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Antoni Werner

Growth of *Heterobasidion annosum* (Fr.) Bref. through bark of one-year-old *Pinus sylvestris* seedlings grown *in vitro*

Abstract: *Pinus sylvestris* L. seedlings grown for 12–17 months in pure culture after inoculation with S and P strains of *Heterobasidion annosum* (Fr.) Bref. were studied with the help of light and transmission electron microscopy. Standardized inoculation procedure and uniform *in vitro* conditions allowed evaluation of the host/pathogen interactions. Necrotic reaction of the bark cells associated with the accumulation of dark-stained material in vacuoles, and lignification of their walls in root regions in proximity to mycelium were the common host reaction. Hypertrophy of the bark cells in areas invaded by hyphae caused a tension that was responsible for strong deformation of the bark cells. Injury to the vascular cambium resulted in formation of barrier zone parenchyma and abnormal xylem elements. High amount of polyphenolic material accumulated in new formed parenchyma cells was an effective chemical "barrier" to the centripetal spread of hyphae. Resin-plugged tracheids and row parenchyma cells rich in polyphenolic material were common in wood areas colonized by the fungus.

Additional key words: pure culture, infection of roots, host reactions, LM, TEM

Address: A. Werner, Polish Academy of Sciences, Institute of Dendrology, Parkowa 5, 62–035 Kórnik, Poland, tel. (4861) 817 00 33, fax (48 61) 817 01 66, e-mail: aswerner@rose.man.poznan.pl

Introduction

The root-rot fungus *Heterobasidion annosum* (Fr.) Bref. is one of the most dangerous pathogens on many coniferous trees in the Northern Hemisphere (Hodges 1969). It consists of three intersterility (IS) groups showing different preferences to the main host trees, namely: pines, spruces and firs (Korhonen 1978, Capretti et al.1990).

Despite frequent failures of artificial infection of young and thin woody roots in field experiments due to strong resistance barriers provided by the living bark and rhytidome (Lindberg 1992, Lindberg and Johansson 1991, 1992, Lindberg et al. 1992), it is assumed that roots of all ages are potentially susceptible to infection. Constitutive and induced resistance barriers provided by the living bark and rhytidome may be overcome either by a long exposure of roots to infective material (Braun and Lulev 1969), a high inoculum potential of the pathogen or by more effective infection procedures (Johansson and Asiegbu 1994, Werner 1991). Results of the recent studies on infection of young nonsuberized, suberized and woody roots of Norway spruce described by Asiegbu et al. (1993, 1994, 1995) and Heneen et al. (1994a, 1994b) and of Scots pine described by Werner (1990) and Werner and Idzikowska (2001) well confirm the above presumption. Moreover, these studies indicate that seedlings of conifers are suitable for studies the host/pathogen interaction and host resistance to *H. annosum* (Johansson and Asiegbu 1994).

In several studies attemps have been made to elucidate the infection process at the tissue and cell structural level. Light misroscope (LM) was used to document fungal colonization of woody roots of Norway spruce (Braun and Lulev 1969), formation of transi-

tion zones in front of invading hyphae in stems and roots of spruces and pines (Shain 1967, 1971, Shain and Hillis 1971, Johansson and Stenlid 1985) and compartmentalization of decay (Shigo 1975, 1979, Tippett and Shigo 1980, Werner 1993). The response of wounded or intact stem and root bark to the fungus was studied intensively by Lindberg (1992), Lindberg and Johansson (1991, 1992) and Lindberg et al. (1992).

Heterobasidion annosum causes a damage to living tissues in conifers root bark, including phelloderm, phloem, vascular cambium and ray parenchyma, and wood (Johansson and Stenlid 1985, Lindberg and Johansson 1992, Werner 1993). Living bark, however, contains antimicrobial compounds that inhibit the growth of the fungus *in vitro* (Alcubilla et al. 1971, 1987, Hart and Shrimpton 1979, Woodward and Pearce 1988) and *in vivo* (Shigo 1975, 1979, Tippett and Shigo 1980, Asiegbu et al. 1998).

Although the S strains of *H. annosum* are less aggressive to pine than to spruce *in vitro* (Werner and Łakomy, unpublished) and *in vivo* (Stenlid and Swedjemark 1988, Swedjemark et al. 1999) they can potentially infect and start the disease on both hosts. Despite the observed host preference, the knowledge about mechanisms responsible for differences in mortality and vertical spread in stems and roots of the host-plants caused by strains of the both IS groups is still unsufficient. Since penetration of the living bark is a critical stage in the life cycle of *H. annosum* (Redfern 1984, Lindberg and Johansson 1991), the study was undertaken to determine the differences in ability to penetrate pine bark between P and S strains.

The purpose of this study was to record the case of mutual interactions between more than one-year-old pine seedlings and *H. annosum* P and S strains during the advanced stage of infection under standardized, axenic, *in vitro* conditions. The evaluation of the differences in host defence reactions after prolonged exposure to P and S strains of *H. annosum* was attempted.

Materials and methods

Plant material consisted of twelve more tolerant Scots pine (*Pinus sylvestris* L.) seedlings that survived for 12–17 months after inoculation with three P strains of *H. annosum in vitro*, and twenty seedlings inoculated with S strain of *H. annosum*. The control were 20 noninoculated pine seedlings grown *in vitro*.

Pine seeds were soaked in water, surface sterilized with 2% $HgCl_2$ for 4 min and rinsed three times for 15 min in sterile distilled water. Seeds were germinated on 0.6% agar (w/v) medium, in dark at 24°C. Subsequently, they were aseptically transferred to 300×30 mm test tubes containing 100 ml agar medium (15 g agar + 100 μg thiamine in l l H_2O) (Pachlewska 1968). The seedlings were incubated in a growth room under fluorescent tubes (Osram L36/W77 Flora) (100 $\mu Em^{-2}s^{-1}$) light for 18 h a day, 80% RH at 24 : 20°C day : night temperatures. After one month, an inoculum disc (5 mm in diameter) of a two-week-old *H. annosum* mycelial mat was placed on the agar close to the seedling. The degree of host resistance was evaluated after the occurence of disease symptoms and the ability of plants to survive in the presence of the pathogen for 15 months, as had been described earlier (Werner 1991, Werner and Idzikowska 2001).

Light microscopy (LM)

Pieces of roots (3–5 mm) were fixed in FAA for 4 h. Dehydration of roots in an ascending ethyl alcohol-tertiary butyl alcohol (TBA) series was followed by embedding in paraffin (Jensen 1962). The specimens were sectioned with an Autocut 2040 Reichert-Jung rotary microtome. Serial transverse and longitudinal sections 8–10 μm thick were stained by the Conant quadruple stain method (Johansen 1940). The colonization of root tissues by the fungus was visualized by staining the sections according to the method of Mallory (Bagiński 1965). The sections were stained for 30 min with Delafield's hematoxylin (Jensen 1962), counterstained in 5% aqueous solution of eosin for 15 min at 37°C, rinsed shortly in distilled water and additionally stained for 10 min with Stirling's violet containing 5 g of crystal violet dissolved in a mixture composed of 88 ml of distilled water, 10 ml of absolute alcohol and 2 ml of aniline. Treatment with potassium iodine was followed by rinsing in water. The excess of violet was differentiated in several changes of aniline. After dehydration in ethanol and xylene the sections were mounted in balsam. The hyphae were stained blue. Phenolic substances were detected by staining with 1% solution of toluidine blue O at pH 4.5 (O'Brien et al. 1964). Tanniferous material was stained by treatment of fresh sections with 1% solution of ferric chloride in 0.1N HCl (Jensen 1962). The Wiesner reaction (phloroglucinol-HCl) was used for detection of lignin (Jensen 1962). Suberine was localized using Sudan B in 70% ethanol or using Sudan IV (Jensen 1962).

Preparation of roots for transmission electron microscopy (TEM)

Main roots were cut into 1–2 mm pieces, fixed in 4% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.2 for 24 h at 4°C and rinsed 3 times for 15 min each in the same buffer. Subsequently, they were postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 h at 4°C and then treated with 2% uranyl acetate for 2 h. The material was dehydrated in an ascending series of ethanols (10% steps 15 min each), and then in a two ethanol-acetone mixtures. The first consisted of 90% ethanol and 90% acetone (1:1) and the second 90% ethanol and 96% acetone (1:1). Dehydration in 96% acetone, pure

acetone and propylene oxide was followed by embedding in Epon 812. Roots were sectioned with glass knives using an LKB Ultratome 3. Ultrathin sections exhibiting a pale gold interference (approximately 60–70 nm thick) were stained in uranyl acetate and lead citrate (Reynolds 1963) and examined with a JOEL JEM 7A transmission electron microscope at an accelerating voltage of 80 keV.

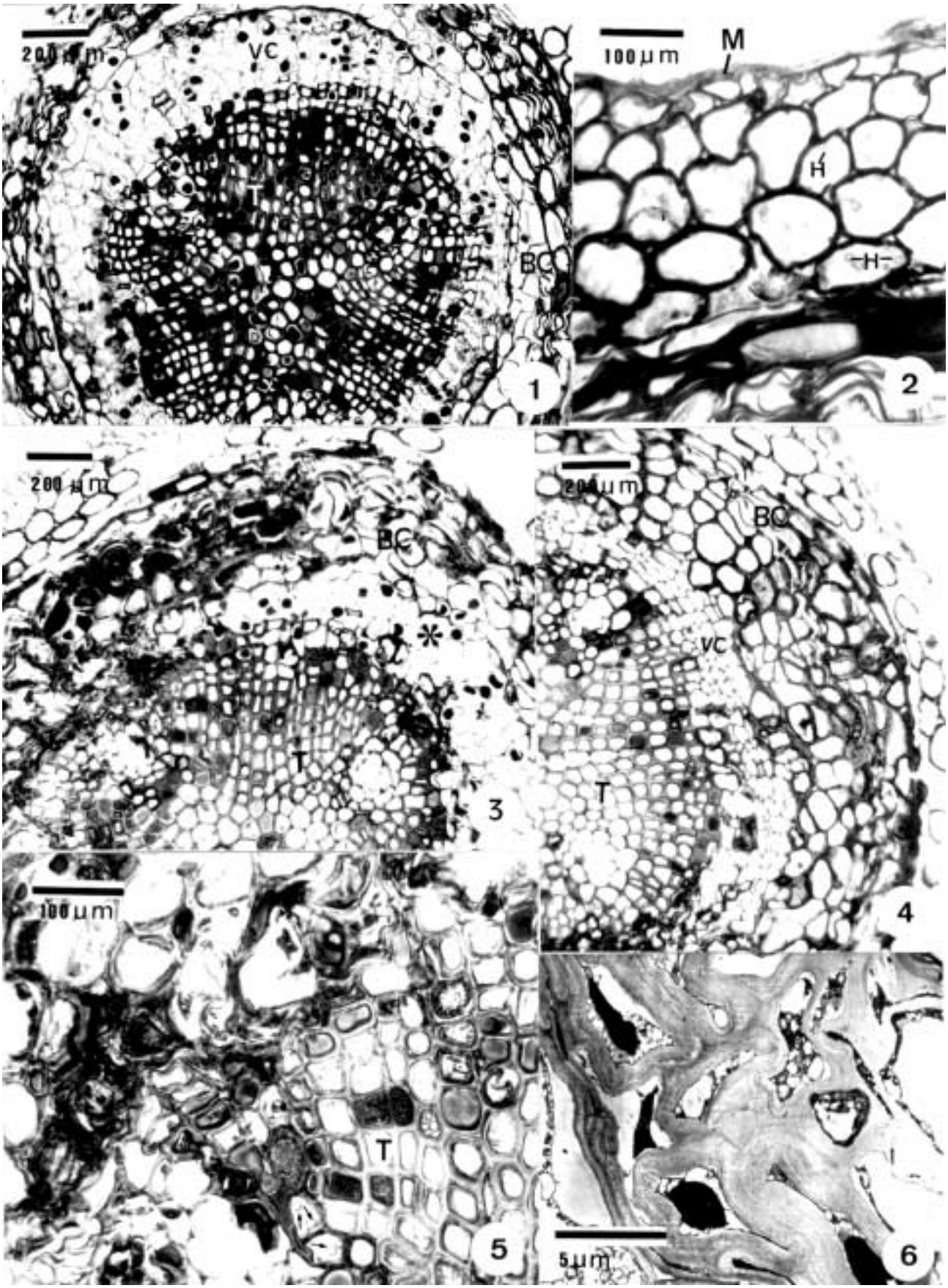
Results

In control, the outer layers of bark were firmly attached to the root, and there were no signs of the phellogen initiation in the bark. Below, there was a three- or four-cell thick layer consisted of thin-walled phloem and parenchyma cells, that contained starch granules and vacuolar phenolic deposits. The subsequent tissue was multilayered vascular cambium. Secondary xylem ring and the primary xylem were positioned centrally (Fig. 1).

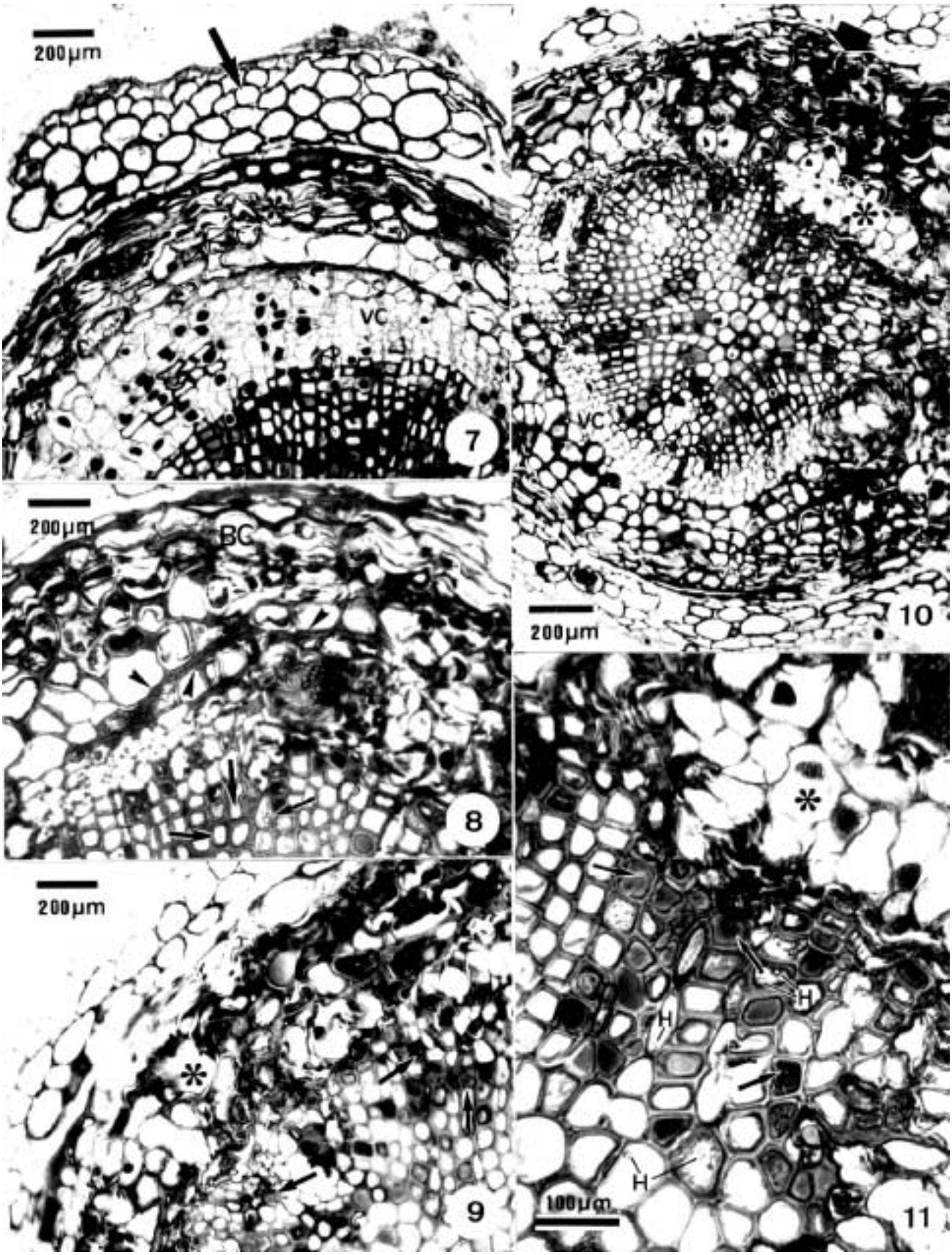
The bark tissues, vascular cambium and wood ring of main roots of Scots pine seedlings inoculated with the S strain of *H. annosum* are shown in transverse sections on figures 2–6. Root sections of pines inoculated with the P strains are shown on figures 7–11. In contrast to the noninoculated seedlings, the bark of infected plants consisted mainly of dead and thick-walled cells. The fungus was localized first in the outer bark. It entered the bark at points adjacent to remnants of the primary cortex that were heavily colonized by the hyphae and were still adhered to the roots (Figs. 2 and 7). Light microscopy (LM) observations disclosed a necrotic reaction of the bark cells in the areas colonized by the S (Figs. 3 and 5) and by the P strains of *H. annosum* (Figs. 8, 9 and 10). The bark cells positioned within the infected areas became necrotic before or while being colonized. Most of the cells were dead and dark-stained due to phenolic globules and melanized material filling the cell lumina (Figs. 3, 8, 9 and 10). Thick walls of the dead cells were lignified and suberized. Specifically thick walls of contiguous cells were seen on transverse sections as discontinuous bands. Despite the bands of the thick-walled cells, in roots of all seedlings inoculated with the P strains and in 17 out of 20 inoculated with the S strain the centripetal spread of the fungus was not stopped and the hyphae were always observed inside tracheids (Figs. 5, 8, 9 and 11). The wood-invading hyphae grew mainly longitudinally, and at this stage of the decay most often entered the adjacent tracheids by crashing tori of the bordered pits and only sporadically by eroded walls. Wood elements of roots colonized by *H. annosum* were obviously less disintegrated as compared to parenchyma and phloem cells. In roots of three seedlings which were not colonized by the S strain, the whole bark was composed of dead and thick-walled cells (Fig. 4). Walls of the cells were stained positively with phloroglucinol-HCl, Sudan B and Sudan IV. Contrary to the transparent content of cells, their walls were green coloured after staining with Toluidine blue O (not shown).

Hypertrophy of cells in the infected bark areas (Figs. 3, 5, 9 and 10) caused a tension, which was apparently responsible for strong deformations in the root concentricity, specifically in outer line of the vascular cambium and wood ring (Figs. 3 and 10). The cambium of the most diseased seedlings was composed of smaller number of cell layers or was partly or entirely desintegrated (Figs. 3, 8, 9 and 10). Discontinuous circles of parenchyma and altered xylem elements formed by the living cambium were observed in roots of the diseased plants (Figs. 3, 10 and 11). The parenchyma cells were thin-walled and nucleate. In contrast to the cell content, on hand-cut sections walls of the parenchyma cells were not stained metachromatically green with toluidine blue, pH 4.5, which suggests that the parenchyma cells were abundant in phenolic material and that their walls were not lignified (not shown). The contrast between the new-formed parenchyma cells and the other bark cells is illustrated in figures 3 and 10. Below the barrier zone parenchyma, several rows of small and deformed xylem elements could be observed. They were particularly frequent in the root areas where the vascular cambium was disintegrated (Figs. 5, 6 and 11). The tracheids adhering to wood elements invaded by hyphae were usually plugged by dark-stained resinous material (Figs. 5 and 11). The content of the row parenchyma cells in the area of resin-impregnated wood was dark-stained, characteristic of a necrotic reaction.

TEM observations showed the inter- and intra-cellular spread of *H. annosum* hyphae in the bark. The hyphae passed from one cell to another by direct perforation of cell walls. At the place of wall perforation, the hypha was constricted. After entrance of the cell, the distal part of the hypha regained its normal thickness (Fig. 12). Contrary to thin-walled cells of the bark, the thicker cell walls were usually slowly decomposed. The thicker walls of dead bark cells were impregnated with osmiophilic material, most probably phenolic in nature. Osmiophilic deposits filling the cells were degraded. Short and elongated segments of intracellular hyphae in close vicinity to osmiophilic deposits were seen (Fig. 13). The parts of the advancing hyphae were occupied by a dense cytoplasm bounded by plasmalemma. The protoplasm contained ribosomes, mitochondria and some lomasome-like structure. Variously sized vacuoles were electron-transparent and contained electron-dense inclusions. Spherical nuclei often contained prominent nucleoli. Great number of small vacuoles were lying close to plasmalemma or remaining contiguous with plasmalemma of physiologically young hyphae. Material of a flocculous appearance adhered to the fungal wall represented sheath material and remnants of destroyed host protoplast.



Figs. 1–6. Light micrographs of *Pinus sylvestris* seedlings 14–17 months after inoculation with the S strains of *Heterobasidion annosum in vitro*.



Figs. 7–11. Light micrographs of *Pinus sylvestris* seedlings 12–17 month after inoculation with the P strains of *Heterobasidion annosum in vitro*

Fig. 1. LM. Transverse section of main root of noninoculated fifteen-month-old seedling of *Pinus sylvestris* grown *in vitro* showing bark cells (BC), vascular cambium (VC), and wood (T)
Fig. 2. Part of the primary cortex superficially, inter- and intracellulary colonized by the hyphae, and attached to periderm. Mycelium (M) and hyphae (H) are seen
Fig. 3. Transverse section of main root showing dark-stained, necrotic cells of the infected bark (BC), barrier zone parenchyma formed in reaction to infection (*). Note lack of the vascular cambium in the root area positioned on the left of the barrier parenchyma
Fig. 4. Transverse section of main root showing strongly lignified and suberized walls of the bark cells (BC). Note lack of hyphae in the bark (BC), undestroyed vascular cambium (VC) and wood elements (T)
Fig. 5. Part of the main root showing hypertrofied, necrotic cells of the bark adjacent to misshapen and misaligned tracheids (big arrow) and several hyphae inside tracheids of the wood formed before the bark invasion by the fungus (small arrows)
Fig. 6. TM. Thick-walled and misshapen tracheids formed in reaction to centripetal spread of the fungus
Fig. 7. Transverse section of main root showing colonized by hyphae cortical cells (arrow), suberized cells of primary periderm and living bark cells (*), vascular cambium (VC) and wood ring
Fig. 8. A section of main root heavily colonized by hyphae. Note necrotic bark cells (BC), strongly lignified walls of the bark cells forming discontinuous bands (arrowheads) and hyphae inside tracheids (arrows)
Fig. 9. Outer part of main root showing hypertrofied (*) and dark-stained necrotic cells of the bark. Note lack of cambium and resin-impregnated tracheids (arrows)
Fig. 10. Transverse section of main root showing discontinuous band of the barrier parenchyma (*) positioned below the necrotic bark (arrow)
Fig. 11. Higher magnification of a portion of Fig. 10 showing thin-walled barrier parenchyma (*) and small, resin-plugged and misshapen tracheids positioned below, resin-impregnated elements of wood formed before infection of the bark (arrows)

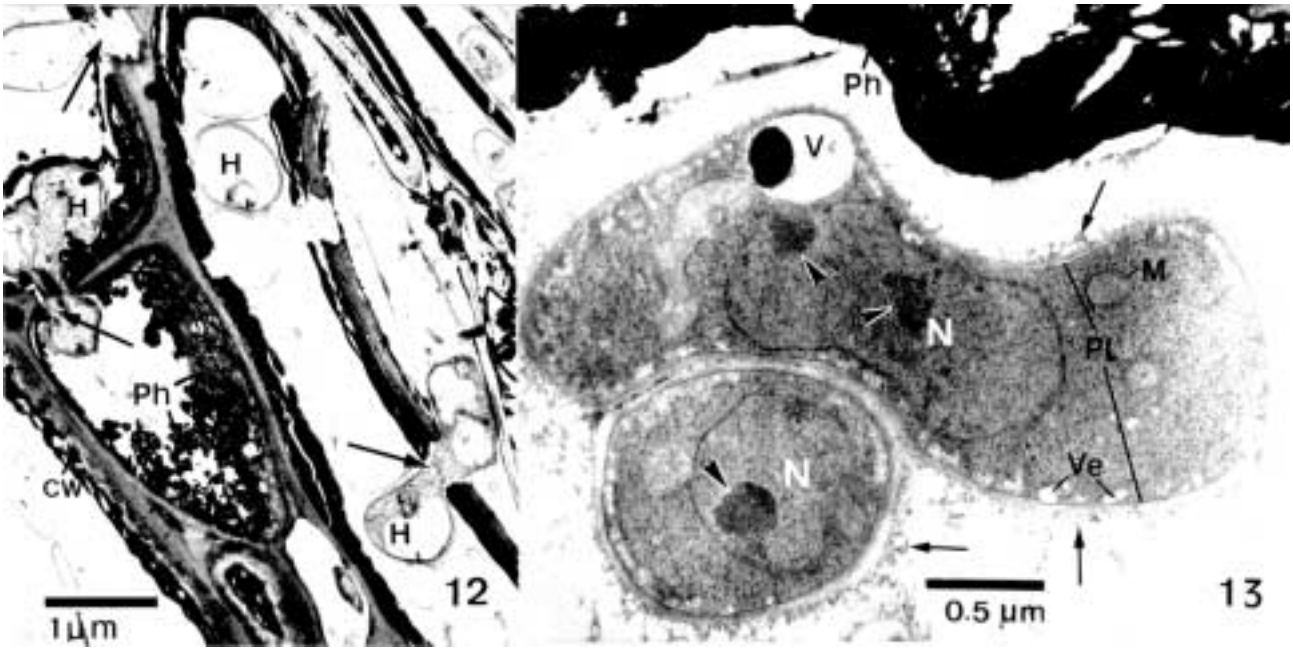


Fig. 12. TEM. Portion of the inner bark of twelve-month-old pine seedling heavily colonized by hyphae (H) showing thickened wall (CW) penetrated by hyphae and osmiophilic material of the phenolic nature (Ph). Note constriction of hyphae (arrows) passing the host cell walls
Fig. 13. Intracellular hyphae inside the bark cell in vicinity of phenolic deposits (Ph) showing numerous vesicles (Ve) located in vicinity of fungal plasmalemma (PL), mitochondria (M), dark-stained globular body situated inside vacuole (V), nuclei (N) and nucleoli (arrowheads). Note flocculous in appearance the sheath material (arrows)

Discussion

Little is known about the pathological anatomy of pine roots infected by *H. annosum in vitro*. The most interesting is the reaction of tissues which are important in formation of mechanical and chemical "barriers" separating injured bark from unaffected wood. Distinctive tissue consisting of thin-walled and unlignified parenchyma created in reaction to *H. annosum* that spread from central xylem was observed by Tippett and Shigo (1980). The anatomy of the barrier zone created by the vascular cambium in response to root invasion by *H. annosum* from the outer bark inward, in the field, was described by Werner (1993). In both studies, the longitudinal polyphenol-rich parenchyma that created a chemical "barrier" was the first-produced cell layer in response to infection. The formation of the barrier zone parenchyma was followed by an abnormal "growth rings" composed of small and compacted tracheids, numerous resin ducts and hypertrofied ray parenchyma.

In both studies the vascular cambium and phellogen participated in separation of decay from living wood. The polyphenol-rich parenchyma and altered xylem elements that bild up the "barriers" are known to protect living cambium and new wood against spread of the pathogen and diffusion of fungal metabolites and autolytic toxic substances formed in the decayed tissues. According to the CODIT model proposed by Shigo (1979), resin production, fungal invasion and decay are compartmentalized due to the above mention "barriers". The "biological sense" of the mechanism in older trees is to keep the spread of decay in check within the "useless" heartwood in order to prolongate the propagation.

In the presented study a quick colonization of thin-walled bark cells contrasted with slow spread of the fungus in areas of bark composed of cells showing thick walls and dark stained contents. Tannins and phenols naturally occurring in the bark and sapwood are known to serve as protectants against invasion of *H. annosum* (Harrs et al. 1981). Subsequent spread of the fungus in the bark and wood is due to its ability to polymerize phenols by oxidation, removing the primary source of inhibition (Stenlid and Johansson 1987). The other source of the potential toxic substances are necrotic cells. In the presented study, the substances formed as a result of the necrotic reaction could not stopped the growth of *H. annosum* – the phenol tolerant fungus, and acted rather as a temporary "barrier" for the fungus. According to Shortle and Cowling (1978), the processes involved in discolouration and decolouration of the infected tissues provide a nonliving, free of toxicity, substrate for subsequent hyphal growth. Contrary, the area of a new formed parenchyma rich in polyphenols was an effective "barrier" to prevent the centripetal spread of mycelium. Since in the studied roots, the zone parenchyma did not form complete circle between the infected bark and non-infected wood the decay and the pathogen spread could not be localized in the bark.

Although in the study by Lindberg (1992) success of infection through the living bark was not related to IS group affiliation in neither spruce nor pine and the growth rates *in vitro* were not related to growth *in vivo*, in the presented study, in the group of 32 pine seedlings that had survived for 17 months after inoculation, 20 seedlings were inoculated with S-strain and the rest were inoculated with P-strains. Within the last group, 9 pines were inoculated with the less aggressive strain. Considering the method used in the study, the observed differences in the host resistance could not be interpreted in terms of differences in inoculum potential, but rather in terms of differences in the host reaction, and in the different pathogenicity of the fungal strains. Strong lignification of cell walls of the bark in three seedlings inoculated with the S strain of *H. annosum* was obviously responsible for the absence of hyphae in cambium and wood. Also in the study by Werner and Idzikowska (2001), higher frequency of papillae formation was observed in the cortical cells of pine seedlings inoculated with the less aggressive P strain. Frequent papillae containing callose in cells of the primary cortex of Scots pine seedlings inoculated with the S strains were observed by Werner and Idzikowska (unpublished). According to Biggs et al. (1984) and Biggs (1986), structural barriers such as the suberized impervious tissue (SIT) and strong lignification can hinder fungal spread in many plants.

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