

Growth and Morphogenesis of Citrus Tissue Cultures Infected with Citrus Tristeza Virus and Citrus Infectious Variegation Virus

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These studies were supported in part by Comisión Asesora de Investigación Científica y Técnica (CAICYT).

We thank J. A. Pina and J. F. Ballester for providing and maintaining the tissue sources used in these studies. We also appreciate the valuable technical assistance of M. E. Martínez, A. Lavina, J. M. Arregui, and V. Ortega.

Accepted for publication 1 December 1988.

ABSTRACT

Duran-Vila, N., Cambra, M., Medina, V., Ortega, C., and Navarro, L. 1989. Growth and morphogenesis of citrus tissue cultures infected with citrus tristeza virus and citrus infectious variegation virus. *Phytopathology* 79:820-826.

Stem segments from citrus tristeza virus (CTV) and citrus infectious variegation virus (CIVV) infected Pineapple sweet orange (*Citrus sinensis*), Mexican lime (*C. aurantifolia*), and Etrog citron (*C. medica*), and uninfected controls were cultured in vitro. Production of roots and regeneration of shoots and buds was reduced as a result of virus infection. The number of explants showing morphogenesis and the amount of rooting and/or regeneration of shoots and buds were affected as compared with the uninfected explants cultured as controls. The effects on morphogenic patterns depended on the virus, the virus strain, and the host. Explants

infected with CIVV produced significantly less primary callus than did the controls, whereas CTV did not affect callus induction. The amount and morphology of secondary callus after periodic subculturing was similar in infected and uninfected tissues. Indexing of callus by ELISA-double antibody sandwich and electron microscopy indicated that CIVV-infected callus was a good host system for virus replication, purification, and cell morphology studies, whereas CTV could not be detected after continuous callus cultures.

Additional keywords: in vitro culture, pathogenesis.

The potential of using plant tissue cultures to study plant virus diseases has been recognized by White, who reported the culture of excised roots of tomato infected with tobacco mosaic virus (TMV) in 1934 (22). Most efforts since then have been devoted to the establishment of long-term virus-infected cell and callus cultures, either by initiation of cultures from infected tissues or by inoculating already established healthy cultures. However, most reports and recent reviews reflect some disappointment in the approach, mainly due to the low virus titers after periodic subculturing (10,18,23).

More recently, callus and cell suspensions of tomato (*Lycopersicon esculentum* Mill.) and potato (*Solanum tuberosum* L.) have been shown to support viroid replication at levels comparable to that in whole plant tissues (13,14,24). Moreover, such tissue culture systems have been used to study viroid replication and pathogenesis (12,21). In these studies, it was demonstrated that viroid infection had a marked effect on morphogenesis of citrus and noncitrus hosts (6).

A recent study found that a number of virus and viruslike agents affecting citrus had a marked effect on the development of bud cultures and the subsequent recovery of whole plants from infected tissues (8).

In the light of the present knowledge about citrus tissue culture methods and the recent advances in the establishment of fast and reliable techniques for detection of citrus tristeza virus (CTV) and citrus infectious variegation virus (CIVV), we tested the potential of using virus-infected tissue cultures to study virus diseases. The objective of this study was to investigate the effect of two citrus viruses, CTV, a closterovirus, and CIVV, an ilarvirus, on morphogenesis and callus cultures of three citrus species.

MATERIALS AND METHODS

Source of tissue and preparation of explants. Seedlings of Pineapple sweet orange (*Citrus sinensis* (L.) Osb.) and Mexican lime (*C. aurantifolia* (Christm.) Swing.), and the citron (*C. medica*

L.) seedling clone Arizona 861-S1 grafted on sour orange (*C. aurantium* L.) were used as the sources of tissue for preparation of explants. Plants were grown in the greenhouse at 18–27 °C for at least 6 mo before they were used. The virus-infected plants had been graft-inoculated with well-characterized isolates of CTV and CIVV at least 6 mo before use.

Two isolates of CTV, T-300 and T-308, were graft-inoculated on Mexican lime and Pineapple sweet orange. Isolate T-300 induced mild symptoms on inoculated Mexican limes and is representative of the common type of CTV found in Spain. Isolate T-308 was obtained from a calamondin (*C. madurensis* Lour.) of unknown origin and induced severe symptoms on Mexican lime, including stunting, vein clearing, leaf yellowing, stem pitting, and occasionally vein corking. However, it never showed the seedling yellows reaction when indexed on sour orange, lemon (*C. limon* (L.) Burm. f.), and grapefruit (*C. paradisi* Macf.). Both isolates were symptomless on Pineapple sweet orange, and so inoculated sweet orange plants were biologically indexed using Mexican lime as an indicator before being used as a source of tissue. Both isolates had been previously indexed (2) and were known to be free of infectious variegation, psorosis, vein enation, cachexia, and exocortis.

A single isolate of CIVV, IV-400, originally from Corsica, was graft-inoculated on Pineapple sweet orange and Arizona 861-S1 citron. The inoculated plants showed characteristic symptoms of leaf distortion, wrinkling, flecking, and chlorotic spots. This isolate was free of tristeza, psorosis, vein enation, cachexia, and exocortis (2).

Stem pieces (10 cm long) were stripped of their leaves and thorns, disinfected by immersion for 10 min in a 2% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween 20 wetting agent, and rinsed three times with sterile water. Internode stem segments 1 cm long were bisected longitudinally and cultured with the cut surface in contact with the medium. In all experiments, at least 20 cultures per treatment were used.

Culture media. The basic nutrient solution (BNS) contained the inorganic salts of Murashige and Skoog (15), *D*-inositol (100 mg/L), thiamine hydrochloride (0.2 mg/L), pyridoxine hydrochloride (1 mg/L), nicotinic acid (1 mg/L), and sucrose (30 g/L).

L). This BNS was supplemented with the amounts of naphthalene acetic acid (NAA) and 6-benzylamino-purine (BA) defined as optimum for morphogenesis and callus culture (5). In all instances, the pH of the media was set at 5.7 ± 0.1 with 0.1 N NaOH. The media were dispensed as 25-ml aliquots into 150×25 -mm culture tubes, capped with polypropylene caps, and sterilized by autoclaving at 121 C for 15 min. The media were solidified by the addition of agar (10 g/L) before autoclaving. All media were cooled as slants.

Culture conditions. Cultures were always maintained under the standard environmental conditions of a culture room at 26 ± 1 C and 60% relative humidity. Cultures exposed to light were exposed for 16 hr daily to $40 \mu\text{E m}^{-2} \text{sec}^{-1}$ provided by daylight-type fluorescent lamps.

Morphogenesis studies. The medium for root initiation contained BNS supplemented with NAA at 10 mg/L for Pineapple sweet orange and Mexican lime and 3 mg/L for Arizona Etrog citron. The cultures were kept in the light. The development of healthy and infected explants and the number of roots produced per explant were recorded periodically.

The medium for bud and shoot regeneration contained BNS supplemented with BA at 3 mg/L for Pineapple sweet orange and Arizona Etrog citron and 1 mg/L for Mexican lime. The cultures were kept in the dark for 8 wk and then transferred to the light. The development of healthy and infected explants and the number of buds and shoots produced per explant were recorded periodically.

Callus culture. The medium for callus initiation contained BNS supplemented with NAA (10 mg/L) and BA (0.25 mg/L). After 5 wk in culture, the production of primary callus was evaluated by weighing the cultures.

Small portions (40–60 mg each) of the primary callus that grew from the cut ends of the stem segment were excised from the original explant and subcultured on fresh medium. The medium for callus subculture had the same composition as the medium used to culture the original explant, supplemented with 10% (v/v) orange juice (7). To evaluate the growth characteristics of healthy and infected callus lines over extended periods of time, at least 20 calli were weighed and tested for virus content at the end of each subculture period. The time interval between two successive subcultures was 4 wk. Callus cultures were always maintained in the dark.

Virus detection. The callus lines derived from infected tissues were indexed by the ELISA-double antibody sandwich (ELISA-DAS) test (3). Callus samples weighed 100–300 mg each. Tissue samples from source plants growing in the greenhouse were also

tested as positive and negative controls. The polyclonal antiserum 879 (kindly provided by S. M. Garnsey, USDA, Orlando, FL) and the monoclonal antiserum 3 DF1 (20) were used in CTV tests, and the polyclonal antiserum 997-FCVV-1 (also provided by S. M. Garnsey) was used in CIVV tests. The immunoglobulins used had been purified as previously described (1). ELISA values were measured against positive and negative standards with an automatic spectrophotometer at 405 nm. The readings were recorded when the positive controls reached the values of 0.8 (CTV) and 1.8 (CIVV).

Virus purification. CIVV was purified by the method of Dauthy and Bové (4) with minor modifications. Callus and young shoots from greenhouse-grown citrons were homogenized in four volumes of extraction medium (0.02 M disodium phosphate and 0.05 M magnesium sulphate, pH 7.5) and four volumes of butanol-chloroform (v/v) at 4 C. After low-speed centrifugation at 3,000 g for 15 min, the supernatant was centrifuged at 100,000 g for 90 min; the pellet was resuspended in 1 ml of buffer (2 mM disodium phosphate and 5 mM magnesium sulphate, pH 7.5) and clarified at 1,000 g for 5 min. The supernatant was subjected to 40,000 g for 4 hr in a sucrose gradient (10–40% sucrose in 0.02 M disodium phosphate, pH 7.5). The fractions collected were centrifuged at 120,000 g for 2 hr, and the pellet was resuspended in 0.02 M disodium phosphate buffer, pH 7.5. The final purified preparation was checked by spectrophotometry, ELISA-DAS, and electron microscopy.

Electron microscopy. Callus samples were fixed in 2% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.3) for 2 hr and washed with the same buffer for 4 hr. After fixing in 2% osmium tetroxide in Veronal acetate buffer (pH 7.3) for 2 hr, the tissues were transferred to Ringer's solution, dehydrated in an ethanol series followed by propylene oxide, embedded in Durcupan resin, and stained with uranyl acetate. Ultrathin sections were prepared, stained with lead citrate (17), and observed under a JEM-100S electron microscope.

RESULTS

Morphogenesis of virus-infected tissue cultures. Root formation on CTV-infected cultures. Uninfected sweet orange explants developed root primordia after 4 wk in culture. Explants infected with the mild isolate (T-300) also developed root primordia and looked similar to the controls, whereas explants infected with the severe isolate (T-308) remained green but did not show any changes. After 10 wk in culture, stem segment explants infected with T-300 and uninfected controls had roots and small

TABLE 1. Morphogenesis on healthy and virus-infected citrus explants cultured in vitro: Root formation after 10 wk in culture and regeneration after 18 wk in culture

Source of tissue			Root formation			Regeneration of adventitious buds and shoots		
Virus	Host	Isolate	Frequency of	Average	Number	Frequency of	Average number	Number of buds
			cultures with					
			roots ^a	of roots ^b	per explant ^c	buds and/or	shoots ^d	per explant ^e
			(%)			(%)		
Citrus tristeza virus	Pineapple sweet orange	Control	50 (20)	1	2	100 (20)	79.15	79.15
		T-300	45 (20)	0.70* ^f	1.6	100 (19)	88.47	88.47
		T-308	0 (16)	0*	0	100 (18)	74.50	74.50
	Mexican lime	Control	35 (20)	2.65	7.6	23 (30)	0.43	1.85
		T-300	6 (17)	0.35*	6	10 (30)	0.13*	1.33
		T-308	0 (20)	0*	0	4 (30)	0.08*	2.00
Infectious variegation virus	Pineapple sweet orange	Control	25 (16)	0.3	1.25	100 (20)	79.15	79.15
		IV-400	0 (16)	0*	0	85 (20)	7.1*	8.35
		Control	95 (20)	4.65	4.9	17 (30)	0.33	2.00
	Arizona 861-S1 citron	Control	95 (20)	4.65	4.9	17 (30)	0.33	2.00
		IV-400	33 (18)	1.33*	4	3 (30)	0.06*	2.00

^aFigures in brackets represent the total number of explants after contaminated cultures were discarded.

^bCalculated by dividing the total number of roots by the number of cultured explants.

^cCalculated by dividing the total number of roots by the number of explants with roots.

^dCalculated by dividing the total number of buds and/or shoots by the number of cultured explants.

^eCalculated by dividing the total number of buds and/or shoots by the number of explants showing regeneration.

^fSignificantly different from the healthy controls ($P = 0.05$). Values were compared within each treatment by analysis of variance.

proliferations of friable callus from cut surfaces, whereas the explants infected with T-308 remained unchanged (Table 1). The gross morphology of the cultures infected with T-300 was similar to that of the controls, whereas the cultures infected with T-308 did not show any development even when kept in culture for a longer time (Fig. 1B).

Uninfected lime explants also developed root primordia after 4 wk in culture. However, explants infected with the mild (T-300) and severe (T-308) isolates did not show any changes until 2 wk later. After 10 wk in culture, all controls had primary and secondary roots, whereas only a small number of explants infected with T-300 produced roots. The roots of the explants infected with T-300 were short and did not show secondary rooting (Fig. 2B). The cultures infected with T-308 had small patches of white and compact callus developing from the bark surface of the explant but did not produce any roots (Table 1).

CTV infection reduced the number of cultures with roots and the average number of roots was less in both hosts. The severity of the isolate and the sensitivity of the host correlated with the observed reduction on rooting efficiency (Table 1).

Root formation on CIVV-infected cultures. Uninfected orange

controls developed root primordia after 4 wk in culture and had well developed roots after 10 wk. Infected explants did not produce roots or root primordia even when kept in culture for longer periods of time (Table 1 and Fig. 3B). Uninfected citron controls produced roots and root primordia after 4 wk in culture, whereas the infected explants did not show any changes until 2 wk later. After 10 wk in culture the controls had well-developed roots (1–2 cm long) whereas only a small number of infected explants developed very small roots or root primordia (Table 1 and Fig. 4B). CIVV infection reduced the number of cultures with roots and the average number of roots and changed the gross morphology of the cultured explants compared to that of the controls.

Bud and shoot regeneration on CTV-infected cultures. All sweet orange explants infected with the mild (T-300) or the severe (T-308) isolates and the uninfected controls produced a large number of adventitious buds and shoots (Table 1). Pineapple sweet orange cultures kept in the dark for 10 wk produced a large number of etiolated adventitious shoots with very small leaves that turned green and expanded after being transferred to the light. All infected and uninfected sweet orange explants produced the same average number of regenerated buds and shoots (Table 1) and were virtually indistinguishable in appearance (Fig. 1A).

Uninoculated Mexican lime explants cultured in the dark for 10 wk produced small proliferations of compact callus containing green buds and very small shoots on transversal cut surfaces. Unlike the development of the Pineapple sweet orange cultures described above, Mexican lime explants did not develop any further after being transferred to the light. Fewer lime cultures

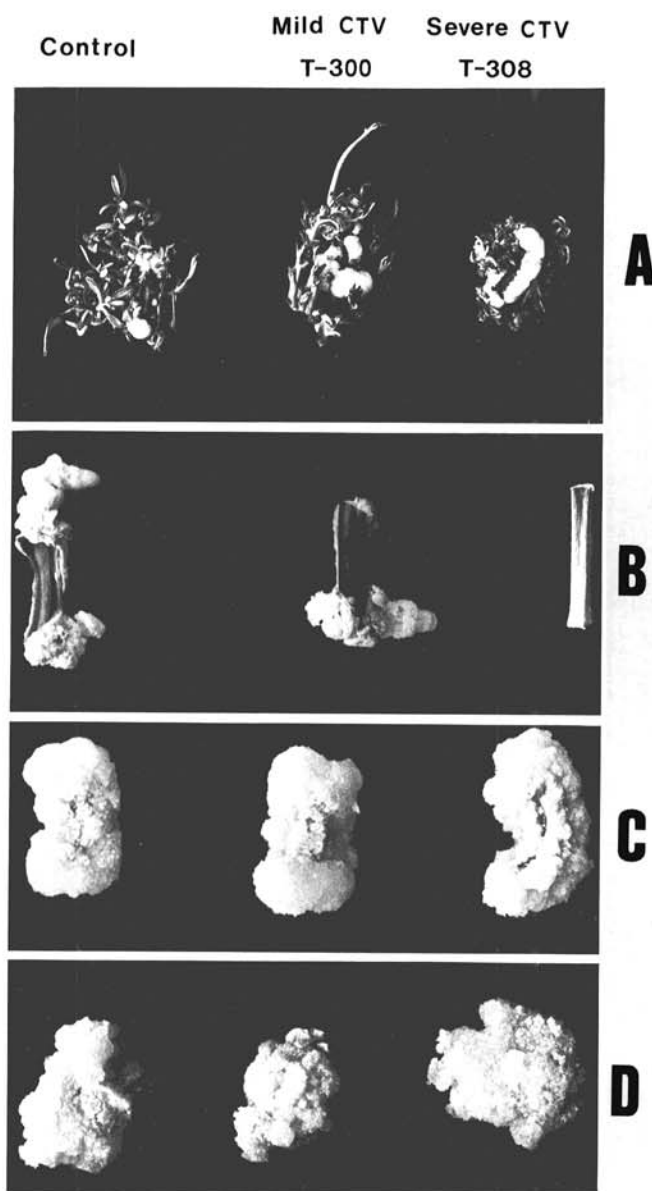


Fig. 1. Morphogenesis and callus cultures on stem segments of Pineapple sweet orange infected with mild (T-300) and severe (T-308) strains of citrus tristeza virus (CTV): **A**, shoot and bud regeneration; **B**, root formation; **C**, callus initiation; and **D**, callus cultures.

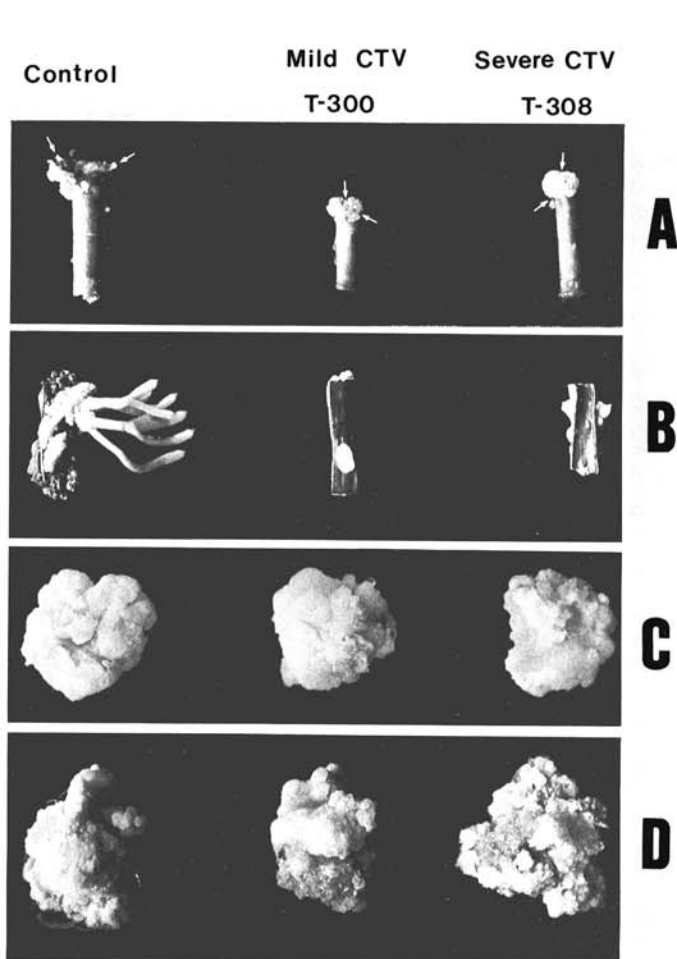


Fig. 2. Morphogenesis and callus cultures on stem segments of Mexican lime infected with mild (T-300) and severe (T-308) strains of citrus tristeza virus (CTV): **A**, shoot and bud regeneration (arrows show regenerated shoots and buds); **B**, root formation; **C**, callus initiation; and **D**, callus cultures.

infected with the mild (T-300) or severe (T-308) isolates regenerated, and the average number of regenerated buds and shoots was less than that of the controls.

The pathogenicity and severity of the strains was reflected only in the decreased number of Mexican lime cultures showing regeneration and the number of regenerated buds and shoots (Table 1 and Fig. 2A).

Bud and shoot regeneration on CIVV-infected cultures. Infected sweet orange explants and uninfected controls cultured in the dark for 10 wk regenerated etiolated buds and shoots. The number of cultures showing regeneration and the number of regenerated buds and shoots was significantly less in infected than in control cultures (Table 1). As described above, the etiolated shoots regenerated from the control explants kept in the dark turned green, and their leaves expanded, after being transferred to the light. Nevertheless, infected cultures produced a lower number of buds and shoots and did not develop any further after being

transferred to the light (Fig. 3A).

Uninfected citron explants produced friable callus from the transversal cuts and smaller proliferations of compact callus from the bark surface (Fig. 4A). A small number of buds were observed on the callus produced on the bark surface after the cultures were kept for 10 wk in the dark, but they did not develop any further after being transferred to the light. Infected cultures produced smaller amounts of the two kinds of callus and a lower number of buds than the controls (Table 1).

The pathogenicity of the virus and the sensitivity of the hosts was reflected in the number of explants showing regeneration, the average number of buds and shoots produced, and the gross morphology of the cultures (Table 1 and Fig. 4A).

Induction and maintenance of callus cultures. *Callus cultures from CTV-infected explants.* Proliferations of primary callus from uninfected and CTV-infected Pineapple sweet orange and

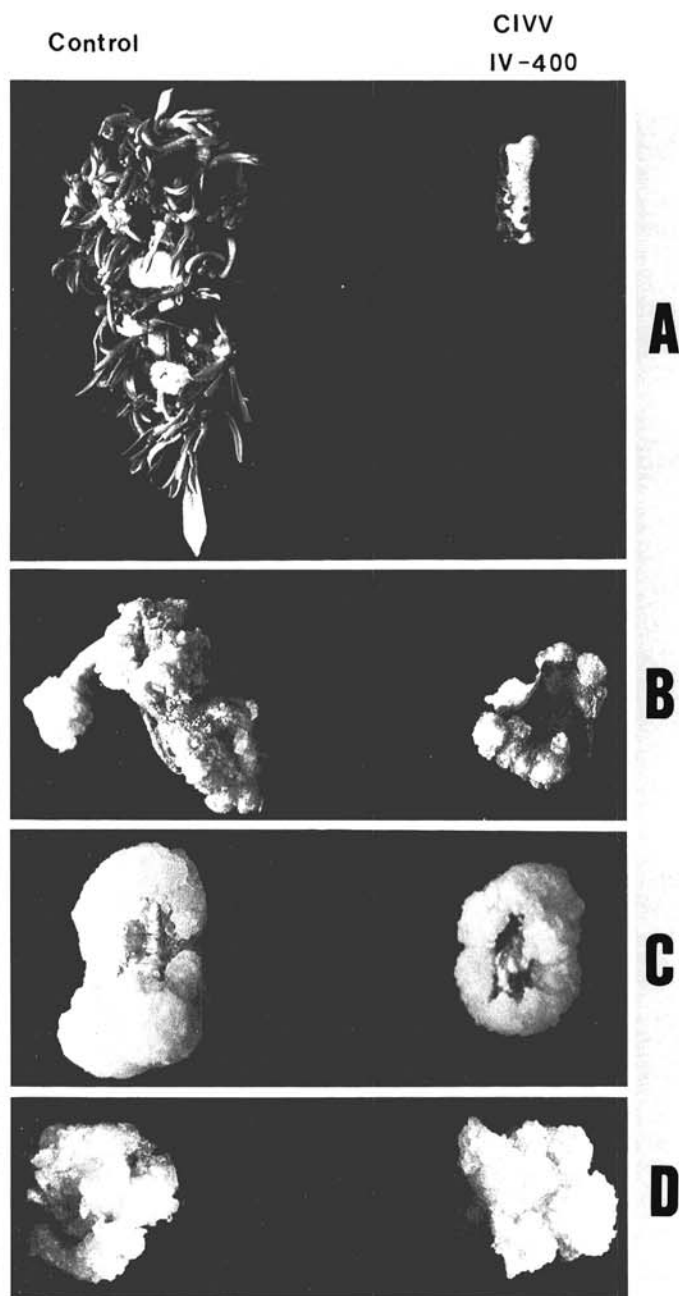


Fig. 3. Morphogenesis and callus cultures on stem segments of Pineapple sweet orange infected with citrus infectious variegation virus (CIVV) (strain IV-400): **A**, shoot and bud regeneration; **B**, root formation; **C**, callus initiation; and **D**, callus cultures.

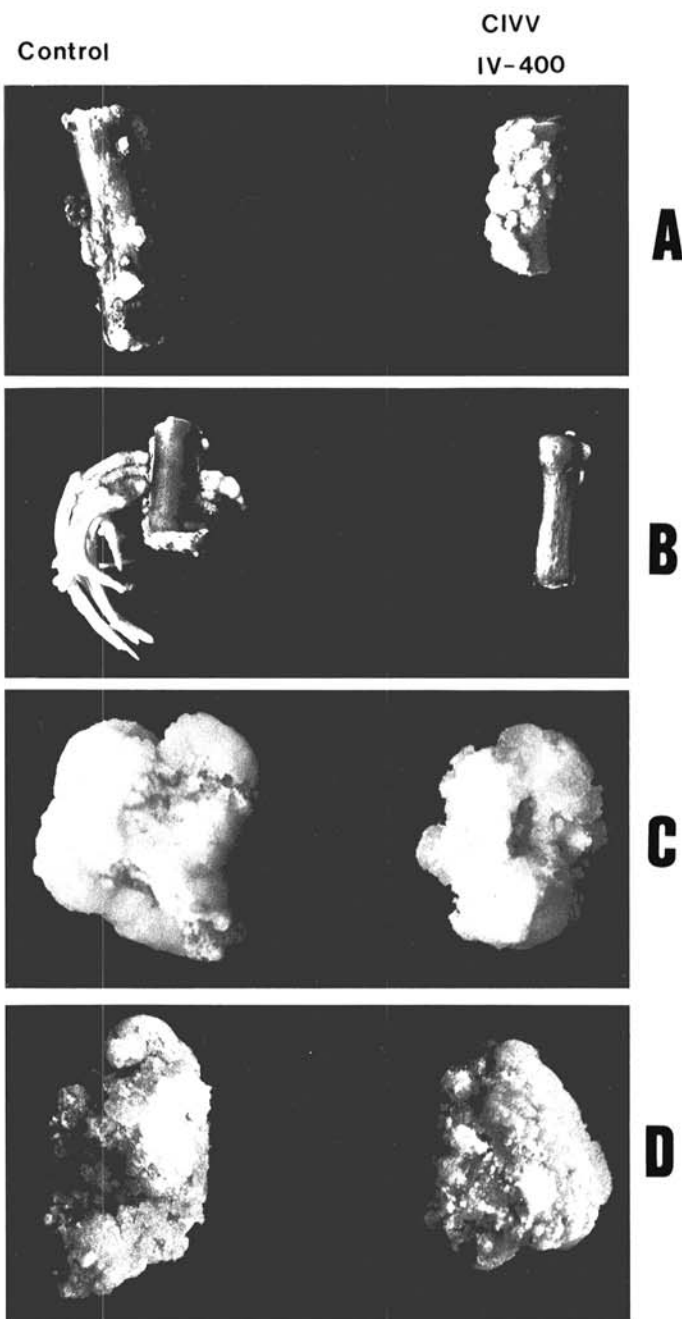


Fig. 4. Morphogenesis and callus cultures on stem segments of Arizona 861-S1 citron infected with citrus infectious variegation virus (CIVV) (strain IV-400): **A**, shoot and bud regeneration; **B**, root formation; **C**, callus initiation; and **D**, callus cultures.

Mexican lime explants started on the longitudinal cut surfaces and were first observed 2 wk after the cultures had been initiated. The growth of primary callus continued during the following 3 wk until the original explants were virtually covered by callus (Figs. 1C and 2C). The primary callus produced from stem segments infected with the mild (T-300) or severe (T-308) isolates had the same size and morphology as that of the controls (Table 2 and Figs. 1C and 2C).

The growth and morphology of secondary callus in the four subcultures studied was similar in uninfected and infected cultures (Table 2 and Figs. 1D and 2D).

Callus cultures from CIVV-infected explants. Proliferations of primary callus from uninfected and infected Pineapple sweet orange and citron Arizona 861-S1 explants also started on the longitudinal cut surfaces 2 wk after the cultures had been initiated and proceeded during the following 3 wk. Stem segments infected with CIVV produced significantly less primary callus than did the controls (Table 2 and Fig. 3C).

The growth of secondary callus produced from CIVV-infected citron did not differ from that produced by the controls in the five subcultures studied (Table 2). However, the production of secondary callus from infected sweet orange explants was significantly lower than in the controls during the first and second subcultures (Table 2 and Fig. 4C).

Callus obtained from infected tissues and from uninfected controls maintained by periodic subculture had the same morphology (Figs. 3D and 4D).

Virus content of callus cultures. *Virus content of callus lines originated from CTV-infected explants.* The number of Pineapple sweet orange and Mexican lime callus lines indexing positive decreased in each subculture. In addition, the ELISA values were lower in callus indexing positive than in the positive controls

using infected plant tissues. Virus was not detected at the end of the third subculture in Pineapple sweet orange callus nor at the end of the fifth subculture in Mexican lime callus (Table 3).

Virus content of callus lines originated from CIVV-infected explants. The number of Pineapple sweet orange callus lines indexing positive decreased in the fourth and fifth subculture. However, in the cultures indexing positive, ELISA values were comparable to those from infected source plant tissues and to the values from the previous subcultures (Table 3).

All citron Arizona 861-S1 callus lines indexed positive in each of five subcultures. The ELISA values were comparable to the values from infected plant tissues and did not vary over the five subcultures (Table 3).

Virus purification. Callus originated from CIVV-infected citron callus produced clearer crude extracts than did mother plant tissues, probably due to the absence of chlorophyll in callus cells. Therefore, the extracts were easily clarified, and the butanol-chloroform clarification used by Dauty and Bové (4) was replaced by milder treatments (see Materials and Methods). Callus tissues gave yields (260/280 ratio: 1.73–1.91) comparable to or higher than those from young leaves from infected citron mother plants growing in the greenhouse (260/280 ratio: 1.78). Spherical particles measuring about 30 nm were detected in negative stained preparations of purified virus.

Electron microscopy. Ultrathin sections of secondary callus from CIVV-infected citron showed crystalline inclusions probably containing virions or protein bodies (Fig. 5B), which were absent from healthy cells (Fig. 5A). Infected callus cells contained endocytic invaginations (Figs. 5C and 5D) of the plasma membrane known as plasmalemmasomes. These invaginations contained vesicular inclusions of different sizes (Figs. 5C and

TABLE 2. Induction and maintenance of callus from healthy and virus-infected citrus explants cultured in vitro

Virus	Source of tissue		Amount of primary callus ^a (g)	Amount of callus produced by periodic subcultures ^b (g)				
				1st	2nd	3rd	4th	5th
Citrus tristeza virus	Pineapple sweet orange	Control	0.78	1.97	2.00	1.17	0.95	...
		T-300	0.83	1.87	2.17	1.29	1.03	...
		T-308	0.83	1.68	2.59	1.27	1.40	...
	Mexican lime	Control	0.86	1.02	1.29	1.22	1.13	...
		T-300	0.74	1.08	0.91	0.93	0.83	...
		T-308	0.84	0.72	1.02	1.24	1.15	...
Infectious variegation virus	Pineapple sweet orange	Control	0.51	0.52	1.75	1.32	1.75	2.81
		IV-400	0.33* ^c	0.31*	1.08*	1.33	1.93	2.28
	Arizona 861-S1 citron	Control	0.42	0.37	0.88	0.93	1.20	1.73
		IV-400	0.31*	0.30	1.27	1.06	1.18	1.89

^aEstimated by weighing the cultures 5 wk after they had been initiated. Figures represent the mean weight of at least 20 cultures.

^bEstimated by weighing the cultures 4 wk after they had been subcultured. Figures represent the mean of at least 15 cultures.

^cSignificantly different from healthy controls ($P = 0.05$). Values were compared within each treatment by analysis of variance.

TABLE 3. Detection by ELISA-double antibody sandwich of viruses in callus cultures maintained over periodic subculturing

Virus	Source of tissue		Number of calli indexing positive in subcultures (%) ^a				
			1st	2nd	3rd	4th	5th
Citrus tristeza virus	Pineapple sweet orange	Control	0(0.006)	0(0.006)	0(0.004)	0(0.000)	NT
		T-300	50(0.408)	21(0.086)	0(0.003)	0(0.008)	NT
		T-308	50(0.488)	36(0.134)	0(0.006)	0(0.009)	NT
	Mexican lime	Control	0(0.007)	0(0.005)	0(0.007)	0(0.008)	NT
		T-300	37(0.425)	40(0.166)	20(0.144)	0(0.010)	NT
		T-308	39(0.299)	35(0.159)	16(0.119)	19(0.031)	NT
Infectious variegation virus	Pineapple sweet orange	Control	0(0.000)	0(0.000)	0(0.001)	0(0.000)	0(0.004)
		IV-400	100(1.584)	100(2.000)	100(1.386)	90(1.230)	60(1.144)
	Arizona 861-S1 citron	Control	0(0.004)	0(0.000)	0(0.007)	0(0.003)	0(0.004)
		IV-400	100(1.800)	100(1.780)	100(1.456)	100(2.000)	100(2.000)

^aIn each subculture, at least 15 calli per treatment were indexed. Data represent the percent of calli indexing positive out of the total number of calli indexed. Figures in brackets are mean ELISA values compared to positive (CTV = 0.800, CIVV = 1.800) and negative (CTV = 0.000, CIVV = 0.000) standards. NT = not tested.

5D). The large vesicles almost completely filled the space of the invagination (Fig. 5E). These vesicles were surrounded by a double membrane (Fig. 5F).

DISCUSSION

Infection with CTV and CIVV had a marked effect on morphogenesis of cultured citrus explants. All virus-host combinations resulted either in a decrease or total impairment of root formation. Root formation on CTV-infected explants reflected the severity of the strains and the sensitivity of the hosts. The severe isolate (T-308) caused total impairment of root formation on both hosts, whereas the mild isolate (T-300) caused a decrease in the number of cultures with roots and the average number of roots. The differences were more marked in Mexican lime, a sensitive host, than in Pineapple sweet orange, a symptomless host.

Infection with CTV also affected the regeneration of adventitious buds and shoots. Mexican lime explants exhibited symptoms of infection proportional to the severity of the two isolates of CTV, whereas Pineapple sweet orange cultures were symptomless. Symptoms of CIVV infection developed in both citron Arizona 861-S1 and Pineapple sweet orange explants.

The effect of CTV and CIVV infection on the growth of in vitro cultures had been reported for cultured buds and subsequent recovery of rooted plants (7). However, the results reported here show that morphogenesis is more markedly affected as a result of virus infection. In addition, the changes in morphogenetic patterns reflect differences in host sensitivity and strain pathogenicity. Therefore, appropriate quantitation of these morphogenic changes may provide additional parameters for characterization of field isolates. Similarly, differences induced by specific strains of a virus may predict the sensitivity of a new host.

As stated in a previous report (7), the effect of virus infection

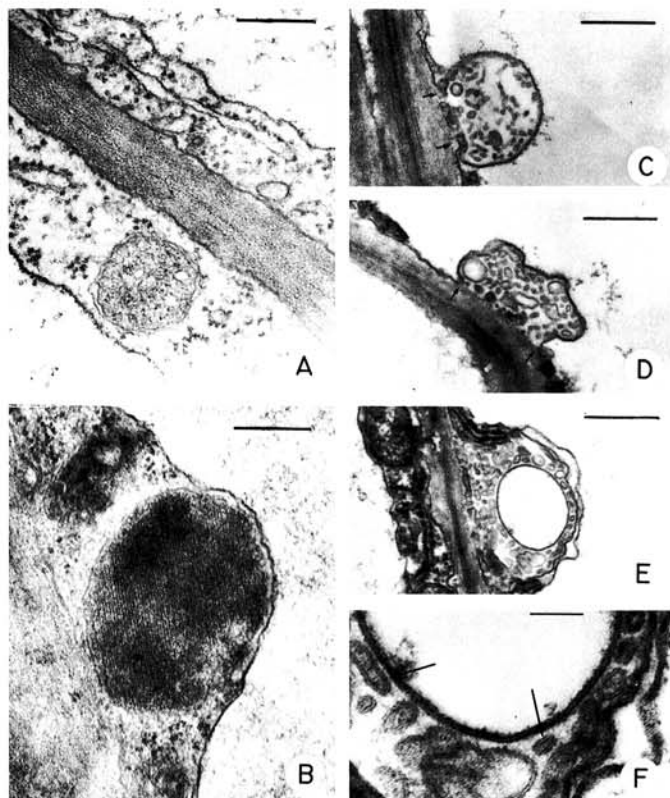


Fig. 5. Electron micrograph of cells infected with citrus infectious variegation virus (CIVV): A, uninfected control; B-F, CIVV-infected callus cells. Infected cells show crystalline bodies (B) and vesicular inclusions (C-F) not present in controls (A). Arrows show plasma membrane invaginations in (C) and (D) and the double membrane surrounding the vesicles in (F). Bar indicates 0.5 μm in A-E and 0.1 μm in F.

on morphogenesis of cultured explants demonstrates the significance of the quality of the mother plant to be used as a source of material in tissue culture experiments. Even latent infections, unnoticed under field and greenhouse conditions, may result in limited morphogenesis.

Growth and appearance of callus lines established from CTV- and CIVV-infected tissues and maintained over periodic subculturing were comparable to those of controls. The low frequency of CTV detection on callus lines from infected tissues and the conclusion that the CTV antigen was present in small amounts are indications that the virus does not replicate well in callus systems. Whether the positive ELISA values obtained during the first and second subcultures were the result of actual virus replication or simply due to the presence of virions or coat protein resulting from the original explant cannot be concluded from the data. In fact, the absence of vascular bundles on long-term established callus cultures should prevent the spread of CTV particles, which are known to be mainly restricted to the phloem. Attempts to graft-transmit CTV from secondary callus to Mexican lime seedlings gave some positive results (two out of 13 calli indexed positive, unpublished results). This is an indication that some infective virions are present in secondary callus. The results of CIVV detection on callus lines from CIVV-infected tissues, and the maintenance of ELISA values comparable to those of infected controls, indicate that the virus replicates well in callus systems. The decrease in the number of Pineapple sweet orange calli indexing positive at each transfer while the ELISA values were still high suggests an uneven distribution of the virus within the tissue rather than a failure of the virus to replicate. The results on virus content of callus cultures initially infected with CTV and CIVV illustrate that different viruses show different patterns of host-tissue interaction. CTV, a closterovirus mainly restricted to the phloem, cannot be detected in callus cultures after a few subcultures, whereas callus lines from CIVV-infected tissues seem to maintain stable virus titers over several subcultures. Response of CTV-infected callus lines is similar to results for tissue cultures initiated from TMV-infected tissues (9,11,16,18) and limits their use for the study of these viruses. The finding that severe strains of CTV were able to invade the ground meristem adjacent to the protophloem of susceptible varieties (19) suggests that it may be feasible to select cells and callus lines able to sustain replication of CTV. The development of techniques to select cells and callus lines replicating CTV was beyond the scope of the present study.

Conversely CIVV, an ilarvirus, replicates in callus lines established from infected tissues. The CIVV-containing callus lines maintain stable virus titers after at least 1 yr of successive subcultures, and they provide a good source of tissue for virus purification. In fact, the yields obtained even with mild purification treatments were comparable or higher than those from plant tissues. In addition, although infected callus resembles the uninfected controls in terms of gross morphology, thin sections visualized under electron microscopy reveal characteristic malformations at the cell level.

In summary, the study of morphogenesis of in vitro cultured citrus explants provides an insight to symptom expression as a result of CTV and CIVV infection. In addition, the quantification of these changes on morphogenesis may be useful as a tool for strain characterization of CTV and CIVV, as well as for host sensitivity evaluation. The utilization of callus cultures is currently restricted to the study of CIVV. However, results of CIVV purification from callus indicate that callus cultures may be used as a tissue source for purification of other virus and viruslike disease-causing agents of unknown etiology. Callus cultures are easy to grow, maintain, and store. Media and environmental modifications that would favor replication and/or accumulation of the agent can be studied. Therefore, provided that the virus replicates, callus cultures may be used as a source of tissue for the study of citrus virus diseases.

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