Growth and Morphogenesis of Citrus Tissue Cultures Infected with Psorosis, Vein Enation, and Cachexia

N. Duran-Vila, V. Medina, J. A. Pina, C. Ortega, M. I. Molins, and L. Navarro

Instituto Valenciano de Investigaciones Agrarias (IVIA), 46113 Moncada, Valencia, Spain.

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ABSTRACT

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Stem segments from Pineapple sweet orange (*Citrus sinensis*) and Etrog citron (*C. medica*) infected with psorosis, vein enation, and cachexia, as well as uninfected controls, were cultured in vitro. Production of roots and regeneration of shoots and buds were modified as a result of 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 differences on morphogenic patterns depended on the disease and the disease isolate. Explants infected with vein enation and cachexia produced significantly less primary callus than the controls, whereas psorosis did not affect callus induction. The amount and morphology of secondary callus after the first subculture were similar in infected and uninfected tissues. Biological indexing of callus indicated that psorosis- and cachexia-infected callus were good host systems for the replication of the disease-causing agents, whereas vein enation could not be detected after continuous callus cultures. The citrus cachexia viroid was detected from infected callus by nucleic acid extraction and sequential polyacrylamide gel electrophoresis. Electron microscopy studies revealed alterations at the cell level on psorosis-infected callus.

Additional keyword: pathogenesis.

The potential of using plant tissue cultures to study plant viruses was recognized by White in 1934 (23). However, many of the studies that followed the initial approach and most of the recent reviews reflect some disappointments with it (9,25). More recently, callus and cell suspension cultures have been shown to be very useful to study viroid replication and pathogenesis (12,14,22,26). Moreover, in vitro culture of viroid-infected explants has also demonstrated the influence of viroids on the morphogenic patterns of infected tissues (6).

With the availability of citrus tissue culture techniques (5), studies were initiated to investigate their potential to examine host-pathogen interactions. With the initial results, the significant influence of disease-causing agents of citrus on the development of buds cultured in vitro and the recovery of rooted plantlets was established (8). The performance of infected cultures was further studied with citrus infected with citrus tristeza closterovirus (CTV) and citrus infectious variegation ilarvirus (CIVV). Both viruses had a significant effect on the morphogenesis of infected tissues, and CIVV was able to persist and replicate on callus cultures over several subcultures (4).

Additional information on the performance of citrus tissue cultures infected with viruslike agents of three citrus diseases is given in this study. The study included the causal agents of citrus psorosis and citrus vein enation, which are two diseases of unknown etiology, and citrus cachexia, which has been recently demonstrated to be caused by a viroid (20).

MATERIALS AND METHODS

Sources of tissue and preparation of explants. Seedlings of Pineapple sweet orange (*Citrus sinensis* (L.) Osbeck), and the citron (*C. medica* L.) seedling clone Arizona 861-S1 grafted on rough lemon (*C. jambhiri* Lush.) were used as the source of tissue for preparation of explants. Infected plants had been graft-inoculated with well characterized isolates of psorosis, vein enation, and citrus cachexia viroid (CCaV) at least 6 mo before use. Plants infected with psorosis and vein enation and the uninfected controls were grown in the greenhouse at 18–27 C. The plants infected with CCaV and another set of uninfected controls were grown at 28–32 C.

Two isolates of psorosis, P-121 and P-123, which were recovered from a single Oroval clementine (*C. clementine* Hort. ex Tan.) after shoot-tip grafting in vitro (16), were graft-inoculated on Pineapple sweet orange. Isolate P-121 caused a shock reaction, vein flecking, oakleaf patterns, and crinkling, whereas P-123 only induced flecking and occasional oakleaf patterns. Both isolates had been previously indexed (2) and were known to be free of tristeza, vein enation, cachexia, and exocortis.

A single isolate of vein enation, V-207 originally found on Parent Washington navel orange, was graft-inoculated on Pineapple sweet orange in which it induced characteristic enations on veins and indexed (2) negative for tristeza, psorosis, cachexia, and exocortis.

A single isolate of CCaV (X-704) originally from California (CA-902), which indexed negative for tristeza, psorosis, and exocortis, was graft-inoculated on Etrog citron. Because it was symptomless on Etrog citron but induced a severe reaction on Parson's Special mandarin grafted on rough lemon (19), all inoculated plants were biologically indexed before being used as sources of tissue for in vitro culture assays.

Stem pieces (10 cm long) were stripped of their leaves and thorns and disinfected, and the explants consisting of stem internodes were prepared as described previously (5). 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), 100 mg/L of *i*-inositol, 0.2 mg/L of thiamine hydrochloride, 1 mg/L of pyridoxine hydrochloride, 1 mg/L of nicotinic acid, and 30 g/L of sucrose. This BNS was supplemented with amounts of naphthalene acetic acid (NAA) and 6-benzylamino-purine (BA), defined as optimum for morphogenesis and callus culture (4,5). In all instances, the media was prepared as described earlier (5).

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

Morphogenesis studies and callus culture. The media and environmental conditions for root initiation and regeneration of buds and shoots were the same as described in earlier studies (4,5). The development of healthy and infected explants and the number of roots, buds, and shoots produced per explant were recorded periodically.

Initiation of primary callus and further maintenance by periodic transfers were accomplished as described earlier (4,5). To evaluate the growth characteristics of healthy and infected callus lines over extended periods of time, at least 20 callus were weighed at the end of four or five successive subculture periods.

Disease indexing. The callus lines derived from infected tissues were biologically indexed essentially as described for citrus trees (2,19). At least 10 calluses were indexed separately for each disease at the end of the first and fifth subculture periods. Inoculations (two inocula per plant) were performed by introducing a piece of callus underneath the bark of the indicator plant, incubating, and reading the symptoms following the system described for indexing of citrus trees. Indicator plants used were: for each psorosis test, one Pineapple sweet orange and one Dweet tangor (*C. tangerina* Hort. ex Tan. × *C. sinensis* seedlings); for each vein enation test, two mexican lime (*C. aurantifolia* (Christm.) Swing.) seedlings; and for each cachexia test, three Parson's Special mandarins grafted on rough lemon.

Viroid detection. Callus samples (10 g) were homogenized in 5 ml of medium (0.5 M sodium sulfite, 1% sodium dodecyl sulfate, 4% 2-mercaptoethanol) containing 15 ml of water-saturated phenol adjusted to pH 7. After centrifugation at 10,000 *g* for 20 min, the aqueous phase was adjusted to contain 35% ethanol-STE (0.1 M NaCl, 1 mM EDTA, 0.05 M Tris-HCl, pH 7.2) and 2 g of cellulose. The mixture was gently agitated overnight, and the cellulose was collected by centrifugation at 6,000 *g* and batch washed three times with 30% ethanol-STE. The cellulose-containing mixture was loaded onto a column and washed further with 2 void vol of 30% ethanol-STE. Nucleic acids were eluted with STE buffer, concentrated by ethanol precipitation and the 2 M LiCl-soluble fraction was collected (21). After ethanol concentration, the pellet was dried and resuspended in 300 μl of TKM (10 mM Tris, 10 mM KCl, 0.1 mM $MgCl_2$, pH 7.4). The preparations were analyzed by sequential 5% polyacrylamide gel electrophoresis (sPAGE) (18).

Electron microscopy. Callus samples were fixed in 2.5% glutaraldehyde in 0.5 M cacodylate buffer, pH 7.0. After postfixing in 1% osmium tetroxide in Veronal acetate buffer, the tissues were dehydrated in an ethanol series that incorporated uranyl acetate and were embedded in London resin (60 C oven for 12 h for polymerization). Thin sections were prepared with a glass knife, mounted on parlodion-coated 200-mesh copper grids, and were stained with lead citrate for 1 min (17).

RESULTS

Morphogenesis of infected tissue cultures. *Root formation on psorosis-infected cultures.* Uninfected sweet orange explants developed root primordia after 4 wk in culture. Explants infected with the mild isolate (P-123) also developed root primordia and looked similar to the controls, whereas explants infected with the severe isolate (P-121) remained green but did not show any changes. After 10 wk in culture, stem segment explants infected with P-123 and uninfected controls had roots and small proliferations of callus from cut surfaces, whereas the explants infected with P-121 remained unchanged (Table 1). The gross morphology of the cultures infected with P-123 was similar to the controls, whereas the cultures infected with P-121 did not show any development even when kept in culture for a longer time (Fig. 1B). Psorosis infection reduced the number of cultures with roots and the average number of roots. The severity of the isolate correlates with the observed reduction on rooting efficiency (Table 1).

Root formation on vein enation-infected cultures. Uninfected sweet orange controls developed root primordia after 4 wk in culture and had well-developed roots after 10 wk. Infection reduced the number of explants with roots and the average number of roots (Table 1). The gross morphology of the explants that produced roots was similar to the uninoculated controls (Fig. 2B).

Root formation on cachexia-infected cultures. Infected citron explants and uninfected controls produced roots and root primordia after 4 wk in culture, and after 10 wk the explants had well-developed roots (1–2 cm long). Infection reduced the number of cultures with roots and the average number of roots (Table 1), but the gross morphology of the explants did not show any changes (Fig. 3B).

Bud and shoot regeneration on psorosis-infected cultures. All sweet orange explants infected with the mild (P-123) or the severe (P-121) isolate and the uninfected controls produced adventitious buds and shoots (Table 1). Infected explants regenerated larger numbers of buds and shoots than the uninfected controls (Fig. 1A). Infection resulted in an increase of the number of regenerating cultures and the numbers of regenerated buds and shoots. The increase in regeneration correlated with the severity of the isolate.

Bud and shoot regeneration on vein enation-infected cultures. Infected sweet orange explants and uninfected controls regen-

TABLE 1. Morphogenesis on healthy and infected citrus explants cultured in vitro

Source of tissue			Root formation			Regeneration of adventitious buds and shoots		
Disease agent	Host	Isolate	Frequency of cultures with roots (%)[a]	Average number of roots[b]	Number of roots per explant[c]	Frequency of cultures with buds and/or shoots (%)	Average number of buds and/or shoots[d]	Number of buds and/or shoots per explant[e]
Psorosis	Pineapple sweet orange	Control	44 (18)	0.66	1.5	70 (20)	5.9	8.5
		P-121	0 (18)	0*	0	76 (17)	29.5*	38.5
		P-123	22 (18)	0.39*	1.75	95 (19)	14.0*	14.8
Vein enation	Pineapple sweet orange	Control	44 (18)	0.66	1.5	63 (19)	6.4	10.2
		V-208	20 (20)	0.25*	1.25	63 (20)	1.9*	3.2
Cachexia	Arizona 861-S1 citron	Control	82 (20)	2.40	3.00	80 (20)	4.17	4.80
		X-704	67 (20)	1.34*	1.99	45 (20)	1.17*	2.65

[a] Figures in parentheses represent the total number of explants after discarding contaminated cultures.
[b] Calculated by dividing the total number of roots by the number of cultured explants. * = Significantly different than the healthy controls ($P = 0.05$). Values were compared within each treatment by analysis of variance.
[c] Calculated by dividing the total number of roots by the number of explants with roots.
[d] Calculated by dividing the total number of buds and/or shoots by the number of cultured explants. * = Significantly different than the healthy controls ($P = 0.05$). Values were compared within each treatment by analysis of variance.
[e] Calculated by dividing the total number of buds and/or shoots by the number of explants showing regeneration.

erated adventitious buds and shoots. The frequency of cultures showing regeneration was not affected as a result of infection, but the numbers of regenerated buds and shoots were significantly less in infected than in control explants (Table 1; Fig. 2A).

Bud and shoot regeneration on cachexia-infected cultures. Infected citron explants and uninfected controls regenerated adventitious buds directly from the explant or from small proliferations of callus developing from the cut ends (Fig. 3A). The regenerated buds did not develop more than a few millimeters in length when kept in culture for longer periods of time. As a result of infection with cachexia, the average of regenerated buds and the number of buds per explant were significantly reduced (Table 1).

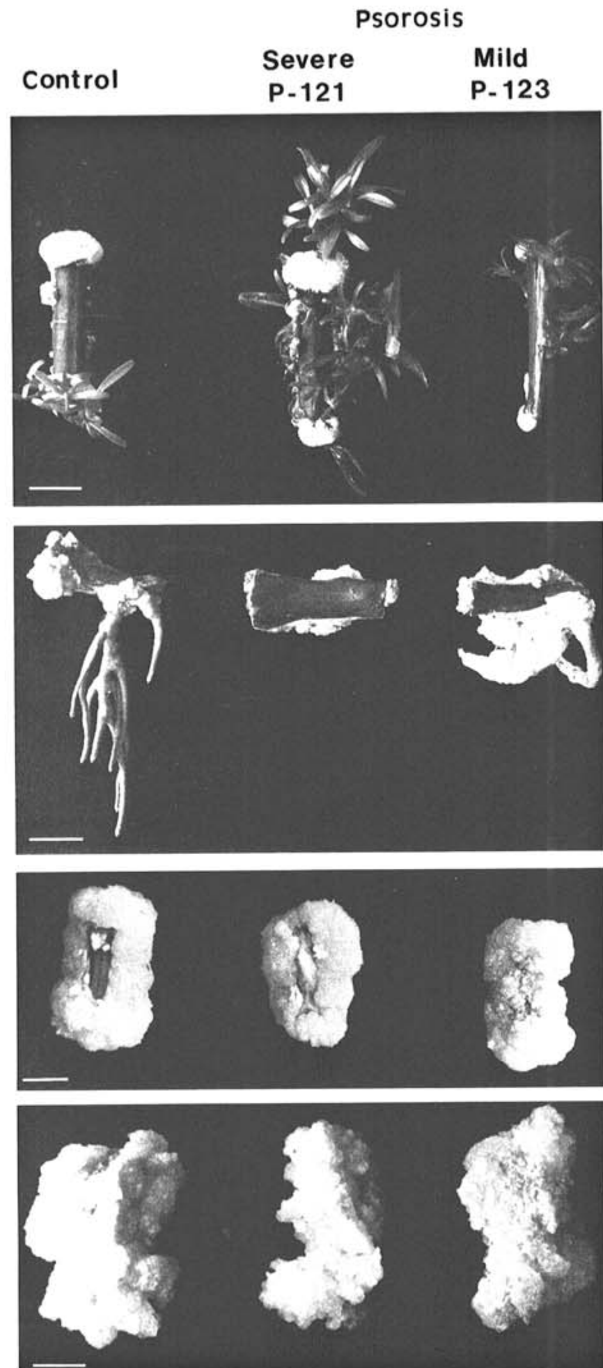


Fig. 1. Morphogenesis and callus cultures on stem segments of Pineapple sweet orange infected with severe (P-121) and mild (P-123) strains of the psorosis agent: **A,** shoot and bud regeneration; **B,** root formation; **C,** callus initiation; and **D,** callus cultures.

Induction and maintenance of callus cultures. *Callus cultures from psorosis-infected explants.* Proliferations of primary callus from uninfected and psorosis-infected Pineapple sweet orange 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 next 3 wk. The amount of primary callus produced in explants infected with the mild (P-123) or severe (P-121) isolate was not significantly different than the uninoculated controls. However, explants infected with the severe isolate produced more primary callus than the explants

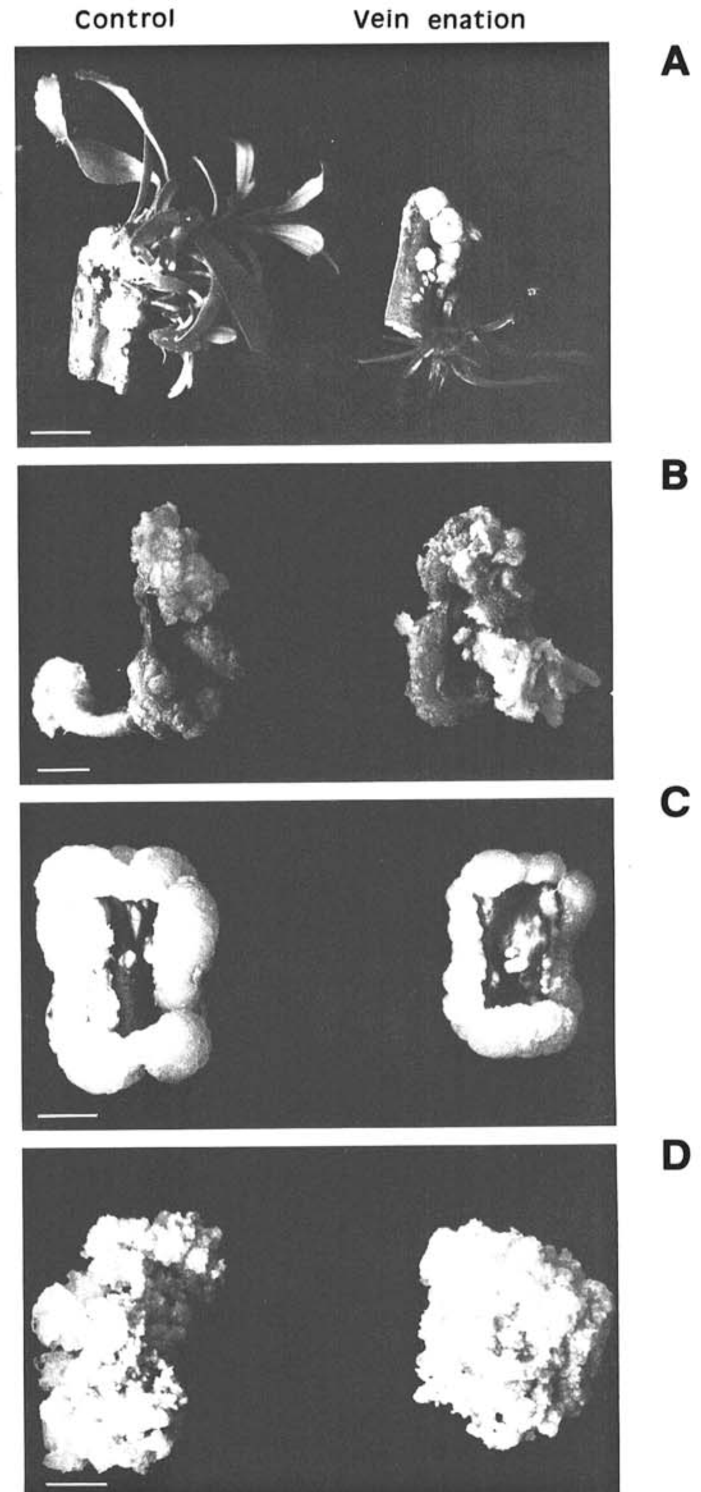
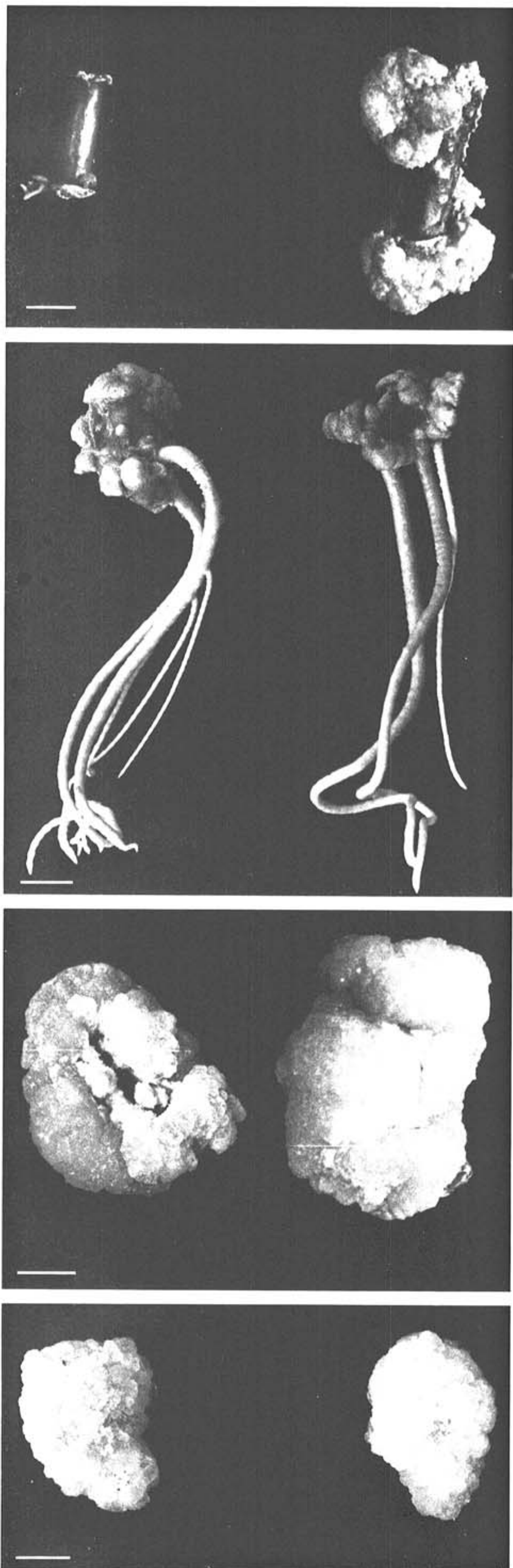


Fig. 2. Morphogenesis and callus cultures on stem segments of Pineapple sweet orange infected with the vein enation agent: **A,** shoot and bud regeneration; **B,** root formation; **C,** callus initiation; and **D,** callus cultures.



infected with the mild isolate (Table 2). Both types of calli had the same morphology as the controls (Fig. 1C).

The growth and morphology of secondary callus during the four subcultures studied were similar in infected and uninfected cultures (Table 2; Fig. 1D).

Callus cultures from vein enation-infected explants. Proliferations of primary callus from uninfected and vein enation-infected Pineapple sweet orange explants also started on the longitudinal cut surfaces 2 wk after the cultures had been initiated. The growth of primary callus continued during the next 3 wk. Infected explants produced significantly less primary callus than the controls (Table 2; Fig. 2C).

The growth and gross morphology of secondary callus produced from infected explants did not differ from the controls in the five subcultures studied (Table 2; Fig. 2D).

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

The growth of secondary callus produced from infected tissues did not differ from the uninfected control in the four subcultures studied (Table 2). Callus obtained from infected tissues and from uninfected controls maintained by periodical subculture had the same morphology (Fig. 3D).

Indexing of callus cultures. *Indexing of callus lines that originated from psorosis-infected explants.* The number of callus lines indexing positive was high in the two assays (Table 3), and the kind of symptoms observed in the inoculated indicators were characteristic of the isolate infecting the plant from which the explants were obtained.

Indexing of callus lines that originated from vein enation-infected explants. Only three out of the 20 callus lines tested at the first subculture indexed positive, and none of them indexed positive after the fifth subculture (Table 3).

Indexing of callus lines that originated from cachexia-infected explants. Nine out of the 10 callus lines tested indexed positive in the first subculture (Table 3). Further tests were done with nucleic acid extraction and sPAGE after each subculture. The CCaV was detected during the six subcultures studied. The viroid titers recovered were comparable to those of the inoculated citron used as a source of tissue to establish the callus lines (Fig. 4).

Electron microscopy. Electron microscopy revealed significant differences between callus cells from healthy and psorosis-infected sources. Callus cells from tissues infected with strain P-121 of psorosis were highly necrotic and presented alterations of the middle lamella (data not shown). Callus cells from tissues infected with strain P-123 of psorosis presented distinctive alterations. Perinuclear blisters, resulting from invaginations of the outer nucleus membrane, were observed. Inside these blisters there were small vesicles containing spherical bodies. Unless they were ribosome agregates, the spherical bodies could be isometric virus particles (Fig. 5).

Cells infected with strain P-123 of psorosis also showed a disrupted tonoplast, poor quality of organelles, and inclusion bodies (Fig. 6).

DISCUSSION

Infection with psorosis, vein enation, and cachexia had a marked effect on morphogenesis of cultured citrus explants. All diseases resulted either in a decrease or total impairment of root formation. Root formation on psorosis explants reflected the severity of the strains. The severe isolate (P-121) caused total impairment of root formation, whereas the mild isolate (P-123)

◀**Fig. 3.** Morphogenesis and callus cultures on stem segments of Arizona Etrog citron infected with citrus cachexia viroid: **A,** shoot and bud regeneration; **B,** root formation; **C,** callus initiation; and **D,** callus cultures.

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

Source of tissue			Production of primary callus	Production of callus over periodical subcultures (g)[b]				
Disease agent	Host	Isolate	(g)[a]	1	2	3	4	5
Psorosis	Pineapple sweet orange	Control	1.07 ab	0.84	2.25	1.47	2.79	...
		P-121	1.25 b	1.03	2.49	1.54	1.67	...
		P-123	1.00 a	1.10	1.93	1.46	2.26	...
Vein enation	Pineapple sweet orange	Control	1.56	0.95	2.27	3.02	2.55	1.94
		V-208	1.18*	0.83	1.94	2.48	2.34	1.95
Cachexia	Arizona 861-S1 citron	Control	1.44	0.71	0.68	0.80	1.13	...
		X-704	1.66*	0.64	0.53	0.63	1.20	...

[a] Estimated by weighing the cultures 5 wk after they had been initiated. Figures represent the mean weight of at least 20 cultures. Values were compared within each treatment by analysis of variance. * = Significantly different than healthy controls ($P = 0.05$). Values followed by the same letter are not significantly different according to Duncan's multiple range test.
[b] Estimated by weighing the cultures 4 wk after they had been subcultured. Figures represent the mean weight of at least 15 cultures. Values were compared within each treatment by analysis of variance. * = Significantly different than healthy controls ($P = 0.05$).

TABLE 3. Detection by biological indexing of psorosis, vein enation, and cachexia in callus cultures maintained over periodical subculturing

Source of tissue			Number of calli indexing positive (%)[a]	
Disease agent	Host	Isolate	1	5
Psorosis	Pineapple sweet orange	Control	0	0
		P-121	89	70
		P-123	94	90
Vein enation	Pineapple sweet orange	Control	0	0
		V-208	15	0
Cachexia	Arizona 861-S1 citron	Control	0	NT
		X-704	90	NT

[a] In the first and fifth subcultures, at least 10 calli per treatment were individually indexed. Data represent the percentage of calli indexing positive out of the total number of calli indexed.

caused a decrease in the number of cultures with roots and the average number of roots. The differences in root formation as a result of psorosis infection were comparable to the effect induced by two strains of CTV (4).

Infection also affected the regeneration of adventitious buds and shoots from cultured explants. Vein enation and cachexia resulted in a decrease of either the number of cultures showing regeneration or the numbers of regenerated shoots and buds. Conversely, psorosis-infected explants exhibited an increase in the number of cultures showing regeneration and the average number of regenerated shoots and buds. The increase in the regeneration potential was proportional to the severity of the two isolates.

As reported earlier for CTV and CIVV infection (4), the results reported here show that morphogenesis is markedly affected as a result of infection. The changes in morphogenic patterns as a result of psorosis infection reflect differences in strain pathogenicity. Therefore, as suggested previously (4), appropriate quantitation of these morphogenic changes may provide additional parameters for characterization of field isolates even in the case, as with psorosis and vein enation, of pathogenic agents whose etiology is not yet well known.

As described for CTV and CIVV (4), the morphogenic changes observed in vein enation- and cachexia-infected tissues result in a decrease or impairment of the ability to regenerate roots and shoots. Yet, in the psorosis-infected tissues, the lower ability to produce roots seems to be coupled with an enhancement of the ability to regenerate shoots. These changes in the morphogenic patterns are similar to what would be expected from an hormonal imbalance resulting in a lower auxin/cytokinin ratio (13). The response of tissue subjected to a very low auxin/cytokinin ratio would illustrate the response of tissues infected with P-121 that show a total impairment of root formation coupled with high

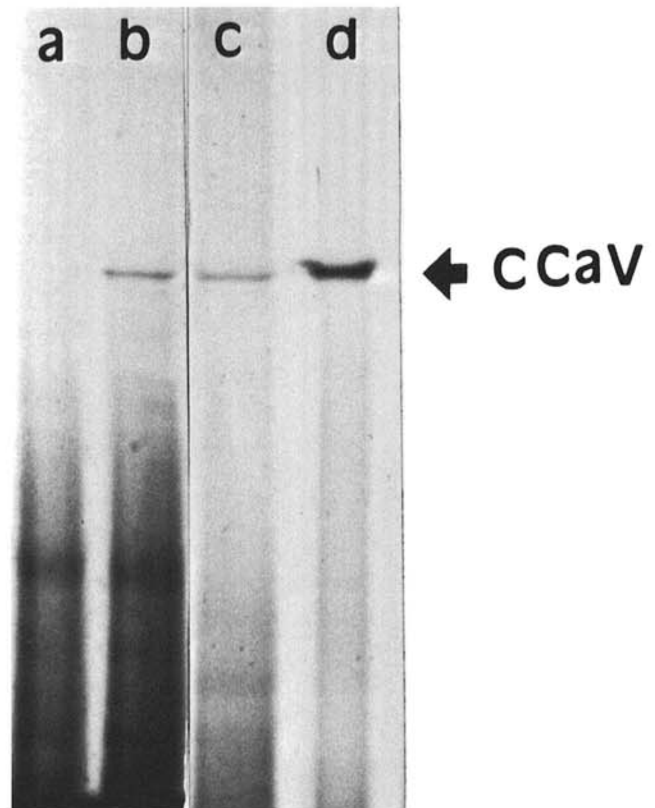


Fig. 4. Detection of citrus cachexia viroid (CCaV) by nucleic acid extraction and sequential polyacrylamide gel electrophoresis: Healthy (a) and infected callus lines (b), as compared to infected citron (c) and cucumber (d) also extracted as positive controls.

regeneration potential, whereas a higher auxin/cytokinin ratio would illustrate the response of tissues infected with P-123 that show reduced root formation coupled with a regeneration potential intermediate between the healthy controls and the P-121-infected tissues. Verification of the relationship between the response observed on psorosis-infected tissues and the ratio of synthesis of endogenous auxins and cytokinins may provide a clue to the possible mechanisms of pathogenesis of psorosis infection.

Growth and appearance of callus lines, established from tissues infected with psorosis, vein enation, and cachexia maintained over periodic subculturing, were comparable to those of controls. As demonstrated for CIVV, the psorosis agent was also able to persist and replicate on callus cultures over several subcultures, whereas the vein enation agent like CTV was difficult to detect even after the first subculture (4). Because CIVV-infected callus has been demonstrated to be a good source for virus purification, the

persistence of the psorosis agent in callus lines established from infected tissues may provide an additional source to attempt the purification of the psorosis agent. As described earlier for other viroids (1,6,14,26), the CCaV was also easily detected over several subcultures.

The results of this study demonstrate that in vitro tissue cultures can be used as tools for plant virology studies. The utilization of callus cultures is, however, restricted to those agents with long-term persistence on such unusual type of plant tissues with poor cell-to-cell interaction. In fact, the observation of thin sections

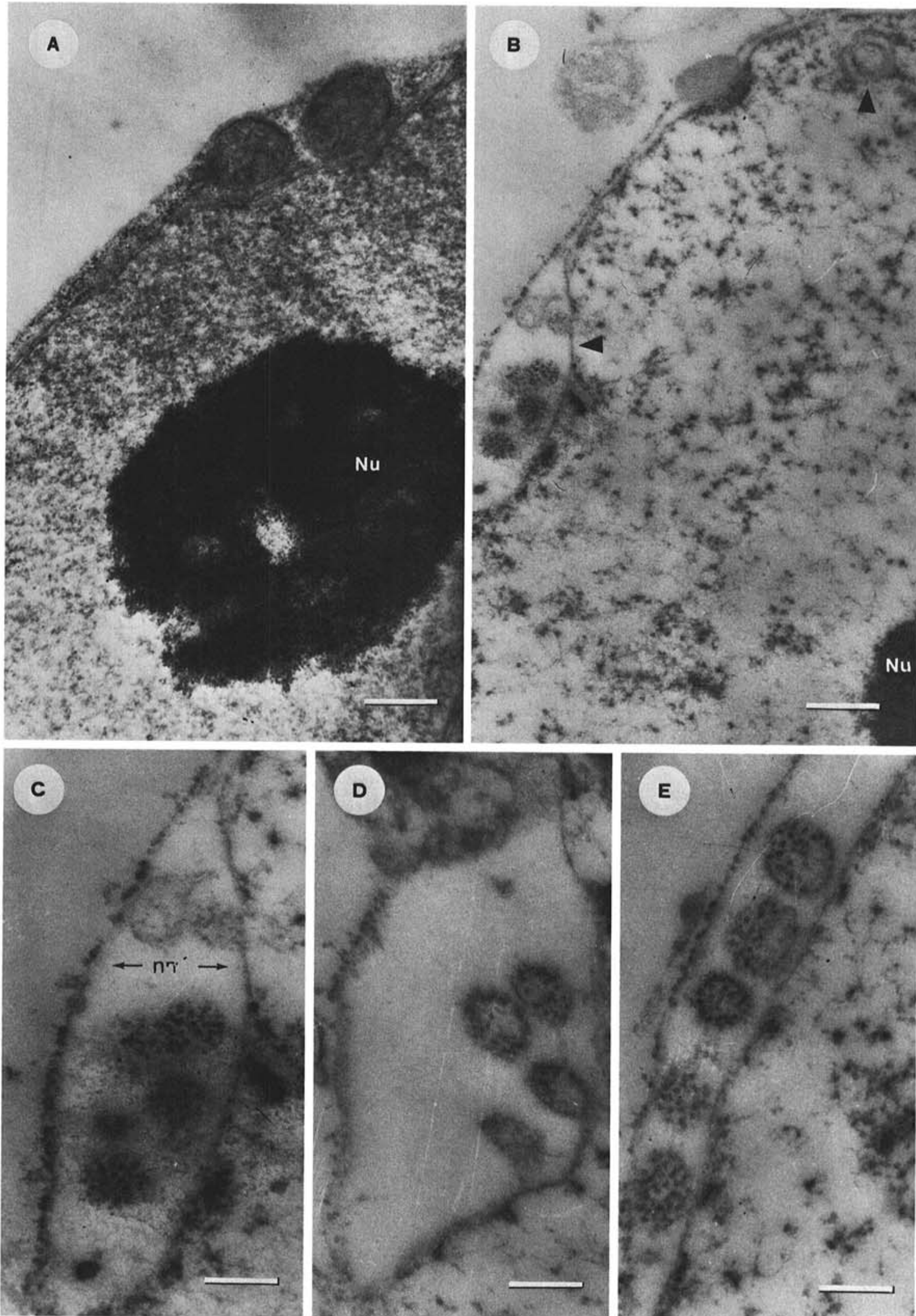


Fig. 5. Electron micrograph of healthy and psorosis (P-123)-infected callus cells: **A,** nucleus of uninfected control (bar = 1 μm); **B,** nucleus of infected cells showing involutions and peripheral blisters around nuclear membrane (arrowheads) (bar = 1 μm); **C,** higher magnification of involutions of nuclear membrane-forming peripheral blisters containing isometric particles (bar = 500 μm); **D** and **E,** vesicles containing isometric particles between the two nuclear membrane layers of different peripheral blisters (bar = 500 μm). Nu = nucleolus; nm = nuclear membrane.

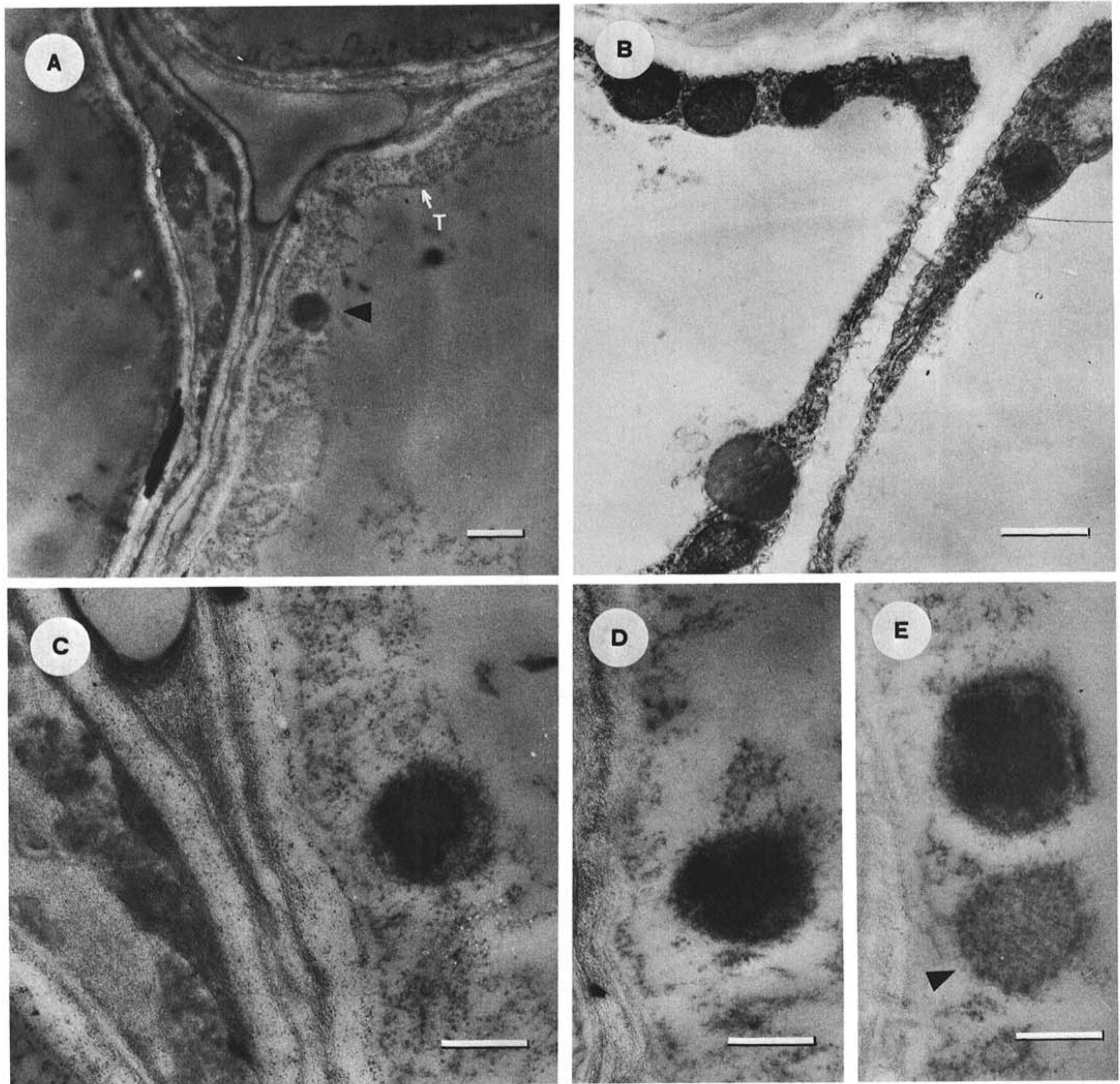


Fig. 6. Electron micrograph of healthy and psorosis (P-123)-infected callus cells: **A,** infected cell showing a disrupted tonoplast (T), poor quality of cell organelles, and inclusion bodies (arrowhead) (bar = 2 μm); **B,** tonoplast of uninfected cell (bar = 2 μm); **C,** higher magnification of disrupted tonoplast observed in infected cells (bar = 1 μm); **D** and **E,** characteristic inclusion bodies or ribosome aggregates (arrowhead) observed in infected cells (bar = 1 μm).

of psorosis-infected callus lines by electron microscopy showed the presence of alterations at the cell level unreported earlier in psorosis-infected tissues.

The peripheral blisters observed in cells of callus infected with the P-123 strain of psorosis resemble the perinuclear blisters described in plants infected with a rhabdovirus, the lettuce necrotic yellow virus (LNYV) (24). Similar alterations have also been described in tissues infected with beet western yellow virus (BWYV) (7), broad bean mottle virus (BBMV) (10,11), and pea enation mosaic virus (PEMV) (3).

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