Altered Pattern of Root Formation on Cuttings of *Gynura aurantiaca* Infected by Citrus Exocortis Viroid

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ABSTRACT

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The ability of cuttings from healthy and citrus exocortis viroid (CEV)-infected *Gynura aurantiaca* plants to form roots was investigated. Roots began to develop 8–10 days and 18–32 days after excision on healthy and CEV-infected cuttings, respectively. It also was observed that the root system of healthy cuttings was composed of a larger number of roots than that of CEV-infected ones. The impaired ability of CEV-infected cuttings to form roots could be related to small amounts of an auxinlike substance(s) with an R_f similar to indoleacetic acid that was found to diffuse from apical buds of CEV-infected *G. aurantiaca*.

Citrus exocortis viroid (CEV), the causal agent of exocortis disease of citrus (10), is a representative member of the viroids, a group of low-molecular-weight RNAs pathogenic to plants. These are the smallest known agents that cause infectious diseases (2).

Although several citrus species are naturally infected by CEV, the most convenient experimental host is the composite, *Gynura aurantiaca* DC, which, after infection, develops epinasty, vein clearing, leaf malformation, and stunting of the plant resulting from the inhibition of internode elongation (15).

Evidence is lacking on how viroids, and particularly CEV, induce the onset of the typical symptoms in their hosts. At the molecular level, larger amounts of some low-molecular-weight proteins have been found in CEV-infected tissue than in healthy tissue (1,3), while at the cellular level, aberrations of the plasma membrane have been identified as the primary cytopathic effect associated with CEV infection in *G. aurantiaca* (12). Finally, from a different standpoint, determinations of plant growth substances have revealed a significant decrease in the endogenous content of gibberellins in CEV-infected *G. aurantiaca* (9).

As an additional physiological effect of CEV infection, it is reported here that cuttings of CEV-infected *G. aurantiaca* developed roots much later than the healthy controls. This delay and its correlation with lower amounts of a diffusible auxinlike substance(s) in CEV-infected material, are the subjects of the present paper.

MATERIALS AND METHODS

Viroid culture. *G. aurantiaca* was used as the host for a severe strain of CEV (9). Inoculation was carried out by slashing the stems with a razor dipped in either buffer (control plants) or a *t*RNA-like preparation obtained by lithium chloride fractionation from infected tissue (11) and concentrated 20-fold on a tissue weight basis by ethanol precipitation. Symptoms appeared 16–20 days after inoculation in 90–100% of the plants.

Root initiation experiments. Cuttings 3.5 cm long and bearing four apical leaves were obtained from matched blocks of healthy and CEV-infected *G. aurantiaca*. Cuttings, standing in small beakers containing distilled water to a level of 1.5 cm, were put into a growth chamber held at 50% relative humidity, illuminated with fluorescent light (2,500 lux at the level of the cuttings) for 18 hr/day, and the temperature was maintained at 30–32 C (day) and 25–27 C (night). Water was added daily to keep the level constant, and every third day it was changed to avoid an excessive microbial growth. Cuttings were checked daily for root initiation and further development. The experiments were repeated six times; four cuttings were used in each experiment.

Diffusion and determination of activities of plant growth substances. An agar diffusion technique, based on the procedure described by Jones and Phillips (6), was used for extracting plant growth substances. Apical buds including 1.5 cm of stem and two expanding leaves were excised from matched blocks of healthy and CEV-infected *G. aurantiaca* plants 30 days after the onset of symptoms in the infected material. The apical buds were put immediately in a vertical position on cylinders (5 mm in diameter × 2 mm high) of 1% special Noble agar (Difco Laboratories, Detroit, MI 48201). Diffusion of growth substances from the sections was carried out in high-humidity boxes for 3 hr at the temperature and diffuse light conditions of the laboratory. The agar blocks, combined in groups of five, were frozen, lyophilized, and stored at −18 C. The dried agar blocks were extracted three times at 4 C with 10 ml of absolute methanol for 1 hr, 2 hr, and overnight. The combined extracts were filtered through paper filters (No. 589²; Schleicher & Schell, Dassel, West Germany) and then taken to dryness under vacuum in a rotatory evaporator at 40

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C. The residue was dissolved in a small volume of absolute methanol, applied to Whatman 3 MM paper (W. & R. Balston Ltd., Maidstone, England), and ascendingly developed in a mixture of isopropanol, NH_3 (25%), and H_2O (80:0.1:19.9, v/v) at 4 C in the dark for a distance of approximately 20 cm. The chromatograms were dried and cut transversely into 10 equal sections that were used for the bioassays. All solvents used were freshly redistilled.

The oat mesocotyl bioassay, which detects auxinlike and gibberellinlike activity (8), was carried out as reported previously (7), with 10 mesocotyl sections of oat (cultivar Brighton) per bioassay vial. Quantification of the activities was done by means of standard curves obtained by bioassaying solutions of known concentrations of indoleacetic acid (IAA) (E. Merck, Darmstadt, West Germany). The activities of auxinlike substances were expressed as the amount of IAA with an equivalent activity in the bioassay. The experiments were repeated three times and five apical buds were used in each experiment.

RESULTS

Root formation on healthy and CEV-infected cuttings of *G. aurantiaca*. Root initiation on healthy cuttings was highly reproducible and consistently occurred between 8 and 10 days in the growth chamber. In viroid-infected cuttings, it was more variable, ranging between 18 and 32 days. To learn whether the delay in root formation in CEV-infected cuttings was dependent on the time that the plants had been showing the symptoms of the disease, cuttings were removed from plants at intervals ranging from 10 to 60 days after the onset of symptoms. The aforementioned variability was not related to whether the cuttings were taken from plants with early or late symptoms of the disease.

Results of a typical experiment are presented in Fig. 1. The healthy and CEV-infected cuttings were removed 30 days after the onset of symptoms in the viroid-infected plant. Roots were apparent at the base of the healthy cuttings after 9 days in the growth chamber (Fig. 1A). Seven days later (day 16) the number and length of the roots had increased and secondary roots also were visible (Fig. 1B). Root initiation on the CEV-infected cutting was not observed until 23 days in the growth chamber; at this time, the healthy one showed a well-developed root system (Fig. 1C). At day 30, roots had increased in number and length on the CEV-infected cutting, but no secondary roots had developed (Fig. 1D). After 37 days in the growth chamber, secondary roots were observed on the CEV-infected cutting (Fig. 1E). At this time, the experiment was terminated. If longer time periods were used, the root length of healthy cuttings remained essentially unchanged whereas that of viroid-infected ones continued to increase.

The root systems of healthy and CEV-infected cuttings of *G. aurantiaca* also differed markedly in the number of roots that formed. One week after root initiation, 20 roots were observed on the healthy cutting (Fig. 1B), whereas on the viroid-infected one only six roots were visible after the same period of time (Fig. 1D). Two weeks after root initiation, the number of roots on the healthy cutting was too high to be estimated quantitatively (Fig. 1C), whereas on the CEV-infected one, only 10 roots were observed (Fig. 1E).

Activities of diffusible plant growth substances. The purpose of these experiments was to investigate whether differences in diffusible plant growth substances could be associated with the observed differences in root formation between healthy and CEV-infected cuttings. The oat mesocotyl bioassay revealed quantitative differences of activity, particularly in the zone of the chromatogram corresponding to R_f values of 0.2 to 0.4. Activity in this zone was approximately from twofold to threefold higher in healthy, than in viroid-infected, material (Table 1). This activity was restricted in some bioassays to the zone of the chromatogram with R_f values of 0.3 to 0.4, and in some other ones was found in a broader zone with R_f values of 0.2 to 0.4 (although, in these last cases, the maximum activity was always detected in the zone corresponding to R_f values of 0.3 to 0.4). Since IAA exhibits maximum activity in this chromatographic system between R_f values of 0.3 to 0.4 and moreover, since no extractable

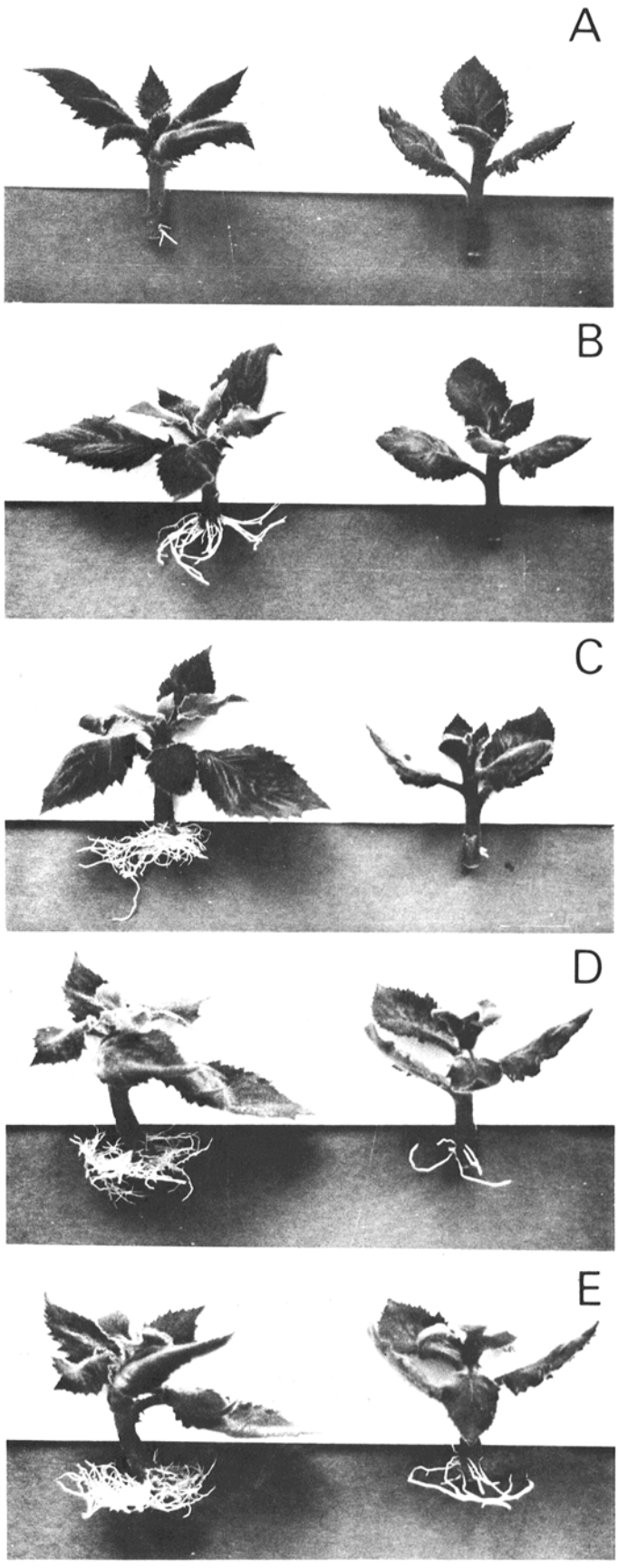

Fig. 1. Cuttings of healthy (left) and CEV-infected (right) *Gynura aurantiaca* after they were removed from the source plant and placed in the growth chamber for **A**, 9; **B**, 16; **C**, 23; **D**, 30; and **E**, 37 days.

TABLE 1. Effect of CEV infection on a diffusible auxinlike substance(s) from excised apical buds of *Gynura aurantiaca*[a]

Experiment	Activities of diffusible auxinlike substances (ng IAA equivalent per gram fresh weight)	
	Healthy[b]	CEV-infected[b]
I	6.0	2.5
II	6.2	3.3
III	7.0	2.5

[a]Zone of chromatogram corresponding to R_f values of 0.2 to 0.4 in the isopropanol-ammonia-water system.
[b]Five excised apical buds were used per experiment.

gibberellinlike activity was found previously in this zone (9), it can be concluded that the observed differences in diffusible plant growth substances are due to an auxinlike substance(s) with an R_f similar to IAA. No further attempts were made to characterize this auxinlike substance.

DISCUSSION

Horst et al (4), studying the effects of infection of some viruses and viroids on the vegetative propagation of chrysanthemum, found a slight delay (2–5 days) in the initiation of roots on cuttings of two chrysanthemum cultivars infected by chrysanthemum stunt viroid. Under our experimental conditions, cuttings of CEV-infected *G. aurantiaca* showed a more pronounced delay (10–22 days) of root initiation compared with the healthy controls. Moreover, it also was observed that infection by CEV caused an alteration in the number and length of the roots that might reflect a difference in physiological vigor between healthy and CEV-infected plants. The initiation of roots on cuttings of numerous plants has long been associated with a stimulus, derived from the leaves, that travels directly downward with little lateral movement, and stimulates the stem to form roots. In most cases, this stimulant was IAA (14). The impaired ability of CEV-infected cuttings to form roots could be explained by assuming that the tissue infected by CEV has a lower sensitivity to a root-forming substance than the healthy one. Alternatively, it could be assumed that lower amounts of a root-forming substance reach the base of CEV-infected cuttings. The results presented here show a decreased level of an auxinlike substance(s) with an R_f similar to IAA in the diffusible plant growth substances from CEV-infected material and favor the second hypothesis. The observed differences in the diffusible auxinlike substances could arise as a consequence of the different pool size of this type of hormone between healthy and CEV-infected tissue. Nevertheless, in previous research (9) we did not find significant alteration, as a result of viroid infection, of the level in *G. aurantiaca* leaves of extractable auxinlike substances with the same R_f as IAA. Among the several hypotheses that can be put forward to explain these results, interference in the transport of IAA and/or increase in an IAA oxidase activity in CEV-infected tissue would seem to be the easiest ones to evaluate experimentally. In this respect, it is interesting that the movement of IAA in pea stem segments is promoted by gibberellic acid (5) and that CEV infection causes a decrease in the content in *G. aurantiaca* leaves of extractable gibberellinlike substances with the same R_f as gibberellic acid has been found previously (9). On the other hand, there are numerous reports on changes in the activity of enzymes involved in auxin degradation following stress situations, particularly infection by plant pathogens (13).

It is also interesting that the impaired rooting of cuttings taken from CEV-infected *G. aurantiaca* did not seem to be a specific peculiarity of the CEV-*G. aurantiaca* system, since preliminary results indicated that, under the same experimental conditions, the behavior of cuttings of CEV-infected tomato was even more dramatic because in some cases, total suppression of root formation was observed.

We want finally to emphasize that the alteration in the pattern of root formation of CEV-infected cuttings of *G. aurantiaca* along with the lower levels of diffusible auxinlike substances reported here, will probably represent only a part of the physiological changes that the viroid triggers in the host plant, and that much remains to be known before an integrated view of the pathogenic processes accompanying viroid infection can be obtained.

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