Effect of Pentachloronitrobenzene on Colonization of Alfalfa Residues by Fungi and Streptomycetes in Soil

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ABSTRACT

Pentachloronitrobenzene (PCNB) at a concentration of 10 μg/g soil produced quantitative and qualitative changes in fungal and streptomycete populations colonizing particles of alfalfa hay in natural soil. At incubation periods from 1 to 32 days, total numbers of fungi in alfalfa particles were reduced when the soil contained PCNB. The proportion of fungi which were tolerant to PCNB was greater in residues recovered from soil containing PCNB than from soil without PCNB. Populations of *Pythium ultimum* and *Fusarium* spp. colonizing alfalfa particles increased in the presence of PCNB in soil, while those of *Rhizopus stolonifer* and *Penicillium oxalicum,* and of streptomycetes, decreased. These changes were correlated with the sensitivity of these microorganisms to PCNB in agar. Alfalfa hay accumulated PCNB to levels 7- to 11-fold higher than the concentration in soil. In sterile soil infested with pairs of microorganisms differing in sensitivity to PCNB, populations of the more sensitive microbes colonizing alfalfa particles decreased and those of the more tolerant microbes increased. Phytopathology 60:1578-1582.

Pentachloronitrobenzene (PCNB) is a common soil fungicide used successfully against certain soil-borne plant pathogens such as *Rhizoctonia solani, Sclerotium rolfsii,* and *Streptomyces scabies* (3). It is also effective in suppressing growth of actinomycetes (4, 5) and species of *Rhizopus* (7, 11). This fungicide is not effective, however, in controlling diseases caused by species of *Pythium* or *Fusarium* (3); in some cases, incidence and severity of these diseases was even increased in soil treated with PCNB (1, 6). This phenomenon has been attributed to the suppression of antagonists of the PCNB-tolerant pathogens (6). Recent evidence, however, indicates that PCNB may temporarily reduce competition for nutrients by suppressing actinomycetes and certain fungi in soil, thus permitting utilization of substrates by the more tolerant fungal pathogens (5).

Little is known regarding the effects of PCNB on saprophytic colonization of organic substrates in soil. In glucose-amended soil without plants, PCNB selectively altered the microbial population (5), suggesting that saprophytic colonization may also be affected. In the present work, the effect of PCNB on saprophytic colonization was studied, using particles of alfalfa hay as substrates for colonization by soil microorganisms.

MATERIALS AND METHODS.—*Preparation of soils and amendments.*—Conover loam from the Michigan State University farm was used in all tests. Water-holding capacity of this soil was 42.7%, organic matter content was 3.8%, and pH was 6.7. The soil contained 7.5% clay, 42.8% silt, and 49.7% sand. The soil was collected from an area which had been fallow for several years and to which pesticides had not recently been applied. Soil was dried with an electric fan and passed through a 40-mesh sieve. For soil amendment, 1 mg technical grade (99% pure) PCNB dissolved in 0.1 ml acetone was added to 15 ml distilled water containing 0.05 ml of a wetting agent, Triton X-100 (alkyl phenoxy polyethoxy ethanol, Rohm & Haas, Philadelphia). The suspension was mixed with 100 g air-dry soil to give a final concn of 10 μg PCNB/g air-dry soil. Soil used as a control was amended with water, acetone, and Triton X-100, without PCNB. Soil moisture content was 15%.

Alfalfa hay was ground in a Wiley mill and passed through a 20-mesh sieve. This material was then passed through a 40-mesh sieve, and the fraction retained on the sieve was used to amend the soil. Thus, only particles between 20- and 40-mesh size were used, so that later they could be recovered from the soil by wet-sieving on a 40-mesh sieve. The alfalfa particles were added to the soil at the rate of 1% (w/w), and were incubated in closed polyethylene boxes for various periods before recovery and analysis.

Enumeration of microorganisms.—Alfalfa particles were recovered from soil by washing the soil through a 40-mesh sieve under running tap water until the particles were free of soil. Five hundred mg washed alfalfa particles from each sample were ground with a pestle and mortar and homogenized with 50 ml sterile water in a Sorvall Omni-Mixer for 2 min at ca. 7,000 rpm. Further dilutions were made in sterile distilled water.

For enumeration of fungi, 0.5 ml of the appropriate dilution was spread over the surface of plates of acidified potato-dextrose agar (PDA) containing 0.1% surfactant (10), and counts were made after 5 days' incubation. At the same time, 0.5 ml aliquots of the diluted alfalfa suspensions were poured on plates containing the same medium supplemented with 100 μg PCNB/ml (4) for determinations of fungi resistant to PCNB. Numbers of the following selected fungi were determined: *Rhizopus stolonifer* (Fr.) Lind, *Penicillium oxalicum* Currie & Thom, and *Fusarium* spp. Populations of *Pythium ultimum* Trow were estimated by

placing washed intact alfalfa particles individually on 20-30 plates of PDA containing 250 μg chloramphenicol/ml. Within 2 or 3 days, *P. ultimum* growth from the particles was evident and results were expressed as the percentage of particles harboring *P. ultimum*. For enumeration of streptomycetes in alfalfa particles, 1 ml of the appropriate dilution was mixed with 15 ml chitin agar (9), and counts were made after 8-10 days. When soil was assayed for fungal and streptomycete populations, a 1:100 soil dilution in sterile distilled water was shaken for 0.5 hr, diluted further, then plated on the selective media.

Six-plate replications were used for all dilution plates. Plates were incubated at 24 C, and results are usually expressed as numbers of propagules per g oven-dry alfalfa hay or soil. At intervals corresponding to those for enumeration of microorganisms, samples of alfalfa in soil were removed by wet-sieving and were assayed for PCNB content by gas chromatography as previously described (2). The soil and alfalfa used were tested by gas chromatography prior to the experiments, and did not contain compounds having retention times similar to PCNB.

RESULTS.—*The effect of PCNB on fungal populations in colonized alfalfa residues.*—PCNB affected the rate of fungal colonization of, and the kinds of fungi colonizing, the alfalfa particles (Table 1). Fungi displayed a pattern of increasing numbers with time, followed by a decrease at 32 days after reaching a peak at 18 days. At all six incubation periods, total numbers of fungi were reduced when the soil contained PCNB. The reduction occurring in PCNB-treated soil ranged from 20 to 80% of soil without PCNB, with the effect greatest during the first days following incorporation of alfalfa.

Four fungi were selected for studying the specific effects of PCNB. Over the period of the experiment, numbers of *Penicillium oxalicum* and *R. stolonifer* in soil containing PCNB were reduced to one-half to one-third those in soil without PCNB. By contrast, population levels of *Fusarium* spp. increased 36-520%, and the percentage of alfalfa particles colonized by *Pythium ultimum* increased 68-200% over those in soil without PCNB. For these four fungi also, the larger differences tended to occur at the earlier sampling times. In a separate experiment, using autoclaved alfalfa, a similar effect of PCNB on *Fusarium* populations was observed. When the above experiments were repeated with sampling done at fewer, selected periods, similar results were obtained.

To test whether the differential effect of PCNB was correlated with relative sensitivity to this fungicide, three isolates each of *Penicillium oxalicum, R. stolonifer, Fusarium* spp., and *Pythium ultimum* were grown on PDA containing different concn of PCNB. An isolate of *Fusarium oxysporum* f. sp. *melonis* (Leach & Currence) Snyd. & Hans. from stock cultures was also included. Agar discs 2 mm in diam from the margins of plate cultures on PDA were used as inoculum. Six-plate replicates were used. *Pythium ultimum* and *Fusarium* spp. were relatively insensitive to PCNB (Table 2). The ED_{50} for these two fungi was greater than 500 μg/ml. By contrast, *R. stolonifer* and *Penicillium oxalicum* were completely inhibited at concn of 1-10 μg/ml, and their ED_{50} values were less than 1 μg/ml and 1-10 μg/ml, respectively.

Effect of PCNB on the proportion of PCNB-sensitive and nonsensitive fungi colonizing alfalfa particles.—Since fungi other than the four mentioned above might also be affected by PCNB, attempts were made to determine the proportion of PCNB-sensitive and tolerant groups colonizing alfalfa. In one approach, alfalfa particles from the experiment described above were poured on the surface of acidified PDA containing a surfactant + 100 μg PCNB/ml agar. After 5 days' incubation, the diam of each colony was measured and placed in

TABLE 1. Effect of pentachloronitrobenzene (PCNB) on fungi colonizing alfalfa residues in soil

Fungi	PCNB in soil[a]	Fungal populations at different days of incubation						
		1	2	4	8	18	32	Mean[b]
Total fungi[c]	+	1.2	27	46	374	1,142	144	289
	−	5.6	68	61	523	1,542	176	396
Penicillium oxalicum[c]	+	0	0	0	8	11	5	8
	−	0	0	0	23	39	10	24
Rhizopus stolonifer[c]	+	0	6	21	37	20	12	19
	−	0	19	41	44	44	25	35
Fusarium spp.[c]	+	0.5	10	18	152	280	110	95
	−	0.1	5	6	106	185	81	64
Pythium ultimum[d]	+	20.0	66	36	42	16	14	32
	−	6.6	33	18	25	8	7	16
PCNB-tolerant (large) colonies[e]	+		38	58	26	21	60	41
	−		4	18	19	14	45	20
PCNB-sensitive (small) colonies[e]	+		59	19	57	61	10	41
	−		87	39	63	73	14	55

[a] + = soil contained 10 μg PCNB/g; − = soil without PCNB.
[b] Means for soil with PCNB differed significantly ($P < .05$) from those for soil without PCNB for each fungal group.
[c] Propagules/g dry alfalfa residue, × 10^4.
[d] Alfalfa particles colonized, %.
[e] % of total fungi. Colonies with diam > 7-8 were classed as tolerant, colonies with diam < 2-3 mm were classed as sensitive, as determined by growth on an agar medium supplemented with 100 μg PCNB/ml.

TABLE 2. Effect of pentachloronitrobenzene (PCNB) on growth of fungi on potato-dextrose agar

Fungus	Incubation time, days	Colony diam, % of growth on agar without PCNB			
		1	10	100	500
		μg/ml			
Pythium ultimum	2	100	100	96	96
Fusarium spp.[a]	5	89	69	60	55
Fusarium oxysporum f. sp. *melonis*	5	86	58	52	52
Penicillium oxalicum	5	59	0[b]	0	0
Rhizopus stolonifer	2	26	0	0	0

[a] Isolates of *Fusarium* spp. were selected randomly from the dilution plates.
[b] No growth occurred.

one of two groups: (i) colonies with diam larger than 7-8 mm; fungi in this group were considered tolerant to PCNB. Typical fungi belonging to this group were fusaria and certain aspergilli. (ii) Colonies with diam smaller than 2-3 mm; fungi in this group were considered sensitive to PCNB. These two groups comprised 60-80% of the total colonies. Colonies intermediate between these two groups were disregarded.

At each of five incubation periods tested, a higher proportion of fungi with larger colonies was isolated from alfalfa particles in soil containing PCNB than in the corresponding PCNB-free soil (Table 1). The mean difference was 2-fold. The opposite trend was evident for fungi with small colonies, the proportion of which from PCNB soil numbered about three-fourths that from soil without PCNB. For both types, the larger differences occurred in the earlier incubation periods, the difference being particularly great in the case of the larger colonies. Thus, fungi tolerant to PCNB were evidently selectively increased in soil containing PCNB, as measured by partial inhibition of colony growth in the presence of PCNB.

The second approach involved an attempt to assess, indirectly, populations of fungi completely inhibited by PCNB. Suspensions of colonized alfalfa particles which had been incubated 2-32 days in soil with and without PCNB were plated on acidified PDA + surfactant, with and without PCNB, at 100 μg/ml. Since PCNB-tolerant fungi included over 90% of the total fungi at all sampling periods, PCNB-sensitive fungi could not be detected with precision by this method. Therefore, a method was devised to detect small numbers of PCNB-sensitive fungi directly. A suspension of colonized alfalfa particles diluted to give 15-20 colonies/plate was spread over sterile cellophane covering a plate of acidified PDA + surfactant + PCNB at 100 μg/ml. It was found in separate experiments that non-coated sterilized cellophane would not affect the response of fungi to PCNB in agar. After 5 days' incubation, when all the tolerant fungi had given visible colonies, the cellophane was transferred to acidified PDA + surfactant without PCNB. The original colonies were then marked on the bottom of the plate and counted. During the next 5 days, the development of new colonies was noted. The new colonies were considered to have been completely inhibited by PCNB.

Combined results of several different experiments showed that when PCNB was present in soil the number of sensitive fungi in alfalfa particles decreased to one-fourth that in PCNB-free soil. A total of 182 sensitive colonies was isolated from particles incubated in soil without PCNB, as compared with 46 from soil containing PCNB. These represented, respectively, 6.4 and 2.2% of the total fungi enumerated. Colonies of *R. stolonifer* which appeared after the transfer to the PCNB-free medium were not included, as it was already known that this fungus was completely inhibited by PCNB.

Newly appearing colonies and colonies emerging before transfer were randomly selected to compare their sensitivities to PCNB in agar containing 100 μg PCNB/ml. Of 20 newly appearing colonies, 9 were completely inhibited on PCNB agar; the average inhibition of growth was 76%. Of 12 original colonies, none was completely inhibited, and the average inhibition of growth was 27%. Most of the tolerant fungi were species of *Penicillium* or *Aspergillus* and a few sterile fungi which differed from those genera originally on the plates.

Concentration of PCNB in soil and alfalfa particles.—The concn of PCNB in soil and alfalfa particles was determined at each of five sampling periods from 2-32 days after commencing the experiment. At the 2nd, 4th, 8th, and 18th day, concn in soil was 8-9 μg/g, and in alfalfa particles, 52-92 μg/g (Table 3). At the 32nd day, the concn in soil had decreased to 2.3 μg/g, and in alfalfa particles, 18 μg/g. Thus, the alfalfa had concd PCNB to levels 7- to 11-fold higher than the concn in soil.

Partial suppression of R. stolonifer and streptomycetes.—The extreme sensitivity of *R. stolonifer* to PCNB in agar raised questions as to why this fungus was not completely suppressed in alfalfa particles in soil containing PCNB (Table 1). The alfalfa hay was found to contain an indigenous population of approx 20,000 propagules of *R. stolonifer*/g (in addition to 1,000 propagules of *Aspergillus* spp. and 2,500 of streptomycetes). This suggested that the incomplete suppression of *R. stolonifer* in the presence of PCNB might be due to its previous establishment in the particles. To test this possibility, alfalfa particles were incorporated into sterile soil either with or without PCNB, thus removing any external source of *R. stolon-*

TABLE 3. Pentachloronitrobenzene (PCNB) concn in soil and alfalfa residues at different times after amending soil with PCNB and alfalfa residues[a]

Incubation time, days	PCNB, μg/g	
	Soil	Alfalfa residues
2	9.0	62
4	8.0	52
8	9.0	54
18	8.5	92
32	2.3	18

[a] PCNB was extracted from soil or alfalfa residue with hexane and isopropyl alcohol, and was assayed by gas chromatography.

ifer. The results showed a pattern of colonization similar to that observed in the previous experiments; namely, the population level of *R. stolonifer* in PCNB-amended soil was reduced to one-half to one-third that in nonamended soil. When sterile alfalfa particles were incorporated into natural soil, with or without artificial infestation with *R. stolonifer*, the difference between the amount of colonization in the presence or absence of PCNB again was no greater than that obtained with nonsterile alfalfa. To test whether the rapid growth rate of *R. stolonifer* might enable it to enter the alfalfa particles before the particles accumulated an inhibitory concn of PCNB, sterilized alfalfa particles were incubated for 17 hr in sterilized soil with PCNB at 10 μg/g, then mixed with *Rhizopus*-infested natural soil containing 10 μg PCNB/g and incubated for 1 or 2 days. Again, suppression of colonization in alfalfa particles was no greater than that which occurred in other experiments.

Isolates of *R. stolonifer* from the alfalfa particles were found to be as sensitive to PCNB in agar as the original isolates. Analysis of individual alfalfa particles showed that *Rhizopus* had colonized all the particles.

A similar partial suppression of streptomycete colonization of alfalfa particles by PCNB was observed. At various time intervals of from 1 to 18 days, particles incubated in soil containing PCNB had about half the number of colonies as those from soil without PCNB. A greater suppression was expected, based on the high sensitivity of streptomycetes to PCNB in agar (4, 5). As with *R. stolonifer*, the alfalfa particles were found to contain an indigenous population of streptomycetes to the extent of about 2,500 propagules/g of tissue. Nevertheless, when autoclaved particles were added to soil, the suppression of streptomycetes was again incomplete, and the isolates colonizing the particles were no more tolerant of PCNB than were the original isolates.

Effect of PCNB on colonization of alfalfa particles in artificially infested soil.—The effect of PCNB on competing pairs of microorganisms, one member being sensitive and the other tolerant to PCNB, was examined in sterilized soil. The pathogenic fungus, *F. oxysporum* f. sp. *melonis*, was chosen as a tolerant fungus, and *P. oxalicum* or either of two isolates of *Streptomyces* isolated from colonized alfalfa were selected as sensitive microorganisms. Growth of the two streptomycetes was completely suppressed in agar containing 10 μg PCNB/ml. These microorganisms did not produce inhibition zones or lytic zones when tested in pairs on different agar media. Sterile soil was artificially infested with each of these microorganisms, and incubated for 6-10 weeks, when population levels were determined. Soils were then mixed in the proportions indicated in Table 4 (footnote a), and were supplemented with PCNB. Subsequently, autoclaved alfalfa particles were mixed with the soils and were allowed to become colonized. The numbers of propagules were determined again after 2-5 days, the fungi on acidified PDA + detergent and the streptomycetes on chitin agar (9).

In four tests, numbers of the sensitive microorganisms colonizing alfalfa were reduced in the presence of PCNB, whereas numbers of tolerant microorganisms were increased. For example, in soil containing PCNB, numbers of *P. oxalicum* were one-third to one-sixth, and streptomycetes, one-ninth to one-twelfth, those in soil without PCNB (Table 4). By contrast, numbers of *F. oxysporum* f. sp. *melonis* in PCNB-amended soil were increased 1.5- to 8-fold over those in soil without PCNB. The increase in *F. oxysporum* f. sp. *melonis* was most pronounced when *P. oxalicum* was the competitor.

Discussion.—Quantitative and qualitative changes in the soil microflora colonizing the alfalfa particles were brought about by PCNB. The decrease in numbers of *Rhizopus* and *Penicillium*, which are PCNB-

Table 4. Effect of pentachloronitrobenzene (PCNB) on colonization of sterilized alfalfa residues incubated in sterilized soil inoculated with pairs of microorganisms with differing sensitivities to PCNB[a]

Test	Incubation time, days	PCNB in soil[b]	Colonies/g alfalfa residues, × 10^5		
			Fusarium oxysporum	*Penicillium oxalicum*	*Streptomyces* spp.
1	2	+	14	4	
		−	4.4	23	
	5	+	35	12	
		−	19	33	
2	2	+	10	2.0	
		−	1.8	8.6	
	5	+	36	34	
		−	4.6	140	
3	3	+	51		14
		−	26		165
4	3	+	9		730
		−	6		6,650

[a] Different volumes of sterilized soil previously infested with *F. oxysporum* were mixed with sterilized soil similarly infested with *P. oxalicum*, *Streptomyces* spp., or with noninfested sterilized soil to give the following initial concn of propagules/g soil. Test 1: *F. oxysporum*, 6×10^4; *P. oxalicum*, 3×10^5. Test 2: *F. oxysporum*, 1.5×10^4; *P. oxalicum*, 7.5×10^4. Test 3: *F. oxysporum*, 8×10^4; *Streptomyces* sp., 1×10^8. Test 4: *F. oxysporum*, 8×10^4; *Streptomyces* sp., 6×10^8. *Fusarium oxysporum* f. sp. *melonis* was tolerant to PCNB, whereas *Penicillium oxalicum* and *Streptomyces* spp. were sensitive.

[b] + = soil contained 10 μg PCNB/g; − = soil without PCNB.

sensitive, and the accompanying increase in numbers of *Pythium* and *Fusarium,* which are tolerant to PCNB, resemble closely changes occurring in parasitic systems wherein disease severity due to *Fusarium* or *Pythium* was increased in the presence of PCNB (1, 6). The selective saprophytic increase in such tolerant fungal pathogens as *Pythium* and *Fusarium* may be significant in terms of increased inoculum potential.

The presence of plant residues appears to enhance the effectiveness of PCNB in bringing about alterations in microbial populations by providing a site for microbial colonization and activity, and for concn of PCNB. The accumulation of PCNB by plant debris may increase its chances of coming into direct contact, in effective concn, with sensitive microorganisms in loci where such organisms are more likely to be abundant, and in a vegetative rather than in a resistant resting condition. Microbial interactions with such pesticides as PCNB might best be sought in organic microhabitats rather than in the soil mass.

PCNB is also taken up and may be concd by mycelia of fungi in soil (8). This affinity could further increase its effectiveness against sensitive soil-borne pathogens, and provide a means for entry into microbial food chains, wherein further concn and opportunity for interactions with specific microorganisms may occur.

The incomplete suppression by PCNB of *R. stolonifer* and streptomycete populations in colonized alfalfa is of interest, since these organisms are very sensitive to PCNB in culture (Table 2) (4). Several factors may be involved. (i) The initial inoculum of *R. stolonifer* and the streptomycetes already present in the alfalfa residues may allow them to escape PCNB, at least in the early stages of colonization; but this does not provide a complete explanation, since suppression was also incomplete with sterilized alfalfa. (ii) PCNB and the microorganisms may exist in different microsites on or within a single alfalfa particle. Even if penetration were restricted, once within the tissues, mycelia could quickly ramify throughout. (iii) Sorption of PCNB may reduce the amount available for biological activity against microorganisms in soil.

Although rates of utilization of substrate were not determined in our study, the alterations in microbial populations brought about by PCNB can be interpreted in terms of the results of Farley & Lockwood (5), who found that PCNB decreases competition for nutrients in soil by suppressing PCNB-sensitive microorganisms, resulting in selective increases of tolerant forms. This interpretation was supported by tests in sterilized soil with pairs of competing microorganisms having different sensitivities to PCNB, but which were not antagonistic to one another in agar tests.

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