THE EFFECT OF PENTACHLORONITROBENZENE ON THE SOIL MICROFLORA

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ABSTRACT

THE EFFECT OF PENTACHLORONITROBENZENE ON THE SOIL MICROFLORA

by James D. Farley

The effect of pentachloronitrobenzene (PCNB) on the soil microflora was studied with the objective of explaining why certain fungi insensitive to PCNB increase following soil treatment with this compound.

Diluted soil suspensions from natural soil were seeded onto selective agar media containing various concentrations of PCNB. Actinomycete numbers were reduced 65% by 1 ppm, 90% by 10 ppm and 99% at 25-200 ppm. Fungal numbers were reduced 27% by 10-500 ppm, but were unaffected by 1 ppm. Bacterial numbers were unaffected at concentrations of PCNB lower than 200 ppm PCNB; at higher concentrations bacterial numbers were greatly reduced. PCNB at 50 ppm partially or completely inhibited the growth in agar of 10 of 14 fungi, 7 of 10 actinomycetes and none of 10 bacteria tested.

PCNB was added to a Conover loam soil in which the metabolic activity of heterotrophic soil microorganisms was minimal. PCNB at 200 ppm did not affect soil respiration, concentrations of sugars or amino compounds and numbers of actinomycetes, bacteria and fungi in such soil. This

indicated that the antimicrobial effects of PCNB observed in agar were not expressed in soil where microbes are relatively inactive.

Actinomycetes and fungi were inhibited by PCNB in soil amended with nutrients, however. Five days after addition of 0.2% chitin and 10 ppm PCNB, there were 70% fewer actinomycetes in PCNB-treated soil than in soil without PCNB; at 10 days, there were 40% fewer, PCNB, at 50 ppm, suppressed respiration by 50% in chitin-amended soil during a 33 day period. In soil treated with 0.2% glucose, numbers of PCNB-sensitive fungi were reduced by about 50% by 100 ppm PCNB. Respiration in soil treated with 1% glucose increased to a maximum of 75 μl/hr/g soil at 3 1/2 days after amendment addition, whereas respiration in soil treated with 1% glucose and 100 ppm PCNB reached a maximum of 58 μl/hr/g soil at the 5th day. The delay of the buildup of a respiring population in PCNB-treated soil was probably the result of the suppression of PCNB-sensitive microorganisms.

Glucose was utilized at a slower rate in soil treated with PCNB than in soil without PCNB, presumably because of the inhibition of PCNB-sensitive fungi and actinomycetes.

Numbers of microbes insensitive to PCNB (e.g., bacteria and Fusarium spp.) increased more in glucose-amended soil treated with PCNB than in glucose-amended soil without

PCNB. Germination of chlamydospores of Fusarium solani f. phaseoli and conidia of Helminthosporium victoriae was greater in soil containing glucose and PCNB than in soil with only glucose. More H. victoriae conidia germinated on PCNB-treated soil amended with alfalfa residue than on alfalfa-amended soil with no PCNB. Since germination of these two fungi in soil was not directly affected by PCNB, it seemed likely that PCNB treatment resulted in increased availability of nutrients to these spores.

The following explanation is proposed for the increase of PCNB-insensitive fungi following soil treatment with PCNB: PCNB decreases competition for nutrients in soil by suppressing actinomycetes and some fungi. The reduced competition allows those fungi insensitive to PCNB to flourish.

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by

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INTRODUCTION

When pentachloronitrobenzene (PCNB) - a narrow spectrum fungicide principally used for the control of damping-off or root rot caused by Rhizoctonia solani - is added to soil, a significant increase in seedling damage by other fungal pathogens insensitive to PCNB often occurs. The two fungi most commonly associated with this increase have been Fusarium spp. (6, 12), and Pythium spp. (13, 16, 22, 46). It has been suggested, although supporting data are generally lacking, that the increased activity of these pathogens following soil treatment by PCNB may be due to the suppression by PCNB of specific microbial antagonists of these pathogens.

The effect of PCNB on the soil microflora was studied with the purpose of explaining why fungi insensitive to PCNB increase following soil treatment with this compound.

LITERATURE REVIEW

When a fungicide is added to soil to control pathogenic fungi, significant alterations in the biophase often occur. Soil fungicides usually make little distinction between the target pathogen and its neighbors (22). Actinomycetes, bacteria and saprophytic fungi, and microfauna as well, may be inhibited or killed outright, and because of reduced biological competition for nutrients unaffected organisms tend to multiply. The well equilibrated and stable biological system of soil is changed, for better or worse. The subject of this literature review concerns the deleterious effects of soil treatment on the soil biophase. The emphasis is on how a soil fungicide (PCNB) promotes disease accentuation and disease exchange.

Disease accentuation: Disease accentuation, also called the 'boomerang effect' (22), involves the disappearance of the dominant pathogen after treatment, which is soon followed by its reappearance in often greater amounts. Kreutzer (22), for example, has observed reinvasion of soils treated with chloropicrin and chlorobromopropene by Rhizoctonia

solani and Pythium spp. Gibson (15) reported that R. solani and Pythium ultimum rapidly recolonized ethyl-mercury phosphate-treated soil, and diseases caused by these pathogens were more severe in treated soil than in untreated soils. R. solani and P. ultimum were noted to be more tolerant to the fungicide than other common soil saprophytes. KenKnight (19) noted that mercury compounds used for soil treatment to control Streptomyces scabies generally caused, instead, a marked increase in scabbing. Certain isolates of S. scabies were very tolerant to mercury compounds and he concluded that these mercury-tolerant isolates were able to increase in treated soil because of reduced competition from mercury-sensitive organisms. For a more complete review of disease accentuation, see Kreutzer (22, 24).

Disease exchange: Disease exchange is a situation in which the dominant pathogen is controlled but a previously unimportant pathogen is elevated to major importance thus becoming the new dominant pathogen. For example, Gibson (14) found that treatment of peanut seeds with organo-mercurial compounds reduced preemergence losses, but damage due to crown rot caused by Aspergillus niger increased. Certain isolates of A. niger were mercury-tolerant and multiplied in the area of mercury-treated seeds. It was speculated that

they increased because of the reduction of competition from mercury-sensitive soil microorganisms.

A soil fungicide often cited as causing disease exchange is PCNB (6, 12, 13, 16, 22, 46). The most common use of PCNB is for the control of damping-off or root rot caused by Rhizoctonia solani. Many workers have treated the soil with PCNB to control R. solani only to find an increase in damping-off or root rot caused by other pathogens. The most common pathogens elevated to new importance by PCNB treatment are Pythium spp. (13, 16, 22, 46) and Fusarium spp. (6, 12).

The first published report of disease exchange caused by soil treatment using PCNB was by Fulton in 1956 (12). Fulton studied the activity of various fungi pathogenic to cotton seedlings in soil treated with various fungicides. In plots treated with PCNB a marked reduction in the pathogenic activity of R. solani and Sclerotium bataticola was evident whereas the incidence of disease in cotton seedlings caused by Fusarium moniliforme and Colletotrichum gossypii increased. Fulton speculated that PCNB decreased microbial competition in the soil, thus allowing the two PCNB-insensitive pathogens to increase their activity.

Bird et al. (6) also tested various fungicides as

control measures for the cotton seedling disease complex, the most important pathogens of which were R. solani, Fusarium spp. and Pythium spp. Bird observed no change in the incidence of damping-off or root rot following PCNB treatment of soil. It was noted, however, that PCNB treatment caused marked decreases in the number of plants infected with R. solani and Pythium spp., whereas the number of plants infected with Fusarium spp. increased with PCNB treatment. Bird thought that PCNB 'conditioned' the soil microflora so as to permit a more rapid development of Fusarium spp. No data were presented to support this hypothesis.

Others have reported an increase in the pathogenic activity of Pythium spp. following soil treatment with PCNB. Garren (13), for example, observed increased pod rot on peanuts caused by Pythium spp. following soil treatment with PCNB for the control of R. solani. Vaartaja et al. (46) also observed disease increases with the use of PCNB to control damping-off of conifers. For example, Dexon (sodium p-dimethylamino-benzenediazo sulfonate) gave significant damping-off control when used alone, but failed when mixed with PCNB. No phytotoxicity was observed and chemical interaction seemed unlikely as these two materials are commonly mixed. Pythium spp. were the organisms most often associated with diseased

seedlings after PCNB and Dexon treatment. They attributed the increased activity of Pythium spp. to the inhibition by PCNB of organisms antagonistic to Pythium, but no conclusive data were presented to verify this conclusion. Kreutzer (22) has also observed disease exchange with sugar beets. PCNB was applied to soil to control R. solani and the activity of Pythium spp. increased.

The only paper which has dealt with PCNB-induced disease exchange in detail is that by Gibson et al. (16). They reported that PCNB was effective as a soil treatment against R. solani damping-off on pine seedlings, but was of little use against Pythium spp. Gibson showed that PCNB not only failed to control disease caused by Pythium spp. but also that appreciable increases in Pythium disease followed use of the fungicide. To investigate the possibility that the increase of disease caused by Pythium might be due to the action of the fungicide on a competitor of Pythium in the soil, soil suspensions were plated onto Czapek-Dox amended with PCNB. When the colonies were three days old, inocula of Pythium were introduced on the side of each plate. Pythium made faster growth through soil plates treated with PCNB than through those untreated. On agar media without PCNB, Pythium was noted to be inhibited in the vicinity of colonies of Penicillium

paxilli, whereas on agar containing PCNB, P. paxilli was inhibited. It was postulated that Pythium increased in PCNB-treated soil because PCNB inhibited an antagonist of Pythium, namely P. paxilli. The addition of P. paxilli to soil to control Pythium disease was unsuccessful, however.

Specificity of PCNB: PCNB has been used to control diseases caused by R. solani, Streptomyces scabies, Botrytis cinerea, Sclerotinia spp., Sclerotium spp., Plasmodiophora brassicae and Tilletia caries (17, 23). It is relatively ineffective against diseases caused by Pythium spp., Fusarium spp., Verticillium albo-atrum, Colletotrichum gossypii, Phytophthora infestans, Aphanomyces euteiches and Thielaviopsis basicola.

There have been several in vitro studies of the effect of PCNB on the germination, sporulation and/or growth of various fungi (2, 10, 11, 32, 34, 36, 37, 45). PCNB has been found to inhibit spore germination and sporulation of some fungi (32, 34). Roy (34), for example, reported that PCNB at a concentration of about 5,000 ppm strongly repressed spore germination of Botrytis cinerea, Fusarium caeruleum, Ascochyta rabiei, Trichoderma viride, Rhizopus nigricans and Alternaria spp. Sclerotial formation of Rhizoctonia solani as well as sporulation of the above mentioned fungi was also almost

completely inhibited by PCNB. Reavill (32) using PCNB in the gaseous phase also found that sporulation of B. cinerea, F. caeruleum and T. viride was repressed. No one, however, has comprehensively studied the effect of PCNB on germination and sporulation on a broader spectrum of pathogenic and saprophytic soil fungi.

PCNB has been reported to inhibit the growth in agar of R. solani (2, 10, 11, 32, 34, 36, 37), Botrytis spp. (2, 31, 32, 34), Rhizopus spp. (34, 45), Trichoderma viride (32, 34, 45), Mucor ramannianus (45), Alternaria sp. (34), Ascochyta rabiei (34), Pencillium spp. (2, 16), Sclerotinia sclerotiorum (2), and Sclerotium rolfsii (2). PCNB was relatively ineffective against Fusarium spp. (2, 32, 34, 45), Pythium spp. (2, 10, 38, 45), Phytophthora spp. (2) and Thielaviopsis basicola (30, 43).

Little work has been done on the effect of PCNB on soil bacteria and actinomycetes. Takahashi et al. (41) found that when 500 ppm PCNB was incorporated into Martin's rose bengal agar, numbers of actinomycetes from soil dilutions were drastically reduced. At 1000 ppm, PCNB also reduced numbers of soil bacteria. Davis (8) also found a very great reduction of numbers of actinomycetes colonies on soil dilution plates with water agar amended with 50 ppm PCNB. Davis also observed

a reduction of bacterial colonies on soil dilution plates treated with 50 ppm PCNB. For the enumeration of soil bacteria, he used soil extract agar, a medium which will also support actinomycetes. Because many actinomycetes were macroscopically indistinguisable from bacterial colonies, both actinomycetes and bacteria were reported as bacteria. Therefore, the reduction of colonies observed may have been due to suppression of actinomycetes rather than bacteria.

PCNB is often cited in the literature as an example of a narrow spectrum soil biocide, affecting only soil fungi (22). Strangely enough there have been no reports dealing with the effects of PCNB soil treatment on actinomycetes and bacteria. However, because of the recent work of Davis (8) and Takahashi et al. (41) showing that PCNB drastically inhibits growth of actinomycetes in vitro, the selective activity of PCNB in soil might be questioned.

Stability of PCNB: PCNB has been reported to have a long lasting effective residue in soil. Scheffer and Haney (35) found that PCNB allowed good seed germination and growth of sweet pea throughout 24 weeks in steamed soil artificially infested with Rhizoctonia solani. Kennedy and Brinkerhoff (20) reported that PCNB controlled R. solani over a 4-6 week period in nonsteamed sandy loam soil at ph 6.0. Other

investigators have reported disease control of R. solani 2-12 months after soil treatment with PCNB (3, 17, 33).

Recent evidence from gas chromatographic assay methods suggested that PCNB may be changed in soil. Chacko (7) has reported that PCNB can be altered in vitro by several soil Streptomyces. Most or all of the PCNB was converted to pentachloroaniline (PCA). Very recently W. H. Ko (unpublished data) found that PCNB was rapidly converted to PCA in soil submerged with water. Apparently, anerobic conditions are essential for conversion. Ashworth et al. (4) suggested that PCNB may be changed in soil into one or more fungitoxic products which may account for the long term disease control by PCNB. Of 100 ppm of PCNB added, only 12-32 ppm could be detected by gas chromatographic analysis after 160 days in a non-sterile field soil. During this time PCNB effectively controlled R. solani root rot on bean seedlings. Unidentified substances were recovered from PCNB-treated soil, but not from untreated soil.

MATERIAL AND METHODS

Chemical and physical properties of PCNB: PCNB is produced by Olin Chemical Corporation under the trade name of Terraclor. Commercial formulations containing 10-75% PCNB are available. A 20% wettable powder was used in most experiments discussed in this thesis. A crystalline preparation (99% purity) was used when PCNB was incorporated into agar media. PCNB is readily soluble in most nonpolar solvents, such as acetone, benzene, toluene, xylene, carbon tetrachloride, carbon disulfide and chloroform, and is less soluble in more polar solvents such as methanol, ethanol and isopropanol. PCNB was dissolved in acetone prior to adding to water in some experiments. It is relatively insoluble in water. The melting point is 142-145 C. The vapor pressure at 20 C is 4 mm of Hg. (32).

Source and preparation of soil: Conover loam from Michigan State University Botany farm was used in all tests unless specified otherwise. Water holding capacity (WHC) of this soil was 42.7% and organic matter content was 3.8%; 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 has been fallow for several years and to which pesticides had not recently been applied. Soil was sieved and stored in closed plastic containers at 15% moisture (35% WHC) at 23-27 C. Such soil will be referred to as natural soil. Unless otherwise stated, soil moisture was maintained at 35% WHC throughout experiments.

Microorganisms used in studies of the effect of PCNB on growth: Fungi used were: Aphanomyces euteiches Drechs., Aspergillus terreus Thom., Fusarium solani (Mart.) Appel & Wr. f. phaseoli (Burk) Snyd. & Hans., F. oxysporum Schlecht. f. melonis (Leach & Currence) Snyd. & Hans., Glomerella cingulata (Ston.) Spauld. & Schrenk, Helminthosporium victoriae Meehan & Murphy, Mucor ramannianus Moller, Pythium ultimum Trow, P. debaryanum Hesse, Rhizoctonia solani Kuhn, Thielaviopsis basicola (Berk. & Br.) Ferr., Trichoderma viride Fr., Verticillium albo-atrum Reinke & Berth. All fungi were maintained on potato-dextrose agar, except H. victoriae, which was grown on V-8 juice agar (per liter: 200 ml V-8 juice, 2 g $CaCO_3$, 20 g agar).

Actinomycetes used were: Actinoplanes philippinensis Couch, Micromonospora sp. Orskov, Nocardia erythropolis (Gray & Thornton) Waksman & Henrici, Streptomyces aureofaciens

Duggar, S. griseus (Krainsky) Waksman & Henrici, S. lavendulae (Waksman & Curtis) Waksman & Henrici, S. scabies (Thaxt.) Waksman & Henrici, S. venezuelae Ehrlich et al., S. viridochromogenes (Krainsky) Waksman & Henrici, Streptosporangium roseum Couch. All actinomycestes were maintained on a nutrient agar (per liter: 10 g maltose, 4 g yeast extract, 4 g dextrose, 20 g agar).

Bacteria used were: Agrobacterium tumefaciens (Smith & Townsend) Conn., Bacillus licheniformis (Weigmann) Chester, B. subtilis Cohn emend. Prazmowski, Corynebacterium fascians (Tilford) Dowson, Escherichia coli (Migula) Castellani & Chalmers, Pseudomonas angulata (Fromme & Murray) Holland, P. fluorescens Migula, Rhizobium trifolii Dangeard, Serratia marcescens Bizio, Xanthomonas phaseoli var sojensis (Hendges) Starr & Burkholder. All bacterial were maintained on potato-dextrose agar (PDA).

Soil amendments: Glucose or chitin and PCNB (20% wettable powder) were usually added in particulate form to moist soil (35% WHC). Chitin amendment was colloidal chitin dried at 100 C and ground by mortar and pestle into a fine powder. Colloidal chitin was prepared by the method of Lingappa and Lockwood (26) with modifications of Lloyd (27). In some experiments glucose and PCNB were added to soil dried

to 1-2% moisture by an electric fan. In these experiments glucose was added in aqueous solution, PCNB as 20% wettable powder.

Nutrient analysis of soil: Carbohydrates and amino compounds were extracted from soil by shaking equal parts of soil and distilled water in a 250 ml erlenmeyer flask on a wrist action shaker for 30 min. The soil suspension was centrifuged at 10,000 X G. for 5 min. and the supernatant was passed through a 0.22 μ Millipore filter and stored at 4 C until analyzed.

Carbohydrates were determined by the anthrone method (29). The anthrone reagent was prepared by dissolving 0.2 g anthrone in 100 ml 7 M sulfuric acid. One ml of the sample was added to 9 ml of the reagent and placed in a boiling water bath for 10 min. Optical density was read at 600 mμ in a colorimeter. A standard curve was made by using glucose at concentrations of 20, 40 and 80 μg/ml.

Amino acids and related compounds were determined by the ninhydrin method (28). Two-tenths g ninhydrin and 0.03 g hydrindantin were dissolved in 7.5 ml methyl cellosolve, followed by the addition of 2.5 ml of 4 N sodium acetate buffer (pH 5.5). One ml of the sample was mixed with 1 ml of the reagent and placed in a boiling water bath for 15 min. The solution was then diluted with 8 ml of 50% ethyl alcohol

and the optical density read at 570 mμ in a colorimeter. A standard curve was prepared by using glycine at concentrations of 4, 8 and 16 μg/ml.

The amount of glucose in extracts of glucose-amended soil was determined by the Glucostat reagent (Worthington Biochemical Corporation). Glucose was extracted from the soil by adding 20 ml of water to 20 g soil and shaking by hand for 1-2 min. The soil suspension was centrifuged at 10,000 X G. for 10 min. and the supernatant was passed through a 0.22 μ Millipore filter. One ml of the sample, diluted such that 1 ml contained 0.05-0.3 mg glucose, was added to 9 ml of the Glucostat reagent, prepared accordingly to the manufacturer's instructions. After 10 min. one drop of 4 M HCL was added to stop the reaction and to stablize the color. The optical density was read at 400 mμ in a colorimeter. A standard curve was prepared by using glucose at concentrations of 20, 40 and 80 μg/ml.

Soil respiration: Oxygen uptake was measured by standard Warburg manometric techniques (44). Six g moist soil was added to each flask and 3 or 4 flasks were used per treatment. Experiments were run for 10-30 days at a constant temperature of 27° C, with KOH (20%) being changed in the center well every 2-6 days. Manometers were read twice daily, the period of oxygen uptake measurements varying from 1/2-3

hrs, depending on respiratory activity of the soil. The rate of oxygen uptake was expressed in µl per g of oven dry soil per hour.

Enumeration of soil microbes: The numbers of propagules of soil bacteria, fungi and actinomycetes were estimated by the soil dilution plate technique. Soil suspensions were prepared by adding the equivalent of 1 g oven-dry soil to 100 ml sterile 0.85% saline solution for bacteria, or to sterile distilled water for actinomycetes and fungi, followed by blending in a Servall Omnimixer for 1 min. at approximately 4,000 rpm. Further dilutions were made with sterile 0.85% saline solution or water. For bacteria and actinomycetes, 1 ml diluted soil suspensions was mixed with 15 ml of molten agar at 43 C in petri dishes. For fungi, 0.5 ml diluted soil suspension was applied to the surface of 10-15 ml cooled agar. Chitin agar (26) was used to support the growth of actinomycetes. A modified soil extract agar containing 25-50 ppm PCNB to suppress actinomycetes was used for the enumeration of soil bacteria (1). This method will be discussed in the results. A modified acidified PDA medium, similar to that of Steiner and Watson (40), was used for enumerating fungi. A detergent, NPX (nonyl phenyl polyethylene glycol ether, Union Carbide Corporation), at a concentration of 1000 ppm, was added to PDA prior to autoclaving to retard fungal growth.

Bacteria were suppressed by acidifying the agar, after autoclaving, by adding 1 ml of 50% lactic acid to 200 ml agar.

Germination of fungal spores on soil: PCNB (200 ppm) in the form of a 20% wettable powder was mixed with air-dried natural soil. Twenty g of the treated soil or soil without PCNB were added to 250 ml flasks and 3 ml of 0.34% glucose solution were pipetted on the soil surface, to give a final concentration of 500 ppm. Soil moistened with distilled water served as the control. The moistened soil was mixed throughly with a spatula and passed through a sieve with 2 mm diam. meshes. At 3-hr intervals after glucose addition the soil was added to a small petri dish (50 X 15 mm). One-1.5 ml of distilled water was added to the soil and a smooth surface was made with a stainless steel spatula. Spore suspensions of Helminthosporium victoriae, washed 3 times by centrifugation in glass distilled water, were then added to the smoothed surface. After incubation at 23-27 C for 6-8 hrs, the spores were stained with phenolic rose bengal, recovered with plastic film and observed microscopically (25).

In other experiments 75 g of moist soil (48% WHC) treated with various concentrations of PCNB (20% wettable powder) were placed in a 150 mm diam. petri dish, and the surface was smoothed. Seventy five mg of moistened, finely

chopped alfalfa residue were placed in a small trough (4 X 0.5 X 0.5 cm) made at the edge of the soil surface, then covered with a thin layer of soil. Spore suspensions of H. victoriae, washed 3 times in glass distilled water, were applied to the soil surface on top and at varying distances from the residue. Spores were incubated at 23-27 C for 6-8 hrs and recovered and observed as previously described.

Stability of PCNB in soil: The stability of 100 ppm PCNB (20% wettable powder) was studied in Conover loam soil (35% WHC) amended with 1% glucose or 0.2% chitin. Treated soil was added to 250 ml flasks and PCNB was extracted after 0, 2, or 4 weeks incubation at 23-27 C.

PCNB was extracted from the soil in the following way: 40 ml of a mixture of hexane and isopropyl alcohol (3:1) were added to 20 g soil in a 250 ml flask. The mixture was shaken on a wrist action shaker for 30 min. After sedimentation, the solvent mixture was decanted. The extraction procedure was repeated once. The combined extracts were diluted with the solvent mixture such that comparisons with a 1 ppm PCNB standard could be made. An Aerograph model 600-D gas chromatograph (Varian Aerograph Co.) with an electron-capture detector was used to detect PCNB in the extract. A column (5 ft. X 1/8 in.) containing 3% SE-30 on 60-80 Chromosorb W was used. Injection, column, and detection temperatures were 260 C, 155 C,

and 170 C, respectively, and the flow rate of nitrogen was maintained at 60 ml/min.

RESULTS

Stability of PCNB in soil: It was necessary to determine whether the microbial effects observed in experiments with PCNB were due to PCNB or some byproduct(s) of PCNB. Experiments were therefore designed to test the stability of PCNB in soil under the conditions of tests done in this research on the effects of PCNB on soil microflora.

The stability of PCNB was tested in Conover loam soil amended with 0.2% chitin or 1% glucose and 100 ppm PCNB. Analyses of soil amended with glucose and PCNB were made 1 and 2 weeks following amendments. Analyses of soil amended with chitin and PCNB, or with PCNB only were made 1, 2, 3, and 4 weeks after treatment. No detectable alteration of PCNB occurred during the time periods tested (Table 1).

The time intervals and amendments are similar to those used in experiments reported in this thesis and thus it appears likely that PCNB and not some byproduct was responsible for the microbial effects to be reported.

The effect of PCNB on soil microbes in agar: The effects of PCNB on growth, germination and sporulation of

Table 1.--Stability of PCNB in Conover loam soil.[a]

Incubation period, weeks	PCNB recovered from soil treated as indicated, %[b]		
	PCNB	Glucose + PCNB	Chitin + PCNB
0	95	95	96
1	95	97	101
2	92	97	98
4	93		98

[a]PCNB (100 ppm) as a 20% wettable powder was added to soil and recovered 0, 1, 2, and 4 weeks later by extracting the soil with hexane and isopropyl alcohol (3:1). The extracts were analyzed for PCNB by gas chromatrography.

[b]Glucose = 1%; chitin = 0.2%.

several soil-borne fungi in agar have been studied (32). Little work, however, has been done on the effects of PCNB on soil bacteria and actinomycetes. In order to get a general idea of the antimicrobial spectrum of PCNB in agar, 2 kinds of experiments were done: 1) The inhibitory effect of various concentrations of PCNB was tested on identified cultures of 10 soil bacteria, 10 actinomycetes and 14 fungi, and 2) diluted soil suspensions from natural soil were seeded onto selective media containing various concentrations of PCNB and the number of colonies of bacteria, actinomycetes and fungi counted.

For experiments with identified soil microbes, diluted spore or cell suspensions were plated onto PDA containing 1, 10, and 50 ppm PCNB. Mycelial disks from agar, rather than spores, were used as inocula for Aphanomyces euteiches, Pythium ultimum, P. debaryanum and Rhizoctonia solani. Agar containing 25 ppm Triton X-100 but without PCNB served as a control. Growth was estimated visually after 3-10 days incubation at 23-25 C and compared with growth on the control.

PCNB was added to agar by the following method: Fifty-100 mg of PCNB (technical grade or an equivalent amount of 20% wettable powder) were dissolved in one ml of acetone in a stoppered test tube. The acetone sterilized the PCNB within two hr. The acetone solution of PCNB was

then added to 100 ml of sterile distilled water to which 0.05 ml of a wetting agent, Triton X-100 (Rohm and Haas Co., Philadelphia), was previously added; PCNB was precipitated as a milky suspension. Suitable amounts of the PCNB suspension were then added to molten agar at 43-50 C. Triton X-100 at the concentrations used, 15-25 ppm in the agar, had no suppressive effect on fungal, actinomycete or bacterial numbers.

PCNB partially or completely inhibited growth of 10 of 14 fungi tested (Table 2). At a concentration of 10 ppm, PCNB completely inhibited growth of H. victoriae and R. solani (isolate 2). A. euteiches, G. cingulata, P. ultimum, R. solani (isolate 1) and T. viride were partially inhibited by 10 ppm PCNB. F. oxysporum f. melonis, M. ramannianus and V. albo-atrum were partially inhibited at 50 ppm. Fungi unaffected by PCNB at 50 ppm were A. terreus, F. solani, P. debaryanum and T. basicola.

The actinomycetes A. philippinensis, Micromonospora sp., S. griseus, S. scabies and S. venezuelae were completely inhibited by PCNB at 50 ppm. Micromonospora sp. was also completely inhibited by 10 ppm and partially inhibited at 1 ppm. N. erythropolis and S. roseum were partially inhibited at 50 ppm. Three other actinomycetes were unaffected. Thus, PCNB at 50 ppm completely or partially inhibited growth of 7

Table 2.--Inhibitory effect of PCNB on microorganisms in nutrient agar.

Microorganism	PCNB (ppm)			
	0	1	10	50
Actinomycetes				
Actinoplanes philippinensis	+[a]	+	-	-
Micromonospora sp.	+	±	-	-
Nocardia erythropolis	+	+	+	±
Streptomyces aureofaciens	+	+	+	+
S. griseus	+	+	+	-
S. lavendulae	+	+	+	+
S. scabies	+	+	+	-
S. venezuelae	+	+	+	-
S. viridochromogenes	+	+	+	+
Streptosporangium roseum	+	+	+	+
Fungi				
Aphanomyces euteiches	+	+	±	±
Aspergillus terreus	+	+	+	+
Fusarium solani f. phaseoli	+	+	+	+
F. oxysporum f. melonis	+	+	+	±
Glomerella cingulata	+	+	±	±
Helminthosporium victoriae	+	+	-	-
Mucor ramannianus	+	+	+	+
Pythium ultimum	+	+	+	±
P. debaryanum	+	+	+	+
Rhizoctonia solani (Isolate 1)	+	±	±	±
R. solani (Isolate 2)	+	±	-	-
Thielaviopsis basicola	+	+	+	+
Trichoderma viride	+	+	±	±
Verticillium albo-atrum	+	+	+	±

[a] +, no inhibition of growth; ±, partial inhibition; -, complete inhibition.

of the 10 actinomycetes. Only Micromonospora sp. was inhibited at concentrations lower than 50 ppm.

Of the 10 bacteria tested none was affected by 50 ppm PCNB. Bacteria tested were: Agrobacterium tumefaciens, Bacillus licheniformis, B. subtilis, Corynebacterium fascians, Escherichia coli, Pseudomonas angulata, P. fluorescens, Rhizobium trifolii, Serratia marcescens, Xanthomonas phaseoli var sojensis.

In experiments using diluted suspensions of natural soil, actinomycete numbers were reduced by 65% at 1 ppm PCNB, 90% by 10 ppm and 99% at 25-100 ppm (Fig. 1). Fungal numbers were reduced 27-40% by 10-500 ppm, but unaffected by ppm. Bacterial numbers were unaffected at concentrations of PCNB lower than 200 ppm PCNB; at higher concentrations bacterial numbers were greatly reduced.

That PCNB inhibits the growth of some soil fungi confirms the reports of others (2, 10, 11, 32, 34, 36, 37, 45). However, the evidence that PCNB dramatically inhibits actinomycetes in agar is contrary to the widely held belief that PCNB is a narrow spectrum fungicide affecting relatively few soil microbes (22). That PCNB inhibits actinomycetes at lower concentrations than it inhibits bacteria appears to conflict with the opinion that bacteria are generally more sensitive to a toxicant than actinomycetes (23).

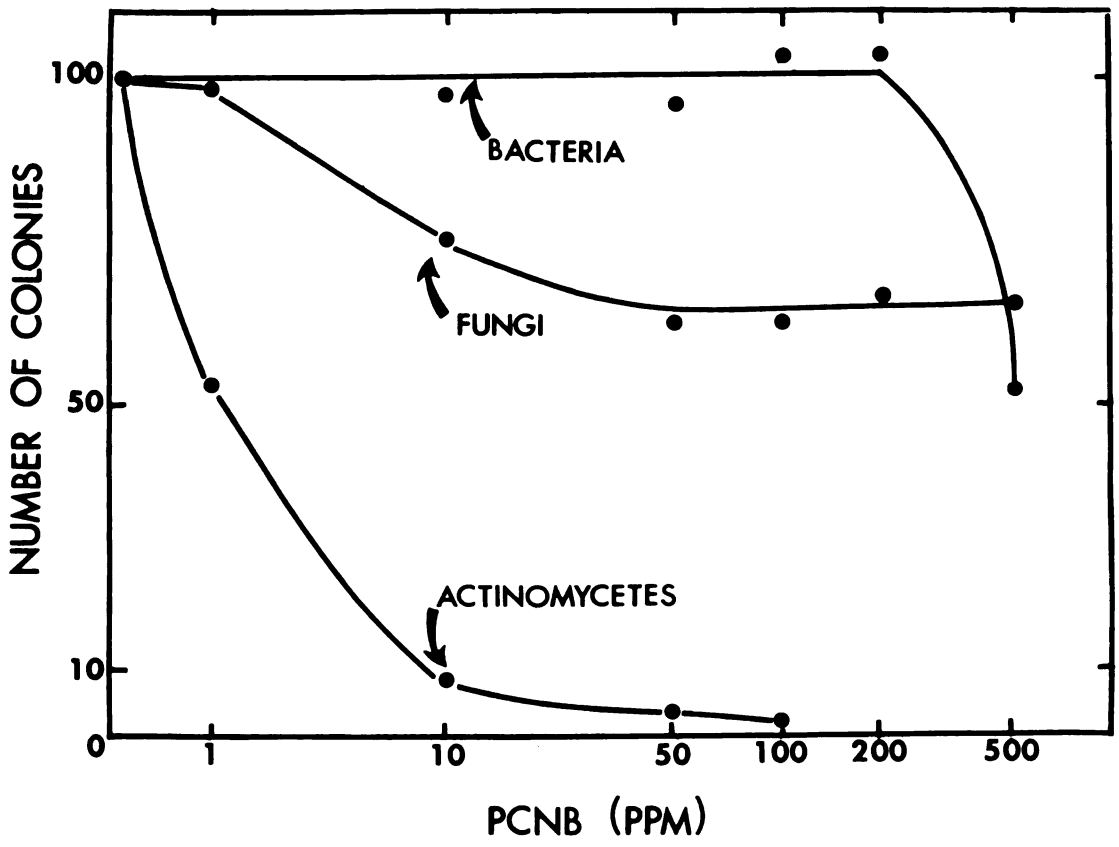


Fig. 1.--Numbers of actinomycete, bacterial and fungal colonies from diluted soil suspensions plated on media amended with various concentrations of PCNB. Chitin agar was used for actinomycetes, soil extract agar for bacteria and acidified PDA + NPX for fungi. For comparison, numbers of colonies were adjusted on the basis of 100 colonies per plate without PCNB.

Selective medium for bacteria: Media used for estimating numbers of soil bacteria also permit the development of actinomycete colonies (26). Since PCNB inhibits actinomycetes, but not bacteria in agar, the use of PCNB in media commonly used for the enumeration of soil bacteria was studied in an attempt to find a medium selective for soil bacteria.

Soil suspensions were prepared as previously described. Six plates were used for each treatment. Plates were incubated at 23-27 C and colony counts were made after 2 weeks incubation. Microscopic examination of all non-sporing colonies was made to ascertain if they were actinomycetes or bacteria. All experiments were repeated at least twice. Indicated differences were significant at the 1% level.

Thornton's standardized agar (42), soil extract agar and sodium albuminate agar (48) were amended with 25-50 ppm PCNB. All media which contained PCNB had more bacterial and fewer actinomycete colonies than media without PCNB (Fig. 2). For example, in a typical test, the numbers of bacteria and actinomycetes on soil extract agar without PCNB were 200 and 70 respectively, whereas there were 270 bacteria and 5 actinomycetes on PCNB-amended soil extract agar (Fig. 3). The increase in numbers of bacteria on PCNB-amended agar was probably the result of reduced competition due to the inhibition of actinomycetes. This was indicated by the

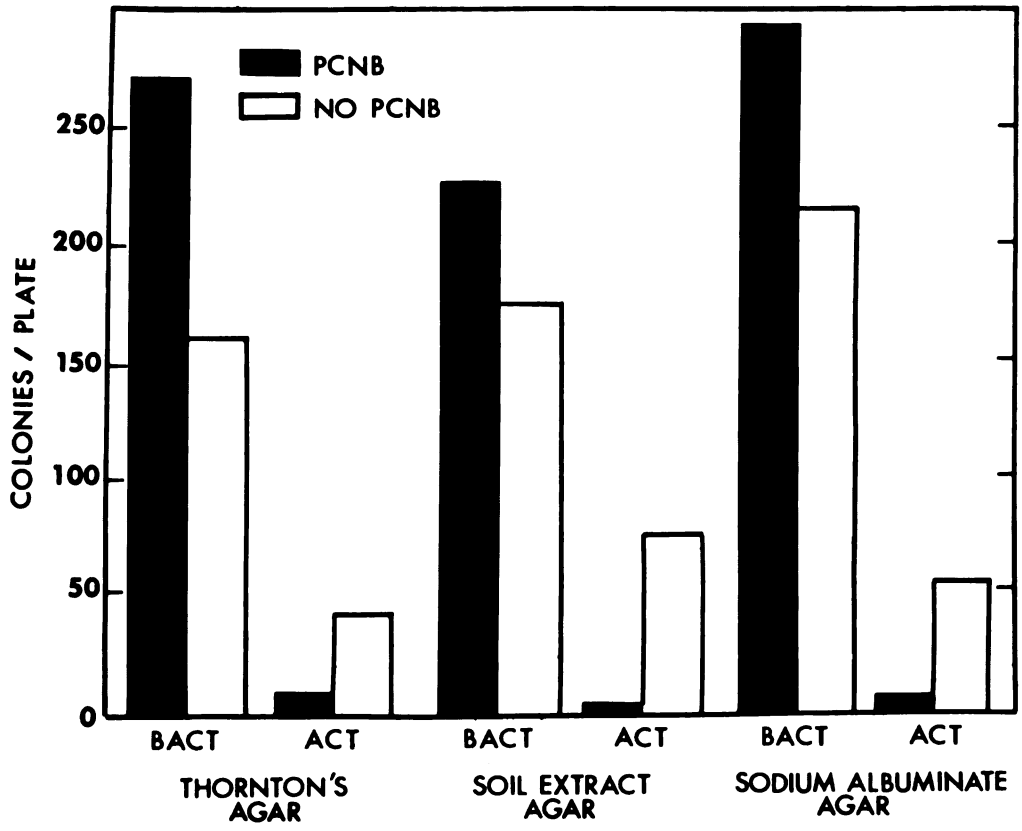
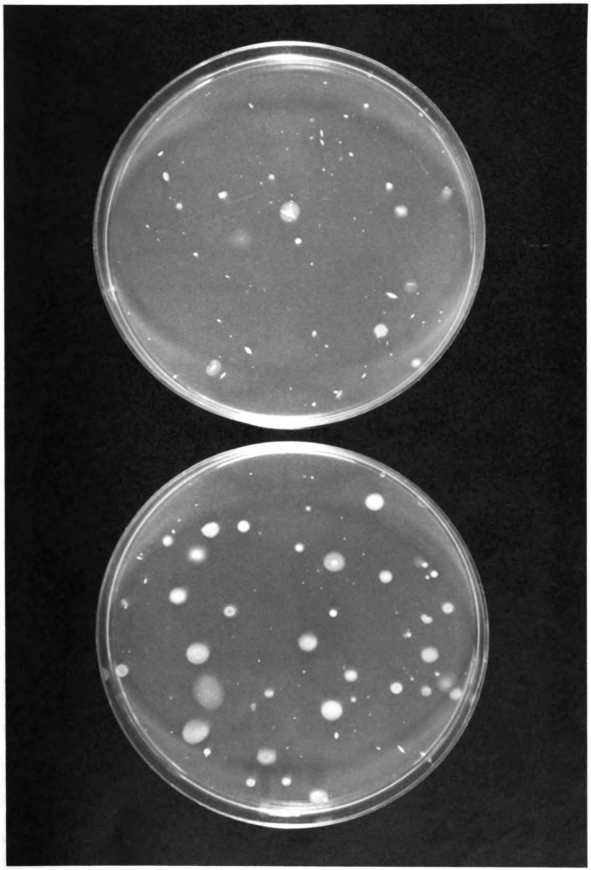


Fig. 2.--The effect of 50 ppm PCNB on numbers of actinomycete (ACT) and bacterial (BACT) colonies on 3 different media seeded with a diluted soil suspension (10^{-5}).

Fig. 3.--Colonies of bacteria and actinomycetes on soil extract agar without or with 50 ppm PCNB. Most of the large colonies on soil extract agar without PCNB (bottom) are actinomycetes. Nearly all the colonies on soil extract agar treated with PCNB (top) are bacteria.



absence of any increase in bacterial numbers on PCNB-amended agar when the distance between colonies was increased by using soil dilutions greater than 10^{-5}.

To establish the usefulness of the PCNB medium with different soil types, soil suspensions of muck, forest, or loam soil were mixed with soil extract agar containing 25-50 ppm PCNB. For each soil type, agar with PCNB had more bacteria and fewer actinomycetes than agar without PCNB (Fig. 4).

Effect of PCNB on microbes in natural soil: Kreutzer (24) has divided soil in which higher plants are growing into three substrate zones; an outer zone, representing the soil external to living roots and beyond the direct influence of their exudates, the rhizosphere zone and the rhizoplane. In the outer zone all utilizable substrates are occupied. This is an area where available energy is low and organisms are approaching or are in a state of quiescence. Soil of this type, which I will call natural soil, was treated with 200 ppm PCNB. If PCNB treatment killed microbes in this 'quiescent zone,' leakage of nutrients from dead cells should prompt microbial growth and multiplication. Soil respiration, population, and nutrient analyses were used as parameters to estimate the effect of PCNB on soil microflora.

Oxygen uptake was measured in natural soil treated

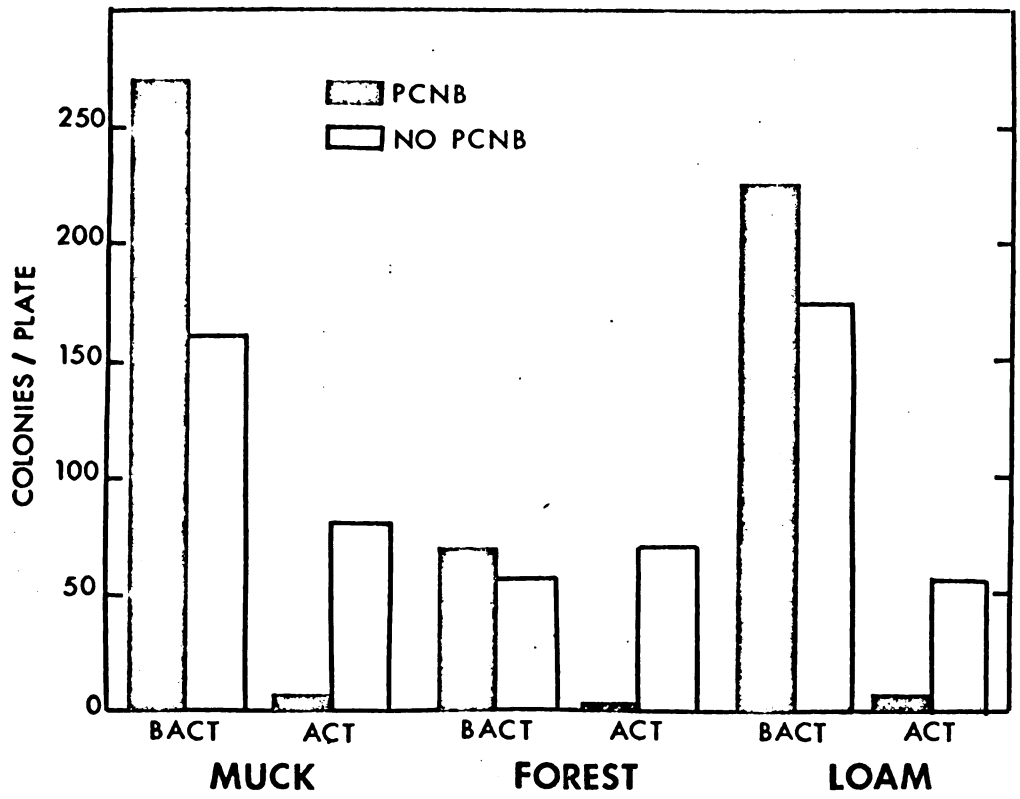


Fig. 4.--The effect of 50 ppm PCNB on numbers of actinomycete and bacterial colonies on soil extract agar seeded with diluted soil suspensions prepared from muck, forest, and loam soils.

with PCNB and in soil without PCNB. Respiration rates were unaffected by PCNB during a 30 day incubation period at 27 C (Fig. 9). Natural soil with or without PCNB took up oxygen at a rate of 0.7 - 2.0 µl/hr/5 g soil during this 30 day period.

The concentration of amino compounds and sugars was determined in natural soil with or without PCNB. Extracts were made at daily intervals for 2 weeks after PCNB treatment. An average of 12 µg of anthrone positive substances (sugars) per g soil and 2.5 µg of ninhydrin positive materials (amino acids and related compounds) per g soil were recovered from natural soil untreated with PCNB. Concentration of nutrients recovered from PCNB-treated soil did not differ from those from untreated soil.

The numbers of actinomycetes, bacteria and fungi in PCNB-treated soil and in soil without PCNB were estimated one and two weeks after PCNB addition. PCNB had no effect on microbial numbers (Table 3).

The results of the respiration, nutrient, and population studies suggest that 200 ppm PCNB does not effect microbes in natural soil.

The effect of PCNB on actinomycetes in chitin-amended soil: The effect of PCNB on the development of actinomycetes in chitin-amended soil was studied to determine if PCNB

Table 3.--Effect of 200 ppm PCNB on numbers of microbes in natural soil.

Microorganisms	Colonies/g soil (X 10^5)[a]			
	7 days		14 days	
	Natural soil	Nat. soil + PCNB	Natural soil	Nat. soil + PCNB
Actinomycetes	83	90	73	71
Bacteria	279	260	200	196
Fungi	0.42	0.48	0.44	0.44

[a]At each time period numbers of microorganisms in natural soil with or without PCNB did not differ significantly (1% level).

inhibits actinomycetes in soil in the presence of a suitable substrate for their growth. Numbers of actinomycetes, soil respiration and the colonization of chitin drops applied to soil surfaces were studied. All experiments were repeated at least twice.

The numbers of actinomycetes were estimated in soil treated with 0.2% chitin and 10 or 100 ppm PCNB and in chitin-amended soil without PCNB 0, 5, and 10 days after soil treatment. Controls were unsupplemented natural soil and natural soil treated with 200 ppm PCNB.

There were about 7×10^6 actinomycetes per g unsupplemented soil. This number remained constant throughout the experiment in natural soil and in natural soil treated with 200 ppm PCNB (Fig. 5). In chitin-amended soil, there were about 3.4×10^7 actinomycetes 5 days after amendment and approximately 8×10^7 10 days after chitin addition. Thus the increase was approximately 5-fold over unsupplemented soil at the 5th day and 11-fold at the 10th day. In soil containing both chitin and PCNB there was approximately 1×10^7 at the 5th day, a 1.7-fold increase over the numbers in natural soil, and 4×10^7 at the 10th day, a 6-fold increase over natural soil. Thus in PCNB-treated soil actinomycete numbers were suppressed by about 70% 5 days after amendment and by about 40% at 10 days.

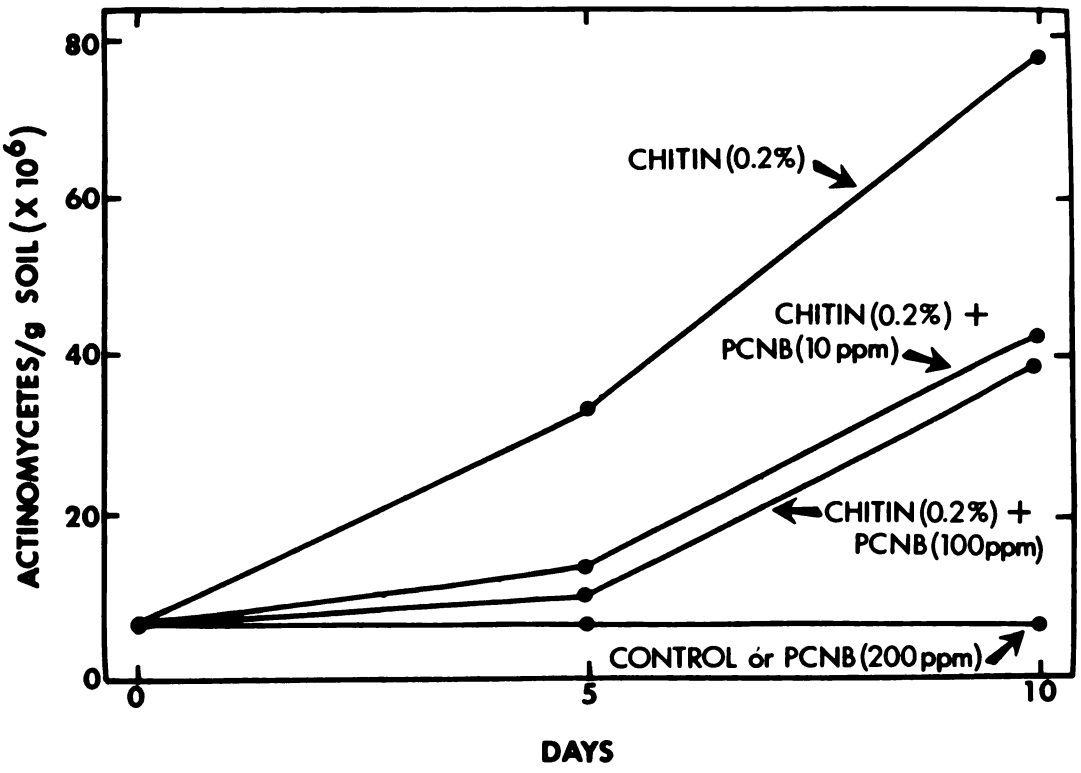


Fig. 5.--Effect of PCNB on numbers of actinomycetes in natural soil and chitin-amended soil.

After ten days the surface of chitin-amended soil was covered with white colonies of actinomycetes, whereas few colonies appeared on the surface of soil treated with chitin and PCNB. To further substantiate this observation, 6 drops of a thick colloidal chitin suspension were placed on the surface of natural soil treated with 0, 10, 50 and 100 ppm PCNB. One week later it was noted that actinomycetes had heavily colonized the chitin drops on natural soil without PCNB, but colonization of drops on PCNB-treated soil was suppressed (Fig. 6). Suppression was progressively greater as the concentration of PCNB increased. The observation that PCNB suppressed colonization of chitin by actinomycetes further indicates that actinomycetes are inhibited by PCNB in soil.

Another approach to estimate the effect of PCNB on actinomycetes was to measure the rate of oxygen uptake in chitin-amended soil treated with PCNB. Chitin was added to soil with 50 ppm PCNB or without PCNB and respiration was measured at daily intervals for 33 days. Natural soil with 200 ppm PCNB and without PCNB served as controls.

PCNB had no effect on respiration in soil treated with chitin during the first 9 days. From the 9th to the 25th day after chitin amendment, soil without PCNB respired more than soil with PCNB (Fig. 7). During this interval

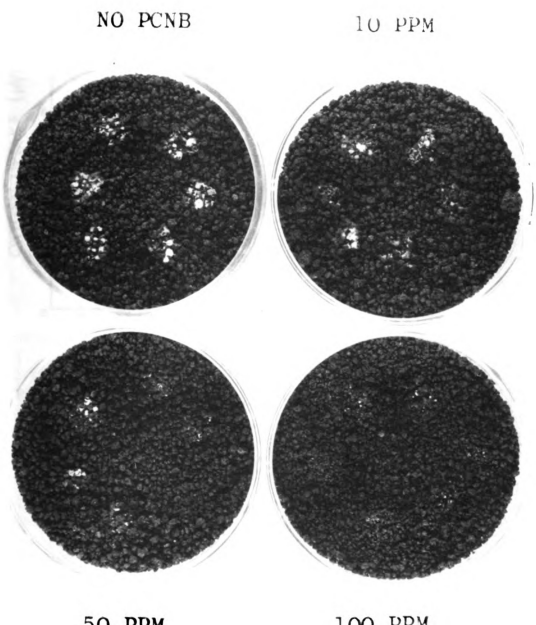


Fig. 6.--Effect of PCNB on the colonization of colloidal chitin by actinomycetes in soil. Six drops of a thick colloidal chitin suspension were applied to the surface of soil treated with PCNB. Pictures were taken after 7 days incubation. White zones on the soil surface are actinomycetes colonizing the chitin.

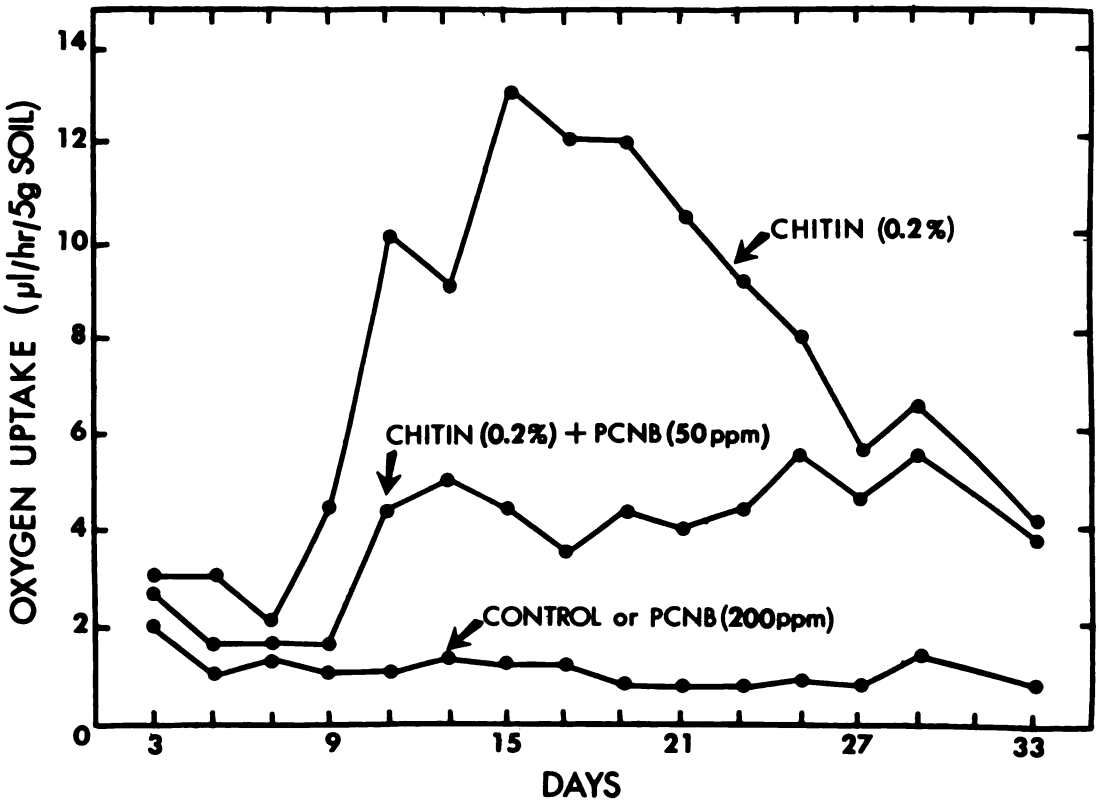


Fig. 7.--Effect of PCNB on oxygen uptake in soil with or without chitin.

chitin-amended soil without PCNB took up oxygen at the average rate of 10 μl/hr/5 g soil, whereas chitin-amended soil with PCNB took up 5 μl/hr/5 g soil. Thus, on the average, PCNB caused about a 50% reduction of respiration in chitin-treated soil. Natural soil treated with 200 ppm PCNB respired at the same rate as natural soil without PCNB during the 33 day period of the experiment.

The effect of PCNB on fungi in glucose-amended soil: To study the effect of PCNB on fungal numbers in soil, soil treated with 100 ppm PCNB was amended with 0.2% or 1% glucose. Controls were unamended natural soil and natural soil plus 100 ppm PCNB. The numbers of fungi were estimated by the soil dilution plate technique 0, 2, 4, 6, 8 and 14 days after amendments.

The numbers of fungi increased more in soil containing 0.2% glucose and PCNB than in soil with glucose only (Fig. 8). Especially striking was the rapid early increase in numbers of fungi in soil treated with glucose and PCNB. The numbers of fungi in this soil more than doubled during the first 2 days whereas the fungal numbers in soil treated with glucose alone did not increase during this period. After 2 days, the rate of increase in the numbers of fungi were nearly the same in both treatments, although numbers in PCNB-treated soil continued to be higher than those in

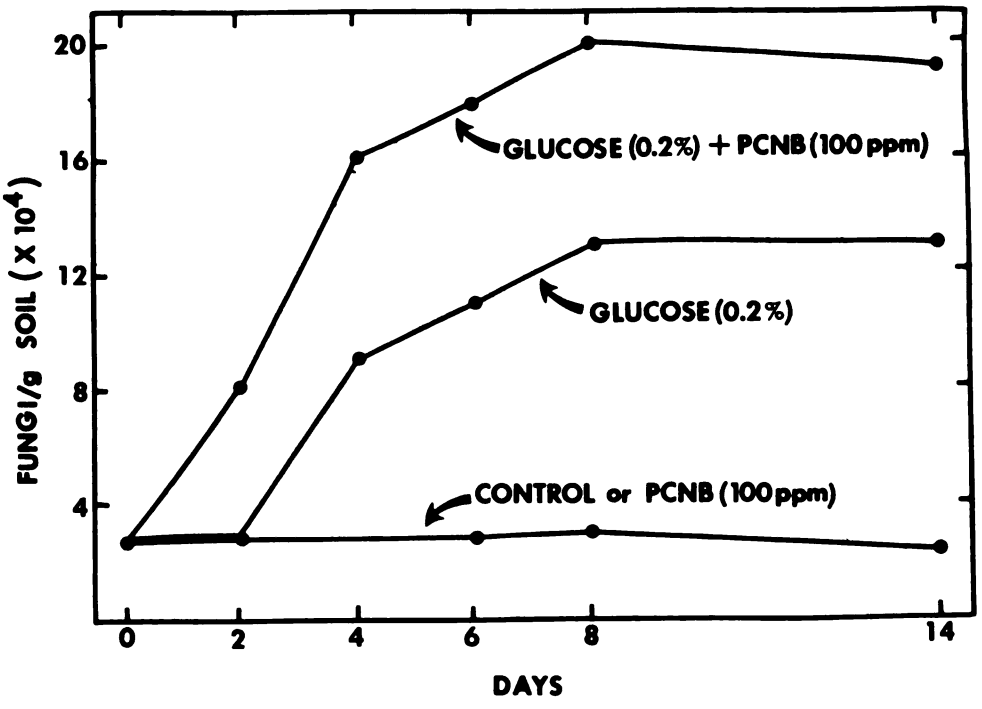


Fig. 8.--Effect of PCNB on numbers of fungi in natural soil and glucose-amended soil. Fungi from soil treated with glucose and PCNB were of 2-3 types. A Fusarium sp. was predominant. Dilution plates from natural soil and soil treated with glucose supported many different fungal genera.

soil amended with glucose only. Similar results were obtained in 5 other experiments using 0.2% glucose and in 3 additional experiments using 1% glucose. Numbers of fungi were unaffected when 100 ppm PCNB were added to natural soil.

The observation that numbers of fungi increased more in soil treated with glucose and PCNB than in glucose only seems to conflict with the results in agar that fungi were inhibited by PCNB. However, nearly all of the fungi from soil treated with glucose and PCNB were of 2-3 types, as indicated by colony growth and pigmentation on agar. One of the types, representing about 50% of the total number of colonies, was a Fusarium sp. which proved to be unaffected by 100 ppm PCNB in agar. The other types could not be induced to sporulate and therefore identifications were not made. Dilution plates from glucose-amended soil without PCNB supported many different types of fungi, not differing largely from those from nonsupplemented natural soil.

Other evidence that Fusaria increase in PCNB-treated soil was obtained with the use of Fusarium oxysporum f. melonis, isolate I-5. One g of sandy soil infested with approximately 1×10^6 chlamydospores of F. oxysporum f. melonis was added to 9 g of dry sandy loam soil. The soil was adjusted to 15% moisture. The infested soil was treated with 0.2% glucose and 100 ppm PCNB or with 0.2% glucose only.

The numbers of fungi were estimated 2 and 7 days after amendment by the soil dilution plate technique using an acidified PDA + TMN (trimethyl nonyl ether of ethylene oxide) (5). The medium was prepared as previously described for the selective medium for fungi, except that TMN was used instead of NPX. On this medium F. oxysporum f. melonis isolate I-5 develops a purplish color, which aids in its distinction from other fungi.

No significant increases in fungal numbers occurred up to 2 days. Seven days after the addition of PCNB and glucose the numbers of F. oxysporum f. melonis propagules were greater and other fungi were fewer in soil treated with glucose and PCNB than in soil treated with glucose only (Fig. 9). For example, in one experiment, 7 days after soil treatment, there were 3×10^5 propagules of F. oxysporum f. melonis per g of PCNB-treated soil and 1.8×10^5 propagules of this fungus in soil treated with glucose only. On the other hand there were 4×10^5 fungi other than F. oxysporum per g of soil treated with glucose and 2×10^5 per g in soil treated with glucose and PCNB. Similar results were obtained in 2 other experiments.

Stimulatory effect of PCNB on F. oxysporum f. melonis and F. solani f. phaseoli: It seemed likely that the increase in the number of propagules of Fusarium was related

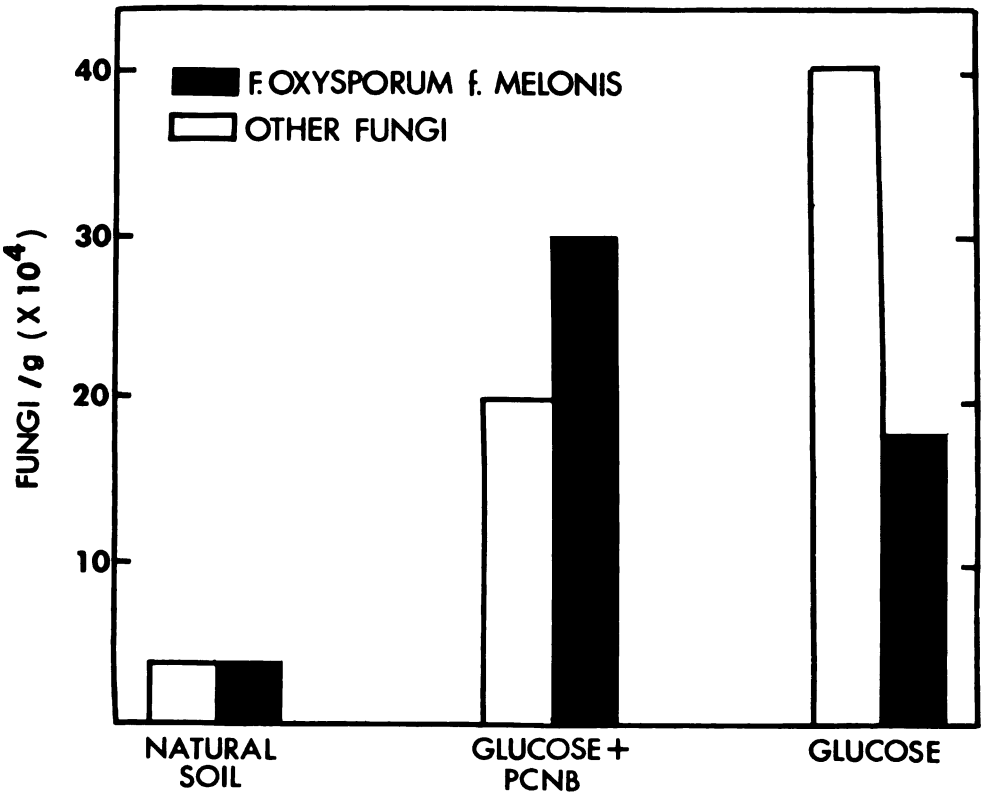


Fig. 9.--Effect of 100 ppm PCNB on numbers of Fusarium oxysporum f. melonis, I-5 and other fungi in natural soil or in soil amended with 0.2% glucose. There were more F. oxysporum f. melonis and fewer other fungi in soil treated with glucose and PCNB than in soil treated with glucose only (1% level).

to the suppression of other fungi PCNB. However there was the possibility that Fusarium spores or mycelia were directly stimulated by PCNB and the increased Fusarial activity was not related to reduced competition. This possibility was tested in 3 ways: 1) In three experiments chlamydospores or conidia of F. oxysporum f. melonis and F. solani f. phaseoli failed to germinate on the surface of natural soil containing 100 ppm PCNB. 2) No increase in germination of chlamydospores or conidia, or in growth of mycelium of either fungus was observed on agar with 100 ppm PCNB as compared with agar without PCNB in 3 tests. 3) In 2 experiments chlamydospores of either fungus were added to autoclaved soil with or without 100 ppm PCNB. The numbers of propagules of Fusarium were estimated by the soil dilution plate technique 3 and 7 days after infestation. There was no evidence of Fusarium stimulation by PCNB; in fact the converse was true, numbers of propagules were usually less in the PCNB-treated soil. For example, in the case of F. solani f. phaseoli, 7 days after infestation there was a 48-fold increase in numbers in PCNB-treated soil over the number originally added and an 84-fold increase in autoclaved soil without PCNB. Results with F. oxysporum f. melonis were similar.

The effect of PCNB on bacterial numbers in glucose-amended soil: Numbers of bacteria were estimated in soil amended with 1% glucose and 100 ppm PCNB or with glucose only at intervals up to 10 days after amendment. Natural soil with or without PCNB served as controls.

In unsupplemented natural soil there were 5×10^6 bacteria per g of soil. During the time intervals tested the number of bacteria were unaffected when 100 ppm PCNB only was added to this soil (Fig. 10). From 1-10 days after amendments numbers of bacteria were greater in soil treated with glucose and PCNB than in soil treated with glucose only. The increase in bacterial numbers with PCNB was especially striking at the 5th day when there were 2.5×10^8 bacteria in soil treated with glucose and PCNB, compared with 7×10^7 in soil treated with glucose only. At the 7th and 10th days there were still more bacteria per g of PCNB-treated soil than in soil without PCNB.

The results with bacteria were very similar to those with fungi, in showing that those soil microbes insensitive to PCNB, i.e., bacteria and certain fungi, such as Fusarium spp., increased more in PCNB-treated soil than in soil without PCNB. PCNB treatment evidently created a partial 'biological vacuum' in glucose-amended soil by the inhibition of actinomycetes and fungi. Those microbes insensitive to PCNB

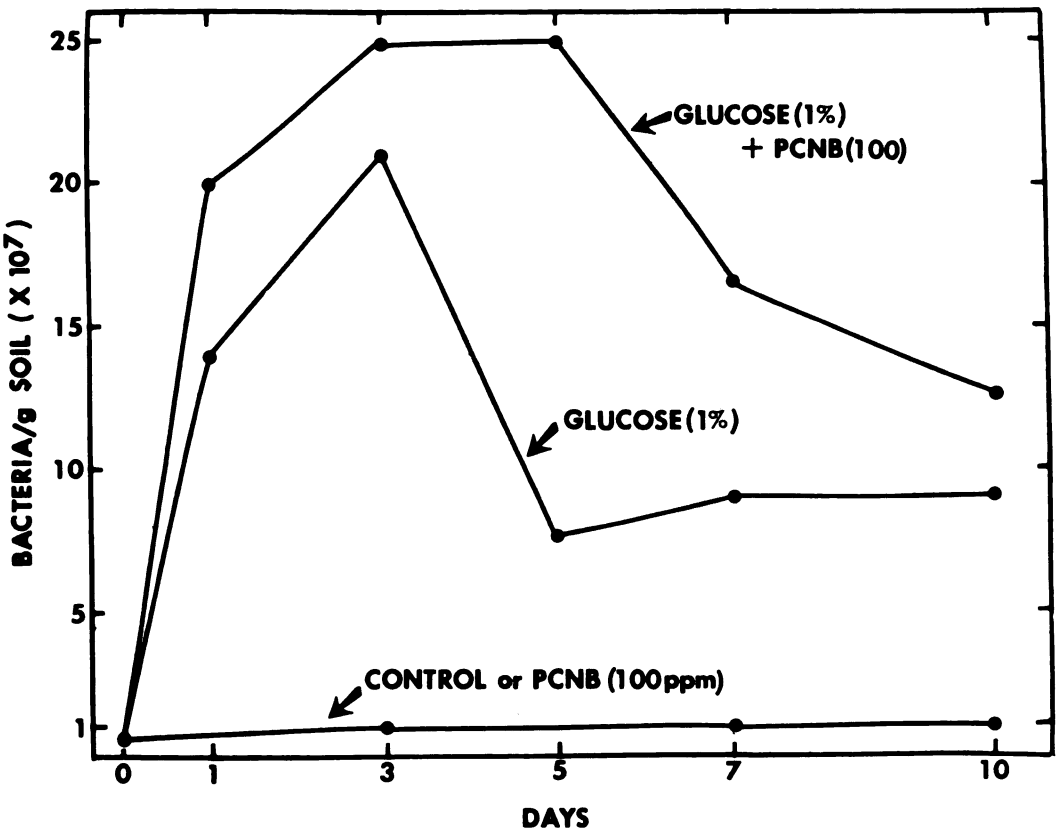


Fig. 10.--Effect of PCNB on numbers of bacteria in soil with or without glucose.

quickly increased to fill such a vacuum.

The effect of PCNB on the rate of glucose utilization in soil: Inasmuch as PCNB partially suppressed the metabolic activity of PCNB-sensitive actinomycetes and fungi in nutrient-amended soil, the rate of utilization of an energy source by soil microbes may be less in PCNB-treated soil than in soil without PCNB. Thus, as another measure of the effect of PCNB on soil microbes, 0.05% or 1% glucose was added to soil with or without 100 ppm PCNB. At varying intervals after glucose addition the amount of glucose remaining was determined.

During the first 2 days, the rate of 1% glucose utilization was the same in the PCNB-treated soil and in soil without PCNB. From the 3rd to the 5th day glucose disappeared faster in glucose-treated soil than in soil treated with glucose and PCNB (Fig. 11). At the 6th day, glucose had almost disappeared from both soils. The lowered rate of glucose utilization in PCNB-treated soil may be related to the suppression of PCNB-sensitive actinomycetes and fungi in that soil.

The effect of PCNB on the rate of glucose utilization was also tested in soil treated with 0.05% glucose and 200 ppm PCNB. Glucose was utilized at a slightly lower rate in PCNB-treated soil than in soil without PCNB (Fig. 12). Similar results were obtained in 4 experiments. The largest

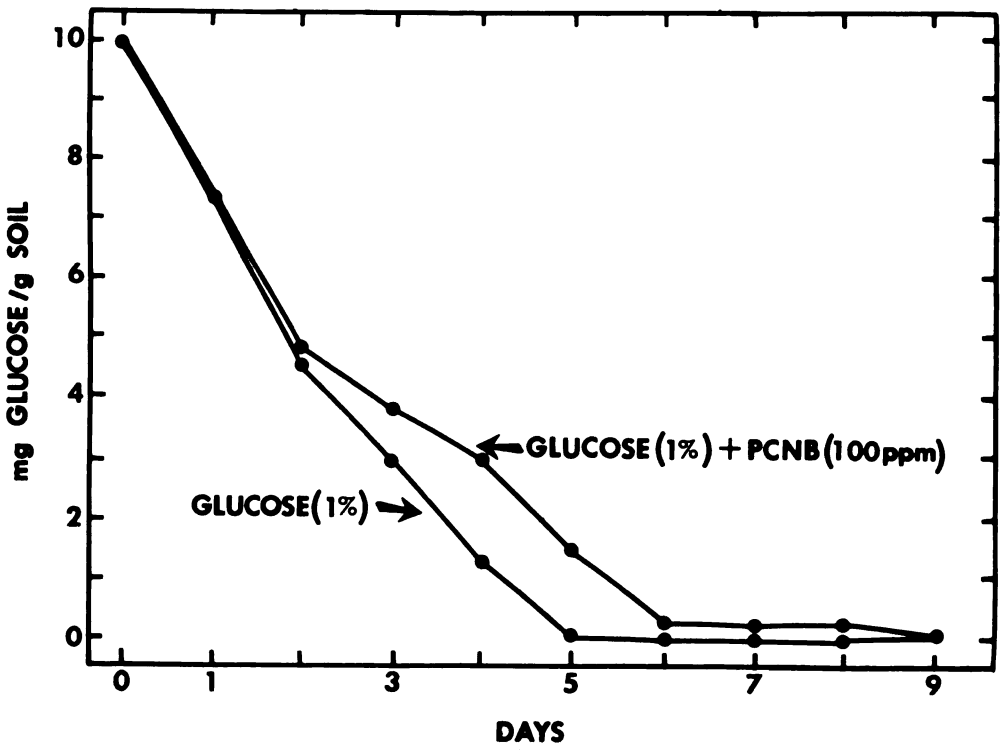


Fig. 11.--Effect of PCNB on the rate of glucose utilization in soil.

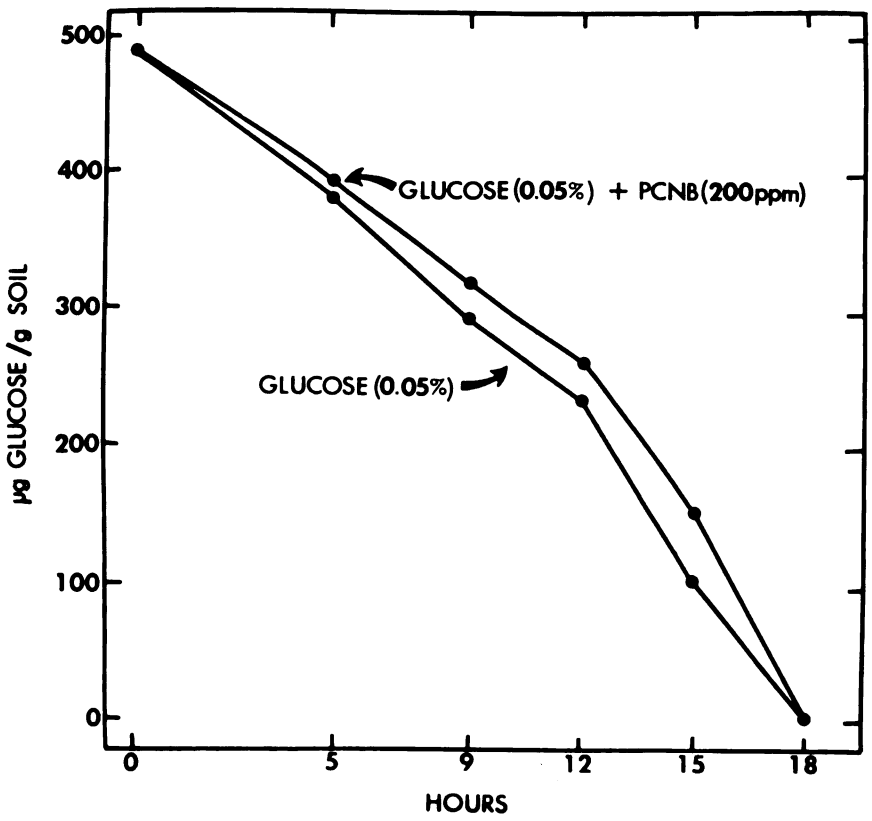


Fig. 12.--Effect of PCNB on the rate of glucose utilization in soil.

difference in the amount of glucose was detected at the 15th hour. For example, in one experiment 100 μg of glucose remained per g in soil treated with glucose only and 150 μg/g in soil treated with glucose and PCNB.

The effect of PCNB on soil respiration in glucose-amended soil: To study the effect of PCNB on the activity of microbes in glucose-amended soil, respiration was measured at daily intervals for an 8 day period in soil amended with 1% glucose and 100 ppm PCNB and in soil treated with glucose only. Natural soil with 100 ppm PCNB and without PCNB served as controls.

For the first 2 days soil treated with glucose and PCNB respired at the same rate as soil treated with glucose only (Fig. 13). Respiration in soil treated with glucose increased to a maximum of 75 μl/hr/g soil at 3 1/2 days. After 3 1/2 days the rate of respiration rapidly declined, until at 7 days, it remained at about 10 μl/hr/g. In PCNB-treated soil oxygen uptake reached a maximum of 58 μl/hr/g at the 5th day, then declined rapidly. Respiration rates in both soils were about the same at the 7th and 8th day.

Evidently, in soil with glucose but no PCNB the population of microorganisms able to utilize glucose increased rapidly and a peak of maximum respiration occurred very quickly. In soil treated with glucose and PCNB, the buildup

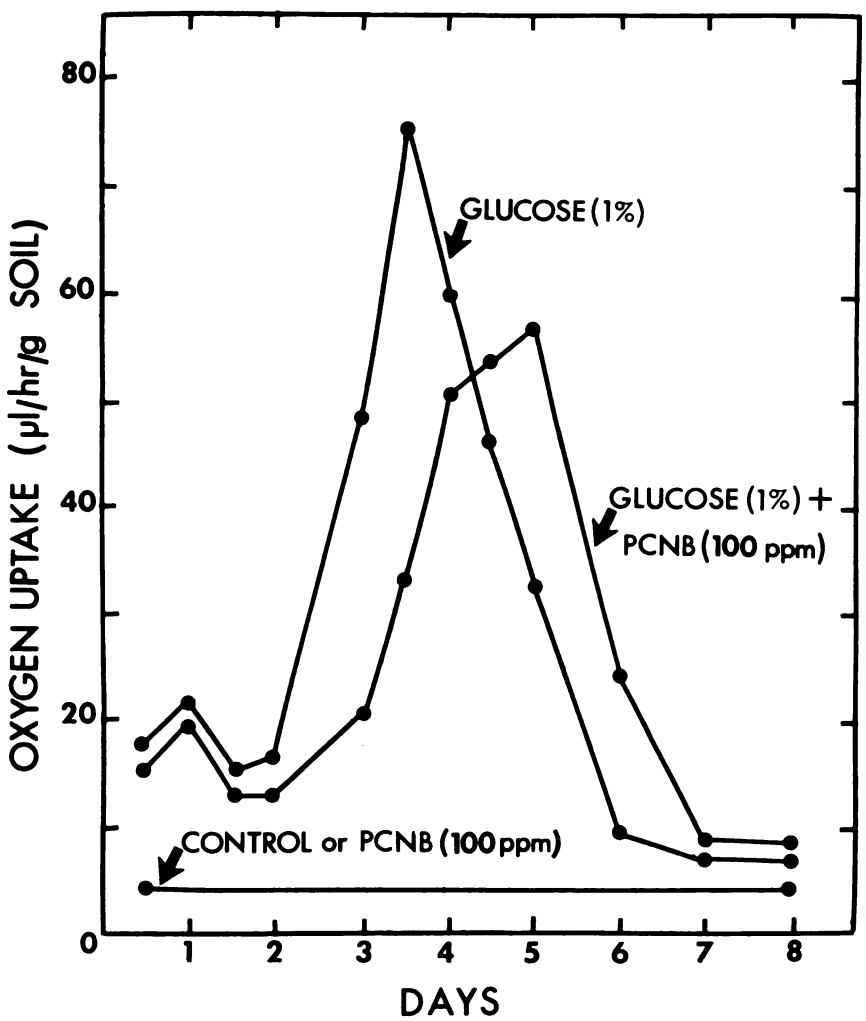


Fig. 13.--Effect of PCNB on oxygen uptake in soil with or without glucose.

of a large respiring population was probably delayed by the suppression of PCNB-sensitive microorganisms. The rapid decline in both soils was probably due to exhaustion of glucose.

Germination of conidia of Helminthosporium victoriae and chlamydospores of Fusarium solani f. phaseoli on PCNB-treated soil: Results from the previous two sections have indicated that competition for glucose may be suppressed in soil containing PCNB. H. victoriae and F. solani f. phaseoli are fungi which are relatively insensitive to soil fungistasis, i.e., their spores require very low nutrient levels to germinate in natural soil (G. W. Steiner, unpublished data). Because very small differences in the amount of available nutrients are reflected in the amount of germination, spore germination of these fungi was used as a bioassay to detect the effect of PCNB on nutrient competition. Two types of tests were conducted: 1) Germination of conidia of H. victoriae and F. solani was tested in glucose-amended soil with or without PCNB, and 2) Germination of H. victoriae was tested in alfalfa residue-amended soil with or without PCNB. Tests with H. victoriae will be discussed first.

Glucose (0.05%) and ascorbic acid (0.05%) were added to soil with or without 200 ppm PCNB. Ascorbic acid was added to insure complete germination on soil (21). Natural

soil with or without 200 ppm PCNB served as controls. After amendment, washed spores of H. victoriae were placed on the surface of the soil at 3-hr intervals for 15 hrs. Germination was determined after 6-8 hrs incubation.

No spores germinated on natural soil with or without PCNB. The germination of H. victoriae spores was greater, at all time intervals, on soil treated with glucose and PCNB than on soil with glucose only (Fig. 14). For example, 72% germination occurred at the 0, 3, and 6 hr intervals in soil treated with PCNB and glucose, whereas germination in soil treated with glucose only declined from 57% at 0 hr to 29% at 6 hr. The difference in germination at time 0 was probably due to the fact that spores took 6-7 hrs for complete germination; during this time period glucose may have been utilized faster in glucose-amended soil than in soil with PCNB and glucose. From the 6th-15th hrs, germination was still higher on PCNB-treated soil than on soil without PCNB.

In another test using conidia of H. victoriae, 75 mg of moistened alfalfa residue were buried in a trough in soil treated with 50, 100, or 200 ppm PCNB, or in soil without PCNB. Washed spores of H. victoriae were applied to the soil surface at varying distances from the residue.

More spores germinated near the residue in soil amended with PCNB than in untreated soil (Fig. 15). For example,

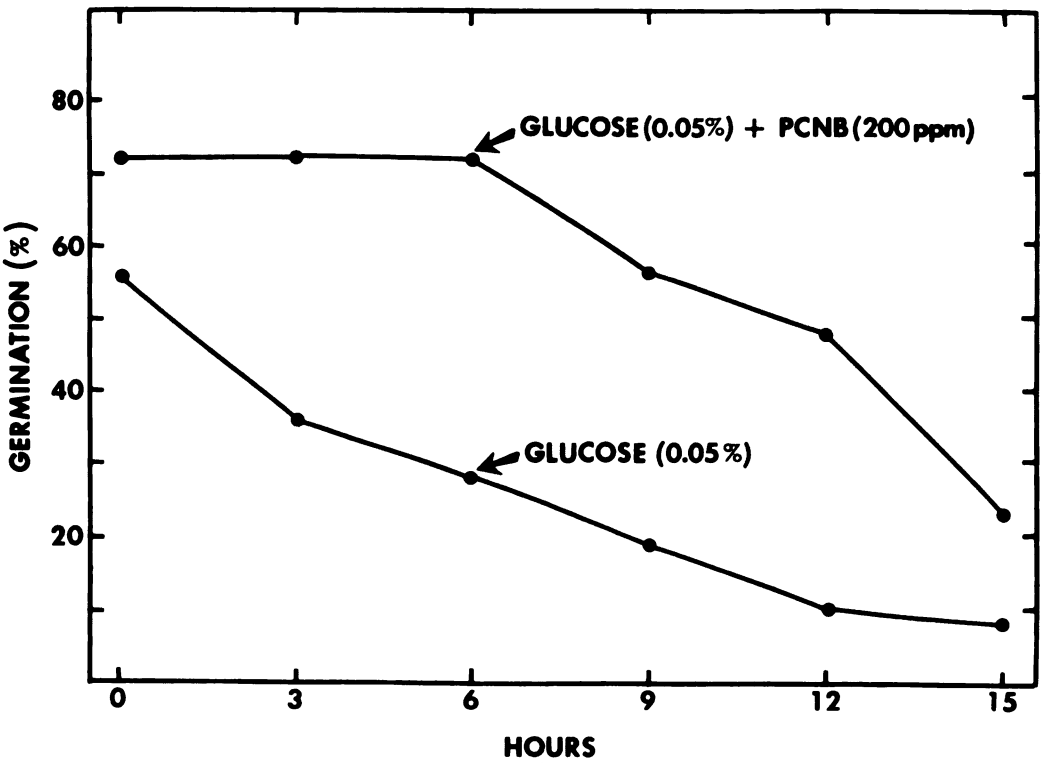


Fig. 14.--Effect of PCNB on germination of conidia of Helminthosporium victoriae on glucose-amended soil. Washed conidia were placed on the soil surface at 3-hr intervals for 15 hrs after soil amendment. No spores germinated on natural soil with or without PCNB.

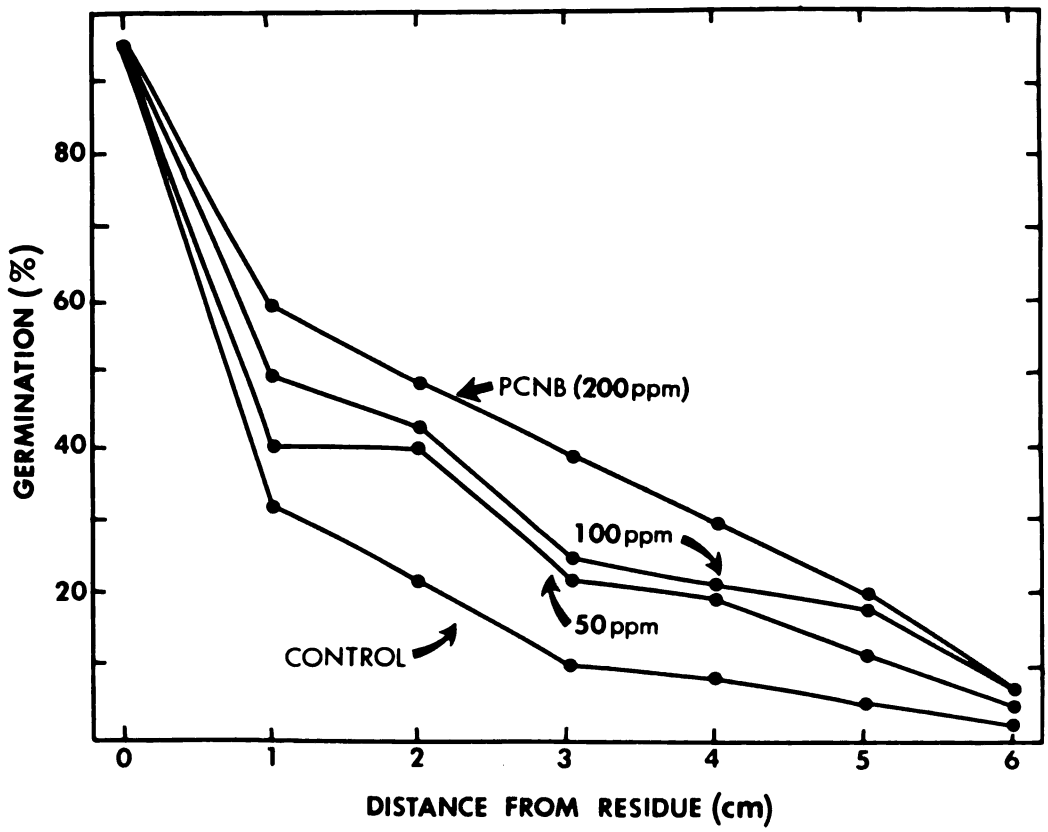


Fig. 15.--Effect of PCNB on germination of conidia of Helminthosporium victoriae on soil. Seventy-five mg of moistened alfalfa residue were buried in a trough in soil treated with PCNB and conidia of H. victoriae were added to the soil surface at varying distances from the residue. Soil without PCNB served as control.

germination at 1 cm was 60, 50, 40, and 31% in soil treated with 200, 100, 50 ppm PCNB and untreated soil, respectively. Differences of this magnitude continued until the 5th cm. No differences in germination were detected directly on top of the residue. These data indicated that nutrients diffusing from the alfalfa residue were utilized more slowly by microorganisms in soil treated with PCNB than in soil without PCNB, thus providing more nutrients for spore germination.

Tests using 1% glucose indicated that the rate of glucose utilization in PCNB-treated soil was slower than that in soil without PCNB (Fig. 11). Chlamydospores of F. solani f. phaseoli were used as a bioassay to confirm these data. Chlamydospores were applied at various time intervals to soil treated with 1% glucose and 200 ppm PCNB or with glucose only. Natural soil with or without 200 ppm PCNB were controls.

No spores germinated on natural soil either with or without PCNB. During the 0-10 day interval 1.5-2 times as many spores germinated on PCNB-treated soil amended with glucose than on soil with glucose only (Table 4). The low germination in the 4-5 day interval may be the result of the production of antibiotic or staling products from soil microbes, or the unavailability of essential mineral elements, since sufficient glucose was present to support germination (Fig. 11). Large numbers of chlamydospores germinated at the

Table 4.--Effect of 200 ppm PCNB on germination of *Fusarium solani* f. *phaseoli* chlamydospores in glucose-amended soil.

Days after amendment of soil	Germination (%)[a]	
	Glucose + PCNB	Glucose
0	96	94
2	56	51
4	5	0
5	0	0
6	58	24
7	54	32
8	68	0
9	29	2
10	0	0
Total	366	203

[a]Data are from one of two experiments with similar results. Two hundred spores were counted in each treatment for each experiment. No spores germinated in natural soil with or without PCNB.

6-9 day interval in PCNB-treated soil even though glucose could not be detected in these soils. Their germination might have been supported by metabolic or autolytic byproducts of microbes.

DISCUSSION

The present study was initiated to clarify some conflicting and controversial aspects of past research on PCNB. Three areas needing clarification were questions of the stability of PCNB in soil, the antimicrobial spectrum of PCNB, and PCNB-induced disease exchange.

The stability of PCNB in soil has been tested by 2 different methods: 1) Determination of the viability of Rhizoctonia solani in soil by seedling tests, and 2) chemical analyses of the extracted fungicide by gas chromatrography. In almost all earlier studies the stability of PCNB has been studied by method 1. Results from these tests indicated that PCNB was relatively persistent in soil (3, 17, 20, 33, 35). However, this method would provide no information as to changes in the chemical structure of PCNB if such changes did not affect the toxicity of the fungicide against R. solani. In other words, the persistence of a degradation byproduct of PCNB may have been estimated, rather than the stability of PCNB itself. A recent unconfirmed report in which gas chromatrography was used for analysis, indicated that PCNB was

changed in soil into one or more fungitoxic products which may account for the long term disease control by PCNB (4).

Results from my research generally confirmed the results from bioassay methods; PCNB was not changed in soil, at least not in 4 weeks. The purpose of stability tests in the present research was to determine if effects on microorganisms were due to PCNB or some byproduct. It is obvious that more work needs to be done in the greenhouse and field over longer periods before the question of PCNB stability is solved. It will be difficult to interpret data from soil treated with PCNB until we are sure that effects observed are due to PCNB and not to some byproduct of PCNB.

PCNB has gained the reputation of being inhibitory to only a few organisms (9, 22, 23). The evidence supporting this premise is that PCNB strongly inhibits some soil fungi but is relatively ineffective against others (23); perhaps more importantly, it is generally regarded as innocuous to soil bacteria and actinomycetes (9).

Two reports that PCNB may affect microbes other than fungi were those of Davis (8) and Takahashi et al. (41), who found that actinomycetes were drastically inhibited in agar by PCNB. Results from my research confirmed these observations, and therefore, were contrary to the generally accepted idea that PCNB acts solely as a selective soil fungicide.

For example, 7 out of 10 species of actinomycetes were partially or completely inhibited in agar by 50 ppm PCNB. More significantly, the number of actinomycete colonies arising from diluted soil suspensions was suppressed by about 90% on agar containing 10 ppm PCNB. Results of respiration and population studies in chitin-amended soil also showed that actinomycetes were inhibited by PCNB. Thus, it was obvious that actinomycetes were greatly affected by PCNB in agar and in soil. It is strange that this fact has generally been overlooked, for PCNB is a very popular fungicide with a hundred or so papers published on its antimicrobial activities (23). The fact that Davis' work is unpublished and Takahashi's paper was published in a Japanese journal probably account for the lack of notice of their works.

In some kinds of experiments actinomycetes were more strongly inhibited by PCNB than in others. For example, actinomycete numbers from a natural soil population in soil dilution plates were reduced 90% by 10 ppm PCNB, whereas numbers of actinomycetes in chitin-amended soil were only reduced 40 to 70% at this concentration. More significantly, only 1 of the 10 identified actinomycetes was inhibited at this concentration. The differences in sensitivity in these tests could be explained in several ways: 1) The heavy inoculum load (mycelia and spores) in chitin-amended soil and

in tests with identified cultures may have been sufficient to overcome the biostatic effect of PCNB. The source of colonies of actinomycetes from natural soil suspensions, on the other hand, was probably a single spore (39). Mycelial growth may have been less affected by PCNB than was spore germination. 3) The 10 identified actinomycetes chosen may not represent a random sample in terms of sensitivity to PCNB. 4) There may be a buildup of a PCNB-tolerant population of actinomycetes in chitin-amended soil treated with PCNB. 5) PCNB may be less effective in inhibiting actinomycetes in soil than in agar.

PCNB inhibited the growth of several fungi in agar and soil. These results generally agree with data from other workers. PCNB is generally regarded as innocuous to soil bacteria. The evidence supporting this premise is that PCNB had no effect on respiration in natural soil (9). This observation was interpreted to mean that PCNB had no effect on actinomycetes or bacteria in soil and that the fungi inhibited by PCNB contributed little to soil respiration. Results obtained in the present work confirmed Vredeveld's (47) observation that the antimicrobial effects of PCNB are not expressed unless microbes are metabolically active. Thus, the testing of the effects of PCNB on soil microbes by measuring soil respiration in unsupplemented natural soil where the activity of heterotrophic microorganisms is minimal

is of little significance. The premise that soil bacteria are unaffected by PCNB was substantiated by the observations in this work that bacteria were unaffected by PCNB in agar or glucose-amended soil; at least at concentrations lower than 200 ppm.

When PCNB is added to soil, an increase in seedling damage by pathogenic fungi not sensitive to PCNB occasionally occurs (6, 12, 13, 16, 22, 46). It has been suggested that the increased activity of these pathogens following soil treatment by PCNB may be due to the suppression of specific fungal antagonists of these pathogens (6, 12, 16, 46). This hypothesis is not unreasonable considering the widely-held view that the inhibitory activity of PCNB was restricted to soil fungi. However, the evidence that PCNB suppresses growth of actinomycetes as well as growth of fungi in nutrient-amended soil, suggested that certain fungi insensitive to PCNB may benefit by soil treatment with PCNB because of reduced competition for nutrients rather than by suppression of specific antagonists.

Two lines of evidence indicated that microbes insenitive to PCNB benefited by soil treatment with PCNB: 1) Bacteria and Fusarium spp., both of which are relatively insensitive to PCNB, increased to a greater extent in PCNB-treated soil amended with glucose than in soil with glucose

only. 2) Conidia of Helminthosporium victoriae and chlamydospores of F. solani f. phaseoli germinated more in PCNB-treated soil with glucose than in soil with glucose only. Since germination of these two fungi was not directly affected by PCNB, it seemed likely that PCNB treatment resulted in increased availability of glucose to these spores.

Two additional lines of evidence indicated that PCNB reduces nutrient competition in soil: 1) Glucose was utilized slower in soil treated with PCNB than in soil without PCNB and 2) the buildup of a respiring population was delayed by PCNB in soil treated with glucose.

In natural soil, the rhizosphere or the vicinity of dead organic matter are zones where the effects of PCNB on the microbial population would be expressed, for they provide energy sources capable of supporting metabolic activity and growth. It would seem likely that one of the factors involved in PCNB-induced disease exchange would be the reduction of competition for nutrients by the inhibition of actinomycetes and fungi in the rhizosphere, thus permitting fungal pathogens insensitive to PCNB to flourish in the infection court. Possibly, by the same means, PCNB-insensitive pathogens could also increase their inoculum density in dead organic matter.

Other factors than microbial competition might also

play a role in disease exchange. For example, the possibility of phytotoxicity due to PCNB and its effects on disease must be considered. In at least 2 instances, disease increases occurred when plants were injured by PCNB (18, Y. Katan, personal communication). In a preliminary experiment in this research, muskmelons were injured by 100 ppm PCNB in soil and the stunted melons were much more susceptible to Fusarium oxysporum f. melonis than melons in soil without PCNB.

In brief summary the results of this research are as follows: 1) PCNB had no effect on soil microbes in nonsupplemented natural soil. 2) PCNB suppressed the activity of sensitive actinomycetes and fungi in soil supplemented with nutrients. 3) The suppression of PCNB-sensitive microorganisms reduced competition for organic carbon sources. 4) Because of reduced competition, PCNB-insensitive fungi and bacteria increased more in nutrient-amended soil with PCNB than in soil without PCNB. These conclusions are illustrated in Fig. 16.

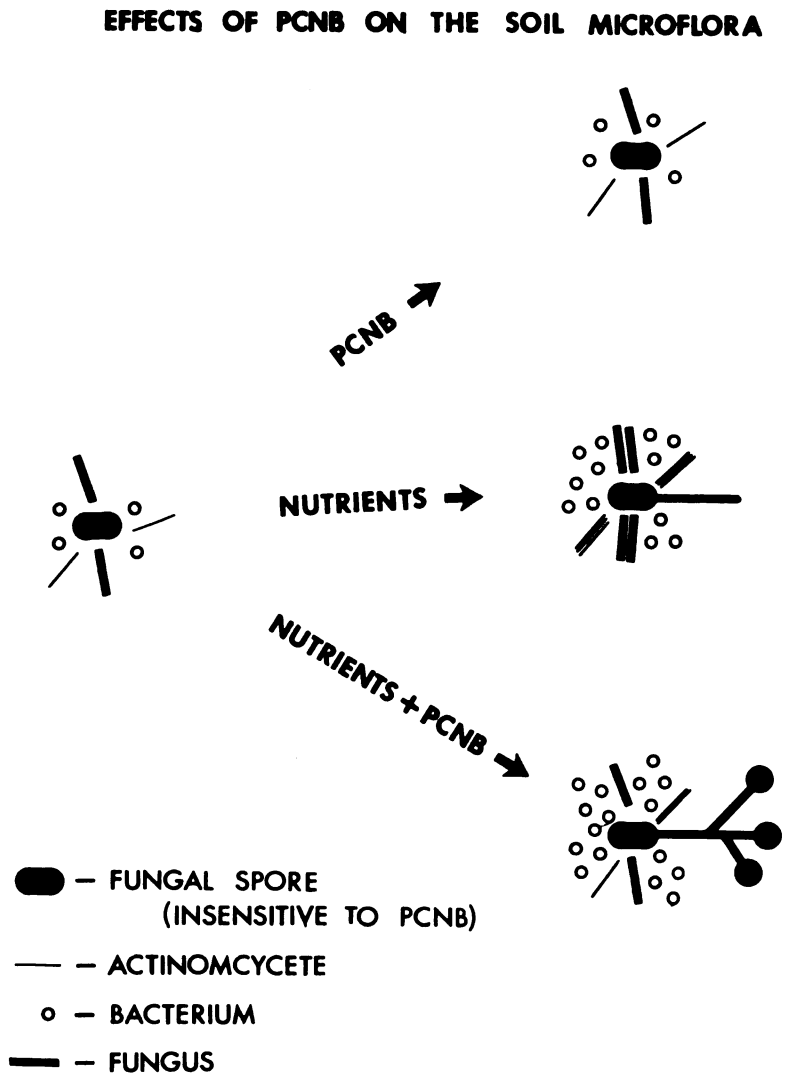


Fig. 16.--A summary of the effect on numbers of actinomycetes, bacteria, fungi and germination and sporulation of a PCNB-insensitive fungus by A) PCNB in natural soil, B) nutrients in natural soil, and C) PCNB in nutrient-amended soil.

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