

## Mode of Inhibition of *Rhizoctonia solani* in Chitin-Amended Soil

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### ABSTRACT

Saprophytic growth and pathogenicity of *Rhizoctonia solani* were reduced in natural, chitin-amended soil during 22 and 42 days of incubation, respectively. Increasing chitin concentrations from 500 ppm to 8,000 ppm resulted in increased inhibition of saprophytic growth during the first 3 days, but did not affect maximum inhibition. Inhibition of saprophytic growth could not be detected in sterilized soil amended with chitin, or in amended soil which was autoclaved or gamma-irradiated after a 14-day incubation period.

Addition of lignin had no significant effect on pathogenicity of *R. solani*, nor did it increase the inhibitory effect of chitin. Both chitin and lignin alone and the two in combination had no significant effect on disease severity of bean caused by *Fusarium solani* f. sp. *phaseoli*. No parasitism or lysis of

*Additional key words:* *Phaseolus vulgaris*, volatile substances, root rot.

*R. solani* hyphae by soil organisms was observed in chitin-amended soil. Addition of glucose or ammonium nitrate to chitin-amended soil did not reduce the inhibition of saprophytic growth of *R. solani*. Volatile substances produced in chitin-amended soil inhibited saprophytic growth of *R. solani* only during the first 12 days of incubation. The fungus was not inhibited in sucrose-amended soil in which CO<sub>2</sub> was produced in greater quantities than in chitin-amended soil. Inhibition was not correlated with competition between *R. solani* and the soil microflora for available nutrients, but seemed to result from the effect of inhibitory compounds of an unknown nature produced by the soil microflora during chitin decomposition. *Phytopathology* 61:1113-1117.

Much information is available regarding the biological control of various soil-borne diseases by soil organic amendments. Populations of microorganisms that decompose hyphal cell walls of various *Fusarium* spp. increased by the addition of chitin and laminarin to soil (16). Henis et al. (8) obtained effective control of *Rhizoctonia* in chitin-amended soil. They demonstrated that the reduction of disease caused by *Rhizoctonia*, the suppression of the saprophytic activity of *Rhizoctonia*, and the increase in counts of soil microorganisms are correlated. The resistance to lysis of *R. solani* hyphae in soil has been reported by several authors (9, 12, 13, 20). Potgieter & Alexander (20) demonstrated that chitinase and  $\beta$ -(1  $\rightarrow$  3) glucanase, which incite lysis of *Fusarium* sp. cell walls, do not affect cell walls of *R. solani*. They relate this resistance to the presence of melanins in the hyphal cell wall of *R. solani*. A similar phenomenon was demonstrated by Chet & Henis (3) in *Sclerotium rolfsii*.

Maurer & Baker (14, 15) suggest that control of pathogens by soil amendments may involve competition for nutrients. They demonstrated (14) that in bean root rot caused by *F. solani* f. sp. *phaseoli*, disease severity decreased in soil amended with chitin and lignin, whereas chitin alone had no effect.

Addition of KNO<sub>3</sub> to these amendments further decreased disease severity, whereas glucose nullified the control of the disease. Similarly, Ko & Lockwood (11) relate soil fungistasis (4) to competition for nutrients between fungal spores and the soil microflora. The possible role of antibiotics in the control of phytopathogenic fungi in chitin-amended soil has been considered

by Henis et al. (8), who observed an increase in the number of antibiotic-producing microorganisms in natural, chitin-amended soil. Volatile substances such as CO<sub>2</sub> (5, 19), produced during decomposition of organic matter in the soil, may also affect fungal activity. The purpose of the present study was to determine the mechanism(s) involved in the inhibition of *R. solani* in chitin-amended soil.

**MATERIALS AND METHODS.**—The soil used was *Rhizoctonia*-free, as tested by the segment colonization and by the disease index methods (21). It consisted of 82.3% sand, 2.3% silt, 15.4% clay, and 0.45% organic matter; it had a pH of 7.4 and a moisture-holding capacity (MHC) of 14.2%. The soil was air-dried and stored until required. It was artificially infested either with *Rhizoctonia solani* Kuehn or with *Fusarium solani* (Mart.) Appel & Wr. f. sp. *phaseoli* (Burk.) Snyder & Hans. Mycelium of *R. solani* [grown on thin layers of yeast extract dextrose broth (YDB) (7) for 6 days] was homogenized in a Waring Blendor for 30 sec and mixed with the soil at a final concentration of 165 mg wet wt/kg. Washed conidia of *Fusarium solani* f. sp. *phaseoli* were added to soil at a final concentration of  $5 \times 10^7$ /kg.

Unless otherwise stated, the soil was amended with 2,000 ppm unbleached chitin (Nutritional Biochemical Co., Cleveland, Ohio), or 4,600 ppm lignin (Indulin A, West Virginia Pulp and Paper Co., 230 Park Ave., New York, N.Y.), or with both. The chitin was ground in a plant mill. Nonamended soil samples served as controls throughout the experiments.

Possible competition between *R. solani* and soil

microflora for available carbon and nitrogen sources was tested by adding to soil various concentrations of glucose and ammonium nitrate, either separately or in combination.

All experiments were replicated 5 times. Those involving bean seedlings were conducted in 14- × 14- × 7-cm plastic boxes, each containing 1,000 g soil and 20 *Phaseolus vulgaris* L. 'Brittle Wax' seedlings. For others, 9- × 9- × 10-cm boxes were used with 500 g soil in each. Soil moisture was adjusted to the required MHC by spraying the soil with the appropriate quantity of water and mixing. The boxes were covered with polyethylene sheets to allow exchange of gases while keeping soil moisture constant. In the greenhouse, boxes were immersed in Wisconsin temperature tanks at 28 C.

Disease was estimated as described by Sneh et al. (21), using an index ranging from 0 (no disease) to 5 (seedling completely girdled).

Saprophytic activity of *R. solani* was estimated after 7 days' incubation, unless otherwise stated, using the following methods: (i) *The slide technique*.—Glass slides were covered on one side with a thin layer of water agar (WA). A 3-mm diam disc from the edge of a 3-day-old culture of *R. solani* grown on potato-dextrose agar (PDA) was placed in the center of each slide, which was then placed vertically in the soil. After an incubation period of 24 hr, the slides were lifted, washed gently with tap water, covered with 0.1% cotton blue in lactophenol solution, washed again, and air-dried. Growth index was determined by multiplying the number of hyphae emerging from each inoculum disc by their average length. Eight slides were used for each treatment.

(ii) *The bean segment colonization method*.—Saprophytic activity of *R. solani* in the soil is positively correlated with the colonization per cent of bean stem segments with this pathogen (21). One hundred segments were used for each treatment. The segments were incubated in the soil for 24 hr, and the colonization of each segment was determined by plating them on chloramphenicol-WA, and incubating for 24 hr before examining microscopically (21). Results are expressed as per cent of segments colonized by *R. solani*.

(iii) *Growth of hyphae through soil*.—One 3-mm diam disc of washed *R. solani* mycelium taken from the edge of a 6-day-old YDB culture grown in petri dishes was placed in the center of each of five petri dishes containing 100 g of soil. After 4 days' incubation, the soil in each petri dish was divided into three concentric zones, 5-40 mm (A), 41-70 mm (B), and 71-90 mm (C) diam from the center. These were transferred separately to 50-mm diam petri dishes, and 5 bean stem segments were placed in each. After 24 hr, the segments were tested for colonization as in the segment colonization method.

*Soil sterilization*.—Soil was sterilized either by autoclaving (121 C at 1 atm for 1 hr on 2 successive days), or by exposure to a dose of 3 Mrad of <sup>60</sup>Co gamma rays. Sterilized samples were streaked on YDA and examined for growth of microorganisms after incubation at 30 C for 48 hr.

*Detection of volatile inhibitory substances in the soil*.—Open 50-mm petri dishes containing 1 ml WA plus 250 ppm chloramphenicol were inoculated with 3-mm diam discs of *R. solani*, covered with thin nylon mesh, and buried vertically in the soil. The nylon mesh prevented direct contact between the soil and the inoculum on the agar, but allowed free exchange of gases between them. After 24 hr, the petri dishes were lifted from the soil, and linear growth of *R. solani* colonies was recorded.

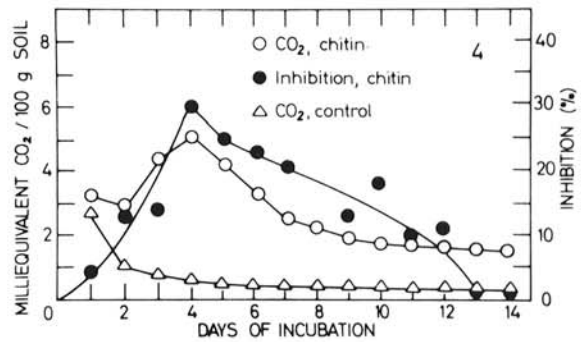
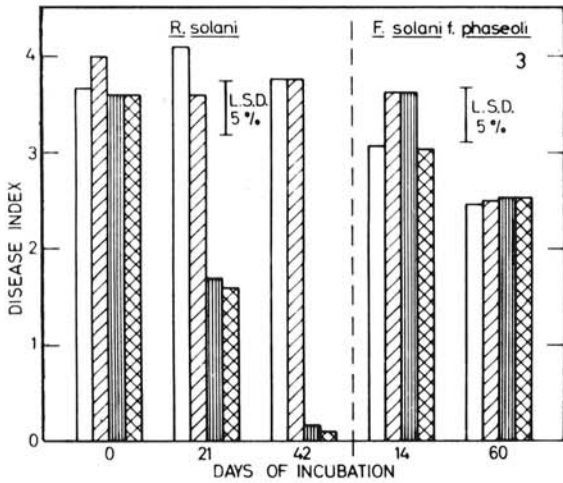
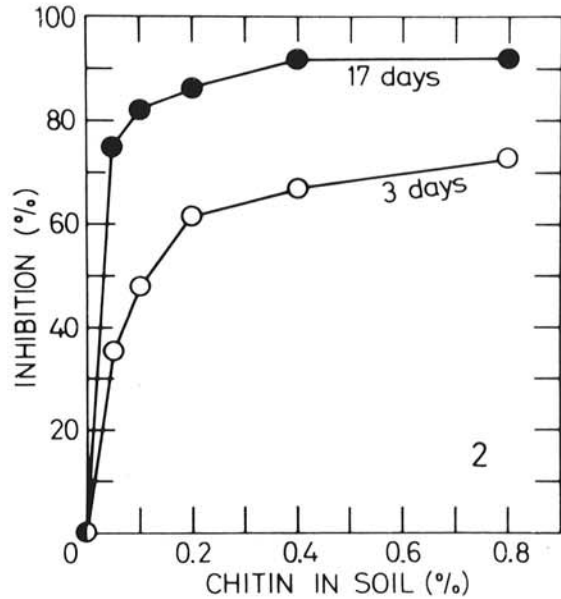
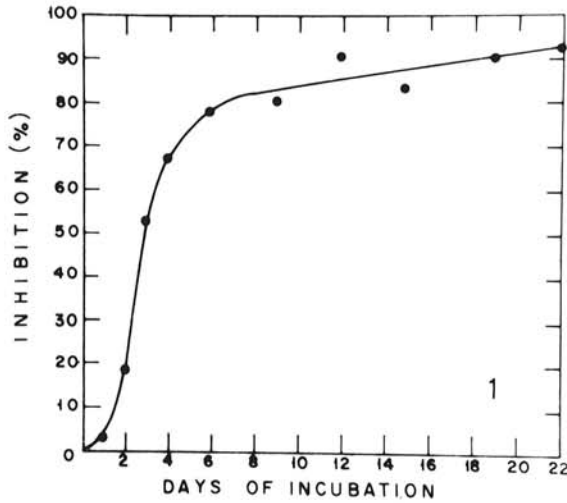
*Carbon dioxide determination*.—One hundred g soil samples were adjusted to 50% MHC and placed in 500-ml Erlenmeyer flasks. A porcelain crucible containing 5 ml 0.5 N NaOH solution was hung from the rubber stopper of each flask. After an incubation period of 24 hr, the solution was titrated with 0.05 N HCl in the presence of 1 ml 25% BaCl<sub>2</sub>, using phenolphthalein as indicator.

*Effect of soil volatiles on the growth of R. solani in liquid cultures*.—Water-saturated air was passed through 1,000 g chitin-amended soil adjusted to 50% MHC from the 3rd to 7th day of incubation. The emerging air carrying soil volatiles was sterilized by passing through a sterile cotton filter. It was used for aerating *R. solani* cultures (grown in 250-ml Erlenmeyer flasks containing 100 ml YDB plus 250 ppm chloramphenicol) at a rate of 1,500 ml/hr for 1 hr/day. After 3 days' incubation, the mycelium was washed with tap water, dried at 65 C for 12 hr, and weighed.

**RESULTS**.—*Inhibition of R. solani in chitin-amended soil*.—Inhibition of *R. solani* in chitin-amended soil as estimated by the slide technique increased rapidly during the 1st week after addition of chitin, then remained constant throughout the experimental period (Fig. 1). In soil amended with as little as 0.05% chitin and incubated for 3 days, saprophytic growth of *R. solani* was inhibited by 35%, whereas in soil amended with 0.8% chitin, inhibition was 73% (Fig. 2). The effect of chitin concentration on inhibition became less obvious after 17 days of incubation.

Inhibition of *R. solani*, as tested by the slide technique, was not affected by a wide range of soil moisture values from 30% to 80% MHC on the 9th day of incubation (Table 1). No lysis of *R. solani* mycelium was observed by extensive microscopic examination made during these experiments.

*Effect of soil sterilization*.—The saprophytic activity of *R. solani* was tested in nonamended and chitin-amended samples which were incubated for 7 days. When tested by the method of hyphal growth through soil, the number of segments (out of five) colonized with *R. solani* in each of the three concentric zones (A, B, and C) was 5, 3, 1 and 3, 0, 0 in nonamended and chitin-amended soils, respectively. When both incubated soils were sterilized either by autoclaving or by irradiation, segments in all zones were completely colonized. Complete colonization was also obtained with soils sterilized before incubation and tested after 7 days under aseptic conditions. Thus, sterilization resulted in complete nullification of the inhibitory effect in chitin-amended soil and in a prominent increase in



□ None    ▨ Lignin 0.46%    ▩ Chitin 0.1%    ▤ Lignin + Chitin

**Fig. 1-4.** 1) Changes with incubation time of percentage inhibition of *Rhizoctonia solani* in soil amended with 2,000 ppm chitin (slide technique). 2) Effect of chitin concentration (per cent) in soil on inhibition of *R. solani* (slide technique). 3) Effect of chitin and lignin on severity of bean seedling disease caused by *R. solani* and *Fusarium solani* f. sp. *phaseoli*. 4) Changes with incubation time of daily production of CO<sub>2</sub> and per cent inhibition of *R. solani* by inhibitory volatiles in chitin-amended soil (buried petri dish technique).

mycelial growth in the nonamended soil. Similar results were also obtained using the slide technique.

**Effect of glucose and ammonium nitrate on inhibition of *R. solani* in chitin-amended soil.**—Possible competition between soil microorganisms and *R. solani* for carbon and nitrogen sources was tested by the addition of glucose and ammonium nitrate 7 days after chitin incorporation (Table 2). Saprophytic activity of *R. solani* was tested by the slide technique and segment colonization. Inhibition of *R. solani* in chitin-amended soil was not nullified by 0.1% glucose, 0.02% ammonium nitrate, or 0.1% ammonium nitrate, either separately or in combination. Similar results were also obtained with the method of mycelial growth through soil. In both chitin-amended and nonamended soils,

addition of glucose at a final concentration of 1%, either alone or with 0.1% ammonium nitrate, resulted in complete suppression of the saprophytic activity of *R. solani*. This was accompanied by an extensive colonization of the bean segments with various fungi, especially phycomyces. Similarly, addition of 0.1% NH<sub>4</sub>NO<sub>3</sub> to both amended and nonamended soils resulted in partial inhibition of *R. solani*.

**Effect of chitin and lignin amendments on bean disease caused by *R. solani* or *F. solani* f. sp. *phaseoli*.**—Soil amendment with chitin, followed by immediate sowing of beans, did not affect the severity of damping-off caused by *R. solani*. When, however, chitin-amended soil was incubated before sowing, disease severity was much reduced (Fig. 3). Twenty-one days' incubation

TABLE 1. Inhibition of *Rhizoctonia solani* in chitin-amended soil at different moisture levels as tested by the slide technique

Incubation time, days	% Soil moisture <sup>a</sup>	Growth index <sup>b</sup>		% Inhibition
		Non-amended soil	Chitin-amended soil	
5	30	644	344	47
	50	618	213	66
	80	705	216	69
9	30	457	48	90
	50	508	62	88
	80	552	35	94
LSD 5%				13.2

<sup>a</sup> Expressed as % of soil moisture-holding capacity.

<sup>b</sup> Calculated by multiplying the number of hyphae emerging from each inoculum disc by the average hyphal length in mm.

reduced disease index to 1.6; 42 days' incubation further reduced it to 0.2 as compared to 4.2 and 3.8, respectively, in the nonamended soils.

Lignin did not significantly reduce disease severity following either incubation period. Combined lignin-chitin amendments were not more effective than chitin alone.

No significant differences in the severity of disease caused by *F. solani* f. sp. *phaseoli* were observed on bean seedlings sown after 14 and 60 days' incubation of chitin or lignin amendments (Fig. 3).

*Production of inhibitory volatile substances in chitin-amended soil.*—Inhibition of *R. solani* by the soil gaseous phase was detected in buried petri dishes after 1-day incubation of chitin-amended soil. Inhibition increased till the 4th day of incubation, after which it gradually decreased, disappearing on the 13th day (Fig. 4).

In another experiment, air containing volatiles collected from chitin-amended and nonamended soils was used for the aeration of YDB cultures of *R. solani*. Mycelial dry weight in these two treatments was 333 and 515 mg, respectively, and 452 mg in the same but un-aerated medium (LSD 5% was 95 mg). However, elimination of the volatiles by continuous aeration of 100-g samples of chitin-amended soil with water-saturated air at a rate of 1,500 ml/hr during the first 5 days did not reduce the 53% inhibition of *R. solani* as tested with the slide technique on the 5th day.

Production of CO<sub>2</sub> in chitin-amended soil followed a similar pattern to that of the inhibitory activity of volatiles (Fig. 4). Therefore, the possibility that CO<sub>2</sub> released during chitin incubation in the soil is the main volatile inhibitory factor (2, 19) was tested, using a sucrose-amended soil as a model system. Soil samples were amended with various amounts of sucrose, ranging from 15 to 65 mg/100 g. These amounts were calculated to yield a little more CO<sub>2</sub> than the daily production in chitin-amended soil throughout the 14-day incubation period. By this technique, the in situ production and distribution of CO<sub>2</sub> in the soil could be expected to resemble that occurring in chitin-amended soil. Carbon dioxide and volatile inhibitory activity were determined every 24 hr. No inhibitory activity could be observed in the sucrose-amended soil throughout the 14-day incubation period.

**DISCUSSION.**—Saprophytic activity and pathogenicity of *R. solani* in bean seedlings were significantly reduced in chitin-amended soil. Inhibition occurred only when nonsterilized soil was amended. Inhibition of saprophytic and/or pathogenic activity of soil-borne pathogens in chitin-amended soil was observed by various authors to last up to 10 weeks (8, 10, 17). During this period, most of the chitin is degraded (18).

There are three principal mechanisms by which a

TABLE 2. Effect of addition of glucose and ammonium nitrate<sup>a</sup> on inhibition of saprophytic activity of *Rhizoctonia solani* in chitin-amended (CA) as compared to nonamended (NA) soil

Added nutrients	Testing method					
	Slide technique (growth index) <sup>b</sup>			% Segment colonization <sup>c</sup>		
	Soil		Inhibition <sup>d</sup>	Soil		Inhibition <sup>d</sup>
%	NA	CA	%	NA	CA	%
None	410	268	35	79	39	51
Glucose (Gl)						
Gl	0.1	469	252	46	75	36
Gl	1.0			0	0	100
NH <sub>4</sub> NO <sub>3</sub>	0.02	434	297	32		
NH <sub>4</sub> NO <sub>3</sub>	0.1			42	19	55
Gl 0.1 + NH <sub>4</sub> NO <sub>3</sub>	0.02	484	219	55		
Gl 0.1 + NH <sub>4</sub> NO <sub>3</sub>	0.1			18	4	78
Gl 1.0 + NH <sub>4</sub> NO <sub>3</sub>	0.1			0	0	100
LSD 5%			15.5	9.5		

<sup>a</sup> Added to soil after 7-day incubation.

<sup>b</sup> Calculated by multiplying the number of hyphae emerging from the inoculum disc by the average length of these hyphae in mm.

<sup>c</sup> Per cent of bean segments colonized by *R. solani*.

<sup>d</sup> Calculated as:  $1 - \frac{\text{Growth or colonization in CA}}{\text{Growth or colonization in NA}} \times 100$ .



pathogen can be inhibited by soil microflora (1): (i) parasitism and lysis; (ii) competition for limiting nutrients; and (iii) production of inhibitory substances. No direct parasitism or lysis of *R. solani* was observed in chitin-amended soil. The addition of glucose or ammonium nitrate did not nullify the inhibitory effect. Therefore, inhibition is not due to competition for available nutrients, and the possible production of substances inhibitory to *R. solani* in chitin-amended soil should be considered.

In the case of bean root rot due to *F. solani* f. sp. *phaseoli*, Maurer & Baker (14) observed reduction in disease severity only by a combined amendment of chitin and lignin. In contrast, chitin alone (as well as the combined amendments) controlled *R. solani* in the present study. Possibly, the effectiveness of any particular amendment may well depend on the nature of the pathogen, the soil type, and its microbial composition.

Concentrations of carbon dioxide produced in sucrose-amended soils, and which are higher than those produced in chitin-amended soil, did not inhibit the growth of *R. solani*. This indicates that carbon dioxide does not play a major role in the inhibition of *R. solani* in chitin-amended soil. Volatile substances produced in amended soils inhibit the growth of soil-borne pathogens either directly (2, 5, 19) or indirectly (6). It seems, however, that in the case of *R. solani*, volatile substances produced in chitin-amended soil (Fig. 4) only play a minor role in the mechanism of inhibition, since they are produced for a relatively short period of time as compared with the period of inhibition of saprophytic activity (8) (Fig. 1). Furthermore, removing the volatiles by continuous aeration of the chitin-amended soil did not increase saprophytic activity of *R. solani*. Thus, the possibility remains that the antifungal activity found in nonsterilized, chitin-amended soil is mainly due to nonvolatile substances produced by the soil microflora during chitin decomposition.

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