ADAPTATION OF FUNGI TO FUNGICIDES

A thesis presented by

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ABSTRACT

When Botrytis allii was grown in the presence of vapour of pentachloronitrobenzene, or isomers of tetrachloronitrobenzene, slow growing mycelium was produced composed of swollen distorted hyphae. Sporulation was reduced or suppressed. Sooner or later variants appeared which had the following characteristics:

(a) in absence of fungicide, hyphae, sporulation, and growth rate very similar to those of parent,

(b) in presence of fungicide, spores germinated more rapidly than those of parent in the same conditions. Growth rate of hyphae from mycelial discs was little affected, and the hyphae were normal in appearance.

Resistant variants grown in liquid medium in the presence of the fungicides utilized carbohydrate more efficiently than did the parent in the same conditions.

Experiments with these variants produced evidence of a common adaptation mechanism as well as for a more specific one.

These variants were used in conjunction with other halogenated nitrobenzenes, with 2:3:5:6 tetrachloroaniline, and with 2,6 dichloro-4-nitroaniline. Resistance to tetrachloronitrobenzenes always conferred some resistance to the other compounds. The 2:3:4:6 tetrachloronitrobenzene resistant strain grew slowly in concentrations of 2,4- and 2,3 dichloronitrobenzene which killed the parent isolate.

The resistant strains showed a graded tolerance to the halogen series of 2,5 substituted nitrobenzenes in the order I > Br > Cl.

The growth of the 2:3:4:6 tetrachloronitrobenzene resistant strain was unaffected by concentrations of benzene vapour which retarded the growth of the parent isolate.

Mycelium of resistant strains retained their resistance to pentachloronitrobenzene, and tetrachloronitrobenzenes for at least 18 months under ordinary cultural conditions in the absence of fungicides.

When Trichoderma viride was grown in the presence of N-trichloromethylmercapto-4-cyclohexene 1,2-dicarboximide (captan) dispersed in agar, growth was abnormal, sporulation was retarded and the growth rate was reduced.

Captan "trained" mycelium grew more rapidly than the parent isolate on agar containing captan, or certain captan analogues, but lost this ability after 2 months sub-culture in the absence of fungicide.

CONTENTS

I INTRODUCTION

The extensive use of fungicides, insecticides, and nematocides in agriculture reflects attempts at control and therapy in plant pathology. A serious threat to these efforts appears in the development of resistance to insecticides, and perhaps a greater potential threat lies in the acquired resistance to fungicides under field conditions. Laboratory studies have shown that resistant strains of many fungi are produced by subjection to sub-lethal doses of toxic substances. This relationship between organisms and toxic substances is manifested in several fields of biology. The phenomenon of acquired resistance may be incorporated in the term adaptation Stanier (1953),

> "In its broadest usage it describes the totality of the various processes of change which confer on an organism fitness to its environment".

The following laboratory studies were designed to investigate the nature and extent of adaptation in Botrytis allii to chlorinated nitrobenzenes and related compounds, and in Trichoderma viride to captan and its analogues.

II REVIEW OF LITERATURE

1. Adaptation

Certain fungicides introduced in the nineteenth century, e.g. sulphur and Bordeaux mixture have been used extensively against powdery and downy mildews, but the literature contains few records of the appearance of strains resistant to their action. Horsfall (1956), stated that to achieve the same control of Phytophthora infestans on potato along the Atlantic seaboard of America with Bordeaux mixture required a threefold increase in spraying compared with that sixty years ago. This might mean the existence of strains resistant to copper. The spore germination studies of Taylor (1953), have shown that spores of Physalospora obtusa from orchards continually sprayed with Bordeaux mixture are more resistant to copper than those from unsprayed orchards. A further instance of acquired resistance is recorded by Littauer and Gutter (1953), who reported that control of Diplodia natalensis on oranges by diphenyl is now adversely affected by diphenyl resistance. Brown (1953), found that control of Botrytis cinerea on lettuce could be obtained by dusting seedlings with pentachloronitrobenzene (PCNB), before planting. Previous to 1954 this compound and the related 2:3:5:6 tetrachloronitrobenzene (TCNB), were the only recommended chemicals for control of Botrytis cinerea under field conditions. A recent paper by Way and Keyworth (1959), on this disease mentions that advisory officers and growers find that the control given by these substances is inconsistent and often

inadequate. It is possible that this is because under some field conditions B.cinerea acquires resistance to these compounds. There are also a number of laboratory studies on adaptation. Mader and Schneider (1947), "trained" cultures of Sclerotinia fructicola to tolerate increased concentrations of copper sulphate in agar media. The variants produced differed in their ability to rot fruit. When transferred to fungicide free media, the tolerance was retained by some variants, but not by others. Jurkowska (1952) investigated the adaptability of Aspergillus niger to copper sulphate. After several generations a resistant strain was obtained which tolerated concentrations of copper sulphate in agar media which were toxic to the parent; this strain was also resistant to zinc and manganese salts. After fifteen transfers on copper free media the resistance was lost. Greathouse et al., (1954), obtained a copper oxinate resistant strain of Aspergillus niger. The susceptibility of this strain to oxine indicated that the resistance was to the copper moiety. Further data for cupric salts are provided by Arakatsu (1954), for yeasts, and Hirt (1949), for Poria xantha.

Stakman et al., (1946), investigated the adaptation of monosporidial lines of Ustilago zeae to arsenic. A threefold increase in tolerance for sodium arsenite was obtained after ten transfers on media containing arsenic, and the ability to grow on arsenic media of the same concentration increased with successive transfers. The resistant variants rapidly reverted when grown

on arsenic free media. Similar studies by Wilson (1947), demonstrated the development of increased resistance to sodium arsenite by Sclerotium rolfsii and S.delphinii. Gattani (1951), "trained" Alternaria spp. to more complex fungicides, "Agrosan G.N.2" (containing tolylmercury acetate), and "Arasan" (containing tetramethylthiurandisulphide) by successive transfers to increasing concentrations of the fungicides in potato dextrose agar. Parry and Wood (1959), obtained resistance to a wide variety of complex organic fungicides. Strains of Botrytis cinerea resistant to ferbam (ferric dimethyldithiocarbamate), and captan (N-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide), were obtained by "training" methods, and these resistant strains did not revert to the parent susceptibility after they had been grown on fungicide free media. It was suggested that more than one mechanism was operative in the "training"of B.cinerea to these fungicides.

Bartlett (1959), obtained strains of Penicillium roqueforti resistant to high concentrations of phenyl mercuric acetate, proflavine, brilliant green, and sodium azide. Resistance developed gradually without evidence of mutation, and most of the resistant strains were unstable and rapidly reverted when grown in drug free media. The resistance of the proflavine resistant strains was reduced under these conditions to a level greater than that of parent.

Brian (1960), investigated the effect of griseofulvin on dry weight and radial growth of surface colonies of Botrytis

allii on liquid media. It was found initially that 5 µg/ml. griseofulvin was inhibitory, but that after a three week period the growth rate increased considerably. Mycelium from these cultures used to inoculate fresh griseofulvin media did not exhibit a long lag phase. After several transfers rapid growth was obtained on media containing 20 µg./ml. griseofulvin. This adaptation was lost when mycelia were transferred to griseofulvin free media. The accumulated evidence of these workers demonstrates the evidence of two distinct behaviour patterns of resistant strains - reversion and non-reversion to parent susceptibility when grown in the absence of the fungicide or drug. There are a number of laboratory studies of adaptation to the chlorinated nitrobenzenes, particularly the three isomers of TCNB, and PCNB. Variants frequently arise in the presence of the vapour of these substances, and characteristically appear as rapidly growing, fan shaped mutants at the colony edge. These variants are almost always non-sporulating, and have been described by Roy (1947), Reavill (1950), McKee (1951), Fushtey (1953), Hewlett (1955), Brook (1952), Brook and Chesters (1957), and Parry (1957).

McKee (1955), showed that Fusarium caeruleum mutates in the presence of 2:3:5:6 TCNB and becomes resistant to it. He suggested that these mutants may be important in the practical control of potato rot induced by F.caeruleum. Parry (1957) showed that the order of toxicity with Botrytis cinerea as a test organism was 2:3:4:6>2:3:4:5>2:3:5:6>TCNB >PCNB. He found

that the 2:3:4:6 isomer was particularly active in suppressing growth, sporulation, germination, and germ tube preliferation. Variants were produced in the presence of 2:3:5:6 TCNB, 2:3:4:6 TCNB and PCNB vapour. These variants were resistant in varying degrees to each of the three substances, i.e. grew faster than parent inocula and produced a normal vegetative mycelium. The variants were unable to sporulate normally in the presence of the fungicide vapours. No variant with normal mycelial morphology was produced in the presence of 2:3:4:6 TCNB vapour, but cultures with considerably increased growth rates were produced. Each of these workers found that variants resistant to TCNB isomer, or PCNB retained their resistance over long periods, even when repeatedly sub-cultured in the absence of fungicide vapour. A characteristic effect of these substances on the test organisms was a suppression of sporulation. Steinberg (1940), observed a similar effect with certain phenanthrene derivatives on Aspergillus niger.

The implication of these experimental studies of adaptation is that a serious threat lies in the development of fungicide resistant strains under field conditions. Parry (1957) concluded from his studies, that the rapid "training" of Botrytis cinerea to the true fungicides was unlikely to be important in the field, but the fungicide stable mutations constituted an important threat.

2. Fungicides

2. 1 Chlorinated nitrobenzenes.

PCNB was developed and introduced by I.G. Farbenindustrie in the late 1930's. It was originally marketed under a number of trade names "Tritisan", a 15% dust for the seed treatment of wheat against bunt, "Brassicol", a 20% dust for use as a soil fungicide, "Folosan", "Botrilex", and "Tilcarex".

2:3:5:6 TCNB was introduced by Bayer Agricultural Ltd., as a selective fungicide, effective for the control of dry rot, Fusarium caeruleum of potato tubers. It was marketed as "Fusarex" a 3% dust, and "Folosan D B 905", a 5% dust.

Brown and Smieton (1940), used PCNB dust as a means of control for Botrytis cinerea on lettuce. They also obtained a degree of control of Plasmodiophora brassicae with PCNB on cabbage and cauliflower. Last (1952), found PCNB and 2:3:5:6 TCNB effective in controlling Botrytis disease and Rhizoctonia attack of lettuce. A related compound, trichlorodinitrobenzene, "Brassisan", was used by Brown (1935), and Smieton (1940), against Botrytis cinerea on lettuce. These compounds however have not found a wide application in agriculture. A recent introduction 2,6-dichloro-4-nitroaniline, is claimed in a Boots Advisory Leaflet to be effective in controlling Botrytis cinerea attack on lettuce. This compound is marketed as "Allisan" and produced by Boots Pure Drug Co., Ltd.

Comparatively little is known about the mode of action of these compounds. Hewlett (1955), established by paper chromatography the presence of ribose in extracts of Botrytis cinerea treated with 2:3:5:6 TCNB, suggesting that the action was presumably due to nuclear poisoning. Further evidence of nuclear disorganisation is provided by Carey and McDonough (1943), who showed that spindle formation in onion is inhibited by paradichlorobenzene. Apart from their fungicidal action these compounds independently affect the growth of the plant, the isomers of TCNB differing in their growth regulating capacity.

2. 2 Captan.

A new class of organic fungicides, arising from the reaction of perchloromethylmercaptan with the alkalimetal salts of amides and imides was introduced by the Standard Oil Development Co. in 1949, and described by Kittleson (1952). Captan was originally marketed under the trade names "SR 406", a 50% dispersible powder and "Orthocide 406". Captan has been applied to a wide variety of plant diseases, Andes and Epps (1956), found captan effective against Sclerotinia laxa on apricot, Venturia inaequalis on peach, and Physalospora obtusa on apple.

Horsfall and Rich (1951), found captan was very active

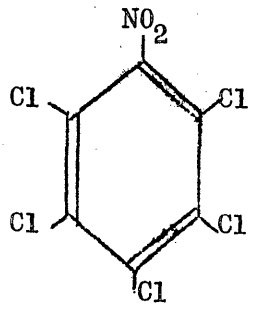
against Stemphylium sarcinaeforme and they considered it had five structural features that could be involved in its fungitoxicity; the CCl_3 group; the sulphur bridge; the diketone; the N heterocycle; and the mobile hydrogens on the carbon atoms alpha to the ketone. Hochstein and Cox (1956), studied the fungicidal action of this compound on the respiration of growing and non-growing conidia of Fusarium roseum. Conidial germination and mycelial growth was observed in the presence of captan. Their manometric studies suggested that captan inhibits growth in fungi by interfering with decarboxylation reactions requiring thiamine pyrophosphate as coenzyme. Rich (1959), studied the chemistry of the fungitoxicity of captan. Various chemicals were tested to determine their ability to antagonise the toxicity of captan in liquid media to Sclerotinia fructicola. Captan at 3×10^{-5} M completely inhibited the growth of the test organism in a liquid medium, this toxicity was reversed by 10^{-2} M l-histidine and 10^{-2} M l-cysteine. The former was effective if added 24 hours after the captan, and the latter after 6 hours but not 24. It was concluded from these observations that S.fructicola can shunt every system poisoned by captan except those needed for synthesis or utilization of histidine.

III MATERIALS AND METHODS

1. Fungicides

1.1 Halogenated nitrobenzenes

These substances are prectically insoluble in water, e.g. the tetrachloronitrobenzenes less than one part in twenty thousand, they are, however, volatile, and were primarily used in the vapour phase. PCNB and the three TCNB isomers were supplied by Boots Pure Drug Co., Ltd. The other halogenated nitrobenzenes were supplied by L. Light & Co.,Ltd.

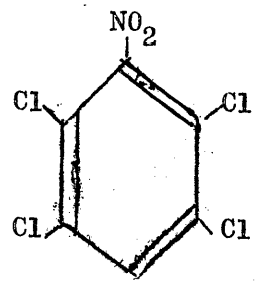


Pentachloronitrobenzene

Colourless polygonal plates.

Recrystallised from acetone

M.P. 146°C.

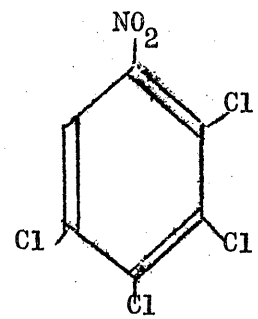


2:3:5:6 tetrachloronitrobenzene

Colourless prisms or needles.

Recrystallised from acetone.

M.P. 99°C.

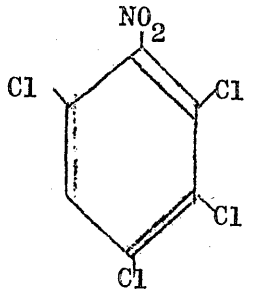


2:3:4:5 tetrachloronitrobenzene

Colourless needles or plates.

Recrystallised from acetone.

M.P. 65-65.5°C.

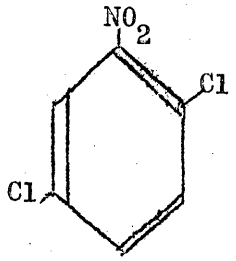


2:3:4:6 tetrachloronitrobenzene

Colourless needles.

Recrystallised from ethyl alcohol.

M.P. 39.5 - 41.0°C.

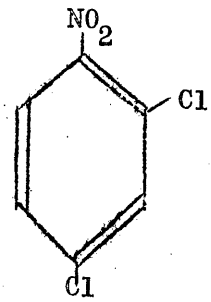


2,5 dichloronitrobenzene

Pale yellow triclinic crystals.

Recrystallised from ethyl alcohol

M.P. 54°C.

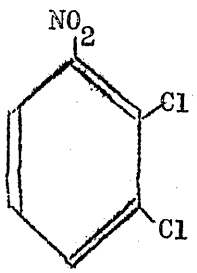


2,4 dichloronitrobenzene

Colourless needles.

Recrystallised from ethyl alcohol

M.P. 33°C.

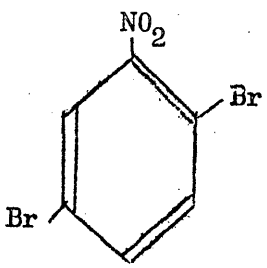


2,3 dichloronitrobenzene

Colourless needles.

Recrystallised from ethyl alcohol

M.P. 61°C.

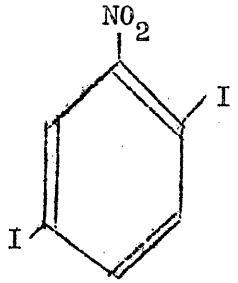


2,5 dibromonitrobenzene

Colourless needles.

Recrystallised from acetone.

M.P. 84°C.



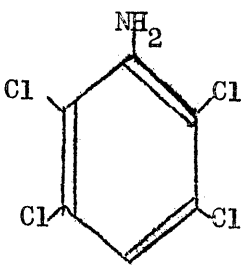
2,5 diiodonitrobenzene

Recrystallised from acetone.

M.P. 109°C.

1.2 Halogenated anilines and nitroanilines.

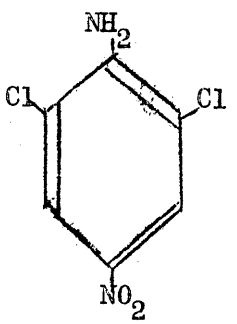
These compounds are also insoluble in water, and were used in the same way as the chlorinated nitrobenzenes. 2,6 dichloro-4-nitroaniline was supplied by Boots Pure Drug Co., Ltd. 2:3:5:6 tetrachloroaniline was prepared by reduction of 2:3:5:6 tetrachloronitrobenzene.



2:3:5:6 tetrachloroaniline

Colourless needles.

Recrystallised from petroleum ether B.P. 60-80°C.
M.P. 109-110°C.



2,6 dichloro-4-nitroaniline

Yellow needles.

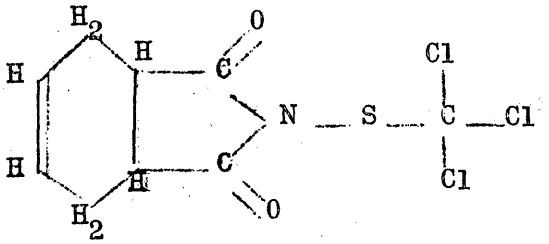
Recrystallised from acetic acid.

M.P. 194 - 195°C.

1.3 Captan and captan analogues.

These fungicides are insoluble in water. They were used dispersed in solid media. They were supplied by A.R. Kittleson of the Esso Research and Engineering Co., (formerly Standard Oil Development Co.).

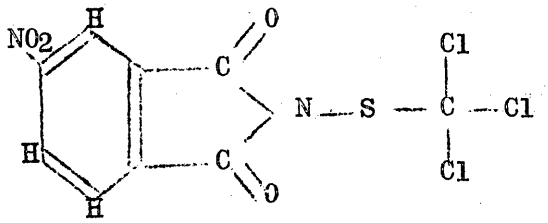
In order to exclude decomposition products the compounds were purified by recrystallisation from an appropriate solvent.



Captan

Recrystallised from acetone.

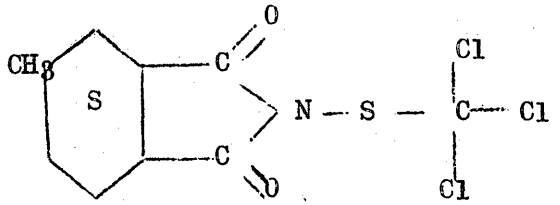
M.P. 172°C.



N-trichloromethylmercapto-4-nitrophthalimide

Recrystallised from benzene

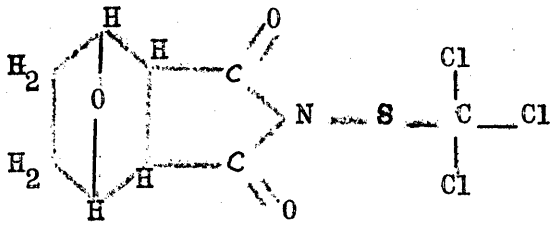
M.P. 147 - 148°C.



N-trichloromethylmercapto-4-methyl hexahydrophthalimide

Recrystallised from n-hexane.

M.P. 128 - 129°C.



N-trichloromethylmercapto-3,6-endoxohexahydrophthalimide.
Recrystallised from benzene.
M.P. 158 - 159°C.

2. Culture media and vessels.

Stock and experimental cultures were grown on glucose peptone agar media in 9.0 cm. Petri dishes. Cultures of Botrytis cinerea and Trichoderma viride for spore germination tests were grown on glucose peptone agar slants in 30 ml. screw capped tubes. Reference cultures were first grown on glucose peptone agar slants in 6 x $\frac{3}{4}$" test tubes, and then covered with sterile liquid paraffin. Liquid cultures were grown in 50 ml. glucose peptone solution in 250 ml. conical flasks on a rotary shaker.

Glucose peptone solution.

KH_2PO_4	0.1%	Peptone	0.2%
$MgSO_4.7H_2O$	0.05%	Glucose	1.0%
$FeSO_4.7H_2O$	0.001%		

Glucose peptone agar.

As for glucose peptone solution, with 2% agar.

Culture vessels other than Petri dishes, and media were sterilised by autoclaving at 15 lbs/in.^{2} for 20 minutes.

Spore germination was examined in films of spore suspensions

on 1.0 cm. diameter 3% agar discs containing 2% sucrose. The agar discs were placed on a 7.5 x 2.5 cm. glass slide and mounted on bent glass rods over 10 ml. of distilled water in 9.0 cm. Petri dishes.

3. Fungi.

Trichoderma viride and Botrytis allii were used as test fungi.

T.viride ..... a sporulating strain isolated from soil.

B. allii ..... a sporulating strain from onion.

The two strains were obtained from the collection in the Plant Pathology Department at Imperial College.

4. Inoculation and incubation.

The inocula consisted either of non-sporulating agar discs, or of spores. The non-sporulating discs were cut from the edges of colonies on agar with a steel disc cutter internal diameter 0.5 cm. The colonies of B.allii were grown at 15°C. for 72 hours so that they were not sporulating when the discs were removed at a radius of 2 cm. from the centre of the colonies. The discs were inoculated with their mycelial surface on the agar.

Spores were washed from the surface of agar slopes with sterile water. These suspensions were passed through two layers of muslin, and then washed by three successive centrifugations at 5,000 - 6,000 r.p.m. for one minute. The spores were suspended in distilled water, and the final concentration of spores adjusted to

500 / 0.02 ml. Nine replicates of each treatment were used.

Cultures on agar media were incubated at 20 ± 2°C., spore germination discs, and liquid cultures at 23°C. Stock cultures other than those grown for disc inocula were incubated at room temperature. Petri dish cultures in the presence of vapours of halogenated nitrobenzenes, anilines, and nitroanilines were kept in separate sets of ten in metal boxes. The Petri dish sets were sealed with "Selotape" to reduce losses through volatilisation.

5. Measurement of Linear growth on agar plates.

Growth along two diameters at right angles to each other was taken and the average of twenty such measurements from ten replicates was recorded.

6. Spore germination assessment.

It was taken that spores had germinated when the length of the germ tube was equal to, or greater than, that of half the short axis of the spore. The behaviour of spores on each agar disc was recorded.

Details of more specialised techniques are given in the appropriate sections of the text.

IV. EXPERIMENTAL RESULTS

1.0 CHLORINATED NITROBENZENES.

Halogenated nitrobenzenes were regarded as insoluble in water, but their volatility enabled them to be used in the vapour phase. This technique was employed by Reavill (1950), Fushtey (1953), Hewlett (1955), and Parry (1957). One ml. acetone solutions of known concentration were allowed to evaporate under sterile conditions, on the inside of the lids of 9.0 cm. Petri dishes. The lids were cooled before adding the acetone because this produced a more even film of fungicide. After evaporation of the fungicide (10 - 15 minutes), the lids were replaced and stored at room temperature for 24 hours, to enzure complete evaporation of the acetone. These lids were transferred to agar plates immediately after these had been inoculated with mycelial discs.

2.0 Effect of PCNB and TCNB on radial growth, hyphal morphology, and sporulation of Botrytis spp.

The effect of these chlorinated nitrobenzenes on radial growth, hyphal morphology, and sporulation of Botrytis spp. was investigated by Hewlett (1955), and Parry (1957). The work of Hewlett (1955), was restricted to PCNB and 2:3:5:6 TCNB. Parry (1957), used PCNB and the three isomers of TCNB. Their results showed that when colonies were grown in the vapour of the fungicides, more than one type of adaptation occurred. One gave rise to

rapid growing variants, "resistant saltants", with normal hyphal morphology, and the other to distorted hyphal forms similar to those produced by the parent strain on introduction to the vapour, but with a higher growth rate. Parry (1957), was unable to obtain a resistant saltant of Botrytis cinerea of B.allii with normal hyphal morphology after three months treatment in the presence of 2:3:4:6 TCNB, nor was any variant of B.cinerea, or B.allii obtained which was able to sporulate in the presence of the fungicides. In contrast, Brook and Chesters (1957), obtained an isolate of B.cinerea which sporulated in the presence of 2:3:4:5 TCNB.

In order to examine the effect of the halogenated nitrobenzenes as a general group of compounds to both types of adaptation, "resistant saltants" and hyphal variants to PCNB and the TCNB isomers were obtained. The production of these resistant strains of B.allii with an analysis of their morphological characters therefore introduces the experimental section of this thesis.

Table 1.

Effect of 10 mg. and 1 mg. PCNB on the linear growth of Botrytis allii

Time after inoculation (hours)	Mean colony diameter (cm.) Control	10mg.	1mg.	Percentage inhibition of growth 10mg.	1mg.
24	1,5	0.6	0.7	92	80
48	2.9 S	0.8	1.1	83	75
72	4.6	1.4 S	1.8 S	63	58
96	6.5	2.2	2.6	63	58
120	6.4	2.8 M	3.3	61	60
168	-	4.2	4.9	-	-
217	-	6.1	6.6	-	-

Mean increase of colony diameter cm./day

Control	10 mg.	1 mg.
1.59	0.62	0.68

S = Start of sporulation

M = First appearance of clearly defined saltant.

The percentage inhibition of growth was calculated from the formula $I = \left(\frac{C - T}{C} \right) 100$. I represents the percentage inhibition of growth, T the daily increase in diameter of treated plates, and C the daily increase in diameter of untreated plates.

2. 1 PCNB

The linear growth of Botrytis allii in the presence of this fungicide is recorded in Table 1. The results obtained are similar to those of Hewlett (1955). The hyphae produced in the first 2-3 days growth, were gnarled, swollen and more deeply pigmented than the pale brown of the parent hyphae. After this initial period the effects on morphology of the hyphae were less pronounced. The outlines of the colonies became lobed and irregular Clearly defined sectors of resistant saltants appeared in 3 out of 10 replicates in the 10 mg. series after 5 days, but saltants were not clearly defined in the 1 mg. replicates. Sporulation was delayed but not suppressed; it was sparse in the 10 mg. replicates, but relatively unaffected in the 1 mg. replicates, Sporulation was not recorded by Hewlett (1955), or Parry (1957), for Botrytis allii at these levels. A lag period, i.e. period before growth was seen, was not evident, but the initial period of growth was characterized by an increasing linear growth rate. The resistant saltants were pale creamy brown in colour, the mycelium of the colonies was closely adpressed to the agar, and the hyphae were not abnormal.

Table 2.

Effect of 10 mg., 1 mg. and 0.1 mg. of 2:3:4:5 TCNB on the linear growth of Botrytis allii

Time after inoculation (hours)	Mean colony diameter (cm.)				Percentage inhibition of growth		
	Control	10 mg.	1 mg.	0.1 mg.	10 mg.	1 mg.	0.1 mg.
24	1.4	0.5	0.5+	0.8	100	100	70
48	2.8 S	0.5	0.5+	1.2 S	100	100	70
72	4.8	0.5+	0.5+	1.9	100	100	61
96	6.9	0.5+	0.5+	2.7	100	100	64
120	8.8	0.5+	0.6	3.8	100	96	61
144	C	0.5+	0.7	5.1	-	-	-
192		0.6	1.0	C	-	-	-
240		0.8 M	1.4 SM		-	-	-
267		1.0	1.7		-	-	-
358		1.8	3.1		-	-	-

Mean increase of colony diameter in cm./day

Control	10 mg.	1 mg.	0.1 mg.
1.65	0.13	0.22	0.77

C = Mycelia covering plates

S = Start of sporulation

M = First appearance of clearly defined saltant

\+ = Slight growth, but less than 0.05 cm.

2. 2 2:3:4:5 TCNB

The linear growth of Botrytis allii in the presence of this fungicide is recorded in Table 2. This compound exerted a greater effect upon the linear growth rate, and hyphal morphology than PCNB. A lag phase was evident for 3 days in the 10 mg. replicates. Very little growth was made in the 10 mg. and 1 mg. treatments during the first 5 days.

The mycelium was pigmented a dark brown, and composed of short swollen cells of irregular outline forming compact nodular masses around the inoculum. Resistant saltants appeared as clearly defined sectors in the 10 mg. and 1 mg. treatments. 1-3 saltants appeared in 4 out of 10 colonies after 10 days in the 10 mg. treatment, and 1-2, in 5 out of 10 colonies after 10 days in the 1 mg. treatment. Sporulation was completely suppressed during the course of the exprinent in the 10 mg. treatment, but appeared after 10 days in the 1 mg. treatment and after 2 days in the 0.1 mg. treatment. Sporulation was recorded by Brook and Chesters (1957), for Botrytis cinerea in the presence of 10 mg. 2:3:4:5 TCNB, but not by Parry (1957), for B.cinerea or B.allii at this concentration.

Table 3.

Effect of 10 mg., 1 mg. and 0.1 mg. 2:3:5:6 TCNB on the linear growth of Botrytis allii

Time after inoculation (hours)	Mean colony diameter (cm.)				Percentage inhibition of growth		
	Control	10 mg.	1 mg.	0.1 mg.	10 mg.	1 mg.	0.1 mg.
24	1.4	0.5	0.5	0.5+	100	100	100
48	2.9 S	0.5	0.5+	0.5+	100	100	100
72	4.9	0.5	0.5+	0.5+	100	100	100
96	7.0	0.5+	0.5+	0.6	100	100	95
120	8.9	0.5+	0.6	0.7	100	95	95
144	C	0.5+	0.7	0.8	-	-	-
192		0.5+	0.7	0.9	-	-	-
240		0.6	0.8	1.2	-	-	-
268		0.6	0.8	1.5	-	-	-
316		0.7	1.0 M	4.3	-	-	-
364		0.9	1.1	6.5	-	-	-
485		1.4 M	1.7	C	-	-	-

Mean increase of colony diameter in cm./day

Control	10 mg.	1 mg.	0.1 mg.
1.69	0.06	0.07	0.43

C = Mycelia covering plates

S = Start of sporulation

\+ = Slight growth, but less than 0.05 cm.

Table 4.

Effect of 10 mg., 1 mg. and 0.1 mg. 2:3:4:6 TCNB on the linear growth of Botrytis allii

Time after inoculation (hours)	Mean colony diameter (cm.)				Percentage inhibition of growth		
	Control	10 mg.	1 mg.	0.1 mg.	10 mg.	1 mg.	0.1 mg.
24	1.4	0.5	0.5	0.5+	100	100	100
48	2.8 S	0.5	0.5+	0.5+	100	100	100
72	4.8	0.5	0.5+	0.6	100	100	95
96	6.7	0.5+	0.5+	0.6	100	100	95
120	C	0.5+	0.5+	0.7	-	-	-
144		0.5+	0.6	0.8	-	-	-
168		0.5+	0.6	0.9	-	-	-
216		0.5+	0.6	1.1	-	-	-
264		0.6	0.6	1.4 M	-	-	-
360		0.7	0.8 M	1.7	-	-	-
456		1.0	1.1		-	-	-
552		1.3 M	1.5		-	-	-
648		1.7	2.2		-	-	-

Mean increase of colony diameter in cm./day

Control	10 mg.	1 mg.	0.1 mg.
1.69	0.06	0.07	0.10

C = Mycelia covering plates S = Start of sporulation

M = First appearance of clearly defined saltant

+ = Slight growth, but less than 0.05 cm.

2. 3 2:3:5:6 TCNB

The linear growth of Botrytis allii in the presence of this fungicide is recorded in Table 3. The response to this fungicide was similar to that described by Hewlett (1955), and Parry (1957). The lag phase was 4 days in the 10 mg. replicates and 2 days in the 1 mg. replicates. In the 1 mg. replicates, 1-2 resistant saltants appeared in 4 out of 10 replicates after 13-20 days. The first hyphae formed were modified in the manner described for 2:3:4:5 TCNB. Sporulation was suppressed during the course of the experiment at all three concentrations.

2. 4 2:3:4:6 TCNB

The linear growth of Botrytis allii in the presence of this fungicide is recorded in Table 4. This compound exerted the most pronounced effect on growth and hyphal morphology of the four substances used. In contrast to Parry (1957), who was unable to obtain resistant saltants, this isomer stimulated the production of a large number of resistant saltants. In the 10 mg. replicates, 1-2 resistant saltants appeared in 4 out of ten replicates after 23-27 days, and in the 1 mg. replicates, 1-3 resistant saltants appeared in 7 out of 10 replicates after 15-27 days. Sporulation was not evident at the three concentrations used.

Summary of results in Tables 1 to 4.

The general pattern of response of Botrytis allii to PCNB and the three isomers of TCNB was basically similar. In the

initial period of growth from the inoculum, gnarled distorted cells were produced which were more deeply pigmented than those of the parent in the absence of the fungicides. These cells tended to form nodular masses of hyphae protruding above the surface of the agar. Sub-surface growth was always less irregular, and the cells were less nodulose. After this initial period, the hyphae formed were less distorted, and swollen. Sporulation was completely suppressed with 2:3:5:6 and 2:3:4:6 TCNB, diminished with 2:3:4:5 TCNB, whereas PCNB had little effect. Resistant saltants were produced after varying periods of treatment. These saltant sectors were initially white but gradually became pale creamy brown. The mycelium was always closely adpressed to the agar, and was similar to that produced by the parent in the absence of the fungicides. The initial period of growth was characterized by an increasing linear growth rate, which approached, but did not reach that of the parent in the absence of fungicides. This suggests that some measure of resistance had been built up by the non-saltant mycelium.

These experiments were repeated using the original Petri dish lids with new cultures. The same results were obtained with original 10 mg., and 1 mg. lids. The 0.1 mg. lids were not so effective in retarding linear growth, as the original lids. This was probably due to volatilisation of the fungicide.

2. 5 Effect of PCNB and TCNB on linear growth of growing cultures of Botrytis allii.

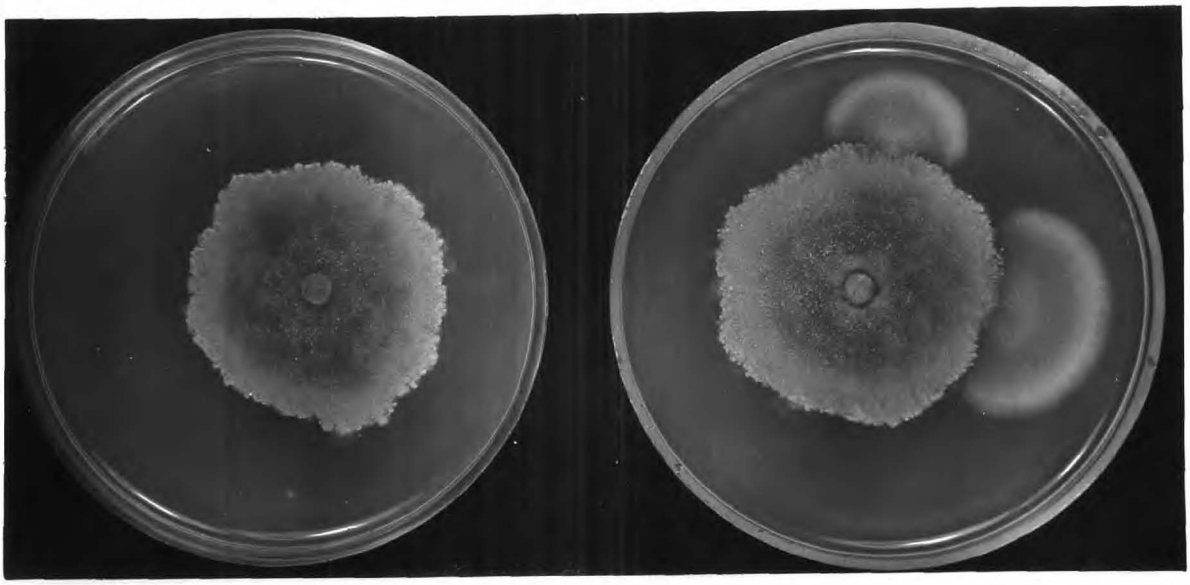
In the previous experiments, recorded in Tables 1-4, the fungicide was introduced into the environment immediately after inoculation.

The effects of the fungicides on established colonies were now studied. The Petri dish lids of colonies of approximately 2, 3, 4 and 5 cm. diameter originating from a non sporulating 0.5 cm. disc were replaced with lids containing 10 mg. of the fungicides. The results obtained are recorded in Table 5.

Table 5.

Effect of PCNB and TCNB on linear growth of growing cultures of Botrytis allii.

Treatment	Lag phase (hours)				Time for appearance of first resistant saltant (days)			
	Colony diameter in cm.							
	2	3	4	5	2	3	4	5
PCNB	-	-	-	-	No clearly defined resistant saltants			
2:3:4:5 TCNB	-	-	-	-	5	5	6	5
2:3:5:6 TCNB	24	12	12	12	8	5	7	5
2:3:4:6 TCNB	72	72	48	48	15	11	11	13



A. B.

Plate 1. Effect of introducing 10 mg. 2:3:4:5 TCNB in growing cultures of Botrytis allii.

Explanation of Plate 1.

The plate shows two colonies A, and B, after 16 days growth in the presence of 10 mg. 2:3:4:5 TCNB. The fungicide was introduced when the diameter of the colonies was approximately 4.0 cm. Subsequent growth was made by non-saltant mycelium in Petri dish A, and non-saltant, and saltant mycelium in Petri dish B. The irregular wavy outline of the colonies was characteristic of all treatments. Petri dish B shows two non-sporulating saltant sectors which arose after 8 days. The pale areas at the edge of the saltant sectors were due to non-pigmented hyphae.

The results recorded in Table 5 show that except in the PCNB cultures, where no clearly defined resistant saltants were seen, the lag phase and time for appearance of resistant saltants compared with cultures where the fungicide was introduced immediately after inoculation, were reduced. The subsequent growth rate and morphology of saltant and non-saltant hyphae in all treatments were similar to those found in the experiments recorded in Tables 1-4. Sporulation stopped on addition of the fungicides, but was resumed in the PCNB replicates after 48-72 hours.

2.6 Germination of spores of Botrytis allii in the presence of PCNB and TCNB.

The spore germination of B.allii in the presence of PCNB or TCNB was investigated on 1.0 cm. diameter, 3% agar discs containing 2% sucrose. Petri dishes with lids containing 10 mg. PCNB or TCNB were used as germination chambers. The results obtained were based on 200-400 spores from each of 9 replicates for each treatment. These results are recorded in Table 6.

Table 6.

Germination of spores of B.allii in presence of PCNB or TCNB.

Time after inoculation (hours)	Percentage germination				
			TCNB		
	Control	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
4	96	88	2	56	0
8	98	95	12	92	0
12	100	100	56	100	7
16	100	100	67	100	24
24	*	*	94	100	47
72	*	*	100	*	95
118	*	*	*	*	100

* = Individual germ tubes indistinguishable.

The results recorded in Table 6 show that the control spores had all germinated after 12 hours, and after 24 hours the germ tubes had formed a rudimentary mycelium in which the individual germ tubes were indistinguishable. The spores in the presence of PCNB germinated as rapidly as the control, and the germ tubes produced were not morphologically different. The TCNB isomers retarded germination and growth of germ tubes. These fungicides also affected the appearance of the germ tubes which were short and bulbous with frequent branching; 1-3 germ tubes were produced in all treatments. Spore viability was not reduced. Table 6 shows that TCNB reduced the rate of germination in the order, 2:3:4:6> 2:3:4:5> 2:3:5:6 TCNB; 2:3:4:6 TCNB also produced the greatest effect on germ tube morphology and subsequent hyphal growth. The behaviour of B.allii spores in the presence of PCNB or TCNB was identical to that reported by Hewlett (1955), for Botrytis cinerea. Similar results were obtained with two different batches of spores.

2.7 Linear growth of PCNB and TCNB resistant saltants in absence of fungicides.

Resistant saltants arising on plates exposed to the vapours of PCNB and TCNB were grown in the absence of these vapours. After 5 days 0.5 cm. diameter discs were transferred to a further set of Petri dishes. The results obtained are recorded in Tables 7-10.

Table 7.

Linear growth of PCNB resistant saltants in absence of fungicide.

Time after inoculation (hours)	Mean colony diameter (cm.) 1	2*	3	4	5	Mean
24	1.2	1.3	1.6	1.5	1.4	1.4
48	2.2	2.6	2.7	2.8	2.6	2.6
72	4.1	4.3	4.3	4.7	4.5	4.4
96	5.9	6.3	6.6	6.6	6.4	6.3
120	7.7	8.3	8.4	8.5	8.2	8.2

Mean increase of colony diameter in cm,/day

1.6

* = Resistant saltant used in subsequent experiments.

Table 8.

Linear growth of 2:3:4:5 TCNB resistant saltants in absence of fungicide.

Time after inoculation (hours)	Mean colony diameter (cm.)					Mean
	1	2*	3	4	5	
24	1.6	1.4	1.5	1.4	1.2	1.4
48	3.0	2.8	3.0	2.8	2.3	2.8
72	4.8	4.6	4.6	4.7	4.1	4.6
96	6.9	6.6	6.7	6.6	6.1	6.6
120	C	8.7	8.9	8.4	7.8	8.5

Mean increase of colony diameter in cm./day

1.7

* = Resistant saltant used in subsequent experiments.

C = Mycelium covering plate.

Table 9.

Linear growth of 2:3:5:6 TCNB resistant saltants in absence of fungicide.

Time after inoculation (hours)	Mean colony diameter (cm.)					Mean
	1	2	3	4*	5	
24	2.0	1.8	1.5	1.5	1.2	1.6
48	3.3	3.3	2.7	2.7	2.5	2.9
72	5.0	4.8	4.3	4.4	3.9	4.5
96	6.8	6.7	6.4	6.5	5.9	6.5
120	C	8.7	8.3	8.5	7.7	8.3

Mean increase of colony diameter in cm./day

1.7

* = Resistant saltant used in subsequent experiments.

C = Mycelium covering plate.

Table 10.

Linear growth of 2:3:4:6 TCNB resistant saltants in absence of fungicide.

Time after inoculation (hours)	Mean colony diameter (cm.)					Mean
	1	2	3	4	5*	
24	1.5	1.3	1.3	1.0	1.2	1.3
48	2.5	2.2	2.4	2.1	2.2	2.3
72	3.9	3.5	3.8	3.2	3.5	3.6
96	5.5	4.9	5.2	4.8	4.8	5.0
120	6.8	6.2	6.7	5.6	5.9	6.2
144	8.3	7.3	8.0	6.9	7.0	7.1
168	C	8.7	C	8.3	8.5	8.5

Mean increase of colony diameter in cm./day

1.2

* = Resistant saltant used in subsequent experiments.

C = Mycelia covering plates.

The results recorded in Tables 7-10 show that the rate of linear growth of the resistant saltants, with the exception of the 2:3:4:6 TCNB resistant saltants was similar. These results were obtained with 20 saltants, 5 from PCNB treatment, and 5 from each of the TCNB treatments. The mean increase in colony diameter in cm./day of the 2:3:4:6 TCNB resistant saltants was always less than that of the parent and other resistant saltants. The 2:3:4:6 TCNB resistant saltants took 6-7 days to reach a diameter of 8.5 cm. The parent and other resistant saltants took 5-6 days to reach this diameter. Hyphal morphology and sporulation were identical with that of the parent in the absence of fungicide.

The resistant saltants marked with an asterisk in Tables 7-10 were subcultured and used in subsequent experiments with halogenated nitrobenzenes, 2,6 dichloro-4-nitroaniline, 2:3:5:6 tetrachloroaniline and benzene. They were regarded as representative examples of resistant saltants arising under the various treatments. These resistant saltants are subsequently referred to in the text as resistant strains, e.g.

PCNB resistant strain

2:3:4:5 TCNB resistant strain

2:3:5:6 TCNB resistant strain

2:3:4:6 TCNB resistant strain

2.8 Linear growth of parent and resistant strains of Botrytis allii in presence of PCNB or TCNB isomers.

The resistant strains were grown in the presence of 10mg. PCNB, or TCNB isomer. The linear growth of these resistant strains is recorded in Tables 11 - 14.

Table 11.

Linear growth of parent and resistant strains of B.allii in the presence of 10 mg. PCNB.

Time after inoculation (hours)	Mean colony diameter (cm.) Parent	Resistant strains PCNB	2:3:4:5	2:3:5:6	2:3:4:6
24	0.6	0.8	1.1	0.9	1.2
48	0.9	1.2	2.2	1.9	2.1 S
72	1.4 S	2.1 S	3.2 S	2.5 S	3.3
96	2.3	3.2	4.0	3.3	4.6
120	2.8 M	4.2	5.7	4.4	6.1
168	4.5	5.7	7.4	5.8	7.8
216	6.3	7.8	C	8.0	C

Mean increase in colony diameter cm./day

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.6	0.8	1.0	0.8	1.0

S = Start of sporulation C = Plates covered with mycelia

M = First appearance of clearly defined saltant.

Table 12.

Linear growth of parent and resistant strains of B. allii in the presence of 10 mg. 2:3:4:5 TCNB

Time after inoculation (hours)	Mean colony diameter (cm.)				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
24	0.5	0.5+	0.7	0.6	0.6
48	0.5	0.5+	1.1	0.7	0.8
72	0.5	0.5+	1.5	1.3	1.2
96	0.5+	0.6	1.9	1.8	1.6
120	0.5+	0.8	2.3	2.2	2.2
144	0.5+	1.0	2.8	2.4	2.5
168	0.6	1.5	2.9	2.7	2.9
216	0.7	2.0	3.7	3.3	3.5
312	0.9 M	3.2	5.2	4.9	5.1
360	1.3	3.8	6.1	5.7	5.7
408	1.8	4.4	7.0	6.4	6.6

Mean increase in colony diameter cm./day

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.11	0.26	0.38	0.34	0.36

+ = Slight growth, but less than 0.05cm.

M = First appearance of clearly defined saltant.

Table 13.

Linear growth of parent and resistant strains of B.allii in the presence of 10 mg. 2:3:5:6 TCNB

Time after inoculation (hours)	Mean colony diameter (cm.)				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
24	0.5	0.5+	0.6	0.8	0.7
48	0.5	0.5+	0.7	1.1	1.3
72	0.5	0.5+	1.1	2.2	2.4
96	0.5+	0.9	2.0	3.4	3.4
120	0.5+	1.3	3.0	4.2	4.6
142	0.5+	1.7	4.2	5.2	5.6
168	0.5+	2.2	5.2	6.2	6.8
216	0.5+	3.2	7.4	8.2	C
266	0.6	4.7	C	C	
360	0.9	7.8			
408	1.1 M	C			
514	1.5				

Mean increase in colony diameter cm./day

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.07	0.5	0.8	0.9	0.9

+ = Slight growth, but less than 0.05 cm.

M = First appearance of clearly defined sektant

C = Plates covered with mycelia.

Table 14.

Linear growth of parent and resistant strains of B.allii in presence of 10 mg. 2:3:4:6 TCNB

Time after inoculation (hours)	Mean colony diameter (cm.)				
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
24	0.5	0.5+	0.5+	0.5+	0.5+
48	0.5	0.5+	0.7	0.6	0.9
120	0.5+	0.6	1.3	1.1	2.0
168	0.5+	0.8	1.7	1.5	2.6
192	0.5+	0.8	1.8	1.7	3.0
240	0.6	1.1	2.2	2.1	3.7
336	0.6	1.5	3.1	3.1	5.3
432	0.9	1.9	3.8	4.0	6.9
480	1.1	2.2	4.1	4.4	7.9
528	1.3 M	2.4	4.4	4.9	8.7

Mean increase of colony diameter in cm./day

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.05	0.09	0.15	0.20	0.37

+ = Slight growth, but less than 0.05 cm.

M = First appearance of clearly defined saltant.

PCNB 10 mg.

The growth of the parent and resistant strains in the presence of PCNB is recorded in Table 11. This table shows that each of the resistant strains grew more quickly than the parent, and the 2:3:4:6 and 2:3:4:5 TCNB resistant strains grew faster than the PCNB resistant strain. Hyphae of the resistant strains were similar in appearance to those of cultures grown in the absence of fungicide. All replicates sporulated after 48 - 72 hours. Erect conidiophores and normal conidia were formed. No saltant sectors appeared in any of the Petri dishes of the resistant strains.

2:3:4:5 TCNB 10 mg.

The growth of the parent and resistant strains in the presence of 10 mg. 2:3:4:5 TCNB is recorded in Table 12. Each of the resistant strains grew morequickly than the parent. The most resistant strain was the 2:3:4:5 TCNB resistant strain. The experiment showed that strains resistant to 2:3:4:6, and 2:3:5:6 TCNB were almost as resistant to 2:3:4:5 TCNB as the resistant strain originally produced under this regime.

2:3:5:6 TCNB 10 mg.

The growth of parent and resistant strains in the presence of 10 mg. 2:3:5:6 TCNB is recorded in Table 13. Table 13 shows that the resistant strains were far more resistant to

2:3:5:6 TCNB than the parent strain.

2:3:4:6 TCNB 10 mg.

The growth of the parent and resistant strains in the presence of 10 mg. 2:3:4:6 TCNB is recorded in Table 14. This table shows that the resistant strains grew more quickly than the parent in the presence of this isomer. The PCNB resistant strain was only slightly more resistant than the parent. The 2:3:4:6 TCNB resistant strain, was clearly more resistant than the parent, PCNB, 2:3:4:5 and 2:3:5:6 TCNB resistant strains.

Summary of results. Tables 11 - 14.

The response of the resistant strains to the vapours of PCNB and TCNB, shows that resistance originating in the presence of one of the fungicides conferred some resistance to each of the other three fungicides. The experiments showed that when B.allii was exposed to either PCNB or TCNB isomer a common adaptation mechanism conferred resistance to each of the four fungicides. The experiments also showed that with 2:3:4:6 TCNB there was some specific adaptation to this fungicide, as the 2:3:4:6 TCNB resistant strain was clearly more resistant to the vapour of this fungicide than the other resistant strains. The activity of the fungicides increased in the order PCNB, 2:3:5:6 TCNB, 2:3:4:5 TCNB, 2:3:4:6 TCNB. The resistance of the strains to the other fungicides increased in the order PCNB, 2:3:5:6 TCNB, 2:3:4:5 TCNB, 2:3:4:6 TCNB.

In the presence of PCNB, the TCNB resistant strains were as resistant as the strain originally developed in the vapour. In the presence of any TCNB isomer, the TCNB resistant strains were more resistant to these vapours than the PCNB resistant strain. In the presence of 2:3:4:5 and 2:3:5:6 TCNB, the three TCNB resistant strains showed the same degree of resistance to these vapours. In the presence of 2:3:4:6 TCNB, the 2:3:4:6 TCNB resistant strain was more resistant to the vapour of this fungicide than either the 2:3:4:5 or 2:3:5:6 TCNB resistant strains.

2.9. Growth of non saltant mycelia taken from colonies of Botrytis allii growing in presence of PCNB or TCNB isomer.

These experiments were carried out to see whether non-saltant mycelia of B.allii which had been growing in the presence of PCNB or TCNB was more resistant to these fungicides than untrained parent mycelia.

After 4 weeks growth in the presence of 10 mg. PCNB or TCNB, 2 mm. discs of non-saltant and saltant mycelia were taken from the colonies and transferred to fresh Petri dishes containing 10 mg. of PCNB or TCNB. The position of the discs with respect to the resistant saltant is shown in Fig. 1.

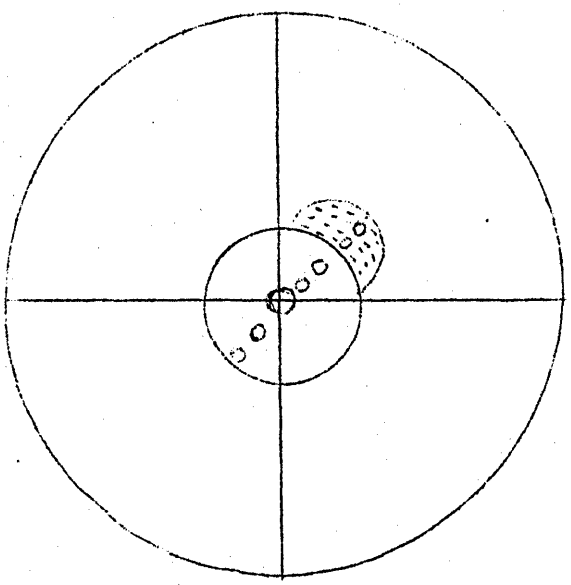


Fig. 1. Diagrammatic representation of Petri dish culture of B.allii after 4 weeks growth in 10 mg. 2:3:4:6 TCNB.

Area regarded as saltant sector.

The results of these experiments are summarized in Tables 15 and 16.

Table 15.

Growth of non-saltant discs in presence of 10 mg. PCNB or TCNB

Fungicide treatment (10 mg.)	Mean increase in colony diameter in cm./day Original treatment				
	None	PCNB	TCNB 2:3:4:5	TCNB 2:3:5:6	TCNB 2:3:4:6
Control	1.5	1.4	1.3	1.4	0.9
PCNB	0.60	0.78	0.56	0.47	0.82
2:3:4:5 TCNB	0.11	0.08	0.18	0.12	0.16
2:3:5:6 TCNB	0.06	0.06	0.17	0.20	0.14
2:3:4:6 TCNB	0.05	0.06	0.08	0.06	0.09

Table 16

Growth of saltant discs in presence of 10 mg. PCNB or TCNB

Fungicide treatment (10 mg.)	Mean increase in colony diameter in cm./day Original treatment			
	PCNB	TCNB 2:3:4:5	TCNB 2:3:5:6	TCNB 2:3:4:6
Control	1.3	1.5	1.4	1.2
PCNB	0.82	0.94	0.75	1.04
2:3:4:5 TCNB	0.22	0.43	0.26	0.34
2:3:5:6 TCNB	0.53	0.84	0.93	0.80
2:3:4:6 TCNB	0.08	0.26	0.17	0.42

The results recorded in Tables 15 and 16 were based on 10 replicates of each treatment. The values for the mean

increase in colony diameter in cm./day were evaluated after 5 days for the untreated control and PCNB treatment, and when the colonies had reached a mean diameter of 2.0 cm. for the TCNB treatments.

Tables 15 and 16 show that the level of resistance of the non-saltant mycelium was always less than that of the saltant mycelium for each treatment. Comparison with the results in Tables 1 - 4 shows that non-saltant mycelium was more resistant to the fungicides than the parent mycelium. Very little resistance had been built up by the non-saltant mycelium. This kind of result was obtained for Botrytis cinerea in the presence of PCNB and 2:3:5:6 TCNB by Hewlett (1955).

2.10. Germination of spores of parent and resistant strains of Botrytis allii in presence of PCNB or TCNB.

Spores were taken from cultures of resistant strains, after sub-culture for 2 weeks in the absence of fungicide. The number of spores was reduced to 200 per 0.02 ml. drop to prevent the mycelia produced from masking ungerminated spores. The percentage germination was determined after 24 hours, and the results recorded in Table 17.

Table 17.

Germination of spores of parent, PCNB and TCNB resistant strains in presence of PCNB or TCNB

Strain	Percentage germination				
			TCNB		
	Control	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
Parent	100	100	92	99	49
PCNB	99	99	88	98	42
2:3:4:5 TCNB	96	95	94	97	66
2:3:5:6 TCNB	93	94	93	92	78
2:3:4:6 TCNB	94	95	87	94	76

The results recorded in Table 17 show that in the absence of PCNB or TCNB, the spores of the TCNB resistant strains were less viable than those of the parent or PCNB resistant strain. This reduction in viability was small, the greatest

reduction, 7%, was shown by the spores of the 2:3:5:6 TCNB resistant strain. The percentage germination of spores of the 2:3:4:5, 2:3:5:6, and 2:3:4:6 TCNB resistant strains after 24 hours in the presence of 2:3:4:5 TCNB was higher than the percentage germination of spores of the parent or PCNB resistant strain in this fungicide. This increased germination was due to the more rapid growth of the germ tubes, as nearly all those spores regarded as ungerminated by definition in section III.6.0, showed 78-93% germination, considered as any spore showing any sign of germination. The effect of the fungicides on germ tube and subsequent hyphal morphology was the same as that found with these fungicides on parent spores.

2.11. Growth of parent and resistant strains of Botrytis allii in liquid medium.

The behaviour of 0.5 cm. non sporulating discs of the parent and resistant strains of B.allii was investigated in 50 ml. glucose peptone medium. Flasks were seeded with parent and resistant strains. Twenty replicates of each strain were prepared, and at intervals two replicates of these shake cultures were taken, and the dry weight of mycelium, and the residual glucose of the medium were found. Glucose was estimated in the following way.

Preparation and standardisation of Fehling's solution for determination of glucose

Solution A

17.320 g. of "Analar" copper culphate, $CuSO_4,5H_2O$ was dissolved in water and made up to 250 ml. in a volumetric flask.

Solution B

86.5 g. of sodium potassium tartrate, $C_4H_4O_6NaK,4H_2O$ was dissolved in 100 ml. warm water and added to 30 g. "Analar" sodium hydroxide, NaOH, dissolved in water. The resulting solution was cooled and made up to 250 ml. in a volumetric flask.

When glucose estimations were carried out, equal volumes of solution A and B were mixed thnroughly by shaking. This solution was standardised against a solution of "Analar"

anhydrous glucose of known concentration.

25 ml. of the freshly prepared and standardised Fehling's solution was pipetted into a porcelain evaporating basin, and diluted with an equal volume of distilled water. This solution was boiled very gently for 30 seconds. The filtered culture medium was slowly run into this boiling solution from a burette until the blue colour disappeared.

Estimations of glucose in the culture medium were initially carried out using 25 ml. aliquots of Fehling's solution, but this was reduced to 5 ml. aliquots during the course of the experiments.

In the following tables residual glucose values lower than 0.0010 g./ml. were not recorded.

The original glucose concentration of the glucose peptone media was 0.010 g./ml.

Determination of dry weight of mycelium

The mycelium was removed from the cultures, washed, drained, placed in pre-weighed aluminium cups, dried at 70°C. for 24 hours, and then reweighed. The "economic coefficients", recorded in Tables 20, 23, 26, 29 and 32 were obtained by dividing the dry weight of mycelium produced by the weight of glucose used.

Table 18

Dry weight of mycelium produced by parent and resistant strains of Botrytis allii in liquid medium.

Time after inoculation (days)	Dry weight of mycelium in g.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
1	0.0146	0.0093	0.0165	0.0201	0.0207
2	0.0391	0.0822	0.0520	0.0672	0.0803
3	0.0964	0.1577	0.1166	0.1399	0.1582
5	0.1714	0.2093	0.1843	0.2247	0.2397
6	0.1815	0.2252	0.1606	0.2403	0.2411
7	0.2281	0.2274	0.2401	0.2476	0.2424
8	0.2197	0.2250	0.2604	0.2265	0.2226
9	0.2246	0.2116	0.2311	0.2162	0.1987

Dry weight of inoculum (mean ten replicates) in g.

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.0011	0.0013	0.0015	0.0015	0.0012

Table 19.

Residual glucose in medium.

Time after inoculation (days)	Residual glucose in g./ml.				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
1	0.0087	0.0093	0.0086	0.0086	0.0083
2	0.0066	0.0055	0.0064	0.0061	0.0059
3	0.0045	0.0032	0.0046	0.0043	0.0029
5	0.0026	0.0015	0.0024	0.0011	-
6	0.0022	-	0.0023	-	-
7	0.0010	-	-	-	-

Table 20.

Economic coefficient.

Time after inoculation (days)	Economic coefficient				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
1	0.21	0.23	0.21	0.27	0.27
2	0.22	0.36	0.28	0.34	0.33
3	0.35	0.46	0.43	0.48	0.45
5	0.46	0.49	0.48	0.50	-
6	0.46	-	0.42	-	-

Table 18 shows the dry weight of mycelium produced, and Tables 19 and 20 the residual glucose and "economic coefficient".

In all readily observable aspects the PCNB and TCNB resistant strains behaved similarly to the parent. After 2 days a compact mycelium formed which enveloped the inoculum. Feathery mycelial strands originated from the colonies. The colour of all the mycelium produced was a pale grey brown. Small mycelial "pellets" appeared in the medium after three days. The colour of the main colony deepened with age, and finally became a golden brown after 6 days. Growth occurred at the glass liquid interface and small black sclerotia like bodies were formed. Sporulation started after 3-4 days. The maximum dry weight of mycelium was recorded at 7-8 days. Glucose was used rapidly and disappeared from the medium after 5-8 days. The value of the "economic coefficient" was between 0,21 and 0,51.

The results in Tables 18, 19 and 20 show that there was no significant difference between the parent and fungicide resistant strains.

2.12 Growth of parent and resistant strains of Botrytis allii in liquid medium containing 10 mg. PCNB or TCNB

The behaviour of 0.5 cm. non-sporulating discs of the parent and resistant strains of B.allii was investigated in liquid medium containing 10 mg. of PCNB or TCNB. Acetone solutions of PCNB or TCNB were put into sterile 250 ml. conical flasks containing 0.5 ml. water. The fungicide was precipitated from solution and after evaporation of the acetone and water (2 days), a fine film of fungicide was left on thebottom of the flask. Fifty ml. glucose peptone solution was added and the flasks seeded with parent and resistant strains. The dry weight, and residual glucose determinations of these shake cultures were carried out as described in section 2.11. The results of these experiments are recorded in Tables 21 to 32.

Table 21.

Dry weight of mycelium produced by parent and resistant strains in liquid medium containing 10 mg. PCNB.

Time after inoculation (days)	Dry weight of mycelium in g.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.0287	0.0197	0.0239	0.0260	0.0331
3	0.0598	0.0421	0.0523	0.0664	0.0686
5	0.0887	0.1443	0.1577	0.1025	0.1725
7	0.1273	0.2065	0.1831	0.1779	0.2465
9	0.2061	0.2362	0.2196	0.2426	0.2287
11	0.1966	0.2248	0.2136	0.2361	0.2351
15	0.2004	0.2011	0.2266	0.2243	0.1942

Dry weight of inoculum (mean ten replicates) in g.

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.0013	0.0014	0.0011	0.0012	0.0014

Table 22.

Residual glucose of medium 10 mg. PCNB.

Time after inoculation (days)	Residual glucose in g./ml.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.0085	0.0086	0.0080	0.0082	0.0082
3	0.0070	0.0078	0.0068	0.0060	0.0075
5	0.0055	0.0042	0.0034	0.0040	0.0031
7	0.0036	0.0013	0.0022	0.0024	-
9	0.0014	-	0.0010	-	-

Table 23.

Economic coefficient. 10 mg. PCNB

Time after inoculation (days)	Economic coefficient				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.38	0.28	0.22	0.29	0.37
3	0.39	0.37	0.32	0.33	0.55
5	0.39	0.49	0.47	0.37	0.50
7	0.48	0.47	0.47	0.47	-
9	0.48	-	0.48	-	-

10 mg. PCNB. Parent and resistant strains

Table 21 shows the dry weight of mycelium produced, and Tables 22 and 23 the residual glucose and "economic coefficient".

In the parent cultures a fine feathery mycelium, pale cream in colour enveloped the original inoculum after 2 days. After 3 to 4 days several small mycelial pellets, resembling the main colony developed in the medium. Growth and sporulation appeared at the glass liquid interface after 3 to 5 days. This mycelium was more deeply pigmented than the floating colonies. Small black sclerotia like bodies were found at the glass liquid interface after 9 days. The main colony sporulated after 6 to 8 days, somewhat later than the controls with no fungicide. Also, fewer spores were produced. The maximum dry weight of mycelium for the parent culture was recorded at 9 days.

The behaviour of the PCNB and TCNB resistant strains was similar in all instances. The maximum dry weight of mycelium was recorded at 7 to 9 days. The differences between the growth of these strains and that of the parent in the presence of PCNB was the more rapid production of mycelium and utilisation of glucose by the resistant strains. The PCNB resistant strain was not more resistant to PCNB in liquid medium than the TCNB resistant strains. The PCNB and TCNB resistant strains showed a slight advantage over the parent strain in production of dry weight and more efficient utilisation of glucose.

10 mg. 2:3:4:5 TCNB. Parent and resistant strains

Table 24 shows the dry weight of mycelium produced, and Tables 25 and 26 the residual glucose and "economic coefficient".

In the parent cultures macroscopic growth was seen after 2 days. The initial period of growth was characterized by a very slow but regular increase in mycelium. The hyphae produced, which formed a small spherical colony were slightly swollen and distorted. After 7 days, small nodules of compact mycelium composed of normal hyphae appeared and these gradually extended over the original inoculum. A number of minute mycelial pelleta were present in the medium at this stage. Growth occurred at the glass liquid interface after

Table 24

Dry weight of mycelium produced by parent and resistant strains in liquid medium containing 10 mg. 2:3:4:5 TCNB.

Time after inoculation (days)	Dry weight of mycelium in g.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.0013	0.0017	0.0033	0.0036	0.0037
3	0.0028	0.0042	0.0306	0.0285	0.0361
5	0.0058	0.0077	0.0962	0.0906	0.0886
7	0.0201	0.0411	0.1374	0.1349	0.1225
9	0.0531	0.0841	0.1518	0.1340	0.1241
11	0.0646	0.0953	0.1569	0.0966	0.1240
13	0.0774	0.1264	0.1166	0.1078	0.1001
20	0.0642	0.0982	0.0929	0.0983	0.1142

Dry weight of inoculum (mean ten replicates) in g.

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.0012	0.0015	0.0011	0.0011	0.0012

Table 25.

Residual glucose of medium. 10 mg. 2:3:4:5 TCNB

Time after inoculation (days)	Residual glucose in g./ml.				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.0100	0.0099	0.0097	0.0097	0.0095
3	0.0099	0.0095	0.0082	0.0081	0.0080
5	0.0094	0.0089	0.0060	0.0056	0.0057
7	0.0081	0.0072	0.0027	0.0033	0.0021
9	0.0046	0.0055	0.0016	0.0012	0.0011
11	0.0022	0.0034	0.0010	-	-
13	0.0016	0.0026	-	-	-

Table 26.

Economic coefficient. 10 mg. 2:3:4:5 TCNB

Time after inoculation (days)	Economic coefficient				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	-	-	0.12	0.14	0.15
3	0.26	0.11	0.33	0.29	0.36
5	0.14	0.11	0.48	0.41	0.41
7	0.20	0.29	0.38	0.40	0.31
9	0.19	0.37	0.36	0.30	0.28
11	0.16	0.29	0.34	-	-
13	0.18	0.34	-	-	-

13 days, with subsequent production of small black sclerotia like bodies and abnormal sporulation. Short aerial hyphae were formed with dilated ends, from which a number of irregular swollen cells were budded. These swollen cells were cometimes septate. They were not easily removed from the parent structure, and may represent abortive conidia. This abortive sporulation was seen on the main colonies after 15 to 20 days. The mycelia and abortive conidia were a pale creamy colour darkening slightly with age.

The behaviour and development of the PCNB resistant strain resembled that of the parent. Macroscopic growth was seen after 2 days and subsequent development was identical with that of the parent cultures. The values for the "economic coefficient" were between 0.11 and 0.37 compared with 0.14 and 0.26 for the parent strain. The maximum dry weight of mycelium recorded after 13 days was almost twice that recorded for the parent strain. The PCNB resistant strain was more resistant to 2:3:4:5 TCNB than the parent strain, and this was shown by the greater production of mycelium and the more efficient utilisation of glucose. In all other readily observable aspects there was no significant difference.

The three TCNB resistant strains behaved similarly to each other, but showed differences from the parent and PCNB resistant strain. There was no corresponding lag phase. A compact pale coloured mycelium developed which enveloped the original inoculum after 2 days. This compact mycelium was con-

posed of closely interwoven hyphae. After 3 days small mycelial pellets, identical in appearance to the main colony appeared in the medium. Growth started at the glass liquid interface after 7 to 9 days, compared with 13 days for the parent. Small black sclerotia like bodies were formed at the glass liquid interface after 9 to 13 days. Abnormal sporulation was seen on the main colonies and at the glass liquid interfaces after 10 to 12 days. The greatest dry weight of mycelium was recorded for the 2:3:4:5 TCNB resistant strain after 11 days. This value was more than twice that recorded from the parent cultures. The "economic coefficients" recorded for the TCNB resistant strains were between 0.12 and 0.48, compared with 0.14 and 0.26 for the parent strain. The highest value, 0.48, was recorded for the 2:3:4:5 TCNB resistant strain. Table 26 shows that at comparable intervals the "economic coefficient" of the TCNB resistant strains was always greater than that of the parent. Table 24 shows that the presence of 2:3:4:5 TCNB reduced the amount of mycelium produced compared with the controls in Table 18. The main difference between the behaviour of the TCNB resistant strains and that of the parent and PCNB resistant strain was the more rapid growth and development of the TCNB resistant strains without prominent hyphal abnormality, the greater production of mycelium, and the more efficient utilisation of glucose. The three TCNB resistant strains were more resistant to 2:3:4:5 TCNB than the parent or PCNB resistant strain. The 2:3:4:5 TCNB resistant strain did

not appear to have any advantage over the 2:3:5:6 TCNB or 2:3:4:6 TCNB resistant strains.

Table 27.

Dry weight of mycelium produced by parent and resistant strains in liquid medium containing 10 mg. 2:3:5:6 TCNB.

Time after inoculation (days)	Dry weight of mycelium in g.				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.0012	0.0013	0.0013	0.0029	0.0027
4	0.0017	0.0013	0.0018	0.0478	0.0526
6	0.0021	0.0035	0.0179	0.0937	0.0867
10	0.0305	0.0378	0.0718	0.1416	0.1316
12	0.0377	0.0521	0.0925	0.0822	0.1214
16	0.0653	0.0770	0.1174	0.1007	0.1202
20	0.0821	0.0956	0.1040	0.0954	0.1168
26	0.0774	0.0931	0.0882	0.0903	0.1262

Dry weight of inoculum (mean ten replicates) in g.

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.0014	0.0013	0.0014	0.0013	0.0011

Table 28.

Residual glucose of medium 10 mg. 2:3:5:6 TCNB

Time after inoculation (days)	Residual glucose in g./ml.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.0100	0.0100	0.0100	0.0099	0.0099
4	0.0100	0.0100	0.0100	0.0073	0.0066
6	0.0099	0.0093	0.0086	0.0042	0.0058
10	0.0081	0.0081	0.0042	-	0.0010
12	0.0075	0.0070	0.0020	-	-
16	0.0060	0.0051	0.0016	-	-
20	0.0022	0.0017	-	-	-

Table 29.

Economic coefficient. 10 mg. 2:3:5:6 TCNB

Time after inoculation (days)	Economic coefficient				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	-	-	-	0.30	0.24
4	-	-	-	0.36	0.31
6	0.12	0.20	0.26	0.32	0.41
10	0.30	0.39	0.24	-	0.29
12	0.29	0.42	0.29	-	-
16	0.33	0.32	0.28	-	-
20	0.21	0.23	-	-	-

10 mg. 2:3:5:6 TCNB. Parent and resistant strains

Table 27 shows the dry weight of mycelium produced, and Tables 28 and 29 the residual glucose and "economic coefficient".

With the parent strain the effect of 2:3:5:6 TCNB on growth was more pronounced than PCNB or 2:3:4:5 TCNB. Macroscopic growth was not observed for 4 to 6 days after inoculation. The hyphae produced in the initial period of growth were swollen and gnarled, and dark brown in colour. After 10 days, pale nodular areas were seen on the inoculum. This paler growth, composed of normal but compact hyphae became the predominant type. Small mycelial pellets appeared in the medium after 10 days. These pellets were composed of pale coloured closely interwoven hyphae identical to the pale coloured hyphae in the main colony. After 12 to 13 days, growth started at the glass liquid interface and subsequently small black sclerotia like bodies were formed. There was no indication of sporulation on the main or subsidiary colonies, but abortive sporulation of the type seen in the 2:3:4:5 TCNB cultures occurred at the glass liquid interface. Table 27 shows that the maximum dry weight of mycelium recorded was 0.0821 g. after 20 days. Compared with the control, Table 18, which recorded a maximum dry weight of 0.2281 g. after 7 days.

The behaviour of the PCNB resistant strain in the presence of 2:3:5:6 TCNB was similar to the parent strain.

Macroscopic growth was seen after 4 to 6 days and the maximum dry weight of mycelium was recorded after 20 days. Table 28 shows that glucose was used at approximately the same rate as for the parent. The initial growth from theinoculum was composed of pale coloured closely interwoven hyphae. The PCNB resistant strain was not more resistant to 2:3:5:6 TCNB than the parent.

With the 2:3:4:5 TCNB resistant strain macroscopic growth was seen after 4 days. During this lag phase the glucose level in the medium remained static. Table 28 shows that this was the same in the parent and PCNB resistant strain cultures. The mycelium produced was initially pale in colour and composed of normal, but compact and closely interwoven hyphae. Small black sclerotia like bodies were formed at the glass liquid interface after 10 to 14 days. Abortive sporulation occurred at the glass liquid interface after 14 to 16 days, but not on the main colony. Glucose was consumed more rapidly than in the parent and PCNB resistant strain cultures. Table 28 shows that the residual glucose had fallen to 0.0020 g./ml. after 12 days compared to 0.0075 g./ml. for the parent, and 0.0070 g./ml. for the PCNB resistant strain. In production of dry weight of mycelium and utilisation of glucose, the 2:3:4:5 TCNB resistant strain showed that it was more resistant to 2:3:5:6 TCNB than the parent or PCNB resistant strain. The 2:3:5:6 TCNB, and 2:3:4:6 TCNB resistant strains showed macro-

scopic growth from the inoculum after 20 hours. The mycelium produced was initially pale in colour, and composed of normal compact closely interwoven hyphae. Abortive sporulation and small black sclerotia like bodies were formed at the glass liquid interface after 14 to 16 days, and abortive sporulation was seen on the main colony after 16 to 20 days. The maximum dry weight of mycelium was recorded after 10 days. Table 28 shows that over 25% of the glucose in the medium was used by the 2:3:5:6 TCNB and 2:3:4:6 TCNB resistant strains before the parent or PCNB resistant strain showed macroscopic growth or had utilised any glucose in the medium.

The 2:3:5:6 TCNB resistant strain was slightly more resistant to 2:3:5:6 TCNB than the 2:3:4:6 TCNB resistant strain, and both these strains were clearly more resistant to 2:3:5:6 TCNB, than the parent, PCNB resistant, or 2:3:4:5 TCNB resistant strains.

Table 30.

Dry weight of mycelium produced by parent and resistant strains in liquid medium containing 10 mg. 2:3:4:6 TCNB

Time after inoculation (days)	Dry weight of mycelium in g.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
3	0.0012	0.0013	0.0017	0.0019	0.0058
7	0.0011	0.0016	0.0085	0.0087	0.0396
11	0.0014	0.0015	0.0246	0.0461	0.0921
13	0.0014	0.0009	0.0430	0.0857	0.1051
15	0.0016	0.0013	0.0542	0.0971	0.1138
22	0.0035	0.0037	0.0813	0.0903	0.1048
28	0.0046	0.0066	0.0892	0.0782	0.1072
32	0.0057	0.0086	0.0888	0.0711	0.1266
43	0.0082	0.0166	0.0755	0.0646	0.0734
53	0.0112	0.0274	0.0865	0.0735	0.0862

Dry weight of inoculum (mean ten replicates) in g.

Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
0.0013	0.0013	0.0014	0.0011	0.0014

Table 31.

Residual glucose of medium. 10 mg. 2:3:4:6 TCNB

Time after inoculation (days)	Residual glucose in g./ml.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
3	0.0100	0.0100	0.0100	0.0100	0.0096
7	0.0100	0.0100	0.0096	0.0094	0.0075
11	0.0100	0.0100	0.0086	0.0072	0.0018
13	0.0100	0.0100	0.0079	0.0046	-
15	0.0100	0.0100	0.0068	0.0018	-
22	0.0095	0.0096	0.0040	-	-
28	0.0094	0.0092	0.0012	-	-
32	0.0086	0.0088	-	-	-
43	0.0074	0.0064	-	-	-
53	0.0056	0.0043	-	-	-

Table 32.

Economic coefficient. 10 mg. 2:3:4:6 TCNB.

Time after inoculation (days)	Economic coefficient				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
3	-	-	-	-	0.22
7	-	-	0.35	0.23	0.32
11	-	-	0.35	0.33	0.23
13	-	-	0.40	0.32	-
15	-	-	0.35	0.24	-
22	0.11	0.12	0.27	-	-
28	0.11	0.13	0.20	-	-
32	0.06	0.12	-	-	-
43	0.06	0.08	-	-	-
53	0.05	0.09	-	-	-

10 mg. 2:3:4:6 TCNB. Parent and resistant strains

Table 30 shows the dry weight of mycelium produced, and Tables 31 and 32 the residual glucose and "economic coefficient".

With the parent the lag phase was 15 to 18 days. This was considerably longer than with the previous TCNB treatments. The original mycelium produced was composed of distorted swollen hyphae, pale brown in colour. Small pale nodulose areas, composed of paler coloured, but normal hyphae appeared on the original inoculum after 32 to 36 days. Small pale coloured mycelial pellets were found after 43 days. Very little growth

took place at the glass liquid interface. The main and subsidiary colonies did not sporulate or produce any structure comparable to the abortive conidia of the previous TCNB treatments.

Table 31 shows that nearly 50% of the glucose had disappeared from the medium but the dry weight of mycelium was only 0.0112 g. This poor utilisation of glucose for production of mycelial dry weight is reflected in the low value of the "economic coefficient".

The growth of the PCNB resistant strain in the presence of 10 mg. 2:3:4:6 TCNB was similar to that of the parent. The lag phase was 15 to 18 days, but the initial growth consisted of creamy coloured nodules on the inoculum composed of closely interwoven hyphae. Mycelial pellets were formed in the medium after 28 days. Small black sclerotia like bodies were formed at the glass liquid interface after 30 to 36 days. There was no sign of sporulation, either on the main colony or at the glass liquid interface. Table 31 shows that over 50% of the glucose in the medium had been used but the dry weight of mycelium was only 0.0274 g. This poor utilisation of glucose gave a low "economic coefficient" of 0.09. The PCNB resistant strain was not more resistant to 2:3:4:6 TCNB than the parent for the first 22 to 28 days, but Tables 30 and 31, show that during the last month of the experiment from 28 to 53 days, the PCNB resistant strain was more resistant than the parent.

The three TCNB resistant strains were all more resistant

to 2:3:4:6 TCNB than the parent or PCNB resistant strain. Growth started from the inoculum after 2 to 3 days in all cultures. Pale nodular areas, composed of closely interwoven, but otherwise normal hyphae formed on the original inoculum. Small mycelial pellets appeared after 3 to 4 days. The hyphae in these pellets were identical with those produced from the original inoculum. Small black sclerotia like bodies formed at the glass liquid interface after 24 to 28 days in the 2:3:5:6 TCNB and 2:3:4:5 TCNB resistant strain cultures, and after 7 to 11 days in the 2:3:4:6 TCNB resistant strain cultures. Aerial hyphae appeared at the glass liquid interface and on the surface of the main colony after 22 to 26 days in the 2:3:5:6 and 2:3:4:5 resistant strain cultures, and after 11 to 13 days in the 2:3:4:6 TCNB resistant strain cultures. These short hyphae terminated in a swollen portion, mostly elongate, but some almost spherical. These structures, which may represent abortive conidiophores were non pigmented, and gave the surface of the mycelium a white powdery appearance. Table 30 shows that the 2:3:4:5, 2:3:5:6 and 2:3:4:6 TCNB resistant strains were moreresistant to 2:3:4:6 TCNB than either the parent, or PCNB resistant strain.

Maximum dry weight of mycelium was recorded after 15 to 28 days, and Table 31 shows that the glucose in the medium had been used after 13 to 28 days. The 2:3:4:6 TCNB resistant strain was more resistant than either the 2:3:4:5 or 2:3:5:6 TCNB resistant strains. Table 31 shows that after 11 days over

80% of the glucose had disappeared from the medium of the 2:3:4:6 TCNB resistant strain, compared with 14% in the 2:3:4:5 TCNB resistant strain cultures, and 28% in the 2:3:5:6 TCNB resistant strain cultures. Table 30 shows that after 11 days the 2:3:4:6 TCNB resistant strain had produced twice as much mycelial dry weight as the 2:3:5:6 TCNB resistant strain and almost four times as much mycelial dry weight as the 2:3:4:5 TCNB resistant strain.

The dry weight of mycelium, and residual glucose of medium of the parent, PCNB and 2:3:4:6 TCNB resistant strain cultures are shown graphically in Figures 2 to 7. These graphs are based on results recorded in Tables 18, 19, 21, 22, 24, 25, 27, 28, 30 and 31.

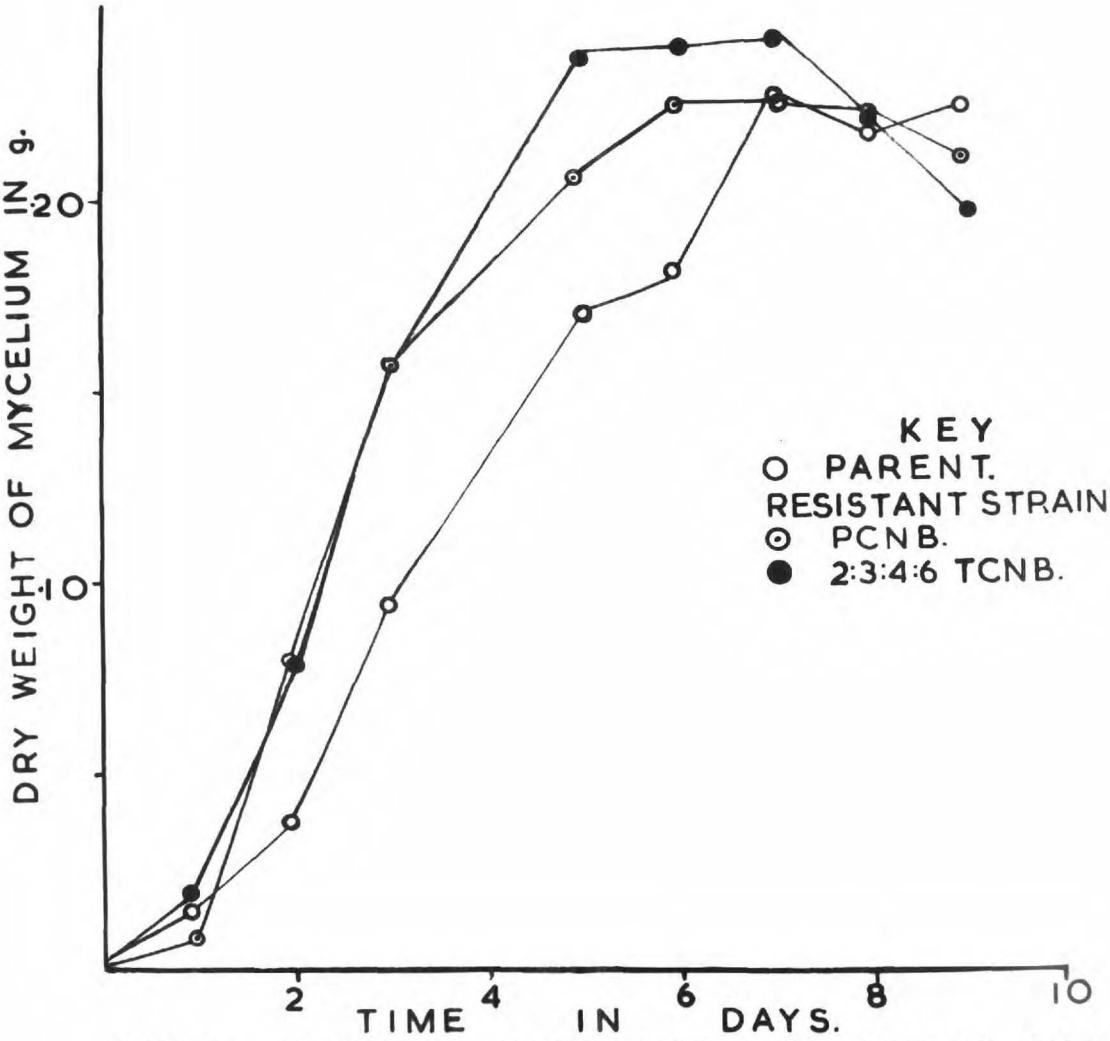


FIG. 2. GROWTH OF PARENT, PCNB, AND 2:3:4:6 TCNB RESISTANT STRAINS OF B.ALLII IN LIQUID MEDIUM.

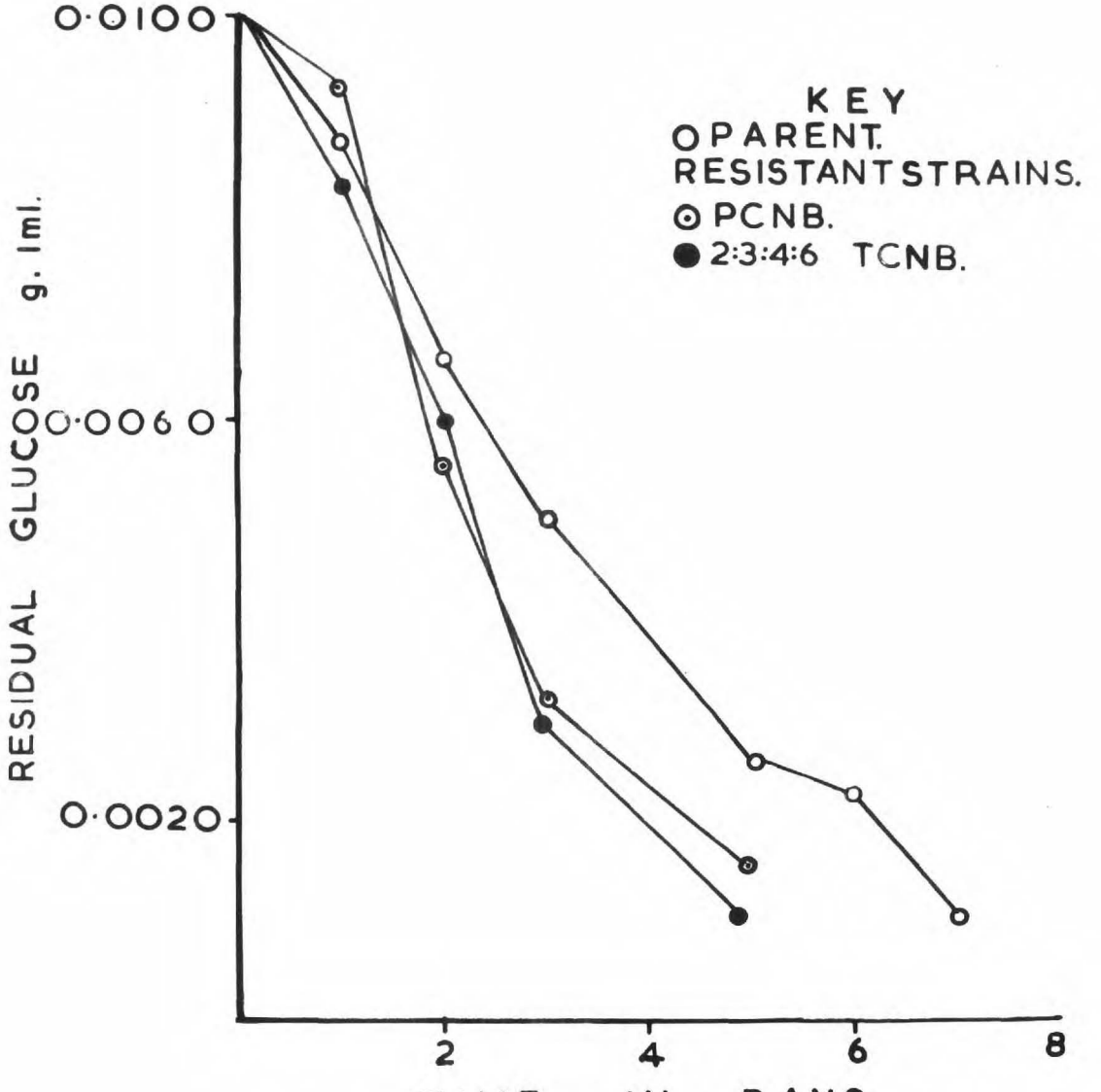


FIG. 3. RESIDUAL GLUCOSE OF MEDIUM.

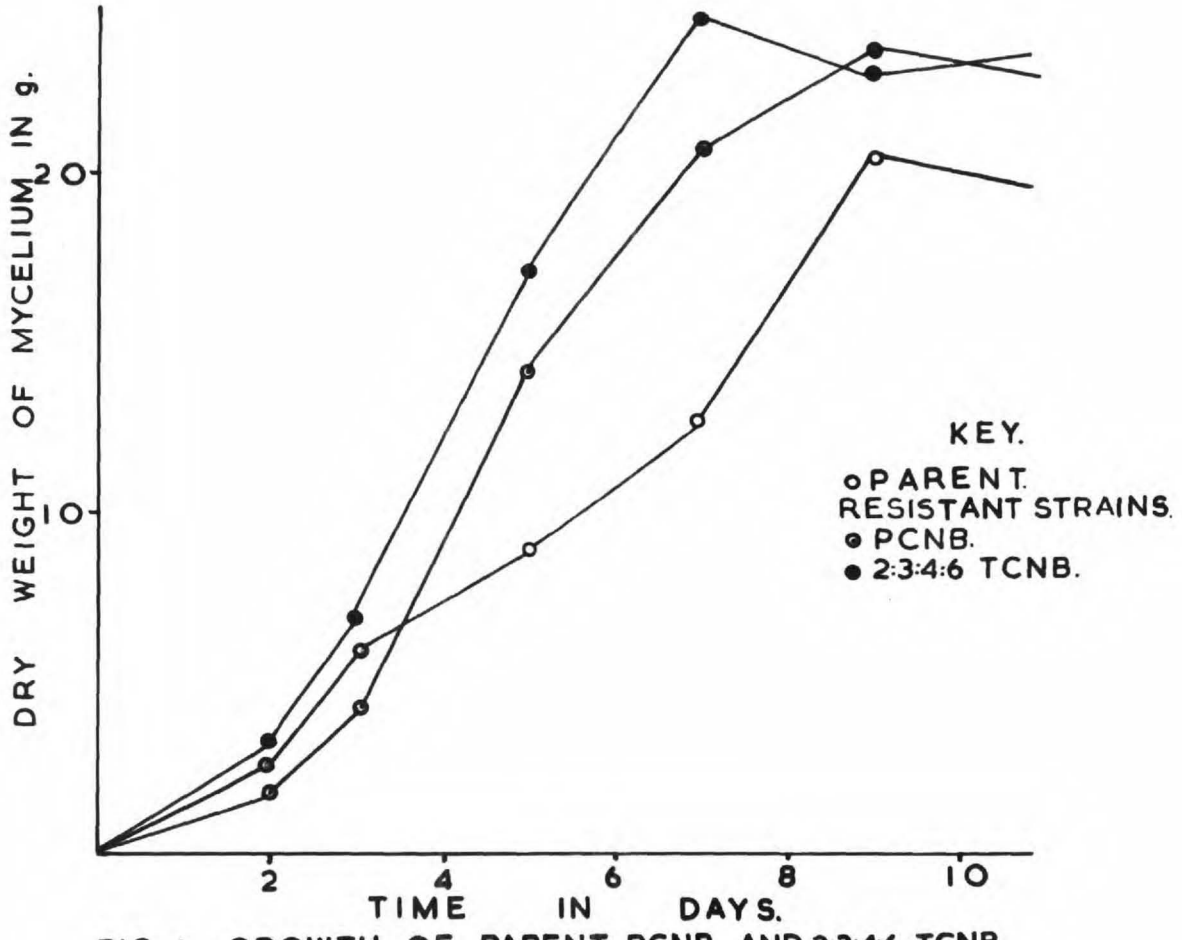


FIG. 4. GROWTH OF PARENT PCNB, AND 2:3:4:6 TCNB. RESISTANT STRAINS OF B. ALLII IN LIQUID MEDIUM CONTAINING 10 mg. PCNB.

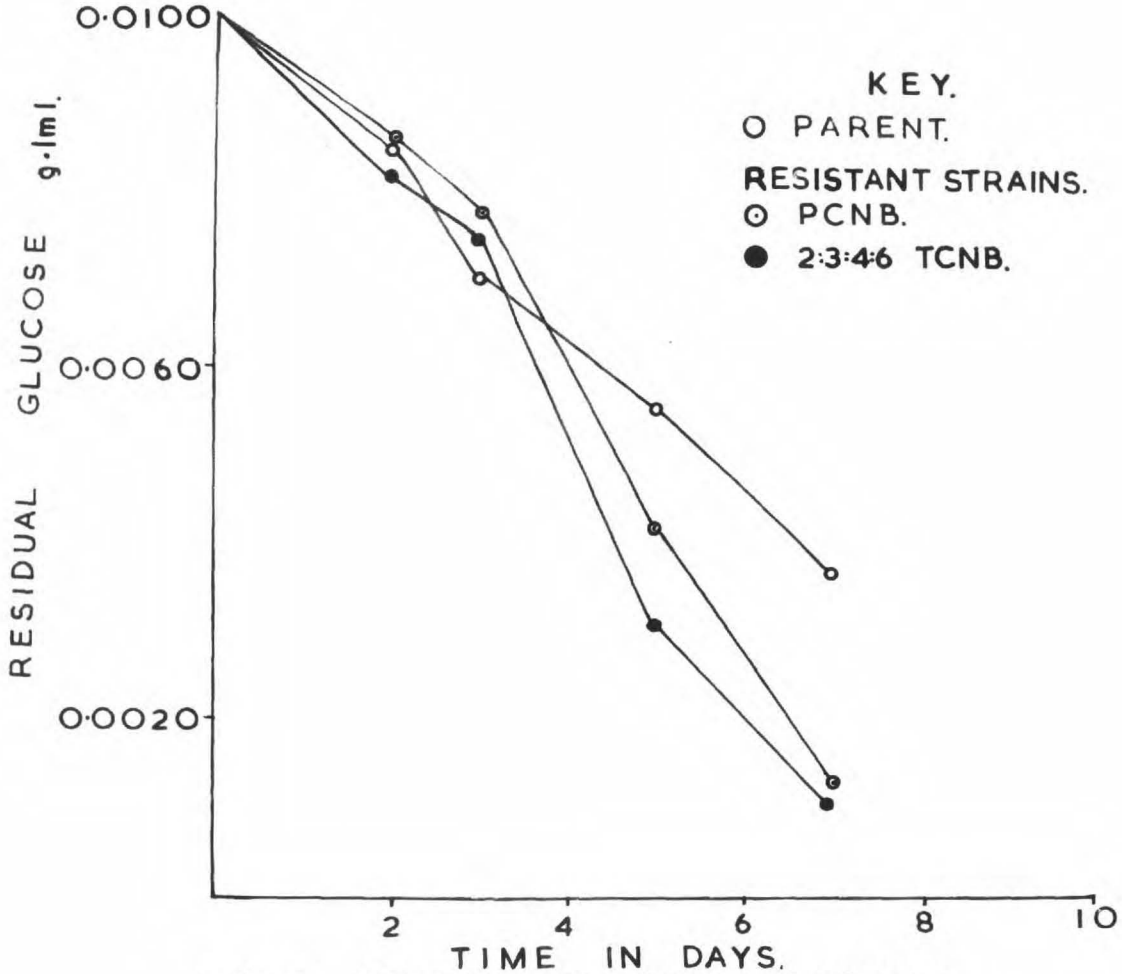


FIG. 5. RESIDUAL GLUCOSE OF MEDIUM (10 mg. PCNB.)

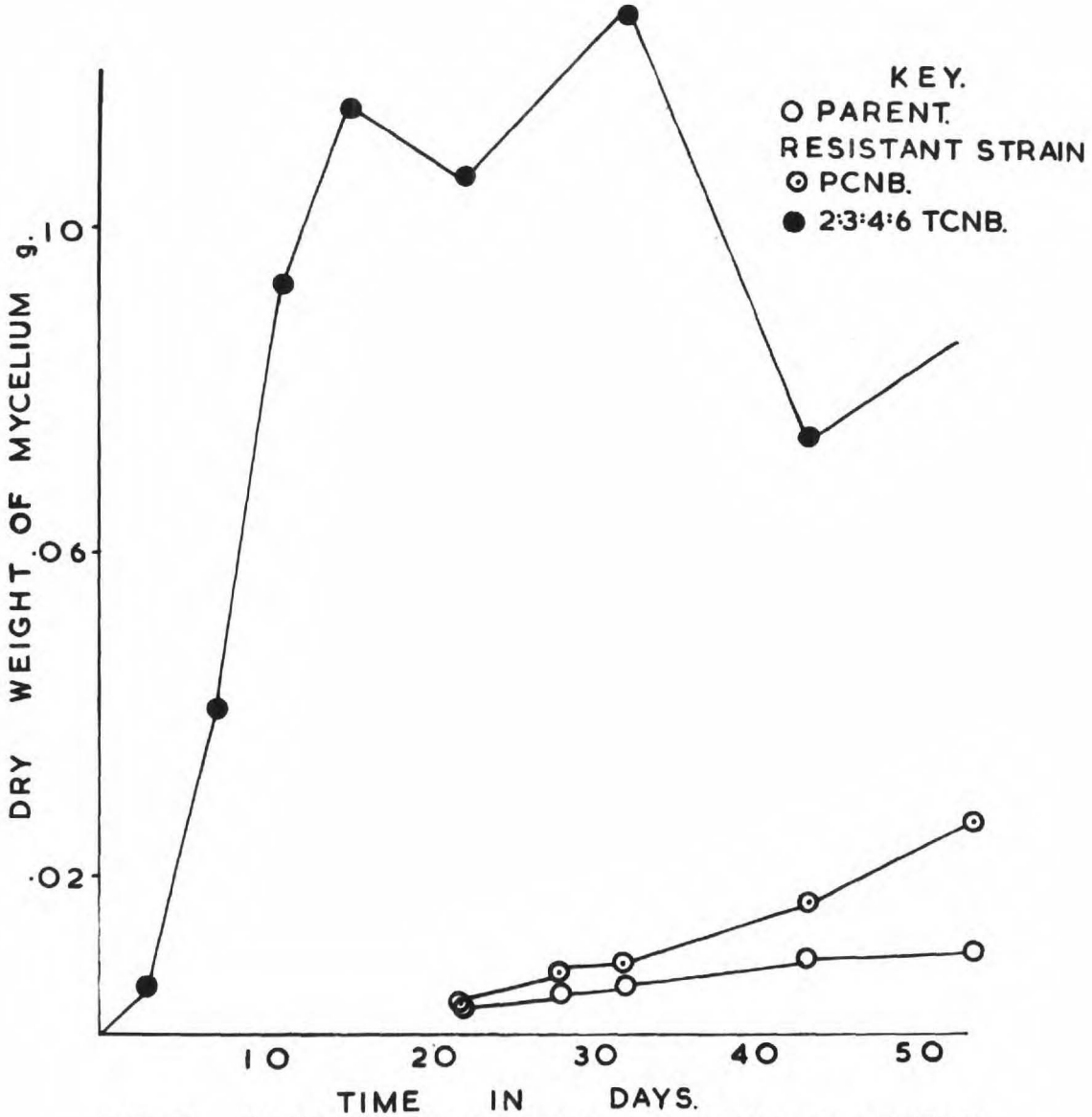


FIG 6. GROWTH OF PARENT, PCNB, AND 2:3:4:6 TCNB RESISTANT STRAINS OF B. ALLII IN LIQUID MEDIUM CONTAINING 10 mg. 2:3:4:6 TCNB.

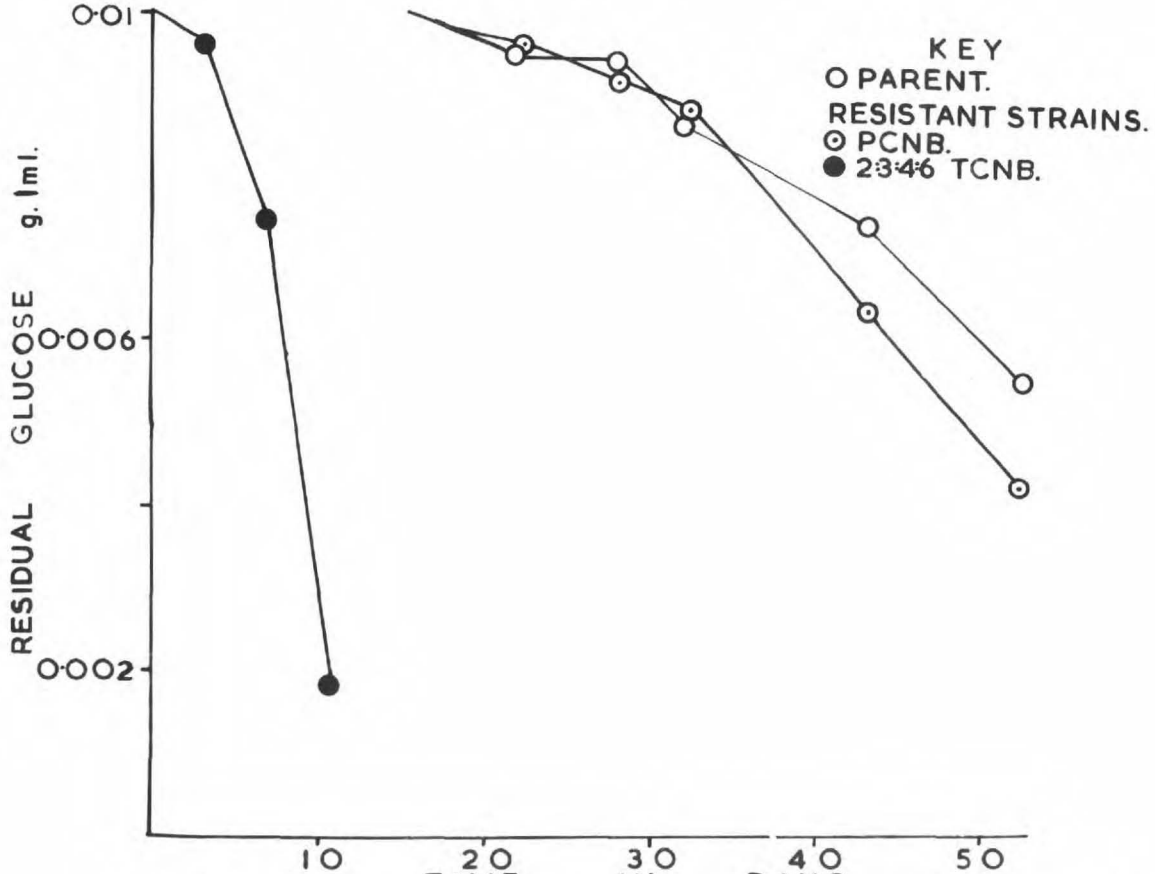


FIG. 7. RESIDUAL GLUCOSE OF MEDIUM (10mg. 2:3:4:6 TCNB).

Summary of results. Tables 18 to 32.

All the resistant strains grew more quickly, produced a greater dry weight of mycelium, utilised glucose more efficiently, and showed less morphological abnormality than the parent strain when grown in the presence of PCNB or TCNB. Resistance to one fungicide resulted in lowered susceptibility to PCNB or the other TCNB isomers. In the presence of 2:3:5:6 TCNB, or 2:3:4:6 TCNB, the most resistant strain was the one which had been exposed to the vapour of that fungicide before the experiment. In the presence of 2:3:4:5 TCNB, the 2:3:5:6 TCNB and 2:3:4:6 TCNB resistant strains were as resistant to this fungicide as the 2:3:4:5 TCNB resistant strain. The 2:3:5:6 TCNB resistant strain was more resistant to 2:3:4:6 TCNB than the 2:3:4:5 TCNB resistant strain. The TCNB resistant strains were as resistant as the PCNB resistant strain when grown in liquid medium containing PCNB. The effect on sporulation of PCNB or TCNB isomer was less than in the vapour phase. The most active isomer was 2:3:4:6 TCNB, and the least active fungicide was PCNB. The order of activity decreased in the series 2:3:4:6 TCNB, 2:3:5:6 TCNB, 2:3:4:5 TCNB, PCNB.

These results substantiated the results obtained in section IV. 2.8, and confirmed the conclusions of Parry (1957), that a common adaptation mechanism to PCNB and the TCNB isomers exists in Botrytis allii.

2.13 Effect of dichloronitrobenzene isomers on linear growth, hyphal morphology and sporulation of parent, PCNB, and TCNB resistant strains of Botrytis allii

Three dichloronitrobenzenes were used in the vapour phase as described for TCNB isomers in section IV. 1.0. 0.5 cm. non-sporulating discs of B.allii were used to inoculate the Petri dishes. The three isomers used were, 2,3 dichloronitrobenzene (2,3 DCNB), 2,4 dichloronitrobenzene (2,4 DCNB), and 2,5 dichloronitrobenzene (2,5 DCNB). The results obtained are recorded in Tables 33 to 37.

Table 33

Linear growth of parent, PCNB and TCNB resistant strains of B.allii in presence of 10 mg. 2,3 DCNB

Time after inoculation (days)	Mean colony diameter in cm.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
47	0.5	0.5	0.5	0.5	0.5+
58	0.5	0.5	0.5	0.5	0.5+
93	0.5	0.5	0.5	0.5	0.6

+ = Slight growth, but less than 0.05 cm.

Table 34

Linear growth of parent, PCNB and TCNB resistant strains of B.allii in presence of 1 mg. 2,3 DCNB

Time after inoculation (days)	Mean colony diameter in cm.				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.5	0.5	0.5	0.5	0.9
3	0.5	0.5	0.5	0.5	1.1
4	0.5	0.5	0.5	0.5	1.3
5	0.5	0.5	0.5	0.5	1.5
7	0.5	0.5	0.5	0.5	1.7
9	0.5	0.5	0.5	0.5+	1.8
13	0.5	0.5	0.5	0.5+	1.8
17	0.5	0.5	0.5+	0.5+	1.8
25	0.5	0.5	0.5+	0.5+	1.8
47	0.5	0.5	0.5+	0.5+	1.9
58	0.5	0.5	0.5+	0.5+	1.9
70	0.5	0.5	0.7	0.5+	2.0
93	0.5	0.5	1.4	0.6+	2.3

+ = Slight growth, but less than 0.05 cm.

Plate 2

A. Growth of 2:3:5:6 TCNB resistant strain of B.allii in presence of 1 mg. 2,3 DCNB. After 100 days.

B. Growth of 2:3:4:5 TCNB resistant strain of B.allii in presence of 1 mg. 2,3 DCNB. After 100 days.

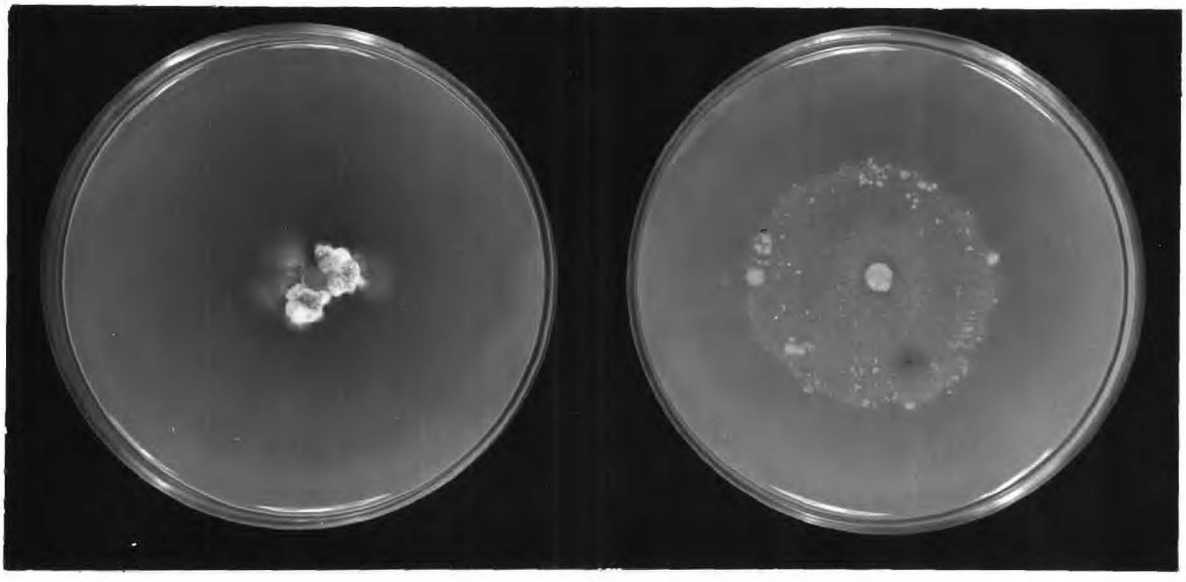


Plate 3

A. Growth of 2:3:4:6 TCNB resistant strain of B.allii in presence of 1 mg. 2,3 DCNB. After 93 days.

B. Growth of 2:3:4:6 TCNB resistant strain of B.allii in presence of 1 mg. 2,3 DCNB. After 93 days.

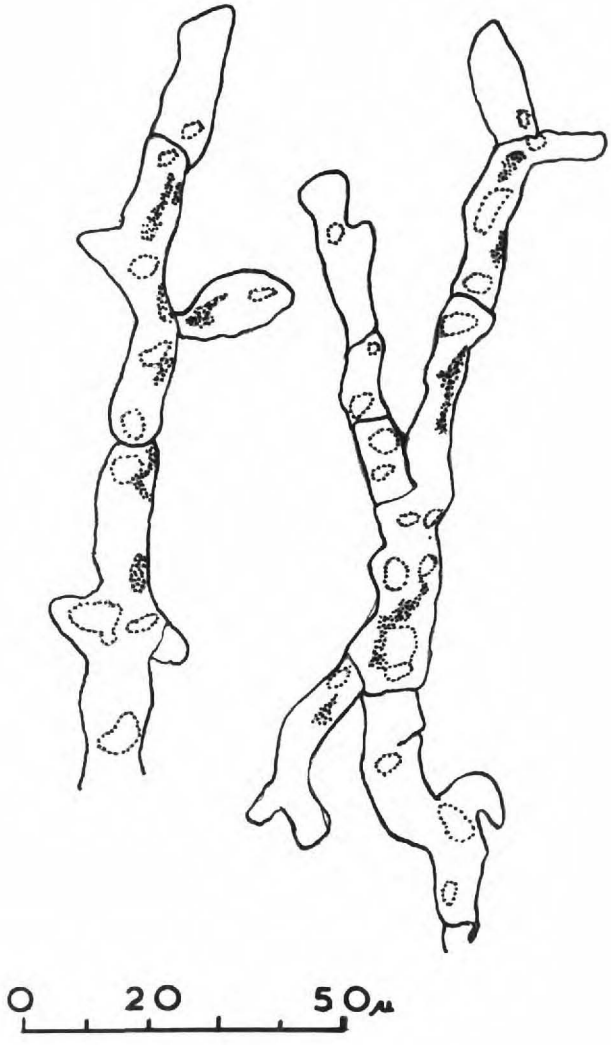


Fig. 8

Abnormal hyphae of 2:3:5:6 TCNB resistant strain.

(1 mg. 2,3 DCNB)

Vacuolated areas enclosed by dotted lines.

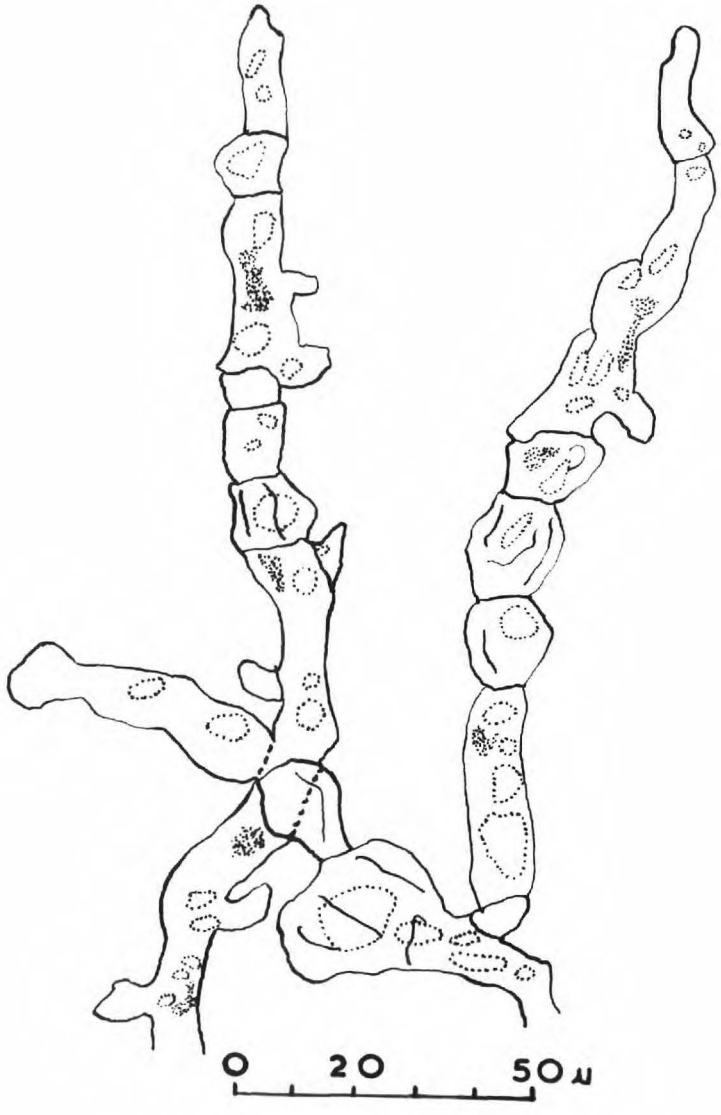


Fig. 9

Abnormal hyphae initially produced by 2:3:4:5 TCNB resistant strain (1 mg. 2,3 DCNB)

Vacuolated areas enclosed by dotted lines.

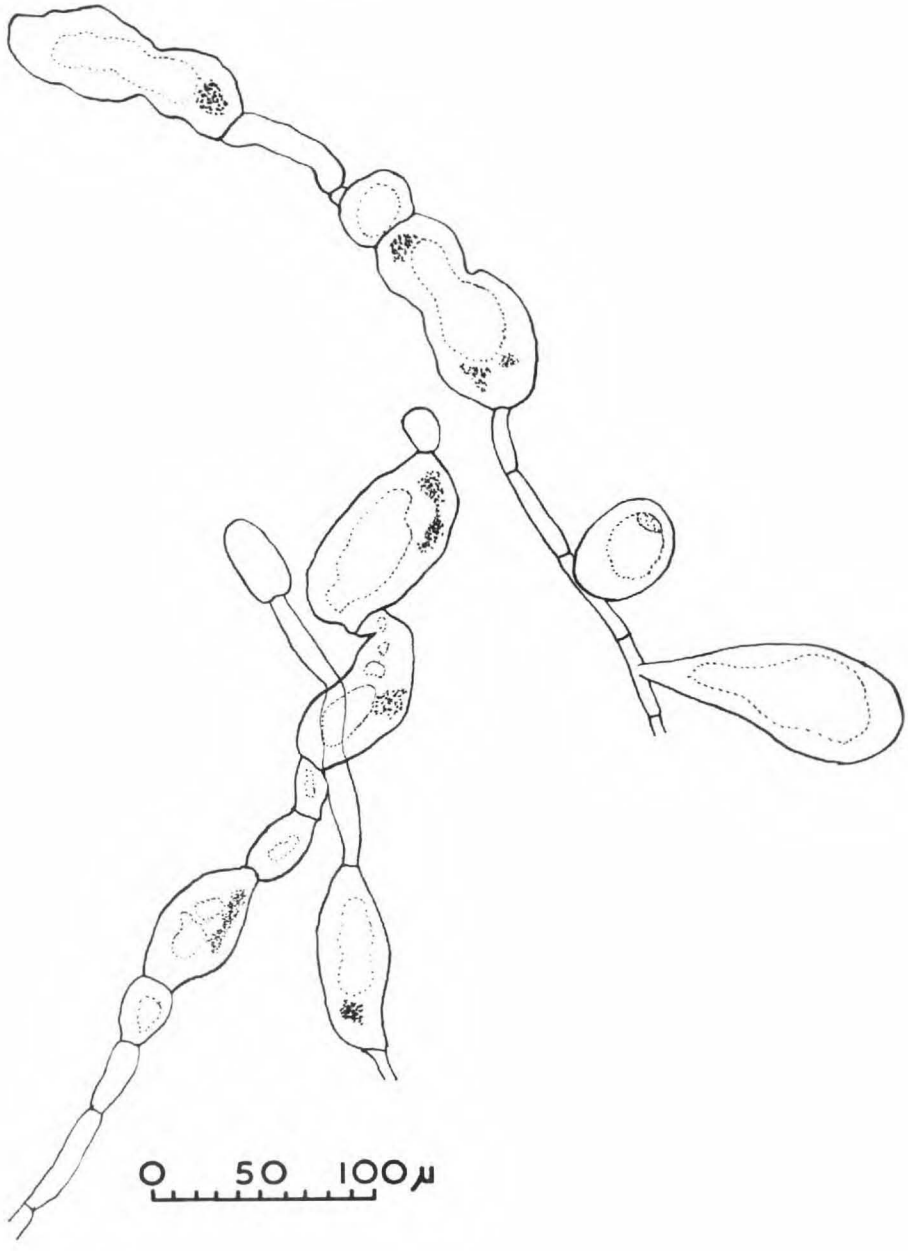


Fig. 10

Abnormal hyphae initially produced by 2:3:4:6 TCNB resistant strain (1 mg. 2,3 DCNB)

Vacuolated areas enclosed by dotted lines

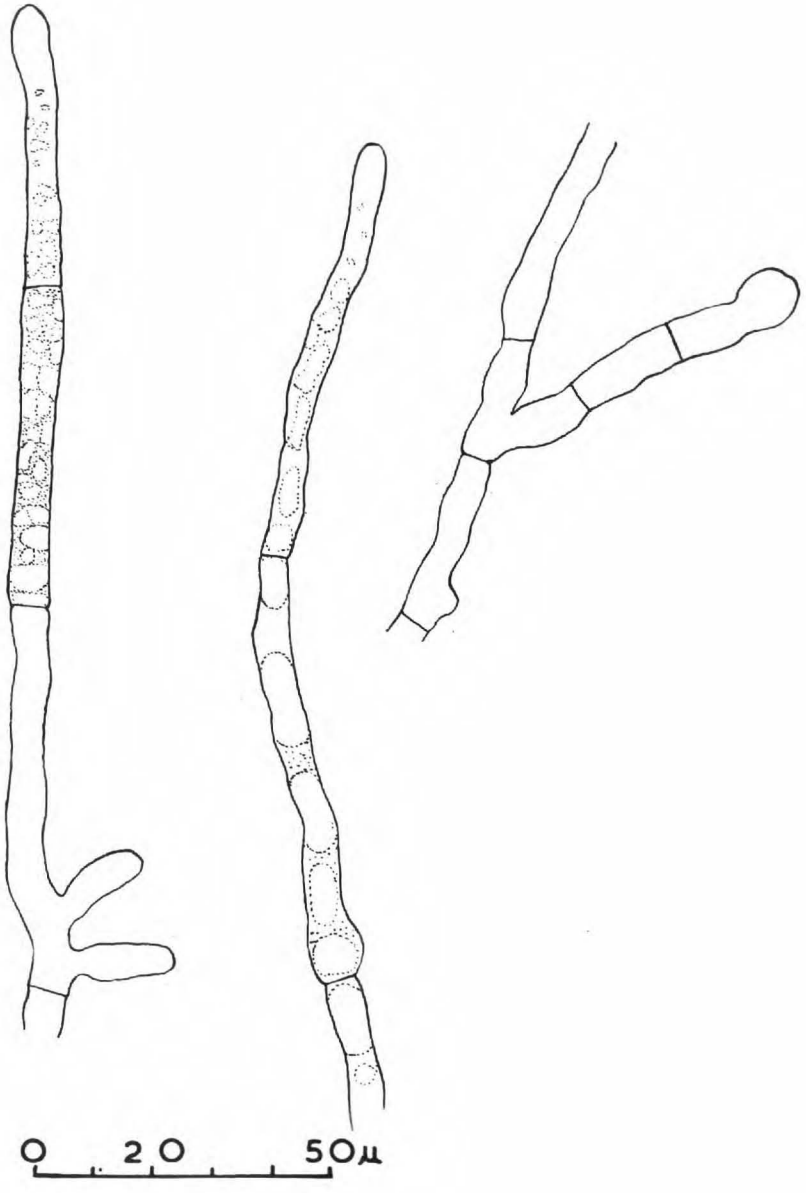


Fig. 11

Typical hyphae of 2:3:4:6 TCNB resistant strain. arising after 17 days (1 ng. 2,3 DCNB)

Vacuolated areas enclosed by dotted lines

2,3 DCNB. Parent and resistant strains

The response of the parent, PCNB, and TCNB resistant strains to 10 mg. and 1 mg. of 2,3 DCNB is shown in Tables 33 and 34.

Table 33 shows that after 93 days there was no measurable growth from the inoculum in the parent, PCNB, 2:3:4:5 TCNB and 2:3:5:6 TCNB cultures in the presence of 10 mg. fungicide. Microscopic examination of the inoculum did not show any hyphae originating from this source. At this point the lids were removed from the plates and replaced with fungicide free lids. After 6 weeks there was no sign of growth. Attempts to subculture from the original inoculum failed. The 2:3:4:6 TCNB resistant strain showed slight growth after 47 days. The hyphae produced during the period 47 to 93 days were pale creamy yellow in colour. These hyphae were swollen and gnarled, and the hyphal walls were wrinkled and corrugated. Extensive areas of vacuolation and granulation were present. Growth was irregular and did not develop evenly from the circumference of the inoculum. This gave the colonies an uneven outline. Seventeen of the 20 replicates of the 2:3:4:6 TCNB resistant strain showed this type of response; 3 plates did not show any evidence of growth after 93 days.

Table 34 shows that after 93 days in the presence of 1 mg. fungicide there was no measurable growth from the inoculum in the parent and PCNB resistant strain Petri dishes. Micro-

scopic examination of the inoculum did not show any newly formed hyphae. The lids were removed from these plates and replaced with fungicide free lids. After 6 weeks there was no sign of growth, and attempts to sub-culture from the original inoculum failed.

Slight growth was seen after 9 days from the 2:3:5:6 TCNB resistant strain inoculum, and after 17 days from the 2:3:4:5 TCNB resistant strain inoculum. All cultures showed some growth from the inoculum. Growth from the inoculum was irregular giving rise to colonies of uneven outline. The pale yellow hyphae produced by the 2:3:5:6 TCNB resistant strain were swollen and gnarled, but less so than the hyphae produced by the 2:3:4:6 TCNB resistant strain in the presence of 10 mg. 2,3 DCNB.

Plate 2 A (2:3:5:6 TCNB resistant strain/1 mg. 2,3 DCNB), shows a typical colony after 100 days, Fig. 8 shows the type of hyphae present.

The pale yellow hyphae initially formed by the 2:3:4:5 TCNB resistant strain were gnarled and swollen. After 70 to 93 days less irregular hyphae were formed. Plate 2 B (2:3:4:5 TCNB resistant strain / 1 mg. 2,3 DCNB), shows a colony with both types of hyphae. Fig. 9 shows the type of hyphae produced initially.

The 2:3:4:6 TCNB resistant strain behaved differently in the presence of 1 mg. 2,3 DCNB. Two types of response were

seen. Eight out of 20 replicates produced a sparse spreading mycelium. The hyphae were normal in size and appearance, but growth stopped after 9 days. The greatest diameter reached was 4.8 cm. and the smallest 1.4 cm. The other 12 replicates showed no macroscopic growth for 7 days. When growth started from the inoculum, gnarled swollen hyphae were formed. These hyphae were pale yellow in colour and showed areas of vacuolation and granulation, Fig. 10. After 17 days small areas of growth appeared within the colonies showing the normal type of hyphae, and at the edge of colonies showing gnarled hyphae. The type of hyphae present in these areas of growth is shown in Fig. 11. These hyphae were creamy yellow in colour and showed areas of vacuolation and granulation. The walls were slightly corrugated, but intercalary swollen portions were absent. Plate 3 shows the two types of colony after 93 days.

No evidence of sporulation was seen in any replicate at either 10 mg. or 1 mg. levels of 2,3 DCNB. Tables 33 and 34 show that the TCNB resistant strains had some resistance to 2,3 DCNB. The 2:3:4:6 TCNB resistant strain survived at concentrations of 2,3 DCNB that were lethal to the parent, PCNB, 2:3:4:5 TCNB, and 2:3:5:6 TCNB resistant strain. The PCNB resistant strain was as susceptible as the parent isolate to the vapour of 2,3 DCNB. Adaptation of B.allii to the tetrachloronitrobenzenes gave some degree of resistance to 2,3 DCNB.

Table 35.

Linear growth of parent, PCNB, and TCNB resistant strains of B.allii in presence of 10 mg. 2,4 DCNB

Time after inoculation (days)	Mean colony diameter in cm.				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.5	0.5	0.5	0.5	0.5
4	0.5	0.5	0.5	0.5	0.5
12	0.5	0.5	0.5	0.5	0.5
20	0.5	0.5	0.5	0.5	0.5+
42	0.5	0.5	0.5	0.5	0.7
53	0.5	0.5	0.5	0.5	2.7
69	0.5	0.5	0.5	0.5	6.4 S
95	0.5	0.5	0.5	0.5	C

\+ = Slight growth, but less than 0.05 cm.

S = Start of sporulation

C = Plates covered by mycelia

Table 36

Linear growth of parent, PCNB, and TCNB resistant strains of B.allii in presence of 1 mg. 2,4 DCNB

Time after inoculation (days)	Mean colony diameter in cm.				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.5	0.5	0.8	1.5	2.1
4	0.5+	0.5+	2.5	2.7	4.4 S
8	0.5+	0.6	3.8	4.0 S	7.2
12	0.5+	0.7	4.9 S	4.0	C
20	0.6+	0.9	6.7	4.0	
42	0.8	2.9	6.7	4.0	
53	1.1	4.4	6.7	4.0	
69	2.6	8.4	8.3	7.8	
95	4.2	C	C	C	

\+ = Slight growth, but less than 0.05 cm.

S = Start of sporulation

C = Plates covered with mycelia

Plate 4

A. Growth of parent isolate in presence of 1 mg. 2,4 DCNB. After 12 days

B. Growth of 2:3:4:6 TCNB resistant strain in presence of 1 mg. 2,4 DCNB. After 12 days.

2,4 DCNB. Parent and resistant strains

The response of the parent, PCNB, and TCNB resistant strains is shown in Tables 35 and 36.

Table 35 shows that after 95 days there was no measurable growth from the inoculum in the parent, PCNB, 2:3:4:5 TCNB, and 2:3:5:6 TCNB resistant strain cultures, grown in presence of 10 mg. 2,4 DCNB. Microscopic examination of the inoculum did not show any newly formed hyphae. At this point the lids were removed and replaced with fungicide free lids. After 6 weeks there was no sign of growth. Attempts to sub-culture from the original inoculum failed. The 2:3:4:6 TCNB resistant strain showled slight growth after 20 days in the presence of 10 mg. 2,4 DCNB. During the period 20 to 53 days, gnarled swollen yellowish hyphae were produced at points around the inoculum giving colonies of irregular outline. These hyphae were identical to those produced by this strain in the presence of 10 mg. 2,3 DCNB. After 53 days less abnormal hyphae were produced. The hyphae produced during the period 53 to 69 days were progressively less abnormal. Sporulation started after 69 days. Sporulation was sparse and did not arise on the mycelium formed up to 53 days. The plates were covered with mycelia after 95 days. Table 36 shows that all the strains produced some growth in the presence of 1 mg. 2,4 DCNB during the course of the experiment. The behaviour of the parent and PCNB resistant strain was similar. Gnarled swollen hyphae, yellow brown in colour were produced, forming

nodular areas around the inoculum. The parent strain produced pale yellow hyphae from the nodular growth after 69 days, but all the hyphae formed during the experiment were abnormal. The PCNB resistant strain produced pale yellow hyphae from the previous growth after 53 days, and finally after 69 days normal hyphae were produced, covering the plates with mycelia after 95 days. Sporulation was not observed in either parent isolate or PCNB resistant strain cultures.

The 2:3:4:5 TCNB and 2:3:5:6 TCNB resistant strains behaved similarly to each other. With the 2:3:4:5 TCNB resistant strain, 7 out of 20 cultures produced a thin mycelium of normal hyphae. This type of growth stopped after 12 days. The maximum diameter reached was 2.7 cm. Small spots of yellowish mycelium slowly formed at the edge, and within these colonies. These spots were composed of abnormal hyphae, showing areas of granulation and vacuolation. They were closely interwoven compared with the loose spreading mycelium previously formed. After 53 days these yellowish hyphae produced fan shaped sectors of sparse mycelium at the edge of the original colony. The hyphae in these sectors were similar to those of the parent isolate in the absence of fungicide, except that they were non pigmented. This type of mycelium covered the plates after 69 days. Sparse sporulation was seen on the latter growth of these sultures after 66 to 84 days. The other 13 cultures immediately produced yellowish gnarled and swollen hyphae

from the inoculum. After 8 days less abnormal hyphae were formed, and during the period 10 to 20 days, normal hyphae were formed. The mycelium covered the plates after 20 days. The outline of the colonies was uneven, but the gradation from one type of hyphae to another was gradual and appeared evenly around the colonies. Sporulation started after 12 days in these cultures, predominantly on the latter growth.

The 2:3:5:6 TCNB resistant strain showed these two types of response. Six out of 20 cultures initially produced the yellowish gnarled and swollen hyphae, these became progressively less abnormal and the plates were covered with mycelia after 8 days. Sporulation started after 8 days, and a moderate crop of spores was produced, mainly on the latter growth. The remaining 14 cultures produced a fine delicate spreading mycelium composed of normal hyphae. The maximum diameter reached by any colony was 2.3 cm. This type of growth stopped after 12 days. Spots of yellowish mycelium developed at the edge and within these colonies. These yellowish areas produced irregular fan shaped areas of normal hyphae after 53 days, which covered the plates with a thin growth after 95 days. Sparse sporulation was seen on this latter growth.

The 2:3:4:6 TCNB resistant strain produced normal colonies of regular outline. The hyphae were pale yellow in colour, but otherwise resembled those produced in the absence of fungicide. The plates were covered with mycelia after 8 to 12

days. Normal sporulation started after 4 days. Plate 4 shows the parent isolate and 2:3:4:6 TCNB resistant strain after 12 days in the presence of 1 mg. 2,4 DCNB.

The results in Tables 35 and 36 show that the strains adapted to PCNB or TCNB isomer were more resistant to 2,4 DCNB than the parent isolate. The 2:3:4:6 TCNB resistant strain grew in the presence of 10 mg. 2,4 DCNB. This concentration killed the parent isolate and other resistant strains.

Table 37

Linear growth of parent, PCNB, and TCNB resistant strains of B.allii in the presence of 1 mg. 2,5 DCNB

Time after inoculation (days)	Mean colony diameter in cm.				
			Resistant strains		
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.5	0.5	0.5	0.5	1.6
3	0.5	0.5	0.5+	1.4	2.4
6	0.5	0.5+	1.2	3.5	5.1 S
8	0.5+	0.5+	2.4	4.7 S	6.1
14	2.3	1.3	4.5 S	8.2	C
20	4.1	3.0	6.7	C	
25	6.6	4.5	C		
37	C	C			

\+ = Slight growth, but less than 0.05 cm.

S = Start of sporulation. C = Plates covered with mycelia.

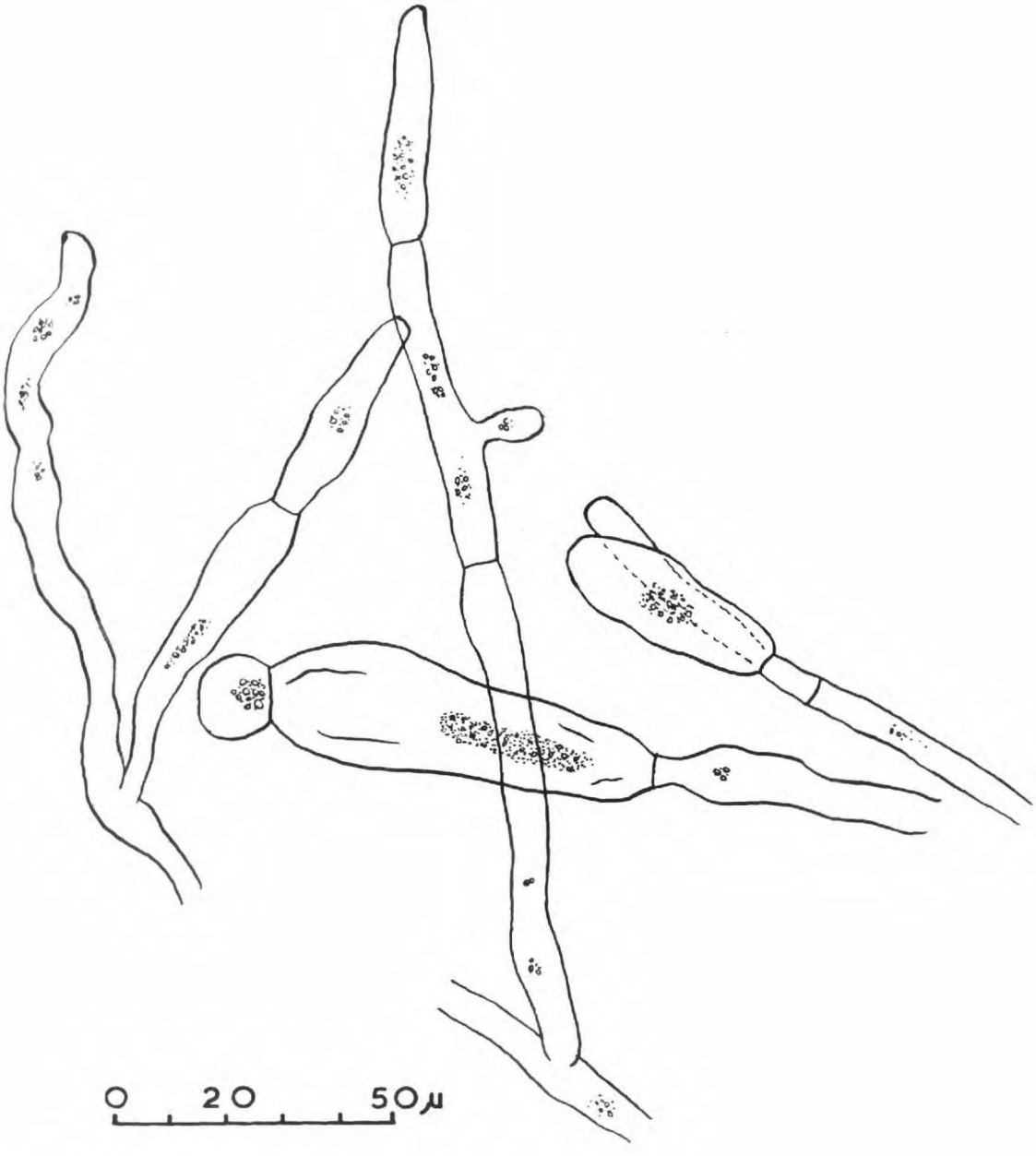


Fig. 12

Hyphae produced by 2:3:4:6 TCNB resistant strain in absence of 2,5 DCNB, after 93 days exposure to 10 mg. 2,5 DCNB

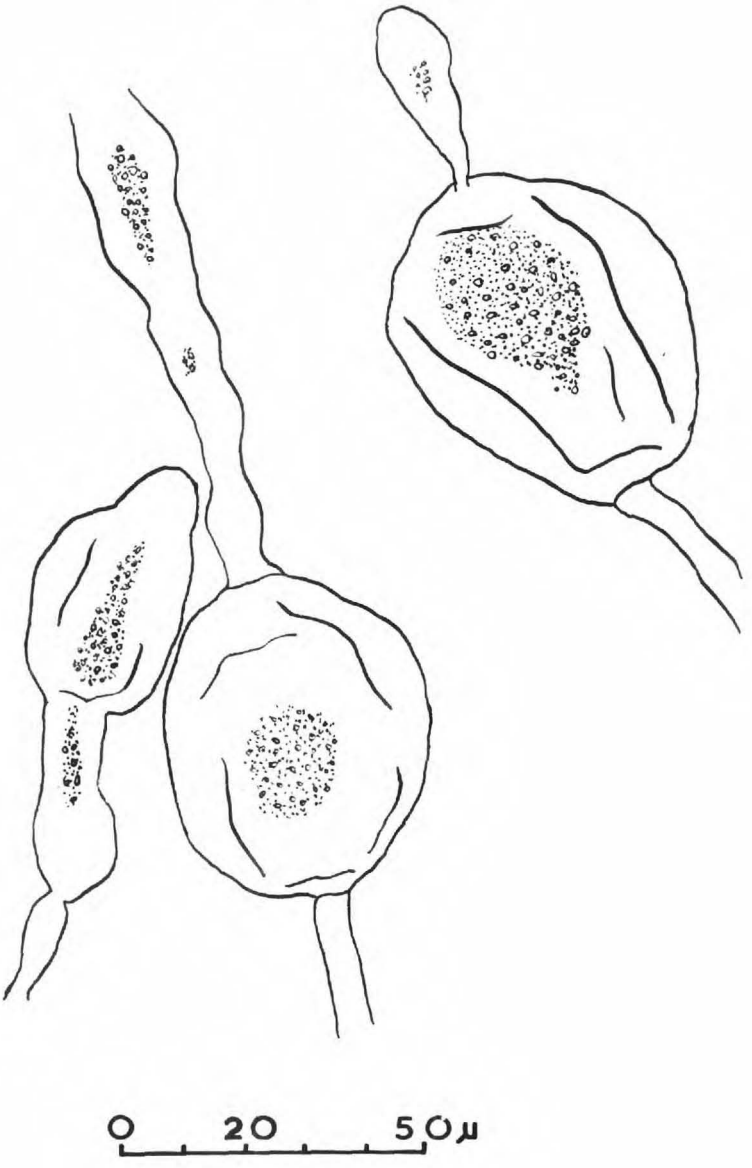


Fig. 13

Abnormal hyphae produced by parent isolate

(1 mg. 2,5 DCNB)

2,5 DCNB. Parent and resistant strains

The growth of the parent, PCNB, and TCNB resistant strains in the presence of 1 mg. 2,5 DCNB is shown in Table 37.

In the presence of 10 mg. 2,5 DCNB, none of the strains showed any growth after 93 days. The lids were then replaced with fungicide free lids. No sign of growth was shown by the parent, PCNB, 2:3:4:5 TCNB, and 2:3:5:6 TCNB resistant strains after 6 weeks. Attempts to sub-culture from the inoculum failed. The 2:3:4:6 TCNB resistant strain started growing after 7 days and reached a mean diameter of 1.9 cm. after 28 days. Hyphae were initially formed at several separate points around the inoculum and gave rise to unevenly shaped colonies. The abnormal hyphae produced, Fig. 12, were pale yellow in colour. Parts of the hyphae were swollen, and these swollen portions often showed conspicuous granulation. The hyphae were closely interwoven, and formed nodular masses of mycelium. Hyphae subsequently produced from these areas were identical to those produced by this strain in the absence of fungicide, though only a sparse mycelium was formed. There was no sign of sporulation on these colonies.

In the presence of 1 mg. 2,5 DCNB, the parent isolate, and PCNB resistant strain behaved similarly to each other. Growth started after 8 days. The hyphae produced Fig. 13, were yellow brown in colour and showed large intercalary portions with wrinkled walls and conspicuous granulation of the cytoplasm.

These hyphae were closely interwoven and formed nodular masses. Irregularly shaped colonies were formed in all replicates. After 14 to 20 days the hyphae formed were less distorted, and after 20 to 25 days normal hyphae were formed, which covered the plates with a sparse mycelium after 25 to 37 days. Slight sporulation confined to the latter growth, was seen on the cultures after 25 to 37 days. The behaviour of the 2:3:4:5 TCNB resistant strain followed the type described for the parent isolate and PCNB resistant strain. In all aspects however, the effect of the fungicide was less marked.

Table 37 shows that the 2:3:5:6 and 2:3:4:6 TCNB resistant strains were almost unaffected by the presence of 1 mg. 2,5 DCNB. The cultures formed regular colonies with normal hyphae, and sporulated abundantly after 8 days in the 2:3:5:6 TCNB resistant strain, and after 6 days in the 2:3:4:6 TCNB resistant strain. The only difference between these cultures and those in the absence of fungicide was a distinct pinkish tint to the immature conidia.

2.14 Effect of 2,5 dibromonitrobenzene, and 2,5 diiodonitrobenzene on linear growth, hyphal morphology, and sporulation of parent and 2:3:4:6 TCNB resistant strain of B.allii.

These two compounds 2,5 dibromonitrobenzene (2,5 DBNB), and 2,5 diiodonitrobenzene (2,5 DINB), were also used in the vapour phase. The results of these experiments are recorded in Tables 38 and 39.

Table 38

Linear growth of parent, and 2:3:4:6 TCNB resistant strain of B.allii in presence of 10 mg. or 1 mg. 2,5 DBNB

Time after inoculation (days)	Mean colony diameter in cm. 10 mg. 2,5 DBNB		1 mg. 2,5 DBNB	
	Parent	2:3:4:6 strain	Parent	2:3:4:6 strain
1	0.5	0.7	0.5	1.4
2	0.5	1.4	0.5	2.2
3	0.5	2.1	0.5	2.7
5	0.5+	3.4	0.5+	4.1 S
10	0.5+	5.3 S	0.7	7.7
14	0.6	6.0	1.0	C
20	0.9	7.8	2.6 S	
25	1.6	C	4.4	

\+ = Slight growth, but less than 0.05 cm.

S = Start of sporulation C = Plates covered with mycelia

Table 39

Linear growth of parent, and 2:3:4:6 TCNB resistant strain of B.allii in presence of 10 mg. or 1 mg. 2,5 DINB

Time after inoculation (days)	Mean colony diameter in cm. 10 mg. Parent	10 mg. 2,5 DINB 2:3:4:6 strain	1 mg. Parent	1 mg. 2,5 DINB 2:3:4:6 strain
1	0.5	1.1	0.5	1.3
2	0.5	1.9 S	0.5+	2.3 S
3	0.5+	3.0	0.6	3.5
5	0.6	4.7	1.5	6.0
10	1.2	7.4	3.1	C
14	2.3 S	C	5.2 S	
20	4.8		7.8	
25	7.9		C	

\+ = Slight growth, but less than 0.05 cm.

S = Start of sporulation

C = Plates covered with mycelia

2,5 DBNB. Parent and 2:3:4:6 TCNB resistant strain

Table 38 shows the growth of the parent and 2:3:4:6 TCNB resistant strain in the presence of 10 mg. or 1 mg. 2,5 DBNB.

At the 10 mg. level the parent initially formed abnormal hyphae of the type first produced in the previous experiments with dichloronitrobenzenes. Less abnormal hyphae

were formed after 14 to 20 days, and eventually after 25 days, the hyphae formed were identical with those produced in the absence of fungicide. The cultures did not sporulate furing the course of the experiment. The hyphae formed by the 2:3:4:6 TCNB resistant strain were similar to those produced in the absence of fungicide, and the plates were covered after 25 days. Moderate sporulation started after 10 days.

At the 1 mg. concentration, the parent first formed abnormal hyphae. These progressively became less abnormal, and after 12 days hyphae indistinguishable from these produced in the absence of fungicide were formed. Moderate sporulation started after 20 days, mainly on the latter growth. The 2:3:4:6 TCNB resistant strain was almost unaffected at the 1 mg. level. The hyphae were normal in colour and appearance. Abundant sporulation started after 5 days.

Table 38 shows that at the concentrations used, 2,5 DBNB had little effect on growth or sporulation of the 2:3:4:6 TCNB resistant strain, whereas with the parent strain, the hyphae formed were initially abnormal and distorted, growth was slow, and sporulation was suppressed or diminished.

2,5 DINB. Parent and 2:3:4:6 TCNB resistant strain

Table 39 shows the growth of the parent isolate, and 2:3:4:6 TCNB resistant strain in the presence of 10 mg. or 1 mg. 2,5 DINB.

At both concentrations the parent strain initially produced abnormal hyphae of the type seen with the other dihalogenatednitrobenzenes. These hyphae became progressively less abnormal, and after 14 days at the 10 mg. level, and 5 days at the 1 mg. level, the hyphae formed were indistinguishable from those produced by the parent isolate in the absence of fungicide. Moderate sporulation started after 14 days at both concentrations. The growth and sporulation of the 2:3:4:6 TCNB resistant strain was comparatively unaffected by the fungicide at both concentrations.

Summary of results in Tables 33 to 39.

The effect of the three DCNB isomers on growth, hyphal morphology, and sporulation of the parent, PCNB and TCNB resistant strains varied considerably at both concentrations used, and depended on the origin of the strains. The parent strain showed least, and the 2:3:4:6 TCNB resistant strain, greatest tolerance to these fungicides. The 2:3:4:5 and 2:3:5:6 TCNB resistant strains were intermediate in their tolerance, and the PCNB resistant strain was almost as susceptible as the parent to these fungicides. At 10 mg. level the strains were far more susceptible to the three isomers of DCNB than to PCNB or TCNB isomers. At the same concentration 2,5 DBNB, and 2,5 DINB exerted considerably less effect on growth, hyphal morphology and sporulation of the parent isolate, PCNB,

and TCNB resistant strains than 2,5 DCNB. The parent and 2:3:4:6 TCNB resistant strain showed a graded tolerance to the halogen series of 2,5 substituted nitrobenzenes in the order I > Br > Cl.

2.15 Linear growth, hyphal morphology, and sporulation of parent, PCNB, and TCNB resistant strains of B.allii in presence of 2:3:5:6 tetrachloroaniline

The fungicide, 2:3:5:6 tetrachloroaniline (2:3:5:6 TCA), was used in the vapour phase as described for TCNB and DCNB isomers. This fungicide was prepared by reduction of 2:3:5:6 TCNB. The results obtained are recorded in Table 40.

Preparation of 2:3:5:6 tetrachloroaniline

Six g. 2:3:5:6 TCNB was mixed with 6 g. granulated tin and added to 30 ml. glacial acetic acid. Twelve ml. concentrated acid was added slowly, and the mixture refluxed for two hours. The resulting mixture was diluted with water to 120 ml., and then made strongly alkaline with 50% sodium hydroxide. This mixture was extracted with 40 ml. benzene, and the benzene extract dehydrated with anhydrous magnesium sulphate. The benzene was slowly distilled off to reduce the volume of solvent. 2:3:5:6 tetrachloroaniline was crystallised out from the benzene, and yielded 4.4 g. The aniline was purified by three recrystallisations from light petroleum ether. Colourless feathery needles of 2:3:5:6 TCA were obtained. M.P. 109 - 109.5°. The final yield

was 4.1 g.

Analysis	Found	Calculated
C	32.1	31.2
H	1.7	1.3
N	5.7	6.1
Cl	59.7	61.4
	99.2	100.0

Table 40

Linear growth of parent, PCNB, and TCNB resistant strains of B.allii in presence of 10 mg. 2:3:5:6 TCA

Time after inoculation (days)	Mean colony diameter in cm.				
		Resistant strains			
	Parent	PCNB	2:3:4:5	2:3:5:6	2:3:4:6
2	0.5	0.5	0.5	2.0	1.7
3	0.5	0.5	0.5	3.2 S	2.7 S
6	0.6	0.6	0.8	6.6	6.0
8	0.8 M	1.0	1.2	C	C
14	2.2	3.7	4.3		

S = Start of sporulation

M = First appearance of clearly defined saltant sector

C = Plates covered with mycelia

2:3:5:6 TCA. Parent and resistant strains

The linear growth of the parent isolate, PCNB, and TCNB resistant strains is shown in Table 40.

The 2:3:5:6 TCNB, and 2:3:4:6 TCNB resistant strains were almost unaffected by this fungicide. The hyphae were identical in colour and morphology to those produced by these strains in the absence of fungicide. Good sporulation started after 3 days. The latter growth during the period 4 to 8 days was characterised by the production of fluffy white aerial hyphae. The 2:3:5:6 TCNB resistant strain grew slightly more quickly than the 2:3:4:6 TCNB

resistant strain. The parent, PCNB and 2:3:4:5 TCNB resistant strains all produced abnormal hyphae after a lag period of 3 to 6 days. The hyphae were similar to those first formed by these strains in the presence of TCNB isomers. After 8 days clearly defined saltant sectors with a more rapid growth rate appeared in the parent cultures. The PCNB and 2:3:4:5 TCNB resistant strains did not produce clearly defined saltant sectors, but the hyphae became progressively less abnormal, and the outline of the colonies became scalloped and irregular after 6 to 10 days. Sporulation was not seen in these cultures during the course of the experiment.

Table 40 shows that the 2:3:5:6, and 2:3:4:6 TCNB resistant strains were almost completely resistant to 2:3:5:6 TCA.

2.16 Linear growth, hyphal morphology, and sporulation of parent isolate, and 2:3:4:6 TCNB resistant strain of D.allii in presence of 2.6 dichloro-4-nitroaniline

The fungicide 2,6 dichloro-4-nitroaniline (2,6 DCNA), was used in the vapour phase as described for TCNB and DCNB isomers. The results are recorded in Table 41.

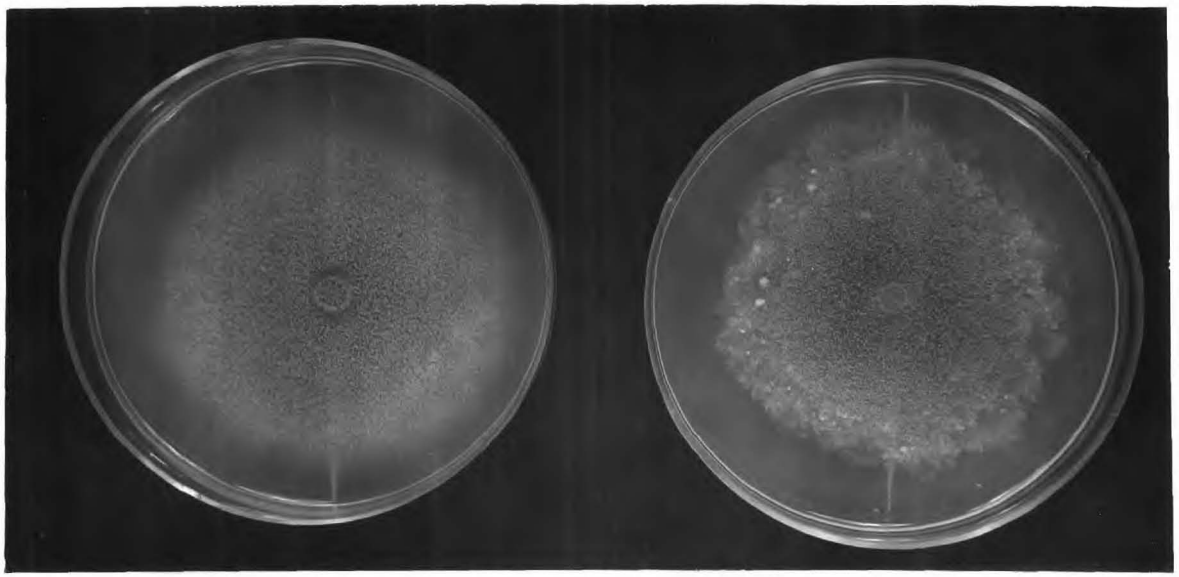


Plate 5

Culture of 2:3:4:6 TCNB resistant strain in 1 mg. 2,6 DCNA after 6 days

Culture of parent isolate in 1 mg. 2,6 DCNA after 6 days

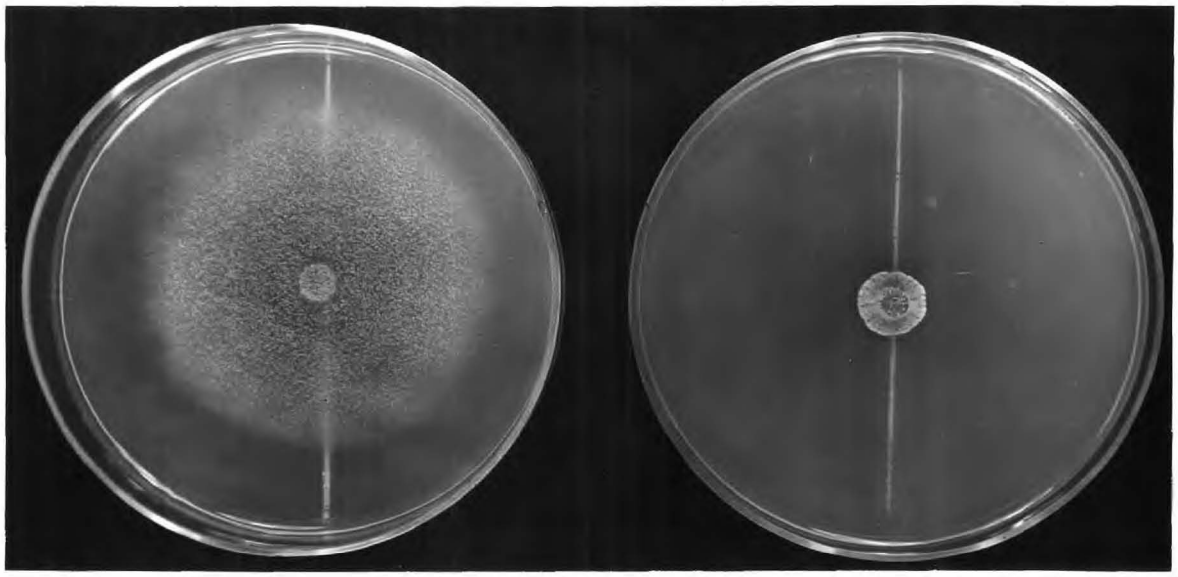


Plate 6

Culture of 2:3:4:6 TCNB resistant strain in 10 mg. 2,6 DCNA after 6 days

Culture of parent isolate in 10 mg. 2,6 DCNA after 6 days

Table 41

Growth of parent and 2:3:4:6 TCNB resistant strain of B. allii in presence of 10 mg. or 1 mg. 2,6 DCNA

Time after inoculation (days)	Mean colony diameter in cm. Parent 10 mg.	Parent 1 mg.	2:3:4:6 TCNB strain 10 mg.	2:3:4:6 TCNB strain 1 mg.
2	0.9	2.6	2.4	2.5
4	1.4	5.2 S	4.4 S	4.9 S
6	1.8	8.1	6.9	7.6
10	2.9 MS	C	C	C
17	5.8			
21	C			

S = Start of sporulation

M = First appearance of clearly defined saltant sector

C = Plates covered with mycelia

2,6 DCNA. Parent isolate and 2:3:4:6 TCNB resistant strain

The growth of parent isolate, and 2:3:4:6 TCNB resistant strain in the presence of 2,6 DCNA is shown in Table 41.

With the 2:3:4:6 TCNB resistant strain, at both concentrations of the fungicide, there was little or no effect upon the rate of growth, hyphal morphology, or sporulation. With the parent isolate at the 1 mg. level there was little effect upon growth rate, hyphal morphology or sporulation compared with controls

in the absence of fungicide. The colonies formed were, however, irregular in outline. Plate 5 shows the parent, and 2:3:4:6 TCNB resistant strain after 6 days growth in the presence of 1 mg. 2,6 DCNA.

At the 10 mg. level, growth of the parent isolate was slow and the hyphae produced were dark brown in colour, frequently branched, and closely interwoven. After 10 days lighter colour fan shaped sectors were produced at the edge of the colonies. Five to 11 of these sectors were produced for each culture. These sectors grew more rapidly than the previous mycelium. Sporulation started after 6 days, sparsely on the growth before sectors appeared, and abundantly on subsequent growth. Plate 6 shows the parent, and 2:3:4:6 TCNB resistant strain after 6 days growth in the presence of 10 mg. 2,6 DCNA.

After 14 days 0.5 cm. discs were taken from the saltant sectors of the parent isolate growing in the presence of 10 mg. 2,6 DCNA. These discs were used to inoculate new Petri dishes, and these cultures were grown in the presence and absence of 2,6 DCNA. The results of this experiment are recorded in Table 42.

Table 42

Linear growth of 0.5 cm. discs removed from cultures of parent isolate of B.allii exposed to 2,6 DCNA in presence of 10 mg. 2,6 DCNA

Time after inoculation (days)	Mean colony diameter in cm.	
	no fungicide	10 mg. 2,6 DCNA
2	1.4	1.2
4	3.1 S	2.5 S
6	5.2	3.8
8	7.4	5.0
10	C	6.6
12		8.8
14		C

S = Start of sporulation

C = Plates covered with mycelia

10 mg. 2,6 DCNA. 2,6 DCNA resistant strain

The results recorded in Tables 41 and 42 show that the 2,6 DCNA resistant strain was more resistant to 10 mg. 2,6 DCNA than the parent isolate, but somewhat less resistant than the 2:3:4:6 TCNB resistant strain. The 2:3:4:6 TCNB resistant strain covered the plates after 8 to 10 days, whereas the 2,6 DCNA resistant strain took 12 days.

2.17 Growth of parent isolate, 2,6 DCNA, and 2:3:4:6 TCNB resistant strains of B.allii in liquid medium

Non-sporulating discs, 0.5 cm. in diameter, of the parent isolate, 2,6 DCNA, and 2:3:4:6 TCNB resistant strains were used to inoculate 250 ml. conical flasks containing 50 ml. glucose peptone medium. Two cultures of the 2:3:4:6 TCNB resistant strain were used; 2:3:4:6 TCNB (1) had been grown in the presence of 2,6 DCNA vapour, and then sub-cultured in the absence of the fungicide; 2:3:4:6 TCNB (2) had not been exposed to 2,6 DCNA vapour. The dry weight of mycelium produced by these shake cultures after varying time intervals was found, and recorded in Table 43.

Table 43

Dry weight of mycelium produced by parent isolate, 2,6 DCNA, and 2:3:4:6 TCNB resistant strains in liquid medium

Time after inoculation (days)	Dry weight of mycelium in g.			
		Resistant strains		
	Parent	2,6 DCNA	2:3:4:6(1)	2:3:4:6(2)
1	0.0202	0.0137	0.0148	0.0166
3	0.1176	0.0904	0.0996	0.1202
5	0.2045	0.1926	0.1975	0.2161
7	0.2272	0.2443	0.2462	0.2386
10	0.2366	0.2427	0.2205	0.2188
14	0.2154	0.2065	0.1966	0.2234

The results recorded in Table 43 show that the dry weight of mycelium produced by these strains was similar. There were no differences in appearance, or behaviour of these cultures.

Table 44

2.18 Growth of parent isolate, 2.6 DCNA, and 2:3:4:6 TCNB resistant strains of B.allii in liquid medium containing 10 mg. 2,6 DCNA

Time after inoculation (days)	Dry weight of mycelium in g.			
			Resistant strains	
	Parent	2,6 DCNA	2:3:4:6(1)	2:3:4:6(2)
3	0.0021	0.0044	0.0511	0.0490
5	0.0064	0.0224	0.0715	0.0630
7	0.0235	0.0842	0.1477	0.1592
10	0.0646	0.2156	0.2530	0.2395
14	0.0932	0.2033	0.2261	0.2308
17	0.0863	0.2161	0.2357	0.1962

Liquid medium containing 10 mg. 2,6 DCNA.
Parent, 2,6 DCNA, and 2:3:4:6 TCNB resistant strains

The dry weight of mycelium produced by these strains in the presence of 10 mg. 2,6 DCNA is shown in Table 44.

The behaviour of the parent isolate, and 2,6 DCNA resistant strain cultures was similar, though more rapid production of mycelial dry weight took place in the 2,6 DCNA resistant strain cultures. Growth from the inoculum was seen

after 3 days. The hyphae produced were pale cream in colour and similar to those produced in the absence of the fungicide. These hyphae were closely interwoven about the original inoculum and formed an almost spherical compact colony. In the parent cultures, a number of small colonies, 40 to 70, and less than 1.5 mm. in diameter appeared after 7 days. The hyphae in these colonies were identical with those in the larger main colony. After 10 to 14 days the main and subsidiary colonies started to develop a black pigment. At this point slight growth was seen at the glass liquid interface. Subsequent growth from the colonies was of pale fawn delicate hyphae. After 12 to 14 days a few conidia were produced, but only at the glass liquid interface. This type of behaviour was shown by the 2,6 DCNA resistant strain cultures. The main and subsidiary colonies turned black after 7 to 10 days. Growth started at the glass liquid interface after 5 days and a few conidia were formed at this surface after 7 days.

The two 2:3:4:6 TCNB resistant strains behaved similarly to each other, but their type of response was completely different from the parent and 2,6 DCNA resistant strain. Growth from the inoculum started after 1 day. The hyphae produced and the colonies formed were identical with those produced by these strains in the absence of fungicide. Smaller subsidiary colonies did not appear in the medium. The mycelium was less compact, and the hyphae were pale fawn brown in colour. Growth and

sporulation started at the glass liquid interface after 3 to 5 days, and the main colony sporulated moderately after 7 to 9 days. The yellow acicular crystals of 2,6 DCNA disappeared from the medium after 2 to 3 days. These crystals were actually enmeshed in the mycelium produced. A similar effect was obtained by dropping cotton wool pellets into shake flasks containing 10 mg. 2,6 DCNA. The parent and 2,6 DCNA resistant strain cultures did not enmesh the fungicide within their mycelium, this probably accounted for the large number of subsidiary colonies in these cultures resulting from mycelial fragments broken from the original inoculum.

Table 44 shows that both the 2:3:4:6 TCNB resistant strains were completely resistant to 10 mg. 2,6 DCNA in liquid medium. Previous exposure to 2,6 DCNA vapour did not give the 2:3:4:6 TCNB resistant strain any advantage over the 2:3:4:6 TCNB resistant strain which had not been exposed to 2,6 DCNA. The 2:3:4:6 TCNB resistant strains grew more rapidly, and produced more dry weight of mycelium than the parent or 2,6 DCNA resistant strain. The 2,6 DCNA resistant strain grew more rapidly than the parent, and produced a maximum dry weight of mycelium, 0.2156 g., after 10 days, compared with the parent, 0.0982 g., after 14 days. Strains of B.allii adapted to 2:3:4:6 TCNB were completely resistant to 2,6 DCNA in liquid medium. Strains adapted to 2,6 DCNA vapour were more resistant to 2,6 DCNA in liquid medium than the parent, but less resistant than the 2:3:4:6 TCNB resistant strain.

2.19 Linear growth, hyphal morphology, and sporulation of parent isolate, and 2:3:4:6 TCNB resistant strain of B.allii in presence of benzene vapour

The behaviour of the parent isolate, and 2:3:4:6 TCNB resistant strain was investigated in benzene vapour; 0.5 cm. non-sporulating discs of these strains were used to inoculate Petri dishes, which were then immediately placed in benzene vapour. The Petri dish lids were supported by three sterile glass rings 1.0 cm. in diameter and 2.0 cm. high, inserted in the agar at the edge of the dishes. This enables the benzene vapour to circulate freely over the surface of the plates. Eight replicates of the parent and 2:3:4:6 TCNB resistant strain were placed in a 17.5 litre desiccator and cooled benzene added to give the required concentration of benzene vapour. In the first experiment recorded in Table 45, the concentration of benzene vapour was increased from 1,000 p.p.m. to 2,000 p.p.m. after 4 days.

The results of these experiments with benzene vapour are recorded in Tables 45 and 46.

Table 45.

Linear growth of parent, and 2:3:4:6 TCNB resistant strain of B.allii in 1,000 and 2,000 p.p.m. benzene vapour

Time after inoculation (days)	Mean colony diameter in cm. Parent	2:3:4:6 TCNB resistant strain	Benzene vapour p.p.m.
1	1.2	1.1	1,000
2	2.2	2.0	1,000
3	4.1	3.4	1,000
4 *	5.3 S	4.1 S	1,000
5	5.5	5.4	2,000
6	5.8	6.7	2,000
7	6.0	7.9	2,000
8	6.6 M	8.4	2,000
9	7.1	C	2,000

S = Start of sporulation

C = Plates covered with mycelia

M = First appearance of clearly defined saltant sectors

* = Benzene concentration increased from 1,000 to 2,000 p.p.m.

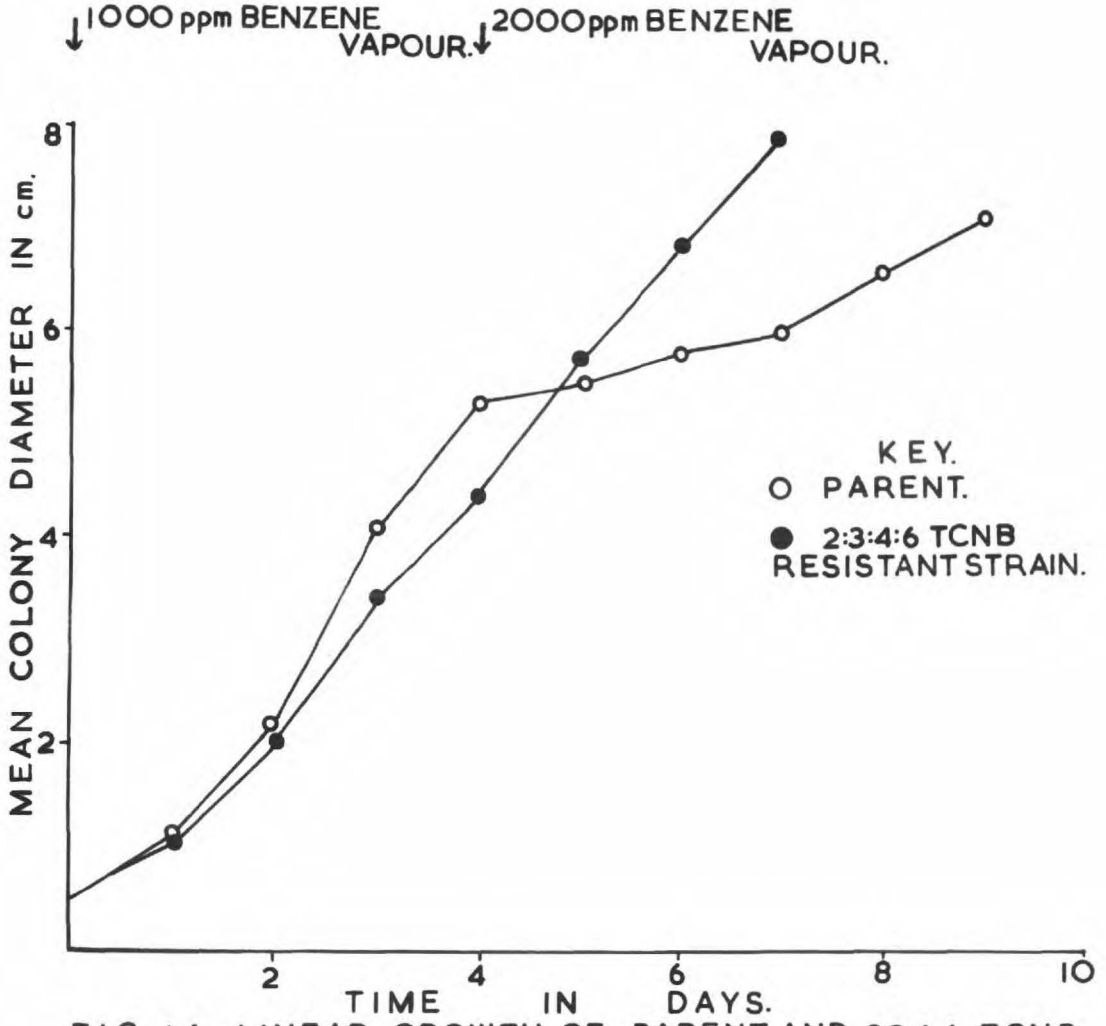


FIG. 14. LINEAR GROWTH OF PARENT, AND 2:3:4:6 TCNB RESISTANT STRAIN OF B. ALLII IN PRESENCE OF BENZENE VAPOUR

1,000 and 2,000 p.p.m. benzene vapour. Parent, and 2:3:4:6 TCNB resistant strain

Table 45 shows the effect of benzene vapour on the linear growth of parent isolate, and 2:3:4:6 TCNB resistant strain. The results are also graphically shown in Fig. 14.

At a concentration of 1,000 p.p.m. benzene vapour, the growth rate, morphology of hyphae, and appearance of colonies were similar to those in the absence of benzene. The 2:3:4:6 TCNB resistant strain was not noticeably affected by increasing the concentrations to 2,000 p.p.m. The parent isolate was, however, affected. The rate of growth was reduced and the hyphae produced were darker in colour and somewhat abnormal. The hyphae were twisted and gnarled and resembled those produced by the parent in the presence of TCNB isomers. After 4 days at the increased concentration of benzene vapour numerous fan shaped sectors appeared in each culture of the parent isolate. The hyphae in these sectors were paler in colour than those produced previously, but did not show morphological abnormalities. Sporulation started after 4 days in both series, but was less in the parent cultures than in the 2:3:4:6 TCNB resistant strain cultures.

Table 46

Linear growth of parent, and 2:3:4:6 TCNB resistant strain of B.allii in presence of 4,000 p.p.m. benzene vapour

Time after inoculation (days)	Mean colony diameter in cm. Parent	2:3:4:6 TCNB resistant strain	Benzene vapour p.p.m.
1	0.7	0.9	4,000
2	0.9	1.7	4,000
3	1.1	2.4	4,000
4	1.2	3.3 S	4,000
5 *	1.4	4.0	4,000
6	2.2	5.1	-
7	3.3	5.9	-
8	4.3	6.8	-
9	6.0	7.9	-

S = Start of sporulation

* = Plates removed from benzene vapour

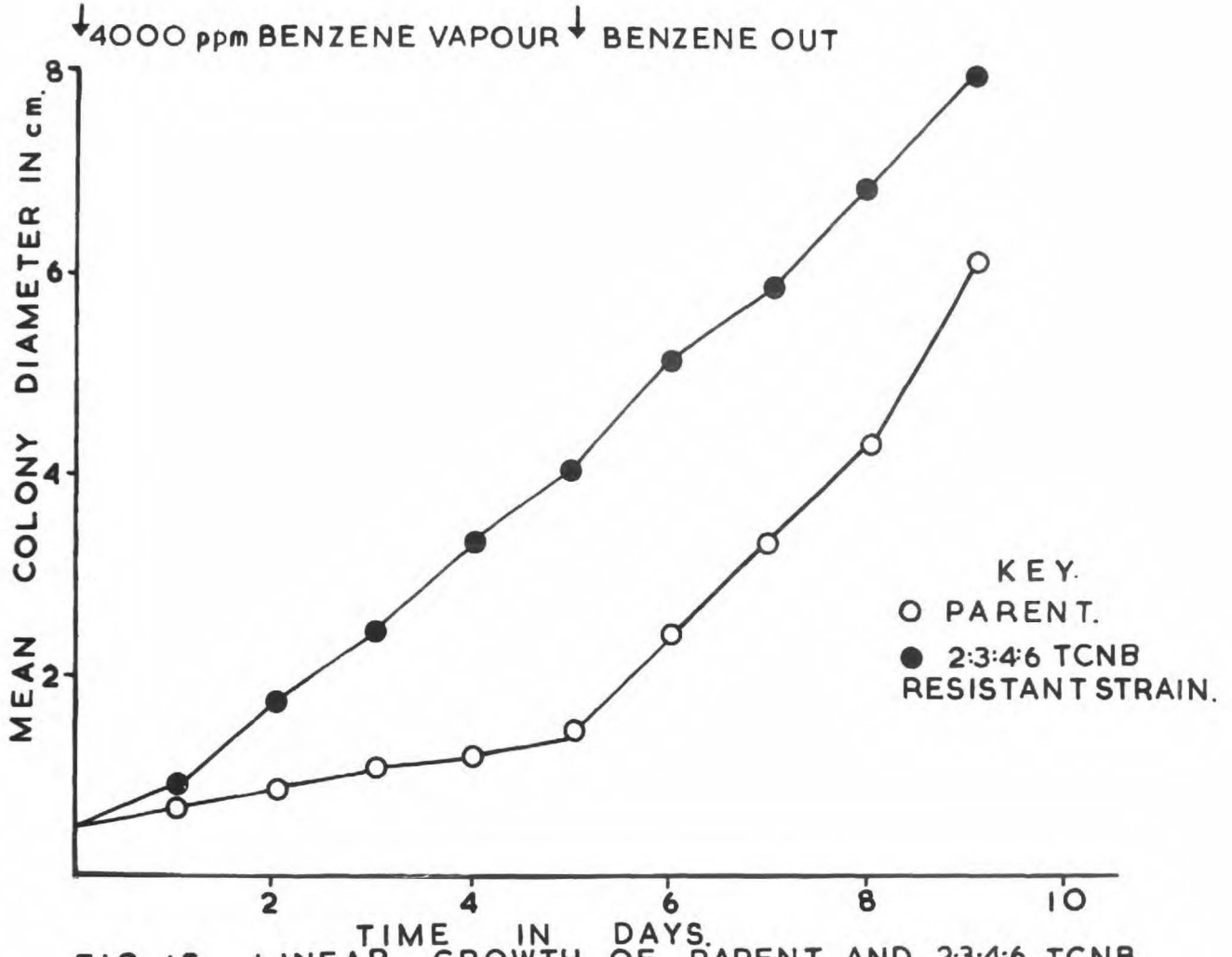


FIG. 15. LINEAR GROWTH OF PARENT, AND 2:3:4:6 TCNB RESISTANT STRAIN OF B.ALLII IN PRESENCE OF BENZENE VAPOUR.

4,000 p.p.m. benzene vapour. Parent, and 2:3:4:6 TCNB resistant strain

Table 46 shows the effect of benzene vapour at a concentration of 4,000 p.p.m. on the linear growth of parent and 2:3:4:6 TCNB resistant strain. The results are also graphically shown in Fig. 15.

Table 46 shows that the rate of growth of the 2:3:4:6 TCNB resistant strain was reduced at this concentration. The appearance of the 2:3:4:6 TCNB resistant strain colonies was identical with that in the previous experiment. The parent isolate grew more slowly, the hyphae were gnarled, twisted, and closely interwoven. Few aerial hyphae were produced, and the colour of the hyphae was darker. Sporulation was reduced, but was seen after 5 days. No saltant sectors appeared in these cultures either during the benzene vapour regime, or after removal to a benzene free atmosphere. After removal from benzene vapour the growth rate rapidly increased, the hyphae produced were normal, and sporulation was abundant. One distinguishing characteristic of these cultures removed from the benzene atmosphere, was the fluffy white aerial mycelium produced on the subsequent growth.

Summary of results, Tables 45 and 46

Benzene vapour at 1,000, 2,000 and 4,000 p.p.m. had little or no effect on growth rate, hyphal morphology and sporu-

lation of the 2:3:4:6 TCNB resistant strain.

Benzene vapour at 1,000 p.p.m. did not affect the growth rate of the parent isolate. At higher concentrations the rate of growth was slower, and abnormal hyphae were formed. Saltant sectors with a higher growth rate than the parent were produced at a concentration of 2,000 p.p.m. benzene vapour. The effects upon hyphal morphology, and rate of growth of non-saltant mycelium were not permanent, but disappeared after transfer to a benzene free atmosphere. At concentrations of benzene vapour which affected the growth and hyphal morphology of the parent, the 2:3:4:6 TCNB resistant strain was highly resistant.

The experiments with PCNB and TCNB resistant strains were carried out over a period of 18 months. During this period there was no reversion of these strains to the susceptibility of the parent, when grown in the presence of PCNB or TCNB vapour.

2.20 Examination of extracts of parent and 2:3:5:6 TCNB resistant strain mycelium for presence of 2:3:5:6 TCA

The results in section IV. 2.15 showed that when the 2:3:5:6 TCNB resistant strain of B.allii was grown on agar in the presence of 10 mg. 2:3:5:6 TCA, the strain was completely resistant to this fungicide, whereas the parent isolate, PCNB, and 2:3:4:5 TCNB resistant strains were susceptible. If the 2:3:5:6 TCNB resistant strain reduced the nitro group of 2:3:5:6 TCNB to an amino group, this might account for the resistance of this strain to 2:3:5:6 TCA vapour. The following experiment was therefore carried out to try and detect the presence of 2:3:5:6 TCA in alcoholic extracts of mycelium and culture medium after growth by the parent isolate and 2:3:5:6 TCNB resistant strain in liquid medium containing 2:3:5:6 TCNB.

Large culture vessels containing 500 ml. glucose peptone medium and 5 g. 2:3:5:6 TCNB were seeded with 10 non-sporulating discs of the parent or 2:3:5:6 TCNB resistant strain. These cultures were allowed to grow for 8 weeks. After 8 weeks the mycelium was separated from the medium by filtration through several layers of muslin, thoroughly washed with distilled water, air dried, placed in a Sohxlet thimble, and then extracted by refluxing with 100 ml. ethyl alcohol for 24 hours. The alcohol extract was then reduced to approximately 5 ml. by evaporation.

The following test was found to be characteristic for

2:3:5:6 TCA, and was applied to 1 ml. portions of the alcohol extract, and to alcohol extracts of culture medium.

0.001 g. 2:3:5:6 TCA was dissolved in 1 ml. ethyl alcohol, and 0.1 ml. 10 N. hydrochloric acid added. This was diluted to 5 ml. and cautiously boiled for 2 to 3 minutes. The mixture was cooled to 10°C with constant stirring. 0.001 g. sodium nitrite in 1 ml. of water was added to this mixture over 30 seconds, with constant stirring. The solution was rapidly filtered, and added to 0.005 g. β -naphthol dissolved in 1 ml. 4 N. sodium hydroxide. An orange brown colour and faint precipitate formed. This was thought to be 2:3:5:6 tetrachlorobenzene-azo-β -naphthol. The results of this experiment are summarised in Table 47.

Table 47

Examination of mycelium and medium extracts for 2:3:5:6 TCA

	2:3:5:6 TCNB resistant strain		Parent		2:3:5:6 TCNB	2:3:5:6 TCA
	mycelium	medium	mycelium	medium		
Reaction	-ve	-ve	-ve	-ve	-ve	+ve

-ve = no orange brown colour

+ve = orange brown colour

The results recorded in Table 47 show that there was no evidence that the parent, or 2:3:5:6 TCNB resistant strain reduced 2:3:5:6 TCNB to 2:3:5:6 TCA.

2.21 Chromatographic examination of carbohydrates in extracts of mycelium and culture filtrate of parent, PCNB, and TCNB resistant strains grown in absence and presence of PCNB or TCNB

This experiment was carried out to determine whether qualitative carbohydrate differences existed in extracts of culture medium and mycelium, when parent, PCNB, and TCNB resistant strains of B.allii were grown in glucose peptone medium containing PCNB or TCNB.

250 ml. conical flasks containing 50 ml. medium were seeded with non-sporulating discs of parent and resistant strains. Each strain was also grown in the presence of 10 mg. PCNB or TCNB. After varying intervals the fungal growth was separated from the medium by filtration through muslin. The filtrate was concentrated to approximately 5 ml. and spotted on No. 1 Whatman chromatography paper. The mycelium was air dried and then extracted with 50% ethyl alcohol by refluxing for 24 hours. The alcohol extract was concentrated to approximately 5 ml. and spotted on chromatography paper. Descending chromatograms were run using propanol/ethyl acetate/water, 7:1:2, as solvent. The following solutions of carbohydrates were used as markers.

1% glucose

1% fructose

2% sucrose

2% mannose

3% ribose

The chromatograms were run for 24 hours at room temperature, dried, sprayed with benzidine trichloroacetic acid, and developed at 100 to 110°C for 3 to 4 minutes. No qualitative differences were found between the parent isolate and resistant strains. Hewlett (1955), found ribose in mycelial extracts of Botrytis cinerea grown in the presence of 2:3:5:6 TCNB, but this carbohydrate was not found in these experiments. The chromatograms showed quantitative carbohydrate differences associated with different rates of utilization of carbohydrate in the medium, by the parent and resistant strains.

3.0 Growth, hyphal morphology, and sporulation of Trichoderma viride in presence of captan

0.5 cm. diameter, non-sporulating discs of T.viride were used to inoculate Petri dishes containing 20, 80, and 500 p.p.m. pure captan dispersed in glucose peptone agar. Captan was added to 200 ml. molten glucose peptone agar contained in medical flats and thoroughly shaken to disperse the fungicide. The agar was poured into Petri dishes and any plates showing uneven dispersion of fungicide were rejected. The results of this experiment are recorded in Table 48.

Table 48

Linear growth of T.viride in presence of 20, 80, and 500 p.p.m. captan

Time after inoculation (days)	Mean colony diameter in cm. Captan p.p.m.			
	Control	20	80	500
1	1.9	0.6	0.6	0.5
2	3.8 S	1.6	1.2	0.5
3	5.6	2.7 S	1.9	0.5+
4	7.3	4.1	2.5 S	0.5+
5	8.8	6.0	3.3	0.5+
6	C	7.5	4.9	0.6
7		C	6.1	0.7
9			7.4	0.8
11			C	0.9* S
13				1.0
25				1.9

Mean daily growth rate in cm./day

Control	Captan p.p.m. 20	80	500
1.7	1.2	0.8	0.06

\+ = Slight growth, but less than 0.05 cm.

S = Start of sporulation. Spores pigmented green

*S = Start of sporulation. Spores pigmented yellow

C = Plates covered with mycelia

Table 48 shows the linear growth of T.viride in agar containing 20, 80, and 500 p.p.m. captan.

There was a noticeable difference between the appearance of mycelium at 20 and 80 p.p.m., and that at 500 p.p.m. At the lower two concentrations, the mycelium was sparse, but hyphal morphology was unaffected. The mycelium was mainly prostrate, but occasional aerial hyphae were formed. Good sporulation started uniformly after 3 to 4 days, and the conidia were pigmented green. At 500 p.p.m. the hyphae were frequently branched and closely interwoven. Aerial hyphae were absent, and the adpressed surface of the mycelium appeared slimy. Uneven sporulation started after 11 days. The conidia were initially coloured yellow, but changed to green after 4 to 7 days. The colonies were circular in outline and growth was regular. No saltant sectors appeared during the course of the experiment. The results in Table 48 show that increased concentration of captan reduced the rate of growth, and retarded sporulation. The mean daily growth rate at 80 p.p.m. was not very different from that of the control or 20 p.p.m. cultures. The mean daily growth rate of T.viride on agar containing 500 p.p.m. captan was much lower than the control.

Growth, hyphal morphology, and sporulation of T.viride in presence of 1,000 and 10,000 p.p.m. captan.

0.5 cm. discs of T.viride were taken from control cultures after 3 days, and from cultures growing on 500 p.p.m. captan after 4 weeks, and used to inoculate agar containing 1,000 and 10,000 p.p.m. captan. The results are recorded in Table 49.

Table 49

Linear growth of T.viride on agar containing 1,000 or 10,000 p.p.m. captan

Time after inoculation (days)	Mean colony diameter in cm. Untrained		Captan "trained"	
	1,000	10,000	1,000	10,000
5	0.5	0.5	0.5	0.5
7	0.5	0.5	0.6	0.6
9	0.6	0.5	0.8 S	0.7 S
12	0.6	0.6	0.8 D	0.7 D
16	0.7	0.6	0.9	0.8
20	0.7 D	0.6 D	1.2	1.0
26	0.8 S	0.7 S	1.6 M	1.1
38	1.0	0.8	2.0	1.3 M
62	1.8	1.4	4.7	3.0
Mean daily growth rate cm./day from start of experiment	0.02	0.01	0.07	0.04

S = Start of sporulation M + First appearance of sectors

D = Discoloured zone present outside limit of radial growth

Table 49 shows that the mean daily growth rate of the untrained and captan "trained" mycelium was different. The captan "trained" mycelium grew three times as quickly as the untrained mycelium at comparable concentrations of captan. Growth of the captan "trained" mycelium started after 7 days, compared with 9 to 12 days for the untrained. Hyphal morphology of untrained and captan "trained" mycelium was similar at both concentrations. The first formed hyphae were pale cream in colour and were composed of short broad cells with thick walls. The hyphal tips were swollen. These hyphae branched frequently and were closely interwoven, but aerial hyphae were absent. The dense mycelium was closely adpressed to the agar surface, and appeared slimy in surface view. The hyphal morphology and appearance of colonies altered during growth. After 9 to 20 days a few aerial hyphae appeared, and the hyphae of the prostrate mycelium were less evident. A yellow brown discolouration of the agar was seen after 12 days with captan "trained" cultures, and after 16 days with the untrained cultures. This discolouration extended 1 to 2 cm. beyond the margin of growth. After 38 days the captan "trained" cultures showed several irregular sectors arising at the edge of the colonies, and these were considered to be saltant sectors. These sectors produced a considerable aerial mycelium, but the growth rate of the prostrate mycelium was the same as that of the surrounding non-saltant mycelium. These sectors did not sporulate during the course of the experiment.

Sporulation was retarded in both sets of cultures at the two concentrations. The spores produced by both captan "trained" and untrained mycelium were usually green in colour, but some yellow conidia were formed which slowly became green.

After 10 weeks growth, 0.5 cm. discs of non-saltant mycelium were taken from the 10,000 p.p.m. captan Petri dishes and sub-cultured on fresh 10,000 p.p.m. captan agar. The results of this experiment are shown in Table 50.

Table 50

Continued growth of captan "trained" mycelium of T.viride on agar containing 10,000 p.p.m. captan

Time after inoculation (days)	Mean colony diameter in cm.
	10,000 p.p.m. captan
2	0.6
8	0.9
12	1.3
17	2.2
38	4.7

Mean daily growth rate in cm./day from start of experiment 0.11

Table 50 shows that the mean daily growth rate of captan "trained" mycelium had increased from 0.04 to 0.11 cm./day when grown in the presence of 10,000 p.p.m. captan dispersed in agar. Occasional sectors appeared but were all of the type described

in the previous experiment. No fast growing saltants appeared in any culture.

3.1 Growth of captan "trained" mycelium of Trichoderma viride on agar containing captan analogues

0.5 cm. discs of the captan "trained" mycelium were taken from the previous 10,000 p.p.m. captan agar cultures after 5 weeks and used to inoculate Petri dishes containing 500 p.p.m. captan, or captan analogue dispersed in agar. The three analogues investigated were;

N-trichloromethylmercapto-4-nitrophthalimide (N)
N-trichloromethylmercapto-3,6-endoxohexahydrophthalimide (E)
N-trichloromethylmercapto-4-methyl,hexahydrophthalimide (H)

The results of this experiment are recorded in Table 51.

Table 51

Linear growth of parent isolate and captan "trained" mycelium of T.viride in presence of 500 p.p.m. captan or captan analogue

Time after inoculation (days)	Mean colony diameter in cm. Control		(H)		(E)		(N)		Captan	
	P	CT	P	CT	P	CT	P	CT	P	CT
1	1.7	0.7	0.5	0.7	0.5	0.5	0.5	0.5	0.5	0.5
2	3.4	0.9	0.5	1.4	0.5	0.5	0.5	0.7	0.5	0.6
3	5.3	1.3	0.7	2.2	0.5	0.5	0.7	0.9	0.6	0.8
4	6.8	2.6	0.9	3.0	0.5	0.5	0.9	1.0	0.7	0.8
5	8.2	3.8	1.3	4.1	0.5	0.5	1.0	1.1	0.8	0.9
6	C	5.7	1.7	4.9	0.5	0.5	1.2	1.2	0.8	1.0
7		7.4	2.3	5.7	0.5	0.5	1.3	1.4	0.9	1.1
9		C	3.0	7.4	0.5	0.5	1.6	1.9	0.9	1.4
11			3.8	C	0.5	0.5	1.8	2.5	1.0	1.8
13			4.4		0.5	0.5	1.9	3.0	1.1	2.3
16			5.6		0.5	0.5	2.2	4.2	1.4	3.0
18			6.3		0.5	0.5	2.4	5.2	1.6	3.7
23			8.4		0.5	0.5	2.8	6.5	2.2	5.3
Mean daily growth rate in cm./day from start of experiment	1.5	1.0	0.3	0.8	-	-	0.1	0.3	0.1	0.2

P = Parent isolate of T.viride

CT = Captan "trained" strain of T.viride

C = Plates covered with mycelia

The results recorded in Table 51 show that for each fungicide where growth was seen, the mean daily growth rate of the captan "trained" mycelium was greater than that of untrained mycelium. The captan "trained" strain grew somewhat less rapidly than that of parent isolate, but this may have been due to residual captan in the inoculum, as subsequent sub-cultures of the 10,000 p.p.m. captan "trained" mycelium showed no difference in the growth rate from that of parent isolate.

No growth was observed in cultures of either captan "trained" mycelium, or parent isolate on agar containing 500 p.p.m. N-trichloromethylmercapto -3,6-endoxohexahydrophthalimide. The results showed that captan "trained" mycelium was more resistant to the analogues, N-trichloromethylmercapto-4-nitrophthalimide, and N-trichloromethylmercapto-4-methyl, hexahydrophthalimide, than parent mycelium.

3.2 Growth of captan "trained" mycelium of T.viride in presence of captan after sub-culture in absence of fungicide

This experiment was carried out to find whether the mean daily growth rate of the captan "trained" mycelium grown on agar containing 10,000 p.p.m. captan remained the same for growth on 10,000 p.p.m. captan agar after 8 weeks sub-culture in the absence of captan, or was reduced to the level of untrained mycelium.

0.5 cm. discs were taken from the 10,000 p.p.m. captan "trained" mycelium and sub-cultured for 8 weeks in absence of fungicide; 0.5 cm. discs were then removed from the last sub-culture and used to inoculate Petri dishes containing 10,000 p.p.m. captan dispersed in agar. The mean daily growth rate recorded was 0.02 cm./day, compared with 0.11 cm./day, Table 50, for previous growth on 10,000 p.p.m. captan agar. The value for the mean daily growth rate was, however, still greater than that for untrained mycelium, which was 0.01 cm./day. This experiment was repeated with similar results. These results show that the ability of captan "trained" mycelium to grow more rapidly than untrained mycelium on captan agar is lost, or greatly reduced after sub-culture in the absence of captan.

3.3 Growth of non-sporulating mycelium of T.viride in presence and absence of captan

During the course of the experiment recorded in Table 49, non-sporulating sectors appeared. 0.5 cm. discs were removed from these sectors and sub-cultured in the absence of captan for 1 week; 0.5 cm. discs were then removed and used to inoculate Petri dishes containing 20 p.p.m. captan dispersed in agar. The results obtained are recorded in Table 52.

Table 52

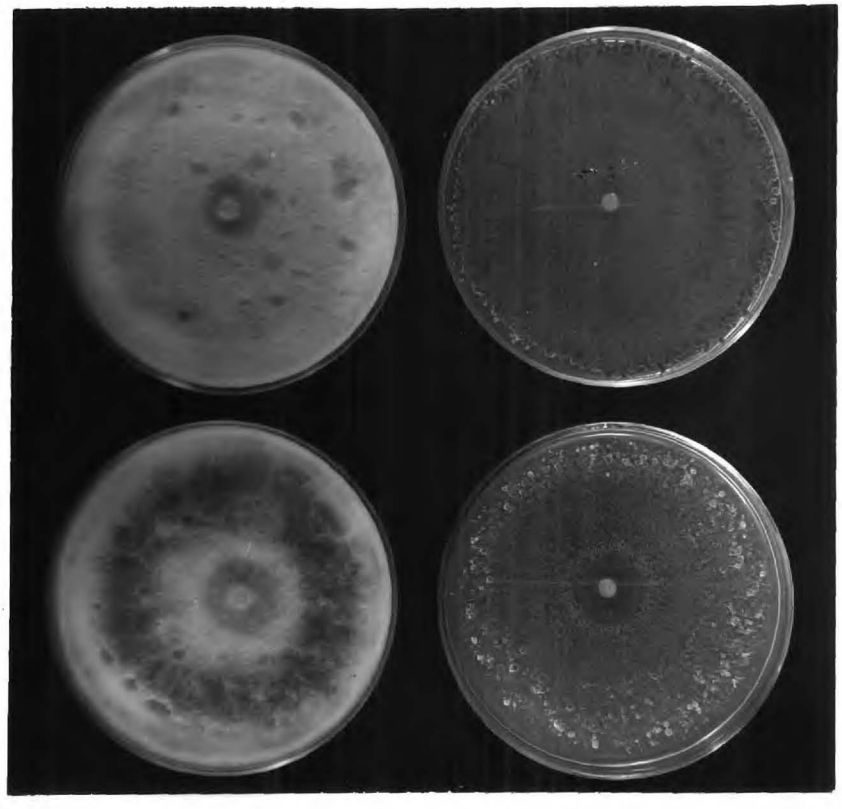
Growth of non-sporulating mycelium of T.viride on agar medium containing 20 p.p.m. captan

Time after inoculation (days)	Mean colony diameter in cm.			
	Captan free agar		20 p.p.m. captan	
	P	NSM	p	NSM
1	1.7	1.8	0.7	0.6
2	3.5 S	3.7	1.7	1.5
3	5.4	5.7	2.8 S	2.9
5	8.3	C	5.2	5.5
7	C		8.5	C

P = Untrained mycelium

NSM = Non-sporulating mycelium

C = Plates covered with mycelia



A. Non-sporulating strain B. Parent isolate

Plate 7

Parent isolate, and non-sporulating strain of T.viride on 20 p.p.m. captan agar, and captan free agar, after 6 days growth.

Table 52 shows that the growth rate of parent isolate, and non-sporulating strain on 20 p.p.m. captan agar and on captan free agar were similar. The main differences between the behaviour of the two strains was the predominantly aerial habit of the non-sporulating strain, and the absence of sporulation, compared with the parent isolate.

Summary of results Tables 48 to 52

Trichoderma viride survived and grew at concentrations of captan up to 10,000 p.p.m. incorporated in glucose peptone agar. The rate of growth of untrained mycelium was lower, the higher the concentration of captan. At 20 p.p.m. there was little or no effect on growth rate, hyphal morphology or sporulation. At 10,000 p.p.m. the growth rate was slow, hyphae were abnormal, and sporulation was retarded. Resistant saltants with a higher growth rate than captan "trained" mycelium did not appear during the course of the experiments. Saltants were produced which formed a predominantly aerial mycelium but did not sporulate either in presence or absence of captan. The higher growth rate of "trained" mycelium, compared with untrained mycelium in the presence of 10,000 p.p.m. captan was lost after sub-culture in the absence of captan. Captan "trained" mycelium grew more quickly than untrained mycelium in the presence of two captan analogues. The N-trichloromethylmercapto-3,6-endoxohexahydrophthalimide at 500 p.p.m. prevented growth of captan "trained", and untrained mycelium.

3.4 Observations on old cultures of T.viride growing in the presence of captan

It was found that old cultures of captan "trained" mycelium of T.viride which had been growing on 10,000 p.p.m. captan agar for several months often smelled strongly. Rich (1959), showed that 10^{-2}M l-cysteine antagonized the toxicity of captan at 3 x 10^{-5}M in liquid medium to Sclerotinia fructicola. Captan reacts with l-cysteine in vitro to give cystine, tetrahydrophthalimide, hydrogen sulphide, carbon disulphide, 2-thiazolidine thione-4-carboxylic acid and hydrochloric acid. The odour of old cultures of T.viride may have been due to the presence of a number of volatile products, but a gas train was made to try and detect the presence of hydrogen sulphide. The apparatus constructed is shown in Fig. 16.

Gas train for detection of hydrogen sulphide

A stream of air was pumped through the apparatus by means of a small "Dynax" pump. A 'T' junction with stopcock (B), allowed a crude regulation of the air stream. The first units (C and C'), were gas washing bottles which contained 100 ml. of 2.5% cadmium acetate with 0.1 ml. of acetic acid. These removed hydrogen sulphide from the incoming air. A second 'T' junction (D), with capillary stopcock allowed a fine regulation of the air stream. Moisture and cadmium acetate spray were removed by two 'U' tubes (F and F'), containing calcium chloride

(8 - 14 mesh). A 'U' tube (G) containing silica gel ($\frac{1}{4}$" to 6 B.S.S.), indicated a dry air stream. The air stream then passed into a saturator (J), which consisted of a roll of chromatography paper, kept moistened by a small water reservoir (L). The air stream then passed through a test chamber (M). This chamber consisted of two pieces of glass tubing (internal diameter 2.5 cm.) with ground glass opposing flanges. A lead acetate paper for detection of hydrogen sulphide was firmly held between these flanges by spring clips. The culture vessel (O), was a 150 ml. conical flask with ground glass neck joint fitted with a gas bubbling head. A second test chamber (P), was placed after the culture chamber. The two test chambers were wrapped in aluminium foil to exclude light. The apparatus ended in a bubbler (Q), containing butyl phthalate. All the glass joints were butted together with polythene tubing.

The lead acetate test papers were prepared in the following manner.

Preparation of lead acetate test papers

Whatman No. 1 filter papers (4.25 cm. circles) were found to be suitable for wet strength and gas porosity. The test circles were prepared by immersion in a 6.5% aqueous lead acetate solution containing 10% glycerol. These circles were drained and allowed to dry by hanging in a desiccator over calcium chloride. The papers were then stored in a 1,500 ml. desiccator over 5 ml. water until required.

The circles were tested in the apparatus and found to detect 1 p.p.m. hydrogen sulphide. A brown stain of lead sulphide appeared when hydrogen sulphide was present. This stain was uniform and not restricted to the downward side of the gas stream.

This apparatus allowed a hydrogen sulphide free moist air stream to reach the culture chamber at a steady rate.

Key to Fig. 16

B .....Stopcock for air regulation

C & C' Cadmium acetate bubblers

D .....Stopcock for fine regulation of air stream

F & F' 'U' tubes containing calcium chloride

G .....' U' tube containing silica gel

J .....Saturator containing roll of filter paper

L .....Water reservoir

M & P ...Test chambers

O .....Culture chamber

Q .....Gas bubbler containing butyl phthalate

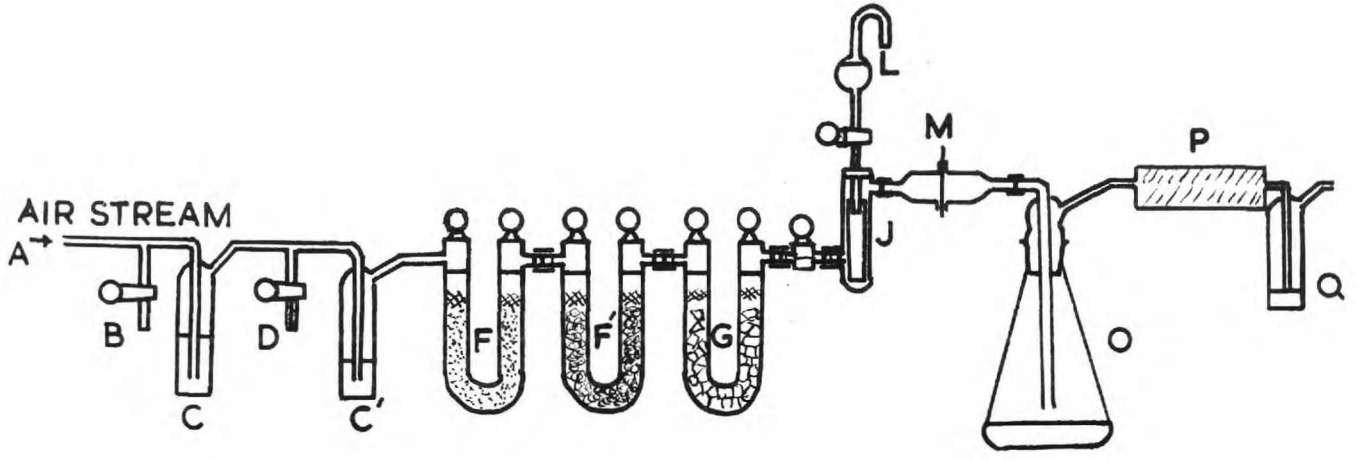


FIG. 16. GAS TRAIN FOR DETECTION OF HYDROGEN SULPHIDE.

Examination of parent and 10,000 p .p.m. captan "trained" mycelium of T.viride growing in presence of 10,000 p.p.m. captan for presence of hydrogen sulphide.

1.0 cm. diameter discs of captan "trained" mycelium of T.viride were removed from cultures containing 10,000 p.p.m. captan after 3 months growth. Similar discs were taken from captan free cultures of the parent after 5 days growth. The discs were transferred to 150 ml. flasks containing 50 ml. 10,000 p.p.m. captan agar. When growth started from the inoculum, the culture chamber was placed in the apparatus and allowed to grow for several weeks. At fortnightly intervals the test circles enclosed in the test chambers (M) and (P), were removed and examined for lead sulphide. The results of these experiments are summarized in Table 53.

Table 53

Examination of T.viride cultures growing on 10,000 p.p.m. captan agar for presence of hydrogen sulphide

Culture chamber	Time after inoculation (weeks)							
	2		4		6		8	
	T1	T2	T1	T2	T1	T2	T1	T2
10,000 p.p.m. captan "trained" mycelium	-ve	-ve	-ve	-ve	-ve	FS	-ve	+ve
Parent	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Blank no inoculum	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

T1 First test circle

T2 Second test circle

-ve Negative test

+ve Positive test

FS Faint stain

The results recorded in Table 53 show that in all tests hydrogen sulphide free air reached the culture chamber. No hydrogen sulphide was detected in the blank test, or in the test with parent mycelium. A faint shadow was seen with the 10,000 p.p.m. captan "trained" mycelium after 6 weeks, and a definite test was seen after 8 weeks. The results show that hydrogen sulphide was not given off by captan, or parent mycelium, but trace quantities were given off by captan "trained" mycelium. The minute quantities evolved were not considered to be of significant importance.

V. DISCUSSION

In this investigation on the effect of the vapour of pentachloronitrobenzene and tetrachloronitrobenzenes on Botrytis allii it was found that more than one type of resistance developed. B.allii became adapted to these substances either by producing non-saltant mycelium with a higher growth rate than that of the parent isolate, or by producing resistant saltants with a much higher growth rate than that of the parent isolate, in the presence of these substances.

The vapour of these chlorinated nitrobenzenes retarded growth, suppressed or reduced sporulation, and produced morphological abnormality in hyphae. These compounds were thus fungistatic, rather than fungitoxic.

In the investigation of the effect of captan on Trichoderma viride it was found that non-saltant mycelium could be"trained" to grow more quickly than the parent in high concentrations of captan, saltant mycelium was produced, but was unable to sporulate either in the presence or absence of captan. This saltant mycelium did not grow more quickly than "trained" mycelium produced under the same conditions.

The results obtained will now be considered more fully.

The production of mycelium resistant to the vapours of pentachloronitrobenzene or tetrachloronitrobenzenes depended upon the

ability to survive the initial period of treatment, when growth was indiscernible. The growth of B.allii in the presence of these vapours was retarded, and the hyphae initially produced were swollen and distorted. Resistant saltants were produced and these were characterized by a much higher growth rate than the parent isolate when grown in the presence of the vapours. The distinction between saltant and non-saltant mycelium was also clearly evident in appearance of hyphae. The morphological characters of saltant hyphae were comparatively unaffected by the vapours. Growth was reduced in B.allii, according to the amount of pentachloronitrobenzene or tetrachloronitrobenzene present. This gradation was always evident, but the reason was not apparent. Hewlett (1955), calculated that the amount of these substances used to saturate the air present in 9.0 cm. Petri dishes with pentachloronitrobenzene, or 2:3:5:6 tetrachloronitrobenzene was only 5×10^{-5} mg. The lowest concentration used in these experiments was 0.1 mg., considerably in excess of this value, but higher concentrations showed an increased effect upon the test organism. The fungicides were purified by recrystallisation and it is unlikely that any impurities were present to influence this gradation.

The parent isolate of B.allii behaved in the same manner to the vapours of pentachloronitrobenzene or tetrachloronitrobenzene, but the degree of effect on hyphal morphology, rate of growth, and sporulation was different

according to the vapour used. The resistant saltants were all characterized by a paler colour, and a higher growth rate than the parent isolate, when grown in the presence of the vapours. These substances always reduced or suppressed sporulation on saltant and non-saltant mycelium. This effect was not permanent, as both types of mycelium sporulated normally when sub-cultured in the absence of fungicide vapour. The effect of equivalent amounts of the fungicides on sporulation and growth was different, and this may have been due to the relative ease with which the vapours were able to penetrate the hyphae.

The resistant saltants produced in the presence of 2:3:4:5 TCNB, 2:3:5:6 TCNB, 2:3:4:6 TCNB, and PCNB vapour were also resistant in varying degrees, to each of the other three substances. This evidence of a common adaptation mechanism suggests that these vapours exert their effect by a similar action. The resistant saltants did not, however, show the same tolerance to each of the other substances. It is possible that the resistant saltants were in some way specifically adapted to the vapour with which they were initially treated. The PCNB, and 2:3:4:6 TCNB resistant strains, sub-cultured from the saltant sectors, clearly showed this effect. The 2:3:4:6 TCNB resistant strain was as resistant to PCNB vapour as the PCNB resistant strain, but the PCNB resistant strain was almost as susceptible as the parent isolate to 2:3:4:6 TCNB vapour, and did not approach the degree of resistance shown by the 2:3:4:6 TCNB

resistant strain. These effects were also apparent in liquid medium. The same pattern of behaviour was found with non-saltant mycelium growing in the presence of the vapours of PCNB and TCNB. These experiments also showed that comparatively little resistance to PCNB or TCNB isomer had been built up by non-saltant mycelium. The results of the initial experiments on production of strains resistant to chlorinated nitrobenzenes, confirmed the conclusions of Parry (1957), and Brook and Chesters (1957), that some differential adaptation to PCNB and TCNB isomers occurred, but showed that some common adaptation mechanism must also be present. This hypothesis was substantiated by the results obtained in liquid medium. Resistant strains produced in response to exposure of the parent isolate to the vapours of PCNB and TCNB isomers were grown in liquid medium containing 10 mg. of the four substances, to see how far resistance to one fungicide conferred resistance to another. The results were assessed on two bases, rate of utilization of glucose and dry weight of mycelium produced. These methods of assessment gave similar results. In the presence of 10 mg. fungicide the resistant strains utilized glucose more efficiently than the parent, and this was reflected in the dry weight of mycelium produced. The same general picture was obtained, that resistance to one of the fungicides always conferred some resistance to the others, but that the extent of the resistance depended upon the origin of the strain. PCNB was the least

fungistatatic member of the penta-, and tetrachloronitrobenzenes, and the PCNB resistant strain was not affected by PCNB, but its growth was retarded by the tetrachloronitrobenzenes in the order, 2:3:4:6 TCNB > 2:3:5:6 TCNB > 2:3:4:5 TCNB, but it still grew better than the parent isolate in the presence of any of these substances. 2:3:4:6 TCNB was the most fungistatic of the four substances, and the 2:3:4:6 TCNB resistant strain was almost unaffected by the other three substances. The 2:3:4:5 and 2:3:5:6 TCNB resistant strains were intermediate in their response to the fungicides, between that of the 2:3:4:6 TCNB resistant strain and the PCNB resistant strain. These two resistant strains were more resistant to the isomer with which they had been originally treated, than to the other fungicides.

The "economic coefficient" for the resistant strains in the presence of PCNB or TCNB isomer reflected their ability and efficiency compared with the parent isolate, in converting carbohydrate supplied into cellular material. The lowest values were given by the parent isolate and PCNB resistant strain in the presence of 2:3:4:6 TCNB. The highest values were recorded in the absence of fungicide, and high values were also recorded by parent isolate and resistant strains in the presence of PCNB. These results emphasized that of the four substances considered, PCNB was the least fungistatic, and 2:3:4:6 TCNB the most fungistatic, and that for the resistant strains, the strain showing the greatest general resistance was

the 2:3:4:6 TCNB resistant strain, and the least general resistance the PCNB resistant strain. The results also showed that hyphal morphology and sporulation were less affected in liquid medium containing the fungicides than by the vapour of these substances. This may have been due to the extremely low solubility of these chlorinated nitrobenzenes in water.

When the resistant strains were grown in the presence of the vapour of dichloronitrobenzene isomers, it was found that the 2:3:4:6 TCNB resistant strain grew slowly in concentrations of 2,3 - and 2,4 dichloronitrobenzene which killed the parent isolate, PCNB, 2:3:4:5 and 2:3:5:6 TCNB resistant strains, and also survived without macroscopic sign of growth, concentrations of 2,5 dichloronitrobenzene, which killed the parent isolate and other resistant strains. These dichloronitrobenzenes were far more effective than PCNB or TCNB isomers at equivalent concentrations in retarding growth. Hyphae produced in the presence of dichloronitrobenzenes were abnormal, and the abnormality though more pronounced was similar to that caused by the tetrachloronitrobenzenes. The abnormal hyphae initially produced were conspicuously distorted. Extensive areas of vacuolation and granulation were present, and the cell walls were often creased and wrinkled. Intercalary swollen portions, resembling chains of chlamydospores were also produced. Increased rate of growth was associated with diminished hyphal abnormality.

A most interesting response was shown by the TCNB resistant strains in the presence of 1 mg. of the dichloronitrobenzenes. Some cultures rapidly produced a thin prostrate spreading mycelium. This growth continued for a few days and then stopped. The hyphae comprising this mycelium were not abnormal. Subsequent growth was always associated with abnormal hyphae produced from spots within, or at the edge of the colony. Less abnormal hyphae were produced from these areas, and eventually hyphae with a much higher growth rate in the presence of these vapours were produced. Other cultures did not start to grow for several days, and the hyphae initially produced were abnormal. There was no obvious explanation as to why replicates of the TCNB resistant strains responded differently to the same treatment. The 2:3:4:6 TCNB resistant strain was clearly the most resistant, and the PCNB resistant strain, the least resistant to the vapour of dichloronitrobenzenes. These laboratory studies showed that the dichloronitrobenzenes were far more effective than the tetrachloronitrobenzenes in retarding growth. They were fungitoxic to the parent isolate, whereas the tetrachloronitrobenzenes only retarded growth and never killed the fungus. If these dichloronitrobenzenes showed low phytotoxicity and were not more toxic than the tetrachloronitrobenzenes to animal life, they may prove more effective under field conditions than TCNB isomers in controlling diseases associated with Botrytis spp. The results with 2,5 halogen

substituted nitrobenzenes showed that when chlorine was replaced with bromine or iodine, the resulting compounds were far less effective in retarding growth of B.allii. Hyphal abnormalities were not as conspicuous with these fungicides as with dichloro-nitrobenzenes. The 2:3:4:6 TCNB resistant strain showed a graded tolerance, at equivalent concentrations to the three 2,5 halogenated nitrobenzenes in the order I > Br > Cl. The reason for this may be associated with the ease with which the halogens are removed from the benzene ring, intrinsic activity of the substances, or ease of penetration of the fungal wall by the vapours.

When parent isolate and the 2:3:4:6 TCNB resistant strain were grown in the presence of 2,6 dichloro-4-nitroaniline, the growth and sporulation of the 2:3:4:6 TCNB resistant strain was scarcely affected, whereas the growth of the parent isolate was retarded and sporulation delayed and diminished. Similar results were obtained in liquid medium. Black mycelial pellets were produced by the parent isolate in liquid medium containing this fungicide. This production of black hyphae was also seen when the parent isolate was grown in liquid medium containing TCNB, though in these cultures the development of black hyphae was associated with growth at the glass liquid interface.

The fungicide 2,6 dichloro-4-nitroaniline (allisan), was recently introduced by Boots Pure Drug Co., Ltd., and in an advisory leaflet by the company was claimed to be particularly

effective against Botrytis cinerea on lettuce. Under laboratory conditions it did not appear to be particularly active against the parent isolate of B.allii, and was without apparent effect on the 2:3:4:6 TCNB resistant strain. This may be due to a number of causes. Hewlett (1955), observed that even within a genus the effect of PCNB and 2:3:5:6 TCNB could not be foretold, as tests with nine different species of Botrytis showed that there was considerable variation both in the degree of retardation of growth, and in the length of time of treatment before cultures resumed slow growth. It is possible that under field conditions allisan exerts its controlling effect upon Botrytis diseases by its presence within the plant system.

Experiments with the parent isolate of B.allii PCNB, and TCNB resistant strains in the presence of 2:3:5:6 tetrachloroaniline vapour, showed that for equivalent concentrations of fungicide, this substance was less effective in retarding growth and suppressing sporulation than the corresponding 2:3:5:6 TCNB. This suggested that the TCNB resistant strains possibly lowered the activity of the tetrachloronitrobenzenes by reducing the nitro group to an amino group. If nitrate reductases were present in the resistant strains they could be associated with the adaptation of B.allii to the tetrachloronitrobenzenes. It was found that 2:3:5:6 tetrachloroaniline formed an orange brown product under diazotisation conditions with β naphthol. This reaction was used as a test for the

presence of 2:3:5:6 tetrachloroaniline in mycelial and culture filtrate extracts of the 2:3:5:6 TCNB resistant strain grown in liquid medium in the presence of large amounts of 2:3:5:6 TCNB. No evidence of 2:3:5:6 tetrachloroaniline was found. This does not invalidate the suggestion of reduction by the resistant strains to a less effective derivative, but it does not appear to be primarily associated with development of resistance in B.allii to the tetrachloronitrobenzenes.

Chromatographic analysis of mycelial and culture filtrate extracts of TCNB resistant strains grown in liquid medium containing the tetrachloronitrobenzenes did not show any qualitative carbohydrate differences, and ribose which was reported by Hewlett (1955), under these conditions with Botrytis cinerea was not found.

The parent isolate of B.allii and the 2:3:4:6 TCNB resistant strain were grown in the presence of different concentrations of benzene vapour. It was found that at the concentrations used, the growth rate of the 2:3:4:6 TCNB resistant strain was hardly affected, whereas the linear growth of the parent isolate was noticeably retarded at 4,000 p.p.m. benzene vapour. This experiment with benzene vapour suggests two possibilities why the TCNB resistant strains, particularly the 2:3:4:6 TCNB resistant strain, are resistant to a wide variety of compounds. The TCNB resistant strains may be resistant to the benzene ring, which is the only chemical

structure common to all the compounds used, or they may be resistant to the penetration of these vapours through the cell wall. An observation possibly associated with this latter suggestion was that when discs were taken from cultures of the 2:3:4:6 TCNB resistant strain, it was found that these discs were much harder to cut than those from the parent cultures. This tougher mycelium was not associated with quantity of mycelium, or with wall thickness.

The TCNB resistant strains retained their resistance to the tetrachloronitrobenzenes during 18 months growth under ordinary cultural conditions and in the absence of these fungicides.

The general resistance of the TCNB resistant strains to a number of compounds, halogenated nitrobenzenes, 2,6 dichloro-4-nitroaniline, 2:3:5:6 tetrachloroaniline, and benzene, conclusively confirmed the hypothesis that some common adaptation mechanism exists in B.allii to the tetrachloronitrobenzenes and associated substances. The results with the tetrachloronitrobenzenes also confirmed the conclusions of Brook and Chesters (1957), and Parry (1957), that some specific adaptation to different isomers may exist.

The results with Trichoderma viride on agar containing captan showed that this fungus could tolerate high concentrations of this fungicide. They also showed that this fungus could be "trained" to grow more rapidly than the parent isolate in the

presence of captan.

The slow growing mycelium initially produced in the presence of captan was composed of prostrate distorted hyphae. Sporulation was retarded, and pigmentation of the spores was affected. Variants comparable to those produced by B.allii in the presence of TCNB with a higher growth rate than the captan "trained" mycelium did not arise during the course of the experiments with T.viride on captan agar. Non-sporulating sectors did appear however, but their growth rate in the presence of captan was not higher than the "trained" mycelium. The captan "trained" mycelium was slightly more resistant to two captan analogues, N-trichloromethylmercapto,4-nitrophthalimide, and N-trichloromethylmercapto,4-methylhexahydrophthalimide, but not to N-trichloromethylmercapto 3,6-endoxohexahydrophthalimide.

Experiments with captan "trained" mycelium of T.viride growing on captan agar to detect the presence of hydrogen sulphide were inconclusive. The minute amounts evolved by this strain showed that hydrogen sulphide was unlikely to be produced as a result of fungal metabolism, or interaction with captan. The results in this section also showed that hydrogen sulphide was not associated with natural decomposition of captan.

The increased tolerance of captan "trained" mycelium to captan was lost after sub-culture in the absence of fungicide.

Significance of experimental results.

The laboratory experiments with chlorinated nitrobenzenes have shown that stable resistant strains of B.allii arise when exposed to the vapour of TCNB isomers or PCNB. These laboratory studies represent extreme experimental conditions which are unlikely to be present under field conditions. It seems improbable that ordinary field conditions would present B.allii with the opportunity to develop resistant strains, since even under laboratory conditions the resistant straind did not develop immediately, and only at the higher concentrations used. However, under certain environmental conditions, such as are present in frames and glass-houses where much higher local concentrations of fungicide vapour may be present, resistant strains could possibly develop. This study suggests that isolates of B.allii or B.cinerea from frames or glass-houses where chlorinated nitrobenzenes have been extensively used should be examined for evidence of stable resistance, as these conditions may represent a focal source, or reservoir of resistant strains accounting for the inconsistent and unsatisfactory results recorded by advisory officers and growers using PCNB and 2:3:5:6 TCNB in controlling diseases caused by Botrytis spp.

One important aspect that arises from this work is the pathogenicity of the PCNB and TCNB resistant strains to

onion. It is possible that under field conditions where these fungicides are used that resistant strains do develop but that they are not as pathogenic to onion as the parent strain.

VI SUMMARY

1. When Botrytis allii was exposed to the vapour of PCNB, 2:3:4:5 TCNB, 2:3:5:6 TCNB, and 2:3:4:6 TCNB slow growing mycelium was produced after varying intervals. The hyphae were swollen and distorted, and more deeply pigmented than those of the parent isolate in the absence of fungicide. Germination of spores of the parent isolate in PCNB or TCNB vapour was retarded, and the germ tubes produced were swollen and abnormal. 2:3:4:6 TCNB was the most fungistatic isomer, and PCNB the least fungistatic substance.

2. Sooner or later resistant variants were produced in the presence of 10 mg. PCNB, or TCNB isomer. The growth rate of these variants was much higher than that of the parent isolate when grown in the presence of PCNB or TCNB isomer. The hyphae of these resistant variants were normal in appearance.

3. The vapour of PCNB and TCNB isomers had less effect on established colonies of the parent isolate of B.allii than on newly inoculated Petri dishes. Lag phase and time for appearance of resistant saltants were reduced. Resistant saltants of PCNB were not so clearly defined.

4. The resistant saltants all grew normally and sporulated in absence of fungicide. The rate of growth of the 2:3:4:6 TCNB saltant in absence of fungicide was lower than that of parent isolate or other resistant saltants.

5. The resistant saltants were sub-cultured in absence of fungicide, (subsequently called resistant strains in text), and then grown in the presence of vapour of each of the other three substances. The resistant strains grew faster in the presence of vapour of all of the substances, than the parent isolate. The 2:3:4:6 TCNB resistant strain showed the greatest general resistance to the vapours, and the PCNB resistant strain the least general resistance to the other vapours.

6. Non-saltant mycelium produced by parent isolate in presence of PCNB or TCNB vapour grew slightly more quickly than untreated mycelium, when grown in presence of PCNB or TCNB vapour.

7. Spores produced by the resistant strains in absence of fungicide did not germinate as well as those of parent isolate in absence of fungicide, but germinated more quickly than those of parent isolate in the presence of PCNB and TCNB vapour.

8. Resistant strains grown in liquid medium in the presence of fungicides utilized carbohydrate more efficiently than did the parent in the same conditions. In liquid medium the most fungistatic substance was 2:3:4:6 TCNB, and the least fungistatic substance PCNB. Resistance to either PCNB or TCNB isomer, always conferred some resistance to the other three compounds. These experiments with resistant strains provided evidence for a common adaptive mechanism as well as for a more specific one.

9. PCNB and TCNB resistant strains were used in conjunction with other halogenated nitrobenzenes, 2,6 dichloro-4-nitroaniline, and 2:3:5:6 tetrachloroaniline. Resistance to TCNB always conferred some resistance to these compounds; these effects were most marked with the 2:3:4:6 TCNB resistant strain. This strain grew slowly in presence of concentrations of 2,3- and 2,4 dichloro nitrobenzene which killed the parent isolate, and other resistant strains.

10. The vapour of the dichloronitrobenzenes produced severe morphological abnormality of the hyphae of parent and resistant strains. More than one type of response to sub-lethal concentrations of these vapours was shown by TCNB resistant strains.

11. The 2:3:4:6 TCNB resistant strain showed a graded tolerance to the halogen series of 2,5 substituted nitrobenzenes in the order I > Br > Cl.

12. The 2:3:4:6 TCNB was unaffected by concentrations of benzene vapour which retarded the growth of the parent isolate.

13. No evidence was found to suggest that the 2:3:5:6 TCNB resistant strain was capable of reducing the nitro group of 2:3:5:6 TCNB to an amino group, and thus reducing the fungistatic potential of 2:3:5:6 TCNB.

14. The PCNB and TCNB resistant strains retained their resistance for at least 18 months under ordinary cultural conditions and in the absence of fungicides.

15. When Trichoderma viride was grown in the presence of captan incorporated in agar, slow growing mycelium was formed, hyphae were abnormal, sporulation was retarded, and pigmentation of the spores was affected. T.viride was "trained" to grow more rapidly on agar containing 10,000 p.p.m. captan after sub-culture for several weeks in presence of increasing concentrations of captan.

16. Captan "trained" mycelium reverted to the parent susceptibility after 8 weeks growth in the absence of captan.

17. Captan "trained" mycelium grew more quickly in the presence of two captan analogues than did the parent isolate.

18. Hydrogen sulphide was found in trace quantities when captan "trained" mycelium was grown on agar containing 10,000 p.p.m. captan. The quantities of hydrogen sulphide produced indicated that this compound was unlikely to be an important breakdown product of captan.

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