First publ. in: Plant cuticles / ed. by G. Kerstiens. Oxford: BIOS, 1996, Chap. 8 (pp. 175-188)

Fungal attachment and penetration

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8.1 Introduction

Many plant pathogenic fungi infect their hosts without using wounds or natural openings, such as stomata. They can penetrate the plant epidermis directly, although its structure is composed in such a way as to deter microorganisms (Juniper, 1991). The outermost layer of the epidermis, the cuticle, is a mixture of the polyester cutin and wax (Holloway, 1993; see Chapter 2) anchored to the wall layers below which consist mainly of carbohydrates and proteins (Carpita and Gibeaut, 1993). The way fungi manage to infect the epidermis has been a matter of interest since the earliest studies on plant diseases. DeBary (1884) and Chester (1946) were the first to review the literature on fungal penetration. More recently, Martin (1964) and, some years later, Van den Ende (1974) concentrated on the degradation of the cuticle and its role in disease resistance. Kolattukudy (1985) was the first to summarize the exciting new facts on the molecular qualities of the enzyme cutinase and the mechanism of cutin degradation. In most recent years, reviews have concentrated on the different tasks of cutinase isozymes (Köller, 1991; Köller *et al.,* 1995) and their importance as a pathogenicity factor during the early stages of infection (Schäfer, 1994). Since cutin is only one hurdle in the obstacle race of the fungus to gain access to the epidermal protoplast, more general reviews may also be of interest to the reader. Elad and Evensen (1995) discuss the mode of infection by *Botrytis* and other reviews deal with the different types of fungal infection structures produced by biotrophic or necrotrophic pathogens (Hardham, 1992; Mendgen and Deising, 1993; Mendgen *et al.,* 1996).

This chapter concentrates on recent results explaining the factors that contribute to the initiation of the penetration process. In addition, observations demonstrating modifications of the plant cell wall during fungal penetration are included.

8.2 Initial contact of the fungus with the cuticle

The leaf surface influences adhesion and growth direction of various fungi by providing factors that can be recognized by spores and germ tubes (Allen *et al.*, 1991; Hoch and Staples, 1991). Cuticles may be smooth (Figure 8.1) or covered with a dense array of wax crystals (Figure 8.2). Water may run off more or less easily from

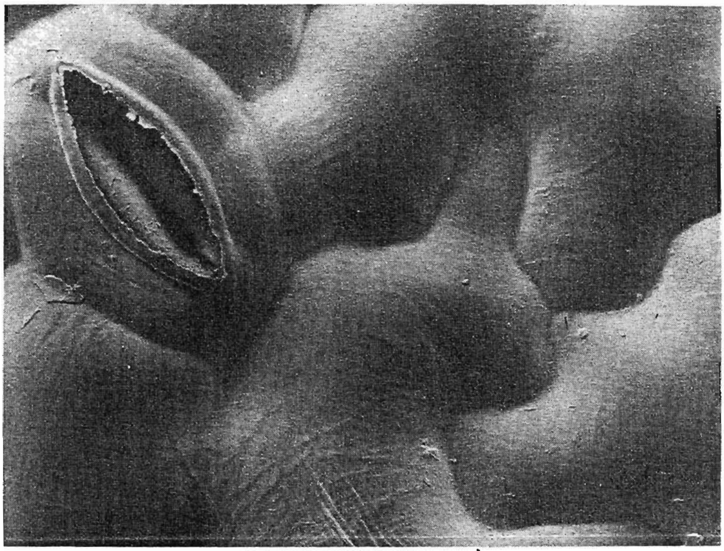

Figure 8.1. The upper leaf surface of Vicia faba, *as seen with a low temperature scanning electron microscope (LTSEM; × 200).*

such cuticles. Depending on the plant, the water droplets on the leaf may keep a neutral or reach a more basic pH after a few minutes (Harr and Guggenheim, 1995; see Chapter 16). Fungi have adapted to the characteristic features of their host plant, irrespective of whether the fungi are endophytic (Viret *et al.*, 1993) or pathogenic. They are able to attach under dry and under wet conditions. Therefore, quite a variety of mechanisms exist that help fungi to keep in contact with their host and to penetrate the cuticle. The different mechanisms of adhesion have recently been reviewed by Nicholson (1996) and cannot be dealt with here in detail. In addition, different mechanisms may prevail in adhesion of aquatic fungi (Hyde *et al.*, 1993; Jones, 1994), nematophagous fungi (Tunlid *et al.*, 1992), insect pathogens (Hajek and St. Leger, 1994) or during human pathogenesis (Dykstra and Bradley-Kerr, 1994).

For urediospores of the rust fungi, after landing on a dry leaf, the most important factor in attachment seems to be the hydrophobic interaction between the cuticles of the plant and of the fungal spore (Clement *et al.*, 1994). During this initial stage of infection, the surface contact between urediospores and the cuticle is mediated by the spines emerging from the spore surface (Figure 8.3). Such a urediospore would have less contact on the rice leaf (Figure 8.4) because of the humps produced by the leaf cuticle. Pycnidiospores of *Phyllosticta ampelicida* germinate only on hydrophobic substrata on which they are firmly attached (Kuo and Hoch, 1996).

8.3 Slime production in advance of and during penetration

Conidia of *Uncinuliella australiana*, a powdery mildew fungus, are coated with a networklike layer of extracellular mucilage. This material spreads from conidial surfaces on to a dialysis membrane and forms an adhesion pad between the underside of a conidium and the membrane surface (Mims *et al.*, 1995).

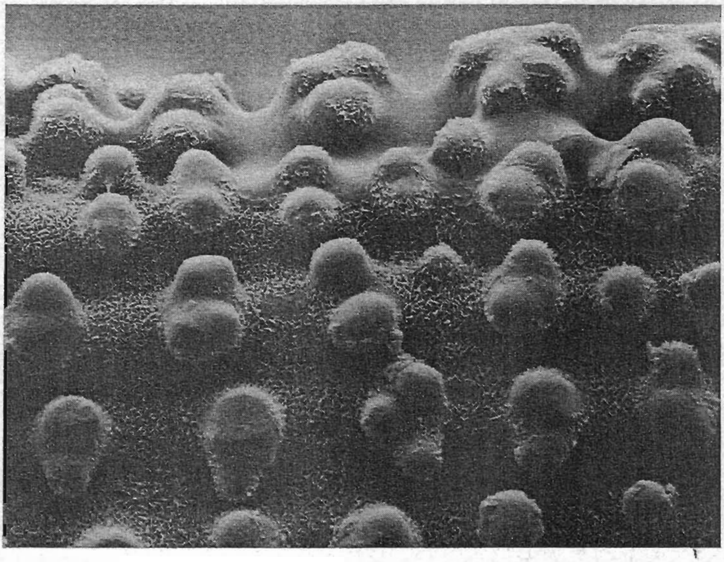

Figure 8.2. *The adaxial leaf surface of* Oryza sativa *as seen with the LTSEM (× 400).*

Conidia of *Erysiphe graminis* release a liquid within 5 min of contact of the spore with the leaf surface (Nicholson *et al.*, 1993). This liquid not only erodes a portion of the leaf cuticle, but also makes the surface of the conidium hydrophilic and the surface of the leaf less hydrophobic. Thus, the fungus prepares the infection court (see also Chapter 13).

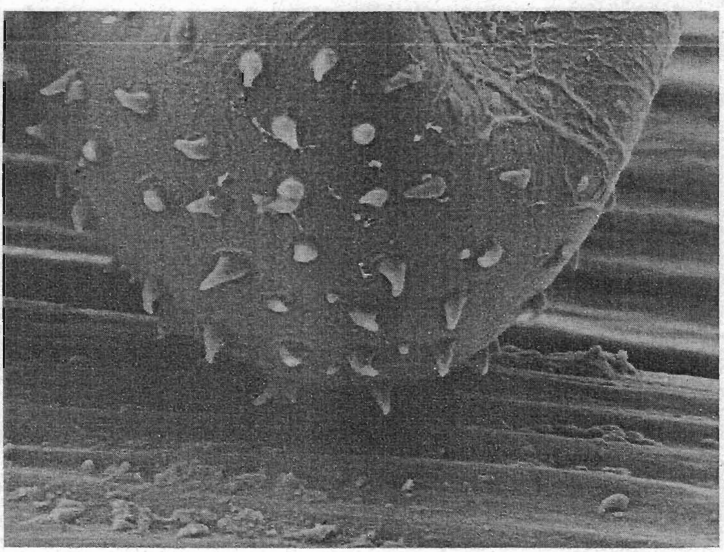

Figure 8.3. *Contact zone between the urediospore of* Uromyces viciae-fabae *and the upper leaf cuticle of* Vicia faba. *Note the spines on the spore surface. (LTSEM micrograph, × 500.)*

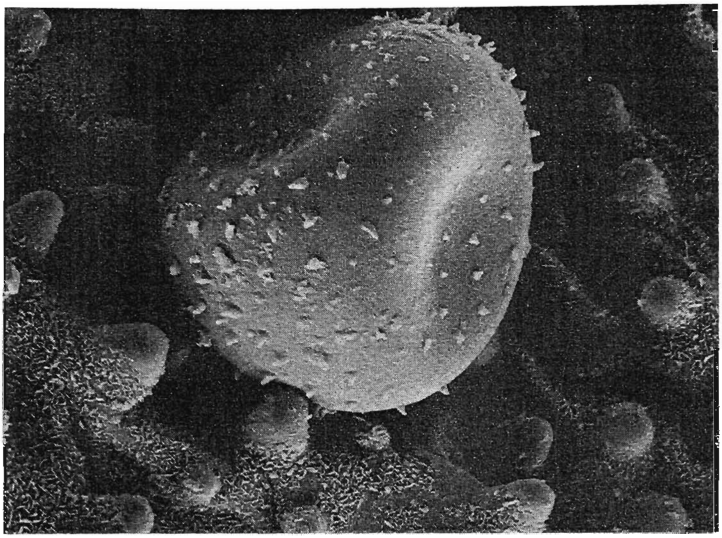

Figure 8.4. *Urediospore of* Uromyces viciae-fabae *on the adaxial cuticle surface of* Oryza sativa, *a non-host. The bumps on the cuticle obviate good contact. (LTSEM micrograph, × 500.)*

In the presence of water, spores of *Cochliobolus heterostrophus* and *Colletotrichum graminicola,* produce a matrix within 20-30 min that accumulates between spores and the plant cuticle as a result of metabolic activities of the fungus (Braun and Howard, 1994; Mercure *et al.*, 1995). A similar situation is observed with fungal endophytes; for example, the beech endophyte *Discula umbrinella* (Viret *et al.*, 1993). This matrix or slime seems to swell with the availability of water on the cuticle. The matrix components are quite heterogenous (Vreeland and Epstein, 1996). In *Nectria haematococca* macroconidia, the mucilage and a 90 kDa glycoprotein are associated with adhesion (Kwon and Epstein, 1993). In *Botrytis* species and in *Colletotrichum lindemuthianum*, glycoproteins typical for spores or infection structures have been characterized with monoclonal antibodies (Cole *et al.*, 1996; O'Connell *et al.*, 1996). In urediospores of *Uromyces viciae-fabae* (Figure 8.5), cutinases and esterases in the matrix of the adhesion pad contributed, among other factors, to the attachment of the fungus to the cuticle (Deising *et al.*, 1992).

A matrix of slime was also observed between European hazelnut leaves and ascospores of *Anisogramma anomala* (Pinkteron *et al.*, 1995). These spores are released from perithecia during periods of rain. Under such conditions, they must adhere rapidly after deposition to prevent being washed off the host. A preformed adhesive attaches these spores and, a few hours later, a more durable adhesive matrix is secreted at the point where the germ tube emerges from the spore. This germ tube immediately penetrates the host while still below the spore. Only the second step in adhesion depends on spore metabolism. Also germlings of *Phyllosticta amphicida* produce a matrix with a specific pattern during their development (Kuo and Hoch, 1995). Similarly, the matrix compostion of *C. lindemuthianum* varies with the development of infection structures and fimbriae project out (O'Connell *et al.*, 1996).

The recent discovery of hydrophobins (Wessels, 1994), which are hydrophobic proteins that mediate the contact of the fungus with hydrophobic parts of the plant

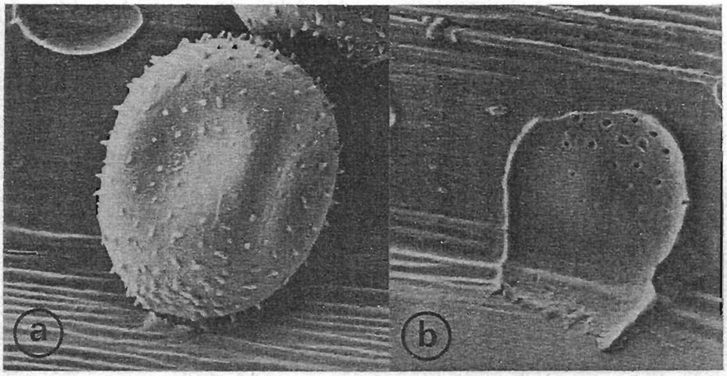

Figure 8.5. *(a) Urediospore of* Uromyces viciae-fabae *with an adhesion pad that mediates contact between plant cuticle and spore about 20 min after wetting the leaf. (b) Removal of the spore leaves the adhesion pad behind. (LTSEM micrographs, × 250.)*

by forming an amphipathic protein layer between fungal hyphae and the hydrophobic plant cuticle, may explain the adhesion process to a certain extent. Coating of glass slides with hydrophobic silicon enhanced adhesion of germ tubes of *Uromyces appendiculatus* to such surfaces (Terhune and Hoch, 1993).

The matrix of slime around spores appears not only typical for plant pathogens. Figure 8.6 shows one of many non-pathogenic microorganisms on an apple leaf. Since all observed microorganisms were surrounded by a matrix, the production of slime is a phenomenon common to many phylloplane fungi, and is not restricted to pathogenic fungi.

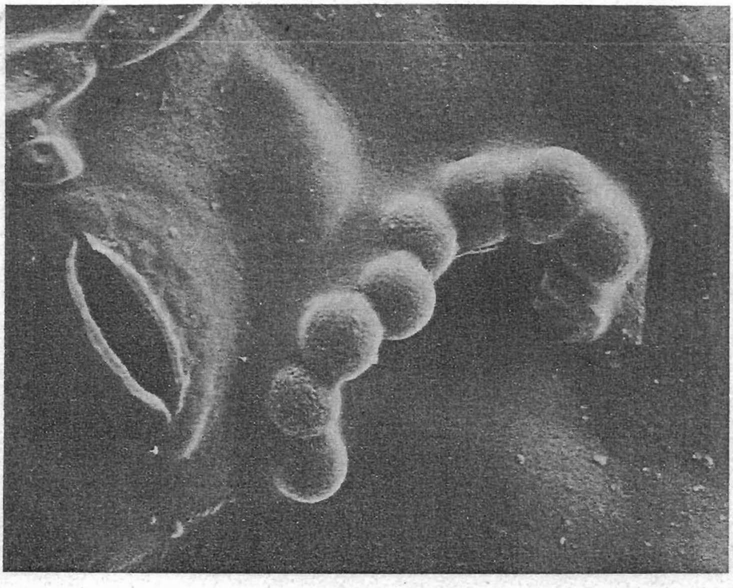

Figure 8.6. *Unknown microorganism on the upper surface of a* Malus *spp. leaf. (LTSEM, × 800.)*

8.4 The influence of water on the infection process

Most infection studies are performed under conditions that do not control the amount of water on the leaf surface. Raspberry cultivars resistant to *Botrytis cinerea* have hairy, waxy canes and their resistance is attributed to greater run off of water (Jarvis, 1994). Thus, the amount of water on the plant surface may influence the interaction of host and pathogens (see also Chapter 14). To study this effect, Williamson *et al.* (1995) dusted rose flowers with dry conidia of *B. cinerea* and incubated them with air of different relative humidities. Conidia germinated in the absence of surface water at 94% relative humidity (r.h.) or above. Germ tubes were smooth, without slime extending over the cuticle and penetrated the epidermis without the formation of appressoria or any structural changes in the cuticle around the germ tube. The authors suggested that no adhesion of the spore may be needed for subsequent infection. Since some germ tubes also had limited contact with the plant surface, penetration of this fungus may require good cuticle contact of the hyphal tip only.

8.5 Morphogenesis of appressorium and penetration hypha

The penetration process of most plant pathogenic fungi is correlated with a series of morphological changes (Figure 8.7), e.g. the differentiation of appressorium and penetration hypha (Mendgen and Deising, 1993). In its simplest form, the germling widens in diameter before penetration occurs and subsequently differentiates a new type of hypha, the penetration hypha with a smaller diameter compared to the germ tube (Figure 8.7). Alternatively, fully developed appressoria may be differentiated (e.g. *Colletotrichum* spp.). This morphogenetic event is preceded by a host signal.

8.5.1 *Signal perception*

Spore germination can be induced by a number of chemical signals (French, 1992). For appressorial development, signals include hardness and hydrophobicity of the substrate (Freytag *et al.*, 1988; Gilbert *et al.*, 1996; Warwar and Dickmann, 1996), heat shock, salts, cyclic AMP (Hoch *et al.*, 1987), starvation (Clay *et al.*, 1994; Jelitto *et al.*, 1994), ethylene (Flaishman and Kollattudkudy, 1994) and components of the cuticle (Kollattukudy *et al.*, 1995). In *Magnaporthe grisea*, the most effective inducers of appressorium development were found to be 1,16-hexadecanedial and 1,16-hexadecanediol (Gilbert *et al.*, 1996). *Uredinales* may recognize topographical signals such as the outer ledge of the stoma or furrows of the cuticle (Hoch and Staples, 1991). Corrêa *et al.*, (1996) suggest that integrin-like proteins are involved in the transmission of such signals from the leaf surface.

8.5.2 *Development of an appressorium*

In a fully differentiated appressorium, the cytoplasm of the germ tube accumulates and a septum separates the appressorial cytoplasm from the highly vacuolated germ tube cytoplasm. The wall of the appressorium may be thickened, or as in the case of *Colletotrichum* spp. and *M. grisea*, enforced with melanin (see below). The appressorium glues to the host surface (Mims *et al.*, 1995). In the case of *M. grisea*, a ring on the base of the appressorium appears to seal the appressorial base to the leaf surface (Howard *et al.*, 1991a).

Deletion of genes expressed during appressorium development of *Colletotrichum gloeosporioides* may interrupt the infection process (Hwang and Kolattukudy, 1995). In *M. grisea*, such a gene coded for a hydrophobin (Talbot *et al.*,

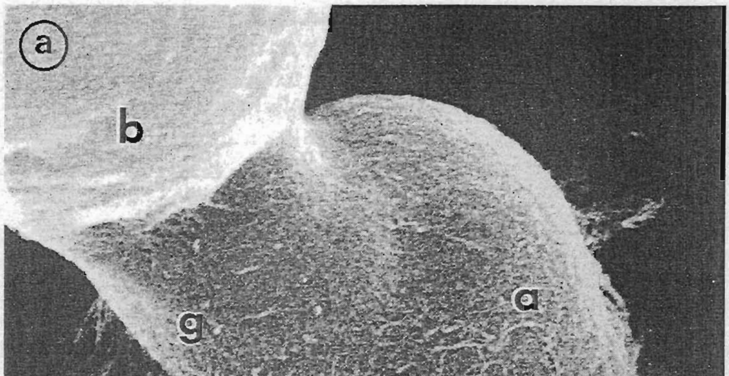

Figure 8.7. *(a) Surface view of a* Uromyces appendiculatus *basidiospore, infecting* Phaseolus vulgaris *(× 5500). (Modified from Gold and Mendgen, 1984.) (b) Cross-section of the* Uromyces vignae *basidiospore, infecting* Vicia faba *(× 5500). (Reproduced from Xu and Mendgen (1994) Endocytosis of 1,3-β-glucans by broad bean cells at the penetration site of the cowpea rust fungus (haploid stage).* Planta ***195****, 282–290, with permission from Springer-Verlag.) (b, basidiospore; g, germ tube; a, appressorium; p, penetration hypha; i, vesicle or infection hypha.)*

1993). Therefore, hydrophobins not only may support adhesion of spores and germ tubes, but also of appressoria.

8.5.3 *Development of the penetration hypha*

In the middle of the appressorial base, the initials of the penetration hypha are characterized by an extremely thin wall, very often surrounded by a cone-like structure. Thus, pressure built up within the appressorium exerts the major force in this place. Vesicles accumulate where penetration hypha development starts. The growing penetration hypha of *Colletotrichum trifolii* does not always grow straight into the host epidermis, as if governed by pressure only, but may turn off in order to avoid the anticlinal walls of the epidermis (Mould *et al.*, 1991). Also, penetration hyphae of *Uredinales* formed in the absence of the host plant, that is on artificial surfaces, still keep their typical shape (Deising *et al.*, 1991). These observations indicate that the penetration hypha itself is able to define its own morphology and direction of growth. The wall of the penetration hypha also is unique. It seems to be modified in such a way as to reduce the elicitation of host defences by masking or modifying the fungal chitin (Deising and Siegrist, 1995).

8.6 Cuticle and cell wall degradation during penetration

The complexity in structure of the cuticle and the plant wall layers below is described in Chapters 2 and 3 by Jeffree and Kolattukudy, respectively. Cuticle and epidermal wall can vary in thickness. In cultivars of rose resistant to *Botrytis cinerea*, the cuticle was thicker as compared to the susceptible cultivar. Under most conditions, this factor seemed to contribute to host plant resistance (Hammer and Evensen, 1994). Studies with transgenic plants having modified wall compositions are not available yet. They would help to elucidate the contribution of the host wall to resist penetration.

The role of fungal enzymes is also unresolved. Since plant pathogenic fungi range from facultative (mostly saprophytic) parasites to obligate parasites entirely dependent on a living host, cell wall-degrading enzymes may enable growth on dead matter and/or contribute to localized penetration into the living cell. This problem was most obvious during the elucidation of the importance of cutinase in pathogenicity. The enzyme is produced by many soil-inhabiting fungi that degrade dead plant material (Heinen, 1963). In plant pathogenic fungi different isoforms of cutinase obviously have different tasks. Conidia of *E. graminis* release cutinase as a component of the conidial exudate to modify the plant cuticle (Pascholati *et al.*, 1993) and in *Uromyces viciae-fabae*, cutinases and esterases contribute to the attachment of the adhesion pad of the spore (Deising *et al.*, 1992). In *B. cinerea* dissolution of the cuticle at the penetration site is suggested by the erosion of the cuticle (Rijkenberg *et al.*, 1980). The enzyme also accumulates around appressoria of *Colletotrichum* spp. (Podila *et al.*, 1995). There, the enzyme may soften the host cuticle in the area of the appressorium. Subsequently the penetration hypha may penetrate by force. Köller *et al.* (1995) have found distinct cutinase isoforms in *Alternaria brassicola,* which may contribute either to saprophytic or pathogenic proliferation. It remains a challenging task to demonstrate a special cutinase isoform at the tip of the penetration hypha. The discussion of this problem is exciting (Kolattukudy *et al.*, 1995; Köller *et al.*, 1995; Schäfer, 1994; Stahl *et al.*, 1994) since the importance of cutinase for infection varied with the conditions used for the infection experiments. We learn from this debate that infection conditions need to be controlled rigorously and have to be as natural as possible.

Work on enzymes that dissolve the wax in the cutin is still completely missing. This work is needed since 'waxases' and cutinases might cooperate during cuticle degradation.

Many components of the plant cell wall are in close contact with or reach into the cutin layer (see Chapter 2). Degradation of polysaccharides may occur by targeted secretion of enzymes. Cellulase production by *Ustilago maydis* occurs during pathogenic development of the fungus only (Schauwecker *et al.*, 1995). *Venturia inaequalis* produces cellulases after contact with cellulose sheets or solvent-extracted apple leaves. Any mechanical disintegration of the leaf substrate resulted in a complete loss of inductive capacity (Kollar, 1994). Presumably, topographic features of the plant surface can induce enzyme production.

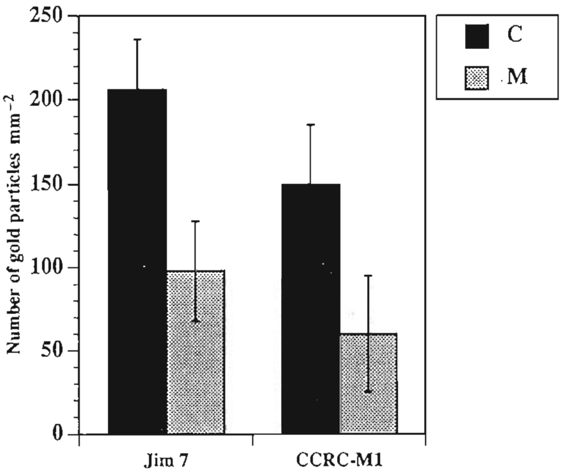

Figure 8.8. Localized degradation of pectin (detected with the monoclonal antibody Jim 7) and xyloglucan (detected with the monoclonal antibody CCRC-M1) in a host wall area, 0.2 µm wide along the penetration hypha of Uromyces vignae. *Degradation is indicated by the number of gold particles bound to the antibodies. (M, enzymatically modified or degraded cell wall; C, normal, non-modified cell wall.) (H. Xu and K. Mendgen, unpublished data.)*

Basidiospores of *Uromyces vignae* obviously penetrate the cuticle with the help of enzymes (Figures 8.7 and 8.8). With antibodies against typical wall constituents, it has been possible to discover localized degradation of pectin and xyloglucans just below the cuticle in the contact zone with the penetration hypha of *Uromyces vignae*. The amount of epitopes for these wall constituents was reduced by 50% (Figure 8.8, H. Xu and K. Mendgen, unpublished data). However, enzymes may be secreted without a role in host wall penetration. Uredospores of the rust fungi germinate without saprophytic phase and produce a number of cell wall-degrading enzymes during stomatal penetration. Only later, during differentiation of haustorial mother cells, do these biotrophic fungi dissolve host walls at very small areas at the tip of the penetration hypha that will give rise to a haustorium. The parasite must be able to handle these enzymes in a way that they are restricted to a tiny area of action. This might be performed by regulated secretion and by binding of the enzyme to the host wall. The high isoelectric point of some cell wall-degrading enzymes (Deising *et al.*, 1995; Deising *et al.*, 1996) suggests that they are bound to the host wall during penetration.

Thus, it is very difficult to prove the role of single wall-degrading enzymes during penetration and subsequent development of plant pathogenic fungi. Although Walton (1994) produced a series of mutants deficient in different enzymes, none of these differed from the wild type in their pathogenicity; for example, deletion of exo β-1,3-glucanase in *Cochliobolus carbonum* led to a restriction in saprophytic growth but did not affect pathogenic development (Schaeffer *et al.*, 1994).

8.7 Turgor pressure

Many fungi such as *Colletotrichum, Magnaporthe, Cochliobolus* or the dikaryon of rusts develop highly differentiated appressoria. *M. grisea* and *C. lindemuthianum* are known to penetrate inert materials such as teflon or epoxy resin (Howard *et al.*, 1991a). A turgor pressure of 8 MPa in appressoria of *M. grisea* (Howard *et al.*, 1991b) enables the fungus to penetrate inert surfaces. Melanization of the appressorial wall seems to be a prerequisite for the ability of the fungus to build up this pressure. Chemically induced melanin-deficient mutants were both unable to form melanized appressoria and apathogenic (Chumley and Valent, 1990; Kubo and

Furusawa, 1991). These observations support the idea of a decisive role of turgor during penetration of host walls by some fungi.

8.8 The cytoskeleton

Since growth of the hyphal tips of *Achlya bisexualis* and *Saprolegnia ferax* proceeds under conditions where turgor could no longer be measured, other factors must contribute to this type of movement (Harold *et al.*, 1995; Money and Harold, 1993). In this case, actin filaments may play the crucial role. Microfilaments grow by the addition of actin monomers to the barbed end that abuts upon the plasma membrane. Elongation is thought to involve detachment of the filament from the membrane and the insertion of a new monomer.

For the acrosomal process of the Thyone sperm, with a diameter of 0.1 μm that contains 140 microfilaments, Cooper (1991) has calculated a force of 60×10^4 dyn cm^{-2} corresponding to a pressure of 0.06 MPa. If the density of filaments would be the same in a fungal hyphal tip, Harold *et al.* (1995) have calculated an actin pressure of 0.004 MPa for a fungal hypha with a larger radius. Since penetration hyphae generally have a smaller diameter, the pressure might differ to some extent at the tip, but the contribution of the actin cytoskeleton to the tip pressure is still only in the range of 1% of considered standard osmotic pressure of 0.4 MPa in fungal hyphae.

8.9 Initial plant defence: cell wall alteration during penetration

Nothing is known about the events during and after cuticle penetration when the fungus contacts the wall layers immediately below. Studies with abraded epidermal surfaces of cucumber (Fauth *et al.*, 1996) and observations with the primary germ tube of *E. graminis* f.sp. *hordei,* which penetrates the epidermal wall of barley only to a certain degree, but never completely, suggest that signals reach the host cell immediately after breach of the cuticle (Kunoh *et al.*, 1991). The time schedule of the first plant cell reactions, measured in cell cultures, is very fast. A few seconds after elicitor binding to parsley cells, Ca^{2+} influx and anion efflux is stimulated that would cause depolarization (Nürnberger *et al.*, 1994). Further reactions include the release of oxygen radicals and peroxide, a phenomenon termed the oxidative burst (Mehdy, 1994). It leads to cross-linking of pre-existing hydroxyproline-rich structural proteins (Brisson *et al.*, 1994). Other plant cell responses in a number of different host–parasite systems include callose synthesis (Kauss, 1995), accumulation of ions (Kunoh, 1990) and the production of hydroxycinnamoyl esters (Matern *et al.*, 1995). The esterification of wall polysaccharides with hydroxycinnamic acids modifies the cell to resist the action of lytic enzymes produced by fungal pathogens (Matern *et al.*, 1995). In addition, the plant may produce proteins that inhibit the action of cell wall-degrading enzymes, such as polygalacturonase-inhibiting proteins (Yao *et al.*, 1995).

Not only the existing cell wall is reinforced. As soon as *Fusarium oxysporum* f.sp. *vasinfectum* starts to penetrate the cells in the meristematic zone of cotton (*Gossypium barbadense*), roots react with the formation of wall appositions. These wall appositions consist of normal cell wall material such as cellulose, xyloglucans, pectin and polygalacturonic acid (Rodriguez-Gálvez and Mendgen, 1995). In addition, large amounts of callose are incorporated. The mechanisms by which pathogenic fungi overcome such wall appositions are mostly unknown.

8.10 Conclusions

We now know most tools a fungus uses to penetrate a plant epidermis. It remains to be elucidated for each host-parasite system which factor contributes to what extent to the penetration process. During these studies, it will be very important to use tests for pathogenicity that are as close as possible to the natural conditions in order to elucidate their importance (Schäfer, 1994).

Acknowledgements

I am indebted to R.J. Howard (Du Pont, Wilmington) for the use of the LT-scanning electron microscope, Christine Struck, Holger Deising, Matthias Hahn and Tim Carver for reading the manuscript. The Deutsche Forschungsgemeinschaft and the Fonds der chemischen Industrie gave financial support.

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