Self-inhibition of Germination of Pycnidiospores of *Mycosphaerella ligulicola* in Relation to the Temperature of their Formation

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SUMMARY

At high concentrations, self-inhibition of germination of pycnidiospores of *Mycosphaerella ligulicola* increased with rise in temperature at which the spores were formed.

Spores formed at 15° (15° spores), which showed little self-inhibition at high concentrations, and 26° (26° spores), which showed marked self-inhibition at high concentrations, were used for experiments. Mixing 15° spores equally with 26° spores increased inhibition of the latter but the former were unaffected. Leachates from 15° and 26° cultures did not affect germination of 26° and 15° spores respectively, but germ tubes of 15° spores showed increased growth. Inhibition of 26° spores was overcome by washing ten times with deionized water and germ tube growth from washed spores was increased in culture leachates. Diffusates collected from dense suspensions of 26° spores did not affect germination. Diffusates from 15° spores prevented germination of 26° spores but not 15° spores where growth of germ tubes was increased. The inhibitory substance from 15° spores was not readily volatile and not affected by high temperature in solution. Volatile inhibitors were not detected from either 15° or 26° spores. Pfeffer 1% glucose solution overcame self-inhibition of 26° spores but 1% glucose or Pfeffer solution alone were ineffective.

INTRODUCTION

A rise in incubation temperature of the chrysanthemum ray blight pathogen (*Mycosphaerella ligulicola*, Baker, Dimock & Davis) between 12 and 27° resulted in an increase in pycnidiospore number which was associated with a decrease in size (Blakeman & Hadley, 1968). Further work (Blakeman & Fraser, 1969) showed that at high concentrations spores formed at 26° showed greater self-inhibition of germination when compared with spores formed at 15°. There appear to be no reports of a connexion between incubation temperature of cultures and self-inhibition of the spores for other fungi. The ability of spores, formed over a range of temperatures, to germinate at different concentrations was examined further and the nature of differences in self-inhibition between spores formed at two selected temperatures was investigated.

METHODS

Spores of *Mycosphaerella ligulicola* were collected by adding 6 ml. of deionized water to the surface of Petri dish potato glucose agar cultures and gently shaking on a rotary shaker for 30 min. The filtrate (referred to as a culture leachate) was removed

by passage through a membrane filter and the deposit of spores which remained on the membrane was normally washed by passing through two or more batches of 20 ml. deionized water. In some cases spores were further washed with up to twenty lots of 20 ml. water. The liquid (referred to as a spore leachate) was collected and concentrated after passage through the membrane. The washed spores were removed by cutting the membrane into four strips and shaking in deionized water in a universal bottle. The concentration of the suspension was measured by haemocytometer and diluted as required. Tests were made to determine the effect on germination of culture leachates and spore leachates. Also dense suspensions of previously washed spores were placed in bijou bottles and shaken gently for 12 hr (spores do not germinate under such conditions). After passage through a membrane filter to separate the spores, the filtrate (referred to as a spore diffusate) was tested for its effect on germination. Germination tests were made on Cellophane (British Cellophane Co., PT300) discs (1.5 cm. diam.), placed on a slide and covered with a coverslip supported on a glass ring which was sealed with petroleum jelly to the slide. All slides were incubated in Petri dish moist chambers for 18, 24 or 48 hr, after which each portion of Cellophane was transferred to a clean slide, mounted in a drop of acid fuchsin stain and covered with a coverslip which was sealed to the slide with nail varnish. Assessment of germination (from samples of at least 100 spores) and length of the longest germ tube from each spore (from samples of 50 germinated spores) were made on the preserved material. Standard deviations for percentage spore germination were calculated using the arcsin transformation and a 't' test was applied to data for germ tube lengths.

The method used to collect volatile materials from spores was as follows. Thirty ml. of dense spore suspension in water was placed in each of four interconnected Dreschler bottles. Filtered air was bubbled through for 6 hr and condensed in a U tube immersed in, or placed 1 to 2 cm. above, liquid nitrogen in a Dewar flask. On transference of the tube, after immersion in the liquid nitrogen, to room temperature, condensed oxygen was given off violently causing loss of any volatiles which might have condensed in it. There was no condensation of oxygen when the tube was placed above the nitrogen where the temperature was above the boiling point of oxygen. Alternatively a mixture of solid carbon dioxide in ethanol was used for the condensation. After collection of volatiles the U tube was sealed with rubber stoppers and stored in a deep freeze. The melted contents of the U tube were subsequently tested for their effect on germination of spores of *Mycosphaerella ligulicola*.

RESULTS

Rise in incubation temperature, from 12 to 27°, of cultures of *Mycosphaerella ligulicola* increased self-inhibition of the spores when tested at concentrations ranging from 0.75×10^6 to 30×10^6 /ml. at 20° (Fig. 1).

Mixing equal numbers of spores formed at 26° with those formed at 15° did not prevent germination of the latter but caused increased inhibition in the former. Suspending 26° spores in leachates from 15° cultures did not overcome self-inhibition. Conversely there was no decrease in germination on suspending 15° spores in leachates from 26° cultures (Fig. 2) and the germ tubes, after 18 hr incubation, were nearly three times as long as those of control spores germinated in water (Table 1). The number of germ tubes arising from each 15° spore was nearly twice that from each 26° spore

regardless of differences in treatments used. No lateral branches arose from germ tubes of 26° spores after 18 hr but a few lateral branches had developed from 15° spores germinated in water and over three times as many from spores suspended in leachates from 26° cultures.

The effect of prolonged washing on germination of 26° spores was investigated. Spores washed ten times showed less self-inhibition when compared with unwashed spores. Resuspending washed spores in a culture leachate resulted in 90 to 100%

Fig. 1

Fig. 2

Fig. 1. Effect of spore concentration on germination of spores of *Mycosphaerella ligulicola* formed at different temperatures. Standard deviations are given for the higher densities.

Fig. 2. Effect of mixed spore suspensions of *Mycosphaerella ligulicola* on germination of 15° (○) and 26° (●) spores; culture leachates on germination of 15° (△) and 26° (▲) spores; comparison with germination of 15° (□) and 26° (■) spores in deionized water.

Table 1. *Effect of culture leachates on germ tube growth of Mycosphaerella ligulicola*

	Spores washed and suspended in			
	15° culture leachate	26° culture leachate	Deionized water	
Test spores	26°	15°	26°	15°
Germ tubes (mean no. per spore)	1.2	2.3	1.2	2.2
Lateral branches (mean no. per spore)	0	1.7†	0	0.5
Mean length (μ) of longest germ tube per spore	7.6*	95.2†	10.8	34.0

When compared with unwashed controls: * $P = 0.01$, † $P = 0.001$.

Measurements made from samples of 50 spores after 18 hr incubation at a concentration of 0.85×10^6 per ml.

germination over the range of spore concentrations tested (Fig. 3). Germ tubes after 48 hr incubation were three times as long after the washing of spores and about fifteen times as long when washed spores were suspended in a culture leachate (Table 2). In the latter the number of germ tubes was nearly twice that of washed or unwashed spores in water. Unwashed spores had no lateral branches after 42 hr but washed spores, suspended in water or culture leachate, had approximately one lateral branch developing from the primary germ tubes from each spore.

Fig. 3

Fig. 3. Germination of unwashed 26° spores (O) of *Mycosphaerella ligulicola* and ten times washed spores suspended in water (●) and in a culture leachate (Δ) at different concentrations. Standard deviations are given for the higher concentrations.

Fig. 4

Fig. 4. Effect of washing on germination (●) and germ tube growth (O) of 26° spores of *Mycosphaerella ligulicola*.

Table 2. Effect on germ tube growth of washing and resuspending 26° spores of *Mycosphaerella ligulicola* in a culture leachate

	Spores washed ten times	Spores washed ten times and resuspended in a culture leachate	Unwashed spores
Germ tubes (mean no. per spore)	1.5	2.8	1.5
Lateral branches (mean no. per spore)	0.8*	1.3*	0
Mean length (μ) of longest germ tube per spore	27.6*	140.0*	9.2

* $P = 0.001$ when compared with unwashed controls.

Measurements made from samples of 50 spores after 48 hr incubation at a concentration of 0.7×10^6 per ml.

Further studies were made by washing 26° spores once, twice, five, ten and twenty times and testing germination at 30×10^6 , 3.4×10^6 and 0.65×10^6 spores/ml. Results (Fig. 4) are given for a concentration of 3.4×10^6 . The trends shown at the other concentrations were similar though of a different order. With rise in the number of washes up to ten, germination and growth of germ tubes increased. Suspending spores washed twenty times in a culture leachate increased germination and germ tube growth only at the lowest concentration tested. Suspending washed spores in spore leachates, obtained from concentrating the collected material from twenty washes (600 ml.) to a volume of 3 ml. in a rotary evaporator, significantly decreased germination only at the highest concentration tested. At a concentration of 3.4×10^6 spores/ml. growth of germ tubes was diminished being similar to unwashed control spores, but at 0.65×10^6 /ml. germ tubes were shorter than unwashed controls (Table 3).

Diffusates from 26° spores (at concentrations of 36×10^6 and 360×10^6 spores/ml.) were shown to increase germination and growth of germ tubes of 26° spores (Table 4), the amount depending on the concentration of the diffuseate (Table 5). Diffusate from 26° spores had no effect on germination of 15° spores (Table 4), but, as with 26° test

Table 3. Effect of culture leachates and concentrated spore leachates on germination of washed 26° spores of *Mycosphaerella ligulicola*

Concentration of test spores ($\times 10^6$) % germination (with standard deviation) Germ tube length (μ)	Spores washed 20 times and germinated in											
	Culture leachates			Spore leachates			Deionized water			Unwashed spores		
	30	3.4	0.65	30	3.4	0.65	30	3.4	0.65	30	3.4	0.65
	44 $\pm$ 6.5	80 $\pm$ 7.5	94 $\pm$ 4.5	26 $\pm$ 6.5	68 $\pm$ 9.5	88 $\pm$ 9.0	55 $\pm$ 5.0	82 $\pm$ 5.0	74 $\pm$ 4.0	36 $\pm$ 6.5	37 $\pm$ 8.5	64 $\pm$ 10.5
	—	63.3†	86.7†	—	28.7	33.2*	—	62.0†	43.6	—	24.5	46.8

When compared with unwashed controls: * $P = 0.05$, † $P = 0.001$. Measurements made after incubation for 48 hr.

Table 4. Effect of spore diffusates on germination and germ tube growth of *Mycosphaerella ligulicola*

Test spores (concentration 1.4×10^4)	15° spores			26° spores		
	15° spore diffusate	26° spore diffusate	Deionized water	15° spore diffusate	26° spore diffusate	Deionized water
Treatment						
% germination after 24 hr	81 $\pm$ 5.5	99 $\pm$ 4.0	99 $\pm$ 2.6	0	94 $\pm$ 7.5	74 $\pm$ 9.0
Germ tube length (μ) after 24 hr	104.8*	43.6*	26.4	0	25.2*	13.6

* $P = 0.001$ when compared with spores germinated in deionized water.
15° and 26° spore diffusates collected from suspensions containing 58×10^6 and 36×10^6 spores/ml. respectively.

spores, the growth of germ tubes was related to the concentration of the diffusate (Table 5).

Diffusates from 15° spores (at 46×10^6 and 58×10^6 spores/ml.) caused a fourfold increase in germ tube growth of 15° test spores when compared with spores germinated in water. This was accompanied by a small decrease in germination possibly due to rapidly spreading hyphae inhibiting spores which had not germinated. On the other hand, diffusate from 15° spores caused complete inhibition of germination of 26° test spores (Table 4). After 48 hr in contact with the diffusate a sample of the spores was removed, washed and transferred to potato glucose agar when only 6% of the spores

Table 5. *Influence of dilution of diffusate from 26° spores (360×10^8 /ml.) on germination and germ tube growth of 15 and 26° spores of *Mycosphaerella ligulicola**

Dilution of spore diffusate	Undiluted		1/2		1/3	
Test spores formed at 15 or 26° (Concentration 1.4×10^6)	15	26	15	26	15	26
% germination (with standard deviation) after 24 hr	99 ± 3.0	94 ± 7.5	98 ± 3.5	85 ± 6.5	98 ± 4.0	81 ± 8.5
Germ-tube length (μ) after 24 hr	92.4*	42.8*	—	—	—	—
Dilution of spore diffusate	1/4		1/8		Deionized water	
Test spores formed at 15 or 26° (Concentration 1.4×10^6)	15	26	15	26	15	26
% germination (with standard deviation) after 24 hr	100 ± 0	88 ± 7.0	99 ± 1.0	79 ± 6.0	98 ± 5.0	79 ± 6.0
Germ-tube length (μ) after 24 hr	28.0*	17.6*	—	—	12.8	6.8

* $P = 0.001$ when compared with spores germinated in deionized water.

Table 6. *Influence of dilution of diffusates from 15° spores (58×10^6 /ml.) on germination and germ tube growth of 26° spores of *Mycosphaerella ligulicola**

Dilution of spore diffusate	Un-diluted	1/2	1/3	1/4	1/8	Deionized water
% germination (with standard deviation) after 24 hr	0	24 ± 8.5	45 ± 8.0	50 ± 3.5	67 ± 12.5	92 ± 4.0
Germ-tube length (μ) after 24 hr	0	10.0*	11.6*	17.2*	11.6*	28.4

* $P = 0.001$ when compared with spores germinated in deionized water.
Concentration of test spores 1.4×10^6 /ml.

grew. Dilution of the diffusate caused a steady increase in germination. Growth of germ tubes of those spores able to germinate was decreased in all dilutions tested as compared with water controls (Table 6).

Investigations were made as to whether the inhibitory effect of diffusates from 15° spores was affected by temperature or was volatile. Samples (1 ml.) of diffusate were placed in bijou bottles, both capped and uncapped, and maintained at temperatures of 2, 20, 37 and 95° for 18 hr. Afterwards the solutions were used at 20° to

determine whether they would support germination. There was a complete inhibition of spore germination using diffusates maintained at temperatures of 2, 20 and 37° in both capped and uncapped bottles, and at 95° in capped bottles only. The diffusate in the uncapped bottle at 95°, which had dried leaving a yellowish deposit, was dissolved in 1 ml. deionized water and added to 26° test spores where it enhanced germination (after 48 hr) and increased growth of germ tubes by ten times (Table 7).

Table 7. Germination of 26° spores of *Mycosphaerella ligulicola* at 20° in diffusate from 15° spores previously maintained for 18 hr at different temperatures in capped (c) or uncapped (uc) bottles

		Temperature (°)								Deionized water control
		2		20		37		95		
		c	uc	c	uc	c	uc	c	uc	
% germination	24 hr	0	0	0	0	0	0	48 ± 11.5	0	53 ± 9.0
	48 hr	0	0	0	0	0	0	100 ± 0.5	0	69 ± 6.5
Germ-tube length (μ) 48 hr		—	—	—	—	—	—	204.0*	—	21.2

* $P = 0.001$ when compared with spores germinated in deionized water.
Concentration of test spores 1.4×10^6 /ml.

Table 8. Germination of spores of *Mycosphaerella ligulicola* after 24 hr at different concentrations in water and nutrient solutions

Spore concentration/ml.	Water	1 % glucose	Pfeffer soln	Pfeffer soln + glucose (1 %)
1×10^5	73 ± 8.0*	53 ± 15.0	79 ± 4.0	84 ± 6.5
15×10^5	13 ± 5.5	3 ± 5.5	41 ± 7.0	94 ± 7.5
30×10^5	0 ± 1.5	7 ± 6.0	3 ± 6.0	99 ± 2.5

* % germination.

Table 9. Germ tube length 48 hr after incubation of spores of *Mycosphaerella ligulicola* at different concentrations in water and nutrient solutions

Spore concentration/ml.	Water	1 % glucose	Pfeffer soln	Pfeffer soln + glucose (1 %)
1×10^5	30.8‡	74.0†	40.8*	290.4†
15×10^5	12.8	15.6	58.8†	260.6†
30×10^5	3.3	11.6†	20.8†	135.3†

When compared with spores germinated in deionized water: * $P = 0.05$, † $P = 0.001$.

‡ Germ tube length μ.

Addition of condensed volatiles obtained from either 15° or 26° spores and collected in a U tube mounted a few cm. above or below the surface of liquid nitrogen or in a mixture of solid carbon dioxide + ethanol caused no change in germination or growth of germ tubes from that of control spores suspended in deionized water.

Tests on the germination of 26° spores at different concentrations in nutrient solutions demonstrated (Table 8) that addition of 1% glucose or Pfeffer solution ($\text{Ca}(\text{NO}_3)_2$, 0.8 g.; MgSO_4 , 0.2 g.; KNO_3 , 0.2 g.; KCl , 0.1 g.; K_2HPO_4 , 0.2 g.; trace-element supplement; made to 1 l. with deionized water) did not overcome the inhibition of germination shown at high spore concentrations. On the other hand provision

of Pfeffer solution + 1 % glucose completely overcame the inhibition of germination even at the highest concentration tested (30×10^6 spores/ml.). The provision of 1 % glucose or Pfeffer solution, separately, in most cases caused some increase in the growth of germ tubes of those spores able to germinate, but much greater increases occurred with Pfeffer solution + 1 % glucose (Table 9).

DISCUSSION

Self-inhibition of germination of spores of *Mycosphaerella ligulicola* has been shown to depend on the incubation temperature of the parent culture. Mixing 26° spores (which show marked self-inhibition related to suspension concentration) equally with 15° spores (in which self-inhibition is much less evident) showed that the inhibitory condition associated with the former did not affect germination of the latter. But the presence of 15° spores in the mixture resulted in a greater inhibition of 26° spores. This suggests that conditions which inhibit germination of 26° spores are associated with both 15° and 26° spores. Either 15° spores are insensitive to their effects or they possess a mechanism to overcome them.

The possibility that the inhibitory condition originated from the parent culture was examined. Leachates from cultures did not decrease the proportion of germinating 15° or 26° spores even at low concentrations but growth of germ tubes of 15° spores was substantially increased. This indicates that the inhibitory condition may originate from the spores themselves. Attempts were made to remove inhibition by washing the spores. A large increase in the proportion of germinating spores, coupled with the development of longer germ tubes (particularly at high concentrations) occurred with ten times washed 26° spores. Washed 26° spores were able to respond to a culture leachate in the same way as unwashed 15° spores, germ tubes showing a further increase in length. More prolonged washing (20 times) resulted in a slightly decreased germination and shorter germ tubes, due possibly to a decrease in endogenous reserves through leaching. The effect of washing might be to remove a germination inhibitor but application of a concentrated leachate obtained from twenty collected spore washings did not inhibit washed spores at the lower concentrations tested. Only at the highest concentration was there some restriction of germination. The length of germ tubes, however, was decreased at all concentrations. It is possible that washing of spores may have resulted in loss of a substrate required for the formation of an inhibitor.

Diffusates collected from dense suspensions of 26° spores did not affect germination but caused enhanced growth of germ tubes of both 15° and 26° spores. Self-inhibition of 26° spores would seem therefore not to be due to water-soluble inhibitors diffusing from spores unless these inhibitors were also rapidly labile. Alternatively a sufficient concentration of inhibitors may not have been attained. Only when tested on 15° spores did diffusates from 15° spores have the same effect as a 26° spore diffusate. The observation that diffusates from 15° spores completely inhibit germination of 26° spores may explain the increased inhibition of the latter when mixed with 15° spores. It also shows the greater sensitivity of 26° spores to inhibitory substances. The active material from 15° spores is not readily volatile and not affected by prolonged exposure to a high temperature in solution. From these results it is clear that the inhibitor causing self-inhibition of 26° spores is different in nature from that produced by 15° spores.

The presence of volatile inhibitors of spore germination was not detected from suspensions of either 26° or 15° spores, though they have been obtained from the mycelium of *Fusarium oxysporum* (Robinson & Park, 1966) and from rust uredospores (Allen, 1955.)

The observation that inhibition of germination of 26° spores at high concentrations can be overcome by provision of a defined nutrient solution might indicate that lack of nutrients was the cause of the inhibition. Nutrients are more likely, however, to overcome an inhibitory condition associated with the spores since germination of spores at high concentrations can take place after prolonged washing of the spores, during which nutrient levels within the spores are likely to be decreased through leaching. The virtual absence of self-inhibition of germination of 15° spores may be attributed to the higher levels of nutrient reserves contained in the larger spores which may, on germination, overcome the action of inhibitors which have been shown to be associated with these spores. Occasional larger spores present in dense suspensions of 26° spores were invariably observed to germinate more readily.

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