INVESTIGATIONS ON PHYTOTOXICITY OF METABOLIC BY-PRODUCTS IN THE CULTURE FILTRATES OF *BOTRYTIS* SPP.

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Non-dialysable and partially thermolabile substances, which cause wilt and necrosis of leaves on cut shoots of bean (*Vicia faba* L.), were produced by both *Botrytis fabae* and *B. cinerea* in liquid shake culture. The filtrates caused browning of the primary root, softening of root tip and stem segments of bean. Culture filtrates of *B. fabae* were always more active than those of *B. cinerea* on a dry weight of mycelium basis. Phytotoxicity of filtrates was affected by the age of mycelia, type of nitrogen source and also by the concentration of glucose and peptone supplied to the fungus. The substances causing wilting and necrosis were most effective against cut shoots at *p*H 4·4. Wilting of cut shoots was accompanied by vascular plugging and browning of the walls of the xylem vessels.

Introduction

The literature on toxins in culture filtrates of pathogenic fungi was reviewed by Ludwig (1960), Wheeler and Luke (1963) and Deverall (1964). There is evidence that some toxins produced *in vitro* are the cause of symptom development in some plant diseases. Meehan and Murphy (1946, 1947) found that the toxin produced by *Helminthosporium victoriae* in culture caused yellowing, chlorosis and necrosis on the shoot system of susceptible oat varieties. A correlation between pathogenicity of isolates of *Periconia circinata* and the production of toxic culture filtrates was reported by Scheffer and Pringle (1961). Evidence was also presented by Russian workers that *Botrytis cinerea* and other pathogenic fungi could produce toxin in different culture media. But different workers claimed different toxic substances. Aksenova (1963) showed that the necrotic spots on the leaves of cabbage could be induced by a polysaccharide fraction isolated from the cultures of *B. cinerea.* In the same year (1963) Ladygina reported that some organic acids synthesized by *B. cinerea in vitro* could produce symptoms on cabbage tissue similar to those caused by the said fungus. However, different pathogenicities of *B. fabae* and *B. cinerea* on bean (*Vicia faba*) might be based on the production by *B. fabae* of a phytotoxic metabolite. With this end in view, the culture

filtrates of two fungi were compared for their effects on stem segments, cut shoots and seedlings of bean. This led to a series of tests on culture filtrates. The effects of cultural factors on phytotoxicity of filtrates and some of the properties of the phytotoxic substances were also studied.

Materials and Methods

The sources and authenticity of cultures of *B. fabae* and *B. cinerea* used in the present investigation were already mentioned in a paper by Purkayastha and Deverall (1965). For culture filtrate experiments, both fungi were grown in different liquid media and their *p*H was adjusted before autoclaving by adding $N/10$ HCl or $N/10$ NaOH. Sterilized medium was taken in conical flasks (100 ml/500 ml flask) and inoculated with 2 per cent water agar blocks (3 mm diameter) containing 4-day old mycelia of either *B. fabae* or *B. cinerea*. The culture flasks were then incubated at 21 ± 1 °C under fluorescent light on a shaking machine for 160 hr at the speed of 200 r.p.m. with an excentric throw of 6 cm. Mycelia were separated by filtering the culture medium through muslin and transferring them to weighed aluminium foil cups. They were dried at 70 °C for 72 hr and after being cooled in a desiccator their dry weights were noted. Filtrates, on the other hand, were centrifuged for 10 minutes at 1,700 g at room temperature (20 °C). The supernatants were combined and tested on bean tissues before and after the adjustment of *p*H.

To determine the phytotoxicity of culture filtrates, three different types of experiment were designed. Firstly, 1 cm segments from the first internodes were kept in McCartney bottles containing culture filtrates and incubated for 68 hr at 18–20 °C in light. The colour and firmness of the segments were recorded following treatment. Secondly, stem cuttings of the same age and length were taken and their cut ends were introduced into specimen tubes ($2'' \times 1''$) containing culture filtrates and incubated for 48 hr, except where otherwise stated, under similar conditions of temperature and light as described. The symptoms (i.e. wilting and necrosis) were noted at an interval of 24 or 48 hr. The average individual symptom per plant was estimated in the following way to assess the severity of damage:

The average development of a particular symptom/plant (i.e. wilting or

$$\text{necrotic index/plant)} = \frac{\text{Total number of leaves showing the symptom}}{\text{Number of test plants}}.$$

Thirdly, roots of seedlings (7- or 8-day old) which were grown under sterile conditions in the laboratory were washed with sterile water. Subsequently, their initial length, firmness and colour were noted and they were immersed in culture filtrates in small culture tubes wrapped with aluminium foil to exclude light. Seedlings were incubated as described for cut shoots and after a given period of incubation final length, colour and firmness of roots were recorded.

EXPERIMENTAL RESULTS

Effect of culture filtrates of Botrytis *spp. on bean tissues*

The fungi were grown in 10 per cent buffered bean leaf extract (10 g fresh weight of leaves/100 ml 0·01 *M* potassium phosphate buffer solution at *p*H 6) for seven days at 20 °C under light on a shaking machine. Both unheated and autoclaved (15 lb./sq. in. pressure for 15 minutes) filtrates were tested on stem segments (1st internode only) and cut shoots of 3-week-old plants. The phytotoxicity of the culture filtrates obtained on 'X' medium†† was also tested on 8-day-old seedlings of bean. Original medium (without fungus) and sterile distilled water used as controls. The results are given in Tables I and II.

TABLE I

Effect of culture filtrates (bean leaf extract medium) of Botrytis *spp. on bean tissues*

Culture filtrates of test organisms	* Final *p*H	Stem segments: Reactions after		Cut shoots: † Wilting index/plant		Cut shoots: † Necrotic index/plant	
		24 hr	68 hr	24 hr	48 hr	24 hr	48 hr
B. fabae							
Unheated	4·5	Firm	Soft	1·0	2·25	1·5	2·5
Autoclaved	4·4	Firm	Firm	1·0	1·5	0·75	1·25
B. cinerea							
Unheated	7·1	Firm	Collapsed	1·0	1·0	0	0·25
Autoclaved	6·7	Firm	Firm	0·25	0·25	0	0·25
Distilled water (control)	—	Firm	Firm	0	0	0	0

* Initial *p*H 6.
† Four replicate plants/treatment.

Culture filtrates of both fungi caused a slow softening and collapse of stem segments. *B. cinerea* was more active than *B. fabae* in this respect. In autoclaved filtrates no softening was observed within 68 hr. This result was similar to those obtained by Deverall (1960) who compared the effect of culture filtrates of the said fungi grown on pectin and cellulosic media. It was also noted that the filtrate of *B. fabae* was more active than those of *B. cinerea* in causing wilting and necrosis in the leaves (Table I) and sometimes shrivelling and drying of leaves and blackening of apical buds and stipules. Autoclaving, however, removed much of the activity.

†† 1 per cent dextrose, 0·2 per cent peptone, 0·15 per cent KH_2PO_4, 0·05 per cent $MgSO_4.7H_2O$, 0·6 per cent $NaNO_3$, 0·05 per cent KCl, 0·3 per cent caseinhydrolysate (acid) and 0·05 per cent yeast nucleic acid.

TABLE II

Effect of culture filtrates ('X' medium) of B. fabae *and* B. cinerea *on the roots of bean*

Treatment		Initial *p*H	Adjusted *p*H	*Average rate of growth of root/ day (mm)	† Colour of root after treatment	Nature of root tip
Culture filtrate (*B. fabae*)	..	3·9	4·4	0	Brown	Soft
Culture filtrate (*B. cinerea*)	..	4·3	4·4	0	Brown	Soft
Medium without fungus (control)	..	5·0	4·4	0·6	White	Firm
Distilled water (control) ..	..	—	—	11·87	White	Firm

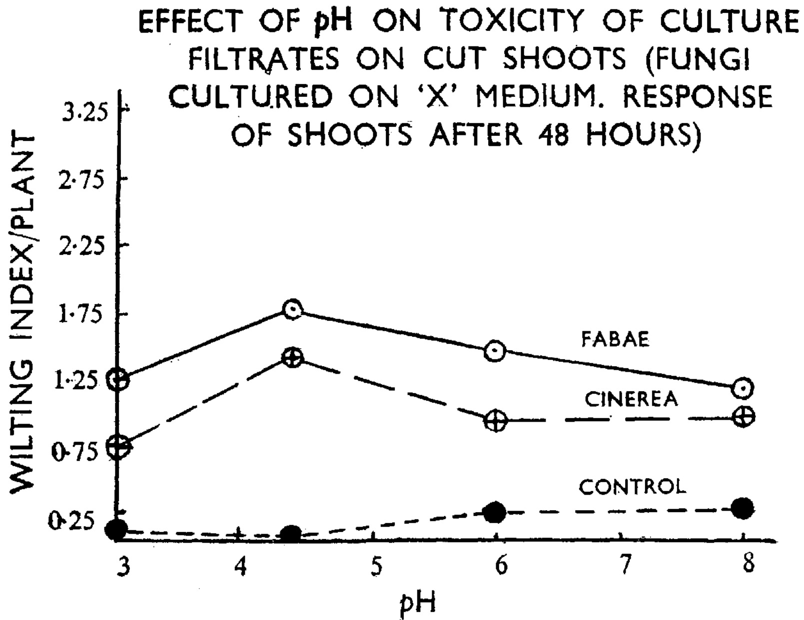
* Four replicates/treatment (treated for 48 hr).
† Original colour was white.

Although the filtrate of *B. fabae* was more phytotoxic than *B. cinerea*, an unsatisfactory aspect of this experiment was the alkaline *p*H drift caused by *B. cinerea* in buffered bean leaf extract medium. It was therefore decided to find out a medium in which both fungi created comparable *p*H conditions in culture and to test the effect of culture filtrates of same *p*H (adjusted) on the seedlings of bean and subsequently on the cut shoots. The 'X' medium was finally selected. The filtrates obtained on the 'X' medium caused three types of symptoms on the seedlings (Table II). Inhibition of root growth, browning of primary root and softening of root tips were the main symptoms. Root growth was also inhibited to the same extent by the original medium so that the only effects of fungal metabolites were discolouration and softening. Both filtrates were equally active in causing these two symptoms. The substance causing softening of root tips was greatly reduced in its activity by autoclaving.

Effect of pH *on activity of culture filtrate toxin*

In order to test the effect of *p*H on the action of culture filtrates of *Botrytis* spp. on cut shoots of bean, two preliminary experiments were conducted. Firstly the *p*H of culture filtrates were adjusted to 3, 4, 5, 6 and 8 and then tested on cut shoots. It was found that the filtrates were most active between *p*H 4 and 5. Therefore, in the second experiment, the *p*H of filtrates were adjusted to 4·2, 4·4, 4·6 and 4·8 before bioassay tests. In this case, the *p*H 4·4 appeared to be the most suitable *p*H for activity of the filtrates of both the species. Hence, in the final experiment only four selected *p*H values including the aforesaid *p*H were taken into consideration. The *p*H values of batches of culture filtrates after seven days' growth of the two fungi on 'X' medium were adjusted to 3, 4.4, 6 and 8 by adding appropriate volumes of *N*/10 HCl

or $N/10$ NaOH. The original culture medium was used as a control. Fig. 1 shows the reactions of cut shoots to the filtrates at different pH values.



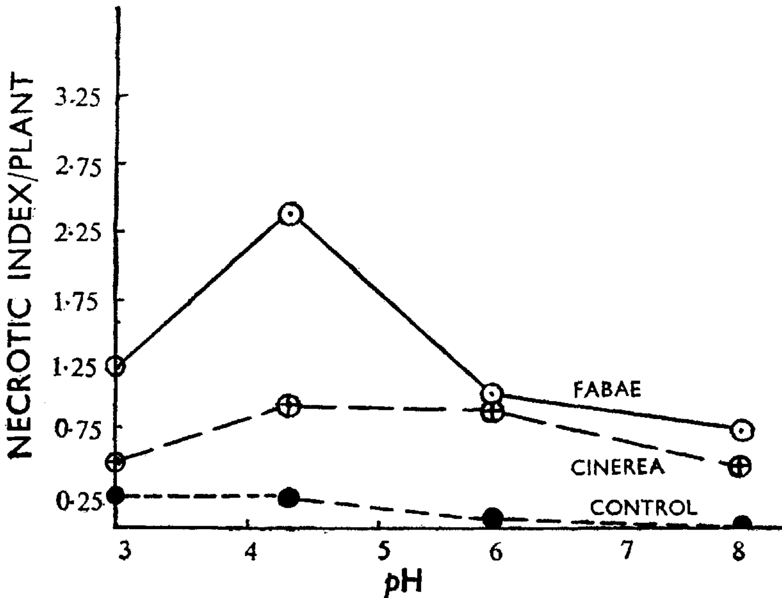


FIG. 1

At *p*H 4·4, the filtrates of *B. fabae* caused more wilting and necrosis than did those of *B. cinerea*, control medium causing slight necrosis. The mycelial dry weight of *B. fabae* was 140 mg and that of *B. cinerea* 395 mg at the time of collection of filtrates. Therefore the toxicity of the filtrate of *B. fabae* greatly exceeded that of *B. cinerea* on the mycelial dry weight basis.

Effect of nutrition and age of mycelia on phytotoxicity of filtrates

To study the effects of carbon and nitrogen nutrition and the age of mycelium on phytotoxicity of filtrates, four series of experiments were performed on both fungi. The basal medium throughout provided 1·5 per cent glucose, 0·2 per cent caseinhydrolysate or peptone, salts and trace elements (0·1 per cent KH_2PO_4, 0·05 per cent $MgSO_4.7H_2O$ (as p.p.m.), Fe 0·2, Zn 0·2, Mn 0·02, Cu 0·04). In the first experiment, the effects of glucose concentrations (range 0·1 to 2·5 per cent) were tested. In the second experiment, different nitrogen sources in the place of caseinhydrolysate were compared, and in the third experiment a range of concentrations of the best nitrogen source were compared. Using best proportions of glucose and nitrogen source, phytotoxicities of filtrates at different stages of mycelial growth were measured.

The growth of each fungus was correlated with the toxicity of their respective culture filtrate. Filtrates of *B. fabae* on 1·5 per cent glucose were more toxic than those of *B. cinerea* on the same amount of glucose, although growth of *B. fabae* was one-third that of *B. cinerea* (Table III). The absence of toxins in culture filtrates of *B. fabae* at other glucose concentrations was also correlated with its very slight growth.

Results (Table IV) showed that peptone was the only nitrogen source on which *B. fabae* grew well and was the best source for growth of *B. cinerea*. Once again phytotoxicity tests revealed that the filtrates from cultures of *B. fabae*, where good growth occurred, were more active than those of *B. cinerea*, despite the fact that *B. cinerea* produced twice as much mycelium as *B. fabae* on peptone.

The culture filtrates of *B. fabae* were slightly more toxic at 0·5 per cent level of peptone. However, greatest toxicity of culture filtrates was produced by both fungi on 1 per cent peptone (Table V). The most important new observation in this experiment was the marked toxicity of the original medium containing 0·5 per cent and 1 per cent peptone. Therefore it was decided not to use peptone concentration exceeding 0·2 per cent in further experiments.

It was also observed that phytotoxicity of filtrate was affected by the age of mycelia (Table VI). The growth-rate of *B. fabae* declined after eight days and the *p*H of the media remained constant. The toxicity of filtrates showed little change after eight days of growth.

TABLE III

Effect of glucose concentrations on the growth of Botrytis *spp. and the phytotoxicity of filtrates*

Fungus	Conc. of glucose %	Initial pH*	Final pH	Dry wt. of mycelia (mg)	Wilting index/ plant †	Necrotic index/ plant †
B. cinerea	0·1	6·0	5·2	63·5	0·33	0
	0·5	5·9	4·7	124·6	0·66	0·33
	1·5	5·7	4·5	201·6	0·66	0
	2·5	5·6	4·3	226·8	1·0	0·66
B. fabae	0·1	6·0	5·9	5·0	0	0
	0·5	5·9	5·4	18·3	0	0
	1·5	5·7	4·4	68·5	1·0	0·66
	2·5	5·6	5·3	18·0	0	0
Medium without fungus (control)	0·1	6·0	6·0	—	0	0
	0·5	5·9	5·9	—	0	0
	1·5	5·7	5·7	—	0	0
	2·5	5·6	5·6	—	0	0

* *p*H before autoclaving being 5·9 in all cases; *p*H of the culture filtrates was adjusted to 4·4 before tests on cut shoots.

† Three replicates/treatment (treated for 48 hr).

TABLE IV

Effect of different sources of nitrogen on toxicity of culture filtrates of Botrytis *spp. on cut shoots (symptoms noted after 48-hr treatment)*

Culture filtrates	Sources of nitrogen	Wilting index/ plant	Necrotic index/ plant
B. cinerea	Sodium nitrate	0·5	1·0
	Asparagine	0·25	0·25
	Peptone (mycological)*	1·0	2·0
	Caseinhydrolysate	1·75	1·5
B. fabae	Sodium nitrate	0·25	0·25
	Asparagine	0	0
	Peptone (mycological)*	2·5	3·0
	Caseinhydrolysate	0·25	0·5
Medium without fungus (control)	Sodium nitrate	0	0
	Asparagine	0	0·25
	Peptone (mycological)	0	0·25
	Caseinhydrolysate	0	0·25

* Maximum mycelial growth in peptone, *B. fabae*—171·6 mg, *B. cinerea*—366·55 mg.

TABLE V

Effect of different concentrations of peptone on the growth of B. fabae *and* B. cinerea *and the phytotoxicity of filtrates (symptoms noted after 48 hr)*

Fungus	Concentration %	*pH of culture filtrate	Dry wt. of mycelia (mg)	†Wilting index/plant	†Necrotic index/plant
B. cinerea	0·1	4·6	301·35	0·5	1·25
	0·2	4·0	331·5	2·0	4·0
	0·5	4·0	630·1	2·75	3·5
	1·0	4·5	367·0	3·5	4·0
B. fabae	0·1	4·7	45·1	1·25	1·75
	0·2	4·3	80·95	1·5	1·25
	0·5	4·0	308·0	3·25	3·0
	1·0	4·5	283·5	3·0	4·0
Medium without fungus (control	0·1	5·8	—	0	0·75
	0·2	5·8	—	1·0	1·0
	0·5	5·7	—	1·75	2·0
	1·0	5·7	—	2·0	2·75

* *p*H of culture filtrates and control medium was adjusted to 4·4 before test on cut shoots.

† Average of four replicates/treatment.

TABLE VI

Effect of age of culture on the growth of B. fabae *and toxicity of culture filtrates on cut shoots*

Age of culture (days)	*Final pH of culture filtrate	Dry wt. of mycelia (mg)	†Wilting index/plant	†Necrotic index/plant
4	4·9	24·86	0	2·0
8	3·9	128·33	2·0	2·25
12	4·0	131·43	1·25	2·25
15	3·9	140·00	1·75	2·5
Medium without fungus (control)				
4	5·2	—	0	2·0
15	5·2	—	0	2·0

* Initial *p*H 5·2; adjusted *p*H 4·4 before test on cut shoot.

† Four replicates/treatment (treated for 48 hr).

Dialysability of phytotoxic substances

A number of precautionary measures were taken to avoid dilution and temperature inactivation and to reveal the effect of salts during the process of dialysis. Dialysis was continued for 24 hr at 5 °C but the bathing fluids around the dialysis tubes were changed five times in this period. The reason for dialysis against the medium was to maintain as far as possible the same concentration of the ingredients present in the culture filtrate except for the fungal metabolite. Dialysis against salts was done because Tribe (1951) and Kamal (1954) showed that salts might act to maintain the activity of some enzymes in culture filtrate. After 24 hr dialysis, the *p*H values of dialysates were adjusted to 4·4 with $N/10$ HCl. Undialysed culture filtrates, the salt solution (0·152 per cent KH_2PO_4, 0·052 per cent $MgSO_4.7H_2O$, 0·6 per cent $NaNO_3$, 0·052 per cent KCl, 0·08 per cent NaCl) and medium 'X' used as controls were treated in the same way. Table VII shows the effects of dialysates and controls on cut shoots.

TABLE VII

Effect of dialysed culture filtrates ('X' medium) of B. cinerea *on cut shoots of bean (symptoms noted after 48 hr)*

Treatment	*Wilting index/plant	†Necrotic index/plant	Colour of cut end of shoot
Undialysed culture filtrate	1·0	1·75	Black
Salt mixture (control) ..	0	2·0	Green
'X' medium (control) ..	0	0·5	Green
Culture filtrate dialysed against water ..	1·25	1·75	Black
Dialysed against salt mixture	0·5	3·5	Black
Dialysed against 'X' medium	0·75	2·75	Black

* *p*H adjusted to 4·4 in all cases.
† Four replicates/treatment.

The toxic substance causing wilting is non-dialysable under the test conditions. Important observations were made on the role of salts as a cause of necrosis. The control salt solution caused marked necrosis. Filtrates dialysed against salt solution caused greater necrosis than either undialysed filtrate or the control salt solution. Therefore, there may be at least two

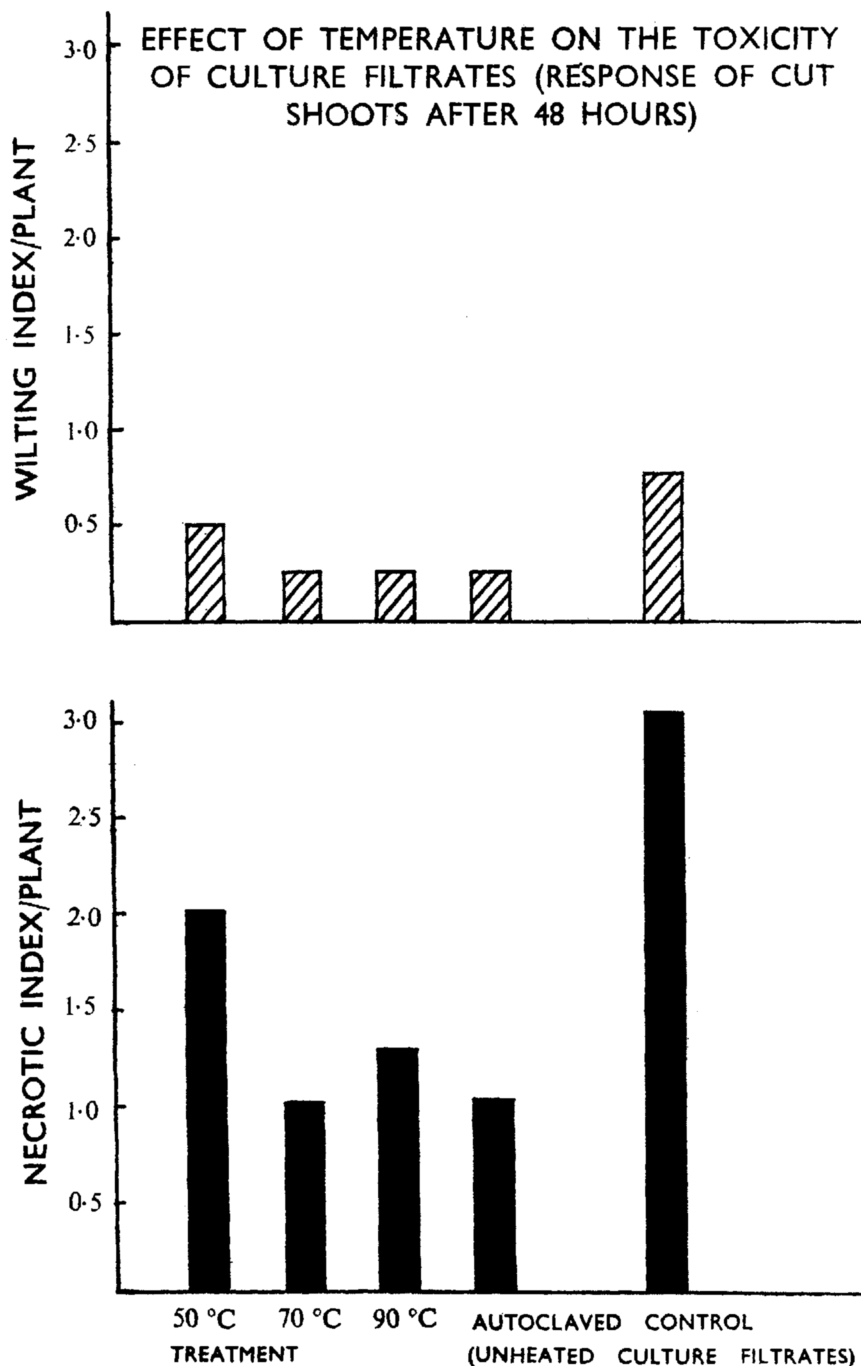


FIG. 2

causes of the symptoms shown by cut shoots in the culture filtrates. The cause of wilting, perhaps followed by some desiccation and necrosis, is a non-dialysable metabolite of the fungus. Components of the medium, probably salts, also cause symptoms of necrosis. This may be the explanation of the toxicity of control media containing high levels of peptone in earlier experiments.

Thermostability of the phytotoxic substances

Aliquots of culture filtrates of *B. cinerea* were heated separately at 50 °C 70 °C, 90 °C, for 20 minutes in each case and also autoclaved for 20 minutes at 15 lb./sq. in. pressure. The *p*H values of treated and untreated culture filtrates were checked prior to their bioassay against cut shoots and found to be 4·3.

The toxicity of the culture filtrate was reduced partially by heat treatment (Fig. 2). The unheated filtrates kept at room temperature (20 °C) showed maximum activity. However, there was a loss of activity from 20 to 70 °C. Higher temperature and autoclaving failed to affect the residual toxic action of the filtrates, which included some ability to induce wilting. The conclusion must be that more than one substance is active in culture filtrate in causing wilting: thermolabile and thermostable substances. Wilting of cut shoots is usually accompanied by vascular plugging and browning of the walls of xylem vessels.

Discussion

The results of present study reveal that both fungi produce toxic culture filtrates although *B. fabae* is more active on the basis of mycelial dry weight. It seems unlikely that toxin production is the characteristic which differentiates the fungi into successful and unsuccessful pathogens of bean. However, the substances produced by the fungi may be important in causing damage to the host. At least two activities of the culture filtrates in causing damage to test tissues of bean were distinguished. First, filtrates of *B. cinerea* were more active than those of *B. fabae* in causing disintegration of pieces of stem tissues. This is in agreement with the results of Deverall (1960) who used culture filtrates of two fungi after growth on pectin and cellulosic media and which were known to contain pectic and cellulolytic enzyme activities. The disintegration of tissues may be caused by these enzymes or by the separate macerating factor found by Byrde and Fielding (1962) in culture filtrate of *Sclerotinia fructigena*. Secondly, the filtrates of *B. fabae* were more active than those of *B. cinerea* in causing wilt and necrosis of leaves on cut shoots of bean.

The anatomical and morphological changes induced in cut shoots of bean by the culture filtrates of *Botrytis* spp. were similar to those described by Scheffer and Walker (1953), Winstead and Walker (1954) and Kamal and Wood

(1956) using other host-parasite combinations. Moreover, in agreement with these workers, the active component of the culture filtrates was found to be non-dialysable and thermolabile to a large extent. The nature of substances responsible for the symptoms may be enzymatic; pectinmethylesterase was implicated by Gothoskar, Scheffer, Walker and Stahmann (1955), protopectinase and polygalacturonase by Kamal and Wood (1956), and cellulase by Husain and Dimond (1960). The most desirable procedure would be to fractionate the culture filtrates by techniques for protein separation, and to discover to which enzyme or enzymes the activity can be ascribed. Towards this end, experiments were performed to detect the cultural conditions in which the highest yield of the substances causing wilt would be given.

Although there are numerous reports of the production by *Botrytis* spp. of cell wall degrading enzymes (Brown 1915; Deverall 1960; Hancock *et al.* 1964*a*, *b*), other discussions of toxic products of *Botrytis* spp. are by Smith (1902), Gentile (1951) and Russian workers. Non-dialysability of the toxic substance in the present work eliminates the involvement of oxalic acid as suggested by Smith (1902), organic acid by Ladygina (1963) and thiourea by Ovcarov (1937). The unidentified toxic component of culture filtrates of *B. cinerea* studied by Gentile (1951) was not urea or thiourea, but was thermostable at 100 °C although it is not possible to compare the degree of toxicity before and after heat treatment in his paper. Aksenova (1963) found that proteins precipitated from culture filtrate of *B. cinerea* by ammonium sulphate were not toxic to cabbage leaves, but that a precipitate obtained by treatment with ethanol was toxic after resuspension in an unspecified volume of water. Thus the toxin may be polysaccharide in nature. However, the greatest difficulty in evaluating the Russian worker is in discovering the concentrations at which substances extracted from filtrates were tested. Most substances present in culture filtrates are likely to be toxic to plants if applied in a sufficiently concentrated form. Another point about the toxin described by Aksenova (1963) and Ladygina (1963) is that they are obtained from culture filtrates after about three months' growth of the fungus. Products of lysed senescent mycelium may be different from products of germ tubes or young mycelium. In tests on toxicity of culture filtrates, it was found that control media cause some wilt and necrosis on cut shoots and inhibited the growth of roots of bean seedlings. Salts in the media were shown to be capable of inducing necrosis and Audus and Quastel (1947) showed that certain amino acids and amines were toxic to the roots of cress seedlings. Therefore there is further indication of need for caution, in evaluating the role of fungal metabolites as toxins which may be important *in vivo*. The reasons for differential phytotoxicity of *Botrytis* culture filtrates and proper identification of the toxic substances which cause wilt and necrosis of leaves on cut shoots remain yet to be ascertained.

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