Effects of Exogenous Auxins on Tomato Tissue Infected With the Citrus Exocortis Viroid

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ABSTRACT

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Leaf disks, stem segments, and callus cultures from healthy and CEV-infected plants of a hybrid of *Lycopersicon esculentum* and *Lycopersicon peruvianum* were cultured in vitro under different hormone regimes. The differences in response observed when the medium was supplemented with auxins, alone or in combination with cytokinins, suggest that the inability of CEV-infected cells to respond to auxins might be involved in the development of the pathogenic syndrome caused by CEV.

Additional key words: auxins and viroid tissues, citrus exocortis viroid, viroid pathogenesis.

Severe stunting, epinasty, and leaf rugosity are the most common symptoms observed in Etrog citron (*Citrus medica* L.), gynura (*Gynura aurantiaca* DC) and tomato (*Lycopersicon esculentum* Mill.) infected with the severe isolate of the citrus exocortis viroid (CEV). It was suggested earlier (9) that the effect of viroid infection resembles a hormonal malfunction that ultimately might implicate an intermediary molecule contributing to symptom expression. In an investigation of endogenous plant growth substances, a significant decrease was detected in the amount of gibberellins in CEV-infected gynura, whereas the amounts of abscisic acid and indolacetic acid remained unchanged (8). On the other hand, we have consistently observed (*unpublished*) that CEV-infected tomato plantlets grown in vitro display a total lack of root formation, whereas healthy plantlets grown under the same conditions form a well-developed root system. This would suggest that an auxin/cytokinin imbalance also might be involved in the abnormal development of CEV-infected plants.

The present study was initiated to evaluate the response of excised tissue from healthy and CEV-infected tomato plants to different hormonal regimes in an effort to evaluate effective hormone levels.

MATERIALS AND METHODS

Source of tissue. The hybrid 741505-45 of *L. esculentum* Mill and *Lycopersicon peruvianum*, kindly provided by D. Pratt (University of California, Davis), was used as the source of tissue. These plants, which are characterized by an indeterminant pattern of growth, provided a continuous source of uniform plant material that developed moderate symptoms when compared to *L. esculentum* 'Rutgers' after being inoculated with CEV. Stable titers of CEV as determined by polyacrylamide gel electrophoresis (PAGE) analysis (6,11) were maintained. The healthy source plants were vegetatively propagated by stem cuttings from a single mother plant and grown under greenhouse conditions. Plants were inoculated by stem puncture with a CEV inoculum as previously reported (10). After symptoms developed in 2–4 wk, tissue was used as a source of CEV-infected material.

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Culture media. The basic nutrient medium contained the inorganic salts of Murashige and Skoog (7) supplemented with 100 mg/L *i*-inositol, 0.2 mg/L thiamine hydrochloride, 1 mg/L pyridoxine hydrochloride, 1 mg/L nicotinic acid, 3% sucrose, and 0.8% agar. For stem segment and leaf disk culture experiments, the basic nutrient medium was supplemented with different amounts of indolacetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), indolebutyric acid (IBA), kinetin (KIN), and 6-benzylamino-purine (BA). For callus induction and maintenance, the basic nutrient medium was supplemented with 2 mg/L IAA, 2 mg/L 2,4-D and 1 mg/L 6-(γ,γ-dimethylallylamino purine (2ip). For callus experiments, the basic nutrient medium was supplemented with different amounts of either IAA or 2,4-D.

All media were autoclaved at 121 C for 15 min and 40-ml aliquots were dispensed into 100 × 25-mm sterile disposable plastic petri dishes.

Tissue culture technique. Leaflets and 10-cm-long stem pieces were surface sterilized with 2% sodium hypochlorite and rinsed three times in sterile distilled water. Leaf disk explants were obtained by using a No. 5 cork borer. Stem segments 1 cm in length were bisected longitudinally.

Leaf disks were placed with the lower leaf surface and cut perimeter in contact with the medium and stem segments with the longitudinal cut surface in contact with the medium. Four leaf explants or five stem segments were cultured in each petri dish and were maintained at 28–30 C. Cultures were exposed 16 hr daily to 0.16 cal · cm^{-2}·min^{1} (measured with an "Eppley" pyranometer), provided by 20-W General Electric F20T12.PL (plant-light) fluorescent tubes. All experiments were repeated twice with at least 20 explants per treatment.

Callus culture technique. Callus was induced by culturing leaf disks from healthy and CEV-infected plants in callus medium following the same techniques described above. After 6–8 wk in culture, the callus that grew from the cut ends was transferred to fresh callus medium. Four individual calli were cultured in each petri dish at 28–30 C in complete darkness and 250-mg pieces were transferred every 6 wk to fresh medium.

The callus used in these experiments had been transferred twice in callus medium before being tested in the media containing IAA or 2,4-D. Callus derived from CEV-infected tissue was also analyzed for CEV (6,11).

RESULTS

Response of healthy and CEV-infected tissue to exogenous auxins. *Indolacetic acid (IAA).* Healthy leaf disks and stem

segments initiated roots when cultured for 1 wk on media containing IAA. Within 2 wk all cultures had well-developed roots (Fig. 1) and showed maximum root differentiation at IAA concentrations greater than 1 mg/L. Higher concentrations of IAA up to 50 mg/L had no effect on the number of roots produced, but caused more browning of the leaf disks and shorter roots than in those exposed to lower concentrations.

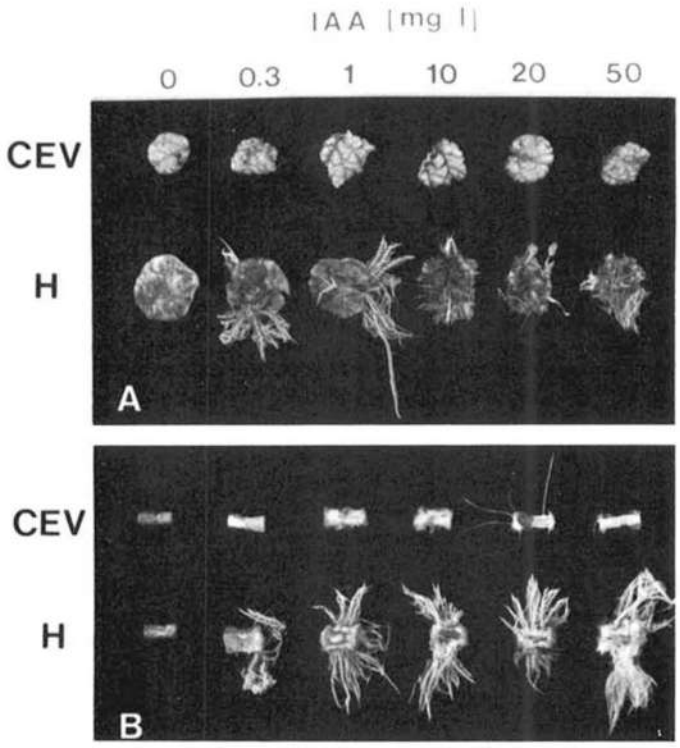

Fig. 1. Response of healthy and CEV-infected tissue to IAA after 2 wk in culture. **A,** Leaf disks, and **B,** stem segments.

TABLE 1. Time required for production of IAA-induced roots on healthy and CEV-infected tomato tissue

IAA (mg/L)	Leaf disks (days to root 90% of the cultures)		Stem segments (days to root 50% of the cultures)	
	Healthy	CEV	Healthy	CEV
0	–	–	–	–
0.3	7	21	7	–
1	7	14	5	20
10	7	14	5	17
20	7	12	5	9
50	7	11	5	12

When CEV-infected leaf disks and stem segments were cultured in IAA-containing media, roots were not observed until after 2–3 wk in culture (Table 1). In general, roots grew to only 2–3 mm and neither their size nor number were increased by greater concentrations of IAA or longer time in culture (Table 2). Concentrations of 50 mg of IAA per liter did not cause browning of the CEV-infected leaf disks as opposed to the effect noted with disks from healthy tissue.

2,4-Dichlorophenoxyacetic acid (2,4-D). Healthy leaf disks and stem segments produced roots when cultured for 2 wk on media containing 0.01–0.1 mg/L of 2,4-D (Fig. 2A). Concentrations of 2,4-D at 0.1–1.0 mg/L resulted in browning of the leaf disks followed by callus formation after 3 wk in culture (Fig. 2B).

CEV-infected leaf disks and stem setments produced very few roots on a small number of cultures (Table 3). Leaf disks did not develop any browning when exposed to the full range of concentrations tested. Callus production in leaf disks and stem segments was observed at 2,4-D concentrations of 0.3 to 1 mg/L after 3 wk in culture and at lower 2,4-D concentrations 1 wk later (Fig. 2 and Table 3).

TABLE 2. Response of healthy and CEV-infected tomato leaf disk tissue to IAA after 3 wk in culture

IAA (mg/L)	Cultures with roots (%)		Amount of rooting[a]	
	Healthy	CEV	Healthy	CEV
Leaf disks				
0	0	0	–	–
0.3	100	85	++	+
1.0	100	100	+++	+
10.0	100	100	+++	+
20.0	100	100	+++	+
50	100	100	+++	+
Stem segments				
0	0	0	–	–
0.3	80	20	++	+
1.0	100	55	+++	+
10.0	100	55	+++	+
20.0	100	60	+++	+
50.0	100	60	+++	+

[a] Amount of rooting: –, no roots; +, 1–10 roots; ++, 10–20 roots; +++, more than 20 roots.

TABLE 3. Response of healthy and CEV-infected tomato leaf disk tissue to 2,4-D after 4 wk in culture

2,4-D (mg/L)	Cultures with roots (%)		Amount of rooting[a]		Amount of callus[b]	
	Healthy	CEV	Healthy	CEV	Healthy	CEV
Leaf disks						
0	0	0	–	–	–	–
0.01	33	0	+	–	–	–
0.03	100	0	+++	–	–	–
0.1	100	25	++	+	+	+
0.3	100	33	++	+	+	++
1.0	87	0	+	–	–	+++
Stem segments						
0	0	0	–	–	–	–
0.01	15	0	+	–	+	+
0.03	52	26	+	+	++	+
0.1	64	15	++	+	+++	++
0.3	65	0	+	–	+++	+++
1.0	0	0	–	–	+++	+++

[a] Amount of rooting: –, no roots; +, 1–5 roots; ++, 5–10 roots; +++, > 10 roots.
[b] Amount of callus: –, no callus, +, small callus; ++, medium callus; +++, large callus.

Other auxins. When healthy leaf disks were cultured for 2 wk in four different media containing 5μM IAA, NAA, IBA or 2,4-D, roots were produced in each of the four treatments. The tissues on IAA, NAA, and IBA containing media had long well-developed roots after 3 wk, whereas the cultures on 2,4-D medium had shorter roots. Small amounts of callus were produced in the tissues grown on media containing NAA and 2,4-D.

CEV-infected leaf disks cultured in the same media also produced roots in the media containing IAA, NAA, and IBA. However, the number of roots was significantly smaller than in the healthy cultures and elongation was severely inhibited. Explants cultured in media containing NAA and 2,4-D also produced small amounts of callus.

Response of healthy and CEV-infected tissue to exogenous cytokinins. *Kinetin (KIN).* After 3 wk in culture in media containing kinetin healthy and CEV-infected leaf disks and stem segments produced bright green callus which eventually gave rise to new buds and shoots. The optimum concentration of kinetin stimulating bud and shoot regeneration was 3 mg/L for leaf disks and 1 mg/L for stem segments in both healthy and CEV-infected tissue (Table 4). At concentrations of kinetin of 3 and 10 mg/L the healthy leaf disks became disrupted in the central area, whereas the infected tissue remained intact (Fig. 3). Kinetin concentrations of 10 mg/L and 30 mg/L were toxic to healthy and CEV-infected stem segments, whereas 30 mg/L was toxic to leaf disks from both healthy and CEV-infected tissue. Essentially no differential response was noted between healthy and viroid infected tissues.

6-Benzyl aminopurine (BA). Healthy and CEV-infected leaf disks and stem segments cultured in media containing BA developed small amounts of bright green callus, which eventually regenerated buds and shoots. The optimum amount of BA to induce regeneration was 1–3 mg/L for leaf disks and 1 mg/L for stem segments of healthy and CEV-infected tissue. As was noted in the kinetin response, disruption of the central part of the leaf disk was also observed only in healthy tissue. BA was toxic when used at 3 mg/L to cultured stem segments of both healthy and CEV-infected tissue, whereas leaf tissue reacted similarly to a 10-fold greater concentration.

Response of healthy and CEV-infected tissue to kinetin in combination with IAA. When healthy and CEV-infected leaf disks

TABLE 4. Response of healthy and CEV-infected tissue to kinetin after 5 wk in culture

Kinetin (mg/L)	Cultures with regeneration of buds and/or shoots (%)		Amount of regeneration[a] of buds and/or shoots	
	Healthy	CEV	Healthy	CEV
Leaf disks				
0	0	0	–	–
1	14	12	+	+
3	62	50	+++	+++
10	21	60	++	++
30	12	6	+	+
Stem segments				
0	0	0	–	–
1	80	72	+++	++
3	30	33	++	++
10	0	0	–	–
30	0	0	–	–

[a] Amount of regeneration: –, none; +, 1–10 buds and/or shoots; ++, 10–20 buds and/or shoots; +++, >20 buds and/or shoots.

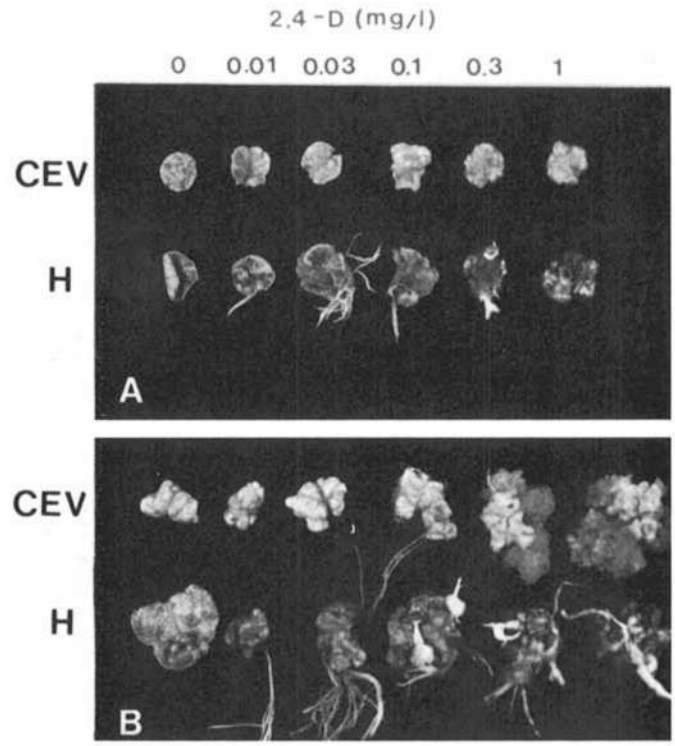

Fig. 2. Response of healthy and CEV-infected leaf disks to 2,4-D. **A,** After 2 wk in culture, and **B,** after 3 wk in culture.

TABLE 5. Response of healthy and CEV-infected tomato leaf tissue to kinetin in combination with indolacetic acid

Kinetin (mg/l)	Cultures with roots (%)		Amount of rooting[a]		Cultures with regeneration of buds and/or shoots (%)	
	Healthy	CEV	Healthy	CEV	Healthy	CEV
No auxin in the medium						
0	0	0	–	–	0	0
0.01	0	0	–	–	0	0
0.03	0	0	–	–	0	0
0.1	0	0	–	–	0	0
0.3	0	0	–	–	0	0
1.0	0	0	–	–	25	30
3.0	0	0	–	–	100	100
1 mg/L IAA in the medium						
0	100	70	+++	+	0	0
0.01	100	10	+++	+	0	0
0.03	83	4	++	+	0	0
0.1	25	0	+	–	0	0
0.3	0	0	–	–	0	0
1.0	0	0	–	–	4	30
3.0	0	0	–	–	25	100

[a] Amount of rooting: –, no roots; +, 1–5 roots; ++, 5–10 roots; +++, >10 roots.

were cultured in media containing low concentrations of kinetin, no effect was observed at concentrations below 1 mg/L (Table 5). At concentrations ranging 1–3 mg/L, healthy and infected tissues respond identically with bud and shoot regeneration.

When similar kinetin concentrations were used in combination with 1 mg/L IAA, roots were produced at concentrations of 0.1 mg/L or below in healthy tissue and at concentrations of 0.03 mg/L or below in CEV-infected tissue. As previously demonstrated with IAA alone, the number of cultures producing roots and the amount of rooting was dramatically lower in CEV-infected than in healthy tissue. In general, roots initiated in CEV-infected tissue grew only 2–3 mm, whereas those initiated in healthy tissue were thicker and grew several centimeters (Table 5).

When kinetin at 1–3 mg/ml was used in combination with 1 mg/L IAA, regeneration of buds and shoots was observed in the absence of rooting. Contrary to the effect noted with kinetin alone, the number of cultures that showed regeneration was significantly greater in CEV-infected than in healthy tissue, and the amount of regeneration per culture was also greater in CEV-infected tissue than in healthy tissue (Table 5).

No differences in amount and quality of induced callus were observed between healthy and CEV-infected tissue.

Influence of auxins on the development of healthy and CEV-infected callus. Callus derived from CEV-infected tissue which supported CEV replication, displayed the same general characteristics of color, texture, and friability as callus derived from healthy tissue (Fig. 4A).

When healthy callus was cultured in media containing IAA or 2,4-D, regeneration of roots was observed after 6 wk in IAA concentrations of 10 to 50 mg/L and 2,4D concentrations of 0.1 to 0.3 mg/L (Fig. 4B). In all cases, the roots were more than 1 cm long and the callus displayed symptoms of toxicity at concentrations of 20 mg of IAA and 0.3 mg of 2,4D per liter.

CEV-infected callus cultured for 6 wk under the same conditions did not regenerate roots in any of the IAA concentrations tested and demonstrated only a weak rooting capacity at 0.1 mg of 2,4-D per liter (Table 6). The callus showed evidence of toxicity at the same IAA and 2,4D concentrations as healthy callus.

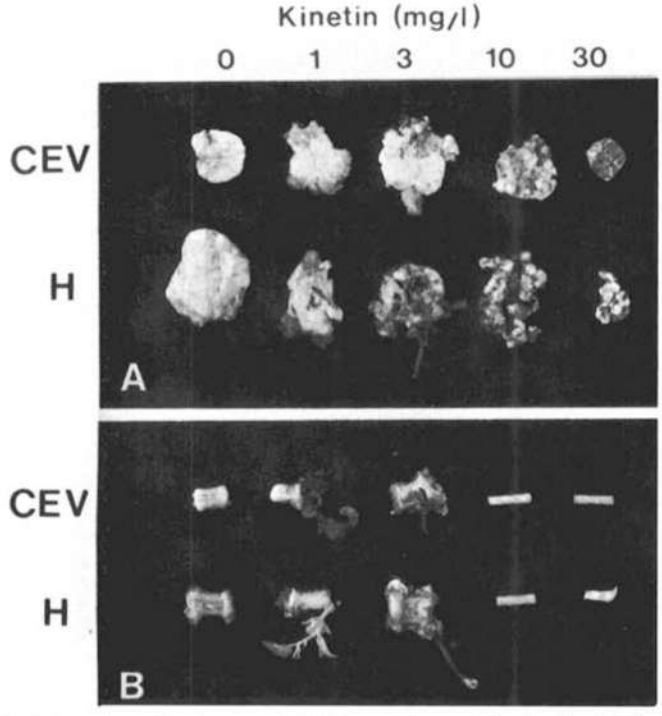

Fig. 3. Response of healthy and CEV-infected tissue to kinetin after 5 wk in culture. **A,** Leaf disks, and **B,** stem segments.

DISCUSSION

In experiments reported here, stem and leaf explants from healthy tomato plants responded to exogenous auxins and cytokinins as expected from the classical studies of Skoog and Miller (5,12). All auxins tested induced root formation, the response was greater for 2,4D and NAA than for IBA and IAA as previously reported for leaf explants of cultivated tomato (4). On the other hand, the effect on CEV-infected tissue was minimal and did not approach the response of healthy tissues even when higher levels of IAA or 2,4D were employed. These data would suggest the

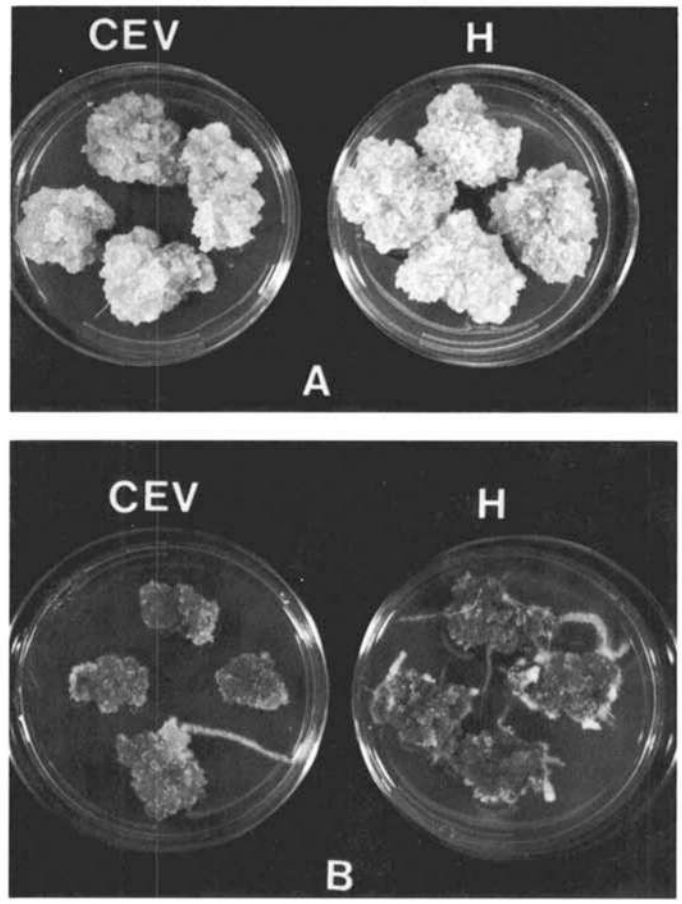

Fig. 4. Response of healthy and CEV-infected callus lines to 2,4D (0.01 mg/L). **A,** Healthy and CEV-infected callus and **B,** rooting induced on healthy and CEV-infected callus.

TABLE 6. Response of healthy and CEV-infected tomato leaf disk callus tissue to IAA and 2,4D after 6 wk in culture

	Cultures with roots (%)		Amount of rooting[a]	
Auxin (mg/L)	Healthy	CEV	Healthy	CEV
IAA				
0	0	0	–	–
10	100	0	+++	–
20	75	0	+++	–
50	37	0	++	–
100	0	0	–	–
2,4D				
0.0	0	0	–	–
0.1	92	20	+++	+
0.3	10	0	+	–
1.0	0	0	–	–
3.0	0	0	–	–

[a] Amount of rooting: –, no roots; +, 1–5 roots; ++, 5–10 roots; +++, > 10 roots.

presence of some aberration in auxin utilization in CEV-infected tissue or a morphological impairment resulting in an inability of the tissue to respond.

When cytokinin was supplied in the absence of exogenous auxins, both healthy and CEV-infected tissues responded with bud and shoot proliferation. The response to BA was the highest and to kinetin next highest, a result that is consistent with those of previous studies (1,4,13).

The ratio of auxin/cytokinin concentrations remains the dominant factor involved in morphogenesis (12) of healthy tomato tissues. In our cultures, rooting increased progressively as the ratio of auxin/cytokinin concentrations was increased, while regeneration of shoots and buds increased with decreasing ratios of auxin/cytokinin concentrations. As anticipated, when increasing auxin/cytokinin combinations were employed, shoot and bud regeneration were significantly reduced in healthy tissues. However, no effect was displayed by viroid-infected tissues. Although the number of cultures that produced roots increased as the concentration of kinetin was decreased, the number of roots was always low in infected tissue and apparently not affected by increasing the ratio of auxin/cytokinin concentrations.

The deviation in the response of CEV-infected tissues to different exogenous auxins and cytokinin regimes suggests that CEV-containing tissue cannot respond normally to exogenous auxins. Although a higher content of endogenous cytokinin in CEV-infected tissue could account for similar results, the inability to observe any differences in response to either kinetin or BA between healthy and CEV-infected tissue does not support this hypothesis. The suggested inability to respond to exogenous auxins was also observed in nondifferentiated CEV-infected callus, which was indistinguishable from healthy callus cultures. This further suggests that the apparent inability of CEV-infected tissue to respond to auxins may reflect an intrinsic property of CEV-infected cells, resulting in the expression of pathogenesis. Although a previous study (8) showed that there were no differences in the levels of endogenous auxins between healthy and CEV-infected tissue of *Gynura aurantiaca*, the inability of infected tomato tissue to respond to exogenous auxins suggests that a similar process may be involved in the abnormal development of CEV-infected gynura.

Results of a recent study in which gynura also was used as a CEV host, however, indicated that lower levels of diffusible auxinlike substances extracted from CEV-infected plants could account for the alteration in the pattern of root formation of CEV-infected cuttings (3). The results of our experiments with in vitro callus cultures in which the tissue is in full contact with the media reduces the probability that diffusion of auxin could be a problem in this system. These data suggest that one or cumulative factors involved in the reduced availability of auxin, such as a lack of recognition of the auxin molecules by the host cell, inactivation of auxin, or impaired synthesis also may play a role in the response noted here. Furthermore, the influence of cytokinin cannot be discounted at this point.

Leaf disks from healthy plants held in phytohormone-free medium underwent tissue expansion to at least twice the initial diameter (Figs. 1, 2, and 3), whereas CEV-infected leaf tissue remained almost unchanged. IAA has been shown to function in the mechanism of cell wall expansion involving a modification in the cell wall structure (2). Therefore, the lack of tissue expansion observed in CEV-infected leaf disks could also reflect a structural anomaly in infected cells resulting from some auxin dysfunction.

LITERATURE CITED

1. Behki, R. M., and Lesley, S. M. 1976. *In vitro* plant regeneration from leaf explants of *Lycopersicon esculentum*. Can. J. Bot. 54(21):2409-2414.
2. Cleland R. 1980. Auxin and H^+-excretion: The state of our knowledge. In: Plant Growth Substances. Fiskood, ed. Springer-Verlag. Berlin and Heidelberg. 527 pp.
3. Flores, R., and Rodriguez, J. L. 1981. Altered pattern of root formation on cuttings of *Gynura aurantiaca* DC infected by citrus exocortis viroid. Phytopathology 71:964-966.
4. Kartha K. K., Gamborg, O. L., Shyluk, J. P., and Constabel, F. 1976. Morphogenetic investigation on *in vitro* leaf culture of tomato (*Lycopersicon esculentum* Mill. cv. Starfire) and high frequency of plant regeneration. Z. Pflanzenphysiol. 77:292-301.
5. Miller, C., and Skoog, F. 1953. Chemical control of bud formation in tobacco stem segments. Am. J. Bot. 40:768-773.
6. Morris, T. J., and Wright, N. S. 1975. Detection on polyacrylamide gel of a diagnostic nucleic acid from tissue infected with potato spindle tuber viroid. Am. Potato J. 52:57-63.
7. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
8. Rodriguez, J. L., Garcia-Martinez, J. L., and Flores, R. 1978. The relationship between plant growth substance content and infection of *Gynura aurantiaca* DC by citrus exocortis viroid. Physiol. Plant Pathol. 13:355-363.
9. Semancik, J. S. 1979. Small pathogenic RNA in plants, the viroids. Annu. Rev. Phytopathol. 17:461-484.
10. Semancik, J. S., Morris, T. J., Weathers, L. G., Rodorf, F., and Kearns, D. R. 1975. Physical properties of minimal infectious RNA from exocortis diseases. Virology 69:669-676.
11. Semancik, J. S., and Weathers, L. G. 1972. Exocortis virus: An infectious free-nucleic acid plant virus with unusual properties. Virology 47:456-466.
12. Skoog, F., and Miller, C. O. 1957. Chemical regulation of growth and organ formation in plant tissue cultured *in vitro*. Eleventh Symp. Soc. Exp. Biol. 11:118-131.
13. Tal, M., Dehan, K., and Heiken, H. 1977. Morphogenetic potential of cultured leaf sections of cultivated and wild species of tomato. Ann. of Bot. 41:937-941.