Phytotoxins in *Rhizoctonia solani*: Isolation and Biological Activity of *m*-Hydroxy- and *m*-Methoxyphenylacetic Acids

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Phytotoxins from culture filtrates of *Rhizoctonia solani* were isolated and identified as *m*-hydroxy- and *m*-methoxyphenylacetic acids via chromatographic and spectroscopic methods. The fungus *R. solani* was isolated from infected soybeans and grown in artificial medium for the isolation of phytotoxins. These toxins and the other synthetic phenylacetic acid derivatives were evaluated for growth-regulating activity and toxic effects in several bioassays systems. It was demonstrated that *m*-hydroxyphenylacetic acid and the newly identified meta methoxy derivative were specific for the soybean isolate of *R. solani* belonging to anastomising group IV even though they may be present in other groups of fungal toxins. This group infects soybeans and decreases nodule formation and yield. An analytical method with high-performance liquid chromatography was devised for the detection of trace quantities of phenylacetic acid and its derivatives. This sensitive analytical method could be applied to screen plants infected with *R. solani*. Because of the ubiquitous nature of this fungus, the screen for these compounds may be used to develop a toxin-mediated bioassay for resistance to rhizoctonia root rot.

The taxonomic identification and the detrimental effects of the soil-borne, root-rotting facultative fungal plant pathogen *Rhizoctonia solani* Kühn (*Thanatephorus cucumeris*) have been well documented (Parmeter, 1970). The effect of this pathogen on the yield components (roots, root nodules, and tops) of soybeans (*Glycine max* L.) inoculated with *Rhizobium japonicum* (Kirchner) Buchanan was reported recently (Orellana et al., 1976). Also, Orellana and Worley (1976) have demonstrated cell dysfunction in root nodules of soybeans grown in the presence of *R. solani*. Pathogenesis from *R. solani* was reported to be partly due to the action of pectinolytic and cellulolytic enzymes produced by the fungus and partly due to nonenzymatic toxic fungal metabolites that vary with the fungal source and the conditions used to grow the pathogen (Sherwood and Lindberg, 1962; Bateman, 1970; Sherwood, 1979). Commonly occurring toxins produced by *R. solani* are phenylacetic acid (PAA) and one or more of its hydroxy derivatives (Aoki et al., 1963; Wu, 1965; Frank and Francis, 1976; Mandava et al., 1978). The presence of PAA and its para and meta hydroxy derivatives (*p*-OHPAA and *m*-OHPAA) in *R. solani* culture has been reported. For example, *Pellicularia filamentosa* (the perfect state of *R. solani*) isolated from clover contained PAA and *m*- and *p*-OHPAAs (Kohmoto and Nishimura, 1974). The rice-infecting fungus, *Hypochnus sasakii* (*T. cucumeris*) produced only *p*-OHPAA as its phytotoxin (Chen, 1958). Other reports on *R. solani* indicate that culture filtrates of *R. solani* contain only PAA and *m*-OHPAA. Aoki et al. (1963) isolated PAA and *p*-OHPAA from sugar beets infected with *R. solani*. The same fungal toxins were confirmed by Wu (1965). Frank and Francis (1976) isolated PAA and *m*-OHPAA from isolates of potatoes infected by *R. solani*. Isolates from rice infected by *R. solani* also contain PAA and *m*-OHPAA (O'Neill, 1976). At low concentrations, phenylacetic acid and its metabolites from plant sources and *R. solani* act as plant growth regulators (Chamberlain and Wain, 1971; Milborrow, 1975; Frank and Francis, 1976).

In continuation of our work on the soybean isolate of *R. solani*, we have isolated a phytotoxic fraction from the culture filtrate. This paper concerns the isolation and identification of *m*-hydroxyphenylacetic acid (*m*-OHPAA) and *m*-methoxyphenylacetic acid (*m*-OMePAA) as phytotoxins. These were the only phenylacetic acids (PAAs) isolated from the culture filtrate (Mandava et al., 1978).

EXPERIMENTAL SECTION

Isolation of Phytotoxins. The isolate of *R. solani* AG IV (Orellana et al., 1976; Orellana and Worley, 1976) used in this investigation was grown in 2 L of Richard's solution (KNO_3, 10 g; KH_2PO_4, 5 g; $MgSO_4$, 2.5 g; $FeCl_3$, 0.02 g; sucrose, 50 g; H_2O, 1 L) fortified with 5% peptone. Cultures were grown for 7–8 weeks at room temperature with 8 h of fluorescent illumination (350 lux) per day and occasional shaking. At the end of this period, the fungal growth (10.8 g) was separated. The culture filtrate was autoclaved at 1.05 kg/cm^2 for 30 min to destroy enzymes and passed through a Seitz filter. The resulting filtrate (0.200 g of solids/1400 mL) was adjusted to pH 8.5 with 1 N NaOH and diluted with acetone (5–7 volumes). The precipitate was removed by filtration (Whatman No. 1 filter paper) and discarded, and the solvent (acetone) was removed in vacuo below 40 °C. The aqueous solution was adjusted to pH 2.5 (1 N HCl) and extracted four times with equal volumes of ether, and the combined ethereal solution was exhaustively extracted with 5% aqueous $NaHCO_3$ solution. The combined bicarbonate layers were adjusted to pH 2.5, and the solution was reextracted with ether. The evaporation of ether left a residue that was purified on a column of silica gel (Unisil, Clarkson Chemical Co., Williamsport, PA) with hexane and ether. The only biologically active fraction obtained, a mixture of phenylacetic acids (compounds I and II; see Results and Discussion section), was from elution of the column with 20% hexane in ether. These phenylacetic acids were separated by high-performance liquid chromatography (LC) on a μ Bondapak C_{18} column (Waters Associates, Inc., Milford, MA) with 1:3 methanol–5% aqueous acetic acid, collected, and recrystallized from anhydrous ether, mp 132–134 °C (compound I) and 72–74 °C (compound II).

Chromatographic and Spectroscopic Methods. Thin-layer chromatography (TLC) was performed on silica gel 60 (F-254) (0.2-mm layer thickness) plates (Brinkmann Instruments, Westbury, NY) with four solvent systems (Table I). Diazotized sulfanilic acid (DSA) as well as iodine staining and H_2SO_4 spray were used to detect re-

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Table I. R_f Values from Thin-Layer Chromatography of Hydroxyphenylacetic Acids and the Unknown Acid from the Culture Filtrate of *Rhizoctonia solani*

compound	solvent system[a]			
	S_1	S_2	S_3	S_4
o-hydroxyphenylacetic acid	0.62	0.51	0.64	0.32
m-hydroxyphenylacetic acid	0.72	0.60	0.43	0.41
p-hydroxyphenylacetic acid	0.78	0.65	0.45	0.47
natural hydroxyphenylacetic acid	0.72	0.60	0.43	0.41

[a] S_1, 1-butanol-pyridine-dioxane-H_2O (105:30:7.5:7.5); S_2, chloroform-95% ethanol-acetic acid (80:20:0.5); S_3, 2-propanol-NH_4OH-H_2O (8:1:1); S_4, toluene-ethanol (75:25).

active spots. For preparative work, 2-mm (layer thickness) silica gel plates were used. Whatman No. 1 filter paper and toluene–acetic acid (5:1) were used for paper chromatography (PC). The LC was performed with a Waters Model ACL-100 Instrument equipped with an M-6000 pump, a U6k injector, and a Model 440 absorbance detector (12.5-μL volume). A reversed-phase μ Bondapak C_{18} column (30 × 0.4 cm i.d.; particle size, 10 μL) and the solvent system methanol–5% aqueous acetic acid (1:3) were used. For gas chromatography (GC), a Hewlett-Packard Model 7620 equipped with a flame ionization detector (FID) was used. A stainless-steel column 1.8 m × 0.16 cm (o.d.) packed with 3% SE-30 on 80/100 mesh Gas-Chrom Q was used to separate methyl esters or the trimethylsilyl (Me_3Si) ethers of the toxins (temperatures: injector port, 250 °C; detector, 300 °C; column, 140 °C; carrier gas, N_2; flow rates: N_2, 90 mL/min; H_2, 40 mL/min; air, 170 mL/min). Proton (^1H) and noise-decoupled carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a JEOL FX-60Q pulsed Fourier transform NMR spectrometer with a dual probe at frequencies of 59.79 and 15.04 MHz, respectively. Deuteriochloroform was used as a solvent unless otherwise stated, and chemical shifts were measured relative to tetramethylsilane (Me_4Si) as internal standard. A Perkin-Elmer (Infracord) spectrophotometer was used for infrared (IR) spectral measurements. The spectra were recorded from chloroform solutions or KBr pellets. A Beckman Model 25 was used to record the ultraviolet (UV) spectra (methanol). Mass spectra (MS) of methyl esters or Me_3Si ethers were recorded on an LKB-9000 mass spectrometer (70 eV) equipped with a gas chromatograph. The test compounds were introduced into the mass spectrometer via a glass GC column packed with 0.75% SE-30. Total ion current monitoring followed GC separation.

Bioassays. *1. Bean Second Internode Bioassay.* This bioassay (Mitchell and Livingston, 1968) was used to evaluate the growth-regulating activity of the toxic components of *R. solani*. Test plants were grown in growth rooms (temperature, 25–30 °C; light, 7.5 klux for 12 h); 6-day-old pinto bean seedlings with second internodes 2–4 mm long were treated with the phytotoxins in lanolin (250 μg/plant). The control plants were treated with lanolin alone. After 4 days, the treated and control plants were compared and the percent increase in internode elongation over the controls was used as a measure of the growth-regulating substances.

2. Lettuce Hypocotyl Bioassay. This bioassay likewise was used to evaluate the growth-regulating activity of the toxins from *R. solani*. Lettuce seedlings were grown in a growth room at (25 °C) under continuous cool-white fluorescent light. At 24–48 h after germination, the seedlings were treated with the test compounds (500 ppm) at 10–50 μL per seedling. After 5 days the root and hypocotyl growth was measured and compared with those of control plants grown in water.

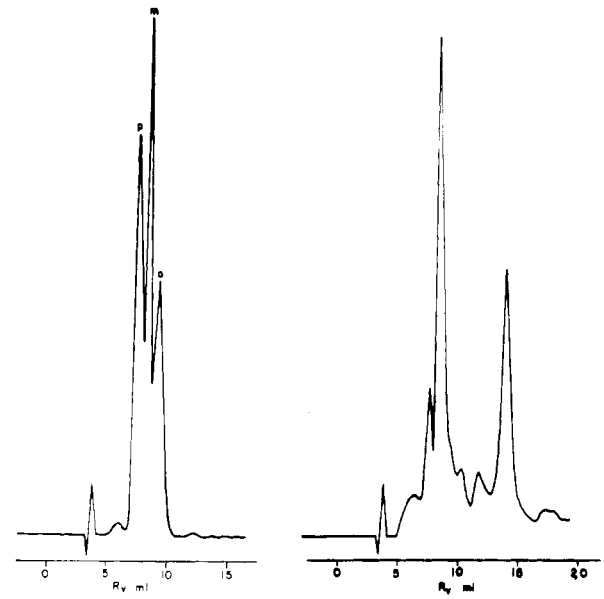


Figure 1. Separation of phytotoxins from *Rhizoctonia solani* by reversed phase LC. (Left) Standard mixture of *o*-, *m*-, and *p*-hydroxyphenylacetic acids. (Right) Hydroxyphenylacetic acids from the fungus. Column, μ Bondapak/C_{18}; solvent system, methanol–5% aqueous acetic acid (1:3).

3. Soybean Wilting Bioassay. "Harsoy" soybean seedlings grown in sterile perlite were used in this system (14- to 18-day-old nodules, 2-mm diameter). The seedlings originated from seed inoculated with a standardized suspension of *R. japonicum* 110 as used previously described (Orellana et al., 1976). After the cotyledons were removed, the roots were surface sterilized with 50% ethanol, rinsed with deionized sterile water, and placed in nitrogen-free Crone's mineral solution containing the test chemicals. Plants in these solutions were maintained under fluorescent light at approximately 7.5 klux for 12 h daily at 26 ± 2 °C. Wilting was recorded at 24-h intervals on a 0–5 (wilting) scale. Results were compared with those from plants grown in Crone's solution alone. Details and results of this bioassay are given.

4. Nitrogen Fixation Assay. Soybean plants (14 days old) with nodules (1–2-mm diameter) grown as described above were used in this study. The reduction of acetylene to ethylene by the plants, which indicates the nitrogenase-catalyzed nitrogen-fixation rates, was monitored by GC (Hardy et al., 1968, 1971). The test plants were placed in nitrogen-free Crone's solution, kept in a growth chamber under fluorescent light (350 lux, 14 h daily, 23–28 °C), and assayed when the plants that had the highest toxin concentration showed initial wilting. The concentrations of the test compounds are given with the results.

RESULTS AND DISCUSSION

Characterization of Phytotoxins. The procedure used to isolate and separate phytotoxins produced by the soybean isolate of *R. solani* in fortified Richard's solution gave two crystalline compounds I and II, which had the physical properties shown in Tables II and III. Compound I was identified as *m*-OHPAA and compound II was identified as its methoxy derivative (*m*-OMePAA) on the basis of spectroscopic data. Of particular interest in this work was the use of (i) LC for detection and separation of PAA derivatives (Figure 1) and (ii) ^1H and ^{13}C NMR for characterization of hydroxy and methoxy PAA isomers.

Earlier reports indicated the presence of PAA, *m*-OHPAA, and *p*-OHPAA in the culture filtrates of *R. solani*

Table II. Physical and Spectral Properties of Toxin Isolated from *Rhizoctonia solani* (Compound I)

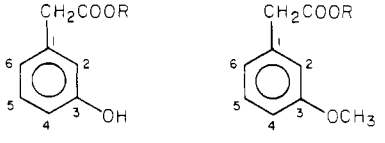
	compound I	compound I, Me ester (Ia)[a]
mp, °C	132-134	
R_f (TLC)	0.4 (75:25, $PhCH_3$-EtOH)	
R_f (PC)	0.22 (5:1, $PhCH_3$-AcOH)	
λ_{max} (MeOH), nm	281 (s) (ϵ 1563), 275 (1719), 216 (s) (5730), 209 (6565)	
ν_{max}, cm^{-1}	3226 (b) (OH), 1700 (C=), 1587, 1408, 1266, 1232, 1150, 970, 880, 770, 704 (meta substitution)	3509 (OH), 1724 (C=), 877, 730, 720, 690 (meta substitution)
^1H NMR, δ (Me_3Si)		3.54 (s) (CH_2), 3.68 (s) (CH_3), 5.90 (s) (OH), 6.60-7.35 (m) (aromatic)
^{13}C NMR, δ (Me_3Si)		41.0 (CH_3), 52.2 (CH_2), 114.5 (C-4), 116.4 (C-2), 121.5 (C-6), 129.9 (C-5), 135.4 (C-1), 156.3 (C-3), 173.0 (C=O)
MS (OMe_3Si & $COOMe_3Si$) *m/e* (% rel intensity)	297 (M^+, 7), 281 (M - 15, 9), 252 (4), 191 (32), 164 (16), 149 (3), 147 (14), 73 (100)	

[a] See structures I and Ia in Figure 2.

Table III. Physical and Spectral Properties of Toxin Isolated from *Rhizoctonia solani* (Compound II)

	compound II	compound II, Me ester (IIa)
mp, °C	72-74	
R_f (TLC)	0.45 (75:25, $PhCH_3$-EtOH)	
R_f (PC)	0.67 (5:1, $PhCH_3$-AcOH)	
λ_{max} (MeOH), nm	280 (s) (ϵ 791), 273, (916), 218 (s) (3665), 203 (7030)	280.5 (s) (ϵ 1379), 274 (1609), 217 (s) (6900), 208 (7700)
ν_{max} cm^{-1}	1715 (C=O), 1600, 1495, 1258, 1150, 1055, 878, 780, 699 (meta substitution)	1730 (C=O), 878, 775, 719, 619 (meta substitution)
^1H NMR, δ (Me_3Si)		3.61 (s) (CH_2), 3.68 (s) (CH_3), 3.78 (s) (OCH_3), 6.69-7.35 (m), (aromatic)
^{13}C NMR, δ (Me_3Si)		41.1 (CH_3), 51.9 (CH_2), 55.1 (OCH_3), 112.5 (C-4), 115.0 (C-2), 121.7 (C-6), 129.7 (C-5), 135.5 (C-1), 159.9 (C-3), 172.1 (C=O)
MS ($COOMe_3Si$), *m/e* (% rel intensity)	238 (M^+, 100), 223 (M - 15, 40), 195 (72), 191 (40), 179 (38), 163 (60), 147 (20), 135 (10), 105 (12), 89 (30), 75 (81), 73 (49)	

[a] See structures II and IIa in Figure 2.



I, R = H
Ia, R = CH_3

II, R = H
IIa, R = CH_3

Figure 2. Structural formulae for *m*-hydroxy- and *m*-methoxyphenylacetic acids and their methyl esters.

on the basis of chromatographic and IR spectral data (Aoki et al., 1963; Wu, 1965; Frank and Francis, 1976), which alone did not unequivocally demonstrate their structures. Now we have provided unequivocal proof of structures (Figure 2) for *m*-OHPAA (I) and *m*-OMePAA (II) on the basis of the following spectroscopic evidence. The ^1H NMR for compound I (*m*-OHPAA) as its methyl ester showed a singlet at δ 3.54 ppm (methylene protons, 2 H), a singlet at δ 3.68 ppm (COOMe, 3 H), a singlet at δ 5.90 ppm (*m*-hydroxyl, 1 H), and an unresolved multiplet (4 H) at δ 6.60–7.35 ppm (a pseudotriplet centered at δ 6.76 ppm, three other signals at δ 7.03, 7.16, and 7.23 ppm, respectively, for aromatic protons). The methyl ester of compound II (*m*-OMePAA) has a similar spectrum except the hydroxyl signal at δ 5.90 ppm was replaced by a methoxy singlet appearing at δ 3.78 ppm (3 H). Comparison of the spectra of Ia and IIa with the spectra of known samples (Aldrich Chemical Co., Milwaukee, WI) of methyl esters of *m*-OHPAA and *m*-OMePAA proved they were identical. The ^{13}C NMR assignments (Table II) of the methyl ester of naturally occurring I are in agreement with the structure for Ia (Figure 2). On the basis of shielding effects of the methylene substituent, the signal at 116.4 ppm was assigned C-2, and the signal at 114.5 was assigned C-4. Similarly, the signal at 135.4 ppm was assigned to C-1 and the signal at 129.9 ppm to C-5 (calculated from shielding parameters). As in phenols (Stothers, 1972), the signal at 121.5 ppm was assigned C-6 and the signal at 156.3 ppm to C-3.

The mass spectrum of the Me_3Si ether of *m*-OHPAA gave a molecular ion at *m/e* 296 (7%). This ether subsequently lost a methyl group to give a fragment ion at *m/e* 281 (9%). The other fragments appeared at *m/e* 252 (4), 191 (32), 164 (16), 149 (3), 147 (14), and 73 (100) (Mamer et al., 1971). The methoxy derivative (*m*-OMePAA) as a Me_3Si ether gave fragments at *m/e* 238 (M^+, 100), 223 (40), 195 (72), 191 (40), 179 (38), 163 (60), 147 (20), 135 (10), 105 (12), 89 (30), 75 (81), and 73 (49). Other spectral information such as UV (showing the benzene chromophore), IR (indicating the functional groups and meta substitution in the benzene ring), and chromatographic (TLC, PC, and GC) data (Tables I–IV) agree with the structures of *m*-OHPAA (I) and *m*-OMePAA (II) (Figure 2).

Biological Activity of the Phytotoxins. Phenylacetic acid and its hydroxylated derivatives have been reported as plant growth-regulating substances in auxin bioassay systems (Chamberlain and Wain, 1971; Milborrow et al., 1975). The natural occurrence of these compounds in tobacco has been reported by Whitman (Whitman, 1973; Whitman and Rauthan, 1973). Phenylacetic acid was re-

Table IV. Comparison of Gas Chromatographic Data on Phenylacetic Acids

compound	retention times,[a] min: Me ester Me_3Si	Me_3Si
o-OHPAA	2.20	3.50
m-OHPAA	2.50	4.02
p-OHPAA	2.90	4.70
o-OMePAA		2.30
m-OMePAA		2.70
p-OMePAA		2.85
natural toxins from *R. solani*		
OHPAA (compound I)	2.40	4.00
OMePAA (compound II)		2.70

[a] Column support: 3% SE-30 on 80/100 mesh silanized Gas-Chrom Q; 1.8 m × 0.16 cm (6 ft × 1/16 in.) stainless-steel column; carrier gas, nitrogen; column temperature 140 °C; injection port temperature, 250 °C; temperature, 300 °C.

Table V. Relative Plant-Growth Promoting Activity of the Isolates from the Culture Filtrate of *Rhizoctonia solani* and Hydroxyphenylacetic Acids in the Bean Second-Internode Bioassay

material	av increase in elongation over that of controls, %[a]
ether extract from the culture filtrate	113
bicarbonate-soluble portion of ether extract containing mixture of *m*-hydroxy- and *m*-methoxyphenylacetic acids	467
m-hydroxyphenylacetic acid	164
o-hydroxyphenylacetic acid	145
p-hydroxyphenylacetic acid	27
phenylacetic acid	133

[a] Six-day-old pinto bean seedlings treated with 25 μg of the test chemical in 250 μg of fractionated lanolin. Controls treated with lanolin only. Three replicates; measurements after 4 days. In this qualitative test, average values of the replicates were used to compare the elongation response to that of the controls (Mitchell and Livingston, 1968).

ported to inhibit root growth of sugar beet at a concentration of 0.05% and of rape and rice seedlings at a concentration of 0.005% (Aoki et al., 1962). All compounds except *p*-OHPAA induced cell elongation of the bean second internodes at 25 μg/plant (Table V). These results support the findings of earlier work on the plant growth-regulating activity of PAAs (Chamberlain and Wain, 1971; Milborrow et al., 1975; Frank and Francis, 1976). In the lettuce seedling bioassay (data not presented), the isolates from the culture filtrate of *R. solani*, as well as all the synthetic hydroxyphenylacetic acids, strongly inhibited root and hypocotyl elongation, compared with the controls. The treated plants grew 2–8 mm from petiole to root hairs, whereas the control plants grew 21–25 mm. This response is similar to that reported by others (Aoki et al., 1963; Nishimura and Sabaki, 1963) for *o*- and *m*-OHPAA; these compounds inhibited root growth in different test systems.

Culture filtrate at 50 ppm (crude undiluted) caused wilting of soybean seedlings in 48 h or less (Table VI). At high dilutions (12 ppm), the active compound(s) in the culture filtrate did not cause significant wilting in test plants (Table VI). The pure chemicals, ortho, meta, and para hydroxy PAAs (Table VII), and their methoxy derivatives (Table VIII) were toxic only at high concentrations. At concentrations as low as 250 ppm, *p*-OHPAA

Table VI. Toxicity Assay of the Culture Filtrate of *Rhizoctonia solani* at Different Concentrations [Nodulated 14-Day-Old Harosoy Soybean Seedlings (Grown in Perlite) Were Immersed in Culture Filtrate after Removal of Cotyledons. Replication: Three Tubes/Concentration, with Three Plants/Tube]

culture filtrate concn,[a] ppm	plant responses rating[b]: 48 h	87 h	111 h	129 h
50	3	3	3	5
25	0	3	3	5
12	0	3	3	4
5	0	0	0	3
1	0	0	0	0
0 (control)[c]	0	0	0	0

[a] Approximate concentration 50 ppm based on the weight of the whole toxic residue. [b] Rated on a 0–5 wilting scale, in which 5 indicates plants killed. [c] Crone's solution.

Table VII. Toxicity Assay of *o*-, *m*-, and *p*-Hydroxyphenylacetic Acids [Nodulated 18-Day-Old Harosoy Soybean Seedlings (Grown in Perlite) Were Immersed in Crone's Solution Containing Test Chemicals and after Removal of Cotyledons. Plants Had 2-mm Nodules. Three Tubes/Concentration with Three Plants/Tube]

	plant response rating[a]: *o*-OHPAA		*m*-OHPAA		*p*-OHPAA[b]	
concn ppm	48 h	72 h	48 h	72 h	48 h	72 h
400	5		4	5	0	3
300	3	5	0	3	0	3
250	3	4	0	3	0	3
200	2	3	0	3	0	0
150	0	2	0	3	0	0
0 (control)[c]	0	0	0	0	0	0

[a] Rated on a 0–5 wilting scale, in which 5 indicates plants killed. [b] Nodules turned brown with *p*-OHPAA at ≥250 ppm. [c] Crone's solution.

Table VIII. Toxicity Assay of *o*-, *m*-, and *p*-Methoxyphenylacetic Acids. [Nodulated 14-Day-Old Harosoy Soybean Seedlings (Grown in Perlite) Were Immersed in Crone's Solution Containing Test Chemicals and after Removal of Cotyledons. Three Tubes/Concentration with Three Plants/Tube]

	plant response rating[a]: *o*-OMePAA			*m*-OMePAA			*p*-OMePAA		
concn ppm	48 h	72 h	92 h	48 h	72 h	96 h	48 h	72 h	96 h
400	5	5	5	5	5	5	5	5	5
200	5	5	5	4	4	4	5	5	5
100	0	0	0	0	0	0	0	0	0
0 (control)[b]	0	0	0	0	0	0	0	0	0

[a] Rated on a 0–5 wilting scale, in which 5 indicates plants killed. [b] Crone's solution.

caused brown discoloration of root nodules, but the significance of this effect is not known. Furthermore, the *o*- and *m*-OHPAAs were more toxic than *p*-OHPAA.

In nitrogen-fixation studies (Table IX), the culture filtrate (50 ppm) suppressed the conversion of acetylene into ethylene by nitrogenase at 1:1 (25 ppm) and 1:4 (5 ppm) dilutions. At the 0.1% level, the synthetic OHPAAs inhibited nitrogen fixation significantly. At a higher dilution, i.e., at 0.005%, these PAA derivatives were less inhibitory. However, *m*-OHPAA was more inhibitory than *o*-OHPAA and *p*-OHPAA at this concentration.

In the previous reports (Aoki et al., 1963; Wu, 1965; Frank and Francis, 1976; O'Neill, 1976), PAA and its hy-

Table IX. Effect of Culture Filtrate of *Rhizoctonia solani* and of Synthetic Hydroxy Isomers of Phenylacetic Acid on N_2-Fixation Rates in Soybeans

material[a]	μmol of N_2 fixation rate,[b] of C_2H_4 gr-nodules^{-1} h^{-1}
culture filtrates	
25 ppm	0
5 ppm	0
m-OHPAA	
0.1%	2.79 ± 0.71
0.005%	51.10 ± 17.3
p-OHPAA	
0.1%	3.52 ± 1.09
0.005%	131.15 ± 53.4
o-OHPAA	
0.1%	1.63 ± 0.47
0.005%	71.57 ± 16.6
0 (control)[c]	118.21 ± 8.6

[a] Test solution adjusted to pH 7.0. [b] Mean plus or minus standard deviation. [c] Crone's solution.

droxy isomers were identified by chromatographic (TLC or PC) data, by UV and IR spectral data, or by both. In the present work, we show by chromatographic and spectral methods the occurrence of *m*-OHPAA and *m*-OMePAA in the culture filtrate of *R. solani* isolated from infected soybean plants. We found the LC method (Figure 1) to be very helpful in separating PAA and all the isomers of hydroxy (Warthen and Mandava, 1977) and methoxy PAA derivatives. This technique can be used routinely to detect and separate all the PAAs present in trace quantities in natural products. The UV and IR data alone did not provide sufficient information for unequivocal elucidation of the structure. The mass spectral data provided general information for the chemical structure and molecular weight. The NMR data, particularly C-13, provided information in the ring substitution. A combination of the chromatographic and spectral data unequivocally supported the structures for *m*-hydroxy- and *m*-methoxyphenylacetic acids as the fungal toxins in the culture filtrate. It is interesting to note that the culture filtrate of the soybean *R. solani* did not contain PAA even though it contained the meta methoxy derivative.

In this study, we observed that the crude culture filtrate was more toxic than the isolated *m*-OHPAA. Similar results were reported in previous investigations (Frank and Francis, 1976). Phenols and other organic acids (e.g., β-furoic, succinic) may be present in the filtrate; and although they may not have toxic or growth-regulating effects in our test systems, these compounds may enhance biological activity by interacting with toxins (Lockhart, 1965). The synergistic effects of *m*-OHPAA and *m*-OMePAA are the subject of current investigations. Our results support the concept that cell dysfunction in soybean nodules infected with *R. solani* (Orellana and Worley, 1976) may be associated with nonenzymic toxins produced by the fungus in vivo. Our results further suggest that *m*-OHPAA and its derivative *m*-OMePAA could be used in tests for selection of *Rhizoctonia*-resistant soybean plants. Although pentachlorobenzene, a common fungicide of *R. solani* (Kohmoto and Nishimura, 1974), is reported as inhibiting the synthesis of *m*-OHPAA, the practical application of this compound has not been studied. The possibility that *R. solani* strains in the four classified anastomosis groups differ in the production of the PAA toxins has not been investigated. In view of the ubiquity of *R. solani* and its implication as a pathogen of many different crop plants, such a study could be undertaken with the LC method described in this paper as a tool to screen for different PAAs at the parts-per-million levels present in different plants infected with *R. solani*. Thus, the identification of these phytotoxins suggests that they can be used for the development of a toxin-mediated bioassay to identify resistance to *Rhizoctonia* root rot in soybean or other crop plants.

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