Leaf-Disc Grafting—A Rapid Indexing Method for Detection of Some Citrus Viruses

R. L. Blue, C. N. Roistacher, G. Cartia and E. C. Calavan

The grafting of rectangular leaf pieces to stems of indicator plants was used for successful transmission of citrus pathogens by Wallace (1947), Garnsey and Whidden (1970) and Calavan *et al.* (1972). Schwarz (1968) transmitted tristeza virus (TV) to 9/20 West Indian limes using leaf-tip grafts held together with commercial masking tape. Cohen (1972) described a method for indexing citrus by grafting triangular leaf pieces containing midribs into triangular holes cut in the leaves of indicator seedlings and covering them above and below with plastic squares held together by clips. He transmitted TV to lime by 17/18 grafts, and citrus exocortis viroid (CEV) to citron by 2/3 grafts. Vogel (1973) independently developed a method of leaf grafting very similar to ours, using a cork borer and tape. He transmitted stubborn disease, CEV, psorosis virus (PV), and cristacortis and cachexia pathogens in a high percentage of attempts. Leaf grafting permits shortening the time from seed planting to index results by using very small seedlings rather than large plants required for bud grafting. We modified the methods of Cohen and Vogel and used a paper punch and tape. This paper compares our leaf-punch method with bud grafting for detection of TV, CEV, vein enation virus (VEV), (PV), and concave gum virus (CGV).

MATERIALS AND METHODS

Leaf-grafting technique. Leaf discs 6 mm in diameter were cut with a paper punch (fig. 1) and secured to punched leaves of indicator plants with tape (Scotch transparent tape No. 600, 0.1 x 33 mm, Minnesota Mining and Manufacturing Co., St. Paul, MN. 55101 or Borden Mystic tape No. 6450, Borden Inc., 1700 Winnetka Ave., Northfield, IL. 60093). The procedure was to: 1) punch a disc out of the midrib area of a leaf of the donor plant; 2) punch a similar hole in a leaf of the indicator plant and attach tape to the underside of the leaf; 3) transfer the disc from the donor to the hole in the leaf of the indicator with a needle and align the midribs; 4) place a piece of tape on top of the leaf and press the top and bottom tapes firmly together; 5) remove the top of the seedling above the uppermost inoculated leaf and shade the plant with cheesecloth for 10 to 14 days. We found that covering leaf-grafted plants with polyethylene bags reduced inoculum survival 38 per cent. The ease and speed of using leaf discs secured by tape without a bag led us to adopt this method.

Donor plants. Inoculum was from previously inoculated screenhouse-grown Valencia sweet orange, unless otherwise stated; all leaves used, except for VEV test 1, were mature, dark green, and were washed and dried off prior to use. Leaf and bud inocula were from the same branches.

Indicator plants. Leaf discs and buds were grafted into separate indicator plants. For TV, three medium (0.6 m) and three large (1.4 m) West Indian lime seedlings were used; also, three small (0.3 m) seedlings were used only for leaf grafts. For PV, five seedlings each of

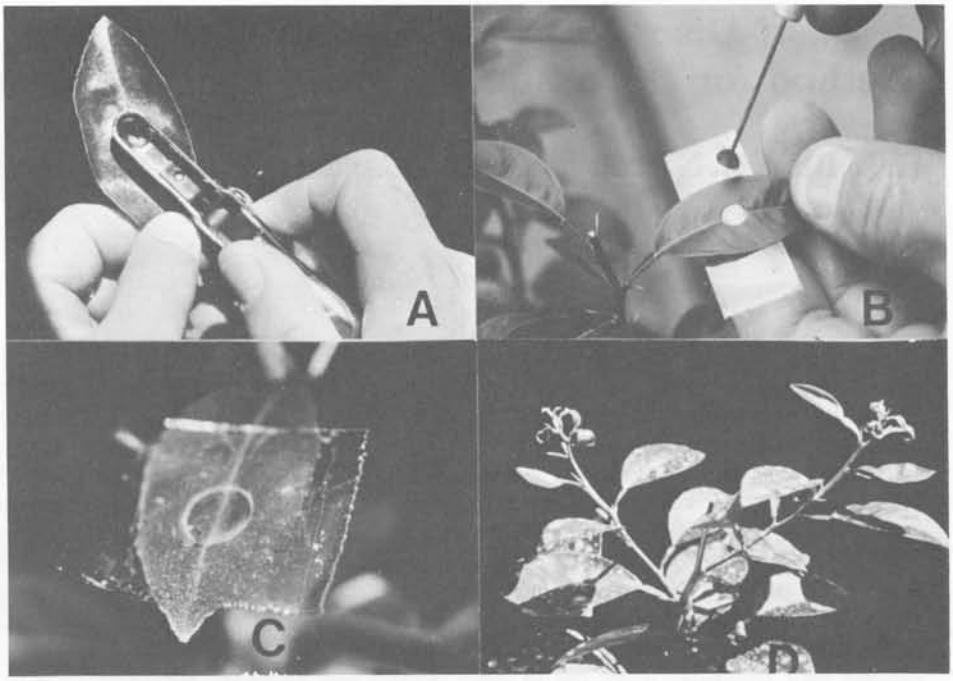

Fig. 1. Leaf-disc grafting and reaction: A) Cutting a leaf disc with a paper punch; B) inserting a leaf-disc from a donor plant into a punched hole in a leaf of an indicator plant, tape on the bottom; C) grafted leaf-disc covered above and below with tape; D) new leaves on shoot near leaf-disc inoculum show epinasty symptoms of tristeza.

Madam Vinous sweet orange and Dweet tangor and for CGV four seedlings each of Pineapple sweet orange and Dweet were used. All PV and CGV indicators were about 0.6 m tall, of pencil thickness and vigorous. For VEV, four small (0.3 m) and four large (1.0 m) lime seedlings were used in test 1 and three medium (0.6 m) and three large (1.4 m) seedlings in test 2. For CEV, four plants of Arizona 861 citron on Rough lemon (1.0 to 1.5 m tall) were used. All medium and large plants were cut back at inoculation to about 20 cm above the soil. Indicators for TV, PV, CGV, and VEV were kept in a room where maximum and minimum temperatures, respectively, averaged 37 and 24° C. Exocortis indicators were kept at temperatures averaging 25 maximum and 19° C minimum. Inoculum survival was recorded at 6 weeks and plants were observed two or three times during each growth-flush period. Each leaf showing tristeza vein clearing was counted and marked. Reactions to other viruses were noted as mild, moderate or severe. Final observations, except for tristeza stem pitting, were made 16 weeks after inoculation, following two complete flushes.

Inoculations. Most inoculations were by three leaf-disc grafts or by two bud grafts. Exceptions were those with CGV and VEV where only two leaf or bud grafts were used. Bud grafts were wrapped with polyethylene tape.

Virus isolates. All isolates were from California except as mentioned.

Tristeza. Six isolates were used, three severe and three mild reacting in lime.

Psorosis. Five isolates were used. PV-208, a moderate bark lesion type, caused a severe reaction in sweet orange. PV-200 and PV-205 originated from typical psorosis-affected trees. PV-201 and PV-203 originated in China and Thailand, respectively. All PV isolates induced shock on sweet orange and protected sweet orange against a challenge by lesion inoculum.

Concave gum. Four isolates were used. CGV-301 and CGV-302 were from sweet orange trees with concavities. CGV-305 was from Cadenera de Carcagente sweet orange from Spain and CGV-304 was from Clementine Monreale mandarin from Siciliy; both carried CEV. All isolates induced oak-leaf patterns and interveinal flecking in sweet orange and mandarin, never induced shock in sweet orange, and did not protect against a challenge by PV lesion inoculum (Roistacher and Calavan, 1965).

Vein enation. Test 1. Two isolates were used. VEV-700 from West Indian lime and Rough lemon originated from a Lisbon lemon; VEV-701 was from the parent Navel orange (Roistacher *et al.*, 1975).

Test 2. Two isolates were used, both from Valencia orange. VEV-701 originated from the parent Navel orange and VEV-702 from a Valencia orange.

Exocortis. Two isolates were used. CEV-808 from Bearss lime was a very mild isolate which induced petiole wrinkle, petiole browning or midrib browning, but rarely epinasty, in citron. CEV-812 was a severe isolate mechanically transmitted from Lisbon lemon to citron.

RESULTS

Tristeza. All grafts survived and all plants developed symptoms. Numbers of leaves showing vein clearing are given in table 1. Small, medium and large plants reacted very similarly to inoculation. For severe strains, bud grafts were as effective as leaf grafts. However, for the mildest strain (TV-511), leaf grafts were more effective than bud grafts. In other tests, especially during cool weather, symptoms appeared sooner and more intensively in plants inoculated by leaf grafts. During the first growth flush 10 weeks after inoculation with TV-511, 6/6 medium or large leaf-grafted plants showed symptoms in 98 leaves compared to 14 leaves in 1/6 bud-grafted plants. When stems were peeled after 32 weeks, pitting was more severe, on the average, in plants inoculated by leaf grafts. All stems were pitted except those of three medium plants inoculated by buds with TV-511 and one large plant inoculated by buds with TV-510.

In another experiment, nine TV isolates, including mild ones, caused definite symptoms following leaf-graft inoculation. A new symptom, not previously observed, appeared in lime inoculated by leaf grafts; leaves on young shoots growing from nodes at the base of inoculated leaves showed strong epinasty (fig. 1D). Vein clearing and cupping associated with leaf epinasty were unusually severe.

Psorosis. All leaf grafts and 95 per cent of the bud grafts survived. Leaf grafts

TABLE 1
NUMBER OF LEAVES FROM THREE WEST INDIAN LIME SEEDLINGS SHOWING TRISTEZA SYMPTOMS 16 WEEKS AFTER INOCULATION

Virus isolate	Small plants inoculated by Leaf	Medium plants inoculated by Leaf	Medium plants inoculated by Bud	Large plants inoculated by Leaf	Large plants inoculated by Bud
Severe isolates					
TV-505	110	98	53	119	123
TV-507	35	43	45	117	148
TV-508	45	81	72	99	83
Mild isolates					
TV-509	44	55	64	55	56
TV-510	43	39	62	68	36
TV-511	34	33	8	66	40

were less effective than bud grafts for PV transmission. At 6 weeks only 10/50 plants inoculated by leaf grafts showed symptoms compared to 46/50 of the bud-grafted group. Table 2 summarizes PV transmission for all isolates. Poor transmission of PV by leaf grafts was also evident in other tests. Leaf-grafted plants that remained symptomless were not protected against challenge by lesion or non-lesion inoculum.

Concave gum. All grafts survived. The four isolates caused similar reactions in Dweet tangor and Pineapple sweet orange. Within 8 weeks 11/32 leaf-grafted seedlings showed symptoms compared to 30/32 bud-grafted seedlings. Table 3 shows final results for CGV transmission. Symptomless inoculated plants challenge inoculated by bud grafts with the same isolates of CGV all developed symptoms, indicating no prior infection from leaf-graft inoculation. Another experiment confirmed the low percentage of CGV transmission from leaf discs.

Vein enation. Test 1. Graft survival was 88 per cent for leaf discs and 90 per cent for buds. There were no differences

TABLE 2
NUMBER OF DWEET TANGOR AND MADAM VINOUS SWEET ORANGE SEEDLINGS (OUT OF FIVE) SHOWING PSOROSIS SYMPTOMS 16 WEEKS AFTER INCOULATION

Virus isolate	Dweet inoculated by		Madam Vinous inoculated by	
	Leaf	Bud	Leaf	Bud
PV-200	2	5	2	5
PV-201	0	5	1	5
PV-203	4	5	5	5
PV-205	2	5	4	5
PV-208	0	5	2	5

TABLE 3
NUMBER OF DWEET TANGOR AND PINEAPPLE SWEET ORANGE SEEDLINGS (OUT OF FOUR) SHOWING CONCAVE GUM SYMPTOMS 16 WEEKS AFTER INOCULATION

Virus isolate	Dweet inoculated by		Pineapple inoculated by	
	Leaf	Bud	Leaf	Bud
CGV-301	3	4	3	4
CGV-302	3	4	4	4
CGV-304	3	4	2	4
CGV-305	2	4	1	4

in graft survival among the different sources of tissue used, i.e., West Indian lime, Rough lemon or Navel orange, greenhouse or screenhouse grown, young or old tissues. This confirms the observation of Wallace (1947) who used leaf pieces placed under bark flaps. However, Calavan *et al.* (1968) reported less survival of grafts from mature leaves than from very young leaves of stubborn-affected orchard trees. At 8 and 16 weeks, respectively, 21/28 and 26/28 plants inoculated by leaf grafts showed symptoms; all bud grafted seedlings showed symptoms within 6 weeks.

Test 2. Eleven of 18 leaf discs grafted into old leaves of large plants survived whereas all 18 leaf grafts to normal leaves on medium-sized plants survived. Most dead grafts were in senescent leaves which dropped early. All bud grafts lived. All eight plants inoculated by bud grafts showed symptoms within 8 weeks for each of the two isolates. However, with VEV-701, 0/4 plants inoculated by leaf grafts showed symptoms. Four of six

plants inoculated with VEV-702 by leaf grafts showed symptoms within 8 weeks.

Exocortis. All grafts survived. Eight weeks after inoculation with mild CEV-808, 0/4 and 3/4 leaf- and bud-grafted plants, respectively, showed symptoms and 1/4 and 4/4 plants, respectively, showed symptoms when inoculated with leaf and bud grafts from severe CEV-812. Table 4 shows final results of CEV inoculations.

TABLE 4
NUMBER OF ETROG CITRON PLANTS (OUT OF FOUR) SHOWING EXOCORTIS SYMPTOMS 16 WEEKS AFTER INOCULATION

Viroid isolate	Reaction to leaf-graft inoculation			Reaction to bud-graft inoculation		
	None	Mild	Severe	None	Mild	Severe
CEV-808 (mild)	2	2	0	0	4	0
CEV-812 (severe)	1	3	0	0	0	4

DISCUSSION

Leaf-graft inoculation was very successful for TV transmission and we recommend the method for tristeza indexing. It was less successful for VEV and unreliable for CEV, CGV, and PV. Leaf grafting was superior to bud grafting for detection of mild TV isolates. The use of small seedlings for leaf grafts saves 4 to 6 months' growing time, considerable labor, and space. By using leaf grafts a number of trees can be indexed on a single medium or large plant without excessive wounding of the stem as with bud or chip grafts. The method is faster than budding, requires less skill, and provides good inoculum survival.

In 1973-75, we inoculated 1,178 leaves of lime, sweet orange, tangor, and citron by leaf grafts from various screenhouse- and greenhouse-grown plants with 97 per cent graft survival. In 1974, we grafted 1,552 leaf discs, mostly into lime, from field trees of Navel, Valencia and other sweet oranges, mandarins, tangelos, grapefruit, lemons, limes and miscellaneous varieties with 90 per cent graft survival. Careful selection and washing of leaves might increase the percentage of successful grafts. When leaf grafts died, plants were effectively reinoculated from refrigerated leaves.

In 1975, we used this technique at Burjasot, Spain and compared two kinds of tape: one transparent amber tinted and the other a white transluscent tape. Eleven days after inoculation 86/87 grafts were alive. However, at 41 days, 39/41 grafts with transparent tape were alive compared to 12/47 grafts with the white transluscent tape. It was evident from this and other tests that the white transluscent tape was toxic and prevented successful leaf grafts. Personal communications from R. E. Schwarz and M. Cohen reported similar toxicity from cellophane tapes. We therefore suggest that tapes other than the two used in these tests be carefully evaluated for any toxic reaction.

The inferior results from leaf grafts for VEV, PV, and CGV indexing might be due to lack of virus in leaf discs of source plants (although virus was present in budsticks) or to failure of the virus to move from the discs into the leaves. Symptomless plants previously inoculated with PV or CGV by leaf grafts almost always reacted when reinoculated by buds, suggesting that virus had not systemically invaded the indicator plants (Roistacher and Calavan, 1965).

Leaf-graft inoculation from plants infected with severe CEV usually resulted in transmission of very mild CEV. Failure to transmit severe CEV from leaf discs could be due to an inhibitor or to strain separation similar to that observed with

PV by Wallace (1957), or that obtained with TV by heat therapy (Desjardins *et al.*, 1959) or by shoot-tip grafting *in vitro* (Roistacher *et al.*, this volume).

Leaf-graft inoculation was tested with other pathogens on a limited scale with varying degrees of success. Tests with *Spiroplasma citri* and tatter-leaf and yellow-vein viruses were generally successful. Further work is needed with these and other pathogens.

LITERATURE CITED

CALAVAN, E. C., E. O. OLSON, and D. W. CHRISTIANSEN
1972. Transmission of the stubborn pathogen in citrus by leaf-piece grafts, p. 11-14. *In* W. C. Price, (ed.), Proc. 5th Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.

CALAVAN, E. C., C. N. ROISTACHER, and D. W. CHRISTIANSEN
1968. Distribution of stubborn disease virus in trees of *Citrus sinensis* and *C. paradisi* at different seasons, p. 145-53. *In* J. F. L. Childs, (ed.), Proc. 5th Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.

COHEN, M.
1972. A leaf insert graft used for virus transmission in citrus, p. 282-84. *In* W. C. Price, (ed.), Proc. 5th Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.

DESJARDINS, P. R., J. M. WALLACE, E. S. H. WOLLMAN, and R. J. DRAKE
1959. A separation of virus strains from a tristeza-seedling-yellows complex by heat treatment of infected lime seedlings, p. 91-95. *In* J. M. Wallace, (ed.), Citrus Virus Diseases. Univ. California Div. Agr. Sci., Berkeley.

GARNSEY, S. M., and R. WHIDDEN
1970. A rapid technique for making leaf tissue grafts to transmit citrus viruses. Plant Dis. Rep. 54:907-08.

ROISTACHER, C. N., and E. C. CALAVAN
1965. Cross-protection studies with strains of concave gum and psorosis viruses, p. 154-61. *In* W. C. Price, (ed.), Proc. 3rd Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.

ROISTACHER, C. N., L. NAVARRO, and T. MURASHIGE
1976. Recovery of citrus cultivars free of several viruses, exocortis viroid, and *Spiroplasma citri* by shoot-tip grafting *in vitro* (this volume).

ROISTACHER, C. N., R. L. WAGNER, and E. C. CALAVAN
1975. The parent navel orange: a healthy centenarian. Citrograph 60:343.

SCHWARZ, R. E.
1968. Transmission of the tristeza virus by a leaf union method. S. Afr. J. Agr. Sci. 11:617-22.

VOGEL, R.
1973. Le cristacortis: une nonvelle maladie a virus des agrumes. Doctor of Natural Sciences Thesis, Univ. Bordeaux II.

WALLACE, J. M.
1947. The use of leaf tissue in graft-transmission of psorosis virus. Phytopathology 37:149-52.

WALLACE, J. M.
1957. Virus-strain interference in relation to symptoms of psorosis disease of citrus. Hilgardia 27:233-46.