ADAPTATION OF BOTRYTIS SPECIES TO CERTAIN FUNGICIDES

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by

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I ABSTRACT

The degree of activity shown against Botrytis cinerea by pentachloronitrobenzene (PCNB), 2:3:5:6-tetrachloronitrobenzene (TCNB), and 2:6-dichloro-4-nitroaniline (DCNA) appeared to depend on the method of application of these fungicides.

When they were applied in the vapour phase, the fungicides retarded the linear growth of the fungus; when they were impregnated in agar, they retarded its linear growth more effectively, and also prevented germination of its spores. The fungus became adapted to them by producing either non-saltant mycelium with distorted hyphae, or resistant saltants with normal hyphae and with a much higher growth rate than that of the parent strain in the presence of fungicides.

Resistance of a strain of the fungus to any one of these fungicides conferred resistance, in varying degrees, to the other two fungicides and mycelia of resistant strains retained their full resistance for a long time and through many non-sporing generations.

About one half or less of the spores produced in the absence of fungicides by fungicide-vapour resistant strains formed resistant colonies; spores produced in the presence of fungicides by the fungicide-vapour resistant strains and those produced by the impregnated-fungicide resistant strains in the presence or absence of fungicides gave rise to resistant colonies, but only about one half or less of such spores appeared to be capable of forming colonies.

Spores which were produced in the absence of fungicides by parent and vapour-resistant strains, as well as mycelia of all strains were equally pathogenic to lettuce. Spores produced by impregnated resistant strains and those produced by vapour-resistant strains in the presence of fungicides appeared to be less pathogenic than the parent strain.

II INTRODUCTION

Fungicide formulations based on certain chlorinated nitrobenzenes and on dichloro-nitro-aniline have been used with appreciable degree of success to control diseases of plants caused by Botrytis species.

Laboratory studies have shown that when these pathogens are grown in the presence of the vapours of, or on agar media impregnated with, the active principles of such fungicies, they grow very little or not at all at first, but later give rise to new forms which are resistant to the fungicides to which the original form was sensitive. These resistant forms are unique in that they arise spontaneously and not as a result of 'training' the parent form by exposure to successively higher concentrations of the fungicides. Furthermore they do not lose their resistance when grown for a long time and through a series of generations in the absence of the fungicides. Some recent investigations have demonstrated the genetical basis of this type of resistance in Hyphomyces solani f. cucurbitae.

The development of resistant strains of pathogens has always been a serious threat to the efforts made to control plant diseases by the use of fungicides. With plant pathogenic fungi, the problem of resistance to fungicides does not yet seem to have become a very

serious one from the pratical standpoint, as is the problem of drug-resistance of many human and animal pathogens in medicine; there have been, however, some reports of such resistance becoming of considerable economic importance. Moreover, a greater variety of chemicals, many of them selective, are now being introduced as plant protectants, and used on a commercial scale. Investigations which aim at a better understanding of the phenomenon of fungicide tolerance therefore seem to be timely.

The present study has been designed to investigate the nature of the resistance of Botrytis cinerea to three fungicides, namely, (i) pentachloronitrobenzene (PCNB), (ii) the 2:3:5:6-isomer of tetrachloronitrobenzene (TCNB), and (iii) 2:6-dichloro-4-nitroaniline (DCNA). These three chemicals have been chosen because they have been used commercially in various fungicide formulations which have given promising results, and because the responses of Botrytis species to them are similar.

III REVIEW OF LITERATURE

1. Adaptation

The earliest accounts of the use of certain non-phytotoxic protectant fungicides, introduced in the nineteenth century, contained information on their specificity; Robertson (1824), for example, noted that sulphur was particularly active against powdery mildews, and Millardet (1885), showed that Bordeaux mixture, ineffective against these fungi, was very active against downy mildews. Sulphur and copper fungicides have now been used very extensively for many years against powdery and downy mildews and other plant pathogens, and it might well be expected that strains of pathogens resistant to them would be produced in a way analogous to the production of drug-resistant human and animal bacterial pathogens in medicine. There are as yet, however, only very few records in the literature, of the appearance of such resistant strains in the field. One such record was that of Taylor (1953), who reported that spores of Physalospora obtusa which were collected from orchards which had been sprayed continually with Bordeaux mixture were less affected by this fungicide in their germination, than were spores collected from unsprayed orchards.

This might be an example of selection, through spores, of strains of the pathogen which were resistant to copper. Littauer and Gutter (1953) also reported that the control of Diplodia natalensis on oranges was adversely affected by resistance of the fungus to diphenyl. Horsfall (1956) had suggested that the apparent reduction in the effectiveness of Bordeaux mixture, after some sixty years of use against Phytophthora infestans on potato along the Atlantic seaboard of America might mean the existence of strains of the pathogen which were resistant to copper, and he considered it almost certain that resistance to fungicides would be a serious problem for the agriculture of the future. Since his writing, one instance at least of such resistance had been reported, and which is of considerable economic importance; diphenyl-resistant strains of Penicillium digitatum, first observed in the laboratory (Farkas and Aman, 1940), were found later to be quite common in lemon groves and packing houses, in Southern California, and such resistant strains were considered to have been responsible for the failure of diphenyl to control decay in shipments from certain lemon packing houses, in which up to ninety per cent of the Penicillium spore population

might be diphenyl-resistant (Harding, 1962). Way and Keyworth (1959) stated that advisory officers and growers found that the control of Botrytis cinerea on lettuce, given by dusting seedlings, before planting, with pentachloronitrobenzene (PCNB) and 2:3:5:6-tetrachloronitrobenzene (TCNB) was inconsistent and inadequate. This could mean that under field conditions, Botrytis cinerea had acquired resistance to these fungicides. More recently, Ogawa and Mathre (1963), reported that isolations of Rhizopus spp. from decaying stone fruits which had been treated with 2:6 dichloro-4-nitroaniline (DCNA) showed high percentages of Rhizopus arrhizus, a strain which was resistant to DCNA, whereas isolations from untreated decaying fruits showed high percentages of Rhizopus stolonifer, a strain which was not resistant to treatment with DCNA.

In certain industrial processes involving the exposure of copper sulphate solutions, fungi have been found which were resistant to this compound (Starkey and Waksman, 1943); in such cases, however, the conditions for obtaining resistant strains of fungi were very different from those in the field; any process of adaptation here was possibly similar to

that in the laboratory, of which there have been a number of studies, the earliest of which was perhaps that of Pulst (1902), cited by Starkey and Waksman (1943). Stakman et al. (1946), investigating the adaptation of monosporidial lines of Ustilago zeae to arsenic, were able to increase the tolerance of the fungus to sodium arsenite from 2400 to 7000 p.p.m. in 10 transfers, the ability to grow on arsenic media of the same concentration increasing with successive transfers; the resistant strains rapidly reverted to the non-resistant form after only four transfers on arsenic-free media. Wilson (1947), obtained similar results when he grew Sclerotium rolfsii and S. delphinii on arsenic media. Hirt (1949), 'trained' Poria xantha to tolerate copper in increasing concentrations with each successive transfer. Mader and Schneider(1948) increased the concentration of arsenic which Sclerotinia fructicola would tolerate from 7000 to 10,000 p.p.m. after eighteen weeks at 5000 p.p.m.; Hirschhorn and Munnecke (1950) 'trained' Ustilago zeae on media containing copper; Gattani (1951) trained Alternaria spp. to the fungicides 'AGROSAN G.N.2' containing organic mercury and 'ARASAN' containing thiram, and concluded that the process of adaptation

here could become important in the field; Parry and Wood (1959) obtained, by 'training' methods, strains of Botrytis cinerea resistant to a wide variety of complex organic fungicides. Leben et al. (1955) obtained a variant of Venturia inaequalis which grew on media containing 5000 times the concentration of antimycin A which was necessary to inhibit growth of the wild type; Brian (1960) obtained a variant of Botrytis allii which was tolerant to griseofulvin on liquid media. The accumulated evidence of these workers showed that most of the resistant strains obtained by 'training' methods rapidly reverted to the non-resistant wild form, when they were grown in the absence of fungicides; exceptions were the results of Mader and Schneider (1948) who showed that only some of the strains of Sclerotinia fructicola which were 'trained' to tolerate high concentrations of arsenic had lost their increased resistance after forty-two weeks in media which contained no fungicides, and those of Parry and Wood (1959) who obtained strains of Botrytis cinerea resistant to ferbam (ferric dimethyl-dithio-carbamate) and captan (N-trichloro-methyl-mercapto-4-cyclohexene-1, 2-dicarboximide), but which did not revert to the parent susceptibility after they

had been grown in the absence of fungicides. They suggested that more than one mechanism was operative in the 'training' of Botrytis cinerea to these fungicides.

More detailed information on adaptation, under laboratory conditions, is available for the chlorinated nitrobenzenes, particularly pentachloronitrobenzene (PCNB), the three isomers of tetrachloronitrobenzene (TCNB), and for 2:6-dichloro-4-nitroaniline (DCNA), because a number of fungi readily produce resistant variants in the presence of these substances. Such variants which appeared spontaneously as fan-shaped, rapidly-growing, saltants, at the edges of colonies, were 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), Parry (1957), Higgons (1962), Sharples (1962), Priest (1960), Georgopoulos (1963b), and Ogawa et al. (1963). Each of these workers found that variants which were resistant to PCNB, TCNB isomer or DCNA, retained their resistance over long periods, even when repeatedly subcultured in the absence of fungicides.

The results of experiments with resistant

strains provided evidence for a common adaptive mechanism as well as for a more specific one; Parry (1957) showed that resistance of Botrytis allii to PCNB or TCNB isomer conferred resistance to the other substances; Priest (1960) confirmed these results and showed further that resistance to 2:3:5:6 TCNB also conferred resistance to DCNA. Georgopoulos (1963b)reported that 12 mutant strains of Hyphomyces solani f. cucurbitae which were resistant to PCNB and 2:3:5:6 TCNB were also resistant to seven more chlorinated nitrobenzenes and to DCNA. Brook and Chesters (1957), however, showed that variant strains of Fusarium caeruleum which were resistant to 2:3:5:6 TCNB were sensitive to the other two isomers, although less so than the parent strain; Hewlett (1955) had already obtained a similar result when she showed that the susceptibility of each of the fungi she used to PCNB differed from their susceptibility to 2:3:5:6 TCNB.

Most of the workers quoted above have made suggestions about the genetical mechanism of adaptation to fungicides, and about the mutagenic properties of fungicides. In most of the studies, the pathogen was 'trained' to successively increasing concentrations of the toxicants. It seemed likely therefore that the resistant lines obtained were the result of multistep

mutations, assuming the modus operandi to have been genetic in the Mendelian sense. Hewlett (1955), however, using monosporidial ascospore cultures, showed that the resistant types were not selected from pre-existing resistant types or from types arising from nuclear rearrangement in an initially heterokaryotic mycelium. Leben et al. (1955) demonstrated that in the lines of Venturia inaequalis which they obtained as resistant to antimycin A, the character for resistance was inherited in a Mendelian fashion and that it was produced by a first-step mutation. Georgopoulos (1963b) also confirmed that the development of resistance to chlorinated nitrobenzenes and to DCNA in Hyphomyces solani f. cucurbitae originated from materials which carried no genes for resistance, that it was definitely of the obligatory one-step pattern, and that no steps for further increase in resistance of this fungus to the fungicides were known; he further demonstrated in a series of studies that tolerance could result from mutation at any one of three independently assorting loci with two alleles each, and that genes for tolerance were not cumulative in their effects.

Reports on the pathogenicity of resistant strains showed that tolerant strains could be as pathogenic, more pathogenic or considerably less

pathogenic than, the non-tolerant parent strain, or they could be non-pathogenic. Hirschhorn and Munnecke (1950) showed that of the two monosporidial lines of Ustilago zeae they obtained as resistant to 9000 p.p.m. arsenic, one remained pathogenic; the other did not, but regained pathogenicity after growing in the absence of fungicide. Hirschhorn (1951) recognized three groups of variants in monosporidial lines of Ustilago zeae resistant to arsenic; the first group lost their pathogenicity to maize temporarily but regained it after growing for not less than sixteen generations in the absence of fungicide; the second group retained its pathogenicity, albeit diminished, even when taken directly from arsenic media; the third group maintained full pathogenicity even after growing for four to eight generations on arsenic media. Hirschhorn (1953) again showed that pathogenicity of formaldehyde-vapour resistant strains of monosporidial lines of U. zeae was unimpaired. Leben et al. (1955) reported the complete loss of pathogenicity in variant strains of Venturia inaequalis resistant to antimycin A and in all progeny carrying the variant genes. Priest and Wood (1961) show that chlorinated nitrobenzene tolerant strains of Botrytis allii were

as pathogenic as the non-tolerant strain. Shatla and Sinclair (1962) reported a very close correlation between pathogenicity and tolerance to PCNB in naturally occurring isolates of Rhizoctonia solani; tolerant strains were pathogenic, susceptible strains were not. Ogawa et al.(1963), working with Gilbertella persicaria reported that resistance to DCNA only slightly diminished pathogenicity; Georgopoulos (1963a) demonstrated that strains of Hyphomyces solani f. cucurbitae resistant to chlorinated nitrobenzenes could be as virulent, or considerably less virulent than, their wild type parents, to Cucurbita pepo seedlings and C. maxima fruits, and that mutation at the same locus for chlorinated nitrobenzene tolerance may give both highly and weakly pathogenic strains. He further established that there was a linkage relationship between a gene (or genes) responsible, in part at least, for reduction in virulence and a chlorinated nitrobenzene tolerant gene. McKee (1951) suggested that mutants of Fusarium caeruleum which were resistant to 2:3:5:6-TCNB may be important in the practical control of potato rot caused by F. caeruleum; Gattani (1951) concluded from his experiments on the 'training' of strains of Alternaria spp. to increased tolerance of 'AGROSAN G.N.2.' and 'Arasan' that the development

of tolerant strains could become important in the field. Parry (1957) also stated that fungicide-stable mutations constituted an important threat in the field, but that the rapid 'training' of Botrytis cinerea to the true fungicides was unlikely to be important in the field.

2. Fungicides

PCNB, developed and introduced by I. G. Farbenindustrie in the late 1930's, was originally marketed under trade names like 'Tritisan', a 15 per cent dust for the seed treatment of wheat against bunt, 'Brassicol', a 20 per cent 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 per cent dust, and 'Folosan DB 905', a 5 per cent dust.

DCNA, recently introduced by Boots Pure Drug Co., Ltd., was claimed in a Boots Advisory Leaflet to be effective against Botrytis spp., particularly Botrytis cinerea on lettuce. It was marketed as 'Allisan'.

All three fungicides have extremely low

solubility in water (Eckert, 1962); their use as fungicides is based partly on their volatility, DCNA being the least volatile. It has been claimed that practical field trials have shown DCNA to be very active and persistent, thus suggesting that vapour action was probably not of significance in the case of this compound (Higgons, 1962). None of these fungicides has found a very wide application in agriculture or horticulture, but they are of considerable academic interest because of their fungistatic action, their specificity, and possibly mutagenic properties. However, PCNB has been used successfully to control Botrytis cinerea on lettuce (Brown and Smieton, 1940; Last, 1952), Rhizoctonia solani (Gibson, Ledger and Bochm, 1961; Livingston, Oshima and Morrill, 1962; Georgopoulos and Wilheim, 1962), Sclerotium rolfsii (Georgopoulos and Thanasoulopoulos, 1960), Plasmodiophora brassicae on cabbage and cauliflower (Brown and Smieton, 1940). TCNB has been used to control Botrytis diseases and Rhizoctonia attack of lettuce (Last, 1952; Brook and Chester, 1958).

DCNA has been used to control Botrytis disease of lettuce, cyclamen, and anemone (Higgons, 1962 ; Crüger, 1962), Rhizopus rot of sweet cherries

(Ogawa, Lyda and Weber, 1963), Rhizopus rot of peaches (Ogawa, Mathre, Weber and Lyda, 1963; Ogawa and Mathre, 1963; Weber and Ogawa, 1963; Cappellini and Stretch, 1962) and Cladosporium fulvum (Higgons, 1962).

Comparatively little is known about the action of the chlorinated nitrobenzenes. The available information suggests that their fungistatic action is due to nuclear poisoning and inhibition of mitosis, this suggestion being supported by the preliminary chromatographic studies of Hewlett (1955), who found ribonin extracts of Botrytis cinerea treated with TCNB, and by the discovery by Carew and McDonough (1943) that in onion, spindle formation was inhibited by para-dichloro-nitrobenzene, a compound related to PCNB and TCNB.

More information is available on the action of DCNA. This compound, like PCNB and TCNB, when used in the vapour phase and in the standard slide germination test, has no activity against germination of spores of Botrytis cinerea, although it retards growth of germ tubes and mycelium (Sharples, 1962). This differential activity against spore germination on the one hand, and hyphal growth of the same pathogen, on the other hand, may be explained partly by the suggestion of Horsfall (1956), that spore germination, unlike

hyphal growth, is independent of mitosis.

When it is impregnated in an agar medium, however, DCNA is very active. Low concentrations, 1 p.p.m. for Botrytis cinerea (Sharples, 1962) and 0.1 p.p.m. for Rhizopus arrhizus (Weber, 1963) of DCNA prevented germination of spores and caused swelling and bursting of germ tubes. Sharples (1962), found that at low dosages (1 p.p.m.), DCNA increased the nucleic acid level of Botrytis cinerea mycelium. A similar result was obtained by Weber and Ogawa (1963), who found that addition of 1 p.p.m. DCNA decreased the protein content of sensitive strains of Rhizopus arrhizus; its ribonucleic acid (RNA) content also increased slightly but the de-oxyribonucleic acid (DNA) content remained similar to that of the untreated control. Addition of 1 p.p.m. DCNA to tolerant strain of the same fungus brought about no change in the level of protein, DNA or RNA contents by comparison with the untreated control.

Also DCNA had no effect on the respiration of the spores. The incorporation of leucine-C^{14} into protein of sensitive strain of R. arrhizus was decreased by 36 per cent by 2 p.p.m. DCNA; glucose C^{14}

metabolism, however, was only slightly affected by the presence of 2 p.p.m. DCNA. They concluded from these results that DCNA had little or no effect on respiration of spores and suggested that it interfered with an anabolic process such as protein synthesis rather than blocking oxidative processes. Sharples (1962) also suggested that DCNA is a structurally non-specific toxicant, exerting its influence by disorganising cell growth and division. Torgeson (1963) has also reported that PCNB did not reduce oxygen uptake of Fusarium oxysporum f. cubense, Sclerotium rolfsii, or Phytophthora parasitica; this suggests a similarity in the mode of action of DCNA and the chlorinated nitrobenzenes.

IV MATERIALS AND METHODS

1.0 Fungicides

The following three fungicides were used:
(i) pentachloronitrobenzene (PCNB) supplied by Bayer Products Ltd.,
(ii) 2:3:5:6-tetrachloronitrobenzene (TCNB) supplied by Boots Pure Drug Co., Ltd., and
(iii) 2:6-dichloro-4-nitroaniline (DCNA) supplied by Boots Pure Drug Co., Ltd.

These three chemicals are practically insoluble in water. They are, however, volatile and readily soluble in acetone and some other organic solvents. Because of these properties, it has been possible to use them in these studies in the (a) vapour phase and (b) agar-imprenation phase. The technique employed is as follows:

1.1 Vapour phase:

1.0 ml. samples of acetone solutions of known concentrations of the fungicides were allowed to evaporate under sterile conditions on the inside of the lids of 9.0 cm. petri dishes, which had been pre-cooled at 10°C. for 24 hours. Such a precooling before adding acetone produced a very even film of the fungicide on the petri dish lid. After evaporation of the acetone, the lids were replaced on the petri dish bottoms and

stored at room temperature for 24 hours to ensure complete evaporation of the acetone before they were used.

1.2 Agar-impregnation phase

0.5 ml. of Triton-X-100, a non-toxic wetting agent, was dissolved in 100.0 ml. ANALAR acetone. 0.30 gm. of the fungicide was dissolved in 20.0 ml. of the acetone-triton-X-100 mixture, and the solution obtained was added to 150.0 ml. distilled water in a 500 ml. beaker, and stirred with a glass rod. A fine suspension of the fungicide in water was obtained. To get rid of the acetone, the suspension was centrifuged at 10,000 r.p.m. at 10°C. for 10 minutes, and was washed three times by further centrifuging with distilled water. It was then re-suspended in 25.0 ml. distilled water to which triton-X-100 had been added at a concentration of 0.05%. The concentration of this final suspension was then obtained by determining the weights of fungicide in 0.5 ml. samples of the suspension dried in aluminium foil cups for 18 hours at 20°C. This period of time was found to be enough for completely drying the samples. The weights were, however, re-determined after a further 6 hours; weights obtained after 24 hours drying have always been almost identical with those obtained after 18 hours drying. The mean

of the weights of five replicates was then taken as the amount of fungicide present in the 0.5 ml. samples. The weights of replicates were always almost identical.

Agar media containing desired concentrations of the fungicide were then prepared by adding a calculated volume of the fungicide suspension to agar medium before autoclaving. When concentrations greater than 250 p.p.m. of the fungicide were desired, the suspension was added to agar medium just before pouring, to prevent coagulation of fungicide by heating.

2.0 Culture media, vessels and glasswares

All chemicals used, unless otherwise stated, were of the ANALAR grade. Experimental and stock cultures were grown on glucose-casein-hydrolysate agar media with or without fungicides. In some experiments, V-8 juice-, potato-dextrose -, potato extract -, cornmeal -, oatmeal -, glucose peptone -, Czapek-Dox-, malt -, starch -, and plain -, agar media were also used. All the agar media were first steamed in a steam bath for 45 minutes, and then sterilized by autoclaving for 20 minutes at 15 p.s.i. pressure. Liquid media, when used, were prepared in the same way as solid media, except that agar was not added to them.

When sterile water was used, distilled water was sterilized in the same way as the culture media. When the fungus was grown in the presence of the vapour of any of the fungicides, 1.0 ml. of a 1.0% acetone solution of the desired fungicide (10mg.) was evaporated on the inside of the lid of a petri dish in the way described in section 1.1. This quantity of fungicide was used because exposure of Botrytis cinerea to its vapour gave well-defined resistant sectors in all cases. When it was desired to impregnate the fungicide in agar medium, an amount of the fungicide suspension calculated to give the desired concentration was added to agar medium to give a total volume of 20.0 ml.; it was then autoclaved and poured into a petri dish.

Stock cultures were maintained under sterile paraffin oil and kept at laboratory temperature.

Culture media:

Glucose-casein-hydrolysate agar	
Glucose	1.0%
Casein hydrolysate	0.4%
KH_2PO_4	0.1%
$MgSO_4.7H_2O$	0.05%
Agar	2.0%
Water	

Potato dextrose agar	
Potato	200g.
Dextrose	20g.
Agar	20g.
Water	to 1000ml.

Potato extract agar

Potato	200g.
Agar	20g.
Water	to 1000ml.

Corn-meal agar

Groundmaize	25g.
Agar	20g.
Water	to 1000ml.

Oat-meal agar

Fine oatmeal	40g.
Agar	20g.
Water	to 1000ml.

Malt agar

Malt extract	15g.
Agar	20g.
Water	to 1000ml.

Starch agar

"Marmite"	5.0g.
Soluble starch	40.0g.
Agar	20.0g.
Water	to 1000.0ml.

Plain Agar

Agar	15g.
Water	to 1000ml.

Glucose peptone agar

Glucose	1.0%
Peptone	0.5%
KH_2PO_4	0.1%
$MgSO_4 \cdot 7H_2O$	0.05%
Agar	1.5%
Water	

Czapek-Dox agar

Sucrose	3.0%
$NaNO_3$	0.3%
K_2HPO_4	0.1%
$MgSO_4 \cdot 7H_2O$	0.05%
KCl	0.05%
$FeSO_4 \cdot 7H_2O$	0.001%
Agar	1.5%
Water	

V-8 juice agar

V-8 vegetable juice (obtained from Campbell's Soups Ltd.)	100.0ml.
Agar	12.0g.
Water	500.0ml.

In those media in which potato was used, the desired weight of potato was scrubbed, boiled for one hour, and strained through muslin by wringing. It was not filtered.

3.0 The Fungus.

A sporulating strain of Botrytis cinerea, freshly isolated from lettuce, was used as the test fungus. The bacteria-free isolate was purified by

re-isolation from single spores. This isolate was described as the parent strain (Pa). When resistant strains were obtained, they were named according to their mode of origin; thus, a strain which was resistant to impregnated DCNA was called 'DCNA-impregnated-resistant' strain, or 'D-imp-r' strain, and one which was resistant to the vapour of TCNB was called 'TCNB-vapour-resistant' strain, or 'T-vap-r' strain. When a heterokaryon was formed between the parent and another strain of the fungus, the heterokaryon was given a name compounded from those of the parent and the resistant strain, the parent part of the name coming first. Thus Pa(P-imp-r) would be the name for a heterokaryon formed between the parent strain and one resistant to PCNB, originating after growth when it was on an impregnated agar medium.

4.0 Inoculation and incubation

The inocula consisted either of non-sporulating mycelial discs, or of spores, of the desired strain. Cultures on agar media and liquid media were incubated at 21°C.. Stock cultures were grown on agar slopes for two days, and then maintained under sterile mineral oil at room temperature. In those experiments in which the number of colonies arising from spore inocula of

known concentrations were to be counted later, the cultures were incubated at 21°C. for the first 36 hrs. after inoculation; they were then transferred to a 10°C. constant temperature room. This procedure allowed for spore germination and colony formation, but prevented too-rapid growth of the colonies formed; the colonies did not coalesce, and recognition of individual colonies was facilitated. Petri dish cultures growing in the presence of the vapours of the fungicides were kept in separate sets of tins.

4.1 Agar disc inocula

When agar discs were used as inocula, they were cut with a 0.5cm. internal diameter cork-borer from the edge of a two-day old colony grown at 21°C. The discs were then inoculated in the centre of the petri dish with their mycelial surface on the agar.

4.2 Spore inocula

The spore-bearing mass was carefully removed with a sterile needle from the surface of a sporing colony without touching the agar beneath it; it was then put in 5.0 ml. sterile distilled water in a McCartney tube and shaken vigorously. The suspension formed was then passed through a double layer of sterile muslin into another sterilized McCartney tube, and its

density adjusted to the desired concentration. This procedure made washing with water by centrifuging unnecessary and therefore avoided the contamination which almost always accompanied such a process. The spore suspension obtained was free of contaminants so that it was not necessary to use any agent which killed or prevented the growth of bacteria.

When it was desired to make single spore inoculations, 0.1ml. spore suspension of density ,2000 spores per ml. was spread evenly, with a glass rod, and under sterile conditions, on the surface of a thin layer of plain agar in a petri dish, and kept at 21°C. After about one hour, when the film of spore suspension would have dried on the surface of the agar, the positions of single spores were marked with a dummy eye-piece, which had been sterilized by flaming with alcohol. The spores were then picked up with a small sterile needle and placed on the surface of an agar medium. By spreading a little volume of the very dilute spore suspension on plain agar, and marking the positions of single spores with sterilized dummy eye-piece, before placing the spores on the surface of nutrient agar, contamination was avoided to a very great extent, although the plain agar plate was exposed to contamination from the air

during the periods when the positions of the spores were marked and when they were transferred to the surface of nutrient agar.

When it was desired to make mass-spore inoculations, 0.05ml. spore suspension of density 1000-2000 spores per ml. prepared in the way already described (this contained 50-100 spores per inoculum) was pipetted on to the surface of the nutrient agar medium and, under sterile conditions, spread very evenly over it with a sterile bent glass rod.

5.0 Measurement of linear growth on agar plates

Growth of the fungus along two diameters at right angles to each other was taken and the mean of the two measurements was recorded for each replicate. The mean of the means of measurements for ten replicates was recorded as the growth of the fungus on the particular medium.

6.0 Counting of colonies on agar plates

When spore inocula were used, the number of colonies which had developed after three days was recorded for each plate, and the mean of the numbers recorded for ten replicates was taken as the number of colonies which had developed for that particular treatment.

7.0 Spore germination

Spores were always obtained from 7-14 days old cultures. The spore mass was carefully removed with a sterilized needle, shaken vigorously with sterile distilled water to which a little Tween 80, a non-toxic wetting agent, had been added, centrifuged and washed three times with sterile distilled water. The spore mass was then re-suspended in sterile distilled water, to give a final concentration of about 50,000 spores per ml. Details of any modification of the technique connected with spore germination experiments are given in the appropriate sections of the text. It was taken that a spore had germinated when the length of its germ tube was equal to, or greater than, that of half its short axis.

V EXPERIMENTAL WORK AND RESULTS

1.0 The fungicides: TCNB, TCNB and DCNA

The properties of the fungicides and the technique which was employed to use them in the vapour phase had been described under 'materials and methods'. Some workers, Higgons (1962), and Sharples (1962) impregnated them in agar media by vigorously shaking 0.5 ml. of acetone solutions of known concentrations of the fungicides with 20.0 ml. of nutrient agar just before pouring plates. Eckert (1962) used ether instead of acetone as the solvent. The same technique has been used in this work in the early part in those experiments in which the test fungus was grown on impregnated agar. It had the drawback, however, in that it was not possible to separate completely the effect, on the fungus, of acetone alone from that of the fungicide. The activity of the fungicide could not be properly determined just by deducting the result obtained for treatment with acetone alone in the control experiments, from that obtained for treatment with the acetone solution of the fungicide, because the latter result may be due to the interaction of the separate effects, on the fungus, of the acetone and the fungicide, and the effect on the activity of

the fungicide, of its solubility in the available amount of acetone. To get over this difficulty, it was necessary to modify the technique; the modification employed has been described in section 1.2 under "material and methods".

2.0 Effect of PCNB, TCNB and DCNA on linear growth of Botrytis cinerea

The effect of PCNB and TCNB on linear growth of Botrytis spp. have been investigated by Hildebrand (1955), Perry (1957), Priest (1960) and that of DCNA by Priest (1960), Higgons (1962) and Sharples (1962). Their results showed that when colonies of Botrytis spp. were grown in the presence of the vapours of these fungicides, there was at first very little growth or no growth at all. Later, two types of adaptation occurred. In the one type, rapid-growing variants, 'resistant saltants', with normal hyphal morphology similar to that of the untreated parent, were produced as sectors from the parent colony (Plate 1). In the other type, colonies with distorted hyphal forms were produced which were similar to those produced by the parent strain on exposure to the vapour of fungicide, but with a slightly higher growth rate (Plate 2).

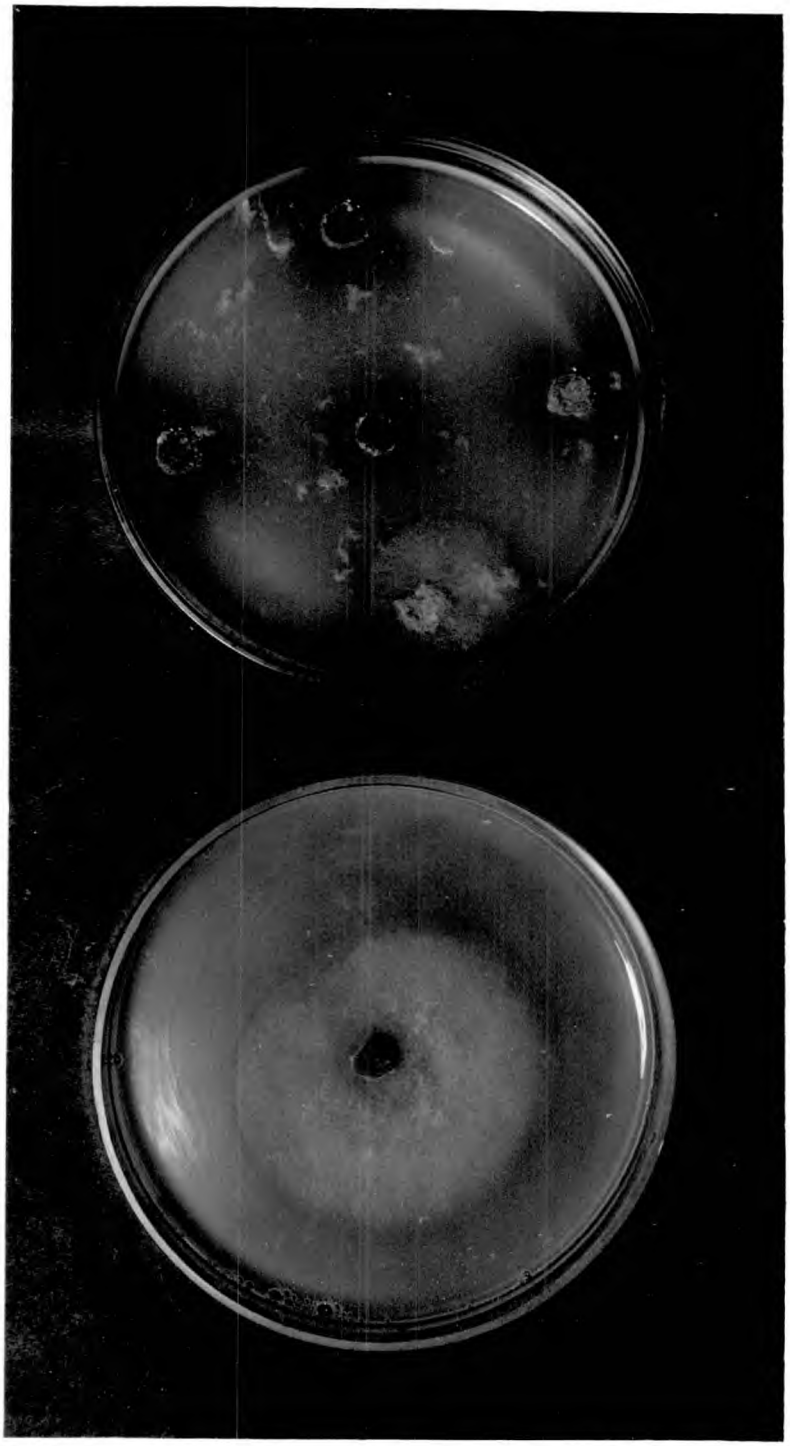
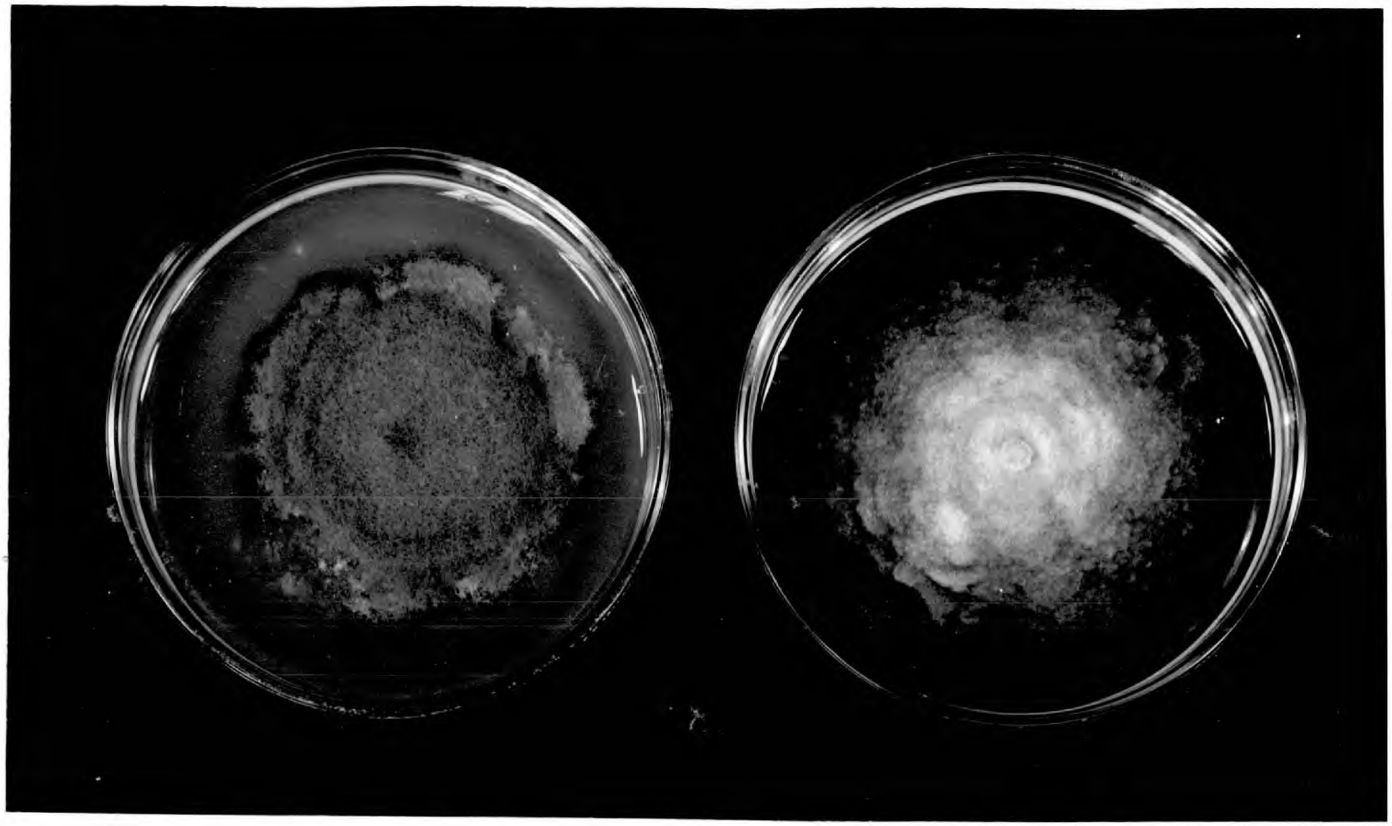


Plate 1 Effect of fungicides on linear growth of Botrytis cinerea

A Resistant saltants formed from one of five slow-growing 12-day old parent colonies growing on agar impregnated with PCNB.

B Resistant saltant formed from a 9-day old parent colony growing in the presence of vapour of PCNB.



A

B

Plate 2 Effect of fungicides on linear growth of Botrytis cinerea

25-day old colonies of parent strain showing non-saltant 'hyphal variants' formed in the presence of vapour of

A DCNA (sporulation observed in colony)

B PCNB (no sporulation)

Higgons (1962), Eckert (1962) have also shown that when Botrytis spp. were grown on agar impregnated with these chemicals, the same types of adaptation occurred. The present study is concerned mainly with the first type of adaptation, in which 'resistant saltants' are produced. In order then to investigate the nature of this type of adaptation to the fungicides, it was necessary to obtain resistant variants both in the vapour and in the agar-impregnated phases. The production of these variant strains of Botrytis cinerea therefore introduces the experimental section of this work. In those experiments in which the fungus was exposed to the vapours of the fungicides, 10mg. samples (1.0ml. of 1.0% acetone solution) of the fungicides were used for each replicate. This was found to be a suitable concentration in the presence of which clearly defined resistant sectors were formed in all cases. Of the three isomers of TCNB, only the 2:3:5:6 isomer has been used in this work; it is the isomer which has been of practical use in the formulation of fungicides (Crüger, 1962).

2.1 Linear growth of Botrytis cinerea in the presence of the vapour of PCNB, TCNB and DCNA

0.5cm. diameter mycelial discs taken from the edges of 2-days old colonies of Botrytis cinerea were inoculated with their mycelial faces downwards, on agar plates exposed or unexposed (control) to the vapours of PCNB, TCNB and DCNA. There were 10 replicates for each treatment. The results obtained are shown in Table 1.

Table 1.

Linear growth of Botrytis cinerea in the presence of the vapour of PCNB, TCNB and DCNA

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Treatment				Percentage inhibition of growth		
	Control	PCNB	TCNB	DCNA	PCNB	TCNB	DCNA
24	0.83	0.12	0.00	0.25	85.5	100.0	69.9
48	1.83	0.22	0.06	0.31	85.0	96.7	83.1
72	3.08	0.36	0.18	0.49	88.3	94.1	84.1
96	4.71	0.46	0.20	0.70M	90.2	95.7	85.1
120	6.95	0.54M	0.25	1.14	92.2	96.6	83.7
144	8.00	1.41	0.44M	1.74	82.4	94.5	77.0
192	C	1.95	0.60	2.36	-	-	-
240	-	3.44	1.64	3.83	-	-	-
336	-	4.51	2.71	4.51	-	-	-
384	-	5.38	3.35	5.46	-	-	-
480	-	5.98	4.63	7.80	-	-	-
504	-	8.00	5.52	C	-	-	-
528	-	C	6.08	-	-	-	-

M = appearance of resistant saltant.

C = plate was covered by mycelia.

These results showed that the three chemicals were fungistatic, rather than fungicidal; they also showed that in the vapour phase, TCNB exerted a greater fungistatic effect on the linear growth rate of Botrytis cinerea than did equal amounts by weight of PCNB or DCNA.

The results were similar to those obtained by Priest (Priest, 1960) working with Botrytis allii. Resistant saltants were obtained, which, in all cases, were similar in hyphal morphology to the parent growing in the absence of the vapour of fungicides. The second type of hyphal variants was also obtained in some of the plates.

Sporulation of both the parent strain and resistant saltants was completely suppressed in plates exposed to the vapours of PCNB and TCNB. In contrast, profuse sporulation was observed in the control plates after 72 hours and in plates exposed to the vapour of DCNA after 120 hours. (Plate 3).

2.2 Linear growth of parent strain and vapour-resistant saltants of Botrytis cinerea in the absence of fungicides

Resistant saltants obtained from plates which had been exposed to the vapours of PCNB, TCNB and DCNA and cultures of the parent strain which had been grown in the absence of fungicides were subcultured

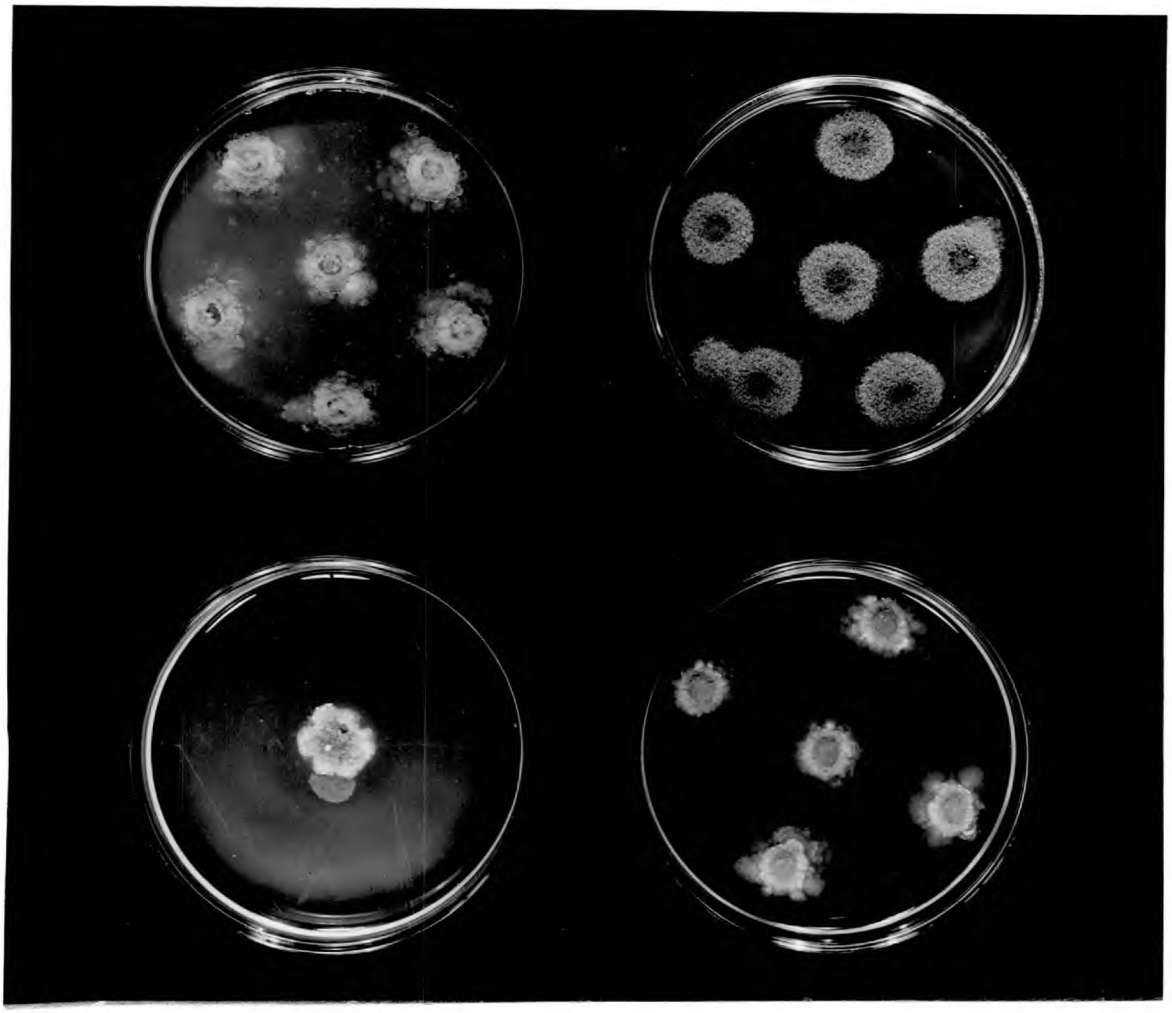


Plate 3 Effect of fungicides on linear growth and sporulation of Botrytis cinerea.

14-day old colonies of parent strain of Botrytis cinerea growing in the presence of

A Vapour of PCNB (no sporulation)

B Vapour of TCNB (no sporulation)

C Vapour of TCNB (no sporulation)

D Vapour of DCNA (sporulation in all colonies)

on agar plates which were not exposed to any vapour of fungicide. After 2 days' growth, 0.5 cm. diameter mycelial discs were taken from the edges of the colonies and inoculated in 10 replicates on agar plates and allowed to grow in the absence of fungicides. The results obtained are recorded in Table 2.

Table 2

Linear growth of parent, P(vap-r), T(vap-r) and D(vap-r) strains of Botrytis cinerea in the absence of fungicides

Time after inoculation (hrs)	Mean increase in colony diameter (cm.) Strain				Percentage growth compared with parent.		
	Parent	P(vap-r)	T(vap-r)	D(vap-r)	P (vap-r)	T (vap-r)	D (vap-r)
24	1.30	0.98	0.82	1.00	75.4	63.1	76.9
48	2.60	1.65	1.48	1.73	63.4	57.0	66.5
72	4.48	2.84	2.74	3.10	63.4	61.2	69.2
96	6.60	4.38	4.18	4.72	66.4	63.3	71.5
120	8.00	5.40	5.35	5.71	67.5	66.9	71.4
144	C	7.35	7.21	7.50	-	-	-
168	-	8.00	8.00	8.00	-	-	-
192	-	C	C	C	-	-	-
216	-	-	-	-	-	-	-

C = plate was covered by mycelia.

The results showed that the linear growth rate of the vapour-resistant saltants was similar, but less than that of the parent strain when they were all grown in the absence of fungicides. The parent strain reached a diameter of 8.0cm. in 5 days whereas the resistant forms took 7 days to attain the same diameter. Sporulation was very poor in the P(vap-r) and T(vap-r) strains, moderate in the D(vap-r) strain, but profuse in the parent strain.

2.3 Linear growth of parent strain and vapour-resistant saltants of Botrytis cinerea in the presence of the vapour of PCNB, TCNB and DCNA

Parent strain and resistant sultants were grown in the absence of fungicides for 2 days. 0.5cm. diameter mycelial discs, taken from the edges of the colonies, were then inoculated on agar plates exposed to the vapours of PCNB, TCNB and DCNA and on plates which were not exposed to vapour of fungicides (control). There were 10 replicates for each treatment. The results obtained are recorded in Tables 3, 4 and 5.

Table 3

Linear growth of parent, P(vap-r), T(vap-r) and D(vap-r) strains of Botrytis cinerea in the presence of vapour of PCNB

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain			
	Parent	P(vap-r)	T(vap-r)	D(vap-r)
24	0.20	0.96	0.90	1.03
48	0.27	1.70	1.50	1.85
72	0.34	3.40	2.81	3.30
96	0.65	4.36	4.12	4.90
120	0.76M	5.50	5.31	5.78
144	1.18	7.70	7.48	7.80
168	1.44	8.00	7.70	C
192	1.68	C	C	C
216	2.04	-	-	-
240	3.24	-	-	-
336	4.11	-	-	-
480	5.65	-	-	-

M = appearance of resistant saltant

C = plate was covered by mycelia.

Table 4

Linear growth of parent, P(vap-r), T(vap-r) and D(vap-r) strains of Botrytis cinerea in the presence of vapour of TCNB.

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain			
	Parent	P(vap-r)	T(vap-r)	D(vap-r)
24	0.01	0.10	1.00	0.98
48	0.03	0.25	1.80	1.75
72	0.15	0.46	2.82	2.97
96	0.20	0.50M	4.19	4.61
120	0.26	0.85	5.20	5.43
144	0.32M	1.18	6.80	7.03
168	0.62	2.08	7.80	8.00
192	0.75	2.35	C	C
216	0.91	4.03	-	-
240	1.64	5.34	-	-
336	2.75	6.39	-	-
480	5.81	7.87	-	-

M = appearance of resistant saltant

C = plate was covered by mycelia.

Table 5

Linear growth of parent, P(vap-r), T(vap-r) and D(vap-r) strains of Botrytis cinerea in the presence of vapour of DCNA

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain			
	Parent	P(vap-r)	T(vap-r)	D(vap-r)
24	0.22	0.91	0.92	1.55
48	0.33	1.79	1.49	3.25
72	0.61	3.00	2.54	4.51
96	0.67	4.27	3.85	7.00
120	0.88M	5.31	4.51	8.00
144	1.04	7.20	6.55	C
168	1.58	7.90	7.78	-
192	2.13	C	C	-
216	3.70	-	-	
240	4.01	-	-	
336	4.73	-	-	
480	7.67	-	-	

M = appearance of resistant saltant

C = plate was covered by mycelia.

These results confirmed the resistance of the saltants to those chemicals in the presence of the vapours of which they originated from the parent strain. When these results were compared with those recorded in Table 2, the inference is that the PCNB-, and TCNB-vapour-resistant strains, P(vap-r) and T(vap-r) grew at the same rate in the presence as well as in the absence of the vapours of fungicides to which they were initially resistant. The DCNA-vapour resistant strain D(vap-r), showed a slightly higher growth rate in the presence than in the absence of vapour of DCNA. Its growth in the absence of fungicide, however, was equal to that of the other resistant strains under the same conditions. In all cases, the growth rate, in the presence of vapour of fungicide, of the resistant strains, was many times higher than that of the parent strain until the latter gave rise to resistant sectors.

The T(vap-r) and D(vap-r) strains grew at the same rate in the presence of the vapours of PCNB and TCNB. The PCNB vapour resistant strain, P(vap-r), grew equally well in the presence of the vapours of PCNB and DCNA; its growth rate in the presence of vapour of TCNB was, however, at first very

small; later it gave rise to resistant saltants. In this way, its response to exposure to vapour of TCNB was very much like that of the parent strain, although resistant saltants appeared more quickly in the P(vap-r) strain than they did in the parent strain.

There was complete suppression of sporulation of all the strains in the presence of the vapours of PCNB and TCNB; the parent and D(vap-r) strains sporulated freely in the presence of the vapour of DCNA.

2.4 Linear growth of Botrytis cinerea on agar medium impregnated with the fungicides PCNB, TCNB and DCNA

Nutrient agar media containing 500 p.p.m. of either PCNB, TCNB, or DCNA were prepared by the method described in section 1.2 under 'materials and methods'. No fungicide was added to the control media. In the experiments first carried out with fungicide-impregnated agar, 0.5ml. of acetone solution containing the appropriate concentration of fungicide was added to 20 ml. nutrient agar medium just before pouring plates; 0.5 ml. acetone alone was used in the control media. This method of

preparing fungicide-impregnated agar media was not satisfactory, for the reasons explained in section 1.0 under 'experimental work and results'. The results of linear growth of Botrytis cinerea on media impregnated with fungicide, but without acetone, are given in Table 6. The concentration of fungicide, 500 p.p.m. has been chosen because the amount, 10mg., of fungicide impregnated in 20 ml. agar to give this concentration was equal to the amount of fungicide deposited on the lid of petri dish in those experiments in which the fungus colony was exposed to the vapour of fungicides. In this way, it was possible to compare the activity of an equal amount of the fungicide both in the 'vapour' and 'impregnated' phases.

Table 6

Linear growth of Botrytis cinerea on agar impregnated with fungicides PCNB, TCNB and DCNA at the concentration of 500ppm.

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Treatment			
	Control	PCNB	TCNB	DCNA
24	1.25	0.00	0.00	0.00
48	2.58	0.00	0.00	0.00
72	4.00	0.00	0.00	0.00
96	6.21	0.20	0.00	0.00
120	7.80	0.30	0.00	0.00
144	C	0.41	0.00	0.00
168	-	1.23M	0.00	0.00
192	-	2.14	0.00	0.00
216	-	3.02	0.00	0.00
240	-	3.60	0.20	0.00
360	-	6.35	2.03M	0.00
480	-	C	3.00	0.15
600	-	-	5.60	1.78M
720	-	-	C	3.21
960	-	-	-	6.31

M = appearance of resistant saltants

C = plate was covered by mycelia.

The response of Botrytis cinerea to the 'impregnated' fungicides was similar in general pattern to its response when it was exposed to their vapours. There was at first little or no growth; later, resistant saltants developed. There were very clear-cut lag periods varying from 3 days in the PCNB treatments to 9 days in the TCNB treatments and over 15 days in the DCNA treatments, when the fungus was grown on agar impregnated with fungicides. All the fungicides appeared to be more active in the 'impregnated' than in the vapour phase. DCNA appeared to be the most active of the three fungicides. This is in contrast to results obtained with the vapour treatments in which DCNA did not appear to be more active than PCNB and was definitely less active than TCNB. Apart from the extensive lag period, there was one other significant different between the 'vapour'- and 'impregnated'- phase treatments with the same fungicides: fewer resistant saltants were formed on impregnated agar than on agar exposed to vapour of fungicide. Whereas about 30% to 40% of plates exposed to vapour of each of the fungicides developed resistant sectors, only about 5% of the DCNA treatments, 9% of the TCNB treatments and 18% of the PCNB treatments developed resistant saltants when colonies were grown on impregnated agar. Again,

about 2 or 3 resistant sectors developed on plates exposed to vapours of fungicides; generally only one such sector was observed in colonies growing on agar impregnated with fungicides.

2.5 Linear growth of parent strain and fungicide-impregnated resistant saltants of Botrytis cinerea in the absence of fungicides

The parent strain and impregnated resistant saltants were grown in the absence of fungicides for 2 days. Mycelial disc inocula were then removed from the edges of the colonies and grown on agar in the absence of fungicides. The results obtained are recorded in Table 7.

Table 7

Linear growth of parent strain and fungicide-impregnated resistant saltants of Botrytis cinerea in the absence of fungicides

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain			
	Parent	P(imp-r)	T(imp-r)	D(imp-r)
24	1.50	1.30	1.25	1.19
72	4.68	3.25	3.27	3.58
120	8.00	6.28	5.95	6.67
144	C	C	C	C

C = plate was covered by mycelia

These results showed that the resistant strains grew at about the same rate in the absence of fungicides, but this rate of growth was a little less than that of the parent under the same conditions. In this respect, the impregnated-resistant strains were similar to the vapour-resistant strains.

2.6 Linear growth of parent strain and fungicide-impregnated resistant saltants of Botrytis cinerea on agar impregnated with PCNB, TCNB and DCNA

Mycelial disc inocula of the parent strain and impregnated resistant saltants were grown on agar impregnated with PCNB, TCNB or DCNA at a concentration of 500 p.p.m. The results obtained are recorded in Tables 8, 9 and 10.

Table 8

Linear growth of parent strain and impregnated resistant saltants of Botrytis cinerea on agar impregnated with 500ppmPCNB

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain			
	Parent	P(imp-r)	T(imp-r)	D(imp-r)
24	0.00	1.25	1.25	1.20
48	0.00	2.41	2.22	2.43
72	0.00	3.30	3.11	3.47
96	0.18	4.65	4.43	4.89
120	0.27	6.01	5.82	6.12
144	0.39	7.53	7.00	7.64
168	0.56M	C	C	C
		-	-	-
		-	-	-
		-	-	-

C = plate was covered with mycelium

M = appearance of resistant saltant.

Table 9

Linear growth of parent strain and impregnated-resistant saltants of Botrytis cinerea on agar impregnated with 500ppm TCNB

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain			
	Parent	P(imp-r)	T(imp-r)	D(imp-r)
24	0.00	0.00	1.27	1.18
48	0.00	0.00	2.00	2.00
72	0.00	0.00	3.21	3.10
96	0.00	0.03	4.59	4.27
120	0.00	0.10M	5.75	5.89
144	0.10	0.50	7.10	7.52
168	0.21	1.30	C	C

C = plate was covered with mycelium

M = appearance of resistant saltant.

Table 10

Linear growth of parent strain and impregnated resistant saltants of Botrytis cinerea on agar impregnated with 500ppm DCNA

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain			
	Parent	P(imp-r)	T(imp-r)	D(imp-r)
24	0.00	1.27	1.15	1.20
48	0.00	2.36	2.02	2.37
72	0.00	4.21	3.89	4.01
96	0.00	5.42	5.31	5.39
120	0.00	6.51	6.12	6.41
144	0.00	7.48	7.15	7.56
168	0.05	C	C	C

C = plate was covered with mycelium.

These results confirmed that the saltantswere truly resistant to those chemicals in response to which they originated from the parent strain; they were now referred to as resistant strains. If these results were compared with those recorded in Table 7, the inference would be that the fungicide-impregnated resistant strains grewat the same rate in the presence as well as in the absence of the fungicide to which they were initially resistant. In these experiments, the parent strain, as was expected, did not grow on agar impregnated with the fungicides. The T(imp-r) and D(imp-r) strains grew at about the same rate in the presence of all the fungicides. The P(imp-r) strain grew at the same rate on agar impregnated with PCNB or with DCNA, but did not grow at first on agar impregnated with TCNB. This result compared very well with the behaviour of the PCNB-vapour-resistant strain P(vap-r) when it was grown in the presence of TCNB vapour. There was complete suppression of sporulation of all the strains on agar impregnated with PCNB or TCNB; all the four strains sporulated on agar impregnated with DCNA.

2.7 Linear growth of parent and both types of resistant strains of Botrytis cinerea in the presence of the vapours of PCNB, TCNB and DCNA

The experiments described in this section were carried out to see whether there was any difference in resistance to fungicide vapour between those resistant strains which were developed in response to vapour treatment and those which developed when the parent was grown on agar impregnated with the fungicides. Mycelial discs, taken from the edges of 2-days old colonies of parent and all the six resistant strains which were grown in the absence of fungicides, were inoculated on agar which was exposed to the vapour of either PCNB, TCNB or DCNA. Control inoculations were on agar which was not exposed to vapour of any of the fungicides. The results obtained are recorded in Table 11.

Table 11

Linear growth of parent, P(vap-r), P(imp-r), T(vap-r), T(imp-r), D(vap-r) and D(imp-r) strains of Botrytis cinerea in the presence of the vapours of PCNB, TCNB and DCNA.

Treatment	Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain						
		Parent	P(vap-r)	P(imp-r)	T(vap-r)	T(imp-r)	D(vap-r)	D(imp-r)
Control	24	0.85	0.87	0.91	0.81	0.78	1.04	0.82
No	72	3.10	2.76	3.00	2.67	2.71	3.00	3.10
Fungicide	120	6.86	5.31	5.87	5.14	5.22	5.69	5.43
	168	C	C	C	C	C	C	C
PCNB	24	0.00	0.90	1.00	0.82	0.81	1.00	0.91
(10mg.)	72	0.27	2.78	3.14	2.71	2.62	3.26	3.16
	120	0.46	5.41	5.91	5 .30	5.41	5.65	5.74
	168	0.89M	C	C	C	C	C	C
TCNB	24	0.00	0.05	0.06	1.01	0.99	0.91	0.89
(10mg.)	72	0.12	0.32	0.29	2.75	2.63	2.85	2.81
	120	0.19	0.78	0.79M	5.31	5.40	5.61	5.43
	168	0.37M	1.35M	1.52	7.85	7.53	C	C
DCNA	24	0.17	0.91	0.85	0.91	0.89	1.51	0.85
(10mg.)	72	0.46	2.86	2.76	2.43	2.44	3.78	3.71
	120	1.00M	5.25	5.80	4.78	4.81	6.52	6.05
	168	1.78	7.80	7.55	7.69	7.59	C	C
	192	2.04	C	C	C	C	-	-

M = appearance of resistant saltant
C = plate was covered with mycelium.

The results showed that there was no difference in resistance to fungicide vapour between the two types of strains resistant to any particular fungicide. Both types of resistant strains obtained in response to treatment with either TCNB or DCNA were resistant to the vapours of all three fungicides. The two types of strains resistant to PCNB treatment were resistant to PCNB and DCNA, but not to vapour of TCNB.

2.8 Linear growth of parent and both types of resistant strains of Botrytis cinerea on agar impregnated with the fungicides PCNB, TCNB and DCNA at a concentration of 500 p.p.m.

These experiments were complementary to those described in the last section; the aim was to find out if the vapour-resistant strains of B. cinerea would also be resistant to fungicides when these were impregnated in agar media. The results obtained are recorded in Table 12.

Table 12.

Linear growth of parent, P(vap-r), P(imp-r), T(vap-r), T(imp-r), D(vap-r) and D(imp-r) strains of Botrytis cinerea on agar impregnated with 500 p.p.m. of PCNB, TCNB or DCNA.

Treatment	Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Strain						
		Parent	P(vap-r)	P(imp-r)	T(vap-r)	T(imp-r)	D(vap-r)	D(imp-r)
Control No Fungicide	24	1.04	0.90	0.91	0.82	0.80	1.02	0.99
	72	3.41	2.68	2.91	2.71	2.68	3.05	2.88
	120	7.30	5.30	5.33	5.03	5.14	6.85	5.97
	168	C	C	C	C	C	C	C
PCNB (500ppm)	24	0.00	0.83	0.91	0.76	0.80	1.08	1.41
	72	0.21	2.69	2.78	2.64	2.71	3.62	3.85
	120	0.50	5.20	5.10	5.00	5.00	5.40	7.20
	168	0.67	C	C	C	C	C	C
TCNB (500ppm)	24	0.00	0.00	0.00	0.85	0.90	0.00	0.87
	72	0.00	0.00	0.00	2.71	2.63	0.00	2.62
	120	0.00	0.23	0.38	5.40	5.39	0.14	5.14
	168	0.02	0.47M	0.56M	C	C	0.33	C
DCNA (500ppm)	24	0.00	1.21	1.00	0.82	0.91	1.21	1.14
	72	0.00	3.72	2.97	2.01	2.36	3.00	3.10
	120	0.00	5.51	5.30	4.90	5.10	5.80	5.70
	168	0.00	C	C	C	C	C	C

These results were similar to those obtained in the previous section; there was no difference in their resistance to impregnated fungicide between the two types of strain resistant to any one fungicide. Here again, strains resistant to PCNB were not initially resistant to TCNB, although their growth rate on agar impregnated with TCNB was higher than that of the parent strain on the same medium.

2.9 Effect of successive subculturing and growth in the absence of fungicides on the linear growth of resistant strains of Botrytis cinerea

In the experiments described in this section, it was desired to find out if successive subculturing and continuous growth for a long time in the absence of fungicides would cause any reduction in, or loss of, resistance of resistant strains of Botrytis cinerea.

Strains which were resistant to the vapours, or to the impregnated phase, of the fungicides were grown for 2 days in the absence of fungicides. They were then inoculated on nutrient agar and allowed to grow for 2 days before the next inoculation was made. Successive inoculations at intervals of two days were

made until 40 generations for the vapour-resistant strains, and 50 generations for the impregnated-resistant strains, were obtained. Every fifth generation in each case was grown in the presence of the fungicide to which the strain was resistant, to find out if there had been any reduction in, or loss of, resistance. The period of two days growth between successive generations was chosen because it allowed for appreciably good growth, but not spore formation. The results obtained are recorded in Table 13.

Table 13

Effect of successive subculturing and growth in the absence of fungicides on the linear growth of resistant strains of Botrytis cinerea.

*	Mean increase in colony diameter (cm.) after 2 days											
	Strain											
	P(vap-r)		P-(imp-r)		T(vap-r)		T-(imp-r)		D(vap-r)		D (imp-r)	
	TREATMENT											
	Cnt	P vap	Cnt	P imp	Cnt	T vap	Cnt	T imp	Cnt	D vap	Cnt	D imp
1st	2.02	2.14	2.03	2.05	2.11	2.18	2.40	2.03	2.45	2.67	2.65	2.63
5th	2.31	2.29	2.05	2.12	2.15	2.10	2.14	2.15	2.41	2.58	2.60	2.61
10th	2.05	2.14	2.14	2.00	2.07	2.13	2.05	2.26	2.48	2.60	2.45	2.52
15th	2.10	2.00	2.61	2.42	2.21	2.20	2.18	2.24	2.41	2.49	2.28	2.29
20th	2.17	2.17	2.30	2.18	2.18	2.09	2.19	2.03	2.39	2.42	2.53	2.19
25th	2.09	2.26	2.19	2.18	2.25	2.23	2.14	2.18	2.40	2.53	2.28	2.31
30th	2.31	2.14	2.31	2.80	2.19	2.14	2.01	2.00	2.18	2.48	2.42	2.50
35th	2.80	2.09	2.25	2.36	2.30	2.25	2.33	2.17	2.41	2.49	2.67	2.63
40th	2.15	2.17	2.41	2.14	2.17	2.21	2.04	2.10	2.26	2.63	2.60	2.52
45th	-	-	2.21	2.24	-	-	2.41	2.18	-	-	2.61	2.40
50th	-	-	2.14	2.18	-	-	2.24	2.30	-	-	2.60	2.53

Cnt = no fungicide treatment
P vap = PCNB applied in vapour phase
T vap = TCNB " " " "
D vap = DCNA " " " "
P imp = PCNB applied in impregnated phase
T imp = TCNB " " " "
D imp = DCNA " " " "
* = generation in absence of fungicide

The results showed that successive subculturing and growth in the absence of fungicides had no effect on the resistance of the resistant strains.

The 40th generation of the vapour-resistant strains and the 50th generation of the impregnated-resistant strain were as resistant when grown in the presence of fungicides as the 1st generations. Non-sporing mycelial discs had been used for inoculation and fungicides were impregnated in agar at the concentration of 500 p.p.m.

2.10 Linear growth of Botrytis cinerea on agar impregnated with fungicides at a graded series of concentration

In the experiments described so far, the fungicides PCNB, TCNB and DCNA had been impregnated in agar at a standard concentration of 500 p.p.m. whenever it was desired to use fungicide-impregnated agar. In view of the very long lag period observed when B. cinerea was grown on such impregnated media and in the light of certain results obtained on spore germination tests (to be described later), experiments were carried out on the linear growth of Botrytis cinerea on agar impregnated with the fungicides at the following concentrations expressed in parts per million (p.p.m.): 0-, 1-, 2-, 5-, 10-, 25-, 50-, 100-, 250-, and 500-ppm. The results obtained are recorded in Table 14.

Table 14

Linear growth of Botrytis cinerea on agar impregnated with PCNB, TCNB or DCNA at a graded series of concentration.

Treatment	Time after inoculation (days).	Mean increase in colony diameter (cm.) Concentration of fungicide in agar (p.p.m.) 0	1	2	5	10	25	50	100	250	500
PCNB	3	4.0	2.4	2.1	0.9	0.4	0.3	0.4	0.4	0.3	0.2
	6	C	5.6	5.4	2.0	0.9	1.0	0.6	0.6	0.7	0.7M
	9	-	C	C	4.6	2.8	2.7	1.6M	1.0	1.3	1.56
	12	-	-	-	6.8	5.0	4.3	5.8	2.3M	2.3M	5.0
TCNB	3	4.0	4.0	4.0	4.0	0.3	0.1	0.0	0.0	0.0	0.0
	6	C	8.0	8.0	8.0	1.2	0.2	0.0	0.0	0.0	0.0
	9	-	C	C	C	2.0	0.3M	0.1	0.1	0.0	0.0
	12	-	-	-	-	3.4	0.9	0.8M	0.6M	0.4M	1.0M
DCNA	3	4.0	3.7	2.7	0.9	0.0	0.0	0.0	0.0	0.0	0.0
	6	C	7.4	6.5	1.8M	0.5	0.0	0.0	0.0	0.0	0.0
	9	-	C	C	5.9	0.9M	0.3M	0.4M	0.3	0.0	0.1
	12	-	-	-	C	3.1	3.3	3.4	0.5M	0.8M	0.6M

M = appearance of resistant saltant

C = plate was covered by mycelium.

These results showed that at concentrations up to 2 p.p.m., PCNB had no effect on the linear growth of Botrytis cinerea. At concentrations of PCNB above 2 p.p.m., and up to 25 p.p.m., there was a steady but slow growth, which was less than the growth in concentrations below 2 p.p.m.. The colonies were lobed with irregular edges; no clearly defined saltants were formed; their morphology was similar to that of 'hyphal variants', the second type of adaptation obtained when the fungus was exposed to the vapour of fungicides. At concentrations of PCNB above 25p.p.m., growth was very slow until resistant saltants were developed.

TCNB had no effect on the growth of the fungus when its concentration was 5p.p.m. or less. At concentrations between 5p.p.m. and 25 p.m.m., colonies were obtained which resembled 'hyphal variants' in their morphology. At concentrations equal to and above 25 p.p.m., there was a long lag period; when growth did take place, it was very slow indeed until resistant saltants were formed.

DCNA, up to a concentration of 2p.p.m. had no effect on linear growth. Concentration of 5p.p.m. or more greatly retarded growth; there was a very

long lag period; resistant saltants were formed in a few of the plates, but not more at any concentration than at others. Very few purely 'hyphal variants' were obtained in the plates; in most of the plates, growth was very slow indeed when it took place.

2.11 Linear growth of resistant strains of Botrytis cinerea obtained from low concentrations of fungicides, in the presence of high concentrations of fungicides

The aim of the experiments in this section was to find out if a resistant strain, obtained in response to treatment with a low concentration of a fungicide, would be equally resistant when grown in the presence of higher concentration of the same fungicide. Resistant strains obtained from PCNB treatment at concentrations 50-, 100-, 250- and 500-p.p.m. were grown on agar containing PCNB at concentrations ranging from 1p.p.m. to 500p.p.m. Resistant strains obtained from treatments with TCNB and DCNA were grown similarly on agar impregnated with various concentrations of TCNB or DCNA. Resistant strains were designated according to their mode of origin; thus one obtained from treatment with DCNA at 5ppm was called RD5 and one obtained from treatment with TCNB at 250ppm was called RT250. The results obtained are recorded in Tables 15, 16 and 17.

Table 15

Linear growth of PCNB-impregnated resistant strains of Botrytis cinerea on agar impregnated with PCNB at various concentrations

	Mean increase in colony diameter (cm.) after 2 days growth Concentration of PCNB (p.p.m.)									
STRAIN	0	1	2	5	10	25	50	100	250	500
RP 50	2.3	2.4	2.3	2.4	2.3	2.2	2.5	2.3	2.3	2.3
RP 100	2.3	2.3	2.3	2.3	2.4	2.2	2.2	2.3	2.3	2.3
RP 250	2.2	2.2	2.3	2.3	2.3	2.4	2.2	2.3	2.5	2.4
RP 500	2.3	2.3	2.3	2.3	2.6	2.7	2.4	2.2	2.5	2.4

Table 16

Linear growth of TCNB-impregnated resistant strains of Botrytis cinerea on agar impregnated with TCNB at various concentrations

	Mean increase in colony diameter (cm.) after 2 days growth Concentration of TCNB (p.p.m.)									
STRAIN	0	1	2	5	10	25	50	100	250	500
RT 50	2.3	2.4	2.2	2.5	2.3	2.4	2.4	2.5	2.4	2.3
RT 100	2.4	2.4	2.3	2.3	2.4	2.5	2.4	2.2	2.3	2.4
RT 250	2.2	2.4	2.3	2.4	2.4	2.5	2.3	2.4	2.2	2.3
RT 500	2.3	2.5	2.4	2.3	2.4	2.5	2.4	2.5	2.5	2.4

Table 17

Linear growth of DCNA-impregnated resistant strains of Botrytis cinerea on agar impregnated with DCNA at various concentrations

STRAIN	Mean increase in colony diameter (cm.) after 2 days growth. Concentration of DCNA (p.p.m.) 0	1	2	5	10	25	50	100	250	500
RD5	3.0	2.5	2.6	2.4	2.4	2.4	2.5	2.5	2.4	2.5
RD10	2.8	2.5	2.6	2.5	2.6	2.7	2.4	2.5	2.5	2.6
RD25	2.5	2.5	2.6	2.5	2.5	2.4	2.5	2.6	2.5	2.4
RD100	2.6	2.6	2.5	2.5	2.5	2.5	2.6	2.4	2.5	2.6
RD500	2.7	2.4	2.5	2.3	2.6	2.5	2.6	2.7	2.5	2.5

These results were recorded after 2 days because growth after 2 days was enough to establish whether or not a colony would be resistant under any particular condition. The results suggested that resistance of Botrytis cinerea to any of the three fungicides was absolute within the limits of the concentrations used in these experiments. A resistant strain obtained on agar medium which contained 5p.p.m. DCNA grew at the same rate on agar containing 500 p.p.m. DCNA as it did on agar which contained no fungicide; similarly a resistant strain developed on treatment with TCNB at 50 p.p.m. grew

at the same rate in agar containing 500 p.p.m. TCNB as it did on agar impregnated with 1 p.p.m. TCNB. A strain which was resistant to a fungicide at any concentration was equally resistant at lower or higher concentrations of the same fungicide within the limits of the concentrations used in these experiments.

2.12 Linear growth of parent and all resistant strains of Botrytis cinerea on agar impregnated with various concentrations of PCNB, TCNB and DCNA

This is a summary, in a tabular form, of the results of experiments on the linear growth of Botrytis cinerea and its resistant forms on agar impregnated with fungicides at concentrations from 1 to 500 p.p.m.. Results are recorded in Table 18.

Table 18

Linear growth of parent and resistant strains of Botrytis cinerea on agar impregnated with PCNB, TCNB and DCNA at various concentrations.

Treatment	concentration (p.p.m.)	Mean increase in colony diameter (cm.) after 5 days growth STRAIN						
		Parent	P vap-r	P imp-r	T vap-r	T imp-r	D vap-r	D imp-r
Control	(0)	7.4	5.3	5.4	4.8	5.2	7.9	8.0
PCNB	1	4.8	5.8	6.0	5.7	6.0	6.6	8.0
	5	2.1	5.8	5.8	5.7	6.0	6.6	7.6
	25	1.2	5.7	5.9	5.1	5.5	6.3	7.5
	100	1.4	5.5	5.6	4.9	5.4	6.2	7.3
	250	1.2	5.1	5.7	4.8	5.3	6.0	7.7
	500	1.2	5.2	5.8	4.9	5.5	6.1	7.9
TCNB	1	8.0	6.8	5.5	5.1	5.0	6.5	7.8
	5	8.0	6.1	5.9	5.1	5.3	6.3	7.8
	25	0.2	4.9	6.1	5.4	6.0	4.6	6.6
	100	0.0	1.0	2.7	5.4	6.0	0.3	5.1
	250	0.0	0.6	1.0	5.4	5.5	0.3	4.3
	500	0.0	0.7	0.6	5.4	5.6	0.3	4.4
DCNA	1	6.8	6.9	6.7	6.3	5.3	7.7	7.6
	5	1.8	6.8	7.0	6.4	5.3	7.8	7.8
	25	0.0	7.2	6.4	6.2	5.3	7.5	6.6
	100	0.0	7.9	7.9	7.1	5.4	7.7	6.5
	250	0.0	7.3	7.4	6.8	5.4	7.6	6.6
	500	0.0	6.8	6.3	5.7	5.5	7.0	6.2

2.13 Summary of results on linear growth of parent and resistant strains of Botrytis cinerea

Botrytis cinerea was susceptible to treatment with PCNB, TCNB or DCNA when these fungicides were applied in the vapour or agar-impregnated phase; its growth rate was at first very slow; but later resistant saltants were formed which grew well both in the presence or absence of fungicides. The growth rate of these resistant saltants was always less than that of the parent in the absence of fungicides. The frequency with which the parent strain formed resistant saltants was much less on agar impregnated with fungicides than on agar which was exposed to the vapour of fungicides. When equal amount of fungicides was used, PCNB, TCNB and DCNA were more active against Botrytis cinerea in their agar-impregnated phase than in their vapour phase. When used in the vapour phase, the order of activity of these fungicides was TCNB > PCNB > DCNA; but when they were impregnated in agar, their activity in descending order was DCNA > TCNB > PCNB. Any resistant saltant, produced in response to treatment with a low concentration of fungicide applied in the impregnated phase, was also resistant to higher concentrations of the same fungicide, and to its vapour;

resistant strains did not lose their resistance when they were grown for up to 40 successive generations in the absence of fungicides. Strains which were resistant to treatment with any one of the fungicides were also resistant to treatment with the other fungicides, except for the PCNB-vapour- and PCNB-impregnated- resistant strains, which were not resistant to TCNB when it was impregnated in agar at concentrations above 25 p.p.m.. They were also not resistant to vapour from 10mg. TCNB. Parent and all the resistant strains sporulated on agar impregnated with DCNA; parent and DCNA-resistant strains sporulated in the presence of the vapour of DCNA. Sometimes, PCNB- and TCNB- resistant strains sporulated in the presence of the vapour of fungicide to which they were resistant.

3.0 Spores of parent and resistant strains of Botrytis cinerea

In the experiments which have been described and recorded so far, successive generations of parent and resistant strains of Botrytis cinerea were maintained by subculturing from young non-sporing colonies, or from non-sporing stock cultures kept under mineral oil at laboratory temperature. In

all the experiments, the inocula were mycelial discs taken from the edges of two-day old, non-sporing colonies. In none of the experiments did any inoculum taken from the parent colony start its growth as a resistant form on agar which was exposed to the vapour of, or impregnated with, fungicides; resistant saltants have always been formed as sectors from the very slow-growing parent colonies. On the other hand, inocula taken from resistant strains have always started their growth as resistant forms on agar treated with the fungicide in the presence of which they were originally obtained as resistant sectors, even when these resistant sectors have been grown for many generations in the absence of fungicides. These results therefore relate to experiments in which no spore, other than the single spore from which the parent strain was originally purified, was involved. It was desired to find out if there was any chance of a parent spore giving rise to a colony which was initially resistant to the fungicides, and also to see if spores from resistant strains gave rise to colonies which would be susceptible when grown in the presence of fungicides, the expectation being that spores produced by resistant strains would always give rise to

resistant colonies, whereas those produced by the parent strain would always form susceptible colonies. The investigation was carried out by analysing results obtained

(a) from a large number of single spore colonies,

(b) by using a modification of the 'Most Probable Number' - 'M.P.N.' technique, and

(c) by using the 'dilution plate' technique.

Both the 'Most Probable Number' and 'dilution plate' techniques will be explained in the appropriate sections. Results obtained with spores produced by the parent strain will be given first.

3.1 Estimation of the percentage of spores of parent strain of Botrytis cinerea which were resistant to PCNB, TCNB and DCNA

(a) Estimation by growing single spore colonies

Spores were obtained from 8-14 day old colonies of parent strain of Botrytis cinerea, which had been grown in the absence of fungicides. A spore suspension containing about 2000 spores per ml. was prepared and samples were spread on plain agar as described in section 4.2 under 'materials and methods';

120 single spores were picked up and placed, five spores per plate, on nutrient agar, and incubated for 60 hours by which time colonies had been formed from the spores. These colonies were then scored for resistance to fungicides by taking mycelial discs from their edges and placing them, mycelial surface downswards, on agar which was either impregnated with 100 p.p.m. of PCNB, TCNB or DCNA or exposed to their vapour. Agar for the control experiments was not treated with fungicides.

None of the 120 single spore colonies was initially resistant to any of the fungicides when they were used either in the vapour phase or in the impregnated phase. As far as could be determined therefore, by this method of investigation, spores of parent strain of Botrytis cinerea formed in the absence of fungicides did not give rise to colonies that were initially resistant. It is recognized that the number of spores tested was very small, but, of course, they had been taken at random from a very much larger number.

(b) Estimation by a modification of the 'Most Probable Number - M.P.N.' technique

The 'Most Probable Number' dilution method

(Cochran, 1950), is a means of estimating, without any direct count, the density of organisms in a liquid. The method consists of taking samples from the liquid, incubating each sample in a suitable culture medium and observing whether any growth of the organism has taken place. The estimation of density is based on an ingenious application of the theory of probability to certain assumptions, namely,
(a) the organisms are distributed randomly throughout the liquid, and
(b) each sample from the liquid, when incubated in the culture medium, is certain to exhibit growth whenever the sample contains one or more organisms. If the culture medium is poor, or if there are factors which inhibit growth, or if the presence of more than one organism is necessary to initiate growth, the 'M.P.N.' method gives an underestimate of the true density. In the mathematical analysis of the results obtained, the probability that there will be no growth in a sample is related to the density of organisms in the original liquid. If the density of organisms were such that all samples taken are sterile, then the estimated density will be zero, and if the density were such that all samples are fertile, then

the estimated density will be infinite. Because the precision of the 'M.P.N.' is very poor when the volume of the sample taken is such that the samples are likely to be all sterile or all fertile, it is usual in practice to take as samples more than one dilution or different volumes of the same dilution in such a way that certain of the samples will be sterile and the others fertile. A volume dilution ratio is chosen in such a way that samples with the smallest volume should be sterile and those with the highest should be fertile.

In these experiments, a suspension of the spores of the parent strain of Botrytis cinerea containing about 100 spores per ml. (determined by haemacytometer counts) was prepared. This dilution of spore suspension was taken to give a sufficiently random distribution of single spores. A dilution factor of '2' was chosen so that the volumes of samples of spore suspension used for inoculation were in the graded series 0.01ml., 0.02ml., 0.04ml.,.....to 0.64ml., 1.28ml.. These samples were then inoculated by spreading thinly over the surface of nutrient agar which was either impregnated with, or exposed to the vapour of, fungicides, or not treated at all with

fungicides. There were 8 replicates for each treatment at each dilution level. The plates were incubated at 20°C., and were examined for growth after 2, 4 and 6 days. If there were any resistant spores in the inoculum, such spores would form colonies in the presence of fungicides; all spores, whether they were resistant or not, would be capable of forming colonies in the control untreated plates. By comparing the estimated density of colonies formed in the presence of fungicides with that of colonies formed in their absence, it would be possible to estimate the percentage of parent spores which were resistant to a particular fungicide.

The results obtained confirmed those with the single spore colonies recorded earlier, because no initially resistant colonies appeared during the first four days germination and growth. After 6 days, resistant colonies, which, expressed as percentages of the estimated number of colonies in the control series, were 0.24, 0.09, 0.05, 0.[illegible], 0.[illegible]6 and 0.00 in the PCNB-vapour-, PCNB-impregnated, TCNB-vapour-, TCNB-impregnated, DCNA-vapour- and DCNA-impregnated-treatments respectively, were obtained; they originated as resistant saltants from very small susceptible parent colonies. It is interesting that the density of the

spore suspension estimated by this method was 10^3.8 per ml.; this was the density of spores incubated in the control plates; the density of the spore suspension, as determined by haemacytometer counts, was about 100 spores per ml.

(c) Estimation by the 'dilution plate' technique

This will be discussed in the next section with the spores formed by resistant strains of Botrytis cinerea.

3.2 Estimation of the proportion of spores produced by the vapour resistant strains, P(vap-r), T(vap-r) and D(vap-r), of Botrytis cinerea, which were resistant to fungicides

The proportion of spores which gave rise to resistant colonies will be expressed in terms of the total number of single spore colonies examined. The spores used in these experiments were obtained from 8-14 day old colonies of the vapour resistant strains P(vap-r), T(vap-r) and D(vap-r) of Botrytis cinerea which had been grown in the absence of fungicides. Sporulation of some of these resistant strains on glucose-casein-hydrolysate agar, the nutrient medium which has been used so far in these experiments, was very poor. This was particularly true of the strain resistant to TCNB. The resistant strains were therefore grown on other nutrient media and examined for

sporulation. Details of the results obtained on sporulation experiments will be given later in another section.

When fairly good sporulation was obtained, it was desired to purify the sporulating strain by single spore isolations; 5 single spores of each vapour-resistant strain were picked up at random and the colonies formed by them were inoculated on different nutrient agar media. At the same time, mycelial discs from these colonies were inoculated on glucose-casein-hydrolysate agar plates which were exposed to the vapour of fungicide to which the strain which produced the spores was resistant. Control plates without fungicide were also inoculated.

At the start of the experiment, it was expected that the spores produced by resistant strains would give rise to resistant colonies. But the results obtained on the linear growth in the presence of the vapour of fungicides of the 5 single spore colonies from each strain showed that this was not so. Of the 5 single spore colonies obtained from the spores of PCNB vapour resistant strain, only 2 were resistant to the vapour of PCNB. Ofthose obtained from the spores of the T(vap-r) strain, 3 were resistant to the vapour of TCNB, and of the 5 colonies from the spores

of the D(vap-r) strain, only 1 colony was resistant to the vapour of DCNA. The linear growth responses of the non-resistant colonies are given in Table 19.

Table 19

Linear growth of non-resistant colonies obtained from certain single spores of the vapour resistant strains P(vap-r), T(vap-r) and D(vap-r) of Botrytis cinerea in the presence of the vapour of PCNB, TCNB or DCNA.

Time after inoculation (hr.)	Mean increase in colony diameter (cm.) Treatment									
	CONTROL (no fungicide)				PCNB vapour		TCNB vapour		DCNA vapour	
	Parent	P vap-r	T vap-r	D vap-r	Parent	P vap-r	Parent	T vap-r	Parent	D vap-r
24	1.67	1.20	0.70	1.60	0.20	0.60	0.00	0.00	0.25	0.20
72	6.90	6.81	4.80	6.99	0.83	1.34	0.20	0.19	0.49	0.85
120	C	C	6.61	C	1.08	1.56M	0.25	0.31	1.22M	1.15
168	-	-	C	-	3.25M	6.20	0.53M	0.51M	2.67	1.90
192	-	-	—	-	5.31	C	1.05	1.12	4.02	2.30M
240	-	-	-	-	6.58	-	2.68	2.81	5.41	2.40
	-	-	—	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-

M = first appearance of resistant saltant.

The results suggest that there was no difference between the behaviour of a colony from the parent strain and those formed by non-resistant spores of the vapour-resistant strains. They all started with little or no growth in the presence of vapour of fungicides; rapid growth took place only after the formation of resistant saltants.

The experiment was repeated with 38 spores from the parent and each of the vapour resistant strains. The results obtained showed again that certain of the colonies formed by spores of resistant strains were resistant to treatment with fungicides, whereas the other colonies were susceptible. An analysis of the proportion of resistant colonies to the susceptible types is given below:-

Strain	No. of spores used	No. forming resistant colonies.	No. forming susceptible colonies
Parent	38	0	38
P(vap-r)	38	17	21
T(vap-r)	38	18	20
D(vap-r)	38	16	22

This problem was also investigated in another way: 25 single spores from each resistant and parent strain were picked up and were then allowed to germinate and grow directly in the presence of the vapour of fungicides. The results obtained are shown below:

Strain	No. of spores used	No. of colonies formed after 2 days	No. of colonies which were resistant	No. of colonies which were susceptible
Parent	25	0	0	-
P(vap-r)	25	15	15	0
T(vap-r)	25	10	10	0
D(vap-r)	25	12	12	0

These results showed that when colonies were derived from spores which initially were allowed to germinate and grow for two days in the presence of the vapour of fungicides, all such colonies were resistant to the vapour of fungicides. When the spores which had not formed colonies in two days were transferred to the surface of media which were not exposed to the vapour of fungicides, such spores formed colonies which, however, were susceptible when grown in the presence of vapour of fungicides. It now became very clear that not all the spores produced by vapour-resistant strains of Botrytis cinerea would give rise to resistant

colonies when grown in the presence of the vapour of fungicides. The latter part of this section deals with the estimation, based on a large number of single spores, of the proportion of 'resistant' spores to 'susceptible' spores, produced by the vapour resistant strains of Botrytis cinerea.

(a) Estimation by growing single spore colonies

150 single spore colonies of each strain, which had been formed in the absence of fungicides, were scored for resistance by measuring the mean daily increases in diameter of the cultures exposed to the vapour of fungicides and comparing them with those of the cultures in the control plates, after 2 days, when a resistant colony would have grown very well in the presence of the vapour of fungicides, and after 6 days, when a resistant colony would have covered the plate when growing in the presence or absence of the vapour of fungicides. There were 3 replicates for each treatment of each single spore colony. A colony was taken to be resistant when its mean daily increase in diameter in the presence of the vapour of fungicide was equal to, or greater than, one half of the mean of the mean daily increases in diameter of all the parent and resistant-spore colonies over the

same period in the absence of the vapour of fungicides. The proportion of spores which gave rise to resistant colonies was expressed as percentage of all the single spore colonies of any particular strain. The results are given below:-

Strain	No of single spore colonies examined	Percentage of colonies which were resistant Treatment PCNB vapour 2 days	PCNB vapour 6 days	TCNB vapour 2 days	TCNB vapour 6 days	DCNA vapour 2 days	DCNA vapour 6 days
Parent	150	0.0	0.07	0.0	0.0	0.0	0.06
P(vap-r)	150	55.9	60.2	-	-	-	-
T(vap-r)	150	-	-	55.6	65.7	-	-
D(vap-r)	150	-	-	-	-	46.3	46.3

These results confirmed the earlier results that not all the spores produced by the vapour-resistant strains of Botrytis cinerea gave rise to resistant colonies when grown in the presence of the vapour of fungicides; it would appear that about 55-60 per cent of the spores produced by the P(vap-r) strain, 55-65 per cent of those produced by the T(vap-r) strain, and 46 per cent of those produced by the D(vap-r) strain gave rise to resistant colonies; the remaining gave rise to susceptible colonies when grown in the presence of the vapour of fungicides. The percentage of resistant colonies after 6 days was slightly higher than that after 2 days in the case of P(vap-r) and T(vap-r) strains;

this was due to the formation of resistant sectors in some of those colonies which initially were not resistant. The method of estimation of proportion of resistant colonies did not make any allowance for the separation of those colonies which one might describe as 'partially resistant' from the truly resistant colonies; these 'partially' resistant colonies were those colonies which were not smooth-edged, and which did not grow so fast as the truly resistant colonies; but they did attain diameter which was about one half of the mean of increases in diameter of colonies which grew in the absence of vapour of fungicides. They were more like the 'hyphal variants' described on page 36 section 2.0 and 2.1 under 'experimental work and results'. If such colonies were excluded from the resistant colonies, the proportion of resistant colonies would be lower than that recorded. The proportion of resistant colonies obtained after 2 days would then be about 51.8 per cent for spores obtained from the P(vap-r) and about 50.9 per cent for spores obtained from the T(vap-r) strains; that for spores obtained from the D(vap-r) strain would still be 46.3 per cent.

(b) Estimation by the 'M.P.N.' technique

This method was not used for spores produced by vapour-resistant strains.

(c) Estimation by the 'dilution plate' technique

The method of estimating the proportion of spores which gave rise to resistant colonies, by growing individual single spore colonies and scoring them for resistance afterwards, was very tedious and it took a long time to do the experiments; and only a relatively small number of single spores could be examined. A method which was quicker and which gave more reliable results was the 'dilution plate' technique. It was based on the observation that in the presence of fungicides, colonies arising from 'resistant' spores could be distinguished from those arising from susceptible ones merely by inspection after three days' incubation at 20°C. (Plate 4). Resistant colonies were about 2-3mm. in diameter and had smooth edges; susceptible colonies were about 0.4mm., in diameter and were lobular with gnarled hyphae. The technique consisted of plating 0.05ml. of spore suspension of density about 2000 spores per ml. by spreading over the surface of nutrient agar with a sterile

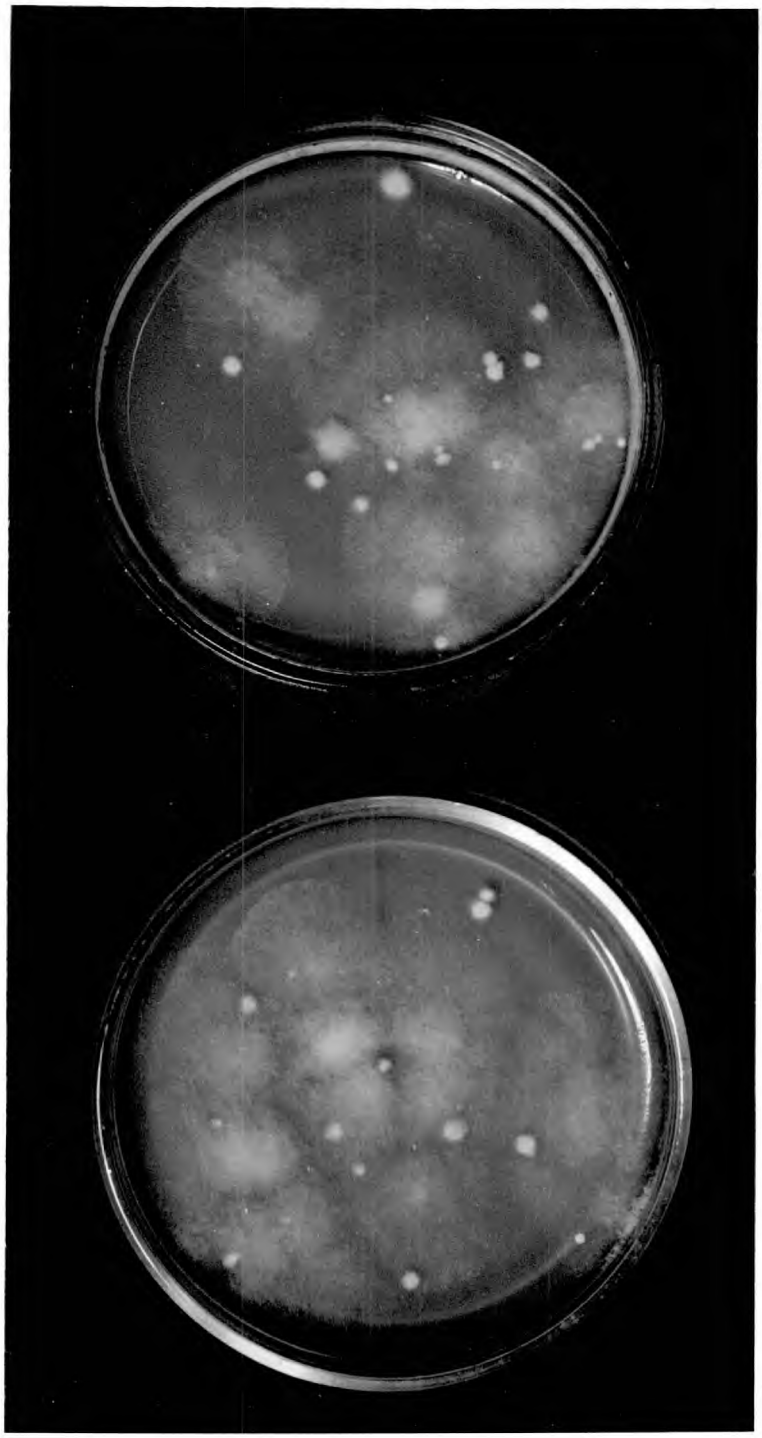


Plate 4 Behaviour of spores produced in the absence of fungicides by vapour resistant strains of Botrytis cinerea.

Resistant and non-resistant colonies formed when spores produced by PCNB vapour resistant strain were inoculated on agar exposed to vapour of PCNB.

glass rod. The plates were then exposed to the vapour of fungicides before incubation. This method has been described on page 31. Using this technique, the spores could be scored at densities of up to 200 colonies per plate; such high densities were not used in these experiments. When the method of raising individual single spore colonies was used for estimating percentage resistance, resistant and susceptible spores could be distinguished only after following their growth rates for about a week, requiring at least 6 petri dishes for each single spore growth. Scoring by the 'dilution plate' technique allowed a much larger number of spores to be classified, and, being based on larger samples, the ratios obtained were more reliable. The results obtained from 'dilution plate' experiments are recorded in Table 20.

Table 20

Estimation by the 'dilution plate' method, of proportion of spores produced by parent, P(vap-r), T(vap-r) and D(vap-r) strains of Botrytis cinerea which gave rise to resistant colonies.

Strain	No. of colonies scored (control) Exp.	Obs.	Treatment PCNB vapour R	S	% resistant	TCNB vapour R	S	% resistant	DCNA vapour R	S	% resistant
Parent	4470	4398	0	4382	0.0	0	4293	0.0	0	4372	0.0
P(vap-r)	4600	4008	2091	2015	50.2	-	-	-	-	-	-
T(vap-r)	1300	923	-	-	-	459	478	49.7	-	-	-
D(vap-r)	4600	3624	-	-	-	-	-	-	1823	1904	50.3

Exp. = expected number of colonies in the control plates.

Obs. = observed number of colonies in the control plates.

R = number of resistant colonies

S = number of susceptible colonies.

These results confirm the earlier observation that only about 50 per cent of the spores produced by vapour-resistant strains of Botrytis cinerea gave rise to resistant colonies. None of the spores of parent strain tested was resistant to any of the vapours of fungicides. (Plate 5) Some of the parent colonies growing in the presence of fungicides later gave rise to resistant saltants, especially in plates exposed to the vapour of PCNB; but the number of resistant saltants formed was very few indeed. This may be due to overcrowding of colonies on the plates. Almost invariably, no resistant saltants were formed in those plates in which parent colonies were exposed to the vapour of DCNA, but the colonies sporulated very well, and when these spores were tested for resistance, none of them gave rise to resistant colonies, although they had been formed in the presence of the vapour of DCNA, and from colonies which were derived from parent spores which germinated and developed in the presence of the vapour of DCNA.

A few of the parent colonies in plates exposed to vapour of TCNB gave rise to resistant saltants. Because of the large number of colonies

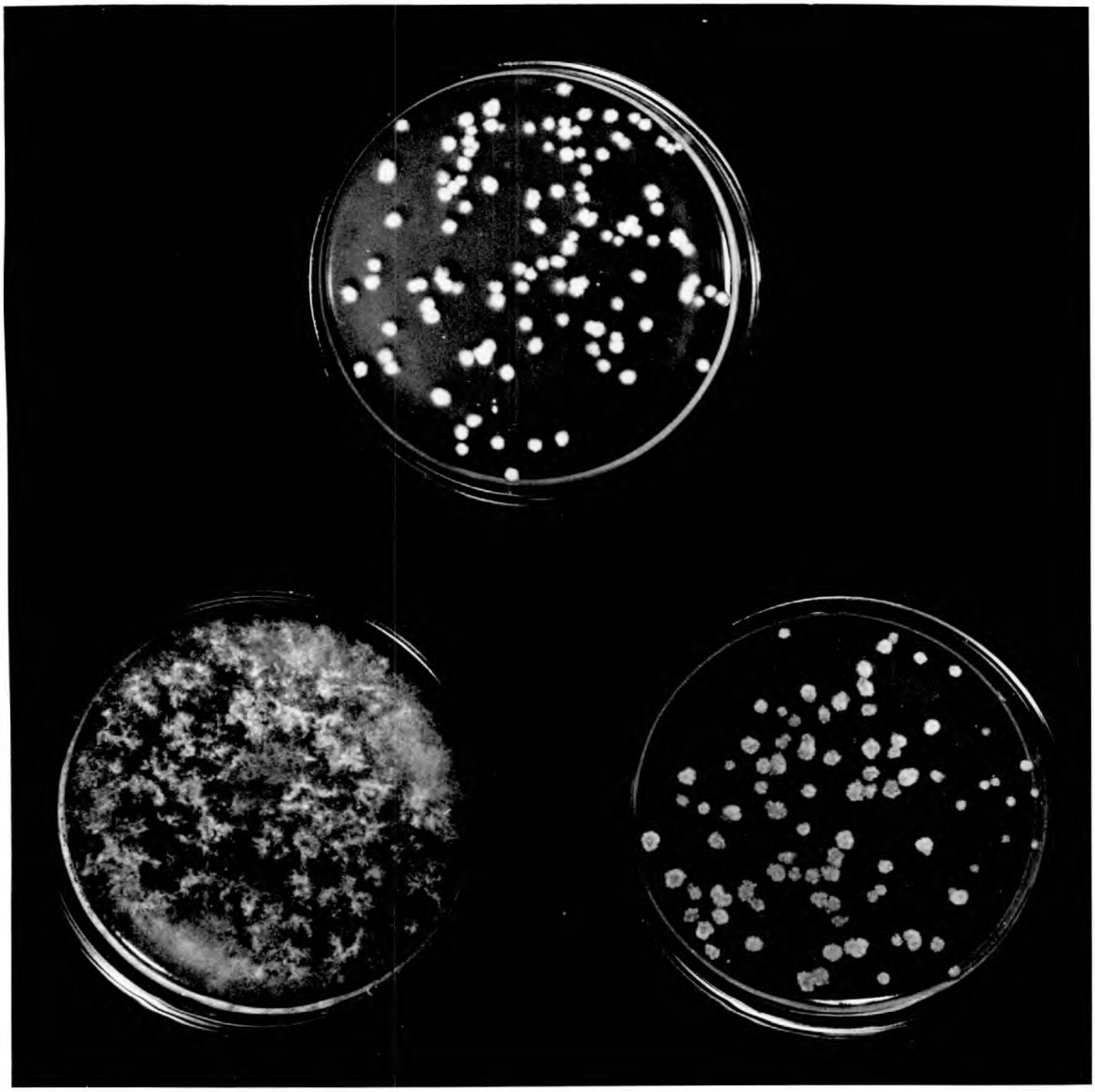


Plate 5 Behaviour of spores of parent strain of Botrytis cinerea in the presence of fungicides.

A. Non-resistant colonies formed in the presence of vapour of PCNB.

B. Non-resistant colonies formed in the presence of vapour of TCNB.

C. Colonies formed in the absence of vapour of fungicide.

on the plate, it was not very easy to estimate how many of the colonies would have given rise to resistant saltants; it appeared, however, that on the whole, the appearance of resistant saltants in plates exposed to vapour of PCNB or TCNB was less frequent when the inoculum was a spore than when the inoculum was a young mycelial disc.

The spores used for inoculation in these experiments were the 'first generation' of spores produced by the vapour-resistant strains of Botrytis cinerea when they were grown in the absence of fungicides. The resistant and susceptible colonies obtained from these 'first generation' spores of vapour resistant strains were subcultured and allowed to grow in the absence of the vapour of fungicides. When they sporulated, these 'second generation' of spores were tested for resistance and the proportion of 'resistant' spores was determined by the dilution plate technique.

The results obtained with the two types of 'second generation spores' will be given separately.

4000 'second generation spores' produced by colonies derived from non-resistant 'first generation spores' of each of P(vap-r), T(vap-r) and D(vap-r)

strains as well as spores produced by parent strain were used. None of the colonies formed from the 4000 spores in each strain was resistant to vapour of fungicide. This result showed that when spores produced by resistant strains gave rise to susceptible colonies, such susceptible colonies did not produce spores which gave rise to resistant colonies; all the spores they produced gave rise in their turn to colonies which were susceptible to treatment with vapour of fungicides. There was no 'reversion' in these 'second generation' spores to the resistant type; all the colonies produced were of the parent, non-resistant type.

The results obtained from experiments with the other type of 'second generation' spores, those produced by colonies derived from 'resistant' 'first generation' spores, are recorded in Table 21.

Table 21

Estimation, by the 'dilution plate' method, of proportion of 'second generation' spores produced by colonies derived from 'resistant' 'first generation' spores of the P(vap-r), T(vap-r) and D(vap-r) strains, which gave rise to resistant colonies.

Strain	No. of colonies scored (control) Exp.	Obs.	Treatment PCNB vapour R	S	% resistant	TCNB vapour R	S	% resistant	DCNA vapour R	S	% resistant
Parent	4000	3892	0	3785	0.0	0	3690	0.0	0	3801	0.0
P(vap-r)	4000	3705	1299	2387	38.0	-	-	-	-	-	-
T(vap-r)	1500	1397	-	-	-	609	917	39.8	-	-	-
D(vap-r)	4000	3723	-	-	-	-	-	-	1723	2438	43.8

Exp. = expected number of colonies in the control plates

Obs. = observed number of colonies in the control plates

R = number of resistant colonies

S = number of susceptible colonies

These results showed that when colonies were formed from the 'resistant' spores of the 'first generation' spores of the resistant strains, such colonies produced spores, some of which gave rise to resistant colonies, but most of which gave rise to susceptible colonies. The percentages of resistant colonies in these 'second generation' spores were less than the percentages in the 'first generation' spores in all cases; their values were 38.0, 39.8 and 43.8 for P(vap-r), T(vap-r) and D(vap-r) respectively, compared with about 50 in Table 20. If the results in Tables 20 and 21 were considered alongside each other, it appeared that only about 19 per cent to 21 per cent of the spores produced originally by the vapour-resistant strains were likely to give rise to vapour-resistant colonies at the 'second generation spore' stage. The proportion of 'resistant' spores among the 'third generation spores' was determined for the P(vap-r) strain, and it was 36.8 per cent. The proportion of 'resistant' 'third generation spores' was not determined for the T(vap-r) and D(vap-r) strains; it seems probable, however, that had this proportion been determined, its value would have lain between 35 per cent and 45 per cent in both cases. It appeared then that with each fresh generation of spores, the number of spores that would give

rise to resistant colonies was progressively reduced by about 55 to 65 per cent.

All the spores used in the experiments described in sections 3.0, 3.1 and 3.2 were produced from colonies which grew in the absence of vapour of fungicides. Sometimes spores were produced in the presence of vapour of fungicides. The parent and the D(vap-r) strains sporulated well when they were grown in the presence of the vapour of DCNA (page 50); occasionally very few spores were obtained when the P(vap-r) and T(vap-r) strains were grown in the presence of vapour of PCNB. The proportion of spores, produced in the presence of vapour of fungicides, which gave rise to resistant colonies, was determined by 'dilution plate' method. 4000 spores of parent strain, produced in the presence of the vapour of DCNA, 2000 spores of P(vap-r) strain produced in the presence of PCNB, and 4000 spores of D(vap-r) strain produced in the presence of vapour of DCNA were used in these experiments. Spores formed by the parent strain gave rise to colonies which were susceptible to treatment with the vapour of DCNA, even though these spores had been formed in the presence of the vapour of DCNA. The spores formed by the P(vap-r) and D(vap-r) strains

gave rise to colonies which were resistant in the presence of the vapour of PCNB and DCNA respectively. (Plate 6) These spores were formed in the presence of the vapour of fungicides. This result is in contrast to the behaviour of spores of the same strain which were formed in the absence of fungicides and in which the percentage of 'resistant' spores was at first 50, but became progressively reduced with each fresh generation of spores. It is significant to note that in experiments with spores produced by the D(vap-r) strain in the presence of vapour of DCNA, the number of colonies observed in the control plates was far less than the number expected; the observed number of colonies was about 50 per cent of the expected number, both in the control untreated plates and in plates exposed to the vapour of DCNA.

3.3 Estimation of the proportion of spores produced by the impregnated resistant strains P(imp-r), T(imp-r) and D(imp-r) of Botrytis cinerea, which gave rise to resistant colonies

Spores were obtained from the impregnated resistant strains P(imp-r), T(imp-r) and D(imp-r), of Botrytis cinerea, which had been grown both on untreated

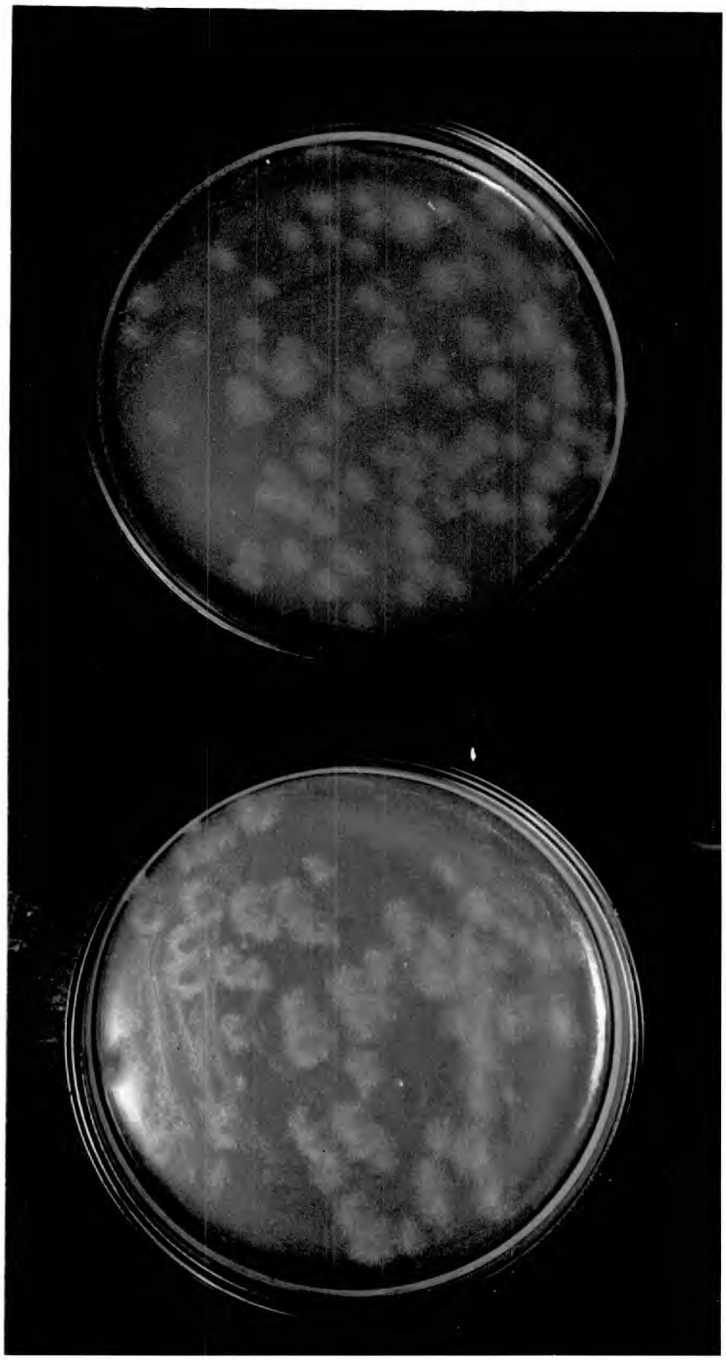


Plate 6 Behaviour of spores produced in the presence of fungicides by vapour resistant strains of Botrytis cinerea.

Only resistant colonies were formed when spores produced in the presence of vapour of PCNB by PCNB-vapour resistant strain were inoculated on agar exposed to vapour of PCNB.

agar, and on agar impregnated with fungicides at the concentration of 250 p.p.m.. All the three impregnated resistant strains sporulated appreciably on agar impregnated with DCNA. The T(imp-r) strain sporulated both in the absence of, and on agar impregnated with, TCNB. Not many spores were formed in either case, however, and there were several plates in which sporulation was not observed. The P(imp-r) strain sporulated fairly well on agar without PCNB, and also sometimes on agar impregnated with PCNB. The parent strain sporulated on agar impregnated with DCNA; the amount of spores produced in such cases was, however, very small, since growth of parent strain on DCNA-impregnated agar was very limited indeed, unless resistant saltants were formed. The results obtained of estimation, by 'dilution plate' method, of proportion of spores which gave rise to resistant colonies, are recorded in Table 22. When spores were obtained from agar impregnated with fungicides, a suffix "(ex-imp)" was added to their description, and when they were obtained from agar without fungicide treatment, the suffix "(ex-o)" was used to describe them. Thus spores which were obtained from P(vap-r) strain which had grown on agar impregnated with PCNB were described as P imp-r (ex P imp), and those obtained from D(imp-r) strain which had grown in the absence of fungicides were described as D imp-r (ex-o).

Table 22

Estimation of the proportion of spores produced by parent and impregnated resistant strains of Botrytis cinerea which gave rise to resistant colonies.

Type of spore	No. of colonies scored (control) Exp.	Obs.	Treatment PCNB impregnated R	S	% resistant	TCNB impregnated R	S	% resistant	DCNA impregnated R	S	% resistant
Parent (ex-o)	2300	2106	0	952	0.0	0	528	0.0	0	18	0.0
Parent (exDimp)	2200	1988	0	870	0.0	0	483	0.0	0	12	0.0
imp-r (ex-0)	1800	1242	1265	0	100.0	-	-	-	-	-	-
imp-r (exPimp)	2000	1682	1334	0	100.0	-	-	-	-	-	-
Timp-r (ex-o)	1500	568	-	-	-	261	0	100.0	-	-	-
Timp-r (exTimp)	1200	429	-	-	-	327	0	100.0	-	-	-
Dimp-r (ex-o)	1500	294	-	-	-	-	-	-	240	0	100.0
Dimp-r (exDimp)	1800	374	-	-	-	-	-	-	296	0	100.0

Exp. = expected number of colonies
Obs. = observed number of colonies
R = number of resistant colonies
S = number of susceptible colonies

These results showed that when spores produced by impregnated resistant strains gave rise to colonies, only resistant colonies were formed, whether these spores were produced in the absence of fungicides or on agar impregnated with fungicides. The number of colonies actually formed was, however, small compared with the number of colonies expected from the density of spore inoculations, as determined by haemacytometer counts. There were several instances in which the number of colonies observed, even in the control plates, was only about 10 to 15 per cent of the actual number expected, especially with the T(imp-r) and D(imp-r) strains. A probable explanation for this result would be that most spores germinated and then stopped growing before they could form mycelium, because spore germination experiments (described later, page 133) showed that spores of resistant strains were not inhibited in their germination by the fungicides to which the strains which produced these spores were resistant.

Not only did spores from parent strain give rise only to susceptible colonies, but the number of colonies formed on impregnated agar was very small compared with the number formed in the control plates,

or even on agar exposed to the vapour of fungicides (page 95). The number of parent colonies formed on impregnated agar was only a fraction of the expected number, ranging from about 45 per cent on agar impregnated with PCNB to 25 per cent on agar impregnated with TCNB and only about 0.9 per cent on agar impregnated with DCNA. Also the number of resistant saltants developed was very small indeed; only 2 saltants were obtained from the 18 colonies on agar impregnated with DCNA, and that was after 68 days after inoculation.

The concentration of fungicides in the agar used in these experiments was 250 p.p.m.. Because of the small number of spores of parent strain which gave rise to colonies on agar impregnated with TCNB and DCNA, it was desired to find out whether there would be any differences in the number of colonies formed by spores of parent strain if agar was impregnated with different concentrations of fungicides. The results obtained in such experiments are shown overleaf.

Effect of concentration of fungicide on the number of colonies formed by spores of parent strain of Botrytis cinerea on agar impregnated with PCNB, TCNB and DCNA

Concentration of fungicides (p.p.m.)	Number of colonies formed Treatment		
	PCNB	TCNB	DCNA
0	456*	496*	344*
1	429*	483*	254*
2	380+	421*	382*
5	390	397+	37
10	393	235+	15
25	375	36	7
50	405	28	13
100	384	55	5
250	363	57	7
500	318	45	8

(i) * = colonies had hyphal morphology like that of parent growing in the absence of fungicides.

(ii) + = colonies had hyphal morphology like that of the non-resistant hyphal variant of the parent.

(iii) All other colonies were of the very slow-growing susceptible type from which resistant saltants may arise later.

A complementary experiment on the effect of different concentrations of fungicides on the number of colonies formed by spores of resistant strains was not done because there was not much difference in the number of colonies formed by spores of resistant strains on agar which contained no fungicide and agar which was impregnated with 250 p.p.m. of fungicide. The results with spores of parent strain showed that PCNB, in the concentrations used, had no very significant effect on the number of spores of parent strain which gave rise to colonies; almost the same number of colonies were observed in the control plates with no fungicide and in plates which contained up to 500 p.p.m. PCNB.(Plate 7) TCNB at concentrations above 10 p.p.m. and DCNA at concentrations above 2 p.p.m. very significantly reduced the number of colonies observed. These results on the effect of concentration of fungicide on the proportion of spores which gave rise to colonies are similar to those on the effect of concentration of fungicide on linear growth of mycelium of parent strain of Botrytis cinerea (page 74) and to those on spore germination (page 125).

Because the number of colonies observed on plates, both with and without fungicides, which were scored with spores of the impregnated-resistant strains

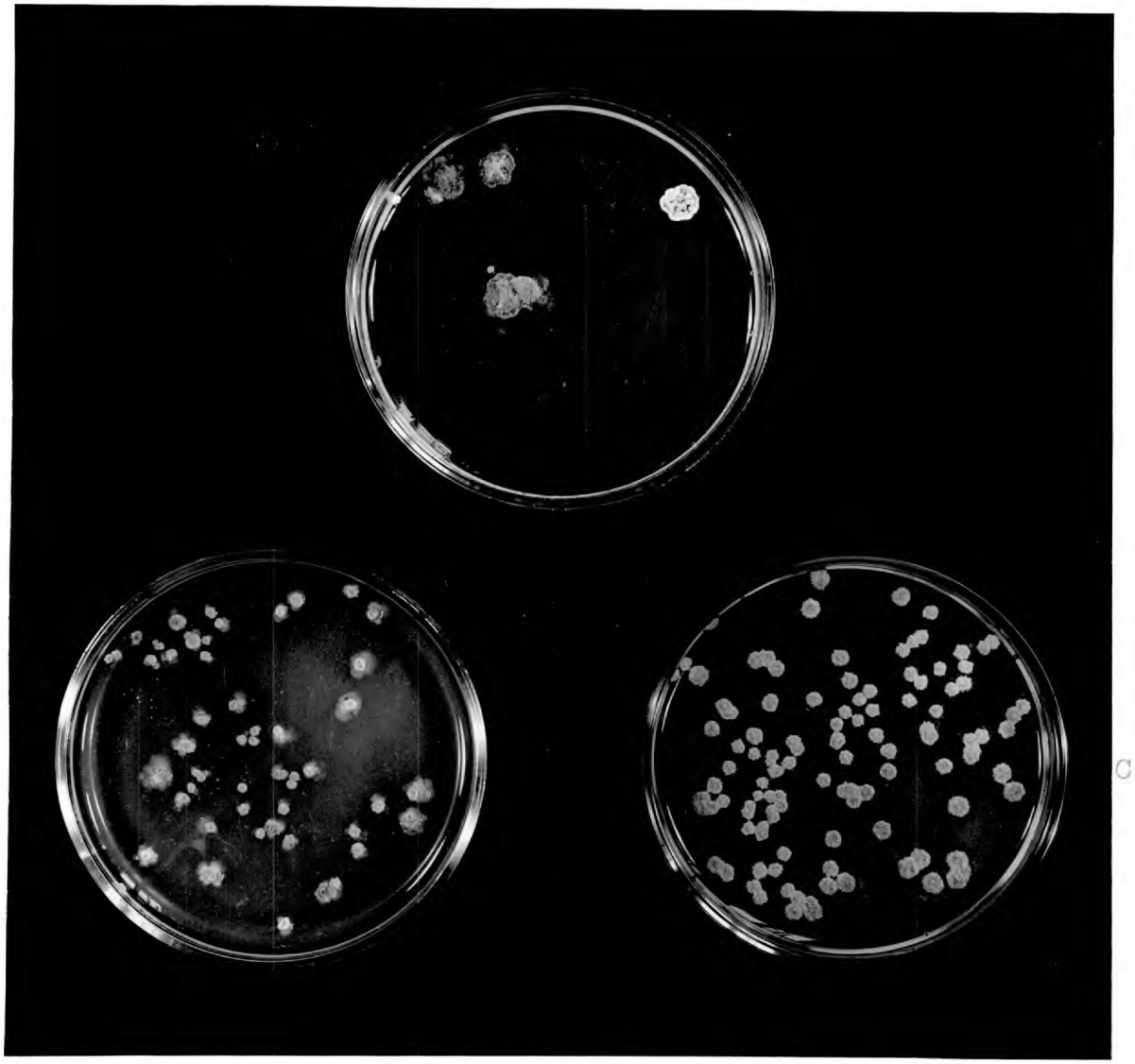


Plate 7 Behaviour of spores of parent strain of Botrytis cinerea in the presence of fungicides.
Non-resistant colonies formed from spores of parent strain when they were inoculated on agar which was

A. impregnated with 50 p.p.m. DCNA (60-day old colonies)

B. impregnated with 100 p.p.m. TCNB (10-day old colonies)

C. impregnated with 500 p.p.m. PCNB (8-day old colonies)

fell far short of the expected number, calculated from the volume and density of spore suspension used for inoculation, and because only colonies of the resistant type were obtained, it was thought possible that these strains might have produced the 'non-resistant' type of spores which, however, were unable to develop in the presence of the 'resistant' type of spores. To investigate this possibility, the following experiment was set up.

Spore suspension of known concentration of the parent, T(imp-r) and D(imp-r) strains were prepared. Samples of suspension of parent spores were mixed with samples of each of the other two suspensions in the ratios of 1:3, 1:1, and 3:1, the parent spore part of the ratio being given first. Thus 9 different samples of spore suspensions were obtained. These were then scored on pla' of nutrient agar with or without fungicides.

The results obtained are given overleaf.

Effect of the presence of spores obtained from the impregnated resistant strains on the development of spores obtained from parent strain

Treatment	No. of colonies observed Type of spore suspension used for inoculation Pa	T-r	D-r	Pa:T-r 1:3	Pa:T-r 1:1	Pa:T-r 3:1	Pa:Dr 1:3	Pa:Dr 1:1	Pa:Dr 3:1
Control	662	559	290	670	543	548	562	546	597
TCNB	540	581	-	598	415	228	-	-	-
DCNA	10	-	233	-	-	-	105	126	149

These results suggested that the development of spores from the parent strain was independent of the presence and concentration of spores obtained from T(imp-r) and D(imp-r) strains. This would suggest that if the impregnated resistant strains were capable of producing non-resistant spores which in behaviour were similar to spores of the parent strain, then the T(imp-r) and D(imp-r) strains could not have produced such non-resistant spores. Had such spores been produced, their development could not have been affected by the presence of the 'resistant' type of spores. A probable explanation, as suggested on page 107, would be that certain of the spores produced by impregnated resistant strains germinated and then quickly stopped growing.

HETEROKARYON

It was desired to find out what type of spores would be produced by heterokaryons formed between parent and the impregnated-resistant strains. The impregnated-resistant strains were chosen because, as far as had been determined, they produced only spores which gave rise to 'resistant' colonies.

0.5ml. of dense spore suspensions, about 200,000 spores per ml., of parent strain was mixed separately and thoroughly with 0.5ml. dense spore suspension of spores from T(imp-r) and D(imp-r) strains. The spore mixture, Pa/T-r and Pa/D-r, were then inoculated on nutrient agar by spreading them carefully with a glass rod over an area about 4.0 cm. diameter. It was expected that heterokaryons would be formed between the parent and each of the two resistant strains. After incubating the cultures for 3 days at 21°C, mycelial discs were taken from the edges of the colonies produced and inoculated on both untreated agar (control) and agar impregnated with PCNB, TCNB or DCNA. They were then scored for resistance by measuring their mean increases in diameter over a period of 6 days. 13 days after the plates inoculated with mixtures of spores had been incubated, when the colonies had already formed spores, the spores were scored for resistance by the dilution plate technique. The results obtained are given in Table 23.

Table 23

Estimation of the proportion of spores produced by heterokaryon between parent and T(imp-r) or D(imp-r) strains of Botrytis cinerea which gave rise to resistant colonies

Type of heterokaryon	Type of colonies	Total no. of colonies and percentage of resistant colonies Treatment CONTROL	TCNB	TCNB	DCNA
Parent spores alone	R	-	0	0	0
	S	-	1103	440	31
	Total	1124	1103	440	31
	% R	-	0.00	0.00	0.00
Parent + T(imp-r) (Pa/T-r)	R	-	84	33	38
	S	-	844	185	62
	Total	705	928	218	100
	% R	-	11.9	4.68	5.4
Parent + D(imp-r) (Pa/D-r)	R	-	150	106	88
	S	-	295	220	179
	Total	628	445	326	267
	% R	-	23.8	16.8	14.0

R = number of resistant colonies observed

S = number of susceptible colonies observed

Total= total number of colonies observed

% R = percentage of the total number of colonies which were resistant.

These results showed again that the number of parent spores which gave rise to colonies on impregnated agar, all of which colonies were susceptible, was small compared with the number of colonies in the control plates.

The percentages of resistant colonies obtained from inoculations with spores of heterokaryons were based on the total number of colonies observed in the control plates and not just on the number of colonies formed on agar impregnated with fungicides. As was seen in the results recorded on page 105 the number of colonies developing from 'resistant' spores obtained from the T(imp-r) and D(imp-r) strains was very similar to the number of such spores forming colonies on the control plates. Such numbers, however, may be very few compared with the total number of spores these resistant strains produced, but they gave a fairly correct value of the number of 'viable' spores. If the heterokaryons had produced both 'resistant' and 'susceptible' spores, the number of 'resistant' spores which formed colonies in the control plates would be about the same as those which formed colonies on plates containing agar impregnated with either TCNB or DCNA. Since 'resistant' and

'susceptible' spores did not appear to interfere with the development of each other, it could be assumed that most of the viable spores produced by the heterokaryons were of the susceptible type. The results on measurement of the linear growth of the hyphae of the heterokaryons showed that the hyphae were resistant to treatment with fungicide. This was perhaps to be expected; any 'resistant factor' in the mycelium of the heterokaryon would allow it to grow in the presence of fungicide.

3.4 Summary of results on the determination of the proportion of 'resistant' spores produced by parent and resistant strains of Botrytis cinerea

All the spores produced by parent strain gave rise to colonies which were susceptible to treatment with fungicides; such colonies may later develop resistant saltants. It did not matter whether such spores were formed in the presence or in the absence of fungicides. When spores were formed by the vapour-resistant strains in the absence of fungicides, about 50 per cent of such spores gave rise to 'resistant' colonies, and the rest formed susceptible colonies. The proportion of 'resistant' spores was progressively reduced at each successive spore generation.

When vapour-resistant strains formed spores in the presence of the vapour of fungicides, such spores gave rise only to 'resistant' colonies. Such spores may, however, not be all viable in the D(vap-r) strain. Spores formed by the impregnated-resistant strains gave rise only to resistant colonies, whether these spores were formed in the absence or in the presence of fungicides; only a small proportion of such spores in the T(imp-r) and D(imp-r) strains, however, were viable. When heterokaryons were formed between the parent and the T(imp-r) or D(imp-r) strains, by allowing a mixture, in equal proportion, of their spores to form colonies, their mycelium appeared to be resistant to fungicides, but most of the spores they produced were of the 'susceptible' type, only about 5-12 per cent (Pa/T-r heterokaryon) and 14-24 per cent (Pa/D-r heterokaryon) of such spores were of the resistant type. Spores of parent and resistant strains did not appear to interfere with the development of each other when they were mixed together.

3.5 Experiments on spore germination

The germination of spores of parent and resistant strains of Botrytis cinerea in the presence

or absence of fungicides was investigated. Spore suspension of the desired strain, with a final concentration of about 50,000 spores per ml., was prepared after washing with sterile distilled water by centrifuging. Germination was recorded in all cases 24 hours after incubation at 21°C. These experiments will be considered under various sections; any experimental details will be discussed in the appropriate section.

A. Germination of spores of parent strain in the presence of the vapour of PCNB, TCNB and DCNA

Coverslips of diameter ½", were fixed with molten Vaseline to slides which were then placed either in moist incubating chambers - plastic boxes lined all over on the inside with damp blotting paper -, or on plain agar in petri dishes on the lids of which 10mg. PCNB, TCNB or DCNA had either been deposited, or not.

Plates of nutrient and plain agar were also prepared which were either exposed, or not, to the vapour of fungicides, but in which slides with coverslips were not placed. Some of these agar plates were exposed to the vapour of fungicides for specified periods of time before they were used for spore germination

experiments. When it was desired to study spore germination in the presence of vapour of fungicides, 0.05ml. of spore suspension was placed without spreading, on the coverslips or directly on the surface of agar, and incubated for 24 hours in the presence or absence of vapour of fungicides, before germination was recorded. The results obtained are given in Table 24.

Table 24

Germination of spores of parent strain of Botrytis cinerea in the presence of the vapour of PCNB, TCNB or DCNA

Time after exposure of agar to vapour of fungicides (days)	Percentage Inhibition of Germination Treatment			
	Control	PCNB	TCNB	DCNA
0	0.0	0.0	55.7	0.0
5	-	0.0	56.0	0.0
10	-	0.0	55.9	0.0
15	-	0.6	56.1	1.0
35	-	0.0	60.0	70.0

These results have been expressed as percentage inhibition of germination and the figures were based on zero for inhibition of germination in the controls. To estimate germination, three individual counts of spores from each of three replicates in at least two separate experiments were made.

All the spores germinated in the presence as well as in the absence of the vapour of fungicides, even when agar had been exposed to the vapour of fungicides for several days before the drop of spore suspension was placed on it. The only exception was the vapour of TCNB which inhibited spore germination by about 55 per cent. But even here, most of the spores which did not germinate did develop germ tubes which, however, were not long enough to regard them as evidence of germination. It appeared then that the vapour from 10 mg. of PCNB or DCNA had no effect on spore germination; germ tubes formed in the presence of vapour of fungicides were, however, much shorter than those formed in their absence. When agar was exposed to the vapour of fungicides for 15 days before inoculation with spore suspension, the amount of fungicide which dissolved in agar did not affect the results on germination of spores, except for DCNA after 35 days. There was no inhibition of spore germination in drops of spore suspension which were placed on coverslips instead of on agar, and which were incubated under the same conditions.

B. Germination of spores of parent strain of Botrytis cinerea in water suspensions of PCNB, TCNB and DCNA

Samples of spore suspension, about 100,000 spores per ml., were mixed separately with equal volumes of graded series of suspension of fungicides in water so that the mixture contained spores at 50,000 spores per ml. in suspension of fungicides in water ranging from 1 p.p.m. to 4000 p.p.m. of PCNB, TCNB or DCNA. 0.05ml. of these spore-fungicide mixtures were then pipetted on coverslips on slides incubated in moist chambers for 24 hours.

There was no inhibition of germination in suspensions containing either PCNB or TCNB up to 4000 p.p.m. Inhibition of germination in suspensions containing 25, 50, 100, 250, 500, 1000, 2000 and 4000 p.p.m. DCNA were 22.0, 0.6, 1.0, 9.3, 18.3, 15.0, 1.3, and 3.9 per cent respectively. The results showed that when spores of parent strain were mixed with suspension of fungicides in water up to 4000 p.p.m., only DCNA inhibited germination very slightly. In one of the experiments, DCNA did not inhibit spore germination at all at any of the concentrations in which it was used. In another series of experiments, the spore-fungicide mixture was inoculated on plain and nutrient

agar, instead of on coverslips. Here again, there was no inhibition of germination at concentrations up to 1000 p.p.m. of fungicide. PCNB at 4000 p.p.m. and TCNB at 2000 p.p.m. inhibited germination by 13.0 and 21.6 per cent respectively, but DCNA at 4000 p.p.m. inhibited germination by 90%, on plain agar, DCNA at 2000 p.p.m. had no effect on germination. These results were similar to those obtained when drops of spore-fungicide mixtures were placed on coverslips, namely, the fungicides PCNB, TCNB and DCNA had no effect on germination of spores of parent strain of Botrytis cinerea when they were mixed with spores in water suspension containing up to 2000 p.p.m. of fungicide.

C. Germination of spores of parent strain of Botrytis cinerea on agar impregnated with PCNB, TCNB or DCNA

Plates of plain and nutrient agar, impregnated with PCNB, TCNB or DCNA at various concentrations, were prepared. In some of the plates, agar was impregnated with acetone solution, instead of water suspension, of fungicides. Such plates contained 5.0 per cent, 1.0ml. in 20ml. agar, of acetone. 0.05ml. spore suspension were placed on the plates before incubation for 24 hours at 21°C. The results obtained are recorded in Table 25.

In these results,

N(o) = nutrient agar impregnated with water suspension of fungicide

N(Ac) = nutrient agar impregnated with acetone solution of fungicide

P(Ac) = plain agar impregnated with acetone solution of fungicide

and treatment with fungicides at concentrations of 0 p.p.m. were the control plates in which there was no fungicide, but which contained 5 per cent acetone in treatments in which acetone was added.

Table 25

Germination of spores of parent strain of Botrytis cinerea on agar impregnated with PCNB, TCNB or DCNA.

Concentration of fungicide (p.p.m.)	Percentage Inhibition of Germination Treatment								
	PCNB			TCNB			DCNA		
	N(o)	N(Ac)	P(Ac)	N(o)	N(Ac)	P(Ac)	N(o)	N(Ac)	P(Ac)
0	0.0	9.3	3.0	0.0	9.3	3.0	0.0	9.3	3.0
1	0.0	9.6	4.8	0.0	2[illegible].0	66.0	0.0	22.7	16.0
2	0.0	16.7	6.9	0.0	74.3	94.3	0.0	67.1	--
5	0.0	43.0	46.9	0.0	85.0	95.7	0.0	94.0	94.6
10	0.0	34.3	48.0	45.1	76.7	97.0	97.3	94.5	97.0
25	0.0	27.7	47.8	79.6	83.6	98.0	99.5	96.5	100.0
50	0.0	74.7	49.3	86.6	86.3	98.5	99.5	96.3	100.0
100	0.6	66.7	49.5	85.8	89.3	98.0	96.7	96.5	100.0
250	0.0	43.0	55.9	90.3	90.0	98.0	100.0	98.5	100.0
500	0.0	87.0	75.0	96.3	91.0	98.8	100.0	100.0	100.0

These results illustrated the high activity of the fungicides when they were impregnated in agar. DCNA more or less completely suppressed germination of the spores when it was impregnated at concentrations of 10 p.p.m. or more; TCNB very effectively suppressed germination at a concentration of 25 p.p.m. or more. PCNB did not prevent germination of spores when it was impregnated in agar; the germ tubes, however, were very short indeed at concentrations above 100 p.p.m.. There was no doubt that impregnation of the fungicides with 5 per cent acetone increased their activity against the spores of Botrytis cinerea; this is particularly true of PCNB when it inhibited germination up to 87.0% in nutrient agar, and up to 75 per cent in plain agar; TCNB which was not active up to 10 p.p.m. without acetone inhibited germination of 94 per cent of spores when acetone was present; DCNA at 5 p.p.m. with acetone inhibited 94 per cent of the spores; it had no effect on spore germination at this concentration without acetone. Apart from results with TCNB at concentrations up to 500 p.p.m., there was not much difference in activity of fungicides whether they were impregnated, with acetone, in plain or in nutrient agar.

D. Germination of spores of parent strain of Botrytis cinerea when spores were mixed with the fungicides PCNB, TCNB and DCNA, suspended in media other than plain water

The big difference in activity, expressed as percentage inhibition of germination of spores, of the fungicides, particularly TCNB and DCNA, when they were suspended in water and when they were impregnated in agar, was very striking indeed. Experiments were devised to investigate how active these fungicides would be if they were suspended in liquid agar or other media rather than in water. 0.1 per cent was found to be the highest concentration of agar in water which would give a suitable liquid medium on cooling after steaming in a steam bath. Agar, fungicide suspension in water, and spore suspension were prepared at three times the required concentrations. When the agar had cooled to about 40°C., all three substances were mixed in equal volumes such that their final concentrations in the triple mixture were: agar, 0.1%; spores, 50,000 per ml., fungicides, a range from 1 to 500 p.p.m. 0.05ml. of the mixture were then placed on coverslips and incubated for 24 hours at 21°C. in humid chambers. Other experiments were set up in which 0.1 per cent

solutions of the following substances in water were substituted for agar: (i) Pectin (ii) Gelatine (iii) Carboxymethyl cellulose (C.M.C.). Some preparations of media had 5 per cent acetone added to them.

The results obtained are recorded in Table 26.

Table 26

Germination of spores of parent strain of Botrytis cinerea when spores were mixed with PCNB, TCNB and DCNA suspended in 0.1 per cent solutions of agar, pectin, gelatine, or carboxy-methyl-cellulose (C.M.C.)

For convenience of presentation, results obtained of treatments with (i) PCNB (ii) TCNB and (iii) DCNA, are given separately.

(i) PCNB treatment

Medium (0.1% solution in water)	Percentage Inhibition of Germination Concentration of PCNB in mixture (p.p.m.) 0	1	2	5	10	25	50	100	250	500
Water alone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Water + 5% acetone	9.3	13.6	-	14.3	-	23.3	-	46.5	67.3	69.0
Agar alone	7.0	39.6	32.5	41.7	38.3	32.3	43.8	52.3	55.8	76.9
Agar + 5% acetone	12.3	36.5	-	36.7	-	46.9	-	48.2	68.0	69.3
Pectin alone	0.0	0.0	0.8	1.7	1.0	1.0	1.6	2.0	1.8	2.3
Pectin + 5% acetone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gelatine alone	8.3	17.2	-	50.0	-	78.6	-	83.0	-	-
Gelatine + 5% acetone	8.3	15.3	-	60.7	-	76.3	-	80.0	-	-
C.M.C. alone	3.7	6.3	-	16.3	-	21.0	-	31.7	-	-
C.M.C. + 5% acetone	3.3	14.3	-	13.0	-	45.3	-	50.0	-	-

(ii) TCNB treatment

Medium (0.1% solution in water)	Percentage Inhibition of Germination Concentration of TCNB in mixture (p.p.m.) 0	1	2	5	10	25	50	100	250	500
Water alone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Water + 5% acetone	9.3	9.6	-	19.0	-	16.3	-	92.0	100.0	100.0
Agar alone	6.7	34.8	35.8	32.8	38.7	70.0	72.7	72.3	97.7	98.0
Agar + 5% acetone	12.3	13.7	-	13.0	-	39.0	-	72.7	98.0	99.0
Pectin alone	0.0	0.7	2.3	0.9	4.3	2.9	5.7	9.5	83.3	91.4
Pectin 5% + acetone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	68.8	70.9
Gelatine alone	8.3	14.0	61.0	62.7	61.7	60.5	55.0	60.8	76.3	78.9
Gelatine + 5% acetone	8.3	14.2	58.5	61.9	62.3	59.5	60.4	63.1	70.8	79.3
C.M.C. alone	3.7	17.2	22.9	23.0	21.5	27.0	19.2	21.7	26.8	60.0
C.M.C. + 5% acetone	3.3	18.3	22.8	20.5	21.6	24.8	21.8	22.4	26.9	58.3

(iii) DCNA treatment

Medium (0.1% solution in water)	Percentage Inhibition of Germination Concentration of DCNA in mixture (p.p.m.)									
	0	1	2	5	10	25	50	100	250	500
Water alone	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Water + 5% acetone	9.3	10.0	9.0	7.0	-	77.3	-	88.0	90.0	93.0
Agar alone	6.7	16.0	-	29.6	-	60.6	-	71.0	97.0	97.0
Agar + 5% acetone	12.3	16.0	-	26.6	-	69.6	-	51.2	83.3	100.0
Pectin alone	0.0	0.7	0.7	1.0	1.0	6.7	9.7	6.0	71.3	79.5
Pectin + 5% acetone	0.0	0.0	0.6	4.6	4.1	4.6	4.6	4.6	69.9	79.4
Gelatine alone	8.3	11.9	60.9	61.8	66.0	71.6	82.5	86.3	94.8	100.0
Gelatine + 5% acetone	8.3	10.8	61.6	60.7	67.1	69.3	78.5	81.4	85.6	93.4
C.M.C. alone	3.7	15.7	28.8	20.7	35.7	43.0	44.4	66.2	82.2	90.0
C.M.C. + 5% acetone	3.3	14.5	21.3	28.5	39.3	44.9	44.8	61.3	79.4	88.2

These results showed that there was no difference in activity of PCNB, TCNB and DCNA when they were suspended in plain water or in dilute solution of pectin in water, except at concentrations of 250 p.p.m. or more when TCNB and DCNA, suspended in pectin, inhibited the germination of more than 50 per cent of the spores. The addition of 5 per cent acetone to water or to pectin solution improved the activity of these fungicides in water especially at concentrations above 25 p.p.m., but not in pectin.

When they were suspended in a solution of carboxy-methyl-cellulose (C.M.C.), PCNB, TCNB and DCNA showed some antifungal activity in preventing germination of spores, especially at concentrations above 100 p.p.m.; addition of 5% acetone to this medium did not significantly improve their activity. The most significant results were observed when these fungicides were suspended in 0.1% solution of agar or gelatine in water. PCNB inhibited the germination of 50 per cent or more of the spores when it was suspended in agar at concentration of 100 p.p.m. and in gelatine at 5 p.p.m.. TCNB and DCNA in agar at 25 p.p.m. or in gelatine at 2 p.p.m. inhibited the germination of spores.

The germ tubes were very long and normal

at all concentrations in water, pectin and carboxy-methyl-cellulose, and at concentrations up to 5 p.p.m. in agar and gelatine; they were short at higher concentrations in agar and gelatine; some of the 'ungerminated' spores in carboxyl-methyl cellulose did develop short germ-tubes.

E. Germination of spores of resistant strains of Botrytis cinerea.

Germination of spores produced by vapour- and impregnated resistant strains was not inhibited by the vapour of PCNB, TCNB or DCNA. When the spores produced by impregnated-resistant strains were allowed to germinate on agar impregnated, at 500 p.p.m. with the fungicides to which the colonies which produced the spores were resistant, inhibition of germination, if any, was always less than 1.0 per cent during 24 hours after incubation. There is a suggestion here that, in view of the fact that many of these spores did not give rise to colony on agar (page 107), most of the spores germinated and then stopped growing before visible colonies could be formed.

3.6 Summary of results on germination of spores

Germination of spores of parent strain of Botrytis cinerea was not inhibited in the presence of

the vapour of PCNB or DCNA; it was inhibited by the vapour of TCNB by about 50 per cent during the first 24 hours; most of the spores, however, germinated later. PCNB, TCNB and DCNA did not inhibit germination when they were suspended in water, or in pectin, or in carboxy-methyl cellulose; but they strongly inhibited germination when they were suspended in a 0.1 per cent solution of agar or gelatine. The greatest inhibition of germination occurred when the fungicides were impregnated in agar.

Germination of spores of resistant strains was not affected by the fungicide to which the colonies which produced the spores were resistant.

3.7 Experiments on sporulation

Resistant strains of Botrytis cinerea either did not produce spores or produced very few spores in comparison with the parent strain when they were grown on glucose-casein-hydrolysate agar. They were then grown on other media, described on pages 22 - 24 under 'materials and methods', to find out which of these media would be suitable for their sporulation.

In one set of experiments, the different strains were grown on the various media in the absence of fungicides; in another set of experiments, they

were grown on glucose-casein-hydrolysate agar medium alone in the presence of fungicides.

In order to estimate the number of spores produced in a culture by any of the strains, sterile distilled water was added to a 12-day old culture in a Petri dish and the surface was gently rubbed with a glass rod to free any spores produced from the mycelium. The suspension was poured into a beaker. After 3 further and similar treatments, all the suspensions from the Petri dish were filtered through a double layer of muslin, centrifuged and resuspended in either 2.0ml., 5.0ml., or 10.0ml. water, depending on the amount of spores obtained. The number of spores produced per plate was then estimated by haemacytometer counts. The mean of five estimates was then taken as the number of spores produced per plate by the strain. A comparison was then made of the number of spores produced by the different strains. The results obtained on sporulation of the different strains are as follows:

(a) Proportion of spores produced on glucose-casein hydrolysate agar by strains of Botrytis cinerea in the absence of fungicides

Strain	Parent	Pvap-r	Pimp-r	Tvap-r	Timp-r	Dvap-r	Dimp-r
Proportion of spores	500	5.0	4.1	0.9	0.3	10.1	7.2

(b) Sporulation of parent and resistant strains of Botrytis cinerea on various agar media

Medium	Parent	Pvap-r	Pimp-r	Tvap-r	Timp-r	Dvap-r	Dimp-r
Glucose-casein hydrolysate	+++	+	++	+	+	++	++
V-8 juice	+++	+	++	+	+	++	++
Potato-dextrose	+++	+	++	+	+	++	++
Potato-extract	+++	+	++	+	+	++	++
Corn meal	+	-	-	-	-	-	-
Oat meal	+	-	-	-	-	-	-
Glucose peptone	+++	-	+	+	+	+	+
Czapek-Dox	+++	+	-	-	-	+	+
Malt	+	-	-	-	-	-	-
Starch	+++	+	+	-	-	+	+

+++ means very good sporulation; ++ means sporulation was fair (1.2 to 5% of spores recorded for parent strain in glucose casein hydrolysate.) + means sporulation was very sparse (1% of spores recorded for parent). - means sporulation was not observed.

(c) Sporulation of parent and resistant strains of Botrytis cinerea on glucose-casein hydrolysate agar medium in the presence of fungicides

Strain	Treatment on agar: Control	Fungicide used as vapour: PCNB	TCNB	DCNA	Fungicide impregnated: PCNB	TCNB	DCNA
Parent	+	-	-	+	-	-	+
Pvap-r	+	+	-	+	+	-	+
Pimp-r	+	+	-	+	+	-	+
Tvap-r	+	-	+	-	-	+	+
Timp-r	+	-	+	-	-	+	+
Dvap-r	+	-	-	+	-	-	+
Dimp-r	+	-	-	+	-	-	+

+ means sporulation, whether sparse or profuse, was observed.

- means sporulation was not observed.

These results show that sporulation of the resistant strains was very sparse compared with that of the parent strain, in the absence of fungicides. This is particularly true of the TCNB vapour- and impregnated resistant strains which did not produce any spores in most of the plates. Sporulation of the resistant strains was not improved by exposure of cultures to X-ray.

All strains sporulated on

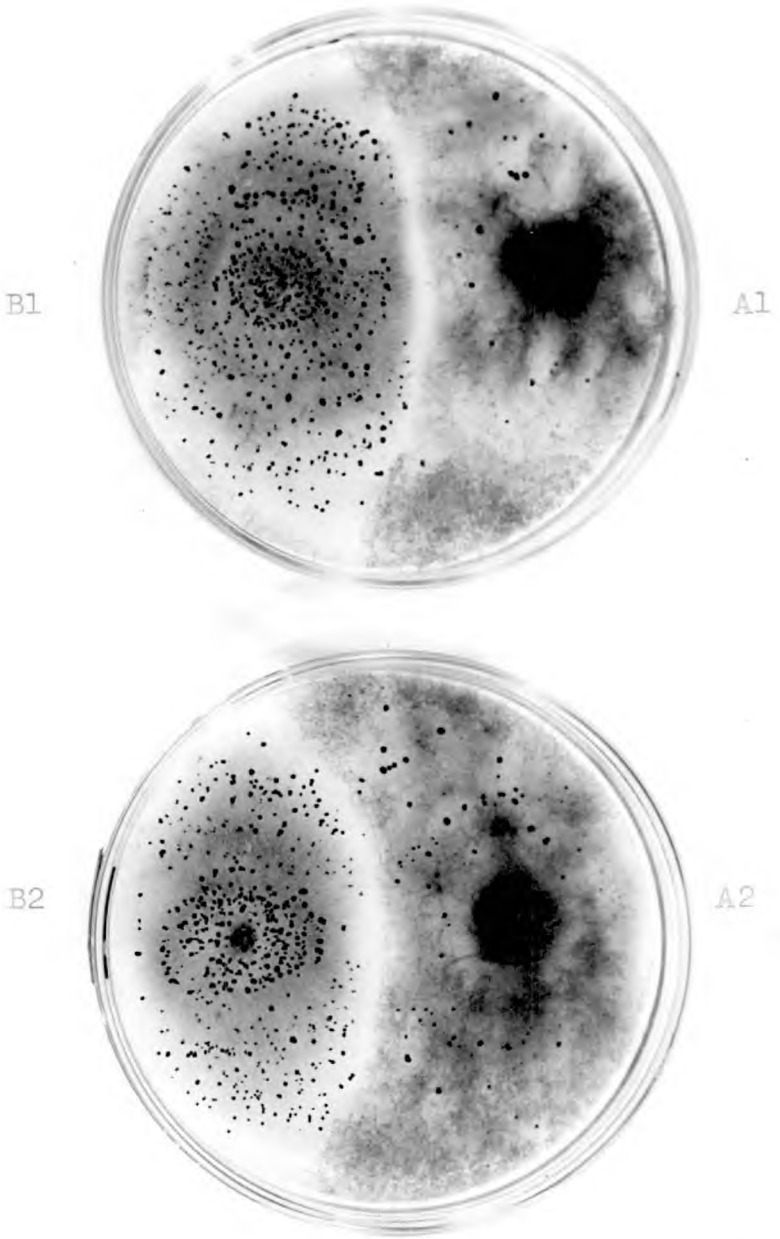


Plate 8 Sporulation in colonies of parent and resistant strains of Botrytis cinerea.

A1 and Colonies of parent strain showing spores.

B1 and Colonies of TCNB-vapour resistant strain (No sporulation).

Both types of colonies were grown in the absence of fungicides.

agar impregnated with DCNA, though only fairly well in the case of PCNB- and TCNB-resistant strains. The TCNB- and PCNB-vapour resistant strains sporulated only very sparsely and infrequently when the respective fungicides were impregnated in agar. The PCNB- and TCNB-impregnated resistant strains sporulated better in the presence of the vapour than on agar impregnated with the respective fungicides; sporulation here, too, however, was very poor indeed compared with sporulation of parent strain in the absence of fungicides. The parent, D(vap-r) and D(imp-r) strains sporulated well on agar exposed to the vapour of DCNA.

4.0 Determination of pathogenicity

Pathogenicity was determined by inoculating detached lettuce leaves with spores or mycelial plugs of the parent and resistant strains. Flattish leaves, about 2-3 inches long, removed from young lettuce plants, were washed first in tap water and then in distilled water; any water left on the surface of the leaves was removed by gently drying leaves between sheetings of blotting paper, care being taken to keep the leaves from drying out; the leaves were then wounded by pressing the smooth, rounded end of a small glass rod, about .3cm. diameter, on their surface. Five

such wounds were made on each half of a leaf. The leaves were then placed on bent glass rods in moist chambers (plastic boxes lined internally on all sides with damp blotting paper).

When mycelial plugs were used for inoculation, parent and resistant strains of Botrytis cinerea were allowed to grow for three days on glucose-casein hydrolysate agar which contained only $1/20$ of the strength of nutrients used in the normal medium. Mycelial discs, about 0.1cm. in diameter, were then removed from the edges of the colonies and placed with the mycelial surfaces downwards, on the wounded 'spots' on the leaves. When spores were used for inoculation, spore suspensions, about 100,000 spores per ml., of the different strains, were prepared and, with an Agla syringe, exactly 0.005ml. of the spore suspension, containing about 500 spores, were dropped on each wounded 'spot'. After inoculation, the leaves were incubated in moist chambers for 4, 5 or 6 days; the number of 'spots' in which lesions developed were then counted for each strain. Inoculation by spraying spores suspended in water or in 0.5 per cent glucose solution on to growing plants was not done because of the very small number of spores produced by resistant strains.

Results

When mycelial plugs were used for inoculation, rot lesions developed in 4 days on all spots inoculated with parent or resistant strains; these lesions were mostly of the fast spreading type, and by the 6th day after inoculation, rot had spread to most of the leaf surface in all cases. When spores were used for inoculation, rot lesions were observed on leaves inoculated with spores produced by parent and the three vapour resistant strains. Of the total of 300 'spots' inoculated with spore suspension of each of the strains, rot lesions developed on 219 inoculated with spores of parent strain, and 218, 242, and 290 inoculated with spores of the PCNB-, TCNB- and DCNA- vapour resistant strains respectively. Rot was observed earlier (in 3 or 4 days) in leaves which were inoculated with spores of parent and D(vap-r) strains, than in those inoculated with spores of the other two strains (5 days after inoculation). About 40-50 per cent of the rot developed on leaves inoculated with spores of P(vap-r) and T(vap-r) strains, about 70 per cent on leaves inoculated with parent strain, and about 85 per cent on leaves inoculated with D(vap-r) strain, were of the fast-spreading type.

It would appear then from these results that spores produced by the vapour-resistant strains were as pathogenic to lettuce as were those produced by parent strain of Botrytis cinerea, the spores of the D(vap-r) strain being even slightly more pathogenic than those of parent strain. This was perhaps not unexpected because about 50 per cent of such spores produced by vapour resistant strains have been shown to behave like spores of the parent type (page 95).

Pathogenicity of the spores produced by impregnated resistant strains was not so easily determined. About 35 to 30 per cent of the 'spots' inoculated with these spores developed typical Botrytis rot lesions, most of which were, however, not of the fast spreading type; about 50 per cent formed rot lesions which soon dried up and did not develop any further. Lesions were not observed in the remaining spots (about 15-20 per cent). The results obtained from inoculations with spores of the P(imp-r), T(imp-r) and D(imp-r) strains were similar. It is not known whether these results meant that spores of the impregnated resistant strains were non-pathogenic or weakly pathogenic when they were compared with spores of parent strain, or that only very few of them formed mycelium (as suggested on page 107) so that the actual level of inoculum was not high enough to form well-developed rot lesions.

VI DISCUSSION

This investigation has shown that the degree of fungistatic activity shown by PCNB, TCNB and DCNA against Botrytis cinerea depended on the method of application of these substances. When they were applied in the vapour phase, the effect of PCNB, TCNB and DCNA on Botrytis cinerea was mainly fungistatic on the mycelium of the fungus; they did not prevent germination of spores, but retarded the growth, of the fungus, which became adapted to them by producing either non-saltant mycelium, 'hyphal variants', with a higher growth rate, or resistant saltants, with a much higher growth rate, than that of the parent isolate, in the presence of the vapour of these fungicides. When they were impregnated in agar, PCNB, TCNB and DCNA became more actively fungistatic. They prevented spore germination, inhibited the formation of colonies from spore inocula, and retarded the growth of the fungus more effectively than the same amount of substance used in the vapour phase.

Resistant strains of Botrytis cinerea which were obtained in the presence of the vapour, or the impregnated, phase of any of the substances were also

resistant to the other phase of the same substance, and all resistant strains retained their resistance after they had been growth for a long time, and through many non-sporing generations, in the absence of fungicides.

Resistant strains which were produced in the presence of the vapour of these substances sporulated very little, or not at all in certain cases, when they were grown in the absence, or in the presence, of fungicides, and only about one half of the spores they produced were resistant in the sense that on germination, they gave rise to colonies which were resistant in the presence of the vapour of fungicides; the other half were not resistant, and the proportion of resistant to non-resistant spores became progressively reduced at subsequent spore forming generations. Resistant saltants which were produced on impregnated agar formed only resistant spores in the presence or absence of fungicides, but these spores, as well as those produced by vapour-resistant strains in the presence of fungicides, which were also all resistant, appeared not to be all viable, only about one half or less of them were capable of forming colonies on inoculation on agar. These, and other results obtained will now be considered more fully.

When young mycelial discs or spores of Botrytis cinerea were used for inoculation on agar, the production of resistant strains in the presence of PCNB, TCNB and DCNA depended upon the ability of the inoculum to survive the initial period of treatment. When the fungicides were applied in the vapour phase, there did not appear to be much difficulty on the part of the inoculum to survive this initial period at the level of application (10mg.), of the fungicides. Spores germinated in the presence of the vapour of PCNB, TCNB and DCNA, and the number of colonies which were produced from spores inoculated on plates exposed to the vapour of PCNB, TCNB or DCNA, was similar to the number on the control untreated plates, although colonies appeared earlier on control than on treated plates, the longest period for appearance of colonies being observed in plates which were exposed to the vapour of TCNB. This was perhaps a reflection of the longer time it took spores to germinate on plates exposed to the vapour of TCNB than on other plates, and the greater activity of TCNB on growth of colonies of Botrytis cinerea. When young mycelial discs were used for inoculation, inocula were also able to survive this initial period; the lag

period before growth was observed varied with the fungicides, being longest with cultures exposed to the vapour of TCNB; sooner and later, however, the fungus responded by producing either non-saltant hyphal variants, or resistant saltants. The response of the fungus to the vapour of fungicides was similar when spores or mycelial discs were used for inoculation, but it seemed that the appearance of resistant saltants was less frequent when spores, rather than mycelial discs, were used for inoculation. Priest (1960) observed that with PCNB and TCNB, the lag phase and time for appearance of resistant saltants of Botrytis allii, in cultures which had grown before they were introduced to the vapour of fungicide, was reduced compared with those cultures where the fungicide was introduced immediately after inoculation. In other words, the vapour of PCNB and TCNB had less effect on established colonies of the parent isolate of B. allii than on newly-inoculated cultures. A similar explanation may be put forward for this difference in the effect of the vapour of the fungicides on colonies which were raised from spores or from mycelial disc inocula in the presence of the vapour of fungicides.

Resistant saltants were observed less frequently in plates inoculated with spores and exposed to the vapour of DCNA, than in plates exposed to the vapour of the other fungicides. Colonies which developed from spores in the presence of DCNA almost always sporulated very well as soon as they attained diameters of about 0.5 to 0.7 cm.; then linear growth appeared to stop and resistant saltans were hardly formed from such colonies after they had sporulated. No spores were formed by the parent strain growing in the presence of PCNB and TCNB. The greater vapour-phase activity of PCNB and TCNB could not alone have been responsible for suppression of sporulation in Botrytis cinerea, because very slow-growing colonies of parent strain on agar impregnated with DCNA, which showed much greater activity than PCNB or TCNB in the impregnated phase, were observed to form spores. Suppression of sporulation by PCNB and TCNB must be an effect peculiar to these substances. Spores which were formed by the parent strain in the presence of the vapour of DCNA were similar in every observable respect to spores which were formed in the absence of fungicides. Exposure of spores of parent strain to the vapour of DCNA did not appear to affect the subsequent

behaviour of the spores. A similar result with vapour of TCNB was obtained by Reavill (1954) who showed that spores of parent isolate of Botrytis cinerea which had been exposed to the vapour of TCNB for 58 days did not show any difference in germination, hyphal morphology and sporulation of colonies produced from them, to spores which were not exposed to vapour of TCNB.

When PCNB, TCNB and DCNA were impregnated in agar, they were more effectively fungistatic than the same amount of substance used in the vapour phase; and the intensity of their activity depended on the concentration of fungicides used. PCNB almost had no effect on spore germination when it was impregnated up to a concentration of 500 p.p.m. in agar; almost all spores of the parent strain germinated at these concentrations of the fungicide, although their germ tubes were very short, compared with those of untreated spores. When spores were inoculated on agar which was impregnated with PCNB, the number of colonies which appeared on plates at different concentrations of PCNB from 1 to 500 p.p.m. were similar to the number on the untreated control plates (page 109).

TCNB and DCNA behaved differently from PCNB. When it was applied at a concentration of more than 10 p.p.m., TCNB inhibited the germination of about 80 per cent of spores of parent strain, and DCNA at concentrations above 2 p.p.m. gave almost 100 per cent inhibition of germination. The average number of colonies which were formed when spores of parent strain were inoculated on agar impregnated with TCNB or DCNA was correspondingly reduced. At concentrations of TCNB of 0 to 10 p.p.m., and of DCNA at 0 to 2 p.p.m., the average number of colonies formed on inoculated plates was similar to the number formed on the control, untreated plates; but at TCNB above 10 p.p.m., the average number of colonies formed up to the highest concentration, 500 p.p.m., used was similar, being only about 9 to 10 per cent of the number formed in control plates, and the number formed on plates which contained above 2 p.p.m. DCNA was only about 4 to 5 per cent of the number formed in the control, untreated plates. There was thus a direct relationship between spore germination and colony-formation on agar which was impregnated with TCNB above 10 p.p.m.; and with DCNA above 2 p.p.m.. The spores of the parent strain which did not form colonies or plates impregnated

with TCNB or DCNA could only have failed to germinate; if they had germinated later, which was most unlikely - because in some experiments on germination, spores did not germinate on plates impregnated with DCNA even 16 days after incubation - then they must have produced germ tubes which quickly stopped growing before they could form rudimentary mycelium. It appeared then that when TCNB or DCNA were impregnated in agar, there was a critical concentration, different for TCNB or DCNA, at which the fungicides showed activity which was high enough to prevent spore germination. Below this critical concentrations, their effect was mainly fungistatic on the mycelium of the fungus.

There was a close similarity between the sets of results obtained from dilution plate inoculations of impregnated agar with spores (page 109), and the results on the linear growth of colonies on agar impregnated with fungicies (page 74), in which concentrations of TCNB above 10 p.p.m. and of DCNA above 5 p.p.m. completely suppressed growth of inocula at least during the first six days of incubation. In plates which were impregnated with 250 p.p.m. and 500 p.p.m. DCNA, or 500 p.p.m. TCNB, some inocula did not show any linear growth even after thirty days,

after which period the plates were discarded. Unfortunately, such inocula, whether they were mycelial discs which showed no growth, or spores which failed to form colonies, at high concentrations of TCNB and DCNA, were not transferred later to untreated agar to determine whether they were still viable or not.

When spores of parent strain were germinated in water suspensions of PCNB, TCNB or DCNA, there was no inhibition of germination even at 4000 p.p.m. of the fungicides. But when these fungicides were imprregnated in plain or nutrient agar, germination of spores was greatly inhibited, almost completely, at certain concentrations of fungicide. 25p.p.m. TCNB under this condition inhibited germination by 80 per cent and 5 p.p.m. DCNA almost completely inhibited germination of spores. A similar result was obtained by Sharples (1962) with DCNA on Botrytis cinerea and by Weber (1963) with DCNA on Rhizopus arrhizus. In all cases, DCNA not only inhibited spore germination, but also caused bursting of germ tubes in some of the few spores which germinated. PCNB did not prevent germination of spores when it was impregnated in agar, although the germ tubes were very short. When PCNB, TCNB and DCNA were suspended in dilute 0.1 per cent water

solutions of agar and of gelatine, all the three fungicides showed activity against spore germination. PCNB at 5 p.p.m. in gelatine and at 100 p.p.m. in agar, TCNB at 2 p.p.m. in gelatine and at 25 p.p.m. in agar, and DCNA at 2 p.p.m. in gelatine and at 25 p.p.m. in agar, inhibited the germination of more that 50 per cent of spores of parent strain. The reason for the highly increased activity of these fungicides when they were impregnated in agar, or were suspended in dilute solutions of agar or gelatine - a process which was similar to impregnation - as shown by their action against spore germination, is not known. It is not merely the suspension of these substances in media other than plain water that made them so active; comparable inactivity was shown when the same quantity of these substances were suspended in dilute solutions of pectin and carboxy-methyl-cellulose. Addition of 5 per cent acetone to the suspending or impregnating medium, which improved the activity of these substances in every other case, did not have much effect on their activity when these substances were suspended in pectin. Also, when 10 mg. of these substances were deposited on the lid of a Petri dish, the vapour from it did not prevent

germination of spores, whereas the same amount of fungicide, 10mg., when it was suspended in 20 ml. solutions of agar or gelatine, or when it was impregnated in 20 ml. agar, gave a concentration of 500 p.p.m., which was by a very long way in excess of the concentration of TCNB or DCNA that was required to give almost complete or even complete inhibition of germination.

Certain propositions may be put forward to explain the apparent differences in behaviour and activity of each of these fungicides when it was used in the vapour and impregnated phases. It is proposed that the two most spectacular changes which occurred in Botrytis cinerea, the production of resistant saltants, and the formation of 'resistant' spores, were two separate changes; that each of them was the permanent, irreversible end point of a series of other changes taking place in the organism, but which were temporary and reversible; that each permanent change is induced to take place when certain critical levels of concentrations of fungicides were reached within the body of the organism. There would thus be two critical levels of concentration of fungicides; the lower critical level would

induce changes that lead to the formation of resistant saltants and the upper critical level would induce changes that lead to formation of 'resistant' spores. Because of the levels of concentrations of fungicides required, any changes which produced 'resistant' spores must have already produced resistant saltants, but the formation of resistant saltants would not necessarily mean that resistant spores would be formed unless the upper critical level of concentration of fungicide were reached.

The concentration of fungicides actually available at the seat (or seats) of toxic action within the body of the organism is not necessarily identical with the concentration measured for fungicides in the circumambient phase surrounding the organism. The former would normally be less than the latter unless equilibria were reached between the two phases - the phase of toxic action within the organism, called the 'biophase', and the external surrounding phase in which the fungicide is present. The concentration of fungicide in the external phase would be governed by the size of this phase, the vapour pressure and the solubility of the fungicide, and its rate of dispersion through the phase. In this and similar studies, the

external phase, that is the space between the lid and the bottom of the Petri dish, was a constant, because 20 ml. agar were always used and the Petri dishes were always of the same size. Therefore the main factors affecting the concentration of fungicide in the external phase would be its vapour pressure, solubility in the medium used, and its rate of dispersion through the medium. Vapour pressure is the expression of the distribution of a substance between the pure (or almost pure) solid or liquid phase, and its vapour; solubility corresponds to the distribution between the pure solid or liquid phase, and its saturated solution. These definitions imply that when different weights of the same substance, for example, 10 mg. and 1 mg. samples, were deposited on the lids of Petri dishes, the amount of substance escaping from the vapour into the external phase and into the medium was not necessarily a direct function of the quantity deposited on the lid of the Petri dish; similarly when substances were deposited in a liquid medium, the available substance might be different from the amount put in. This may perhaps explain the results obtained by Priest (1960) when he observed that the vapour from 10 mg. PCNB or TCNB always showed greater

activity on Botrytis cinerea than the vapour from 1.0 mg., whereas the calculations of Hewlett (1955) showed that the amount of these substances needed to saturate the air present in the Petri dish with PCNB or TCNB was 5×10^{-5}mg.. The suggestion here is that distribution equilibrium between the solid and the vapour was not reached at the 1.0 mg. level of application of the fungicides during the period when observations were made; when 10 mg. of the fungicide was used, more material was released to the space in the Petri dish, hence greater activity. This view is perhaps supported by the observation also of Priest (1960) that when Petri dish lids were removed several days after the fungicide had been deposited on them, and were replaced on fresh bottoms, the vapour from them still inhibited the growth of fungus.

When the fungicides were impregnated in agar or suspended in dilute solutions of agar or gelatine, the chances were that these substances were more soluble in such media than in plain water, and there was a higher distribution equilibrium between the solid and the saturated solution than when plain water was used; in other words, more of the substance was available for transportation to the 'biophase' when the fungicides

were suspended in solutions of agar or gelatine, or impregnated in agar, than when they were suspended in plain water.

When these fungicides were used in the vapour or impregnated phase in equal amounts, the actual quantity of substance available for transportation to the biophase would depend on whether the distribution equilibrium of the substance between the solid and the saturated solution on the one hand was greater than, equal to, or less than the corresponding equilibrium between the solid and the vapour. In other words, it would depend on whether they were more soluble in the medium used than they were volatile. DCNA is the least, and TCNB the most, volatile of the substances used. TCNB is also more soluble in water than PCNB. It was possible that when these substances were used in the vapour phase, the available amount of TCNB which could move to the 'biophase' was greater than the corresponding amounts of PCNB or DCNA, in spite of the fact that equal weights of the three substances had been deposited on the lids of Petri dish. This point alone, apart from any specific intrinsic toxicity of the fungicide, would give TCNB an advantage over the other two fungicides

when they were used in equal weights in the vapour phase. TCNB was always observed to be the most active of the three substances against Botrytis cinerea when they were used in the vapour phase.

When these substances were impregnated in agar or gelatine, it was possible that they were more soluble in these media than they were in plain water. Richardson and Miller (1960) determined the fungistatic activity of certain insecticides, and found that the dosage response curve for one of these insecticides, lindane, against Rhizoctonia solani, had a constant slope extending far beyond lindane solubility in water. They attributed this to supersaturation of the assay medium by lindane. McGowan (1952, 1954) proposed the general theory that if undissolved solids were present in an assay medium, it would be possible to have 'contact toxicity' by which the active compound actually dissolves the 'biophase', and this would give rise to a biophase concentration of the substance greatly in excess of that achieved by an aqueous partition. Eckert (1962) pointed out that either one or both of these findings by Richardson and Miller, and by McGowan, could explain the ED_{50} values in excess of the solubility which he obtained

for certain chlorinated nitrobenzenes, including PCNB and TCNB. He stated further that several chlorinated nitrobenzenes formed supersaturated solutions after heating to 60°C. and then allowing these solutions to return to room temperature, and that certain low-melting compounds, including TCNB, formed persistent oily dispersions in water following heating to a high temperature and then cooling to room temperature. These were essentially the conditions under which impregnated media were prepared, and solid particles of PCNB and TCNB were almost always observed at concentrations of these substances above 100 p.p.m.; either supersaturation, proposed by Richardson and Miller, or 'contact toxicity' theory, proposed by McGowan might explain the very high activity of PCNB, TCNB and DCNA, when they were impregnated or suspended in agar or gelatine. The point then is that when PCNB, TCNB and DCNA were impregnated in agar, it was possible that more substance was available for translocations to, or actually reached, the biophase, than when they were used in the vapour phase. If this were so, the proposition now put forward is that when these substances were used in the vapour phase, the concentrations in the biophase were high enough to

reach the lower critical level, suggested, which induced in the organism permanent changes that led to the formation of resistant saltants, but were not high enough to reach the upper, suggested, critical level that would induce changes leading to the production of resistant spores. When the substances were impregnated in agar, enough material was available to allow the concentration of fungicide in the biophase to reach the upper critical level. When the concentration of substances in the biophase were below the lower critical level, it is suggested that the toxic action of the fungicides on the fungus were governed by a physical mechanism, for example, adsorption of the toxic agent to certain cell surfaces, dissolution of the fungicide in certain cell lipoids, coagulation of certain proteins, and that such changes as were produced in the organism were only of a temporary reversible nature, which were lost when the fungus was grown in the absence of fungicides. These changes could induce response in the fungus which led to the slow growth of the colony or to the production of non-saltant mycelium. When the fungus was removed from the vapour of fungicides, the toxic effect was lost and growth was again normal. Reavill (1954) showed

that when slow-growing mycelium of Botrytis cinerea which have been exposed to the vapour of TCNB were removed to untreated agar, there was a small carry-over of the toxic effect of the fungicide which, however, was lost between the third and sixth day of growth in the absence of fungicide. A similar observation was made in this study. When the concentration of fungicide in the biophase were up to the lower critical level, then changes took place within the organism which greatly reduced or suppressed its growth until and unless it was able to produce resistant saltants. The organism would not be induced to produce resistant saltants until this lower critical level of concentration were reached. Beyond this critical level, any further increase in concentration of the fungicide in the biophase would not affect the nature of the resistant saltant, hence saltants formed at any one concentration would also be equally resistant at higher concentrations when the fungicides were impregnated in agar. This increase in concentration of fungicide in the biophase would, however, bring about changes in the physiology of the spores and in the spore-forming apparatus of the fungus. Certain spores may fail to germinate and the spore-forming

reactions may be affected in such a way that some spores that were formed would be of the resistant, and the others, of the susceptible, type. Such changes, however, were only temporary and not permanent. The germinated 'susceptible' spores would form only non-resistant colonies, and the resistant spores would give rise to colonies in which the proportion of resistant spores formed at each spore-forming generation would gradually diminish in the absence of fungicides. When the concentration of fungicide in the biophase reached the upper critical level, then changes which took place were such that only potentially resistant spores were formed and the colonies produced from these did not revert to the formation of the parent-type non-resistant spores. When saltants were formed in the presence of the vapour, the level of concentration was above the lower, but below the upper, critical toxic level; when these strains were grown in the absence of fungicides, they produced both resistant and non-resistant spores, the proportion of the former depending on how much extra toxic agent had penetrated into the biophase. With successive growth and spore-formation in the absence of fungicides, ability to form resistant spores was gradually

lost. When these vapour resistant strains were grown in the presence of vapour, or on impregnated agar, they were disposed to absorb more fungicide into the biophase so that if the upper critical level of concentration were now reached, permanent, irreversible changes took place so that only resistant spores could be formed, and colonies which were produced from them did not lose this ability, nor did they revert to the original parent-type state when they were grown in the absence of fungicides.

Priest (1960), Parry (1957) and Hewlett (1955) have already referred to evidence which suggest that there is a common adaptation mechanism on the part of Botrytis species to PCNB and TCNB. The parent isolate of B. cinerea or B. allii behaved in the same manner to PCNB and TCNB. The response of Botrytis cinerea to DCNA is also similar in that in the presence of the three fungicides, slow-growing hyphal variants or fast-growing resistant saltants were produced. The difference in the effects of these fungicides on sporulation and on spore germination and certain difference in the behaviour of the resistant saltants would also suggest that there exists some differential adaptation mechanism on the part of

the fungus to these fungicides. DCNA did not suppress sporulation of Botrytis cinerea when it was used in the vapour or impregnated phase provided the fungus grew, and the parent spores produced in the presence of the vapour of DCNA were not different, so far as is known, from those produced in its absence. PCNB and TCNB on the other hand completely suppressed sporulation of parent strain. Priest (1960) and Reavill (1954) also obtained similar results with PCNB and TCNB. Reavill (1954) showed further that the effect of TCNB on sporulation of Botrytis cinerea is not affected by the state of development of the fungus before it was introduced to the vapour of the fungicide. Sporulation stopped as soon as the fungus was introduced to the vapour of TCNB. Priest (1960) showed that when resistant strains produced on exposure of parent strain to the vapours of PCNB and TCNB were grown in liquid medium in the presence of fungicides, resistant strains utilized glucose more efficiently than the parent strain, but PCNB-resistant strain utilized glucose less efficiently than the TCNB-resistant strains.

Results which were obtained from experiments in which the fungicides were used in the vapour phase

would suggest that DCNA was probably the least active of the three fungicides, against Botrytis cinerea, whereas results of experiments in which the fungicides were used in the impregnated phase would suggest that DCNA is the most active substance. Higgons (1962) observed that the low volatility of DCNA as compared with TCNB was at first thought to be a disadvantage as it was believed that compounds of this type mainly act in the vapour phase in the control of Botrytis cinerea on lettuce, but he found that practical field trials have shown that DCNA is both very active and persistent, indicating that vapour action is probably not of significance in the case of DCNA. Priest (1960) observed that when DCNA was used in the laboratory (in the vapour phase alone), it did not appear to be particularly active against the parent isolate of Botrytis allii, and suggested that it is possible that under field conditions, DCNA exerts its controlling effect upon diseases caused by Botrytis spp. by its presence within the plant system. Results which were obtained in this study with agar impregnated with fungicides suggest that the fungicides PCNB, TCNB and DCNA might well be present within the plant system, and might be persistent in their effect.

Parry (1957) and Priest (1960) pointed out that laboratory studies represented extreme experimental conditions which are unlikely to be present under field conditions, and that results which were obtained in the laboratory about the formation of resistant strains were not likely to be repeated, at least to the same degree, on the field, but that under certain environmental conditions, such as are present in frames and glasshouses, where much higher local concentrations of fungicide vapour may be present, resistant strains could possibly develop. Since their writing, no naturally-occurring PCNB- or TCNB-resistant isolate of any organism has been reported to be isolated, but Ogawa and Mathre (1963) reported the isolation of Rhizopus arrhizus, though infrequently, which were resistant to DCNA, from rotten peach fruits which had been previously treated with DCNA. The inability to isolate any strain which is naturally resistant to PCNB or TCNB may have been due to the effect of these fungicides on the suppression of sporulation, not only of the parent, but also of the resistant, strains. Spores were hardly observed on any strain growing in the presence of PCNB or TCNB, but resistant strains sporulated in the presence of DCNA.

The results of experiments on pathogenicity of 'resistant' spores of resistant strains were not conclusive; it is suggested that more investigation should be carried out on this aspect of the study.

VII SUMMARY

A. Linear growth of Botrytis cinerea in the presence of PCNB, TCNB and DCNA

1. When young mycelial discs of Botrytis cinerea were inoculated on agar which was exposed to the vapour of, or impregnated with, PCNB, TCNB or DCNA, there was at first very little, or no, growth of the mycelium. Later the fungus became adapted to these fungicides by producing either non-saltant mycelium, 'hyphal variants', with a higher growth rate, or resistant saltants, with a much higher growth rate, than that of the parent isolate, in the presence of fungicide.

2. Equal amounts of each of the fungicides were much more effectively fungistatic on the fungus when they were applied in the impregnated phase than when they were used in the vapour phase.

3. When spores of Botrytis cinerea were inoculated on agar which was exposed to the vapour of these fungicides, colonies which were all susceptible were formed in numbers almost equal to colonies arising on agar which was not exposed to the vapour of fungicides.

4. When spores of the fungus were inoculated on agar which was impregnated with fungicides, susceptible

colonies were also formed, but the number of such colonies, compared with colonies formed on control agar without fungicides, depended on the concentration of fungicides in agar. When PCNB was impregnated in agar, there was only little difference between the number of colonies observed on agar which contained the highest concentration used, 500 p.p.m., of the substance, and agar which contained no fungicides. When TCNB or DCNA were impregnated in agar, almost the same number of colonies were observed in control plates without fungicides as in plates containing up to 2 p.p.m. of TCNB or of DCNA. In plates which contained more than 2 p.p.m. and up to 10 p.p.m. TCNB, the number of colonies which were formed was always less than, but about equal to or more than one half of, the number formed in the absence of fungicides. In plates which contained more than 10 p.p.m. TCNB or more than 2 p.p.m. DCNA, the number of colonies formed was very much reduced, being only about 10 per cent in plates containing TCNB, and about 4 per cent in plates containing DCNA, of the number formed in plates without fungicides.

5. Resistant saltants were formed less frequently on agar which was impregnated with fungicides than on agar which was exposed to the vapour of fungicides.

They also appeared less frequently when spores were used for inoculation than when mycelial discs were used.

6. Strains of the fungus which were resistant to anyone of the fungicides when it was applied in the vapour or in the impregnated phase were also resistant to the other phase, of the same fungicide; resistant saltants which developed on agar which was impregnated with fungicides at a particular concentration were also resistant in the same degree to lower or higher concentrations of the same fungicide, and to its vapour.

7. Resistant saltants did not show any diminution of their resistance to fungicides when they had been grown for a long time, and through many series of successive non-sporing generations, in the absence of fungicides.

8. The resistance developed to any of PCNB, TCNB or DCNA by Botrytis cinerea also conferred resistance to the other two fungicides. TCNB- and DCNA-resistant strains were also resistant in the same degree to all the three fungicides; PCNB-resistant strains were also resistant in the same degree to DCNA; they were not initially resistant to TCNB, but they grew better than the parent strain in the presence of this fungicide.

B. Sporulation

9. Parent strain did not sporulate in the presence of PCNB or TCNB in either phase of application of these fungicides; it sporulated little, whenever it grew, on agar impregnated with DCNA; its sporulation did not appear to be affected by the vapour of DCNA.

10. Resistant strains sporulated only very little or not at all, on different media, in the presence of PCNB or TCNB; they sporulated slightly in the presence of DCNA and in the absence of fungicides.

C. Behaviour of spores.

11. Spores which were obtained from parent strain in the presence of DCNA or in the absence of fungicides, always gave rise to colonies which were susceptible to treatment with fungicides.

12. The behaviour of spores which were produced by resistant strains depended on the origin of the strains which produced them, and the conditions under which these spores had been produced. When spores were produced in the presence of fungicides by vapour- or -impregnated-resistant strains, only resistant colonies were observed to develop when these spores were inoculated on agar which contained, or which was exposed to the vapour of, fungicides; but only about one half

or less of the number of such spores appeared to be viable. Spores which were produced by impregnated resistant strains in the absence of fungicides also behaved in a similar way. When spores were produced in the absence of fungicides by the vapour-resistant strains, only about one half of such spores which were produced during the first spore-forming generation gave rise to resistant colonies; the other half produced non-resistant colonies; the proportion of resistant-colony-forming spores was progressively reduced at subsequent successive spore-forming generations.

13. Spores of parent and resistant strains germinated in the absence of fungicides.

14. Germination of spores of parent strain was not inhibited in the presence of the vapour, or suspension in water alone or dilute solution of pectin, of fungicides; it was inhibited when fungicides were suspended in dilute 0.1 per cent solutions of agar or gelatine, or when they were impregnated in agar.

15. Germination of 'resistant' spores produced by resistant strains was not inhibited by any treatment.

16. Spores of parent and resistant strains did not appear to interfere with the germination or with the development of each other when they were mixed together.

17. When heterokaryons were formed between the parent and the TCNB- or DCNA- impregnated resistant strains, by allowing a mixture, in equal proportion, of their spores to form colonies, their mycelium appeared to be resistant to treatment with fungicides, but most of the spores, about 88 to 95 per cent in parent-TCNB-resistant heterokaryon, and about 76 to 86 per cent in the parent-DCNA-resistant heterokaryon, gave rise to susceptible colonies.

D. Pathogenicity

18. Spores of PCNB-, and TCNB-vapour resistant strains were as pathogenic to lettuce as, and spores of the DCNA-vapour resistant strain were slightly more pathogenic than, the spores produced by the parent strain. Observation on pathogenicity of spores produced by impregnated resistant strains was not very conclusive; on the whole, however, it appeared that when these spores were pathogenic to lettuce, they were less so than spores of the parent strain.

19. Mycelia of parent and resistant strains were pathogenic to lettuce.

E. General

20. When they were impregnated in agar or suspended in dilute solutions of agar or gelatine, the order of

activity of the three fungicides against Botrytis cinerea was DCNA > TCNB > PCNB; but when they were used in the vapour phase, the order of activity was TCNB > PCNB ≥ DCNA.

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