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Initial stages of host-pathogen interaction between *Pinus sylvestris* seedling roots and the P-, S- and F-intersterility group isolates of *Heterobasidion annosum*

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Abstract: Prepenetration and penetration phenomena after inoculation of roots of *Pinus sylvestris* seedlings grown *in vitro* with the P-, S- and F-group isolates of *Heterobasidion annosum* were observed using scanning electron microscop. There were no differences in the behaviour of hyphae and in the appearence of mycelia formed by the three IS-group isolates. Four types of the root penetration by hyphae were observed. In the first, the entrace of hypha into root was achieved throught tiny pore formed in walls of the cortical cells. In the second, swellings resembling appressoria were formed by the hyphal tips at the points of contact with the root surface. In the third, the hyphae penetrated cells through holes in eroded areas of roots, and in the fourth by the natural crevices at points of the cortical cell junctions. It is concluded that the different frequencies of the four types of penetration observed after inoculation with isolates of the three IS group should be carefully explained in term of their different pathogenic and saprobic capabilities on pine.

Additional key words: adhesion, hyphal growth, penetration, root surface, SEM

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Introduction

Heterobasidion annosum (Fr.) Bref. is one of the most important specialized pathogens causing great losses to the production of timber, primarily in managed coniferous forests in the temperate and boreal regions of the world (Hodges 1969). Due to its adaptation to living inside standing trees, it has been described as "obligatory symbiotic" in the sense of being obligatory parasitic (Worral et al. 1983). The species consists of three intersterility (IS) groups showing different preferences to host trees (Korhonen 1978, Capretti et al. 1990).

Although *H. annosum* is considered to destroy woody roots and stems, it has been shown that this pathogen is also able to infect primary and thin woody

roots of Scots pine (Werner 1990, 1993). Recognized as a useful model for studying resistance mechanisms (Johansson and Asiegbu 1994), nonsuberized and suberized roots were consequently used to study infection process, host-pathogen interaction (Asiegbu et al. 1993, 1994, 1995, Heneen et al. 1994a, 1994b, Werner and Idzikowska 2001) and host resistance (Werner 1991a, 1991b). In spite of this, seedlings of conifers have never been used to test the host preference in *H. annosum* complex. Recently, however, variation between the IS-group isolates in virulence, expressed in mortality rate of pine and spruce grown *in vitro* was described by Werner and Łakomy (2002). The P isolates displayed similar virulence on both hosts, while S isolates caused higher mortality of spruce seedlings and significantly lower mortality of pine seedlings. Isolates of the F group were less virulent, but killed more spruce than pine seedlings. Statistically significant interaction host-plant × IS group, which contribute to over 47 % of the explained variance provided strong evidence for the occurrence of intraspecific variation in *H. annosum* similar to that observed in nature.

Contrary to many agricultural pathosystems, knowledge about expression of virulence, physiological specialization and specifically host-parasite recognition in *H. annosum* complex is still insufficient. Attachment to the host is presumed to be the first step in host-pathogen recognition and in establishing the parasitic interaction. The role of mucilage in adhesion and germination of *H. annosum* spores, colonization and penetration has been reviewed recently by Asiegbu et al. (1998). The mucilage is particularly abundant in the slimy root region, where the cortical cells slough-off and infection takes place (Werner and Idzikowska 2001). To date, however, there is no information concerning the differences in the behaviour of mycelia of the three intersterility groups on the root surfaces of host and non-host-plants. Since studies on the prepenetration phenomena seem to be of a great importance for a better understanding the host preference, a model system has been developed to study the external and internal colonization of conifer roots by the pathogen in uniform *in vitro* conditions (Werner 1991a).

Scanning electron microscope (SEM) studies on the penetration of nonsuberized roots of *Picea abies* (L.) Karst. by hyphae of the S-group isolate were described by Asiegbu et al. (1993). Similar observations on the penetration of woody roots of *Picea abies* through broken phellem cells and intact bark were described by Heneen et al. (1994b).

The objective of this study was to document the differences in the mode of penetration and establishment of mycelia of the P, S, and F isolates of *H. annosum* on the root surfaces of *Pinus sylvestris* L. seedlings.

Materials and methods

Plant and fungi

Pinus sylvestris L. seedlings from the provenance of Bolewice (52°28'N and 16°03'E) were used in the study. *Heterobasidion annosum* was represented by three strains, one each of the P-, S-, and F-intersterility groups (W2, 96043 and 96067, respectively).

Growth condition and inoculation procedure

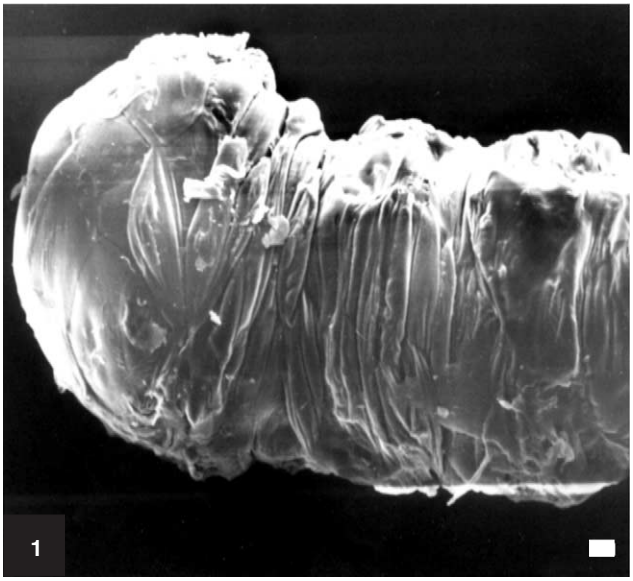
Sterile pine seedlings were grown under conditions described by Werner and Idzikowska (2001). Two-month-old seedlings grown *in vitro* were aseptically transferred from test tubes into Petri dishes containing a sterile mixture of perlite and peat (3:1 v/v). Subsequently, they were inoculated with several pieces (5 mm in diameter) of two-week-old mycelia of the fungi growing on malt extract agar at 24°C in the dark, and incubated in a growth room under fluorescent tubes (Osram L36/W77 Flora) (100 $\mu Em^{-2}s^{-1}$) light 18 hours a day, 80% RH at 24:20°C day: night temperatures for 24 hours, 3, 7, and 14 days. Uninoculated seedlings served as the control.

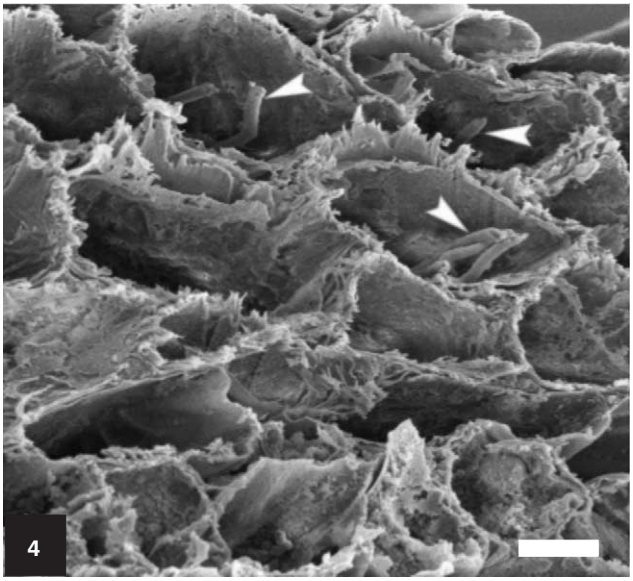
Root preparation for scanning electron microscopy (SEM)

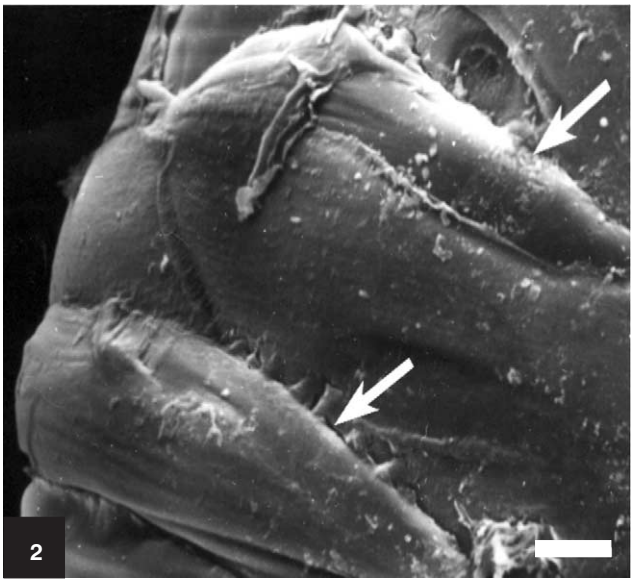
Small pieces (3–5 mm) of apical parts of roots were collected after 24 hours and 3, 7, and 14 days after inoculation. Each sample consisted of 10 root pieces inoculated either with P, S or F isolate. A total of 120 root pieces were studied by SEM. The samples were fixed in 2.5% glutaraldehyde in 0.5 M cacodylate buffer at pH 7.2 for 24 h and postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 h at 4°C. The specimens were then washed in distilled water, dehydrated, and critical point dried in a Balzers CPD-030 unit using CO_2 as a transition fluid. Afterwards they were mounted on aluminium stubs and coated with gold (12–15 nm thick) using a Balzers SPD-050 sputter coater. Finally, the roots were observed in a Philips 515 scanning electron microscope at 15 keV.

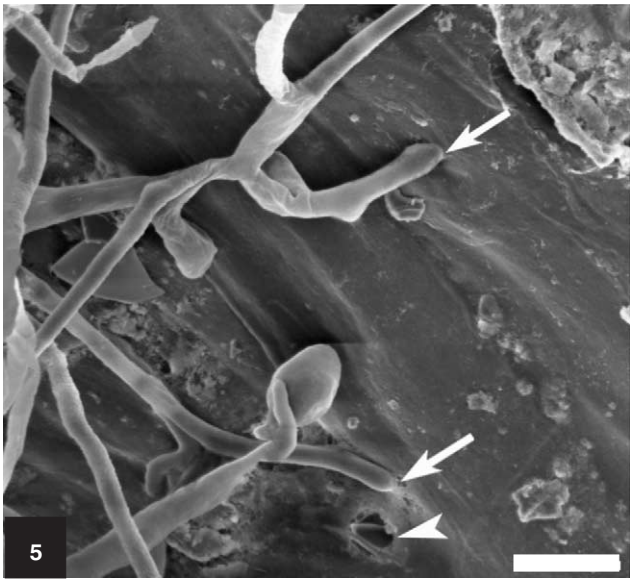
Results

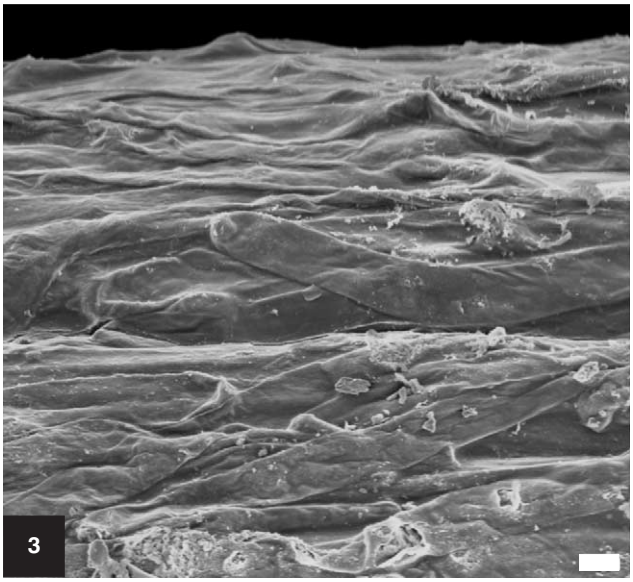
In apical and subapical parts of short roots, the cortical cells were oriented transversely to the long root axis and firmly juxtaposed (Figs 1 and 2). By contrast, they were oriented longitudinally in proximal part of roots, where some of them began to slough-off (Fig. 3). The cortical cells of the main and first-order lateral roots were as a rule oriented longitudinally through the length of the roots. The root surface was not smooth. In young roots, the protuberances of the cortical cells and depressions in points of the cell junctions flattened with age. Besides the presence of *H.*

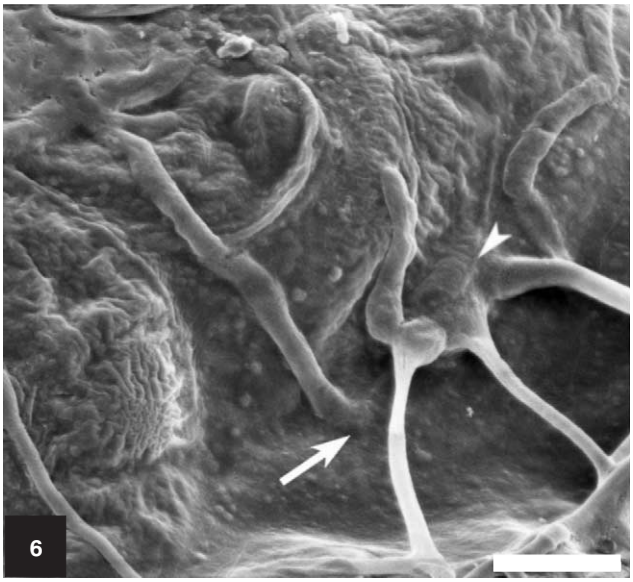




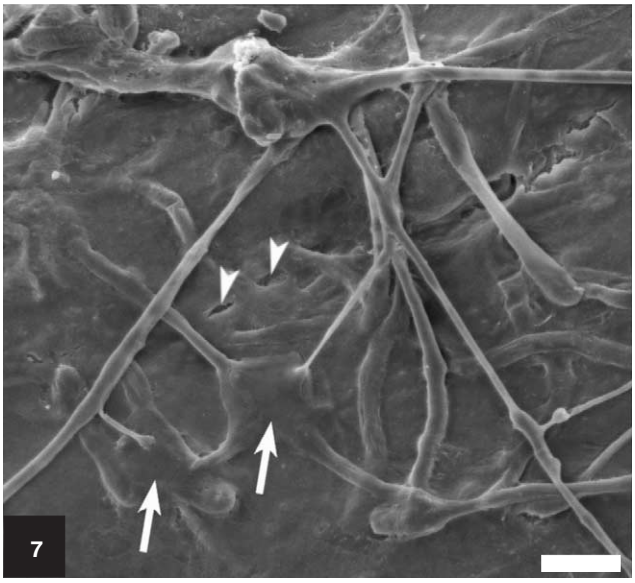


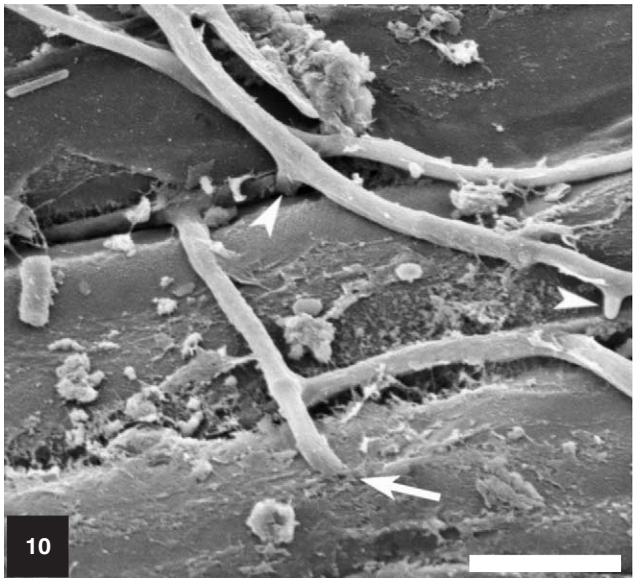


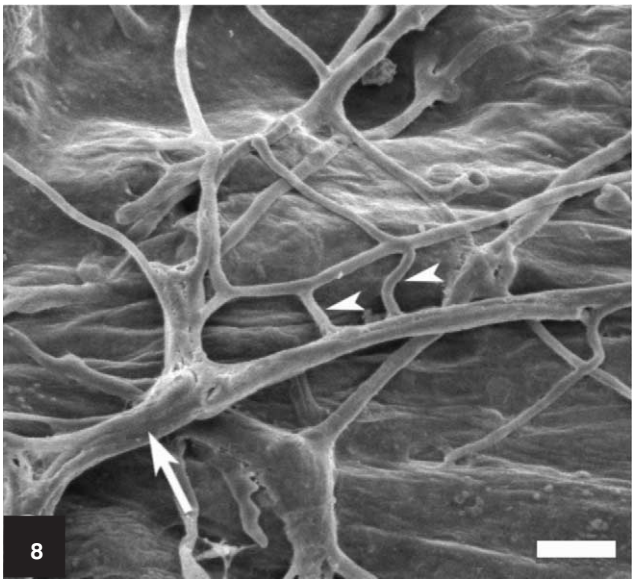


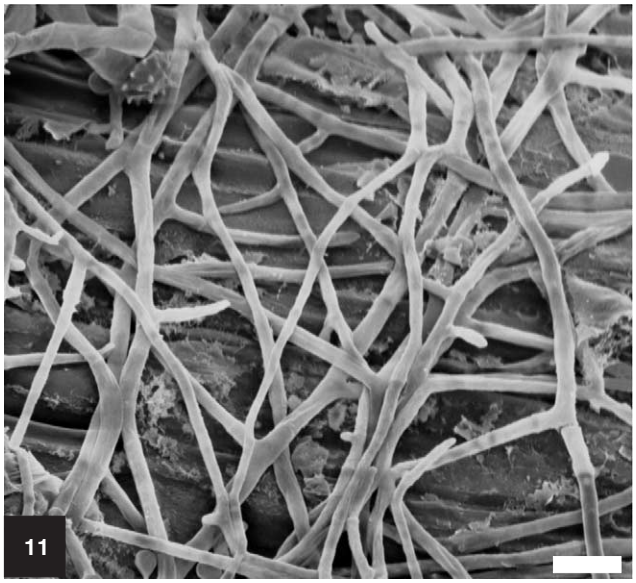


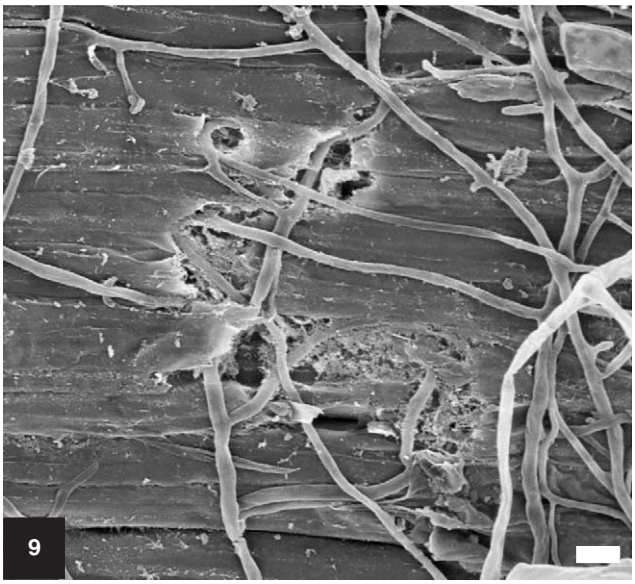
Figs. 1–6. Explanations on page 61

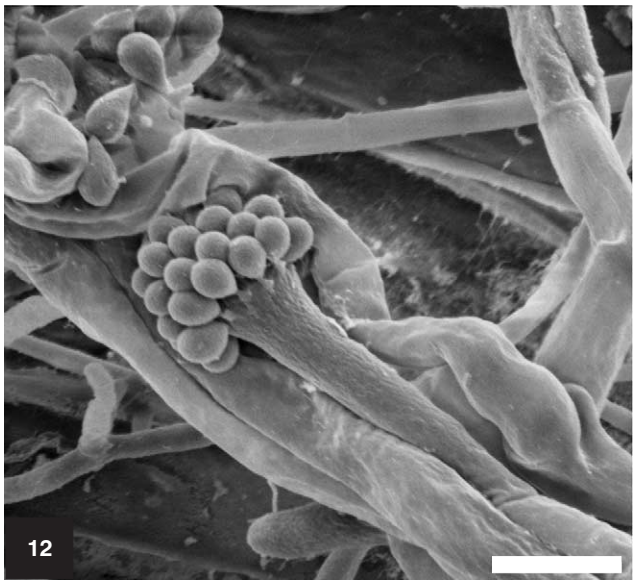












Figs. 7–12.

Figs. 1–6. SEM micrographs of roots of *Pinus sylvestris*: uninoculated and inoculated with P and S isolates of *Heterobasidion annosum*
Fig. 1. Uninoculated short root. Note the cortical cells oriented transversely to the long root axis
Fig. 2. Part of a short root showing proturberances of the cortical cells and depressions in points or the cortical cell junctions (*arrows*)
Fig. 3. Longitudinally oriented cortical cells in proximal part of root
Fig. 4. Transverse section through first-order lateral root showing hyphae inside outer cortical cells (*arrowheads*). Three days after inoculation with P isolate
Fig. 5. Hyphal tips firmly adhered to the root surface (*arrows*). Note a hole in a vicinity of hypha (*arrowhead*). Three days after inoculation with P isolate
Fig. 6. Swellings of hyphal tips (*arowhead*) and direct penetration the root by hypha (*arrow*). Three days after inoculation with S isolate (*bars* 1–6 = 10 μm)
Figs. 7–12. SEM micrographs of *Pinus sylvestris* roots inoculated with S and F isolates of *Heterobasidion annosum*
Fig. 7. Disappearing of the hyphal tips in root mucilage (*arrows*). Note small holes in the root surface (*arrowheads*). Seven days after inoculation with S isolate
Fig. 8. Mycelium of the S isolate on the root surface. Hyphal strand (*arrow*) and anastomoses (*arrowheads*) are seen. Seven days after inoculation
Fig. 9. The penetration of hyphae into the root through holes in eroded cortical cell walls. Seven days after inoculation with F isolate
Fig. 10. Hyphae growing in grooves between cortical cells and crossing the cells. Note direct penetration the root by hypha (*arrow*) and ramifications formed by hyphae at points of the cortical cell junctions (*arrowheads*). One day after inoculation with F isolate
Fig. 11. Mycelium of the S isolate on the root surface. Seven days after inoculation
Fig. 12. Conidiophore of *Heterobasidion annosum* with attached conidia. Two weeks after inoculation with F isolate (*bars* 7–12 = 10 μm)

annosum hyphae on the surfaces of inoculated roots, they were generally sterile. Various materials accumulated on the root as it aged: these were mostly debris, small plates and patches of a material which looked as if it had been extruded from the root. In older parts of roots there were more materials resembling mucilage on the root surfaces and deep cracks between cortical cells were observed.

Within 24 hours elongated and branched hyphae emanating from the inocula were observed on the root surfaces. Penetration sites were detected within 3–7 days after inoculation.

Four types of the root penetration by hyphae were observed.

In the first, the hypha adhering the root surface tapered and the hyphal tip directly perforated the cortical cell wall (Fig. 4). Penetration was achieved through tiny pore, most probably due to enzymatic erosion of the wall materials. Occasionally, small holes, from 0.5 to 2.0 μm in diameter were observed in the vicinity of other hyphae (Fig. 5). These may be an indicative of uncompleted perforation and further sucking out of infective hyphae due to usage a vacuum during fixation procedure for SEM.

In the second type, a single or several hyphae became swollen and formed a structure resembling appressorium, which was tightly adpressed to the root surface (Fig. 6). In most cases the swollen hyphal tips simply disappeared in the mucilage covering the roots (Fig. 7). The lack of further growth of the hyphae on the root surface may suggest that the penetration was completed.

In the third type, a severe degradation of the cortical cell walls (more advanced in the second week after inoculation) preceded the penetration. The entrance of hyphae into roots was achieved simply through holes in eroded cell wall (Fig. 9).

In the fourth, the hyphae entered the roots through crevices in points of cell junctions or cracks between sloughing cortical cells. Frequently, the hyphae growing across the cortical cells formed short branches just at these points. The newly formed hyphae immediately entered the roots or continued their growth along the natural crevices (Fig. 10).

From days 7 to 14, the number of hyphae increased forming more or less compact mat. It consisted of interwoven hyphae usually devoid of clamp-connections and forming frequent anastomoses (Figs 8 and 11).

Two weeks after inoculation numerous conidiophores bearing conidia (Fig. 12) made it impossible to observe the infection points. Although there were no distinct differences either in the arrangement of hyphae and in the appearance of mycelia or in frequency of conidiophores formed by the isolates of the three IS groups, the first mode of the penetration was most often observed after inoculation with the P isolate. The second type was dominat on roots inoculated with the S isolate, while the third type was the most numerous after inoculation with the F isolate. The entrance of hyphae into roots through the crevices was observed in similar frequency after inoculation with the isolates each of the IS-group of the fungus.

Discussion

The results of this study did not provide uncontrovertible evidence of various behaviour of hyphae of the three IS-group isolates of *H. annosum* on the sur-

faces of pine roots. The observations with the help of SEM technics make it impossible to count precisely the real number of penetration points. Nevertheless, after having examined 120 root pieces we are convinced about a more frequent direct penetration of the cortical cell walls by the hyphae of the P-group isolate. Degradation of wall materials limited to a tiny pore, suggests a high facility to accomplish the penetration of pine roots. By contrast, the formation of structures resembling appressoria may suggest a necessity of a mechanical force and an enzymatic action to complete the cell wall perforation (Bracker and Littlefield 1973).

The establishment of infection caused by pathogenic fungi strongly depends on properties of the host surface. The significance of the sculpture of the leaf surface in successful infection of the obligate parasites was demonstrated in a series of elegant studies of Dickinson (1949a, b, c, 1969). In the case of rusts and mildew spores, the sculpture of cuticle and epidermis influence the behaviour of germ tubes and the development of infection structures. The developmental stages of the infection structures, such as appressorium, infection peg, vesicle and infection hypha correlate with the structure and position of the guard cells and stomata (Werner 1982). In the present study, the sculpture of the root surface influenced both the growth of hyphae along depressions in points of the cortical cell junctions and their ramification, and consequently the frequency and success of infection.

Although our investigations provided no direct evidence on the role of root exudates in the host preference, the entrance of hyphae of F isolate into roots was most frequently completed through holes in the eroded root areas. The fact, that in most species of the obligate parasites, chemical components of cuticle or specific volatile substances are also involved in the morphogenesis of germ tubes and initiation of the infection structures (Allen 1957), suggests a need of both the physical-contact stimuli and the host exudates to establish the infection (Endo and Amacher 1964, Flentje et al. 1963). Fungi, which do not directly penetrate cuticle such as the rusts and powdery mildews, instead form appresoria over stomata. Necrotrophs, however, have evolved another strategy to gain access to plant tissues. In this group of fungi, the ability to neutralize potentially toxic compounds increases a probability of successful infection. Moreover, there is only little evidence about compunds favouring infection by *H. annosum* on the root surfaces (Asiegbu 2000). Cosidering higher saprobic capabilities and a low preference of the F-group isolates to pine, the more frequent penetration of the studied F isolate in strongly eroded root areas may be explained in terms, either of a higher tolerance to potentially toxic compounds or a lower ability to induce hypersensitive reaction and/or a low ability to penetrate an intact cortical cells of non-host plant.

Most of plant's exudates are known to act as elicitors involved in the phenomena of specificity and host-parasite recognition (Albersheim and Anderson-Prouty 1975). There is also evidence that during adhesion of *H. annosum* spores to host surface, several compounds of mucilage, including polysaccharides and proteinaceous material play a key role in the prepenetration phenomena (Asiegbu et al. 1998). In the pathosystem studied here, the establishment of infection following direct penetration was more dependent on the attachment of infective hyphae to the root surface after inoculation with the P and S isolates. Recently, an adhesion of the infective hyphae of the P stains of *H. annosum* to pine roots, preceding penetration was described by Werner and Idzikowska (2001). The same adhesion of hyphae of S strains before crossing down several layers of the pine cortex was observed by Werner (unpublished).

In conclusion, since the four types of penetration were observed after inoculation with the same isolate and similar situation on *P. abies* roots inoculated with S isolate was described by Asiegbu et al. (1993), these may be also related to differences in structure and/or thickness of the cortical cell walls. On the other hand, the different frequencies of the four types of penetration found after inoculation with each isolate can be only carefully explained in term of their different pathogenic and saprobic capabilities on pine.

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