Colonization of Stem Segments and Chitin Particles by Rhizoctonia solani in Soil

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ABSTRACT

Incubation of bean (*Phaseolus vulgaris* 'Brittle Wax') stem segments and chitin particles in *Rhizoctonia*-free soil, resulted in a significant suppression of subsequent colonization by *Rhizoctonia solani*. Autoclaving, extracting the previously incubated stem segments and chitin particles with ethanol, or sterilizing the chitin particles with gamma irradiation allowed them to be colonized to nearly the same level as the nonincubated segments and particles. The ethanol extracts of incubated stem segments and chitin particles contained thermostable substances inhibitory to *R. solani*. The colonization of bean stem segments by *R. solani* was not correlated with content of soluble nutrients. Suppression of colonization of *R. solani* observed with incubated stem segments and chitin particles is ascribed either to competition for degradation products between the established microflora in these substrate units and *R. solani* or to the production of unknown antifungal substances, or both.

Rhizoctonia solani did not survive after incubation for 1 month in a wet, natural sandy-loam soil, but did survive in sterile soil kept aseptically under the same conditions. The decrease observed in colonization of chitin particles colonized with *R. solani*, when incubated in chitin-amended soil, did not differ significantly from that in particles incubated in nonamended soil. A significantly greater decrease in colonization of chitin particles by *R. solani* was observed when the particles were incubated in sterile soil infested with antibiotic-producing microorganisms than in soil infested with nonantibiotic-producing microorganisms. Survival of *R. solani* in plant debris and incidence of damping-off of onion seedlings caused by this fungus in naturally infested soil were much higher after an incubation period of 360 days in air-dried soil than in soil kept at 50% of its moisture-holding capacity.

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Fresh organic matter added to soil is soon colonized by soil microorganisms. The rate of substrate colonization by any particular fungus depends on factors such as the inoculum level, saprophytic ability of the fungus, and the antagonistic microorganisms exploiting the substrate (2, 4, 5, 15).

Stanier (24) suggested that antibiotic substances may be produced in microenvironments in concentrations which could influence microbial composition. This was demonstrated by Wright (26) in wheat straw inoculated with spores of *Trichoderma viride* and incubated in soil. Wastie (25) observed a positive correlation between competitive colonization in soil and growth rate of *Rhizoctonia solani*, but not with its resistance to antibiotics. Rao (20), however, did find such a positive correlation between saprophytic ability and resistance to antibiotics in *R. solani*.

Fusarium oxysporum (18) and *R. solani* (16, 21, 25) are frequent and early colonizers of fresh plant tissues in soil. Upon depletion of available food

reserves, the composition of the microflora inhabiting the plant debris particles changes in accord with the new environmental conditions (21). Fresh organic matter previously incubated in natural soil was poorly colonized by *F. oxysporum* (18).

Papavizas & Davey (17) and Sneh et al. (23) demonstrated an increase in colonization of stem segments by *R. solani* during 3 to 4 days of incubation in soil, followed by a decline in colonization of the previously colonized substrate units; i.e., decolonization. Complete decolonization was observed in oat straw after 21 days of incubation and to a somewhat lesser extent in bean and cotton segments. Similar results were observed by Pitt (19) with wheat straw. On the other hand, *R. solani* was able to survive in pigweed (*Amaranthus* sp.) stems buried in soil for 1 year (2).

Several authors (3, 8, 9) have shown that *R. solani* persists in soil in particles of organic debris as mycelium and sclerotia. However, hyphae colonizing the particles of plant debris seem to lose their protoplasm with time; it was found that only 33% of the hyphae within the particles and 44% of the sclerotia on the particles survive after 7 months (3).

The present work deals with the effect of incubation of plant segments and chitin particles in *Rhizoctonia*-free soil on their subsequent colonization by *R. solani*, decolonization of substrate units from *R. solani*, and the correlation between decolonization and disease incidence. Chitin was included because of its reported role in biological control of several plant pathogens (2, 7, 22).

MATERIALS AND METHODS.—The isolate of *Rhizoctonia solani* Kuehn and the soil type used, methods for preparation of the inoculum, and the experimental conditions used in these studies have been described (22).

Incubation and colonization of substrate units.—Nonsterile 5-mm stem segments of 4-week-old bean (*Phaseolus vulgaris* L. 'Brittle Wax'), cotton (*Gossypium hirsutum* L. 'Acala 4-42'), mature oat straw, and unbleached chitin particles (9 to 25 mm^2) (Nutritional Biochemical Co., Cleveland, Ohio) were incubated in *Rhizoctonia*-free natural soil for 5 days. They were subsequently transferred to fresh soil infested with 165 mg of homogenized mycelium of *R. solani* per kg, incubated for 24 hr, and lifted and washed under running tap water. In each treatment, 100 of 150 incubated segments were transferred to petri dishes (5 segments/plate) containing tap-water agar plus 250 µg/ml chloramphenicol, incubated for 24 hr, then examined for the presence of hyphae typical of *R. solani*. Results were expressed as per cent of segments colonized with *R. solani* (22). In some experiments, stem segments and chitin particles were sterilized by autoclaving (121 C at 1 atm for 20 min). Chitin particles were also sterilized by exposure to a dose of 3 Mrad of gamma rays from a ^{60}Co source, carried out at the Soreq Nuclear Research Centre. The effect of removing nutrients and inhibitory compounds on colonization of substrate units with *R. solani* was studied by shaking bean segments and chitin particles in 95% ethanol for 30

min and washing thoroughly with tap water until no ethanol was detected in the washing water by the iodoform test (12) prior to incubation in soil.

To inhibit growth of bacteria and actinomycetes in the substrate units during their colonization by *R. solani*, segments and chitin particles which had been previously incubated were placed in 250 µg/ml chloramphenicol solution for 20 min.

Nutrient determination and microbial counts in the segments.—Samples of bean stem segments weighing 2 g were treated in a Waring Blendor with 50 ml water for 3 min. The suspension was filtered through a Büchner funnel using Whatman No. 1 filter paper under partial vacuum; the filtrate tested for the presence of carbohydrates with the anthrone reagent (14); and amino acids and related compounds, with the ninhydrin reagent (13). Solutions of D-glucose and L-alanine, respectively, were used as standards. Stem segment samples weighing 2 g were treated with 100 ml sterile water in a Waring Blendor; The resulting suspension was diluted; and aliquots were mixed with Martin's rose bengal agar (7) for counts of fungi and with soil extract agar (7) for bacteria and actinomycete counts.

Extraction of inhibitory compounds.—One hundred and fifty stem segments or chitin particles were shaken in 40 ml of 95% ethanol for 30 min, the extract was evaporated, and the residue was dissolved in ethanol. Aliquots were applied to sterile, 50-mm diam discs of Whatman No. 1 filter paper, placed in petri dishes of the same diameter, and the solvent was evaporated to dryness. One ml of Czapek's broth containing 250 µg/ml chloramphenicol was added to each petri dish, and a 3-mm 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 filter paper disc. After incubation for 48 hr at 28 C, the filter paper discs were stained with cotton blue-lactophenol, washed with tap water, and dried. The fungal colony appeared as a blueprint on the white filter paper. Solvent solutions similarly treated were used as controls; a second set of controls, in which the solvent was omitted, gave similar results. Per cent inhibition was calculated as $(1 - \frac{A}{B}) \times 100$, where A = colony diameter in the treatment and B = colony diameter in the control.

Decolonization.—The process of decolonization is measured by the decline in amount (%) of substrate units harboring *R. solani* on further incubation, estimated as follows: Sterile 1- X 1-cm squares of thin nylon net (30 mesh) or chitin particles were placed in petri dishes containing PDA and *R. solani* cultures for 3 days. The growing hyphae colonized the net squares and the chitin particles. These colonized units were incubated in a *Rhizoctonia*-free soil either nonamended or amended with 0.2% (w/w) chitin for different periods. At the end of incubation, 100 substrate units were recovered from each treatment and rinsed with tap water, and colonization was determined as described above for stem segments. Results were expressed as percentage of nylon squares or chitin particles harboring *R. solani*.

A sandy loam (pH 7.6) soil naturally infested with

TABLE 1. Colonization of stem segments of various plants and of chitin particles by *Rhizoctonia solani* in soil

| | Colonization (%) | | |
| | | Incubated[a] in | |
Substrate	Not incubated	Nonamended soil	Chitin-amended soil
Cotton	80	32	18
Bean	80	8	5
Oat straw	32	5	0
Chitin particles	20	0	0
LSD .05 9.8			

[a] The segments were incubated 5 days in *Rhizoctonia*-free soil and transferred to a fresh soil artificially infested with homogenized mycelium of *R. solani* at a rate of 165 mg/kg soil. One hundred substrate units were used for each treatment.

TABLE 2. Carbohydrates and amino acids and related compounds in bean stem segments after various treatments

Treatment of segments[a]	Carbohydrates μg/g	Amino acids and related compounds μg/g
Fresh	4,230	7,000
Fresh, ethanol-treated	2,200	3,850
Fresh, autoclaved	5,100	7,300
Incubated[b]	1,320	700
Incubated[b], ethanol-treated	880	350

[a] In each experiment, 2-g bean stem segments were treated in three replicates.
[b] Incubated in *Rhizoctonia*-free soil for 5 days.

R. solani was placed in plastic boxes (14 X 14 X 7 cm) and sown with five successive crops of onions at 3-week intervals in order to obtain a high inoculum density and percentage of plant debris particles colonized with *R. solani*. Soil samples were incubated for different times as indicated. The percentage of plant debris particles in which *R. solani* survived was determined as described by Boosalis & Scharen (3). Disease incidence was determined by sowing 100 onion seeds in each plastic box containing 1,000 g of naturally infested soil. The percentage of seedlings killed by postemergence damping-off was recorded over a period of 21 days during which the boxes were immersed in Wisconsin temperature tanks at 28 C. Pre-emergence damping-off was calculated by comparing the per cent emergence of onion seedlings in the treatment to that in noninfested control soil. This experiment was replicated 4 times.

RESULTS.—*Effect of incubation of stem segments and chitin particles in Rhizoctonia-free soil on their subsequent colonization by R. solani.*—Segments or chitin particles incubated in *Rhizoctonia*-free soil, then subjected to colonization with *R. solani* in artificially infested soil, were less colonized than segments and particles not previously incubated (Table 1). Incubation in chitin-amended soil resulted in less colonization when compared to nonamended soil.

When ethanol-treated fresh bean segments and chitin particles were incubated in *R. solani*-infested soil, despite the resulting significant reduction in soluble anthrone- and ninhydrin-positive compounds (Table 2), no significant reduction in their subsequent colonization by *R. solani* was observed (Fig. 1). The amount of soluble nutrients in segments previously incubated in *Rhizoctonia*-free soil was lower than that in fresh segments. Although ethanol treatment further reduced their soluble nutrient content (Table 2), ethanol-treated incubated segments and chitin particles were readily colonized by *R. solani* (Fig. 1).

Ethanol treatment also markedly reduced the microbial counts in incubated segments. Counts before treatment were 1.7×10^5/g and 3.5×10^9/g;

and after treatment, 0/g and 3.2×10^3/g for fungi and for bacteria and actinomycetes, respectively.

Chloramphenicol treatment increased colonization of incubated bean segments from 25 to 45%, but had no effect on colonization of incubated chitin particles (Fig. 1).

Autoclaving both fresh bean segments and chitin particles and gamma irradiation of chitin particles did not affect their subsequent colonization by *R. solani*, but increased colonization of incubated segments (Fig. 1).

The antifungal activity of extracts of incubated bean stem segments and chitin particles.—*Rhizoctonia solani* was inhibited by extracts of bean segments and chitin particles which had been incubated previously in *Rhizoctonia*-free soil (Table 3). Inhibitory activity of extracts was not affected by prior autoclaving of stem segments or chitin particles.

Decolonization of chitin particles colonized with R. solani.—Some decolonization was observed after 10 days of incubation of chitin particles in natural *Rhizoctonia*-free soil. After 28 days, the particles were completely decolonized (Fig. 2). No decrease in survival of *R. solani* in chitin particles mixed with sterile soil and kept under aseptic conditions was observed. A similar trend was obtained with colonized squares of nylon net (Fig. 2). No lysis of the fungal hyphae was observed.

When chitin particles colonized with *R. solani* were incubated for 12 days in either chitin-amended soil or nonamended natural soil, ca. 36 and 42% of the particles in the amended and nonamended soil, respectively, remained colonized by *R. solani*.

Effect of microorganisms on decolonization of chitin particles colonized by R. solani in autoclaved soil.—Isolates of bacteria, actinomycetes, and fungi obtained from chitin-amended soil were tested for antibiotic production (7) and added to sterile soil samples in groups of five. Chitin particles colonized with *R. solani* were incubated in these soils for 55 days. The results (Fig. 3) indicate that microorganisms which do not produce antibiotic substances affect survival of *R. solani* in the chitin particles less than do antibiotic-producing ones. Thus, antibiotic-producing actinomycetes and bacteria reduced colonization to 74 and 66%, respectively, but
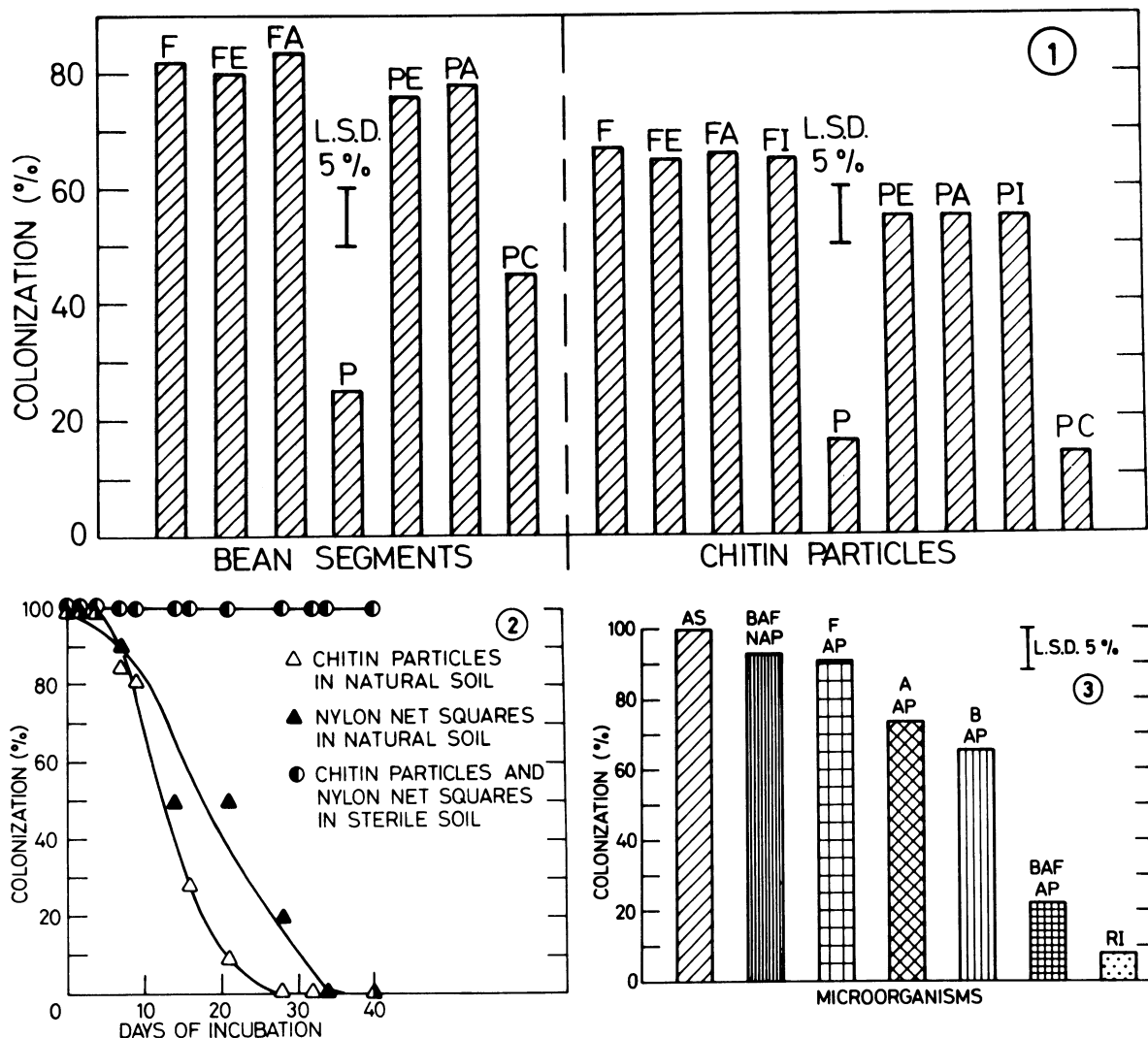
Fig. 1-3. 1) Effect of various treatments on colonization of bean stem segments and chitin particles with *Rhizoctonia solani.* F = fresh; P = incubated in *Rhizoctonia*-free soil for 5 days; A = autoclaved; I = irradiated; E = ethanol-treated; C = chloramphenicol-treated. 2) Decolonization from *R. solani* of chitin particles and nylon net squares incubated in natural, *Rhizoctonia*-free, and sterile soils. 3) Decolonization from *R. solani* of chitin particles in autoclaved soil inoculated with various soil microorganisms. Five isolates of each group (bacteria, actinomycetes, and fungi) were added, either separately or in combination. The incubation period was 55 days; AS = autoclaved soil; B = bacteria; A = actinomycetes; F = fungi; AP = antibiotic-producing; NAP = nonantibiotic producing; RI = inoculated with natural soil (2 g/kg).

nonantibiotic-producing microorganisms reduced it only to 94%. When all the antibiotic-producing groups were added together to the sterile soil, only 22% of the chitin particles remained colonized with *R. solani*. Decolonization was most prominent in sterile soil which was infested with a small amount of natural soil (2 g/kg). Under these conditions, only 8% of the chitin particles remained colonized.

Effect of soil moisture on decolonization of plant debris particles and on damping-off of onion caused by R. solani.—Plant debris particles colonized with *R. solani* and incubated for 110 days in naturally infested, air-dried soil were only slightly decolonized, whereas at 50% MHC, the proportion of plant debris

particles harboring *R. solani* declined from about 50% at zero time to 10% after 110 days (Fig. 4). Most of the decolonization occurred during the 1st month. The chitin amendment had no significant effect on decolonization.

Colonization of plant debris declined from 69% at zero time to 21% after 360 days in soil kept at 50% MHC, but only to 60% in soil kept dry (3% MHC). All the onion seeds sown in this soil at zero time and maintained under irrigation in the greenhouse for 21 days died of pre-emergence damping-off. After 360 days of incubation under wet and dry conditions, soil samples were sown with onion seeds and tested again for disease incidence. It decreased in the wet soil, but
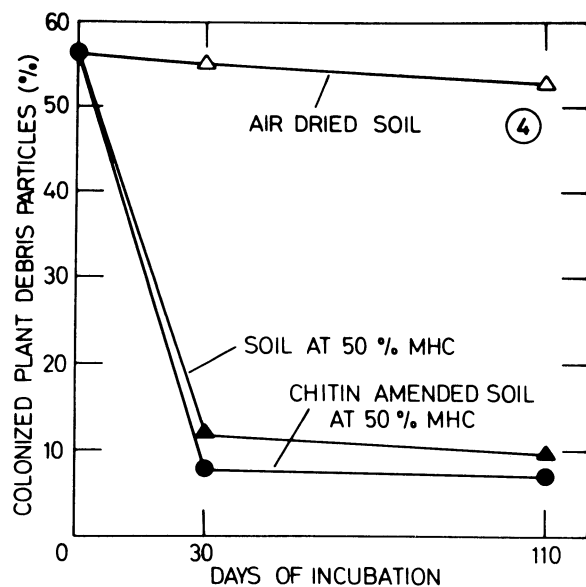
NOT EMERGED

PRE-EMERGENCE DAMPING-OFF

POST-EMERGENCE DAMPING-OFF

HEALTHY SEEDLINGS

Fig. 4-5. 4) Effect of incubation time, soil moisture, and chitin amendment on decolonization of plant debris particles from *Rhizoctonia solani* in natural soil. **5)** Damping-off of onion seedlings in a soil naturally infested with *R. solani* after 360 days' incubation; A = noninfested soil (control); B = infested soil kept at 50% moisture-holding capacity; C = air-dried infested soil; e = per cent emergent seedlings.

TABLE 3. Effect of ethanol extracts of bean stem segments and chitin particles on *Rhizoctonia solani*

Extract origin[a]	Inhibition[b] (%)	
	Incubated[c]	Nonincubated
Nontreated bean segments	48	16
Autoclaved bean segments	48	37
Nontreated chitin particles	68	2
Autoclaved chitin particles	65	2

[a] An extract from 25 segments or particles was evaporated in each petri dish.

[b] Calculated by comparing the diameter of the *R. solani* colony in the treated material to that of the control. Colony diameter in the control containing the solvent did not differ from that without the solvent.

[c] Incubated 5 days in *Rhizoctonia*-free soil before extraction.

remained high in soil incubated under dry conditions, as only 1% of the seeds sown in this soil produced healthy plants (Fig. 5).

DISCUSSION.—Colonization by *R. solani* of plant segments and chitin particles previously incubated in *Rhizoctonia*-free natural soil was less than that of nonincubated ones. This is in accordance with the results obtained by Park with *F. oxysporum* using segments of wheat straw (18), and *R. solani* may also be classified as a "pioneer colonizer" (18).

The decrease in colonization of substrate units previously incubated in *Rhizoctonia*-free soil by *R. solani* is not due to competition for available water and alcohol-soluble nutrients, as their removal did not reduce colonization with *R. solani*. Ethanol treatment of incubated bean segments and chitin particles restored their colonization to that of fresh material. In addition to removal of available nutrients and inhibitory substances, ethanol treatment greatly suppresses fungi, bacteria, and actinomycetes in the incubated segments. Thus, decrease in colonization by *R. solani* of incubated substrate units probably results either from the production of inhibitory substances or from competition between *R. solani* and the established microflora for degradation products released from the substrate units during microbial decomposition (6), or from both.

Baker et al. (1) noted that the genetic potential, the sum of the antagonistic abilities of the soil microorganisms towards *R. solani*, and the soil environment affect the survival of *Rhizoctonia* in soil. In some of our experiments, *R. solani* failed to survive after 30 days in wet natural soil, but did survive when incubated in autoclaved soil. Loss of viability of *R. solani* in natural soil resulting from microbial activity was not due to lysis of the mycelium, as demonstrated previously (22). Huber et al. (10), using the plate profile technique, noted hyphal death of *R. solani* without lysis, defining it as "bacterial necrosis".

Antibiotic-producing microorganisms caused a marked decolonization of chitin particles previously colonized with *R. solani* and incubated in autoclaved soil, whereas inoculation with natural soil resulted in
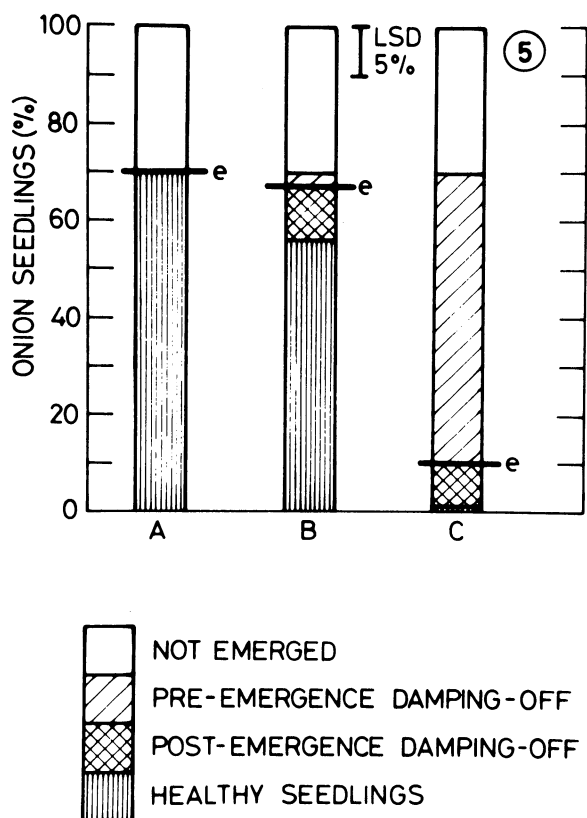
a considerably greater decolonization. It seems that decolonization is not due to one or a few specific species in the soil, but rather to the activity of many microorganisms. Ko & Lockwood (11) noted that antibiotic substances cause hyphal death.

It is important to obtain fast decolonization of plant residues from soil-borne phytopathogenic fungi. We have previously demonstrated that oat straw is easily decolonized (23) and is useful, when applied as a soil amendment, in decreasing the saprophytic and pathogenic activity of *R. solani* (7). Possibly the effect of crop rotation on damping-off of various seedlings caused by *R. solani* depends, among other factors, on the rate of decolonization of the pathogen from the residues of the crops used.

It seems that the biological control of *R. solani* by chitin amendment is not due to decolonization of plant debris particles harboring *R. solani*, as addition of chitin to soil had no significant effect on decolonization. Inhibition of growth of *R. solani* in soil (22), rather than loss of viability of fungal mycelium colonizing substrate units, seems to be the mechanism involved.

Soil moisture is also a significant factor in the process of decolonization. Pronounced decolonization of plant debris and decrease in damping-off of onion seedlings caused by *R. solani* was observed in wet soil (50% MHC), but not in an air-dried soil. Thus, maintaining a naturally infested soil under the moist conditions suitable for microbial activity, for a period of time and in the absence of a susceptible host, may result in a decrease in the occurrence of damping-off of various crops caused by *R. solani*.

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