.5 ± 0.1	4.4 ± 1.2	3.9 ± 1.2	6.8 ± 0.9	8.6 ± 1.1
15	0.2 ± 0.0	0.3 ± 0.1	3.7 ± 0.6	4.0 ± 0.8	6.4 ± 1.1	7.4 ± 1.3
50	0.4 ± 0.1	0.2 ± 0.1	5.4 ± 0.0	5.3 ± 0.9	4.0 ± 0.6	4.5 ± 1.0
90	0.5 ± 0.1	0.4 ± 0.2	5.0 ± 0.0	4.8 ± 0.9	2.6 ± 0.1	3.3 ± 0.5
150	0.3 ± 0.0	0.4 ± 0.1	5.0 ± 0.0	4.0 ± 1.4	2.6 ± 0.4	2.4 ± 0.3

[a] Values are given as mean ± standard deviation of each fruit ($n = 4$) analyzed in triplicate.

and low concentrations in citric acid and corresponded to acidless lemon, lime, and orange. The third one (G3), where intermediate values of citric acid and fructose concentrations were observed, corresponded to acidic orange fruit.

DISCUSSION

Three different profiles were distinguished on the basis of titratable acidity: acidic (lime and lemon), intermediate (acidic orange), and acidless (**Figure 1**). Acidic lime and lemon were the most acidic fruit reaching maximum values of 1.0 and 0.8 meq g^{-1} f.p., respectively, at 105 days of development. Our results were consistent with those of Ziena (*19*) and Sadka et al. (*16*), who measured lime and lemon titratable acidity (respectively, 1.0 and 0.9 meq g^{-1} f.p.). We have also showed a lack of titratable acidity for the three acidless varieties ($\leq$0.1 meq g^{-1} f.p). The difference in titratable acidity, between acidic and acidless varieties, took place early in fruit development (20 days for lime and 50 days for lemon and orange).

Titratable acidity of citrus juice is known to be due largely to citric acid (*2*). Citric acid is synthesized in the mitochondria of juice cells via the Krebs cycle and is stored in the vacuole (*1*). In acidic lemon, lime, and orange, citric acid accounted for 79, 71, and 45% of the seven detected organic acids, respectively. These percentages were lower than those reported in the literature, which were estimated on a limited number of organic acids (citric, malic, and quinic acids) (*20*, *21*).

Our results also showed that citric acid was not always the major organic acid since it was quinic acid during the first 50 days after fruit-set (**Figure 2**). A high concentration of quinic was also reported in growing tissues (*22*). Weinstein et al. (*23*) believed that quinic acid is a precursor of the synthesis of flavonoid compounds in plants. Leuschner et al. (*24*) highlighted that quinic acid also could play a role for the biosynthesis of aromatic amino acids. Our results showed that a decrease in

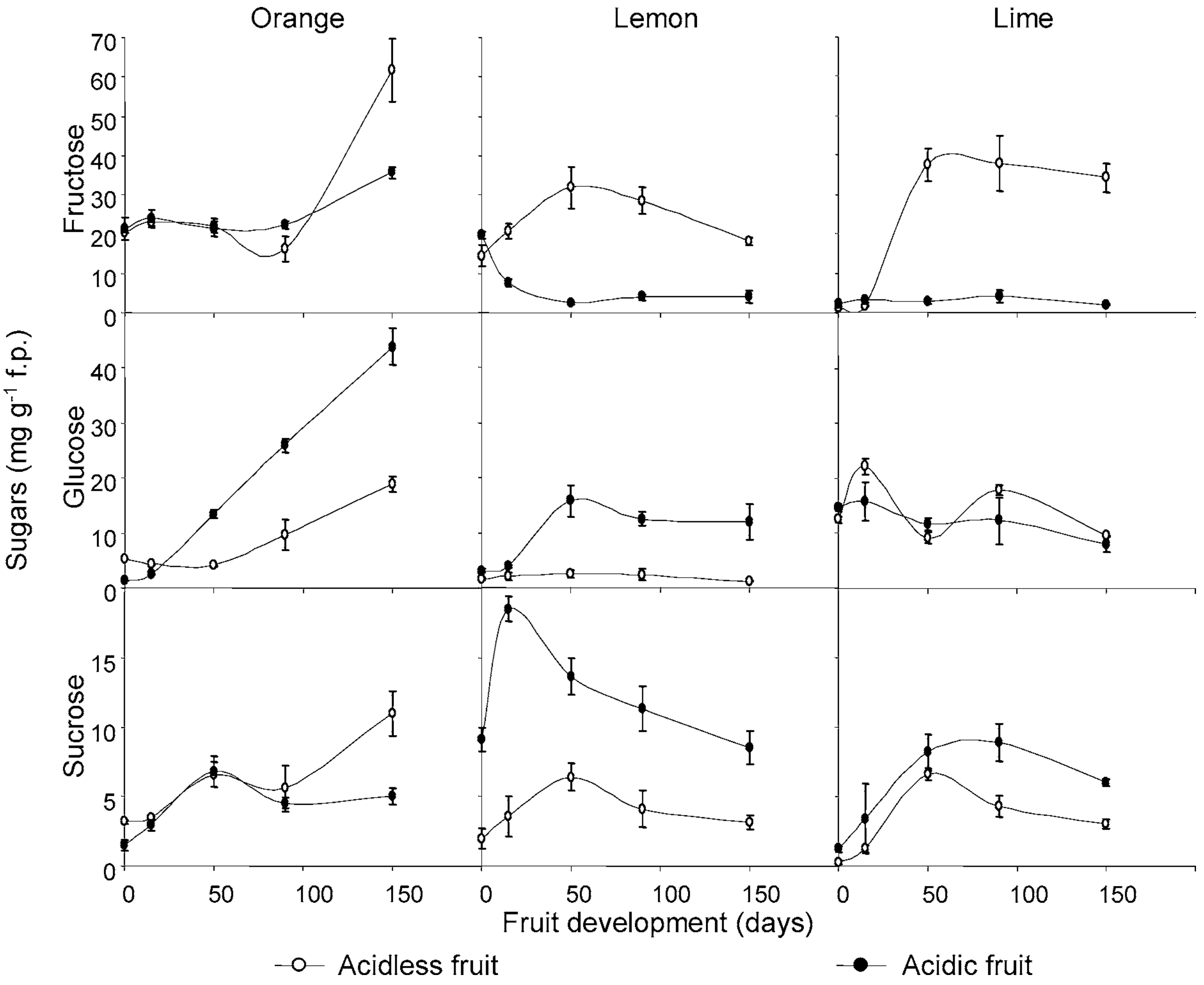

Figure 3. Change in sugar concentrations in citrus fruit pulp during fruit development of acidic (●) and acidless (○) varieties. Each point on the graph shows the mean and standard deviation of three measurements done on each of the four samples.

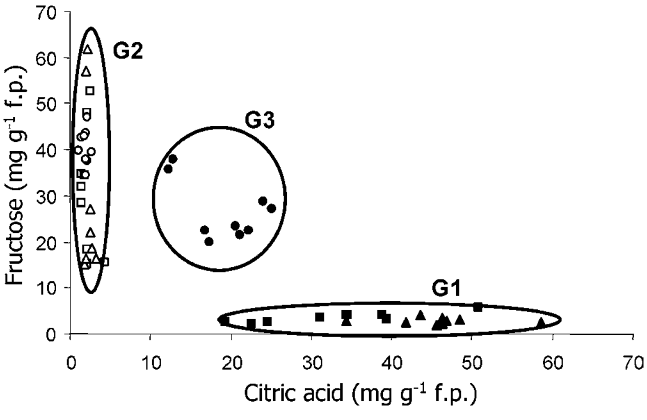

Figure 4. Fructose concentrations vs citric acid concentrations in citrus fruit pulp of Tahiti lime (▲), Villafranca lemon (■), Salustiana orange (●), Brazil sweet lime (△), Sweet lemon (□), and Iaffaoui orange (○).

quinic acid is concomitant to an increase in citric acid (**Figure 2**). Both organic acids having the same precursor, the phosphoenolpyruvate (PEP), we could hypothesize that PEP could be preferentially used for quinate synthesis in the early fruit development and for citrate synthesis afterward.

The major sugars of citrus fruit pulp are fructose, glucose, and sucrose (**Figure 3**). Fructose was the only sugar for which a significant difference could be observed between all acidic and acidless varieties. Fructose was the major sugar of mature acidless lime (34 mg g^{-1} f.p.) as previously reported by Tzur et al. (*25*). Fructose concentrations were significantly higher in the two other acidless varieties, with maximal values of 50 and 55 mg g^{-1} f.p. for lemon and orange, respectively. The fructose concentrations of acidic lemon (6 mg g^{-1} f.p.) and lime (3 mg g^{-1} f.p.) were lower but consistent with the data obtained by Tzur et al. (*25*) and Echeverria (*26*). For acidic orange, we measured 25 mg g^{-1} f.p., and no data were available in the literature with which to compare. When plotting fructose versus citric acid concentrations, we have shown that the most acidic fruit (G1) were characterized by the highest citric acid concentration and the lowest fructose concentration (**Figure 4**). A high level of citric acid is accumulated in acidic lemon and lime vacuoles. This accumulation is accompanied by a large influx of protons that is mediated by the vacuolar H+-ATPase (V-ATPase) (*27*). This influx of protons reduces the vacuolar pH and provides a driving force for additional citric acid uptake (*28*). On the contrary, acidless fruit (G2) were characterized by the highest level of fructose and the lowest level of citric acid. Two hypotheses might explain the difference in citric acid concentration. First, a citric acid synthesis was linked to a failure in its uptake. Echeverria et al. (*29*) have previously reported that the tonoplast-bound H^+-pyrophosphatase (V-PPase) of sweet lime is responsible of the small pH gradient (vacuolar pH is about 5.0) and the lack of citric acid accumulation. An activation of the phosphoenolpyruvate carboxykinase (PEPCK) could occur, and citric acid should be transformed in fructose via the gluconeogenetic pathway, as Famiani et al. (*30*) have reported in soft fruit. Berüter (*31*) have also reported that PEPCK was involved in low acidity of acidless apple. Second, a lack of citric acid synthesis, as has been suggested by Sadka et al. (*16*), and fructose should be directly synthesized from glucose. In G3, acidic orange showed intermediate concentrations of both citric acid and fructose, which might be due to a normal activity of V-ATPase and a higher activity of PEPCK or direct fructose synthesis.

By the Candidate gene approach, we plan to identify genes involved in citric acid accumulation and affected in acidless varieties. The expression of these identified genes will be verified during the fruit development of the acidic orange variety.

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