Components in Unbleached Commercial Chitin Stimulate Pythium ultimum in Sugarbeet Spermosphere

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ABSTRACT

Severe pre-emergence damping-off occurred when sugarbeet seeds coated with commercial chitin were planted in soils either naturally or artificially infested by *Pythium ultimum*. Uncoated seeds were not damaged in two of three infested soils. In noninfested soils, coated seeds were not damaged. No pre-emergence damping-off occurred when seeds were coated with bleached chitin, then planted in infested soil. Glucose and nitrogenous compounds were found in the water extract of unbleached chitin. Unbleached chitin, its water extract, as well as glucose and peptone, stimulated germination of sporangia of *P. ultimum* in pure culture both in the presence and absence of natural soil. Bleached chitin did not stimulate germination of sporangia under the same coniditions. Sporangia germinated more rapidly, and in greater numbers in the spermosphere of sugarbeet seeds coated with unbleached chitin, than in that of uncoated seeds. It is suggested that unbleached chitin applied to seeds provides nutrients that increase the germination of sporangia of *P. ultimum* in the spermosphere, and enhance subsequent colonization of the seeds by the pathogen.

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Products designated as 'chitin' have provided some hopes for biological control of certain soilborne plant pathogenic fungi. When incorporated into soil, chitin reduced severity of diseases caused by *Fusarium* spp. and *Rhizoctonia solani* (2, 5, 6, 10, 12). Damping-off of vegetable plants caused by *Pythium debaryanum* was not controlled by chitin amendments (10). Reduction of disease by chitin may be correlated with a decrease in soil populations of the pathogens (2, 5, 7), possibly due to antifungal compounds produced in chitin-amended soils (15, 16). Chitin added to soil consistently increased the population of bacterial species (11, 14), actinomycetes (2, 6, 7, 12, 14), and Phycomycetes (6).

Considering this information, an attempt was made to control seedling diseases by coating seeds with chitin. The research presented in this paper describes the response of *Pythium ultimum* Trow. to sugarbeet seed coated with commercial chitin.

MATERIALS AND METHODS.—The soil used in this study was a dark-colored peat soil (pH 7.2) collected from the uncropped hedgerow (poplar) of a cultivated field in the sugarbeet area of Bavois, Canton Vaud, Switzerland. Preliminary tests showed that little damping-off could be obtained in this soil, which contained less than 10 *P. ultimum* propagules per gram, as measured by Stanghellini and Hancock's quantitative method of isolation (17). In the same area, similar field soil was infested by 350-600 propagules of *P. ultimum* per gram. The latter soil was used in some tests. A sandy-loam-clay soil, and a clay soil from Orny and Lonay, Canton Vaud, respectively, were used in several comparative tests.

The *P. ultimum* isolate used in this study was obtained in 1969 from a diseased sugarbeet (*Beta vulgaris* 'KWZ Mono') seedling collected from a commercial crop in Bavois. The isolate was stored on corn meal agar at 20 C, and transferred monthly to fresh medium. The host plant used in this study was sugarbeet cultivar KWZ Mono.

Unbleached chitin was obtained from Fluka, Buchs, Switzerland. This product, designated "chitin purum", consisted of small, brownish flakes. In some tests, Pflatz and Bauer (Serlabo, Paris, France) and BDH (BDH Chemicals Ltd. Poole, England) unbleached chitins were used. Bleached chitin was prepared according to the method of Lingappa and Lockwood (9). The process was stopped after three to four washings in 95% ethanol. All other chemicals used were of "purissimum" quality, except peptone (Fluka), Difco Bacto yeast extract, and quartz powder (99.8% purity, Fluka).

To study the effect of seed coating on the disease, 200-g samples of fresh soil were poured into petri dishes, 9 cm in diameter, 5 cm in height. Total water content of the soil was 42-55%. The soil was infested by mixing 25 g of a suspension prepared by blending, in sterile, distilled water, cultures of *P. ultimum* grown on 1.5% water agar 10-13 days in darkness at 20 C. Infested soil contained 200-400 sporangia per gram of dry soil. Control soil was moistened with 21 ml of sterile, distilled water. Dishes were covered with glass lids, and stored overnight in a growth chamber. Seeds were coated by rapidly dipping undamaged, monogerm seeds in 2.2 ml of sterile, distilled water plus one of the following mixtures: chitin, unbleached or bleached, 1 g; bleached chitin 0.75 g + glucose or peptone 0.75 g; bleached chitin 0.75 g + glucose 0.375 g + peptone 0.375 g; glucose 2 g + quartz powder 4 g; peptone 3 g; glucose 2 g + peptone 2 g. Ten seeds were planted at a depth of 0.7-0.9 cm in each dish. Dishes were incubated in a growth chamber at 20-22 C, under 4,400 lx

produced by a row of alternate Sylvania Grolux F40 and Philips TL 40w/33 fluorescent tubes, with 14-hour daily photoperiods. Four days after planting, the lids were removed, seedlings were counted, and the dishes were placed in Ward plastic seed trays (Vatter Samen, Köniz-Bern, Switzerland), 57 × 29 × 7.5 cm containing 350 ml of deionized water. Transparent, plastic, 14-cm high Ward covers were placed on the trays. Temperature and light intensity were 25 C and 3,500 lx, respectively, during the daily illumination periods under the plastic covers. Every 4-5 days, the weights of the dishes were adjusted to their original values by a mist of sterile, distilled water. Seedlings were counted, and dead plants were removed. Preliminary tests indicated that no more seedlings would emerge 16 days after planting with any seed treatment. Three dishes were used per treatment. Frequent isolations were made from killed seeds or seedlings to check if *P. ultimum* was the cause of the damping-off.

Similar tests involving unbleached chitin were made in the same type of soil naturally infested by *P. ultimum*. Experiments were repeated until simple randomization analysis of variance gave comparable results at $P = 0.05$.

The effect of chitin and other chemicals on the germination of *P. ultimum* sporangia was studied in pure culture, and in soil with or without sugarbeet seeds. Water extracts of unbleached chitin were obtained by blending 2 g of the commercial product in 200 ml of distilled water for 5 minutes in a Virtis homogenizer. The suspension was filtered through a 0.45-μm Millipore membrane, and the filtrate was concentrated to 5 ml at 45 C under vacuum. Chitin (15 mg) was placed inside a ring, 13 mm in diameter, drawn at the surface of 10- to 13-day-old fungal cultures on water agar. Four rings were used in each of three replicate cultures. In another experiment, blotting paper disks (12.7 mm in diameter) moistened with 0.1 ml of the desired solution were placed on the culture medium. Microscopic counts of germinated sporangia were made inside a 3.6-mm-wide circular strip traced around the disks or around the rings, 2 and 8 hours after the supply of the chemicals to the colonies. Fifty sporangia were counted in each of three rings per experiment. Cultures were kept at 20 C in darkness.

Studies including soil were made with similar *P. ultimum* colonies. Ten grams of fresh soil mixed with 2, 20, or 200 mg of unbleached chitin, or with 200 mg of bleached chitin, glucose, or peptone, were gently spread onto the agar cultures. After 2, 8, and 24 hours of incubation at 20 C in darkness, the soil was removed by a spurt of sterile, distilled water, and 100 sporangia per dish in three dishes per treatment were examined for germination.

The study of the effect of coating seeds with chitin on germination of sporangia in the spermosphere was made as follows: Glass vials (10 × 1 cm) were filled to a depth of 4 mm with natural soil mixed 10 days earlier with a mycelial mat of the fungus which was produced on water agar and contained a large number of sporangia and oospores. Three sugarbeet seeds, pelleted with unbleached chitin, as described above, were placed on the top of the soil column and covered with 3 mm of infested soil. The system was moistened with 0.15 ml of sterile, distilled water. Uncoated seeds, and infested soil alone, were used as controls. Vials were plugged and kept at 20 C in darkness. Eight replicates were prepared in each test. After 2, 4, 8, and 24 hours of incubation, sporangia in two vials of the spermosphere soil were examined by the method of Stanghellini and Hancock (18). Values recorded represent the mean of two counts of 150 sporangia per vial. Each experiment on the effect of chitin and other chemicals on the germination of sporangia was repeated three times. Similar tests were made in naturally infested soil.

Analysis of the water-soluble carbohydrate and nitrogen content of unbleached chitin was made on a water extract prepared as described above, except that the filtrate was evaporated to dryness at 45 C under vacuum, and the residue was dissolved in distilled water to a volume of 2 ml. Total carbohydrate content was established colorimetrically by the phenol-sulfuric acid method (3). Nitrogen was determined by the micro-Kjeldahl method with titration of ammonia in boric acid (13). Qualitative determination of sugars was made by thin-layer chromatography using a butanol-acetic acid-water (4:1:1, v/v) solvent system. Sugars were detected with a naphthoresorcinol-sulfuric acid reagent.

RESULTS.—Initially, coating tests were conducted with unbleached chitin. Significantly more pre-emergence damping-off was observed when coated seeds were planted in Bavois soil artificially infested with *P. ultimum* than when uncoated seeds were planted in this soil (Table 1). Little difference was observed between uncoated seeds planted in noninfested vs. infested soil. Moreover, in Orny soil, little difference was noted between uncoated seeds planted in noninfested or artificially infested soil. Coating of seeds stimulated the disease in infested soil, but not enough to be significantly different. Significantly more disease occurred in artificially infested than in noninfested Lonay soil when both coated and uncoated seeds were tested. However, the increase in damping-off was much more severe with coated seeds.

Bavois soil was chosen for a study of the mode of action of seed coating on disease stimulation because when it was artificially infested with *P. ultimum*, damping-off was significantly increased when seeds coated with unbleached chitin were planted. The various coating mixtures tested were selected according to the well-

TABLE 1. Effect of coating sugarbeet seed with unbleached chitin on the emergence of sugarbeet seedlings in three soils either infested or noninfested with *Pythium ultimum*

Soil source	Infestation with *P. ultimum*	Seedling emergence (%)[a] 16 days after the planting of: Uncoated seeds	Coated seeds
Bavois	noninfested	84 x[b]	77 x
	infested	73 xy	14 z
Orny	noninfested	73 xy	84 x
	infested	65 xy	41 y
Lonay	noninfested	82 x	82 x
	infested	40 y	11 z

[a]Calculated from the means of four experiments, three dishes of 10 seeds per treatment in each treatment group.

[b]Percentages not followed by the same letter represent means significantly different, $P = 0.05$.

TABLE 2. Effect of coating sugarbeet seed with chitin (bleached and unbleached), glucose, and peptone on the emergence and survival of sugarbeet seedlings in soil infested or noninfested with *Pythium ultimum*

	Emergence and survival of seedlings 16 days after planting			
	Noninfested soil[a]		Infested soil[a]	
Seed Coating	Seedling emergence (%)[b]	Seedling survival (%)[b]	Seedling emergence (%)[b]	Seedling survival (%)[b]
No coating	80	77	71	54
Unbleached chitin	74	64	15 z[c]	7 z
Bleached chitin	73	70	54	31 z
Bleached chitin + glucose	78	71	36 z	19 z
Bleached chitin + peptone	79	79	61	48
Bleached chitin + glucose + peptone	75	42	54	24
Glucose + quartz	63	63	46	40
Peptone	73	67	63	43
Glucose + peptone	84	82	59	40 z

[a]Soil from a poplar hedgerow bordering a commercial sugarbeet field near Bavois, Canton Vaud, Switzerland; chosen for this experiment because when it was artificially infested with *P. ultimum* significantly more damping-off occurred when seeds were coated with unbleached chitin before being planted.
[b]Calculated on the mean of five experiments, three dishes of 10 seeds per experiment.
[c]The letter "z" following a mean indicates that it is significantly different ($P = 0.05$) from the corresponding mean for noninfested soil in at least three of five experiments.

TABLE 3. Effect of chitin and other compounds on the germination of *Pythium ultimum* sporangia

		Germination of sporangia after:			
Medium	Compound added	2 hours (%)[a]	4 hours (%)[a]	8 hours (%)[a]	24 hours (%)[a]
Water agar[b]	Unbleached chitin[c]	91		98	
	Unbleached chitin water extract[d]	94		97	
	Bleached chitin[c]	6		20	
	Glucose, 1% solution[d]	65		75	
	Peptone, 1% solution[d]	84		96	
	Blotting paper disk	16		18	
	No compound added	17		17	
Water agar	Unbleached chitin, 0.2 mg/g of soil	0		6	8
+	Unbleached chitin, 2 mg/g of soil	2		14	14
soil layer[e]	Unbleached chitin, 20 mg/g of soil	54		55	55
	Bleached chitin, 20 mg/g of soil	3		3	5
	Glucose, 20 mg/g of soil	66		67	67
	Peptone, 20 mg/g of soil	62		68	71
	No compound added	0		0	0
Soil[f]	Sugarbeets[g]	2	17	33	39
	Sugarbeet seeds coated with unbleached chitin	44	77	77	79
	No compound added	0	0	0	0

[a]Represents the mean of three experiments.
[b]One-hundred and fifty sporangia examined in each experiment.
[c]Fifteen milligrams of chitin placed inside a ring, 13 mm in diameter.
[d]One milliliter moistening a blotting paper disk, 12.7 mm in diameter.
[e]Three-hundred sporangia examined in each experiment.
[f]Six-hundred sporangia examined in each experiment.
[g]Three seeds in 550 mm^3 of moist soil.

known stimulating effect of sugars and simple organic nitrogen compounds on propagules of *P. ultimum* in soil (4, 20). The effects of the different chemicals on emergence and survival of sugarbeet seedlings are presented in Table 2.

Emergence was severely decreased by unbleached chitin in infested soil, but not in the noninfested control soil. Similar results were obtained with two other trademarked chitin preparations. Bleached chitin did not affect emergence in infested soil. The only coating mixture that significantly stimulated pre-emergence damping-off was bleached chitin plus glucose. Survival of seedlings 16 days after planting usually followed the emergence pattern, with more postemergence damping-off with all coating mixtures in infested soil. No difference in emergence of seedlings after seed coating with unbleached chitin was observed in naturally or artificially infested soils. Isolations made in several experiments always gave *P. ultimum*. Exploratory experiments using similar methods showed that incorporating 0.5, 5, or 50 mg of unbleached chitin per gram of fresh infested soil did not stimulate damping-off when seeds were planted 1 or 4 days later.

Chemical analysis of the water-soluble extract of unbleached chitin gave 0.75% carbohydrate and 7% nitrogen content in dry, unbleached chitin. Separation of sugars by thin-layer chromatography produced a dense spot at the same R_f as that of the standard glucose. Traces of a few other unidentified sugars were also present. A similar analysis of the bleached chitin water extract only gave traces of carbohydrates.

The effects of various chemicals on the germination of *P. ultimum* sporangia either in pure culture or in soil, with or without sugarbeet seeds, are summarized in Table 3. Readings at more than 8 hours (without soil) and 24 hours (with soil) are not reported, because new sporangia were formed after those periods in the presence of several chemicals. On water agar, germination of sporangia was stimulated by unbleached chitin, by its water extract, and by glucose and peptone. Bleached chitin stimulated germination only slightly. Similar observations were made when a soil layer mixed with the chemicals covered the surface of water agar. In this case, 20 mg of unbleached chitin per gram of soil was necessary to stimulate germination of sporangia. In soil, no germination occurred in the absence of seeds. Sporangia germinated more rapidly and at a higher rate in the vicinity of seeds coated with unbleached chitin than near uncoated seeds.

DISCUSSION.—The stimulating effect of unbleached chitin on damping-off and germination of sporangia suggests that disease stimulation may be due to rapid stimulation of pathogen sporangia in the vicinity of the treated seeds. Stanghellini and Hancock (19) showed that bean seed exudates stimulated sporangial germination of *P. ultimum* in soil contiguous to seed, within 3-4 hours after sowing. Evidence has also been provided that sugars, amino acids, and seed exudates stimulate rapid germination of *P. ultimum* sporangia in soil (1, 18). Since similar chemicals, e.g., glucose, were found in the water soluble extract of unbleached chitin, it can be assumed that they contributed to the stimulation of damping-off. The data presented here indicate that unbleached chitin, sold under various designations of purity, contains impurities that stimulate germination of *P. ultimum* sporangia in soil. Pure chitin (i.e., *N*-acetyl-glucosamine polymer) apparently was not involved in the activities recorded in this study, since bleached chitin did not have the stimulating effect of unbleached chitin. Pure chitin failed to control or stimulate pre-emergence damping-off caused by *P. ultimum*. Rather, it stimulated postemergence damping-off, as shown in Table 2.

Glucose was a major contaminant in the commercial unbleached chitin used in this study, and it probably played an important role in stimulation of *P. ultimum* near sugarbeet seeds coated with unbleached chitin. Bean seedling disease caused by *Rhizoctonia solani* was reduced by incorporation of unbleached chitin into soil (5). More recently, exogenous glucose was shown to repress virulence of *R. solani* on cotton seedlings (21). Although substances which reduced saprophytic growth of *R. solani* were found in soil amended with unbleached chitin (16), the repressive effect of glucose (probably present in most commercial chitins) on the virulence of *R. solani* should be taken into consideration. The data presented here should encourage those using chitin in soil microbiological studies to pay more attention to the purity of that compound. To the author's knowledge, many published works describing the effect of chitin on soil microflora did not state precisely the type of chitin used. When obtained with unbleached chitin, such results should be compared with those obtained by using chemicals existing as impurities in commercial chitin; e.g., glucose and nitrogenous compounds.

One may wonder why uncoated seeds escaped the disease in infested soil (Table 2), although sporangia germinated to some extent in their spermosphere, as indicated in Table 3. This might be due to the unique environmental conditions of these experiments, which allowed the seedlings to grow more rapidly than in natural field conditions. It is known that mung bean seed exudation is more abundant at 12 and 42 C than at intermediate temperatures (8). At 20-25 C, exudation from sugarbeet seeds might not be high enough to permit rapid development of *P. ultimum* sporangia and subsequent damping-off. Unbleached chitin placed around the seeds provided abundant nutrients for fast sporangial germination and colonization of the seeds. A rapid nutritional stimulation of *P. ultimum* propagules is of first importance for a successful colonization of fresh plant tissue (20).

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