Chemical Dissolution of Cellulose Membranes as a Prerequisite for Penetration from Appressoria of *Colletotrichum lagenarium*

By K. SUZUKI,* I. FURUSAWA, N. ISHIDA AND M. YAMAMOTO

Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

(Received 28 April 1981; revised 14 September 1981)

The relationship between protein synthesis during appressorial formation and the ability of appressoria to form penetration hyphae in nitrocellulose membranes was investigated for the plant pathogenic fungus *Colletotrichum lagenarium*. Cycloheximide treatment before appressorial pigmentation inhibited the formation both of penetration hyphae and of haloes, the latter resulting from the partial degradation of cellulose membranes; but treatment after appressorial pigmentation did not inhibit halo formation. When spores were transferred to 32 °C before appressoria became pigmented, both pigmentation and penetration were prevented. In the presence of 1 mM-3,4-dihydroxyphenylalanine (DOPA) at 32 °C, pigmentation was restored, but the formation of haloes and penetration hyphae was still prevented. Penetration into cellulose membranes pretreated with cellulase was observed from 20–30% of appressoria which had been formed in the presence of cycloheximide at 24 °C or in the presence of DOPA at 32 °C. The results suggest that there are two phases of protein synthesis following spore germination, only the first of which is involved in halo formation.

INTRODUCTION

In some plant pathogenic fungi, formation of appressoria is a prerequisite for invasion of host plants (Emmett & Parbery, 1975). A relationship between appressorial formation and protein and RNA synthesis has been reported for a few plant pathogens (Dunkle *et al.*, 1969; Miehl & Lukezic, 1972; Staples & Yaniv, 1976), but whether protein synthesis is involved in the process of penetration from appressoria has not been established.

Formation of appressoria in *Colletotrichum lagenarium* is temperature sensitive: appressoria form when spores are incubated at 24 °C, but do not form at 32 °C (Ishida & Akai, 1969). A polypeptide with a molecular mass of 95 kilodaltons was specifically associated with appressorial formation, and when its synthesis was inhibited by cycloheximide, appressoria matured in structure, but seemed to lack the ability to penetrate artificial nitrocellulose membranes (Suzuki *et al.*, 1981).

This paper reports the effects of temperature and cycloheximide on the formation of penetration hyphae and of haloes resulting from the partial degradation of the nitrocellulose membrane. The significance of protein(s) synthesized during appressorial formation on the ability to form penetration hyphae in membranes is discussed.

METHODS

Spores. *Colletotrichum lagenarium* (Pass.) Ellis and Halsted was cultured on potato sucrose agar medium at 24 °C for 7 d. Spores on the mycelial mat were collected with a brush and washed three times by centrifugation with ice-cold distilled water (Furusawa *et al.*, 1977).

Spore germination. The spores were suspended in deionized sterile water at about 1×10^5 spores ml⁻¹. Drops of the spore suspension were placed on glass slides in humid Petri dishes at various temperatures.

Observations on the penetration of nitrocellulose membranes. A nitrocellulose membrane (2 × 2 cm), prepared from Visking cellulose tubing, was coated with a uniform suspension of spores and soaked in 2 ml sterile distilled water in a Petri dish (4.5 cm diam.). After various incubation times, the water was exchanged for 2 ml of a solution of 3.55 µM-cycloheximide or 1 mM-3,4-dihydroxyphenylalanine (DOPA). Removal of cycloheximide was done as described previously (Suzuki *et al.*, 1981). After incubation the membranes were stained with lactophenol cotton blue or ZnCl₂/KI solution.

Partially denatured nitrocellulose membranes were prepared by treatment with 2% (w/v) cellulase (Onozuka R-10, Kinki Yakult, Japan) for 6 h at 30 °C.

Table 1. *Effect of 3.55 µM-cycloheximide on formation of penetration hyphae and haloes by C. lagenarium on nitrocellulose membranes*

Spores were incubated at 16 °C on nitrocellulose membranes. At the indicated time, spores were transferred to 24 °C, cycloheximide was added and incubation continued until 60 h.

Incubation time before cycloheximide addition (h)	Percentage of spores with pigmented appressoria when cycloheximide added	Percentage of appressoria with:	
		Penetration hyphae*	Haloes*
6	0	0.9 (0)	4.1 (0)
12	12.8	1.1 (0)	7.4 (0)
18	50.3	2.7 (0)	41.0 (0.8)
24	75.0	9.0 (6.3)	62.7 (8.1)
30	81.5	36.3 (32.6)	64.7 (36.6)
36	86.8	44.4 (43.4)	63.0 (44.5)

* The numbers in parenthesis are the percentages of appressoria with penetration hyphae or haloes at the time of addition of cycloheximide.

Table 2. *Effect of 3.55 µM-cycloheximide treatment before and after appressorial pigmentation on formation of penetration hyphae and haloes by C. lagenarium on nitrocellulose membranes*

Cycloheximide was added after 12 h (expt 1) or 24 h (expt 2) prior incubation at 16 °C. After incubation in the presence of cycloheximide for the indicated time at 24 °C, the membranes were washed to remove cycloheximide and incubated in water for a further 48 h at 24 °C.

Expt no.	Length of cycloheximide treatment (h)	Percentage of appressoria with:	
		Penetration hyphae	Haloes
1 (12 h prior incubation)	0	70.3	83.0
	6	39.0	48.6
	12	20.3	28.4
	18	12.1	19.1
	24	5.3	9.4
2 (24 h prior incubation)	0	72.2	80.6
	6	62.9	70.1
	12	57.2	69.6
	24	50.8	62.9

RESULTS

When spores were incubated on nitrocellulose membranes at 16 °C, there was little overlap between the phases of appressorial formation, pigmentation and penetration, all of which were delayed relative to 24 °C. By 12 h appressoria had attained full size, but were unpigmented, while by 24 h they were structurally mature and dark pigmented. After 24 h at 16 °C, transfer of spores to 24 °C permitted the synchronous formation of penetration hyphae which were accompanied by the formation of haloes indicating partial digestion of the membranes.

After various incubation times at 16 °C, cycloheximide (3.55 µM) was added to spore suspensions. Formation of penetration hyphae was almost completely inhibited irrespective of the time of cycloheximide addition (Table 1). Halo formation was inhibited by addition of cycloheximide within 12 h of incubation, whereas it was not affected by addition of cycloheximide at and after 24 h regardless of complete inhibition of formation of penetration hyphae (Table 1). When cycloheximide was added before appressoria had matured in structure and pigmentation (12 h at 16 °C) and removed after they had so matured, haloes and penetration hyphae were rarely observed even after a further 48 h incubation (Table 2, Expt 1). However, if cycloheximide was added after appressoria had coloured (24 h at 16 °C)

Table 3. *Effects of temperature on appressorial pigmentation, lateral germination from appressoria and penetration by C. lagenarium, following 12 h preincubation at 16 °C*

Temperature (°C)	Percentage of pigmented appressoria*	Percentage of lateral germination from appressoria*	Percentage of appressoria with:	
			Penetration hyphae†	Haloës†
16	94.3	2.4	61.1	66.0
20	96.7	2.1	72.3	73.4
24	95.7	2.4	75.0	83.5
28	72.7	14.3	51.0	66.2
32	16.3	31.0	3.7	12.4

* Spores were incubated at 16 °C for 12 h, transferred to the temperatures shown and incubated for a further 24 h on glass slides.

† Spores were incubated at 16 °C on nitrocellulose membranes for 12 h, transferred to the temperatures shown and incubated for a further 48 h.

Table 4. *Effects of temperature and DOPA on appressorial pigmentation and the formation of penetration hyphae and haloës by C. lagenarium on nitrocellulose membranes following 12 h preincubation at 16 °C*

Spores were incubated on nitrocellulose membranes at 16 °C for 12 h, followed by incubation for a further 48 h at 24 or 32 °C in the presence (+) or absence (–) of 1 mM-DOPA.

Temperature (°C) to which spores transferred after 12 h	Presence of DOPA	Percentage of:		
		Pigmented appressoria	Penetration hyphae	Haloës
24	—	94.8	79.8	83.2
	+	97.6	72.4	76.6
32	—	12.9	4.1	14.0
	+	92.9	0	3.8

Table 5. *Effect of temperature on the formation of penetration hyphae and haloës by C. lagenarium on nitrocellulose membranes following 24 h preincubation at 16 °C*

Spores were incubated at 16 °C for 24 h on nitrocellulose membranes then transferred to the temperatures shown and incubated for a further 36 h.

Temperature (°C)	Percentage of appressoria with:	
	Penetration hyphae	Haloës
16	61.1	66.0
20	72.2	72.3
24	71.4	73.0
28	67.7	74.9
32	66.2	73.1

and removed after a further 24 h incubation, both haloës and penetration hyphae were observed (Table 2, Expt 2). These results suggest that protein synthesis involved in halo formation occurs during maturation of appressoria.

When spores were incubated at 16 °C for 12 h and transferred to a temperature within the range 16 to 32 °C before appressoria had coloured, percentage formation of haloës and penetration hyphae decreased at 32 °C (Table 3). In addition, the percentage of appressorial pigmentation decreased at 32 °C and about 30% of appressoria developed germ tubes laterally from appressoria when incubated on glass slides (Table 3).

In order to clarify the relationship between appressorial pigmentation and the ability to penetrate cellulose membranes, appressorial pigmentation was induced at 32 °C by the addition of DOPA (1 mM), a known melanin precursor (Bull, 1970). Pigmented appressoria

did not germinate laterally but neither penetration hyphae nor haloes were regularly observed (Table 4), suggesting that protein synthesis involved in halo formation might be temperature sensitive and not occur at 32 °C. The percentage of halo formation was dependent on the timing of the temperature shift from 16 to 32 °C. Treatment with DOPA at 24 °C did not affect the formation of penetration hyphae and haloes (Table 4). On the other hand, if spores were incubated at 16 °C for 24 h (appressoria mature in structure and pigmentation) and transferred to a temperature within the range 16 to 32 °C, the formation of haloes and penetration hyphae was not affected. Therefore, following a 24 h preliminary incubation the formation of haloes and penetration hyphae was not temperature-dependent (Table 5).

To determine whether halo formation is essential for penetration or not, spores were incubated on nitrocellulose membranes which had been treated previously with 2% (w/v) cellulase for 6 h at 30 °C. From 20–30% of appressoria which were formed in the presence of cycloheximide (3.55 µM) at 24 °C or those coloured in the presence of DOPA (1 mM) at 32 °C, penetration into cellulose membranes previously treated with cellulase occurred, whereas penetration was rarely observed into membranes which were not treated with cellulase.

DISCUSSION

The formation of appressoria is necessary for infection by many plant pathogenic fungi (Emmett & Parbery, 1975). To date, studies have been concerned mainly with the relationship between macromolecular synthesis and appressorial morphogenesis (Dunkle *et al.*, 1969; Miehe & Lukezic, 1972; Staples & Yaniv, 1976), and there is little information available on the involvement of protein synthesis in host penetration by appressoria.

Our results suggest that the requirement for protein synthesis during penetration from appressoria can be divided into two phases. One phase occurs during appressorial maturation and may be closely connected with halo formation. The other occurs after the structural maturation of appressoria and seems to be required for the elongation of penetration hyphae, because treatment with cycloheximide after appressorial maturation with pigmentation inhibited the appearance of penetration hyphae but not of haloes. Appressoria which lack the ability to penetrate – those formed in the presence of cycloheximide (Suzuki *et al.*, 1981) or those coloured in the presence of DOPA at 32 °C – successfully penetrated membranes previously treated with cellulase. This suggests that the formation of haloes is caused by the partial digestion of cellulose and is probably essential for the development of penetration hyphae. Appressorial formation and pigmentation were temperature sensitive and did not occur at 32 °C. Results in this study suggest that appressorial pigmentation was not directly connected with the synthesis of proteins necessary for halo formation, because at 32 °C penetration hyphae were not observed even if appressorial pigmentation was induced by DOPA.

We have already reported that a polypeptide with a molecular mass of 95 kilodaltons was specifically synthesized only when appressoria were formed (Suzuki *et al.*, 1981). As shown in Tables 3 and 4, the early phase of protein synthesis necessary for halo formation might be temperature sensitive and be inhibited at 32 °C. It is probable that the 95 kilodalton polypeptide (Suzuki *et al.*, 1981) might be cellulase itself or another related molecule which results in halo zones around the penetration sites.

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