CHAPTER 5

Infection strategies

C. STRUCK AND K. MENDGEN

5.1 INTRODUCTION

Successful colonization of the habitat plant, including uptake of nutrients and reproduction, greatly depends on an efficient mode of infection. Plant parasitic fungi have developed various strategies to enter their hosts and to establish direct contact with them. For example, the pre-penetration phase of an obligate biotrophic leaf pathogen is critical because unfavourable environmental conditions could disturb the development of the fungal structures.

Establishment of contact with the host protoplast is a prerequisite for colonization. Thus, conidia of the leaf pathogen *Erysiphe graminis* initiate physiological contact with the host cell by forming primary germ tubes from 15 to 60 min after inoculation (Kunoh *et al.*, 1977). Formation of appressorial germ tubes and appressoria occurs in 4–8 h (Masri and Ellingboe, 1966). Basidiospores of *Uromyces appendiculatus* need a minimum time of 4–6 h for penetration and vesicle formation (Gold and Mendgen, 1984), whereas the epidemiologically more important uredospores of the obligate rust fungi germinate very quickly – within less than one hour under favourable climate conditions. On the other hand, infection 'cushions' of the soil-borne saprophytic pathogen *Rhizoctonia solani* on cotton take 21 h to be formed before host cell penetration occurs (Armentrout and Downer, 1987).

The severity of the developing epidemic correlates with both the environmental conditions during the early infection phases and the resistance of host plants. In addition, the invasion of the different plant organs, such as leaves, stems, roots and fruits, requires the development of specialized infection structures. The form and shape of these structures (germ tubes, appressoria, infection hyphae and haustoria) is pathogen-specific. A review by Aist (1976) concerning penetration and infection of plant parasitic fungi concentrates on the cytological aspects and describes different penetration types and several specialized fungal structures associated with infection. Progress on biochemical and molecular genetic techniques in combination with a variety of cytochemical techniques has led to a better understanding of the complexities of the early fungus/plant interaction. Some aspects of the molecular mechanisms of fungal pathogenicity to plants have been reviewed recently (Schäfer, 1994; Knogge, 1996). New facts about morphogenesis and penetration mechanisms of plant pathogenic fungi have been summarized by Mendgen *et al.* (1996).

In this chapter we shall focus on various ways in which different plant parasitic fungi enter their hosts and establish direct contact with them. We shall distinguish

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several steps in the plant infection process: attachment to and recognition of the plant surface; germination of propagules and penetration of the plant surface; and colonization of the plant tissue. The influence of environmental conditions on the infection process and the resulting amount of sporulation will be discussed.

5.2 ATTACHMENT OF FUNGAL SPORES

The initial step of establishing infection is the adhesion of fungal propagules on the plant surface. Recent advances concerning these earliest events of fungal infection are reviewed by Mendgen (1996) and Nicholson (1996). A binding process is essential to resist displacement by wind or water. Conidia of many fungi are dispersed in water. Thus, non-adhered spores remain within the water droplets and may be washed away.

Of special importance is attachment of zoospores of soilborne oomycetes living in water-saturated soil to make sure that the pathogen is not washed away by water before invasion of the host tissue can occur. Zoospores of *Phytophthora cinnamoni* that adhere to a surface during the first 3–4 min of encystment remain strongly attached. It was found that, although the adhesive material was still present, cell adhesivness had declined rapidly 5 min later (Gubler *et al.*, 1989).

Considering the differences in surface composition and properties (e.g. hydrophobicity) of aerial plant organs and roots, it seems obvious that fungi use different mechanisms to bind to the host surface. One important factor is the amount of water available during infection. In some cases, the hydration of fungal propagules leads to a rapid release of mucilage that is involved in a passive, non-specific adhesion to a variety of substrates. For example, the spores of the rice blast fungus *Magnaporthe grisea* release a carbohydrate-containing adhesive material from the tip region of the germ tube as a result of hydration expansion (Howard *et al.*, 1991a). About 20 min after hydration, conidia of *Cochliobolus heterostrophus* secrete a material at the spore tips that serves as non-specific adhesive (Braun and Howard, 1994).

Ungerminated conidia of *Colletotrichum graminicola*, the causal agent of anthracnose (leaf blight of corn), begin to adhere within minutes of their contact with the leaf surface. Adhesion is shown to be essential to the success of disease establishment (Mercure *et al.*, 1994). The spores produce a water-soluble glycoprotein-rich material with properties to protect spores against unfavourable conditions such as dry periods (Mercure *et al.*, 1995). Weak adhesion of germlings of *Botrytis cinerea* to hydrophobic substrates occurs immediately upon hydration. In a second stage, strong attachment of viable conidia occurs to either hydrophobic or hydrophilic substrates which involves secretion of a sheath of material (Doss *et al.*, 1995).

Recently, it has been reported that attachment of pycnidiospores of *Phyllosticta ampelicida*, the causal agent of black rot of grape, is a prerequisite for germination. In this case, attachment depends on low wettability of the substrate but not on metabolic processes associated with the conidia. Thus, it seems that the process of adhesion involves hydrophobic and ionic interactions (Kuo and Hoch, 1996). The uredospores of the rust fungus *Uromyces fabae* form an adhesion pad (Fig. 5.1) and release a cutinase and two non-specific esterases after contacting the host cuticle. Apparently, adhesion of the pads is improved by these enzymes. The spores had reduced ability to attach to the leaf surface when these enzymes were inactivated (Deising *et al.*, 1992).

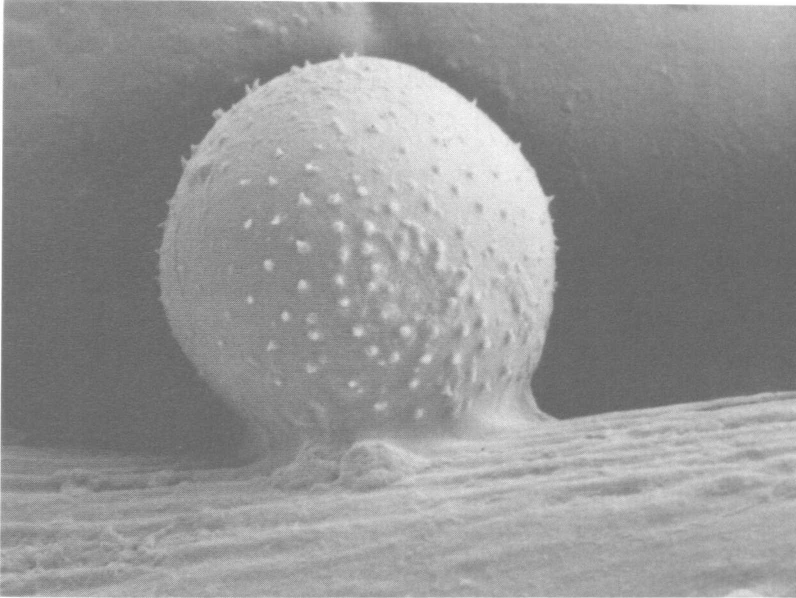

Fig. 5.1 Urediospore of *Uromyces fabae* with an adhesion pad, which mediates contact to the leaf surface (magnification × 3000).

In contrast to propagules of most fungi, conidia of barley powdery mildew (*Erysiphe graminis*) begin the process of adhesion in the absence of free water in a wide range of high relative humidity. Carver *et al.* (1995) showed that the extracellular material released by germlings of *E. graminis* f.sp. *avenae* sticks the fungus firmly to the leaf surface. The conidia of *E. graminis* f.sp. *hordei* produce an esterase-containing liquid in response to a non-specific contact stimulus with the barley leaf surface or to a moistened cellophane surface (Nicholson *et al.*, 1988). Furthermore, this exudate contains cutinase activity (Pascholati *et al.*, 1992), which alters the cuticle surface and may help the fungus to penetrate the leaf surface more efficiently.

5.3 GERMINATION PROCESS: INFLUENCE OF ENVIRONMENTAL CONDITIONS

The infection mechanisms of pathogenic fungi are highly variable. The morphogenetic and physiological differentiation of the invading fungus depends on the mode of penetration. The infection process consists of a number of morphologically more or less distinguishable stages: spore germination, formation of an appressorium and a penetration hypha that penetrates the cuticle and the cell wall. Within the host tissue, pathogenic fungi develop infection hyphae and some biotrophs form haustoria.

During the early phases of the infection process before invading the plant tissue, the developing fungus greatly depends on favourable environmental conditions such as surface moisture, relative humidity, temperature and light. In some cases

also, the supply of nutrients on the leaf surface (Derridj, 1996) may have an influence on germination. Germination of conidia of *Botrytis fabae* is stimulated by the release of nutrients from epidermal cells (Rosall and Mansfield, 1980; Harrison, 1988). In addition, spores of numerous fungal species are stimulated by specific volatile substances released by their host plants (French, 1992).

One factor affecting the infectivity of spores is the duration of wetness periods. For *Venturia inaequalis*, Mills (1944) listed the durations of continuous leaf wetness at different temperatures that permit successful infection. Infection of groundnut by *Puccinia arachidis* requires a minimum of 6 h of leaf wetness. These values decreased linearly as temperature increased from 15 to 25°C (Butler and Jadhav, 1991). It is also important that plant disease incidences depend on the ability of propagules to survive dry periods. The spores must withstand variations in humidity, determining the success of infection or even the survival of spores. Becker and Burr (1994) studied this effect of discontinuous wetting intervals on the viability of conidia and germlings of *V. inaequalis* and showed that viability of germinated conidia decreased by 20% in the first 15 min of a dry interval, whereas ungerminated conidia were not affected by dry intervals of 24 h. Interestingly, dry-inoculated conidia of *Botrytis cinerea* germinated in the absence of water to form short germ tubes only (Fig. 5.2), which appeared to penetrate the leaf surface directly (Williamson *et al.*, 1995; Cole *et al.*, 1996).

The infection of *Phytophthora infestans* is strictly dependent on moisture and temperature. Germination of sporangia with production of a germ tube takes place in the presence of free water or dew at 25°C; indirect germination by releasing motile zoospores following encystment and germination requires water and a temperature of 10–15°C (Robertson, 1991).

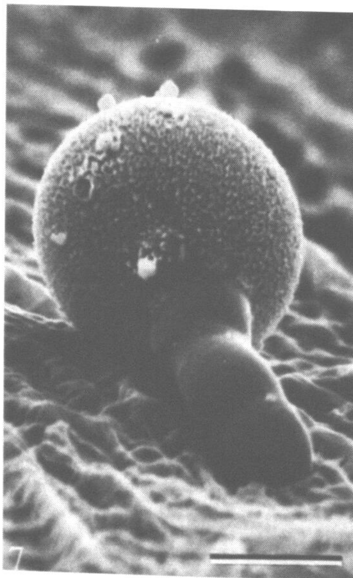

Fig. 5.2 Germinated conidium of *Botrytis cinerea* showing short germ tube without appressorium (scale bar 5 μm). (From Williamson *et al.*, 1995, with permission.)

The infection efficiency of urediospores of the soybean rust *Phakopsora pachyrhizi* decreases on unwetted soybean leaves during sunshine conditions. Uredospores of *P. pachyrhizi* began germinating 1.5 h after dew was provided but no disease developed after dew periods of less than 6 h (Melching *et al.*, 1989). Urediospores drying for four days after an initial dew period showed some infectivity but this was 50% less compared with spores that had not been exposed to an initial dew period. After eight days on dry foliage, uredospores did not cause any more lesions during a following dew period (Melching *et al.*, 1989).

Spores of *Puccinia recondita* f.sp. *tritici* and *P. striiformis* did not germinate after a short dry period. However, as soon as the fungus had reached the substomatal chamber and differentiated substomatal vesicles, the pathogen became independent of wetness conditions on the leaf surface (Vallavielle-Pope *et al.*, 1995). Numerous similar studies describing effects on moisture duration and temperature on disease development have been published – for example on *Botrytis cinerea* (Bulger *et al.*, 1987), *Colletotrichum gloeosporioides* (Chakraborty *et al.*, 1990), *C. acutatum* (Wilson *et al.*, 1990) and *Phytophthora porri* (Smilde *et al.*, 1996). Recent findings on the influence of micrometeorological parameters such as free moisture and relative humidity, focusing on aspects of modelling leaf wetness, have been reviewed by Huber and Gillespie (1992).

5.4 ENTERING THE PLANT TISSUE

Fungal pathogens may penetrate through wounds, through natural openings such as stomata and lenticels, through stigmas, or by direct penetration. Entering of a host plant requires the recognition of possible openings or the breaching of both cuticle and cell wall.

5.4.1 Entering via wounds

Post-harvest diseases caused by *Botrytis* or *Monilinia* are often the result of infections through wounds which result from handling injuries during or after harvest. Although these and other wound-infecting fungi are able to penetrate the cuticle and cell wall directly, some factors of the wound, such as humidity or nutrients, may stimulate the spore germination. In the case of *B. cinerea*, it is assumed that nutrients released from wounds of various host plants lead to greater susceptibility to infection (Harrison, 1988).

Studies of *Phialophora malorum*, the causal agent of side rot (a post-harvest disease of pears), demonstrated that infection depends on the relationship between wound size and hydrostatic pressure in immersion tanks. Infection of wounds < 1 mm in diameter generally depended on immersion depth, whereas at wound diameters $>$ 1 mm infection took place at all immersion depths. Furthermore, it is shown that wound exudates stimulate spore germination (Sugar and Spotts, 1993).

The sporangia and zoospores of *Phytophthora infestans*, the cause of potato late blight, infect potato tubers through any surface not completely suberized, wounds and lenticels (Robertson, 1991), while on potato leaf surface most of the penetrations occur in the stomatal leaf complex and epidermal cells adjoining the stomatal guard cells. These spores can directly penetrate the outer cell wall of epidermal cells (Gees and Hohl, 1988).

5.4.2 Entering via stigmas

Claviceps purpurea, the causal agent of ergot of cereals and grasses, is highly organ-specific. It exclusively attacks the flowers of grasses. The highest infection rates were observed during the few hours of floret opening (Shaw and Mantle, 1980). Infection of rye florets by *C. purpurea* starts from spores germinating on the surface of pistils. They produce an external mycelium, and infection hyphae preferably pass the epidermal cell layer of the lower part of the ovary and grow intercellularly in the anticlinal epidermal cell walls (Tudzynski *et al.*, 1995).

Dicaryotic hyphae of *Ustilago maydis*, the common smut fungus of *Zea mays*, are able to infect all above-ground meristematic plant tissue of maize and form the characteristic galls. In the field, however, maize is generally infected through stigmas. Snetselaar and Mims (1993) observed that infection hyphae grow rapidly across the stigma surface and develop hyaline appressorium-like structures before entering epidermal cells of the stigma.

5.4.3 Entering via stomata and lenticels

Pathogens invading the host through stomata and lenticels enter by penetrating the cell wall with more or less specialized infection structures before the invading hyphae reach the underlying tissue.

Zoospores of pathogenic oomycetes enter their host plants through both stomata and lenticels. Penetration through these natural openings requires that the fungus can find them. It is assumed that fungal zoospores have receptors able to detect signals that influence the direction of motility but our knowledge about chemotaxis of fungal zoospores is limited (Hardham, 1992).

Zoospores of *Plasmopara viticola*, the downy mildew of grape, penetrate through stomata on the undersides of leaves. Secondary zoospores of *Spongospora subterranea*, the causal agent of powdery scab of potatoes, enter roots or the tuber tissue through lenticels and stomata. Zoospores of the downy mildew *Pseudoperonospora humuli* swim towards open stomata of hop leaves. It seems likely that this fungus uses both the physical stimulus of the open stoma and chemical stimuli indicating active photosynthesis (Royle and Thomas, 1973).

In contrast to zoospores which localize their penetration site actively, some fungi have evolved a non-motile way of orientation. In the plasma membrane of germ tubes of *U. appendiculatus* mechanosensitive ion channels have been identified which might be involved in the perception of topographical signals leading to oriented growth and localization of stomata (Zhou *et al.*, 1991).

5.4.4 Direct penetration of cuticle and host cell wall

The most common type of entry into leaves, roots and fruits is by direct penetration through the plant surface with minor modifications of the hyphae. In some cases the fungus only penetrates the cuticle and grows between cuticle and cell wall. *Rhynchosporium secalis* (Oudem.) J.J. Davis (the causal agent of barley leaf scald) and *Venturia inaequalis* (causal agent of apple scab) develop such subcuticular mycelia.

The saprophytic pathogen *Botrytis cinerea* is responsible for various plant diseases of leaves and fruits and is able to infect leaves, stems and fruits. In the case of the post-harvest decay of fruits, the developmental stage of the fruit is important for a

successful invasion. The green fruit is less susceptible than mature fruit. The fungus is unable to breach the cuticle of green fruits, but penetrates through their stomata. In contrast, direct penetration occurs in ripe nectarines (Fourie and Holzl, 1995).

In many fungi, the infection sites are not found in a random pattern on their host surface. *Pythium ultimum*, a soilborne rot disease of roots and leaves, mainly penetrates the elongation and maturation regions near root tips but rarely the mature root parts and never the meristem or the differentiation zone just behind. *Fusarium oxysporum*, causal agent of vascular wilts with a wide host range, infects plants through the roots by direct penetration. Rodriguez-Galvez and Mendgen (1995) observed that *F. oxysporum* f.sp. *vasinfectum* penetrates preferentially in the meristematic zone of cotton roots, and 40% less in the elongation and root hair zone. No further infection occurs as soon as root branching starts.

5.4.5 Appressorium formation

In many fungi, wall penetration is initiated by the differentiation of appressoria. From a pore in the appressorial base a penetration hypha (or infection peg) grows directly through the cuticle and cell wall into the host tissue or, in the case of dicaryotic stages of rust fungi, through the stomatal aperture into the substomatal chamber. Some directly penetrating plant pathogens such as *Colletotrichum* species produce an appressorial cone (Landes and Hoffmann, 1979). This new cell wall structure surrounding the germ-pore may function to focus the penetration pressure on the small area of the pore (O'Connell and Bailey, 1991).

Appressoria attach firmly to their substrate and increase the area of contact between the fungus and the host. Depending on the species, appressoria are positioned over stomata or they develop in random distribution over the leaf surface – in some fungi preferentially over anticlinal cell walls of epidermal cells, as shown for *Cochliobulus sativus*. This preference may be related to the availability of nutrients and moisture.

To place the appressorium in the optimal site for penetration, a recognition event is required which includes topographical, chemical or environmental signals. *In vitro* studies of germinating spores of *C. sativus* indicate that both chemical and topographic signals given by anticlinal cell wall junctures over epidermal cells are involved in the appressorium induction (Clay *et al.*, 1994).

Appressorium formation by urediospore germ tubes of the bean rust *Uromyces appendiculatus* is induced by physical differences in the topography of the leaf surface, such as stomatal lips of guard cells (Fig. 5.3), or by defined ridges of 0.5 μm height formed on an artificial surface (Kwon and Hoch, 1991). In addition, Allen *et al.* (1991) showed that many rust fungi exhibit species-specific responses on membranes with defined topographies.

Surface contact on host leaves or artificial substrates was found to be essential for the formation of appressoria by germ tubes of *Magnaporthe grisea* (Xiao *et al.*, 1994). Furthermore, a high surface hydrophobicity and light favoured the formation of appressoria but these factors are not essential (Jelitto *et al.*, 1994). Gilbert *et al.* (1996) reported evidence for the regulation of appressorium formation of *M. grisea* by chemical signals: they found that plant cutin monomers of host plants and non-host plants induced infection structure formation.

Host surface wax components have been shown to induce germination and appressorium differentiation in *Colletotrichum gloeosporioides* causing rot in some

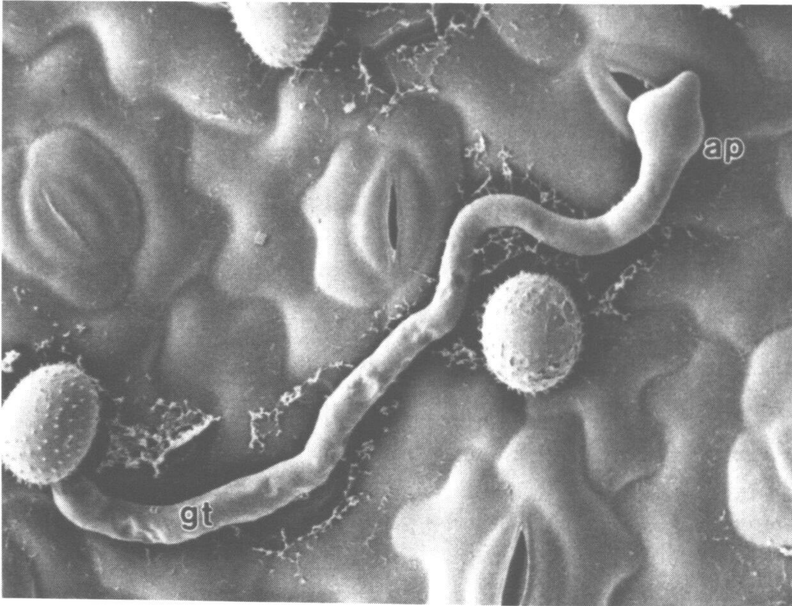

Fig. 5.3 Urediospore germling of *U. fabae* penetrating the stomatal pore from an appressorium (magnification × 690): **gt** = germ tube; **ap** = appressorium.

tropical fruits. Hwang and Kolattukudy (1995) cloned three genes which were expressed in appressorium-forming conidia induced by avocado surface wax. Disruption of one of these genes caused failure to penetrate the host tissue.

5.4.6 The penetration process

The mechanism of penetration has been discussed for a long time. Penetration may be the result of either mechanical force or enzymatic digestion by means of hydrolytic enzymes secreted by fungal pathogens. A combination of both mechanisms is most likely but the contribution that each one makes is unclear.

Early studies on *Botrytis cinerea* suggested that mechanical forces are involved in penetrating the plant surface (Miyoshi, 1895). Another example of a mechanical penetration process is that of *C. lindemuthianum* while penetrating a layer of resin (Mercer *et al.*, 1971).

A well studied example of penetration by mechanical force is that of the rice blast fungus *Magnaporthe grisea* (Howard and Valent 1996). Cytological and genetic studies indicate that high turgor pressure generated in the appressorium enables the fungus to penetrate plant cuticle and cell wall. In mature appressoria, cell walls are melanized. Experiments with melanin synthesis inhibitors (Uehara *et al.*, 1995) and with melanin-deficient mutants (Chumley and Valent, 1990) show that non-melanized appressoria have lost the ability to penetrate the plant surface as well as artificial membranes. Melanin lowers the porosity of appressoria and thus helps to increase the osmotic pressure – up to 80 bars in appressoria of *M. grisea* (Howard *et al.*, 1991b). Turgor measurements showed that, in wild-type conidia and in conidia of melanin-deficient strains, the pressure generated is the same; in contrast to the

conidia, wild-type appressoria generated much higher pressures than appressoria of a melanin-deficient mutant (Money and Howard, 1996).

(a) Mechanisms for penetration of the cuticle

Penetration of plant surfaces might be supported by cuticle and cell wall degrading enzymes like cutinases, cellulases and pectinases synthezised and secreted by the fungus (for review see Mendgen and Deising, 1993; Mendgen, 1996). Essential to an understanding of cuticle and cell wall degrading enzymes is a knowledge of the complex structure of these surface barriers. The first barrier to be breached is the plant cuticle. It consists of two lipid polymers, which may occur in any ratio, embedded into wax. The dominant structural component is the lipid polyester cutin, a polar cross-linked polymer containing predominantly C16 and C18 fatty acids. It is readily solubilized by alkaline hydrolysis. A very insoluble residue remains after saponification – the polymethylene polymer cutan. For detailed reviews, see Köller (1991), Jeffree (1996) and Riederer and Markstädter (1996).

The diversity of the physical structure and chemical composition varies not only in different plant species. Even within the same species, it depends on the type of tissue, environmental conditions and the age of the plant.

Cuticle penetration by enzymatic digestion would require cutin-degrading cutinases. Many plant pathogenic fungi produce cutinase but the importance of this enzyme for pathogenicity is disputed. The first cutinase studied in plant pathogens is that of the pea pathogen *Fusarium solani* f.sp. *pisi* (*Nectria haematococca*). Kolattukudy and co-workers demonstrated the secretion of this enzyme during penetration of the host cuticle (Shaykh *et al*, 1977). Inhibitors of cutinase as well as antibodies to it prevented fungal infection on intact host surfaces but no effect has been found on wounded cuticle (Maiti and Kolattukudy, 1979; Köller *et al.*, 1982). Furthermore, insertion of a cutinase gene derived from *F. solani* f.sp. *pisi* to the cutinase-deficient wound parasite *Mycosphaerella* spp. enabled the transformants to infect intact surfaces of papaya fruits (Dickman *et al.*, 1989).

Results of a promoter analysis with transformants of *F. solani* f.sp. *pisi* showed that plant cutin monomers regulate the induction of the cutinase gene (Bajar *et al.*, 1991; Kämper *et al.*, 1994). However, other results have questioned the importance of cutinase for penetrating the host cuticle: the disruption of cutinase genes gave no indication for any involvement of cutinases in pathogenicity of *F. solani* (Stahl and Schäfer, 1994). Furthermore, quantitative and microscopical analyses of the infection of *F. solani* showed that the wild type as well as the null mutant invaded the pea stem by direct penetration (Stahl *et al.*, 1994). These results suggest either that cutinases were not important in plant infection, or that different cutinases, as yet not characterized, were required. The results by both groups suggest that the significance of cutinases depends on experimental conditions used for inoculation. Therefore, Schäfer (1994) emphasized that the pathogenicity must be tested under natural conditions defined as precisely as possible. Interestingly, the *F. solani* strain containing the disrupted cutinase gene exhibited a tendency to penetrate the host mostly via stomata (Rogers *et al.*, 1994).

Recently, more experiments have put into question the importance of cutinase for infection. Two isoenzymes of cutinase with different enzymatic properties were purified from *Alternaria brassicola* (Yao and Köller, 1995). The disruption of one of the cutinase genes had no effect on pathogenicity of the null mutants. A cutinase-deficient

mutant of *Botrytis cinerea* was constructed to determine whether the fungus required cutinase for successful penetration. However, the ability to penetrate *Gerbera* flowers and tomato fruits was unaltered compared with the wild-type (VanKan *et al.*, 1997). So far, in no case has it been possible to demonstrate that all genes responsible for cutinases or esterases with cutinolytic activity were inactivated. Nevertheless, cutinases may have additional roles during the infection process. Release of cutinase by the obligately parasitic fungus *Erysiphe graminis* appears to be involved in quite different tasks. Cutin monomers coated on glass slides act as a signal triggering the development of the appressorial germ tube of *Erysiphe graminis* f.sp. *hordei*. Furthermore, inhibition of cutinase activity results in decreasing germling development on barley leaves. It seems that the cutin monomers that are released by the action of cutinase of powdery mildew spores act as a signal for germ tube development (Francis *et al.*, 1996). However, it is assumed that higher plants have evolved mechanisms for perception of molecules released by enzymatic activity of pathogens and use these chemical signals for the induction of defence reactions (Dixon *et al.*, 1994). It has now been shown that application of cutin monomers partially protects leaves of a highly susceptible barley cultivar against *E. graminis* (Schweizer *et al.*, 1996a). Furthermore, activation of defence-related genes could be observed on the mRNA level (Schweizer *et al.*, 1996b).

These examples of the many tasks of cutinase may explain the problems that arise when the infection process is claimed to rely on single virulence (or avirulence) factors. Degradation of the cuticle by cutinases may be responsible for adhesion phenomena of early fungal structures on the host surface, for penetration of the cuticle, involvment in releasing signal molecules and recognition of the host surface by the pathogen, as well as induction of defence mechanisms. It may be that none of these factors is recognized during infection studies under laboratory conditions.

(b) Penetration of the cell wall

After breaching the host cuticle, the next barrier to the invading fungus is the cell wall. In mature plant cells, this mainly consists of two layers: the primary and the secondary cell wall. The structural complexity of the primary cell wall is described in Carpita and Gibeaut (1993) and Carpita *et al.* (1996). In most flowering plants it consists of chains of β-1,4-linked glucose interwoven with a xyloglucan polymer embedded in a matrix of pectin. In contrast, the cellulose microfibrils of primary cell walls of poaceaes contain chains of β-1,4-xylose, instead of xyloglucan, which are connected by arabinose and less frequently by glucoronic acid. Both types of primary cell wall are associated with protein components. The major structural protein is extensin, a hydroyprolin-rich glycoprotein (Showalter, 1993).

Degradation of this complex structure of the cell wall requires several enzymes, including cellulases, xylanases, pectic enzymes and proteases which act synergistically to degrade the polymers efficiently. Penetration by obligately biotrophic parasites, such as rust fungi and powdery mildew or some hemibiotrophs, requires only minor damage of the cell wall. Secretion of cellulytic enzymes of these pathogens are either developmentally regulated or triggered by environmental signals (Mendgen, 1996).

Cellulase activity of *Uromyces fabae* germlings is shown to be strictly regulated by differentiation. It increases during appressorium formation and reaches a maximum during development of infection hyphae and haustorial mother cells (Heiler *et al.*,

1993) Also, the production of the pectic enzymes pectin methylesterase and polygalacturonate lyase (Deising *et al.*, 1995) and extracellular proteases (Rauscher *et al.*, 1995) of this rust fungus depends on the differentiation of infection structures. Using antibodies against epitopes present in pectin, polygalacturonic acid, xyloglucan and callose, Xu and Mendgen (1997) studied the modification of the *Vicia faba* cell wall components during penetration of epidermal walls by the cowpea rust fungus *Uromyces vignae*. Compared with non-infected areas, the density of pectin and xyloglucan epitopes was reduced. A similar reduction was observed after treatment of non-infected leaves with xylanase, cellulase and pectinase, which indicates that the fungus degrades the plant cell wall at the penetration site.

During its biotrophic phase, the mycelium of *Venturia inaequalis* does not macerate the host tissue and is restricted to the area between the cuticle and the outer epidermal cell wall. Low amounts of cell wall-degrading enzymes are necessary to remove physical barriers and to release nutrients. Kollar (1994) detected a pattern of 12 cellulase isoenzymes that are produced constitutively in very low amounts *in situ* as well as in *in vitro* cultures of different isolates of *V. inaequalis*. This complex cellulytic system, with low variability, appears to be correlated with virulence of the fungus or may give flexibility with properties that contribute to the performance of the enzyme.

The adaptations enabling the hemibiotrophic *Claviceps* spp. to infect their hosts during the brief receptive period of anthesis ensure that the fungus obtains sufficient nutrients to produce its survival structures. Cell wall degrading enzymes might play an important role during the first stages of infection before establishing a stable host–parasite interface in the grass ovaries. Tenberge *et al.* (1996) described two putative polygalacturonase genes of *Claviceps purpurea* that are expressed in axenic culture as well as *in planta* from very early stages of infection up to the beginning of sclerotia formation. During this early phase, the fungus grows mainly intercellularly in the host pistil towards the ovarian tissue. The action of polygalacturonases might loosen the middle lamella and make the cell wall more accessible for other fungal enzymes.

5.5 STRATEGIES FOR COLONIZATION OF HOST TISSUE

Necrotrophic parasites can obtain nutrients from dead plant tissues. These fungi establish themselves inside the host tissue by releasing toxins and macerating enzymes, which disrupt cell integrity and cause cell death immediately. In contrast, plant-infecting biotrophic fungi colonize and draw nutrients only from living tissue. During infection, some of these pathogens form specialized physiological and morphological adaptations such as haustoria which represent the host–parasite interface specialized in nutrient uptake.

5.5.1 Colonization supported by enzymes

Fungal enzymes can play important roles during any or all stages of infection: initial penetration as described above, supporting the spread through the host tissue and later by providing a food source. The set of macerating enzymes and some detoxifying enzymes may play a role in the infection process. One interesting and well studied example is the pisatin demethylase of the mating population VI of *Nectria haematococca*, pathogenic on pea. The fungus is able to detoxify the pea phytoalexin

pisatin by a cytochrome P450-mediated demethylation (Matthews and VanEtten, 1983). Recently, it has been shown that mutants of *N. haematococca* deficient in pisatin demethylase (Pda$^-$) created by transformation-mediated gene disruption are less virulent on pea compared with the Pda$^+$-isolates but the lack of Pda does not eliminate the virulence of the pathogen on pea (Wasmann and VanEtten, 1996). Furthermore, after incorporation of the *Pda* gene into a Pda$^-$ isolate of *N. haematococca*, the virulence increases (Ciuffetti and VanEtten, 1996).

The group of cell wall degrading enzymes, including cellulases, xylanases, pectic enzymes and proteases, are important factors for the parasite in both spreading through the host tissue and obtaining nutrients. Vast amounts of these enzymes are secreted by saprophytes and necrophytes and are found to be regulated by substrate induction or product repression. Although it is well known that most plant parasites secrete cell wall degrading enzymes, the importance of those enzymes for their pathogenicity is poorly understood. Gene disruption experiments constructing specific mutants lacking one or more cell wall degrading enzymes have been used in order to understand better the role of these enzymes in pathogenicity.

Mutants of the maize pathogen *Cochliobolus carbonum* that specifically lacked a functional gene for a xylan-degrading enzyme showed 85–94% reduced glucanase activity but growth of this strain was indistinguishable from the wild type in media containing corn cell walls or xylan as the sole carbon source (Apel *et al.*, 1993).

To examine the role of proteases in plant pathogenicity, the *ALP1* gene (which encodes two extracellular serin proteases of *C. carbonum*) was disrupted. The total protease activity in the null mutants was reduced by 35–45%. Both the *in vivo* growth on medium containing collagen and the disease phenotypes of *C. carbonum ALP1* null mutants were indistinguishable from the wild type strain, indicating that these proteases are not required for pathogenicity (Murphy and Walton, 1996).

The role of pectinases, including pectate lyases, polygalacturonases and pectin methylesterases, have been studied in many fungal and bacterial plant pathogens. They are involved in the degradation of pectin, the main compound of the middle lamella and primary cell walls and thus able to cause maceration. During recent years, pectinase-encoding genes have been cloned from different fungal pathogens, such as *C. carbonum* (Scott-Craig *et al.*, 1990), *Fusarium moniliforme* (Caprari *et al.*, 1993), *Sclerotinia sclerotiorum* (Reymond *et al.*, 1994) and *Glomerella cingulata* (Templeton *et al.*, 1994). All subsequent studies with pectinase-deficient mutants indicate that the gene products are not essential for pathogenicity (Scott-Craig *et al.*, 1990; Bowen *et al.*, 1995). The possibility that additional genes compensate the loss of those pectinases cannot be excluded. In the case of *F. solani* f.sp. *pisi*, various genes encoding pectate lyases have been found (Guo *et al.*, 1995a,b). A new pectate lyase has been described (Guo *et al.*, 1996) which is synthesized uniquely in infected host tissue. *In planta* induced pectate lyases could be responsible for pathogenicity or enhanced virulence but the issue requires further studies.

5.5.2 Colonization supported by toxins

It is generally accepted that fungal toxins support the progress of diseases caused by necrotrophic fungi. There is great diversity of fungal toxins in structure as well as in their modes of action. In many cases, toxins are very effective in causing tissue damage but their role in pathogenicity is not fully understood. Many attempts have been made to distinguish between toxins that are essential for infection and those

that are involved as virulence factors. Progress has been obtained by the use of molecular genetic approaches.

The non-host selective toxins typically affecting fundamental processes potentially have activity on host as well as on non-host plants. One interesting example of these toxins is a group of phytotoxic peptides of the barley pathogen *Rhynchosporium secalis* that are known to induce necrosis of barley leaves, as well as leaves of other cereals and some dicotyledonous plants (Wevelsiep *et al.*, 1993). One of these necrosis-inducing proteins, called NIP1, seems to be a product of a fungal avirulence gene that was found to induce a race-specific defence response in barley carrying the resistance gene *Rrs1* (Hahn *et al.*, 1993). Furthermore, transformation of a race virulent on barley with the *nip1* gene yielded avirulent mutants (Rohe *et al.*, 1995). In summary, the NIP1 protein, which was detected originally as a non-specific phytotoxin, has been shown to function as elicitor in the recognition process of plants that results in the onset of the defence response.

In contrast, the host-selective toxins, most of which are produced by species of the genera *Alternaria* and *Cochliobolus*, are determinants of specificity. The function of some of these compounds is regulated by single host plant genes (Walton, 1996), making them ideal subjects for examining host parasite specificity.

Another interesting example of phytotoxins is the *Alternaria* toxin group. In this genus, nine host-specific toxins are known that differ structurally as well as in their mode of action. In all cases, direct or indirect disfunctions of the host cell plasma membrane have been observed (Otani *et al.*, 1995). The toxin of *A. alternata* f.sp. *lycopersicon* (AAL) is known as a pathogenicity factor for *Alternaria* stem canker disease of tomato. Only cultivars of tomato that were susceptible to the pathogen were highly sensitive to the toxin. The precise site of action is unknown. However, AAL is an interesting exception in the group of host-specific toxins. AAL and the host-unspecific *Fusarium* toxin, fumonisin, function as inhibitors of ceramide synthesis in several plants and animals. Therefore, inhibition of ceramide synthase is not the basis of host specificity (Gilchrist *et al.*, 1995). Some results in research on the AAL toxin led to the assumption that this toxin is involved in apoptosis of the host plants (Wang *et al.*, 1996). The authors observed fragmentation of nucleosomal DNA, a typical characteristic of apoptosis in animals. They therefore concluded that the fundamental elements of apoptosis are conserved in plants and that the lesions formed in *Alternaria* stem canker disease of tomato are results of this process. It seems likely that AAL affects the signaling pathway leading to apoptosis in plants and can be used to characterize this process in more detail.

5.5.3 Colonization strategies by biotrophic pathogens

In contrast to necrotrophs, biotrophic plant pathogens obtain their nutrients from living host plant cells. A prerequisite for nutrient movement between host and pathogen is the development of a functional interface which promotes the transfer. In some fungi, this contact zone is represented by intercellular hyphae (e.g. *Cladosporium fulvum*, *Taphrina* spp.) or intracellular hyphae (e.g. *Ustilago maydis*, *Claviceps purpurea*) (Spencer-Phillips, 1997). The obligate biotrophs, including downy mildews, powdery mildews and rust fungi, produce haustoria – specialized infection structures that bring the fungus into close contact with the plant symplast.

Among the parasitic fungi, many species do not function strictly as biotrophs or necrotrophs; they have evolved both modes of tropism. For example, *Colletotrichum*

lindemuthianum and *Phytophthora infestans* are so-called hemibiotrophic fungi that obtain their nutrients from the living host for a limited period after infection before the host cell dies. A predominantly biotrophic phase until reproduction occurs has been observed in some parasites, such as *Septoria* spp., *Claviceps* spp. and *Venturia inaequalis* (Parbery, 1996).

The biotrophic way of life requires a high degree of adaptation to the living host metabolism to ensure the supply of nutrients from the living plant cell (without seriously disturbing the host cell metabolism) and the avoidance of host defence reactions. One of the most intensively studied examples of this topic is the powdery mildew fungus, *Erysiphe graminis*, reviewed by Aist and Bushnell (1991), because the haustoria of this species provide the only interface with the host cell, while the mycelium develops on the leaf surface.

The fact that obligate biotrophs, such as downy mildews, powdery mildews and rust fungi, do not develop at all or only very poorly in culture has led to the assumption that these fungi require special nutrients or signals from the host (Smith, 1900; Bushnell, 1972). The mechanism of the transport process across the host parasite interface is not well understood. For instance, it is not clear whether special modifications of this process exist within haustorial structures. It has been hypothesized that substrate uptake requires an active mechanism driven by the activity of the plasma membrane H^+-ATPase. Cytochemical investigations indicated the presence of H^+-ATPase activity in the haustorial membrane of *Erysiphe pisi* (Spencer-Phillips and Gay, 1981), *Puccinia poarum* (Woods and Gay, 1987) and *P. punctiformis* (Baka *et al.*, 1995). The first direct biochemical evidence for the existence of a plasma membrane-bound H^+-ATPase in the rust fungus *U. fabae* was presented by Struck *et al.* (1996), who also showed that, compared with ungerminated urediospores and germ tubes, ATPase activity is four to six times higher in the haustoria.

Experimental evidence for nutrient transfer has been obtained for powdery mildew haustoria. Studies on primary infections of *E. graminis* showed that the transfer of ^{32}P- and ^{35}S-labelled compounds from host to pathogen depended on the successful establishment of the primary haustorium (Slesinski and Ellingboe, 1971; Martin and Ellingboe, 1978). Other biochemical investigations have concentrated on soluble photosynthates such as carbohydrates and amino acids. A demonstration of nutrient uptake by haustoria of the powdery mildew fungus, *E. pisi*, has been achieved by isolating haustorial complexes from infected leaves exposed to $^{14}CO_2$ (Manners and Gay, 1978, 1982).

Early studies on the rust fungus *Uromyces phaseoli*, after exposure of infected leaves to radiolabelled amino acids, gave evidence for solute transport into haustoria (Mendgen, 1979).

Heath and Skalamera (1997) emphasized that haustoria might be more important for fungal amino acid uptake than for carbohydrate uptake. The first evidence for this speculation comes from studies on molecular components of haustoria of *Uromyces fabae*, showing that one of various haustorium-specific genes encodes a putative amino acid permease which was detected exclusively in plasma membranes of haustoria (Hahn *et al.*, 1997).

5.6 CONCLUSIONS

Apart from favourable environmental conditions and physiological qualities of the host plant, the astonishing complexity of the fungal infection process and the

multiplicity of infection strategies are the most influential factors for the spread of plant diseases and the development of severe epidemics.

Our understanding of fungal infection mechanisms has been significantly advanced through the use of molecular techniques, which provide the tools to characterize all stages of the interaction between plants and pathogens – from the earliest recognition event to colonization of plant tissue. Nevertheless, research in recent years has shown that a more detailed knowledge of physiological, biochemical and biophysical processes gained through genetic approaches and results obtained with 'conventional' techniques will yield the biological knowledge that can bring practical benefits for the protection of plants from pathogen attack.

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