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## Early stage development of IS-group isolates of *Heterobasidion annosum* on *Abies alba* roots – scanning electron microscopical studies

Received: 19 May 2005, Accepted: 04 November 2005

**Abstract:** The growth of hyphae and prepenetration phenomena on *Abies alba* roots after inoculation with the P-, S- and F-group isolates of *Heterobasidion annosum* were observed using scanning electron microscope. Elongated hyphae emanating from the inocula grew indiscriminately across and along root tips and entered the cortical cells randomly, while in subapical root areas they quite often grew along grooves at points of cell junctions. The ridges, folds and depressions seen on the root surface obviously directed the hyphal growth and their further entrance into roots through natural crevices or cracks. Although hyphae of all the isolates could penetrate the roots directly through small openings, the hyphae of the F isolate penetrated preferably more eroded and older parts of roots. A peculiar habit of the direct penetration, characterized by formation a structure resembling infection peg, was observed only after inoculation with the P and S isolates. It is suggested that specifically frequent penetration the roots in eroded areas by the hyphae of the F isolate may be one of the factors connecting with its lower pathogenic capabilities.

**Additional key words:** adhesion, behaviour of hyphae, prepenetration phenomena, root surface.

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### Introduction

In Europe, *Heterobasidion annosum* (Fr.) Bref. consists of three intersterility (IS) groups showing preferential specialization to host trees (Korhonen et al. 1998). In central Europe, the F isolates cause no damage in *Abies alba* Mill. stands and are found on stumps, logs and sporadically on old dead trees (Łakomy et al. 2000). Also in Slovenia the F isolates showed rather saprobic capabilities (Munda 1994). By contrast, they

are pathogenic to *A. alba* in Italy (Capretti et al. 1990) and to *A. cephalonica* Loud. and *A. borisii-regis* Mattf. in Greece (Tsopelas and Korhonen 1996). Isolates of this group can also inhabit other coniferous trees, including spruces and pines (Korhonen et al. 1998).

In field inoculation experiments, the F isolates showed similar virulence to S isolates on pine and significantly lower virulence than S and P isolates on spruce. Similar virulence of the three IS groups on fir suggests a lower preference of the F group to its main

host than in the case of the other IS groups (Werner and Łakomy 2002 a).

*Heterobasidion annosum*, although recognized as specialized pathogen of woody roots and stems, it is capable of parasitizing on conifer roots of all ages (Werner 1990, 1993, Asiegbu et al. 1998). Hypersensitive reaction (HR) and subsequent death of infected tissues result in the saprobic colonization of roots. In the studied pathosystem, the effectiveness of HR seems to have no effect on the penetration and colonization of the pine cortex by the P (Werner and Idzikowska 2001) and the S isolates (Werner – unpublished). Nevertheless, results of the *in vitro* inoculation experiments on pine and spruce seedlings with isolates of the three IS groups provided evidence for the occurrence of host preference in *H. annosum* complex, similar to that observed in nature (Werner and Łakomy 2002b).

There is no information in the literature concerning physiological aspects of the host preference of the F group to fir. Scanning electron microscope (SEM) observations by Werner et al. (2005) on the colonization of Scots pine roots inoculated with isolates of the three IS groups of *H. annosum* showed the ability to penetrate the roots variously by hyphae of all the isolates. Direct penetration of spruce roots and formation of appressoria preceding the penetration by hyphae of S isolate was also observed by Asiegbu et al. (1993). Nevertheless, a higher ability to enter an intact pine roots by hyphae of P isolate in contrast to preferential entering eroded roots by hyphae of F isolate allows to relate the differences with various host preference.

In many biotrophic fungi, the principal factors determining the hyphal behaviour are physical and chemical stimuli derived from the host. These are involved in the expression of virulence and host-plant specificity (Dean 1997; Tucker and Talbot 2001). The knowledge, however, about the relationship of these signals with the host preference in *H. annosum* complex only begin to emerge (Asiegbu 2000). Analogically to biotrophs, if roots of conifers released a variety of chemical and/or topographical signals, the fungi varying in the host preference would respond to these signals variously.

The purpose of this study was to document the prepenetration phenomena on the roots of *A. alba* seedlings after inoculation with the P, S and F isolates of *H. annosum* and to find out whether the low host preference of F isolates to fir observed in nature could manifest itself at the initial stage of host-pathogen interaction.

## Materials and methods

### Plant and fungi

*Abies alba* originated from Śnieżka Forest District, 50°55'N and 15°46'E; altitude – 400–600 m). *Heterobasidion annosum* was represented by three strains, one each of the P-, S-, and F-IS groups. The P (95107), S (96083) and F (96067) strains originated from Polish stands and were isolated during 1995–1996 year from dead trees of Scots pine, Norway spruce and common fir, respectively. The strains were assigned to the IS groups according to their ability to heterokaryotize homokaryotic tester mycelia (Korhonen 1978). Each isolate was paired with several homokaryotic tester strains as was described by Werner and Łakomy (2002 b). The fungi were grown on malt extract agar (Difco) at 24°C in the dark.

### Growth conditions and inoculation procedure

Fir seeds were germinated on perlite and peat (3:1 v/v) at 24°C. Two-month-old seedlings were removed from the substrate. Root systems of the seedlings after cleaning in distilled water were surface sterilized with 70% ethyl alcohol and additionally in 2% HgCl<sub>2</sub> (about 10–15 second in each) and washed in sterile distilled water (3 × 15 min). Then, the seedlings were inoculated with several discs (5 mm in diameter) of a 2-week-old mycelial mat of the fungi in a modified vertical Petri dish system described by Unestam and Stenström (1989) in a growth room under fluorescent tubes (Osram L36/W77 Flora) (100 μEm<sup>-2</sup>s<sup>-1</sup>) light 18 hours a day, 70% RH at 24:20°C day:night temperatures. Ten plants were inoculated with each of the isolate. Uninoculated seedlings grown in the same conditions served as control. The experiment was repeated twice.

### Root preparation for scanning electron microscopy (SEM)

Small pieces (3–5 mm) of apical and subapical parts of roots were collected after 12 and 24 hours and 3, 7, and 14 days after inoculation. Each sample consisted of 10 roots inoculated either with P, S or F isolate. A total of 150 roots were studied by SEM. The samples were fixed in 2.5% glutaraldehyde in 0.5 M cacodylate buffer at pH 7.2 for 24 hours and postfixed in 2% OsO<sub>4</sub> in 0.1 M cacodylate buffer for 2 hours at 4°C. The specimens were then washed in distilled water, dehydrated in a series of ethanol (10% steps 15 min each), and critical point dried in a Balzers CPD-030 unit. Afterwards, they were coated with gold (12–15 nm thick) using a Balzers SPD-050 sputter coater and observed in a Philips 515 scanning electron microscope at 15 keV.

## Results

In apical and proximal parts of main and first-order lateral roots, the cortical cells were oriented longitudinally (Figs 1 and 2). The protuberances of the root surfaces, conditioned by the shape and arrangement of the cells, flattened with the age of roots (Fig. 2).

During the first hours after inoculation, the hyphae emanating from the inocula grew indiscriminately across or along root apices and adhered to the root surfaces (Fig. 3). Some of them began to branch out dichotomously forming a loose mycelium (Fig. 4). In proximal parts of roots the hyphae grew preferably along in grooves between the cortical cells (Fig. 5).

The entrance of the fungi into the outer cortical cells was mostly observed within three days after inoculation (Fig. 6). In the root patches less colonized by mycelia, the individual points of penetration were also seen a week later. Through all the time some germinated conidia and elongated germ tubes were occasionally found on the root surfaces. The behaviour of the germ tubes did not vary from that of the hyphae emanating from mycelia (Fig. 4).

Three penetration routes of roots were observed.

In the first, called "direct", slight swellings or narrowings of the hyphal tips at contact with the root surfaces were observed (Fig. 3). The adhesion and further disappearing of hyphae in the roots are indicative of the cell wall perforations limited to small openings corresponding with the diameter of the penetrating hyphae. The formation of a structure resembling infection peg was observed occasionally only after inoculation with the P and S isolates. A hypha running parallelly to the root surface changed the direction of its growth to upright just over the point of penetration. The angle between the hypha and the 'upright' hyphal tip was seldom less than 90°. The penetration was preceded either by an extensive erosion of cell wall around the hyphal tip (Fig. 7), or the entrance into the root was accomplished through a crack in the root surface (not shown). In older parts of roots, the penetration was mostly achieved through holes in a weakened or eroded walls of the cortical cells. This route of root penetration was most frequently observed after inoculation with F isolate (Fig. 8). In some roots inoculated with S isolate, the hyphae were immersed in an abundant mucilage and their tips disappeared in it (Fig. 9).

The ridges, folds and depressions seen on the root surface obviously directed the hyphal growth or even influenced the ramification of hyphae and their further entrance into root through natural crevices or cracks. Hyphae growing along cortical cells often branched out at right angle. The new branches could penetrate the root directly when crossing the cortical cells (Fig. 10), however, most of them entered the roots *via* crevices. In the case of repeatedly ramified

hyphae, some branches grew toward the crevices and entered them side by side, while the others after having crossed out the cortical cell continued their growth along the depressions at the cortical cell junctions and disappeared in them (Fig. 11).

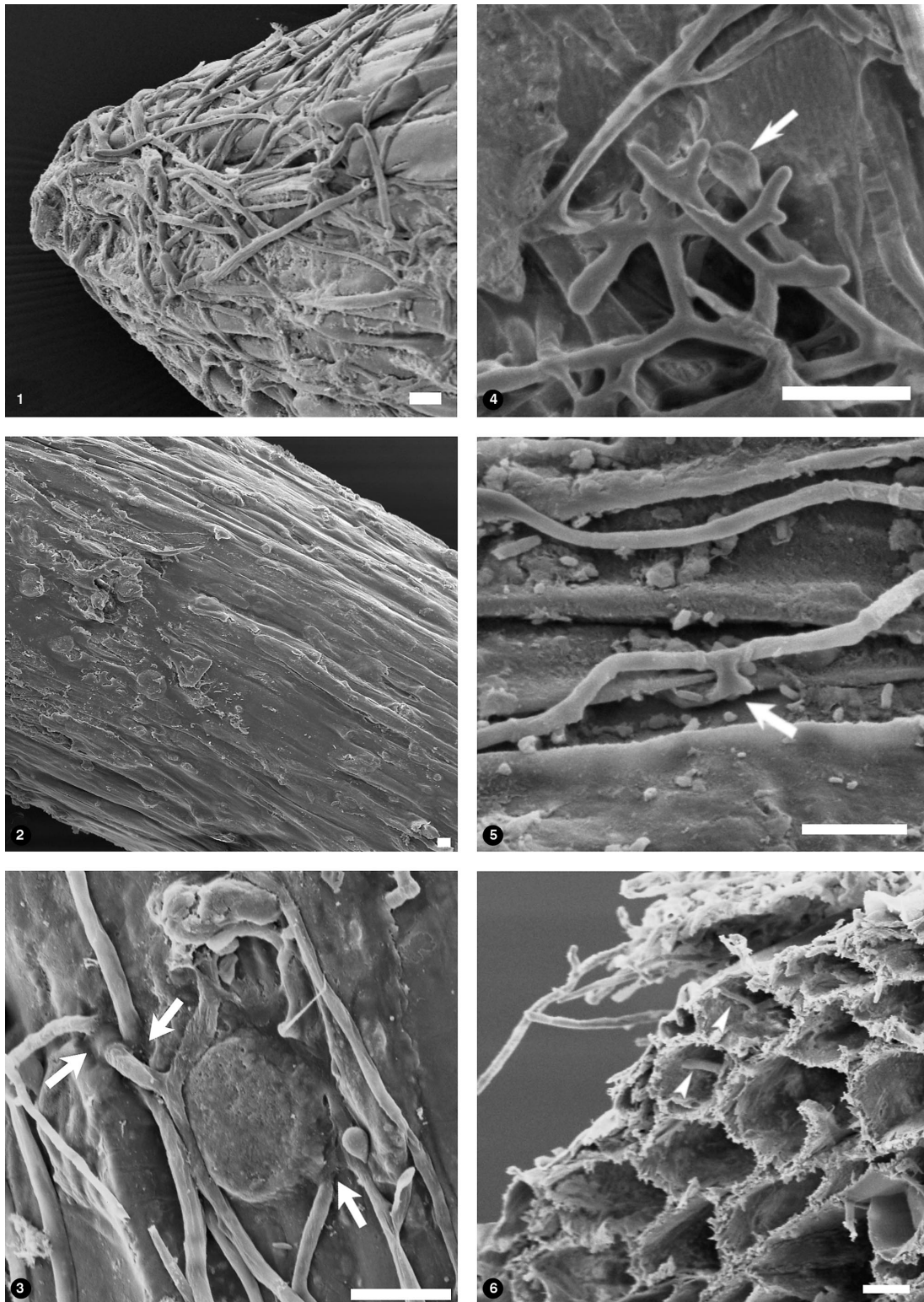
In contrast with control (uninoculated seedlings), a great number of conidiophores and conidia were observed on the inoculated roots two weeks after inoculation (Fig. 12), and there were no distinct differences between the three isolates in the ability to sporulate on fir roots.

## Discussion

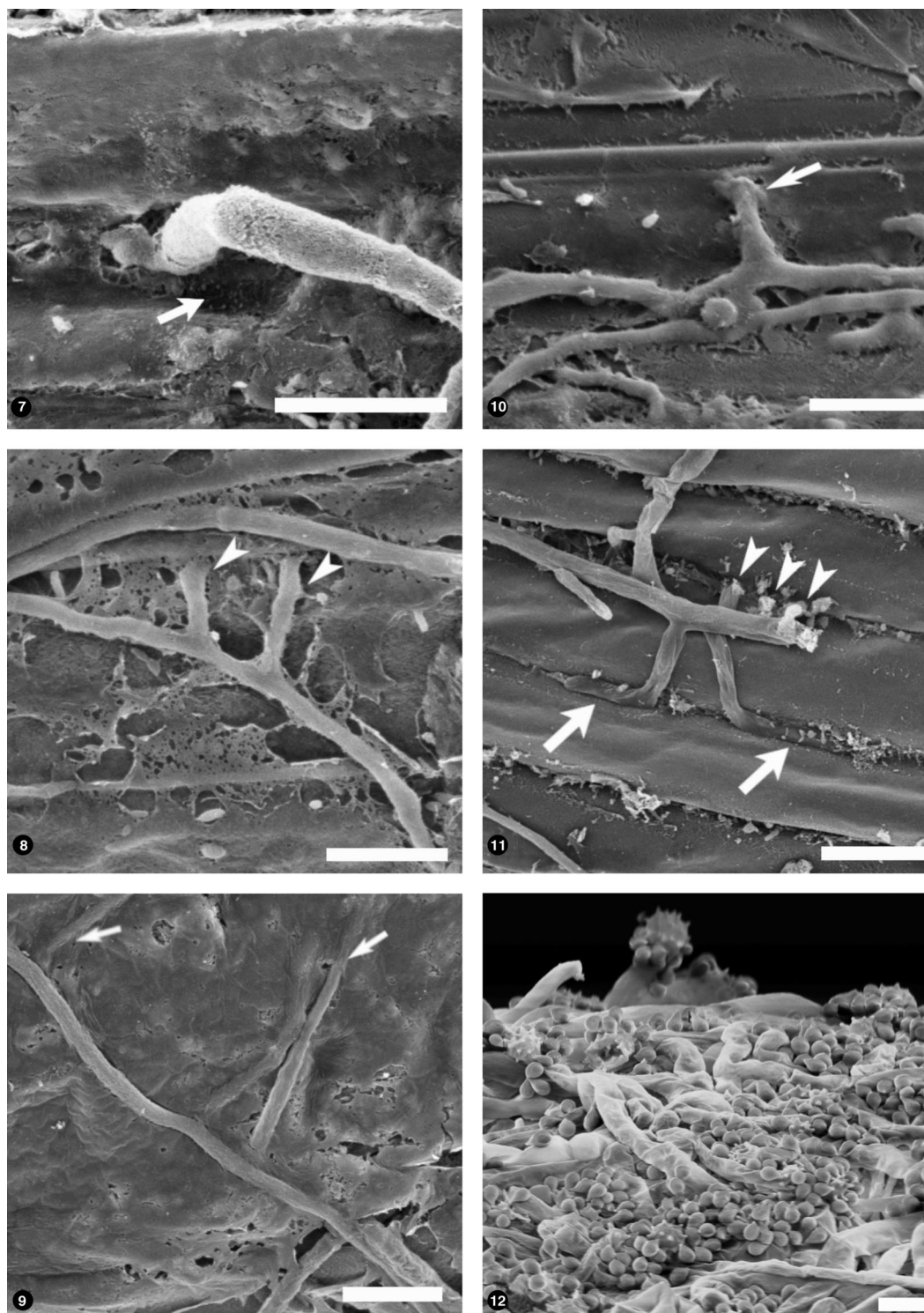
In the observations described above a large number of the running hyphae showed more or less preferential growth in grooves between the cortical cells. As the majority of crevices and cracks was positioned in these points, the topographic signal could increase the root infection through natural openings. Similarly as in the case of pine roots inoculated with isolates of the three IS groups (Werner et al. 2005), the root topography stimulated ramification and multiplication of hyphae, and in a consequence could increase success of infection.

In contrast to biotrophs, infection by necrotrophs is preceded by secretion of cell wall-degrading enzymes and toxins (Schafer 1994). The extensive erosion of the cortical cells observed in the older parts of roots was most probably related with a great amounts of these substances released by hyphae forming a compact mycelium in the proximal parts of roots. In the study performed by Werner and Idzikowska (2001), an advanced degradation of the cortical cells in root areas colonized externally by hyphae of *H. annosum* coincided with heavy internal colonization. In these areas, the hyphae entered the roots rather by splitting the adjacent cortical cells due to breakdown of middle lamella compounds than by the cell wall perforation and the precise count of the penetration points appeared to be quite impossible (Werner 1991). In eroded cortex the hyphae grew inter- and intracellularly, and there were no differences in the structure of bore holes formed by hyphae crossing inner cortical cells and those formed by hyphae entering the roots from outside (Werner 1990; 1991; Werner and Idzikowska 2001). This suggests that hyphae penetrating the roots directly through tiny pores perforated the cortical cell walls enzymatically. The P and S strains of *H. annosum* are known to secrete cellulose- and pectin-degrading enzymes (see Asiogbu et al. 1998 for extensive review). A high pectinolytic activity of *H. annosum* at an early stage of infection has been also confirmed by Johansson and Stenlid (1985).

In the SEM studies by Werner et al. (2005), hyphae of the F isolate preferentially entered older and eroded parts of pine roots. It is noteworthy that



Figs 1–6. SEM micrographs of *Abies alba* roots: uninoculated and inoculated with P, S and F isolates of *Heterobasidion annosum*. Fig. 1. Root apex showing hyphae of F isolate growing along and across longitudinally oriented cortical cells, 12 hours after inoculation. Fig. 2. Proximal part of uninoculated root showing longitudinally oriented cortical cells. Fig. 3. Apical part of root showing hyphae of F isolate, 3 days after inoculation. Note hyphae attached to the root surface and disappearance of the hyphal tips in cortical cells (arrows). Fig. 4. Proximal part of root showing dichotomously branched hyphae forming a loose mycelium, 3 days after inoculation with P isolate. Note germinating conidium (arrow). Fig. 5. Hyphae of S isolate growing in grooves between the cortical cells, 6 days after inoculation. Arrow indicates point of penetration. Fig. 6. Transverse section through first-order lateral root showing hyphae of P isolate penetrating the outer cortical cells (arrowheads), 3 days after inoculation. Scale bars: 1–6 = 10  $\mu$ m



Figs 7–12. SEM micrographs of *Abies alba* roots inoculated with P, S and F isolates of *Heterobasidion annosum*

Fig. 7. Hyphal tip resembling infection peg and penetrating cortical cells at right angle to the root surface. Note an eroded area around the infection peg (arrow), 3 days after inoculation with P isolate. Fig. 8. Branches of a ramified hypha of F isolate penetrating root through holes in eroded root area (arrowheads), 7 days after inoculation. Fig. 9. Abundant mucilaginous material covering the root surface. Note disappearance of the hyphae of S isolate in the mucilage (arrows), 3 days after inoculation. Fig. 10. Hyphae growing along cortical cells. Note short branch of hypha addressed to the root surface (arrow), 7 days after inoculation with S isolate. Fig. 11. A ramified hypha on the root surface. Note short branches entering the root side by side through a crevice (arrowheads) and long branches showing a tendency to grow along depressions and disappearing at point of cell junction (arrows), 7 days after inoculation with F isolate. Fig. 12. A part of root heavily colonized by hyphae of F isolate showing numerous conidiophores and conidia, 2 weeks after inoculation. Scale bars: 7–12 = 10  $\mu$ m

hyphae of the same isolate showed a higher ability to direct penetration fir roots than pine roots. Consequently, it is suggested that the F isolate showed a higher preference to fir, albeit similar to the P- and S-group isolates, while specifically frequent entering more weakened areas of fir and pine roots by the F isolate may reflect rather its higher saprobic than pathogenic peculiarities.

The direct penetration with formation a structure resembling infection peg, observed only after inoculation with the P and S isolates, may be indicative of necessity of a higher physical force to complete the perforation. The hyphae forming infection pegs were seen as being tensed over the root surface. It is not clear, whether the detachment of the hyphae from the root was an artifact caused due to fixation procedure or a pressure exerted by the infection peg on the root surface.

It would seem that the abundant mucilaginous matrix covering the root surfaces and embedding the hyphae of S isolate may be related with an unknown property of S-group isolates and/or host reaction on their enzymatic activity. Similar abundance of mucilage on Scots pine roots inoculated with the another S isolate was also observed by Werner et al. (2005). Spores and hyphae are known to produce mucilaginous materials with adhesive properties following surface contact. Various enzymatic activities have been detected in mucilage released by spores of many fungi (Dean 1997). Some enzymes are necessary for attachment to the plant surface, initiation and establishing of infection (Deising et al. 1992). Seedlings of conifers were used to study the potential role of mucilage in adhesion of spores, germ tube development and pathogenesis (Asiegbu et al. 1995a; b; Snape et al. 1994). Polysaccharides, proteins and glycoproteins are known to be principal components of mucilage. However, the composition of this material is heterogeneous and according to Nicholson (1996), there is no evidence for a common adhesive compound or mechanism of attachment.

## Acknowledgements

This study was financially supported by the Polish Academy of Sciences and the Polish Committee for Scientific Research, grant No 5 P06H 004 15. We wish to thank Mrs Anna Błaszowskiak for assistance during the course of the study.

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