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4/15/52

A REVIEW OF LABORATORY TESTS FOR THE DETERMINATION OF CITRUS ROOTSTOCK VARIETIES

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EVER since the announcement that sweet oranges on sour rootstocks were dying from a disease, which subsequently has been called "quick decline," growers have sought some means of determining the type of rootstocks their oranges were growing upon. They wish to know so they may fortify themselves against economic loss by taking immediate steps to obtain trees on disease resistant rootstocks for replacmeent of trees that may die. On the other hand, if they could be assured that their trees are on a resistant stock they could proceed with future plans with confidence.

As a public service to the citrus growers of California and elsewhere, Citrus Leaves *publishes herewith a chemical method of determining varieties of rootstock. It is understood that any grower, with a high school education in chemistry, can carry out these tests himself.*

—The Editor

THERE is a great need for the development of a dependable method for determining the identity of citrus rootstocks. This need has become pronounced because of variations in the susceptibility of several commercially used citrus rootstocks to virus and fungus diseases, and because of the desirability of selecting rootstocks superior for particular soil and climatic conditions.

It is impossible to identify a rootstock variety with certainty from the appearance of the bud union, stock, and scion except in certain combinations of stocks and scions, as in the case of trifoliate stock overgrowth. Inducing leafy shoots to develop from the rootstock and observing the shape of the leaves and their characteristic odor when crushed will give additional aid in identification. The chemical methods applied by Halma and Haas[1] have been used with success in identifying sweet orange and sour orange stocks. The same tests have been used with less confidence in attempting to distinguish other stocks. Marloth[2] and Bacchi[3] have both applied these methods with some success. This paper is a report of the experiences of the author using these methods.

In order to obtain a satisfactory bark sample for these identification tests, select an area just below the bud union and scrape free of foreign matter with the back of a knife blade, being very careful not to penetrate the bark. With a sharp knife remove lengthwise of the trunk a spindle-shaped piece of bark measuring approximately 2½ to 3 inches in length and 1 inch at its greatest width. Since it has been found that diseased trees do not always give a typical reaction, it is helpful, when testing diseased trees, to test an additional sample from a healthy tree in the same grove. The variety of the top and any other pertinent data, such as striations, sandwiches, overgrowths of the bud union and also its relation to the ground should be noted.

The sample should be dried for at least 24 hours in an oven at 65° C., then run through a grinder or broken up with a mortar and pestle so that the resulting powder will pass through a 40-mesh sieve.

At the present time, four chemical solutions are used in colorimetric tests. They are Almen's reagent and solutions of ferric chloride, ammonium molybdate, and titanous chloride.[4] Determination of the degree of fluoresence in ultra-violet light is also used as a supplementary test.[5]

The procedure used in the laboratory for making colorimeric tests is to place 0.75 grams of the dried, ground bark in a beaker with 20 cc. of distilled water. Allow the suspension to stand for 30 minutes, agitating occasionally, and then filter. Finally wash the residue with an additional 10 cc. of distilled water. Of the approximately 25 cc. of filtrate thus obtained, 10 cc. are used for the Almen test and 5 cc. for each of the three remaining tests. All tests are made in standard pyrex test tubes.

Almen test: To 10 cc. of the bark extract add 2 drops of the potassium hydroxide solution and then 8 drops of a saturated solution of copper sulfate. This is shaken thoroughly and 8 drops of the Almen reagent are added and the solution is then boiled for 10 to 15 seconds. The resulting color is noted 2 to 3 hours later when it has developed maximum intensity and the precipitate which formed during the reaction has settled to the bottom of the test tube.

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1/Halma, F. F. and A. R. C. Haas. Identification of certain species of citrus by colorimetric tests. Plant Physiology 4:265-268. 1929.

2/Marloth, R. H. Notes on colorimeteric tests for citrus species. Jour. Pomology and Hort. Sci. 14:1-8. 1936.

3/Bacchi, Osvaldo. Identificacao colorimetrica em citrus. Bragantia 3:179-192. 7 figs. 1943. (In Portuguese)

4/Almen's reagent is made by weighing 110 grams of pure mercury in a beaker and transferring this to a clear glass bottle to which is then added 110 cc. of red fuming nitric acid C.P. (sp. gr. 1.60) and covering the mouth of the bottle with an inverted beaker and placing this in a hood. After the action has ceased (from 3 to 4 hours) dilute with 220 cc. of distilled water. The ordinary C.P. nitric acid cannot be used for this purpose. This test requires a potassium hydroxide solution containing 30 grams of C.P. KOH in 100 cc. of distilled water.

The ferric chloride reagent is a saturated solution of C.P. $FeCl_3$ in distilled water. (125 gms. $FeCl_3$ in 29 cc. H_2O.)

The ammonium molybdate reagent is made by dissolving 100 grams of molybdic acid in a mixture of 144 cc. of ammonium hydroxide (sp. gr. 0.90) and 271 cc. of distilled water. Pour SLOWLY with constant shaking into a COOL mixture of 489 cc. of nitric acid C.P. (sp. gr. 1.42) and 1148 cc. of distilled water.

Optional ammonium molybdate reagent: Solution A. Ten grams of ammonium molybdate are dissolved in 40 ml. of water and to this solution are added 8 ml. of 15 M ammonium hydroxide. If a precipitate appears, the solution is filtered. Solution B. Forty ml. of concentrated nitric acid, sp. gr. 1.42, are mixed with 60 ml. of distilled water. When ready to use, the proper quantity of Solution A is mixed with twice its volume of Solution B. Solution A is added to Solution B very slowly and with rapid stirring.

Titanous chloride is used as a 20 per cent solution in distilled water and is obtainable in this concentration from chemical suppliers.

5/The ultra-violet lamp used in these tests should be a Shannon laboratory and general purpose ultra-violet fluorescent lighting unit or its equal; it is equipped with two snap-on filter holders, with a five-inch heat resisting, red purple, rounded filter, and a 3⅛-inch, square, ultra-violet filter, and with spill ring louvre; complete with spot lamp.

Ferric chloride test: Add 2 drops of the ferric chloride solution to 5 cc. of the bark extract. This is shaken at once and observed during the first 15 minutes. The immediate result is indicative of its degree of turbidity and color with the reaction reaching its peak at the end of this time. Sweet orange usually shows an immediate turbidity with a heavy precipitate within 15 minutes. Sour orange usually remains clear during this interval but an occasional sample may show some cloudiness.

Ammonium molybdate test: Three to 4 drops of the ammonium molybdate reagent (either the original or optional reagent) are added to 5 cc. of the bark extract and the solution is then boiled for 20 to 30 seconds. Within 3 to 5 minutes the reaction gives its most typical color and should be read and recorded during that period. The tests are then set aside for 24 hours to allow the solutions to clear, thus making it possible to study the results and match the colors. Rough lemon is an exception to this procedure. The immediate reaction (3 to 5 minutes) is typical but at the end of 24 hours the color may not be strong enough to be conclusive. In this case it is necessary, if a 24 hour check is desired, to reduce the bark extract by adding an equal amount of water and taking 5 cc. of this solution. To this are added 3 drops of the reagent. The result thus obtained is a clear blue, paler than that obtained with the first method. The optional reagent gives slightly better results when used with the diluted bark extract than does the original reagent.

Titanous chloride test: Two drops of titanous chloride solution are added to 5 cc. of the bark extract and then shaken. The reaction is completed within an hour.

An ultra-violet lamp has been in use for several years for determining the degree of fluorescence from bark and other extracts and solutions. While the results with extracts of citrus bark have been variable, the chart at the bottom of the next page shows differences with ten varieties and indicates that the method will serve as a useful supplementary test. Specimens of bark of known identity have shown the degrees of fluorescence recorded in the following chart.

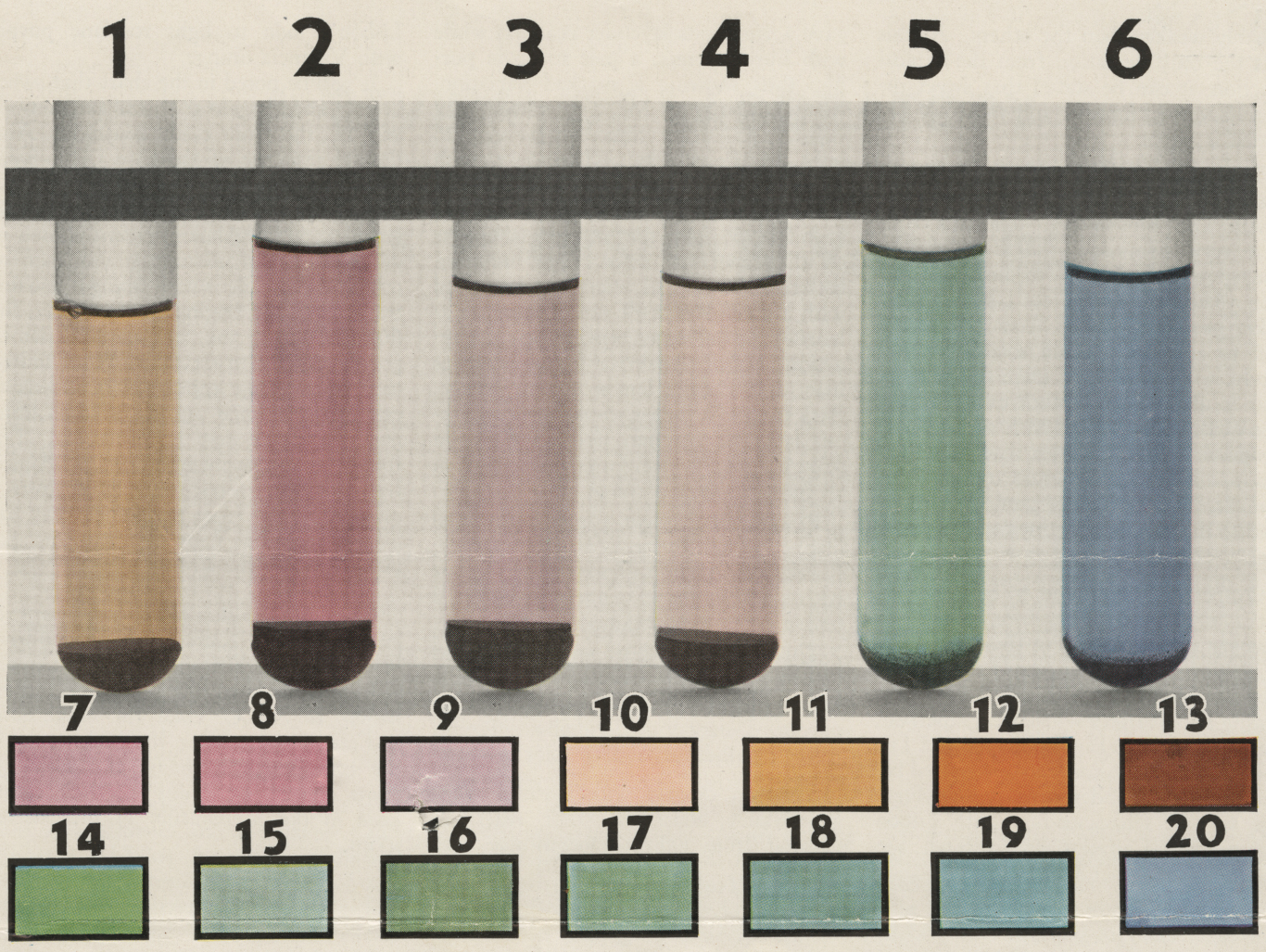
With the results obtained from testing known rootstocks a chart has been pre-

COLORIMETRIC CHART FOR THE DETERMINATION OF ROOTSTOCK VARIETIES

ALMEN TEST	FERRIC CHLORIDE	AMMONIUM MOLYBDATE	TITANOUS CHLORIDE	ULTRA VIOLET (Fluorescence)	VARIETY
Sour Group					
Cinnamon (1)	Brown to Dark Brown, Very Clear	Light Sulfate Green (5) to Light Cendre Green	Olive Green	Poor	Sour orange *C. Aurantium*
Ochraceous Buff (11) to Zinc Orange (12)	Very Dark Reddish Brown, Clear	Methyl Green (18) to Tyrolite Green (17)	Olive Green	Poor	Bittersweet *C. Aurantium*
Ochraceous Buff (11)	Very Dark Brown Clear	Motmot Blue (19) to Methyl Green (18)	Light Brown, Green Tint, Clear	Fair	Cuban shaddock *C. limon*
Brick Red (13)	Nearly Black, Clear	Sulfate Green (16)	Dark Green, Brownish Tint, Very Clear	Poor	Eureka lemon *C. limon*
Grapefruit Group					
Pale Vinaceous (4) Lilac	Pale to Straw Yellow, Cloudy	Methyl Blue (6)	Light Brown, Pink Tint	Good	Rough lemon *C. limon*
Persian Lilac (8)	Straw Yellow Cloudy	Venetian Blue (20)	Weak Very Light Brown S1. Irradiant	Excellent	Sweet lime *C. aurantifolia*
Persian Lilac (8)	Light Amber Clear	Pale Sulfate Green (15)	Brownish Cream with Pink Tint	Excellent	Trifoliate *Poncirus trifoliata*
Vinaceous Lavender (9)	Straw-yellow, Cloudy	Light Sulfate Green (5)	Pale Pinkish Irradiant	Fair	Tangerine *C. reticulata*
Vinaceous Lilac (3)	Cream to Buff Yellow – Cloudy	Light Sulfate Green (5)	Yellow to Light Brown	Good	Grapefruit *C. paradisi*
Sweet Group					
Light Rosolane Purple (2)	Brown, Turbid	Cendre Green (14)	Vivid Green	Fair to Poor	Sweet orange *C. sinensis*
Pale Rosolane Purple (7) to Persian Lilac (8)	Cream to Light Brown Turbid	Pale Sulfate Green (15) to Methyl Green (18)	Weak, Pinkish Irradiant	Good	Mandarin *C. reticulata*
		Methyl Green (18) to Sulfate Green (16)	Deep Yellowish Green	Fair	Tangelo
Light Congo Pink (10)	Dark Brown, Turbid	Cendre Green (14)	Light Brown Greenish Tint, Cloudy	Poor to Excellent	Citrange

All the above colors with a number following in parenthesis are terms assigned by Ridgway.

It should be emphasized that the colors shown in this chart were obtained with the reagents made as described. Any modification of these reagents may change the color value.



pared which gives the normal colors and the degree of fluorescence to be expected for a given rootstock or for a group of similarly reacting rootstocks. A sample of bark of unknown identity can thus be tested with the four chemicals and an ultra-violet lamp, and from the reactions obtained its probable identity determined.

The procedure is simplified by first testing the unknown with the Almen reagent as this indicates its probable identity and places it in one of the three groups as shown on the chart. The remaining tests are used, generally, to confirm or modify the conclusions drawn from the Almen test and assist in separating the species within one of the color groups.

The color plate shows the color reactions obtained with the Almen and ammonium molybdate tests of the varieties mentioned. These colors were painted from test solutions observed by resting the test tubes on white paper and holding them immediately beneath the shade of a fluorescent lamp.[6] In order that they might be accurately described and that a definite name might be given to each, they were matched as nearly as possible with the colors shown in Ridgway's "Color Standards and Nomenclature."[7] These can be considered only approximate because of the variations in bark samples and the differences in the eyesight of individuals performing the test.

Color Number	Ridgway Color Name	Plate Number
1	Cinnamon	XXIX
2	Light Rosolane Purple	XXVI
3	Vinaceous Lilac	XLIV
4	Pale Vinaceous Lilac	XLIV
5	Light Sulphate Green	XIX
6	Methyl Blue	VIII
7	Pale Rosolane Purple	XXVI
8	Persian Lilac	XXXVIII
9	Vinaceous Lavendar	XLIV
10	Light Congo Pink	XXVIII
11	Ochraceous Buff	XV
12	Zinc Orange	XV
13	Brick Red	XIII
14	Cendre Green	VI
15	Pale Sulphate Green	XIX
16	Sulphate Green	XIX
17	Tyrolite Green	VII
18	Methyl Green	XIX
19	Motmot Blue	XX
20	Venetian Blue	XXII

INTENSITY OF FLUORESCENCE OF BARK EXTRACTS IN ULTRA-VIOLET LIGHT

Bark material from	No. of Samples	Excellent	Good	Fair	Poor
Grapefruit	18	4	13	1	0
Sweet Orange	10	0	0	6	4
Sour Orange	9	0	0	1	8
Mandarin	7	1	6	0	0
Sampson tangelo	5	0	1	2	2
Rough lemon	12	2	8	2	0
Shaddock	4	0	0	3	1
Citrange	4	1	1	1	1
Dancy tangerine	2	0	0	2	0
Trifoliate	5	4	1	0	0

The colors used for the above chart were selected, in most cases, from average color reactions of several varieties of each species. The sweet orange varieties tested were Koethen, Madam Vinus, Bessie, Oroville, Indian Orchard and Homosassa. These gave a light rosolane purple color reaction with the Almen test. Tests were also made using several scion varieties with a given rootstock. Bessie sweet with Eureka lemon top gave a color no-

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6/Dazor P-2324-16 fluorescent desk lamp, office model with two 15W. T8 bulbs 18 inches long, daylight, code F 15T8D.

7/Ridgway, Robert. Color standards and nomenclature. 44 pp., 53 pls., 1115 colors, Washington, D.C.

ticeably weaker than the others. As this was the only deviation from the typical reaction, it can be stated that sweet orange is very consistent and the color obtained is stable.

The sour orange varieties used were Standard, African, Brazilian, Paraguay, Seville and Bittersweet. The first four were very consistent in their color reaction, giving a cinnamon to pinkish cinnamon. The reactions of the last two were alike, producing colors from ochraceous buff to zinc orange and sometimes, but rarely, to brown. The scion did not appear to affect the rootstock except in the case of Brazilian sour, which with a navel top gave the lightest color reaction of the group, and with a Valencia top gave the darkest. This difference could be attributed to the variation in the samples rather than to the effect of the scion.

Grapefruit was found to be inconsistent in its reaction. Therefore, in order to discover a color typical of this species, all available grapefruit varieties were used. Duncan was tested with each of the following scion varieties—Marsh grapefruit, Valencia orange, Eureka lemon and Lisbon lemon. The color reactions varied between light lavender-violet with Marsh top, to pale vinaceous-lilac with Lisbon lemon top. Camulos with Marsh top gave a vinaceous lavender reaction while with Eureka lemon a pale rosolane purple, thus showing a marked variation. Limited tests with McCarty varied only slightly, producing vinaceous lavender to light vinaceous lilac. The tests with C.E.S. #343 grapefruit did not conform with those listed above. This strain has been found to give the most erratic results of all grapefruit tested. The variation between the several scion varieties used with a given rootstock was striking. Valencia gave an orange vinaceous color reaction; Lisbon lemon, a light vinaceous lilac, and Eureka lemon a zinc orange. Bark from a Windsor scion gave a vinaceous lavender reaction; from a Claysom scion a light purplish-vinaceous and from an Imperial scion a congo pink.

Rough lemon gave a pale vinaceous-lilac reaction with Valencia orange, Eureka lemon, and Lisbon lemon tops, but with a Marsh grapefruit top a pale laelia pink reaction was obtained.

The mandarins, Cleopatra, King, and Onoco, gave similar reactions with colors ranging from pale laelia pink to liseran purple. Dancy was decidedly different, giving a pallid purple-drab color reaction.

Sampson tangelo with various scions gave fairly consistent color reactions varying from light vinaceous-lilac with Lisbon lemon to light rosolane purple with Valencia orange.

It will be noted in the chart that sweet orange and tangelo give similar reactions and are therefore easily confused. Also the fluorescence of these two is usually the same, being classified as fair. However, if the solution which was previously tested with the Almen reagent is used in determining fluorescence, it will then be noted that while sweet orange remains the same as the original sweet filtrate, the fluorescence of the tangelo increases to *good* and often to *excellent*. From limited tests the citrange appears to be inconsistent in the Almen and fluorescent tests, and, therefore, may be confusing. Trifoliate is also inconsistent in the Almen test, but with ferric chloride it gives a color different from any other that has been tested. The fluorescence of this species is usually the strongest of all.

The author wishes to express his appreciation to L. J. Klotz for his encouragement and suggestions and to other department staff members for their comments; to A.R.C. Haas for suggestions and details regarding the technique of the colormetric methods, and to L. J. Klotz and H. D. Evans for their assistance in the preparation of this manuscript.