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Infection of beech leaves by the endophyte *Discula umbrinella* (Berk. & Br.) Morelet [teleomorph: *Apiognomonia errabunda* (Rob.) Höhnel]: an ultrastructural study

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Summary

The aim of this study was to detect the mode of penetration and colonisation of beech leaves by the symptomless endophyte *Discula umbrinella*, a fungus known to induce only sporadically anthracnose of beech leaves.

In a first step, appropriate methods were developed to carry out the investigation. Light microscopy was useful only for preliminary observations of the endophyte on the leaf surface and at the subcuticular level. To a large extent, however, only electron microscopy could provide all the necessary information. Only high pressure freezing was useful to study the ultrastructure of the conidia of *D. umbrinella*, since the extracellular matrix present around the conidial cell wall was completely dissolved after conventional chemical fixation. On the other hand, cryofixation of the beech leaf resulted in an only partial preservation of the tissues, thus making this method inadequate to detect the endophyte in the tissues and to study its interaction with the host. Therefore, subsequent studies of the infection process on the beech leaf were carried out using chemical fixation. Low temperature scanning electron microscopy was shown to be the most adequate method to study the endophyte on the leaf surface.

To facilitate the detection of the fungus in the tissues, disease symptoms were induced by exposing the leaves to stress conditions. On detached beech leaves, and to a lesser extent on whole infected plants, the fungus induced conspicuous necrotic spots. A reaction of the host was apparently involved, since the symptoms were limited to the infection drops.

Ultrastructural studies of the conidia of *D. umbrinella* revealed the presence of a fibrillar extracellular matrix composed of (glyco)-proteins and polysaccharides. The inner part of the conidial cell wall is electron transparent and contains mainly chitin. *D. umbrinella* from different hosts are morphologically distinguishable by the shape and size of their extracellular matrices, which may be indicative of an adaptation to different leaf surface morphologies.

In vitro cytological investigations demonstrated clearly that *D. umbrinella* is able to penetrate the host, directly or after the formation of an appressorium, and to colonise after a period of latency the whole tissue, inducing necrotic spots. Colonisation of the beech leaf is apparently mediated by enzymes which may be activated by host signals, but the action of mechanical forces cannot be excluded. The infection process started with a specific adhesion of the fungal conidia to the host surface. Polysaccharides such as mannans and glucans or their moieties and (glyco)-proteins present in the extracellular matrix of the conidia are clearly involved in the binding process, since the ablation of the fibrils inhibited the adhesion to the host surface. It is possible that

compounds present on the leaf surface are specifically recognised by the extracellular fungal matrix. When contact with the host is established, leaf compounds that act as signals for the fungus possibly induce spore germination.

This study demonstrates that endophytes which are present in host tissues without causing disease symptoms may be latent pathogens, their pathogenic character being expressed under particular conditions only.

Résumé

Le but de ce travail est de détecter le mode de pénétration et de colonisation de feuilles de hêtre par l'endophyte *Discula umbrinella*, un champignon induisant sporadiquement l'anthracnose du hêtre.

Dans un premier temps, les methodes appropriées à cette études ont été développées. La microscopie photonique n'apporte des informations utiles que pour des observations préliminaires de la surface des feuilles et au niveau subcuticulaire. Seule la microscopie électronique, au sens large du terme, permet d'obtenir des informations détaillées. La cryofixation sous haute pression s'avère être la méthode la mieux adaptée au maintien de l'ultrastructure des conidies de *D. umbrinella* puisque la matrice extracellulaire présente autour des conidies est complètement dissoute par la fixation chimique conventionelle. D'autre part, la cryofixation de matériel végétal reste un problème. En effet, le tissu n'est que partiellement préservé, rendant la méthode inadéquate à la détection de l'endophyte et à l'étude de l'interaction avec son hôte. Ainsi, toutes les études impliquant la feuille de hêtre ont été conduites en fixant le matériel conventionnellement. Pour l'observation de l'endophyte à la surface de la feuille, la microscopie à balayage à basse température est la méthode la mieux adaptée.

Pour faciliter la détection du champignon dans le tissu, des symptômes ont été induits en soumettant les feuilles à des conditions de stress. L'infection de feuilles détachées de la plante ainsi que de plantes entières provoque des nécroses macroscopiquement visibles. Toutefois, l'hôte semble réagir, puisque les symptômes ne s'étendent pratiquement pas au-delà des gouttes d'infection.

L'étude ultrastructurale des conidies de *D. umbrinella* démontre la présence d'une matrice fibrillaire extracellulaire composée de (glyco)-protéines et de polysaccharides. La partie interne de la paroi cellulaire des conidies est transparente aux électrons et contient principalement de la chitine. Différents isolats de *D. umbrinella* provenant de différents hôtes se distinguent morphologiquement par la forme et les dimensions de leur matrice extracellulaire, pouvant indiquer une adaptation à différentes surfaces de feuilles.

Les travaux cytologiques *in vitro* démontrent clairement que *D. umbrinella* peut pénétrer son hôte directement ou en formant un appressorium et, après une période de latence, coloniser tout le tissu, induisant des nécroses. La colonisation de la feuille de hêtre semble être dirigée par des enzymes pouvant être activés par des signaux de l'hôte, toutefois l'intervention de forces méchaniques ne peut pas être exclue. L'infection commence par l'adhésion spécifique des conidies à la surface de la feuille. Des polysaccharides, tels que des mannanes et des glucanes ou leurs dérivés ainsi que des (glyco)-protéines présents dans la matrice extracellulaire des conidies sont clairement

responsables de l'adhésion, puisque l'ablation des fibrilles inhibe complètement ce méchanisme. Il est possible que des composés présents à la surface de la feuille soient spécifiquement reconnus par des substances extracellulaires du champignon. Lorsque le contact avec l'hôte est établi, des composés de la feuille, jouant probablement le rôle de signaux pour le champignon, induisent la germination.

Cette étude démontre que les endophytes vivant dans les tissus de leur hôte sans provoquer de symptômes peuvent être des pathogènes latents exprimant leur pathogénicité que dans des conditions particulières.

Zusammenfassung

Ziel dieser Arbeit ist es, das Eindringen und die Kolonisationsmechanismen des Endophyten *Discula umbrinella* auf Buchenblätter zu untersuchen.
In einem ersten Teil mussten die Methoden auf diese Untersuchung angepasst werden. Das Lichtmikroskop ergab zufriedenstellende Resultate nur für Beobachtungen der Wirtsoberfläche und im subkutikularen Bereich des Gewebes. Detailinformationen konnten nur mit Hilfe der Elektronenmikroskopie erzielt werden. Ultrastrukturelle Studien der Konidien von *D. umbrinella* konnten nur nach Kryofixation unter hohem Druck befriedigend durchgeführt werden, da extrazelluläre Komponenten nach konventioneller chemischer Fixation aufgelöst werden. Das Hochdruckgefrieren von pflanzlichem Material bleibt allerdings ein Problem. Nur einzelne Stellen der Proben bleiben gut erhalten, was den genauen Nachweis des Endophyten im Blattgewebe hindert und somit die Untersuchung der Interaktion mit dem Wirt nicht erlaubt.
Um das Finden des Pilzes im Wirtsgewebe zu ermöglichen, wurden Symptome induziert, indem die Blätter unter Stressbedingungen gestellt wurden. Sowohl auf von der Pflanze abgetrennten Blättern, als auch auf ganzen Pflanzen kann der Pilz den Wirt besiedeln und sichtbare Nekrosen induzieren. Dabei findet eine Reaktion durch die Pflanze statt, weil sich die Symptome auf der Fläche der Infektionstropfen beschränken.
Ultrastrukturelle Studien der Konidien von *D. umbrinella* zeigen das Vorhandensein einer extrazellulären fibrillären Matrix, welche aus (Glyko)-proteinen und Polysacchariden besteht. Der innere Teil der Konidienzellwand ist elektronendurchsichtig und enthält Chitin. Verschiedene Isolate von *D. umbrinella* aus verschiedenen Wirten unterscheiden sich in der Morphologie der extrazellulären Matrix, was eine Anpassung an unterschiedliche Oberflächenbeschaffenheiten andeutet.
In vitro zeigen zytologische Untersuchungen, dass *D. umbrinella* in seinen Wirt ohne Bildung eines Appressoriums einzudringen vermag. Ein Appressorium kann aber auch gebildet werden, und nach einer Latenzzeit des Pilzes kann das ganze Gewebe unter Bildung von Nekrosen kolonisiert werden. Die Kolonisation des Buchenblattes findet wahrscheinlich durch enzymatische Einwirkungen statt, obwohl die Mitwirkung mechanischer Kräfte nicht ausgeschlossen werden kann. Der Infektionsprozess beginnt mit dem spezifischen Haften der Konidien auf der Wirtsoberfläche. Polysaccharide wie Mannane und Glukane oder ihre Derivate und (Glyko)-proteine, die in der extrazellulären Konidienmatrix vorhanden sind, sind ebenfalls im Haftungsprozess beteiligt, da eine Auflösung dieser Schicht das Haften völlig unterbindet. Es ist möglich, dass Substanzen, die auf der Wirtsoberfläche vorhanden sind, spezifisch von extazellulären Komponenten

des Pilzes erkannt werden. Wenn der Kontakt mit dem Wirt vorhanden ist, induzieren wahrscheinlich Blattkomponenten die Keimung der Sporen.

Diese Arbeit zeigt auch, dass ein symptomloser Endophyt unter gewissen Bedingungen als latenter Pathogen lebt.

1. INTRODUCTION

Traditionally, the presence of fungi in the foliage of plants has been related to evident disease symptoms followed by the decay of the plant tissue. However, many reports have now mentioned the frequent occurrence of fungi in healthy, symptomless leaves and indicate that microorganisms can colonize plant organs without causing apparent disease (e.g., Petrini, 1991). These microorganisms have been referred to as "endophytes". The definition of endophytes given by De Bary (1866) include all organisms able to colonise internal plant tissues and has subsequently been restricted by Carroll (1986) to mutualistic asymptomatic infections, excluding pathogenic fungi and mutualistic associations like mycorrhizae. Recently, Petrini (1991) has extended the use of the term endophyte to all organisms present in plant organs that at some time of their life can colonise a host asymptomatically. This includes latent pathogens and endophytes with a relatively long epiphytic phase.

Fungal endophytes have been isolated from many different plant species, including marine algae (Cubit, 1974), mosses and ferns (Petrini, 1986; Petrini et al., 1992), Ericaceae (Petrini, 1985), grasses (Clay, 1988; Bacon and De Battista, 1991), coniferous trees (Carroll and Carroll, 1978; Sieber, 1988) and broad-leaved trees (Sieber and Hugentobler, 1987; Fisher and Petrini, 1990; Sieber et al., 1991). It is now generally accepted that all living plants probably contain endophytic fungi (Petrini, 1986; 1991).

Taxonomically, the endophytes so far isolated belong mainly to ascomycetes and deuteromycetes (Petrini et al., 1992). A reason for the lack of other taxonomic groups may be the use of unselective isolation media. In fact, Bills and Polyshook (1992) have demonstrated that the use of different media for the isolation of endophytes influence the species richness and the number of isolates.

The clavicipitaceous grass endophytes are distinguished from other endophytes, because they form non-pathogenic, systemic associations with their host (Bacon and De Battista, 1991) and are mainly seed-borne (Clay, 1988). In contrast, endophytes of coniferous and broad-leaved trees, as well as most other endophytes, are transmitted mainly horizontally by spores. In these models the influence of climatic conditions on the colonisation of the hosts is evident (Carroll, 1988).

Endophytes are regularly isolated from healthy plant organs but often the same endophytes can cause disease symptoms when their hosts are stressed (Millar, 1980; Andrews et al., 1982). For example, Stone (1987) has described a latency period of several years for *Rhabdocline parkeri*, a common endophyte of Douglas fir. The fungus

lives intracellularly in the epidermis cells of the host until the onset of needle senescence. For this fungus, however, there is no evidence that it will ever become pathogenic. Wilson (1992) has reported the formation of disease symptoms on infected, axenically grown oak trees after inoculation with the endophyte *D. quercina.* Even in some grass endophytes a necrotrophic phase can be observed, as in *Epichloë typhina* infecting *Dactylis glomerata* (Sampson, 1933).

On the other hand, some pathogens can live latently in plant organs without causing any apparent modification of the tissues (Nathaniels and Taylor, 1983; Kulik, 1984; Davis and Fitt, 1990; Sinclair, 1991). *Stagonospora nodorum* is the most frequent endophyte of wheat leaves, but is also a known pathogen on the same host (Sieber et al., 1988) and *Fusarium moniliforme* is present in young maize plants long before the outbreak of any disease (Leslie et al., 1990). Endophytes have probably evolved from pathogens or vice-versa, since some endophytes are taxonomically closely related to virulent pathogens of the same or related hosts (Carroll, 1988). In pine needles, the endophytes *Lophodermium pinastri* and *L. conigenum* belong to the same genus as the pathogen *L. seditiosum* (Minter et al., 1978) and in Douglas-fir the needle pathogens *Rhabdocline weirii* and *R. pseudotsugae* (Parker and Reid, 1969) are taxonomically closely related to the endophyte *R. parkeri.* Therefore, it is almost impossible to draw a clear line between endophytes and latent pathogens. Recently, Petrini (1991) has suggested that the interaction between a given fungus and its host does not correspond to a static event, but the association may switch from one type of symbiosis to the other during the lifetime of the fungus. Good evidence for this hypothesis is provided by the fact that endophytes become saprobes or pathogens only when the host tissues are stressed, in other words when the substrate conditions for the fungus change.

Grass endophytes as well as some endophytic fungi of coniferous trees have been described as mutualistic symbionts, because of their positive effects on the host (Clay, 1988; Carroll, 1986). Field observations report a higher fitness, generally due to reduced herbivory as well as lower infection rates by pathogenic fungi in plants colonised by some endophytes (Carroll, 1986). Carroll (1986) has observed a high mortality of gall midge larvae of the genus *Contarinia* on Douglas fir after spraying with the endophyte *R. parkeri. Lophodermium conigenum* and *L. seditiosum* are both isolated from needles of *Pinus sylvestris* (Minter et al., 1978), but the pathogen *L. seditiosum* is absent when the needles are colonised by the symptomless *L. conigenum* (Minter and Millar, 1980). The best studied example of antagonism is provided by the clavicipitaceous grass endophytes (Clay, 1991a; 1991b). These endophytes are obligate biotrophs, living most of their life without damaging their hosts, although most species are potential pathogens that induce sterilisation of the flower panicles (Siegel and Schardl, 1991). Several antiherbivore

effects have been reported in relation to the presence of grass endophytes. Endophytes of the genus *Acremonium*, present in *Lolium* and *Festuca*, are the causal agent of toxic syndromes in grazing mammals and may cause serious economical losses (e.g., Read and Camp, 1986; Fletcher, 1983). It has been reported that the toxic effects are provoked by alkaloids produced by the fungi (Bacon et al., 1986). Mutualistic defensive effects have also been observed against grazing insects (Clay, 1988; Dahlman et al., 1991). Such positive associations, however are always dependent on the presence of a third species interacting antagonistically with the host (Thompson, 1982). Therefore, the assumption of a mutualistic association between endophytes and their hosts is based on the fact that endophytes obtain nutrition and protection from the host, while providing the host improved survival, growth and/or reproduction rates (Clay, 1991a). Evidence for direct positive effects mediated by the endophyte to the host, as reported for mycorrhizal or rhizobium symbiosis, is so far only known for grass endophytes, where infected plants present several physiological advantages as compared to non-infected ones (Arachevaleta et al., 1989; Hill et al., 1990).

In general, a large number of endophytes can be isolated from a single plant species or from the same tissue (Petrini, 1986), but only few fungal taxa are dominant in each host. Indeed, several fungal species are specifically reported only for a given host, for instance *Hypoxylon fragiforme* (Pers.: Fr.) Kickx in wood of *Fagus sylvatica* L. in Europe (Chapela and Boddy, 1988) and *Fagus grandifolia* Ehrh. in North America (Chapela, 1989), *Discula umbrinella* (Berk. et Br.) Morelet in leaves of *Fagus sylvatica* L. in Europe (Sieber and Hugentobler, 1987) or *Rhabdocline parkeri* in Douglas-fir in the Pacific Northwest (Sherwood-Pike et al., 1986). Petrini and Fisher (1990) have isolated endophytes from twigs of *Salix fragilis* and *Quercus robur* from the same site and have demonstrated that endophytes can be specific at the species level. Sieber et al. (1991) have found a similar specificity in leaves and twigs of *Alnus rubra*. Carroll and Petrini (1983) have even postulated specificity at the tissue level, since different strains of the same endophyte, isolated from different parts of the same conifer, vary in their substrate utilisation capacities, suggesting that several endophytes may co-exist within a single leaf. For example, *R. parkeri* is confined to the epidermal and hypodermal cells, while *Phyllosticta* sp. occurs intercellularly in the mesophyll of the same Douglas-fir needle (Stone, 1987). Isozyme electrophoresis and other biochemical analyses have demonstrated host-specificity for isolates of the grass endophyte *Atkinsonella hypoxylon* (Peck) Diehl (Leuchtmann and Clay, 1989). A similar host-specificity has been also reported for *Melanconium* spp. from different species of *Alnus* in Europe and Canada

(Sieber et al., 1991). These reports, based on diagnostic methods, indicate that host specificity may be a frequent trait in endophytic fungi.

Higher plants are exposed to a wide range of microorganisms, including viruses, bacteria and fungi, but only a small number of pathogens are able to attack plants successfully. Therefore, defence mechanisms by the host, as well as specific recognition of the host surface may be active, probably mediated by finely-tuned signals (Callow, 1987; Brett and Waldron, 1990). The colonisation of the plant tissue by fungi depends on a complex sequence of events, including recognition of the host surface by spores, germination and penetration. These processes have been described for many plant pathogenic organisms (e.g., Bourett and Howard, 1990; Li and Heath, 1990; Staples, 1985), as well as for some symbiotic mycorrhizal-root associations (Duddridge, 1986) and are based on physical or chemical signals produced or recognised by the fungi (Hoch and Staples, 1991). Boller (1989) has indicated that elicitors produced by a pathogen act as primary signals and induce a defence response of the host, such as a hypersensitive reaction or the production of phytoalexins. Extracellular enzymes, especially polygalacturonases, have been shown in fungi to act as phytoalexin elicitors (Amin et al., 1986). Boller (1989) has postulated that these enzymes are the primary signals acting on the pectin present in the plant, which becomes a secondary signal for the defence response. Other chemical signals are often mediated by plant extracts (Stockwell and Hanchey, 1983; Stewart et al., 1989). On the other hand, the cell wall of plants contains also glycoproteins and many polysaccharides (Brett and Waldron, 1990) that may recognise or be recognised by fungi. Thus, compatibility as well as defence mechanisms may be mediated by compounds present on the surfaces of both plant and microorganism that influence directly the success of the infection. As physical host recognition, Hoch and Staples (1991) have reported that certain plant pathogens exhibit some kind of tropic response. As typical thigmosensitive reaction, the induction of appressoria in the rust *Uromyces appendiculatus* is caused by irregularities on the host surface as small as 0.5 µm in height, which corresponds roughly to the height of the stomatal guard cells (Hoch et al., 1987). Allen et al. (1991a) have demonstrated that two-thirds of the 27 rust species they investigated are thigmosensitive. The involvement of host-endophyte mediated signals has so far been reported only for *Hypoxylon fragiforme*, a xylariaceous endophyte of beech (Chapela et al., 1991). These authors have described the activation of ascospores by two monolignol glucosides extracted from the bark of the host. Consistent with the presence of a host signal, the highest activation of ascospores of *H. fragiforme* has been obtained on beech leaves compared to 16 other plants and inorganic materials (Chapela et al., 1991). This indicates that in endophyte symbioses, recognition mechanisms may play an important role for the infection process.

A large number of recognition mechanisms have been reported in plant-bacteria (e.g., Diaz et al., 1989; Michiels et al., 1991), plant-fungus (e.g., Hamer et al., 1988), fungus-fungus (Manocha and Chen, 1990; 1991) and mycorrhizal symbioses (e.g., Bonfante-Fasolo and Spanu, 1992). Cell-to-cell contact is the first step in the process of host colonisation by a microorganism and is often mediated by cell adhesion molecules (CAM; Edelman, 1984), mainly lectins, glycoproteins that show specific binding activities to complementary carbohydrates (Sharon and Lis, 1989; Pustzai, 1991). Colloidal gold conjugated with WGA and ConA lectins to label specifically some cell wall carbohydrates has been successfully used in electron microscopy for yeasts (e.g., Horisberger and Vonlanthen, 1977), fungi (e.g., Benhamou, 1991), as well as for pathogenic and endomycorrhizal associations (e.g., Bonfante-Fasolo and Spanu, 1992). Adhesion to the host surface is generally strongly related to the presence of a conspicuous extracellular matrix around the fungus, mainly composed of glycoprotein and carbohydrates (Bonfante-Fasolo and Perotto, 1986; Mendgen et al., 1985; Nicholson and Epstein, 1991). The involvement of the extracellular matrix in the adhesion process has been demonstrated by TonThat and Epstein (1991) with adhesive-reduced mutants of *Nectria haematococca*, in which a different extracellular matrix was observed than in the controls. Using immunogold labelled lectins, Bonfante-Fasolo and Perotto (1986) have demonstrated the presence of mannose and glucose in the extracellular matrix of ericoid mycorrhiza. In addition, Hamer et al. (1988) have reported that ConA inhibits the adhesion of *Magnaporthe grisea*. Prior to germination this fungus produces at the spore tip a ConA-binding mucilage, which is responsible for the attachment to the host surface. Proteins or glycoproteins are apparently also involved in the adhesion to the host in bean rust germlings (Epstein et al., 1985). In addition, the extracellular matrix of fungi may contain esterases, invertases, glucosidases, peroxidases (Louis and Cooke, 1985), and cutinases (Kolattukudy, 1985), which are most likely involved in the digestion of the physical barriers of the host during infection. Contact with cutin may be the first signal that induces cutinases in spores. When fungal spores land on the leaf surface, the cutinase genes may be turned on to produce the enzymes needed by the germinating spores to penetrate the cuticle (Kolattukudy, 1985). Many approaches to investigate the first contact between microorganisms and their hosts have been used, but so far they have never been applied to endophytes.

APPRESSORIA

Pathogenic fungi are known to overcome the host barriers either by penetrating the cuticle directly or by growing through the stomata or other apertures, such as hydathodes or lenticels (Allen et al., 1991b). Upon reaching the penetration site, most pathogens form an appressorium or penetrate subcuticularly. Criteria to define appressoria are their capacity to adhere to the host surface, to germinate and to penetrate the host. In most cases, "Appressorium" can be considered a synonym of "infection structure" (Emmett and Parbery, 1975). Morphologically a high variability can be observed, from the evident swelling of the germ tube tip in rusts and in *Phytophtora infestans* (Coffey and Gees, 1991) to the only slightly swollen and almost unoticeable appressoria of *Pythium* sp. (Emmett and Parbery, 1975).
Subcuticular penetration after appressoria formation has been reported, e.g., for *Rhynchosporium secalis* on barley (Jones and Ayres, 1974) and for *Mycosphaerella pinodes* on pea leaves (Clulow et al., 1991). In *Venturia inaequalis* on apple leaves both subcuticular penetration and the formation of appressoria have been reported (Smereka et al., 1987). In both penetration forms enzymatic activities, particularly cutinase, esterase and pectinase activities have been observed (Kollattukudy, 1985). Mechanical forces may also be involved, as reported by Howard et al. (1991) for *Magnaporthe grisea* and by Deverall (1981) for *Botrytis cinerea*. In many plant pathogens a combination of enzymatic and mechanical effects has also been observed (e.g., Kollattukudy, 1985; Howard et al., 1991).

MICROSCOPY

Evidence for the presence of endophytic fungi in plants has so far been provided principally by isolation of the fungi after surface sterilisation of the tissue (Petrini, 1991). At the cytological level, only few investigations have been carried out to characterise the first infection and colonisation events. Carroll (1986) has mentioned that the detection of endophytes under the microscope is a laborious and difficult task, particularly when they reside intracellularly in tissues such as leaf epidermis or wood. Some histological studies, especially on the grass endophyte *Acremonium*, have been carried out (Clark et al., 1983). Hinton and Bacon (1985) have localised the endophyte intercellularly within *Festuca arundinacea*, with no obvious alterations of the cell morphology. Recently, White et al. (1991) have studied the anatomy of stroma formation in the same endophyte on different grasses and Siegel et al. (1987) have described some specific ultrastructural

features in hyphae which are formed only when the endophyte is in contact with the appropriate host. In woody-plant endophytes only few models have been studied. Suske and Acker (1989), using immunogold labelled *Lophodermium piceae* antiserum, have detected the endophyte inter- and intracellularly in the tissue of symptomless needles of Norway spruce (*Picea abies* (L.) Karst.) and have reported that the infection is carried out by only few hyphae localised in the near-surface tissues as long as the needles are symptomless. Heavy colonisation by *L. piceae* has been detected only in brown needles. Similar results have been reported for *Rhabdocline parkeri* in Douglas fir needles by Stone (1987; 1988), the first author who has demonstrated clearly that a symptomless endophyte can penetrate directly into the host after the formation of an appressorium. The colonisation of the tissue is limited to a single epidermal cell in which the fungus may remain latent intracellularly for several years. During this time the needles are symptomless, until the onset of senescence, when the endophyte colonises the whole tissue. Both examples from coniferous trees suggest that under particular conditions, such as a modified nutrient supply, the endophyte may switch from an asymptomatic to a pathogenic phase.

DISCULA UMBRINELLA

A number of investigations on the role played by microoganisms in the development of disease have been carried out following the onset of serious forest decline (Sanasilva-Report, 1986) on beech (*Fagus sylvatica* L.), a common and economically important tree in Europe. *Discula umbrinella* (Berk. and Br.) Morelet, the anamorph of the ascomycete *Apiognomonia errabunda* (Rob.) von Höhnel (Morelet, 1973; Sutton, 1980) is frequently found in the leaves of a number of trees, including *Fagus sylvatica* L., and *Quercus* spp. (von Arx, 1970; Monod, 1983). *D. umbrinella* has been reported as the causal agent of the beech, oak and sycamore leaf anthracnose (Monod, 1983; Butin, 1989; Morelet, 1989). Recently, Redlin (1991) has described *D. destructiva* Redlin as the causal agent of the dogwood anthracnose.

Sieber and Hugentobler (1987) have isolated 60 endophyte species from leaves of beech collected at several sites in Switzerland, recovering *D. umbrinella* as a symptomless endophyte from virtually all beech leaves investigated. The high colonisation rates by the fungus are indicative of a possible, though still unknown, ecological importance of this fungus and leaves open questions on the kind of interaction between endophytes and their hosts. Morelet (1989) has described the appearance of brown to black spots on the abaxial leaf surface of beech, in which conidia are formed. The teleomorph of the fungus

has been regularly found on dead leaves fallen on the ground (Kloidt, 1989; Morelet, 1989). On adult trees, under natural conditions, Morelet (1989) has observed only a benign development of the disease. The biological cycle of this common beech endophyte has been described by Morelet (1989), without any consideration of its endophytic phase: the fungus survives during the winter, either by forming pycnidia on infected organs on the tree or perithecia in abscised dead leaves. On the ground the fungus overwinters probably forming only perithecia, since Kloidt (1989) found the anamorph only in October and November, while the teleomorph was present throughout the whole year in the freshly fallen beech litter. In the spring the fungus is dispersed apparently both by conidia and ascospores in wind-blown rain. Secondary infections during the summer take place essentially by conidial dissemination (Morelet, 1989). Humidity and temperatures around 20^0 C are favourable for the development of the infection (Toti, 1993). Rainfall, as a vector of the conidia to the leaves, has been reported in *R. parkeri* (Stone, 1987) and *D. quercina* (Wilson, 1992).

OBJECTIVES OF THIS INVESTIGATION

The ecology of beech and oak endophytes has been studied quite extensively (Sieber and Hugentobler, 1987; Wilson, 1992), but the mechanisms of penetration into the host are so far unknown. The endophyte population of beech is comparatively well known (Sieber and Hugentobler, 1987), although the biological significance of these fungi is still unclear. Beech seedlings can be produced comparatively easily (Muller and Bonnet-Masimbert, 1989; Muller et al., 1990) and axenic beech seedlings can thus be obtained using appropriate treatments. In addition, *D. umbrinella* grows well in culture, sporulates well and the conidia germinate readily within 24 h on nutrient media in moist chamber. In addition, this endophyte is widespread, since it can be isolated from symptomless leaves almost everywhere in Europe.

This study has been undertaken to

- characterise the ultrastructure of the conidia of *Discula umbrinella*, using scanning and transmission electron microscopy. Particular attention has been given to define optimal fixation methods, since it has been demonstrated that conventional methods may dissolve extracellular structures in fungi.
- define and localise histochemically the different cell wall components of the conidia in relation to adhesion to the host. Investigations have been carried out to demonstrate the importance of adhesion for the infection and the relationship between adhesion and germination.

- develop a suitable *in vitro* infection model, using axenic beech seedlings, to study the interaction between the endophyte and its host.
- study the first contact between the endophyte and its host, to detect possible specific host/endophyte recognition mechanisms and the factors responsible for a successful infection.
- characterise the penetration of *D. umbrinella* into the beech leaf at the light microscopy and ultrastructural level and to develop reproducible electron microscopical procedures to detect hyphae of *D. umbrinella* in the beech leaf.
- describe the kinetics of the colonisation process and the interaction between the symbionts after penetration of the host by the fungus.
- determine the conditions needed for the appearance of symptoms induced by *D. umbrinella* on its host.

2. MATERIAL AND METHODS

2.1. Production of axenic beech seedlings

Beech seeds were collected in autumn by spreading nets under trees at about one meter above the ground, to avoid contamination by soil fungi and bacteria. The seeds were sorted, empty ones eliminated (floating test) and the fertile ones air dried at room temperature to a relative humidity of approx. 8%. The moisture content was computed on a fresh weight basis after drying 24 h at 108° C. After this treatment, the seeds can be stocked for at least two years at -4° C without loss of germination capacity (Muller and Bonnet-Masimbert, 1989).

To produce axenic seedlings, the seeds were peeled completely by removing the pericarp and the testa. Seeds showing disease symptoms were placed in 90 mm Petri dishes, containing malt agar (MA: malt extract, Difco, 1%; agar, Serva, 2%), to check for the presence of fungi or bacteria.

The healthy seeds were surface sterilised for 5 min under permanent stirring in a saturated solution of CaOCl, washed five times with sterile tap water and placed under sterile conditions in Petri dishes (MA). The plates were incubated at 4° C, usually during six to eight weeks, until the seeds germinated. Regular checks were made to eliminate the contaminated seeds and only axenic seeds were used in further experiments.

Axenic seeds were placed with sterile tweezers into sterilised glass containers filled with vermiculite, to which 40 ml sterile tap water was added to a final relative humidity (rH) of 20%. Care was taken to cover the cotyledons with a thin layer of vermiculite. The containers were then closed with a sterile cover and placed at 20° C, 80% rH with a photoperiod of 16 h light / 8 h dark (50 $\mu E \cdot m^{-2} \cdot s^{-1}$). The seedlings were watered with autoclaved tap water without any fertiliser. In all experiments only the two primary leaves, produced approx. one month after seeding, were used. Additional details can be found in Toti et al. (1992b).

2.2. Strains used and induction of sporulation

The *D. umbrinella* strains used were isolated by Toti (1993) from leaves or seeds of beech (*Fagus sylvatica* L.), oak (*Quercus rubra* L.) and chestnut (*Castanea sativa* Mill.), collected during the summer 1989 in France, Switzerland and Canada (Tab. 1). Sporulation was induced by exposing well-developed cultures for two to three weeks to UV-light (PHILIPS TL 40W/05) until the first conidia were formed. A similar, more effective method consisted of adding pieces of surface sterilised beech leaves taken from axenic seedling onto the agar, thus reducing considerably the sporulation time. Acervuli on the mycelium produced a grey-pink mass of conidia.

Tab. 1: *D. umbrinella* isolates used in the experiments.

Strains	Host	Place of collection
LT096, LT098, LT099	*Fagus sylvatica* L. (leaves)	Strassbourg, Alsace (France)
LT117	*Fagus sylvatica* L. (leaves)	Benglen, Zürich (Switzerland)
FP7, LT215	*Fagus sylvatica* L. (seeds)	Ferrette, Alsace (France)
LT190	*Quercus rubra* L. (seeds)	Cap-de-la-Madeleine (Quebec, Canada)
LT002, LT079	*Castanea sativa* Mill. (leaves)	Cureglia, Ticino (Switzerland)

2.3. Infection and induction of necroses

2.3.1. Conidial suspension and application

Conidia were harvested by gently scraping off the conidial masses with a sterile needle and suspending them in autoclaved tap water or in buffer solutions. The optimum pH for the infection was determined by suspending the conidia in Tris-HCl, Hepes or phosphate buffers (pH 5-7). The density of the conidial suspension was adjusted with a Helber chamber to 5-10 x 10^6 conidia x ml^{-1}. To infect beech leaves, two different methods were

used. The first technique consisted of spraying the leaves with a Ninhidrin-dispenser fitted with a Millipore filter (0.2 µm, SCHLEICHER & SCHUELL) on the air supply to avoid contaminations. The spray distributed droplets of about 0.2-0.5 µl regularly on the leaf surface. Alternatively, 2 µl drops were placed on the leaf surface with a sterilised 100 µl syringe (HAMILTON). The spots on the leaf surface where the suspension was applied was marked, thus allowing a correlation between the infected area and the formation of necrotic spots. For each experiment a negative control, consisting of the medium without conidia, was applied to the leaves. Germination controls were carried out by streaking 50 µl of the suspension on MA plates. After 24 h incubation at room temperature the percentage of germinated conidia was assessed by counting at 200x magnification.

2.3.2. In vitro models

2.3.2.1. Infection of leaf discs

The technique described by Redlin and Stack (1985) was slightly modified. Two cm leaf discs were cut out with a cork borer from leaves infected by spraying. On droplet-infected leaves, the discs were tacked before infection and one droplet containing 1.6, 4.8, or 10 x 10^6 conidia x ml^{-1} was placed in the middle of the sample. Six infected leaf discs and a non-infected disc as a control were inserted each into 2.5 cm holes cut out in MA plates. Leaf discs were used only for preliminary experiments.

2.3.2.2. Infection on detached leaves and whole plants

Leaves taken from axenically grown seedlings were detached from the plant, infected with either one of the two methods described under 2.3.1 and incubated, adaxial side uppermost, inside 90 mm diam. petri dishes containing MA in which the agar had been cut to form a well slightly larger than the leaf. This method allowed to keep a constant rH of 100% in the moist chamber and was used throughout the experiments, since reproducible results were obtained. Inoculation of the abaxial side of the leaves was also carried out to assess potential penetration through the stomatal apertures.
Leaves still attached to the seedlings were infected by applying the conidial suspension to plants incubated in large boxes with a wetted filter paper on the ground.

Petri dishes and boxes containing the infected leaves were sealed with Parafilm and incubated at 20° C, 80% rH, with a photoperiod of 16 h light (50 $\mu E \cdot m^{-2} s^{-1}$) / 8 h dark.

2.3.3. Host specificity

To study host-specificity by the endophyte, 2 cm diam. leaf discs of *Fagus sylvatica* L. (host), *Corylus avellana* L., *Carex sp.* (non-hosts), and *Castanea sativa* Mill. (non-host for beech isolates) as well as detached whole leaves of *Fagus sylvatica* and *Quercus rubra* L. (non-host for beech isolates) were sprayed with a conidial suspension and incubated as described before.

2.3.4. Monitoring of disease progress and re-isolation

In all experiments the infected leaves were checked macroscopically every day for the presence of necrotic spots or any other modification. Necroses were scored as follows: 0, no necrosis; 1, very small brownish surface modification of the leaf at the places of infection drops; 2, evident brown dots; 3, brown spots covering the entire infection drop; 4, brown to black spots expanding over the infection drops; 5, necroses visible on either face of the leaf; 6, leaf completely necrotic. Re-isolation was performed to check for the presence of the fungus in the host tissue. The infected leaves were surface sterilised in 96% ethanol (1 min), 4% sodium hypochlorite (2 min) and 96% ethanol (30 sec) after the method described by Sieber and Hugentobler (1987), cut in small pieces and placed on MA supplemented with 50 ppm Terramycin (PFIZER).

2.4. Cytological localisation of the fungus by light microscopy

2.4.1. Equipment

A LEITZ-ORTHOPLAN microscope equipped for bright field, phase contrast, polarisation and Nomarski interference microscopy was used. Micrographs were taken with an ORTHOMAT camera on Kodak T-Max 100 or Tri-X Pan 400 film for black and white prints and Kodak Ektachrome 160 or 400 for colour slides.

2.4.2. Clearing and fixation of infected leaves

For light microscopy, whole leaves were cleared in 1N KOH at 45° C for several days, with regular changes of the solution, until colourless, rinsed 3x in bidistilled water, bleached 5 min by adding a few drops of H_2O_2 to the third change of rinsing water and rinsed again (2x) in bidistilled water (Stone, 1986). Alternatively, the clearing method described by Wolf and Fric (1981) was used. This included clearing the leaves in an ethanol-chloroform (75:25, v/v) mixture containing 0.15% trichloroacetic acid (TCA) during 60 min at 70° C, with several changes of the solution.
After appropriate staining, the leaves were dehydrated in 50, 70, 95 and 100% ethanol; 50% ethanol / 50% xylol and 100% xylol (10 min each step), and preserved in a solution of 5 ml acetic acid : 20 ml glycerine : 75 ml bidistilled water. For light microscopic observations the cleared, dehydrated leaf pieces were mounted in DePex-mounting medium, containing 58.4% xylene (BDH limited Poole, England). Cleared or fresh whole leaves were stained after one of the following protocols.

2.4.3. Staining procedures

Cotton blue in lactophenol. In all experiments a standard microscopical control procedure was used to monitor the development of the fungus on the leaf surface and during the first penetration events. Samples from the infected leaves were cut with a 2 mm diam. cork borer, placed on clean glass slides, mounted in a drop of lactophenol cotton blue, covered with a cover slip and observed at 400x or 1000x magnification in bright field microscopy (Rawlins, 1933).

Coomassie Brilliant Blue R 250. The samples were immersed in a solution of 0.6% (w/v) coomassie blue dissolved in 99% methanol for 5 to 55 min and washed thoroughly with methanol. This protein-specific dye was used to differentiate haustoria in the tissue (Wolf and Fric, 1981).

Resorcinol blue. 200 ml of a 1.5% aqueous resorcin solution with 3 ml ammonium hydroxyde were prepared and used at 1:5 (v/v) dilution in bidistilled water. After a staining time of 10 min, a specific blue staining of callose (β, 1-3- glucan) is obtained (Eschrich and Currier, 1964).

Trypan blue. 0.05% trypan blue was dissolved in lactophenol and the samples stained for 10 min at 45° C, followed by a destaining in hot lactophenol, to differentiate the fungus on the leaf surface (Stone, 1986).

Fast green (acid fuchsin-lactophenol). This dye was used to differentiate between fungal structures on the leaf surface and those that are subcuticular or intracellular. Samples were fixed in (1:2) acetic acid : ethanol for 24 h, cleared in lactophenol for 24 h, stained for 30 min in 0.1% (w/v) acid fuchsin in lactophenol, rinsed briefly with lactophenol and stained for 5 min in fast green, rinsed again with lactophenol and mounted in a mixture of glycerol, bidistilled water and Tween 20 [90:9:1, (v/v); Myers and Fry, 1978].

Periodic acid-Schiff reaction (PAS-reaction). This protocol was applied on whole leaves as well as on hand-made semi-thin sections of fresh infected leaf pieces. Samples were fixed in 70% (v/v) ethanol for 10 min, placed for 30 to 40 min in 400 mg periodic acid in 45 ml bidistilled water with 5 ml of a fresh solution of 0.2 M sodium acetate, rinsed in bidistilled water and stained for 5 to 10 min in Schiff's reagent, rinsed again with bidistilled water, immersed in SO_2 water and finally washed with bidistilled water. The stained specimens were preserved in 70% ethanol until observation in bidistilled water for light and fluorescence microscopy (Ruthmann, 1966; modified by Smolka and Wolf, 1983).

2.4.4. Observations of the first infection structures

To study the formation of the first infection structures, the conidia were suspended either in bidistilled water (pH 5.5) or in 50 mM Hepes (pH 5) and applied by the drop infection method to the leaf surface. Correlation between enzyme synthesis and germination of the conidia, was investigated using a solution of 1 M PMSF (Phenylmethylsulfonyl fluoride), an inhibitor of serine protease and other enzymes (Sekar and Hageman, 1979), dissolved in 1% isopropanol, diluted to 1 mM in water.

Detached axenic leaves were infected with 0.4 µl droplets of the appropriate conidial suspension (isolate LT099) and incubated as described above. Four discs each of 2 mm diam. were taken from the leaves and observed in cotton blue at 0, 2, 4, 6, and 24 h after infection. The frequency of conidia presenting particularities on the host surface were recorded. The average counts of 4 samples were calculated and expressed as percentage of the total number of counted spores. As a control for the induction of different

development forms by the beech leaves, dialysis membranes and Teflon-coated slides were inoculated.
To detect any correlation between conidial germination and the attachment of the conidia on the leaf surface, the infected leaves were rinsed for 5 sec at 0.5 KPa as described by Toti et al. (1992b) before the samples were taken at 0, 2, 4, 6, 8, 24 h after infection and processed as above.
Germination rates of the fungus were assessed on MA 24 h after infection as described under 2.3.1.

2.4.5. Effects of leaf extracts on the germination of conidia

Host-specific induction of germination was assessed by incubating conidia in leaf extracts of beech, oak, chestnut and barley (*Hordeum vulgare* L.). For the extraction, 0.5 g leaf (fresh weight) was ground in liquid nitrogen (LN_2) and suspended in 10 ml bidistilled water. This solution was stirred during 4 h at 4^0 C and centrifuged for 15 min at 12,000 *g*. The extracts were filter-sterilized (0.2 µm filter) and stored at 4^0 C.
Conidia were suspended in 1 ml of the appropriate leaf extracts, 2 µl of the suspensions were placed on Teflon-coated slides in a moist chamber for 24 h at room temperature without cover slip. Germination was assessed at regular intervals. Conidia incubated either in bidistilled water, in 1% (w/v) aqueous Malt extract, or in Hepes (pH 5) were used as controls. The germination rate of the conidia suspended in water was carried out on MA plates as comparison to the Teflon-coated slides.

2.5. Fluorescence microscopy

2.5.1. Equipment

Fluorescence microscopy of PAS-staining and tetramethyl-rhodamin-isothiocyanate (TRITC)-labelled lectins was carried out with a LEITZ-ORTHOPLAN equipped with an ORTHOMAT camera and a fluorescence vertical illuminator (PLOEMOPAK 2.1 a. 2.2) at following settings: Leitz filter A (UV), excitation 330 nm, emission 380 nm for PAS; Leitz filter N2, excitation 530 nm, emission 560 nm for TRITC. Fluorescein isothiocyanate (FITC)-labelled lectins were observed under a ZEISS AXIOMAT, filter

487715, excitation 546 nm, emission 590 nm. All micrographs were taken on Kodak Ektachrome 400.

2.5.2. TRITC- and FITC-labelled lectins

2.5.2.1. Labelling of conidia

To demonstrate the presence of polysaccharides on the conidial surface, TRITC or FITC-labelled wheat germ agglutinin from *Triticum vulgaris* L., known to bind specifically N-acetyl-D-glucosamine (GlcNAc), the monomer of chitin, and Concanavalin A from *Concanavalia ensiformis*, specific for D(+)-mannose and D(+)-glucose, were used. Conidia were suspended either in bidistilled water, in Hepes or in 10 mM phosphate buffered saline (PBS, pH 7.4, SIGMA). Two µl of each suspension were incubated for 30 min on Teflon-coated slides, in a moist chamber in the dark after addition of 1 µl of either TRITC-ConA or TRITC-WGA or FITC-WGA (SIGMA) at concentration of 100 $\mu g \cdot ml^{-1}$ in 10 mM PBS. After incubation, the liquid was gently removed with a Hamilton syringe and substituted by Hepes, PBS or bidistilled water.
To assess changes in the sugar composition of the extracellular sheath of the conidia and germ tube during the germination process, labelling was also performed on conidia incubated for 24 h in 20 mM Tris-HCl buffer (pH 7) under the conditions described.
For negative controls, conidia were incubated in unlabelled lectins for 1 h and then transferred to a solution containing the fluorochrome-labelled lectins. In addition, fluorochrome-labelled lectins were saturated with their specific haptens for 1 h before application to the conidia. The haptens used were, for ConA: 30 mM D(+)-Mannose or 30 mM D(+)-Glucose, or both of them; for WGA: 30 mM N-acetyl-D-glucosamine.

2.5.2.2. Histology of the conidial surface

To study the composition of the extracellular conidial sheath, conidia were treated with enzymes or chemicals prior to application of the labelled lectins. Either proteinase K (EC 3.4.21.14, MERCK, 1 $mg \cdot ml^{-1}$, 1 h incubation at 25° C), pronase E (EC 3.4.24.4, FLUKA, 10 $mg \cdot ml^{-1}$, 1 h incubation at 25° C), snail enzyme (helicase,10 $mg \cdot ml^{-1}$, 1 h or 2.5 h incubation at 25° C; gift of L. Ferenczy, Dept of microbiology, University of Szeged, Hungary), or a mixture of snail enzyme and novozyme 234 (snail enzyme as above, novozyme 234, Novo Nordisk AB, 2 $mg \cdot ml^{-1}$, 1 h and 2.5 h incubation at 25° C)

were added to the conidial suspension. All enzymes were dissolved in PBS. To test the effect of chemical treatments, conidia were also suspended for 1 h at 25° C in 100 mM HCl (pH 4.0) and 100 mM NaOH (pH 13.0). After incubation, suspensions were washed by centrifugation 3 x 5 min (15,000 *g*) with PBS and labelled as described above.
The effects of the treatments on germination were tested by streaking the treated conidia on MA as described under 2.3.1., and the adhesion to the host surface was tested as described by Toti et al. (1992b)

2.6. Transmission electron microscopy (TEM)

2.6.1. Material

Fresh conidia from beech, chestnut and oak isolates were compared and the influence of different enzyme treatments on the composition of the conidial sheath was assessed, in an attempt to relate the structure and the composition of the extracellular sheath with the binding mechanisms.
Infected leaves at different stages of the infection, from the initial colonisation to the onset of the necroses, were fixed for transmission electron microscopy. Detached leaves as well as whole plants were used and were either spray- or drop-infected under the *in vitro* conditions described above. Since the fungus could be recovered regularly from the necrotic spots and around the diseased area, samples were always taken at the border between the green, symptomless tissues and the necroses. Samples were excised from the leaves with a cork borer, 2 mm diam. To achieve a better penetration of the fixing chemicals, razor blade sections (0.2-0.8 mm wide) were made through the necrotic spots under the dissecting microscope. All samples were placed in sterile Eppendorf tubes, containing either 1 ml of the fixative used for the chemical fixation or 1-hexadecene (FLUKA) for cryofixation and evacuated in a desiccator or in a sterile syringe until they sunk in the solution.

2.6.2. Conventional chemical fixation

Samples were fixed in 6% aqueous glutaraldehyde (GA) in 0.2 M sodium cacodylate buffer, pH 7.4, 1:1 (v/v) for 2 h at 4° C, washed 3 x 10 min in the buffer (0.1 M), postfixed 1 h at 4° C in 2% osmium tetroxide (OsO_4) in the same buffer 0.2 M, 1:1 (v/v),

washed again in the buffer (0.1 M) and dehydrated in 2,2-dimethyloxipropane (DMP) containing one drop 0.1 N HCl per ml DMP for 25 min at room temperature. The specimens were then washed 3 x 5 min in anhydrous acetone (dehydrated in $CaCl_2$) and embedded in resin. With infected leaves, better results were obtained when dehydration was made in a graded series of ethanol 30, 50, 70, 80, 95, 100% (2 x 15 min for each step).

2.6.3. High pressure freezing (HPF)

2 mm leaf discs were evacuated in 1-hexadecene (FLUKA) in a desiccator to substitute intercellular gases, placed in the cavity of a cylindrical aluminium platelet and sandwiched with a second platelet, which matched the specimen thickness as closely as possible (Fig. 1). The space between the sample and the platelets was filled with 1-hexadecene. The specimen sandwiches were frozen under high pressure (Moor, 1987) with LN_2 in a high pressure freezing machine (BALZERS HPM 010) under a pressure of 2,500 bar obtained after 15 msec and maintained for 650 msec. The frozen specimens were immediately transferred to liquid nitrogen (LN_2) for storage (Studer et al., 1989; Michel et al., 1991).

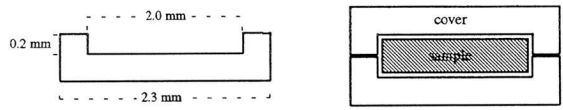


Fig. 1: Aluminium sandwiches used as sample holders for HPF.

The cryofixed samples were placed with the aluminium sandwiches in Eppendorf tubes containing 1.5 ml 2% OsO_4 in anhydrous acetone as substitution medium, pre-cooled at –90° C. For the substitution, a fully automatic BALZERS freeze substitution machine FS 010 (Müller et al., 1980) was used. The samples were kept at -90° C, -60° C and -30° C for 8 h at each step and 1 h at 0° C to allow a better fixation of the internal structures. The specimens were then washed 3 x 5 min in anhydrous acetone and embedded (Studer et al., 1989).

2.6.4. Embedding

Epon/Araldite (Luft, 1961). The following three methods were used:

1) The fixed, dehydrated samples were infiltrated stepwise with a 30% and 70% mixture of plastic in acetone at 4° C, followed by 100% plastic at room temperature (12 h for each step).
2) The samples, dehydrated in ethanol, were incubated for 1 h in 1:1 (v/v) ethanol/propylene oxide and 1 h in 100% propylene oxide before stepwise embedding in 1:1 (2x), 2:1 (2x) and 3:1 (2x) plastic dissolved in propylene oxide for 15 min at each step, followed by embedding in 100% plastic at room temperature overnight (Larcher et al., 1988).

With conidia, in both procedures the suspension was centrifuged at 10,000 ***g*** to obtain a visible pellet of conidia, to loose as little as possible material during the subsequent steps. This procedure was possible only until a 3:1 plastic/solvent concentration was reached. The conidial pellet, embedded in the 100% plastic, was polymerised directly in the Eppendorf tubes for 12 h or overnight at 60° C.

3) The flat-embedding method between two Teflon-sprayed microscope slides, described by Howard and O'Donnell (1987) was applied on the fixed leaf sections after the 100% Epon/Araldite mixture step. This method allows a rapid selection of the embedded sections by phase contrast light microscopy, at 400x magnification, after polymerisation (12 h or overnight at 60° C). The microscope slides were separated, useful sections selected, cut out with a sharp razor blade, and stacked on the top of an Epon/Araldite block with a drop of quick-binding cyanoacrylate glue (CYANOLYT, universal rapid glue, 3M). This allowed to orientate the sections as desired.

Low viscosity Spurr medium (Spurr, 1969):

Chemically fixed or cryofixed infected leaf samples or conidia were embedded stepwise in 1:3, 1:1 (2x), 3:1 (2x) Spurr medium in propylene oxide for 1 h at each step, followed by 100% plastic mixture. Polymerisation was completed after 12 h or overnight at 70° C.

Ultra-low viscosity medium VCD/HXSA (Mascorro et al., 1976):

Chemically fixed infected leaf samples were embedded stepwise for 2 h each step in 1:3, 1:1, 3:1 (v/v) VCD/HXSA plastic dissolved in ethanol and polymerised 12 h at 60° C.

2.6.5. Sectioning and staining of the sections

Sectioning

The polymerised resin blocks containing the sample were first trimmed with a razor blade under the dissecting microscope in the shape of a pyramid. A second trimming was performed with a glass knife on a REICHERT ULTRACUT E microtome. With a diamond knife (DIATOME) semi-thin sections of about 0.6 µm were cut, collected with a loop and placed on a clean microscope slide. The slides were heated to dry, stained with Epoxy tissue stain (JBS-Em services Inc.), a commercial solution of toluidine blue and basic fuchsin (Hayat, 1989), heated again and washed under running bidistilled water. Sections were examined under phase contrast microscopy at 1000x magnification. Blocks that contained useful material, as assessed by study of the semi-thin sections, were used to prepare ultra-thin sections of about 75 to 85 nm thickness, which were placed on collodion or formvar, carbon-coated, 100 mesh copper-grids for conventional observations or 200 mesh gold-grids for histochemistry and gold labelling. Overviews of sections were possible only on formvar, carbon-coated, copper single slot grids, on which the sections were placed with a loop, after elimination of the water drop with a piece of filter paper. During this operation tweezers holding the copper support were fixed on a magnet holder to facilitate the collection of sections and the elimination of water drops.

Staining

The ultra-thin sections mounted on the grids were double stained in aqueous 2% (w/v) uranyl acetate for 7 min, washed in a series of six drops bidistilled water, contrasted in lead citrate after Reynolds (1963) for 7 min and thoroughly rinsed under running bidistilled water.

2.6.6. Histochemistry

2.6.6.1. PA-TCH-SP staining for polysaccharides

The PA-TCH-SP reaction of Thiéry (1967), modified by Ruel and Joseleau (1991) consists of the specific oxidation of vicinal, free alcohol groups of sugars (vic-glycols) into aldehydes which are in turn visualised by silver proteinate. The reaction is positive

only for 1-2, 1-4, and 1-6 linked glucans, leaving many vic-glycols which are accessible to a periodic oxidation (Roland and Vian, 1991). The reaction is negative for chitin (Valk van der et al., 1977; Fevre and Rougier, 1980), an unbranched polysaccharide of β, 1-4 linked GlcNAc.

Ultra-thin sections of cryofixed and chemically fixed conidia of different beech isolates, chemically fixed infected leaves (2 mm discs), and cryofixed, enzymatically digested conidia as described under 2.5.2.2., were placed on collodion, carbon coated, 200 mesh gold grids.

The sections were stained for polysaccharides as follows: grids supporting the sections were placed, section undermost for 90 min in 5% (w/v) aqueous periodic acid, rinsed 4 x 5 min with bidistilled water and for 48 h in 0.2% (w/v) thiocarbohydrazide in 20% (v/v) acetic acid. The grids were then rinsed in a degrading series of 20% (2 min), 10% (5 x 5 min), 7% (2 min), 4% (2 min) acetic acid, followed by a washing (3 x 2 min and 2 x 5 min) in bidistilled water. The polysaccharides were visualised by incubating the sections in 1% (w/v) silver proteinate in bidistilled water for 30 min in the dark. Specimens were washed thoroughly with bidistilled water and examined without post-staining. In TEM pictures, silver particles of about 5 nm indicate the presence of polysaccharides. Since the silver is present in the metallic form in the reaction, a nucleation induced by the electron beam can occur, increasing the particle size (Ruel, personal communication).

2.6.6.2. Lectin gold-labelling

To localise the presence of sugars on the conidial surface and in the cell wall, investigations on Epon/Araldite embedded material were carried out with gold-labelled lectins following the method described by Benhamou and Ouellette (1986). For direct labelling, following chemicals were used:

Buffers:
- either 20 mM, 50 mM or 100 mM Tris-HCl (pH 7), containing 0.04% (w/v) polyethylene glycol 20,000 (PEG);
- either 10 mM, 50 mM, 100 mM, 150 mM PBS (pH 7.4), containing 0.02% (w/v) PEG .

Gold-labelled lectins (all purchased from SIGMA):
- WGA, 10 nm gold-labelled;
- ConA, 5 nm, 10 nm, 20 nm gold-labelled.

To optimize the concentrations of the labelled lectins in the buffers, preliminary experiments with dilutions ranging from (v/v) 1:1, 1:2, 1:3, 1:5, up to 1:10 were performed. The optimal concentrations vary according to the lectins, the buffers and the

samples used, and are 1:2 (v/v) in 100 mM PBS/PEG for 10 nm gold-labelled WGA and 1:10 (v/v) in the same buffer for 10 nm gold-labelled ConA or 1:2 (v/v) in 20 mM Tris-HCl/PEG for 20 nm gold-labelled ConA.

Cytochemical controls

1) Incubation of the sections with a lectin-gold complex previously neutralised by incubation for 1 h with a 30 mM solution of the corresponding sugars. GlcNAc or the more specific N,N'-diacetylchitobiose for WGA and D(+)-mannose, D(+)-glucose, or both for ConA.
2) Incubation of the sections with the lectin for 1 h and washing with the buffer before treatment with the lectin-gold complex.

Ultra-thin sections were mounted on collodion, carbon coated, 200 mesh gold grids, placed for 5 min in the buffer, incubated with the gold-labelled lectins for 40 to 60 min, rinsed 4 x 2 min in the buffer and subsequently in bidistilled water. The grids were post-stained in uranyl acetate/lead citrate. To improve labelling, a careful pre-treatment of 30 min in 4% (w/v) sodium metaperiodate ($NaJO_4$), followed by a thorough washing of the grids with bidistilled water was performed (Bonfante-Fasolo and Spanu, 1992).

2.6.7. Electron microscopy

Micrographs were taken on a HITACHI transmission electron microscope H-600, at 100 kV on AGFA, SCIENTIA 23D 56 planar films. The films were developed 3.5 min in GEVATONE G5c at 20° C. The different step used for TEM are summarised in Fig. 2.

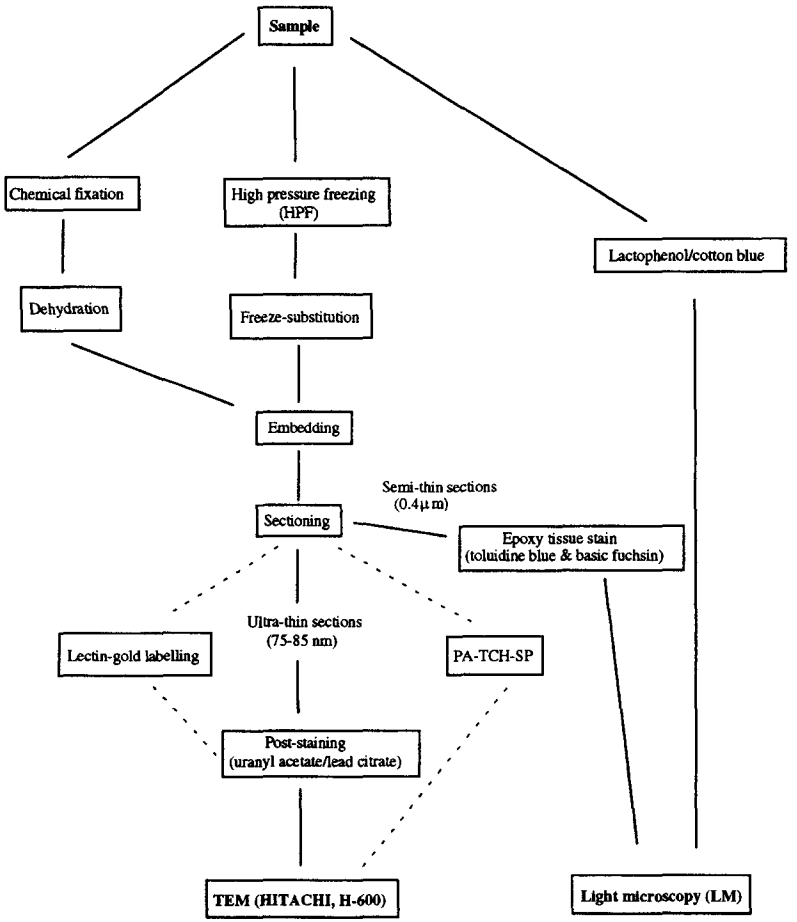


Fig. 2: Flow-diagram summarising the different methods used for transmission electron microscopy and light microscopy.

2.7. Scanning electron microscopy (SEM)

2.7.1. Material

Detached leaves were infected on the adaxial or abaxial side. The samples used for scanning electron microscopy consisted of 2 mm discs excised from the leaf with a cork borer and fixed by conventional fixation or high pressure freezing.

2.7.2. Conventional fixation

The same fixation method described for TEM (2.6.2.) was used until dehydration. Dehydrated samples were washed 3 x in anhydrous acetone and critical point dried in liquid CO_2 (BALZERS KPD 030). The samples were mounted on aluminium stubs in conductive carbon cement (LET C, NEUBAUER), the infected side uppermost or broken and mounted vertically to observe the inside of the tissue, and sputter-coated with platinum or gold, 65 to 70 nm thickness in an argon atmosphere (BALZERS MED 010).

2.7.3. Fixation by high pressure freezing (HPF)

The same method reported under 2.6.3. was also applied to fix material for SEM. After freeze substitution in OsO_4/acetone and washing in anhydrous acetone, the samples were critical point dried, mounted on stubs in conductive carbon cement and treated as described under 2.7.2.

2.7.4. Observations of the tissue interface

The method described by Honegger (1985) was used to study drop-infected, detached leaves, 5, 6, 10 and 20 days after infection. The leaves were sectioned with a sharp razor blade under the dissecting microscope to expose necrotic and intact tissue in the same section. They were then immersed in an aqueous saturated solution of protease-containing detergent ("Ariel", Procter and Gamble, manufacturers, containing a *Bacillus subtilis* protease) for 5 to 8 h at 30° C, transferred to bidistilled water and thoroughly

washed, 6-8 times for 10 min. Subsequently the specimens were fixed with 1% OsO_4 in 1/16 M phosphate buffer (pH 7.1) for 6-10 h at room temperature, washed again in the buffer, dehydrated in a graded series of acetone (50%, 70%, 90%, 96%, 100% and anhydrous 100%, 2 x 15 min each step), critical point dried and mounted vertically on the stubs. The material was finally sputter-coated with gold-palladium (Au-Pd) 65 to 70 nm thickness and examined with a CAMBRIDGE Stereoscan microscope S-4 or with a HITACHI S-700.

2.7.5. Low temperature scanning electron microscopy (LTSEM)

Conidia were suspended either in autoclaved water or in 50 mM Hepes buffer (pH 5.7). Two µl drops of the appropriate suspension were applied to detached leaves and the leaves were incubated for 8, 12, 16, 24, 36, 48 h under conditions reported under 2.3.2.2. In some experiments, leaves were infected at the abaxial side and samples were taken 8, 16 and 34 h after infection. Four samples, 2 mm diameter, were used for light microscopy and four 5 mm diameter discs, washed 5 sec under running water at a constant pressure of 0.5 KPa (Toti et al., 1992b) were used for LTSEM, after the water on the sample surface was gently removed with a piece of filter paper.

The specimens were mounted on aluminium stubs with 1% methylcellulose (Methocel, MC, medium viscosity, FLUKA) in water. This operation had to be executed as fast as possible to avoid desiccation of the conidia on the leaf surface. The samples were plunged-frozen in LN_2 (Scheidegger et al., 1991). and transferred onto the cold stage in the preparation chamber of the BALZERS SCU 020 (Müller et al., 1991). Some samples were fractured with a microtome at -120° C, others were partially freeze-dried in the preparation chamber for 10 min at -80° C in a high vacuum ($P < 2 \cdot 10^{-4}$ Pa; Müller et al., 1990). After raising the pressure to 2.2 Pa, the specimens were sputter-coated with gold to reach 15 nm thickness and transferred with a manipulator (Müller et al., 1991) through the sliding vacuum valve onto the SEM cold stage in a PHILIPS SEM 515 at 12 kV. Temperature in the SEM was kept below -120° C.

2.7.6. Electron microscopy

The platinum- or gold-coated samples were examined with a field emission scanning electron microscope HITACHI S-700 at 25 kV accelerating voltage. Micrographs were taken on Kodak T-Max 400 films developed for 8 min in Kodak TMY 120 developer. The different methods used for scanning electron microscopy are summarised in Fig. 3.

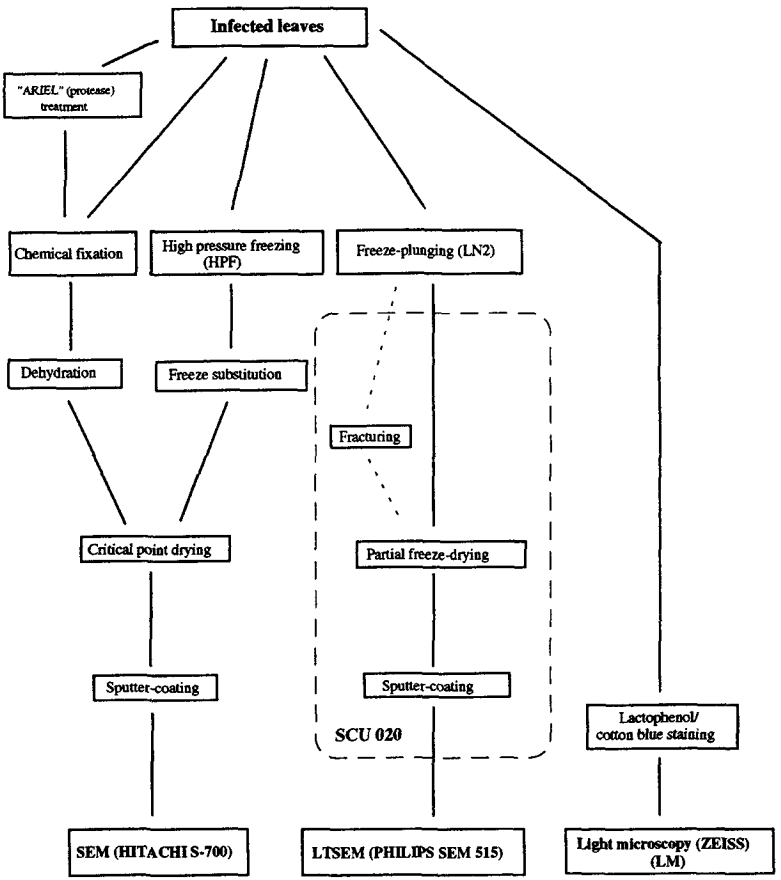


Fig. 3: Flow-diagram highlighting the methods used for scanning electron microscopy.

3. RESULTS

I. METHODOLOGY

Summary
Light microscopy was used only to observe the endophyte at the leaf surface after cotton blue staining. The conidial extracellular matrix was apparent only after high pressure freezing; it dissolved completely during conventional chemical fixation. Cryofixation of the beech leaf, however, resulted in only a partial preservation of the tissues. Therefore, conidia were studied using high pressure freezing and the infection process in the beech leaf using chemical fixation. LTSEM proved to be the best adapted method to observe the endophyte on the leaf surface.

3.1. Light microscopy investigations

Cotton blue staining and the PAS-reaction provide satisfactory information on the first infection and penetration events (Plate 1). The other staining procedures allow only fungal structures at the surface of the tissue to be differentiated, without providing any additional clue on the colonisation of the host. Clearing with KOH is very effective but apparently also clears or damages fungal structures, because no fungus could be detected on the leaf surface or in the epidermis after staining. The KOH clearing was described by Stone (1986) to clear Douglas fir needles containing endophytes, but in beech leaves it also affects the fungus present probably only in the epidermal layers of the leaf at the initial stage of infection. A reduction of the clearing time does not improve results. In tissue cleared with ethanol-chloroform-TCA the mycelium can be observed on the leaf surface, but no invading structures are detected.
Coomassie brillant blue R 250, and trypan blue allow a differentiation of the mycelium on the leaf surface but the penetrating structures can not be seen clearly. Resorcin blue, a dye for callose, considered to be a response of the plant against fungi, stains only the leaf veins, but no other staining reaction can be seen in the host tissue, suggesting that callose is not involved or at least not detectable during the early colonisation events of the tissues. It is not surprising to find a positive reaction of the leaf veins, since Mohr and Schopfer (1985) have reported the presence of callose in the sieve pores of woody plants.

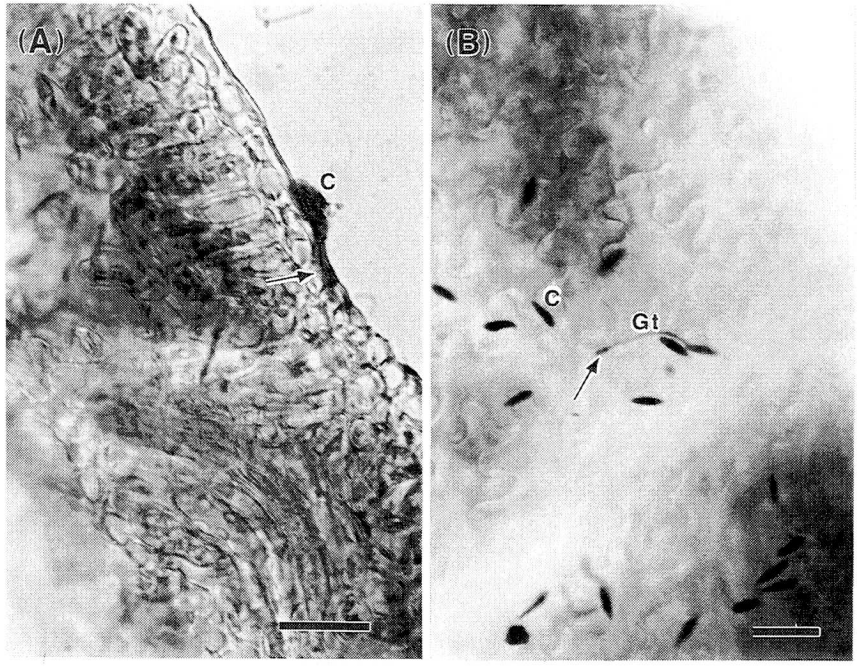


Plate 1: Staining methods used in light microscopy to differentiate the fungus in infected leaves.
A, PAS-reaction on free-hand section, one month after infection (necrotic stage). The conidia and the germ tube are stained. Penetration into the host can be seen (arrow).
B, Cotton blue. 24 h after infection (no symptoms visible). Only the conidia are stained. A swelling at the tip of the germ tube suggests the presence of an appressorium (arrow).
C, conidia; Gt, germ tube. Scale bars: A, 10 µm; B, 20 µm.

Fast green staining differentiates superficial hyphae from those that have penetrated into the tissue: superficial hyphae are stained green to blue-green, as opposed to those present within the tissue which are pink to red (Myers and Fry, 1978). Investigations of necrotic tissue, in which the endophyte was present within the mesophyll, were successful, but no conclusive results were obtained from observations of freshly infected plant material.

The PAS-reaction for polysaccharides stains fungal structures pink and can also be observed under the fluorescence microscope. However, the observation of fresh material is hampered by the presence of polysaccharides contained in the plant cells and reacting with the PAS dye. On free-hand sections good results are obtained (Plate 1A), but conclusions on the succession of the penetration events are very difficult. Plate 1A shows a germ tube that penetrates subcuticularly into the host (arrow), but any further interpretation is not possible.

Using cotton blue staining, a good differentiation of the fungal structures on the leaf surface is obtained and yields the most interesting information that can be obtained by light microscopy during the early infection events. It is the most reproducible and fastest staining method that can be used on total preparations of infected leaves (Plate 1B). However, the light microscope, with its relatively limited power of resolution, makes the interpretation of the results uncertain, not only because of the dimensions of the object investigated but also because of the difficulty of detecting the fungus inside the leaf tissue.

3.2. Electron microscopy

3.2.1. Preparation procedures for TEM

The first step in the preparation methods for TEM is the fixation of biological material to preserve the structures of the living cells with minimal alteration. Chemical fixation is the most widely used method and cryofixation, a purely physical immobilisation of the material, has been developed for a better preservation of the tissues (Studer et al., 1989). The superiority of this method has been reported by Howard (1981) for fungal structures and is evident after observation of ultra-thin sections of cryofixed conidia of *D. umbrinella* (Plate 2). In particular the conspicuous extracellular fibrillar sheath observed after cryofixation is almost completely dissolved after conventional fixation, leaving remnants that are usually called mucilage (Jacobi et al., 1982; Gold and Mendgen, 1984; Mims and Richardson, 1989). The inner, electron dense layer of the cell wall presents a

diffuse and deformed structure in chemically fixed conidia, compared to the corresponding, well preserved, cryofixed double plasma membrane (Plate 2). On cryofixed conidia, the mitochondria, vacuoles and the nuclei as well as the cytoplasm are much better preserved than after chemical fixation (Plate 2).

The fixation of plant material is particularly critical. Large vacuoles, cell wall and plastids can cause difficulties at every step of the preparation. The cell walls represent a barrier to the penetration of chemicals and resins and the intercellular spaces, filled with gases, prevent the infiltration of the tissues by the fixatives. The liquid phase contained in the vacuoles hampers cryofixation; on the other hand, conventional fixation induces modifications that are difficult to control during the relatively slow penetration of chemicals (Roland and Vian, 1991). No reproducible method could be obtained with high pressure freezing for beech leaves. The infiltration of the tissue with 1-hexadecene, an indispensable step for the optimal transmission of pressure and temperature (Studer et al., 1989), is difficult because of the thick cell wall and the large intercellular spaces present in the mesophyll (Plate 3A, B). The plant structures are damaged by the high pressure applied and only restricted areas are well preserved. The use of 1-decene, tetradecene, 1-pentadecene or 5% aqueous methanol, instead of 1-hexadecene as infiltration media has yielded no better results. Michel (1991) obtained good results only with young apple leaves but not with tobacco and barley leaves, thus indicating that cryofixation cannot be yet applied to all types of tissues. The success of this method is apparently dependant on sample-specific parameters, for example on the presence of natural cryoprotectants in the tissue. For the purpose of my work, the few well-preserved spots on the sections were insufficient to study the penetration events by the endophyte, since the fungus is sparsely present in the host tissue. Plate 3A shows the best results obtained after high pressure freezing of beech leaves. Compared to the chemically fixed material (Plate 3B), the ultrastructure of the tissue, especially the chloroplasts, the vacuoles and the cell wall are evidently better preserved. A commonly encountered problem after freeze substitution is the separation of the cytoplasm from the cell wall that occurs generally between the plasma membrane and the wall matrix (Hoch, 1991). Artefacts caused by the embedding procedure are visible using both fixation methods (Plate 3) and are probably due to weak bonds between the resin and membrane lipids, which are broken during sectioning (Michel, 1991). Embedding in low (Spurr, 1969) or ultra-low viscosity epoxy resins (Mascorro et al., 1976) yielded similar results. In addition, sectioning difficulties never encountered using Epon/Araldite had to be faced with these resins. The best results were obtained after a dehydration in a series of ethanol, combined, for infected leaves, with the flat-embedding method of free-hand sections. This method allows a useful pre-selection of the best sections and a precise orientation of the samples.

After consideration of all these aspects, I have decided to use cryofixation for ultrastructural observations and histochemistry of the fungus and chemical fixation to study the interaction of *D. umbrinella* with its host.

Plate 2: Comparative results of the fixation methods used with conidia of *D. umbrinella* (isolate LT099 and FP7). A, C, chemical fixation (GA-OsO_4-Epon); B, D, cryofixation (HPF-freeze sustitution-Epon).
A, After chemical fixation only residues of the mucilaginous sheath are present (arrowhead) and the plasma membrane is not clearly visible.
B, The fungal cell wall presents an external fibrillar sheath maintained only after HPF and the plasma membrane is well preserved (double arrowhead).
C, D, Longitudinal section at the excision scar (double arrowhead, plasma membrane). F, fibrillar or mucilaginous sheath; M, mitochondria; N, nucleus; V, vacuole. Scale bars: 0.5 μm.

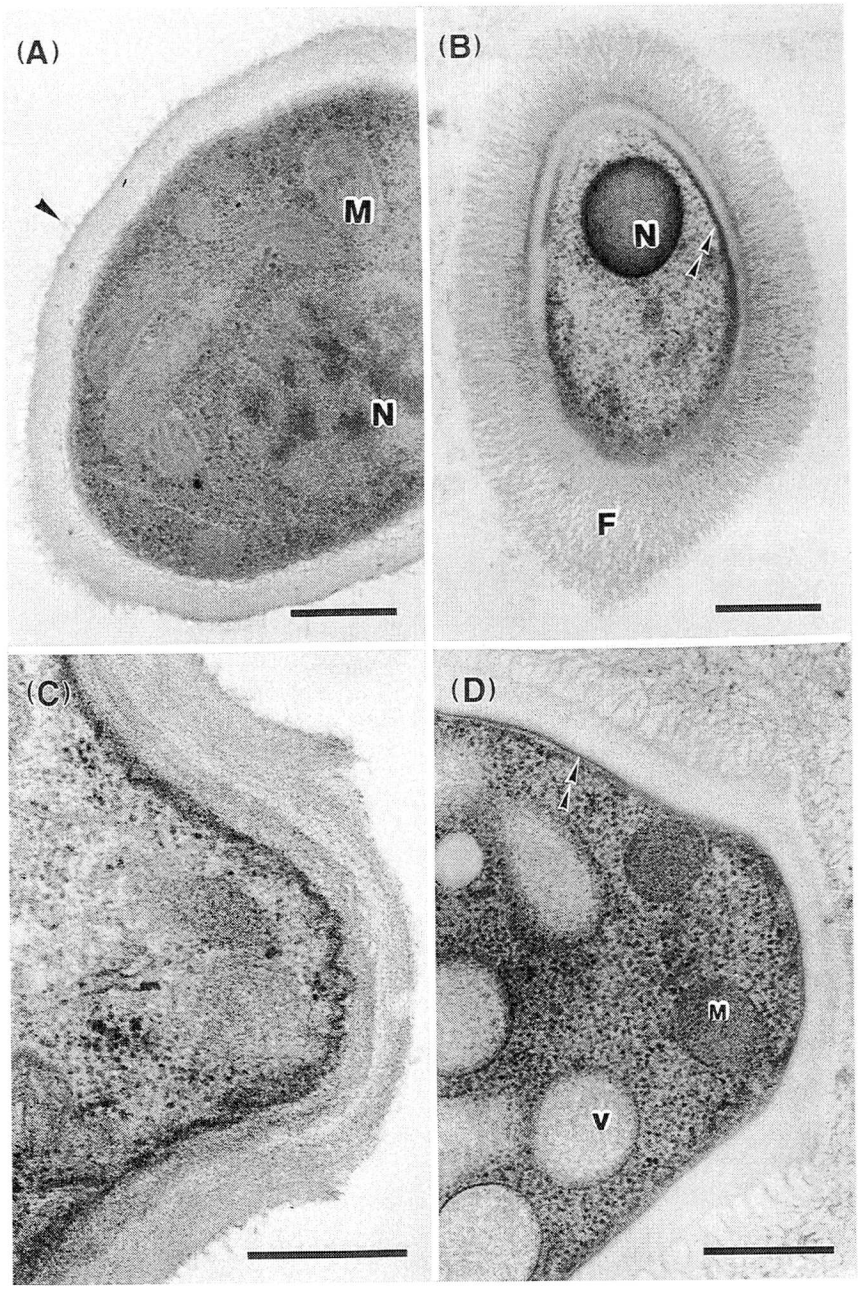
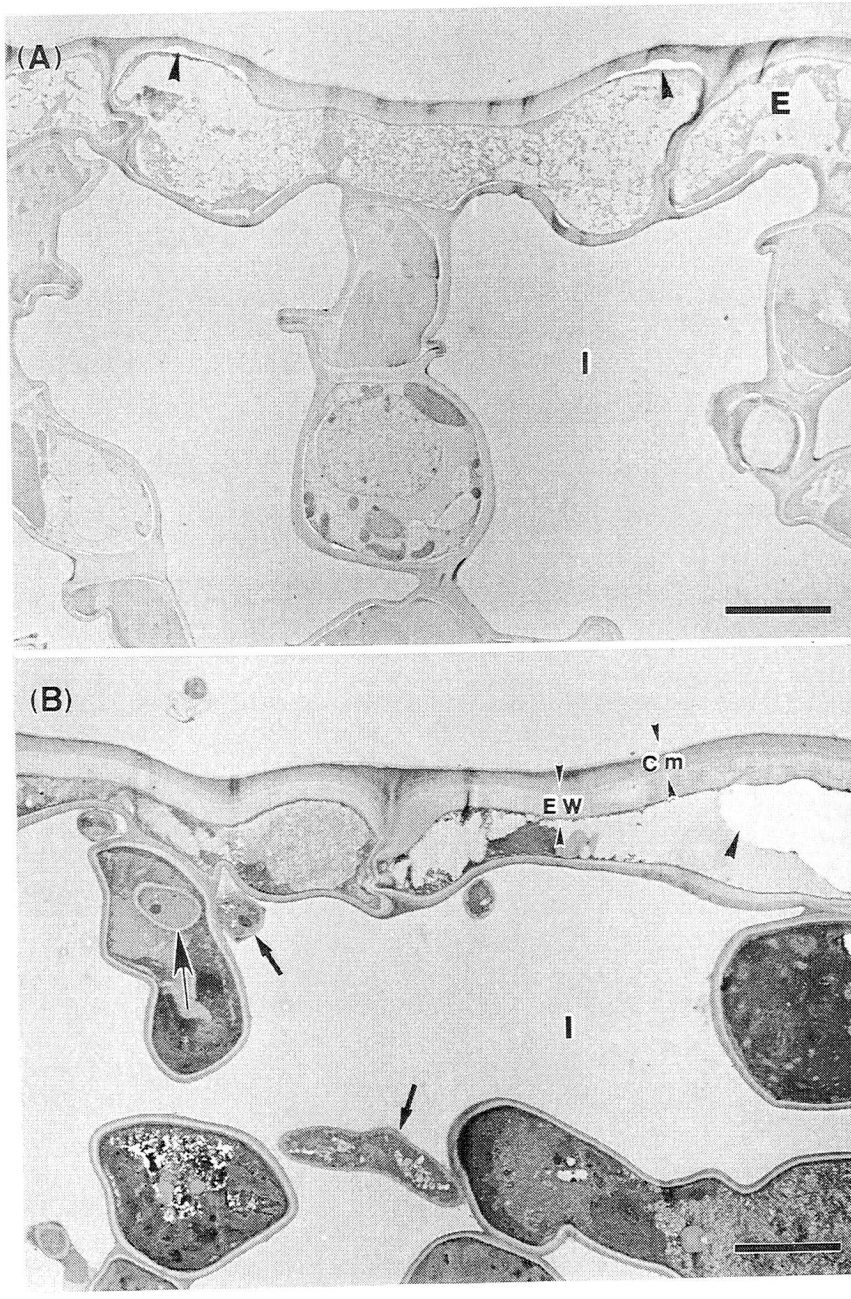

(A)
M
N
(B)
N
F
(C)
(D)
M
V

Plate 3: Results of the fixation methods used with infected beech leaves.
Cross sections through the adaxial side of the leaves, 15 days after infection. A, cryofixation (HPF-freeze substitution-Epon). B, chemical fixation (GA-OsO_4-Epon).
A, Generally a better preservation can be observed after HPF. The endophyte is absent from this section (arrowheads, embedding artefacts).
B, The beech leaf presents very large intercellular spaces which are difficult to fill with the fixatives and the embedding medium. Embedding artefacts are present after both fixation methods (arrowheads). Hyphae occur inter- (small arrows) and intracellularly (large arrows) in the mesophyll and the cells present a modified ultrastructure probably due to the fungus. Cm, cuticular membrane; E, epidermis; EW, epidermis cell wall; I, intercellular space. Scale bars: 5 μm.


(A)
E
I
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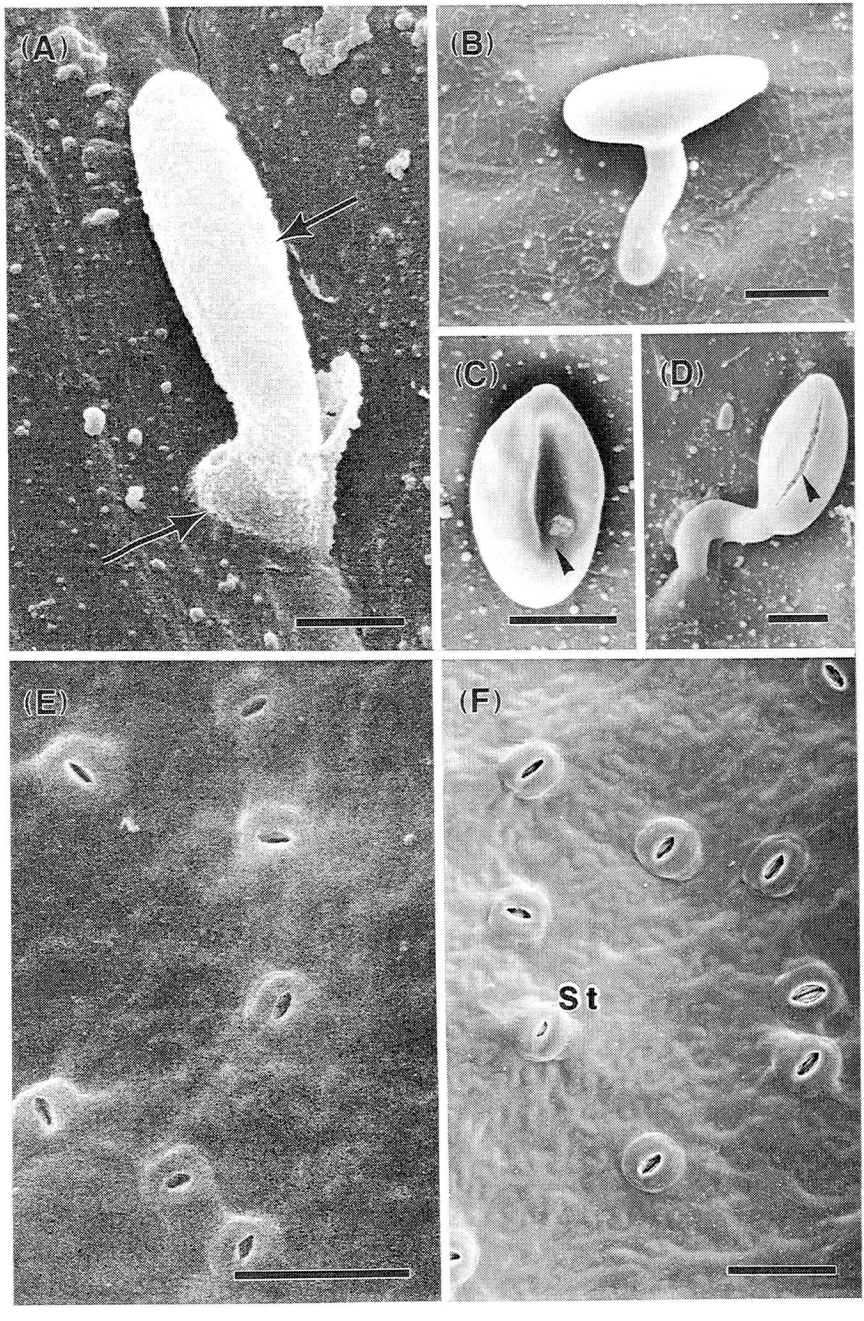
C m
E W
I

Plate 4: A, E, Conventional SEM method (GA-OsO_4-CPD) compared with LTSEM (B, C, D, F).
A, Conidia of *D. umbrinella* on the leaf surface. The conidium is shrunken (arrows). 16 h after application of 2 µl drops, suspension in Hepes (pH 6.5), at 4 x 10^6 conidia x ml^{-1}, isolate LT096.
B, The conidium presents a smooth surface.
C, D, Regularly observed methodological artefacts are a desiccation of the conidia (C, arrowhead) and electron-beam damages (D, arrowhead). 12 h after infection with 2 µl drops, suspension in Hepes (pH 5) at 7 x 10^6 conidia x ml^{-1}, isolate FP7.
E, F, The abaxial surface of the beech leaf is better preserved after LTSEM. St, stomata. Scale bars: A, 1.5 µm; B, C, D, 4 µm; E, F, 40 µm.


(A)
(B)
(C)
(D)
(E)
(F)
St

3.2.2. Scanning electron microscopy

Contrarily to the conventional preparation methods for scanning electron microscopy, which eliminate the cellular water during critical point drying (CPD), LTSEM involves the direct examination of frozen-hydrated material at low temperature. The superiority of preservation using LTSEM has now been described for many biological specimens (Read and Jeffree, 1991) and has proven to be advantageous also in this study.

After critical point drying the conidia of *D. umbrinella* are shrunken (Plate 4A) as compared to the cryofixed ones, on which a smooth surface can be seen (Plate 4B). Cryofixation before CPD does not yield better results, since deformation of the cell wall occurs during CPD. Artefacts, however, can be obtained also with LTSEM. For example the slit, surrounded by a halo on the cryofixed conidial surface (Plate 4D, arrowhead) is a typical electron-beam damage (Read et al., 1983) never observed on conventionally treated specimens. Another artefact ocurring during preparation is the collapse of the conidia resulting from desiccation before fixation or from a too slow manipulation before freezing in liquid nitrogen (Plate 4C, arrowhead).

Using LTSEM, the leaf surface is better preserved than after CPD. On the abaxial surface, the structure of the stomata and the compartmentation of the epidermal cells are clearly delimited (Plate 4E, F), probably because external compounds, such as waxes, otherwise modified by conventional fixation, are completely maintained. Scheidegger et al. (1991) have used different preparation methods to examine the effects of ozone on birch leaves and have obtained the best preservation of stomatal apertures using LTSEM. The beech leaf surface reveals a smooth epicuticular wax layer. The preservation of such structures for histological investigations on fungus-plant interactions is obviously of great importance.

LTSEM is beyond any doubt the best adapted method to study the first contact between a plant and a fungus among the techniques tested here. Important information can be obtained on extracellular mucilaginous material and extremely fragile structures, present on the surface of fungal cells.

II. PATHOGENICITY

Summary

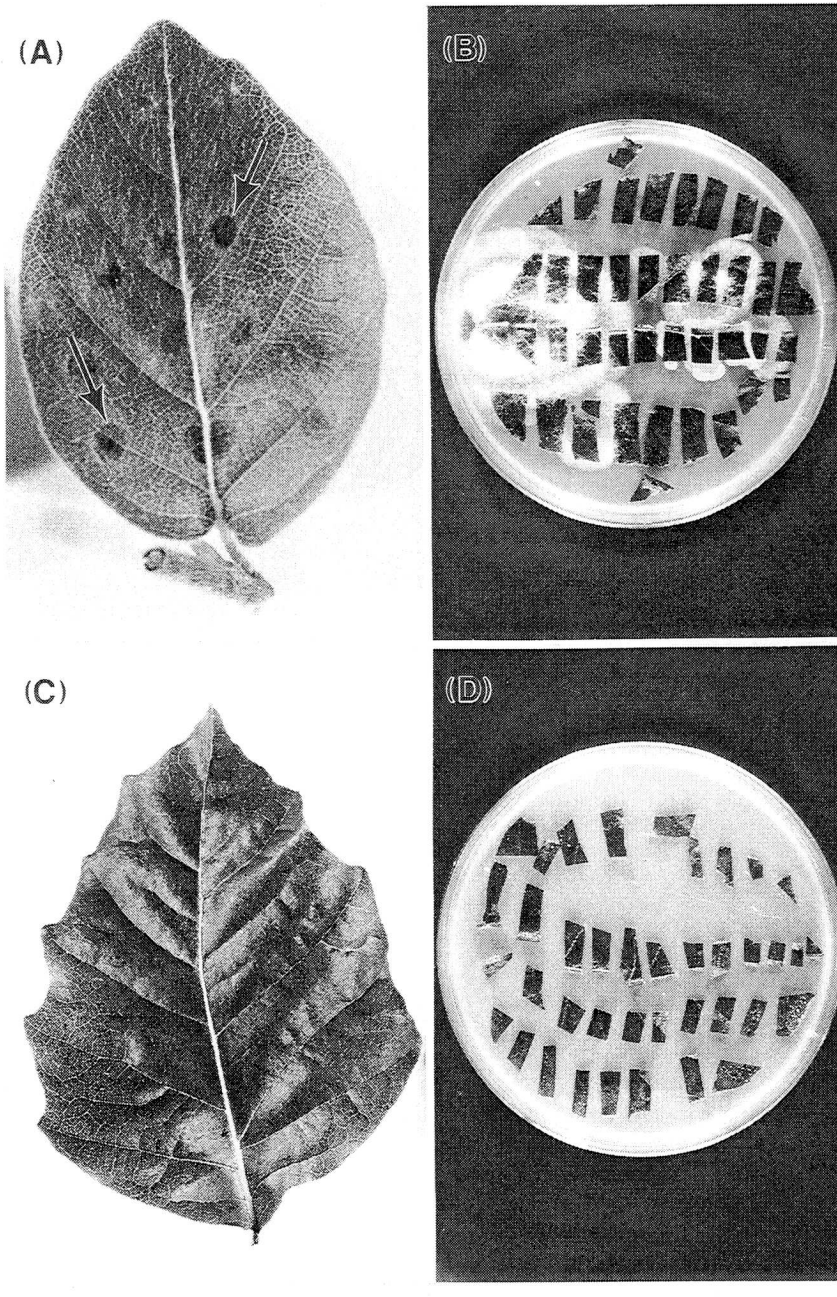
The induction of symptoms observed on detached beech leaves, as well as on whole infected plants suggests that at least under the *in vitro* conditions used in this study, the fungus is able to overcome the external host barriers and induce conspicuous necrotic spots. A reaction of the host is involved, since the symptoms are limited to the infection drops. Reduced penetration frequencies have been described for certain plant pathogens (e.g., Huang, 1989; Leslie et al., 1990) or for other latent infections (Fisher and Petrini, 1992).

3.3. Formation of necrosis on beech leaves

After infection of beech leaves with *D. umbrinella* disease symptoms are regularly formed on the leaf surface (Plate 5A, arrows). At the beginning the spots are light brown and visible only around the infection drops. Later they cover the entire drop surface and become dark brown to black, visible also on the reverse of the tissue. Finally the symptoms expand beyond the surface covered by the drop but the growth is limited and large areas of the leaf remain symptomless even after a long incubation time. Similar observations have been made by Wilson (1992) for *Discula quercina* on oak leaves, where the isolation of the fungus was successful only 2 mm away from the site of inoculation, thus pointing to an only weak virulence by the endophyte and a corresponding minimal reaction by the leaf. The long time needed for the appearance of necroses on the beech leaves supports this hypothesis. Necroses are visible after a period ranging from two days to three weeks, depending on the conidial concentration, the physiological stage of the leaves, the pH values of the suspension and the mode of infection on detached leaves (Schulthess, 1992). On whole plants, the onset of symptoms takes several weeks and the development of necroses is slow, thus indicating the involvement of an active defence mechanism by the plant, which seems to be at least partly suppressed under stress conditions, e.g., after the leaves have been detached. The location of the symptoms is always related to the position of the infection drops, and the only endophyte isolated after surface sterilisation is *Discula umbrinella* (Plate 5B). Uninfected control leaves remain unaltered for several weeks in the moist chambers without apparent modifications (Plate 5C), which is an indication of the survival of the

leaves after excision from the plant. In some cases roots were even formed on the petiole, thus suggesting the high adaptability of the beech leaf to extreme conditions.

Plate 5. Necroses on beech leaves after inoculation with *D. umbrinella*, 15 days after infection (isolate LT096).
A, Infected leaves with necrotic spots (arrows) at the place of drop application (2 µl drops of 6.7 x 10^6 conidia x ml^{-1}, suspended in Hepes (pH 7.5).
B, Re-isolation from the infected leaf after surface sterilisation.
C, Non-infected control without necrotic spots.
D, Re-isolation from the control leaf.


(A)
(B)
(C)
(D)

3.4. Infection conditions

Different infection methods yield highly variable results of the onset of necroses and therefore of the penetration frequency of the endophyte into the host.

The concentration of the conidial suspension influences positively the whole infection process (Fig. 4). Higher infection frequencies were obtained either by increasing the conidial density or by spraying rather than by dropwise application of the conidial suspension (Tab. 2). Thus, to increase the likelihood to detect the fungus in the tissue and to provide better conditions for further microscopical investigations I applied relatively high densities of conidia. The relatively big drops (2 µl) formed after infection with syringe remain almost intact during the first five days of the experiment on the leaf surface, in contrast to the small drops (0.2-0.5 µl) produced by the spray, which dry off shortly after application. I have observed that conidia remaining in suspension in an Eppendorf at room temperature do not germinate even after one week. Using drop-infection, it is possible that conidial germination is low, because conidia remain in suspension in the large drops, thus reducing considerably the infection frequencies. In contrast, the contact with the host surface induces the germination of the spores present in the dried spray drops and consequently a larger number of conidia may penetrate into the host. Schulthess (1992) has observed that more necroses are formed when the drops are dried off before incubation in the moist chamber, therefore confirming the importance of the direct contact of the conidia with the host surface for the infection process. In addition, the most important factor for a successful infection, after the physical contact between the conidia and the host surface has been established, is a 100% rH in the incubation chamber.

Variations of the pH show that in Hepes, at pH 5, necroses are detected the second day after infection and at pH 6 after six days. At pH 7 and in water (pH 6) the symptoms are very weak (Fig. 5). The use of different buffers, such as phosphate or Tris-HCl, does not influence significantly the infection process (Schulthess, 1992). On the other hand, pH conditions influence directly the latency period; water as suspension medium prolongs the latency over 9 days (Fig. 5). Using water to suspend conidia, the appearance of necroses occurs generally only after several weeks, independently on the fungal strains used. The differential expression of symptoms in water and Hepes both at the same pH suggests a positive effect of the buffer, probably because the buffer provides constant conditions for the development of the fungus.

Tab. 2: Isolations of *D. umbrinella* 5, 7 and 9 days after infection with a suspension containing either 1.6, 4.8 or 10 x 10^6 conidia ml^{-1}, isolate LT096. Beech leaf discs were either sprayed or drop-infected. The values represent the average percentage of leaf pieces containing *D. umbrinella* from 12 leaf discs per treatment, each cut in 8 pieces. Re-isolation from the non-infected control discs was always negative.

Time (days)	Conidial concentration					
	1.6 x 10^6		4.8 x 10^6		10 x 10^6	
	spray	drops	spray	drops	spray	drops
5	75	41	100	58	100	100
7	91	58	100	58	100	75
9	100	66	100	66	100	91

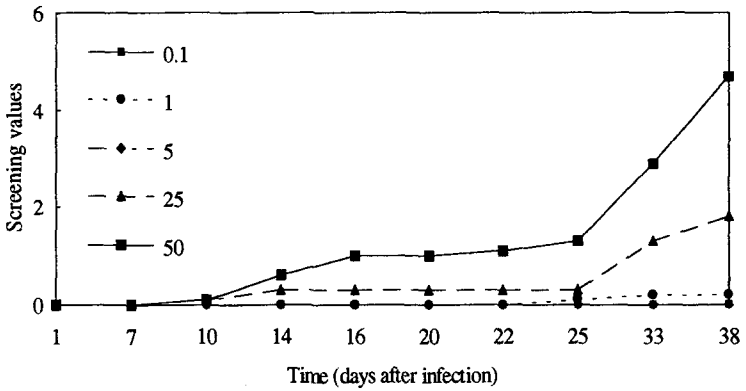


Fig. 4: Development of the symptoms on detached leaves over 38 days at different conidial concentration.
The leaves were infected with eight 0.2 µl drops containing either 0.1, 1, 5, 25, or 50 x 10^6 conidia x ml^{-1}. Conidia of isolate FP7 were suspended in 50 mM phosphate-buffer, pH 6. Each infection site was scored according to a scale from 0 (intact leaf) to 6 (necrotic). The values plotted represent the median value of 24 infection sites on three leaves.

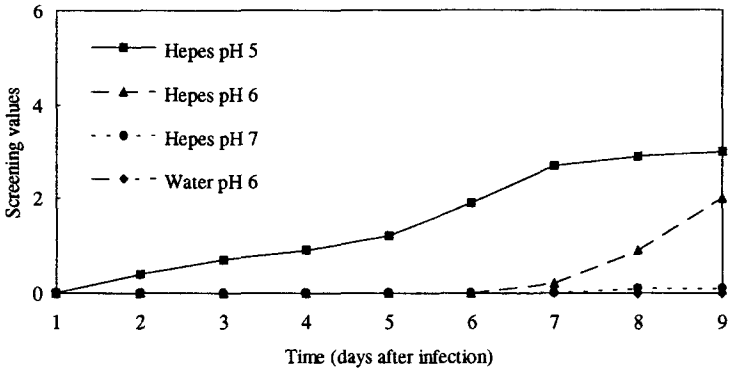


Fig. 5: Development of the symptoms on detached leaves over 9 days at different pH of the conidial suspension.
Each leaf was infected with eight 0.2 µl drops, containing approx. 20 x 10^6 conidia x ml^{-1}. Conidia of isolate FP7 were suspended either in Hepes or in sterile water. Each infection site was scored according to a scale from 0 (intact leaf) to 6 (necrotic). The values plotted represent the median value of 16 infection sites on two leaves.

3.5. Host specificity and leaf topography

Infection of different hosts and non-hosts of *D. umbrinella* with the endophyte results in a differential interaction. Ten days after infection, *D. umbrinella* can be isolated only from beech and chestnut leaves, both Fagaceae (Tab. 3). The fungus is apparently able to penetrate and colonise only closely related plants and may be considered at least partly host specific. A second experiment, performed on detached whole leaves of oak and beech have given similar results. In addition, *D. umbrinella* produces necrotic spots only on beech, whilst two weeks after infection the oak leaves are still unaffected. This suggests a differential resistance mechanism of the two hosts against *D. umbrinella.* Cross infections of axenic beech, chestnut and oak leaves with isolates originating from the three different hosts indicate a fair degree of specificity at the strain level, since the development of necroses is more pronounced when the different hosts are infected with isolates derived from the same species (Schulthess, 1992). Toti et al. (1992b) have reported a differential adhesion of the conidia to the beech leaf surface as compared to

chestnut and oak, to the non hosts barley and to an artificial surface, and have suggested that host specificity is of paramount importance for a successful infection.

Tab. 3: Isolation from spray-infected 2 cm leaf discs of different host and non host plants, 10 days after infection (isolate LT099, 10 x 10^6 spores x ml^{-1}). The values represent the average number of leaf pieces containing *D. umbrinella,* out of a total of 36 infected discs for each plant, as a percent of the total number of plated leaf pieces (8 pieces for each disc).

	Hosts		Non hosts	
	Fagus sylvatica	*Castanea sativa*	*Corylus avellana*	*Carex sp.*
Infected	95	92	0	0
Control	0	0	0	0

Stomatal openings in beech leaves are present only on the abaxial side (Denffer et al., 1978). Infection of the abaxial side of the leaves was performed to assess the potential penetration of the fungus through the stomatal apertures. Independently of the infection mode, a higher colonisation of the host is achieved when the spores are applied to the abaxial leaf side (Tab. 4), thus suggesting that *D. umbrinella* can penetrate the leaf also through the stomatal opening. In rusts, stomatal penetration is the only way for the fungus to penetrate the host and is the result of a very fine thigmotropic reaction of the fungus (Hoch and Staples, 1991). For *D. umbrinella* stomatal penetration obviously enhances colonisation but is not a prerequisite for a successful invasion of the tissue (see 3.13.2), since the endophyte can be re-isolated also after an infection at the adaxial side of the leaf.

Tab. 4: Isolation from spray-infected, detached beech leaves and whole plants, 22 days after infection of either adaxial or abaxial side of the leaves (isolate LT099, spray-infected with 10 x 10^6 conidia x ml^{-1}). Five leaves per treatment were infected. The values represent the average of leaf pieces containing *D. umbrinella* expressed as a percent of all leaf pieces. Each leaf was cut in 18 pieces after surface sterilisation.

Leaf side	Detached leaves		Whole plants	
	Infected	Controls	Infected	Controls
Abaxial	52	0	42	0
Adaxial	25	0	8	0

III. ULTRASTRUCTURE OF *DISCULA UMBRINELLA*

Summary

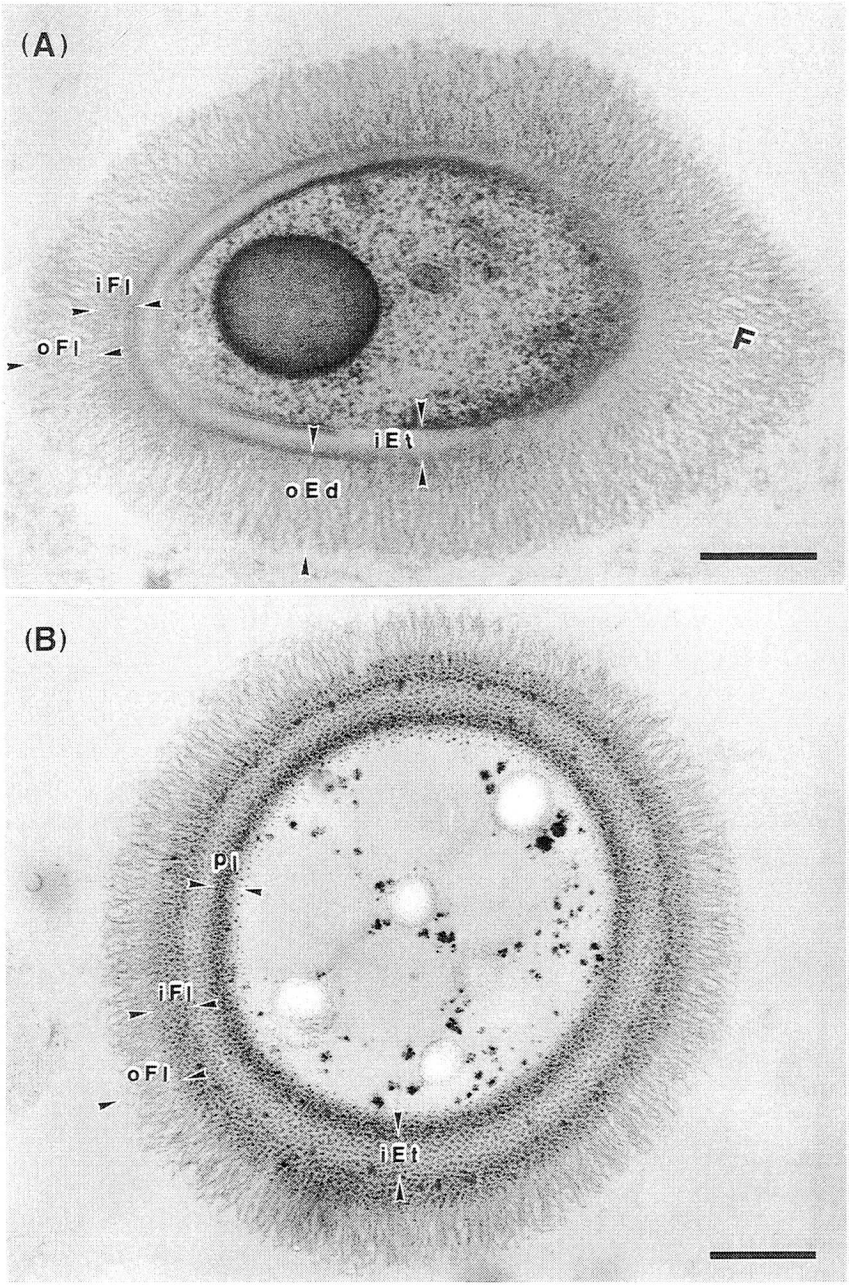
The conidia of *D. umbrinella* present a fibrillar extracellular matrix, responsible for the attachment to the beech leaf surface, composed of (glyco)-proteins and polysaccharides, among others, mannose and glucose. The inner part of the conidial cell wall is electron-transparent and composed mainly of chitin.

Cryofixation of *D. umbrinella* isolates from different hosts reveals variation in the structure of the extracellular matrix, indicating probable adaptations to different leaf surface morphologies.

3.6. Ultrastructure

The conidia of *D. umbrinella* are unicellular, and the inner part, surrounded by the plasma membrane, contains the cell organelles embedded in a granular cytoplasm (Plate 6). Many mitochondria, the nucleus and vacuoles are the principal organelles visualised by TEM. The cell wall is composed of an inner, electron-transparent layer attached to the plasma membrane and an outer electron-dense layer composed of parallel fibrils, divided in an inner dense and an outer looser part (Plate 6A). The PA-TCH-SP reaction shows better the bilayered structure of the extracellular sheath and a weaker reaction of the inner electron-lucent layer. The plasmalemma is also PA-TCH-SP positive, as opposed to the inner electron-transparent cell wall layer that shows only a weak reaction (Plate 6B). Conidial cell walls, like all other walls of Eumycota, are likely to be built up mainly by chitin and β-1,3 glucans. These compounds are electron-transparent and PA-TCH-SP negative (van der Valk et al., 1977; Fevre and Rougier, 1980) and thus cannot be detected with these particular preparative procedures. The weak positive reaction of the inner layer is probably due to the presence of other polysaccharides, in which chitin microfibrils are probably embedded, as reported for *Schizophyllum commune* by van der Valk et al. (1977). The outer extracellular fibrillar sheath as well as the plasma membrane are polysaccharide-rich.

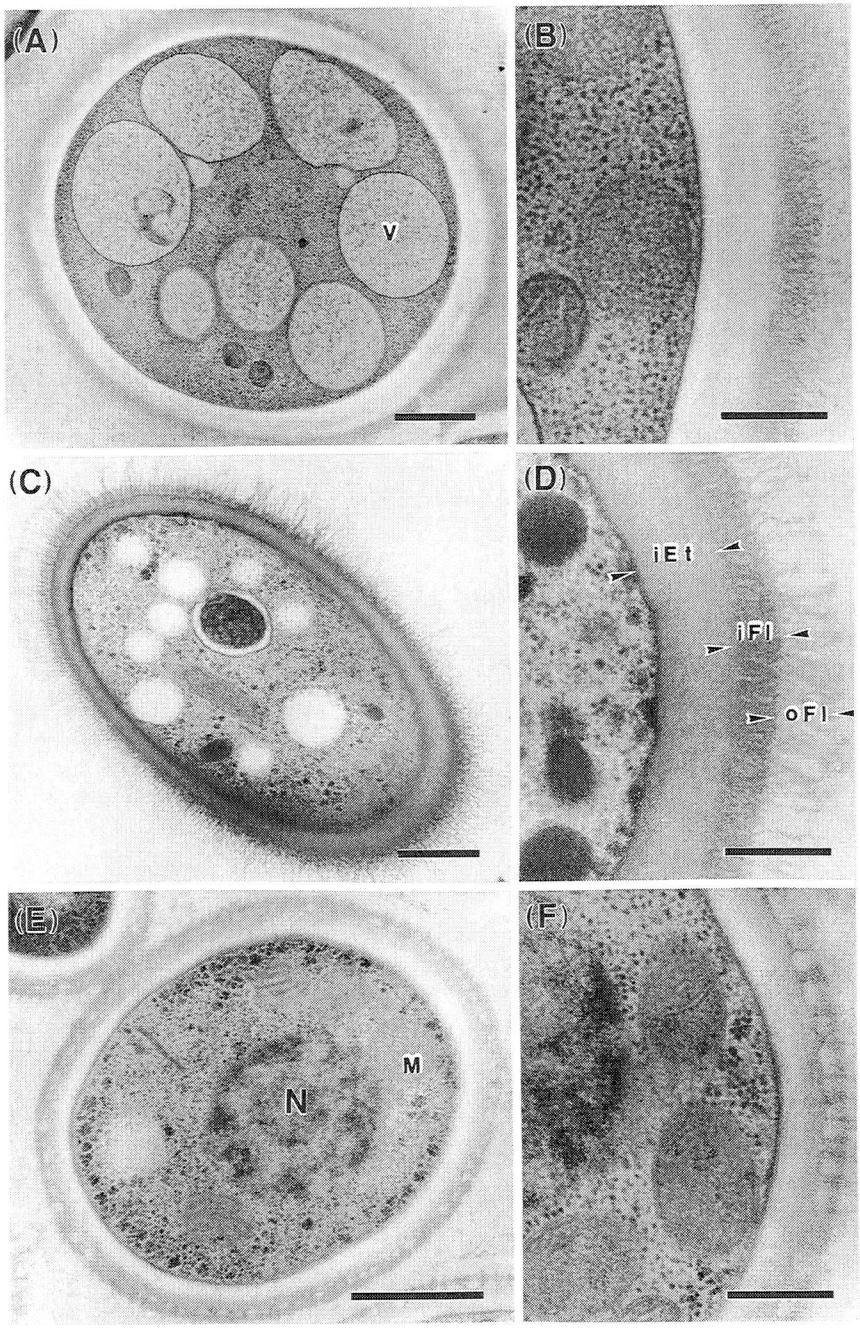
Plate 6: Conidial ultrastructure of *D. umbrinella*, isolate LT099 (HPF-freeze substitution-Epon).
A, The cell wall is divided in an electron-transparent inner layer (iEt) and an electron-dense outer layer (oEd) composed of parallel fibrils. These are divided in a dense, short inner part (iFl) and a longer, more spaced outer part (oFl).
B, PA-TCH-SP staining of the same material. The inner, electron-transparent layer (iEt) reacts to a lesser extent than the plasma membrane (Pl) and the extracellular matrix. The inner fibrillar layer (iFl) is more PA-TCH-SP-positive than the outer layer (oFl). F, fibrillar sheath; iEt, inner electron-transparent layer; iFl, inner fibrillar layer; oEd, outer electron-dense layer; oFl, outer fibrillar layer; Pl, plasma membrane. Scale bars: 0.4 µm.


(A)
iFl
oFl
F
iEt
oEd
(B)
Pl
iFl
oFl
iEt

3.7. Ultrastructure of different host isolates

Toti et al. (1992a), using morphometric methods with the conidia of *D. umbrinella*, have detected two different groups of isolates, which are strongly related to their host origin. The first group comprises beech isolates with clearly larger conidia as compared to the other isolates. The second group of isolates is derived from oak and chestnut trees and has smaller conidia. A comparative study at the TEM level shows distinct ultrastructural features of the conidial cell walls in selected isolates of both groups (Plate 7). As reported before, the external sheath of the beech isolate is composed of parallel fibrils, about 0.2-0.4 µm long, arranged at the surface of an electron-transparent inner layer (Plate 6 and Plate 7A, B). The conidia of oak isolates show an inner electron-dense layer and external fibrils which are longer than those of the beech isolates and clearly bilayered. The inner electron-transparent layer is about 0.1 µm thick and very dense, as compared to the less dense structure of the external part, with about 0.2 µm long fibrils (Plate 7C, D). The strain isolated from chestnut has the most distinct ultrastructure. The inner layer is thin (0.08 µm) and the outer layer contains a lace-like fibrillar structure not observed in strains derived from other hosts. The conidia of the beech isolates are larger (Toti et al., 1992a). Ultrastructural differences in the sheath could be an adaptation of the fungus to recognise different leaf morphologies. Alternatively, the polysaccharides composition of the sheath may be different among isolates, thus causing differential host-specific adhesion. No reports on host specific ultrastructures of the outer sheath of the spore cell wall within the same fungal species has so far been published, perhaps because the extracellular sheath is destroyed by the chemicals used for the conventional fixation methods (Plate 2A). However, TonThat and Epstein (1991) have demonstrated the presence of a similar extracellular matrix which is present in wild-type *Nectria haematococca* strains after incubation in host extracts but is absent in adhesion-reduced mutants, indicating induction mechanisms mediated by the host, probably responsible for a certain host-specificity.

Plate 7: Ultrastructure of different strains of *D. umbrinella* (HPF-freeze substitution-Epon).
A, B, isolate FP7 from beech; **C, D,** isolate LT190, from oak; **E, F,** isolate LT079 from chestnut. Note the distinct sizes of the conidia and the ultrastructure of the fibrillar sheath for each strain. iEt, inner electron-transparent sheath; iFl, inner fibrillar layer; M, mitochondria; N, nucleus; oFl, outer fibrillar layer; V, vacuole. Scale bars: A, C, E, 0.8 µm; B, D, F, 0.4 µm.


(A)
V
(B)
(C)
(D)
iEt
iFl
oFl
(E)
M
N
(F)

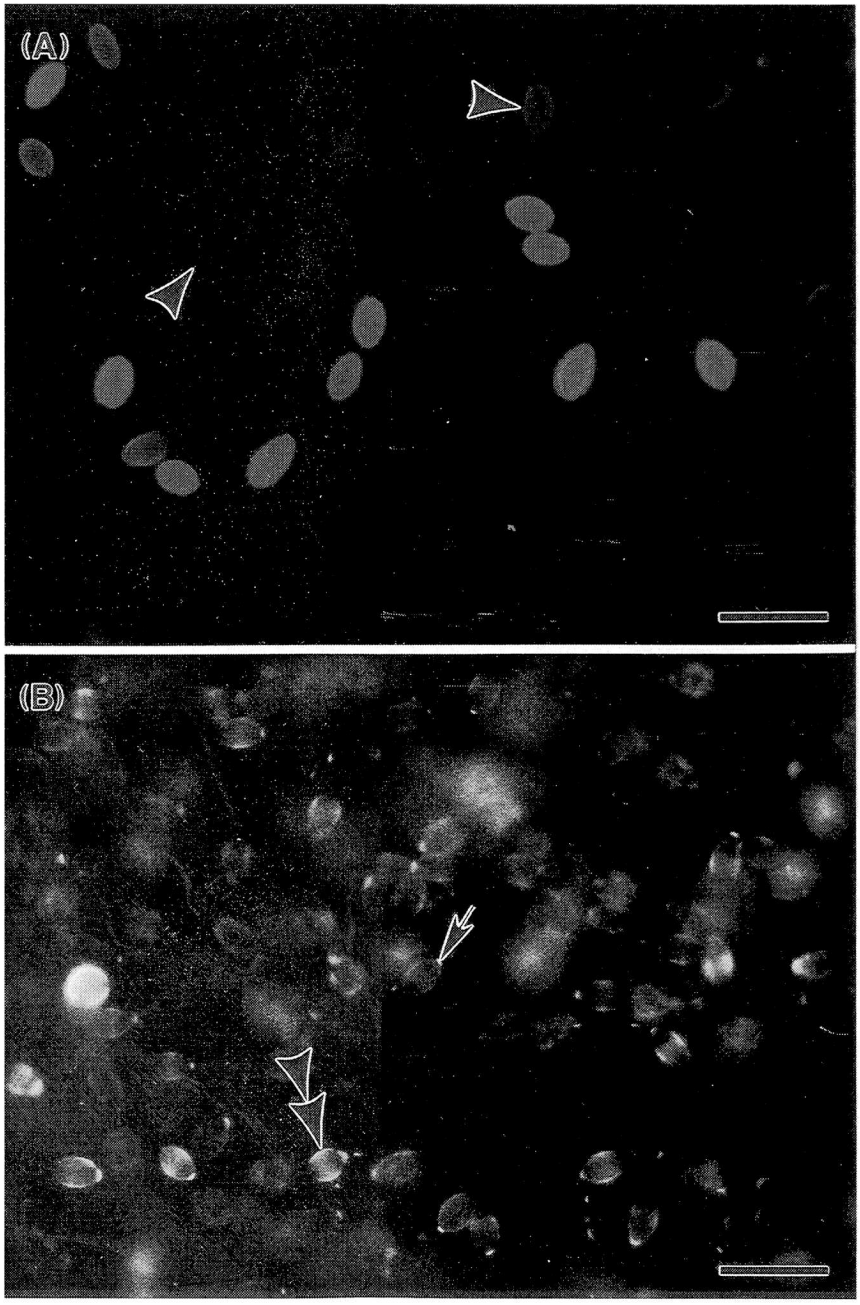
3.8. Lectin fluorescence

3.8.1. ConA and WGA-labelling

More information on the nature of the fibrillar layer and its potential involvement in the specific recognition of the host surface has been obtained using fluorochrome-labelled lectins. The conidia of *D. umbrinella* show an intense fluorescence when incubated in TRITC-labelled ConA (Plate 8A) which can be observed throughout the whole germination process (Tab. 5). Hamer et al. (1988) have also demonstrated the presence of ConA binding sites in the mucilaginous sheath of the conidia of *Magnaporthe grisea.* They have reported that the extracellular mucilage of this fungus is responsible for the adhesion to the support.

Using FITC-labelled wheat germ agglutinin, specific for the chitin monomer, GlcNAc, a weak fluorescence can be observed on the excision scar and at the germination sites on the conidial walls in most untreated spores (Plate 8B). Tab. 5 shows that WGA-TRITC fluorescence can be seen only on damaged and on some of the germinated conidia, suggesting that the fibrillar layer contains neither chitinous, nor non-chitinous GlcNAc. Van der Valk et al. (1977) demonstrated a better accessibility by chitinase to chitin after removal of glucans on hyphae of *Schizophyllum commune,* supporting the lack of fluorescence obtained with WGA-TRITC without pre-treatment. The fluorochrome had access to the excision scar and to the newly formed germ tube, a site of *de novo* synthesis of chitin and a cell wall type which differs considerably as regards to its structure from conidial walls (Gull and Trinci, 1971). Similarly, Furch and Pambor (1979) have reported that in *Phycomyces blakesleeanus* spore germination is related to a decrease of neutral sugar content in the cell wall and an increase of chitin and proteins.

Plate 8: Fluorochrome-labelled lectins on conidia of *D. umbrinella* (isolate FP7).
A, TRITC-labelled ConA. The whole conidia fluoresces and some conidia present a weaker reaction (arrowheads).
B, FITC-labelled WGA. Only the excision scar (arrow) and partly the conidial cell wall (double arrowhead) react positively. Scale bars: 24 µm.


(A)
(B)

Tab. 5: Fluorescence of conidia (isolate FP7) labelled with ConA- and WGA-TRITC lectins, after 24 h incubation in 20 mM Tris-HCl-buffer (pH 7) on Teflon-coated slides. Scale used to estimate the fluorescence: ++ high ; + weak; (+) very weak; 0 no fluorescence.

	ConA-TRITC	WGA-TRITC
Not germinated	+	0
Not germinated, damaged	+	+
Germinated:		
conidia	+	+
germ tube	+	(+)
Fluorescence controls:		
saturated lectins	0	0
unlabelled lectins	0	0

3.8.2. Chemical and enzymatic treatments

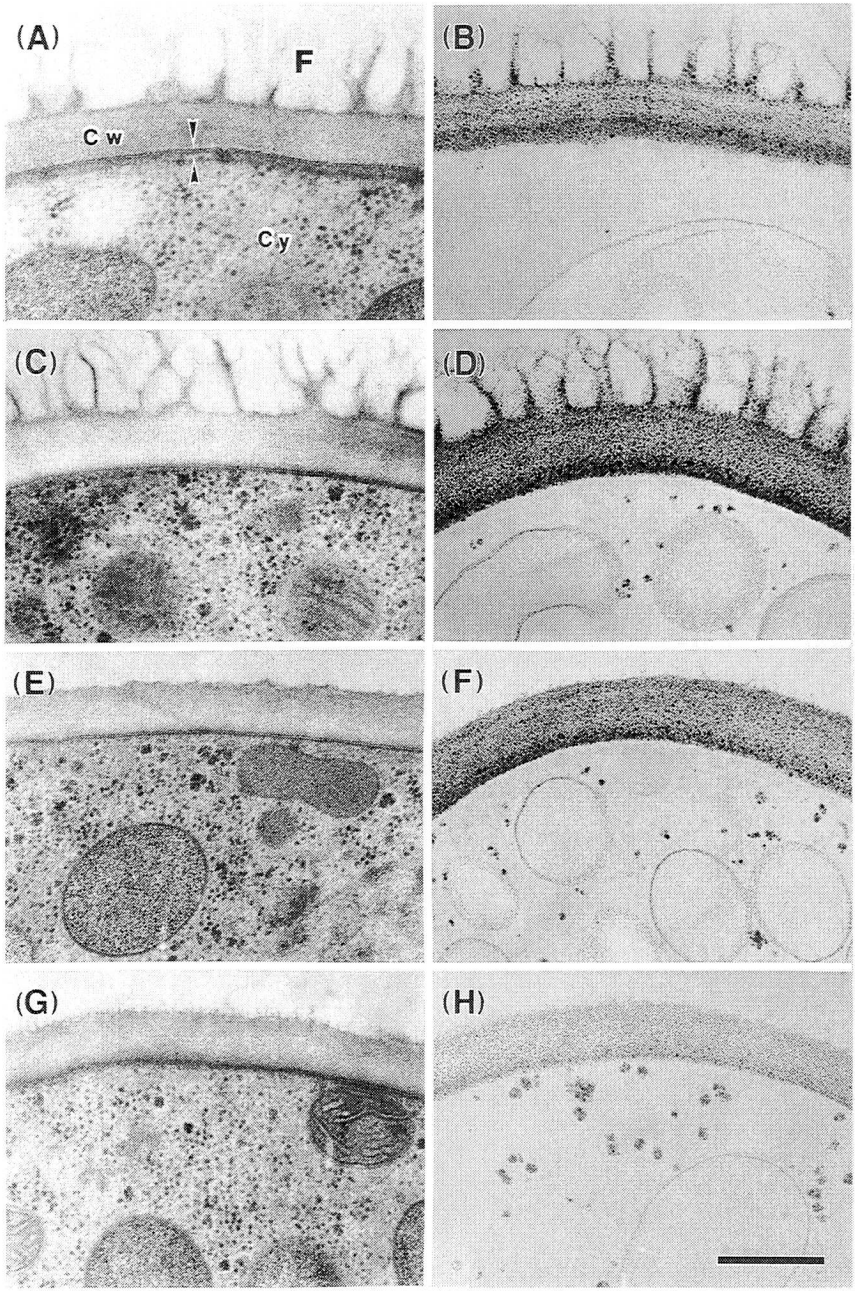
Tab. 6 shows that a strong fluorescence can be observed almost always after TRITC-ConA treatments on all conidia. Enzymatic ablation or chemical treatment of the spores reduced only slightly the ConA binding pattern, thus suggesting that mannose, glucose or their conjugated forms are present in all layers of the external sheath and in the cell wall. WGA labelling, on the other hand, gave more selective results. Fluorescence with WGA-FITC gives a weak local fluorescence in untreated conidia (Plate 8B), while after enzymatic treatment the whole conidial cell wall fluoresced strongly. Enzymatic digestion and treatment with NaOH results in a more generalised and strong fluorescence of the cell walls, especially for the damaged spores. These results seem to confirm the absence of chitin at the surface of the conidia and its presence in the deeper cell wall layers. All controls, using saturated conjugated lectins or after incubation of the conidia with unlabelled lectins before labelling, gave negative results.

Saturation of the sugar residues present on the conidial surface with the corresponding lectins results in a sharp inhibition of the adhesion of the conidia to their host surface (Viret et al., 1993). Partial digestion of the conidia with snail enzyme, a mixture with strong chitinase and glucanase activity (Peberdy, 1985) also results in a strong reduction of attachment (Tab. 6). Both observations suggest that sugar residues and proteins, possibly glycoproteins, present on the conidial surface, are involved in the attachment of the conidia to the host surface. Digestion of the conidia with proteases eliminates the adhesion to the host (Tab. 6) and electron microscopy shows that the fibrillar layer present on the untreated conidia has completely disappeared after this treatments (Plate 9E-H). Proteinase K affects more strongly the structure of the cell wall, as shown by the less intensive PA-TCH-SP reaction (Plate 9H). Nevertheless, the treatments seem to reduce germination only slightly, supporting that undigested, deeper layers of the conidia are involved in the germination process, as already suggested by the WGA-TRITC labelling (Tab. 5). Only incubation time of 2.5 h in Snail enzyme and Snail+Novozyme result in the almost total digestion of the conidial wall (Tab. 6).

If conidia are incubated 1 hr in water (Plate 9A), the structure of the fibrillar layer is slightly different from that observed in the conidia illustrated in Plate 6, which suggests that some compounds of the fibrils are water soluble. Snail enzyme modifies only slightly the structure of the sheath, indicating that chitin and glucan are not the major components of the fibrils (Plate 9C, D). PA-TCH-SP stained the fibrillar sheath and the plasma membrane more intensely than the cell wall and showed a more diffuse distribution of polysaccharides in and among the fibrils, as compared to the control (Plate 9B).

Taken together, these observations demonstrate that the external sheath is composed of polysaccharides and proteins that cover the chitin present in deeper conidial wall layers and at the same time suggest an active involvement of the fibrillar sheath in the adhesion process.

Plate 9: Effects of different treatments on the external sheath of the conidia of *D. umbrinella,* isolate FP7 (HPF-freeze substitution-Epon).
A, C, E, G, Uranyl acetate / lead citrate-staining. B, D, F, H, PA-TCH-SP staining.
A, B, Conidia suspended 1 h in water. The fibrils are partly dissolved (see also Plate 11).
C, D, 1 h digestion with snail enzyme.
E, F, 1 h digestion with pronase E.
G, H, 1 h digestion with proteinase K. Both proteinases digest the fibrillar sheath. Cw, cell wall; Cy, cytoplasm; F, fibrillar sheath; between arrows, plasma membrane. Scale bars: 0.4 µm.


(A)
F
C w
C y
(B)
(C)
(D)
(E)
(F)
(G)
(H)

Tab. 6: Fluorescence microscopy of conidia (isolate FP7) incubated in TRITC- and FITC-labeled lectins after different pretreatments and controls.

	ConA-TRITC[a]	WGA-FITC[a]	Germination (%)[b]	Attachment[c]
Controls				
Fresh conidia in water	+/s	(+)/w	98	+
Fresh conidia in Hepes (50 mM)	+/s	(+)/w	99	+
Fresh conidia in PBS (10 mM)	+/w-s	(+)/w	99.5	+
Treatments				
Pronase E	+/w	+/s	99	-
Proteinase K	+/w	+/w-s	95.5	-
Snail enzyme (1 h)	+/w-s	+/w-s	80	-
Snail enzyme (2.5 h)	+/s	+/w-s	n.t	-
Snail+Novozyme (1 h)	+/w-s	+/w-s	77	-
Snail+Novozyme (2.5 h)	+/s	+/s	n.t	-
HCl	(+)/s	0	86.5	n.t
NaOH	+/s	+/s	86	n.t

[a] Scale used for the semi-quantitative analysis of the fluorescence (estimated over at least 100 conidia): 0, no fluorescence; (+), less than 40%; +, up to 100% fluorescent conidia; s, strong (whole conidial surface fluoresce); w, weak fluorescence (local fluorescence of the conidial cell wall)
[b] Germination: percent of germinated conidia counted over 200 after 16 h incubation on MA plates.
[c] Attachment: +, adhesion; -, no adhesion to the host surface.
n.t, not tested.

Plate 10: WGA-gold labelling on cross-sections of fresh cryofixed conidia (isolate LT099), incubated in gold-labelled WGA (10 nm) diluted (1:2, v/v) in 100 mM PBS/PEG.

A, Only the electron-transparent inner layer is labelled (arrow).

B, Detail of the cell wall, only the inner electron-transparent layer is labelled (iEt between arrowheads; colloidal gold particles, arrow).

C, Negative control on the same material (WGA-gold preincubated in N,N'-diacetyl-chitobiose).

D, Detail of the cell wall. iEt, inner electron-transparent sheath; iFl, inner fibrillar layer; oFl, outer fibrillar layer. Scale bars: A, C, 0.9 μm; B, D, 0.3 μm.

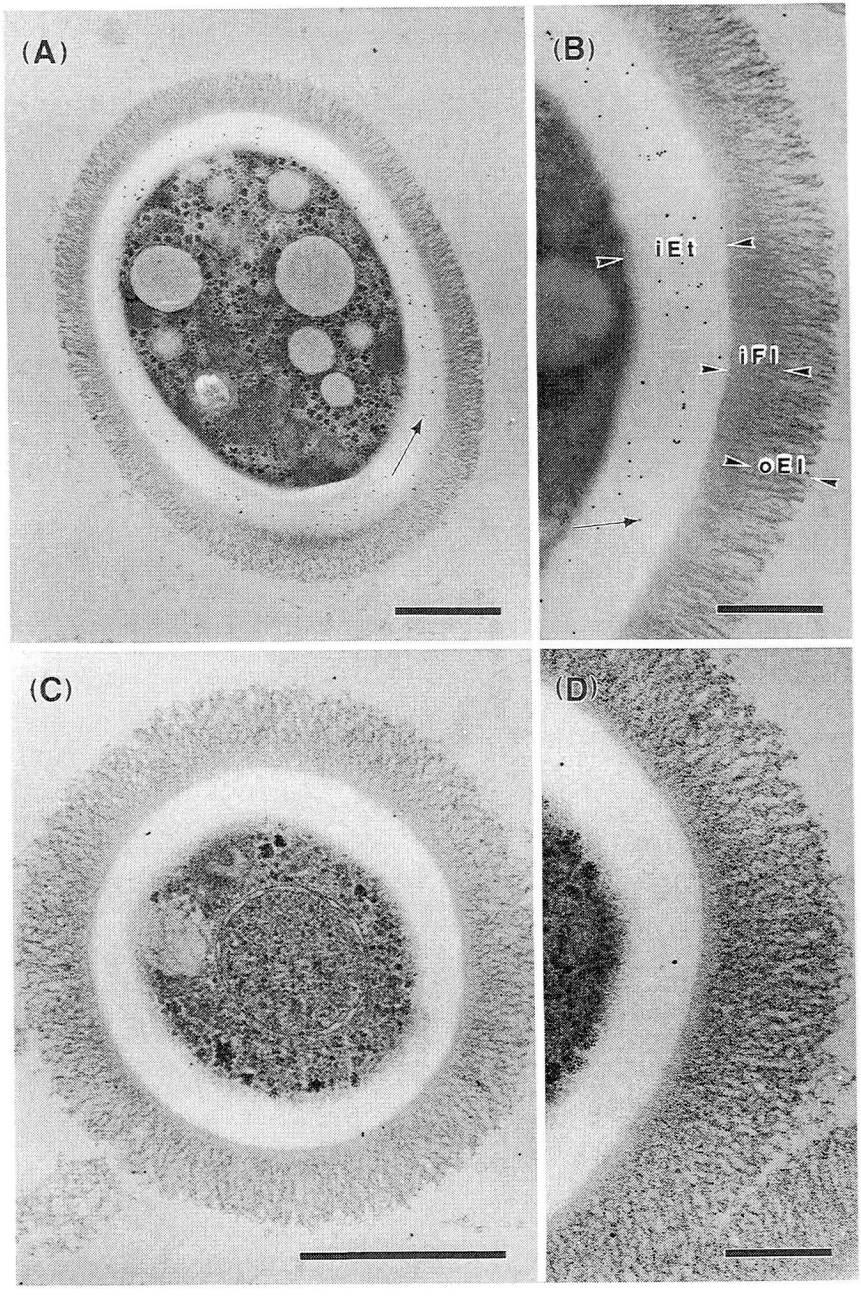
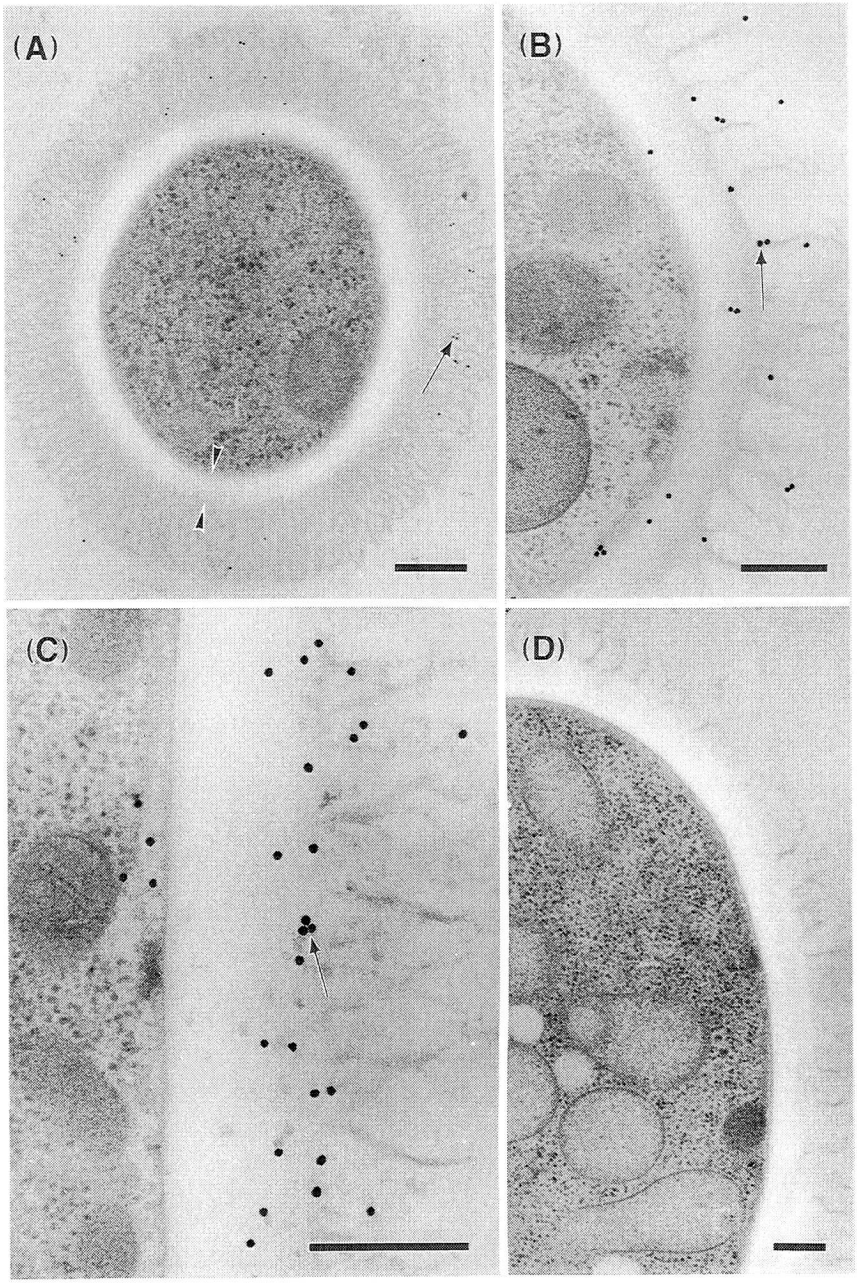

(A)
(B)
iEt
iFl
oEl
(C)
(D)

Plate 11: ConA-gold labelling on cross-sections of cryofixed conidia (isolates LT099 and FP7).

A, The outer fibrillar layer is weakly labelled (arrow) and the inner electron-transparent layer (between arrowheads) is not labelled at all. Sections incubated in ConA-gold (10 nm) diluted (1:10, v/v) in 10 mM PBS/PEG.

B,C, Labelling is concentrated on the fibrils (arrows). Conidia incubated 1 h in water, cryofixed, sectioned and labelled with ConA-gold (20 nm) diluted (1:2, v/v) in 20 mM Tris-HCl/PEG.

D, Negative control. Sections incubated 1 h in ConA, washed and labelled subsequently with ConA-gold. Scale bars: 0.3 μm.


(A)
(B)
(C)
(D)

3.9. Location of surface sugars with gold-labelled lectins

In *D. umbrinella* both gold labelled WGA and ConA bind selectively to different parts of the conidial cell wall. The WGA labelling is strictly limited to the inner electron-transparent layer (Plate 10A, B), in contrast to ConA, which can be detected mainly in the electron-denser external layers of the cell wall, particularly in the dense inner fibrils (Plate 11B, C). The negative labelling controls are always negative. Thus, as suggested by observations mentioned above, chitin is located only in the electron-transparent layer of the cell wall and the fibrillar sheath, especially the inner dense part contains also mannose and glucose. These results are consistent with the fluorescence labelling obtained under 3.8.1 and with previous reports from the mycological literature (e.g. Bonfante-Fasolo and Perotto, 1986; Bonfante-Fasolo et al., 1990).

IV. INFECTION

Summary

In vitro cytological investigations of *D. umbrinella* on beech leaves demonstrate clearly that this endophyte is able to penetrate the host, either directly or after the formation of an appressorium, and can colonise the whole tissue after a period of latency, inducing necrotic spots. The infection process begins with a specific adhesion of the fungal conidia to the host surface. When contact with the host is established, spore germination is induced possibly by compounds present on the leaf or in the leaf tissue. Polysaccharides such as mannans and glucans or their moieties and (glyco)-proteins present in the extracellular matrix of the conidia are clearly involved in the binding process, since ablation of the fibrils inhibits adhesion to the host surface. Complex, finely tuned signals originating from one or both of the symbionts may be involved in the recognition mechanisms. Colonisation of the beech leaf is principally mediated by enzymes, may be activated by host signals although mechanical forces are also involved.

3.10. Adhesion

In *D. umbrinella,* Toti et al. (1992b) have reported a differential attachment of the conidia to the beech leaf, as compared to other, different surfaces. Plate 12A and B illustrate conidia adhering to the leaf surface after a strong washing under running water at 0.5 KPa. Ungerminated conidia (Plate 12A, arrows) show a thin slimy layer at one end, which could be responsible for the adhesion. Some germinating spores are firmely attached to the host surface at the end of the germ-tube (Plate 12B, arrowhead). However, no conspicuous adhesive film, as reported by Nicholson et al. (1989) for *Colletotrichum graminicola,* can be seen after LTSEM. The principal factor responsible for the adhesion may be the dense extracellular fibrils present in cross-sections of the conidia (Plate 6A). This structure cannot be observed at the LTSEM level, because of the high density of the fibrils and their small dimensions (approx. 0.02 µm large and 0.4 µm in length). Moreover, in the microscope these fibrils may be surrounded by a thin frozen water film which is responsible for the smooth appearance of the conidial surface. In addition, high resolution imaging at the LTSEM level requires a very thin metal coating (Hermann and Müller, 1991). The necessary platinum- or gold-coatings used in the present study in order to improve the conductivity of the probe were too thick to allow high resolution.

Cross sections of hyphae on the host surface at later infection stages reveal the presence of an extracellular matrix which is partially dissolved by chemical fixation (Plate 12C). This matrix may function as a bond between hyphae. At the interface with host tissue, the matrix seems to be concentrated at the edges of the contact site between the host cell wall and the hyphae which can stimulate some cell wall deformation (Plate 13A, arrowhead), suggesting the onset of a mechanical or enzymatic effect. Since the extracellular matrix is present around almost all hyphae at the host surface and is reduced as soon as the fungus enters into the mesophyll, it may indicate a high level of adaptation related to the protected environment of the host. On the other hand the mucilage may be the centre of enzymatic activities, concentrated at the contact site with the host cell wall in the mesophyll.

PA-TCH-SP staining of the same tissue demonstrates the presence of large amounts of polysaccharides in the mucilaginous sheath around hyphae (Plate 13B). The cell wall of the hyphae reacts also positively to the PA-TCH-SP staining. This is not surprising since fungal cell walls contain principally polysaccharides, among proteins and lipids (Ruiz-Herrera, 1992). A particularly strong reaction can be observed at both contact angles of the hypha with the host cell wall, thus suggesting once again that polysaccharides may be actively involved in the adhesion process.

Plate 12: Adhesion of *D. umbrinella* to the leaf surface (isolates FP7 and LT098).
A, After washing the conidia are still attached to the host surface prior to germination (arrows).
B, The tip of the emerged germ tube seems to bind to the host surface (arrowhead). LTSEM after washing from the leaf, 16 h after application of 2 µl drops, suspension in Hepes (pH 5.7) at 7.5 x 10^6 conidia x ml^{-1}.
C, Cross-section of hyphae on the host surface. An extracellular matrix, partly dissolved by the chemical fixation, surrounds the hyphae. GA-OsO_4-Epon, spray infection, 9 days after application, suspension in water at 6 x 10^6 conidia x ml^{-1}. EM, extracellular matrix; H, hyphae; HCW, host cell wall. Scal bars: A, B, 3.5 µm; C, 0.9 µm.

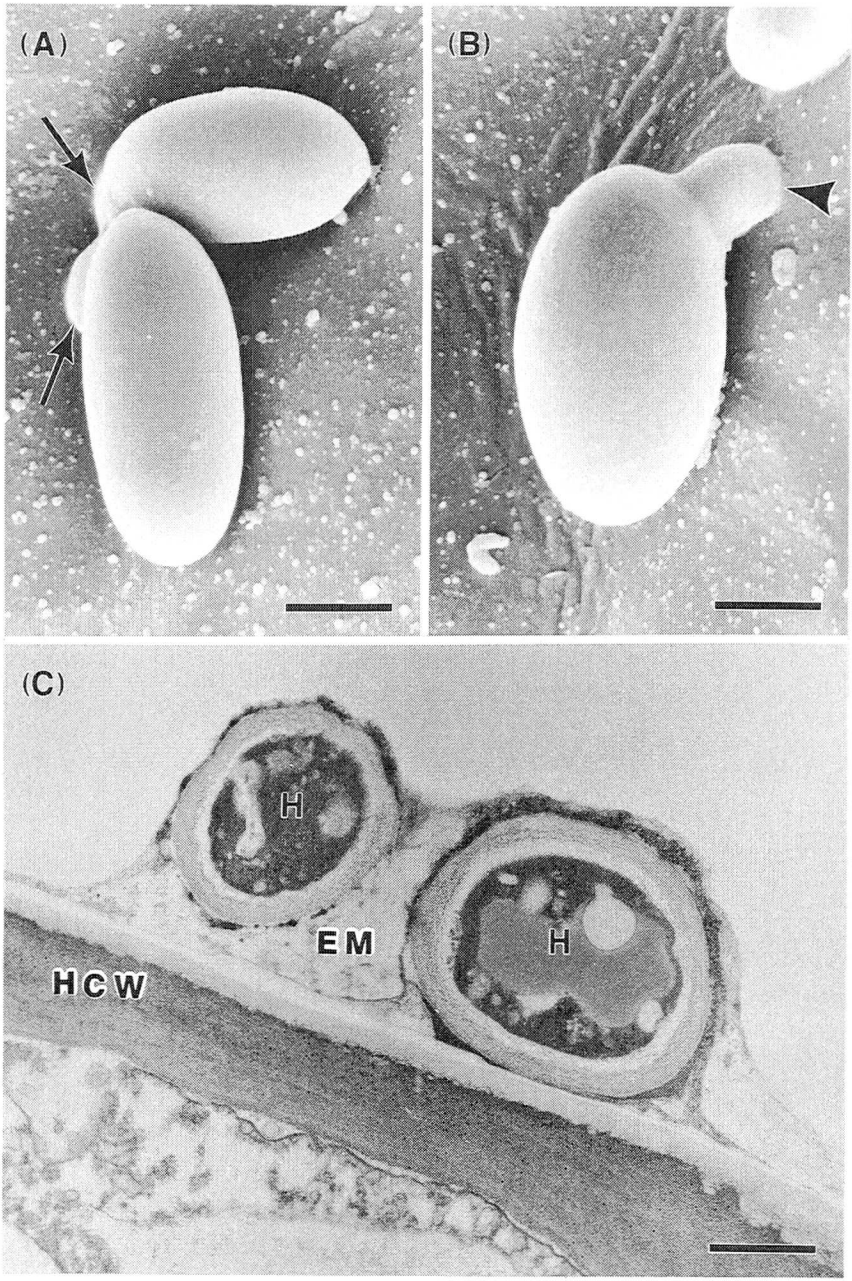
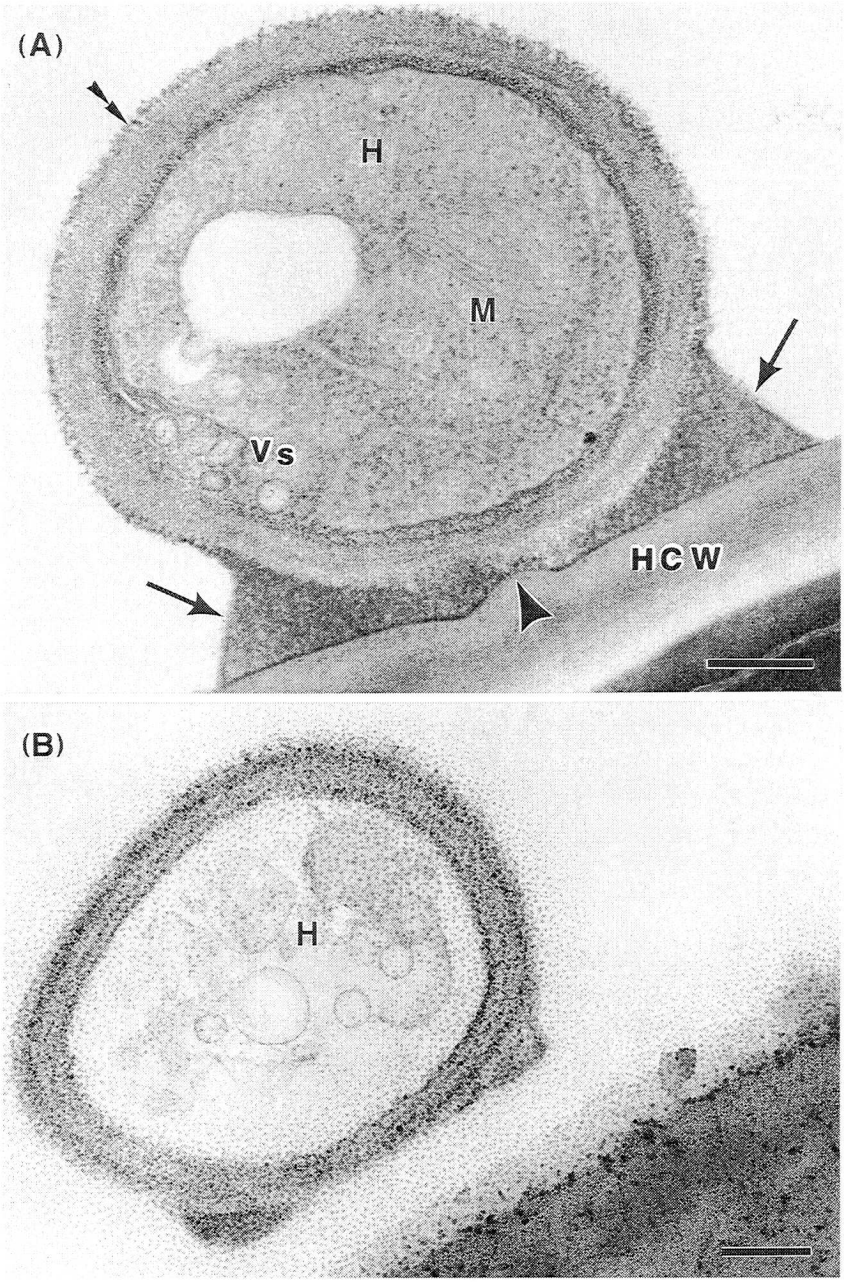

(A)
(B)
(C)
H
EM
H
HCW

Plate 13: Adhesion of *D. umbrinella* in the mesophyll (isolate LT215).
A, The extracellular matrix is concentrated at the edges of the contact site to the host cell wall (arrows) which is deformed (arrowhead). Around the hypha the extracellular matrix (double arrowheads) is restricted. GA-OsO_4-Epon, 24 days after application of 2 µl drops, suspension in Hepes at 5.8 x 10^6 conidia x ml^{-1}.
B, PA-TCH-SP reaction on the same tissue. The matrix around the hyphae contains polysaccharides labelled by the electron-dense silver particles. H, hyphae; HCW, host cell wall, M, mitochondria. Vs, vesicles. Scale bars: 0.3 µm.


(A)
H
M
Vs
HCW
(B)
H

3.11. Germination

Germination begins with the formation of a germ tube and subsequent hyphal elongation after contact of conidia with the substrate. The germination process in *D. umbrinella* starts 2 h after the spores have been applied to a supporting surface. The germ tubes emerge either at the excision scar, on the side, or on both ends of the conidia (Plate 14). Germination is related to an accumulation of organelles such as mitochondria and vacuoles at the site of germination, for example at the tip of the excision scar (Plate 2D). As expected, the fastest and highest germination rate is observed on MA, a complex medium on which 100% of the spores germinate after 24 h. On Teflon-coated slides very few spores are able to germinate (Fig. 6), as compared to incubation of the conidia on MA plates. On the beech leaf surface the germination rate is very variable, depending on the suspension medium and the experimental setup, but is relatively low (Tab. 7). However, an induction of the germination process can be observed when conidia are incubated in leaf extracts, such as beech, chestnut, oak or barley, even without direct contact to the support (Fig. 6). Malt extract increases the germination only slightly in comparison to the controls in water and Hepes. Leaf extracts, particularly of beech and chestnut, may thus contain a factor necessary for the development of *D. umbrinella*, which may be also responsible for the host specificity reported during the adhesion (Toti et al., 1992b). This kind of host specificity is not limited to *D. umbrinella*. Cruickshank and Wade (1992) have shown that the number of appressoria formed by *Monilinia fructicola* on apricots increases in presence of apricot juice, indicating that host chemistry may induce conidial germination. In *Hypoxylon fragiforme*, a xylariaceous beech endophyte, Chapela et al. (1991) have described a mechanism, termed eclosion, mediated by the host and is a necessary requirement for the germination of the ascospores.

Plate 14 illustrates the germination process at different times after application to the beech leaf surface. After 8 h a short germ tube (2 to 4 µm) spreading from the excision scar binds to the host surface. On some conidia the germ tube can emerge from the side and an appressorial swelling at the end of the germ tube can be also observed (Plate 14A). After 12 h the elongation of germ tubes reaches about 10 to 12 µm. Germination at both spore ends can be seen and some hyphae penetrate into the host epidermis (Plate 14B). After 24 h, more than 25 µm-long hyphae are visible on the leaf surface; some conidia have not yet germinated and only few fungal structures penetrate into the epidermal cells, "tunnelling" under the cuticula (Plate 14C, D, arrowheads). Penetration under the cuticula was confirmed by TEM investigations (Plate 17).

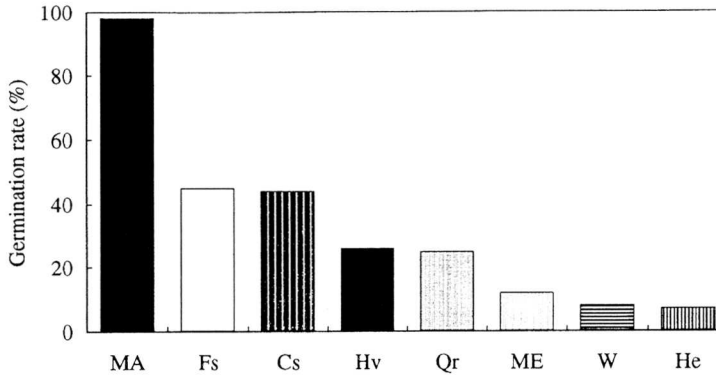


Fig. 6: Percentage of germinated conidia (N=200), 24 h after incubation on Teflon coated slides in moist chambers. The conidia (isolate FP7) are suspended in beech (Fs), chestnut (Cs), barley (Hv) and oak (Qr) leaf extract, as well as in 1% Malt extract (ME), water (W) and Hepes at pH 5 (He). To check the germination capacity of the conidia, the suspension in water has been placed on malt agar plates (MA).

Conidia of *D. umbrinella* present different development strategies on the host surface which can be observed already under the light microscope. Following types can be observed:

I. No germination (Plate 15A).

II. Germination with a long superficial hypha (Plate 15B).

III. Germination with differentiation of an appressorium at the end of the germ tube (Plate 15C).

IV. Presence of a halo around the germ tube tip (Plate 15D).

Forms I. and II. usually never penetrate the host surface. The presence of halos in *D. umbrinella* indicates a lifting of the cuticle by the fungus (Plate 16), suggesting a subcuticular penetration. At the LTSEM or light microscopical level the penetration can not be assessed with absolute certainty (Plate 15), but it is confirmed by TEM, where some hyphae are located between the cuticule and the epidermal cell wall (Plate 17) or penetrate with an appressorium (Plate 18). These different types are detected only when the conidia are in contact with the host surface and never on artificial supports such as dialysis membranes or Teflon-coated slides (Tab. 7). Aist and Israel (1977) have reported different developmental forms of *Erysiphe graminis* f. sp. *hordei* on the leaf

surface after light microscopical investigations and have found that only about 44 % of the appressoria-forming hyphae successfully penetrate the host.

Incubation of the conidia in PMSF results in a strong inhibition of the fungal development on the host surface; spores treated with PMSF, however, germinate normally on MA (Tab. 7). This indicates that on MA a *de novo* synthesis of compounds needed for germination may take place. Page and Stock (1974) have suggested that spore germination of *Microsporum gypseum* is initiated by a protease released by fungal vesicles.

Tab. 8 shows that the largest number of invading structures (III and IV) is found when the conidia are suspended in Hepes at pH 5, the pH-optimum for adhesion to the host surface (Toti et al., 1992b). Cruickshank and Wade (1992) report similar results for the induction of appressoria by *M. fructicola* on apricot. Forms III and IV are already present 2 h after application. During the first 6 h of the infection process, the number of penetrating structures (III+IV) increases in both water and Hepes. At time 6, independently of the medium, the form IV is present at the highest percentage, indicating that at this stage of the infection *D. umbrinella* penetrates principally subcuticularly. At time 24 the different germination types are more or less balanced, with a slightly higher amount of not penetrating forms (I+II), as compared to the conidia forming appressoria (III) or penetrating subcuticularly (IV). Hepes seems to favor the development of more penetrating structures.

Tab. 7: Germination types observed on dialysis membrane (isolate LT215), Teflon-coated slides (isolate FP7), as well as on the host surface, suspending the conidia either in water or in PMSF (isolate LT099), 24 h after application. The values are the average percentage of 6 counted leaf or membrane discs (2 mm diam.) or Teflon-coated fields (4 mm diam.). I. not germinated, II. germinated, III. germinated with appressoria, IV. germinated without or with a short germ tube and presence of a halo. Gr, germination rate (II+III+IV); MA, germination on malt agar plates, estimated over 200 conidia.

Supports	I.	II.	III.	IV.	Gr (%)	MA(%)
Dialysis membrane (LT215)	100	0	0	0	0	98
Teflon (FP7)	90	10	0	0	10	97
Beech, suspension in water (LT099)	34	37	9	20	66	99
Beech, suspension in PMSF (LT099)	100	0	0	0	0	98

Tab. 8: Different germination types observed on beech leaf surface (isolate LT099). 0 to 24 h after infection, application of 0.4 µl drops, conidia suspended in Hepes (pH 5) or in water (pH 5.5) at 7-8 x 10^6 conidia x ml^{-1}. Values are the average of 12 counts (two repetitions), expressed in percent of the total number of conidia. I. not germinated, II. germinated, III. germinated with appressoria, IV. germinated without or with a short germ tube and presence of a halo. Gr, germination rate (II+III+IV).

Time (h)	Hepes					Water				
	I.	II.	III.	IV.	Gr (%)	I.	II.	III.	IV.	Gr (%)
0	100	0	0	0	0	100	0	0	0	0
2	96.3	0.2	1.9	1.6	3.7	99.3	0.2	0.5	0	0.7
4	72	0	1	27	28	85	12.4	1.4	1.2	15
6	42.2	0.6	0.4	56.8	57.8	60	8	1	31	40
24	22	35	20	23	78	34	37	9	20	66

Plate 14: Germination of *D. umbrinella* on the beech leaf surface after washing under running water (isolate FP7).
A, 8 h after application. A short germ tube binds to the host surface (note the appressorial swelling at the end of some germ tube).
B, 12 h after application. The hyphae are longer, germination at both ends of the conidia can be observed and some hyphae penetrate directly into the host (arrow).
C, **D**, 24 h after application. Some of the hyphae are developing subcuticularly arrowheads). LTSEM, conidia suspended in Hepes (pH 7), applied in 2 µl drops, at 7.5 x 10^6 conidia x ml^{-1}. Scale bars: 4 µm.

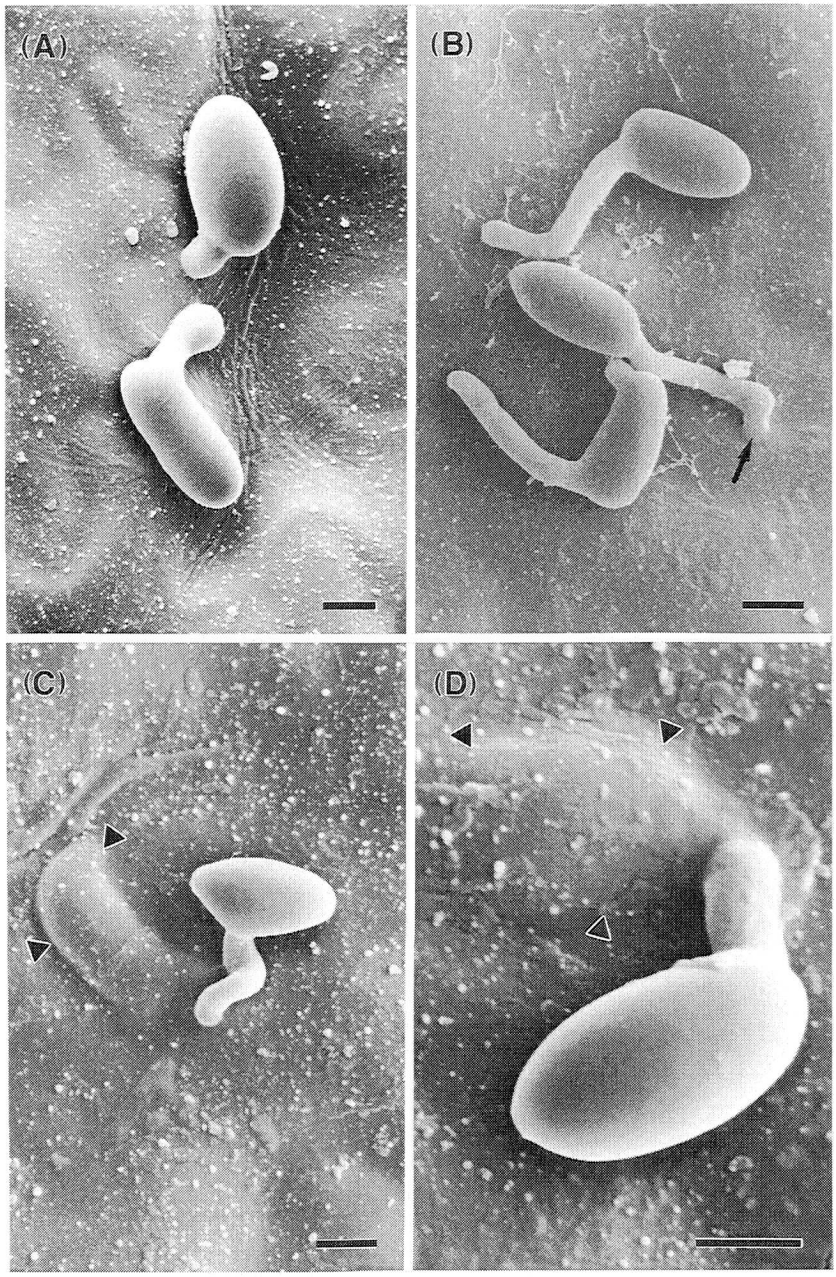
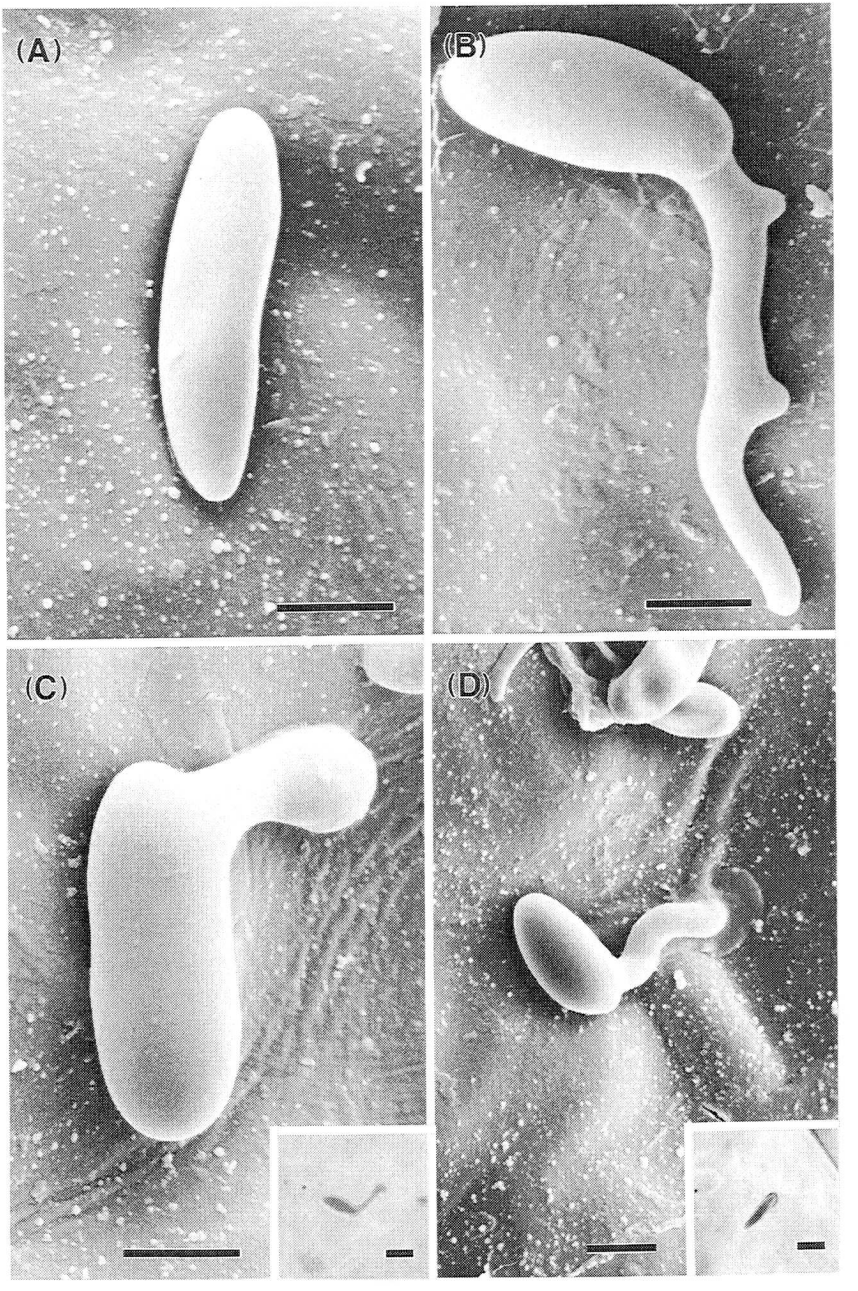

(A)
(B)
(C)
(D)

Plate 15: Different germination types on the leaf surface (isolate FP7).
A, Not germinated (I).
B, Germinated (II).
C, Germinated with an appressorium at the tip of the germ tube (III). Inset: LM micrograph of an appressorium.
D, Germinated, with halo formation, at the tip of a long (inset) or short germ tube (IV). LTSEM, 16 h after infection with 0.4 µl drops, suspended in water at about 7 x 10^6 conidia x ml^{-1}. Scale bars: 4 µm; insets, 7 µm.


(A)
(B)
(C)
(D)

3.12. Germination types in relation to adhesion

To obtain more information on the correlation between germination and adhesion, I washed the leaves under running water at a constant pressure of 0.5 KPa (Toti et al., 1992b) before counting the different germination types. The germination process does not seem to be directly involved in the binding process (Tab. 9). In this experiment, the majority of attached conidia did not germinate and only a very small number of conidia had formed appressoria or halos. This, however, may also depend on the experimental design used.

Tab. 9: Different germination types observed on the beech leaf surface after washing under running water (isolate LT099).
0 to 24 h after infection, application of 0.4 µl drops, conidia suspended in water at 7-8 x 10^6 conidia x ml^{-1}. The values are the average of 6 counts, expressed in percent of the total number of conidia. I. not germinated, II. germinated, III. germinated with appressoria, IV. germinated with the presence of a halo. Gr, germination rate (II+III+IV). The binding is expressed in percent and represents the number of conidia still attached after washing in relation to the conidial concentration applied.

Time (h)	Germination forms				Gr (%)	Binding
	I.	II.	III.	IV.		
0	100	0	0	0	0	9
2	97.6	1.2	1.2	0	2.4	22
4	98	1.5	0.5	0	2	24
6	99.4	0.3	0.3	0	0.6	20
8	96.2	1.8	1.3	0.7	3.8	28
24	95.9	3.3	0.3	0.5	4.1	32

3.13. Penetration

The beech leaf is bifacial, consisting of an adaxial epidermis, the mesophyll divided in palisade and spongy parenchyma and an abaxial epidermis with stomata. The leaf is protected toward the outside by a cuticle. The cuticle can be classified as "type 3" (Holloway, 1982): the outer region is amorphous, covered by the epicuticular wax layer and the inner region is mainly reticulate, separated from the epidermis cell wall by a pectin layer. Together, the amorphous and reticulate parts constitute the cuticular membrane. Plant cuticles are composed of two major lipid components: cutin, an insoluble polymeric material and waxes, soluble long-chain aliphatic compounds within which the cutin is embedded (Kolattukudy, 1985). The stomatal guard cells are covered by a cuticular layer which forms the guard cell lips. In addition to providing a barrier to diffusion, the cuticle also protects against microorganisms. The structural importance of the epicuticular wax layer has been reported by Juniper (1991). Electron microscopical investigations on beech leaves have so far been carried out only in relation to tolerance to air pollutants (Stirban et al., 1988) and photosynthetic activity (Larcher et al., 1988).

3.13.1. Infection at the adaxial surface

A. Direct penetration

At the adaxial side of the leaf two distinct infection types are observed. *D. umbrinella* can either penetrate directly, developing a subcuticular hypha (Plate 16), or can form an appressorium at the end of the germ tube prior to penetration (Plate 15C).
The SEM-micrographs presented in Plate 16 show the relationship between the presence of halos around conidia and subcuticular penetration into the host (Plate 15D). The halos formed by *D. umbrinella* are not related to the papilla reported by many authors (e.g., Aist and Israel, 1977; Koga et al., 1990) in which callose accumulates during an early phase of plant defence. In the beginning the fungus is present only on the host surface and in the reticulate part, underneath the amorphous layer of the cuticular membrane (Plate 17A, B). The superficial hyphae are always surrounded by mucilage (see also Plate 12C). As soon as the fungus penetrates the tissue, this extracellular sheath is detectable only around intercellular hyphae and not around intracellular ones (Plate 21A). A similar observation has been made by Bonfante-Fasolo (1988) in ericoid mycorrhiza, where the mucilaginous sheath decreases as soon as the fungus penetrates the cells.

Since the cuticle may be considered the first host barrier to penetration by pathogens, this part of the cell wall has to be first digested by the fungus. Indeed, an enzymatic activity is suggested by the presence of electron-transparent material, due to the loss of cuticular compounds around the hyphae (Plate 17, see also Plate 22A). The cuticular membrane is mainly composed of cutin embedded in extracellular waxes (Holloway, 1982; Kolattukudy, 1985). However, the major physical strength of the cuticle is provided by the polymeric structure of the cutin and not by the monomeric wax fraction (Köller, 1991). Generally fungi may readily produce cutinases (Kollatukudy, 1985). It would therefore be expected that *D. umbrinella* produces cutinases, enabling it to penetrate into the host, as already demonstrated in other models. The subcuticular presence of a fungus during the initial infection processes, described as "tunnelling hyphae", has been reported by Clulow et al. (1991) for *Mycosphaerella pinodes* on pea and by Jones and Ayres (1974) for *Rhynchosporium secalis* on barley. Subcuticular penetration without formation of appressoria has been reported for *Venturia inaequalis* by Smereka et al. (1987), and the cutinase responsible for the degradation of the apple leaf has been purified by Köller and Parker (1989). Other examples of direct penetration and the correlated secretion of cutinase have been observed in *Fusarium* spp. (Parry and Pegg, 1985).

The cell walls of the epidermal cells underneath the subcuticular hyphae are almost completely digested, and the cytoplasm appears completely electron-dense compared to the intact parenchyma cells (Plate 17). Such a reaction can be explained by the accumulation of phenolic compounds, as the result of an hypersensitive response against the fungus, which is actively isolated from the host by a barrier of dead cells (Plate 17). Indeed, the fungus continues to grow along the degenerated epidermis cell, without penetrating it. A similar plant reaction has previously been described by Freytag and Hahlbrock (1992) for potato leaves infected by *Phytophthora infestans.*

Plate 16: Evidence for the subcuticular penetration of *D. umbrinella* in beech leaves (isolate LT 099). SEM-micrographs of symptomless leaves.
A, B, At this infection stage, the conidia are tunnelling underneath the cuticle, showing the halos (arrowheads) observed with light microscopy. GA-OsO_4-CPD, 16 h after application of 2 µl drops, suspension in Hepes (pH 5) at 5 x 10^6 conidia x ml^{-1}. Scale bars: 1.4 µm.

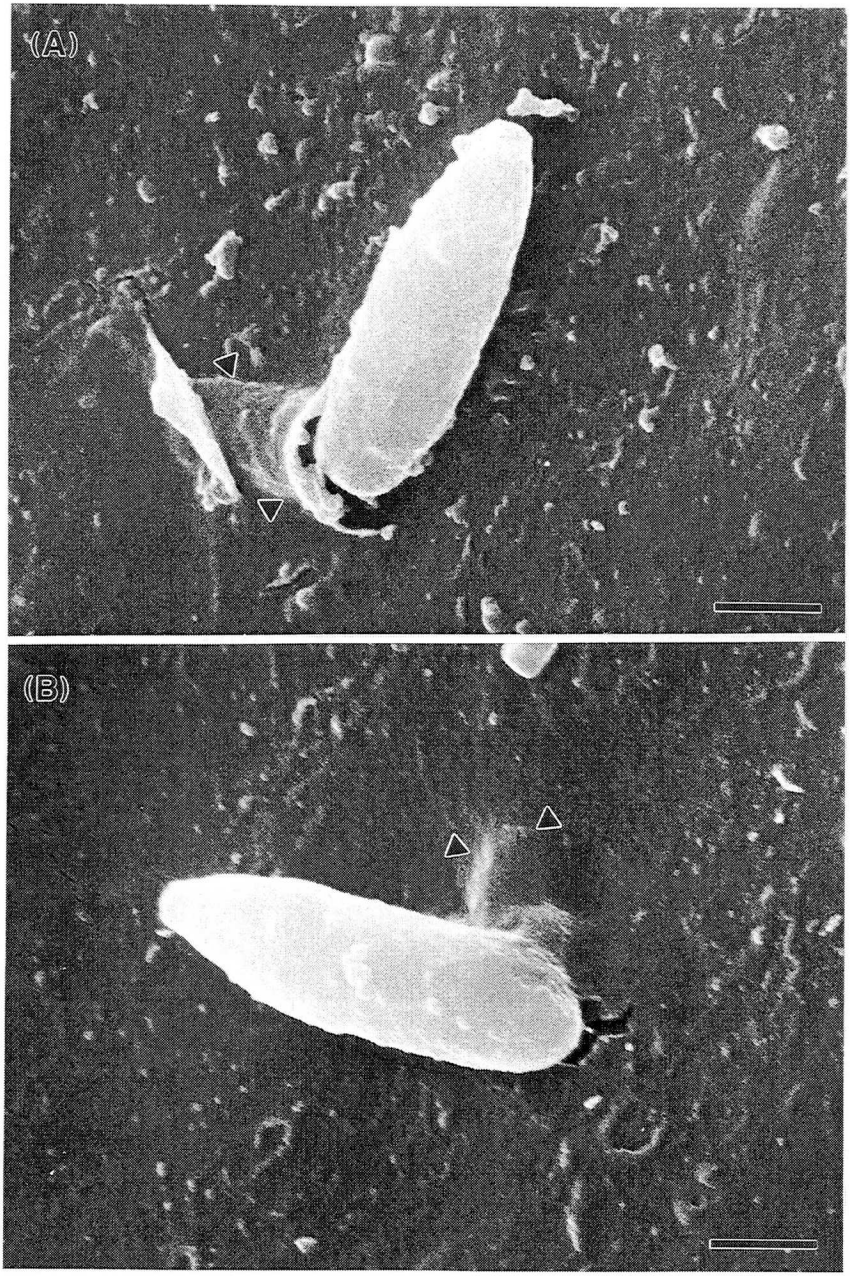

(A)
(B)

Plate 17: Evidence for the subcuticular penetration of *D. umbrinella* in beech leaves (isolate LT098). TEM-micrographs of partly necrotic leaves.
A, B, Penetrating hyphae are mostly present in the reticulate part of the cuticular membrane. The epidermal cell underneath appears completely destroyed, while the palisadic parenchyma cells are unaffected. The host tissue is apparently enzymatically degraded. GA-OsO_4-EPON, 9 days after spray-infection with 12 x 10^6 conidia x ml^{-1}, suspension in water. aC, amorphous cuticular membrane; E, epidermis cell; ECW, epidermis cell wall; H, hyphae; P, palisade parenchyma; rC, reticulate cuticular membrane; arrow, plasma membrane. Scale bars: 1 µm.

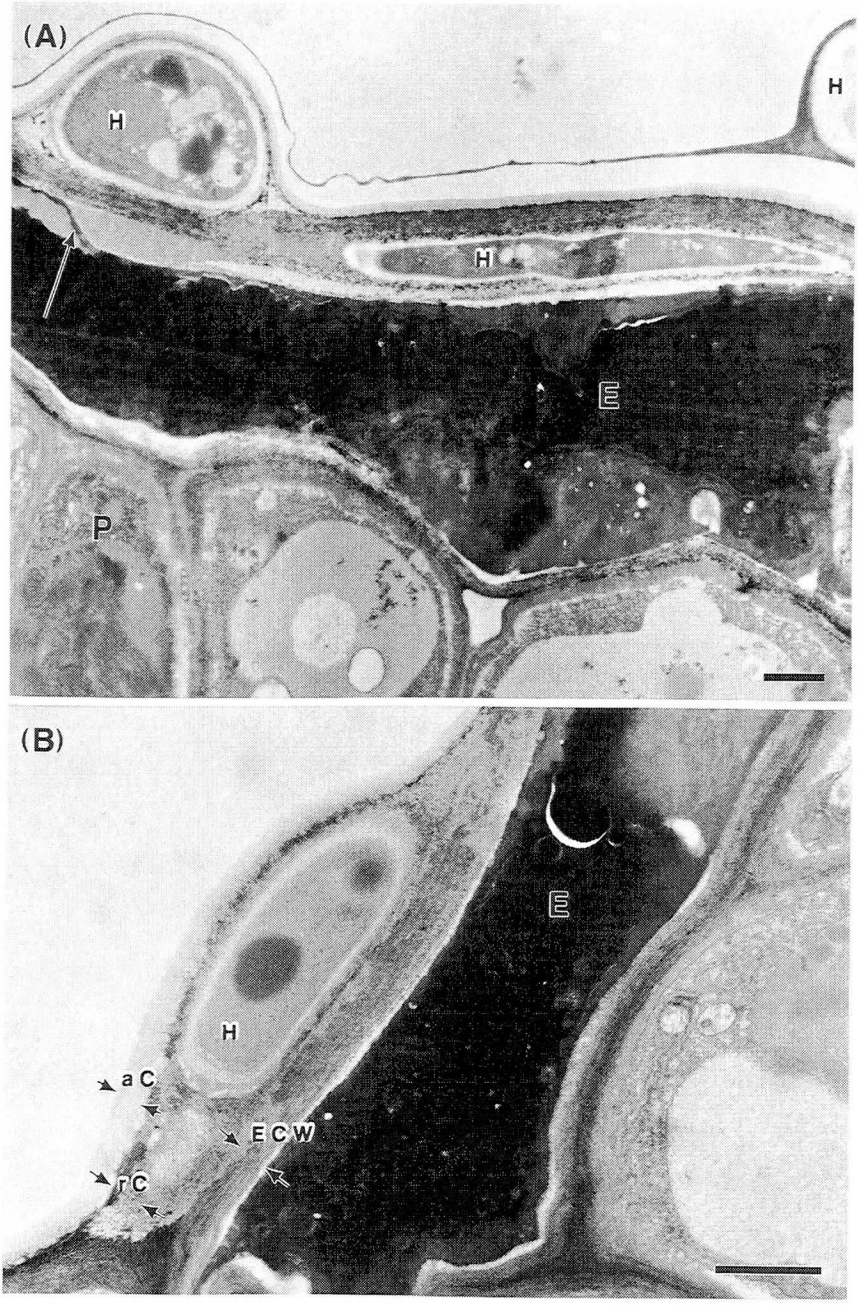

(A)
H
H
H
E
P
(B)
E
H
aC
ECW
rC

Plate 18: Penetration of *D. umbrinella* after the formation of an appressorium on serial cross sections (isolate LT098).
A, B, From the appressoria present on the leaf surface, a penetration peg emerges. The cuticular membrane and the epidermal cell wall of the host are at least partly dissolved. A mechanical effect is apparently also involved (B, arrows). A septum separates the penetration peg and the growing hypha, which is parallel to the epidermal cell.
Inset: the fungal cell wall is very thin at the point of penetration (double arrowhead). GA-OsO_4-EPON, 9 days after spray-infection at 12 x 10^6 conidia x ml^{-1}, conidia suspended in water. Ap, appressoria; Cl, chloroplast; Cm, cuticular membrane; E, epidermal cell; ECw, epidermal cell wall; H, hyphae; Pp, penetration peg; S, septum; V, vacuole. Scale bars: 1.6 µm, inset, 0.3 µm.

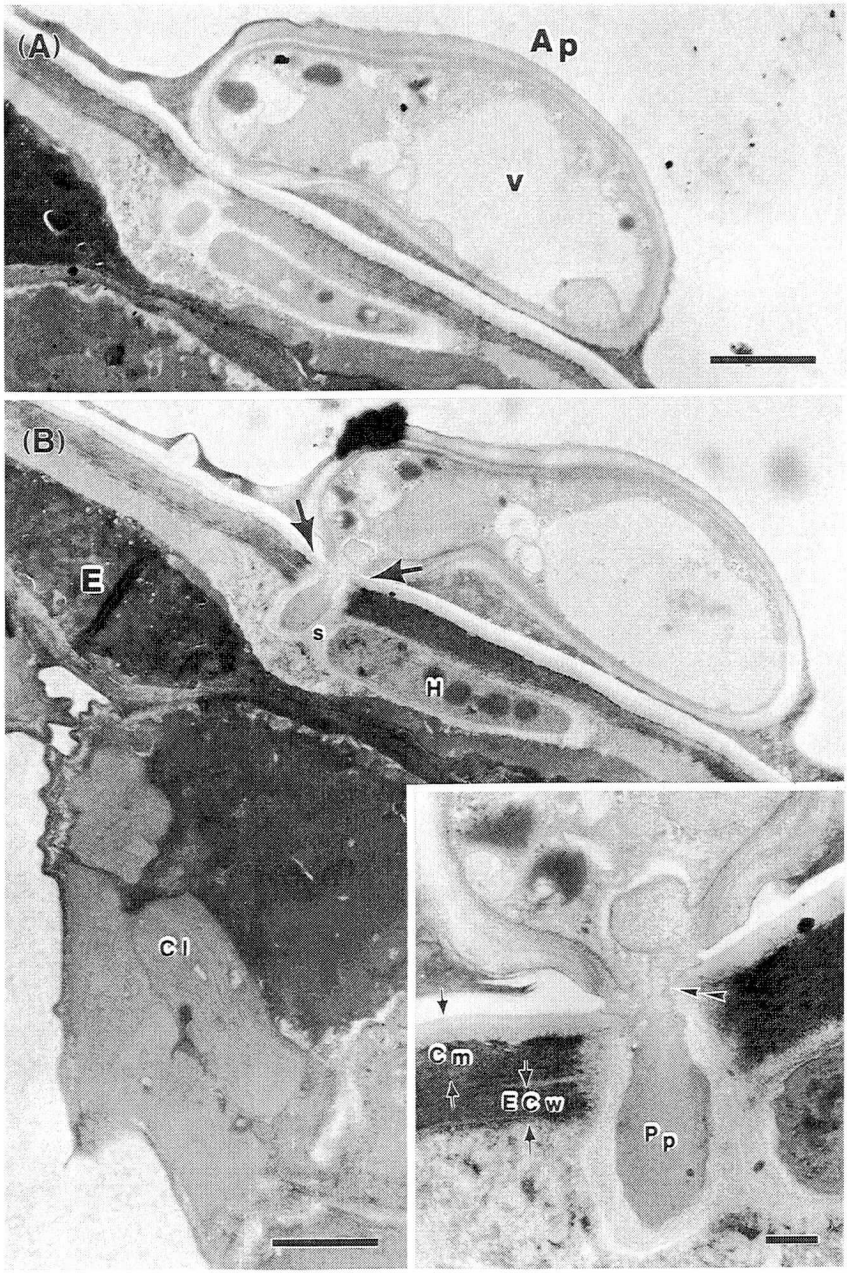

(A)
Ap
v
(B)
E
s
H
Cl
Cm
ECw
Pp

Plate 19: Penetration of *D. umbrinella* after the formation of an appressorium (isolate FP7).

A, The penetration peg penetrates only the cuticular membrane and the hypha grows between the cuticle and the epidermis cell wall.

B, Detail of the penetration peg.

C, Detail of the hyphal tip. The fungus starts to digest the epidermis cell wall (arrow). GA-OsO_4-EPON, 15 days after infection, with 2 µl drops, conidia suspended in water at 6 x 10^6 conidia x ml^{-1}.

D, WGA-gold labelling: cross-sections of an appressorium with peg penetrating the cuticular membrane, gold-WGA (10 nm) particles (arrows) bind to the inner sheath of the penetration peg (WGA-gold diluted 1:2 (v/v) in 100 mM PBS/PEG, 27 days after infection on whole plants, conidia suspended in water at 12 x 10^6 conidia x ml^{-1}). Ap, appressoria; Cp, cuticular peg; ECw, epidermal cell wall; H, hypha; M, mitochondria; Pp, penetration peg. Scale bars: A, 2.8 µm; B, C, D, 0.8 µm.

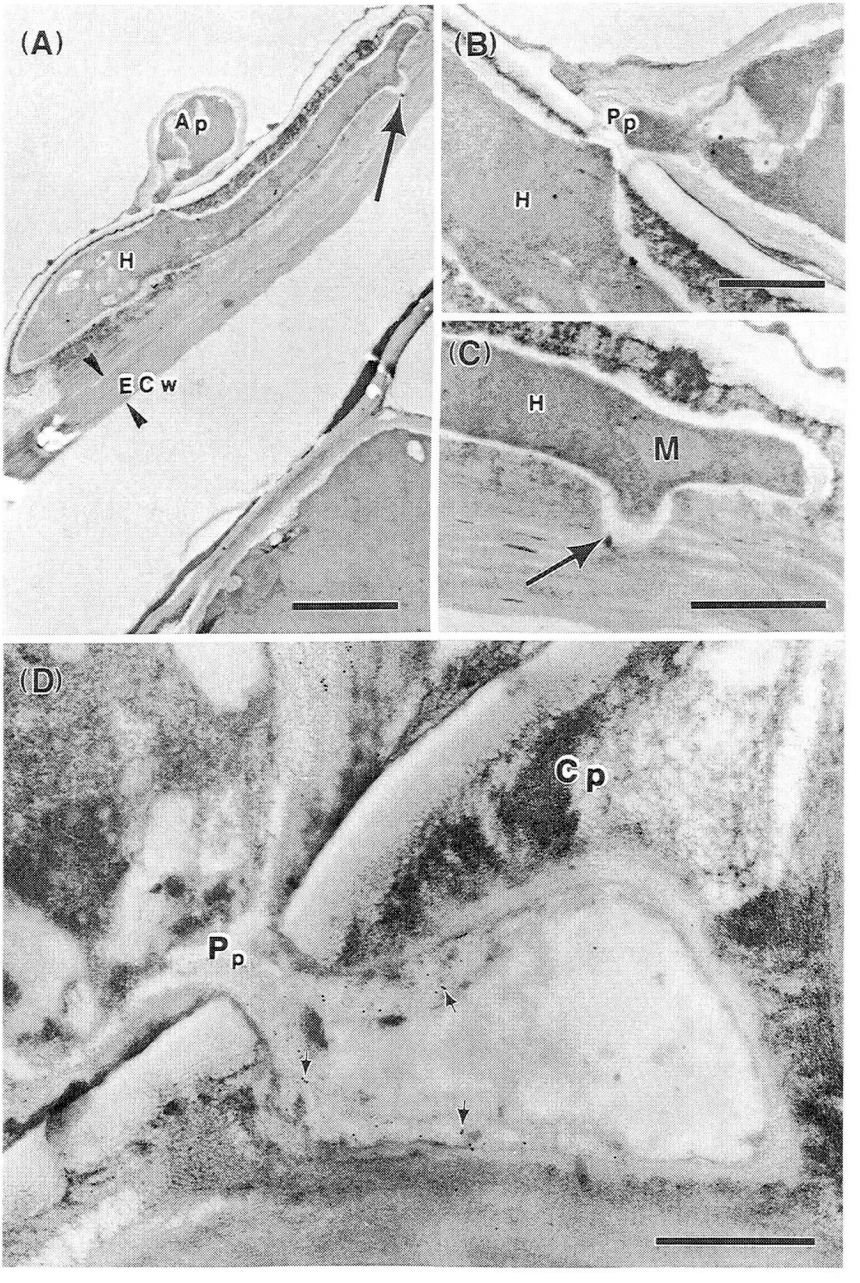
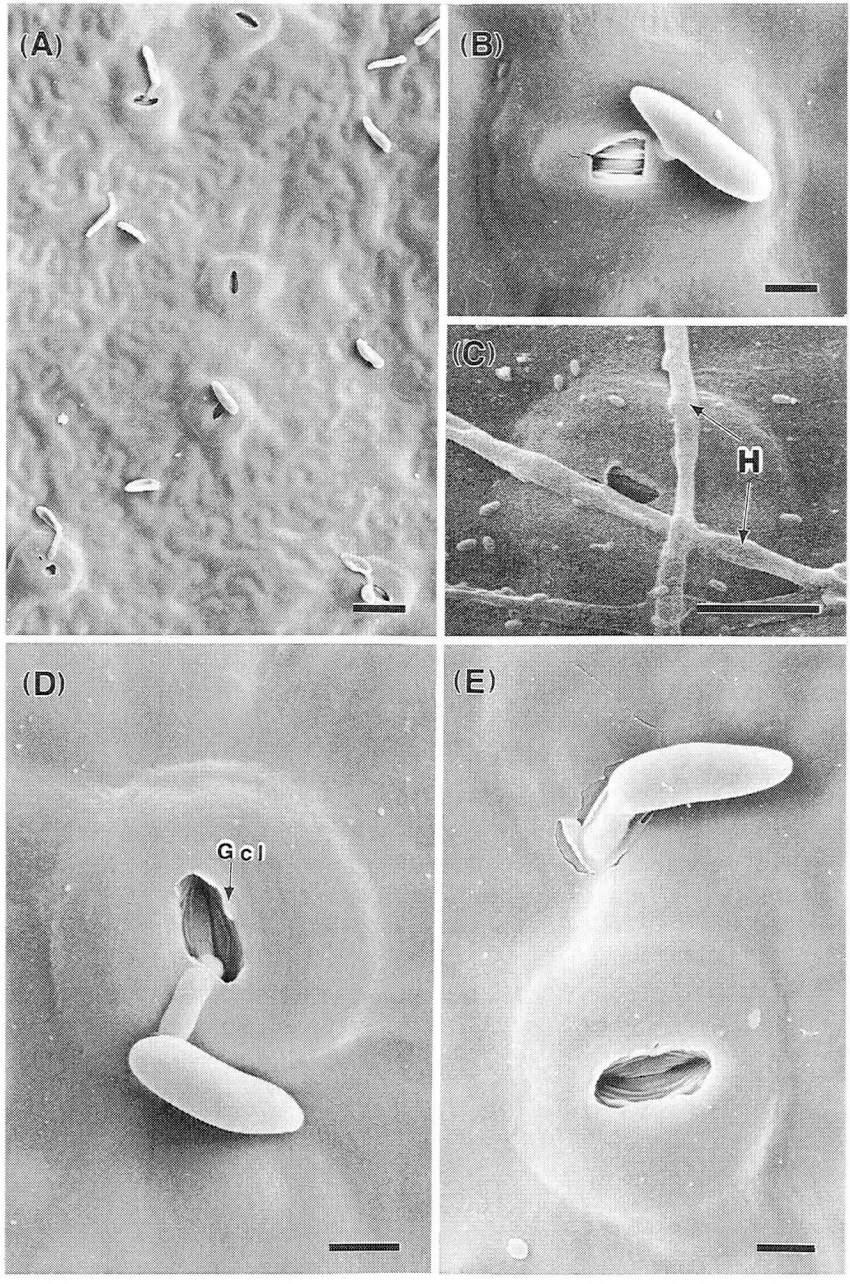

(A)
Ap
H
ECw
(B)
Pp
H
(C)
H
M
(D)
Cp
Pp

Plate 20: Infection at the abaxial side of the leaf (isolate LT099 and FP7).
A, Overview.
B, D, The conidial germ tube has penetrated through the stoma after digesting the cuticle of the guard cell lips.
E, Subcuticular penetration beneath a stomatal aperture. LTSEM of infected leaves washed under running water 16 h after application, of 2 µl drops, suspension in water at 7-8 x 10^6 conidia x ml^{-1}.
C, The hyphae can grow over a stoma without penetrating the host. GA-OsO_4-CPD, 7 days after infection by spraying the leaf with 10 x 10^6 conidia x ml^{-1}, suspended in water. Gcl, guard cell lips; H, hyphae. Scale bars: A, 20 µm; B, C, D, E, 4,5 µm.


(A)
(B)
(C)
H
(D)
Gcl
(E)

B. Penetration with the formation of an appressorium

The second penetration type involves the formation of an appressorium at the tip of the germ tube, which can be observed by light microscopy as well as in LTSEM micrographs (Plate 15C). The penetration through the host cell wall is visible on serial cross sections of appressoria, showing the presence of a penetration peg that pierces not only the cuticular membrane but also the epidermal cell wall (Plate 18). At the site of penetration the fungal cell wall is very thin and the extracellular matrix around the fungus is no longer present. A degradation of the leaf tissue around the penetration peg is evident. The cuticle is usually attached to the epidermal cell wall by a layer of pectinaceous material (Kolattukudy, 1985) so that this material must be degraded by the fungus. A pectinase activity has been detected in *D. umbrinella* by Toti et al. (1991) and Toti (1993). On the other hand, a mechanical effect on the host cell wall can not be excluded and may also play a role during the invasion process, since the walls of the epidermal cell underneath the penetration peg is slightly deformed (Plate 18B, arrows).

Alternatively, the peg emerging from the appressorium can penetrate only the cuticle and form subcuticular hyphae along the epidermal cell (Plate 19A). Digestion of the epidermal cell wall occurs in a second step at the end of the hypha, which branches to continue the colonisation process (Plate 19A).

Two distinct infection types have been already described for *V. inaequalis* by Smereka et al. (1987). These authors have reported that the conidial germ tubes of the apple scab fungus can either terminate in an appressorium or penetrate directly, developing a subcuticular stroma.

Investigations using gold labelled WGA demonstrate the presence of chitin in the fungal cell wall throughout the infection process. The WGA-gold labelling pattern of the appressorium and of the penetration peg present a weak labelling of the inner appressorium wall and of the peg (Plate 19D). Similar results have been reported by Bonfante-Fasolo et al. (1990) for endomycorrhizal-root associations, on which gold-labelled WGA particles are only to be found in the cell wall of the penetration peg and not in the infection hyphae. The same authors have also observed that the chitin labelling depends on the hyphal wall thickness. Moreover, Mendgen et al. (1988) have reported that chitin of thick-walled hyphae seems to be protected by compounds related to glucans.

3.13.2. Infection at the abaxial surface

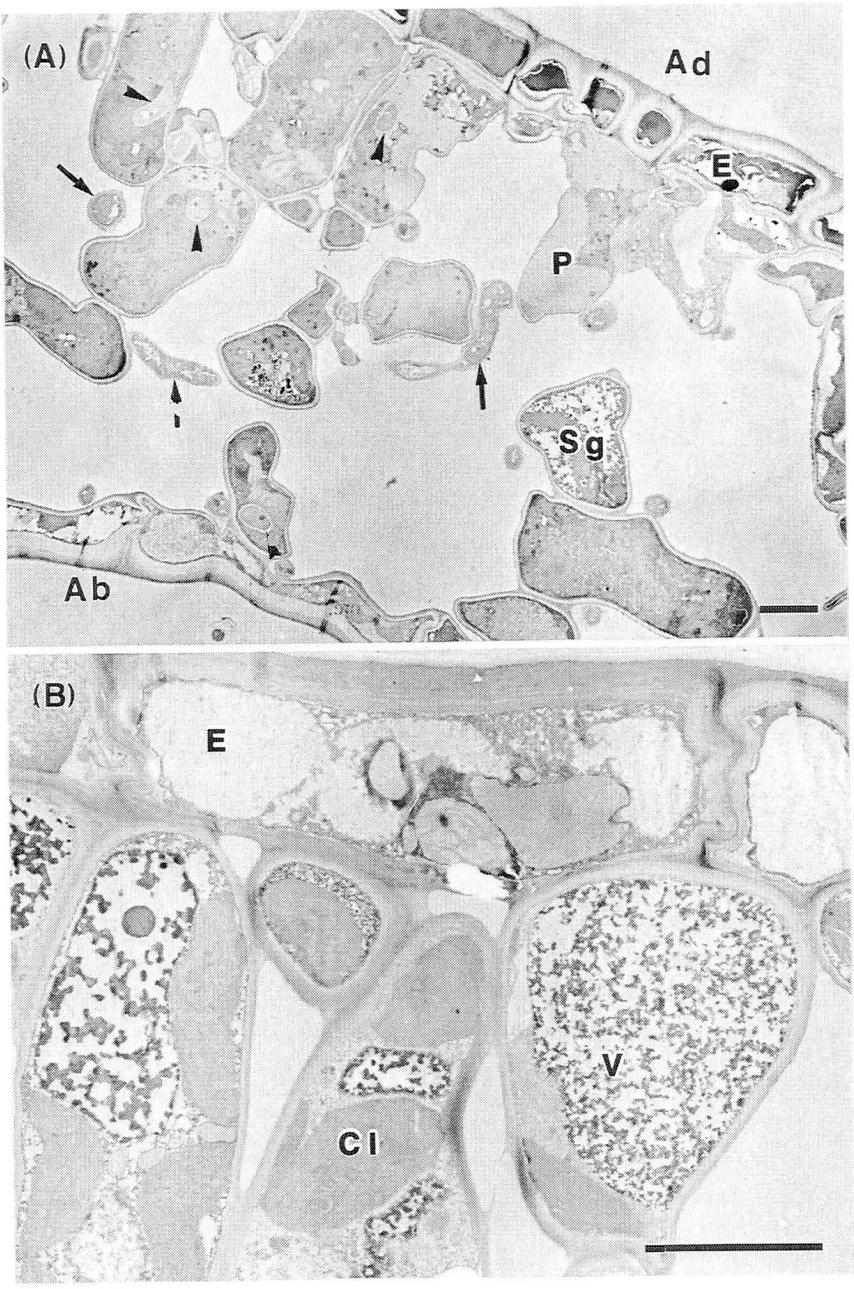
Application of the conidia on the abaxial side of the leaf results in a better colonisation of the leaf by the fungus, suggesting that *D. umbrinella* may use the stomatal apertures to penetrate the host surface (Tab. 4). A detailed analysis of the contact side shows that germ tubes invade stomata indirectly by penetrating the cutinised stomatal lip (Plate 20). Some hyphae even grow over the stomata without penetrating the leaf (Plate 20C). The fungus apparently induces closing of the guard cells, partly covered by the cuticular layer of the lips which, however, can be digested by the conidial germ tube (Plate 20B, D). Therefore, even the colonisation of the host tissue through the stomata may require an enzymatic activity by the fungus. In *D. umbrinella* a sensory recognition of the stomatal apertures, reported for many rust fungi or for the downy mildew on grape (Hoch and Staples, 1991) does apparently not trigger penetration into the host.

3.14. Post penetration events

The colonisation events by *D. umbrinella*, that include the adhesion to the host surface, the germination and the penetration of the cell wall, are observed throughout the first 24 h. A precise time course for the germination and penetration cannot be described since these events occur apparently at random during the first 24 h after the fungus makes contact with the host and adheres actively to its surface (Tabs. 8, 9). The adhesion to the host is detected immediately after application, with a maximum 16 h after application (Toti et al., 1992b) and the germination process starts 2 h after contact. Since we could demonstrate an activation of germination by the beech leaf extracts it is possible that the induction of this process is mediated by a signal from the leaf which may be accessible to the fungus only under particular unknown conditions, which would explain the very variable germination rates measured on the leaf surface during the first 24 h. Observations 24 and 48 h after infection have revealed that while the length of the hyphae on the host surface increases, no additional penetration takes place. This suggests that penetration must occur during the first 24 h after contact with the host and that hyphae which do not penetrate during this time will never colonise the beech leaf. The first symptoms are observed after 48 h but again may vary, depending on the infection conditions. Therefore between 24 and 48 h the leaf may defend itself against the fungus, maintaining the hyphae in latency at the subcuticular or epidermal level, and so avoiding a colonisation of the mesophyll. A reaction by the plant can still be observed between the

necrotic tissue and the green, healthy tissue up to 23 days after infection (Plate 17, Tab. 10), even when the centre of the necrotic zone is completely colonised by the fungus.
Initial penetration events, related to a symptomless expression of the disease has been reported by Clulow et al. (1991) for *Mycosphaerella pinodes* on *Pisum sativum.* They suggested that this early phase of infection, which lasts 48 h, is probably biotrophic. The same authors describe the first symptoms after 48 h. This sequence corresponds approximately to the infection process of the beech leaf by *D. umbrinella.*
During the necrotic phase of the infection *D. umbrinella* grows progressively through the whole leaf section (Tab. 10). Six days after infection, when the first symptoms are observed in this experiment, the hyphae can be found only intercellularly, except in the adaxial epidermis, and are mainly concentrated in the adaxial layers of the leaf section. The first intracellular hyphae are found in the mesophyll 10 days after infection, and the fungus colonises the whole tissue only 15 days after infection. After 23 days, no more hyphae can be observed at the adaxial subcuticular level and the number of hyphae on the leaf surface is relatively low, indicating that *D. umbrinella* is now concentrated in the deeper parts of the host tissue, particularly in the intercellular spaces of the spongy parenchyma. The presence of the fungus in the host tissues results in a loss of turgescence and a disorganisation of the cell organelles, compared to the uninfected controls (Plate 21). The adaxial epidermis cells reveal an electron-dense appearance, confirming the hypersensitive reaction mentioned before.

Plate 21: Presence of *D. umbrinella* in the beech tissue during the necrotic phase of the infection (isolate LT215), 25 days after infection (2 µl drops with conidia suspended in Hepes (pH 7), at a concentration of 5 x 10^6 conidia x ml^{-1}, GA-OsO_4-EPON).
A, The whole tissue is invaded by the fungus. The hyphae are mainly located in the large intercellular spaces (arrows), but are also present intracellularly (arrowheads). Extracellular mucilage is evident only around the intercellular hyphae in contact with the host cell wall. The general organisation of the tissue is maintained but the cells are less turgent, as compared to the control.
B, Control. Ab, abaxial side; Ad, adaxial side; Cl, Chloroplast; E, epidermis; P, palisade parenchyma; Sg, spongy parenchyma; V, vacuole. Scale bars: 5 µm.


(A)
Ad
E
P
Sg
Ab
(B)
E
V
Cl

Tab. 10: Time development of the infection from the onset of necrosis (6 days after application) to 23 days after infection. Semi-thin sections (0.2 µm) of chemically fixed, Epon embedded 2 µl drop-infected leaves with isolate FP7 suspended in water at a concentration of 6 x 10^6 conidia x ml^{-1}. The samples were taken at the boundary between the necrosis and the healthy tissue. The values represent the average number of hyphae counted in the different layers of the leaves, on 5 semi-thin sections each and expressed in mm^2 after measuring the average surface of the 5 sections.
Hypersensitive reaction: -, no reaction; +/-, less than 50 % of the epidermal cells; +, over 50 % of the epidermal cells; ++, whole epidermis react hypersensitively.

Tissue	Days after infection					
	6	7	8	10	15	23
Adaxial surface	3.3	4.8	9.7	11.5	28.1	3.6
subcuticular	0.28	1	0.4	2.8	3.8	0
Adaxial epidermis						
intracellular	0.48	1.5	1	1	3.8	9.1
intercellular	0.95	2.1	0.5	2	2.6	0
Palisade parenchyma						
intracellular	0	0	0	0.8	3.1	10.9
intercellular	0.85	2.1	2.1	5.5	11.1	7.2
Spongy parenchyma						
intracellular	0	0	0.5	0.6	2.4	5.5
intercellular	0.85	5.3	7.8	13.9	34.1	30.9
Abaxial epidermis						
inner cell surface	0.2	1.3	1.3	2.1	2.6	9.1
intracellular	0	0	0	1	0.7	9.1
subcuticular	0	0	0	0	0.5	0
Abaxial surface	0	0	0	0	4.2	0
Hypersensitivity	+/-	+	+	++	++	++

maximum number of hyphae found in the different parts of the leaf sections

maximal number of hyphae found at each time over all leaf parts

The hyphae of *D. umbrinella* can digest the epidermal cell wall and after a complete digestion of the cytoplasm they continue to grow into the parenchyma (Plate 22A). Once the hyphae are present intracellularly in the epidermis cell, they start to actively digest the cell content (Plate 22B). In the mesophyll the hyphae are first observed in the intercellular spaces (Tab. 10), but the fungus also grows intracellularly (Plate 23). Intracellular penetration and colonisation are related to the local decomposition of the cell wall by a penetration peg emerging from the hyphae.

At later stages haustoria grow from a haustorial mother cell and penetrate the host cell wall (Plate 23D). In general intracellular fungal penetration correlates with the destruction of the internal structure of the host cell, which is always electron-dense and corresponds to the necrotic phase of the infection (Plate 23). Stone (1986) has reported the same results for *Rhabdocline parkeri*, which suggests a similar life strategy for both endophytes. As long as the hyphae are intercellular, the cells undergo no recognisable morphological modifications at the TEM-level, probably because the fungus is latent in the superficial leaf layers.

PA-TCH-SP staining of penetrating hyphae in the mesophyll shows an accumulation of positive-stained material around penetration pegs (Plate 24B, C) and at the tip of an emerging peg (Plate 24A), in other words where a high enzymatic activity can be expected. This accumulation of PA-TCH-SP-positive material may correspond to degradation products of the cell wall such as cellulose monomers. Cellulase activity was demonstrated for *D. umbrinella* by Toti (1993), but the involvement of other enzymes in the colonisation process must be assumed as well. A large zone containing PA-TCH-SP-positive material is present around the intracellular hyphae (Plate 24A) but is not detectable around intercellular ones (Plate 24B, C). This suggests that an enzymatic activity in the extracellular fungal matrix takes place only at the direct contact zone with the host. The PA-TCH-SP staining of internal hyphae (Plate 24) results not in a clear distinction between the thick inner chitin layer and the extracellular matrix. Bonfante-Fasolo (1982) has reported a similar PA-TCH-SP reaction of conidia and internal hyphae of VA-mycorrhizae and has postulated that the chitin polymerisation in the fungal cell walls is not yet complete during this colonisation phase.

Plate 22: Post penetration events in the mesophyll of beech leaves (isolate FP7).
A, A subcuticular hyphae crossing the epidermal cell wall and growing further between two palisade parenchyma cells. Arrowheads: places of presumptive high enzymatic activity.
B, Intracellular hyphae in the epidermis cell. The whole cell content is digested and only residues of the cytoplasm (arrow) remains around the hyphal tip. GA-OsO_4-EPON, 15 days after infection of 2 µl drops suspension in water at a concentration of 6 x 10^6 conidia x ml^{-1}. E, epidermis; H, hyphae; P, palisade parenchyma; W, epicuticular wax. Scale bars: 2 µm.

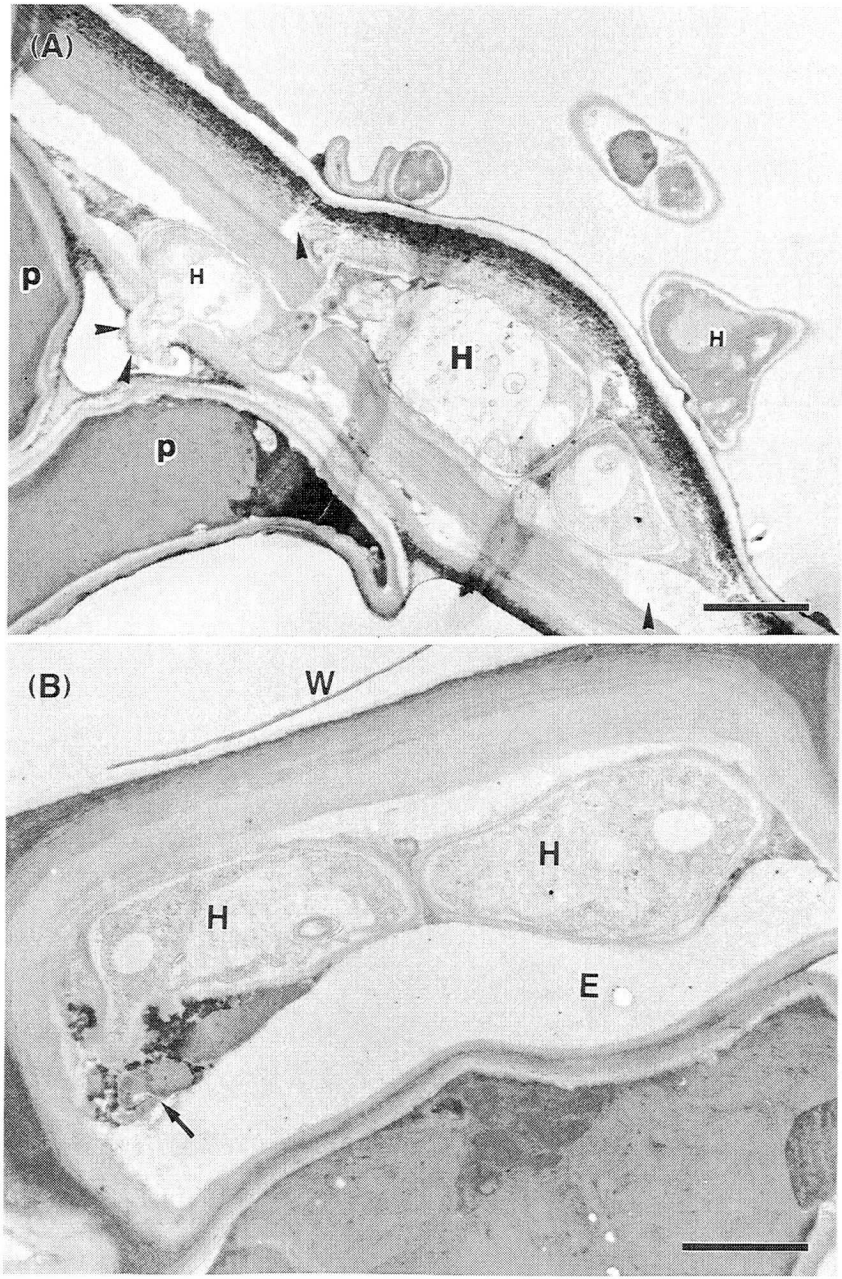

(A)
p
H
H
H
p
(B)
W
H
H
E

Plate 23: Post penetration events observed in the mesophyll of beech leaves (isolate LT215).
A, B, Initial stages of cell wall digestion (arrows).
C, Cell wall penetration.
D, Haustorium formation after cell wall penetration. GA-OsO_4-EPON, 2 µl drop infection with conidia suspended in Hepes (pH 7), at a concentration of 5 x 10^6 conidia x ml^{-1}, 25 days after infection. HC, haustorium mother cell; HA, haustorium. Scale bars: 1 µm.

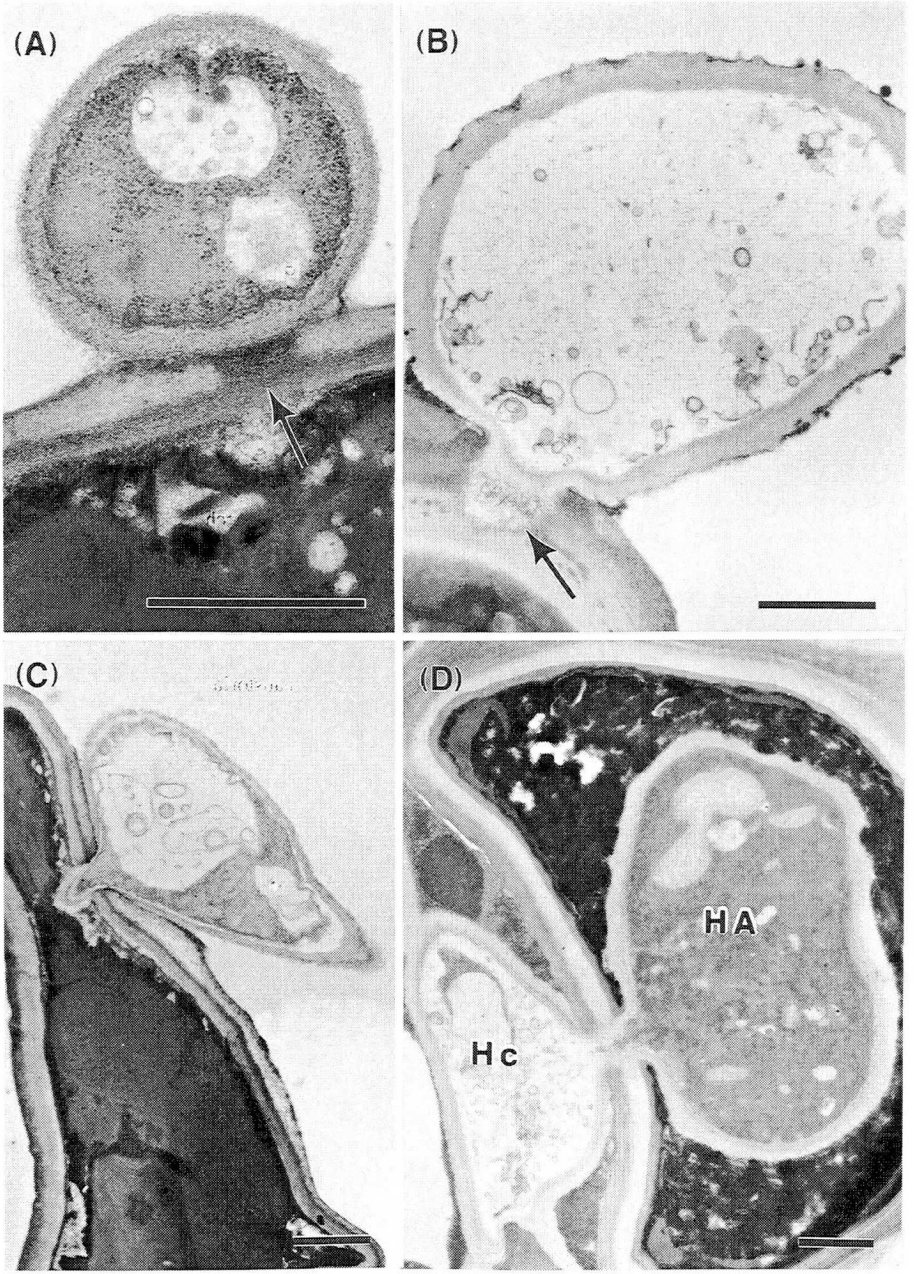
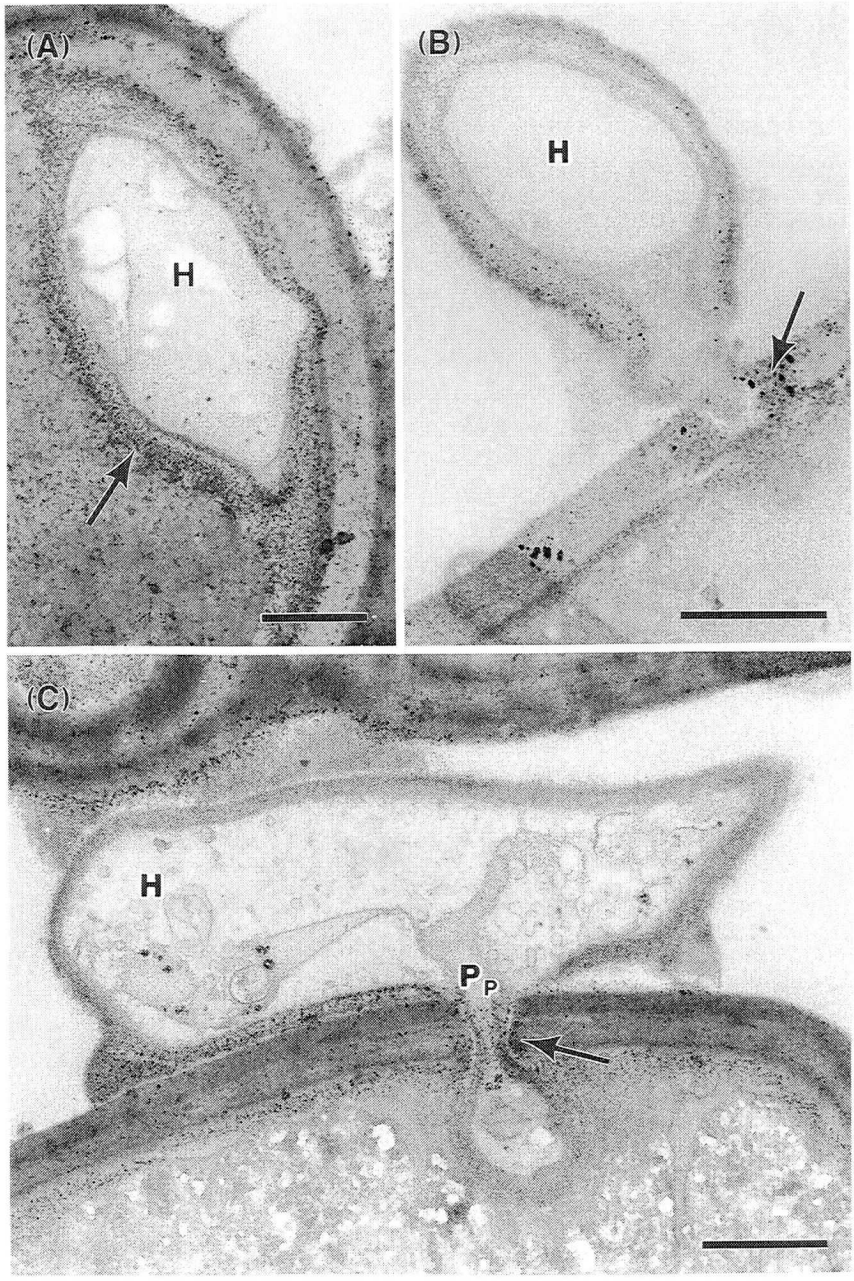

(A)
(B)
(C)
(D)
HA
Hc

Plate 24: PA-TCH-SP staining of infective structures in the mesophyll of beech leaves (isolate LT215).
A, Intracellular hypha. An accumulation of PA-TCH-SP positive material is present around the hyphal wall (arrow), indicating an enzymatic activity of the fungus.
B, **C**, Intercellular hyphae penetrating cells of the spongy parenchyma. An accumulation of polysaccharides can be observed at the point of penetration and around the penetration peg (arrows). GA-OsO_4-EPON, 25 days after infection of 2 µl drops, suspension in Hepes (pH 5) at a concentration of 5 x 10^6 conidia x ml^{-1}. H, hyphae; Pp, penetration peg. Scale bars: 1 µm.

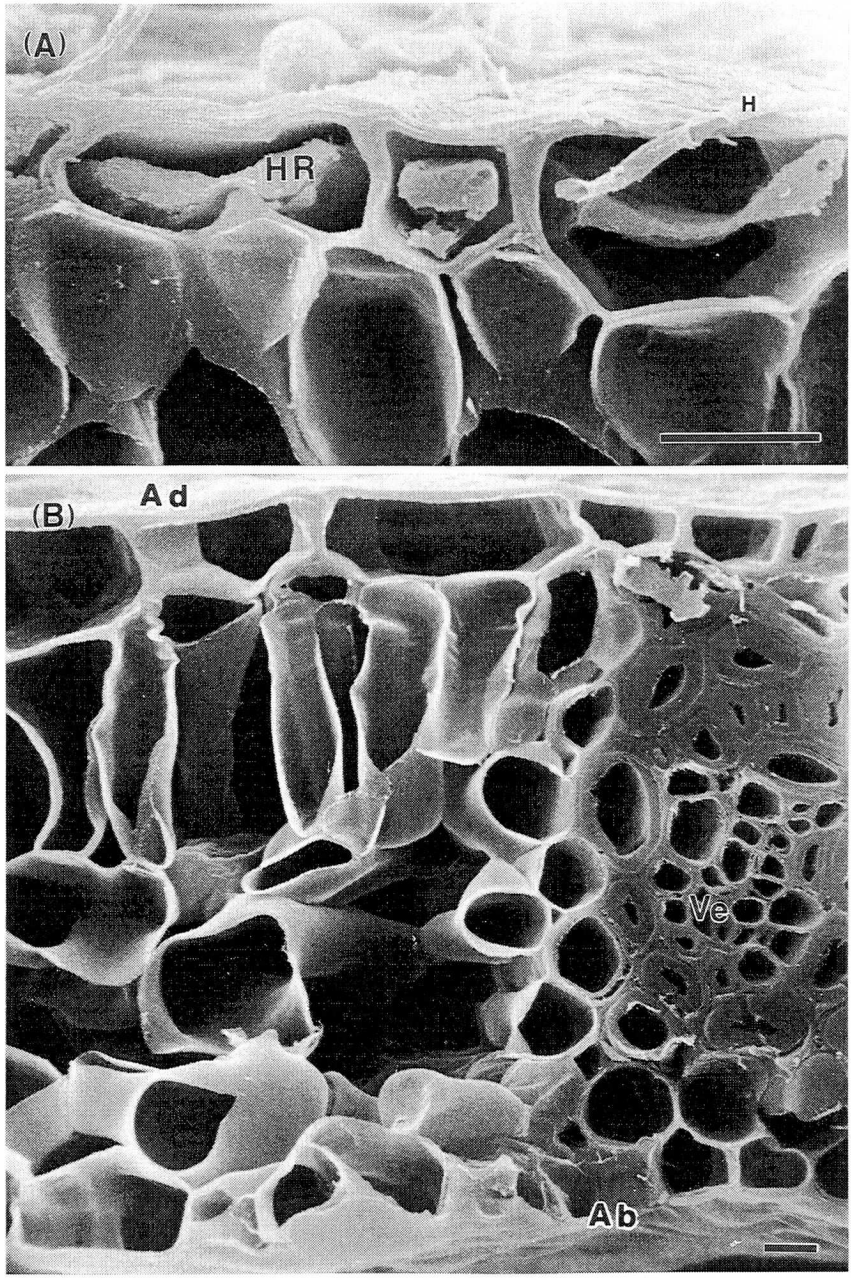

(A)
H
(B)
H
(C)
H
P
P

3.15. Plant reactions

3.15.1. Hypersensitivity (HR)

The accumulation of electron-dense compounds in the epidermal cells suggests a HR of the host and has been mentioned in relation to the reaction of the plant against *D. umbrinella* (Plates 17, 18, Tab. 10). A HR is characterized by the rapid death of the invaded cell, the production of phytoalexins (Mansfield, 1986) and the accumulation of phenolics and lignin (Beardmore et al., 1983), isolating a fungal structure from healthy tissues. HR has been reported for potato leaves infected with *Phytophthora infestans* by Freytag and Hahlbrock (1992), for powdery mildew on cereals (*Erysiphe graminis*) by Aist and Bushnell (1991) and for several other host pathogen interactions (e.g. Koga et al., 1988; 1990). After detergent-protease treatment on hand made sections of infected leaves, the whole cytoplasmic content of the cells is dissolved and only the plant cell wall and the fungal structures are left. An electron-dense cytoplasmic content in the epidermis cells is also visible and must be related to the presence of the fungus in the tissue, since uninfected controls never present a similar structure (Plate 25). The cytoplasmic material seen in infected material is probably phenolic because phenols are not dissolved by proteases. Freytag and Hahlbrock (1992) have also reported an accumulation of phenolic compounds, especially lignin metabolites, that cause a brown coloration of areas of potato leaves infected by *P. infestans*. The cells underneath the hypersensitive sites are still alive (Plates 17, 18), since a normal structural organisation can be observed, even when brown necrotic spots are present at the leaf surface. Only the cells invaded by the endophyte present a desorganisation of the organelles. Aist and Bushnell (1991) have already shown that the hypersensitive reaction is often limited to the cells where the fungal structures are present, but the remaining tissue is intact. Time scale observations of the infection process of *D. umbrinella* on semi-thin sections reveal a host reaction only when the hyphae are already subcuticular.

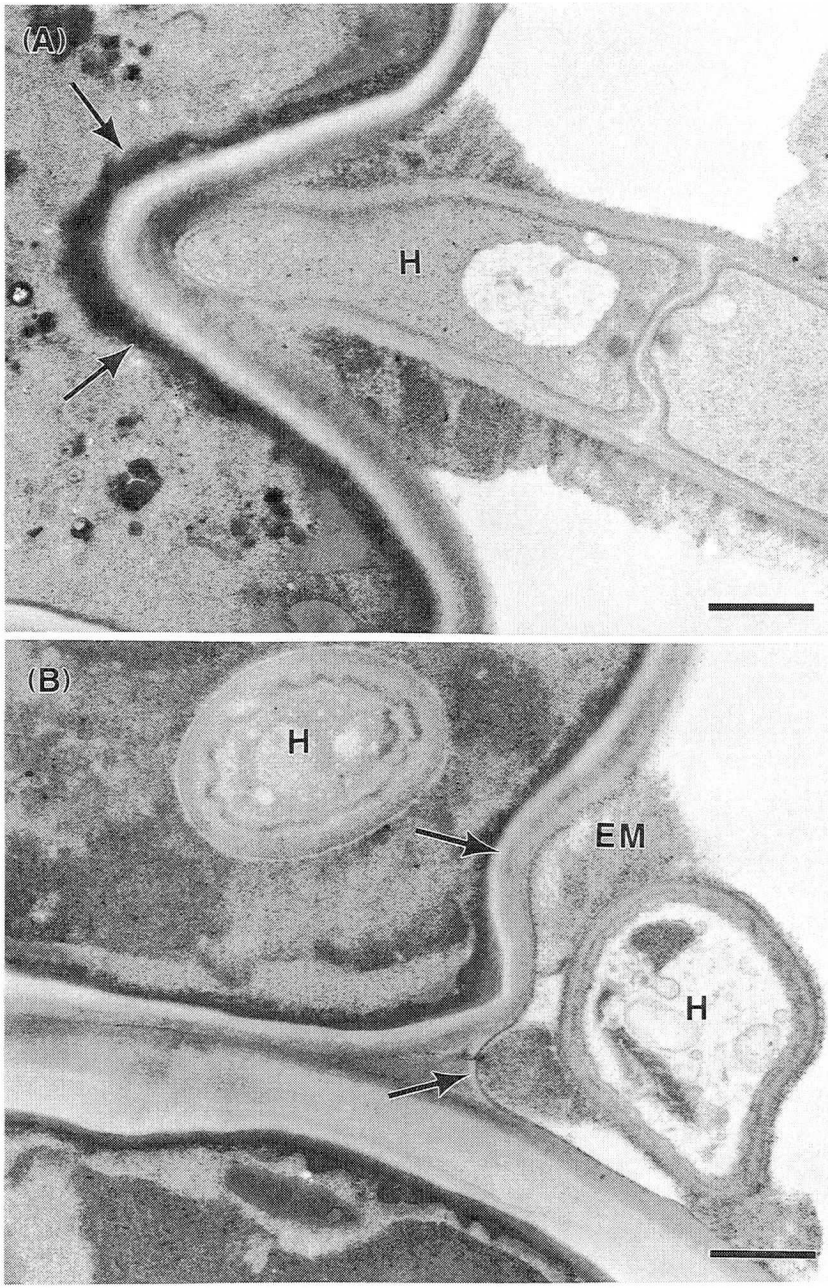
Plate 25: Hypersensitive reaction (isolate FP7).
A, The adaxial epidermis cells contain a compound resistant to protease digestion. The parenchyma cells underneath are still alive and fungus free.
B, Uninfected control. The electron-dense cytoplasmic content of the epidermal cells is not present. SEM-micrographs of detergent-protease treated, hand made sections, OsO_4-CPD, 6 days after infection of 2 µl drops, suspension in water, at 8 x 10^6 conidia x ml^{-1}. Ab, abaxial surface; Ad, adaxial surface; H, hyphae; HR, hypersensitive reaction; Ve, vessel. Scale bars: 4.5 µm.


(A)
H
HR
(B)
Ad
Ve
Ab

3.15.2. Mechanical effect

The enzymatic activities described for *D. umbrinella* (Toti et al. 1991; Toti, 1993) suggest that the infection process may be primarily mediated by the chemical digestion of the host walls. However, as already suggested, during the penetration of the host the involvement of mechanical forces cannot be excluded. Several observations suggest a combined enzymatic activity and mechanical pressure, on the leaf surface (Plates 16, 18) as well as on mesophyll cell walls (Plates 23C, 26). In the mesophyll, cell walls are clearly deformed by growing hyphae (Plate 26). The combined effects of enzymatic activities and mechanical pressure to overcome host surface barriers have been previously reported in several plant pathogens (e.g., Kolattukudy, 1985; Howard et al., 1991).

Plate 26: Mechanical deformation of the host cell wall (isolate LT215).
A, Effect of mechanical pressure on the cells of the palisade parenchyma (arrows).
B, The cell wall deformations are restricted to the extracellular matrix present only at the contact sites with the cell wall, due most probably by sectioning artefacts. Cross sections of infected necrotic leaves, GA-OsO_4-Epon, 25 days after infection, of 2 µl drops, suspension in Hepes (pH 5) at 5 x 10^6 x ml^{-1}. EM, extracellular matrix; H, hyphae. Scale bars: 0.6 µm.


(A)
H
(B)
H
EM
H

4. DISCUSSION

4.1. Methodology

Infection of axenically grown seedlings has several advantages. While under field conditions the same leaf can be colonised by different endophytic species (Sieber and Hugentobler, 1987), on axenic seedlings only the fungus applied is present in the host tissue. Using material collected in the field, antibodies against the endophyte are necessary to recognise specifically the fungus in the host tissue (Suske and Acker, 1989). The whole infection process, however, can be followed in detail only *in vitro* under controlled conditions. The *in vitro* model used in this study allows a reproducible infection of the host tissue, otherwise impossible under field conditions. Necroses are of great importance to localise the endophyte in the leaf at the ultrastructural level. In fact, as long as symptoms are not present, very few hyphae can be detected in the tissue, because of the low probability of sectioning symptomless leaves exactly where the endophyte lies.

The light microscopical investigations carried out in this study have provided only limited information. The hyaline conidia of *D. umbrinella* are visualised only after staining and further, the relatively small dimensions of the fungal structures require high microscopic resolution. The light microscope can be used only to visualise some specific fungal compounds or host reactions, as evidenced by the PAS-staining.

The information obtained with conventional SEM is clearly of inferior quality than that obtained with LTSEM. However, a confirmation at the TEM level is always necessary for a conclusive interpretation of the results, especially to explain the penetration of the endophyte into the host. Cryofixation is the best method to study fungal ultrastructure but is only partly useful for investigations of plant material. The rapid immobilisation of material by high pressure freezing (micro- to milliseconds) allows a better control of modifications in the tissue than aldehyde fixation (Michel et al., 1991). As a consequence the structures are better preserved. The only limiting factor is the formation of ice crystals by cellular water, which occurs when water is not frozen in the amorphous state (vitrification); this prevents the preservation of the liquid phase characters (Dubochet et al., 1982).

In contrast to cryofixation, the conventional chemical fixation causes uncontrollable ultrastructural modifications because the chemicals applied act on the membranes, which loose their selective permeability. The penetration rates of the fixatives are tissue-specific

and are particularly slow in plant and fungal cell walls (Mersey and McCully, 1978), because of their thickness compared to animal cells that have no cell wall. During the relatively slow process of penetration several modifications, results of defence mechanisms, occur, inducing artefacts especially at the molecular level (Zierold, 1982).

4.2. Pathogenicity

The labour-intensive preparation of material needed for electron microscopy permits only few repetitions of the same experiment. However, all experiments have been reproducible throughout the present work. This study demonstrates that an otherwise symptomless endophyte can become pathogenic. It is therefore difficult to restrict the term endophyte to mutualistic symptomless fungi as it has been proposed by Carroll (1986). The definition proposed by Petrini (1991) to include also latent pathogens, which at some time in their life can inhabit plant tissue without causing apparent symptoms, seems to be more useful.

Symptomatic expression of endophytes has never been described on axenically infected plant material *in vitro*. *D. umbrinella* can cause sporadic beech and oak leaf anthracnoses. The formation of necroses that may be related to the presence of endophytes has been reported in other examples. Redlin (1991) has characterised *Discula destructiva* as the causal agent of dogwood anthracnose and Wilson (1992) has observed the formation of disease symptoms on oak leaves infected with *D. quercina*. The clavicipitaceous grass endophytes can also present a weak pathogenicity since Clay (1988) has described the presence of stromata, the reproductive organs of the fungus, on inflorescences or leaves of infected grasses, which inhibit seed production. Symptomless infections have been reported for many plant pathogens (Petrini et al., 1989; Davis and Fitt, 1990; Sinclair, 1991). For example *Stagonospora nodorum* is the most common endophyte isolated from symptomless wheat leaves (Sieber et al., 1988) and is also known to be an important pathogen of *Triticum* spp. In addition, some endophytes isolated from trees are taxonomically closely related to pathogens of the same host. For example, in Douglas fir *Rhabdocline parkeri* is closely related to two other virulent pathogens, *R. weirii* and *R. pseudotsugae* (Carroll, 1986) and in pine the endophytic species *Lophodermium pinastri* and *L. conigenum* belong to the same genus as the pathogen *L. seditiosum* (Minter et al., 1978). Therefore, the appearance of symptoms, caused by *D. umbrinella in vitro*, opens several questions about the significance of this endophyte, considered by Sieber and Hugentobler (1987) as symptomless. Similar observations have been made recently by Wilson (1992) for *D.*

quercina. A controlled, well balanced interaction between both symbionts is probably the case in the forest ecosystem; a pathogenic reaction will set in only when the host is stressed.

The infection conditions modify considerably the effects of the fungus on the leaf. A high conidial concentration or a buffered suspension medium at pH 5 provides the most favourable conditions for the infection process, Toti et al. (1992b) have demonstrated a maximal attachment of conidia to the host surface at pH 5. In other words, a correlation between adhesion and infection can be assumed. In addition, the different developmental forms, particularly the differentiation of appressoria and halos are found mostly at pH 5.

D. umbrinella demonstrates a moderate specificity, but a high host selectivity at the family level, a phenomenon already described in endophytes (Petrini, 1986; Petrini et al., 1987). For instance, *Coleophoma empetri* (Rostr.) Petrak and *Phyllosticta pyrolae* have been recorded on hosts belonging to the Ericaceae and Pyrolaceae, two closely related families of the subclass Ericales (Petrini, 1986). Carroll and Petrini (1983) have demonstrated that strains of the same fungus isolated from different host parts differ in their ability to digest their substrates, suggesting even a tissue specificity for endophytes at the strain level. Host specificity has been also reported for grass endophytes (e.g., Leuchtmann and Clay, 1990; 1992), for many plant pathogenic fungi (e.g., Bourett and Howard, 1990; Allen et al., 1991b) and some mycorrhizal associations (e.g., Duddridge, 1986). Toti et al. (1992a) using morphometric measurements, and Hämmerli et al. (1992) using RAPD markers on *D. umbrinella* isolated from beech, chestnut and oak have differentiated groups that correspond strongly with their host origin. Taken together these results confirm a strong adaptation of the fungus at the species level and suggest that subtle host recognition mechanisms may underlie a successful establishment of the interaction.

4.3. Ultrastructure of *Discula umbrinella*

The structure of the extracellular matrix is apparently dependent on the host origin of the isolate and could be related to the recognition of the host by a fungus. A comparable cell wall ultrastructure has been described for the ericoid endophyte isolated from *Erica vagans* by Duclos et al. (1983) and by TonThat and Epstein (1991) for the conidia of *Nectria haematococca*. In *N. haematococca* the fibrillar, extracellular matrix has been observed only after a 5 h incubation of the spores in zucchini fruit extracts prior to germ

tube emergence and absent from two adhesion-reduced mutants. This indicates that adhesiveness is correlated with the appearance of new cell wall layers and that conidia undergo morphological changes prior to germination.

The presence of polysaccharides in the conidial cell wall of *D. umbrinella,* evidenced by the PAS-, and by the PA-TCH-SP-reaction is consistent with the fungal cell wall composition discussed by Ruiz-Herrera (1992). This author has mentioned that polysaccharides represent about 80% of the cell wall dry weight and that other compounds are proteins (3-20%), lipids, pigments, and inorganic salts. A differential distribution of polysaccharides in the conidial cell wall layers similar to that demonstrated by the PA-TCH-SP-reaction in *D. umbrinella* has been also reported in endomycorrhizae, where a differential glycoconjugates distribution has been demonstrated in the hyphal cell wall (Bonfante-Fasolo and Perotto, 1986; Gianinazzi-Pearson et al., 1986; Gianinazzi and Gianinazzi-Pearson, 1992; Bonfante-Fasolo and Spanu, 1992). The presence of an extracellular mucilaginous sheath containing polysaccharides has also been described for bacteria (Dazzo, 1984) and pathogenic fungi (Ruel and Joseleau, 1991; Hamer et al., 1988).

Lectins and their specific haptens or lectin-like interactions, related to extracellular fungal matrix are frequently assumed to be recognition determinants in microorganism-plant interactions (Hohl and Balsiger, 1986; Manocha and Chen, 1990; 1991; Nordbring-Hertz, 1988; Gubler and Hardham, 1988). In *D. umbrinella* the fibrils seem to contain mannose, glucose or their conjugates, since a strong fluorescence can be obtained with ConA-TRITC. The ConA-gold labelling, however, demonstrates that these sugars are not present in large amounts in the extracellular matrix. A more consistent labelling pattern is seen on conidia incubated for 1 h in water. On fresh conidia, electrically charged glycoproteins present in the extracellular matrix may interact positively or negatively with labelled lectins. After incubation in water, these reactions are partly weakened, thus resulting in a stronger labelling. Hamer et al. (1988), have applied colloidal gold ConA to the conidia of *M. grisea,* and have found a consistent binding pattern at the apex which correlates with adhesion to the support. The extracellular fungal matrix of mycorrhizae, rich in mannose binding sites, has been even reported as a prerequisite for a successful invasion of the roots (Bonfante-Fasolo, 1988). Contrarily to the ConA labelling pattern, the strong WGA-fluorescence obtained only after incubation of the conidia in different enzymes and NaOH suggest that chitin is internally located and covered by alkali-soluble manno- or glycoproteins, as in most Eumycota (Gooday and Trinci, 1981). This hypothesis is confirmed by the WGA-gold labelling pattern and consistent with the localisation of chitin, hidden by non-chitinous cell wall compounds found in endomycorrhiza (Bonfante-Fasolo et al., 1990).

The variation in ultrastructure of the conidial sheath of *D. umbrinella* isolated from oak, chestnut and beech leaves, may be one of the factors that explains host-specificity. The formation of host specific physiological strains has been postulated by Petrini et al. (1982) and reported for endophytes by Sieber et al. (1991), Sieber-Canavesi et al. (1991). Leuchtmann et al. (1992) have reached similar conclusions with different species of *Phyllosticta*. So far however, no ultrastructural investigations have been carried out to fully explain this phenomenon, probably because the extracellular matrix is completely dissolved after conventional chemical fixation and no differences can be observed. The different conidial ultrastructure derived from different hosts also opens some questions on the taxonomy of the fungus. Although only small differences in morphology (Toti et al., 1992a), biochemistry, physiology (Toti, 1993) and ultrastructure can be seen among isolates from different hosts, the combination of all characters would suggest that the establishment of sibling species may be justified and therefore that electron microscopic investigations, using cryofixation, can provide new taxonomic information.

4.4. Infection

The life cycle of *D. umbrinella* has been described by Morelet (1989), based on field observations and certain steps are still doubtful. So far no investigations have demonstrated whether the fungus penetrates into the leaf tissue or colonises systemically the leaves from infected wood pieces close to the buds. Butin (1989) has reported that beech anthracnose can be observed only at intervals of several years, although *D. umbrinella* can be regularly isolated from symptomless leaves. Clavicipitaceous grass endophytes, the best studied host-endophyte system, are mainly seed-borne (Clay, 1988). Since *D. umbrinella* has been regularly isolated from beech seeds (Toti, 1993), seed-borne transmission cannot be excluded, although *D. umbrinella* has never been isolated from leaves of seedlings growing from infected seeds. Wilson (1992) reports the same observation from acorns infected by *D. quercina*, indicating that a systemic seed-borne infection of the leaves is less probable than a direct penetration by the conidia. However, we could isolate *D. umbrinella* from the bud scales and the twig pieces contiguous to the buds but never from the rolled up leaves enclosed by the scales (Toti et al., 1993). This suggests that hyphae could grow into the leaves from the twigs and scales when the climatic and ecological conditions are favourable. Nevertheless, infection seems to be principally mediated by the anamorph. In fact, Hämmerli et al. (1992), using RAPD markers could detect up to four different individuals of *D. umbrinella* within the same leaf and at the same time a remarkable homogeneity among beech isolates.

Observations by Stone (1987; 1988) on *Rhabdocline parkeri* and by Wilson (1992) on *D. quercina* suggest a transmission of the spores by rainfall. The importance of rain has so far not been explored for the infection by *D. umbrinella*, but water and air are probably the determinant transmission factors in *D. umbrinella*, since grazing insects, such as *Rhynchenus fagi*, seem to play no role (Toti, 1993).

Attachment of spores to the host surface has been demonstrated for a number of plant pathogenic, hyperparasitic and mutualistic fungi (e.g., Duddridge, 1986; Hamer et al., 1988; Manocha and Chen, 1990). The conidia of *D. umbrinella* adhere to the host surface with an increase of attachment over 16 h (Toti et al., 1992b). Germination is not a prerequisite for the attachment to the host surface, since the adhesion can be observed before conidia start to germinate, about two hours after inoculation. Thus conidia must contain preformed adhesive compounds before contact with the host has been established. In *Nectria haematococca* TonThat and Epstein (1991) have reported that adhesiveness is correlated with the appearance of new conidial wall layers, induced by incubation in extracts of the host. In *D. umbrinella* cell wall modifications, induced by host extracts, which would improve the adhesion have not been ultrastructurally assessed. However, the increase of binding until 16 h after infection suggests that the adhesion may be improved by germination of conidia or a host-mediated activation mechanism may be involved. In addition, a selective adhesion of conidia to the host as compared to non-hosts has been observed. This result suggests the involvement of an active recognition mechanism, specific to the beech leaf, since only a small number of conidia are able to bind to artificial surfaces, such as Teflon coated slides (Toti et al., 1992b). Adhesion and penetration into the host are certainly strongly related, since the binding increases slightly during the first 24 h after infection. Taken together these results suggest that the attachment is a prerequisite for the colonisation of the host by *D. umbrinella*, as it has been reported for plant pathogens (Nicholson and Epstein, 1991).
Enzymatic treatments of the conidia of *D. umbrinella* inhibit completely the adhesion to the host surface. Since proteinase K and protease E dissolve the extacellular matrix, the fibrils must be responsible, at least partly, for the adhesion. Extracellular mucilage related to adhesion mechanisms has also been found in the rust *Gymnosporangium juniperi-virginianae* (Mims and Richardson, 1989). Helicase, which has a strong chitinase and glucanase activity does not dissolve the fibrils, but the binding to the host surface is suppressed. In other words, not only proteins but also glucans and/or chitin may play a role in the adhesion process. The proteins present in the conidial cell wall of *D. umbrinella* are probably glycoproteins or mannoproteins as reported by Barkai-Golan and Sharon (1978) for different yeasts, using FITC-labelled lectins. Glycoproteins are

also the major compounds of the extracellular mucilage in endomycorrhiza (Bonfante-Fasolo and Perotto, 1986).

The extracellular mucilage may not only function as adhesive; it could also contain some enzymatically active proteins such as cutinases or pectinases (Kolattukudy, 1985). Nicholson and Epstein (1991) have demonstrated an esterase activity in the extracellular sheath of the conidia of *Erysiphe graminis* which may degrade leaf surface waxes. Kolattukudy and Soliday (1985) have reported a similar enzymatic activity in the mucilage of *Colletotrichum graminicola.* Esterase, pectinase and cellulase activity, reported for different isolates of *D. umbrinella* (Toti et al., 1991; Toti, 1993), are probably present in the extracellular sheath or may be activated by the host. Hypothetically, pectinases or esterases present in the extracellular matrix could act as primary signals which are sensed in the leaf by receptors, such as cutin or pectin. The presence of a signal from the host is consistent with the induction of germination by beech and chestnut leaf extracts. However, since these leaf extracts contain all the cell compounds of the plant tissue, the level of specificity is not clear and fractioning of the extracts is necessary to detect the inductors. Purnell (1971) has found sugars, amino acids, proteins, salts, and phenolics to be fungal stimulants in the leachates of several leaf surfaces. Chapela et al. (1991) have demonstrated the presence of specific recognition mechanisms mediated by the plant. These authors have extracted monolignol glucosides from the beech bark and shown that they are specific recognition messengers for the endophyte *Hypoxylon fragiforme.* In addition, they have observed the highest germination rates on beech leaf squares compared to 16 other plant surfaces. In *D. umbrinella* the penetrating forms with an appressorium or the formation of a halo, present only on the host surface, compared to artificial supports give further evidence for the production of an induction signal by the host.

As demonstrated by TEM, the halo observed at the end of the germ tube corresponds to a subcuticular penetration into the host. The halo, defined by Aist (1983) as a locally modified region of the host cell wall around a penetration side and described by Webster (1986) to be an enzymatic softening of the cuticle, is probably indicative of the subcuticular presence of *D. umbrinella.* On the other hand, the differentiation of an appressorium at the end of the gem tube, a common feature in plant pathogens, was demonstrated in ultra-thin sections to allow direct penetration into the host. In TEM, cross-sections through appressoria and sub-cuticular hyphae point to an enzymatic decomposition of the cuticle and epidermis cell wall rather than to the involvement of mechanical forces. In plant pathogens both mechanical and enzymatic activities are known (Kolattukudy, 1985; Howard et al., 1991). In *D. umbrinella* a mechanical pressure during penetration cannot be excluded, but is difficult to demonstrate conclusively, since the cell wall deformation observed could also be a consequence of a

general loss of turgescence during the infection process. There is good evidence that the cuticle represents only a partial barrier, which can be overcome by *D. umbrinella* without induction of a reaction mechanism by the host at the moment of penetration. Similar observations have been made for *Venturia inaequalis* on apple leaves (Valsangiacomo and Gessler, 1988). The advantages provided by two different penetration strategies in these fungi is unclear. In *D. umbrinella,* factors inducing the formation of an appressorium, rather than direct subcuticular penetration, are unknown. Contact stimuli, based on the physical topography of the leaf surface have been reported by Hoch et al. (1987) for rusts as a determinant factor for the formation of appressoria and may also play a role in *D. umbrinella,* although no evidence for a thigmosensitive reaction could be observed. LTSEM observations demonstrate that *D. umbrinella* conidia germinating close to a stoma trigger a local closing of the stomatal opening by the cuticular layer of the guard cell lips.

Only few cytological investigations have been carried out on asymptomatic fungal colonisation of plant tissues. Electron microscopical studies have been performed on conifer needles (Stone, 1987; 1988; Suske and Acker, 1989) and on the grass endophyte *Acremonium coenophialum* (Hinton and Bacon, 1985) but never on broad-leaved trees and under *in vitro* experimental conditions. Stone (1986) has shown a direct penetration by the endophyte *R. parkeri* into Douglas fir needles after the formation of an appressorium, indicating that endophytes are able to colonise their host in ways similar to those used by pathogens. The Douglas fir needles (Stone, 1986) and the beech leaves show an electron-dense cytoplasm in the cells invaded by the endophyte, or adjacent to invaded cells. In the beech leaves this cell content could not be dissolved by protease treatment. It can therefore be assumed that phenols, such as lignin-like compounds reported for pathogens to be related with hypersensitive defence reactions (e.g., Freytag and Hahlbrock, 1992), are present in such cells. The hypersensitive reaction may release toxic factors from the dying cells (Aist and Bushnell, 1991) and provides a physical barrier against fungal invasion. A response mechanism of the host can be at least postulated also in the case of *D. umbrinella / F. sylvatica* symbiosis, since the hyphae grow parallel to the colonised epidermis cells or to the cuticular membrane. This infection phase can be observed between 24 h and the onset of the first necroses. Thus the hypersensitive reaction limits the colonisation of the mesophyll by the fungus which remains latent in the superficial layers of the tissue. The development of disease symptoms under the experimental setup used in this study is understandable since the leaves are stressed. These results point out that under field conditions only a few number of conidia may successfully penetrate the host. Since in the mesophyll hyphae are mainly located in the very large intercellular spaces with little or no interaction with the plant, the host and the endophyte may exist in a mutualistic or neutral, well balanced

association until leaf fall. The extremely long latency of several years reported by Stone (1987; 1988) for *R. parkeri*, during which the hyphae are confined to the intracellular spaces of the host epidermis is obviously reduced to the summer months in *D. umbrinella*. This would explain the absence of symptoms as long as the leaves are not stressed or are still attached, even if the endophyte can be regularly isolated (Sieber and Hugentobler, 1987). An epidemic development will then take place only when the climatic and ecological conditions are particularly favourable to the fungus.

This first study under *in vitro* conditions demonstrates that the endophyte *D. umbrinella* may also be considered a latent pathogen of beech which causes disease symptoms when the host is stressed. The colonisation of the beech leaf by *D. umbrinella,* is, in general terms, not different from that reported previously for coniferous endophytes (Stone, 1986). The mutualistic symbiosis described for grass endophytes (Bacon and De Battista, 1991; Clay, 1991a; 1991b) or for endophytes of woody plants (Caroll, 1986; 1988; 1991) are always related with the positive interaction with predators of the same host. I could not see any positive effects by the endophyte in the colonised beech leaf. A neutral or mutualistic interaction, however, cannot be excluded, since the endophyte is mainly localised in the large intercellular spaces of the beech tissue and does not cause any symptoms as long as the host is not stressed. Therefore, fungal activities directed against a third organism, e.g., grazing insects or pathogens, could be beneficial to the host.

4.5. Outlook

The recognition mechanisms suspected to be responsible for the infection of the host represent an aspect which has been only briefly approached in this study, despite its importance in plant-fungi interactions. Since several promising results have been obtained using specific lectin-labelling, further basic mechanisms could be studied on beech leaf protoplasts. To better understand the adhesion process, light microscopical observations of carbohydrate-coated agarose beads on protoplasted beech cells as well as fluorochrome-labelled lectins could demonstrate the presence or absence of lectins or polysaccharides in beech leaves. Hohl and Balsiger (1988) and Guggenbühl (1991) have successfully studied cell-cell interactions on soybean protoplasts infected with *Phytophtora megasperma* f.sp. *glycinea*. Moreover, staining procedures specific for host reactions could enhance the presence of phenolic compounds and confirm the hypersensitive reaction observed in TEM-micrographs. Fluorescence reactions, such as

anilin blue for callose or toluidine blue for lignin (Kovats, 1990) could be particularly advantageous.

The specific recognition of the host is another open field of research which could be successfully studied. Incubation in beech leaf extracts and subsequently in fractions of the extract could show eventual cell wall modifications at the TEM level, as reported by TonThat and Epstein (1991) in *Nectria haematococca.* Such a result, completed with analysis of non-adhesive mutants, would help to explain the mechanisms behind specific adhesion to the host.

During the first infection steps, thigmotropism has also to be considered, because it could be responsible for the formation of appressoria in *D. umbrinella.* LTSEM and TEM of conidia growing on artificial replicas of leaf structures, as reported by Wynn and Staples (1981) could provide evidence for the involvement of eventual thigmosensitive recognition. For the confirmation of the presence, and location, of enzymes in the conidial extracellular matrix, gold-labelled, previously purified or available enzymes such as pectinases, cutinases or esterases should be applied. Interesting results have been obtained with this method by Ruel and Joseleau (1991) with fungal wood decay. Gold-labelled antibodies raised against enzymes could also be used to demonstrate conclusively that penetration of the host is enzymatic.

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