Production of Antifungal Substances Active Against Rhizoctonia solani in Chitin-Amended Soil

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ABSTRACT

A correlation between inhibition of saprophytic activity of *Rhizoctonia solani* in chitin-amended soil and the increase in antifungal activity of *n*-butanol soil extracts as compared to nonamended soil was observed. In addition to *R. solani*, extracts of chitin-amended soil inhibited the growth of 17 other species of fungi; two species of *Fusarium* were not affected. Addition of active soil extracts resulted in inhibition of saprophytic activity of *R. solani* in natural but not in autoclaved soil. Multiplication of *Saccharomyces cerevisiae*, a polyene-sensitive organism, was not more inhibited in a chitin-amended than in a nonamended soil, suggesting that polyenes are not the active agents. The antifungal factors in the *n*-butanol extract of chitin-amended soil were separated by thin-layer chromatography into 23 ethanol-soluble active fractions. Active fractions were also obtained from ethanol extracts of bacteria, streptomycetes, and fungi isolated from chitin-amended soil and from nonamended check soil. On separation, the active substances behaved like polar lipids but their phosphorus content (0.05 to 0.7%) was much lower than that of phospholipids. It is postulated that the active factors may be nonspecific cell constituents of microbial origin.

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Chitin, like other organic amendments, causes an increase in the soil microbial populations, especially bacteria, actinomycetes, and phycomycetes (9, 11, 14). Henis et al. (8) demonstrated increased numbers of antibiotic-synthesizing microorganisms in chitin-amended soil. This was accompanied by a decrease in saprophytic activity of *Rhizoctonia* and reduction of disease severity, suggesting the possibility that *Rhizoctonia*-inhibiting substances are produced.

Antibiotic production in the particulate fraction of soil organic matter may affect the soil microflora (8, 13, 17). Manninger et al. (10) and Soulides (15) provided indirect evidence that production of antibiotics in natural soil is of ecological significance. Antibiotic substances have been detected in inoculated sterile soil, to a lesser extent in natural soil heavily amended with organic matter (2, 6, 23), but not in nonamended natural soil.

Results of previous experiments indicated that inhibition of saprophytic activity of *Rhizoctonia solani* in chitin-amended soil was caused by inhibitory substances (14). In the present study, evidence is given for the role of antifungal substances in suppressing the saprophytic activity of *R. solani* in chitin-amended soil. Particular attention was given to the polyene antibiotics in view of their widespread distribution and their specific ultraviolet absorption spectrum which allows for their easy detection in soil and culture extracts.

MATERIALS AND METHODS.—Soil type, cultures of *Rhizoctonia solani* Kuehn, and experimental conditions employed in this study were as previously described (14). Soil was amended with 0.2% (w/w) of unbleached ground chitin. All the reagents used were of analytical reagent grade. Unless otherwise stated, experiments were performed in five replicates. Soil microorganisms were isolated on soil extract agar (SEA) (3), and supplemented with 0.2% chitin in place of glucose. Colonies of soil actinomycetes antagonistic to *R. solani* were detected by the method of Henis et al. (8).

Extraction of inhibitory compounds from soil and cultures.—Aqueous extracts of soil samples were obtained either by displacing soil water with ethanol (12) or by shaking the soil for 2 hr with water or with 0.1 M phosphate buffer, pH 7.0, at a ratio of 1:1 w/v, followed by filtration through Whatman No. 1 filter paper and an HA Millipore filter (0.45 μ). Aliquots of 0.9 ml of the filtrate were mixed with 0.1 ml of tenfold concentrated Czapek's medium and tested for inhibitory activity toward *R. solani*.

n-Butanol extracts were obtained by shaking the soil with *n*-butanol at a ratio of 3:2 (w/v) for 2 hr followed by filtration through Whatman No. 1 filter paper. The extracts were evaporated to dryness in a Rotavapor (Büchi, Switzerland) at 55 C, and the dry residue was dissolved in ethanol or chloroform.

Cultures of soil organisms were grown on soil extract broth (SEB) (3), with 0.2% chitin replacing glucose. Aliquots of 100-ml medium contained in 250-ml Erlenmeyer flasks were inoculated with the cultures and incubated with shaking at 30 C for 4 days. The cultures were harvested by centrifugation at 10,000 *g* for 15 min, the precipitates extracted with ethanol, and the extracts subjected to chromatographic analysis.

Inhibition of R. solani and other fungi by soil and culture extracts.—Aliquots of the soil and culture extracts were applied to 50-mm **diam** discs of Whatman No. 1 filter paper and placed in petri dishes of the same diam; and the solvent was evaporated to

dryness. One ml of Czapek's broth containing 250 μg/ml chloramphenicol was added, and a 3-mm disc from the edge of a 3-day-old culture of *R. solani* grown on potato-dextrose agar (PDA) was then placed at the center of each filter paper disc. After 48 hr of incubation at 28 C, the filter paper discs were stained with cotton-blue-lactophenol solution, washed with tap water, and dried. The fungal colony appeared as a blue print on the white filter paper. Solvent samples alone, similarly treated, were used as controls. These did not differ from controls without solvent. Percentage inhibition of fungal growth was calculated from the formula $(1 - \frac{A}{B}) \times 100$, where A is the colony diameter grown in presence of the extract; and B, the colony diameter of the control.

Saprophytic activity of R. solani in soil.—This was tested by the segment colonization method, in which the percentage of bean stem segments colonized by *R. solani* was determined and by hyphal growth through soil, where soil samples taken from concentric zones at various distances from the inoculum disc were tested for the presence of *R. solani*, using bean stem segments as baits (14).

Thin-layer chromatography (TLC) of soil and culture extracts.—Aliquots of soil and culture extracts were evaporated to dryness in a Rotavapor, and the residue was dissolved in *n*-butanol, chloroform, or ethanol. Aliquots (0.3 ml) of these solutions were applied to Silica Gel G plates as a thin streak along the starting line, dried, and run in ethanol-ammonia-water, 8:1:1; chloroform-methanol-water, 100:15:1 and 65:25:4; and in petrol ether-diethyl ether-acetic acid, 90:10:1 (16). A vertical section of each plate was sprayed with 0.5% $KMnO_4$ solution; and 10 min later, with 0.1% bromphenol blue solution (1). Horizontal strips of silica gel layers of the unsprayed sections containing zones parallel to those which reacted positively to $KMnO_4$ were collected separately and extracted with *n*-butanol, the extracts evaporated to dryness, and the residues dissolved in ethanol.

Chemical and physical tests.—The presence or absence of amino groups was determined by spraying the TLC plates with ninhydrin (16). Phosphorus was determined according to the method of Chapman & Parker (4). Ultraviolet absorption spectra of the ethanol extracts of actinomycete cultures were recorded using a Beckman DB spectrophotometer.

Detection of substances inhibitory to Saccharomyces cerevisiae in chitin-amended soil.—This yeast was chosen as the test organism because of its sensitivity to polyene antibiotics, and because its growth in soil can be quantitatively estimated. In preliminary experiments, growth of *S. cerevisiae* was completely inhibited by the polyene antibiotic trichomycin (TEVA Ltd., Jerusalem) at 0.2 units/ml, as compared to 5 units/ml required for complete inhibition of *R. solani*. The test medium was yeast-dextrose agar (YDA) composed of (g/liter) glucose, 20; Bacto peptone, 5; Bacto yeast extract (Difco), 5; and Bacto agar (Difco), 20, in distilled water. Samples of 500 g of soil were inoculated with washed suspensions of cells of *S. cerevisiae* grown on YDA, at a final concentration of 10^3 cells/g of soil. The soil was mixed with 10 ml yeast-dextrose broth (YDB), 2.5 mg diphenyl (dissolved in 0.1 ml ethanol), and 100 mg chloramphenicol. After incubation at 30 C for 24 to 48 hr, the soil was diluted with 0.1% agar in sterile tap water; and 1-ml aliquots were taken for counting in YDA supplemented with 250 μg/ml chloramphenicol added to prevent overgrowth of soil bacteria and actinomycetes. In order to prevent growth of filamentous fungi during the incubation period, 0.1-ml aliquots of 10 μg/ml diphenyl solution in ethanol were applied to discs of sterile filter paper placed on the inside of the covers of the inverted petri dishes. Counts were made after an incubation period of 48 hr at 30 C.

RESULTS.—Linear growth of *R. solani* was inhibited 20% and 52% with *n*-butanol extracts on filter paper discs obtained from nonamended and chitin-amended soils, respectively. No inhibition was observed with aqueous extracts of these soils. Autoclaving the soil (121 C at 1 atm for 20 min) before extraction, did not affect the inhibitory activity of the *n*-butanol extract.

In comparing inhibition of saprophytic activity of *R. solani* in chitin-amended soil and the antifungal activity of an *n*-butanol extract of this soil to that of a nonamended one, we found both greater after 21 days than after 4 days' incubation (Table 1). This may indicate that the same factors can be involved in both inhibition phenomena.

In another experiment, *n*-butanol residues obtained from chitin-amended soil were more active than similar extracts of nonamended soil in inhibiting linear growth of *R. solani*. Equal amounts (3.2 mg dry wt/disc) inhibited growth by 52% and 8%, respectively.

The activity of extracts from chitin-amended soil towards several filamentous fungi was tested by the filter paper disc technique. With the exception of *Fusarium solani* f. sp. *phaseoli* and *F. moniliforme*, all (including *F. oxysporum* and *F. solani*) were

TABLE 1. Inhibition of the saprophytic activity of *Rhizoctonia solani* in chitin-amended soil after 4 and 21 days, correlated with growth inhibition by residues of *n*-butanolic extracts of the soil

Days of incubation	Amendment	Inhibition of colonization[a] (%)	Extract residue (dry wt), mg/300 g soil	Growth inhibition by residue[b] (%)
4	None	0	35	4
4	Chitin	40	41	15
21	None	0	35	3
21	Chitin	90	114	51
LSD 5%		8		

[a]In each treatment, 100 bean segments were incubated in the soil for 24 hr and examined for colonization with *R. solani*.

[b]Residues of extract of 20 g soil were applied to each filter paper disc.

TABLE 2. Inhibitory activity[a] of extracts obtained from chitin-amended and nonamended soils towards various fungi

Fungus	% Inhibition by extracts from Chitin-amended soil	Nonamended soil
Rhizoctonia solani	81	16
Trichoderma lignorum	82	50
Aspergillus sclerotiorum	80	40
Achlya bisexualis	46	21
Sclerotium rolfsii	43	8
Sclerotinia sclerotiorum	100	81
Epicoccum sp.	66	50
Penicillium sp.	59	37
Gliocladium roseum	55	18
Alternaria tenuis	21	24
Chaetomium sp.	45	0
Geotrichum sp.	29	0
Fusarium oxysporum f. sp. *lycopersici*	33	12
Fusarium solani f. sp. *phaseoli*	0	0
Fusarium solani	16	8
Fusarium moniliforme	0	0
Cladosporium sp.	36	20
Rhizopus nigricans	70	50
Cunninghamella sp.	46	46
Bipolaris sorokiniana	77	52

[a]Tested on filter paper discs in petri dishes, each containing 0.3 ml concentrated extract representing 60 g soil.

TABLE 3. *Rhizoctonia solani* hyphal growth and bean segment colonization in natural and sterile nonamended soils supplemented with extract residues of chitin-amended soil and infested with *R. solani*

Treatment	Hyphal growth through soil[a] Natural soil A	B	C	Sterile soil A	B	C	Segment colonization[b] (%) Natural soil	Sterile soil
None	4.5	2.6	0	5	5	5	64.0	100
Chloroform[c]	3.0	2.2	0	5	5	5	65.0	100
Soil extract[c]	0.3	0	0	5	5	5	9.0	100
LSD 5%							10.5	

[a]Tested for presence of *R. solani* in concentric zones at various distances from the inoculum disc. A = 5-40 mm; B = 41-70 mm; C = 71-90 mm. The data are expressed as the average colonized segments from 5 in each zone.

[b]In each treatment, 100 bean segments were incubated in the soil for 24 hr and examined for colonization with *R. solani.*

[c]Soil extract residue of 6 kg soil was dissolved in 30 ml chloroform and mixed with 100 g soil; and the chloroform evaporated; this sample was then mixed with 900 g soil. Chloroform alone was used as control. *n*-Butanol was not used because it could not be evaporated completely.

inhibited to a varying extent by the soil extracts (Table 2).

Effect of n-butanol extracts obtained from chitin-amended soil on growth of R. solani in natural and sterile nonamended soils.—Preliminary recovery experiments of active extracts added to nonamended soil indicated that only a small part of the inhibitory substances could be extracted with the technique used. Therefore, the residue obtained from 6 kg chitin-amended soil by *n*-butanol extraction was dissolved in 30 ml chloroform, added to 100 g soil, and the chloroform evaporated; the residue was then mixed with 900 g of natural nonamended soil or soil sterilized by autoclaving. Saprophytic activity of *R. solani* was found to decrease in the natural, but not in the sterile, soil (Table 3).

Thin-layer chromatography of extracts from soil and cultures.—Five fractions of the crude extract of chitin-amended soil were separated by chromatography with ethanol:ammonia:water (8:1:1). Each fraction was collected and chromatographed twice, using the same solvent system. Values of R_F, yields, and inhibitory activity of the various fractions are shown in Table 4. Considerable amounts of material were lost during separation and purification. All fractions reacted positively with $KMnO_4$ and bromphenol blue. Fraction 5 was stained yellow by bromothymol blue, but did not react with benzidine or ninhydrin. Fraction 3 reacted positively with ninhydrin, and fractions 1 and 2 reacted with benzidine.

Fractions 1, 2, and 4 had no inhibitory effect on *R. solani* at concentrations up to 1 mg/disc. However, fraction 5 (1 mg/disc) inhibited linear growth of *R. solani* by 52%, as compared to 26% inhibition observed with the same fraction from nonamended soil. Fraction 5 was the most abundant and active. It was found to be insoluble in water, partially soluble in petroleum ether, and soluble in methanol, ethanol, *n*-propanol, *n*-butanol, amyl alcohol, acetone, ethyl acetate, diethyl ether, benzene, xylene, and chloroform. It was further separated into three fractions as follows. Nine ml of petroleum ether were mixed with 1 ml of a concentrated ethanolic solution of fraction 5. After centrifugation at 5,000 *g* for 10 min, the sediment was washed 3 times with a solution of petroleum ether:ethanol (9:1). The supernatant was twice evaporated to dryness and dissolved in ethanol. After the second evaporation, the dry material was extracted with petroleum ether. The soluble fraction was designated as No. I. The remaining fraction, which was insoluble in pure petroleum ether, was designated as No. II, and the sediment as No. III. Fractions I, II, and III were further separated with chloroform-methanol-water to fractions Ia-Ih, IIa-IIe, and IIIa-IIIe, respectively. Extractions were also carried out with distilled water instead of the active fractions. *n*-Butanol extracts of distilled water served as control. The chromatographed extracts did not yield any $KMnO_4$-positive spots, and were nontoxic to *R. solani*. Fraction Ih was the largest one obtained. It was further separated on petrol ether-diethyl ether-acetic acid (90:10:1) into five fractions designated as Iha to Ihe. These separations finally yielded 22 fractions (Table 4) which could not be separated further by any of the four different solvent systems used.

Cultures of bacteria (14 isolates), actinomycetes

TABLE 4. Fractions obtained by thin-layer chromatography of residues from *n*-butanol extracts of 20 kg chitin-amended soil and their activity towards *Rhizoctonia solani*

Solvent system[a]	Fraction no.	R_F	Dry weight[b] (mg)	Inhibition growth of fungus[c] (%)
EAW	1	0.00	332	0
(8:1:1)	2	0.18	36	4
	3	0.63	180	40
	4	0.71	178	0
	5	0.86	510	52
CMW	I a[d]	0.25	0.50	70
(100:15:1)	I b	0.36	0.70	74
	I c	0.45	1.20	72
	I d	0.50	1.80	54
	I e	0.56	0.80	60
	I f	0.64	3.90	60
	I g	0.75	6.80	64
	I h	0.90	35.90	70
CMW	II a	0.46	0.90	74
(100:15:1)	II b	0.59	1.20	60
	II c	0.67	1.60	50
	II d	0.82	1.70	60
	II e	0.86	11.30	60
CMW	III a	0.00	0.80	46
(65:25:4)	III b	0.17	4.30	68
	III c	0.23	7.70	60
	III d	0.53	3.10	70
	III e	0.80	2.60	64
PE DE AA	Iha	0.00	3.20	78
(90:10:1)	Ihb	0.04	2.60	40
	Ihc	0.06	4.20	44
	Ihd	0.15	3.20	64
	Ihe	0.82	4.90	66

[a]E = ethanol; A = ammonia; W = water; C = chloroform; M = methanol; PE = petroleum ether; DE = diethyl ether; AA = acetic acid.

[b]The dry weight of the crude extract from 20 kg soil was 3,460 mg, obtained by extracting soil with *n*-butanol at a ratio of 3:2 (w/v) and evaporated to dryness.

[c]One mg of each fraction was applied to each filter paper disc. Per cent inhibition calculated as $(1 - \frac{A}{B}) \times 100$, where A = colony diam in treatment; B = colony diam in control.

[d]Fraction 5 was separated into three main subfractions I, II, and III, according to solubility in petroleum ether. These were further separated to fractions Ia-Ih, IIa-IIe, and IIIa-IIIe, respectively. Fraction Ih was further separated to Iha-Ihe.

(22 isolates), and fungi (11 isolates), obtained from chitin-amended soil, were grown on SEB, extracted, and chromatographed. All extracts yielded fractions of R_F value identical to fraction 5 and were inhibitory to *R. solani* when applied at a concentration of 1 mg dry residue/disc.

None of the fractions was sufficiently pure to permit determination of physical properties. Similarly, none of their ultraviolet spectra showed any specific absorption at any wavelength. Phosphorus content of these fractions ranged between 0.05 and 0.7%.

Production of inhibitory substances in chitin-amended sand.—In order to minimize the relatively high background of inhibitory activity towards *R. solani* obtained with extracts of nonamended soil, quartz sand was mixed with 2,000 μg/g chitin, adjusted to 50% moisture-holding capacity with a solution of Czapek's medium without glucose, and autoclaved. An amount of 10^9 cells of bacteria (isolated from chitin-amended soil), or 500 mg of natural soil, were added to 100 g of the sterile mixture. Sterile chitin-amended sand was used as a control. After a 7-day incubation period, the inoculated sand samples and the control were tested for saprophytic activity of *R. solani* (using the method of hyphal growth through soil) for yields of residues of *n*-butanol extracts and for inhibitory activity of these residues towards *R. solani*. In the noninoculated control, the fungus spread throughout the petri dishes but remained restricted to the central zone in the soil-inoculated and in the bacteria-inoculated sand. Residue yields of ethanol extracts of control, bacteria-inoculated sand, and soil-inoculated sand were 6, 26, and 24 mg/100 g sand, and percentage inhibition of *R. solani* (1 mg/disc) was 8, 48, and 62, respectively.

The possible role of polyene antibiotics in the inhibition of R. solani in chitin-amended soil.—Ultraviolet spectra of ethanol extracts of actinomycete cultures of 30 different colonies antagonistic to *R. solani*, isolated from chitin-amended soil, were examined. All the extracts showed distinct peaks at 360, 380, and 403 nm (Fig. 1) characteristic of polyene antibiotics of the heptaene group (21). Soil was amended with chitin and then inoculated 10 days later with the polyene-sensitive yeast *S. cerevisiae*. After incubation for 48 hr, the count of *S. cerevisiae* was 173×10^3/g as compared with 148×10^3/g in nonamended soil, indicating that there is no polyene antibiotic production in amended soil.

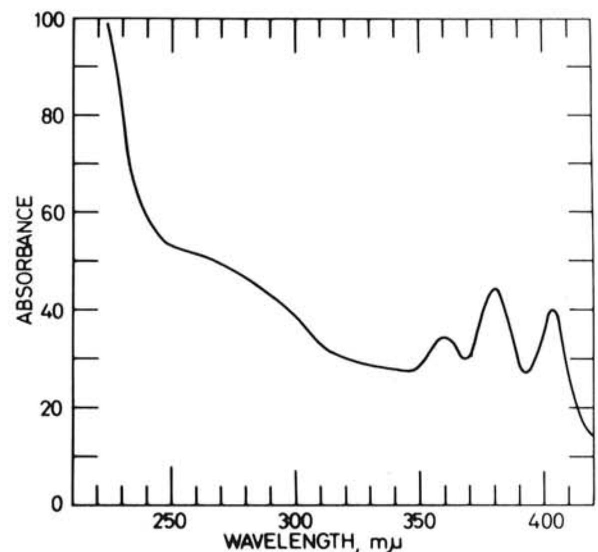

Fig. 1. Ultraviolet spectrum of ethanol extracts of actinomycete cultures.

In another experiment, nonamended soil was inoculated with 10^4 cells/g of the yeast, mixed with trichomycin at concentrations of 0.5 and 25 units/g soil, and incubated for 24 hr at 30 C. At the higher trichomycin concentration, which is 125-fold higher than that required for complete inhibition of the yeast, growth was inhibited only by 50% (42×10^4 cells/g as compared to 62×10^4 cells/g with 0.5 units and 81×10^4 cells/g in the control). Thus, the role of this polyene antibiotic in the inhibition of *R. solani* in chitin-amended soil could not be demonstrated by this method.

DISCUSSION.—*n*-Butanol extracts of chitin-amended soil contain substances inhibitory to fungal growth. Smaller amounts of similar substances having a much lower specific activity are found in natural nonamended soil. Saprophytic activity of *R. solani* was inhibited in natural soil mixed with *n*-butanol extracts of chitin-amended soil, whereas no inhibitory effect on *R. solani* was demonstrated in autoclaved soil. This is in accordance with the reported loss of inhibitory activity of chitin-amended soil which occurs upon autoclaving (14), in spite of the thermostability of the antifungal activity. Possibly, inhibition of *R. solani* in chitin-amended soil is counteracted by the increased presence of available nutrients (5, 20, 22) in autoclaved soil (18). Suppression of saprophytic activity of *R. solani* in chitin-amended soil was positively correlated with growth inhibition in media containing the *n*-butanol extracts. The inhibitory compounds could be extracted from chitin-amended quartz sand inoculated with soil microorganisms. Finally, growth of *F. solani* f. sp. *phaseoli* was not affected by *n*-butanol chitin-amended soil extracts, and, as shown in a previous study (14), the severity of bean root rot caused by this fungus was not decreased in chitin-amended soil.

These findings indicate that inhibitory substances which develop in chitin-amended soil play a major role in the inhibition of the saprophytic activity of *R. solani*, whereas lysis and competition for available nutrients are less important (14). The presence of antifungal substances in amended soil has been demonstrated (19).

All the actinomycetes isolated from chitin-amended soil synthesized polyene antibiotics in culture. However, the possibility that polyene antibiotics are involved in the inhibition of *R. solani* in chitin-amended soil seems unlikely due to the lack of inhibition of the polyene-sensitive yeast *S. cerevisiae*.

In their solubility properties and behavior in TLC, the active substances resemble polar lipids (16). The *n*-butanolic extract of the chitin-amended soil was separated by TLC into 26 fractions, apparently still impure, hence not permitting evaluation of their precise chemical composition.

Henis & Inbar (7) found three fractions of antifungal polar lipids in extracts of cultures of *Bacillus subtilis*. We found substances having the same R_F values on chromatographic analysis in extracts of chitin-amended soil. It seems that part of the lipid fraction synthesized by the enriched soil microflora of chitin-amended soil, including bacteria, actinomycetes, and fungi, possesses antifungal activity.

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