Some Biological Properties of Trichothecin, an Antifungal Substance from *Trichothecium roseum* Link

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SUMMARY: The production of trichothecin by strains of *Trichothecium roseum* was greatly increased when nutrients such as yeast extract, malt extract or corn-steep liquor were added to Czapek-Dox medium containing 5 % (w/v) glucose. Ammonium salts, especially ammonium tartrate, were better than nitrate as source of nitrogen, and high trichothecin titres were also obtained with asparagine or glycine as source of nitrogen. Maximum yields of the order of 100 mg./l. were obtained on a modified Czapek-Dox medium containing ammonium tartrate instead of sodium nitrate and including 5 % (w/v) glucose and 1 % (v/v) of corn-steep liquor, in about 30 days, with 4 cm. depth of medium.

Trichothecin had no antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* or *Bacterium coli*. It was active against each of the twenty-seven species of fungi examined. The growth of *Penicillium digitatum*, the most sensitive species investigated, was completely inhibited at 0.64 mg./l., and 50 % germination of its spores took place at trichothecin concentrations between 0.30 and 0.75 mg./l., according to the age of the spores and other factors.

Antagonism between *Trichothecium roseum* Link and certain plant-pathogenic fungi was reported by Whetzel (1909), Boning (1933), Koch (1934) and Greaney & Machacek (1935). Koch (1934) stated that *T. roseum* actively parasitized the stroma of *Dibotryon morbosum* in the black-knot disease of various hosts. The influence of *Trichothecium roseum* on the pathogenicity of *Helminthosporium sativum* in experiments with cereals has been observed by Greaney & Machacek (1935). Antifungal activity of culture filtrates of *Trichothecium roseum* against *Botrytis allii* was reported by Brian & Hemming (1947). The substance responsible for this antagonism was isolated from culture filtrates of the fungus and its chemical properties described (Freeman & Morrison, 1948), and was given the name 'trichothecin'. The work described here is concerned with the most favourable conditions for the production of trichothecin, and with its biological properties. We first observed that a ten-fold dilution of the culture filtrate of a strain of *Trichothecium roseum* on beer-wort completely suppressed the germination of *Penicillium digitatum* spores. In cylinder-plates the growth of *Aspergillus niger* mut. *schimani*, *Paecilomyces varioti*, *Fusarium graminearum* and *Penicillium hagemii* was inhibited, though less markedly than that of *P. digitatum*. Antagonism of *Trichothecium roseum* towards *Penicillium* spp., both on liquid and solid media, was frequently observed, the mycelia of the latter being ultimately completely overgrown by *Trichothecium roseum*.

EXPERIMENTAL

Strains of Trichothecium roseum. Four strains of *T. roseum* were used; they were isolated in this laboratory from specimens of cut wood collected locally. When freshly isolated on beer-wort agar medium all showed the characteristic

macroscopic appearance of *T. roseum*. The colonies, which were initially white and thinly floccose, rapidly developed a rose pink conidial zone. The erect conidiophores carried terminal clusters of two-celled oval conidia about $20 \times 10 \mu$, each with a nipple-like projection at the point of attachment. Microscopically the various strains were indistinguishable; their various colonial appearances were as follows.

Strain no. F109. Colonies on beer-wort agar were thinly floccose, white at first, rapidly becoming pink and velvety as conidia developed. On liquid Czapek-Dox medium (5 % (w/v) glucose) supplemented by 1 % (v/v) corn-steep liquor (CSL), initially produced thick smooth convoluted felt, deep pink in colour. After a number of subcultures on wort agar, the mycelium on liquid medium became largely sterile, compact, white and convoluted with a few small pink conidial areas. This change coincided with a decrease from 30 to 5 mg./l. in the trichothecin production on the above medium. The original

Table 1. *Maximum trichothecin production and other data of strains of Trichothecium roseum*

Strain no.	Source	Date isolated	Maximum trichothecin production under conditions described in Table 7 on medium 4 cm. deep (mg./l.)
F109	Sycamore (<i>Acer pseudoplatanus</i>) log	June 1945	25
F198	Unidentified wooden log	August 1946	150
F227	Dead stem of <i>Rosa canina</i>	December 1946	110
F271	Sycamore (<i>Acer pseudoplatanus</i>) log	January 1948	20

activity was restored when sodium nitrate was replaced by ammonium tartrate as nitrogen source, but the mycelium remained largely sterile, although conidia developed normally on wort agar.

Strain no. F198. Colonies on wort agar were thin, pale pink with numerous areas of sterile floccose mycelium. After 3–4 subcultures on wort agar only white floccose sterile mycelium was produced. No antifungal activity was detected when this strain was grown on Czapek-Dox glucose CSL medium, but high concentrations of trichothecin were obtained with the ammonium tartrate-containing medium.

Strain no. F227. Colonies on wort agar were similar to those of strain F109, but with occasional floccose sterile areas. On liquid glucose CSL Czapek-Dox medium the mycelium was mainly pink with heavy development of conidia, and the surface was much rougher than that of strain F109. There was occasional development of loose, white floccose areas with delayed conidial production. On the ammonium tartrate-containing medium similar but heavier growth took place.

Strain no. F271. Colonies on wort agar and conidial production were similar to strain F109. On the ammonium tartrate-containing medium the mycelium resembled that of strain F109 in texture, but conidial development took place evenly over the whole surface.

Assay of trichothecin

(a) *By inhibition of germination of Penicillium digitatum conidia.* The basis of the method was described by the Committee on Standardization of Fungicidal Tests of the American Phytopathological Society (1947), and its application to conidia of *Botrytis allii* by Brian & Hemming (1945). *Penicillium digitatum* conidia were used as test material and Czapek-Dox medium containing 5 % (w/v) glucose at pH 4.2 was employed as germination medium. In preliminary tests, considerable variations were observed in the sensitivity towards the antifungal compound of different batches of spore suspensions (Table 2). This variation

Table 2. *Variation in sensitivity to trichothecin of Penicillium digitatum spore suspensions*

No. of culture	Age of culture (days)	Percentage germination of spores in presence of trichothecin (mg./l.)					ED 50 (mg./l.)
		1.25	0.63	0.31	0.16	0.08	
1	7	10	60	100	—	—	0.75
2		1	10	50	80	100	0.29
3		5	50	100	—	—	0.63
4		10	60	100	—	—	0.75
5		5	30	70	100	—	0.44
6		5	20	50	80	100	0.31
7	14	1	40	80	100	—	0.53
8		0	20	50	100	—	0.38
9		5	50	100	—	—	0.63

was overcome by use of a parallel series of standard concentrations of trichothecin with each set of assays, from which the concentration of the substance which permitted 50 % germination (ED 50) was determined for each batch of spores. The ED 50 was determined graphically by plotting the probit corresponding to the percentage germination against the logarithm of the concentration (Finney, 1947) and reading the ED 50 on the linear response curve thus obtained. The ED 50 is cited in the tables in mg./l.

(b) *By a cylinder-plate method.* The method was an adaptation of the cylinder-plate diffusion technique (Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey, 1941), in which a porcelain cylinder was embedded in beer-wort agar medium, sown with *P. digitatum* spores, in a Petri dish. The cylinder was filled with the test solution and the plate incubated for 3 days at 25°. The dose-response curve for trichothecin (Fig. 1), for concentrations in the range of 1.56–200 p.p.m., shows that there is a linear relationship between diameter of zone of inhibition and the logarithm of trichothecin concentration.

The method compares unfavourably with the spore-germination method. It is less precise because of the diffuse nature of the boundaries of the zones of inhibition, and because the zones are too large to allow both standard and test solutions to be set up on the same 9 cm. plate. It is also slow, since 3 days must elapse before results are obtained.

The data summarized in Tables 3 and 4 were obtained by this method.

Factors which influence trichothecin production in liquid media

The influence of a number of factors on trichothecin production in liquid media was investigated. The basal medium was Czapek-Dox containing 5% (w/v) glucose, sterilized by autoclaving in 350 ml. quantities (giving 17 mm. depth of medium) in 'Glaxo' bottles. The initial pH was 5.0. The cultures were

Fig. 1. Relationship between trichothecin concentration and diameter of zone of inhibition of *Penicillium digitatum* in cylinder-plate assay method. $\circ$ — $\circ$ zone diameter plotted against trichothecin concentration (mg./l.). $\times$ — $\times$ zone diameter plotted against log concentration. Each diameter represents the mean reading from four plates.

inoculated with a suspension of *Trichothecium roseum* spores from about 7-day beer-wort agar slopes. The cultures were incubated at 25° in the dark, and samples of the filtrate assayed at intervals. In the absence of added organic nutrients growth was slow and sparse. To produce rapid growth and heavy development of mycelium nutrients such as yeast extract, malt extract, beer wort and CSL were necessary. In preliminary experiments CSL appeared to be most effective in stimulating development of trichothecin.

Corn-steep liquor concentration. The effect of CSL concentrations in the range 0.1–2% (v/v) at initial pH values of 4.0, 6.1 and 7.6 was determined (Table 3). In the absence of CSL only traces of activity were produced. Addition of 0.1% (v/v) resulted in a striking increase of activity, but further increase of CSL above 0.2% (v/v) produced little further increase in activity. Within the

range pH 4.0–7.6, initial pH had no significant effect on trichothecin production. In the subsequent experiments the various media contained 1 % (v/v) of CSL.

Table 3. *Influence of corn-steep liquor concentration on production of trichothecin by Trichothecium roseum strain F109 in 'Glaxo' bottle cultures*

Trichothecin was assayed by the cylinder-plate method.

pH	CSL (%, v/v)	Period of incubation (days)			
		4	6	8	12
		Concentration of trichothecin (mg./l.)			
4.0	0	—	—	—	Trace
	0.1	—	4.2	20.0	34.0
	0.2	—	6.8	17.0	50.0
	0.5	1.2	6.8	14.0	40.0
	1.0	Trace	7.8	12.0	34.0
	2.0	Trace	10.0	12.0	40.0
6.1	0	—	—	1.4	4.2
	0.1	—	1.8	3.5	12.0
	0.2	1.2	6.0	7.8	41.0
	0.5	2.0	6.0	16.0	41.0
	1.0	—	6.0	14.0	60.0
	2.0	Trace	10.0	14.0	50.0
7.6	0	—	—	—	—
	0.1	2.0	6.0	6.0	20.0
	0.2	3.8	10.0	7.8	40.0
	0.5	Trace	3.5	8.8	30.0
	1.0	Trace	2.7	6.0	34.0
	2.0	Trace	4.7	11.0	60.0

Carbon source. Comparison of glucose, sucrose, lactose and glucose + lactose as carbon sources (Table 4) showed that maximum trichothecin production took place with sucrose and that lactose was unsatisfactory.

Table 4. *Effect of source of carbon on trichothecin production by Trichothecium roseum strain F109 in basal medium enriched by various sugars*

Trichothecin was assayed by the cylinder-plate method.

Carbon source and % (w/v) conc.	Period of incubation (days)			
	6	10	13	16
	Trichothecin concentration (mg./l.)			
Glucose, 5	3.4	2.4	5.2	3.1
Sucrose, 5	12.0	9.0	16.0	4.7
Lactose, 5	< 1.0	< 1.0	< 1.0	—
Glucose, 2.5; lactose, 2.5	2.0	2.2	3.1	—

— Not tested.

Nitrogen source. The nature of the nitrogen source in the medium had considerable influence on the production of trichothecin. *T. roseum* strain F227 was grown on a series of media based on the Czapek-Dox formula, containing 5 % (w/v) of glucose and 1 % (v/v) of CSL, in which the normal nitrogen source,

NaNO_3 , was replaced by the equivalent amounts of KNO_3 , NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$, ammonium tartrate, asparagine, urea and glycine (Table 5). The ammonium ion was superior to nitrate as a source of nitrogen, except when supplied in the form of ammonium sulphate, in which case the pH rapidly fell to about pH 2, presumably because of the liberation of sulphuric acid. Ammonium tartrate was the best nitrogen source for trichothecin production and was used in the preparation of trichothecin on a relatively large scale. Asparagine and glycine media both produced high titres of trichothecin, but the maxima were reached more slowly than with ammonium tartrate. Only a trace of activity developed when the medium contained urea as source of nitrogen. In a separate experiment, a medium containing the equivalent concentration of acetamide gave trichothecin production of the same order as that obtained with nitrate nitrogen.

Table 5. *Effect of nitrogen source on production of trichothecin in cultures of Trichothecium roseum strain F227*

Sodium nitrate in the Czapek-Dox formula was replaced by equivalent concentrations of other nitrogen sources in media containing glucose (5 %, w/v) and CSL (1 %, v/v). Trichothecin was assayed by the spore-germination method. Each result represents the mean of three cultures.

Nitrogen source	Conc. (g./l.)	Period of incubation (days)							
		4	7	10	13	4	7	10	13
		Trichothecin concentration (mg./l.)				pH			
Potassium nitrate	2.5	—	—	2.4	3.3	6.2	6.1	6.3	6.5
Ammonium nitrate	0.6	2.6	25.0	56.0	50.0	4.3	5.9	6.5	6.9
Ammonium sulphate	1.5	2.5	8.0	5.1	5.0	3.5	2.4	2.0	2.1
Ammonium tartrate	2.2	—	25.0	34.0	77.0	6.0	5.5	5.5	6.5
Asparagine	1.4	—	—	6.8	35.0	6.5	6.4	6.3	6.9
Urea	0.7	—	—	0.7	—	7.0	7.5	7.3	8.0
Glycine	1.8	—	—	18.0	55.0	6.0	6.6	6.5	6.8

— Not tested.

Glucose concentration. The effect of initial concentrations of glucose in the range 1–20 % (w/v) was determined in a medium containing 1 % (v/v) CSL and Czapek-Dox salts in which sodium nitrate was replaced by the equivalent quantity of ammonium tartrate (Table 6). With low concentrations (1 or 2 % (w/v)) of glucose, trichothecin concentrations rose to a maximum after 6 and 10 days, respectively, and thereafter fell equally rapidly to zero. The highest concentration was obtained with 5 % (w/v) glucose and was only slightly less with 10 % (w/v) initial glucose. With 20 % (w/v) initial glucose, both the rate of trichothecin production and the maximum concentration were much lower than with 5 and 10 % (w/v) initial sugar concentrations.

Depth of medium. The maximum practicable depth of medium for trichothecin production and isolation was determined in the medium used in the previous experiment, containing 5 % (w/v) initial glucose. The results (Table 7) show that although trichothecin concentration attained a maximum more slowly in the deeper media it was at least as high as in the shallower media.

Biological properties of trichothecin

Trichothecin had no antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* or *Bacterium coli*, at a concentration of 400 mg./l. (saturated aqueous solution) and pH 7.0, as determined by the cylinder-plate method (Abraham *et al.* 1941). Its antifungal activity is exhibited against Fungi

Table 6. *Effect of initial glucose concentration on trichothecin production by Trichothecium roseum strain F227*

The fungus was grown on the following medium: ammonium tartrate, 2.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; K_2HPO_4 , 1.0 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; KCl, 0.5 g.; corn-steep liquor, 10 ml.; glucose, 10–200 g.; water to 1000 ml.; pH 5.0. Trichothecin was assayed by the spore-germination method.

Glucose initial concentration % (w/v)	Trichothecin concentration (mg./l.) after (days)						pH after (days)					
	4	7	10	13	17	24	4	7	10	13	17	24
1	19.0	20.0	11	2.5	—	—	5.0	6.5	7.1	7.7	—	—
2	24.0	30.0	39	25.0	13	3	5.4	6.6	7.3	7.7	8.5	9.1
5	24.0	45.0	33	34.0	61	29	5.1	6.7	7.2	7.3	8.3	8.6
10	27.0	41.0	45	50.0	38	33	4.8	6.5	7.0	7.3	8.4	8.2
20	1.3	4.7	13	26.0	33	35	4.1	6.0	6.7	7.0	8.2	8.1

— Not tested.

Table 7. *Effect of depth of medium on trichothecin production by Trichothecium roseum strain F227*

Medium as in Table 6, with 5 % (w/v) glucose. Trichothecin was assayed by the spore-germination method.

Depth of medium (cm.)	Volume of medium per bottle (ml.)	Period of incubation (days)						
		6	9	13	16	20	27	36
		Trichothecin concentration (mg./l.)						
0.5	100	32.0	26.0	17	28	—	—	—
1.0	200	14.0	—	72	71	56	—	—
2.0	400	6.3	25.0	55	58	74	91	57
4.0	800	2.5	9.4	30	72	91	111	72

— Not tested.

Imperfecti, Zygomycetes and Ascomycetes, and the growth of the twenty-seven species of fungi so far examined was inhibited in some degree. Tests in the presence of a range of concentrations of trichothecin (0.13–80 mg./l., Table 8) revealed wide differences in the sensitivity of different fungi to trichothecin. The growth of *Penicillium digitatum*, much the most sensitive species examined, was completely inhibited by 0.64 mg./l. In contrast, the growth of *P. notatum*, *P. nigricans janczewskii* and *Trichoderma viride* was not completely inhibited at a concentration of 80 mg./l. Growth of *Trichothecium roseum* strain F227 was itself partially inhibited by a trichothecin concentration of 80 mg./l.

Germination of *Penicillium digitatum* conidia was virtually suppressed by

1.25 mg./l. of trichothecin; the ED₅₀ varied from 0.30 to 0.75 mg./l. according to the age and other properties of the conidia (Table 2). The corresponding figures for *Botrytis allii* spores are 6.25 and 3.12 mg./l.

Table 8. *Effect of trichothecin on the growth of certain fungi*

The fungi were seeded on to the surface of beer-wort agar containing varying trichothecin concentrations. After 5 days' incubation at 25° growth was compared with that obtained on control plates containing no antifungal substance. No growth, 0; trace of growth, 1; slight growth, 2; good growth, 3; abundant growth (as control), 4.

Laboratory collection no.	National Collection of Type Cultures no.	Organism	Relative growth at tricho- thecin concentrations (mg./l.)				
			80	16	3.2	0.64	0.13
			Relative growth				
Fungi Imperfecti							
F 153	982	<i>Aspergillus fumigatus</i> Fres.	0	3	4	4	4
F 160	3308	<i>A. niger</i> van Tiegh.	0	0	4	4	4
F 181	1859	<i>Cephalosporium longisporum</i> Petch	0	1	4	4	4
F 182	2278	<i>Cladosporium herbarum</i> Link	0	0	3	4	4
F 197	—	<i>Fusarium graminearum</i> Schwabe	0	0	3	3	3
F 180	1357	<i>Helminthosporium sacchari</i> Butl.	0	1	2	3	4
F 17	—	<i>Paecilomyces varioti</i> Bain.	0	3	4	4	4
F 117	583	<i>Penicillium caseicola</i> Bain.	0	1	4	4	4
F 229	3539	<i>P. citrinum</i> Thom	0	1	4	4	4
F 196	—	<i>P. digitatum</i> Sacc.	0	0	0	0	3
F 119	593	<i>P. expansum</i> Link	0	3	4	4	4
F 132	584	<i>P. lilacinum</i> Thom	0	1	3	4	4
F 140	3958	<i>P. meleagrinum</i> Biourge	0	0	3	4	4
F 211	—	<i>P. nigricans</i> — <i>janczewskii</i> series	1	3	4	4	4
F 143	4222	<i>P. notatum</i> Westling	1	4	4	4	4
F 135	983	<i>P. oxalicum</i> Currie & Thom	0	0	4	4	4
F 133	588	<i>P. roqueforti</i> Thom	0	1	4	4	4
F 234	591	<i>P. spinulosum</i> Thom	0	0	4	4	4
F 214	6436	<i>Stachybotris atra</i> Corda	1	4	4	4	4
F 5	—	<i>Trichoderma viride</i> Pers. ex Fries	1	3	4	4	4
F 227	—	<i>Trichothecium roseum</i> Link	2	4	4	4	4
Ascomycetes							
F 177	4020	<i>Chaetomium convolutum</i> Chivers	0	0	2	4	4
F 268	3411	<i>Neurospora crassa</i> Shear & Dodge	0	0	0	4	4
B 55	742	<i>Saccharomyces carlsbergensis</i> Hansen	0	0	1	4	4
Zygomycetes							
F 266	1904	<i>Mucor erectus</i> Bain.	0	1	2	3	4
F 264	3155	<i>Syncephalastrum racemosum</i> (Colm) Schroet.	0	3	4	4	4
F 265	1127	<i>Thamnidium elegans</i> Link	0	0	3	4	4

DISCUSSION

The isolation of trichothecin and demonstration of its antifungal properties provide fairly conclusive evidence that this compound is responsible for the antagonism exhibited by *Trichothecium roseum* towards other fungi. Several other crystalline compounds have been isolated from *T. roseum* culture filtrates and will be described elsewhere, but all were devoid of antifungal or anti-

bacterial activity. Trichothecin is extracted from the filtrates by chloroform, which removes the whole of the antifungal activity. None of the fractions obtained chromatographically, with the exception of the trichothecin-containing fractions, exhibit any antifungal activity. The stability of trichothecin at normal temperatures and over a wide range of pH (Freeman & Morrison, 1948) suggests that the compound may persist for sufficiently long in nature to influence the growth of other fungi. In this connexion the frequent occurrence on dead wood of characteristic, virtually pure cultures of *T. roseum* is of interest. Concentrations of trichothecin of the order of 80 mg./l. partly inhibit growth of *T. roseum* in culture, and old cultures of the fungus slowly lose their antifungal activity.

The antifungal activity originally observed in Czapek-Dox-5 % (w/v) glucose medium proved to correspond to about 10 mg./l. of trichothecin. The bulk of the work described above was undertaken with the object of improving the yield to provide sufficient quantities of the compound for chemical analysis and degradation studies. No evidence of the mode of antifungal action of trichothecin is yet available; it has, however, been shown that its action is not inhibited by an excess of cysteine (cf. Cavallito & Bailey, 1944).

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