European Journal of Plant Pathology **109:** 599–605, 2003.

Extracellular matrix, esterase and the phytotoxin prehelminthosporol in infection of barley leaves by *Bipolaris sorokiniana*

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Accepted 18 March 2003

Key words: adhesion, cryo-SEM, *Hordeum vulgare,* immunofluorescence, prehelminthosporol

Abstract

Light microscopy and cryo-scanning electron microscopy showed that hyphae of *Bipolaris sorokiniana* adhered to the wax surfaces of barley leaves by means of an extensive extracellular matrix (ECM). Prehelminthosporol, the major non-host specific phytotoxin formed by *B. sorokiniana* was immunolocalized in large amounts in the ECM surrounding the hyphae. Similarly, esterase activity involved in degradation of the cuticular wax surface was found in the ECM. Therefore, it appears that the ECM is not only important for adhesion of the fungus to its host, but also functions as a sink of phytotoxins and lytic enzymes important for infection of the host plant.

Abbreviations: ECM – extracellular matrix; PHL – prehelminthosporol.

Introduction

A layer of wax crystals in the shape of platelets, tubes, rods and other forms is located above the cutin layer in plant leaves (Baker, 1982). The wax layer reduces water loss, controls gaseous exchange and reduces leaching of nutrients. The external wax layer also provides a habitat for various microorganisms and serves as a barrier against entry of fungal pathogens (Baker, 1982; Isaac, 1992). To infect plant leaves pathogenic fungi must be able to adhere to the surface and prepare for penetration of the plant tissues. The adhesion may involve the formation of extracellular matrix (ECM) (Braun and Howard, 1994a), as well as production of lytic enzymes, e.g. esterases and cutinases (Deising et al., 1992).

Extracellular matrix occurs within many groups of fungi and has been reported to coat several fungal structures such as appressoria, haustoria, conidia and infecting hyphae (Nicole et al., 1994; Tunlid et al., 1992; Apoga and Jansson, 2000). The ECM may serve different functions such as protection from desiccation and protection from toxic compounds of host origin (Nicholson and Epstein, 1991). However, the ECM has often been implicated in fungal adhesion to surfaces.

Several *Bipolaris* species adhere non-specifically to surfaces (Braun and Howard, 1994b; Pringle, 1981; Evans et al., 1982). Two-layered ECM of germ-tubes of *Bipolaris maydis*, *B. zeicola* and *B. turcicum* have been visualized by histological staining (Evans et al., 1982; Braun and Howard, 1994b). The inner layer, which probably contains proteins (Evans and Stempen, 1986; Braun and Howard, 1994b), has been shown to be responsible for adhesion of conidia to surfaces (Braun and Howard, 1994b). Apoga and Jansson (2000) showed that the inner layer of *B. sorokiniana* germ-tubes consists mainly of proteins, and the outer layer of carbohydrates. By using a mutant of *Cochliobolus heterostrophus*, defective in the outer germ-tube ECM layer, Zhu et al. (1998) showed that the two layers may exist independently and still adhere to the leaf

surface; thus the inner layer may be mainly responsible for the adhesion.

Ultrastructural studies of root infection by *B. sorokiniana* revealed a fibrillar ECM beneath hyphae in contact with the root surface (Carlson et al., 1991b). A similar fibrillar ECM has also been observed when the fungus grows on artificial surfaces (Apoga and Jansson, 2000). However, the mechanisms behind adhesion of *B. sorokiniana* to leaf surfaces have not been studied. We have earlier proposed that release of the hydrophobic phytotoxin prehelminthosporol (PHL) by *B. sorokiniana,* may assist in softening the leaf wax layer (Nilsson et al., 1993), thereby facilitating plant infection.

The objective of the current investigation was to study the involvement of PHL in pre-penetration events. Cryo-scanning electron microscopy (cryo-SEM) was used to examine barley leaves inoculated with *B. sorokiniana.* Antibodies against PHL were used to localize extracellular PHL in cultures on a dialysis membrane. In addition, we investigated the ability of *B. sorokiniana* to produce esterases on artificial surfaces.

Materials and methods

Organisms

The *B. sorokiniana* isolates (Tellus 1, A18 and K247) were maintained on agar slants at 4 °C. The fungus was cultured on a defined agar medium (Carlson et al., 1991a) for 14–28 days to produce inoculum. Conidial suspensions were obtained from plate cultures by washing with sterile distilled water. Hyphae were removed by filtration through a nylon screen (100 μm mesh) and the resulting suspension was concentrated by centrifugation (200*g*, 1 min). The conidia were used to inoculate dialysis membranes, barley leaves or glass cover slips.

Seeds of barley (*Hordeum vulgare* L. cv. Harry) were surface-sterilized in 4.4% (v/v) NaClO and placed in glass beakers on top of expanded clay pellets in a nutrient solution containing 1 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 2.0 mM KNO_3, 0.25 mM NaH_2PO_4, 0.1 mM Fe^{3+}(EDTA), 5.0 μM $MnCl_2$, 10.0 μM H_3BO_3, 0.25 μM Na_2MoO_4, 0.5 μM $CuCl_2$. The plants, derived from surface-sterilized seeds, were grown aseptically for 10–14 days in a growth chamber using 16 h light (18 °C) and 8 h dark (16 °C).

Inoculation of leaves with B. sorokiniana

The abaxial surface of the first or second leaf of the barley seedlings was inoculated with conidia of *B. sorokiniana* using a sterilized cotton swab, incubated for 1–3 days and grown under the same conditions as above.

Antibodies against PHL

Polyclonal antibodies against PHL were raised in rabbits immunized with a PHL-hexon conjugate (Åkesson et al., 1996). The anti-prehelminthosporol-hexon (anti-PH) IgG was isolated from the serum and the specificity of the purified antibodies was determined using indirect enzyme-linked immunosorbent assays (ELISAs).

Detection of PHL in ECM using immunofluorescence

In order to detect PHL in hyphal ECM, a modified dialysis membrane technique (Nordbring-Hertz, 1983) was used together with an immunofluorescence technique. Pieces of sterile washed dialysis membrane (2 × 2 cm), placed on top of 1% (w/v) water agar surfaces in Petri dishes were inoculated with conidia and were incubated at room temperature (22 °C) for 5 days. For immunofluorescence labelling of the fungal cultures, the membranes were transferred to Petri dishes with Nescofilm (Nippon Shoji Kaisha, Osaka, Japan) on the bottom of the plate. The dialysis membrane with mycelium was placed on a drop of phosphate buffered saline (PBS, pH 7.4) and treated in a stepwise fashion with 400 μl of the appropriate solutions as described below. Because of the hydrophobic properties of the Nescofilm, water solutions remained on the strip and could easily be decanted.

The mycelium was rinsed once with PBS followed by incubation in PBS containing 1% (w/v) bovine serum albumin (BSA, fraction V, Sigma) for 30 min at room temperature. After incubation in anti-PH IgG (1 : 800) in PBS at 6 °C overnight, the membranes were rinsed for 5 × 4 min in PBS. Sheep anti-rabbit IgG, conjugated to the fluorochrome Cy 3 (Sigma, 1 : 100) in PBS, was added and the mycelium was again rinsed in PBS (6 × 4 min). The membranes were mounted in glycerol–PBS (1 : 2) and viewed with a fluorescence microscope (Zeiss standard) equipped with rhodamine specific filters that could also be used for Cy 3. The

specificity of the staining reaction was investigated by (a) incubation with the Cy 3 conjugated sheep anti-rabbit IgG alone and (b) incubation with anti-hexon (anti-H) IgG followed by the secondary antibodies.

Cryo-SEM

To avoid organic solvents, cryo-SEM was employed for examination of the waxy leaf surface. Leaf pieces infected by *B. sorokiniana* were attached to a specimen holder using a clamp and were immediately frozen by plunging into a liquid nitrogen slush. The frozen specimen was transported in a vacuum transfer chamber from the slush freezer to the cold stage of a cryo-transfer system (Oxford CT 1500) mounted on a Philips 515 SEM. Contaminating ice was removed by sublimation at −88 °C for 7 min. The specimen was transferred under vacuum to the preparation chamber and sputtercoated with gold, before being returned to the SEM stage and examined at an accelerating voltage of 20 kV at −150 °C.

Esterase assay

Esterase activity was assessed by a method in which indoxyl acetate served as the substrate for nonspecific carboxylic acid esterases (Barnett and Seligman, 1951; Deising et al., 1992). Substrate hydrolysis results in the accumulation of pigmented crystals of indigo blue at the site of hydrolysis. A 100 µl droplet of spore suspension was placed on a glass cover slip and incubated in a moisture chamber for 19 h. The coverslip was then rinsed with distilled water and 100 µl of a filtered substrate solution (9.3 mM indoxyl acetate in 20 mM Tris–HCl buffer (pH 8.0), containing 0.99 M NaCl and 44.6 mM $CaCl_2$), was then added. A control was included in which the germlings on coverslips were incubated at 90 °C for 1 h, followed by incubation in the substrate solution. The samples were examined for indigo blue after 4 h incubation in the dark at room temperature.

Results

Hyphal ECM on barley leaves

Wax crystals on the leaf surface were visible using the cryo-SEM technique (Figures 1–5). ECM was present around hyphae growing on the leaves (Figures 1 and 2). The ECM was produced at the parts of the hyphae seen in close contact with the leaf surface and could be several hyphal diameters wide (Figure 1) or thinner (Figure 2). Hyphae with thin ECM was easily removed and carried with them wax platelets from the leaf surface (Figure 2). After removal the hyphae left tracks on the leaf where the smooth cutin layer was exposed (Figure 2).

Growth on leaf surfaces

Tropic growth of hyphae was observed in the grooves between epidermal cells of barley leaves (Figure 3). However, hyphae often crossed the epidermal ridges and were then attached to the ridges by the ECM (Figures 1 and 2). Hyphae differentiated into appressoria which were either simple, as for the isolates Tellus 1 and K247, or complex (Figure 3), as for isolate A18. A wax-free zone surrounded the appressoria (Figure 3).

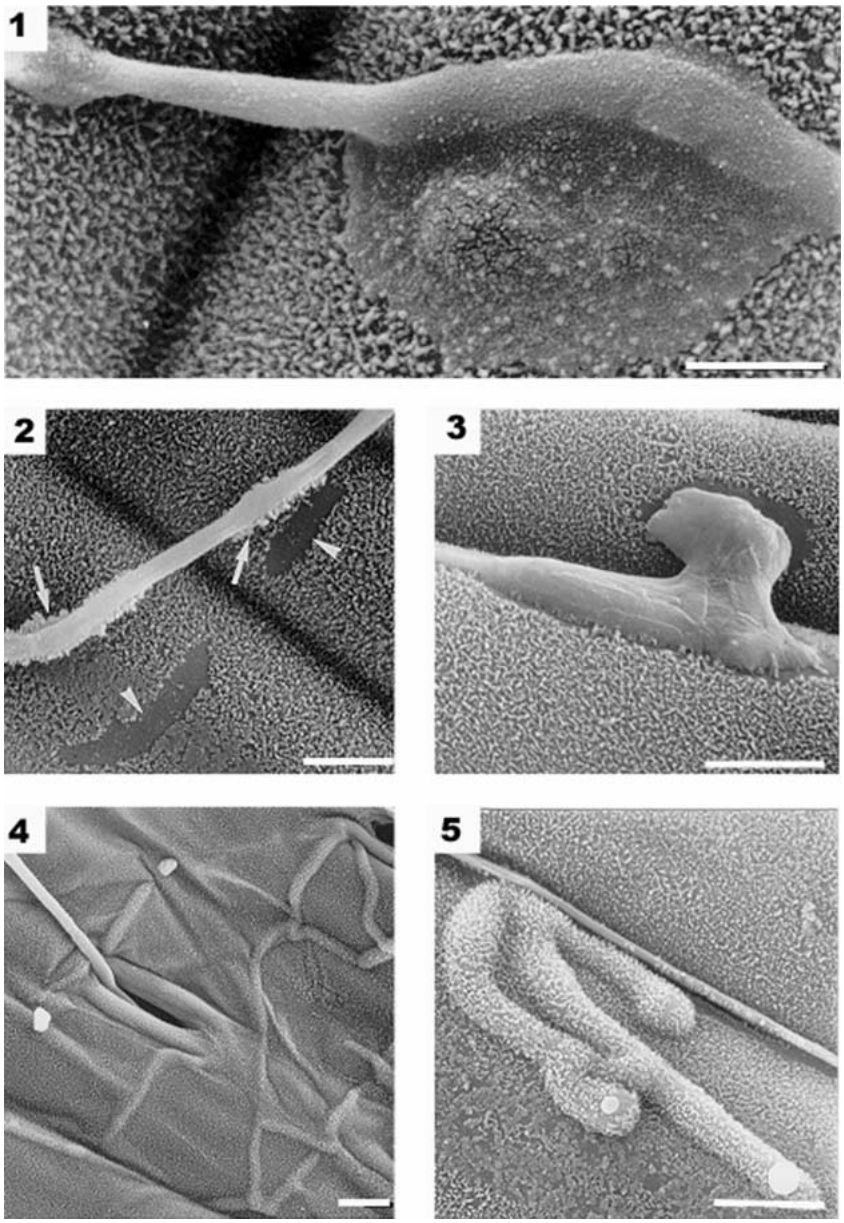
Subcuticular growth of *B. sorokiniana* was pronounced 3 days after inoculation of the leaves (Figures 4 and 5). The advantage of this mode of growth may be a way for the fungus to protect itself against desiccation until the time of reproduction. Conidiophore-like hyphae were often produced from these hyphae, but no conidia were seen. Sometimes hyphae growing out through the stomata were observed (Figure 4).

Labelling of PHL in hyphal ECM

Antibodies raised against the PHL–hexon conjugate were used for localization of PHL in a culture of *B. sorokiniana* grown on dialysis membrane. Heavy labelling was observed in a zone around peripheral hyphae (Figure 6) corresponding to the zone that was visualized by staining the ECM with Indian ink (not shown). This suggests that the phytotoxin PHL and the other closely related compounds produced by *B. sorokiniana,* are released and concentrated in the ECM. Substitution of the PHL antibodies with anti-hexon antibodies showed a very low or non-existing labelling of the ECM (Figure 7). Similarly, omission of the primary antibodies resulted in absence of labelling of the sheath (data not shown).

Esterase activity in hyphal ECM

Incubation of coverslip cultures of the fungus in drops of indoxyl acetate as esterase substrate resulted in

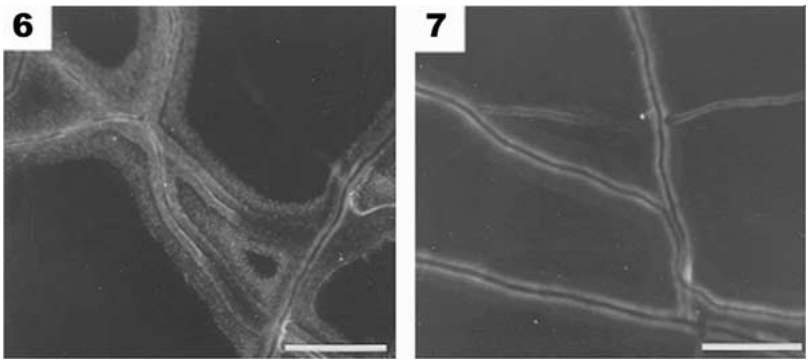


Figures 1–5. Cryo-SEM of barley leaves infected by *B. sorokiniana*. 1. Hyphal ECM of *B. sorokiniana* (isolate K247) was produced by hyphae in contact with the leaf surface and could be several hyphal diameters wide. 2. Hyphae with thinner ECM were easily removed, carrying wax platelets from the leaf surface (arrows). Note the smooth wax-cutin surface on the leaf (arrowheads). 3. The appressoria of the fungus (isolate A18) were composite, resulting from aggregation of hyphae. Note the wax-free zone surrounding the appressorium, and ECM. 4 and 5. Subcutical hyphae of *B. sorokiniana* (isolate K247) were visible 3 days after inoculation. Hyphae penetrating the leaf surface later developed into conidiophores. Note hypha protruding through stomata in Figure 4. Bars = 10 μm.

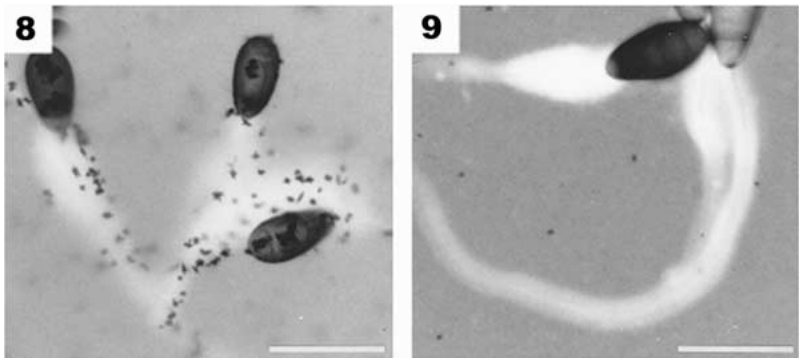
formation of indigo blue crystals in the hyphal ECM (Figure 8), but the crystals could also later be seen in the surrounding medium. Esterase activity of conidia and hyphae was also seen. No crystals were formed in the heat-treated controls (Figure 9).

Discussion

The phytotoxin PHL is a cell membrane-disrupting sesquiterpene metabolite of *B. sorokiniana*, which affects not only plant cells, but also bacteria and other



Figures 6 and 7. Immunofluorescence labelling of hyphae of *B. sorokiniana* growing on dialysis membrane. 6. Fluorescent hyphal ECM was seen after incubation with anti-PH IgG and Cy 3-conjugated sheep anti-rabbit IgG showing presence of the phytotoxin prehelminthosporol in the ECM. 7. Incubation with anti-H IgG followed by Cy 3-conjugated sheep anti-rabbit IgG (control) showed virtually no labelling of the ECM. Bars = 50 μm.



Figures 8 and 9. Histochemical labelling of esterase activity. 8. Crystals of indigo blue show esterase activity in hyphal ECM. 9. No crystals were seen in heat treated controls. Bars = 50 μm.

fungi (Olbe et al., 1995; Åkesson and Jansson, 1996). PHL is an important virulence factor in *B. sorokiniana* and there is a correlation between PHL production and plant infection by different isolates of the fungus (Apoga et al., 2002). We previously showed using immunolocalization and transmission electron microscopy (Åkesson et al., 1996) that PHL was specifically localized to the Woronin bodies in the fungal cells. Nilsson et al. (1993) showed that PHL was present already in non-germinated conidia, but that its release increased substantially upon conidial germination in liquid cultures. In the current paper PHL, was collected and concentrated in the ECM covering the hyphae. This aggregation of the toxin is probably a way for the fungus to achieve a concentrated action of PHL at the sites of cell wall penetration. Despite PHL having amphiphilic properties and might function as a detergent (Åkesson and Jansson, 1996), we have not been able to prove any dissolution or softening of the wax layer by PHL. The major effect of PHL during leaf infection appears to be its membrane disrupting properties, and with the ECM as a sink of the toxin this may be a great advantage for the fungus during the infection process.

Thigmotropic growth of germ-tubes of *B. sorokiniana* was observed on leaf replicas by Clay et al. (1994), as well as in many other phytopathogenic fungi (Staples and Hoch, 1997). We observed a tropic growth of hyphae in grooves between epidermal cells of barley leaves. However, hyphae often crossed the epidermal ridges and were then attached to the ridges by the ECM (Figure 1). A close contact of germ-tubes and hyphae with the cuticular surface may be necessary for directed growth. The extensive growth of hyphae on the leaf surface before appressorial formation and penetration of the host by *B. sorokiniana,* suggests that appressoria are mainly formed when the nutrients stored in the conidia are

depleted. However, no appressoria were formed on the artificial surfaces used, indicating that appressoria also need a thigmotropic signal to be formed. Studies on appressorial formation by *B. sorokiniana* on leaf replicas showed that appressoria were more frequent over grooves formed by the juncture of anticlinal walls of epidermis cells (Clay et al., 1994).

Areas without wax platelets underneath hyphae removed from plant surfaces have been observed in several studies of fungus–leaf interactions. These wax-free areas have been explained by a dissolution or utilization of wax platelets in contact with the fungus (Staub et al., 1974), or simply by an adherence of the platelets to the hyphae (Lewis and Day, 1972). A clearing zone of the wax surface was observed around the appressoria indicating enzymatic activity, and is probably an effect of esterase activity. Hydrolytic enzymes such as cutinases and esterases may also assist in adhesion of fungal propagules to the plant surface (Deising et al., 1992), and Lin and Kolattukudy (1980) isolated cutinases from *B. sorokiniana.* Changes in the wax layer of barley leaves were observed after treatment with an esterase preparation from *Erysiphe graminis* (Kunoh et al., 1990). Their untreated control leaves contained an amorphous component of the wax that was not observed in our controls. The localization of esterases to the ECM, shown in this investigation, probably helps the fungus to soften or digest the wax layers of the leaf and is thus important for the pre-penetration events. A similar localization of lytic enzymes (proteases and phosphatases) in conidial ECM of the nematophagous fungus *Drechmeria coniospora* has also been reported (Jansson and Friman, 1999). Like the accumulation of PHL in the ECM, action of esterases (and possibly other lytic enzymes) is concentrated in the sites of fungal penetration and this may also facilitate infection by the fungus.

Acknowledgements

We thank Agneta Persson for technical assistance and Rolf Odselius at the Electron Microscopy Unit, University Hospital, Lund University for help with the cryo-SEM. We also wish to thank K. Bailey (Agriculture Canada Research Station, Saskatoon, Canada) and R.W. Stack (Plant Pathology, North Dakota State University, USA) for kindly supplying the fungal isolates. The work was financed by grants from the Swedish Council for Forestry and Agricultural Research and the Swedish Natural Science Research Council.

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