A COMPARISON OF THE MODE OF ACTION OF CERTAIN NEW CHLORO-NITROBENZENE PREPARATIONS WITH THAT OF STANDARD FUNGICIDES

By R. Y. Roy

Department of Botany, Benares Hindu University

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I. Introductory

In recent years a number of chloronitro-derivatives of benzene have been used in the control of certain horticultural plant diseases. The first paper in this connection was by Smieton (1939) who showed that a preparation in the form of a powder ('Brassisan') containing 20% of trichlorodinitro-benzene gave a fair control of club-root disease of cabbage. The dust was incorporated into seedboxes or worked into the upper soil layers in the open after the manner of a top-dressing or applied, diluted with soil, to the dibble holes when the plants were set out in their final positions. The measure of disease control was comparable to that obtained by the standard method in which mercuric chloride is used (Preston, 1931), and like the latter has the demerit of causing an appreciable check to the growth of the plants. Another derivative, pentachloronitrobenzene, also administered in the form of a powder ('Folosan'), was less checking upon plant growth but was also distinctly inferior in control of the disease.

Following upon this work, Brown (1935) and later Smieton and Brown (1940) reported extensive experiments in which 'Folosan' was used with considerable success for the control of certain lettuce diseases. Incorporated in the top soil of seedbeds or dusted on to the seedlings soon after emergence, it gave a good control of damping-off as caused by *Rhizoctonia solani* but not as caused by *Pythium* spp. Applied later as a dust to growing seedlings, and at the appropriate times, it was very effective in controlling the "Red-leg" disease caused by *Botrytis cinerea*. It was also of value in reducing the damage caused by mildew (*Bremia Lactucæ*) and in this case the effect obtained was strongly influenced by the nature of the diluent ('filler') used. With lime as filler the fungicidal effect was much less than with talc. For none of these purposes was the preparation containing trichlorodinitrobenzene usable on account of the serious phytocidal damage produced.

The use of "Folosan" has been reported by Hawker and co-workers (1944) in connection with diseases of Gladiolus. With the Hard Rot disease (*Septoria Gladioli*) and Core Rot (*Botrytis sp.*) dusting of the corms with "Folosan" gave promising results (Hawker, 1944 and 1946); with the Dry Rot disease (*Sclerotinia Gladioli*) control

was more difficult (Hawker, Bray and Burrows, 1944) and treatment with Folosan was only sometimes effective.

Within the last few years another derivative has been tested by Brown and Montgomery (1948). This is one of the tetrachloronitrobenzenes. This newest preparation is about equal to the original "Folosan" in the control of *Botrytis* disease of lettuce and the *Rhizoctonia* diseases of lettuce and potatoes, and is superior in that it produces still less phytocidal damage so that it can be used in practice under conditions where the older preparation was liable to cause a significant check to plant growth.

In all the foregoing researches the fungicides used were in the form of commercial powders, and as much of the work was carried out with growing plants in the open, standardisation of the dose was not easily attainable. Attention was also primarily paid to disease control and not to the immediate effect of the chemical substances upon the fungi concerned. An investigation of the latter, under laboratory conditions, was therefore taken up, in which the two substances penta- and tetra-chloronitrobenzene were compared with each other and with two standard fungicides, Bordeaux mixture and "Shirlan". In this work attention was directed to the effects produced on the germination, mycelial growth and sporulation of a number of fungi. The following is an account of this work.

II. Materials Used

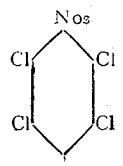
1. *Fungicides*

The two *benzene derivatives* are marketed under trade names, of which the following are the particulars:—

"Folosan" is a powder containing 20% of pentachloronitrobenzene with talc as filler.

"Folosan D.B. 905" is a powder containing 5% of a tetrachloronitrobenzene with china-clay as filler.

For the purposes of this work it was highly desirable to have the active principles free from the filler and in the early stages this was achieved by dissolving them out in acetone or in benzene, in which both are freely soluble. For brevity the two active principles will be referred to henceforth as PC for pentachloronitrobenzene and TC for tetrachloronitrobenzene. They are crystalline, PC being in the form of pale yellow polygonal crystals, those of TC white and acicular. The melting points of PC and TC crystals as obtained in the manner described were 140° C. and 98° C. respectively. According to Beilstein (1922) the isomeric form of TC which has its melting point at 99° C. is the 2, 3, 5, 6 type, of formula



The melting point of PC as given by Beilstein is 144–45° C. which agrees reasonably well with that determined by the writer.

Later a liberal supply of the active principles (Commercial grade) was obtained through the courtesy of Messrs. Bayer Products. These preparations were essentially similar in appearance to those obtained by extraction from the commercial powders and gave the following melting points:

PC—144° C.; TC—99° C.;

i.e., more or less identical with the figures given by Beilstein. Both active principles are soluble to a very slight degree in water. In an experiment 0·0022 g. of PC and 0·0024 g. of TC were each shaken up in 1 litre of distilled water without going completely into solution. The solubility of each in water is therefore less than 0·00024%. Accurate determination of solubility in water was difficult on account of the somewhat volatile nature of both substances, as it was impossible to evaporate down a large quantity of water containing traces of each without the serious risk of losing the latter in the process. A saturated solution of each was shaken up with benzene, and the latter then evaporated, but the amount of dry substance left indicated a solubility of not more than 0·0001%. Though the effects to be described later indicate clearly that both substances must be soluble in water to some extent, the degree of their solubility is very low. Saturated solutions in water are faintly acidic, the respective pH's for PC and TC being 6·58 and 6·7. Both substances are freely soluble in benzene, paraffin oil and acetone. TC is soluble in hot alcohol whereas PC dissolves with difficulty and precipitates on cooling.

PC and TC are somewhat volatile, more particularly the latter. A comparison of their volatility was obtained by exposing weighed quantities of each to different temperatures. TC was found to be about 4–5 times more volatile than PC.

Bordeaux mixture.—This has been used throughout in the proportions 10 lb. $CuSO_4 \cdot 5H_2O$; 15 lb. $Ca(OH)_2$: 100 gallons water as suggested by Martin (1944). As 1 gallon is equal to 10 lb., the above formula represents a 1% solution of copper sulphate and will be referred to as 1% Bordeaux mixture. Five grams of copper sulphate were dissolved in 50 c.c. distilled water and 7·5 g. calcium hydroxide suspended in 450 c.c. distilled water. Both were kept in well stoppered bottles as stock solutions. When required the standard mixture was prepared by mixing in the ratio of 1 : 9. Before using, the mixture was tested for its alkalinity with red litmus paper. Lower concentrations were made by simple dilution with water.

Salicylanilide (Trade name "Shirlan").

A 1% suspension by weight was used as standard. Once prepared it was not used for longer than a week. The suspension is almost neutral but on the alkaline side (pH 7·05).

2. *Fungi*

The fungi used in the course of this work are as follows:—

1. *Botrytis cinerea* Pers. from lettuce (Stock culture, Imperial College).
2. *Fusarium cæruleum* (Lib.) Sacc. from potato (Rothamsted Experiment Stat.)
3. *Ascochyta rabiei* (Pass.) Lab. from *Cicer arietinum* L. (Lyallpur, Pakistan).
4. *Trichothecium roseum* Link from soil (Stock culture, Imperial College).
5. *Trichoderma viride* Pers. from soil (Stock culture, Imperial College).
6. *Rhizopus nigricans* Ehrenberg (Stock culture, Imperial College).
7. *Alternaria sp.* from tomato seed (Slough).
8. *Pythium de Baryanum* Hesse from potato (Stock culture, Imperial College).
9. *Phytophthora parasitica* Dast. from tomato (Slough).
10. *Rhizoctonia solani* Kuhn from seakale (Slough).

Stock cultures of the above fungi were maintained in tube slants on potato, oatmeal or 2% malt agars. In some respects a preference was shown for one or other of these media. Thus sporulation of the *Ascochyta* was best on oatmeal agar, and the *Phytophthora* grew best on malt. The cultures were incubated at 20-25° C.

III. Germination of Spores in Drops Containing Fungicide

In a work of this description it was necessary to ensure that the drops of standard size should occupy a standard area. They must therefore spread over the glass and the limits of their spread be fixed. These results were achieved by the following experimental technique.

Fresh coverslips were first rubbed with a detergent ("Vim") and then washed thoroughly under the running tap. They were then boiled for 10 minutes in a mixture of sulphuric acid and potassium dichromate, again washed in running tap water, then in three changes of distilled water and stored in alcohol. With coverslips which were being reused, and which therefore were coated with vaseline on one side, the abovs treatments were preceded by immersion in xylol for at least 24 hours.

In order to ensure that drops of fluid do not spread indefinitely over the glass surface Montgomery and Moore (1934) developed a method of cutting circles on glass slides. A more convenient method is that of Peterson (1941) which has been followed with a slight modification. The method is as follows: A number of drops of vaseline are laid on a glass slide. The coverslip is picked up by forceps, heated by flaming and laid on the vaseline drop. The latter is melted by heat and runs readily, so that the coverslip settles down on the slide with a continuous vaseline layer below. The vaseline forms a slight rim round the edge of the coverslip and this effectively prevents any drop

laid on the coverslip from spreading farther than its edge. Usually 3 such coverslips were fixed to a slide; with wide slides it was convenient to attach 6 coverslips, in two rows of 3 each.

The following three methods of setting up preparations for the study of spore germination were adopted.

(1) One drop of PC or TC in acetone was pipetted on to the surface of each coverslip on a slide. This solution spread immediately over the surface, the acetone evaporated and left a fine layer of crystals on the coverslip. One drop of spore suspension was then added.

(2) 0·005 g. of PC or TC crystals, the minimum quantity which could be conveniently weighed on the balance available, was distributed as equally as possible between 3 coverslips, on each of which a drop of spore suspension in water or nutrient had been placed. The crystals and spore suspension were then thoroughly mixed by means of a needle.

(3) The suspension of fungicide (PC or TC or Bordeaux mixture or Shirlan) was shaken up vigorously and drops pipetted directly on to the coverslips. To these were added drops of the spore suspension. The effect here is approximately to halve in each case the concentration of the fungicide.

The slides so prepared were supported on pieces of cork on petri-dish lids of 11 cm. diameter, then covered with lids of 8·5 cm. diameter. This allowed a marginal space into which water was poured so as to maintain a saturated atmosphere inside. One slide only, carrying 3 or 6 coverslips, was kept in each dish. The preparations were incubated at 20° or 25° C.

(*a*) *Botrytis cinerea*

Table I gives the summary of 9 experiments regarding the effect of PC and TC on germination of *Botrytis* spores in water after 60 hours. All the 3 methods described gave similar results as is shown in Table I. To each preparation was added a drop of spore suspension in water which was the same for each experiment. The figures given in the Table are the average of 3 replicates in each case and are in terms of the smallest division of the eyepiece micrometer, which is equal to 9·4 microns. The latter statement applies to all Tables which relate to spore germination.

The conclusions to be drawn from Table I are as follows:—

(1) Depression of growth by PC and TC is shown more distinctly on average germ tube length than on percentage germination.

(2) TC is more effective than PC.

(3) There is great variability in percentage germination with regard to TC, *e.g.*, in Experiment 2, the percentage germination is 4·8 whereas by the same method in Experiment 1 it is 64·6. Whether this variability is due to the fungus itself or to the fungicide cannot be definitely said.

TABLE I

Effect of PC and TC on germination of Botrytis *spores in water after 60 hours*

Preparation of coverslip	% Germination	Av.g.t.l* of germinated spores, 50 counted	Av.g.t. l* per 100 spores sown
Method 1 :			
Exp. 1 Control (1 drop acetone evaporated)	91·7	34·6	31·7
1 drop 1% PC (acet. evpd.)	87·6	10·4	9·1
1 ,, 1% TC (,, ,,)	64·6	1·8	1·2
Exp. 2 Control (1 drop water after evaptg. 1 drop ac et.)	68·5	11·6	7·9
1 drop 1% PC· acet. evap.+1 drop water	70·3	5·3	3·6
1 drop 1% TC ,, ,, ..	4·8	0·64	0·03
Exp. 3 Control (similar to 2) ..	85·6	42·7	..
2 drops 1% PC acet. evap.+1 drop water	..	Max. 3	..
2 drops 1% TC ,, ,, ..	..	11·1	..
Method 2 :			
Exp. 4 Control ..	91·5	36·6	33·4
ca. 0·002 g. PC ..	90·8	9·8	8·8
ca. 0·002 g TC ..	42·9	1·6	0·68
Exp. 5 Control ..	75·9	..	..
ca. 0·002 g. PC ..	80·0	..	..
ca. 0·002 g. TC ..	56·2	..	..
Exp. 6 Control ..	78·5	..	..
ca. 0·002 PC ..	77·7	..	..
ca. 0·002 g. TC ..	0	0	0
Method 3 :			
Exp. 7 Control (1 drop water) ..	78·9	28·6	22·5
1 drop 1% PC in water ..	69·6	10·2	7·09
1 ,, 1% TC ,, ..	7·4	3·6	0·26
Exp. 8 Control (1 drop water) ..	88·6	8·02	7·1
1 drop 1% PC in water ..	84·1	2·8	2·3
1 ,, 1% TC ,, ..	5·1	Max. 2	..
Exp. 9 Control (1 drop water) ..	75·6	..	..
1 drop 1% PC in water ..	77·5	..	..
1 ,, 1% TC ,, ..	24·9	..	..

* g.t.l. = germ tube length.

In order to compare the effect of PC and TC on spore germination with that of the standard fungicides the writer set up experiments on germination in which all were included.

Table II gives the result of two such experiments after 60 hours. Bordeaux mixture and Shirlan were used in the freshly made up condition. PC and TC were used in the first experiment from 1% solution in acetone and in the second from 1% suspension in water.

Column 1 of Table II gives the method of preparing the coverslips. The concentration shown in that column in the case of the standard fungicides was obtained by mixing 1 c.c. spore suspension in water with 1 c.c. of double the concentration given and then pipetting

2 drops (about 0·1 c.c.) of the mixture on to each of the 3 coverslips. In the case of the new fungicides 1 drop of water was pipetted, after the evaporation of acetone containing PC or TC, on to each coverslip in the first experiment before adding to each 1 drop of spore suspension. In the second experiment 1 drop of spore suspension was added to 1 drop of 1% PC or TC in water.

Column 2 gives the amounts of copper, salicylanilide, PC and TC in micrograms spread over an area of about 2 sq. cm. As the drops (aqueous) of Bordeaux mixture and Shirlan are approximately twice the size of those of PC and TC (in acetone) and as 2 drops were used in the former case as against 1 drop in the latter, the amount given in the column represents what is present in 0·1 c.c. and 0·025 c.c. of the standard and the new fungicides respectively.

TABLE II

Comparative effect of PC, TC, Bord. Mix. and Shirlan on germination of Botrytis *spores in water*

Preparation of coverslip	Mcg.*	Exp. 1		Exp. 2	
		%germ.	Av.g.t.l.	% germ.	Av.g.t.l.
Control (1 drop water+1 drop sp. sus.	0	68·5	11·6	88·6	8·02
0·0025% Bordeaux Mix. ..	0·64	0	0	0	0
0·00125% do ..	0·32	3·1	Max. 2·5	0	0
0·00063% do ..	0·16	9·7	..	31·6	27·1
0·00032% do ..	0·08	24·1	..	42·8	10·04
0·00016% do ..	0·04	35·5	30·2	59·2	15·6
0·00008% do ..	0·02	..	..	68·3	23·3
0·00004% do ..	0·01	..	..	81·7	70
0·01% Shirlan ..	10·0	0	0	0	0
0·005% do ..	5·0	8·4	Max. 2·0	0	0
0·0025% do ..	2·5	68·4	5·9	52·5	3·6
0·00125% do ..	1·3	65·6	13·3	66·6	8·4
0·00063% do ..	0·65	72·6	18·2	94·8	14·2
1% PC ..	250	70·3	5·2	81·1	2·8
1% TC ..	250	4·8	0·64	5·1	Max.2·0

* Mcg = amount of fungicide in micrograms $\left(1 \text{ Mcg.} = \frac{1}{10^6} \text{ g.}\right)$

Table II shows the following results:—

(1) Bordeaux mixture and Shirlan are very effective in reducing percentage germination even at low concentration.

(2) Bordeaux mixture is more effective than Shirlan in reducing germination of spores but less effective in reducing germ tube length.

(3) PC in the dose applied is inferior to the standard fungicides in reducing germination percentage but equally good if not better in its effect on germ tube length. The greater effectiveness of TC over PC is again brought out.

According to Branas and Dulac (1933) very alkaline Bordeaux mixture on quick desiccation does not lose its effectiveness as there is enough lime uncarbonated to dissolve a toxic amount of copper. The writer tested whether Bordeaux mixture which in the fresh condition was very effective on spore germination (see Table II) retained its efficiency or not. Two drops from a 5 c.c. pipette of various concentrations of Bordeaux mixture and Shirlan were added to each of 3 coverslips and dried down in a vacuum desiccator within 3 hours. A germination test was carried out which shows that Bordeaux mixture after desiccation is as effective as in the freshly made up condition in checking *Botrytis* spores from germinating whereas Shirlan loses its effectiveness to some extent.

The effect of nutrient was examined in a series of experiments in which water was replaced by a lettuce extract. This was prepared by boiling 250 g. of lettuce leaves for about half an hour, squeezing through muslin cloth and finally making the volume up to a litre. Afterwards the extract was sterilised in the usual way. Bordeaux mixture and Shirlan were dried on the coverslips as mentioned before and 2 drops of spore suspension, made in lettuce extract, added. In the case of PC and TC a weighed quantity was mixed with 2 drops of spore suspension on each coverslip. Table III gives a summary of 3 experiments on this point.

In the experiments of Table III the Bordeaux mixture and Shirlan were first of all dried down on the coverslips. In 2 further experi-

TABLE III

Effect of PC, TC, Dried Bord. Mixture and Dried Shirlan on germination of Botrytis *spores in Lettuce Extract*

Preparation of coverslip	Mcg.	Exp. 1 (16 hours)		Exp. 2 (16 hours)		Exp. 3 (16 hours)		(60 hours)	
		% germ.	Av.g.t.l	% germ.	Av.g.t.l	% germ.	Av.g.t.l	% germ.	Av.g.t.l
Control ..	0	100	>100	100	>100	100	>100	100	>100
PC ..	ca. 2000	100	14·8	100	16·5	100	19·8	100	> 50
TC ..	ca. 2000	..	6·5	34·1	5·6	20·8	Max.8	..	Max.17
1% Bord. Mix. ..	254			0	0	0	0	0	0
0·5% do ..	127					0	0	0	0
0·1% do ..	25·4			100	>100	100	>100	100	>100
0·01% do ..	2·54	100	100						
0·001% do ..	0·254	100	100						
1% Shirlan ..	1000			0	0	0	0	13·1	Max.4
0·5% do ..	500					..	short	82·9	2·9
0·1% do ..	100			75·8	3·0	23·7	Max.6	96·5	4·8
0·01% do ..	10	100	4·9						
0·001% do ..	1	100	>100						

ments the fungicides were not dried down, except that 2 concentrations of Bordeaux mixture were tested in 1 experiment both in the fresh and the dried condition. For the sake of uniformity 1 drop of water was added to the dried stuff before adding a drop of spore suspension. The results of both the experiments are shown in Table IV. Column 1 gives the mode of preparation of coverslips, to each of which 1 drop of spore suspension in lettuce extract was added. The amount of fungicides in micrograms is given in column 2.

TABLE IV

Comparative effect of Fresh and Dry Bordeaux Mixture, Fresh Shirlan. PC and TC on germination of Botrytis *Spores in Lettuce Extract*

Preparation of coverslip	Mcg.	% germ	Av.g.t.l
Experiment 1. (60 hours)			
Control (1 drop water) ..	0	100	>100
1% PC in water ..	500	100	57·1
1% TC in water ..	500	24·6	28·4
1% Bordeaux mixture dried ..	127	0	0
1% do fresh ..	127	0	0
0·2% do dried ..	25·4	100	>100
0·2% do fresh ..	25·4	0	0
0·02% do fresh ..	2·54	100	>100
0·2% Shirlan ..	100	0	0
0·02% do ..	10	100	>100
Experiment 2. (60 hours)			
Control (1 drop water) ..	0	100	>100
0·1% Bordeaux mixture ..	12·7	0	0
0·05 do ..	6·4	100	>100
0·1% Shirlan .	50	6·1	..
0·05% do ..	5	12·2	..

The following points emerge from Tables III and IV.

(1) Germination of spores in lettuce extract is enhanced to such an extent that germ tubes form a weft even after 16 hours. This state of affairs is conventionally represented by the symbol > 100. After 60 hours the mass of mycelium is visible to the naked eye.

(2) Bordeaux mixture and Shirlan which stopped spore germination in distilled water at very low concentrations allow 100% germination in 0·05% and 0·02% respectively (Table IV). Drying of Bordeaux mixture or of Shirlan still further reduces their efficiency in the extract.

(3) The new fungicides do not lose their effectiveness in the extract, *i.e.*, the degree of reduction in percentage germination or in average germ tube length obtained in presence of lectuce extract is much the same as in water.

Two experiments were set up in which the nutrient used was malt extract. The coverslips were prepared exactly as in Table III,

Two further experiments were done in 2% malt extract with the standard fungicides only. In the first of these the fungicides were fresh; in the second fresh and dried were compared. The method followed and the quantity of the fungicides employed were similar to those described for Table IV.

The following points were obvious from the 4 experiments in malt extract:—

(1) Just as with lettuce extract malt strongly increases germination in the controls.

(2) Spores germinate in concentrations of Bordeaux mixture and Shirlan which gave complete inhibition in water. Desiccation definitely reduces the efficiency of both fungicides.

(3) The new fungicides show the same features as in lettuce extract, *viz.*, their activity is comparable to that shown in water.

The effect of acidity on the activity of the various fungicides was investigated to some extent. Table V records an experiment in this connection.

TABLE V

Effect of PC, TC, Bordeaux Mixture and Shirlan on germination of Botrytis *Spores in Malic Acid*

Preparation of coverslip	Mcg.	0·1% Malic acid 40 hours % germ.	Distilled water 40 hours % germ.
Control (1 drop water) ..	0	78·4	75·6
1% PC in water ..	500	29·4	77·5
1% TC in water ..	500	0	24·9
0·1% Bord. Mix. ..	12·7	13·6	0
0·01% do ..	1·3	84·7	0
0·001% do ..	0·13	95·8	12·9
0·1% Shirlan ..	50	0	0
0·01% do ..	5	0	0
0·001% do ..	0·5	91·5	74·8

A comparison of the third and fourth columns of Table V shows that while the amount of malic acid used had no material effect upon percentage germination in the controls, it distinctly increased the efficiency of PC and TC, but reduced that of Bordeaux mixture. With Shirlan no obvious effect was seen. The effect on Bordeaux mixture just mentioned is in agreement with the results of Goldsworthy and Green (1938) who found that ionic copper in quantities sufficient to be toxic to the conidia of *Sclerotinia fructicola* could be entirely inactivated by the addition of an equivalent amount of malic acid, this being contrary to what had been reported by Mc Callan and Wilcoxon (1936).

Thus with regard to the properties of the new fungicides, the main results which emerge from the foregoing experiments with spores of *Botrytis cinerea* are:—

(1) The effect of PC and TC is more marked on the average germ tube length than on percentage germination.

(2) PC is less effective than TC both as regards percentage germination and germ tube length.

(3) Weight for weight both Bordeaux mixture and Shirlan are more effective than PC or TC on germination of *Botrytis* spores in water.

(4) The effectiveness of Bordeaux mixture and Shirlan is less in nutrients such as lettuce or malt extract. On the other hand, PC and TC are relatively unaffected by such nutrients.

(5) The activity of Bordeaux mixture is distinctly reduced in presence of malic acid. On the other hand, PC and more particularly TC are rendered more active in the same circumstances.

(*b*) *Fusarium cæruleum*

Germination of spores of *Fusarium* in contact with the fungicides was studied in distilled water only. The effect of PC and TC on germination was examined in 2 ways, *viz.*, (1) by mixing weighed amounts of the material with the spore drop, (2) by taking 1 drop of a 1% suspension of PC or TC in water and mixing with the spore drop. Table VI contains a summary of 2 experiments, the first with the new fungicides only and the second with all. To each coverslip of the new fungicides and control, set up as shown in the table, was added 1 drop of spore suspension, whereas to the coverslips of the standard fungicides were added 2 drops after mixing the fungicide and spore suspension as mentioned for *Botrytis* in Table II.

TABLE VI

Effect of PC, TC, Bordeaux Mixture and Shirlan on germination of Fusarium *spores in water*

Preparation of coverslip	% Germ.	Av.g.t.1
Experiment 1. (24 hours)		
Control ..	91·6	31·4
ca. 0·002 g. PC ..	90·9	18·3
ca. 0·002 g. TC ..	85·3	12·5
Experiment 2. (60 hours)		
Control (1 drop water) ..	97·2	37·8
1% PC in water ..	97·2	36·0
1% TC in water ..	94·7	15·1
0·00125% Bordeaux Mixture ..	0	0
0·00063% do ..	72·5	5·6
0·00032% do ..	91·0	8
0·05% Shirlan ..	8·2	Max. 5
0·025% do ..	12·3	..
0·0125% do ..	17·9	5·2
0·0063% do ..	24·3	6·4
0·0032% do ..	37·2	12·5

Comparision of Tables II and VI shows that whereas Bordeaux mixture is about equally effective in repressing the germination of *Fusarium* as of *Botrytis*, both PC and TC are strikingly ineffective on *Fusarium*, the only effect noted being a slight reduction of germ tube length by the latter. Shirlan occupies an intermediate position, being less effective on *Fusarium* than on *Botrytis*.

(*c*) *Rhizopus nigricans*

As the germination of spores of *Rhizopus* in distilled water was very irregular, the effect of the fungicides on spore germination was tested in the presence of a trace of nutrient. To each coverslip set up in the manner in Column 1 of Table VII was added a drop of spore suspension in dilute turnip extract. The latter was prepared by adding 1 c.c. of the extract to 99 c.c. distilled water.

TABLE VII

Effect of PC, TC, Bordeaux Mixture and Shirlan on germination of Rhizopus *spores in a trace of nutrient*

Preparation of coverslip	Mcg.	40 hours % Germ.
Control (1 drop water) ..	0	100, weft
1% PC in water ..	500	3·4
1% TC in water ..	500	0
0·01% Bordeaux Mixture ..	1·3	0
0·005% do ..	0·65	2·7
0·001% do ..	0·13	20·3
0·01% Shirlan ..	5	0
0·005% do ..	2·5	1·3
0·001% do ..	0·5	25

Though a rigid comparison cannot be made with the results shown in Table II for *Botrytis*, because no nutrient was added for the latter as it was for *Rhizopus*, yet it appears that Shirlan, PC and TC are more repressive on *Rhizopus* than on *Botrytis* germination. The effect of the nutrient added in the experiment of Table VII would presumably be to reduce fungicidal action, if it had any effect at all. Bordeaux mixture was somewhat less effective on the germination of *Rhizopus* than of *Botrytis*, but this may be due to the action of the nutrient which was added in the experiment with *Rhizopus*.

(*d*) *Ascochyta rabiei*

The spores of this fungus have been reported by Sattar (1933) to germinate better in an acidic medium than in water. Nevertheless the spores of this particular isolate germinate well in distilled water, so the effect of the new fungicides was studied both in water and in 0·025% malic acid.

Two drops of each concentration of the standard fungicides as given in Table VIII were quickly dried on the coverslips and then 2 drops of spore suspension in acid were added.

TABLE VIII

Effect of PC, TC, Bordeaux Mixture and Shirlan on germination of Ascochyta *spores in malic acid*

Preparation of coverslip	% Germ. in distilled water	Preparation of coverslip	Mcg.	% Germ. in 0·025% malic acid
Experiment 1. (50 hours)		Experiment 2. (50 hours)		
Control ..	69·8	Control ..	0	76·7
		PC ..	ca. 2000	0
1% PC in water ..	20·4	TC ..	ca. 2000	0
		0·01% Bord. M. ..	2·54	0
1% TC do ..	1·3	0·005% do ..	1·27	73·5
		0·01% Shirlan ..	10	0
		0·005% do ..	5	0
		0·001% do ..	1	79·1

Table VIII shows the following points:—

(1) PC and TC are effective in reducing spore germination in distilled water but much more so in the presence of acid. In the latter case both gave complete inhibition. This behaviour is parallel to that shown by *Botrytis* spores (Table V), which were more sensitive to these fungicides in presence of acid.

(2) Shirlan is more effective than Bordeaux mixture in an acidic medium.

(*e*) *Trichothecium roseum*

The spores of *Trichothecium* like those of *Rhizopus* germinated very poorly (ca 38%) in distilled water. Therefore the effect of all the fungicides on germination was studied in dilute turnip extract. The method followed was exactly the same as described for *Rhizopus*.

Experiments perfomed show that TC, as usual, is more effective in checking germination of spores. Of the standard fungicides Bordeaux mixture is superior to Shirlan.

(*f*) *Trichoderma viride*

Germination of spores of *Trichoderma* was tested both in distilled water and in dilute turnip extract in contact with the fungicides. Table IX gives a summary of 3 experiments, the first with the new fungicides in distilled water, the second with all the fungicides in the same medium and the third with all in dilute turnip extract. On account of the small size of the spores and the presence in turnip extract of small particles of much the same size and appearance as spores, the germination percentage in this medium was difficult to determine. The method followed was the same as for *Rhizopus*.

In all the fungi studied prior to *Trichoderma* it was seen that TC was more effective than PC but here the reverse happens, *i.e.*, PC has a greater retarding effect on germ tube length than TC.

TABLE IX

Effect of PC, TC, Bordeaux Mixture and Shirlan on germination of Trichoderma *spores in water and turnip extract*

Preparation of coverslip	Av.g.t.l	Preparation of coverslip	Mcg.	% Germ.	Preparation of coverslip	Av.g.t.l
Experiment 1. (50 hours)		Experiment 2. (50 hours)			Experiment 3. (50 hours)	
Control ..	9·0	Control ..	0	94·6	Control ..	>100
ca. 0·002 g. PC ..	5·6	1% PC ..	250	70·7	1%PC ..	12·3
ca. 0·002 g. TC ..	8·6	1% TC ..	250	79·3	1%TC ..	> 50
		0·01 % Bord. Mix. ..	1·3	0	0·1 % Bord. Mix. ..	0
		0·001% do ..	0·13	32	0·01% do ..	6·6
		0·01 % Shirlan ..	5	3·8	0·1% Shirlan ..	0
		0·001% do ..	0·5	100	0·01% do ..	> 50

(g) *Alternaria* sp.

Germination of *Alternaria* spores was studied in distilled water, dilute turnip extract and 2% malt. Table X gives the summary of

TABLE X

Effect of PC, TC, Bordeaux Mixture and Shirlan on germination of Alternaria *spores in water, malt and turnip extracts*

Preparation of ocverslip	Mcg.	% Germ.	Preparation of coverslip	Av.g.t.l
Experiment 1. (60 hours)			Experiment 2. (48 hours)	
Control ..	0	64·5	Control ..	>100
ca. 0·002 g. PC ..	..	13·2		
ca. 0·002 g. TC ..	..	2·1	ca. 0·002 g. PC ..	24·9
0·001 % Bord. Mix.	0·254	0		
0·0005% do ..	0·13	0	ca. 0·002 g. TC ..	7·1
0·0001% do ..	0·026	61·3		
0·01 % Shirlan ..	10·0	0		
0·001% do ..	1·0	70·5		

Preparation of coverslip	Mcg.	% Germ	Av.g.t.l
Experiment 3. (48 hours)			
Control ..	0	100	>100
ca. 0·002 g. PC ..	..	89·1	15·3
ca. 0·002 g. TC ..	..	77·2	4·7
0·1% Bord. Mix. ..	12·7	0	0
0·05 % do ..	6·4	7·9	Max. 4·0
0·01 % do ..	1·3	38·1	5·6
0·005% do ..	0·65	100	Max. 30
0·1 % Shirlan ..	50	13·0	8·2
0·05% do ..	25	22·4	11·0
0·01% do ..	5	100	>100

Preparation of coverslip	Mcg.	% Germ.	Av.g.t.l	% germ.	Max.g.t.l
Experiment 4. (24 hours)				(84 hours)	
Control ..	0	100	>100	100	>100
ca. 0·002 g. PC ..	..	91·5	11·04	..	30
ca. 0·002 g. TC ..	..	80·6	3·2	..	10
0·5 % Bord. Mix. ..	127	0	0	0	0
0·1 % do ..	25·4	Trace	..	6·5	6
0·05% do ..	12·7	do	..	16·6	..
1·01% do ..	2·54	91·4	15·7	..	70
0 % Shirlan ..	1000	0	0	0	0
0·5% do ..	500	0	0	0	0
0·1% do ..	100	10·4	..	44·8	50

4 experiments, (1) in water with all the fungicides, (2) in 2% malt with PC and TC only, (3) and (4) in dilute turnip extract with all the fungicides, the last differing from (3) in the fact that standard fungicides were desiccated on the coverslip whereas they were fresh in (3). The amount of fungicide in microgram is given in each case for the standard fungicides.

The conclusions to be drawn from Table X are as follows:—

(1) PC and TC are very effective in reducing percentage germination of spores in water. In turnip extract percentage germination is not appreciably affected but reduction in germ tube length is very marked.

(2) Bordeaux mixture and Shirlan are very effective even in very low concentration. So far as the effects of desiccation and nutrient are concerned the behaviour is similar to that shown by *Botrytis cinerea.*

IV. Germination of Spores in Vapour of Fungicides

As the new fungicides are volatile in nature, germination of spores of 3 fungi, *viz. Botrytis cinerea, Fusarium cæruleum* and *Trichoderma viride,* was studied in presence of vapour. Of the two standard fungicides, Bordeaux mixture is well known to act protectively by contact only, but in the case of Shirlan it was not known whether it might act by its vapour. Tests were therefore carried out with *Botrytis* spores to determine whether Shirlan exerted any such effect.

The general method was as described earlier, except that the fungicide was placed on the lower Petri dish, not in physical contact with the spore drops. To obviate the risk that the powdered fungicide might blow about and thereby fall into the drops of spore suspension, it was moistened with water so as to make a kind of paste. This was the method adopted with PC and TC. With Shirlan a 1% solution was used.

In view of the negative result obtained with Shirlan, it was decided to test this compound under conditions where, if present, the vapour could more easily reach an effective concentration, *viz.* in the limited space of a Van Tieghem cell. The Shirlan solution (0·2 c.c.) was placed in the bottom of the cell, and the spores exposed to a possible vapour effect in hanging drop culture.

The results of 7 experiments are collected in Table XI. The various experimental conditions, fungi used, etc., are shown in the Table.

The following points can be concluded from Table XI.

(1) The effect on spore germination by the vapour of Shirlan is negligible.

(2) PC and TC show the same effects on spore germination and germ tube length by the action of their vapours as by contact.

TABLE XI

Effect of vapour of PC, TC and Shirlan on germination of Botrytis *spores*

Fungus	Fungicide	Experimental conditions	% Germ.	Av.g.t.l of germinated spores	Av.g.t.l. per 100 spores sown
Botrytis—					
Exp. 1 ..	0	20°C., 60 hrs. sealed	91·8	12·3	11·3
	0·05 g. PC	do	80·3	3·0	2·4
	0·05 g. TC	do	59·5	Max. 2	..
Exp. 2 ..	0	20°C., 24 hrs. Van Tieghem	82·8	17·5	14·5
	0·2 cc. Shirlan	do	75·3	16·3	12·3
Exp. 3 ..	0	20°C., 24 hrs. sealed	76·6	12·0	9·2
	0·05 g. PC	do	66·4	3·7	2·4
	0·05 g. TC	do	11·2	1·4	0·16
	0·3 cc. Shirlan	do	68·5	11·0	7·5
Exp. 4 ..	0	20°C., 24 hrs. not sealed	54·4	11·6	6·3
	0·05 g. PC	do	42·6	6·3	2·6
	0·05 g.TC	do	44·1	3·4	1·5
Exp. 5 ..	0	8°C., 24 hrs. sealed	70·9	2·3	1·6
	0·05 g. PC	do	6·8	1·6	0·10
	0·05 g. TC	do	0	0	0
Fusarium—					
Exp, 6. ..	0	20°C., 24 hrs. sealed	92·9	29·6	27·5
	0·05 g. PC	do	91·6	17·3	15·8
	0·05 g. TC	do	89·2	11·2	9·9
Trichoderma—					
Exp. 7 ..	0	20°C. 48 hrs. sealed	..	9·7	..
	0·05 g. PC	do	..	6·2	..
	0·05 g. TC	do	..	9·2	..

(3) Even when the dishes are not sealed the effect of PC and TC on germ tube length is nearly as pronounced as when they are sealed.

(4) The variable effect of TC on germination already noted also comes out in these experiments (Compare experiments 1 and 4 with 3).

(5) Depression of percentage germination by the vapour of TC and PC is most marked at low temperature.

(6) It was shown earlier that PC is less volatile than TC but that nevertheless it is more effective than TC in repressing germination of *Ttichoderma* when present in the spore drop. The above experiment shows this to be true also when the spores are subjected to the vapours of these compounds.

(7) The effect of vapour on *Fusarium* is similar to what was observed when PC and TC were in contact.

V. Effect of Fungicides on Linear Growth

1. *Fungicides mixed with Nutrient Medium*

(a) Botrytis cinerea

This was studied in lettuce extract agar. The method of preparing the latter has already been described. 67 cc. of the extract was placed in a 150 cc. flask and 1 g. agar added. After sterilising in the usual way 0·067 g. PC was added while the contents were still hot, the concentration of the fungicide thus being 0·1%. The agar was then poured into 3 sterilised petri-dishes, each therefore having about 20 cc. of medium. A similar triplicate set was set up with the same percentage concentration of TC, and 3 others as controls. In later experiments it was found more convenient to add weighed amounts of the fungicides to the sterilised petri-dishes and then to pour the hot medium over the fungicide, with a certain amount of rocking to distribute the fungicide evenly over the plate. After inoculating with a loopful of spore suspension the dishes were incubated at 20° C. The growth of colony in each dish was measured at 2 diameters (at right angle to each other) in millimetres after 2 days and again at 3-day intervals. The amounts of growth after 5, 23 and 35 days are given in Table XII. Each figure represents the average of 3 replicates.

Table XII

Effect of PC and TC, mixed with the solid medium, on spore germination and growth of Botrytis

	5 days	23 days	35 days
Control ..	81·6±0·33	Petri-dish full after 6 days	..
0·1% PC ..	19·8±0·92	5·2±2·5	53·1±1·3
0·1% TC ..	No spread*	12·3±0·6	33·1±9·3

* By this is meant that though germination has begun the colony has not advanced to the naked eye beyond the limits of the original inoculum.

The reason for the standard error being very high in the case of TC after 35 days is that hyphæ of a different kind arose in one of the dishes. It will be shown later that these hyphæ are specially resistant to the fungicide.

The following conclusions are drawn from Table XII.

(1) The new fungicides are so effective in reducing colony growth that even after a month the petri-dishes were not full whereas the control dishes became full after 6 days.

(2) PC is less effective than TC.

The greater effectiveness of TC over PC is further emphasised in a number of experiments in which equal concentrations of the commercial dusts were added to the nutrient medium. As the respective concentrations of PC and TC in the dusts are 20% and 5% the greater repression of growth shown in Table XIII by TC takes place in spite of the concentration of the latter being only one-quarter that of PC. The record of one such experiment is shown in Table XIII.

TABLE XIII

Effect of Commercial dusts PC and TC, mixed with the solid medium, on spore germination and growth of Botrytis

	20° C.		8° C	
	6 days	16 days	6 days	16 days
Control ..	85 (full)	..	18±0	85 (full)
0·5% PC dust ..	7±0·76	18·5±2·01	No spread	8·1±0·92
0·5% TC dust ..	No spread	5±0·86	do	No spread

It will be noticed again that the repressive effect of the fungicide is accentuated at the lower temperature.

In the experiment of Table XIV the effect of the fungicides was determined on young mycelium of *Botrytis*. This was cut out in a standard manner by means of a cork borer from the growing edge of a 4 days' old culture. The results therefore give the effect on growth only and not on germination and growth as in the 2 preceding Tables. Two media were used, lettuce extract agar and glucose-peptone agar (5 g. glucose, 5 g. peptone, 0·5 g. magnesium sulphate, 1·0 g. potassium dihydrogen phosphate, 15 g. agar and 1 litre distilled water).

TABLE XIV

Effect of PC and TC on colony growth of Botrytis

	Glucose-peptone agar		Lettuce extract agar	
	6 days	24 days	6 days	24 days
Control ..	90 (full)	..	90 (full)	..
0·1% PC ..	44·3±2·0	62·5±2·9	39±6·0	66·1±6·8
0·1% TC ..	6·0±0·28	28·8±7·2	5·5±0·95	21·5±1·7

This table confirms the results of Tables XII and XIII and shows that there is no material difference between the 2 media in respect of the magnitude of the effect.

An effect over and above what is shown in Table XIV is that the fungicides PC and TC alter the manner of growth of the fungus. Colonies in the controls grow in regular circles and have profuse aerial mycelium. In presence of the fungicides (and, as will be seen later, in their vapours) growth is distinctly lobed and there is a suppression of aerial mycelium. These differences are shown in Figs. 1, 2 and 3.

The effects of PC and TC on colony growth were compared with those of varied doses of Bordeaux mixture and Shirlan when similarly added. Copper sulphate and calcium hydroxide were sterilised separately at 10 lb. pressure for 10 minutes whereas Shirlan was steamed for 15 minutes. Equal quantities of 4% malt extract and 1% Bordeaux mixture or 1% Shirlan were mixed, thus giving 2% malt with 0·5% Bordeaux mixture or Shirlan. A range of concentrations of Bordeaux or of Shirlan was set up in this way. The mixtures were then steamed on 2 successive days. PC and TC were used in 2 ways, *viz.*, (1) by mixing weighed amounts with the medium in the petri-dish and (2) by placing 0·2 cc. of 1% PC or TC in acetone in the petri-dish and allowing the solvent to evaporate. Three dishes of the 10 used for controls were similarly treated with acetone without the fungicide. After inoculation the dishes were incubated at 20° C. The first measurement was taken after 4 days and then after every 2 days. The amount of colony growth (mm.) after 4, 10 and 16 days is given in Table XV, the figures in the table being averages of 3 replicates excepting for the control, which had 10.

TABLE XV

Comparative effect of PC, TC, Bordeaux Mixture and Shirlan on germination and colony growth of Botrytis

	4 days	10 days	16 day
Control	61·8±1·1	90 (full) after 6 days	(Full)
0·5 % Bord. Mix.	0	Trace	Trace
0·1 % do ..	6·5±1·0	66·5±12·5	2 dishes full after 12, 3rd, 90
0·01% do ..	63·6±1·0	90 (full) after 6 days	(Full)
0·5 % Shirlan ..	0	0	0
0·1 % do ..	0	0	0
0·01% do ..	19·6±0·44	90±0 (full)	Full
0·25% PC ..	8·1±0·44	17·3±0·44	26·3±0·1
0·01% PC ..	11·3±0·4	33·4±3·5	52·4±3·1
0·25% TC ..	0	4·1±0·53	9·6±2·7
0·01% TC ..	4·2±0·37	6·6±0·23	7·2±0·38

The conclusions to be drawn from Table XV are:—

(1) A trace of germination takes place at the highest concentration of Bordeaux mixture used,

(2) For comparable quantities, Shirlan is more effective than Bordeaux mixture.

(3) With PC and TC pronounced reduction of growth is given by the highest and lowest concentrations added, the former not producing much greater effect than the latter. PC is as usual not so effective as TC.

(*b*) *Other Fungi*

Table XVI gives a summary of the effects of the new fungicides on linear colony growth of *Fusarium cæruleum, Rhizopus nigricans, Ascochyta rabiei, Trichothecium roseum, Trichoderma viride, Alternaria* sp., *Rhizoctonia solani, Pythium deBaryanum* and *Phytophthora parasitica* after mixture with the nutrient medium. In the case of non-sporing fungi the inoculum was cut by a cork borer from the edge of a growing colony. The figures in Table XVI are in mm. and are averages of 3 replicates in each case. Colony growth of some of the above mentioned fungi is illustrated in Figs. 4–15.

TABLE XVI

Effect of PC and TC on Colony Growth of Various Fungi

Fungus	Medium	No. of days	Control	0·25% PC	0·25%TC
Fusarium ..	Potato agar	10	39·8±0·66	38·8±0·58	17·1± 1·0
	do	20	70·5±0·76	68·5±1·6	30·6± 0·44
	Malt agar	10	43·2±2·7	28·0±0·74	13·5± 0·53
	do	20	77·1±1·16	60·1±1·01	28·3± 1·36
Rhizopus ..	do	3	85 (full)	0	0
	do	25	do	0	0
Ascochyta ..	Potato agar acidified by malic acid	7	9·1±0·72	0	0
		21	52·7±1·49	12·8±1·14	0
Trichotheeium ..	Malt agar	4	14·6±0·43	6·7±0·36	0
	do	20	79·5±1·16	26·4±0·66	8·8± 1.36
Trichoderma ..	do	2	40·5±1·02	9·4±0·2	25·3± 0·37
	do	6	90 (4 days)	42·6±0·72	90 (full)
	do	8	do	67·1±1·45	do
Alternaria ..	do	8	56·5±1·32	11·3±0·44	8·3± 0·72
	do	22	90 (16 days)	25·0±3·95	36 ±19·4
Rhizoctonia .. (seakale strn.)	do	2	18·1±1·3	7·1±1·10	12·7± 0·22
	do	14	88 (10 days)	32·8±2·18	62·2± 2·86
Rhizoctonia .. Potato (strn.)	do	2	37 ±2·56	8±1·59	18·9± 0·97
	do	14	90 (6 days)	49·5±1·46	71·2± 3·58
Rhizoctonia .. (seakale strn.)	do	2	19·7±0·66	0	13·2± 0·23
	do	14	88 (8 days)	29·3±2·53	64·7± 0·25
Pythium ..	Potato agar	1	43·5±0·76	41·2±1·2	30 ± 0·28
	do	3	90 (2 days)	90 (full)	90 (full)
	Potato sprout Extract agar	6	85 (full)	79·8±1·3	21 ± 6·1
	do	14	85 (full)	85 (7 days)	55·6±18·3
Phytophthora ..	Malt agar	12	82·5±2·78	77·3±9·6	53·1± 1·85
	do	20	88 (16 days)	88 (16 days)	81 ± 0·76

The following conclusions are to be drawn from Table XVI.

(1) All the fungi excepting *Phytophthora* are considerably retarded in their colony growth by one or the other of the new fungicides.

(2) TC is more effective than PC on most of the fungi, but the reverse is the case with *Trichoderma* and both strains of *Rhizoctonia*.

(3) Both the new fungicides completely inhibit colony growth of *Rhizopus* in a nutrient medium. This closely agrees with what was found in spore germination tests of the fungus in turnip extract.

(4) The effectiveness of the fungicides increases with increase of acidity in the medium as was also found in spore germination tests. This is illustrated very clearly by the linear growth of *Fusarium*, *Pythium*, etc. With *Fusarium* on potato agar there is no difference in the growth of control and PC treated dishes either after 10 or 12 days whereas on malt agar (pH 4·6) the difference between the 2 is highly significant. With *Pythium* the check to growth by either fungicide is very slight on potato agar (pH 6·2) but is distinct with TC on potato sprout extract agar (pH 5·5). The markedly repressive effect on the growth of *Ascochyta* may also be associated with the acidity of the medium used, but no comparison with a non-acidified control was made.

2. *Nutrient in Vapour of Fungicides*

(*a*) *Botrytis cinerea*

The method of experiment was the same as in the tests where spore germination was being examined. The fungicide was introduced into the lower (larger) lids of the petri-dishes, either in the form of moistened crystals or in solution in acetone. In the latter case the solution was prevented from spreading beyond the limits of the smaller petri-dish component by means of a ring of paraffin-wax. Alternatively the experiments were carried out in desiccators.

Table XVII gives the results of 5 experiments of which the first 4 were carried out at 20° C. and the last one at 8° C. The figures in the Table give the average diameters (mm.) of triplicate cultures.

TABLE XVII

Effect of vapour (air-tight condition) of PC and TC on spore germination and colony growth of Botrytis

Exp.	No. of days	Control	PC		TC	
			Amount	Diam (mm.)	Amount	Diam. (mm.)
1	6	(85) full	1 cc. 0·1%	19·1±0·66	1 cc. 0·1%	6·3± 0·44
	12	do	1 cc. 0·1%	35·6±2·90	do	27·7±18·13
2	7	do	0·02 g.	17·5±0·57	0·02 g.	6·3± 0·6
	22	do	do	55·5±2·08	do	16·1± 1·63
3	6	do	do	17·6±0·67	do	7 ± 0·28
	21	do	do	49·8±4·6	do	56·1± 20·5
4	6	do	0·04 g.	20·3±1·09	0·04 g.	5 ± 0·57
	22	do	do	48·5±3·25	do	10·8± 0·88
5	21	do	0·6 g.	11·1±0·92	0·6 g.	6± 1

The following points emerge from Table XVII:—

(1) As can be seen by comparing Table XVII with Table XII the new fungicides are effective in reducing colony growth by their vapour almost to the same extent as when mixed with the medium.

(2) A quantity as small as 0·001 g. (Exp. 1) can effectively reduce colony growth even on a highly nutrient medium.

(3) At lower temperature, *viz.*, 8° C., the retarding effect of both fungicides on colony growth (Exp. 5) is very pronounced and the difference between PC and TC dishes is not so marked as it is at the higher temperature, *i.e.*, 20° C.

In the experiments of Table XVII the fungicidal vapours were contained either by sealing the dishes with paraffin wax or by carrying out the experiments in desiccators. These precautions were shown not to be necessary in as much as similar results were obtained when experiments were carried out under ordinary conditions of petri-dish culture. Four experiments of this kind are summarised in Table XVIII. The amount of PC and TC added to each petri-dish was 0·04 g. in all cases.

TABLE XVIII

Effect of vapour (ordinary condition of petri-dish culture) of PC and TC on colony growth of Botrytis

Exp.	No. of days	Control	PC Colony growth in mm.	TC Colony growth in mm.
1 ..	4	71·6 ± 1·30 (nearly full)	10·8 ±0·44	No spread
	12	Full	35 ±2·78	6·0 ± 0·07
	24	do	54·8 ±2·17	36 ±20·03
2 PC, TC as vapour	7	88 (full)	15·75 ± 0·48	No spread
do	22	do	37 ±2·88	24·5 ± 13·62
PC, TC added to medium	7	do	15·9 ±0·89	No spread
do	22	do	35·5 ±2·15	9·7 ± 3·06
3 ..	9	do	22·7 ±0·74	6·2 ± 0·25
	23	do	42 ±2·0	55 ±18
4 8°C ..	7	28·6 ± 0·44	7·3 ±0·16	4·8 ± 0·16
	14	74·6 ± 0·44	15·1 ±0·72	6 ± 0

It will be seen that the degree of suppression was much the same as in those experiments (Table XVII) in which the dishes were sealed and that the effect of the vapour is as great as that shown when the fungicide was incorporated in the medium (Table XVIII, Exp. 2).

Six experiments were done with the commercial dusts, both at 20° C. and 8° C., the quantity being 0·1 g. in each dish. The method employed was similar to that described for Table XVIII. The results were similar to those already pointed out in connection with Table XIII, *viz.*, that the greater effect of TC over PC is still shown, even

though the amount of active principle of the former was only quarter that of the latter. The magnitude of the retarding effect appears to be actually greater for the commercial dusts than for the active principles only, perhaps because the former, being in a much finer state of division, give off vapour more readily from the increased surface.

(*b*) *Other Fungi*

Table XIX records the behaviour of 6 fungi when exposed to the vapours of PC and TC in unsealed petri-dishes. The medium used was 2% malt agar except for *Ascochyta*, where it was potato agar.

The results of Table XIX follow the same lines as in Table XVI in which the fungicides were included in the medium. The greater sensitiveness of *Rhizoctonia* and *Trichoderma* to PC than to TC is again shown.

TABLE XIX

Effect of vapour of PC and TC (unsealed petri-dishes) on colony growth of various fungi

	No. of days	Control	PC	TC
Rhizoctonia ..	4	36 ±0·5	9·3±0·49	16·9±0·30
	16	90 (8 days)	35·9±5·32	82·8±0·92
Trichothecium ..	4	14·1±0·72	6·6±0·16	0
	20	79·3±1·16	31 ±2·78	14·1±3·9
Ascochyta ..	7	22 ±0	9·3±0·16	No spread
	21	71·1±1·96	26·6±0·6	8·1±1·59
Trichoderma ..	2	48·8±0·44	10·4±0·62	31·1±0·56
	4	90 (full)	33·1±1·16	99 (full)
	6	90 (full)	63·5±1·89	do
Alternaria ..	4	27·1±0·16	9·1±0·16	5·1±0·16
	12	67·3±0·88	25·1±1·09	7·6±0·52
Rhizopus ..	3	88 (full)	0	0
	9	do	0	0

VI. EFFECT ON SPORULATION AND SCLEROTIAL FORMATION

The new fungicides have a very marked effect on suppressing the formation of spores or sclerotia. So far as *Botrytis* is concerned sporulation and sclerotial formation are completely checked by both fungicides either by contact or by vapour (Figs. 13–15). In the case of those fungi which are not appreciably checked in colony growth by the fungicide, there is also considerable reduction in the formation of spores. Thus with *Fusarium* and *Trichoderma*, which are insensitive to PC or TC as regards mycelial growth, there is a pronounced reduction of sporulation in presence of either of these fungicides.

The method adopted for spore estimation was to wash off the spores in a standardised manner, *viz.*, by rubbing with the finger after flooding the dish with a measured amount of water. This process was repeated a number of times with fresh additions of water. The various washings were added together, and the concentration of spores in the

final suspension estimated by counting with a hæmocytometer. Triplicate counts were made in each case. The records so obtained were then corrected for the total amount of water used in each case.

With some of the fungi, such as *Botrytis* and *Alternaria* the spores tended to run together into clumps. This was prevented by adding about 1 c.c. of "Teepol", a wetting agent, to each 100 c.c. of water used.

TABLE XX

Effect of PC and TC on production of spores in various fungi

	Control		PC		TC	
	Diam.	Spore count	Diam.	Spore count	Diam.	Spore count
Botrytis ..	85	46, 40, 50 Av. 45	57·5	0, 0, 0	76	0, 0, 0
Fusarium ..	84	20, 14, 16; Av. 17	79	1, 2, 0	40·5	0, 1, 0
Trichoderma ..	90	262, 236, 275 Av. 258	90	0, 1, 0	90	4, 7, 2
Trichothesium ..	77·5	54, 62, 72; Av. 63	25·2	4, 9, 5	13·7	0, 0, 1
Alternaria ..	85	124, 112, 109; Av. 115	35·5	22, 18, 17 Av. 19	9·5	0, 0, 0

Comparative figures for 5 fungi are given in Table XX. No attempt was made to count the number of zoospores or oospores in the case of *Phytophthora* and *Pythium*.

In the case of *Ascochyta* pycnidial formation is suppressed by both fungicides. Only one pycnidium was found in the dish exposed to the vapour of PC and this on crushing was found to contain no pycnospores.

Sclerotial formation of *Rhizoctonia* is completely inhibited by both PC and TC (Figs. 10–12).

In the above respects the fungicides PC and TC stand in strong contrast to Bordeaux mixture and Shirlan. Whenever the latter allow of growth, they equally allow of sporulation. Thus in the experiment of Table XV the presence of 0·1% Bordeaux mixture cut down the growth rate to approximately one-tenth of that of the control, but by and by all the plates become indistinguishable in the matter of sporing. The same applies to colonies, the growth of which was checked by Shirlan.

VII. DEVELOPMENT OF RESISTANT STRAINS

It was noticed from time to time during the course of this work that new hyphæ developed from the suppressed colonies after a certain interval (2–4 weeks). Such hyphæ grew vigorously in spite of the fungicide and filled the petri-dish within a few days. Isolations were

made from those new hyphæ and a comparison of their rate of growth in presence of the fungicides and of other characters with those of the original cultures showed them to be different. Development of such hyphæ has been seen in cultures of *Botrytis*, *Rhizoctonia* and *Alternaria* and these will be described in that order.

(*a*) *Botrytis*

Resistant hyphæ arose in plates exposed to the vapour of TC or when the latter was mixed with the medium. Isolates of such resistant hyphæ gave rise to a strain in which there was complete absence of spores except at the point of inoculation but in which sclerotia, formed in concentric rings, were the outstanding feature (Fig. 22). The rate of growth of such hyphæ in the presence or absence of the fungicide was different from that of the parent as is shown in Table XXI.

TABLE XXI

Effect of PC and TC on colony growth of Parent and Saltant of Botrytis

	Parent			Saltant		
	2 days	4 days	6 days	2 days	4 days	6 days
Control ..	26·1±0·88	58±0·57	88 (full)	26·8±0·16	48·8±0·60	68·3±0·44
0·25% PC	9±0·28	18±0·76	26·6±1·69	25·1±0·44	45·6±0·01	68·5±0·57
0·25% TC	0	0	0	27·4±0·30	51·7±0·37	71·8±1·09

It is seen from the above table that the saltant strain grows less rapidly than the parent. The difference in amount of growth between the two after 4 days is highly significant, the value of "*t*" being 11·08. In presence of the amounts of fungicide applied the saltant strain grew as well as in the control, in which respect it differs markedly from the parent. This difference is most clearly shown in presence of TC.

The effect of TC and to a less extent of PC on the growth of this saltant strain is to reduce or eliminate sclerotial formation (Figs. 16–18).

A comparison was made between the sclerotial strain which arose in the manner described and an ordinary sclerotial strain of *Botrytis cinerea* isolated from onion bulbs by Dr. Peiris of Imperial College. The effect of PC and TC on these 2 strains is as shown in Table XXII.

It is clear that the 2 strains are widely different in their response to the new fungicides, the one from onion being as much retarded in growth as the sporulating strain used in this work (Figs. 19–21). The resistance of the saltant strain is therefore not connected with the fact that it is of sclerotial nature.

TABLE XXII

Effect of PC and TC on colony growth of a saltant and an ordinary sclerotial strain of Botrytis

	Sclerotial strain from onion		Saltant strain	
	2 days	6 days	2 days	6 days
Control ..	35·6±0·72	69·3±4·56	28·3±0·33	80·3±0·66
0·05 g. PC ..	11·3±0·44	41·6±1·87	31±0·28	78·1±1·74
0·05 g. TC ..	0	6·6±0·06	30·5±0·28	77·8±2·31

The saltant strain was observed in culture for 5 months, during which time it was re-cultured about 5 times. It still retained its resistant character.

(*b*) *Rhizoctonia*

New hyphæ have arisen on several occasions in cultures of this fungus exposed to the action of PC (Fig. 11). Isolations have given rise to a saltant strain. The growth behaviour of this as against that of the parent is shown in Table XXIII.

TABLE XXIII

Effect of PC and TC on colony growth of parent and saltant of Rhizoctonia

No. of days	Parent			Saltant		
	Control	PC	TC	Control	PC	TC
2	20·8±0·25	0	13·3±0·25	29·8±0·25	21·5±1·5	22·6±0·4
4	43·8±0·25	10·6±0·4	25·1±0·4	62·3±0·75	44·3±0·25	37·3±0·75
6	67·5±0·1	12·1±0·1	36·5±0·5	90 (full)	65±0·5	59·8±0·25

The greater susceptibility of the parent strain to PC and TC is clear from the table, though the difference between parent and saltant is less pronounced than was shown by the corresponding *Botrytis* strain.

Culturally (on malt agar) the saltant strain of *Rhizoctonia* differs from the parent in having a higher growth rate (*cf.* Table XXIII, Control columns; also from separate determination, $69 \cdot 1 \pm 0 \cdot 3$ against $38 \cdot 6 \pm 0 \cdot 33$ after 88 hours on 2% malt agar), in forming more numerous and larger sclerotia (Figs. 23 and 24) and in colouring of medium, *viz.*, tawny-olive (Ridgway's colour chart, Plate XXIX, 17″–O–Y) as

against clay colour in 1-month old culture of saltant and parent respectively. There was no significant difference between hyphal diameter of the 2 strains (7·3 μ as average of 50 counts for parent compared with 7·5 μ for saltant).

(*c*) *Alternaria*

The resistant hyphæ of this fungus gave cultures which were slower growing than the parent, as the following figures, derived from quadruplicate cultures on malt agar after 5 days, show:—

$$\text{Parent } 32{\cdot}1 \pm 1{\cdot}31$$

$$\text{Saltant } 26{\cdot}2 \pm 1{\cdot}24$$

$$t = 3{\cdot}27 \quad P \ll 0{\cdot}02$$

Experiments were set up to find out the comparative resistance to PC and TC. The insensitiveness of the saltant to PC and TC was quite obvious from the experiments done.

The saltant strain is less actively sporulating than the parent (the average number of spores per count by hæmocytometer method being 115 for the parent and 40 for the saltant strain) and shows dark ivy green (Ridgway, Plate 47) as compared with deep greyish olive (Ridgway, Plate 46) for the parent after 16 days on 2% malt agar.

VIII. Attack on Plants

(*a*) *Botrytis*

Attack of *Botrytis* on lettuce was studied in 3 ways. The first was to spray or dust the plants with the various fungicides and after Bordeaux mixture and Shirlan had dried, 2 cc. of spore suspension in turnip extract was sprayed over the leaves. The second method was to make a wound at the base of young leaves and to place on this a piece of inoculum cut by a cork-borer from a young growing and sporing colony of *Botrytis*. Afterwards 2 drops of 1% Bordeaux mixture or Shirlan or 2 drops of a suspension in water of PC or TC crystals were placed by the side of each inoculum. In the third method the plants were sprayed or dusted with fungicides as in Method 1, but the inoculum, taken from a growing culture, was placed on the intact surface of a leaf at the base of the lamina.

The lettuce plants used (ranging in height from 2″ to 6″ in the various experiments) were dug up and placed in moist glass dishes with their roots dipping in water.

The results of these experiments are collected in Table XXIV.

Notes on Table XXIV.—Under Method 1 "Severely", "Moderately" and "Slightly" attacked mean respectively—over $\frac{3}{4}$, $\frac{1}{4}$–$\frac{3}{4}$, and less than $\frac{1}{4}$ of the total leaves attacked.

Under Method 3, the same phrases relate respectively to lesion covering about half the leaf, lesion a cm. wide and small lesion not exceeding 0·4 cm.

TABLE XXIV

Effect of PC, TC, Bordeaux Mixture and Shirlan on Botrytis (*sprayed on lettuce seedling*)

	Fungicide	Total plants	% killed	%Attacked			% healthy	Sporulation on plants
				Severly	Moderately	Slightly		
Method 1								
T.E. only sprayed ..	None	10	0	0	0	0	100	..
Spores in T.E. sprayed	None	20	100	0	0	0	0	Present
do ..	PC	16	0	0	75	12·5	12·5	Absent
do ..	TC	16	0	0	19	44	37	do
do ..	1% Shirlan	27	26	29	41	4	0	Present
do ..	1% Bord. Mix.	20	25	40	35	0	0	do
Method 2								
Agar only ..	None	20	0	*	*	*	100	..
Agar+Mycelium ..	,,	20	100				0	Present
do ..	PC	20	20				80	Absent
do ..	TC	33	12				88	do
do ..	1% Shirlan	20	30				70	Present
do ..	1% Bord. Mix.	20	40				60	do
Method 3		Tota leaves						
Malt agar only ..	None	15	0	0	0	0	100	..
Malt Agar+Mycelium	,,	15	0	100	0	0	0	Present
do ..	PC (Dusted)	15	0	0	0	47	53	Absent
do ..	PC (Vapour)	15	0	0	100	0	0	do
do ..	TC (Dusted)	15	0	0	0	0	100	do
do ..	TC (Vapour)	15	0	0	0	40	60	Absent
do ..	1% Shirlan	15	0	53	0	0	47	Present
do ..	1% Bord. Mix.	15	0	73	0	0	57	do

* Inoculation by this method leads either to no attack or to progress through the stem so that all parts above die.

Table XXIV clearly shows that PC and TC are decidedly better than Bordeaux mixture and Shirlan for checking infection by *Botrytis*. Furthermore plants and parts of plants which are attacked in presence of Bordeaux mixture or Shirlan develop spores of *Botrytis*. They do not do this in presence of PC or TC.

In control experiments (with 8–10 plants in each case) in which the fungicides only were applied, slight spotting of the youngest leaves was caused by Bordeaux mixture, discolouration of the leaves resulted where a deposit of the Shirlan had laid; no effect was seen resulting from the action of PC or TC.

(*b*) *Rhizoctonia*

The effect of PC and TC on the attack of seakale leaves was tested both by dusting the leaves beforehand or by enclosing them in presence

of the apour. Malt agar inocula of the fungus were placed on the intact epidermis.

From the results obtained it is clear that both PC and TC distinctly repress *Rhizoctonia* attack, especially when dusted over the leaf surface prior to inoculation. There is, as in previous comparisons, an indication that PC is the more effective against this fungus.

IX. Summary

1. The two substance pentachloronitrobenzene (PC) and tetrachloronitrobenzene (TC) are soluble in acetone, benzene, and to a less degree in alcohol, and very slightly soluble in water. The degree of solubility was not accurately determined but was found to be not more than 0·0001% for both substances.

2. Both PC and TC are volatile, the latter being approximately 4–5 times more so than the former.

3. PC and TC, in terms of solid material used, are less effective than Bordeaux mixture and Shirlan in repressing germination of spores of *Botrytis cinerea* in water though the former have a marked retarding effect on average germ tube length. With spores of the same fungus in nutrient (lettuce and malt extracts) PC and TC had much the same or even greater relative effect in water, whereas Bordeaux mixture and Shirlan (especially after desiccation) show considerably reduced effectiveness.

4. The activity of PC and TC was intensified in presence of acid (malic), the latter having the opposite effect upon Bordeaux mixture.

5. Similar results were obtained with spores of *Fusarium cæruleum*, *Ascochyta rabiei*, *Trichothecium roseum*, *Trichoderma viride*, *Rhizopus nigricans* and *Alternaria* sp. The majority of these fungi, including *Botrytis*, are more sensitive to TC than to PC, but the converse is true for *Trichoderma*. Spores of *Fusarium* and *Trichoderma* are distinctly less sensitive to both chemicals than those of the others.

6. Germination of spores of *Botrytis*, *Fusarium* and *Trichoderma* was studied in presence of the vapours of PC, TC and (possibly) of Shirlan. The same effect was found with the new fungicides as when they were mixed in the spore drop. No such effect was given by Shirlan. At 8° C. the effect of PC and TC was much greater than at 20° C.

7. The effect of the 4 fungicides on linear colony growth was examined for *Botrytis*, the fungicides being included in the nutrient medium. Both PC and TC, and especially the latter, much repressed colony growth, even at such very low concentrations as were quite ineffective with Bordeaux mixture and Shirlan.

8. The linear growth of all the fungi tested under 5 together with *Phytophthora parasitica*, *Pythium de Baryanum* and *Rhizoctonia solani* were examined in relation to the retarding effect of PC and TC when added to the medium. All these fungi excepting *Phytophthora* were

greatly retarded by either PC or TC. The latter was the more effective, except for—*Trichoderma* and *Rhizoctonia* which were more actively repressed by PC.

9. Colony growth of *Botrytis* is about equally retarded in presence merely of the vapour of PC and TC as it is when the fungicide is incorporated in the medium. The magnitude of the effect is practically the same whether the containers are sealed or unsealed; it is more pronounced at lower temperature; and it is as pronouncedly shown when the active principle is diluted as in the commercial dusts.

10. The response of 6 other fungi to the vapours of PC and TC was essentially similar to that shown when these fungicides were included in the medium.

11. Exposure of developing *Botrytis* cultures to the action of PC and TC (either incorporated in the medium or as vapour only) has a distinct effect upon the type of growth produced, *viz.*, cultures so treated, in addition to retardation of growth, show lobed margin, are lacking in aerial mycelium and are devoid of spores and sclerotia. Similar effects were observed with *Fusarium*, *Ascochyta*, *Trichothecium Trichoderma*, *Alternaria* and *Rhizoctonia*. This reduction of sporulation can occur under conditions where the effect on growth is not at all marked.

12. In some of the fungi, *viz.*, *Botrytis*, *Rhizoctonia* and *Alternaria*, saltant hyphæ from time to time arose when the cultures were subjected to the action of PC or TC. Such saltants proved resistant to the fungicide. The saltant from *Botrytis* was of sclerotial character and it was shown to differ markedly from a sclerotial isolate occurring naturally.

13. PC and TC were superior to Bordeaux mixture and Shirlan for checking the infection of lettuce by *Botrytis*.

14. Inoculation experiments on seakale leaves in presence of PC and TC showed that *Rhizoctonia* was markedly checked by both of them.

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XII. Explantation of Plates

Plate XII.

FIGS. 1–3. *Growth of Botrytis after* 24 *days on lettuce extract agar.*

Fig. 1. Control, showing aerial mycelium.

Fog. 2. PC (mixed in the medium); growth lobed and aerial mycelium absent.

Fig. 3. TC (mixed in the medium); features as in Fig. 2.

FIGS. 4–6. *Growth of Trichothecium after* 29 *days on malt agar.*

Fig. 4. Control.

Fig. 5. PC (mixed in the medium); shows reduced growth.

Fig. 6. TC (mixed in the medium); shows reduced growth.

FIGS. 7–9. *Growth of Trichoderma after* 12 *days on malt agar.*

Fig. 7. Control shows spores.

Fig. 8. PC (mixed in the medium); shows zones and absence of spores.

Fig. 9. TC (mixed in the medium); shows absence of spores.

FIGS. 10–12. *Growth of Rhizoctonia after* 25 *days on malt agar.*

Fig. 10. Control shows sclerotia.

Fig. 11. PC (mixed in the medium); growth restricted, sclerotia absent; saltant showing (top left-hand).

Fig. 12. TC (mixed in medium); sclerotia absent.

PLATE XIII

FIGS. 13–15. *Growth of Botrytis after* 28 *days on Glucose-peptone agar.*

Fig. 13. Control; aerial mycelium, spores and sclerotia present.

Fig. 14. PC (vapour); spores and sclerotia absent.

Fig. 15. TC (vapour); similar to PC. Saltant hyphæ showing on lower edge.

FIGS. 16–18. *Growth of saltant of Botrytis after* 25 *days on glucose-peptone agar.*

Fig. 16. Control; with sclerotia in concentric zones.

Fig. 17. PC (vapour); sclerotia much fewer.

Fig. 18. TC (vapour); complete suppression of sclerotia.

FIGS. 19–21. *Growth of Botrytis (sclerotial strain from onion) after* 25 *days on glucose-peptone agar.*

Fig. 19. Control; Sclerotia present.

Fig. 20. PC (vapour); growth lobed, sclerotia suppressed.

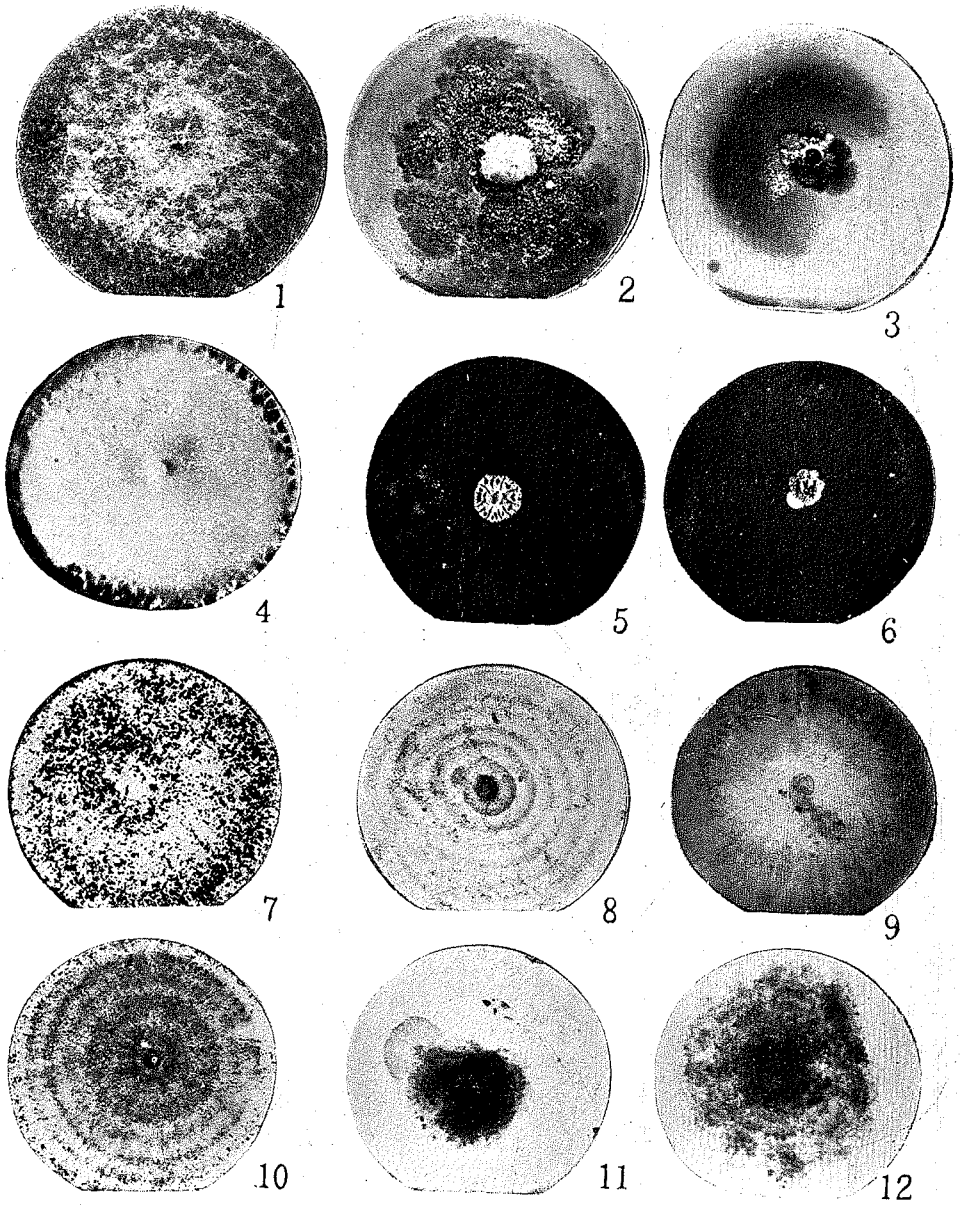
Fig. 21. TC (vapour); growth much restricted.

FIG. 22. *Growth of saltant of Botrytis after* 35 *days on glucose-peptone agar,* showing sclerotia in three concentric zones.

FIGS. 23–24. *Growth of saltant and parent of Rhizoctonia after* 32 *days on malt agar.*

Fig. 23. Shows saltant with numerous and larger sclerotia.

Fig. 24. Shows parent with minute sclerotia.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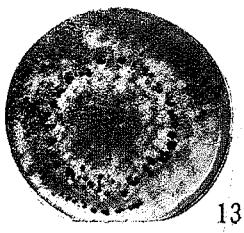
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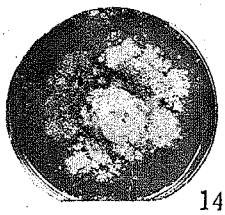
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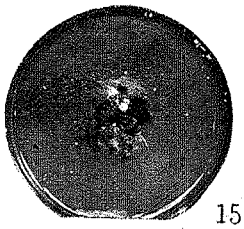
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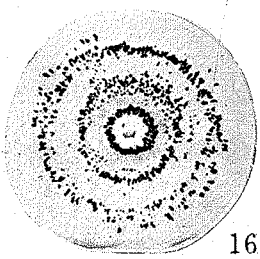
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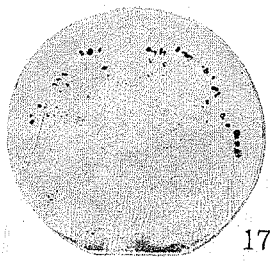
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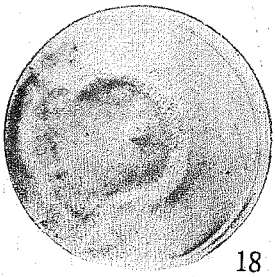
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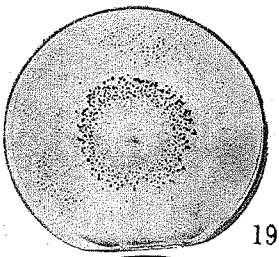
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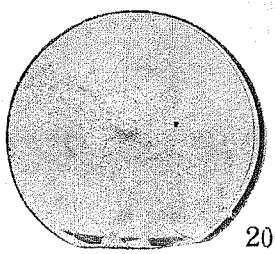
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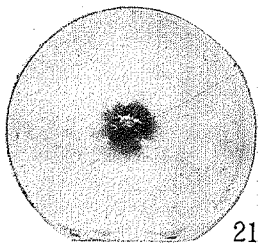
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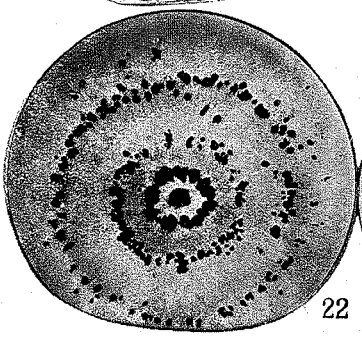
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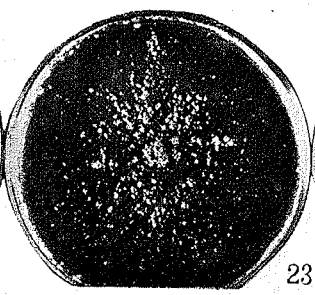
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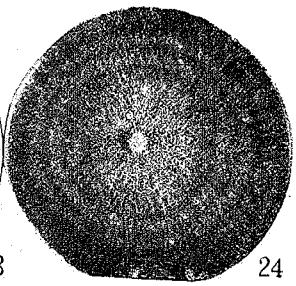
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