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ON SOIL FUNGI

Thesis for the Degree of Ph. D.

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THESIS

This is to certify that the

thesis entitled

Selective Action of Pentachloronitrobenzene on Soil Fungi

presented by

Nicholas G. Vredeveld

has been accepted towards fulfillment
of the requirements for

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Robert P. Scheffer

Robert P. Scheffer
Major professor

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ABSTRACT

SELECTIVE ACTION OF PENTACHLORONITROBENZENE

ON SOIL FUNGI

by Nicholas G. Vredeveld

Pentachloronitrobenzene (PCNB) reduces damping off and root rot diseases of many plants caused by Rhizoctonia solani. A companion soil fungus Pythium ultimum is not affected in field and greenhouse tests. Laboratory studies showed that differential toxicity of PCNB to the two organisms could explain the observations made in the field and greenhouse. PCNB was presented to the fungus both in solution and as a gas. The dosage-response curve derived from growth inhibition studies using PCNB in solution indicates that PCNB has a single mode of action. The inhibitory efficiency of PCNB as a gas on agar or liquid culture appears to be about the same as that in solution. Vapor inhibition studies suggest that very little of the chemical is involved in the inhibitory process.

Bioassay and spectrophotometric measurements of filtrates show that R. solani absorbs much more PCNB from the medium than does P. ultimum. These methods also show that a portion of the PCNB absorbed by R. solani was not extractable into petroleum ether and detectable by a colorimetric procedure.

It is suggested that this may represent the amount required for inhibition.

Warburg studies indicate that total gas exchange by R. solani was not affected by PCNB. Also through growth studies, PCNB was shown not to act as an antimetabolite competing with the vitamin inositol. By measuring incorporation of labelled L-leucine C^{14} into the mycelial protein (TCA insoluble fraction) PCNB was shown not to inhibit protein synthesis on a short term basis.

SELECTIVE ACTION OF PENTACHLORONITROBENZENE ON SOIL FUNGI

by

Nicholas G. Vredeveld

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INTRODUCTION

Pentachloronitrobenzene (PCNB), sold in this country as Terraclor, is widely used as a soil fungicide in greenhouse and field. It is very effective in reducing damping off and root rot damage incited by Rhizoctonia solani Kuehn. A distinctive characteristic which makes it of particular value is the long-lasting effective residue, which remains in the soil for 3 to 12 months (2).

Species of Pythium are associated with R. solani as a cause of root rot and damping off of plants. There are many reports showing that PCNB is not effective in controlling Pythium spp. under field conditions (26, 11). There are data indicating that damping off can be more severe with PCNB than without it, presumably because Pythium is freed of competition by other soil microorganisms (11). At the present time it is necessary to formulate a mixture of fungicides which will effectively control these soil diseases (25, 28).

I have made an attempt in this study to explain the field data on the basis of differential toxicity of PCNB to R. solani and Pythium ultimum Trow. Dosage-response data soon showed that R. solani is sensitive while growth of P. ultimum is little affected by PCNB. The study was extended

in an effort to explain the fundamental basis of the differential toxicity. Included also are data on the significance of vaporization in fungicidal action, because this should help to explain the peculiar nature of PCNB as a soil fungicide. These were accomplished by presenting the inhibitor to the growing fungi direct to the solution and as a gas, attempting to determine the most efficient state and the minimum dosage which would cause a growth reaction by the fungus R. solani. The differential uptake of PCNB by R. solani and P. ultimum was investigated first with a bioassay method which used R. solani as the sensitive agent to detect the unabsorbed chemical, then followed with a chemical method which analyzed colorimetrically the amount of inhibitor taken up by the fungus as well as that remaining in the medium. Metabolic reactions of R. solani to PCNB were examined by comparing oxygen uptake of the fungus with and without PCNB in the medium. Inhibition of protein synthesis over a period of time was calculated by counting radioactivity of precipitated protein which had incorporated tagged leucine.

LITERATURE REVIEW

PCNB was first synthesized by Emile Jungfleisch in 1868 as part of a series of synthetic procedures for chlorobenzene compounds (15). He produced the pale yellow polygonal crystals by heating pentachlorobenzene with fuming nitric acid. By 1922 PCNB was listed in Beilstein's handbook (3) as a compound which was being produced by the chemical industry in Germany. Later I. G. Farbenindustrie developed it as a potential fungicide and introduced it in the early 1930's under the name Brassasan in England, where it was distributed by Beyer Products Ltd. In Germany it was called Tritisan.

The first reported field work on PCNB was by Brown in 1935 (5). He used it on lettuce in cold frames and found that it inhibited damping off of the seedlings caused by R. solani and also gave some control of Botrytis disease. By 1939 the name Brassasan was limited to the tetrachloro derivative because it was found to be superior for cabbage; the trade name Folosan was given to PCNB. PCNB has also been called Brassicol, Botrilex, and Tilcarex. Other field work has shown that PCNB will also inhibit or retard Plasmodiophora brassisae Wor. on cabbage (27), Streptomyces scabies (Thaxt.) Waks. and Henrici on potatoes (13), Botrytis cinerea Pers. on

lettuce (5) and other soil microorganisms.

The first laboratory work with PCNB was published by Roy in 1951 (25). Along with a number of other fungicides he experimented with PCNB, testing it for inhibition of spore germination and colony growth on cultures of various fungi. He reported that at a concentration of about 1.5 mg of pure PCNB per nutrient drop of spore suspension there was repression of spore germination in Botrytis cinerea Pers., Fusarium coeruleum (Lib.) Sacc., Ascochyta rabiei Ell. and Ev., Trichoderma viride Wein. and Fawcett, Rhizopus nigricans Ehr., and Alternaria sp. Colony growth was retarded when PCNB was added to the nutrient agar at 0.1% in cultures of B. cinerea, T. viride, Alternaria sp. and R. solani but not in cultures of F. coeruleum. He also reported vapor phase action against colony growth with 10 mg per petri dish cover and an inhibition of spore germination when a great excess of crystals was used. Roy noted that PCNB acted as an anti-sporulant on fungi where no growth inhibition occurred.

Mary Reavill continued Roy's work in the same laboratory (23). Again she was interested in comparing the effects of PCNB and homologs on several fungi and used the chemical in the gaseous phase in excess of minimum requirements. Accompanying this post-war interest in PCNB by the British was growing interest in the United States. The residual tenacity of PCNB was reported by Last (19), using R. solani on lettuce and by Arndt (2) with R. solani on cotton. These reports stimulated still further research. Olin Mathieson

Corporation obtained authority from Beyer to make PCNB and began to test it in the U.S. as Mathieson #275; later it was marketed as Terraclor.

Scheffer and Haney (26) tried PCNB as a soil treatment for root rot control of ornamentals in the greenhouse. They found that root rot under these conditions was caused by several fungi, including P. ultimum and that PCNB was not effective in control. A mixture of PCNB and Captan gave good control for a short time. Further experiments showed that PCNB would effectively control R. solani in the soil for many months after application, but that root rot caused by P. ultimum was not inhibited in the least. Sowell (28) also found that R. solani and Pythium spp. causing belly rot of cucumbers was controlled by a mixture of PCNB and Captan.

Recent work concerned with the mode of action of PCNB has been carried out by Eckert who worked with an isolate of R. solani which required about 10 ppm of PCNB for 50% inhibition of growth (ED 50). He compared the fungistatic effect of a number of homologs of PCNB with their oil to water solubility ratio and found the inhibitory effect to be proportional to the solubility ratio. From these data he concluded that the mode of action of all the chloronitrobenzenes was the same. Elsaid and Shatla (18) in Louisiana are approaching the problem by use of strains of R. solani with natural and adaptive tolerance to PCNB. Most wild-type isolates are relatively sensitive to PCNB.

Working on the metabolic problem Torgeson (30) recently studied the possible effect of PCNB on the respiration of several fungi. He found it had no affect on total gas exchange by Fusarium oxysporium f. cubense E. F. Sm., Sclerotium rolfsii Sacc., and Pythophthora parasitica Dastur. R. solani was not used as a test fungus in these studies. There have been no other attempts to elucidate this mode of action, either before or since the present work was started. Furthermore, there have been no comparative studies of the effect of PCNB on susceptible and resistant organisms.

MATERIALS AND METHODS

R. solani was isolated from a lesion on a damped off pine seedling growing in a flat of untreated soil in the greenhouse. This isolate was not known to have previously been in contact with soil fungicides. It was transferred to potato dextrose agar (PDA) plates and cultured at room temperature. The mycelium had the characteristic light brown coloring and branching. Small irregular dark brown sclerotia were scattered on the plate. After several days a hyphal tip consisting of three or four cells was cut off with a sterile razor blade, transferred to a PDA slant, and incubated at room temperature. The fungus was propagated in this manner throughout the series of experiments.

P. ultimum was isolated from a Croft lily bulb and handled similarly to *R. solani*. It was identified in culture by Dr. E. S. Beneke as being *P. ultimum*. In this case a hyphal strand was taken from a plate culture, transferred to a PDA slant and was also propagated on PDA slants throughout the experiments.

To determine that the selected isolates of *R. solani* and *P. ultimum* were parasitic and cause disease in plants, they were tested for pathogenicity by inoculating them into soil planted with sweet peas and observing pre-emergence

damping off of the seedlings. Ten 4 inch pots filled with loam soil were steamed in an autoclave under 0 pressure. Agar from petri plates containing mycelium of R. solani was cut into small pieces and mixed thoroughly with the soil in four of the pots; agar containing mycelium of P. ultimum was also cut and mixed with soil in four additional pots. Two pots of steamed soil were used for controls. Ten seeds were planted just below the surface in each pot and given normal greenhouse environmental conditions. Percent germination was noted when control plants attained 2-3 inches of height.

Of the seeds planted in the R. solani inoculated pots only 8% germinated and grew to two inches above the soil. In the P. ultimum inoculated pots 15% of the seeds germinated and emerged. The control pots had 90% germination and emergence. It was concluded that both isolates were pathogenic and could legitimately be used as representative isolates of the pathogenic soil population of R. solani and P. ultimum.

Table 1. Effect of Rhizoctonia solani and Pythium ultimum isolates on sweet pea germination and seedling survival in soil

Fungus inoculum	Planted	Emerged	Killed
Control	20	18	2
R. solani	40	3	37
P. ultimum	40	5	35

Technical grade PCNB (Olin Mathieson Corporation) contains 0.5% acetone-insoluble materials. These were removed by dissolving the crystals in warm acetone, cooling the acetone and then re-crystalizing the dissolved portion by putting it in water, filtering, and drying. This procedure was repeated until the melting point of the crystals rose from 141°C to 144-145°C. approaching the M.P. of pure PCNB (146°C). Crystalline material was dissolved in absolute ethanol at 10 mg/ml (10,000 ppm). One ml of PCNB-ethanol solution was suspended in 100 ml sterile water containing 0.001% Triton X-100 to assist suspension. The mixture was transferred to the medium at various concentrations as indicated in the results. Captan used in certain experiments for comparative purposes was a 50% wettable powder.

RESULTS

Preliminary experiments:

Selection of suitable media: A reasonably well-defined medium that supports rapid growth of both R. solani and P. ultimum was needed. Accordingly, 3 known media were selected for comparison. The three were Czapeks solution, an inorganic nitrogen medium; potato dextrose broth, a fortified natural substrate; and casein-hydrolysate solution (7) a fairly defined medium. The casein-hydrolysate solution contained the following in g/l: casein-hydrolysate, 1.5; yeast extract, 1.0; potassium monobasicphosphate, 1.5; magnesium sulfate, 1.0; glucose, 15.0; and trace elements in Hoagland's AZ solution, 0.5 ml (24). Cultures were grown in 250 ml Erlenmeyer flasks, each containing 40 ml medium. Standard procedures of sterilization and aseptic technique were followed. Each flask was inoculated with two 6 mm disks of 5 day old mycelium grown on PDA. Cultures were incubated at 28° C in the dark, using a reciprocal shaker at 90 shakes per minute. These conditions were sufficient to cause splashing and folding of the liquid surface and adequate aeration. After 5 days growth, cultures were harvested by filtering the fungal contents of each flask through a pre-weighed dried paper in a Buchner funnel under vacuum. The

paper and mycelium were dried in an oven at 100° C for 24 hours and weighed. Triplicate flasks were used for each determination. Average weights for 10 agar discs which had been similarly dried showed good precision and were therefore not considered a variable in mycelial weights.

Growth of R. solani in the 3 solutions, expressed as mg dry weight, was as follows: in casein-hydrolysate, 355; in PDA 372; in Czapek's solution, 24. Even though PDA gave more growth, casein-hydrolysate medium was chosen for use throughout the rest of the experiments because it gave adequate growth, was a relatively well defined medium, and growth was more uniform. P. ultimum grew well on each of the three media producing approximately 80 to 95 mg dry weight, under the conditions of cultivation used for R. solani.

Method of transfer of cultures: Precision in establishing growth curves often requires special techniques in starting cultures, especially for fungi such as R. solani which ordinarily do not sporulate. In these cases cultures are often started from disks cut from colonies on agar. This method was compared with use of macerated fungus suspensions prepared by two methods.

The first maceration method tried was used by Strecker (29) for growth studies of R. solani. The mycelial mat was placed in flasks containing physiological saline and sterile 0.5 cm glass beads, and the flasks were rotated until fragments were small enough to transfer by pipet. To test

this method, I macerated 2 g of mycelium in 20 ml saline, and transferred 0.5 ml to each culture flask. Disadvantages of the method were the frequency of contamination and the difficulty in controlling the grinding action.

An alternative method of maceration using the Waring blender was tried. A metal Waring blender cup containing 1 ml water was covered with aluminum foil, tightly capped and sterilized. After cooling, 20 ml of 0.9% sterile saline and approximately 2 grams (wet weight) of 5 day old mycelial mat were added under aseptic conditions. This mixture was blended for 30 seconds, resulting in a preparation suitable for transfer by pipet. 0.5 ml (approximately 10 mg dry weight) was placed in each culture flask containing 40 ml of casein-hydrolysate solution, and incubated for 5 days.

To determine the effect of inoculum volume, two other sets of flasks were inoculated with 0.1 ml and 2.5 ml of fungal suspension.

Table 2. Effect of type and amount of inoculum on growth of Rhizoctonia solani in casein-hydrolysate medium for 5 days.

Type of inoculum	Amount of inoculum ml	Mycelial Growth dry wt mg	Standard deviation
2 disks 6 mm	-	240	23
glass bead maceration	0.5	385	10
Waring blender maceration	0.1	386	7
Waring blender maceration	0.5	379	9
Waring blender maceration	2.5	370	13

Results (Table 2) show that cultures started with macerated inoculum grew faster and more uniformly than did cultures started with solid disks of inoculum. The amount of macerated inoculum within wide limits did not affect growth of the cultures. The Waring blender method of maceration was used in later work because it was much easier to handle.

P. ultimum when inoculated with a suspension of blended mycelial mat responded with about the same amount of growth as it had when a disk inoculum was used. Because of its coenocytic nature partial maceration of the mycelium may have resulted in enough destruction to counteract any advantage of increasing the number of growing points. For uniformity of work the Waring blender method of maceration was used in later works.

Growth curves for R. solani and P. ultimum in casein-hydrolysate solution: Many batches of casein-hydrolysate solution gave consistently good growth of R. solani and adequate growth of P. ultimum. Therefore this medium was used in growth curve determinations and in subsequent experiments with R. solani and P. ultimum. Fifteen flasks were started with 0.5 ml each of a suspension of R. solani mycelium prepared in the Waring blender as described above. Three flasks were chosen at random for harvest on each of five successive days.

Results (Fig. 1) show a typical growth curve. The daily increment increase in growth was about 8 mg for the

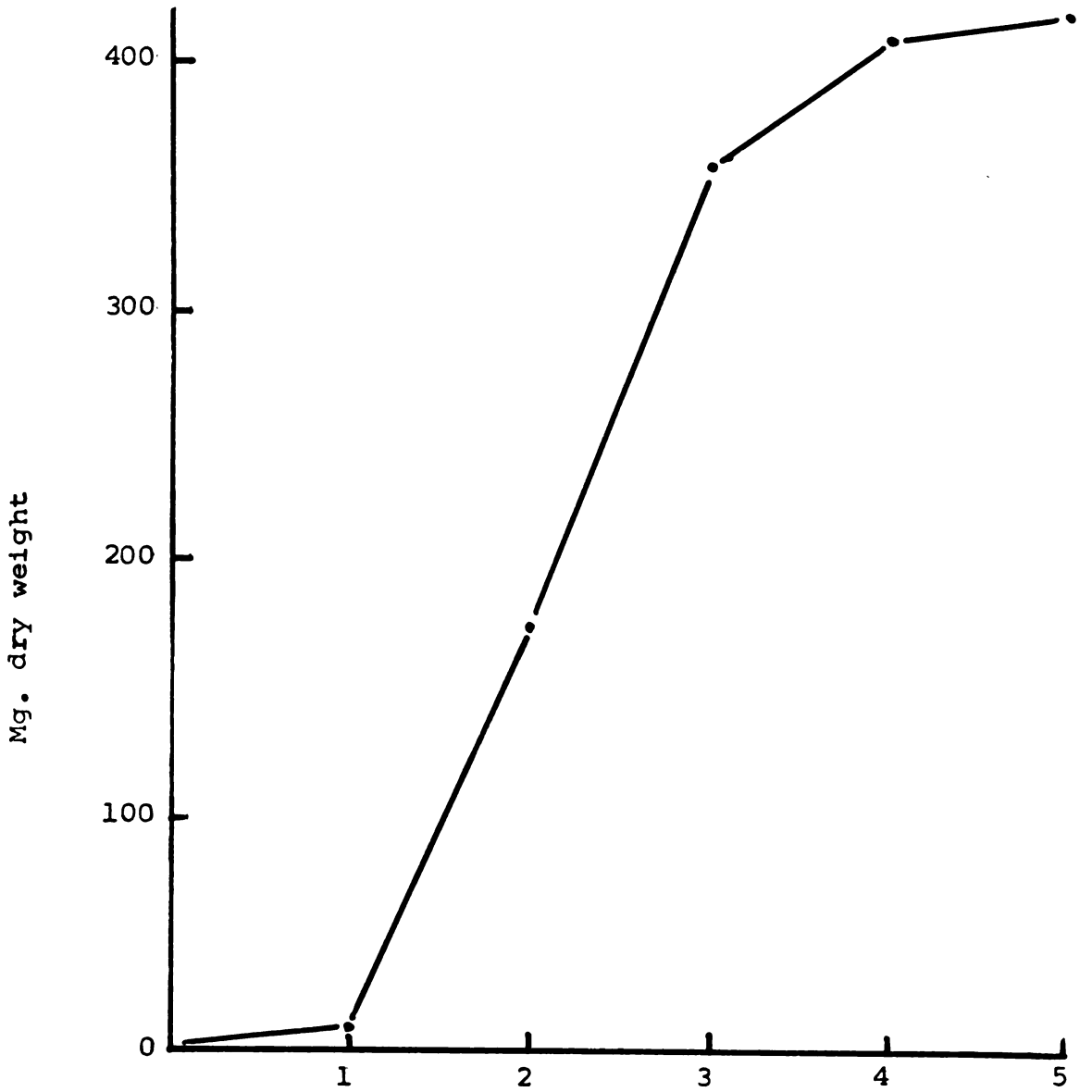


Fig. 1. Growth curve of Rhizoctonia solani in casein-hydrolysate medium, liquid shake culture, at 23-26° C.

first day, 167 for day 2, 185 for day 3, 52 for day 4, and 8 mg for day 5. The log phase of growth appears to occur during days 2 and 3.

Exactly the same methods were used to determine a growth curve for P. ultimum. Results (Fig. 2) again show a typical growth curve. The increment in growth was 8 mg for the first day, 8 for day 2, 22 for day 3, 26 for day 4, and 14 mg for day 5. The P. ultimum culture appeared thick when it was suspended in 40 ml of medium and tended to grow in sheets either on the glass or the liquid surface. When it was harvested and dried, however, the accumulated weight seemed small.

Pentachloronitrobenzene dosage-response relations

Relative sensitivity of R. solani and P. ultimum to pentachloronitrobenzene and Captan: A Captan-PCNB combination used in soil will prevent root rot and damping off of many plants (26, 28). To separate the individual effects of each fungicide a test was run to check relative sensitivities of R. solani and P. ultimum as measured by growth and by the ability of the fungus to revive after pre-exposure. Roughly 250 ppm of fungicide in soil are recommended for field control. Therefore that amount of active Captan and PCNB were added directly to 2% PDA, mixed well, autoclaved, and 30 ml added to each 9 cm petri dish. A 6 mm disk of mycelium and agar taken from the outer edges of a 5 day old colony growing on PDA in a petri plate was placed with mycelium down in the

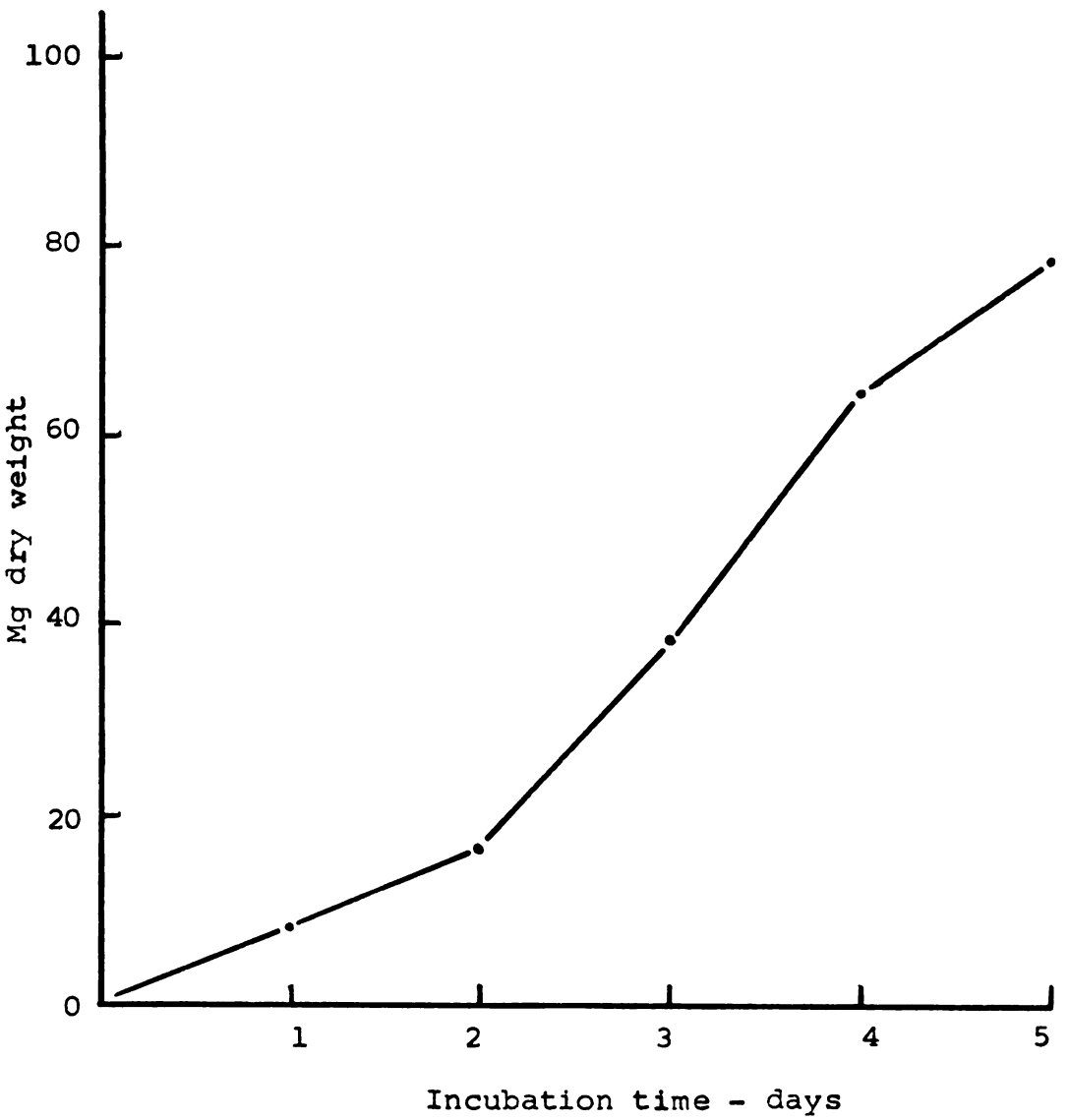


Fig. 2. Growth curve of Pythium ultimum in casein-hydrolysate medium, liquid shake culture, at 23-26° C.

center of each new plate. The plates were incubated in the dark at 28° C. Growth of the colonies was measured as the diameter at the widest point. Control colonies completely filled the 9 cm petri plates in 3 days, and data were taken at that time.

Table 3. Effect of PCNB and Captan (250 ppm each) on growth of Rhizoctonia solani and Pythium ultimum on agar in petri plates.

Treatment	Colony diameter in cm[a]	
	R. solani	P. ultimum
Control	9.0	9.0
PCNB	2.8	8.2
Captan	3.2	1.2

[a]Colonies were measured after three days growth.

Results (Table 3) show that growth of R. solani was inhibited by both PCNB and Captan. On the other hand, P. ultimum was hardly affected by PCNB, but was inhibited by Captan. This is indicative of PCNB's differential action on the two fungi. It seems probable that the success of the mixture in the field depends on the sensitivity P. ultimum to Captan.

After these readings had been made, agar disks were cut and lifted from the margins of colonies from each treatment and transferred in the usual way to fungicide-free PDA in petri plates. After 2 days incubation time, R. solani

colonies which had been transferred from the PCNB treated cultures grew as well as the colonies from the control cultures (Table 4). This indicated that the growth inhibiting effect of PCNB was temporary and was therefore a static effect. It does not kill and therefore have a cidal effect on the cells.

Table 4. Growth of Pythium ultimum and Rhizoctonia solani colonies that were transferred from agar containing fungicides to agar free of fungicide.

Pre-exposure to	Colony diameter in cm[a]	
	R. solani	P. ultimum
No fungicide	5.6	5.0
PCNB	5.2	5.1
Captan	1.0	0

[a]Colonies were grown on PDA and diameters were measured after 2 days growth.

Similar results were noted by Reavill in her work with PCNB (23). The P. ultimum mycelium was not affected by the PCNB but it was certainly inhibited and killed by Captan (Table 4). R. solani may not have had every cell killed by Captan but must have been severely damaged. The effects of Captan on R. solani very likely are fungicidal.

Morphological effects of Pentachloronitrobenzene on R. solani: The R. solani hyphae which were able to make a little growth in the presence of a sub-inhibitory level of PCNB showed considerable morphological derangement. The cells were approximately one third of their usual length.

The diameter of the cells varied considerably so that the hyphal margins were very irregular. There was an increase in frequency of branching with a great number of the branches consisting of only a short bud-like projection. Hyphae with this stunted branching growth developed into tight beady mats rather than the diffuse growth of the control. A greater than normal concentration of highly refractive material was visible in the protoplasm. Mycelium in control cultures normally produces a brown coloration after the onset of the stationary growth phase (6). Treatment by PCNB in most cultures tended to give premature formation of this brown color.

Pentachloronitrobenzene dosage-response for R. solani and P. ultimum: The dosage-response experiment was done with two purposes in mind. First because of its acute sensitivity to the presence of PCNB, I decided for future experiments to use R. solani as an assay organism to detect and measure PCNB concentration in a solution. A dosage-response curve, once constructed could be used as a reference upon which the future R. solani growth could be plotted for estimation of unknown PCNB concentration. Second, an accurate dosage-response curve will shed light upon the mode of action of a chemical (12). Since the growth curve experiments indicated that R. solani and P. ultimum has a log phase lasting from about day 2 to day 6, I decided to harvest at day 5 where the curve begins to flatten but also before the possible variable effect of metabolic products becomes

too great. Three cultures were prepared with casein-hydrolysate medium, PCNB, Triton X-100 and inoculum for each of the following levels of PCNB: 0, 0.1, 0.5, 1.0, 5.0, 10.0, and 100.0 ppm. The amount of Triton X-100 increased along with the PCNB ethanol solution. To be sure that it was having no effect on growth it was added to the control culture in the same amount as the culture with 100.0 ppm of PCNB. The solubility of PCNB is in the range of 1 ppm. Therefore the PCNB was in a suspension in all the cultures with concentrations of 5.0 ppm or more.

Table 5. Effect of PCNB concentration on growth of Rhizoctonia solani and Pythium ultimum in liquid shake culture.

Concentration ppm	Dry wt. mg[a]	
	R. solani	P. ultimum
0	379	68
0.1	321	-
0.5	79	-
1.0	34	-
5.0	22	-
10.0	23	59
100.0	21	71

[a]Cultures were harvested after 5 days growth in casein-hydrolysate medium at 23-26° C.

The results (Table 5) indicate that R. solani is most sensitive to a change in concentration of PCNB between 0.1 ppm

and 1.0 ppm. The point at which it is inhibited by 50% of the control culture is about 0.3 ppm with nearly complete inhibition at a little more than 1.0 ppm. It appears that even at greater than inhibitory amounts, the fungus is able to double its weight during the 5 day period. This could be due to delayed action (assuming that PCNB affects new cells only) or to a flattening of the curve for log phase growth.

If the data are plotted on a probit scale as the percent inhibition of the control culture on the ordinate against the log function of the concentration of PCNB on the abscissa, a straight line is produced which extends to about 95% inhibition (Fig. 3) (14). This suggests that the chemical acts on the fungus at one primary site, and does not have several modes of action that start at different concentrations. This isolate of P. ultimum was not affected by PCNB even at 100 ppm (Table 5). It can be concluded that the damage incited on the lily bulbs in the presence of PCNB was because P. ultimum is not sensitive to this chemical at all.

Effect of Pentachloronitrobenzene on the growth curve of R. solani: Cultures in the dosage response experiments described above were harvested on the 5th day when the log growth of the control began to diminish. The possibility remains that PCNB could change the nature of the growth response. Therefore an experiment was devised to obtain a series of growth curves of R. solani in the presence of several concentrations of PCNB. PCNB was added to the inoculated casein-hydrolysate medium at 0, 0.1, 1.0, and 3.0 ppm.

Fifteen flasks were started with each concentration of PCNB. Previously stated conditions of shaking, temperature, and harvest were used.

Growth inhibition occurred at 0.1 ppm PCNB but the growth curve had essentially the same shape as the control curve (Fig. 4). At 1.0 ppm only a faint log stage was evident. At 3.0 ppm no growth occurred. Apparently at partially inhibitory concentrations of PCNB the lag phase is not longer than the control and the log phase of the treated cultures parallels the control growth curve.

Pentachloronitrobenzene dosage-response of other fungi and yeasts: For comparative purposes I ran dosage response experiments on Fusarium oxysporum f. lycopersici (Sacc.) Snyder and Hansen, Botrytis cinerea, and Sclerotinia scleratiorum (Lib.) de Bary hoping to find an assay organism more suitable than R. solani. The fungi were tested in casein-hydrolysate medium using shake culture at 25° C.

Growth of F. oxysporum was not affected by PCNB in concentrations up to 50 ppm. However, there was considerably more red pigment in the liquid medium of treated cultures after removal of the fungus, while the control cultures left a purple pigment. There was a decrease in color intensity as PCNB concentration increased.

B. cinerea was inhibited by PCNB but was not as sensitive as R. solani. The slope of the growth curve was much flatter and 50% inhibition occurred at approximately

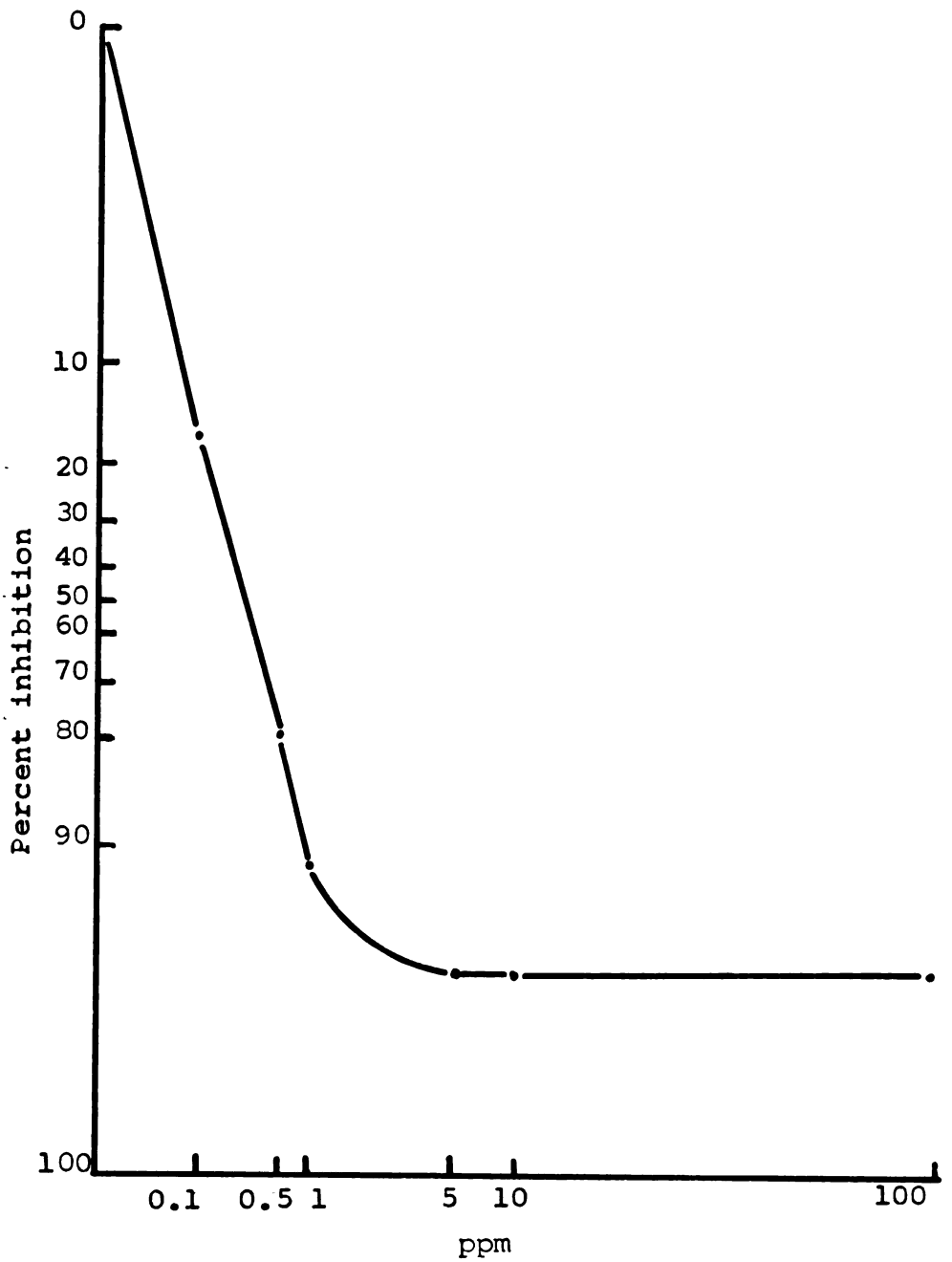


Fig. 3. Growth response of Rhizoctonia solani to increasing concentrations of PCNB. The ordinate is percent inhibition of growth on a probit scale, while the abscissa is the log of PCNB concentration.

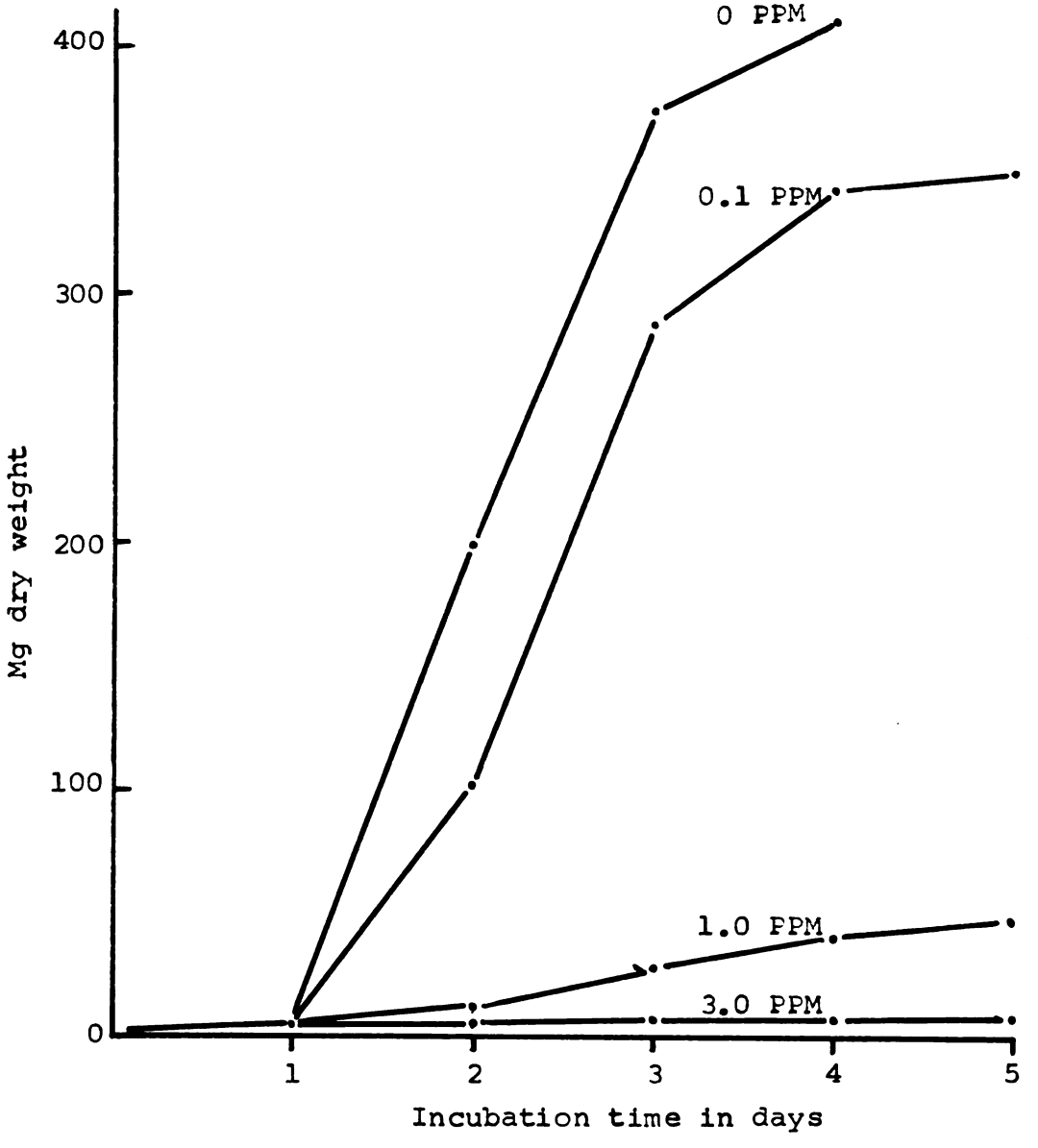


Fig. 4. Effect of 3 concentrations (ppm) of PCNB on growth of Rhizoctonia solani in liquid shake culture using casein-hydrolysate medium.

5.0 ppm PCNB. Thus Botrytis will tolerate about 10 times more PCNB than will R. solani. Previous experiments (25) have shown that spore germination is affected by PCNB.

S. sclerotiorum was only slightly less sensitive than R. solani to PCNB. The 50% inhibition point was about 0.5 ppm making the curve a little flatter than that of R. solani.

The yeasts Schizosaccharomyces octosporus Beijer, Dipodascus uninucleatus Biggs, and Endomyces fibuliger Lindner also were tested for sensitivity to PCNB. All three grew as well in the 5.0 ppm PCNB as in the controls cultures and were considered insensitive to PCNB.

R. solani was the most sensitive organism found.

Effect of initial pH on dosage-response: Since permeation into membrane is best accomplished by a nearly non polar molecule one can assume the PCNB might be able to permeate very well. However, a change in pH of the medium could change the membrane so that permeation of PCNB was affected, therefore I grew R. solani at several pH levels in the presence and absence of 0.5 ppm of PCNB. The pH of the casein-hydrolysate medium was varied from its initial 5.4 by titration with HCl and NaOH to give values of 3, 6, and 9. Three 125 ml flasks containing 40 ml were set up at each pH level of titrated medium plus PCNB and 3 of each pH level but without PCNB were inoculated and incubated as usual.

Table 6. Effect of pH on the fungistatic action of PCNB (0.5 ppm) to Rhizoctonia solani.

pH	Growth[a] (mg dry wt) in PCNB	Control	% inhibition by PCNB
3	8	31	25
6	64	398	17
9	80	337	24

[a]Liquid shake culture, casein-hydrolysate medium, harvested after 5 days.

The percent inhibition (Table 6) of PCNB at the 3 pH levels were considered to be the same. It appears that any effect of pH on the nitro group does not exist or is not enough to over ride the generally high permeability provided by the chlorine groups.

Sublimation as a factor in Pentachloronitrobenzene action: The fact that PCNB vaporizes at normal growing temperatures has suggested to others that fungal growth could be inhibited at a distance from the source of the chemical (25) and that this factor makes PCNB effective in the soil. In an effort to understand more fully the significance of vapors in fungal growth inhibition, I carried out a series of experiments designed to show its effect, determine its efficiency, and analyze the role played by the soil. The initial step was to determine whether or not PCNB can sublime enough to inhibit fungus growth.

Three ml of an acetone solution of technical grade PCNB at 10 mg/ml was pipetted onto the inner surface of the larger valve of the petri dish. The acetone was allowed to evaporate at room temperature causing the PCNB to crystallize onto the surface of the glass. Mycelial disks of R. solani were seeded on the center of an agar filled smaller valve. When the valves were matched and sealed with masking tape the PCNB passed as a vapor into the air space above the fungus. Care was taken not to allow crystals to fall on the agar surface. Cultures were incubated in the dark at 28° C. Colony diameters were measured when the mycelium in controls reached the plate margin. This required 3 days during which time the treated plates grew an average of 2.1 cm. It is clear that PCNB crystals can vaporize enough to act at a distance. This may be an important property of PCNB as a soil fungicide.

If a quantity of PCNB crystals are placed within a closed volume at a constant temperature, PCNB gas will be generated until the atmosphere is saturated. It is plain from the last experiment that the concentration of PCNB gas at its saturation point is sufficient for growth inhibition. I attempted to see if a less than saturated atmosphere would also inhibit growth. 0.01, 0.1, 1.0, and 10.0 mg of PCNB in acetone solutions were pipetted onto petri dish tops. After allowing the acetone to evaporate, the tops were placed on inoculated PDA plates and sealed with masking tape to reduce vapor loss and incub ated as usual. The data (Table 7)

indicate that 1.0 mg was the smallest amount of PCNB able to produce an atmosphere inhibitory to the fungus. According

Table 7. The effect of various quantities of PCNB in vapor form on the diameter of Rhizoctonia solani colonies on agar in a constant air volume.

PCNB, mg	Growth in cm.
0	9.0
0.10	8.6
0.1	8.8
1.0	3.1
10.0	2.5

to Reavill (24), the vapor pressure produced by PCNB gas is 4 mm of Hg. Calculating from Avogadro's principle ($PV = nRT$), where the volume is 34 ml and the temperature is 301° K, 1.0 mg of PCNB is in the gaseous state when the saturation point is reached. This indicates that the fungus requires a minimum of an atmosphere saturated with PCNB gas to cause inhibition.

If 1.0 mg of PCNB is the minimum needed to maintain a saturated atmosphere and the fungus is drawing off molecules from the equilibrium system, a less than saturated situation will soon develop. In a less than saturated atmosphere the fungus would be able to take up fewer and fewer molecules. The very little growth which occurs at 1.0 mg per petri plate seems to indicate that very few molecules are necessary to inhibit but that a saturated atmosphere is necessary to

provide even the few that are utilized.

Effect of soil on pentachloronitrobenzene vapor action: It would seem that soil might act as a barrier to vapor movement. This would, on the one hand, reduce the distance through which the PCNB gas could inhibit, while on the other hand it could help to contain the gas in small pockets where a saturated atmosphere could be maintained. Possible differences in wet and dry soil as barriers to vapor movement were also tested. Sandy loam soil was put through a screen sieve (16 mesh), mixed well and divided into two portions. One was allowed to air dry at room temperature. Water was added to the other until it had become saturated. Three 50 ml aliquots of wet soil were placed on the larger valve of petri dishes which had previously been coated with 30 mg PCNB crystals. Three more were placed in non-PCNB plates. The dry soil was divided similarly. The smaller valves containing agar and inoculum were placed so the rims were resting on the soil. Additional control plates with and without PCNB, but with no soil were also prepared. In the plates without soil they were propped up with wooden blocks to maintain the same distance between PCNB and agar as with the soil plates. During incubation water was added from time to time to keep a high soil moisture content.

Colonies in the non soil control as well as colonies in the wet and dry soil controls reached 9.0 cm at three days but the colonies in plates with both PCNB and wet soil,

and PCNB and dry soil were held to 4.0 and 4.3 cm respectively. The non-soil PCNB colony grew 2.3 cm. The soil showed ability to reduce the action of the fungicide, but there was no difference between the wet and dry soil. This indicates that the soil is able to hold PCNB gas, thus reducing the distance through which the crystals can inhibit the fungus. It appears that soil may adsorb some of the PCNB w hich would be a factor in the soil's ability to maintain an inhibitory level of PCNB residue long periods of time. There was some bacterial contamination on agar above the soil, but the contaminants did not have a significant effect on fungal growth.

Effect of pentachloronitrobenzene vapor on fungus growth in bubble cultures: I felt that if a way could be devised to present PCNB gas to the fungus without the requirement of a saturated atmosphere the most exact estimate of an inhibitory dose could be made. Such a system would have to provide only the amount of gas that the mycelium could absorb at a specific time and be able to do this over a period of time. The system I devised to accomplish this amounted to an air stream which carried PCNB gas to a liquid culture of R. solani and bubbled it into the medium. The PCNB presented to the fungus was probably in the same state as when it was added to the medium directly.

Air was drawn from the room by use of a vacuum line, through a liter bottle of 400 ml of water; then through a

stoppered glass chamber (6" x 2" diameter) of known weight on whose bottom 1 gram of PCNB had been crystalized (Fig. 5). From this point the gaseous mixture passed through a sterile cotton plug and into a 4 liter culture flask containing 2 liters of medium and 4 ml of inoculum. The bubbling provided good agitation to the cultures. Air free of PCNB was bubbled through the control flask. Rapid growth made it possible to harvest on the third day, reducing the risk of contamination. PCNB in the vapor chamber was weighed again to determine the amount lost by vaporization. The chamber was held in an atmosphere above $CaCl_2$ for several hours before the initial weighing and again before the second weighing in order to remove water.

Dry wt. of fungus growth in the control culture was 2505 mg, as compared with 36 mg in the PCNB gas treated flask. Twelve mg of PCNB was lost from the crystals. Assuming all of the PCNB (lost from the flask containing crystals) remained in the solution, the total concentration in the culture flask at the end of the third day was about 6 ppm. This meant that each day the system was producing gas at a rate of 2 ppm in the solution.

The possibility that some PCNB was lost as vapor in the air stream was tested also by bioassay. Two culture flasks were set in series which allowed the exhaust from flask No. 1 to pass through the medium of flask No. 2. A PCNB set and a control set were arranged by using 500 ml flasks with 150 ml casein-hydrolysate medium each (Fig. 6).

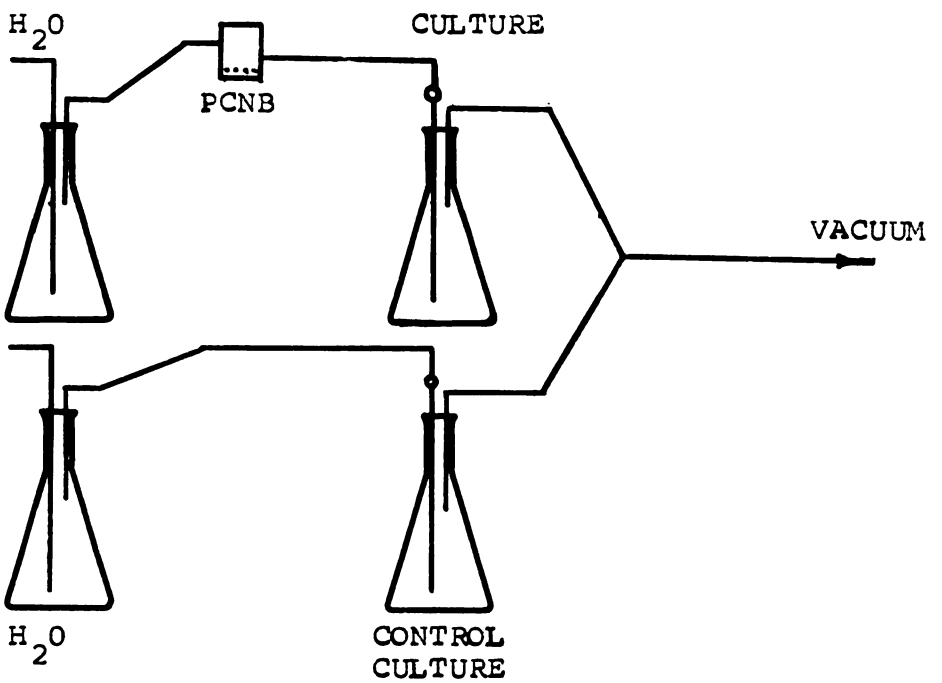


Fig. 5. The system used to supply PCNB vapor by passing moist air over PCNB crystals at room temperature and bubbling it through the culture flask.

The control cultures grew to 346 mg (dry wt.) in the first flask and 308 mg in the second flask, while the first treated culture grew only 49 mg and the second treated culture grew 163 mg. The PCNB loss in the chamber was 2.3 mg. The conclusion was that the amount of PCNB that escaped from the first treated flask to the second in the series was not more than 0.3 ppm.

To compare the efficiencies of both methods of adding PCNB to the culture flask, a third control flask was placed in the system. Crystalline PCNB at 1 ppm was added to this reference flask along with the inoculum. This culture was also bubbled with air as in the control culture. The flasks were 500 ml size containing 150 ml medium, with an air tubing system designed as shown in Fig. 7. The cultures were harvested the third day and the chamber containing crystals weighed to determine PCNB loss. The control culture produced 325 mg (dry wt.) of mycelium; the reference culture of 1 ppm PCNB, 142 mg; and the PCNB vapor culture, 23 mg. The crystal chamber lost 1.8 mg PCNB, which amounts to about 2 ppm, added to the solution each day, or as final concentration of PCNB in the cultures of 7 ppm. The method of cultivation apparently reduced the efficiency of the reference flask because the percent inhibition by 1 ppm PCNB, as estimated from the dosage-response curve, should have been about 90% (30-50 mg of mycelium). Also, the air stream may have taken some PCNB out of the culture flask. At room temperature PCNB in the chamber appears to be sublimating at a rate

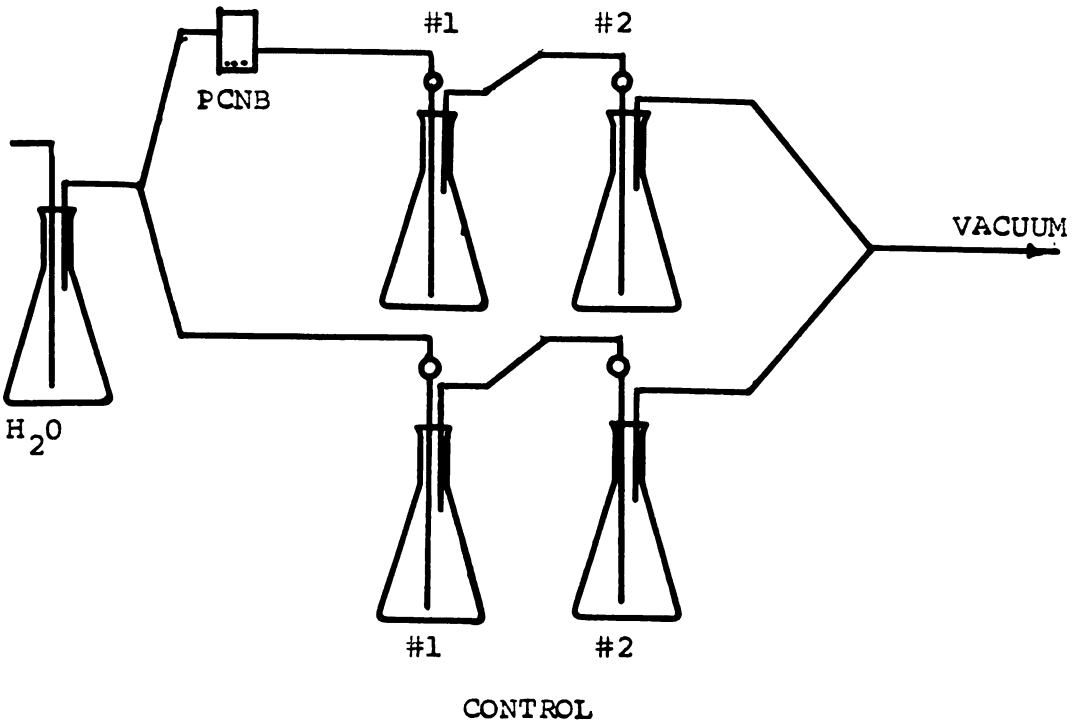


Fig. 6. Arrangement of flasks in series to assay the amount of PCNB vapor lost to the vacuum source.

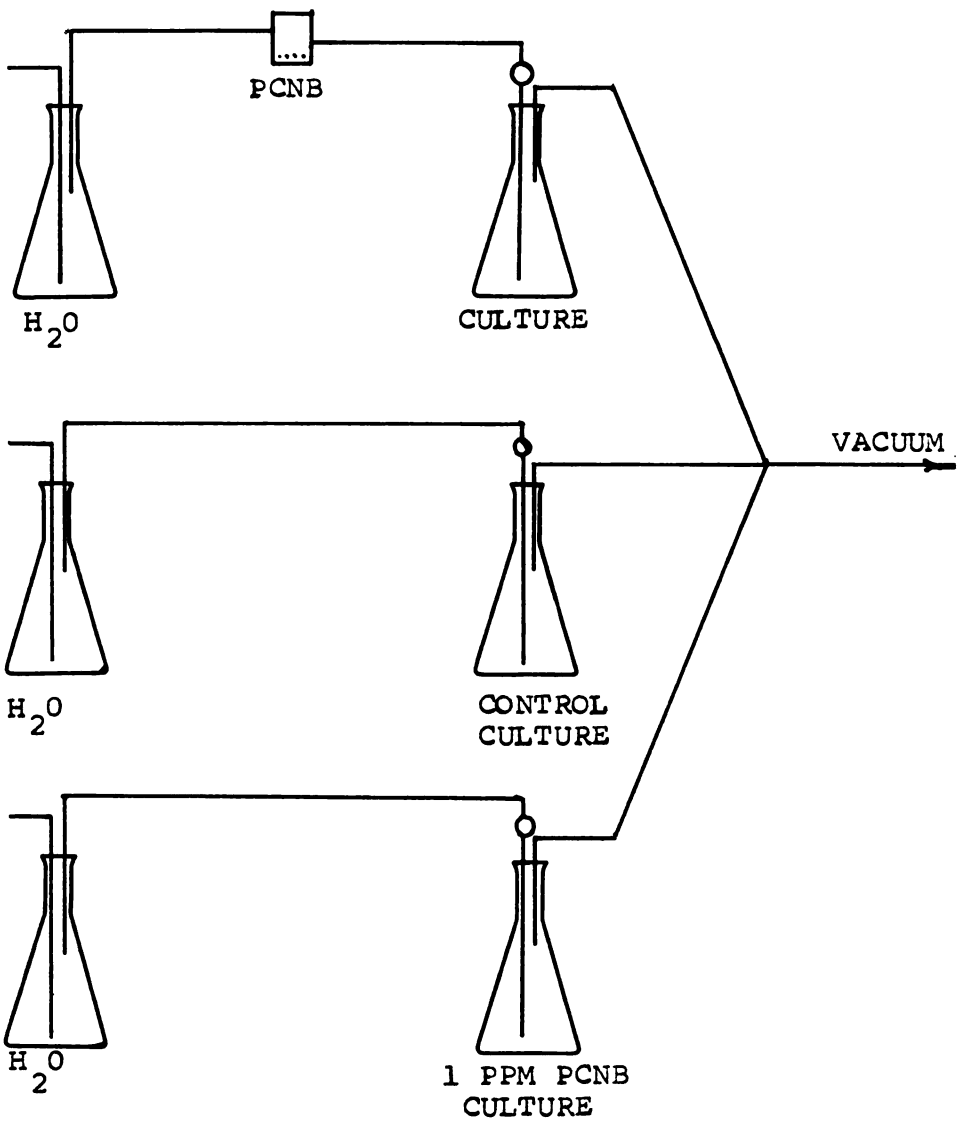


Fig. 7. The system used to provide both PCNB vapor and PCNB crystals in solution to bubble cultures (right).

greater than that required for 50% inhibition of fungus growth. One PCNB crystal chamber was placed on the lab bench along with the cultures at room temperature, and one in the refrigerator at 3° C (Fig. 8). Two controls were included; one at 0 ppm and one at 1 ppm with PCNB crystals added directly to the culture medium. The cultures were harvested on the third day and the vapor chambers weighed. The reference culture with 1 ppm PCNB grew to 65 mg dry wt (Table 8).

Table 8. The effect of PCNB gas concentration (as controlled by temperature of sublimation chamber) on growth of Rhizoctonia solani in bubble culture[a].

Treatment conditions Temperature of chamber °C	Rhizoctonia solani mg dry wt.	PCNB used mg	PCNB used ppm
25 C sublimation chamber	17	1.5	10
3 C sublimation chamber	46	0.5	3
Bubble culture control	123	0	0
Reference control[b]	65	-	1

[a]Cultures were grown in casein-hydrolysate medium at room temperature with aeration by bubbling. Harvest was at day 3.

[b]PCNB (1.0 ppm) added directly to medium as crystals.

This is roughly 50% of the growth in the bubble culture control (Table 8). The PCNB crystal chamber at room temperature again gave off more than a minimum dose of PCNB vapor. The chamber in the refrigerator produced 0.5 mg PCNB gas,

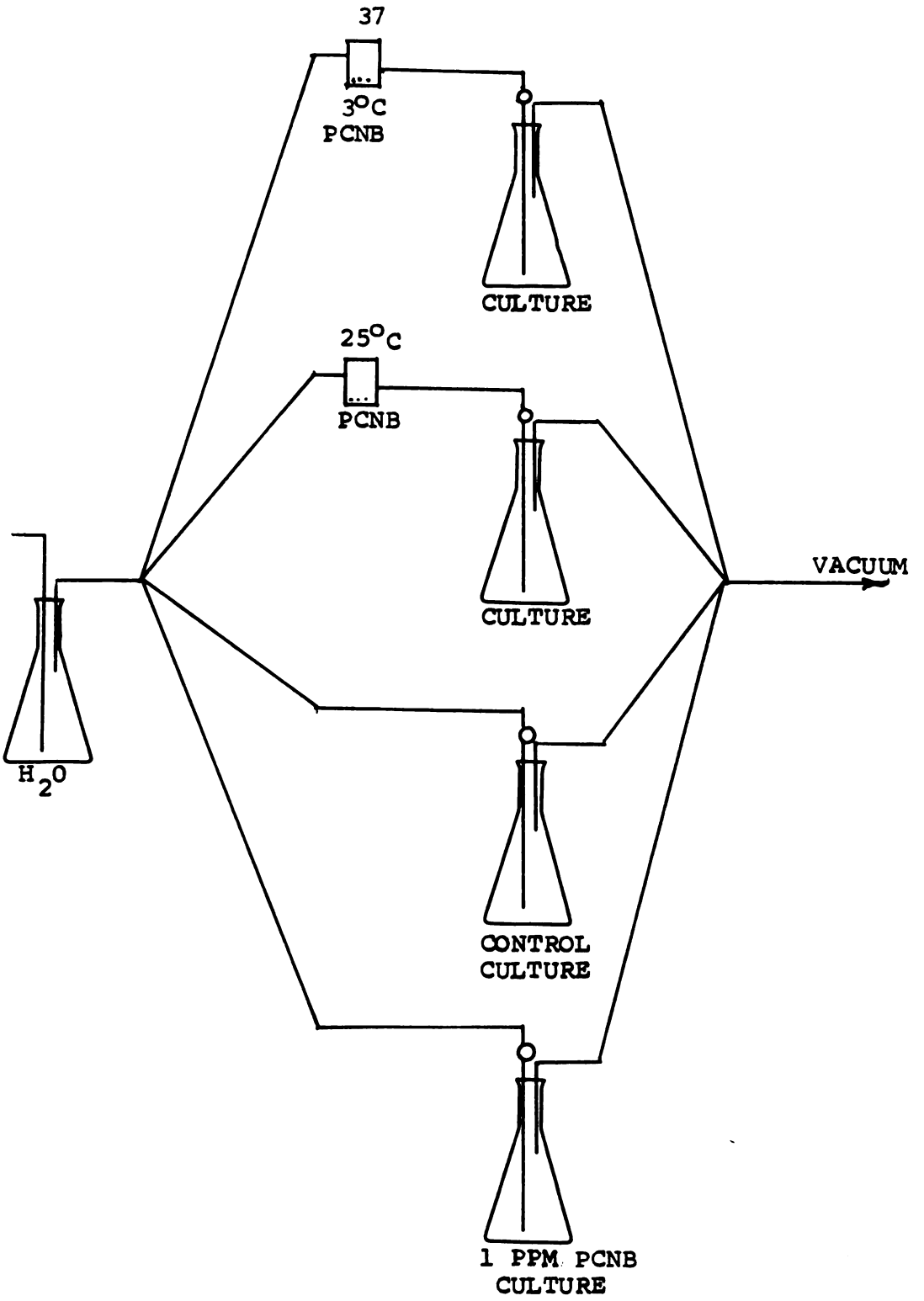


Fig. 8. Arrangement of flasks used to provide varying concentrations of PCNB vapor by placing crystals at different temperatures, and passing the vapor through cultures (right).

amounting to 3 ppm at the end of the third day, which was calculated as 3 ppm in the culture medium at the end of the third day, assuming no vapor loss to the air stream. This amounts to 1 ppm per day. Growth inhibition by PCNB vapor at this concentration is about 40% or roughly comparable to the inhibition obtained by adding PCNB crystals directly to the culture medium (Table 8).

Comparative uptake of pentachloronitrobenzene by R. solani and P. ultimum: Several possibilities could account for the lack of response by P. ultimum to PCNB. The fungus could detoxify the chemical either intra- or extracellularly, or it could simply "ignore" the compound and fail to absorb it. In an attempt to eliminate one of these possibilities, I grew P. ultimum in the presence of PCNB and estimated the residual concentration of PCNB in the culture filtrate by a bioassay method using R. solani as the assay organism. P. ultimum was grown in two 500 ml Erlenmeyer flasks containing 100 ml casein-hydrolysate medium. One flask received PCNB at 2 ppm while the other served as a control. The cultures produced nearly equal growth (181 mg dry weight for PCNB treated culture and 165 mg for the control). After removing all fungal growth, 50 ml of each filtrate was sterilized by filtering through an ultra fine fritted glass filter and 20 ml was transferred to each of three 250 ml sterile flasks. Twenty ml of fresh medium was added to each flask. As controls, PCNB was added to additional flasks of

casein-hydrolysate solution at 0, 0.5, and 1.0 ppm. To assay the residual PCNB, each flask was inoculated with R. solani and incubated for five days.

R. solani in the control cultures produced 373 mg (dry weight) at 0 ppm of PCNB; 112 mg at 0.5 ppm; and 79 mg at 1.0 ppm. In the culture medium containing P. ultimum and PCNB filtrate, R. solani produced 225 mg dry wt. and in the culture medium containing P. ultimum filtrate plus residual PCNB it grew only 52 mg. The unamended medium controls when plotted on a probit scale as percent inhibition gave a straight line comparable to Fig. 3. A comparison of R. solani inhibition (77%) with the reference standard (Fig. 3) indicates that 0.85 ppm PCNB was present. P. ultimum mycelium appeared to have removed about 0.15 ppm PCNB from the medium or had inactivated it through detoxification.

For comparative purposes, another experiment was run to determine whether R. solani removed PCNB from the medium. Since R. solani will not grow in the presence of PCNB, a non-amended medium was used to produce mycelium. The mycelial mat was harvested at day 3, during its most active growth state, divided into two parts (183, and 190 mg dry weight). Each mycelial mat was added to 100 ml of medium in two 500 mlflasks, each containing 1.0 ppm PCNB. These replacement cultures were allowed to incubate in the PCNB medium for another 24 hours. The cultures were then filtered and the filtrates were sterilized. Forty ml of each filtrate was placed in each of three 250 ml flasks, and

inoculated with R. solani as the assay organism. The appropriate controls were included.

After 5 days R. solani growth in the control culture with no PCNB was 432 mg. In the culture filtrate an average of 193 mg was produced and in the culture filtrate with residual PCNB 150 mg. The percent inhibition of R. solani which can be attributed to the residual PCNB in the filtrate (18%) was plotted on the straight line curve (Fig. 3). An estimated 0.2 ppm were present in the medium, indicating that the R. solani mycelium had removed about 0.8 ppm from the medium.

Because of the complicating effect of nutrient depletion and accumulation of staling products in the filtrate a method was devised which consisted of reacting equal sized mycelial mats of both fungi with PCNB in a buffered glucose solution. Two day old R. solani mycelium was washed in a buffer solution. A 1 g (wet weight) mycelial mat was added to each of 3 flasks containing 40 ml of a solution composed of 1.5% glucose, 0.15 % KH_2PO_4, and 1 ppm PCNB. Also 1 g mycelial mats were added to three other flasks of PCNB. After a 24 hour incubation period the mats were harvested, dried, and weighed. The reaction filtrate was charged with the other elements of the casein-hydrolysate medium so that the final concentration was approximately equivalent to normal casein-hydrolysate medium plus the residual PCNB. This medium was sterilized through an ultra fine fritted glass filter, inoculated with R. solani and incubated for 5 days

along with inoculated controls containing 0 and 1 ppm PCNB. The reduction in PCNB detected by the growth of R. solani was calculated per gram dry weight of the fungus.

R. solani cultures grown without PCNB produced 225 mg dry wt. while the cultures treated with 1 ppm PCNB produced 80 mg. R. solani grown in the R. solani filtrate produced 171 mg while the culture grown in the R. solani filtrate containing the residual PCNB grew 172 mg. Lack of inhibition indicates that the R. solani mycelium removed all of the PCNB from the buffered glucose filtrate.

In a fourth experiment, the ability of P. ultimum to remove PCNB from the glucose salt solution was tested. The procedure was identical with the above experiment. The assay organism R. solani produced 320 mg in control cultures without PCNB and 91 mg in 1 ppm in control culture with PCNB. The R. solani grown in the P. ultimum filtrate with no PCNB reached 306 mg while the culture in the P. ultimum filtrate with residual PCNB grew 117 mg. The percent inhibition due to the residual PCNB is about 72%, which when plotted on the straight line curve (Fig. 3) estimates the residual PCNB at 0.7 ppm, indicating the P. ultimum mycelial mat removed or inactivated 0.3 ppm from the solution.

A comparison of the results of the last 2 experiments shows that R. solani inactivates or absorbs a larger amount of PCNB from the medium than does P. ultimum. However, the bioassay method was far from quantitative. A more quantitative colorimetric method was devised, based on the spectrophotometric analysis of PCNB developed by Ackerman for crop and

soil residues (1). It was not possible to use the ultra violet absorption to measure PCNB in a spectrophotometer because the absorption peak at 210 mu was too small for accuracy. I modified the colorimetric method for application to media and mycelial homogenates. R. solani and P. ultimum culture mats grown for 3 days in casein-hydrolysate medium were divided into 3 gm portions (wet weight) and transferred aseptically to 500 ml flasks containing 100 ml casein-hydrolysate medium. PCNB (1.0 ppm) was added to these flasks as well as to a non-inoculated control flask. Incubation continued for 1, 4, 8, and 24 hour periods. The culture mats were removed from the medium and both medium and mycelium were analyzed for PCNB.

The medium from each flask was extracted in a glass stoppered 500 ml separatory funnel with 100 ml petroleum-ether for 60 minutes. The petroleum-ether layer was then concentrated in a vacuum oven at room temperature to about 25 ml. The concentrate was placed on a 3 x 40 cm column containing 10 cm of activated Florisil topped by 2 cm of anhydrous sodium sulfate which had been prewashed with 75 ml petroleum-ether. The filtrate was collected in flask. The column was eluted with 150 ml 16% diethyl-ether-petroleum-ether solvent. The total eluate was pooled and concentrated by vacuum to 5 ml. A 5 ml standard solution of PCNB (10 ug/ml) in petroleum-ether was placed in a similar flask and analyzed as a reference standard. A reagent blank control was also used.

PCN3 was acetylated by adding 1 ml of 0.5 N ethanolic potassium hydroxide and 1 ml acetone to each flask. The flasks were heated for exactly 7 minutes at 80°C in a water bath and then were cooled in a water bath at 0°C. The preparations were then diazotized and color was developed by addition of 20 ml of the color reagent and 25 ml of petroleum-ether.

The color reagent was prepared by dissolving 0.35 gm of 1-naphthylamine in 88 ml of glacial acetic acid and 200 ml of triple distilled water. 7.5 g of procaine hydrochloride was added and the mixture was dilutedto 1000 ml with water and stored in a dark bottle.

The reaction flasks were stoppered after addition of the color reagent and were shaken for 15 minutes on a reciprocal shaker. The solutions were transferred to 60 ml separatory funnels. The aqueous layer was passed through a 1 cm layer of Celite 545 under vacuum into a 25 mm cuvette. Absorbance was measured in a Coleman Jr. spectrophotometer at 525 mu against water as a blank set at 0 absorbance. Absorbance was corrected by subtracting the reagent blank.

The mycelial mats were ground with a mortar and pestle using fine white sand to assist fracturing the hyphae. The whole amount was transferred to a 500 ml flask and extracted with 100 ml of petroleum-ether by shaking for 60 minutes. The solids were allowed to settle and the supernatent was decanted through glass wool. The petroleum-ether extract of mycelium was handled exactly as the culture filtrates.

A reference standard curve was made by reacting 5 ml sample of petroleum-ether containing 0, 10, 30, 50, 70, and 100 ug each of PCNB. This curve was used as a reference for the standard of 50 ug which was run along with the determinations. A 50 ug standard sample gave an absorbance of 0.432. In the reaction the PCNB is acetylated by the ethanol forming pentachlorophenetole, water, and potassium nitrite. The potassium nitrite diazotizes the procaine hydrochloride which then couples with the 1-naphthylamine to form a magenta color (Fig. 9).

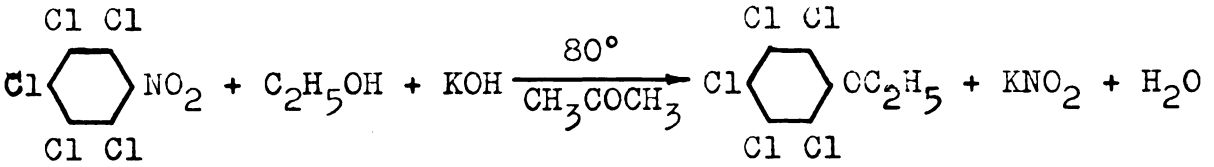
The results (Figs. 10 and 11) show that culture filtrates of both species of fungi diminished in residual PCNB with time and that the filtrate of the R. solani culture contained less PCNB at any time level than did that of P. ultimum. Also they show that the mycelial mats continue to absorb PCNB for the medium over a period of time. They absorb at a fast rate the first hour and at a decreasing rate to 24 hours. R. solani absorbed considerably more PCNB than did P. ultimum.

The data indicate that P. ultimum does not ignore or detoxify (in a way to prevent extraction and detection of the nitro group) the PCNB in the medium but removes it from the medium by absorbing it. The absorption by R. solani, however, is greater per wet weight than that of P. ultimum and this greater absorption may be partially responsible for the sensitivity of R. solani to PCNB.

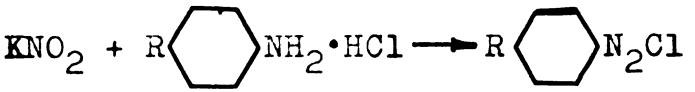
The yield in the extraction from the medium at 0 time amounted to about 85%. The total amount extracted from the mycelial mat plus the culture filtrate of P. ultimum cultures approximated this yield at most reaction times. Extraction of R. solani filtrates and mycelial mats yielded amounts of PCNB which progressively decreased with reaction time. Since the PCNB yield was determined by the number of nitro groups on the PCNB molecule, available in an extractable form, it can be argued that the PCNB molecule is either entering into a reaction with cell constituents in which the nitro is involved, or that the whole molecule is being tied up in a form to make it unextractable by this process.

Effect of pentachloronitrobenzene on the metabolism of R. solani: The report (17) that Lindane (gamma isomer of Hexachlorocyclohexane) a compound relatively analogous to PCNB, causes inhibition of growth in yeast by interfering with inositol metabolism suggests that PCNB may act in the same way. Therefore I added inositol to PCNB treated cultures in a 1 to 1 ratio at 0.1, 0.5, and 1.0 ppm and in a 50 to 1 ratio with a 1.0 ppm PCNB culture in case inositol were absorbed very slowly. Control cultures without PCNB but with 1.0 ppm inositol and a series of cultures with 0, 0.1, 0.5, and 1.0 ppm PCNB were included. None of the inositol containing cultures were significantly different from the cultures treated with PCNB only. Either the PCNB does not compete with inositol in the fungus metabolism or the inositol added to the

Acetylation



Diazotization



Coupling

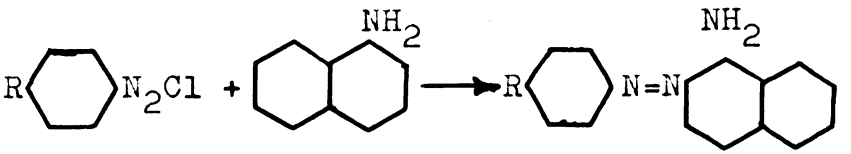


Fig. 9. PCNB color reaction, by Ackerman.

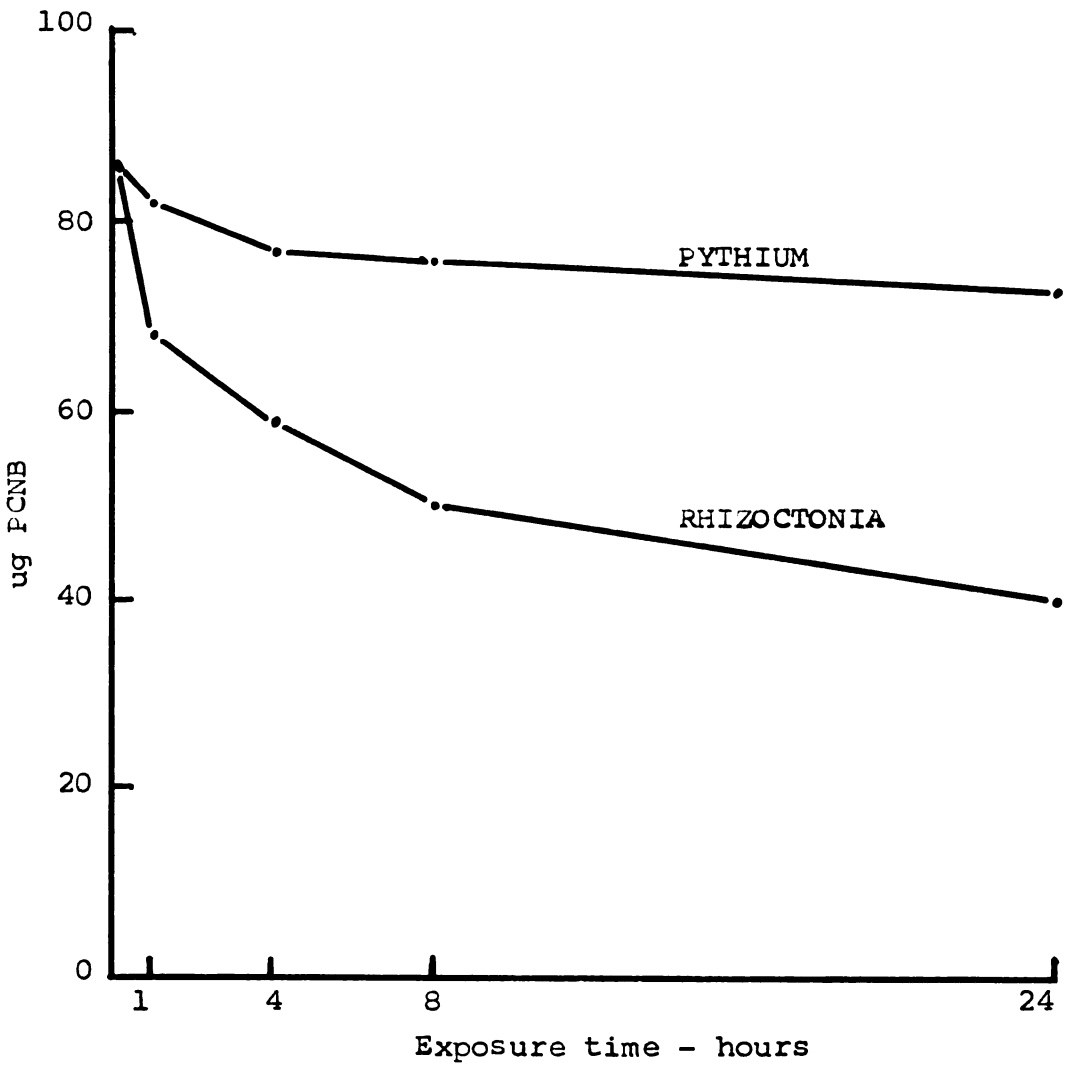


Fig. 10. Comparative uptake of PCNB by Pythium ultimum and Rhizoctonia solani, as determined by residual concentrations in the medium. Three-day old mycelial mats were placed in one ppm PCNB solutions and left for the times indicated. PCNB concentrations were determined colorimetrically.

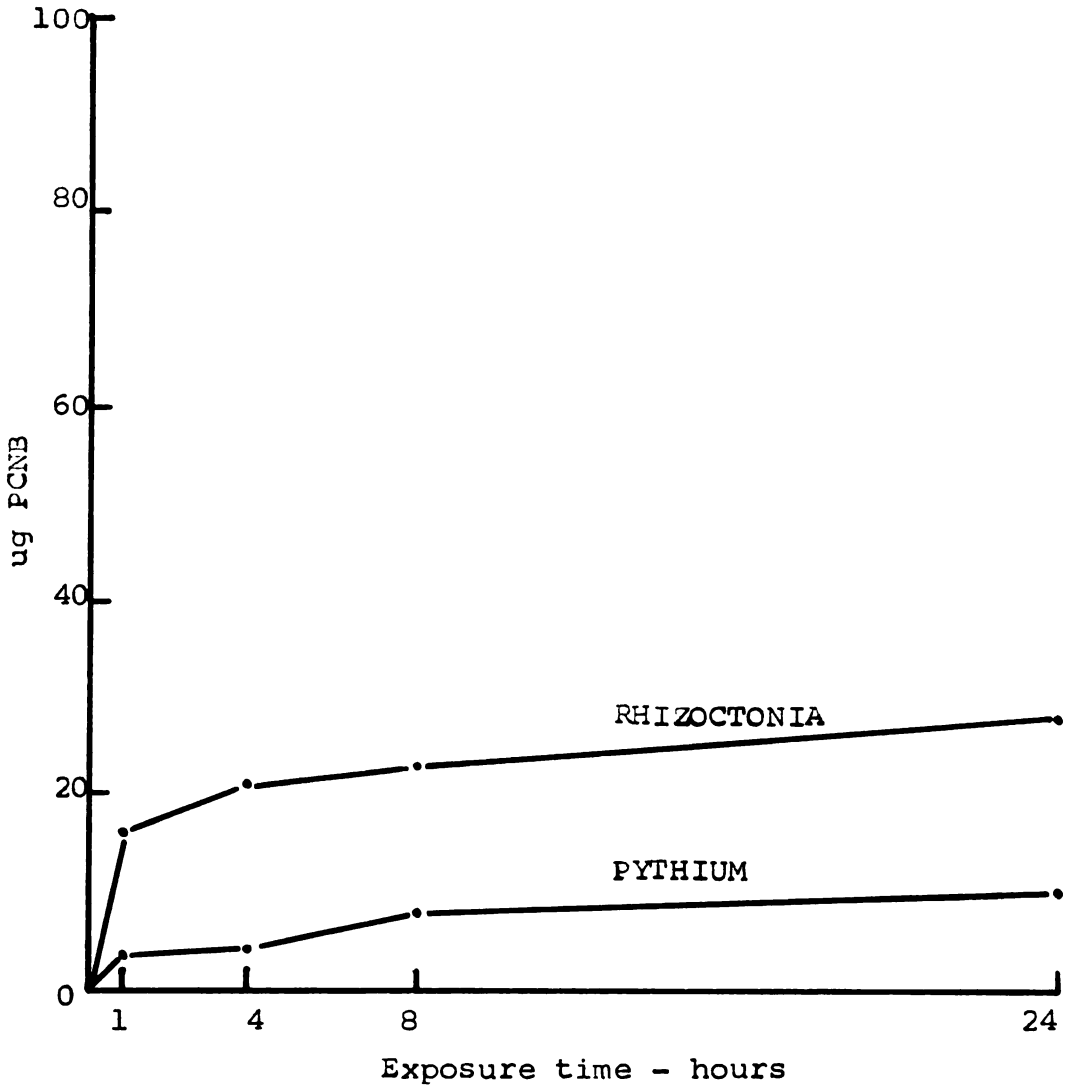


Figure 11. Comparative uptake of PCNB by mycelial mats of Rhizoctonia solani and Pythium ultimum. Values are total ug PCNB extracted from mycelial mats of equal weight (wet) after exposure to 1 ppm solutions of PCNB for the times indicated.

medium could not reach, in adequate amounts, the site of action to counteract the competition of PCNB.

To determine whether PCNB inhibition was due to an interference with respiration, oxygen uptake by R. solani was measured manometrically, using the Warburg apparatus (21). R. solani was grown in casein-hydrolysate medium for 3 days, until the mid-log growth stage was reached. The mat was washed 3 times in m/30 phosphate buffer at pH 6.0 and was separated into portions of about 100 mg fresh weight so as to be approximately 20 mg dry weight. The mats were teased apart with tweezers to give small particles of fungus tissue and placed in a Warburg flask containing 1.8 ml of 0.2 M glucose and m/30 phosphate buffer (pH 6.0). 0.2 ml of 10% KOH was placed in the center well, along with a filter paper wick. 0.2 ml phosphate buffer containing 0.01 % ethanol and 0.0001% Triton X-100 was placed in the side arms of all reaction vessels, including the controls. Treated vessels contained in addition 1 and 5 ppm of PCNB. Duplicate flasks were used for each PCNB and control treatment. The usual thermobarometer flask was included in each experiment. Water bath was 30°C, and a 20 minute equilibration time was allowed. Three readings were taken at 30 minute intervals. After the first reading the contents of side arms were added to the reaction mixture. After the final reading, the fungus was completely removed from the Warburg flasks, transferred to weighing cups, and dried at 100°C in a ventilated drying oven for 24 hours before weighing. Microliters of oxygen per 10 mg dry weight was calculated as:

$$\text{ul } O_2/10 \text{ mg} = \frac{\text{change in manometer reading x flask K}}{10 \text{ mg wt}}$$

All the mycelial mats absorbed oxygen at an even rate throughout the entire experiment (Fig. 12). PCNB did not inhibit oxygen uptake by R. solani within an hour of the time it was placed in contact with the fungus.

In order to insure that there was sufficient time for a reaction between PCNB and R. solani, a modified procedure was used. Cultures were allowed to grow 48 hours, then 1 and 3 ppm PCNB was added and cultures were incubated for an additional 24 hours. Mats were removed and washed as previously described. Again, 1 and 3 ppm PCNB was added to the reaction mixture in Warburg flask, along with the fungus growth. Flasks for control mats and a thermobarometer were also run as previously described.

Table 9. Oxygen uptake by R. solani cells pre-treated with PCNB[a], as compared with non-treated controls.

Treatment ppm PCNB[b]	ul O_2/mg at times (hrs) indicated[c]		
	0.5	1.0	1.5
0	0.98	1.86	2.47
1	0.87	1.68	2.31
3	1.03	1.89	2.81

[a] 1 and 3 ppm of PCNB added to 48 hour culture of R. solani and incubated for 24 hours.

[b] Reaction mixture in Warburg flask contained 2.0 ml of 0.2 m glucose, m/30 phosphate buffer, 0.01% ethanol, 0.0001% Triton X-100, and 0.1, or 3 ppm PCNB.

[c] Values are averages of 2 flasks.

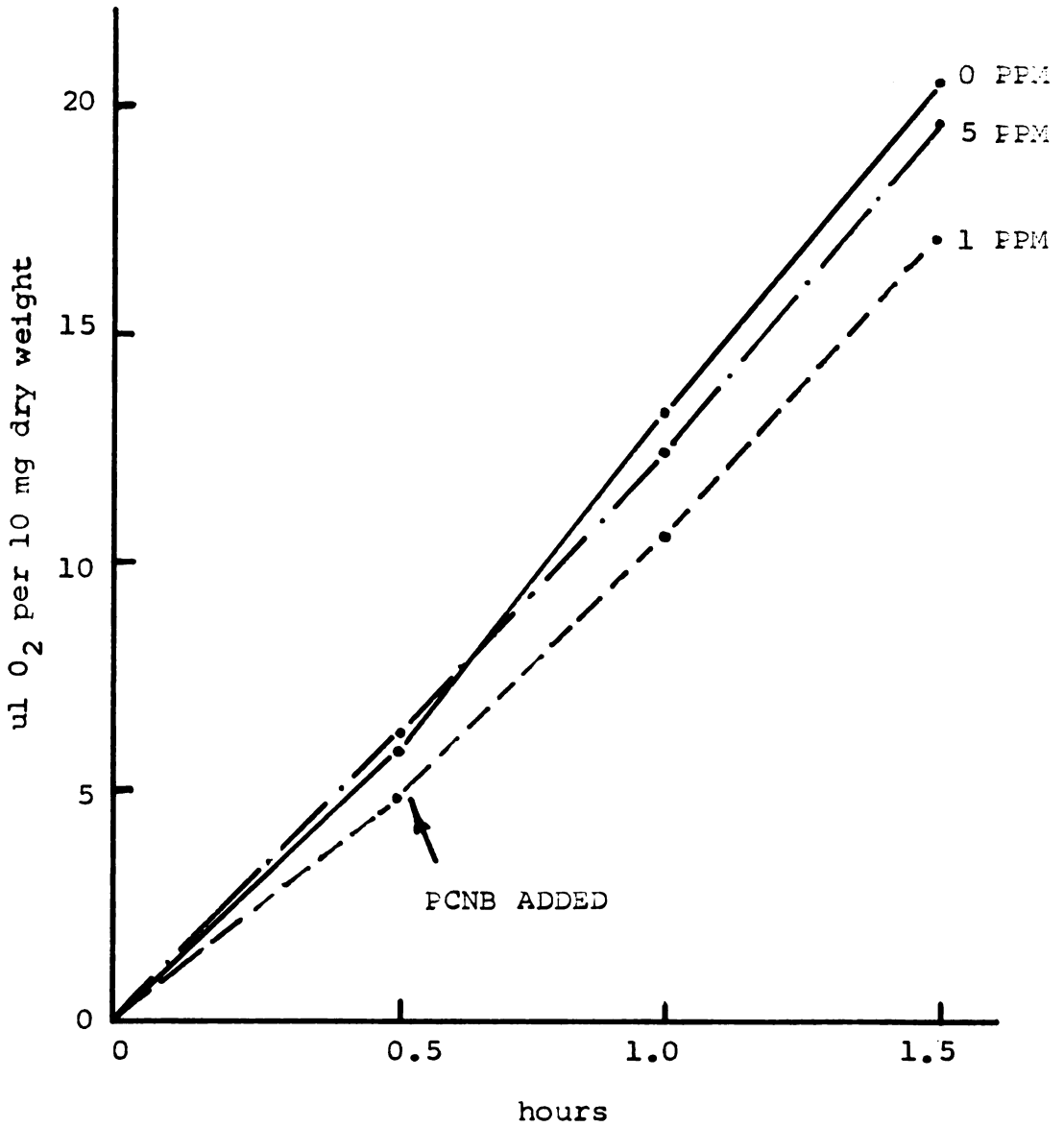


Fig. 12. Oxygen uptake by Rhizoctonia solani in the presence of fungistatic levels of PCNB. Reaction mixture contained 0, 1, and 5 ppm at pH 6.0. Temperature was 30°C.

Even under these conditions PCNB did not inhibit oxygen uptake by R. solani (Table 9). The mycelium pre-treated with 1 or 3 ppm PCNB for 24 hours was respiring at the same rate as the control.

Abnormalities in hyphal morphology (as noted earlier) seem to suggest incomplete synthesis of protoplasm and cell wall material at various places on the hyphal surface. A possible reason for this is a deficiency in protein production. An experiment was designed to measure the effect of PCNB on protein synthesis by R. solani. L-leucine (C^{14}) and PCNB were added to R. solani mycelium and the amount of the amino acid incorporated into the trichloroacetic acid (TCA) insoluble fraction was determined by radioactivity of the precipitated protein. Approximately 2 g wet weight of 3 day old R. solani was placed as a diffuse mat into 125 ml Erlenmeyer flasks containing 30 ml of casein-hydrolysate medium. PCNB was added to the treated flask at 10 ppm. Then 600 lamda of a 0.04 M L-leucine solution, containing C^{14} (1 uc/ml) was added to each flask. The amount added measured 130,000 counts per minute. A small vial, with 0.2 ml of 10% KOH solution and a filter paper wick was suspended above the reaction mixture with a wire from the lip of the flask to absorb metabolized CO_2. Flasks were sealed. The top was covered with aluminum foil and the flasks were shaken for various time periods.

After the stated reaction times (Table 10), the KOH vials were sealed. The reaction mixtures were filtered

through nylon cloth in a Buchner funnel and the mycelium was washed three times with 30 ml of physiological saline. Mycelium was placed in 10 ml 8% TCA in a test tube and ground with a motor driven teflon ball. The suspension was centrifuged at 2000 RPM, the supernatent was removed, and the pellet was washed three times in 5 ml 8% TCA. The solid material was transferred by spatula to a planchette and dried under an infra-red light on a level surface, giving a layer about 0.5 mm thick. Activity was counted in a RIDL 2 pi gas flow chamber for 5 minutes, with results reported as counts per minute per flask less background counts. The KOH solution used to absorb CO_2 was precipitated with 0.4 ml saturated $Ba(OH)_2$ solution and filtered through a 1 in. diameter #42 Whatman filter paper in a glass funnel. The paper was then placed in the planchette for drying and counting.

Table 10. Incorporation of L-leucine (C^{14}) into TCA insoluble protein of Rhizoctonia solani mycelium treated with PCNB for various times.

Hours	CM pellet[a]	
	PCNB	Control
0.5	94	83
1.0	136	142
2.0	168	193
4.0	224	216
8.0	406	522

[a]Pellet contained all TCA insoluble parts of 2g (wet wt.) 3 day old mycelial mats. L-leucine and PCNB were added to casein-hydrolysate solution with mycelium and incubated for stated hours.

Results showed no inhibition of amino acid incorporation into the TCA insoluble fraction from the mycelial mat until about 8 hours exposure (Table 10). By 4 hours the carbon dioxide captured in the potassium hydroxide vials began to show some radioactivity which indicates that the amino acid was being utilized in pathways other than protein synthesis (Table 11).

Table 11. Metabolism of L-leucine (C^{14}) to CO_2 by mycelium of Rhizoctonia solani treated with [2] PCNB for various times.

Hours	CM vial of CO_2[a]	
	PCNB	Control
0.5	0	0
1.0	0	0
2.0	0	0
4.0	16	12
8.0	21	33

[a]Respired CO_2 from 2 gm of 3 day old mycelial mat in casein-hydrolysate[2] solution with L-leucine and PCNB added. CO_2 was collected in 10% KOH on a paper wick in a mat suspended in atmosphere above culture.

DISCUSSION

Garrett (10) amply discusses the parallel roles that R. solani and Pythium spp. take in the soil. Both are described as being able to compete with other organisms because of fast germination from resting cells and rapid growth on residual soil carbon sources. Each of these two fungi can grow toward and damage roots and stems of agriculturally important plants. The desirability of inhibiting both organisms is clear. PCNB was soon shown to control damping off and root rot under certain conditions, but the results on ornamentals in the greenhouse were disappointing (26). PCNB, a known inhibitor of R. solani, appeared not to affect the total damage picture on the plant roots. The assumption was made that root rot damage in the presence of PCNB was a result of P. ultimum infection. This fungus was isolated from roots of plants in PCNB treated soil (11).

The comparative toxicity of PCNB to R. solani and P. ultimum in vitro was studied with the hope of explaining the peculiar results of soil treatment tests. Early experiments with growth inhibition on agar and in shake culture confirmed the assumption that PCNB was toxic to R. solani but not to P. ultimum.

Gibson's report (11) that addition of PCNB to soil actually increased the incidence of damping off disease in pine seedlings appears disconcerting and in contrast to the reported effect of PCNB on this disease. However in view of the data showing the differential toxicity of PCNB to R. solani and P. ultimum, it is possible that the R. solani was inhibited by the chemical and that the P. ultimum was now able to thrive on the seedlings with no competition from R. solani. Gibson also found that PCNB inhibited growth of Penicillium paxilli Bain. which he felt normally acted as a competitor for Pythium spp. Reavill's report (23) that PCNB has an inhibiting effect on Trichoderma viride may add a further facet to the competition question. It is generally believed that T. viride inhibits other fungi in the soil (10). It is possible that this is due to antibiotic metabolites given off by T. viride. If this is true, the demise of T. viride could encourage infection by P. ultimum.

The dosage response data for several other soil fungi were included in this work to see if these also will correlate with field data. S. sclerotiorum was very sensitive to PCNB, which is in accord with the field data on the fungus. Kendrick and Middleton showed control of S. sclerotiorum with PCNB. There are many reports of other species of this genus which have been inhibited to field experiments. B. cinerea is also relatively sensitive, again confirming field data on gray mold of lettuce. There are reports by Kendrick (16), Martinson (20), and Jamalainen (18) which

indicate that Fusaria spp. are not affected in the field by PCNB. Again the dosage-response data with Fusaria confirm the field results.

R. solani was equally sensitive to PCNB in the vapor form and in solution. Many effective soil disinfectants have tended to be a gas or vapor at normal temperatures and have had their effectiveness attributed to the penetration a gas has in soil. The effectiveness of PCNB to susceptible organisms might then be attributed in part to its small but definite vapor pressure (4 mm Hg. at 25° C). Brinkerhoff in Oklahoma (4) found that R. solani causing damping off and sore shin on cotton seedlings was inhibited by PCNB during warm weather but that it failed to control the disease in cool weather. Since the PCNB saturation point in air is dependent on temperature there would be less PCNB in the gaseous state in the cooler weather; perhaps not enough to inhibit the fungus under cool conditions.

Data from vapor phase experiments on agar and in liquid bubble cultures show that PCNB gas is about as efficient in its inhibitory action as is PCNB added directly to media as crystals. Thus the evidence from both field and laboratory experiments indicates strongly that vaporization is an important part of the action of PCNB in the soil. In fact we could even postulate that the main effect of the compound is madeby the gas phase. However, a new question now arises: how can the few crystals in the soil vaporize enough to maintain the saturated atmosphere needed for inhibition and

still be so persistant in the soil? The data on the effect of soil on PCNB action seems to indicate some adsorption of PCNB to the soil particles. The fungus therefore, may be affected by the PCNB vapor and by the PCNB adsorbed to soil particles.

The peculiar behavior of PCNB when used as a soil treatment can be explained by its differential effect on R. solani and P. ultimum. However, the question of its mode of action against R. solani is still open, as is the explanation of the resistance of P. ultimum to the compound. The mode of action problem has been approached from two different angles. One is with the use of homologs of PCNB, attempting to identify the particular atomic configuration which imparts toxicity or permeation ability to the molecule. The other approach is to compare the characteristics which susceptible fungi share and in this way produce a generalized principle of toxicity. Both approaches have had only limited success. The homologs (especially the tetrachloro) have varied from each other and from PCNB in their inhibitory pattern to such a degree that no logical principle could be seen (24). For example, the deletion of the Cl atom on carbon No. 6 made the molecule inhibitory to P. ultimum when the compound was used in high concentrations (8). This is not explainable on the basis of Eckert's oil to water solubility ratio. Horsfall feels that the shape of the molecule affects activity and makes the logical assumption that the chloro groups on the

benzene ring impart permeation ability while the nitro groups are the toxic moities (14).

PCNB susceptible fungi are found randomly in all taxonomic groupings. PCNB is reported to affect a few Actinomycetes, one Myxomycete, several Phycomycetes, a number of Ascomycetes and two Basidiomycetes. Among green plants, Oxalis sp. and onions are known to be sensitive while the flavor of potatoes is adversely affected (22). The diverse list of sensitive organisms indicates that the chemical affects a cellular activity which is rather generally found in the plant kingdom. This problem was approached by more detailed comparative studies of a susceptible and a resistant fungus, R. solani and P. ultimum.

Since P. ultimum was shown not to have a detoxifying mechanism against PCNB, it seems unlikely that other resistant organisms have such a mechanism. Far from detoxifying PCNB, P. ultimum actually absorbs a limited amount from the medium. R. solani, however, absorbed about 3 times more PCNB from the medium than did P. ultimum. The greater amount absorbed by R. solani could be significant in its susceptibility to PCNB. However, the amount absorbed by the R. solani mycelial mat seems to be more than the amount needed to inhibit growth. The absorption to weight ratio is one part PCNB to 20,000 parts dry fungus; from growth studies, one part PCNB will inhibit 30,000 parts (dry wt.) of fungus. Considering the conclusion made from the vapor phase experiments, in which only a small portion of the molecules

in a saturated atmosphere could be used to inhibit growth, it would seem that more is adsorbed than is required for inhibition. A critical point here may be the amount of PCNB which was removed by the fungus from the medium but which could not be accounted for by extraction procedures. Possibly the non-retrievable portion is the amount of PCNB that actually is needed to cause inhibition (1:30,000 dry weight). The fact that the nitro group is not detectable in the missing PCNB, clearly leaves open the possibility that the nitro group is entering into the inhibitory reaction.

The accuracy which has been achieved in the dosage-response experiments allows some general conclusions on the mode of action question. Horsfall (14) discusses the linear versus the non-linear dosage-response curve and concludes that in a case such as the tetramethyl thiuram disulfide (TMTD) curve the non-linear character indicates a double mode of action. Sijpesteijn and van der Kerk (31) conclude that the two modes are oxidation and chelation. Each mode has its own minimum concentration where spore germination is affected. Since these differ from each other, the point where the action of the second mode begins results in a change in the slope of the dosage-response curve. If this reasoning is acceptable, then the linear PCNB response indicates a single mode of action against R. solani.

Eckert (8) tested the toxicities of a homologous series of chloronitrobenzenes which varied only by the number and position of the Cl group. He concluded that each of the

series acted at a common site, because all of the compounds in his series had a nearly equal relationship between their water solubility and the dose required for an ED 50. He found that 2, 3, 4, 5-tetrachloronitrobenzene was inhibitory to P. ultimum. If it is true that all the compounds in his series act in the same manner then the tetrachloro compound must be able to permeate the P. ultimum membrane better than PCNB. This would seem to be in accord with the data presented here showing that P. ultimum does not absorb as much PCNB as the susceptible R. solani.

If PCNB affected protein structure in general it might be expected to affect the enzymes involved in respiration. The oxygen uptake studies with R. solani and PCNB show that oxygen is neither being utilized at a faster or a slower rate than normal and consequently the Kreb cycle as well as other systems of oxidation are not the sites of action of PCNB.

Several antibiotics are now known to inhibit protein synthesis at one site or another (9). This possibility with PCNB was explored by the use of C^{14} labelled amino acids. PCNB apparently does not affect protein synthesis in short term experiments, since treated and control mycelium incorporated equal amounts of labelled amino acid into a TCA insoluble fraction. After 4 hours exposure some carbon atoms of the amino acid were being oxidized and given off as labelled CO_2, indicating that the amino acid was being

metabolized in ways other than in protein synthesis. From this I would conclude that the slight difference in amino acid incorporation at 8 hours was due to inhibition of another system or systems linked in some way with protein synthesis.

SUMMARY

Rhizoctonia solani was inhibited by 1.0 ppm pentachloronitrobenzene (PCNB) while *Pythium ultimum* was not affected by several times this concentration. PCNB was shown to inhibit growth of *R. solani* in culture when added to the media in acetone solution or when added as a gas. Soil placed adjacent to PCNB crystals appeared to absorb the chemical and retard its action against fungi in the vicinity. *R. solani* absorbs more PCNB from the media than *P. ultimum* absorbs. Some of the PCNB absorbed by *R. solani* is unextractable and may be reacting with the mycelium substance. PCNB in sub-inhibitory amounts causes derangement of the hyphal cells and premature pigment formation but does not affect respiration or protein synthesis on a short term basis.

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