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Elution of Fungitoxic Compounds from Cowpea Leaves Infected with Cucumber Mosaic Virus*

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Summary

When cowpea primary leaves were inoculated with cucumber mosaic virus (CMV), detached from their petioles, immersed in water and incubated on a shaker, the leaf ambient fluid demonstrated a characteristic spectrum with a maximum peak at 285 nm, with a descrete peak at 318 nm, and with minima at 304 and 250 nm. When the fluid was extracted with ether and analysed by TLC, 3 main spots were detected under ultraviolet light. Each of the substances separated from the spots on TLC, having a charactristic UV absorption spectrum with a maximum peak at 285 nm, had fungitoxicity; germ-tube growth of *Pyricularia oryzae* was inhibited by the applications of over 25 μg/ml, and germination of the spore was completely prevented by a 250 μg/ml application. These substances also had phytotoxicity at the concentrations affecting the fungus.

Introduction

Cowpea primary leaves respond very rapidly to infection with cucumber mosaic virus (CMV) and produce tiny necrotic local lesions up to 24 hr after inoculation under usual conditions. A previous work has shown that when leaves of cowpea were inoculated with CMV, detached before lesion appearance, vacuum-infiltrated with water, and incubated in water, fungitoxic substances were eluted in quantity from the infected tissues into the leaf ambient fluid during incubation (3). The method proposed in the previous paper takes advantage of the fact that antifungal compounds are induced and accumulated continuously in tissue following virus multiplication and are able to be extracted in quantity without killing the tissue. On the other hand, the method may be useful not only as an extraction method of antifungal compounds from plant tissues infected with virus but also for investigations of relationship between virus multiplication and virus elicited compounds.

* A part of this work was reported in *Physiological Plant Pathology* in 1981.

The present report describes in detail some chemical characteristics of the infected-leaf ambient fluid, and illustrates fungitoxicity and phytotoxicity of the substances separated from the fluid.

Materials and Methods

Fully expanded primary leaves of cowpea (*Vigna sinensis* (L.) Endle. var. *sesquipedalis*, cv. Kurodane sanjaku) were rubinoculated with purified CMV (Yellow strain) at 50 to 100 μg/ml in 0.01 M phosphate buffer, pH 7.0. Control leaves were rubinoculated with buffer only. The inoculated leaves were cut from their petioles, washed with sterile water several times and immediately each of the 10 leaves was placed in 200 ml Erlenmeyer flasks with 50 ml of distilled water. The flasks containing the leaves were placed in a reciprocal shaker operating at approximately 90 cycles per min at 27°C for 30 hr. As a comparable experiment, the intercellular spaces of the detached leaves were filled with water by vacuum infiltration before incubation, as done in the previous experiments (3), and these leaves were incubated in the same way. After incubation, the leaf ambient fluids were collected and spectra characteristic were recorded with a spectrophotometer (Shimazu multipurpose recording spectrophotometer MSP–5000), and then extracted with ethyl ether. The ether fraction was vacuum-concentrated at 40°C and analysed by thin layer chromatography (TLC), on silica gel plates developed with a mixture of chloroform which contained 2% V/V ethanol and methanol (100: 20 or 100: 4).

For bioassay of antifungal activity of the substances separated by TLC, *Pyricularia oryzae* Cav. (Isolate, F67–54) was used as in the previous work (3). In the first, developed silica plates were dried and sprayed with a suspension of spore of the fungus in potato decoction. In the second, substances separated from spots of first developed TLC were redeveloped on TLC under the same conditions for further purification, and each substance separated was dissolved in water. The aqueous solution was mixed with spore suspension. Drops of the mixture were placed on glass slides and incubated in a moist plastic box for 9 h at 25°C. Subsequently germination and germ-tube growth of the spore were observed under microscope. For the test of phytotoxicity of the substances separated here, rectangular pieces, ranging in size from 4 to 9 mm^2, were excised from cowpea primary leaves, and floated on the aqueous solution of the substances with various consentrations. As a control, the leaf pieces were floated on water. Each of them was incubated at 25°C for 48 hr, and whether or not specific necrosis occurs in the marginal areas of the leaf pieces was observed under a microscope.

Results

Spectra characteristics of the ambient fluid of CMV-infected cowpea leaves were shown in Fig. 1; with maximum peak at 285 nm, with a discrete peak at 318

nm, and with minima at 304 and 250 nm. Such a characteristics spectrum did not occur in comparable fluid from cowpea leaves inoculated with buffer only and from tobacco (*Nicotiana tabacum* cv. ky57) leaves inoculated with CMV or buffer only. Thus, the characteristics of the ambient fluid of CMV-infected cowpea leaf are described below.

Initial pH of water used for the incubation of leaves was 6.5 and that of the leaf ambient fluids after incubation were moved slightly to acidic side ranging from 5 to 6. When the leaf ambient fluid was acidified to pH 2.1 with 0.1 N HCl, it had an absorption spectrum with a maximum at 285 nm only but not with a discrete maximum at 318 nm. When the pH of the fluid was changed to strong basic side (pH 10.5) with 0.1 N NaOH, on the contrary the peak at 285 nm disappeared and only a peak at 318 nm remained (Fig. 2). Thus it was suggested that the appearance of both peaks at 285 and 318 nm depended greatly on the pH of the fluid.

When the fluid was dialyzed using cellulose tubing (Visking Company, 16/32) against distilled water over night, the dialyzate lost the characteristic absorption spectrum (Fig. 3). When the fluid was extracted with ether, the main peaks of the spectrum were transfered to ether fraction (Fig. 4). When the leaf ambient

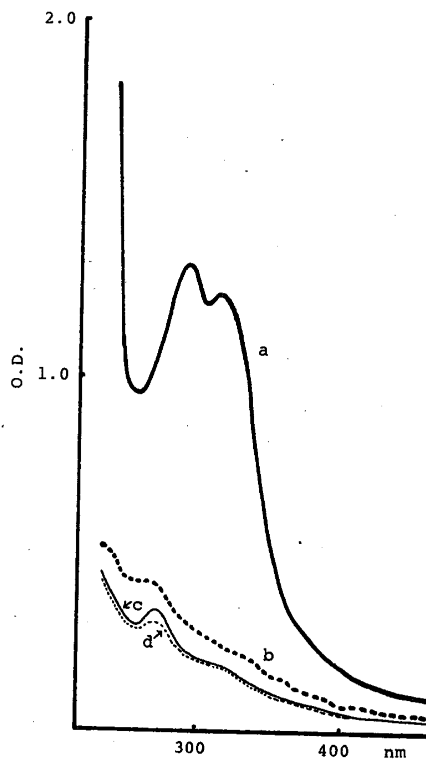


FIG. 1

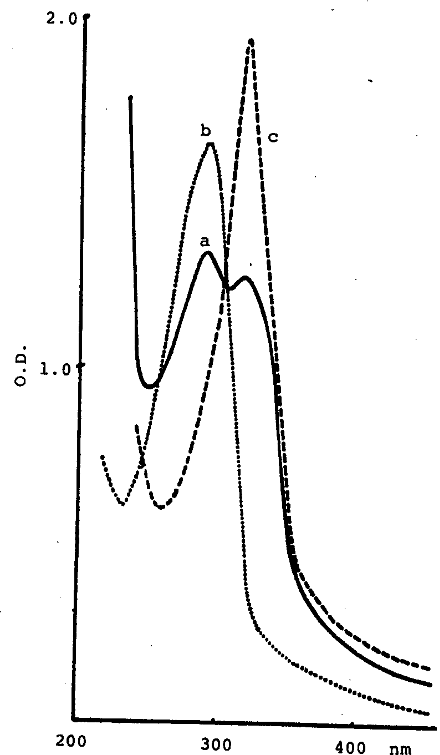


FIG. 2

FIG. 1. Spectra of ambient fluids of inoculated (a) and uninoculated cowpea leaves (b), and those of inoculated (c) and uninoculated tobacco leaves (d).

FIG. 2. Spectra of infected leaf ambient fluids at acidic and basic sides. a; original fluid b; at pH 2.1 c; at pH 10.5

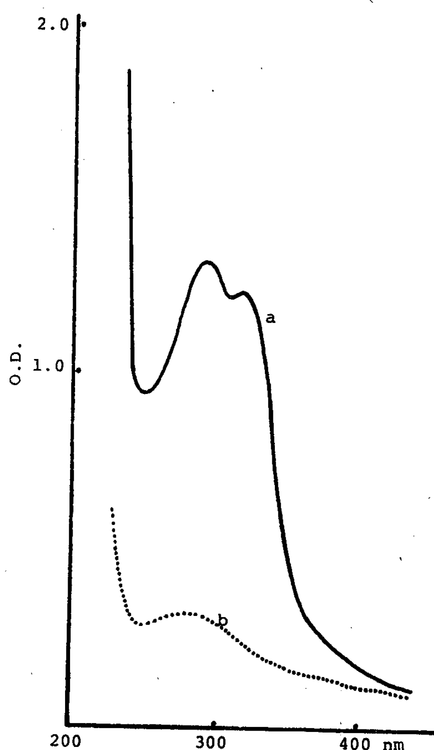


Fig. 3

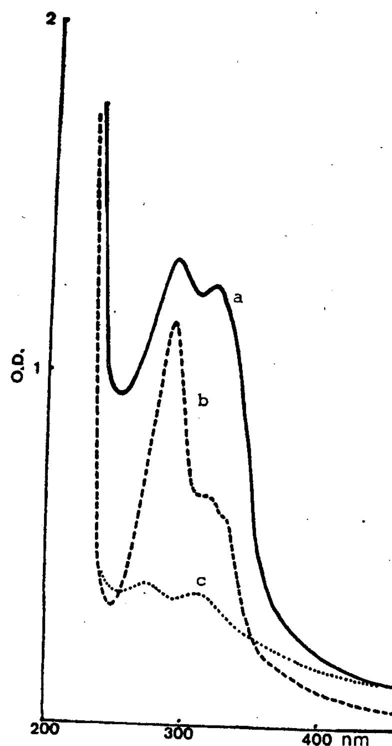


Fig. 4

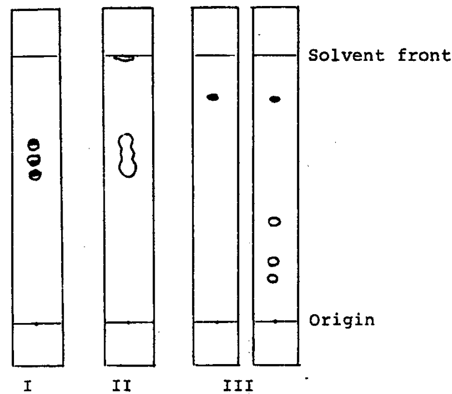
Fig. 3. Spectrum of infected leaf ambient fluid dialyzed against water. a; original fluid (before dialysis). b; after dialysis

Fig. 4. Spectra of ether and water fractions by ether extraction of infected leaf ambient fluid. a; original fluid b; ether fraction c; water fraction after extracted with ether

fluid was extracted with ether, vacuum-concentrated and analysed by TLC, 3 main spots were detected on the plate under light of wavelength 254 nm (Fig. 5 I). The R_f values of the spots were 0.56, 0.62 and 0.69. When the plate was sprayed with 5% $FeCl_3$, the zones corresponding to the spots described above or those in the vicinity of them revealed to be purple to blue in color. On the other hand, when the developed TLC plate was sprayed directly with a suspension of spores of *P. oryzae*, inhibition zones were revealed as white areas devoid of mycelium in the area corresponding to the spots described above (Fig. 5 II).

In the previous report, the inoculated leaves were vacuum-infiltrated with water and filled their intercellular spaces with water to facilitate the elution of substance from the cells. However, it was confirmed that even though the process of vacuum-infiltration was excepted, the yield of the substances eluted into leaf ambient fluid was little affected (Table. 1). Thus, in the extraction of this experiment the process of vacuum-infiltration was excepted.

The main 3 spots detected on TLC plate by ultraviolet light were removed by scraping the silica gel from the plate and they were eluted with ether. The spectra of those compounds eluted in ether showed essentially similar patterns; with a maximum peak at 285 nm. Next when the substances obtained by



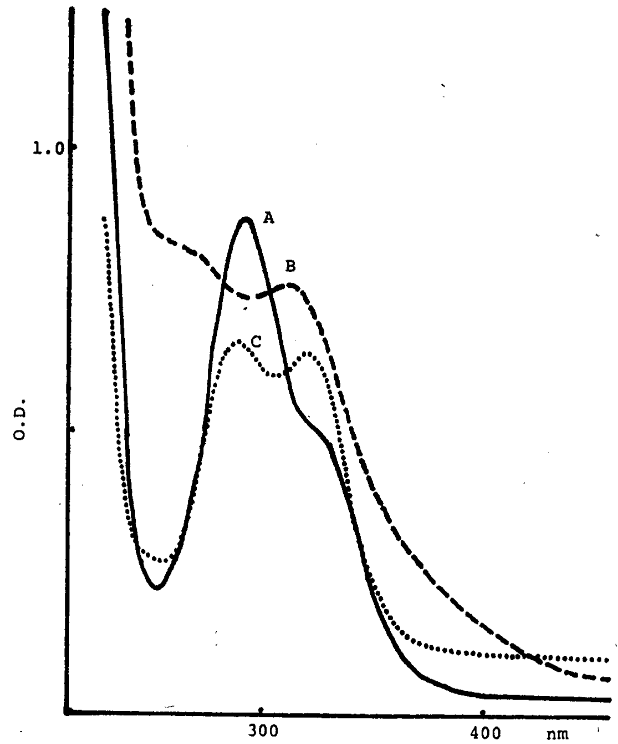


FIG. 5 FIG. 6

FIG. 5. Schematic representation of thin-layer chromatograms (TLC) of ether extracts from the ambient fluid of CMV-infected cowpea leaves. I. The extracts were subjected to TLC and developed in a mixture of chloroform which contained 2% v/v ethanol, and methanol (100:20); white areas show the spots appeared under ultraviolet light (wavelength 254 nm), and black areas show the spots colored after spraying with 5% $FeCl_3$. II. Fungal growth inhibition zone revealed on TLC plate corresponding to I. III. TLC of phaseollin (left) and a mixture of the extract and phaseollin (right). They were subjected to TLC on silica in a mixture of chloroform which contained 2% v/v ethanol, and methanol (100:4), and the plates were sprayed with 5% $FeCl_3$. The upper spots (black), phaseollin, exhibited slight blue color, and the under three spots (white) exhibited a purple color. Any spots did not occur on the TLC of extracts from the comparable fluids of uninoculated leaves.

FIG. 6. Spectra of aqueous solutions of the substances separated by TLC. A; From R_f 0.62, B; From R_f 0.69, C; From R_f 0.56

TABLE 1. *Amounts of the Substances Eluted into the Ambient Fluids of the Inoculated Leaves Vacuum-Infiltrated or Uninfiltrated with Water before Incubation*

	Yield* of substances from		
	R_f** 0.56	R_f 0.62	R_f 0.69
Vacuum-infiltrated with water	2.8	2.5	1.8
Excepted the infiltration process	2.7	2.6	1.6

* mg per 200 inoculated leaves (about 45g)

** Position on TLC developed in a mixture of chloroform which contained 2% v/v ethanol, and methanol (100 : 20)

removing the ether under a vacuum were dissolved in distilled water, the each solution showed a slightly different absorption spectrum (Fig. 6). When aqueous solutions of 3 substances separated by twice TLC developments were mixed with the spore suspension, germination of spores and germ-tube growth were significantly inhibited according to the concentrations of the substances; germ-tube growth was inhibited by applications of over 25 μg/ml (Fig. 7) and germination of the spore was completely prevented by a 250 μg/ml application (Fig. 8).

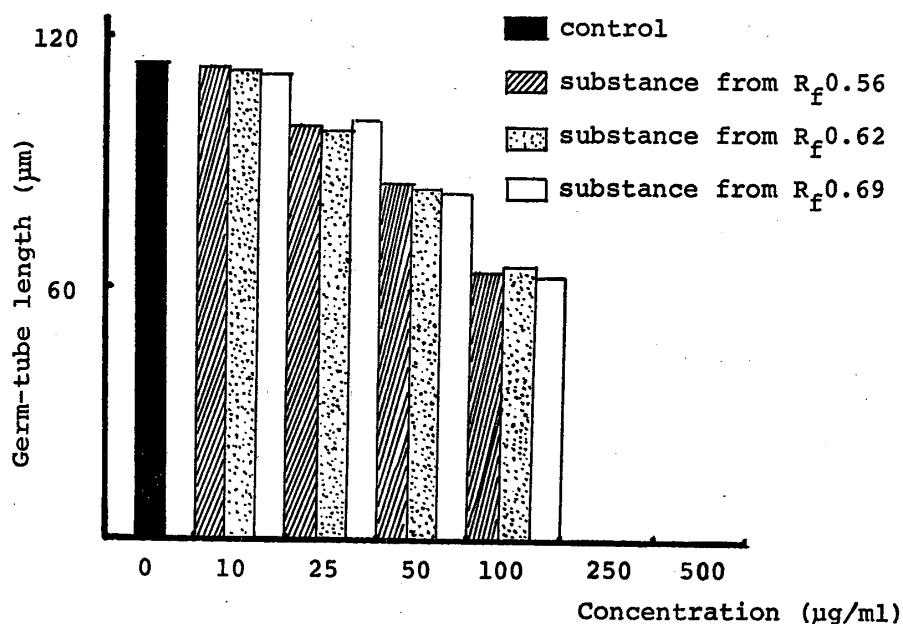


Fig. 7. Effect of the substances separated by TLC on the germ-tube growth of *Pyricularia oryzae.*

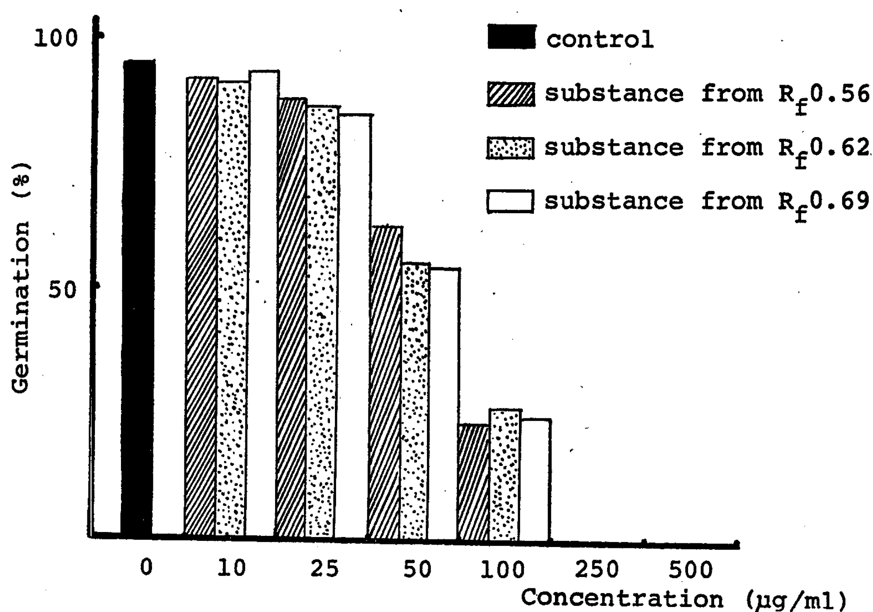


Fig. 8. Effect of the substances separated by TLC on the germination of *Pyricularia oryzae.*

When phaseollin alone and a mixture of the present extraction and phaseollin were developed on TLC, the R_f values of thee substances were clearly different from each other. Thus, it was confirmed, judging from the R_f value on TLC, that each of the main substances extracted from the leaf ambient fluids of CMV-infected cowpea was different from phaseollin (Fig. 5 III).

Phytotoxic activity: The phytotoxic effect of the substances separated here was tested by using cowpea leaf tissues. As described in Materials and Methods, the fragments of cowpea leaves were floated on aqueous solutions of the substances at concentrations ranging from 0 to 500 μg/ml. The necrosis appeared in the marginal area of the leaf fragments at concentrations of 200 to 500 μg/ml. The width of the necrotic area was 136 μm at 200 μg/ml, 252 μm at 300 μg/ml and 526 μm at 500 μg/ml. The results showed that each compound induces cell death at all concentrations which completely inhibited spore germination of *P. oryzae.*

Discussion

In the previously proposed method for the extraction of fungitoxic substances from CMV-infected cowpea leaves, the infected leaves were vacuum-infiltrated with water after inoculation and incubated (3). In the present experiments, the process of vacuum-infiltration was omitted, however, the yields of fungitoxic substances eluted into the ambient fluid of the infected leaves were almost the same as that in the case of the previous method. The fact shows that water entered gradually into the intercellular spaces of the leaves during incubation. It is noteworthy that the yields of the antifungal substances eluted into the leaf ambient fluid on the present or the previously reported incubation methods were considerably higher than that extracted directly by ethanol from the intact infected leaves (3).

In fact, the fungitoxic substances were eluted into leaf ambient fluids from just before the appearance of the initial symptom until the necrotic lesions were matured (2). These substances were not only fungitoxic but also phytotoxic, and therefore it seemed that the accumulation of the substances in the cell might have induced cell death. If there could be excretion of the harmful substances, the infected cells might survive for longer time than being in intact leaves and become to continue the production of such substances, and also the virus multiplication might continue, for as long as the infected cells survived. This phypothesis is supported by an evidence that the virus activity of leaves incubated in water was always higher than that of the intact infected leaves (3).

In this experiment also, the fungitoxic activities of each substance separated by TLC were bioassayed by using *P. oryzae* (race F67–54) as examined previously (3). The 3 substances separated here showed a similar antifungal activity; germ-tube growth of the spore was inhibited at concentrations of over 25 ppm and germintation was completely prevented at 250 ppm. These values showed that

fungitoxicity of each substance was stronger than those obtained in previous experiments (3) in spite of the fact that the same fungus was used. This may imply that the purity of each substance was higher than that of the previous samples owing to the fact that each one was developed twice on TLC.

From data concerning the pattern of absorption spectrum, its change by varying the pH values and coloration by $FeCl_3$ on TLC analysis, the main substances separated from the ambient fluid of the infected leaves might be phenolic compounds. However they were not compounds as chlorogenic acid, caffeic acid and ferulic acid (unpublished data). The pattern of the ultraviolet absorption spectrum of the infected leaf ambient fluid was very similar to that of ethanolic solutions of extracts, containing phaseollin, from bean (*Phaseolus vulgaris*) hypocotyls infected with *Colletotrichum lindemuthianum* (1). Therefore, first we assumed that the main of fungitoxic substances separated here are phaseollin or phaseollin like compounds which has been isolated generally from fungal-infected leguminous plants. However neither of them was phaseollin at least, judging from their R_f values on TLC, thus they may be other isoflavonoids. Now we are examining whether or not the substance obtained from each spot on TLC is composed of a simple compound or a mixture, and furthermore identifying the acculate chemical structures of these substances.

Acknowledgements

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