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Chemical Studies on Stubborn-Affected Marsh Grapefruit and Washington Navel Oranges

It is generally assumed that stubborn is a virus disease, although its cause is still not clearly established (3). Presence of acorn-shaped fruit is the most specific expression of stubborn on orange trees. On grapefruit, blue albedo has been considered a valuable indicator of stubborn, although nonspecific. Stubborn symptoms on citrus trees can be very severe, but an indicator plant for stubborn, such as Mexican lime for tristeza, is not known yet.

Tests for virus diseases are based on specific anatomical or chemical changes that are induced by the virus in the host plant. One way of detecting these changes has been successfully opened by Childs, Norman, and Eichhorn (6, 7) for exocortis when they developed a staining technique giving a "specific color reaction in the phloem ray cells of the bark of exocortis-infected trifoliate orange." Advantages of this colorimetric test over the indicator plant method reside in the fact that it provides a specific test before the morphological symptoms are visible on the indicator plant.

One other approach to the problem would be to detect an early virus-induced, biochemical modification, as for instance the presence in the virus-infected plant of a compound that would be absent in the healthy one, such as the phenols noticed by Martin and Morel (10) in potatoes and tobacco affected with virus.

This paper reports studies on organic acids and amino acids in the juice from normal and stubborn-affected Marsh grapefruit and Washington Navel oranges, as a first step towards this approach.

Materials

The grapefruit for these studies were kindly supplied by Dr. J. B. Carpenter. The blue albedo fruit, as well as the normal fruit, came from the same old-line Marsh grapefruit tree, numbered J.B.C. 436; it should be considered to have stubborn.

The fruit, picked on March 16, 1960, were packed in clean new vermiculite and shipped by air parcel post to Versailles (France) where they arrived on March 22, 1960. Four normal fruit weighed an average of 342 g whereas 13 blue albedo fruit weighed an average of 186 g.

The Washington Navel oranges used in the studies to be reported came from two 11-year-old trees, designated A and C, in a 10-acre block in the Sidi Slimane district in Morocco. Tree A had been given the same cultural treatment as those in the remainder of the block, but tree C was given a yearly "rich additional application" of fertilizer, starting when the tree was 4 years old. Fruit from each tree was picked in 1959 and sorted into 2 categories, normal fruit and stubborn fruit (very small size or misshapen). Tree A yielded 33 normal fruit (average weight, 197 g) and 80 acorn fruit (average weight, 188 g); tree C yielded 341 normal fruit (average weight, 158 g) and 440 acorn fruit (average weight, 104 g) (6). We chose 6 normal fruit (average weight, 199 g) and 8 acorn fruit (average weight, 170 g) from tree A, and 9 normal fruit (average weight, 167 g) and 17 acorn fruit (average weight, 94 g) from tree C.

Methods

PREPARATION OF FRUIT JUICE.—*Marsh grapefruit.*—The peeled fruit were homogenized in a Waring Blendor and the homogenate centrifuged. The supernatant fluid was kept frozen for one month at -10°C before being worked up. Before use, the unfrozen juice was filtered in a folded filter paper. This filtrate is referred to as juice and all results are expressed per 100 ml of this juice.

Washington Navel oranges.—Each fruit was cut into 2 parts—a peduncular half and a stylar half. The normal fruit were cut into 2 equal parts. The stubborn-affected fruits were cut at the level of the acorn constriction, so that the peduncular half corresponded to the part of the fruit where the peel had a normal thickness, whereas the stylar half corresponded to the narrow-skinned part of the fruit. The juice was extracted with a lemon squeezer. Juices from similar half-fruits were combined and filtered, first over a cheesecloth, then over folded filter paper. Results are expressed per 100 ml of this filtrate, referred to as juice.

Free acidity.—Aliquots of juice were titrated with 0.1 NaOH in the presence of phenolphthalein in the case of the grapefruit, and in the presence of phenol red in the case of the oranges. From titration curves, it was found that phenol red was a better pH indicator than phenolphthalein in the sense that the pH zone of the color change corresponds more closely with the equivalent point.

TOTAL ACIDITY AND SEPARATION OF SOLUBLE CONSTITUENTS INTO THREE GROUPS.—The constituents of the juice were separated into 3 groups: (a) acidic components, including the organic acids; (b) basic components, including the free amino acids; and (c) neutral components, including the soluble sugars, by passing the juice successively through a column of Permutite 50, H^+ form, (a strongly acid, sulfonated polystyrene cation exchange resin) and a column of Dowex 2, CO_3^{--} form (a quaternary ammonium, strongly basic, anion exchange resin).

The basic components are retained on Permutite 50, and the acidic ones on Dowex 2. The neutral substances, including the sugars, are not retained, and end up in the effluent from the Dowex 2 column. Total acidity was determined on an aliquot of the effluent from the Permutite 50 column, before passing it through the Dowex 2 column. The titrations were done with the same pH indicators used for free acidity determinations.

The amino acids were eluted from the Permutite 50 with 1 N NH_4OH and the ammonium salts of the acid components from the Dowex 2 by 0.5 N CO_3 $(NH_4)_2$.

The sugar solution, the amino-acid solution, and the organic acid ammonium-salt solution were respectively taken to dryness under vacuum at 25°C. Excess NH_4OH or $CO_3(NH_4)_2$ was thereby eliminated. The sugars were redissolved in a small volume of water. So were the amino acids and the organic-acid-ammonium salts. The latter were passed furthermore through a Permutite 50 column to free the organic acids from their ammonium salts.

Thus one obtains 3 solutions: an amino-acid solution, a free organic-acid solution, and a sugar solution.

SEPARATION AND DETERMINATION OF ORGANIC ACIDS.—The organic acids were separated by partition chromatography on a silicic acid column (1). They were eluted from the column by mixtures of tertiary butanol in chloroform; the eluate was collected with a fraction collector, and the fractions were titrated with 0.005 N NaOH.

The great amount of citric acid present in certain citrus-fruit juices and its percentage, very high in comparison with that of other organic acids present, render it generally difficult to detect and determine the other organic acids. We overcame the problem by separating the organic acids into 2 groups: one containing only malic, citric, and isocitric acids; the other containing fumaric, glutaric, itaconic, succinic, lactic, alpha-ketoglutaric, aconitic, malonic, oxalic, and glycolic acids (2).

The nature of the organic acids separated on silicic acid column was furthermore checked and confirmed by two-dimensional paper chromatography (4).

SEPARATION AND DETERMINATION OF THE FREE AMINO ACIDS.—The amino acids were separated by two-dimensional paper chromatography (9). The purple spots obtained after spraying with ninhydrin were eluted and the optical density measured at 570 mu (11).

SEPARATION OF SOLUBLE SUGARS.—The sugars were separated by one-dimensional paper chromatography (8).

TOTAL SOLUBLE NITROGEN.—Total soluble nitrogen was determined on the amino-acid solution by the Kjeldahl method.

Results

FREE ACIDITY, TOTAL ACIDITY, AND ORGANIC ACIDS.—Data for grapefruit are summarized in Table 1; those for oranges in Table 2. In the grapefruit, there seemed to be little difference in acid content between

TABLE 1. ACIDITY DATA ON GRAPEFRUIT JUICE FROM NORMAL AND BLUE-ALBEDO FRUIT (FIGURES ARE MILLI-EQUIVALENTS (MEQ) PER 100 ML OF JUICE)

	Normal fruit	Blue albedo fruit
Free acidity	21.25	21.80
Total acidity	27.10	28.30
Citric acid	21.72	25.60
Malic acid	0.65	0.49
Oxalic + glycolic acids	0.14	0.20
Aconitic + malonic + alpha-keto-glutaric acid	0.13	0.19
Succinic acid	0.05	0.06

TABLE 2. Acidity data on juices from Washington Navel oranges (figures are in milli-equivalents (meq) per 100 ml of juice)

	Tree A				Tree C			
	normal fruit		acorn fruit		normal fruit		acorn fruit	
	peduncular half	stylar half	peduncular half	stylar half	peduncular half	stylar half	peduncular half	stylar half
Free acidity	17.00	17.20	17.40	18.70	23.40	21.80	23.80	30.20
Total acidity	22.70	22.00	24.20	24.70	29.00	24.20	29.70	37.00
Citric acid	18.96	18.56	20.56	21.21	26.77	22.64	27.61	35.17
Malic acid	3.18	3.14	3.13	3.13	1.77	1.38	1.80	1.19
Oxalic + glycolic acids	0.18	0.13	0.19	0.22	0.20	0.14	0.19	0.17

normal and albedo fruit. With respect to the oranges, total acidity was higher in acorn fruit of tree C than in normal fruit and this was mainly due to the higher citric acid content of the stylar half of the acorn fruit. The same trends seemed to show up in the case of tree A, but to a much smaller extent. As a matter of fact, there was a greater difference in acidity between tree A and tree C than between normal and acorn fruit from tree A.

Besides citric, malic, oxalic, and glycolic acids, the following acids have been chromatographically identified and tentatively estimated in the oranges, but so far no significant differences concerning these acids have been found between the normal and the stubborn-affected fruit: succinic acid (0.05 meq/100 ml juice) alpha-ketoglutaric acid (0.01 meq/100 ml juice), aconitic + malonic acids (0.07 meq/100 ml juice), and isocitric acid (traces). Quinic, shikimic, and glyceric acids have not been looked for. A few acids, present in microtrace amounts, have not been identified yet.

Total soluble nitrogen and amino acids.—Tables 3 and 4 summarize the results concerning total soluble nitrogen and amino acids, respectively, for the grapefruit and the oranges. The blue albedo grapefruit contained much less arginine, proline, and gamma-amino-butyric acid than the normal fruits. In the case of the oranges, the total soluble nitrogen was higher in the acorn fruit than in the normal ones. There was an increase in arginine, aspartic acid, alanine, and gamma-amino-butyric acid in the stylar half of the acorn fruit and a decrease of most of these acids in the peduncular half of the same fruit.

Besides the amino acids listed in Tables 3 and 4, the following free amino acids have been identified in grapefruit and oranges, stubborn-affected or not: lysine, asparagine, glutamine, glycine, threonine, tyro-

TABLE 3. Total soluble nitrogen, and nitrogen of various amino acids in grapefruit juice from normal and blue albedo fruit (figures are milligrams of nitrogen per 100 ml juice)

	Normal fruit	Blue albedo fruit
Total soluble nitrogen	105.2	111.7
Arginine	53.2	30.8
Proline	17.5	13.0
Aspartic acid	11.9	9.5
Alanine	14.0	11.9
Gamma-amino butyric acid	13.3	6.7
Serine	6.3	5.6
Glutamic acid	2.6	2.1

TABLE 4. TOTAL SOLUBLE NITROGEN, AND NITROGEN OF VARIOUS AMINO ACIDS IN ORANGE JUICE (FIGURES ARE MILLIGRAMS OF NITROGEN PER 100 ML OF JUICE)

	Tree A				Tree C			
	normal fruit		acorn fruit		normal fruit		acorn fruit	
	peduncular half	stylar half	peduncular half	stylar half	peduncular half	stylar half	peduncular half	stylar half
Total soluble N	95.00	72.00	137.00	83.00	81.00	83.00	115.00	100.00
Arginine	17.0	25.2	11.2	29.4	19.0	19.5	16.7	33.5
Proline	8.0	7.9	11.2	11.2	12.1	15.4	15.0	10.9
Aspartic acid	3.4	2.6	3.1	3.6	6.0	2.3	3.0	7.3
Alanine	2.1	1.9	2.8	3.0	4.2	1.9	3.1	5.9
Gamma-amino-butyric acid	2.1	2.6	1.7	3.0	3.7	2.4	3.0	6.3
Serine	2.3	2.3	1.9	3.1	2.4	2.4	3.0	4.0
Glutamic acid	1.1	0.9	1.4	0.8	1.6	0.9	1.4	1.6

sine, valine, leucine, phenylalanine, and cysteine. Asparagine was present in great amounts, but has not been estimated yet. Tyrosine, valine, leucine, phenylalanine, and cysteine were present in very low amounts. Three undetermined compounds—I, II, and III—have been detected on the ninhydrin sprayed chromatograms from the orange juices. They could not be detected in grapefruit juices. Compound I moves close to asparagine. Compound II lies between threonine, glutamine, and alanine. Compound III has an Rf value of 0.98 in the phenol solvent. Two undetermined compounds, IV and V, have been noted in trace amounts on the chromatograms from grapefruit juices, but not on those from orange juices.

Soluble sugars.—Fructose, glucose, and sucrose were separated by paper chromatography. From the size of the spots on the developed chromatograms, it was very apparent that the blue-albedo-grapefruit juice contained less sucrose than the normal one, and that the stylar juice from the acorn oranges also contained much less sucrose than the normal stylar juice. Work is underway to check, with accurate sugar determination, the preceding rough estimations.

Discussion and Conclusion

From the data presented, it appears that stubborn induces rather clear-cut chemical modifications in the juice from affected grapefruit and oranges. That the chemical differences between the normal and affected fruit are due to stubborn seems highly probable, since the affected fruit showed severe stubborn symptoms. However, the results obtained so far indicate that the chemical modifications induced by stubborn respectively on oranges and on grapefruit are not similar.

The observed chemical differences are only quantitative, not qualitative. But one should remember that the so-called "normal" fruits come from the same tree as the affected fruits. The probability of finding a qualitative difference, the only one of real value in trying to establish a virus test, would be greater if one were to look for differences between diseased material and 100 per cent healthy material. At the present time, it is difficult, if not impossible, to find such healthy material, at least in Morocco.

Tree C has received during 7 years a "rich additional application" of fertilizer. Tree A did not receive it. The data on organic acids and amino acids show that the chemical modifications induced by stubborn are greater on tree C than on tree A. Thus it seems as if applying more fertilizer has resulted in enhancing the effects of the disease.

We think that the ultimate chemical work intended to find a virus test for stubborn, or for any other citrus virus disease, should not be done on fruit, because it may take a long time before a tree bears a fruit. But, in a preliminary phase, work on severely affected fruit can open the way to a test that would have to be worked out on vegetative material. In the case of stubborn, the use of fruit can be helpful because the disease affects the fruit so very much.

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