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# Adhesion of conidia and germlings of the plant pathogenic fungus *Bipolaris sorokiniana* to solid surfaces

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Soon after coming in contact with its host, the plant pathogenic fungus *Bipolaris sorokiniana* produces an extracellular material that appears to be important for adhering conidia and germlings to the host surface. To further understand this step of the infection, the adhesion of *B. sorokiniana* to artificial solid surfaces was examined. On a hydrophobic (polystyrene) surface adhesion occurred in two stages, the first by conidia and the second by germlings. Conidial adhesion occurred shortly (0–1 h) after hydration. The conidia were easily detached by increasing the shear force and including detergents in the washing buffer. As conidia were hydrophobic, these observations indicate that conidial adhesion to polystyrene is due to weak, hydrophobic interaction. The second stage of adhesion was accompanied by conidial germination and occurred 1–2 h after hydration and contact with the surface. Concomitant with the delayed adhesion, the fungus produced an extracellular matrix (ECM). The adhesion of germlings was firm and surface-unspecific since they adhered to both hydrophobic and hydrophilic (glass) surfaces. Except for strong bases, hydrochloric acid and broad-specificity proteases (including Pronase E), none of the hydrolytic enzymes, electrolyte solutions, ionic and hydrophobic detergents and organic solvents removed germlings from the solid surfaces. The adhesion of germlings incubated in the presence of the protein glycosylation inhibitor tunicamycin or the lectins Con A (Concanavalin A) and GNA (from *Galanthus nivalis*) was significantly reduced, which indicates the involvement of surface glycoproteins in this process. The surface proteins of germlings were labelled with $^{125}$I, extracted and analysed by two-dimensional gel electrophoresis. This revealed about 40 surface proteins over a wide pH range (4–10) with molecular masses between 10 and 100 kDa.

## INTRODUCTION

Adhesion to the host surface is thought to be an important step in fungal infection of plants (Epstein & Nicholson 1997). For example, Jones & Epstein (1990) showed that an adhesion-deficient mutant of the fungus now called *Haematonectria haematococca* had lower virulence than the wild type. In the grape pathogen *Phyllosticta ampelicida* adhesion of conidia is an absolute requirement for germination and subsequent infection (Kuo & Hoch 1996). It has also been suggested that the thigmotrophic growth in many leaf pathogens is dependent on the ability of the fungus to grow in close association with the plant surface (Epstein & Nicholson 1997, Staples & Hoch 1997). Furthermore, the appressorium of the rice-blast fungus *Magnaporthe grisea* has to be tightly attached to the host surface during penetration of the plant cuticle to withstand the enormous turgor pressure needed to penetrate the host surface (Howard *et al*. 1991).

Conidial adhesion can be accomplished by several mechanisms. *M. grisea* contains pre-synthesized material in the conidial apex that is released upon hydration, at the time when the tip of the conidium becomes anchored to the substratum (Hamer *et al*. 1988). *H. haematococca* synthesizes a spore tip material that is temporarily associated with attachment of the spore to the host plant (Jones & Epstein 1989). Many other fungi produce conidial mucilages when contacting a substratum (Sela-Buurlage, Epstein & Rodriguez 1991, Clement *et al*. 1993, Nicholson & Kunoh 1995, Kuo & Hoch 1995). Conidial attachment of *Botrytis cinerea* is mediated by interactions between the very hydrophobic conidial surface and a hydrophobic substratum, like leaf cuticle or polystyrene (Doss *et al*. 1993). In addition, attachment of urediospores of *Uromyces viciae-fabae* and some other fungi, at least in part, are known to involve hydrophobic forces (Young & Kauss 1984, Hamer *et al*. 1988, Doss *et al*. 1993, Kuo & Hoch 1996). The adhesion of germ tubes and appressoria of most plant pathogenic fungi, is associated with the production of an extracellular matrix (ECM) (Evans, Stempen & Frasca 1982, Chaubal, Wilmot & Wynn 1991, Ben-Naim & Yaacobi 1974, Doss *et al*. 1995, Cole, Dewey & Hawes 1996, Kuo & Hoch 1995, Apoga & Jansson 2000). However, little is known about the actual mechanisms and molecules involved in the adhesion of fungal pathogens to their host surface.

*Bipolaris sorokiniana* (syn. *Helminthosporium sativum* teleomorph *Cochliobolus sativus*) is a severe pathogen on grasses and causes root rot and leaf spot diseases mainly in barley, wheat, and oat. The fungus is unspecific regarding host range and the location of infection of the plant. *B. sorokiniana* is

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known to produce a number of phytotoxic metabolites, and it has recently been shown that the *in vitro* production of the toxin prehelminthosporol correlates with the degree of virulence of the fungus (Apoga 2000). Despite several studies on the mechanism of infection and pathogenicity factors of the fungus, there is little knowledge on the early interactions between the pathogen and the host substratum. However, it is known that conidia of *B. sorokiniana* soon after contacting a barley leaf surface, release a conidial mucilage, as observed using Cryo-SEM (Apoga & Jansson 2000). Furthermore, it has repeatedly been observed that the germ-tubes of *B. sorokiniana* are surrounded by an extracellular matrix (ECM), and it has been suggested that this material is important in adhering the fungus to the host surface (Pringle 1981, Evans *et al*. 1982, Carlson *et al*. 1991a, Apoga & Jansson 2000).

In the present study, the adhesion of conidia and germlings of *B. sorokiniana* to solid surfaces has been examined in detail. Furthermore, evidence is presented indicating the involvement of extracellular glycoproteins in the adhesion of germ-tubes.

## MATERIALS AND METHODS

### *The fungus*

The strain of *Bipolaris sorokiniana* (isolate Tellus) was isolated from diseased barley (*Hordeum vulgare* cv. 'Tellus') in Sweden (Landskrona, W Weibull AB) by Carlson *et al* (1991a) and is stored in the culture collection of the Department of Microbial Ecology, Lund University. Conidia were collected from 7 to 14 d old colonies grown on a defined agar medium (Carlson *et al*. 1991b).

### *Germination experiments*

Droplets (50 µl) of a conidial suspension ($2.0 \times 10^4$ conidia $ml^{-1}$) in water or 2.4% (w/v) potato dextrose broth (PDB, Difco) were applied on pre-cleaned glass multi-well slides (Kebo, Sweden) or on the surface of polystyrene Petri dishes. The numbers of germinated conidia on the solid surfaces were counted in an inverted light microscope. Germination was also studied in bulk medium by incubating 25 ml of the conidial suspension in 50 ml Falcon tubes on a bottom-up-bottom mixer. The significance of the difference between germination on surface and in bulk medium was assessed by ANCOVA test (analysis of covariance with germination as dependent variable, and the incubation time as co-variable). To test the effects of Pronase E on germination, the enzyme was added to conidia incubated in 10 mM Tris buffer (pH 7.4) containing 0.02% (w/v) PDB.

### *Adhesion assay and detachment experiments*

Conidia were suspended in 2.4% PDB or water and applied onto the glass or polystyrene surfaces as described above.

**Table 1.** Detachment of germlings of *Bipolaris sorokiniana* adhered to solid surfaces (polystyrene or glass) by chemicals and detergents[a].

| Treatment[b] | Detachment (%)[c] Polystyrene | | Glass | | ECM[d] |
|---|---|---|---|---|---|
| Strong bases | | | | | |
| KOH (1 M) | 67.3 ± 6.8 | (7)*** | 39.0 ± 10.1 | (18)*** | ± |
| $NH_4OH$ (10%, v/v) | 57.6 ± 11.8 | (4)*** | 64.0 ± 7.2 | (4)*** | + |
| TEA (10%, v/v) | 61.1 ± 14.7 | (7)*** | 58.9 ± 13.8 | (26)*** | + |
| Other chemicals | | | | | |
| HCl (1 M) | 4.2 ± 8.0 | (12) | 17.3 ± 8.3 | (18)** | + |
| LiCl (5 M) | 6.7 ± 12.2 | (13) | 4.2 ± 12.2 | (23) | + |
| Urea (4 M) | 16.1 ± 7.3 | (8) | 6.3 ± 11.4 | (14) | + |
| DMSO (10%, v/v) | −0.6 ± 9.7 | (13) | 6.6 ± 8.0 | (19) | + |
| Detergents (1.5%, w/v) | | | | | |
| CHAPS | 10.9 ± 5.1 | (6) | 5.5 ± 5.2 | (11) | ± |
| CTAB | 4.3 ± 5.8 | (6) | 4.3 ± 5.8 | (4) | ± |
| SDS | 9.1 ± 8.7 | (6) | 8.8 ± 10.9 | (8) | + |
| Tween 20 | 4.8 ± 9.9 | (6) | 6.4 ± 11.1 | (8) | + |
| Triton X 100 | nt | | 6.5 ± 9.7 | (7) | + |
| DTAB | nt | | 7.5 ± 6.6 | (8) | + |
| Controls | | | | | |
| MOPS (10 mM, pH 7.2) | 11.0 ± 4.8 | (9) | 4.9 ± 8.0 | (9) | + |
| Water | 0.5 ± 10.3 | (6) | 6.3 ± 7.2 | (29) | + |

[a] Conidia were germinated in 2.4% PDB on glass or polystyrene surfaces for 3.5 h, then washed with MOPS or water. Germlings adhered to the surfaces were treated overnight at room temperature (continuously agitating at 75 rev $min^{-1}$) with the chemicals and detergents. Thereafter, the samples were washed 2 × 30 ml MOPS or water.

[b] TEA, triethylamine; CHAPS, 3-((3-chloramidopropyl)-dimethyl-ammonio)-1 propane-sulfonate; CTAB, cetyldimethylammonium bromide; SDS, sodium dodecyl sulphate; DTAB, dodecyltrimethyl-ammonium bromide; MOPS, 3-(N-morpholino) propanesulphonic acid.

[c] The percentage of detached germlings was calculated according to the numbers of germlings attached on a surface before and after treatments. Mean ± SD (*n*). nt = not tested. The significance of difference between the treatment and corresponding control was tested using ANOVA with $^{**}P < 0.005$ and $^{***}P < 0.001$. Controls were treatments of germlings with water or MOPS buffer on corresponding surface. MOPS was control for detergent treatments and water for treatments with chemicals.

[d] ECM was labelled with Au/Ag and examined with a light microscope. Owing to extensive labelling background on polystyrene, only the samples on the glass surface were examined. +ECM, was present and did not differ from control; ±ECM, was present but did differ in appearance from control. Controls were germlings treated with MOPS buffer or water.

**Table 2.** Detachment of germlings of *Bipolaris sorokiniana* adhered to glass surface by different enzymes[a].

| Enzymes[b] | Buffer | Total concn (mg ml$^{-1}$) | Germlings detached[e] | ECM[f] |
|---|---|---|---|---|
| Proteases | | | | |
| Protease | Tris/HCl (10 mM, pH 7.4) | 1.1 | + | ± |
| Pronase E | Tris/HCl (10 mM, pH 7.4) | 1.1 | + | ± |
| Collagenase | Tris/HCl (10 mM, pH 7.4), $CaCl_2$ (4 mM) | 11.0 | − | + |
| Leucine aminopeptidase | Phosphate (60 mM, pH 7.2) | 5.6 | − | + |
| Pepsin | HCl (10 mM, pH 2.0) | 12.2 | − | + |
| Trypsin | Tris/HCl (20 mM, pH 8.0) | 11.8 | − | + |
| Exo-polysaccharidases | | | | |
| β-N-Acetylglucosaminidase | Citrate/phosphate (10 mM, pH 4.5) | 1.25 U[d] | − | + |
| α-Amylase | Phosphate (10 mM, pH 7.0) | 11.0 | − | + |
| Cellulase | Acetate (50 mM, pH 5.0) | 10.0 | − | + |
| Chitinase | Phosphate (10 mM, pH 6.0) | 9.4 | − | + |
| β-Galactosidase | Tris/HCl (5 mM, pH 7.4) | 1000 U[d] | − | + |
| α-Glucosidase | Phosphate (10 mM, pH 6.8) | 12.2 | − | + |
| β-Glucosidase | Acetate (10 mM, pH 5.1) | 12.2 | − | + |
| α-Mannosidase | Acetate (10 mM, pH 4.5) | 4.0 U[d] | − | + |
| Endo-polysaccharidases | | | | |
| endo-β-Galactosidase | Acetate (50 mM, pH 5.8) | 1.0 U[d] | − | + |
| N-Glycosidase A | Acetate (10 mM, pH 5.0) | 0.01 U[d] | − | + |
| Others | | | | |
| Novozyme 234[c] | Phosphate (10 mM, pH 6.8) | 10.5 | + | ± |
| Laminarinase | Acetate (10 mM, pH 5.5) | 7.5 | − | + |
| Neuraminidase | Acetate (10 mM, pH 5.5) | 6.0 | − | + |
| Lipase | Tris/HCl (10 mM, pH 7.4) | 14.0 | − | + |
| Esterase | Tris/HCl (50 mM, pH 8.0) | 8.7 | − | + |

[a] Conidia were germinated in 2.4% PDB on a glass surface for 3.5 h, washed in appropriate buffer and treated with the enzymes for 2 h at 25 or 37 °C (according to manufacturer's recommendations). Thereafter, the samples were washed 2 × 30 ml with a buffer.

[b] Protease from *Streptomyces griseus* (Sigma); pronase E (Merck); α-amylase, endo-β-galactosidase and N-glycosidase A (Boehringer Mannheim); and Novozyme 234 (Novozyme A/S, Denmark. Other enzymes were obtained from Sigma.

[c] Containing chitinase, cellulase and protease activity.

[d] Units (U) ml$^{-1}$, as given by the manufacturer.

[e] + Detachment of germlings. − No detachment of germlings in comparison with controls as observed in light microscope. Two controls for each treatment were set up, germlings treated with buffer alone and germlings treated with heat denaturated (95 °, 10 min) enzyme. Experiments were performed with three replicates.

[f] ECM was labelled with Au/Ag and examined using light microscope. + ECM was present and did not differ from control, ± ECM was present but differed in appearance from control. The control was germlings treated with buffer only.

Following incubation for 0–4 h, the surfaces were washed by adding 30 ml of PBS (10 mM sodium phosphate buffer, pH 7.4 and 0.15 M NaCl), 1.5% (v/v) Triton X 100 (in PBS), or water. After agitation (100 rev min$^{-1}$, 5 min) washing buffer was decanted and the attached conidia and germlings were fixed in 3% (v/v) glutaraldehyde (in PBS) and counted using a video equipped light microscope. To investigate the strength of the adhesion, adhered conidia and germlings were washed with 30 ml of PBS or water, varying the rate of agitation (0, 100, or 200 rev min$^{-1}$) and the number of washing (0, 1, 3, or 6 times). In the detachment experiments, adhered germlings were treated with various chemicals, detergents and lytic enzymes as described in Tables 1–2. After washing, the remaining germlings were counted (per unit area) using a video equipped light microscope.

### *Hydrophobicity test*

The hydrophobicity of conidia was assessed using a two-phase system (Rosenberg, Gutnick & Rosenberg 1980). Conidia ($2.3 \times 10^6$ conidia ml$^{-1}$) were mixed with *n*-octanol, and the samples were vigorously vortexed for 2 min. The two phases were allowed to separate and the numbers of conidia present in the water phase were counted (Fuchs–Rosenthal counting-chamber). Hydrophobicity was expressed as the percentage of conidia present in the organic phase related to the total number of added conidia.

### *Visualization of ECM*

ECM of germlings was labelled with colloidal gold followed by silver enhancement (Au/Ag) and visualized using a light microscope. In some experiments, the samples were also stained with calcofluor white (CFW) and acid fuchsin (Apoga & Jansson 2000).

### *Inhibitors and lectins*

Conidia were suspended in 0.02% PDB containing different biochemical inhibitors and lectins at different concentrations (see below). After incubation for 3.5 h, germination, adhesion and hyphal length were quantified. The percentage attachment was calculated by relating the number of adhered germlings to the total number of germinated conidia. Average germ tube length was determined from 100 germlings of four replicates.

The following inhibitors were used: nikkomycin Z (Calbiochem), an inhibitor of chitin synthesis (used at concentrations 0.002, 0.02, and 0.2 μg ml$^{-1}$); tunicamycin

(Calbiochem), inhibitor of protein glycosylation (0.1, 1.0, 10, and 50 μg $ml^{-1}$); hygromycin (Calbiochem), inhibitor of protein translation (0.05, 0.5, 5.0, and 50 μg $ml^{-1}$); brefeldin A (Sigma), inhibitor of glycoprotein transport (0.1, 1.0, and 10 μg $ml^{-1}$); and sodium azide ($NaN_3$), inhibitor of respiration (2, 20, and 200 μg $ml^{-1}$).

The lectin Concanavalin A (Con A) was tested at concentrations 0.4, 2.0, 10, 50, 250, 1000 μg $ml^{-1}$, the *Galanthus nivalis* lectin (GNA) at 125, 250, 500, 750, 1000 μg $ml^{-1}$, and wheat germ agglutinin (WGA) at 250, 500, 750, 1000, 2000 μg $ml^{-1}$ in 0.02% PBD. PBS, the buffer normally used for lectin assays, was not used in this experiment because PBS itself inhibited the adhesion. Hapten experiments were done by pre-incubating (45 min) Con A with 2.5 mg $ml^{-1}$ of mannose, methyl-α-mannopyranoside, glucose, methyl-α-glucopyranose or galactose. All hapten sugars were tested separately for their effect on germling adhesion.

### *Labelling and extraction of germling surface proteins*

Approximately $4.5 \times 10^6$ conidia were germinated in 2.4% PDB for 4 h at room temperature on a bottom-up-bottom mixer. The germlings were pelleted by centrifugation, washed and re-suspended in 600 μl PBS (ice-cold). Surface proteins of the germlings were radiolabelled by adding 130 μl of $^{125}$I-labelled Bolton–Hunter reagent (18.5 MBq) (Thompson, Lau & Cunningham 1987). After incubation on ice (30 min), 1 ml lysine (1 mg $ml^{-1}$ in PBS) was added to stop the reaction, and the sample was washed $3 \times 1$ ml PBS and 1 ml of deionized water. The germlings were suspended in 300 μl of an IEF dry strip rehydration medium (2 M thiourea, 8 M urea, 20 mM Tris base, 4% 3-((3-chloramidopropyl)-dimethylammonio)-1 propane-sulfonate (CHAPS), 65 mM DTT, 2% Immobiline DryStrip gel (IPG) sample buffer, pH 3–10 non-linear (NL). All chemicals were from Pharmacia Biotech. PMSF (phenylmethylsulphonyl fluoride) was added (final concentration 2 mM) and the germlings were homogenized using an ultrasonication probe (Vibra-cell model VC 50) on ice for $2 \times 15$ s. The homogenate was centrifuged, and the protein concentration in the supernatant was adjusted to about 0.5 mg $ml^{-1}$ using the rehydration medium as dilutant.

### *Two-dimensional gel electrophoresis*

Proteins were focused in the first dimension using non-linear pre-cast immobilized pH gradient gel strips (IPG, 18 cm, pH 3–10 NL, Pharmacia Biotech). Dry gel strips were rehydrated with the protein samples (170–190 μg protein per strip), and isoelectric focusing was performed in a horizontal electrophoresis apparatus (Multiphor II, Pharmacia Biotech) (300 V, 1 h; 3500 V for a total of 50–80 kVh). After focusing, the IPG strips were equilibrated in a SDS equilibration solution (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS and trace of bromophenol blue) supplemented with 2% DTT (v/v) followed by equilibration for another 20 min in the SDS equilibration solution supplemented with 4.5% (v/v) iodoacetamide. The equilibrated strips were applied to an acrylamide gradient gel (ExcelGel SDS gel, Pharmacia Biotech). The gels were silver-stained (silver staining kit, Pharmacia Biotech). Before exposure to X-ray films, the gels were soaked in a storage solution (ethanol/glycerol/water, 75:10:165 (v/v/v)).

## RESULTS

### *Germination of conidia*

When incubated in water, the germination of *Bipolaris sorokiniana* conidia was significantly higher on solid surfaces (glass or polystyrene) than in the bulk medium ($P < 0.001$, ANCOVA) (Fig. 1). A similar difference in germination between the surface and bulk medium was not observed when the conidia were incubated in PDB.

### *Adhesion of conidia*

Conidia of *Bipolaris sorokiniana* adhered to the polystyrene surface shortly (0–1 h) after hydration but not to the glass surface (Figs 2–3). The adhered conidia were easily detached when increasing the shear force by agitation or by repeating

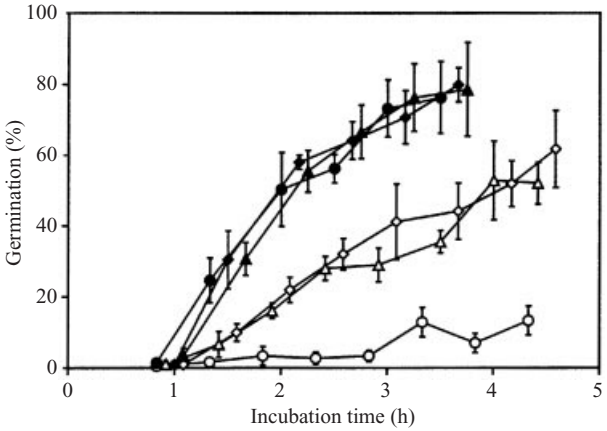
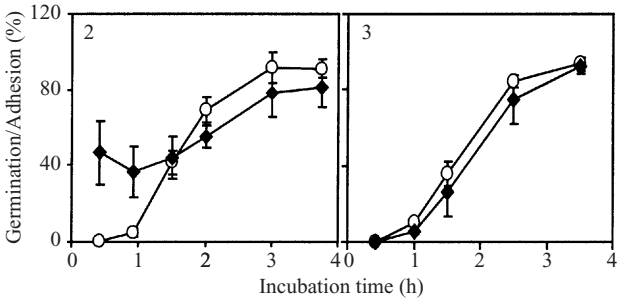
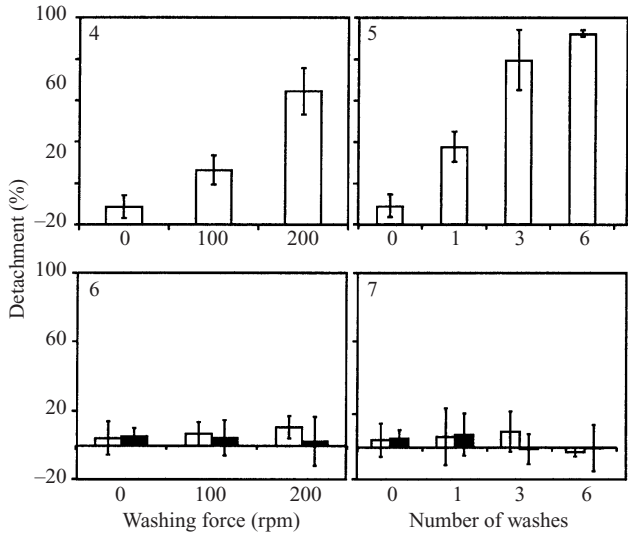



**Fig. 1.** Germination of conidia of *Bipolaris sorokiniana* in water (empty symbols) and PDB (filled symbols). The conidia were suspended in a bulk medium (●) (○), incubated on a glass surface (△) (▲), or a polystyrene surface (◇) (◆). The percentage of germination was calculated by relating the number of germinated conidia to the total number of added conidia. Values indicate means ± SD, $n = 3$.




**Figs 2–3.** Adhesion (◆) and germination (○) of conidia of *Bipolaris sorokiniana* on solid surfaces. Conidia were suspended in 2.4% PDB and incubated on a polystyrene (**Fig. 2**) or a glass surface (**Fig. 3**). The percentage of adhered conidia was calculated by relating the number of attached conidia to the total number of added conidia. Values indicate means ± SD, $n = 8$.




**Figs 4–7.** Detachment of conidia (**Figs 4–5**) and germlings (**Figs 6–7**) of *Bipolaris sorokiniana* from solid surfaces using PBS as the washing buffer and varying the washing force or number of washes. Conidia were incubated on a polystyrene surface for 30 min prior to the washings. Conidia were germinated for 3.5 h on polystyrene (empty bars) and glass (filled bars) surfaces before being subjected to the washing procedures. Values indicate means ± SD, where $n = 4$ for conidia and $n = 3$ for germlings.

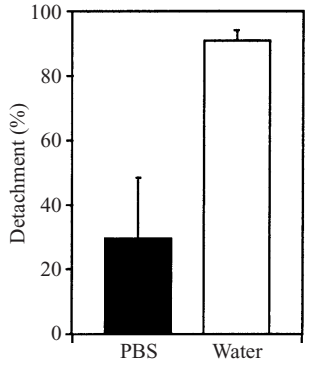
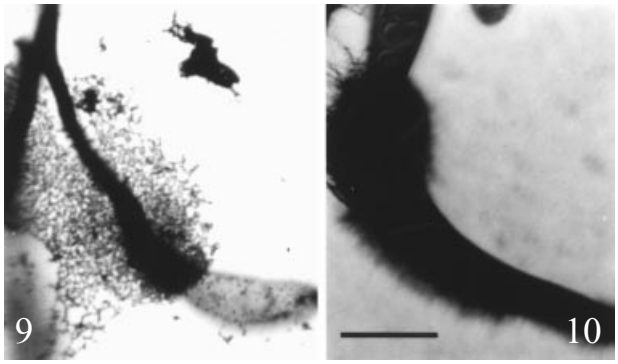



**Fig. 8.** Detachment of conidia from polystyrene surface. Conidia of *Bipolaris sorokiniana* were incubated on the surface for 30 min, then washed with PBS (filled bar) or water (empty bar). Values indicate means ± SD, $n = 4$.




**Figs 9–10.** The effects of 1 M KOH on extracellular matrix (ECM). The ECM of 3.5 h old *Bipolaris sorokiniana* germlings was labelled with Au/Ag. **Fig. 9.** 1 M KOH treatment. **Fig. 10.** Control, germlings treated with water, Bar = 25 μm.


**Figs 11–12.** The effects of Pronase E on the extracellular proteins of the germlings of *Bipolaris sorokiniana*. **Fig. 11.** ECM proteins partially digested with pronase E (2 h at 37 °C). **Fig. 12.** Control, germlings treated with buffer alone. ECM was labelled with acid fuchsin Bar = 10 μm.

the washes (Figs 4–5) or by adding the detergent Triton X-100 to the washing buffer (data not shown). In addition, more conidia were detached from the polystyrene surface when washed with water than with PBS (Fig. 8).

Conidia exhibited higher affinity to the hydrophobic hydrocarbons than to water. Thus, 99.9 ± 0.1% ($n = 4$) conidia partitioned into *n*-octanol in the phase distribution test.

### *Adhesion of germlings*

Germinated conidia adhered to both the glass and polystyrene surfaces (Figs 2–3). In contrast to the conidia, the adhered germlings were not detached from the solid surfaces by increasing the shear force or volume of the washing buffer (Figs 6–7). Attempts were made to remove the adhered germlings from the surfaces by treatments with various chemicals and detergents (Table 1). Strong bases, such as 1 M KOH, 10% $NH_4OH$ or 10% triethylamine (TEA) removed a significant fraction of the germlings from both of the used surfaces (Table 1). Other chemicals, except 1 M HCl, did not detach the adhered germlings. When comparing all treatments, detachment was not influenced by the surface used (polystyrene and glass) ($P < 0.05$, two-way ANOVA).

The effects of these chemicals on the structure of the ECM of germlings were also examined by light microscopy and various staining techniques. The ECM was present in all samples, visualized by Au/Ag staining, although the appearance of the ECM layer was affected in those treated with KOH, CHAPS, and CTAB. The ECM of the germlings treated with KOH were more heterogenous and stained less intensively compared to the ECM of the controls (Figs 9–10).

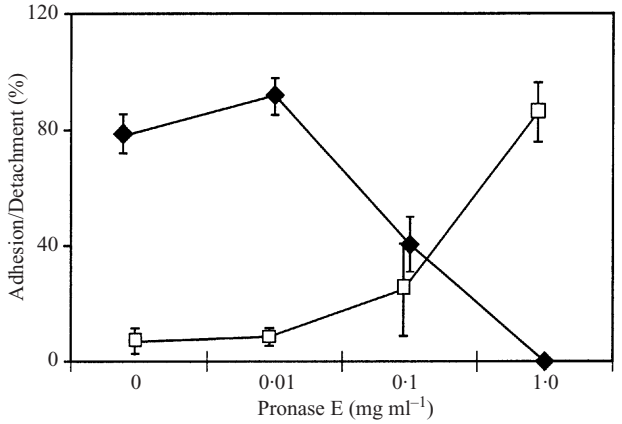



**Fig. 13.** The effects of Pronase E on adhesion (◆), and detachment (□) of germlings of *Bipolaris sorokiniana*. Values indicate means ± SD, $n = 4$.

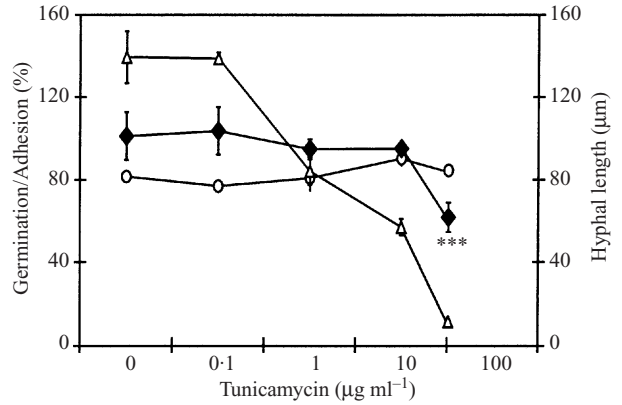
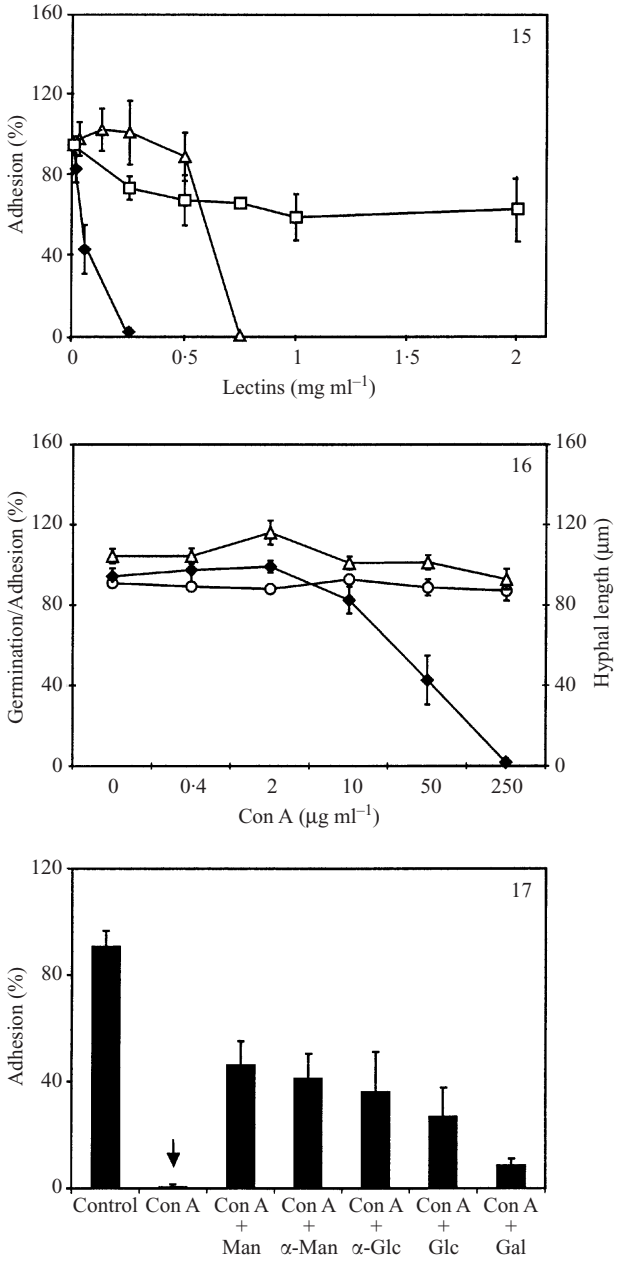



**Fig. 14.** The effects of tunicamycin on germling adhesion (◆), conidial germination (○), and hyphal length (△) of *Bipolaris sorokiniana* on a glass surface. Values are means ± SD, $n = 4$. *** indicates significant ($P < 0.001$) difference in germlings adhesion between tunicamycin treated sample and control (no tunicamycin).

The intensity of labelling of germlings treated with detergents was weak or non-existent unless the preparations were pre-washed with a weak acid (0.01 M HCl). The ECM of acid-treated germlings labelled intensively with Au/Ag, while there was no labelling with CFW (data not shown).

### *Effects of enzymes*

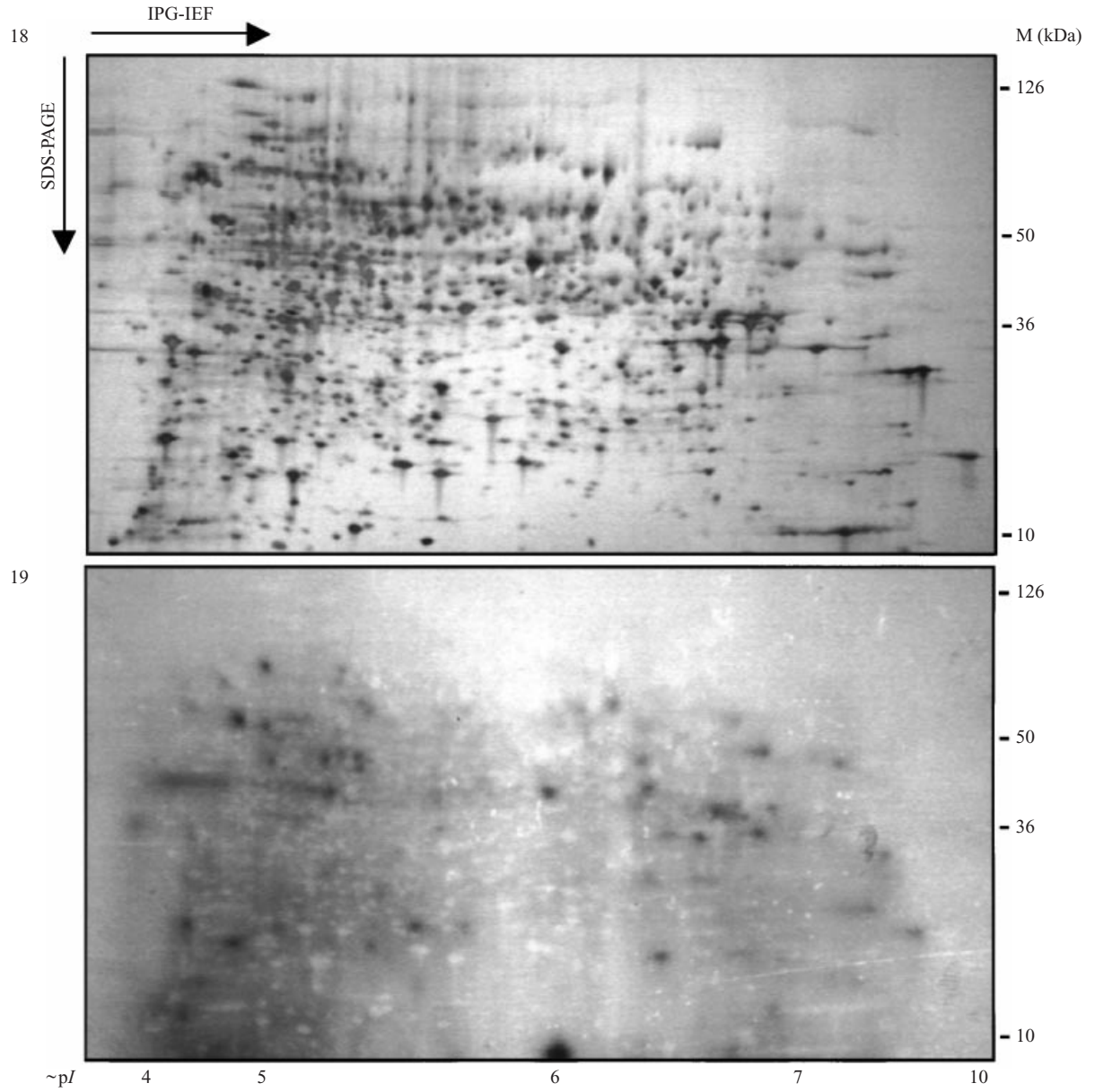
To obtain information on the chemical composition of the germling adhesives, a number of enzymes were tested for their ability to remove adhered germlings from the glass surface. Of all enzymes tested, including proteases, exo/endo-polysaccharidases, and lipase, only two proteolytic enzymes, a broad-specificity protease and Pronase E, removed germlings from the surface (Table 2). Other proteases having higher substrate specificities, like trypsin, pepsin, or collagenase, did not affect germling adhesion. Novozyme, a cell wall degrading enzyme containing cellulase, protease, and chitinase activities (according to the manufacturer) also detached germlings. This was probably due to a protease activity since pure cellulase or chitinase did not disrupt the adhesion (Table 2). Microscopic observations of Au/Ag stained germlings after the enzyme




**Figs 15–17.** The influence of lectins on adhesion of *Bipolaris sorokiniana* to glass surface. **Fig. 15.** Conidia were suspended in the lectin (Con A (◆), GNA (△), WGA (□)) solutions. **Fig. 16.** The effects of Con A on germination (○), germling adhesion (◆) and hyphal length (△). **Fig. 17.** The effects of Con A pre-incubation with sugar haptens mannose (Man), methyl-α-mannopyrannoside (α-Man), methyl-α-glucopyranoside (α-Glc), glucose (Glc), and galactose (Gal.) Values are means ± SD, $n = 4$.

treatments showed that all contained an ECM layer. However, the appearance of the ECM for the broad-specificity protease treated germlings was abnormal. Staining with acid fuchsin, a protein specific stain, revealed a loss of proteinaceous ECM components (Figs 11–12).

The ability of Pronase E to remove germlings from the surface was dependent on the enzyme concentration (Fig. 13). Furthermore, when the conidia were germinated in the presence of Pronase E, germling adhesion was reduced and there was no adhesion at an enzyme concentration of




**Figs 18–19.** Protein profile of 4 h old germlings of *Bipolaris sorokiniana* resolved by 2-D gel electrophoresis. Surface proteins were labelled with $^{125}I$ before being extracted. **Fig. 18.** Silver staining. **Fig. 19.** Autoradiogram.

1 mg $ml^{-1}$ (Fig. 13). At the concentration of Pronase E that completely inhibited adhesion, neither germination nor hyphal growth was affected (data not shown). Treatment with heat-denatured enzyme (95 °, 10 min) did not influence adhesion, germination, or hyphal growth (data not shown).

### *Effects of inhibitors and lectins*

Different biochemical inhibitors were added to the medium to examine their ability to reduce germling adhesion. Inhibitors were tested at 10-fold increasing concentrations until levels were reached where the germination, hyphal growth, or adhesion was significantly reduced. Except tunicamycin, a protein glycosylation inhibitor, none of the inhibitors used (brefeldin A, hygromycin, nikkomycin, sodium azide) significantly reduced germling adhesion ($P < 0.001$, ANOVA) (Fig. 14). At the concentration of tunicamycin that inhibited adhesion, the germination of conidia was unaffected. However, at this concentration the hyphal growth was reduced, and microscopic examinations revealed that germ-tubes were abnormally swollen and that several germ-tubes had burst. Nevertheless, the tunicamycin-treated germlings had ECM that labelled with Au/Ag and CFW. Visually the ECM did not differ from that of the control (not shown).

As the inhibitor of protein glycosylation reduced germling adhesion, we also tested whether treatments with the lectins Con A, GNA, and WGA affected this process (Fig. 15). WGA, N-acetylglucosamine binding protein, initially decreased the germling adhesion by approx. 25 %, at 500 μg $ml^{-1}$. However, attachment was not decreased further with increasing lectin concentration. GNA with binding specificity to terminal mannose of glycosides inhibited germling adhesion (Fig. 15) and decreased germination by 45 % at the highest concentration used (not shown). The addition of Con A

(possessing binding specificity to α-mannose and α-glucose residues) to the medium inhibited germling adhesion with no inhibitory effect on conidial germination or hyphal growth (Figs 15–16 ). Pre-incubation of Con A with the sugar haptens recovered the adhesion by maximum 46% (Fig. 17). Galactose, the carbohydrate having no specificity to Con A was significantly weaker ($P < 0.05$, ANOVA) in ability to recover the adhesion to the test surface.

### *Surface proteins*

The extracellular proteins of 4 h old germlings were $^{125}$I-labelled, the proteins extracted, and resolved by two-dimensional electrophoresis (Fig. 18). The autoradiogram of the gel reveals the presence of about 40 surface proteins over wide pH (4–10) and Mw ranges (10–100 kDa) (Fig. 19). The experiment was repeated several times and similar patterns for both silver stained gels and proteins in the autoradiogram were obtained.

## DISCUSSION

The adhesion of *Bipolaris sorokiniana* to a hydrophobic polystyrene surface occurred in two stages: the first by conidia, and the second by germlings. The initial conidial adhesion was weak since attached conidia were easily removed by increasing the shear force of the washing buffer. Several observations suggest that the conidial adhesion was due to hydrophobic interaction. First, the conidial surface was hydrophobic, and several studies have demonstrated a correlation between cell-surface hydrophobicity and adhesion to polystyrene (Doss *et al*. 1993, Hazen & Hazen 1987, Kuo & Hoch 1996). Second, including salt in the washing buffer increased conidial adhesion. Salts are known to increase the strength of the hydrophobic interaction including those between fungal cells and solid surfaces (Ben-Naim & Yaacobi 1974, Young & Kauss 1984). Third, the detergent Triton X-100, which interferes with hydrophobic binding, disrupted the adhesion of the conidia to the polystyrene.

The conidial adhesion of *B. sorokiniana* appeared to be selective to hydrophobic surfaces, since no adhesion was observed to hydrophilic glass surfaces. A similar preference for adhesion to hydrophobic compared to hydrophilic surfaces has been observed for conidia of a number of different plant pathogens including the ascomycetes *B. cinerea* (Doss *et al*. 1993) and *Colletotrichum* spp. (Young & Kauss 1984, Sela-Buurlage *et al*. 1991, Mercure, Leite & Nicholson 1994), as well as the rust *Uromyces vicae-fabae* (Clement *et al*. 1994). All these species, except *B. cinerea*, have been reported to release mucilage that is thought to assist conidial adhesion. *B. sorokiniana* also releases a conidial mucilage on the contact with a leaf surface, but it is not known whether this material is involved in adhesion (Apoga & Jansson 2000).

For several plant pathogens, it has been shown that the germination of conidia is stimulated by contact with or after adhesion to a solid-surface. Conidial adhesion is required to stimulate germination in *Magnaporthe grisea* and *Phyllosticta ampelicida* (Liu & Kolattukudy 1999, Kuo & Hoch 1996) whereas solid-surface contact is sufficient for inducing germination in *Colletotrichum* (Kim, Li & Kolattukudy 1998). In *B. sorokiniana*, adhesion was not needed for conidial germination since germination occurred in the bulk media. However, germination was stimulated on a solid surface when the fungus was incubated in water, indicating that surface contact (or adhesion) can stimulate germination.

The second stage of adhesion of *Bipolaris sorokiniana* to solid surfaces was accompanied by germination and release of extracellular material, which has also been observed for germling adhesion in *Cochliobolus heterostrophus* and *Botrytis cinerea* (Braun & Howard 1994a, Doss *et al*. 1995). The germling-associated adhesion of *B. sorokiniana* appeared to be surface unspecific since it occurred on both polystyrene and glass surfaces. Furthermore, germling adhesion was strong, increased washing force and harsh chemical treatments like 5 M LiCl, 4 M urea, and different detergent solutions, did not detach the germlings from the surfaces. A similar resistance to chemical treatments has been shown for adhered germlings of *Puccinia sorghi* and *B. cinerea* (Chaubal *et al*. 1991, Doss *et al*. 1995).

Production of an extracellular matrix has commonly been related to fungal adhesion (Jones 1994, Braun & Howard 1994b, Epstein & Nicholson 1997), but the molecular structure of fungal adhesives is not well known. A number of reports have indicated that fungal adhesives consist of high molecular weight glycoproteins (Kuo & Hoch 1995, Chaubal *et al*. 1991, Jones 1994, Epstein & Nicholson 1997, Ding *et al*. 1994, Sugui, Leite & Nicholson 1998, Hughes *et al*. 1999). Such glycoproteins can probably also be modified after secretion from the cells. For example, it has been suggested that extracellular transglutaminase activity polymerizes the adhesive glycoprotein of *H. haematococca* (Kwon & Epstein 1997). In the present study, evidence was obtained that germ tube adhesion of *B. sorokiniana* is mediated by extracellular glycoproteins. This conclusion was drawn from the observation that treatment with a broad-specific protease and Pronase E detached adhered germlings, and digested, at least partly, the ECM of the germlings as visualized by microscopy. In addition, treating the germlings with tunicamycin reduced adhesion, which indicates that N-glycosylated proteins are involved in adhesion (Elbein, 1987). Similar sets of inhibitor experiments have shown that the adhesion and differentiation of appressoria of the oomycete *Phytophthora palmivora* is mediated by surface glycoproteins (Bircher & Hohl 1997).

The carbohydrate portion of fungal glycoproteins contain α-mannosides and α-glycosides that can bind to the lectin Con A. Treating germ tubes of *Bipolaris sorokiniana* with this lectin significantly decreased adhesion, that has also been observed in a number of other plant pathogenic fungi (Hamer *et al*. 1988, Kwon & Epstein 1993, Bircher & Hohl 1997, Shaw & Hoch 1999, Mercure *et al*. 1994). As reported earlier, Con A binds to the cell wall and not to the ECM of *B. sorokiniana* (Clay, Enkerli & Fuller 1994, Apoga & Jansson 2000). Although the mechanisms of the effects of Con A on adhesion are not known, the above observations indicate that the Con A binding compound(s) which is involved in adhesion, is localized to the cell wall or its close vicinity of *B. sorokiniana*.

The proteins present in the ECM of *B. sorokiniana* germlings were analysed using two-dimensional gel electrophoresis.

Before electrophoresis and extractions, ECM proteins were radiolabelled with $^{125}I$, which is a method commonly used for analysing surface proteins (Richardson & Parker 1985, Thompson *et al*. 1987). As discussed above, the adhesives of *B. sorokiniana* are highly insoluble, therefore, the cells were extracted with a buffer developed to solubilize a wide range of proteins (Pasquali, Fialka & Huber 1997, Rabilloud 1998). At least 40 labelled proteins were detected on the 2-D gels indicating a very complex pattern of surface proteins. Some of them can be involved in germ tube adhesion. However, apart from the adhesive nature, the ECM may possess properties that prevent desiccation of the fungus or increase its tolerance to toxic substances, and it may contain enzymes that can degrade the tissues of the host plant (Nicholson, Hipskind & Hanau 1989, McRae & Stevens 1990, Doss 1999). To further investigate the role of the ECM proteins of *B. sorokiniana* in adhesion and other processes is a challenge for the future.

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