Distribution of Stubborn Disease Virus in Trees of *Citrus sinensis* and *C. paradisi* at Different Seasons

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THE PER CENT OF transmission and perpetuation of stubborn disease from severely affected mother trees has ranged from 0 to 100 in numerous experiments by the authors during the past decade. Other workers also noted normal trees among inoculated plants (5) and among the progeny of diseased trees (4, 7). Calavan and Christiansen (2) suggested that normal trees occur among inoculated plants and bud progeny of stubborn trees because of the irregular distribution of the pathogen within the host. With the advent of a rapid-indexing method for stubborn disease (3), it became possible to determine the distribution of virus within stubborn-diseased trees by the reaction of inoculated indicator plants. This paper reports results of grafting sensitive indicator plants at different seasons with tissues from various parts of stubborn trees.

Materials and Methods

INOCULA.—The main sources of inocula were 3 severely diseased nucellar-clone trees: a 6-year-old pink Marsh grapefruit (*Citrus paradisi* Macf.) on sour orange (*C. aurantium* L.) rootstock, a 5-year-old Frost Washington navel sweet orange [*C. sinensis* (L.) Osb.] on Troyer citrange [*C. sinensis* x *Poncirus trifoliata* (L.) Raf.] rootstock, and an 8-year-old Campbell Valencia sweet orange on Troyer citrange rootstock. These grapefruit, navel, and Valencia source trees (designated G, N, and V, respectively) all had shown fruit, leaf, and shoot symptoms normally associated with stubborn disease (1). They had been graft-inoculated with virus from trees used in previous experiments; the G source from tree 29-36, and the N and V sources from tree C-189 (2). Tree V is also infected by tristeza virus. Other inocula used were columellas and anthers obtained from naturally infected 5-year-old Frost Valencia trees on trifoliate orange [*P. trifoliata* (L.) Raf.] rootstock. Inocula were collected October 11, 1965, January 19, 1966, May 3, 1966, and August 15, 1966. The portions of trees G, N, and V used as inocula are indicated in Figure 1.

Ten branches were cut from each tree on each collection date to obtain all the branch parts used for inocula, except the expanding leaves,

columella, and anthers. Grafting procedures were as follows: leaf pieces and wood shields were inserted under flaps of bark; trunk bark patches replaced bark pieces removed from the indicator plants; bud grafts, internodal bark shields, and root bark were inserted into T-cuts; and side grafts were inserted into diagonal clefts in the stem of indicator plants. The upper and lower sides of midveins in leaf pieces were scraped. All grafts were wrapped with plastic tape or rubber bands. Side grafts and portions of supporting stems were enclosed in polyethylene sleeves for

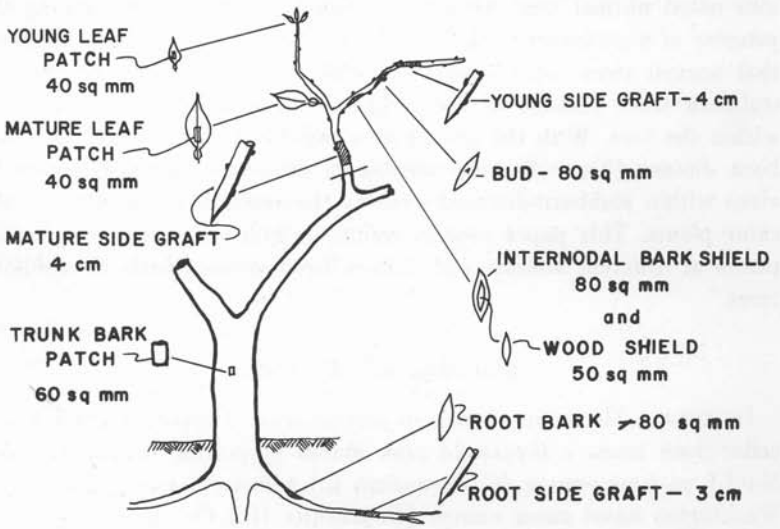

FIGURE 1. *Sketch showing kinds of pieces taken from stubborn trees G (grapefruit), N (navel), and V (Valencia) for graft-inoculation of indicator plants.*

2 weeks. Except for side grafts, 2 contiguous and similar sections from a source tree were used to inoculate each indicator plant. No plant was inoculated with grafts from more than 1 branch or root. Side grafts were limited to 1 per plant; the side graft from each shoot was supplemented by a bud graft from that shoot.

INDICATOR PLANTS.—Madam Vinous sweet orange was selected as the indicator variety because it consistently gave a strong and typical reaction to stubborn virus. Indicator plants for the October and January series were produced by propagating 1 bud from a Madam Vinous seedling on each Rough lemon (*C. jambhiri* Lush.) seedling at the time of inoculation. Ten replications were made for each type and major source

of inoculum on the October and January dates. Replications of May and August inoculations were changed to 5 inoculations in Rough lemon seedlings budded with Madam Vinous and 5 inoculations in Madam Vinous seedlings. Grafts were examined for vitality after 3-4 weeks. After 4 weeks, plants in the October and January series were bent to force the Madam Vinous buds; after 12 weeks the Rough lemon tops were cut off about 3 cm above the upper grafts. Plants in the May and August series were bent at 3 weeks and cut off at 4 weeks after inoculation. A single Madam Vinous shoot was grown near an inoculation site on each indicator plant until it showed symptoms of stubborn disease or attained a height of 1 m. Symptomless shoots 1 m tall were cut back to 20 cm to force growth of a new shoot. Ten non-inoculated control plants for each series were similarly treated. A side graft from each mature shoot used as inocula from trees N and V was grafted into a Rough lemon seedling and grown to serve as an indicator of disease perpetuation. All plants were examined weekly for symptoms.

The plants were grown in sterile U.C. mix, to which minor elements had been added, in a glasshouse free of insects and *Phytophthora* spp. harmful to citrus. Temperature ranges in the glasshouse for the 3-month period following inoculation averaged 21-29, 20-31, 20-34, and 19-33°C, respectively, for the October, January, May, and August series.

A supplemental experiment compared the effectiveness of bark and wood grafts from an Atwood navel orange tree infected with stubborn disease, vein-enation, and tristeza viruses. Separate wood and bark portions of 50 internodal shields from 25 branches were inserted into T-cuts in Hinckley sweet orange seedlings, using one piece of inoculum per plant.

Results

Survival of bud, bark, and side grafts was nearly 100 per cent. However, both grafts died in 12 per cent of the plants inoculated from expanding leaves, and in 25 per cent of those inoculated from mature leaves. All pieces of wood and most columella grafts seemed dead. The possibility of tissue union between indicator plants and grafts that died was not verified; nor was the fate of the anthers determined.

REACTION TO VARIOUS INOCULA.—Results are listed by type, source, and date of graft in Table 1. With few exceptions, positive reactions were severe. All inoculated normal plants cut back to 20 cm remained symptomless. Type and source of inocula influenced the probability of reaction but not the incubation period or symptomatology. Grafts from

TABLE 1. REACTION OF MADAM VINOUS SWEET ORANGE SEEDLINGS AND BUDLINGS TO INOCULA FROM VARIOUS PARTS OF STUBBORN-DISEASED GRAPEFRUIT, NAVEL, AND VALENCIA ORANGE TREES COLLECTED IN OCTOBER, JANUARY, MAY, AND AUGUST

Kind of graft	Number of indicator plants with stubborn symptoms[a]											
	Source of inoculum and month grafted											
	Grapefruit				Navel				Valencia			
	Oct.[b]	Jan.[b]	May[c]	Aug.[c]	Oct.[b]	Jan.[b]	May[c]	Aug.[c]	Oct.[b]	Jan.[b]	May[c]	Aug.[c]
Leaf patch, young	3	0	0	5	2	7	4	3	0	0	3	5
Leaf patch, mature	0	0	0	0	0	0	0	0	0	0	0	0
Bud	1	0	8	0	1	2	4	1	0	1	5	0
Bark shield, internodal	0	0	4	2	2	1	3	0	0	0	2	0
Wood shield, internodal	0	0	0	0	0	0	0	0	0	0	0	0
Side graft + bud, young	9	4	7	8	7	9	9	8[e]	1	2	8	2[e]
Side graft + bud, mature	8	6	9	9[e]	7	6	10	9[e]	1	2	10	4
Side graft, root[d]	0	0	0	0	2	0	0	0	0	0	0	1
Bark patch, trunk	0	0	0	0	7	0	4	9	0	0	0	4
Bark, root[d]	0	0	0	0	0	0	0	0	0	0	0	0
TOTALS	21	10	28	24	28	25	34	30	2	5	28	16

a. Of 10 plants inoculated in each category.
b. Indicator plants: Madam Vinous budded on Rough lemon, 10 per treatment.
c. Indicator plants: Madam Vinous seedlings, 5 per treatment, and Madam Vinous budded on Rough lemon, 5 per treatment.
d. Sour orange rootstock for grapefruit; Troyer citrange for navel and Valencia.
e. Only 9 indicator plants grew.

grapefruit transmitted the virus from 36 (90 per cent) of the branches used; those from navel, 37 (93 per cent); and those from Valencia, 21 (53 per cent). Combined results from grapefruit, navel, and Valencia inocula show that symptoms developed in leaves and shoots (Fig. 2) of 65 per cent of all plants inoculated with a side graft plus a bud. Symp-

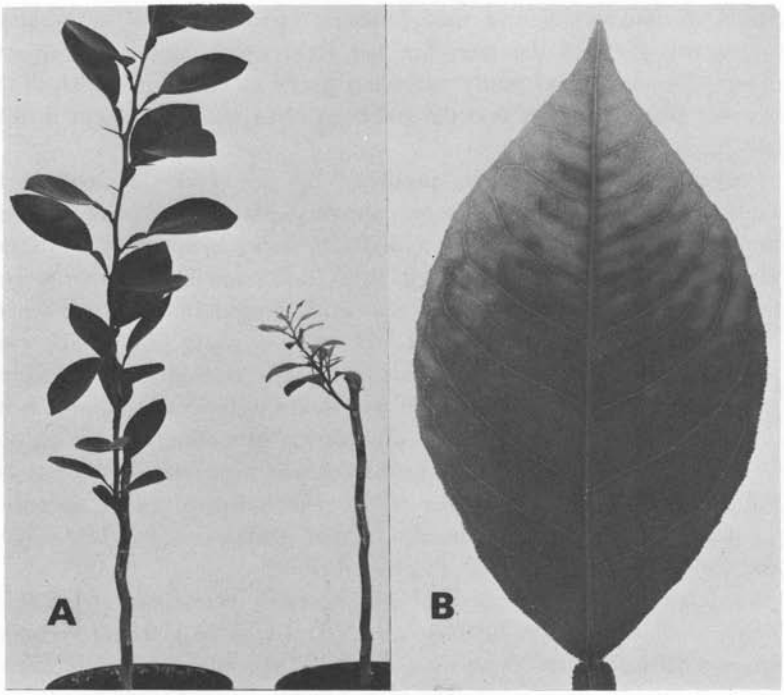

FIGURE 2. *A. Madame Vinous sweet orange seedlings 17 weeks after bud-graft inoculation from stubborn tree N (navel); one seedling is symptomless, the other severely diseased and stunted. B. Immature leaf of inoculated Madame Vinous seedling with typical marginal mottling reaction to stubborn virus.*

tom expression ranged from 10 per cent of plants inoculated from Valencia in October to 95 per cent of plants inoculated from navel orange in May.

The per cent of indicator plants reacting to the various types of grafts was as follows: side graft plus bud from mature shoot, 69; side graft plus bud from young shoot, 63; bud, 19; expanding-leaf patch, 27; internodal bark shield of mature shoot, 12; bark patch from scion trunk, 20; and side graft from root section, 3. Inoculation by grafts of columella

from green Valencia fruits in October caused reactions in 2 plants (20 per cent); no reaction resulted from columella grafts made in January, May, or August. No symptoms appeared in the plants inoculated with mature leaf patches, root bark, wood shields, or anthers. All non-inoculated control plants remained normal.

These data show that side grafts from shoots were the most effective method of inoculation and that stubborn virus was graft-transmitted from many parts of the tree, but not from wood, mature leaves, or anthers. The failure of many surviving grafts to transmit the virus to indicator plants suggests that the stubborn virus was not present in the graft pieces.

PROPAGATIONS.—The shoots produced by side grafts derived from navel branches collected in October, January, May, and August showed symptoms in 7/10, 7/10, 10/10, and 10/10, respectively, and those from Valencia showed symptoms in 2/10, 2/10, 6/10, and 7/10, respectively. Stubborn shoots were produced by side grafts from 34/37 (92 per cent) of the navel branches and from 12/21 (57 per cent) of the Valencia branches from which the virus was concurrently transmitted to Madam Vinous indicators (see above). Stubborn shoots were also produced from side grafts of 5/19 (26 per cent) of Valencia branches from which no transmission to Madam Vinous indicators was apparent. These results confirm the irregular distribution of the virus indicated by the inoculation data and discount the possibility that graft-union barriers interfered with virus movement into inoculated plants.

SEASONAL VARIATION.—Seasonal differences in transmission from tree N were small, except that January inoculations with bark from the trunk were ineffective whereas those with young-leaf patches were highly effective. The number of transmissions from grapefruit were similar in October, May, and August, but were much lower in January. The number of transmissions from the Valencia varied from 2 in October to 28 in May. Total transmissions from each of the 3 source trees were highest in May and next highest in August.

Seasonal ranges in transmission by certain types of grafts, from trees G, N, or V, were as follows: side graft of young shoot plus bud, 50 per cent in January to 80 per cent in May; side graft of mature shoot plus bud, 47 per cent in January to 97 per cent in May; patch of expanding leaf, 17 per cent in October to 43 per cent in August; bud graft, 3 per cent in August to 57 per cent in May; internodal bark shield, 3 per cent in January to 30 per cent in May; bark patch from scion trunk, 0 per cent in January to 43 per cent in August; side graft from roots, 0 per

cent in January and May to 7 per cent in October. These results indicate considerable seasonal variation in virus distribution, especially in the Valencia tree, and suggest that distribution within shoots is more general in May than in October or January, that possibly there is more virus in trunks and roots in August and October than in January or May, and that the virus builds up seasonally in young leaves and shoots.

SEASONAL EFFECT ON INCUBATION PERIOD.—After inoculation in October, January, May, and August, definite symptoms were first noted at 15, 11, 8, and 8 weeks, respectively. Of the plants reacting in these respective series, 50 per cent developed symptoms within 20, 20, 12, and 12 weeks after inoculation. The rapid development of symptoms during late spring and early summer was associated with high temperatures and extended daylight. The total hours above 25, 30, and 35°C, respectively, during the 3-month periods following inoculation were 604, 89, and 16 hours in October to January; 751, 135, and 14 hours in January to April; 1,019, 618, and 102 hours in May to August; and 907, 415, and 41 hours in August to October.

SUPPLEMENTAL EXPERIMENT.—Stubborn symptoms appeared in 35 (70 per cent) of the plants inoculated with internodal bark of Atwood navel. Vein enation was found in 45 (90 per cent) and tristeza in 45 (90 per cent) of the plants. Many plants inoculated with bark grafts developed symptoms of stubborn disease in 6-7 weeks, but none of the 50 plants inoculated with wood shields showed symptoms of any virus disease in 5 months. This evidence indicates that the stubborn virus is absent from or is not readily transmitted from young wood of infected branches.

Discussion

This work supports the conclusion of Fawcett (6) that stubborn disease is a virosis and suggests that the virus multiplies in young leaves and shoots. The possibility that the pathogen might be an obligate fungus is not eliminated, but is not supported by the failure of patches from mature leaves and of wood shields with cambium to transmit the disease.

The failure of many inoculated indicator plants and propagations from diseased trees to develop symptoms in six months does not, by itself, prove they have escaped infection, because stubborn-disease symptoms are variable (1), and the possibility of mild or delayed reactions exists. However, in previous experiments by the authors, plants that remain normal in the glasshouse four months after inoculation rarely, if ever, develop stubborn symptoms in two to three years in the orchard. In one

experiment, four inoculated sweet orange seedlings that were symptomless in the glasshouse are vigorous and indistinguishable from three non-inoculated seedlings after 43 months in the orchard. Conversely, eight seedlings that reacted within four months in the glasshouse are stunted and continue to show stubborn symptoms in the field. From time to time, relatively mild reactions have been noted in plants inoculated from trees having severe symptoms. However, all attempts to transmit stubborn disease virus from non-inoculated plants, from normal inoculated plants, and from apparently normal branches of diseased trees have failed to produce even mild symptoms. The overall results of inoculation and indexing work from 1957 through 1966, involving several thousand indicator plants and about 100 stubborn source trees, clearly indicate that stubborn virus is distributed irregularly in many trees and may be lost, at least temporarily, from many portions of the tree. The very low percentage of transmission from buds grafted in August, October, and January contrasts sharply with the May results, implying that stubborn virus is inhibited or lost in most buds during much of the year.

Conclusions and Suggestions

The extensive replication and seasonal repetition, including springtime collection of graft pieces, is very important if not required for indexing budwood trees for stubborn disease. Side grafts appear to be the most dependable method of inoculation for indexing work. The possibility of transmitting or perpetuating stubborn virus is reduced during the winter months.

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