

# A 90-kDa Glycoprotein Associated with Adhesion of *Nectria haematococca* Macroconidia to Substrata

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*Nectria haematococca* mating population I macroconidia incubated in water are nonadherent. Within 10 min after incubation in zucchini fruit extract or potato-dextrose broth, *N. haematococca* macroconidia adhere at the spore apices, produce material at the spore apices which is labeled with concanavalin A (ConA), and produce a 90-kDa glycoprotein which is labeled with ConA. Neither the ConA labeling nor the 90-kDa glycoprotein are observed on spores incubated in water. Spores incubated in V8 broth or in defined media do not produce ConA-labeled material at the spore apex, do not become adherent, and do not produce a 90-kDa glycoprotein; however, these spores germinate as efficiently as spores incubated in the adhesion-inducing media. Thus, the production of the ConA-labeled material at the site of attachment and the production of the 90-kDa glycoprotein are specifically associated with adhesion and are not generally associated with the process of germination. Furthermore, ConA prevents adhesion, but not germination, of spores. Thus, the same compound that labels the material at the site of adhesion also blocks adhesion.

**Additional keywords:** attachment, *Fusarium solani* f. sp. *cucurbitae* race I, spore, squash.

Many plant pathogenic fungi adhere to their hosts before penetration (Epstein *et al.* 1987; Gubler and Hardham 1988; Hamer *et al.* 1988; Young and Kaus 1984). In some species, spore adhesion is important for dissemination, prevention of fungal displacement, morphogenesis, and chemical communication between the pathogen and host (Nicholson and Epstein 1991). Using adhesion-reduced mutants, Jones and Epstein (1990) provided experimental evidence that macroconidial adhesion is a virulence factor for the cucurbit pathogen *Nectria haematococca* (Berk. & Br.). Despite the apparent importance of adhesion in pathogenicity of plant pathogenic fungi, the biochemistry of adhesion has not been well characterized.

We study spore adhesion in *N. haematococca* mating population I (anamorph: *Fusarium solani* f. sp. *cucurbitae*

race I). In the field, the macroconidia are water disseminated. Macroconidia harvested in water from a culture plate are nonadherent (Jones and Epstein 1989). After a 5-min exposure to a zucchini fruit extract, the macroconidia become adhesion-competent and will adhere non-specifically to polystyrene and other substrata. The macroconidial surface undergoes major ultrastructural changes hours before germ tube emergence (Caesar-TonThat and Epstein 1991).

In the present study, we investigated biochemical changes associated with the conversion of macroconidia from a nonadherent to an adherent phenotype. Our data indicate that *N. haematococca* macroconidia produce a 90-kDa glycoprotein that is associated with the development of adhesion-competence. This compound may be the adhesive material.

## RESULTS

### Macroconidial tip mucilage and adhesion.

Under a dissecting microscope, macroconidia incubated in water did not appear to attach to a polystyrene substratum. Macroconidia incubated in zucchini extract appeared attached via either or both of the spore apices, or along the length of the spore. Macroconidia incubated in zucchini extract were adherent and produced macroconidial tip mucilage (MTM) which labeled with FITC-ConA. In zucchini extract-treated macroconidia, FITC-ConA labeled both spore apices (Fig. 1A) but not the body of the macroconidium. Thus, the macroconidial tip mucilage was spatially associated with adhesion.

Macroconidia were labeled similarly regardless of whether macroconidia were fixed before incubation in FITC-ConA. Macroconidia incubated in water were not labeled with FITC-ConA. FITC-ConA-labeled MTM was observed after only a 1-min incubation in zucchini extract. However, labeling was more pronounced after either 10 or 30 min in zucchini extract than after 1, 2, 3, 4, or 5 min of incubation. Thus, the presence of FITC-ConA-labeled MTM is temporally correlated with the induction of adhesion competence (Jones and Epstein 1989).

Adhesion-competent macroconidia produced the MTM regardless of whether the macroconidia were physically attached to a substratum or suspended in an adhesion-inducing medium. Sometimes, the FITC-ConA-labeled MTM dissociated from a macroconidium, particularly when macroconidia were not fixed. Consequently, dissociated MTM labeled with FITC-ConA was observed on the glass substratum from treatments where macroconidia

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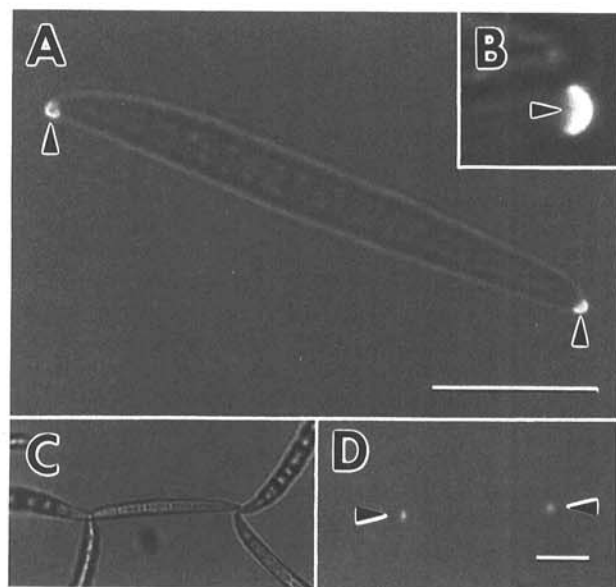
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were incubated with zucchini extract (Fig. 1B) but not water. The negative control, zucchini extract without macroconidia, was not labeled.

When adherent macroconidia were physically dislodged from a polystyrene or a glass substratum, the macroconidia agglutinated at their apices (Fig. 1C, D). Thus both cell-substratum adhesion and agglutination appear mediated by macroconidial tip mucilage.

Of the six FITC-conjugated lectins tested at 20  $\mu\text{g/ml}$ , only FITC-ConA labeled the MTM (Table 1). Lentil and pea lectins also labeled the MTM, but only at a higher concentration of 2 mg/ml. Because ConA, lentil lectin, and pea lectin recognize  $\alpha$ -D-mannose and  $\alpha$ -D-glucose, the data suggest that the MTM contains either  $\alpha$ -D-



**Fig. 1.** *Nectria haematococca* macroconidia were incubated in zucchini extract for 30 min, fixed unless indicated otherwise, rinsed, and stained with 2 mg/ml FITC-concanavalin A (ConA), and examined by fluorescence (A, B, D) and light (C) microscopy. A, FITC-ConA labeled mucilage emanating from both tips of a macroconidium. B, FITC-ConA labeled macroconidial tip mucilage attached to the substratum. The conidium has been dislodged. C, D, Adhesion-competent but detached macroconidia agglutinate, apparently by macroconidial tip mucilage, labeled here with FITC-ConA. Arrowheads indicate the macroconidial tip mucilage. Bar = 20  $\mu\text{m}$ .

**Table 1.** Fluorescein isothiocyanate (FITC)-conjugated lectins used to label macroconidial tip mucilage (MTM) of *Nectria haematococca*<sup>a</sup>

Lectin	Specificity	Lectin concentration <sup>b</sup>	
		20 $\mu\text{g/ml}$	2 mg/ml
Concanavalin A	$\alpha$ -D-Man, $\alpha$ -D-glu	++	++
Lentil	$\alpha$ -D-Man, $\alpha$ -D-glu	—	+
Pea	$\alpha$ -D-Man, $\alpha$ -D-glu	—	±
Soybean	D-galNAc	—	—
Wheat germ	D-gluNAc	—	—
Coral tree	$\beta$ -D-Gal, D-galNAc	—	—

<sup>a</sup>To induce adhesion competence, macroconidia were incubated in zucchini extract for 30 min. Then macroconidia were fixed, rinsed, incubated in 2 mg/ml FITC-conjugated lectins, and examined by fluorescence microscopy.

<sup>b</sup>MTM labeling; ++ strong labeling; + moderate labeling; ± weak labeling; — no labeling.

mannose and/or  $\alpha$ -D-glucose. Lectins with primary specificities for *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and  $\beta$ -D-galactose did not label the MTM.

To further indicate if mannose or glucose residues in the MTM were binding to the FITC-ConA, we added potential haptens to the adhesion-competent macroconidia and looked for loss of ConA-FITC labeling of the MTM.  $\alpha$ -Methyl mannoside was the most effective competitor for ConA-FITC labeling of the MTM (Table 2). Other haptens, such as mannose and  $\alpha$ -methyl glucoside, blocked FITC-ConA labeling of the MTM, but only at 50 mM, the highest concentration tested.

We used FITC-ConA labeling of MTM to determine if MTM is correlated with either adhesion or germination. *N. haematococca* macroconidia both adhered and germinated comparatively poorly when incubated in either water or in Czapek Dox medium (Table 3). In contrast, macroconidia germinated well but adhered poorly when incubated in either minimal media or in V8 broth. Macroconidia both adhered and germinated comparatively well in either of two preparations from zucchini fruits or in potato-dextrose broth. Macroconidia incubated in either of the two zucchini fruit preparations appear to produce the most MTM, since the MTM in these treatments was labeled most intensely. Macroconidia incubated in either 1× or 0.1× potato-dextrose broth appear to produce somewhat less MTM because these treatments were labeled less intensely. Macroconidia incubated in any of the media that did not induce adhesion did not appear to produce any MTM. Thus FITC-ConA labeling of MTM is qualitatively correlated with adhesion.

#### Effect of ConA on adhesion.

ConA inhibited adhesion-competent macroconidia from adhering to polystyrene (Fig. 2). Macroconidial adhesion was reduced as concentration of ConA was increased. Incubation of ConA with  $\alpha$ -methyl mannoside significantly ( $P = 0.05$ ) reduced the inhibition of ConA on adhesion (Table 4). Germination was not affected by ConA at any tested concentration (Fig. 2), suggesting that ConA spe-

**Table 2.** Compounds competing with FITC-ConA for labeling of the macroconidial tip mucilage (MTM) on *Nectria haematococca*

Compounds <sup>a</sup>	Loss of FITC-ConA labeling of MTM (mM) <sup>b</sup>			
	1	5	10	50
<i>N</i> -Acetyl-D-glucosamine	—	—	—	—
Glucose	—	—	—	—
Mannose	—	—	—	+
$\alpha$ -Methyl glucoside	—	—	±	+
$\alpha$ -Methyl mannoside	—	+	++	++
$\beta$ -Phenyl glucoside	—	—	—	—
Sucrose	—	—	—	—
Tryptophan	—	—	—	—

<sup>a</sup>The indicated compounds were incubated in phosphate-buffered saline (pH 6.5), containing FITC-ConA,  $\text{CaCl}_2$ , and  $\text{MnCl}_2$  for 30 min. At the same time, macroconidia were incubated in zucchini extract on a glass coverslip. Then macroconidia were fixed, rinsed, incubated in 2 mg/ml FITC-ConA and the indicated compounds, and examined by fluorescence microscopy.

<sup>b</sup>++ complete inhibition of labeling; + moderate inhibition of labeling; ± partial inhibition of labeling; — no inhibition of labeling.

**Table 3.** Effect of incubation media on FITC-ConA labeling of the macroconidial tip mucilage (MTM), macroconidial adhesion, and germination of *Nectria haematococca* macroconidia

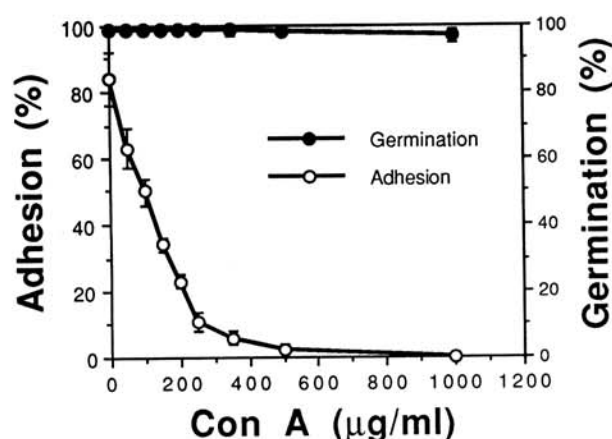
Incubation medium <sup>w</sup>	FITC-ConA labeling of MTM <sup>x</sup>	Macroconidial adhesion (%) <sup>y</sup>	Germination (%) <sup>y</sup>	90-kDa band present <sup>z</sup>
Zucchini fruit extract	++	65 a	99 a	++
Zucchini fruit exudate	++	75 a	95 a	++
Potato-dextrose broth (PDB)	±	56 a	98 a	++
0.1× PDB	+	71 a	98 a	++
V8 juice broth	—	6 b	98 a	—
Minimal medium	—	10 b	89 a	±
Czapek Dox	—	17 b	0 b	—
Water	—	19 b	0 b	—

<sup>w</sup>See Materials and Methods section for media preparation.

<sup>x</sup>Macroconidia were incubated in the indicated medium on glass coverslips for 30 min before macroconidia were fixed, rinsed, and then stained in 2 mg/ml FITC-ConA. ++ strong labeling; + moderate labeling; ± weak labeling; — no labeling.

<sup>y</sup>Macroconidia were incubated on polystyrene in the indicated medium for 30 min and 6 hr, for the adhesion and germination assays, respectively. Values are the means of three independent, replicated trials. Treatments followed by the same letter are not significantly different by the Student-Newman-Keuls multiple range test at  $P = 0.05$ .

<sup>z</sup>See Figure 3 for summary of methods and partial results.



**Fig. 2.** Effect of ConA on adhesion and germination of *Nectria haematococca* macroconidia. Macroconidia were incubated on polystyrene in zucchini extract amended with ConA for 30 min and 6 hr for the adhesion and germination assays, respectively. Values are the means of five independent, replicated trials. Error bars represent one standard deviation.

cifically prevents adhesion and not general spore metabolism.

#### Association of 90-kDa glycoprotein with adherent macroconidia.

A preparation enriched for spore surface compounds was separated by SDS-polyacrylamide gel electrophoresis, blotted, and probed with ConA. Blots of extracts from both nonadhesive spores incubated in water (Fig. 3, lane 1) and adhesive spores incubated in ZE (Fig. 3, lanes 2–5) had five common bands with apparent molecular masses of 65, 39, 34, 19, and 16 kDa. Fainter bands at 150 and 35 kDa also were seen in preparations from adhesive and nonadhesive macroconidia. Only adhesive macroconidia produced a ConA-labeled band with an apparent molecular mass of 90 kDa. The 90-kDa band was observed after macroconidia were incubated in ZE for as little as 5 min (lane 2) and was more pronounced after incubating for 10, 30, or 60 min (lanes 3–5, respectively). The 90-kDa band also was produced by macroconidia incubated in potato-dextrose broth (lane 6), but was only faintly present

**Table 4.** Effect of ConA with or without  $\alpha$ -methyl mannoside on adhesion of *Nectria haematococca* macroconidia

Treatments <sup>a</sup>	Adhesion (%) <sup>b</sup>
Control	56 a
25 mM $\alpha$ -Methyl mannoside	50 b
250 µg/ml ConA	13 d
25 mM $\alpha$ -Methyl mannoside + 250 µg/ml Con A	40 c

<sup>a</sup>All treatments contained 0.75× zucchini extract, 0.125× PBS, 12.5 µM  $\text{CaCl}_2$ , and 12.5 µM  $\text{MnCl}_2$ . Treatments were incubated for 30 min before conducting the adhesion assay.

<sup>b</sup>Macroconidia were incubated on polystyrene for 30 min. Values are the means of three independent, replicated trials. Treatments followed by the same letter are not significantly different by the Student-Newman-Keuls multiple range test at  $P = 0.05$ .

on macroconidia which were incubated either in V8 broth or minimal media (lanes 7 and 8, respectively). Zucchini extract and potato-dextrose broth controls (lanes 9 and 10, respectively) lacked the band, demonstrating that the 90-kDa band was from the macroconidia and not from the adhesion-inducing media.

Macroconidia adhered to a polystyrene substratum and produced a 90-kDa band when incubated in either zucchini extract, zucchini exudate, or potato-dextrose broth (Table 3). Macroconidia did not adhere and did not produce a 90-kDa band when incubated in V8 broth, minimal medium, Czapek Dox medium, or water. Interestingly, macroconidia incubated in V8 broth and minimal media germinated as well as macroconidia incubated in media in which the macroconidia became adherent. Thus the 90-kDa band is correlated specifically with the induction of adhesiveness and not the induction of germination.

Labeling of the 90-kDa band with ConA indicates the band is glycosylated. To further characterize the 90-kDa band, adhesion-competent macroconidia were incubated with proteases. The 90-kDa band was removed efficiently by either pronase E or proteinase K (Fig. 4). The band was reduced in intensity by trypsin. Thus, the 90-kDa band is a glycoprotein.

We examined the effect of 500 µg of proteases per milliliter on adherent conidia. Pronase E and proteinase K caused adherent macroconidia to detach (Table 5). Con-



idia treated with either pronase E or proteinase K were significantly ( $P < 0.01$ ) less adherent than their heat-denatured controls. However, pronase E was more effective than proteinase K; pronase E- and proteinase K-treated conidia adhered 10 and 78% of their heat-denatured controls, respectively. Trypsin did not cause adherent conidia to detach.

Two-dimensional (2-D) gel electrophoresis indicated that the 90-kDa band contains two ConA-binding compounds with isoelectric points of 5.5 and 6.5 (Fig. 5). As expected, the adhesion-incompetent macroconidia incubated in water did not produce any compounds with a 90-kDa molecular weight.

## DISCUSSION

Within minutes after exposure to its plant host extract, *N. haematococca* macroconidia become adhesion competent (Jones and Epstein 1989), produce ConA-labeled mucilage at the spore apex, adhere at the macroconidial apex, and produce two ConA-labeled 90-kDa glycopeptides. In media that do not induce adhesion competence but do support germination, the macroconidia neither produce ConA-labeled macroconidial tip mucilage nor the 90-kDa glycopeptides. Thus the production of the 90-kDa band is associated with the induction of adhesion competence. Pronase E and proteinase K both remove adherent macroconidia from polystyrene and the 90-kDa band; trypsin is relatively ineffective in removing adherent macro-

conidia from polystyrene and the 90-kDa band. Thus, both the 90-kDa band and the adhesive material are proteinaceous, sensitive to pronase E and proteinase K, and relatively insensitive to trypsin. The fact that ConA specifically inhibits adhesion is the strongest evidence that at least one of the 90-kDa glycopeptides might be the fungal adhesive compound. However, further work is required to conclusively demonstrate that the 90-kDa glycopeptides 1) are present in the macroconidial tip mucilage and 2) have adhesive properties. Nonetheless, regardless of whether the 90-kDa glycopeptides merely are associated

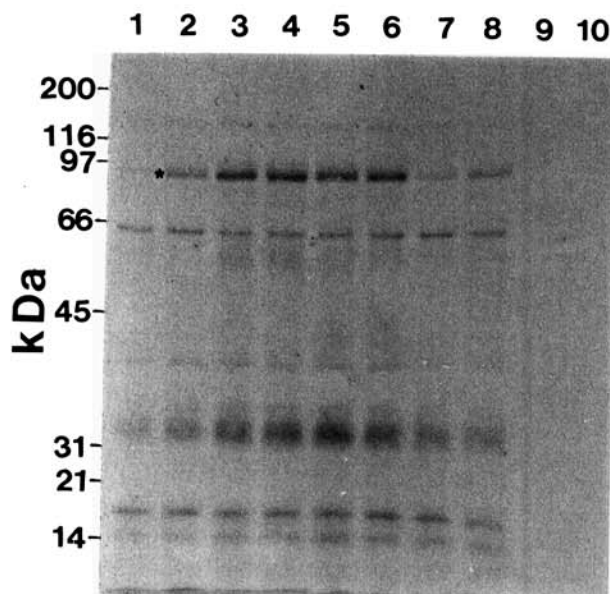


Fig. 3. Cell surface compounds were extracted from *Nectria haematococca* macroconidia and examined on a western blot probed with ConA. The molecular weights of standards are shown on the left side. The asterisk indicates the 90-kDa band present only in treatments in which the macroconidia adhered to the substratum. Lane 1, macroconidia were incubated in water. Lanes 2–5, macroconidia were incubated in zucchini extract for 5, 10, 30, and 60 min, respectively. Lanes 6–8, macroconidia were incubated for 30 min in potato-dextrose broth (lane 6), V8 juice broth (lane 7), and minimal medium (lane 8). Lanes 9 are 10 are zucchini extract and potato-dextrose broth controls, respectively.

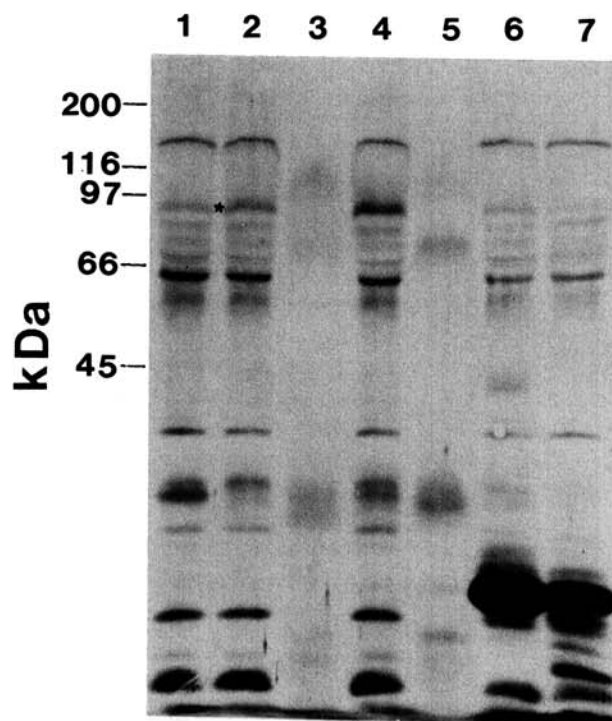


Fig. 4. After adherent *Nectria haematococca* macroconidia were treated with 500 µg/ml proteases for 30 min at 23° C, cell surface compounds were extracted and examined on a Western blot probed with ConA. The molecular weights of standards are shown on the left side. The asterisk indicates the 90-kDa band associated with adhesion. Lane 1, no enzyme control; 2, heat-denatured pronase E; 3, pronase E; 4, heat-denatured proteinase K; 5, proteinase K; 6, trypsin and 500 µg/ml trypsin inhibitor; 7, trypsin.

Table 5. Effect of proteases on adhesion of *Nectria haematococca* macroconidia

Treatments <sup>a</sup>	Adhesion (%) <sup>b</sup>
Control	73 ± 6
Pronase E	7 ± 6
Heat-denatured pronase E	71 ± 4
Proteinase K	61 ± 4
Heat-denatured proteinase K	78 ± 3
Trypsin	95 ± 0.3
Trypsin inhibitor	94 ± 0.03
Trypsin and trypsin inhibitor	95 ± 0.3

<sup>a</sup> Conidia were incubated for 30 min in polystyrene dishes containing zucchini extract. After the zucchini extract was decanted, the adherent conidia were incubated for 1 hr in 10 mM HEPES (pH 7.5), 2 mM CaCl<sub>2</sub> and 500 µg/ml of the indicated compounds.

<sup>b</sup> Values are the means ± SD of two replicates. LSD (0.05) = 9. LSD (0.01) = 13. The experiment was repeated with similar results.

with the development of adhesion competence or actually are the adhesive material, we note that the production of macroconidial tip mucilage, adhesion, and the production of the 90-kDa band are very early events in the germination process; after addition of a suitable medium, these events occur within 10 min. Germ tubes do not emerge before three or more hours (data not shown).

The estimation of the molecular weight of the 90-kDa band may be dependent on the polyacrylamide concentration. We calculated an apparent molecular mass of 86 and 90 kDa in 10 and 12.5% gels, respectively. Such acrylamide concentration-dependent variation in molecular weight estimates maybe due to the carbohydrate portion of the glycoprotein (Hames 1990).

Several lines of evidence suggest that the *N. haematococca* adhesive compound is a glycoprotein. First, proteins in the fungal wall and extracellular matrix generally are glycosylated. Second, pronase E and proteinase K but not heat-denatured controls removes adherent spores. Third, the adhesive compounds on plant pathogenic fungi appear to be glycoproteins (Estrada-Garcia *et al.* 1990; Gubler and Hardham 1988; Gubler *et al.* 1989; Sing and Bartnicki-Garcia 1975). More specifically, ConA apparently binds to the adhesive material on conidia of *Magnaporthe grisea* (Hamer *et al.* 1988), and encysting zoospores of *P. palmivora* (Sing and Bartnicki-Garcia 1975).

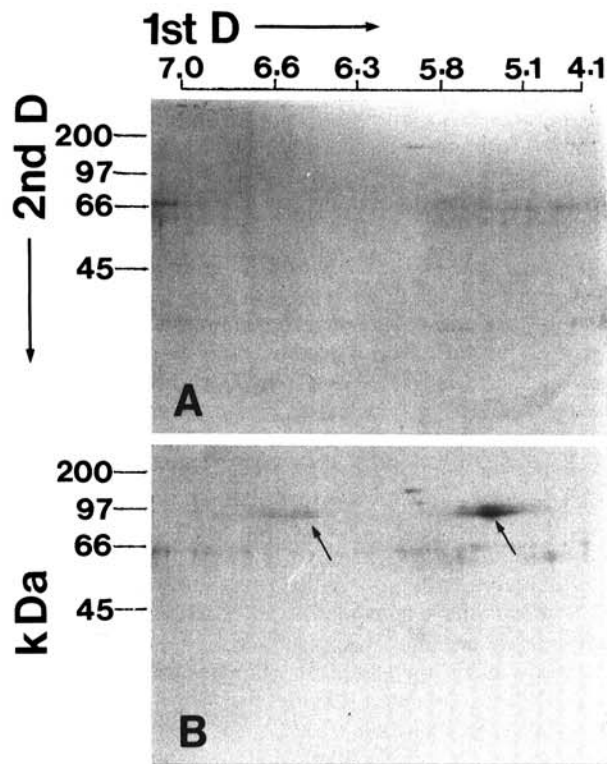


Fig. 5. Cell surface compounds were extracted from *Nectria haematococca* macroconidia, separated by two-dimensional gel electrophoresis and examined on a Western blot probed with ConA. Isoelectric points and molecular weights of standards are shown on the horizontal axis and vertical axis, respectively. Macroconidia were incubated for 30 min in either A, water or B, zucchini extract. Arrows point to the two 90-kDa compounds.

The fact that adhesion is induced by zucchini extract and by potato-dextrose broth but not by V8 broth suggests that while the adhesion-inducing compound is not host specific, it also may not be present in all plants. Although potato-dextrose broth is an efficient inducer of adhesiveness and of the 90-kDa band, it is a less effective inducer of ConA-labeled macroconidial tip mucilage. An organic carbon source is not always required for the induction of adhesiveness in plant pathogenic fungi. For example, only water is required for adhesion of conidia of *M. grisea* (Hamer *et al.* 1988) and *Colletotrichum musae* (Sela-Buurlage *et al.* 1991), and urediospores and germlings of rust fungi (Beckett *et al.* 1990; Chubal *et al.* 1991; Epstein *et al.* 1985).

Our data indicate that the development of adhesion competence is not a prerequisite for germ tube emergence since V8 broth and a defined minimal medium support germination but do not support either the production of macroconidial tip mucilage or adhesion. The adhesion-inducing compound(s) will have to be identified before we can determine if the same compounds that induce adhesion competence also support germ tube emergence.

## MATERIALS AND METHODS

### Preparation of macroconidia and media.

Macroconidia of *N. haematococca* were stored, produced, and harvested as previously described (Jones and Epstein 1989, 1990). Briefly, macroconidia were seeded onto potato-dextrose agar petri dishes and incubated at 20° C for 7 days under fluorescent light. After flooding the culture dishes with distilled water, the macroconidia were collected, washed twice in 30 ml of water by centrifugation at 200 × g for 2 min, and resuspended in water.

The macroconidia of *N. haematococca* were incubated in various media. Zucchini fruit extract (ZE) was prepared as described previously (Jones and Epstein 1989). Zucchini exudate was made by submerging whole, firm fruits in water (100 g of fruit in 100 ml of water) at 23° C for 2 hr. After removing the fruits, the exudate was autoclaved. Minimal medium contained 20 mM glucose, 2.7 mM asparagine, 2.9 mM K<sub>2</sub>HPO<sub>4</sub>, 3.7 mM KH<sub>2</sub>PO<sub>4</sub>, 5.3 mM Na<sub>2</sub>SO<sub>4</sub>, 0.73 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.6 mM KCl, 0.54 mM CaCl<sub>2</sub>, 0.38 mM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> · 6H<sub>2</sub>O, 0.18 mM MnSO<sub>4</sub>, 0.1 mM ZnSO<sub>4</sub> · H<sub>2</sub>O, 0.032 mM CuSO<sub>4</sub> · 5H<sub>2</sub>O; to prepare the medium, an autoclaved mineral solution was added to a filter-sterilized solution of glucose and asparagine. Potato-dextrose broth, V8 broth, and Czapek Dox were prepared as described by Tuite (1969).

### Adhesion and germination assays.

One milliliter of macroconidial suspension containing 1–2 × 10<sup>5</sup> spores was added to 7 ml of either ZE or other media in a 5-cm-diameter polystyrene petri plate (Fisher) and incubated at 23° C. For adhesion assays, spores were incubated, generally for 30 min, then the dishes were gently swirled, decanted, and rinsed twice with 5 ml of water. The recovered nonadherent spores were suspended in a counting medium with a final concentration of 0.02% Tween 20 and 100 mM KCl. The number of nonadherent macroconidia recovered from the petri dishes and the

number of spores originally added to the petri dishes were determined with a particle counter (Elzone model 80, Particle Data Inc., IL) equipped with a 150- $\mu$ m-diameter orifice and a 1-ml sampling tube.

For germination assays, macroconidia were incubated as described above, but for 6 hr. Percentage of germination was assessed *in situ* at 160 $\times$  with a Zeiss microscope.

#### FITC-lectin labeling.

To induce adhesion, 50  $\mu$ l of a suspension containing approximately  $2.5 \times 10^5$  macroconidia was incubated on a glass coverslip (Fisher) with 200  $\mu$ l of ZE. Generally, after 30 min, the zucchini extract was wicked away and the adherent macroconidia were fixed in 3% formaldehyde, buffered with 50 mM piperazine-*N,N'*-bis(2-ethane sulfonic acid, (PIPES), pH 7.0. After a brief rinse in phosphate-buffered saline (PBS), the coverslip was put cell-side down on a glass slide containing 50  $\mu$ l of a fluorescein isothiocyanate (FITC)-conjugated lectin. The lectins listed in Table 1 were obtained from Sigma and used in a concentration series between 20  $\mu$ g and 2 mg per milliliter in a solution of 0.125 $\times$  PBS with 12.5  $\mu$ M  $\text{CaCl}_2$  and 12.5  $\mu$ M  $\text{MnCl}_2$ . Following a 30-min incubation in darkness, the macroconidia were examined using a Nikon photomicroscope with fluorescence optics. Images shown in Figure 1 were recorded on Kodak Tri-X Pan 400 film at 1600 ASA.

To examine agglutinated macroconidia, we rinsed an adhesion assay plate and then gently removed adherent spores with a camel hair brush. The dislodged macroconidia were transferred to a coverslip, and incubated with FITC-concanavalin A (ConA) as indicated above.

Although ConA binds specifically to  $\alpha$ -D mannosyl and  $\alpha$ -D glucosyl residues, other haptens also can compete for ConA (Goldstein and Poretz 1986). To determine which haptens might be competing for ConA, compounds listed in Table 2 were incubated for 30 min with 2 mg per milliliter of FITC-ConA before macroconidia were added.

#### Effects of ConA on adhesion and germination.

Because FITC-ConA binds to macroconidial tip mucilage, we determined if ConA blocks adhesion of macroconidia. For the experiment shown in Figure 2, ConA was dissolved in zucchini extract by sonicating briefly. Adhesion and germination assays were conducted as described above. To confirm that ConA was specifically affecting adhesion, ConA and its hapten  $\alpha$ -methyl mannoside were dissolved in PBS after a brief sonication. For the experiment shown in Table 3, the final incubation mixture contained 0.125 $\times$  PBS, (pH 6.8), 12.5  $\mu$ M  $\text{CaCl}_2$ , 12.5  $\mu$ M  $\text{MnCl}_2$ , and 0.75 $\times$  ZE.

#### Effect of proteases on adhesion.

After conidia were incubated for 30 min in ZE, the ZE was decanted and the adherent conidia were incubated at 23 $^\circ$  C for 1 hr in 10 mM (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethane sulfonic acid]) (HEPES), pH 7.5, 2 mM  $\text{CaCl}_2$  and 500  $\mu$ g/ml of either pronase E, proteinase K, or trypsin (all from Sigma). Controls included: 1) no enzyme; 2) heat denatured (45 min, 90 $^\circ$  C) pronase E or proteinase K;

- 3) 500  $\mu$ g/ml trypsin inhibitor and 500  $\mu$ g/ml trypsin; and
- 4) 500  $\mu$ g/ml trypsin inhibitor.

#### Preparation of macroconidia for Western analyses.

*N. haematococca* macroconidia (approximately  $4 \times 10^6$  per dish) were incubated at 23 $^\circ$  C in 8.5-cm-diameter polystyrene petri dishes containing 15–20 ml of media. Six to ten dishes were used for each experimental treatment. Unless indicated otherwise, conidia were incubated for 30 min. After the medium was decanted, the dishes were rinsed twice with 10 ml of water. Adherent macroconidia were gently dislodged with a camel hair brush while submerged in water and then collected and centrifuged at 800  $\times$  g for 5 min. Spore pellets were suspended in PBS, pH 6.8, transferred to a microcentrifuge tube, and centrifuged at 8,000  $\times$  g for 3 min. While this technique was developed for the recovery of adherent macroconidia, stereo and standard microscopy was used to monitor spore collection.

When indicated, a 100- $\mu$ l spore pellet was incubated at 23 $^\circ$  C for 20 min in 500  $\mu$ l of 500  $\mu$ g/ml of either pronase E, proteinase K, or trypsin and 10 mM Tris buffer (pH 7.8), 2 mM  $\text{CaCl}_2$  and 0.05% Triton X-100. Controls included: 1) no enzyme; 2) heat denatured (30 min, 90 $^\circ$  C) pronase E or proteinase K; and 3) 500  $\mu$ g/ml trypsin inhibitor and 500  $\mu$ g/ml trypsin.

#### Gel electrophoresis and Western blots.

A 50- to 100- $\mu$ l macroconidial pellet was incubated at 100 $^\circ$  C for 5–10 min in an equal volume of Laemmli buffer (625 mM Tris-HCl [pH 6.8], 2% SDS, 5%  $\beta$ -mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue). For control treatments of media without macroconidia, 5 ml of either zucchini extract or potato-dextrose broth were freeze-dried and then solubilized in 400  $\mu$ l of Laemmli buffer. Biotinylated high (45–205 kDa) and low (18.5–106 kDa) molecular weight standards (Bio-Rad), and 10- and 30- $\mu$ l aliquots of control and sample supernatants, respectively, were separated by SDS-polyacrylamide gel electrophoresis; 10% gels were run overnight at a constant 60 V.

A cell surface-enriched fraction also was separated by two-dimensional (2-D) gel electrophoresis (Dunbar 1987). For the isoelectric focusing gel, the macroconidial pellet was extracted at 100 $^\circ$  C for 10 min in 50 mM (2-[*N*-cyclohexyl amino]ethane sulfonic acid) (CHES) buffer (pH 9.0), 2% SDS, 2%  $\beta$ -mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue. The extractant was isoelectrically focused with a Bio-Rad Protean II xi apparatus; manufacturer's recommended procedures were followed. For 2-D gels, water- and zucchini extract-treated macroconidia were run simultaneously.

Blotting onto nitrocellulose (Towbin *et al.* 1979) was performed at a constant 450 mA for 3 hr at 23 $^\circ$  C. After blots were blocked with 0.1% gelatin in Tris-buffered saline with Tween 20 (TBST: 10 mM Tris-HCl [pH 7.4], 140 mM NaCl, 0.5% Tween 200) for 1 hr, the blots were incubated with ConA-biotin (Sigma) (20  $\mu$ g/ml in TBST) for 1 hr. After washing three times with 200 ml of TBST, the blot was treated with 2  $\mu$ g/ml streptavidin-conjugated alkaline phosphatase (either Bio-Rad or Pierce, Rockford, IL) for 30 min. Following three washes with TBST, color

was developed using 5-bromo-4-chloro-3-indoyle phosphate (BCIP) and para-nitro blue tetrazolium chloride (NBT).

### Experimental design.

Adhesion and germination assays were conducted with either two or three replicates. Except when indicated, for quantitative data, results from two to five independent replicated trials were analyzed by an analysis of variance as a randomized complete block design with experimental trials as blocks. All experiments with data which were evaluated visually were performed at least three times.

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