Efficiency of Mechanical Transmission of Citrus Tristeza Virus

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ABSTRACT. Effects of inoculation site, wounding procedure, age of receptor plant, cambial activity, and pre- and postinoculation environment on transmission efficiency of citrus tristeza virus (CTV) were evaluated. Stem-slash inoculation was generally more efficient on mature stems 5 to 8 mm in diameter than on tender new growth. Other forms of bark wounding were also effective, but leaf inoculation was not. Transmission of CTV was obtained to fresh and callused cuttings of Etrog citron which were slash-cut inoculated and then rooted. Transmission efficiency was not correlated with cambial activity and preconditioning of receptor plants. Use of cool glasshouse conditions to increase tissue for inoculum production improved transmission efficiency.

A number of isolates of citrus tristeza virus (CTV) have been mechanically transmitted to citrus or citrus relatives by the stem-slash method (1, 2, 4). Development of an *in vitro* assay for CTV has facilitated characterization of CTV, promoted safe international exchange of isolates, and provided a potential for efficient long-term storage of CTV isolates (4). However, the stem-slash method has several drawbacks which hinder its more widespread use for tristeza assays. Each assay requires five or more healthy, potted citrus receptor plants 6 to 12 months old, which must be grown for at least 2 months in a temperature-controlled greenhouse after inoculation. These assays are based on systemic infection and do not provide quantitative information unless a dilution end point assay is performed. Infection rates often have been variable in repeated assays of the same preparations, suggesting that host or procedural variables have some effect. Early studies indicated that the concentration of intact, flexuous CTV particles in the inoculum was correlated positively to transmission success and that gentle extraction and concentration procedures were essential to obtain infectious preparations (3).

Attempts to find rapidly-reacting herbaceous indicators for CTV have not been successful (2).

We have conducted a series of experiments in an effort to improve efficiency of mechanical transmission assays for CTV on citrus indicators. Investigations on different inoculation procedures and inoculation sites, on use of fresh or recently rooted cuttings as receptors, and on the effect of environment on receptor plant susceptibility and transmission efficiency are presented here.

METHODS AND MATERIALS

Plant materials and growing conditions. Plants were container-grown in a sterilized potting mix and fertilized and sprayed as needed to promote vigorous, healthy growth. Unless otherwise noted, receptor plants were clonally propagated cuttings of the RMA-861 selection of Etrog citron. Mature, potted plants were well-established cuttings grown in individual containers for approximately 6 months prior to inoculation. These plants were usually 75 cm or more in height with a stem diameter of 5 to 8 mm (5 cm above the soil line).

Fresh cuttings were freshly cut sections of citron stem 5 to 8 mm in diameter and 10 to 15 cm long with all

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but the terminal leaf removed. Callused cuttings were prepared as above except they were incubated in moist perlite or similar media for 2 to 4 weeks prior to use. These cuttings usually had one or more small roots on the callused lower cut and a shoot of new growth 5 to 10 cm long (fig. 2A). Calamondin seedlings were used in one test on dormancy.

An air-cooled, partly shaded glasshouse, as previously described (2), was used for most studies. Night temperatures were 21 to 23 C, whereas maximum day temperatures varied from 22 to 26 C in winter and spring to 30 to 35 C in summer. For some studies, infected plants used to increase infected tissue and inoculated receptor plants were incubated in an air-conditioned (24 C), clear plastic cell within the glasshouse. A programmed, artificially illuminated growth chamber adjusted for a 12-hr photoperiod was used to condition plants for two experiments. In one test plants were subjected to a 16 C day and 4 C night, and in the second, temperatures were reduced from 21/10 C to 16/10 C to 16/4 C (day/night) in a weekly sequence.

Virus isolates and preparation of inoculum. The T-4 and T-3 isolates of CTV were used for most studies (2). These isolates produce distinct vein clearing, leaf cupping, stem pitting and stunting in Etrog citron receptors under moderate temperature conditions. Foliar symptoms are partially masked in summer.

Inocula for most experiments were obtained by concentrating extracts from diced, young bark tissue on sucrose step gradients or cushion gradients as previously described (2). Gradient fractions were usually evaluated by enzyme-linked immunosorbent assay (ELISA) to locate those most likely to contain concentrations of intact particles. Gradient fractions used as inoculum were normally diluted 1:1 in 0.05 M Tris buffer, pH 7.8, to reduce sucrose concentration below 300 mg/ml. In some tests, a further 1/5 dilution of the stock inoculum was also assayed.

Inoculation procedures. Stem-slash inoculations were made with disposable scalpels. Inoculum was pipetted onto the blades prior to cutting (fig. 1A) and cross-cut inoculations were made in the bark perpendicular to the axis of the stem (fig. 1B). Normally, 90 cuts were made per plant, but other numbers were used for some tests when longitudinal cuts parallel to the stem axis were tested. The length and number of longitudinal cuts were adjusted to equal the aggregate cut length created by the cross-cut treatment under comparison.

Several modifications of bark-flap inoculation were used. In the basic procedure, three cuts were made on the stem to create a flap 3-5 mm across and 20-25 mm long. The upper edge was teased away from the stem and a drop of inoculum placed in the trough created. The flap was then pulled away from the stem, allowing the drop of inoculum to flow down both the stem and bark surface exposed (fig. 1D). In some cases, additional wounding was done including rubbing the inner bark surface with a cotton swab carrying carborundum, pricking the face of the inoculum-covered flap with a small needle, or crushing the flap with serrated forceps or pliers (fig. 1E). Wounded areas in some tests were wrapped with Stericrepe®, self-adhering crepe rubber tape.

Leaf-cut inoculations were done by making cross or longitudinal cuts in the leaf midrib with an inoculum-coated scalpel blade. Leaf abrasion inoculations were made by applying a drop of inoculum to carborundum-dusted leaves and rubbing it over the leaf surface with a fingertip. In several instances, leaves were infused with inocula by syringe injection into the midrib (fig. 1C) and wounded.

Except where noted, all inoculations were done at temperatures from 20 to 24 C and plants were incubated

after inoculation in a greenhouse held at temperatures from 24 to 28 C during the day.

Evaluation. Inoculated plants were inspected visually for symptoms after new flushes of growth. Most tests were terminated 5 to 6 months after inoculation and symptomless plants were assayed by ELISA for CTV infection. The ELISA procedure used was the conventional double sandwich procedure as previously described (2).

RESULTS AND DISCUSSION

Wounding. Results of several experiments on the relationship of wounding procedures and site to CTV transmission are summarized in table 1. In experiment 1, there was an association between the degree of wounding and the percentage of infected plants obtained (table 1, treatments 1-4). This was less evident in experiment 2, where a relatively high rate of transmission occurred at the lower wounding rate (table 1, treatment 1).

Infection rates for cross-cut stem inoculation were higher when plants were inoculated on the older main stem (table 1, treatment 4) than when inoculated on young stems of smaller diameter (treatment 10). Since less total cut surface per cross cut is ex-

TABLE 1
EFFECT OF WOUNDING SITE AND WOUNDING PROCEDURE ON THE MECHANICAL TRANSMISSION OF CITRUS TRISTEZA VIRUS TO CITRON RECEPTOR PLANTS

Treatment no.	Inoculation site and procedure	Experiment and dilution of inoculum				
		1		2		3
		None	1/5	None	1/5	None
	Mature stem					
1	10 cross cuts/W[y]	1/5[z]	0/5	3/5	1/5	—
2	30 cross cuts/W	3/5	1/5	—	—	—
3	90 cross cuts/W	5/4	1/5	—	—	—
4	90 cross cuts/NW	5/4	3/5	3/5	1/5	7/10
5	9 long cuts/W	5/5	0/5	—	—	0/10
6	Bark flap/W[x]	5/5	0/5	5/5	2/5	—
7	Bark flap/crushed/W	5/5	1/5	2/5	1/5	—
8	Bark flap/pricked/W	5/5	2/5	—	—	—
9	Bark flap/removed	—	—	2/5	0/5	—
	Young stem					
10	90 cross cuts/W	1/5	0/5	—	—	5/10
11	3 long cuts	—	—	—	—	1/5
	Leaves[w]					
12	Leaf abrasion	0/5	0/5	0/5	0/5	—
13	Leaf crushed	0/5	0/5	—	—	—
14	Leaf injected/crushed	2/5	0/5	0/10	0/10	—
15	Long cut/midrib	—	—	—	—	1/10
16	Cross cut/midrib	—	—	—	—	0/10
	Roots					
17	90 cross cut/NW	0/5	0/5	—	—	—

[z]Number of plants positive for CTV over number inoculated; status of symptomless plants verified by ELISA.
[y]W = stem wounds wrapped with rubber tape after inoculation (fig 1F), NW = no wraps applied.
[x]For bark-flap inoculation, cuts were made to create a flap and the top of the flap was opened slightly to receive a pipetted drop of inoculum and then the flap was fully opened (fig. 1D) and treated as indicated. Crushing (treatment 7) was done with serrated jaws of small pliers, (fig. 1E) pricking (treatment 8) was done to inoculum-wetted cambial surface with a small needle.
[w]For leaf inoculation, inoculum was pipetted to surface of a carborundum-dusted leaf and applied by finger tip (treatment 12); applied to serrated jaws of pliers used to crush areas on the leaf (treatment 13) or injected into the leaf (fig. 1C) which was subsequently crushed with inoculum-free pliers (treatment 14). See text for additional details on inoculation technique.

posed on the smaller diameter stems, we tried to determine if the difference was related solely to cut surface area exposed. Longitudinal cuts along the stem axis were made in young stems with a total length of 45 cm calculated to equal the aggregate length for 90 cross cuts on the mature stems. Results with long cuts did not indicate that failure to infect young stems (table 1, treatment 11) was solely a function of wound area exposed.

Several variations of bark-flap inoculation (fig. 1D & E) were tested and all appeared relatively effective (table 1, treatments 6-8). There was no clear indication that wounding in addition to that made in creating and opening the bark flap improved transmission. In fact, transmission occurred when the bark flap was removed after the initial inoculation process (treatment 9). This suggested that at least some of the infection sites were

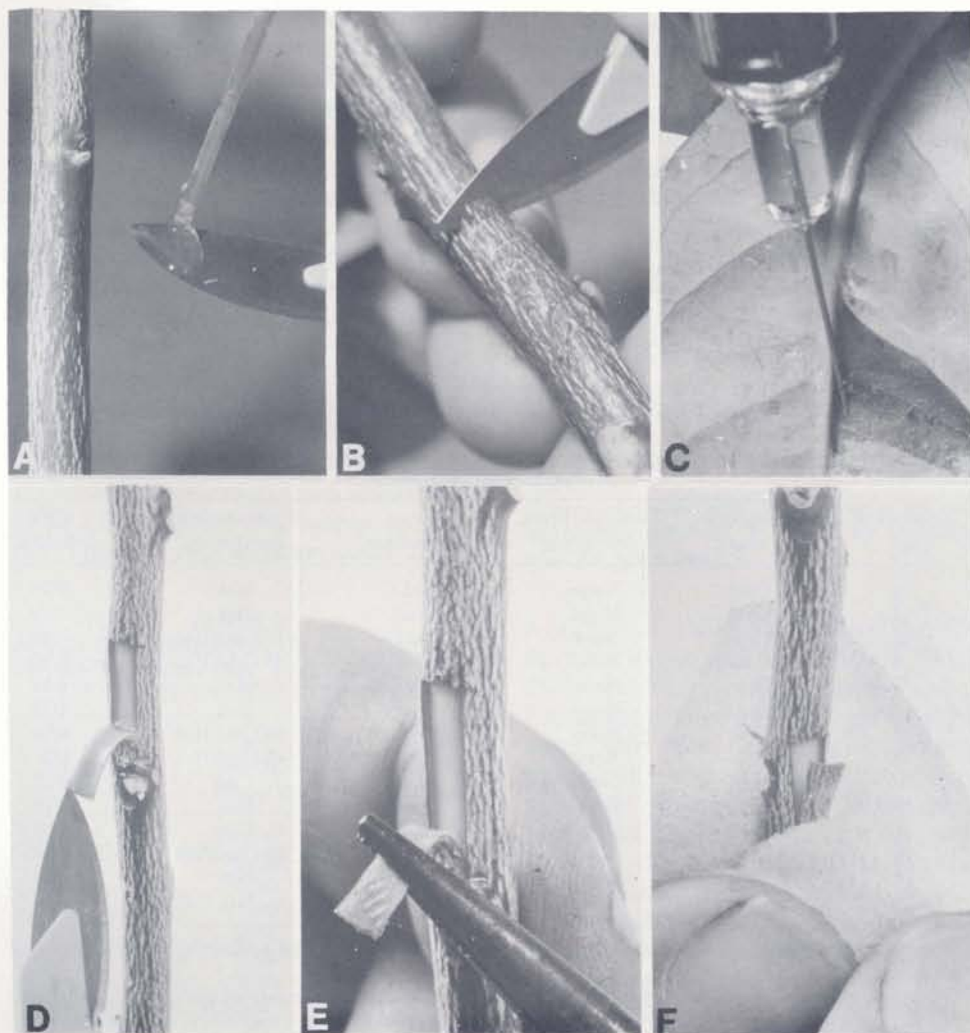


Fig. 1. Inoculation procedures for mechanical transmission of citrus tristeza virus. A) Application of inoculum to knife blade. B) Slash-cut inoculation of receptor plant stem. C) Syringe infusion of citrus leaf with inoculum (infused area was also wounded). D) Pulling bark flap away from xylem in presence of inoculum (drop applied to wedge created when top of flap was first teased open). E) Crushing bark flap moistened with inoculum as in D. F) Wrapping inoculated bark flap back in original location with self-adhesive tape.

at the margin of the cuts or in cambial tissues remaining on the stem. The effectiveness of bark-flap inoculation was significant for subsequent work with fresh or callused cuttings, since only small areas of stem were needed for inoculation and no unprotected wounded surfaces were exposed to the soil.

Various types of leaf inoculation were tested, but none proved highly effective (table 1, treatments 12-16). The limited positive results obtained do, however, indicate that cells receptive to mechanical inoculation with CTV are present in leaves. The results also indicate that extensive wounding is necessary. The single test done with roots (treatment 17) indicated that these were not efficient receptor sites for mechanical inoculation.

Temperature effects. Several experiments were conducted to evaluate the effect of temperature on different phases of the transmission process. The results are summarized in table 2. These tests indicated that inocula prepared from plants grown under cool conditions were more effective than inocula taken from plants grown under very warm conditions, even when young flush tissue was used. Growing receptor plants under warm conditions, however, did not adversely affect susceptibility. Similarly, there was no clear indication that inoculating under warm conditions adversely affected results, but the number of comparisons was limited.

The effect of postinoculation incubation conditions was also not clear cut. Holding plants under warm greenhouse conditions after inoculation did not prevent infection. The length of time plants were exposed daily to high temperature depended on the weather and on the time of the year (day length) when experiments

TABLE 2
EFFECT OF TEMPERATURE CONDITIONS ON MECHANICAL TRANSMISSION OF CITRUS TRISTEZA VIRUS

Treatment no.	Temperature conditions: Inoculum increase[z]	Receptor plant conditioning[y]	Inoculation[x]	Postinoculation incubation[w]	CTV infection[v]
1	Cool	None	Hot	Hot	6/10
2	Cool	None	Cool	Hot	3/10
3	Cool	None	Cool	10 Cool/Hot	7/10
4	Cool	None	Cool	40 Cool/Hot	6/10
5	Hot	None	Hot	Hot	1/10
6	Hot	None	Cool	Hot	1/10
7	Hot	None	Cool	10 Cool/Hot	0/10
8	Hot	None	Cool	40 Cool/Hot	2/9
9	Cool	Cool	Cool	Cool	3/10
10	Cool	Cool	Cool	Hot	3/10
11	Cool	Hot	Cool	Cool	4/10
12	Cool	Hot	Cool	10 Cool/Hot	6/10
13	Cool	Hot	Cool	Hot	7/10
14	Cool	Hot	Cool	Hot (GC)	3/10

[z]Temperature conditions for citrus plants used as source of tissue for preparing partially purified inoculum.
[y]Plants to be used as receptors for inoculation tests were held under normal greenhouse conditions (none), in an exceptionally hot greenhouse environment (hot),or in an air-conditioned chamber (cool) for at least 1 month prior to inoculation.
[x]"Cool" inoculations done in an air-conditioned lab (22 C); "hot" inoculations done in a warm greenhouse (32 C).
[w]Plants held in warm greenhouse area for 40 days or more postinoculation (hot) or in an air-conditioned chamber (cool) for days specified and then moved into warm greenhouse as indicated.
[v]Number of plants infected over number inoculated. Treatments 1-8 and 9-14 were inoculated from different inoculum sources.

were conducted. Although the maximum temperature exceeded 32 C many days, the period it was above 30 C was often less than 6 hr. In only one case (table 2, treatment 14) were plants held under a constant hot regime. Extremely hot conditions should probably be avoided for inoculation and incubation, but it appears that the main temperature effect is on the inoculum increase plants.

Dormancy. Cambial activity can fluctuate in individual plants over time depending on growth patterns, nutrition, and stress. We predicted that presence of young cambial cells or protophloem may be essential to establish CTV infection by stem wounding, and that the degree of cambial activity could be an important but easily overlooked variable affecting host susceptibility. Two separate experiments were conducted to evaluate the relationship of plant dormancy to CTV susceptibility, and the results are summarized in table 3. These results indicate that dormant plants are at least as susceptible to infection as actively-growing plants. Whereas, an active cambium is essential to perform techniques such as the bark-flap procedure (fig. 1D), it apparently is not essential for successful stem-slash inoculation.

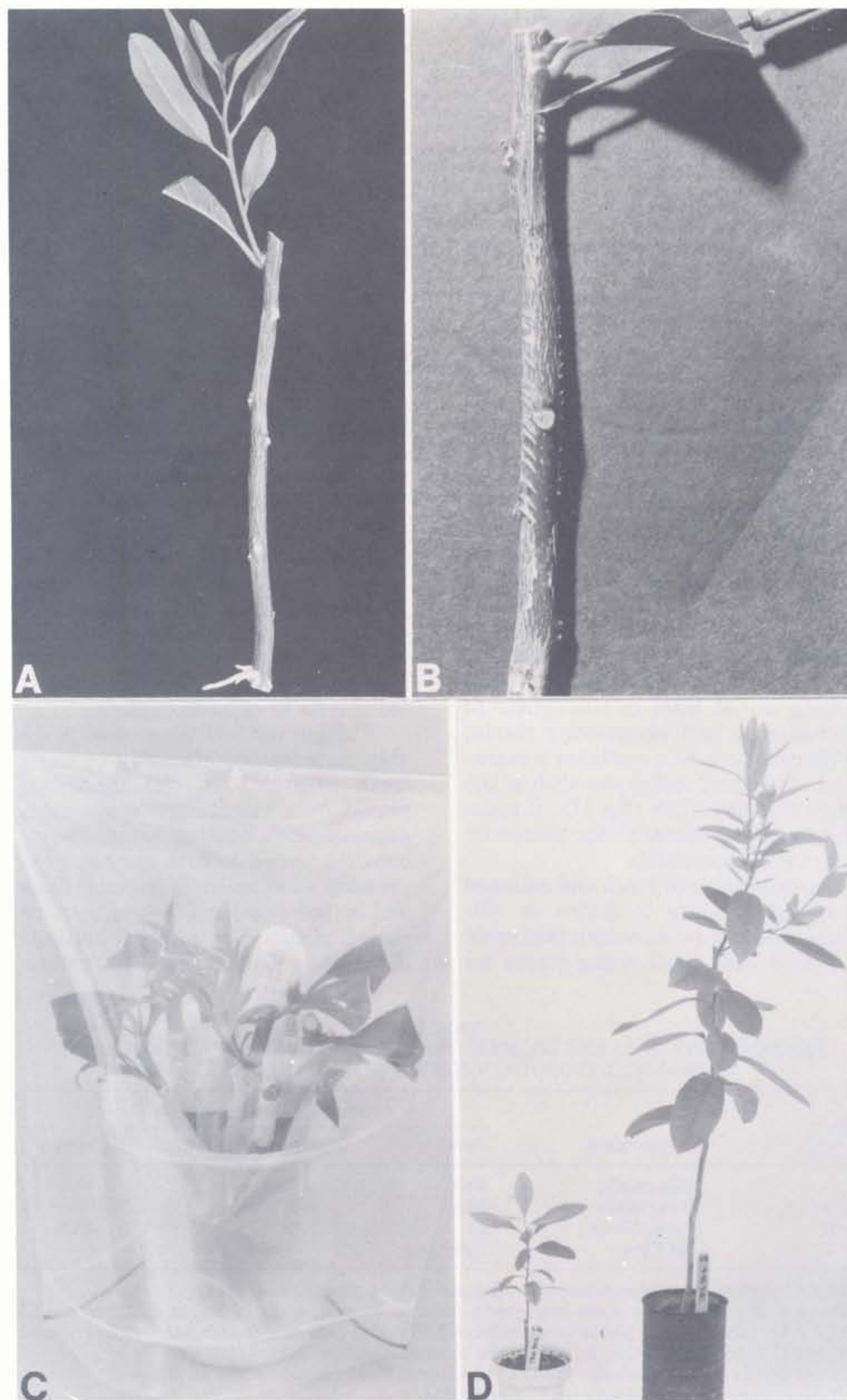
Inoculation of fresh and callused cuttings. Failure to devise an efficient inoculation procedure that could be used with small young plants led to the evaluation of other ways to speed the CTV assay process. Initial attempts to slash-cut inoculate and simultaneously root freshly prepared citron cuttings failed because of the high mortality among freshly wounded cuttings placed directly into rooting media. Some surviving cuttings also failed to root and/or form new shoots. To circumvent these problems, cuttings were prepared several weeks prior to inoculation and allowed to callus (fig. 2A). Freshly inoculated cuttings were held in a humid, soil-free environment (fig. 2C) until the inoculation wounds could heal (fig. 2B). Several tests were conducted with fresh or callused cuttings and the results are summarized in table 4. Infection was obtained consistently in callused cuttings, but at somewhat lower rates than for comparably inoculated, well-established potted cuttings. Fresh cuttings, inoculated by a combination of bark-flap and slash-cut methods, and allowed to heal before planting yielded comparable results to callused cuttings.

Further modifications could probably increase the efficiency of using fresh cuttings, but the incubation period from inoculation to symptom expression is a limiting factor. The incubation period to first symptom expression was similar in fresh cuttings and in inoculated and topped, mature potted plants. Use of fresh cuttings did greatly reduce plant production

TABLE 3
RELATIONSHIP OF PLANT DORMANCY TO SUSCEPTIBILITY TO INFECTION BY MECHANICAL INOCULATION WITH CITRUS TRISTEZA VIRUS

Expt. no.	Receptor plant	Dormancy-inducing factor		
		None[z]	Temperature[y]	H_2O stress[x]
1	Calamondin	8/10[w]	9/10	—
1	Etrog citron	2/10	9/10	—
2	Young Etrog	4/9	3/10	8/10
2	Old Etrog	1/10	3/10	—

[z]Plants grown in warm greenhouse with adequate water and nutrients.
[y]Plants conditioned for 3-4 weeks in a growth chamber. In expt. 1, plants held at 4 C 12 hr and 16 C for 12 hr daily; in expt. 2 plants were conditioned (in weekly sequence) from day/night temperatures of 21/10 C to 16/10 C and then to 16/4 C prior to inoculation (see text).
[x]Plants held in warm greenhouse (28-32 C) under continuous moisture stress for 3 weeks.
[w]Number of plants infected over number inoculated.


A
B
C
D

time and greenhouse space requirements (fig. 2D). It will be especially advantageous where large numbers of assays must be done unexpectedly, for quantitative studies where large populations of uniform receptors are required, and for facilities where temperature-controlled glasshouse space is limited.

In summary, stem-wounding procedures remain the most effective means to transmit CTV mechanically to citrus. Bark-flap procedures may have advantages over stem slashing in some situations, especially where fresh cuttings are used. Minor differences in inoculation efficiency between procedures could not accurately be determined with the limited number of replications used. We used fewer replications because our purpose was to look for major effects among the largest number of treatments possible.

Temperature effects are apparently more significant during growth of the virus increase tissue than in the inoculation or incubation processes. Significant quantitative differences in infectivity between different CTV preparations remain difficult to determine. Assays of several dilutions of each preparation were helpful, but increased the work involved.

Use of fresh citron cuttings as receptors in place of mature potted plants can significantly reduce plant production time and space requirements. The two CTV isolates used induce visible symptoms in Etrog citron and in calamondin, and infection could be read visually. Mild isolates of CTV may not cause detectable symptoms in citron, and for these isolates, use of cuttings of more reactive hosts or verification of infection by ELISA would be necessary (4). ELISA is also useful where infection must be verified as rapidly as possible.

TABLE 4
COMPARISON OF SUSCEPTIBILITY OF LARGE POTTED PLANTS, CALLUSED CUTTINGS, AND FRESHLY MADE CUTTINGS OF ETROG CITRON TO INFECTION BY MECHANICAL INOCULATION WITH CITRUS TRISTEZA VIRUS

		CTV transmission[y]		
Expt. no.	Method of inoculation[z]	Potted plants	Callused cuttings	Fresh cuttings
1	BF	5/5	2/5	0/5[x]
1	SS	3/5	1/2[x]	0/5[x]
2	SS + BF	29/40	6/17	9/15
3	SS + BF	11/20	1/8	2/8
4	SS	9/10	4/9	—
5	SS	7/20	9/20	—

[z]BF = inoculation on bark flap, SS = stem slash—90 cuts for potted plants in Expt. 1-3, 30-50 for all other slash treatments indicated.
[y]Number of plants infected over number infected. Potted plants were well-established plants of Etrog citron in separate containers grown from cuttings. Basal stem diameter was 5-8 mm. Callused cuttings were stem cuttings 5-8 mm in diameter and 15 cm long made 25-30 days prior to inoculation which had formed callus plus one or more roots and a flush of growth. Fresh cuttings were of similar dimensions, freshly prepared prior to inoculation.
[x]All, or a large proportion of plants died before assays could be made.

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Fig. 2. Use of fresh or newly callused Etrog citron cuttings as receptors for stem-slash inoculation with citrus tristeza virus. A) A callused citron cutting ready for inoculation after 3 weeks in moist rooting medium. B) A cutting inoculated immediately after preparation and shown here after 7 days' postinoculation incubation in a moist chamber. Note healing of inoculation cuts. C) Incubation of inoculated cutting in moist chamber within constant chamber cabinet. D) Comparison of inoculated fresh cutting (after postinoculation incubation and transplanting to soil) and inoculated large potted citron plants (right) at time of first symptom expression. Potted large plant was cut back following inoculation to force new growth.

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