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# PHYTOTOXIC SUBSTANCES IN ROOT EXUDATES OF CUCUMBER (*Cucumis sativus* L.)

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**Abstract**—The addition of activated charcoal to a nutrient solution for the hydroponic culture of cucumber resulted in significant increases in the dry weight of the plant and fruit yield. Hydrophobic root exudates were collected at different growth stages with Amberlite XAD-4 resin and bioassayed with lettuce seedlings. The exudates at the reproductive stage were more phytotoxic than those at the vegetative stage. The exudates contained organic acids such as benzoic, *p*-hydroxybenzoic, 2,5-dihydroxybenzoic, 3-phenylpropionic, cinnamic, *p*-hydroxycinnamic, myristic, palmitic, and stearic acids, as well as *p*-thiocyanatophenol and 2-hydroxybenzothiazole, all of which, except 2-hydroxybenzothiazole, were toxic to the growth of lettuce.

**Key Words**—Activated charcoal, allelopathy, autotoxicity, *Cucumis sativus* L., hydroponics, *Lactuca sativa* L., nutrient solution, organic acid, phytotoxicity, root exudates.

## INTRODUCTION

Allelopathy and autotoxicity due to root exudates of plants are important in agricultural and ecological problems such as the replant failure of horticultural crops, the selection of companion crops in mixed cropping, crop rotation, and growth reduction in some fruit vegetables during fruit enlargement (Putnam, 1986; Rizvi and Rizvi, 1992; Sarobol and Anderson, 1992; Tucker, 1981; Young, 1984). We recently reported that the growth of tomato in hydroponic culture was inhibited by organic substances that arose from the root exudates and were removed from the nutrient solution by adsorption on activated charcoal (Yu et al., 1993). The present study deals with the phytotoxicity of root exudates

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of cucumber plants and the identification of the phytotoxic substances. Cucumber is a vegetable cultivated throughout the world. However, poor growth due to successive cropping has frequently been observed. The phenomenon has been attributed to the buildup of pests, nutritional disorder, or other unknown factors (Takahashi, 1984). It has also been suggested that cucumber has allelopathic potential and some accessions of cucumber severely inhibit the growth of cucumber and weeds (Gaidamak, 1971; Lockerman and Putnam, 1979, 1981a,b; Putnam and Duke, 1974; Putnam, 1986).

## METHODS AND MATERIALS

*Reagents.* All the reagents and solvents used were commercially available and used without further purification except for pyridine, which was dried over $CaH_2$ and distilled before use. *p*-Thiocyanatophenol was prepared according to the directions of Bordwell and Boutan (1956): $^1$H NMR ($CDCl_3$) $\delta$ = 6.88 (2H, d, $J$ = 8.8 Hz) and 7.45 (2H, d, $J$ = 8.8 Hz); MS (70 eV) $m/z$ (relative intensity) 151 (100), 123 (23), 96 (49), 65 (28), 39 (40), and 27 (21).

*Growth Tests.* Cucumber plants (*Cucumis sativus* L., cv. Tokiwa, Sakata Seed Co.) were cultivated by means of hydroponic systems as described previously (Yu et al., 1993). Each system was mainly composed of a culture bed (94 × 94 × 5 cm), a nutrient solution tank (150 dm$^3$), a pump with a time switch, and an inlet with an air-siphon. The nutrient solution was periodically recycled by the pump. Six cucumber seedlings at the two-leaf stage were transplanted to each bed and cultivated for about two months. The initial nutrient solution contained (in mmol/dm$^3$) $Ca(NO_3)_2$, 3.0; $KNO_3$, 6.0; $NH_4H_2PO_4$, 1.0; $MgSO_4$, 1.5 and (in mg/dm$^3$) $FeCl_3$, 15; $H_3BO_3$, 3; $MnCl_2$, 2; $ZnSO_4$, 0.22; $Cu(NO_3)_2$, 0.05; $Na_2MoO_4$, 0.02. The pH and electrical conductivity (EC) at 25°C of the solution were 5.5 and 1.9 dS/m, respectively. The nutrient solution was not renewed during cultivation. Nutrients and water consumed by the plants were periodically compensated on the basis of chemical analysis (Yu et al., 1993) of the nutrient solution collected weekly or biweekly. The hydroponic culture was carried out twice, from June 26 to August 22 and from September 6 to November 13, 1992, with and without the addition of 600 g of granular activated charcoal (Takeda Chemical Industry Co., Shirasagi $W_2C$, 4–8 mesh) to the nutrient solutions (150 dm$^3$) in the tanks. Each treatment was triplicated in the first cropping and duplicated in the second cropping. At the end of cultivation, the plants were divided into root, shoot, and fruit, dried at 80°C, and weighed.

*Collection of Root Exudates.* The residual nutrient solutions (10 dm$^3$) of the hydroponic cultures were passed through columns packed with 15 cm$^3$ of Amberlite XAD-4 resin (Sigma Chemical Co.) as an adsorbent of hydrophobic

substances. Prior to use, the resin was cleaned by treatment with hot water, followed by Soxhlet extraction with methanol, acetone, and diethyl ether, each for 24 hr (Tang and Young, 1982). The columns, after use, were washed with 200 $cm^3$ of water and treated with 100 $cm^3$ of methanol as an eluent. The eluates were concentrated to 10 $cm^3$ by evaporation in vacuo at 40°C.

In another experiment, the root exudates of cucumber were collected at different growth stages with a continuous trapping system similar to that devised by Tang and Young (1982). A column of the system was packed with 15 $cm^3$ of the precleaned XAD-4 resin. Three cucumber seedlings at the two-leaf stage were transplanted in a pot (12 $dm^3$) filled with 10 $dm^3$ of the nutrient solution with the same composition as described above. The nutrient solution was aerated and continuously recycled at the rate of 1 $dm^3$/hr by means of an air pump. The pot was maintained at 28°C by day and at 20°C by night in a growth chamber under natural light. The cultivation was carried out from June 12 to September 2, 1992, and the column of the system was replaced by a new column every 10 days during cultivation. The detached columns were washed with 200 $cm^3$ of distilled water and then treated with 200 $cm^3$ of methanol. The methanol solutions were concentrated to 3 $cm^3$ by evaporation in vacuo at 40°C. Concurrently, an additional trapping system was employed as a control, in which no cucumber plant was transplanted.

*Phytotoxicity Bioassay.* The assay was carried out according to the method of Tang and Young (1982). Thus, an aliquot of a test solution was applied with a micropipet to a 3.5-$cm^2$ disk of filter paper in a 5.5-cm-diameter Petri dish. After evaporation of the solvent, the disk was wetted with 0.2 $cm^3$ of distilled water. Eight lettuce seeds (*Lactuca sativa* L., cv. Shisuko; Takii Seed Co.) were placed on the disk. The seeds were incubated in a moisture-saturated box at 24°C for 50–60 hr. The mean root length of the resulting seedlings was selected as a growth index. Bioassay with cucumber seeds was also carried out in a similar manner, except that the disk area, the amount of water added, incubation time, and temperature were 19.6 $cm^2$, 3 $cm^3$, 120 hr, and 28°C, respectively.

*Identification of Phytotoxic Substances.* The methanol eluate of the pot experiment, after evaporation to dryness, was dissolved in 20 $cm^3$ of water. The resulting solution was adjusted to pH 8.0 with 0.1 mol/$dm^3$ NaOH and extracted three times with 30 $cm^3$ of ethyl acetate. The aqueous layer was then adjusted to pH 2.0 with 1.0 mol/$dm^3$ HCl and extracted three times with 30 $cm^3$ of ethyl acetate. The extracts from aqueous solutions at pH 8 and pH 2 were separately dried over anhydrous $CaSO_4$ and evaporated in vacuo to give 10 $cm^3$ of the concentrates, which we hereafter call neutral (NF) and acidic (AF) fractions, respectively. The residual aqueous layer was neutralized and passed through a column filled with 15 $cm^3$ of the XAD-4 resin. The column was eluted with 50

$cm^3$ of methanol, and the eluate was evaporated in vacuo to give 10 $cm^3$ of the concentrate, which we hereafter call the water-soluble (WF) fraction.

Aliquots (1 $cm^3$) of the NF, AF, and WF were evaporated in vacuo to dryness, and to the residues were added 0.2 $cm^3$ of pyridine and 0.2 $cm^3$ of *N*, *O*-bis(trimethylsilyl)trifluoroacetamide containing 1% of trimethylchlorosilane. The resulting and original solutions were analyzed with a Shimadzu GC-14A gas chromatograph and a Hitachi M-80 gas chromatograph–mass spectrometer (GC-MS). A TC-5 capillary column (60 m, GL Science) and a OV-17 column (2% Uniport HP 80/100, 2 m) were used for the gas chromatograph with He as carrier gas. The column temperature was elevated from 100°C to 270°C at the rate of 5°C/min. The ionization voltage in the electron impact mode of the mass spectrometer was 70 eV.

## RESULTS AND DISCUSSION

*Effect of Activated Charcoal on Growth of Cucumber.* Table 1 shows the dry weights of organs and the fruit yields at the end of cultivation in the absence and presence of activated charcoal. The addition of the charcoal resulted in significant increases in dry matter production and fruit yield. During the experiments, the concentrations of nutrients were periodically regulated to be as constant as possible. Figure 1 shows changes in pH, EC, and the concentrations of some major nutrient elements during cultivation. The changes were virtually independent of the addition of the charcoal. Similar results were also obtained for $Mg^{2+}$ and $SO_4^{2-}$. Hence, the effect of the charcoal on the growth of cucumber plants was not attributable to changes in the composition of inorganic nutrients but to phytotoxic organic substances, which were adsorbed on the charcoal, although cucumber plants grown without charcoal exhibited no apparent symptoms of autotoxicity except their leaves were relatively small.

TABLE 1. EFFECTS OF ACTIVATED CHARCOAL ON GROWTH OF CUCUMBER PLANT[a]

| Cropping | Treatment | Dry weight (g/plant) | | | | Fruit yield (kg/plant) |
|---|---|---|---|---|---|---|
| | | Root | Shoot | Fruit | Total | |
| 1 | Control | 6.4 | 95.2 | 50.7 | 152.4 | 1.18 |
| | Charcoal | 11.2** | 123.5** | 73.1** | 207.8*** | 1.80*** |
| 2 | Control | 5.8 | 94.5 | 19.0 | 119.3 | 0.43 |
| | Charcoal | 8.4 | 131.7* | 48.8* | 189.4** | 1.11* |

[a] *, **, and *** refer to significant differences compared with the control at 0.05, 0.01, and 0.001 levels by Student's *t* test, respectively.

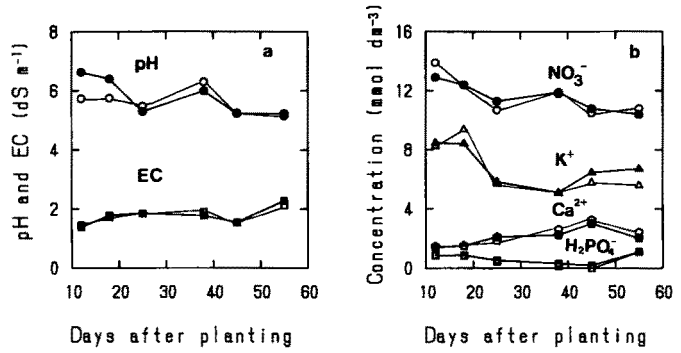


FIG. 1. Changes in pH, EC, and the concentrations of a few major ions in nutrient solutins during the first cropping of cucumber plants in the absence (open circles) and presence (solid circles) of activated charcoal.

Hydrophobic substances in the residual nutrient solutions after cultivation were collected by passing 10 $dm^3$ of the solutions through Amberlite XAD-4 resin columns and eluted by methanol. The eluates were concentrated to 10 $cm^3$, and the concentrates were bioassayed with cucumber and lettuce. Figure 2 shows the effects of the volume (V) of aliquots of the concentrates on the mean root length (MRL) of the seedlings after incubation. The MRL values for the solution treated with charcoal were significantly larger than those for the untreated solution. Furthermore, the MRL value for the latter decreased with increasing V value. These results support our presumption that cucumber plants exude phytotoxic substances that are adsorbed on activated charcoal. Figure 2 also shows

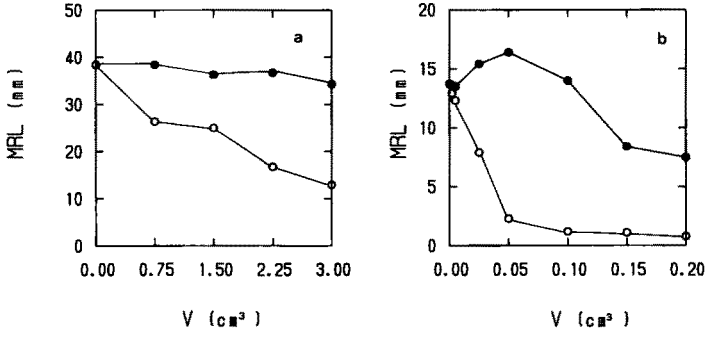


FIG. 2. Plots of the mean root lengths (MRL) vs. the volume (V) of concentrated eluates assayed with cucumber (a) and lettuce (b) for residual nutrient solutions with (solid circles) and without (open circles) charcoal.

that the bioassay with lettuce is much more sensitive than that with cucumber, so that the former was used for the subsequent experiments.

*Phytotoxicity at Different Growth Stages.* Pot experiments with root exudate trapping systems provided several XAD-4 resin columns through which nutrient solutions were passed for about 10 days. We treated the columns with methanol and concentrated the eluates to 3 $cm^3$ to give solutions containing root exudates at difference growth stages. Figure 3 shows changes in the MRL values of lettuce with sampling time. The MRL values were virtually constant for the initial periods of 40 days (vegetative growth stage), then decreased rapidly, and approached a very small constant value at the reproductive stage. The solutions from the control pot gave a virtually constant MRL value throughout the experiment. The results suggest that phytotoxic substances are mainly exuded at the reproductive stage. Similar phenomena have been reported on yellow fieldcress (Yamane et al., 1992) and tomato (Yu et al., 1993).

*Identification of Phytotoxic Substances.* The eluate from the XAD-4 resin in the pot experiment was evaporated to dryness, and the residue was fractionated into three fractions: NF, AF, and WF. Table 2 shows the results of bioassay with lettuce for these fractions at various volume values. The ratio value in the table refers to the ratio of the MRL value for the root exudates to that for the control. Growth inhibition was observed for NF and AF but not for WF. Thus, NF and AF were used for the GC and GC-MS analyses to identify compounds involved.

The NF gave a number of GC peaks (Figure 4), among which the main

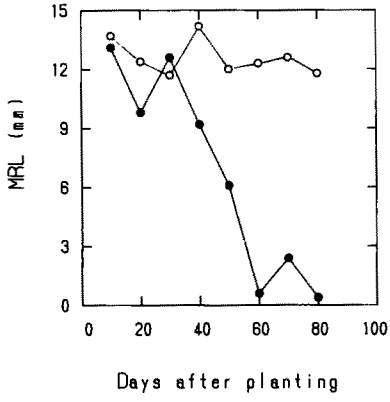


FIG. 3. Mean root lengths (MRL) of lettuce seedlings assayed for root exudates at different growth stages of cucumber plants. Open circles: control; solid circles: root exudates. Three replications with eight seeds for each were carried out at 20 $mm^3$ of test solutions.

TABLE 2. RATIOS OF MEAN ROOT LENGTHS FOR NEUTRAL (NF), ACIDIC (AF), AND WATER-SOLUBLE (WF) FRACTIONS FROM ROOT EXUDATES TO THOSE FROM CONTROL AT VARIOUS VOLUMES OF TEST SOLUTIONS

| Volume ($mm^3$/disk) | Ratio | | |
|---|---|---|---|
| | NF | AF | WF |
| 5 | 0.96* | 0.83* | 0.94 |
| 10 | 0.90* | 0.82* | 0.93 |
| 20 | 0.66* | 0.63** | 0.97 |
| 40 | 0.37** | 0.34*** | 0.91 |
| 80 | 0.19*** | 0.09*** | 1.03 |

"*, **, and *** refer to significant differences compared with the control at 0.05, 0.01, and 0.001 levels by Student's $t$ test, respectively.

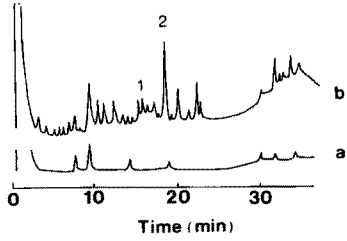


FIG. 4. Gas chromatogram of the neutral fraction (NF): (a) control; (b) root exudates. Analytical conditions: column, OV-17 (2 m); oven temperature was raised from 100°C to 270°C at 5°C/min; detector and injector temperatures, 300°C; the flow rate of carrier gas (He): 30 $cm^3$/min. Peak 1, $p$-thiocyanatophenol; peak 2, 2-hydroxybenzothiazole.

peak (peak 2) was attributed to 2-hydroxybenzothiazole by the comparison of the mass spectrum [$m/z$ (rel. intensity) 151 (100), 123 (49), and 96 (66)] and the retention time (18.1 min) with those of the authentic sample. Similarly, the peak 1 was attributed to $p$-thiocyanatophenol. The contents of $p$-thiocyanatophenol and 2-hydroxybenzothiazole in NF were 0.7 and 8.0 mg/g root exudate, respectively. The other peaks in Figure 4 have not been identified at present. 2-Hydroxybenzothiazole has been separated from the rhizosphere of coffee tree (Waller et al., 1986), although there is no report showing that cucumber exudes the compound. There is also no report that $p$-thiocyanatophenol is separated from the rhizosphere of plants, including cucumber.

The GC analysis of the AF, after trimethylsilylation, gave a number of peaks (Figure 5), among which nine were assigned to trimethylsilylates of organic acids such as benzoic, 3-phenylpropionic, cinnamic, $p$-hydroxybenzoic, 2,5-

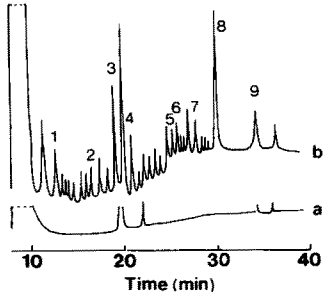


FIG. 5. Gas chromatogram of the acidic fraction (AF) after trimethylsilylation: (a) control; (b) root exudates. Analytical conditions: column, GL Sciences TC-50 (60 m); oven temperature, 100°C for 2 min and then raised to 270°C at 5°C/min; detector and injector temperatures, 300°C; the flow rate of carrier gas (He), 1 $cm^3$/min. Peak 1, benzoic acid; peak 2, 3-phenylpropionic acid; peak 3, cinnamic acid; peak 4, *p*-hydroxybenzoic acid; peak 5, 2,5-dihydroxybenzoic acid; peak 6, myristic acid; peak 7, *p*-hydroxycinnamic acid; peak 8, palmitic acid; peak 9, stearic acid.

dihydroxybenzoic, myristic, *p*-hydroxycinnamic, palmitic, and stearic acids, based on the comparison of their GC-MS data with those of authentic samples. These compounds were not detected in the AF of a control, indicating that they were the constituents of root exudates. They have frequently been separated from the root residue, root exudates, and rhizosphere of plants (AlSaadawi et al., 1983; Pérez and Ormeño-Nuñez, 1991b; Rice, 1984; Tang and Young, 1982; Yamane et al., 1992), although not from the root exudates of cucumber. The mean rates of release for these compounds at the vegetative and reproductive stages were calculated on the basis of the amounts of the compounds detected and the days needed for the collection (Table 3). Significant amounts of the fatty acids, as well as benzoic acid, were released at both stages, whereas the other aromatic carboxylic acids were mainly released at the reproductive stage. The calculated rates of release for the aromatic carboxylic acids were similar to those for allelochemicals observed for other plants, ranging from less than 1 μg/day to several micrograms per day (Pérez and Ormeño-Nuñez, 1991a,b; Tang and Takenaka, 1983; Yamane et al., 1992).

Table 4 shows the phytotoxicity of the identified compounds at various concentrations. Most of the compounds exhibited significant phytotoxicity at concentrations higher than 0.1 mmol/$dm^3$. Among them, *p*-thiocyanatophenol was the most phytotoxic. 2-Hydroxybenzothiazole had no inhibitory effect on the growth of lettuce. The inhibitory effect of phenolic acids such as ferulic and *p*-hydroxycinnamic acids on the growth of cucumber plants and their nutrient uptake has been reported (Blum et al., 1985; Blum and Dalton, 1985; Holappa and Blum, 1991; Lyu et al., 1990).

TABLE 3. IDENTIFIED ORGANIC ACIDS IN ACIDIC FRACTION (AF) AND MEAN RATES OF RELEASE FOR THEM AT DIFFERENT GROWTH STAGES

| Peak | m/z | Organic acid | Release rate (μg/day/plant) | |
|---|---|---|---|---|
| | | | Vegetative | Reproductive |
| 1 | 194 | Benzoic | 1.5 | 1.5 |
| 2 | 222 | 3-Phenylpropionic | ND[a] | 0.7 |
| 3 | 220 | Cinnamic | ND | 1.6 |
| 4 | 282 | *p*-Hydroxybenzoic | ND | 1.5 |
| 5 | 370 | 2,5-Dihydroxybenzoic | 0.1 | 0.4 |
| 6 | 300 | Myristic | 1.4 | 1.5 |
| 7 | 308 | *p*-Hydroxycinnamic | ND | 0.9 |
| 8 | 328 | Palmitic | 2.6 | 5.0 |
| 9 | 356 | Stearic | 0.5 | 1.1 |

[a]ND: not detected.

TABLE 4. EFFECT OF IDENTIFIED COMPOUNDS AT VARIOUS CONCENTRATIONS ON ROOT LENGTH (mm) OF LETTUCE

| Compounds | Concentration (mmol/dm$^3$) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.01 | 0.05 | 0.10 | 0.50 |
| Benzoic acid | 11.2a[a] | 11.2a | 10.1a | 6.3b | 3.4c |
| *p*-Hydroxybenzoic acid | 11.2a | 9.6a | 9.6a | 6.3bc | 4.7c |
| 2,5-Dihydroxybenzoic acid | 11.2a | 11.3a | 11.1a | 9.2ab | 8.2b |
| 3-Phenylpropionic acid | 11.2a | 10.5a | 9.2a | 6.9b | 2.4c |
| Cinnamic acid | 11.2a | 10.2a | 9.6a | 6.4bc | 5.4c |
| *p*-Hydroxycinnamic acid | 11.2a | 11.0a | 9.9a | 9.6a | 7.8b |
| Myristic acid | 11.2a | 10.4a | 9.2ab | 8.3ab | 8.4b |
| Palmitic acid | 11.2a | 11.0a | 10.9ab | 10.3ab | 6.9b |
| Stearic acid | 11.2a | 10.9a | 9.4ab | 9.3ab | 8.1b |
| *p*-Thiocyanatophenol | 11.2a | 9.7a | 9.4b | 6.8c | 0.8d |
| Mixture[b] | 11.2a | 10.3a | 8.2ab | 7.7.b | 6.9b |

[a]Numbers with different letters within a row refer to significant difference at 0.05 level according to Duncan's multiple-range test.
[b]The mixture of the above 10 compounds, each concentration being one tenth of those cited.

The above results clearly indicate that autotoxic and allelopathic compounds were accumulated in the nutrient solutions for cucumber, especially at the reproductive stage of growth. However, as shown in Figures 4 and 5, we identified only some of compounds involved in NF and AF, and most of them

remained to be identified. The identified compounds were less than 5% by weight of the collected exudates. Furthermore, the fractions might contain nonvolatile compounds that could not be detected by gas chromatography. Thus, it is reasonable to consider that not only the identified but also unidentified compounds are responsible for the phytotoxicity of the fractions. It is also possible that the effect of the phytotoxic compounds is additive, synergistic, or antagonistic (Leather and Einhellig, 1986; Rice, 1984). It is important to examine the effect of mixed compounds on the growth of cucumber plants. These problems remain to be solved.

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