DEFENCE RESPONSES OF NON-MYCORRHIZAL AND MYCORRHIZAL SEEDLINGS OF *Pinus sylvestris* L. TO FUNGAL PATHOGENS.

A thesis submitted for the degree
of Doctor of Philosophy
in the University of Oxford

by

Pierluigi Bonello
Green College
Hilary Term, 1991

ABSTRACT

Pierluigi Bonello
Green College

Doctor of Philosophy
Hilary Term

Defence Responses **of** Non-mycorrhizal and Mycorrhizal Seedlings of *Pinus sylvestris* L. to Fungal Pathogens.

The defence mechanisms expressed in roots of *Pinus sylvestris* seedlings challenged with fungal pathogens were investigated, and a comparison was made between the expression of defences in non-mycorrhizal and mycorrhizal seedlings.

Papillae were formed by cortical cells of non-mycorrhizal seedlings infected with *Cylindrocarpon destructans*. Histochemical evidence was obtained for pectic materials comprising an important polysaccharide component of these structures, and for the deposition of polyphenolic compounds also. Proton induced X-ray emission (PIXE) microanalysis indicated that insoluble calcium levels were elevated in papillae relative to normal cell walls. Although papillae appeared important in protecting cortical cells against penetration by fungal hyphae, a primary role for the wall appositions in the resistance of seedlings of Scots pine against root pathogens could not be proven.

Although phytoalexins were not detected in the roots of Scots pine seedlings following infection with *C. destructans*, the mean content of an abietic acid fraction (comprising six compounds, of which only dehydroabietic acid could be positively identified), increased from 5.2 to 9.7mg g^{-1} dry weight. This fraction exhibited some antifungal activity.

Pathogenesis-related proteins induced *de novo* by infection could not be detected, but several constitutive apoplastic proteins, including some with chitinase activity, appeared to increase in the needles of root-infected seedlings.

The formation of ectomycorrhizae with *Pisolithus tinctorius*, *Suillus bovinus* and *Hebeloma crustuliniforme* did not itself induce papilla formation in the roots of *P. sylvestris*. Evidence was obtained to suggest that the response was suppressed when mycorrhizal seedlings were challenged with *C. destructans*.

Results highly suggestive of the induction of systemic resistance in *P. sylvestris* seedlings, consequent upon mycorrhizal infection, were obtained. In seedlings grown *in vitro* the survival rate of mycorrhizal seedlings challenged aerially with *Botrytis cinerea* was 37.5% compared with 7.1 in seedlings grown gnotobiotically. However, the physiological mechanisms by which this protection was imparted remain to be determined.

ACKNOWLEDGEMENTS

My first and warmest thanks must go to my inimitable supervisor, Dr. Ray B. Pearce. He must surely be commended for being able to withstand, for three long years, the constant battering of my questions, enquiries, complaints, anxieties. He will certainly miss my ritual "three knocks" on his door, by which I think he must have been able to recognize me every time, even before seeing my face. I owe to him all I know about plant pathology. He has given me the opportunity to mature as a scientist by instilling in me the passion and the rigour necessary for research. He will certainly be missed very much.

I owe many thanks to the following people, who have assisted me during my stay at Oxford: Dr. Caroline Mohammed, whose help was essential for the protein work; Drs. Frank Watt and Geoff Grime (Nuclear Physics), for giving me the opportunity to carry out the very interesting work on PIXE microanalysis; Miss Linda Lockhart for some preliminary gas chromatography; Dr. William Greenaway, for his invaluable help with gas chromatography and mass spectrometry; Mr. John Baker and Mr. Tony Allen, the overworked, but always jovial, departmental photographers; Mr. Cledwyn Merriman, whose help was often essential for electron microscopy; Mr. John Kemp and Mr. Ian Abbott for their help in solving many technical problems; the OFI library staff, for being always willing to guide me through their labyrinths; and Mrs. Cheryl Howes, Dr. Kit Bailey, Mrs. Julie Smith and Mr. Peter Franklin for their help with inexplicable computer matters.

I would like to thank my colleagues Jane Carter, Fortunato Garza, John Hall, Dr. Haroula Kargiolaki, Patrick Olngotie, Rekha Wadekar, and Andrew Wong for their friendship and for bearing with my grievances (sometimes!). They will all be missed.

I must also thank my sponsors, the "Fondazione Ing. Aldo Gini" (Padova, Italy) which, by giving me financial support, have made a substantial contribution for the completion of this project.

Finally, but most importantly, I would like to thank the members of my family: my wife Rosie, whose constant support has been essential for maintaining my psyco-physical equilibrium, especially in the final stages of my work; my wonderful little daughter Isabella, who brought me joy every single day; my father Luciano, my mother Edda, my sister Daniela and my brother Luca for being always close to me, in good times, and in times of psycological and financial hardship.

TABLE OF CONTENTS

page

LIST OF FIGURES

page

spectrometry as dehydroabietic acid (see Fig. 3.23).

(WA: water agar). Each value in a) and b) is the mean of 6 and 4 replicates, respectively.

and 2 are needle extracts from control and seedlings infected with *C. destructans*, respectively; lane 3 is a laminarinase preparation (Sigma). The development of a strong, fairly uniform background does not allow for any meaningful interpretation. Only the commercial preparation of laminarinase (asterisk) can be identified. Several areas of inhibition can be seen in the plant extracts, corresponding to some of the chitinase bands in Fig. 5.3b (arrows).

LIST OF TABLES

CHAPTER 1

General Introduction

1.1 Defence mechanisms in plants

1.1.1 General

Plants defend themselves by deploying various protective mechanisms which can be conceptually divided in two major categories: constitutive (preformed or pre-infectional) and induced (post-infectional). Each of these two broad categories can be subdivided into two more groups, according to the nature of the defence mechanism, i.e. into structural (morphological) and biochemical defences, although it is obvious that structural changes are a consequence of biochemical processes (Aist, 1983; Heath, 1980).

1.1.1.1 Constitutive defences

1.1.1.1.1 Structural

Starting from the interface between the host plant and the external environment, various anatomical features are or may be involved in protection from disease. On the aerial portion of the plant, hydrophobic materials, such as suberin, cutin and waxes on 'herbaceous' or succulent organs (e.g. leaves, fruits, etc.), and rhytidome and periderm on woody parts, can all play a very important role as physical barriers. In particular, secondary surfaces like the rhytidome form a 'wall' beyond which a pathogen can seldom penetrate, unless external factors cause preliminary disruption of their continuity. For instance, wounds inflicted on small branches

by extreme weather conditions (e.g., ice and snow cracks), or by the insect *Phloeosinus aubei* Perris, belonging to the Scolitidae (Coleoptera), are essential for infection of various cypresses with the canker agent *Seiridium cardinale* (Wag.) Sutton & Gibson (Phillips & Burdekin, 1982; Covassi *et al.*, 1975).

Physiologically normal lignification and/or suberization of the outermost tissues, such as the epidermis and the bark, may afford remarkable mechanical protection. In many cases, lignification and suberization of cell walls are also effective barriers to enzymic degradation, perhaps the most important mechanism of fungal invasion (Collmer & Keen, 1986). At the hypogeous level, the rhizodermis of the primary root is cutinized to a limited degree (Arrigoni, 1980) and this makes it rather vulnerable to pathogens. On the other hand, the secondary root presents external barriers similar to the periderm of the stem, which confer effective protection against soil-borne pathogens (apart from a few fungi like *Armillaria* spp., which are specifically adapted to penetrating these multicellular barriers).

Within the primary root, the endodermis represents an important preformed tissue defence, in addition to expressing other physiological functions (e.g., it contributes to the regulation of water and solute transport to the vascular tissues - Arrigoni, 1980). The suberization of its walls, and especially the Casparian band on the anticlinal ones, may act as a barrier, at least temporarily, against fungal invasion, e.g. by *Gibberella zeae* on maize (Akai & Fukutomi, 1980).

However, this is of a certain significance only in some plants (monocots and some dicots) where the roots do not exhibit secondary growth: in addition to suberization, the endodermal cells may also display a rather pronounced thickening of their radial and inner tangential walls, which often become lignified (Arrigoni, 1980). This can clearly represent an even more effective barrier to fungal spread towards the stele, often actually limiting the damage by some wilt pathogens (e.g. *Fusarium* sp.) (Akai & Fukutomi, 1980).

1.1.1.1.2 Biochemical

Preformed chemical inhibitors of fungal growth can fall into a number of groups. The main subdivision is perhaps between high and low molecular weight compounds. In the former category, certain enzymes of host origin, notably glucanases and chitinases, may degrade fungal cell walls, which probably disrupts the apical growth of invading hyphae and can result in strong fungal inhibition (Schlumbaum *et al.*, 1986; Pegg & Young, 1981 and references therein).

The other group is mainly represented by aromatic compounds, especially soluble phenols and their derivatives. Typically these chemicals are stored in vacuoles and are released upon rupture of the tonoplast caused by invading agents. They may express antimicrobial activity either in their original state or upon chemical modification into more active configurations: e.g. phenols into free quinones and phenoxy radicals, and/or into polyphenolic compounds like lignins and tannins, these processes being mediated by enzymes

such as phenoloxidases and peroxidases. Such metabolism of phenolic compounds may result in the inhibition of certain fungal hydrolases, e.g. polygalacturonases or cellulases, which allegedly play a major role in pathogenesis (Collmer & Keen, 1986).

Constitutive phenolics are often stored in the plant cell as glucosides, and become more effective inhibitors after separation of the active aglycone from the sugar, a process mediated by glucosidases of either fungal or host origin. One example is presented by two stilbene glucosides (a class of compounds present in many species of the Coniferales), isorhapontin and astringin, and their respective aglycones, isorhapontigenin and astringenin (Woodward & Pearce, 1988[a]). The aglycones are more active than the glucosides from which they derive, and their accumulation in challenged tissues is related to increased activity of ß-glucosidase, which, in this study, was reported to be of fungal origin.

Stilbenes have also been detected in Angiosperms, such as grapevine (Langcake & Pryce, 1976), groundnut (Aguamah *et al.*, 1981; Ingham, 1976) and mulberry (Takasugi *et al.*, 1978).

Other important categories of antifungal compounds in the plant kingdom include saponins, unsaturated lactones, mustard oils and cyanogenic compounds (Schlosser, 1980).

1.1.1.2 Induced defences

1.1.1.2.1 Structural

Despite the fact that the anatomy of epi- and hypogeous portions of higher plants is different, induced structural

barriers can be quite similar in both.

The periderm, which comprises the phelloderm, the phellogen and the phellem (or cork) (Arrigoni, 1980), is a secondary, peripheral tissue. It is present in both the secondary stem and root. Here, the most important response at the tissue level is the so called 'corking out' mechanism. Simply put, wounds of any origin, including lesions inflicted by some pathogens (e.g. *Armillaria* sp., *Heterobasidion annosum)*, may be quite rapidly isolated (and the necrotic tissue excluded) by a layer of cells, which resume cambial activity and become an integral part of the damaged phellogen. This new cambium produces new periderm (necrophylactic periderm), on the lesion side, effectively sealing the wound and expelling the possible inoculum (*cf.* Mullick, 1977; Biggs *et* al., 1984).

In the sapwood, yet another barrier is produced by cell division, again subsequent to wounding, or to invasion by pathogens (e.g. wood decay fungi). This barrier represents 'wall 4' in the CODIT model of Shigo and Marx (1977) and is laid down by the vascular cambium in the vicinity of the wound. The suberization of these cells appears functional in checking a potentially pathogenic invasion, e.g. *Stereum gausapatum* in *Quercus robur* (Pearce & Rutherford, 1981).

The vessels can also respond to pathogens, both in secondary and primary tissues, by forming gel or gum plugs and occlusions called tyloses. Tyloses are bag-like extensions of the vessel parenchyma cell walls, which protrude into the vessels through the membranes of the connecting pits. They

often occupy the whole vessel lumen, actually plugging it, and represent a very effective barrier to the systemic spread of vascular wilt pathogens like *Fusarium oxysporum* and *Verticillium albo-atrum* (Ride, 1983), and other diseases, like *Stereum gausapatum* in *Quercus* spp. (Pearce & Rutherford, 1981), especially when they become encrusted with polyphenolic materials and/or suberin (Pearce & Holloway, 1984; Mai & Abawi, 1987).

In primary tissues, structural barriers are expressed at the cell rather than at the tissue level, since there is no cambial activity present which could produce multicellular barriers.

The only type of structural response known in the plant cell is the apposition of new wall material in the form of papillae and lignitubers. An additional defence mechanism, which cannot be strictly categorized either as a structural or as a biochemical response, is provided by the hypersensitive response.

Cell wall appositions

These can be regarded as the 'classical' type of induced structural response at the cell level. Generally, they consist of a rather rapid thickening of the cell wall brought about by the deposition of new material on the internal side of the wall, ahead of and around the penetrating peg of the fungus. In its most typical form, that of a more or less hemispherical

sheath around the hyphal tip, this structure is called 'papilla'. These can vary quite dramatically, ranging from a generalized thickening of the cell wall to a rather elongated structure, which, when lignified, is alternatively termed 'lignituber' (Beckmann, 1980; Fellows, 1928).

The phenomenon was first described last century by de Bary (1863), who noticed a correlation between the appearance of these structures and infection failures in certain host-pathogen combinations. It was subsequently noticed by many researchers that this process was widespread in the plant kingdom, and, as a consequence, it has received much attention by plant pathologists.

Papillae are very heterogeneous in both their structure and chemical composition. Indeed, papillae can be highly complex morphologically, sometimes showing finger-like projections invaginating the host plasmalemma, and often displaying a variety of microbodies, including membrane fragments, vesicles, and apparently hydrophobic droplets (Aist, 1976).

The main chemical component of the matrix in which all these particles are embedded is usually assumed to be callose, a ß-1,3 linked glucan polymer that is present in all higher plants. This interpretation is the result of histochemical studies carried out on a large variety of species (Aist, 1976).

Many other chemicals have been recognized as constituents of cell wall appositions, as diverse as protein, peroxidases, pectin, cellulose, suberin, gums and silicon (Aist, 1976).

Perhaps the most important to plant pathologists is lignin, because this complex phenolic polymer appears to confer extra resistance to fungal penetration (Vance *et al.*, 1980; Ride & Pearce, 1979; Edwards & Ayres, 1981). The last two authors investigated papilla formation in leaf epidermal cells of *Quercus* spp. infected by the powdery mildew fungus *Microsphaera alphitoides.* The lignification of such structures appeared to confer resistance to the responding leaves, although some degree of variation in speed and intensity of the lignification process itself occurred in different cells of the same leaf, both in resistant and susceptible species. This may account for the odd infected plant in otherwise healthy beds of resistant oak seedlings. The correlation between papilla lignification and cell resistance appears to be consistent with the fact that the disease is most common on seedlings and young shoots of mature plants (Edwards & Ayres, 1981), in which papillae do not lignify, or do so to a lesser extent than in mature leaves and shoots.

The detection of lignin in resistant structures is not always straightforward. Mayama and Shishiyama (1978), for example, were not sure whether the aromatic compounds they detected in barley leaves (by fluorescent microspectrophotometric analysis) were lignin or not, because of negative histochemical tests. Histochemical specificity for lignin, cellulose, callose, pectin, etc., has also been questioned, but despite their known limitations there are no better histochemical tests than the classical ones (Sherwood

& Vance, 1976), such as the Maule test or Phloroglucinol HCl for lignin (the latter, more specifically, for cinnamaldehyde end-groups), Zinc-Chlor-Iodine for cellulose, and Ruthenium red for pectic substances (O'Brien & McCully, 1981). Degradative chemical tests are much more reliable, both qualitatively and quantitatively (Ride, 1983), but they are not always applicable.

The composition of inorganic elements in papillae has only been investigated in crop species (Aist *et al.*, 1988; Heath, 1979[a], 1981a,b; Kunoh *et al.*, 1986). Calcium and silicon accumulate insolubly in papillae of various plants, especially cereals. It has been suggested that both silicon and calcium may strengthen the wall apposition mechanically, making it tougher to fungal penetration. On the other hand, because calcium is a mediator in polysaccharide synthesis, its higher concentration in the papilla area may just reflect the increase in synthetic activity required for the renewed cell wall growth (Kunoh *et al.*, 1986; Kohle *et al.*, 1985).

Papillae have been observed in primary roots of higher plants in response to an array of fungal pathogens, including *Phytophthora* spp. especially. Apart from a few studies on dicotyledonous forest species, like *Acacia pulchella* (Tippett & Malajczuk, 1979), these investigations were mainly carried out on crop plants, both dicots and monocots, such as *Capsicum* sp., *Solanum* sp., *Malus* sp., *Zea mays*, etc. (Hinch & Clarke, 1982).

1.1.1.2.2 The hypersensitive response

Following fungal challenge in incompatible plant-pathogen interactions, a relatively small, necrotized tissue area around the infective structure of the fungus can frequently be observed, and, more or less at the same time, the growth of the fungus is halted. This phenomenon was first described almost 90 years ago by Ward (1902) who was working on the interrelations between *Puccinia graminis*, a rust fungus, and *Bromus* spp., but was named as the 'hypersensitive response' by Stakman only in 1915 (Ingram, 1978).

Kiraly (1980) gives the following more rigorous definition of this type of response: "Hypersensitivity means that a host plant or a non-host plant is more than normally sensitive to an infecting agent and reacts with an early tissue necrosis. A hypersensitive, versus a normosensitive, reaction is a characteristic of a plant resistant to an infecting pathogen in an incompatible plant-pathogen relationship. In fact, this relationship can exist between non-host species and species specific parasites."

After Ward's pioneering studies, a wealth of work has been carried out on this subject, mostly on rust infected cereals. This led to the initial belief that the hypersensitive response was the causal mechanism for host resistance, because the death of infected cells could not conceivably allow for the growth and activity of rust fungi, which are typical biotrophs. However, the discovery that this phenomenon was also apparent with some facultative parasites shed some doubt over this early interpretation, since the dead

host cytoplasm in this type of response should not be, *per se*, inhibitory to the growth of saprophytes.

The hypersensitive reaction has also been associated with the accumulation of induced antimicrobial compounds, formed *de novo* in response to pathogenic challenge, termed 'phytoalexins' (see also page 12). This may account for the hypersensitive reaction as a cause of resistance even in plant-facultative parasite interactions (De Wit, 1987). In fact, the hypersensitive response can sometimes be attributed to the degenerative action of the phytoalexins on the host itself, leading to necrosis of the affected areas (e.g. Franich *et al.*, 1986).

From all this, the understanding of the role of the hypersensitive response in resistance appears confused, and a wealth of studies have suggested that it can be either one of the causal mechanisms of resistance in plants, or simply a consequence of it, depending on the particular host-pathogen combination examined (Ingram *et al.*, 1976; Ingram, 1978).

Although, as mentioned above, most of the research work on the hypersensitive response has been conducted on agricultural species, some examples from forest trees exist. An early report of the hypersensitive response in *Pinus sylvestris* attacked by a *Peridermium* sp. (Hutchinson, 1935) has been followed by other more recent observations. For example, slash pine seedlings (*Pinus elliottii* Engelm. var. *elliottii)* exhibited hypersensitivity in response to *Cronartium fusiforme* attacks, but only at the bark level

(needles appeared not to be able to respond in any way - Miller *et al.*, 1976). The hypersensitive response was also observed in the bark of *Pinus monticola*, at the base of infected needles under attack by blister rust (Hare, 1972), and in the association *Quercus velutina* (black oak) - *C. fusiforme* (Dwinell, 1969).

1.1.1.2.3 Biochemical

1.1.1.2.3.1 Low molecular weight compounds

As a rigorous definition, low molecular weight, pathogen induced antimicrobial compounds formed *de novo* from remote precursors are termed 'phytoalexins' (Mansfield, 1983; Bidwell, 1978; Kuc', 1972; Deverall, 1976).

Although phytoalexins are, by definition, formed from remote precursors, the metabolic pathways involved in their biosynthesis commonly operate in the healthy plant to produce chemically related secondary metabolites like, for instance, phenolic substances (see below). The physiologically normal metabolic pathways and the ones that lead to phytoalexin biosynthesis may diverge only at certain key points (Bidwell, 1978): in terms of energy allocation this is certainly more advantageous to the plant than the formation of entirely new compounds.

Most of the phytoalexins so far discovered come from herbaceous or, more generally, crop species; their chemical composition is extremely diverse throughout the range of plants studied, and ranges from hydroxyflavans in *Narcissus*

bulbs, to polyacetylenic compounds in safflower stems (Mansfield, 1983). Investigations have been carried out also on, and phytoalexins detected in, woody angiosperms such as *Ulmus glabra, Tilia europaea, Acer* spp., etc.. Very often they are derivatives of aromatic compounds, including lignans, flavonoids, and alkaloids (Kemp & Burden, 1986).

A number of phytoalexin-like compounds have also been reported from the Coniferales. Although derived from rather incomplete evidence, most authors consider pinosylvin, and its derivatives pinosylvin monomethyl ether, pinosylvin dimethyl ether, and the phenylpropanoid derivative eugenol dimethyl ether (the first two usually found in the sapwood, the other two in needles after attack by *Peridermium pini* - Kemp & Burden, 1986) as the most important phytoalexins in *Pinus sylvestris* and other species of the Pinaceae (Shain, 1967; 1971; 1979). However, recent observations have shown that pinosylvin is constitutively present in this species, at least in tissues other than the sapwood, e.g. in the needles (Sandermann *et al.*, 1989; Pearce, personal communication), strongly suggesting that pinosylvin should not perhaps be considered a true phytoalexin.

Other compounds regarded as conifer phytoalexins are pinocembrin, a flavanone present in the sapwood of *Pinus radiata* and *P. taeda,* and taxifolin, another flavonoid present in roots of *Pseudotsuga menziesii* (Kemp & Burden, 1986).

Benzoic acid may also be considered a conifer phytoalexin: it has been shown to accumulate in infected

needles of *Pinus radiata*, and to be strongly fungistatic against *Dothistroma pini*, a major pathogen of this pine. Benzoic acid may also cause some of the symptoms observed on this plant when attacked by this pathogen (Franich *et al.*, 1986).

1.1.1.2.3.2 High molecular weight compounds

Pathogenic induction of high molecular weight compounds, i.e. polypeptides and proteins, has in recent years also come to the attention of plant pathologists, notably with studies on Pathogenesis Related-proteins (PR-proteins, Antoniw *et al.*, 1980).

PR-proteins are produced in a number of plant species as a response to abiotic and/or biotic agencies. They are proteins that either appear in greatly increased amounts, or appear *de* novo in some microbially challenged plants, or in plants subjected to certain types of physical and chemical disturbances, including, among others, heat stimuli (Wildon *et al.*, 1989) and gaseous pollutants (Sandermann *et al.*, 1989). There are also reports of PR-proteins induced by nematodes (Hammond-Kosack *et al.*, 1989), although most of the work has been carried out on virus-infected plants and, since the early 80's (Gianinazzi *et al.*, 1980; de Wit & Bakker, 1980), on fungus-challenged plants.

The PR-proteins so far investigated exhibit a range of common features: they often are acid-extractable (down to pH 2.8, Antoniw & White, 1983), low molecular weight proteins, and have an extracellular localization (Hammond-Kosack *et al.*,

1989; Parent & Asselin, 1984; Redolfi, 1983).

Almost all of the species that so far have shown this phenomenon are Angiosperms. Indeed, the first reports regarding the occurrence of PR-proteins, in the early 1970's, concerned crop plants, notably tobacco (Gianinazzi *et al.*, 1970; Van Loon & Van Kammen, 1970). During the 1980's, and up to the beginning of the 1990's, much work has been conducted on a number of other species, including parsley (Knogge *et al.*, 1987), cucumber (Metraux *et al.*, 1988), tomato (Christ & Mosinger, 1989; Garcia Breijo et *al.*, 1990), bean (Janicka, 1987), and barley (Bryngelsson & Green, 1989), and several PR-proteins have been purified and characterized. Perhaps the most interesting results indicate that some of the observed PR-proteins exhibit chitinolytic and 1,3-ß-glucanohydrolytic activities (Boller *et al.*, 1983; Boller & Metraux, 1988; Garcia Breijo *et al.*, 1990; Kauffmann *et al.*, 1987; Legrand *et al.*, 1987; Metraux *et al.*, 1988), which may confer antifungal properties (Schlumbaum *et al.*, 1986). Therefore, these proteins may be involved in plant protection, at least against fungal pathogens, even systemically (Redolfi, 1983; Kuc', 1987; Ye *et al.*, 1989). In relation to this last aspect, the presence of PR-proteins in tissues located at some distance from the site of the primary disturbance has been attributed to the spread of a hypothetical chemical inducer and not to the translocation of the proteins themselves (Van Loon & Antoniw, 1982). This is strongly reminiscent of the Systemic Induced Resistance mechanisms so far suggested (see page 17), and indeed the two concepts have already been associated (Ye

et al., 1989).

Once again practically nothing is known about Gymnosperms in this respect. The only case in which PR-proteins have been detected in Gymnosperms involves *Pinus sylvestris*. In that study, PR-proteins (named as stress proteins) were detected in mature needles of plants subjected to ozone treatment (Sandermann *et al.*, 1989). No investigation has yet considered the possible appearance of these proteins in conifers undergoing fungal attack.

1.1.2 Defences in roots of woody plants

Most of what has been said for secondary tissues in general can be applied here for the secondary roots, where the responses to the major root- and butt-rotting fungi, such as *Heterobasidion annosum* (Tippett & Shigo, 1980), and *Phaeolus schweinitzii* (Woodward & Pearce, 1988[a,b]) have been investigated, although available information is far less complete than it is for the stem.

The literature on the defences in primary roots of forest trees against soil-borne pathogens is quite scanty. For a number of reasons, not least perhaps because of the technical difficulties involved in these studies, only a few reports exist of responses to fungal invasion in feeder roots. The hypersensitive response has only been observed in some ectomycorrhizae produced *in vitro* (Duddridge, 1986[a,b]; Molina, 1981), in which it has often been considered a symptom of incompatibility, although macroscopically normal mycorrhizae

were usually formed. The few existing examples of papilla formation in primary roots of forest species refer to ectomycorrhizae (Duddridge, 1986[a,b]; Duddridge & Read, 1984[a,b]; Nylund *et al.*, 1982; Nylund & Unestam, 1982), and to non-mycorrhizal roots of *Acacia pulchella* (Tippett & Malajczuk, 1979).

No studies of constitutive or induced low molecular weight antimicrobial compounds in primary roots of forest trees are known, apart from those on the production of antibiotics by mycorrhizal roots (*cf.* 1.3.2).

1.2 Systemic induced resistance

Systemic induced resistance (SIR), also termed acquired resistance, acquired immunity, immunization and induced sensitization, is a process comparable (in outcome, but not in nature) to the immunization phenomenon in animals. Essentially, if a potential host is pretreated with a hypovirulent strain of a pathogen, a nonpathogen, or a heat-killed or attenuated pathogen (Kuc' *et al.*, 1975), it may subsequently display resistance to a virulent strain, or even to a different parasite, and this resistance will be expressed systemically, i.e. all over the organism, including parts that were not directly pretreated.

According to Kuc' (1983), a restricted necrosis or injury is a *sine qua non* condition for SIR elicitation, and it appears that the key to SIR is a low level, persistent metabolic stress. This stress may even be abiotic in nature:

in numerous examples, natural and synthetic chemicals have been reported to induce systemic resistance (Kuc', 1987; Dean & Kuc', 1987).

The dynamics of SIR is poorly understood, although the literature on the subject can give some clues. The elicitors could be quite similar to the ones involved in the more general phytoalexin accumulation process (e.g. plant cell wall fragments released upon fungal degradative action, glucans of fungal origin, etc.), and even systemic electric disturbances, caused by injury or disease, may be involved (Dean & Kuc', 1987; Wildon *et al.*, 1989). SIR expression appears to consist of a potentiation, or a more prompt deployment, of known defence mechanisms, such as papilla formation, lignification, induction of phytoalexins and plant hydrolases, (Kuc', 1987; Dean & Kuc', 1987), including PR-proteins (Ye *et al.*, 1989).

If the dynamics of SIR is still imperfectly understood, its outcome is quite promising, with a reduction in diseased area of over 90% in certain cases (Dean & Kuc', 1987), and with convincing evidence of disease protection in the field (Caruso & Kuc', 1977[b]).

Furthermore, SIR is persistent in time (up to the plant's lifetime in certain cases), and can be transmitted from stock to scion in grafted plants (Kuc', 1983; 1987).

Once again, the bulk of the literature on this subject regards crop species, and there are no reports of investigations on forest trees.

1.3 Mycorrhizae and defence

1.3.1 General

Mycorrhizae are mutualistic associations between some fungi and primary roots of plants. In nature mycorrhization appears to be the rule, rather than the exception (Harley & Smith, 1983). Mycorrhizae are found in the four divisions of the plant kingdom in which plants have some sort of root system: in the Bryophyta and the Pterydophyta, which have rhizoids, the Gymnospermae, and the Angiospermae, which produce true roots (Gellini, 1973; Harley & Smith, 1983).

Anatomically, mycorrhizae are placed in two main groups: ectomycorrhizae and endomycorrhizae. According to the type of association, the fungi may belong to the Zygomycotina (vesicular-arbuscular (VA) and some other endomycorrhizae), or to the Ascomycotina and Basidiomycotina (usually, but not always, ectomycorrhizae). This classification is far from being rigorous, however: in fact the same mycorrhizal fungus may behave either as an ectomycobiont or as an endomycobiont, according to the particular host and ecological situation with which it has to cope (Duddridge, 1987). Since ectomycorrhizae are the predominant form (Harley & Harley, 1987) on the host species used in this thesis, *Pinus sylvestris*, other groups will not be considered for further discussion (for more comprehensive and exhaustive information see Harley & Smith, 1983).

The ectomycorrhizal association brings about a radical modification of both the root and fungal anatomy, acquiring

morphology and physiology of its own.

Ectomycorrhizae are characterized, as all mycorrhizae, by a very intimate anatomical relationship between the two partners. In this group the fungus normally penetrates the root tissues only intercellularly, inducing a dramatic change in the appearance of the rhizodermal and cortical cells and changing its own growth pattern from hyphal to pseudoparenchymatous. This fungal pseudoparenchyma envelopes cortical and rhizodermal cells, almost separating them from each other, although symplastic continuity is maintained (Harley & Smith, 1983). This structure is termed the Hartig net. Another special characteristic of an ectomycorrhiza is the presence of the mantle, a fungal pseudoparenchyma which covers the root as a sheath and which can be viewed as a continuation of the Hartig net on the root surface. In a few cases direct penetration of host cells can be observed (Nylund *et al.*, 1982; Garza, unpublished).

Quite often ectomycorrhizae show an extensive network of hyphae (extramatrical mycelium) and hyphal strands or rhizomorphs, radiating from the infected roots into the soil, growing outwardly for considerable distances, and representing a very important means of spread of the fungus to other potential hosts (Brownlee *et al.*, 1983). This is perhaps the most important way for the spread of an ectomycobiont, spores being not always so efficient (Fox, 1983).

These symbioses are traditionally seen as fine examples of mutualistic relationships, although, in reality, there

certainly is a gradient (Wilcox, 1983) between pure parasitism of the fungus on the plant (e.g. all fungal pathogens) and pure parasitism of the plant on the fungus (e.g. orchids at the germination stage).

In the most classical and simplistic view, the fungus, usually a typical biotroph, exploits the amino acid and carbohydrate production by the plant. In respect to the latter, Harley and Smith (1983) suggest that the fungus may modify, at the Hartig net level, the activities of the plasmalemma of the host, causing an inhibition of cell wall development, the fungus being able to utilize cell wall precursors in the form of mono- or oligosaccharides. In return, by increasing the absorption of mineral nutrients (especially phosphates) and water, the fungus proves to be useful to the plant. The extramatrical fungal hyphae practically represent an extension of the phytobiont, and serve the important function of increasing greatly the volume of soil which can be explored and utilized by the plant's root system.

It is well known that mycorrhization confers a remarkable competitive advantage to the establishment and good growth of forest seedlings and mature plants (e.g. Stenstrom & Ek, 1990). Among the proposed sources of this ecological advantage are the nutritional benefits described above, but an apparently enhanced protection against soil-borne pathogens may also be involved.

1.3.2 Defence mechanisms

As Zak (1964) stated: "mycorrhizal fungi may conceivably afford protection to the root by (a) utilizing surplus carbohydrates, thus reducing attractiveness of the root to pathogens; (b) providing a physical barrier; (c) secreting antibiotics; and (d) favouring, along with the root, protective rhizosphere organisms". In the same paper the author hypothesized that fungal antibiotic metabolites may be transferred to the host and translocated therein to other non-mycorrhizal parts, thus offering a more generalized protection of the host. Indeed, Krywolap *et al.* (1964) demonstrated that an antibiotic substance was translocated to the needles of *Pinus strobus* and *P. resinosa* from *Cenococcum graniforme* mycorrhizae. Although not characterized, it appeared that the same chemical was present in extracts from pure cultures of the fungus. Rather more interestingly, Marx (1972) also postulated that, in response to the mycobiont, the host itself may produce certain substances which may have an inhibitory effect on pathogens, even by translocation to other organs.

Among the antibiotics a major role is probably played by certain volatile compounds, such as fungistatic monoterpenes, which are produced in much larger quantities in mycorrhizal than in non-mycorrhizal roots, and perhaps by some antifungal diterpenes (Toyota & Hostettmann, 1990). Oxalic acid, whose production by *Paxillus involutus* is remarkably stimulated by the presence of the host *Pinus resinosa*, seems also to be a candidate for fungistasis/fungitoxicity (Duchesne *et al.*, 1989). This antibiosis, along with the different range of

exudates actually diffusing from the mycorrhiza into the soil (the 'mycorrhizosphere'), modifies the rhizosphere microflora both qualitatively and quantitatively, bringing about a general suppression of feeder-root pathogens (Katznelson *et al.*, 1962; Perrin & Garbaye, 1983; Duchesne *et al.*, 1987).

More recently the production of chitinase has been described in ectomycorrhizae, as an induced response in roots of *Picea abies* (L.) Karst. inoculated with *Amanita muscaria* (Sauter & Hager, 1989). An induced change in chitinolytic activity was also recorded for the endomycorrhizal association *Allium porrum-Glomus versiforme* (Spanu *et al.*, 1989). In this study, immunocytochemical localization studies of the chitinase indicated that a role for this enzyme in the control of the mycorrhizal infection is probably to be excluded. To date, nothing is known about the possible role of these mycorrhiza-induced chitinases in mycorrhiza-pathogen interactions. However, these responses are reminiscent of the concept of PR-proteins, which, *per se*, has never been investigated in roots.

A mechanical effect of the mantle was also demonstrated by Marx and Davey (1969[a,b]) and Marx (1970), who were working with *Phytophthora cinnamomi*. They concluded that this is a very effective barrier to pathogenic invasion. They also suggested that an important role is played by the Hartig net in stopping infections that are already in progress, but did not clarify the mechanisms by which this barrier may be afforded.

Despite the work described above, the protection of roots against potential pathogens by the mycorrhizal association has received rather little attention during the past decade.

1.4 Plant-fungus specificity

A host-parasite association is said to be specific when the parasite can infect only a particular host, whether it is at the species (species-specificity) or at the population level (e.g. cultivar-specificity).

For all major soil-borne fungal pathogens, such as *Pythium* spp., *Phytophthora* spp., *Fusarium* spp., etc., the host range is quite wide. Indeed, many of these pathogens may thrive even in the absence of a host because, being facultative parasites, they also have the possibility of surviving in the soil by feeding on dead organic matter (saprotrophy).

Phytophthora cinnamomi, an Oomycete, is a good example of a non-specific pathogen: it attacks over 950 species and cultivars, albeit mainly those of woody plants (Zentmyer, 1980), distributed over a large number of families, among both Gymnosperms and Angiosperms. *Phytophthora* spp. are soil-borne but they can usually infect aerial parts as well (by rain-splash dispersal), displaying low specificity also at the tissue level.

A low specificity is also observed in ectomycorrhizal associations. Most mycorrhizal fungi can form mycorrhizae with a variety of hosts, and *vice versa* (Duddridge, 1987). The

clearest and most striking example of the latter case is the so called 'mycorrhizal succession', the concept that different fungi can form mycorrhizae at different times and locations on the same root system (Gibson & Deacon, 1988; Last *et al.*, 1984; Mason *et al.*, 1983).

True ectomycorrhizal specificity is thus rare. A classic example is *Suillus plorans* and *Pinus cembra* (Moser, 1978). *Suillus grevillei* and *Larix* spp. is also considered to be a specific relationship, although the mycobiont has been shown to form mycorrhizae *in vitro* with *Pinus sylvestris* and *Pseudotsuga menziesii* as well (Duddridge, 1986[a,b]). These results suggest that *Suillus grevillei-Larix* spp. might then be a case of ecological, rather than physiological, specificity, and indeed the same conclusions might possibly be inferred also for the *S. plorans-P. cembra* combination. Despite the fact that in Duddridge's experiments *P. sylvestris* and *P. menziesii* became mycorrhizal with *Suillus grevillei*, it was clear that mycorrhization did not occur very easily; and electron microscopy showed that the symbionts may not even be completely compatible physiologically, because the atypical hosts produced wall appositions and displayed signs of hypersensitive response which are usually symptomatic of incompatibility (see page 10). One explanation for the successful syntheses *in vitro* may lie in the high exogenous carbohydrate levels usually present in the synthesis systems (Duddridge, 1986[a,b]), although this has not been clearly demonstrated so far.

In naturally occurring compatible associations there seem

to be some sort of recognition phenomena involved, which come into play only once the fungal hyphae are in contact with the root surface (Duddridge, 1987). Contact (thigmotropic) stimuli are certainly important because chemotropic signals through root exudates, which promote growth of the fungus towards the root, are non-specific. Once the mycobiont has been recognized, it becomes attached to the root surface much more tightly in compatible than in incompatible associations. In the former case, polysaccharides, especially pectic substances, seem involved in cementing the fungal hyphae to the host cell walls (Anderson, 1985).

The ectomycorrhizal relationship is clearly a fine balance between the two organisms, with the process appearing to be controlled mainly by the host (Nylund *et al.*, 1982). These authors noticed that papilla formation was associated with early penetration stages of *Piloderma croceum* in senescent cells within the live cortex of *Pinus sylvestris*. They also observed direct penetration of the same type of cells, in a manner which suggested enzymic lysis of the cell wall. They reached the conclusion that "the intercellular growth pattern of the fungi is thus not a consequence of fungal inability to penetrate host tissue, but a result of host control of the mycobiont". In fact, penetration was never observed in healthy young cells, suggesting that these are capable of warding off a potentially damaging fungal attack, therefore leading to mycorrhizal equilibrium.

Other factors are also likely to take part in the regulation of the mycorrhizal equilibrium. The mycorrhizal fungus must be able to defend itself from the plant's general responses to invading organisms. Avoiding intracellular penetration in ectomycorrhizae might be one way for the fungus to bypass cellular defences in the first place. In endomycorrhizae, and some ectomycorrhizae, other processes may operate. General biochemical plant defences are often based on the metabolism of phenolic compounds: the fungus may be able to circumvent these and other biochemical responses, for instance by producing superoxide dismutases and catalases, capable of opposing the activity of the superoxide anion, hydrogen peroxide, and of such host enzymes as peroxidases, laccases, etc. (Anderson, 1985). These last enzymes are involved in the production of phenoxy radicals (antimicrobial activity), and of lignin-like materials (by condensation). The latter are, as described earlier, very important components of induced structural barriers.

Additionally, several other factors, known to have a role in plant-pathogen interactions, may also be involved, including the abilities to metabolize/detoxify antimicrobial compounds, to suppress genetically their formation/accumulation, or to avoid their elicitation (Anderson, 1985; Heath, 1981[a]).

1.5 Aims and objectives

From the above it is apparent that very little is known about the responses of the primary root system of woody plants to pathogens, despite the fact that 'damping off' of tree seedlings in forest nurseries can be quite a serious problem (Gradi, 1980; Phillips & Burdekin, 1982; Peace, 1962; Smith *et al.*, 1988).

The present research was undertaken in order to understand more fully the nature and role of the antimicrobial defences of the feeder roots of conifers. Roots are commonly exposed to a wide variety of potential pathogens, often under conditions that may be more conducive to fungal infection than the aerial surfaces (e.g. pH conditions favourable to pathogens, waterlogging).

Besides analysing non-mycorrhizal roots, the occurrence of host responses in the regulation of the mutualistic mycorrhizal association was also examined, and their possible contribution to the reported enhanced resistance of mycorrhizal roots to infection discussed.

Furthermore, the role of SIR in forest seedlings, particularly in relation to mycorrhizal plants, was investigated. Protection of seedlings by mycorrhization may be expressed systemically, but this has never been clearly established.

1.5.1 The host

Pinus sylvestris is a temperate, tendentially microthermal conifer, typically suited to continental climates and to sub-alkaline (calcareous), well drained soils. It is rather xerophylous, especially in its southernmost areal limit, but it can also adapt to fairly moist, sub-acidic soils (e.g. some parts of the heathlands in Lombardy, Italy). Like most other Pinaceae it is heliophylic, albeit not to a very high degree (less than *Larix decidua* Mill., for instance) (de Philippis, 1957).

Silviculturally, *P. sylvestris* plays a secondary role in the British Isles, despite its rather widespread presence in the Scottish highlands. However, *P. sylvestris* is well represented, and has considerable economic importance, in other European areas, like the central-eastern Alps, where it also constitutes an important factor in soil protection (e.g. the Alto Adige region in Italy).

This host was chosen for the present study because:

a) it is a good candidate as a member of the Coniferales, because of its widespread use in forestry;

b) it is rather well represented in the literature on Forest Pathology, which means there is a relatively extensive database to refer to;

c) it forms associations with a wide range of mycorrhizal fungi rather easily under controlled (including *in vitro*) conditions.

1.5.2 The pathogen

Cylindrocarpon destructans (Zins.) Scholt. is the anamorph of *Nectria radicicola* Gerlach & Nilsson, an ascomycete in the order Hypocreales. *Nectria radicicola,* a fungus displaying a large variability (Samuels & Brayford, 1990), is soil-borne, and is considered to be a weak pathogen (Ehle, 1988); these characteristics are also common to its anamorph. *C. destructans* and other species of the same genus, are generally found on, and in, the seeds and roots of a large variety of plants: from ornamentals like Gerbera (Kaewruang *et al.*, 1987) (pathogenic), to crop plants like potato (Gindrat & Pilloud, 1985) (pathogenic and saprophytic), soybean (Lee, 1984) (saprophytic), ginseng (Matuo & Miyazawa, 1984) (pathogenic), and grapevine (Grasso, 1984) (pathogenic), to conifer seedlings, like *Pinus sylvestris* (Chakravarty & Unestam, 1987[a]; Dahm & Strzelczyk, 1987) (pathogenic and saprophytic), *Pinus pithyusa* (Kizikelashvili, 1985) (pathogenic), and *Abies alba* (Dahm & Strzelczyk, 1987) (pathogenic and saprophytic). *Cylindrocarpon* spp. have also been isolated from aerial organs of declining, mature *Quercus* spp. (Ragazzi *et al.*, 1989) (pathogenicity unclear), and, as a secondary parasite, from declining *Larix decidua* (Horn, 1985).

From these and other reports it appears that the species of the genus *Cylindrocarpon* are soil fungi exhibiting very low host specificity, and behaviour ranging from saprophytic to parasitic, and pathogenic. Pathogenicity often arises from stressful circumstances for the host, such as anomalous soil

conditions (like drought with *Quercus* spp. - Ragazzi *et al.*, 1989), air pollution (like with *Larix decidua* - Horn, 1985), established disease, or light starvation (Chakravarty & Unestam, 1987[a]).

This fungus, therefore, generally exhibits low levels of pathogenicity. This is a desirable characteristic for this study, because highly pathogenic microorganisms may not allow the host to express its potential defences, either because the speed of invasion is too high, giving the host insufficient time to react, or because the pathogen may actually suppress the defence mechanisms of the host.

1.5.3 Mycorrhizal fungi

The following ectomycobionts were selected for this study: *Hebeloma crustuliniforme* (Bull. ex St. Am.) Quel., *Pisolithus tinctorius* (Pers.) Coker & Couch, and *Suillus bovinus* (Fr.) O. Kunz. The choice was dictated by the relative ease with which these microorganisms can be grown and maintained in pure culture, their established position in the mycorrhizal research field (as evident from the previous paragraphs), and the fact that they all easily form ectomycorrhizae with *P. sylvestris*.

CHAPTER 2

General materials and methods

2.1 Plant material

2.1.1 Identity and origin

Pinus sylvestris seeds [Forestart, Shrewsbury, U.K. (Identity Numbers: 86(2039)lot2F and 88(2039)F; region and country of provenance: Altyre, Scotland, U.K.], were stored in a glass jar at 4°C until use.

2.1.2 Cultural conditions

Seeds were surface sterilized in 30% H_2O_2 for approx. 30 min. They were then plated out on sterile 0.8% deionized water agar (Oxoid No.3) and germinated at approx. 20°C in a growth cabinet with a 16h photoperiod. Lighting was provided by 3X30W Gro-Lux fluorescent tubes (Thorn) plus 2X25W incandescent bulbs. Total photon flux density, measured at the centre of the growth cabinet floor with a Silicon Cell Quantum Sensor for Photosynthetically Active Radiation (Delta-T Devices Ltd, Cambridge, UK), was 16µmol m^{-2} s^{-1}. A Solartron 7040 (Schlumberger) current detector was used.

Either of the two following methods was used for seedling establishment, depending on the specific experiment.

2.1.2.1 Glass jar system (Fig. 2.1)

Non-contaminated seedlings were transferred to sterile 250ml glass jars containing approx. 100ml of 0.8% deionized

Figure 2.1. Growth and inoculation systems for *P. sylvestris* seedlings. Both the petri dish and the glass jar contain 0.8% deionized water agar. The fungal inoculum plugs (*C. destructans*) are visible in both the petri dish (3 plugs) and the glass jar (agar surface).

water agar (10-20 seedlings per jar). Alternatively, 500ml glass jars (Kilner jars, Ravenhead, UK) containing approx. 200ml of 0.8% deionized water agar were used (30-40 seedlings per jar). In both cases care was taken in delicately embedding the root systems in the agar. The jars were then loosely capped and maintained in the growth cabinet described above until ready for use.

2.1.2.2 Petri dish systems (Fig. 2.1)

A derivation of a method developed by Duddridge (1986[a,b]) was employed. Aseptically germinated Scots pine seedlings were introduced into sterile plastic petri dishes (diameter = 9cm), containing approx. 25ml of 0.8% deionized water agar, through a notch cut on the rim of the dish with a hot scalpel, so that only the roots were enclosed in the chamber. Care was taken in embedding the roots in the agar medium. The plates were then sealed with PVC tape. The notch, through which the seedling hypocotyl was placed, was sealed with lanolin. The petri dishes, with the seedlings upright, were kept in a covered propagator, on a window sill, for a week, at the end of which they were uncovered. They were then kept on the window sill for some time before use to check for growth of contaminants on the root systems. Contaminated plates were discarded.

2.2 Fungal material (inoculum)

2.2.1 Identity and origin

2.2.1.1 Pathogens

5 pathogenic fungi were used at various stages of the work, of which 3 are root pathogens (*Cylindrocarpon destructans*, *Pythium oligandrum* and *Phytophthora citricola*) and 2 foliar pathogens (*Cladosporium cucumerinum* and *Botrytis cinerea*).

Cylindrocarpon destructans (Zins.) Scholt., isolated from the collar region/roots of seedlings of *Larix* x *eurolepis* Henry & Flood (isolate n. 86/1279/110.43, identified by CMI), *Pythium oligandrum* Drechsler, isolated from the roots of *Picea sitchensis* (Bong.) Carr. seedlings (isolate n. 87/1109/143.6, identified by CMI), and *Phytophthora citricola* Sawada, isolated from seedling roots of *Picea sitchensis* (isolate n. 87/1053/115.5, identified by CMI), were kindly provided by the Forestry Commission Northern Research Station, Roslin, Midlothian, U.K..

Cladosporium cucumerinum Ellis & Arthur and an isolate of *Botrytis cinerea* Pers ex Pers, from an infected tomato, were from stocks maintained in the Forest Pathology Laboratory of the Oxford Forestry Institute.

2.2.1.2 Mycorrhizal fungi

Pisolithus tinctorius (Pers.) Coker & Couch was from stocks maintained in the Forest Pathology Laboratory of the Oxford Forestry Institute. *Hebeloma crustuliniforme* (Bull. ex St. Am.) Quel. and *Suillus bovinus* (Fr.) O. Kuntz were kindly

provided by Prof. D. Read (University of Sheffield, U.K.).

2.2.2 Cultural conditions

2.2.2.1 Pathogens

C. destructans, *P. oligandrum* and *Phytophthora citricola* were maintained on 2% malt extract (Oxoid) agar; *C. cucumerinum* was maintained on potato dextrose agar (PDA - Oxoid); *B. cinerea* was maintained on RMBX medium (see 2.4 for recipe). All fungi were grown at 25°C.

2.2.2.2 Mycorrhizal fungi

All mycorrhizal fungi were generally maintained at 25°C on modified Melin-Norkrans (MMN) medium as described by Marx (1969[a,b]), except that glucose was used in place of sucrose (see 2.4 for recipe).

2.3 Inoculation procedures

2.3.1 Host-pathogen interactions

C. destructans was chosen because of its relatively weak pathogenicity on *P. sylvestris* (see chapter 1), a useful characteristic aiding the detection of host defence mechanisms. Conversely, *P. oligandrum* and *P. citricola* were selected for their virulence, a more appropriate feature for determining levels of protection and/or resistance.

2.3.1.1 Glass jar systems (Fig. 2.1)

Between four and eight weeks from the time of transfer of the pine seedlings, the jars were inoculated with 5 plugs each

of 2 weeks old mycelial cultures of *C. destructans*, by placing the plugs, mycelium down, on the surface of the medium. The jars were then incubated in the growth cabinet for a period ranging from 2 to 4 weeks, at the end of which seedlings were collected for examination.

2.3.1.2 Petri dish systems (Fig. 2.1)

The petri dish systems were usually inoculated with *C. destructans*, *Phytophthora citricola*, or *Pythium oligandrum*, at the time of seedling transfer. Generally, 3 plugs (diametre = 10mm) of mycelial culture taken from the margins of actively growing colonies were placed, mycelium down, on the surface of the water agar. Differences in timing of inoculation, and/or in number and size of inoculum plugs, are noted for specific experiments.

2.3.2 Host-mycorrhizal fungus interactions

The petri dish system described in the previous paragraph was used for this study, with the following modifications. The petri dishes contained a modified MMN medium (glucose omitted, 10^{-1} original concentration, and with a 0.8% agar content),

After approx. 3 more weeks of incubation to check for contaminated seedlings (which were to be discarded) the plates were opened and a number (10-20) of 4mm plugs of mycelial cultures of mycorrhizal fungi were placed, mycelium down, on the agar surface, on both sides of the root systems (essentially as described in 2.1.2.2). The plates were then sealed again and plants grown on the window sill until needed.

Figure 2.2. A petri dish system for the synthesis of ectomycorrhizae in a peat-vermiculite medium (from Duddridge, 1986[a,b]). The inoculum plugs are not visible because the reverse of the plate is pictured to show the lateral roots of a *Pinus sylvestris* seedling mycorrhizal with *Pisolithus tinctorius* (arrows).

Ectomycorrhizae were also synthesized according to a partially modified version of the method of Duddridge (1986[a,b]) in petri dish systems containing a peat-vermiculite mixture (Fig. 2.2). Sieved peat (P) (particles < 3.35mm), and expanded vermiculite (V) (particles > 3.35mm) were mixed in the following proportion: P:V (v:v) = 0.25. To this P-V mixture, a modified MMN solution (1/10 strength, glucose and agar omitted) was added in the ratio MMN:P-V (v:v) = 0.4. The petri dishes were inoculated with several 10mm plugs of mycelial cultures at the time of insertion of the pine seedlings (see also 2.1.2.2).

2.4 Media for fungal cultures

Two media were used which were not commercially available: modified Melin-Norkrans (MMN) medium (Marx, 1969[a,b]), for mycorrhizal fungi, and RMBX (modified by Ride, after Last & Hamley, 1956) for the culturing and sporulation of *Botrytis cinerea*. Recipes as follows:

MODIFIED MELIN-NORKRANS medium

		Concentration of mineral elements
Agar	15.00 g	
$CaCl_2$	0.05 g	4.5×10^{-4} M
$FeCl_3$ (1% sol.)	1.2 ml	7.4×10^{-5} M
Glucose	10.0 g	
KH_2PO_4	0.5 g	3.67×10^{-3} M
Malt Extract	3.0 g	
$MgSO_4 \cdot 7H_2O$	0.15 g	6.0×10^{-4} M
NaCl	0.025g	4.27×10^{-4} M
$(NH_4)_2HPO_4$	0.25 g	1.8×10^{-3} M
Thiamine HCl	100.00 µg	
Deionized water	to 1.00 l	

(N.B.: glucose substituted for sucrose in original formulation)

RMBX medium

Agar		30.00 g
Casein hydrolysate		3.00 g
Dextrose		10.00 g
KCl		0.52 g
KH_2PO_4		1.52 g
$MgSO_4 \cdot 7H_2O$		0.52 g
$NaNO_3$		6.00 g
Peptone		2.00 g
Yeast extract		0.50 g
Deionized water	to	1.00 l

2.5 Preparation of fungal spore suspensions

Spore suspensions of *Cladosporium cucumerinum*, *Cylindrocarpon destructans* and *Botrytis cinerea* were prepared as follows.

Sporulating petri dish cultures were flooded with approx. 10ml of autoclaved Czapek Dox liquid medium (Oxoid) (*Cladosporium cucumerinum* and *Cylindrocarpon destructans*), or autoclaved deionized water (*B. cinerea*). Conidia were dislodged using a flame-sterilized loop previously dipped in Tween 80, a surfactant which helps in wetting the spores. The crude suspensions were then filtered through 4 layers of muslin. *C. cucumerinum* and *C. destructans* conidia were used in bioassays for the detection of antifungal compounds on thin layer chromatography (TLC) plates (Homans & Fuchs, 1970), on which spore suspensions were sprayed using a laboratory spray gun (Shandon Southern Products Ltd, U.K.). *B. cinerea* conidia were used for aerial inoculations of Scots pine seedlings (see 6.2.1).

2.6 Chemicals

Deionized water used for media preparation was obtained ufrom a deionizer (Elga, U.K.), with or without coupling to a reverse osmosis system (Elgastat Spectrum, cartridge type SC 32, Elga, U.K.). Water for HPLC work was equivalent to double distilled, purified with a Milli-Q water system (Millipore Corp., U.S.A.).

Chemicals were generally of Analar grade or otherwise of the highest grade available.

Solvents for HPLC work were of HPLC grade (HyPerSolv, BDH, U.K.); solvents for extraction and routine work were glass redistilled from Analar or GPR grade by the author.

2.7 Procedures for cleaning glassware

Glassware was either washed manually using the phosphate-free detergent Decon 90 (Decon Laboratories Ltd., U.K.), or machine-washed in a Gallay Lab 901 automatic washer using the phosphate-free, Hamo Solid 02 (Hamo AG, CH) detergent. In either case the glassware received two or more final rinses with deionized water obtained as described in 2.6.

CHAPTER 3

Defence responses to pathogenic infection in non-mycorrhizal primary roots of *Pinus sylvestris*

3.1 Introduction

3.1.1 Structural defences at the cell level

Cell walls - general

Far from being inert structures, plant cell walls play an integral part in cellular metabolism. The mechanical function, albeit the most important, is just one of their many characteristics, among which is their role in cell-cell communication, both within the plant itself and with non-self cells, including plant pathogens (Ralton *et al.*, 1987).

Due to its principal function, i.e. mechanical, the cell wall can be plastic or hard, elastic or stiff, according to its specific role in the various tissues of the organism. Most importantly in the context of this work, in possibly all stages of its life it is highly dynamic (Arrigoni, 1980; Bidwell, 1978).

Cell walls - role in plant defence

In view of a possible role in plant defence, the chemical composition of the cell wall is obviously important, since a pathogenic organism can be adversely affected by its immediate environment, especially when faced by such toxic, or metabolically recalcitrant chemicals as phenolic, polyphenolic (including lignin) or suberin-like compounds. Some lytic enzymes associated with the cell wall and the intercellular spaces, like glucanases and chitinases (the subject of chapter

6), which can be detrimental to fungal pathogens (Schlumbaum *et al.*, 1986), may also be important in disease resistance.

Mineral elements may play a role in plant-pathogen interactions: in the context of the present work two are of particular and direct interest. Calcium is essential for wall metabolism because it takes part in the synthesis of various polysaccharides, to the point that a severe deficiency brings about rapid deterioration and death of the affected plant (Bidwell, 1978). It is also implicated in localized disease resistance mechanisms, e.g. in the hardening of cell walls in areas of attempted penetration, together with a mediating role in the deposition of callose and the lignification of the cell wall (Kohle *et al.*, 1985; Aist *et al.*, 1988; Kunoh *et al.*, 1986; Bayles & Aist, 1987).

Silicon appears to be a facultative micronutrient in general, although some cereals, such as rice and maize, seem to thrive in silicon-rich soils, to the point that up to 20% of their dry weight may be represented by this element (Bidwell, 1978). In cereals it also seems to be involved in resistance mechanisms, with a role in the mechanical toughening of the cell wall **(Kunoh & Ishizaki, 1975).**

The dynamism of the cell wall, expressed mainly in primary walls, is clearly of the utmost importance when structural defence mechanisms are considered, particularly in primary tissues such as the feeder roots of conifer seedlings where structural barriers can only be expressed at the cell

level. The capability of a cell to oppose a pathogen by synthesizing new wall materials can be determinant for the outcome of the interaction (Vance & Sherwood, 1976).

Wall appositions have been implicated as one of the causal mechanisms for resistance in a number a studies (Aist, 1976; Aist, 1983; Edwards & Ayres, 1981; Ride & Pearce, 1979; Sherwood & Vance, 1980; Vance & Sherwood, 1976), relating almost invariably to herbaceous species.

These and other studies have analysed the ultrastructure and the histochemistry of these formations, sometimes also in relation to Systemic Induced Resistance (SIR) (*cf.* chapter 6) (Aist, 1976; Aist, 1983; Hammerschmidt & Kuc, 1982; Hinch & Clark, 1982; Medeghini Bonatti *et al.*, 1985; Sherwood & Vance, 1976; Smart *et al.*, 1986; Vance *et al.*, 1980). In a few cases the elemental composition of wall appositions, and of cell walls in general when challenged with pathogens, has also been analysed (Aist *et al.*, 1988; Heath, 1981[b]; Kunoh *et al.*, 1986).

Cell wall appositions, variously termed papillae or lignitubers when lignified (Fellows, 1928), have therefore been implicated in antimicrobial defence in a range of plant species, in which they are believed to provide an inducible barrier to cellular penetration by the potential pathogen (Aist, 1976; 1983; Ride, 1983).

Although a papilla response has been demonstrated in the leaves of *Quercus* spp. (Edwards & Ayres, 1981), which are woody angiosperms, the majority of studies on this defence mechanism have been conducted on the aerial parts of

herbaceous crop species, belonging, in particular, to the family Graminaceae (Sherwood & Vance, 1976; 1980; Vance & Sherwood, 1976). In these and other cases papillae typically comprise a callose matrix, variously incorporating pectic materials, cellulose, suberin, gums, protein (including peroxidase enzymes), and the elements calcium and silicon (Aist, 1976; 1983). Commonly, lignification of papillae confers extra resistance to penetration (Edwards & Ayres, 1981; Ride & Pearce, 1979; Vance *et al.*, 1980).

Although there have been a few reports of cell wall appositions in the roots of woody dicotyledonous plants, such as *Acacia pulchella* (Tippett & Malaiczuk, 1979), and *Malus* sp. (Mourichon & Salle', 1981), little is known of this potential defence mechanism in the roots of Gymnosperms. Wall appositions have however been found in ectomycorrhizae of various coniferous species (Duddridge, 1986[a,b]; Nylund *et al.*, 1982), although their role in these plant-fungus interactions remains unclear.

3.1.2 Biochemical defences

Various potentially antifungal, low molecular weight compounds, are produced by plants. General information on this topic has already been given in chapter 1, and good reviews on this subject have been published by Boller (1987), Cruickshank (1980), Darvill & Albersheim (1984), Deverall (1976), Kuc' (1972), Mansfield (1983), and Schlosser (1980).

A major distinction among these compounds is on the basis of their molecular weight. Compounds are conventionally

considered as low molecular weight when their MW < 1000. This group comprises the major antibiotics of plant origin, usually phenolic in nature or derivation (Mansfield, 1983). They may be present before microbial attack (constitutive compounds) or appear only subsequently to it. Microbial attack is known to induce *de novo* production of antibiotics, termed phytoalexins, in many plants (Mansfield, 1983).

Some proteins (i.e. high molecular weight compounds) may also show antifungal activity and possibly be involved in defence. Hydrolytic enzymes like chitinases and ß-1,3-glucanases may be particularly relevant in this context (Boller, 1987). Being the subject of chapter 6 they will not be discussed here any further.

True phytoalexins (*cf.* chapter 1) have not often been reported in the Pinaceae. Perhaps the most convincing phytoalexin described in this family is benzoic acid, which was detected in the needles of *P. radiata* (Franich *et al.*, 1986). The author is not aware of any report of true phytoalexins in *P. sylvestris*, but the presence of various constitutive antifungal compounds has been demonstrated, including the stilbene pinosylvin and its derivatives in the wood (Kemp & Burden, 1986) and needles (Sandermann *et al.*, 1989), and the diterpenic resin acid, abietic acid, in the wood (Shain, 1971; Henriks *et al.* 1979) and roots (Fries, *et al.*, 1987).

Most of the work on *P. sylvestris* has been conducted on aerial organs, and only few examples exist of work on the root system of this conifer (Fries *et al.*, 1987; see also chapter

1).

This chapter describes papillae in the cortical tissues of primary roots of *Pinus sylvestris*, formed in response to infection by the root pathogen *Cylindrocarpon destructans*. Cell wall polymers in the papillae have been characterized by a range of established histochemical techniques, and the principal elements present have been mapped and quantified using proton induced X-ray emission (PIXE) microanalysis. This relatively new technique, conceptually similar to the more widely used electron microprobe analysis (*cf.* Heath, 1981[b]), utilizes a finely focussed beam of fast protons to induce the emission of characteristic X-rays from the atoms in the sample. By analysing and quantifying the emitted X-rays, the elements in the sample can be identified and the concentrations can be determined.

Because of the much greater mass of the proton compared with electrons, the primary bremsstrahlung background, which limits the sensitivity of the analogous electron microprobe analysis, is absent. This results in an improvement of two to three orders of magnitude in analytical sensitivity and parts per million of elements above and including Na in the periodic table can be detected (Campbell & Johanssen, 1988).

In addition to detecting characteristic X-rays, the energy of backscattered protons can be measured, and this can be used to give information on the concentrations of light elements (C, N, O) in the sample matrix. By scanning the beam across the sample and correlating the element signals with the instantaneous beam position, maps of the element distributions in the sample can be obtained (Grime & Watt, 1990; Watt &

Grime, 1987).

This chapter also describes how the extent of lignification of non-mycorrhizal, primary root cell walls, and the concentration of soluble phenols, change in response to attack by *C. destructans in vitro*.

An assessment of the role of papilla induction in resistance of Scots pine roots has also been attempted. This experiment was undertaken to test the hypothesis that, upon fungal infection, roots of resistant seedlings express a more intense papilla response than susceptible seedlings. For this experiment *P. oligandrum* was used in place of *C. destructans*. *P. oligandrum* causes higher death rates on Scots pine seedlings, a characteristic that was considered helpful in the quantitation of this phenomenon.

Work was also conducted to assess whether *P. sylvestris* produces any phytoalexins, and/or whether there is any change in constitutive antifungal compounds, when challenged with *Cylindrocarpon destructans in vitro*. In the event that any antifungal compounds were detected, their identification and quantification would be attempted, and their role in defence evaluated.

3.2 Materials and methods

3.2.1 Structural defences

3.2.1.1 Plant material and inoculation procedures

Pinus sylvestris seedlings were grown and inoculated as described in 2.1.2.1 and 2.3.1.1, except for the experiment involving PIXE, in which one batch of seedlings was grown on 0.8% deionized water agar supplemented with $10mg \cdot l^{-1}$ of $Na_2SiO_35H_2O$ (sodium metasilicate, BDH). Controls consisted of uninoculated seedlings grown under the same conditions.

3.2.1.2 Light and electron microscopy, and PIXE

Root segments of pine seedlings were processed according to one of the following schedules (all steps carried out at room temperature):

whole-mounts: root segments were used fresh and stained as described in Tables 3.2, 3.3, 3.4;

sections:

a) for TEM: fixation for 45min in the following solution: 4% (v:v) glutaraldehyde, 1% (w:v) paraformaldehyde, 0.2M sucrose, 0.02M $CaCl_2$ in 0.1M sodium cacodylate buffer (pH 7.0); tissues were then rinsed 3 X 10min in buffer followed by dehydration in a graded ethanol series.

b) for ultracytochemistry, light microscopy, and PIXE: fixation for 45min in 5% (v:v) glutaraldehyde in deionized water, followed by 3 X 10min rinses in deionized water, followed by dehydration in a graded ethanol series.

The roots were then infiltrated for 2 X 1h periods in fresh LR White resin (The London Resin Co. Ltd, U.K.), and polymerized in gelatine capsules at 60°C for 24h. The blocks were cut on a Reichert Om U2 ultramicrotome using glass knives.

Light microscopy

3µm (nominal) sections were cut for optical microscopy.

The histochemical tests used for the detection of the various classes of cell wall and papilla components were as listed in Tables 3.2, 3.3, 3.4.

Epifluorescence microscopy was carried out using a Leitz Orthoplan fluorescence microscope fitted with a 200W mercury vapour lamp. Incident illumination from the exciter filters UG1 (plus a k430 barrier filter for the detection of Calcofluor fluorescence and autofluorescent compounds) or BG12 (plus a k530 barrier filter for aniline blue-induced fluorescence of callose-like compounds) was employed (O'Brien & McCully, 1981). Sections were observed dry for detection of aromatic compounds by autofluorescence, or mounted in water (after staining with Calcofluor White M2R New for the detection of cellulose), or in aniline blue staining solution (0.05% in phosphate buffer, pH 8.5) (O'Brien & McCully, 1981). Fresh root segments were never observed dry.

Transmission electron microscopy

For anatomical observations ultrathin sections (gold interference colour) were picked up on 200 mesh copper grids coated with pioloform, contrasted with uranyl acetate and lead citrate, and carbon coated.

For ultracytochemical tests the sections were processed as follows:

aldehyde detection: bismuth test, part of the periodic acid-alkaline bismuth method for carbohydrates (Lewis & Knight, 1977). The grids were floated for 30-60 min at room temperature on an alkaline bismuth staining solution prepared as follows: 400mg of potassium sodium tartrate were dissolved in 10ml of 2N NaOH, and this solution was added dropwise to 200mg of bismuth oxynitrate with constant stirring. 1ml of this stock solution was diluted in 50ml of deionized water just before use.

acidic (pectic) polysaccharides: colloidal-iron test; the sections were floated free on the staining solution described below and picked up on the copper grids; or were picked up on uncoated gold grids and subsequently stained (Lewis & Knight, 1977). The colloidal-iron staining solution was prepared by adding, dropwise, a small volume of a concentrated (10-30%) $FeCl_3$ solution to 200-300ml of vigorously boiling distilled water, to a final concentration of 0.5-1%. When the solution turned dark red it was allowed to cool, and was then dialysed against distilled water for 3 days, changing the water twice daily. The staining solution was prepared just

before use by mixing 5ml of colloidal-iron stock with 9ml of distilled water and 6ml of glacial acetic acid (to give a final pH of approx. 1.8). Sections were stained for 1h at room temperature.

The sections for the ultracytochemical tests and their controls (i.e., sections from unchallenged seedlings) were neither contrasted nor coated. Specimens were observed with a 'JEOL 2000 EX' TEM.

PIXE imaging and analysis

Elemental maps and point determinations were obtained from representative structures using the Oxford Scanning Proton Microprobe (SPM) (Department of Nuclear Physics, University of Oxford). This uses a 1.7MeV tandem accelerator (Grime *et al.*, 1990) to produce beams of 3MeV protons which can be focussed to a minimum spot diameter of 0.3μm. Emitted X-rays were detected using a lithium-drifted silicon detector, and backscattered protons were measured using a silicon surface barrier detector.

Papillae were located in unstained sections by their autofluorescence.

Sections (3μm nominal) were placed on glass slides which had been previously coated with pioloform and dried on a hot plate. Squares of pioloform supporting the relevant sections were then cut, floated off the glass slides in deionized water and placed on aluminium target stubs, purpose-built for the SPM machine. The targets were then lightly carbon coated prior

to PIXE microanalysis.

Precise targeting of the beam was achieved by locking its position on selected points of images of the sample derived either from the total emitted X-rays, or from the corresponding elemental maps. Calibration of the instrument was not necessary for this study, as this is done very rarely using a glass standard provided by the National Institute for Science and Technology, U.K. (former National Bureau of Standards). The high precision attainable in the routine quantification of elements is ensured by the set up of the machine according to very strict physical parameters.

From sections of challenged seedlings point analyses (beam spot diameter < 1μm) were obtained from 1 papilla from each of 2 seedlings grown in deionized water agar culture, each seedling taken from a different jar, plus 2 normal cell walls taken from the same specimens. The same sampling scheme was used for seedlings growing in Si-enriched culture.

From sections of unchallenged (control) seedlings similar point analyses were obtained from 1 normal cell wall from each of 2 seedlings grown in deionized water agar culture, each seedling taken from a different jar. The same sampling scheme was used for seedlings growing in Si-enriched culture.

Point analyses of the resin background (blanks) were also performed on two sections.

3.2.1.3 Estimation of lignification

P. sylvestris seedlings were cultured as described in 3.2.1.1, except that they were grown for 3 months, required to

obtain a reasonable root development, prior to inoculation and incubation for 4 weeks with *C. destructans*. Controls consisted of uninoculated seedlings grown under the same conditions.

Seedlings were harvested, their whole root systems placed in Eppendorf tubes and desiccated on a Edwards "Modulyo" freeze dryer. The dry biomass was processed using an ionization difference spectra method, a partial modification of the original methods by Ride (1975) and Langcake and Wickens (1975).

Sample preparation

5mg (dry weight) of sample were ground in 3% sodium dodecyl sulphate (SDS) (5ml) in a mortar, transferred to a tapered centrifuge tube, and made up to approx. 10ml. The suspension was centrifuged for 5min at approx. 1500g, and the supernatant discarded. The residual solids were washed with 3% SDS (5ml), and centrifuged. The residue was washed with water (5ml), centrifuged and washed with the following solvents sequentially: ethanol, acetone, ether (5ml), spinning between each treatment, and air dried.

Removal of soluble phenols with cold NaOH

The dry residue was treated with 3ml of 0.5M NaOH (16h) at room temperature. This suspension was then diluted to 10ml with water and the solids spun down (dilution assists the separation of the solids). The residue was washed (water, 5ml) and spun down.

Removal of lignin with hot NaOH

2ml of NaOH (0.5M) were added to the residue and the suspension was heated in a stoppered tube for 16h at 80°C. After cooling, the residue was spun down. 1.6ml of supernatant were taken, adjusted to pH 7-9 with 2.5M HCl (approx. 7 drops), and made up to 2.5ml with water. 0.5ml of this were diluted with 2.5ml of 0.06M NaOH. The UV spectrum of this last fraction (pH 12) was compared with that of the fraction at pH 7-9, running the spectrum from 220 to 400nm.

3.2.1.4 Estimation of total soluble phenols

Plant material and inoculation procedures were as in 3.2.1.3. Dry biomass was extracted in pure MeOH (solvent:dry biomass = 100:1 (v:w)). 3 replicates each for infected and control material were used.

The following is a modification by Pearce (personal communication) of the original method by Wong and Preece (1978). 150µl of sample (methanol extract) were diluted to 1.5ml with water, to obtain a 10% methanol solution. To this was added 0.75ml of Folin-Ciocalteau's reagent (Sigma, 2N, diluted 2X with water) and, after standing for 3 min, 0.75ml of 1M Na_2CO_3. After shaking, the tubes were left for 1h, and the optical density (OD) measured at 725nm, against a control blank, prepared using pure methanol in the reagent mixture. The assay was calibrated against a standard curve using gallic acid (Fig. 3.15).

3.2.1.5 Relationship between papilla response and resistance

110 seedlings established in 0.8% deionized water agar petri dish systems (*cf.* 2.1.2.2) were inoculated with mycelial plugs of *Pythium oligandrum* cultures taken from the margins of actively growing colonies, 1 month after transfer to the petri dishes. The plugs (diameter = 10mm, 3 per plate, mycelium down) were placed in positions that were consistent in relation to the tip of the main root, one on each side of the tip about 0.5cm distant from it and one the same distance in front of the tip. The systems were then incubated on a window sill. A seedling was considered wilted when the hypocotyl appeared shrivelled.

As the seedlings died they were harvested and stored in fixative (as described above for light microscopy), at 4°C, until needed. When the point of maximum wilting rate [as determined by the maximum partial mortality (maximum slope of the total mortality curve] was reached, 6 surviving seedlings and 6 of the most recently wilted ones were sampled and their roots were processed for light microscopy as described above.

3µm thick sections were cut starting from about 3-5mm behind the root apex. 100 serial sections per root were cut and stained with toluidine blue, and examined for papillae by light microscopy. The total number of papillae throughout the 100 sections was counted, the mean number of papillae was calculated for resistant and susceptible seedlings, and the difference between the two means tested for significance using the Student-t test.

3.2.2 Biochemical defences

3.2.2.1 Plant material and inoculation procedures

P. sylvestris seedlings were grown, inoculated and incubated as described in 2.1.2.2 and 2.3.1.1. Controls consisted of uninoculated seedlings grown under similar conditions.

3.2.2.2 Detection of antifungal compounds

After 1 month of incubation the seedlings were harvested carefully, and their whole root systems immediately extracted in redistilled MeOH overnight at 4°C [solvent:fresh weight (v:w) > 10]. This crude extract was filtered under reduced pressure (two thicknesses of Whatman No. 1 filter paper) and diluted with water to give a 60% methanol solution. This solution was partitioned twice against an equal volume of redistilled $CHCl_3$. The bottom fraction ($CHCl_3$) was termed the "organic" phase, while the top fraction was the "aqueous" phase. Both phases were brought to dryness in a thin film rotary evaporator, at 38-40°C, under reduced pressure. The dry residues from the O and A phases were redissolved in redistilled ethyl acetate and MeOH respectively, to a final concentration corresponding to 0.5g fresh weight ml^{-1}. An analytical thin layer chromatography (TLC) plate with a 0.2mm thick layer of silica gel (DC-Alufolien Kieselgel 60 F_{254}, Merck) was loaded with 400µl of each extract and developed in a $CHCl_3$:MeOH = 4:1 (v:v) solvent system. At the end of development the plate was dried with a cold air stream and immediately sprayed (Homans & Fuchs, 1970) with a freshly

prepared spore suspension of *C. cucumerinum*, as described in 2.5. The plate was then incubated in the dark, under humid conditions, at 25°C for 2-3 days, after which it was observed for detection of antifungal activity.

3.2.2.3 Isolation of antifungal compounds

After bioassay the major zone exhibiting antifungal activity (in the O phase fraction) was purified as follows. An analytical TLC plate was loaded with 1ml of O fraction on a 3cm strip and run as described above. After development the band running at Rf = 0.64 was scraped off the TLC plate and the silica gel powder suspended in 5ml of redistilled MeOH with vigorous shaking on a rotamixer for 1min. The silica gel was spun down and the supernatant collected. This procedure was repeated, the two supernatants combined and evaporated to dryness under reduced pressure as described above. The dry residue was then redissolved in MeOH to a concentration of 500mg fresh weight equivalent ml^{-1}.

3.2.2.4 Gas chromatography and mass spectrometry of antifungal compounds

The extract from 3.2.2.3 was treated to obtain methyl ester (ME) and trimethylsilyl (TMS) derivatives for gas chromatography as follows. To the sample, a solution of 50% pyridine-50% dimethylformamide dimethylacetal (Sigma) was added, and the mixture heated at 60°C for 30min. This was then brought to dryness in a freeze-drier under N_2 to finally yield the methyl ester derivatives of the sample. To neutralize all

remaining reactive groups, the sample was treated with a solution of 50% pyridine-50% BSTFA [bis(trimethylsilyl)-trifluoroacetamide (Sigma)] (which contains 1% trimethylchlorosilane - TMCS). This mixture was then heated at 100°C for 1h to yield the trimethylsylilate ether derivatives of the sample.

The derivatized samples were separated and analysed in a Finnegan 1020 automated GC-MS system (incorporating a Data General Nova 3 computer). The GC system was fitted with a 25m X 0.32mm i.d. silica column coated with 0.5µm of immobilized polydimethylsiloxane (Thames Chromatography - Maidenhead, U.K.) and a splitless injector with a flush 30s after sample injection to remove residual gases. The end of the column was introduced directly into the mass spectrometer analyser chamber. The system operated under the following conditions:

- He pressure: 926.1 mbar (13lbs in^{-2})
- injector temperature: 310°C;
- GC temperature gradient: 75-310°C, at 3°C min^{-1}.

The mass spectrometer was set to scan at 40-650 AMU per nominal second with an ionizing voltage of 70eV. The filament was switched on 250s after the injection of the sample (0.4-1µl) into the GC (Greenaway *et al.*, 1990).

3.2.2.5 Spectrophotometric characterization of antifungal compounds

Spectra of partially purified compounds in methanol solution were recorded using a Varian spectrophotometer (Series 634), connected to a Varian recorder (model 9176). The

reference blank was pure methanol.

3.2.2.6 Identification of terpenes on TLC plates

Terpenes were identified by spraying TLC plates, loaded and developed as described above, with antimony pentachloride (Sigma). The plates were then heated at 120°C until the coloured spots appeared (approx. 10min) (Stahl, 1969).

3.2.2.7 Quantification of antifungal compounds

Seedlings were harvested, their whole root systems placed in Eppendorf tubes and desiccated on a Edwards "Modulyo" freeze dryer. 4 replicates each for the infected and control material were used. The dried biomass was comminuted coarsely using small scissors and extracted in pure MeOH [solvent:dry weight = 100:1 (v:w), corresponding to 10mg dry weight ml^{-1}], at 4°C, for 2h. After extraction the tubes were centrifuged, the supernatant collected and stored in Eppendorf tubes at approx. -20°C until use.

An abietic acid (AA) standard was prepared from a commercial preparation (Sigma) (see results). This was obtained by preparative TLC using a glass plate coated with a 0.5mm thick layer of silica gel GF (Uniplate, Analtech Inc., U.S.A.). 10 lanes were loaded with 10mg each of the commercial compound. Materials running at Rf = 0.64 (see results) were eluted and combined. The total eluate was evaporated under reduced pressure to yield 7.5mg of a dry, partially purified fraction (called AA/R2).

The fractions thus obtained were separated by high

performance liquid chromatography (HPLC) using a Waters 600 multisolvent delivery system connected to a Waters LC 455 spectrophotometer (variable wavelength detector). Data were recorded and analysed with a Waters 740 data module (integrator). Samples were loaded, after being spun in a Microspin-12 microfuge (Sorvall Instruments) to eliminate all particulates, using a Rheodyne 7125 sample injector (20µl loop), and separated on a Nova-Pak C18-Radial Pak (100X8mm ID) reverse phase column (Waters Assoc. USA) mounted in a Waters RCSS module. The stationary phase consisted of a C18 phase bonded on a fully end-capped spherical silica support (4µm particle size). Precolumn inserts (Guard-Pak: µBondapak C18, Waters) were used to protect the column (Drago-Toscano, 1989). A linear methanol/water gradient (table 3.1) was used. Column temperature was maintained at 30°C, and eluting compounds were detected at 254nm.

Preparative HPLC was performed, as required, using the set up described above, the only difference being in the use of a 200µl loop in the sample injector.

Table 3.1

Solvents and gradient used for HPLC separation of plant extracts

Time (min)	Flow (ml/min)	water (%)	methanol (%)	Gradient curve
initial	1.5	50	50	*
4.00	1.5	15	85	6(i.e. linear)
14.00	1.5	0	100	6
22.00	1.5	0	100	6
32.00	1.5	100	0	6
37.00	1.5	100	0	6
42.00	1.5	50	50	6

Abietic acids in root extracts were quantified against an external standard of the AA/R2 fraction (125µg ml^{-1}).

3.2.2.8 Estimation of the antifungal activity of abietic acid *in vitro*

A germination test with spores of *Cylindrocarpon destructans* was carried out to assess the antifungal activity of a commercial preparation of abietic acid (AA) (Sigma). Sporulation of *C. destructans* was induced by growing the fungus on full strength Czapek Dox agar (Oxoid) supplemented with 0.1% yeast extract. The petri dishes were incubated at approx. 25°C, in the dark, for 3 weeks, after which they were placed on a window ledge to be exposed to daylight. After 4 more weeks the cultures were utilized for the preparation of a spore suspension (10^6 spores ml^{-1}) in autoclaved deionized water, as described in 2.5.

Test solutions were prepared by first dissolving AA in dimethylsulphoxide (DMSO) (Sigma) (*cf.* Skipp & Bailey, 1977), followed by dilution with deionized water to a concentration of 8mg AA ml^{-1} (8% DMSO). Part of this solution was then diluted by adding deionized water to obtain the following series of concentrations: 8, 4, 2, 0.8, 0.4, 0.2, 0.08, 0.04 mg AA ml^{-1}.

The actual test was set up by combining, on a glass slide, 25µl of spore suspension with 25µl (measured with an air-displacement automatic pipette) of each test solution, thus halving their concentrations to 4, 2, 1, 0.4, 0.2, 0.1, 0.04, 0.02 mg AA ml^{-1}. Contents of DMSO were therefore also

halved. A similar dilution series containing only DMSO was used as a control. Spore germination was assessed 24h later.

3.2.2.9 Preparation of fungal extracts for TLC and HPLC analysis

Cultures of *C. destructans* were grown on a 2% malt extract broth prepared as follows: a 2% malt extract (Oxoid) solution in deionized water was heated in a boiling water bath for approx. 30min, vacuum filtered twice with a double thickness of Whatman No. 1 filter paper, and autoclaved; 3 replicates (100ml each in 250ml conical flasks) were inoculated, or left uninoculated as controls. Each flask was inoculated with 5 mycelial plugs. The flasks were incubated in the dark at 25°C for 4 weeks. After incubation, 2ml aliquots of crude culture liquid from each flask were saved for HPLC analysis and stored at approx. -20°C until use. HPLC of these samples was carried out as described in 3.2.2.7.

The remaining culture liquid from inoculated or control flasks was pooled, filtered through a double thickness of Whatman #1 filter paper, and concentrated 20X by freeze drying. These concentrated crude culture filtrates were extracted for TLC analysis following a procedure essentially identical to that used for the plant extracts: 2ml of concentrated (20X) filtrate were extracted in 50ml of 60% aqueous MeOH and partitioned twice against an equal volume of $CHCl_3$. The two phases, "organic" (O), and "aqueous" (A), were brought to complete dryness and redissolved in 2ml of ethyl acetate and 2ml of MeOH respectively. The A phase did not

redissolve completely: a large amount of undissolved materials (presumably carbohydrates) remained in the vessel of the rotary evaporator. 400µl aliquots of extracts (corresponding to 8ml of the original culture filtrate) were loaded onto a silica gel TLC plate and developed with a $CHCl_3$:MeOH = 4:1 (v:v) solvent system. The developed plate was bioassayed as described in 3.2.2.2.

3.3 **Results**

3.3.1 Structural defences

3.3.1.1 Morphology of the infection process

Two populations of challenged pine seedlings were apparent from 2 weeks after challenge with *C. destructans*. 4-5% of challenged seedlings proved susceptible to this pathogen and died within this time; the remainder were resistant, remaining alive indefinitely after challenge.

In susceptible individuals the pathogen penetrated deeply into the roots, extensively colonizing the stele and largely destroying the integrity of the stele tissues (Fig. 3.1). In resistant seedlings there was no penetration of the stele by the fungus. Pathogen invasion appeared to be halted in the cortex. In these seedlings papillae were present in cortical and, occasionally, rhizodermal cells, adjacent to fungal hyphae (Fig. 3.2), and at sites of attempted cellular penetration (Fig. 3.3). Although some host cortical cells were penetrated in resistant seedlings, this normally took place in the absence of papilla formation. Papillae were only rarely present in such cases, and less than 1% of the total number of

papillae observed showed evidence of successful penetration (Fig. 3.4).

As seen in whole mounts of both susceptible and resistant roots, single hyphae of *C. destructans* typically made multiple penetration attempts. Papillae were observed underlying unsuccessful penetration sites (Fig. 3.3).

3.3.1.2 Fine structure of papillae

Pine papillae were not clearly visible in unstained sections and were best detected by either fluorescence microscopy (autofluorescent papillae) or staining with toluidine blue.

Papillae were hemispherical to elongated in section, and in some cases were bifurcated; viewed from above (in whole mounts) they were typically sub-circular, often with clear digitiform projections, and with an evident central hole underlying the fungal appressorium, apparently representing the penetration peg (Fig. 3.5).

There was some heterogeneity of papilla ultrastructure. Most papillae had a layered structure (Fig. 3.6), although instances were observed of papillae in which microbodies appeared embedded in an amorphous matrix (Fig. 3.7). Cytoplasm was rarely seen to have accumulated around papillae, this apparently occurring only during the early formation stages (Fig. 3.7).

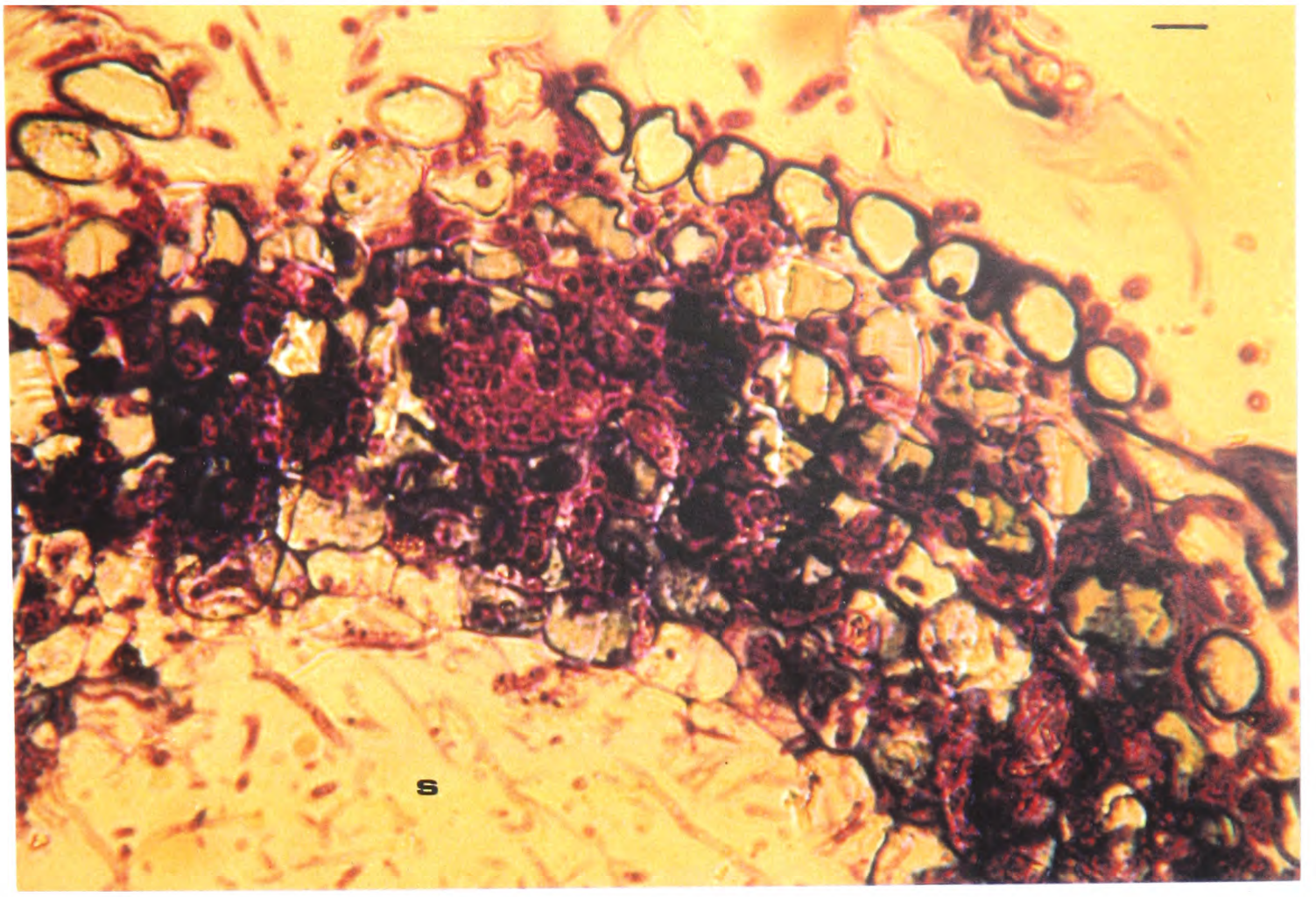


Figure 3.1. A susceptible (dead) root of *P. sylvestris* showing full colonization of the cortex by *C. destructans*. The stele tissues (s) are completely missing and have been replaced by a loose web of fungal hyphae. Section stained with toluidine blue. Scale bar: 10µm.

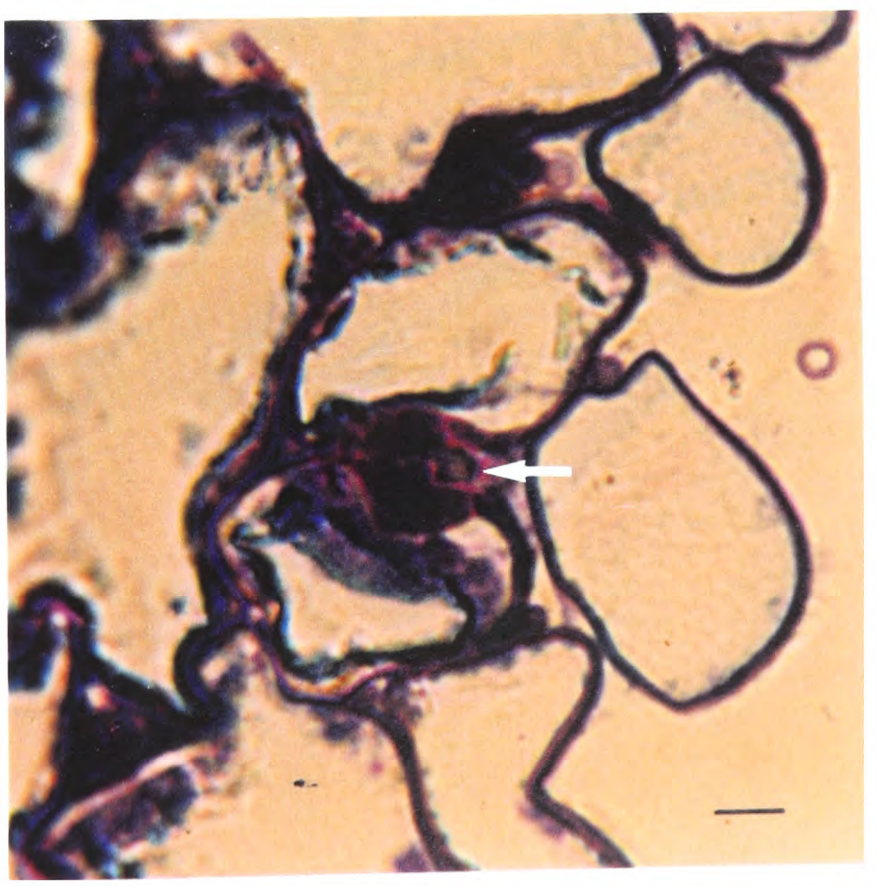


Figure 3.2. Typical, adjacent papillae with interposed hypha (arrow) in the second layer of cortical cells. Toluidine blue staining of the section suggests the presence of pectic materials (magenta colouring). Scale bar: 5µm.

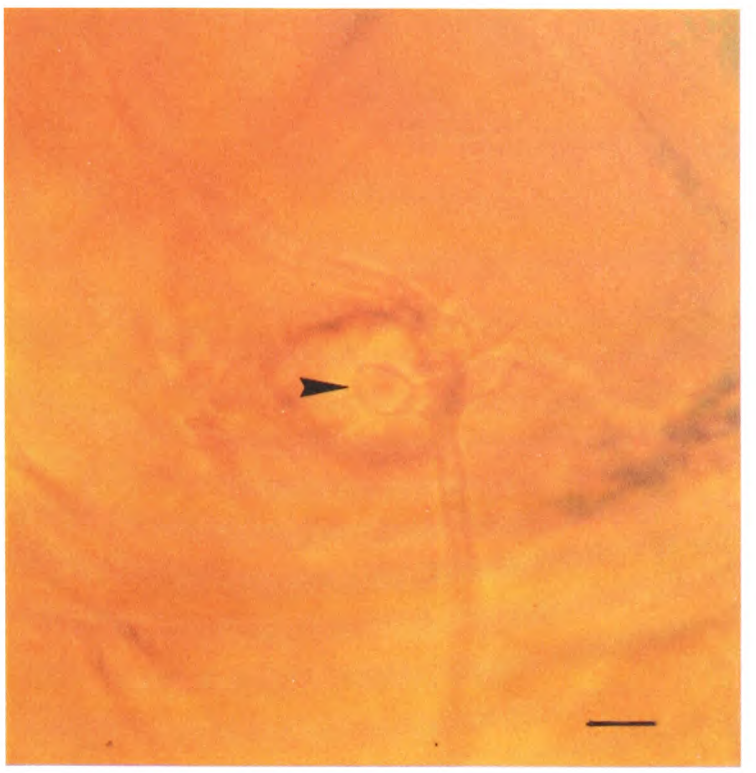


Figure 3.3. An unstained, fresh whole-mount of an infected root. The fungal appressorium (arrow) and the underlying papilla are evident. The penetration peg is also visible at the centre of the appressorium. Scale bar: 5µm.

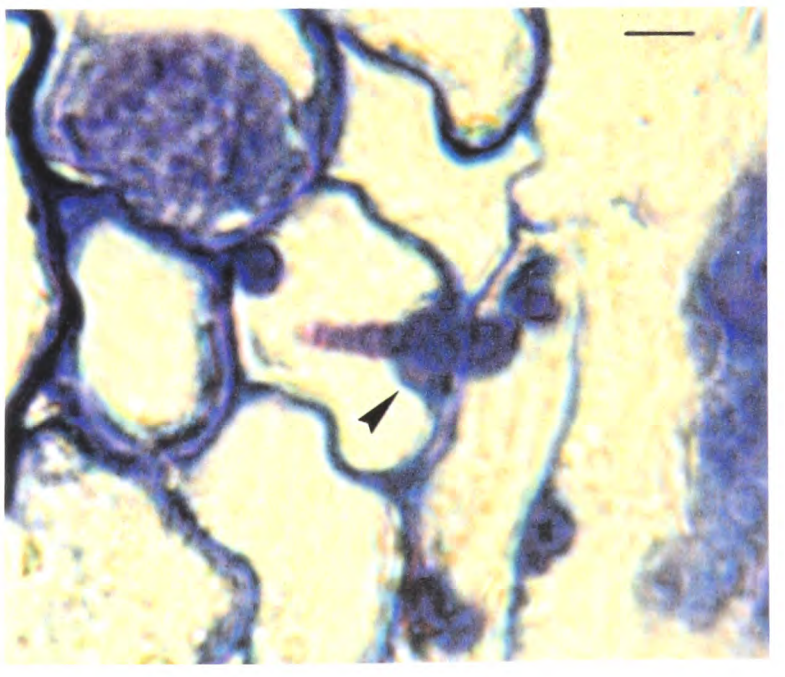


Figure 3.4. A dome-shaped papilla (arrow) with an invading hypha, which is apparently encapsulated by additional papilla material. Toluidine blue-stained section. Scale bar: 5µm.

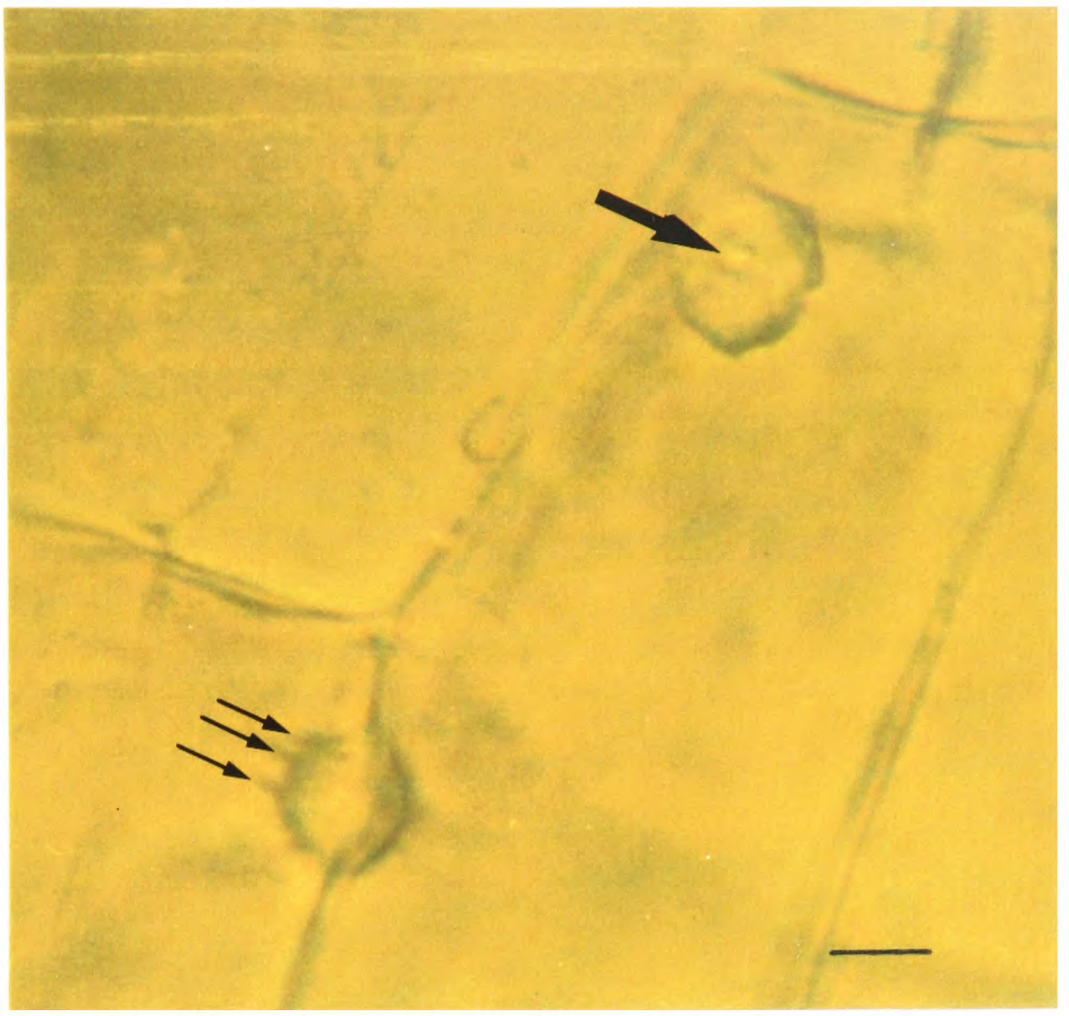


Figure 3.5. Papillae in sub-rhizodermal cortical cells. The penetration peg is visible in one of them (large arrow). The two abutting papillae, formed in two adjacent cortical cells (analogous to Figure 3.2), show typical digitiform projections (small arrows). Unstained, fresh whole-mount. Scale bar: 5µm.

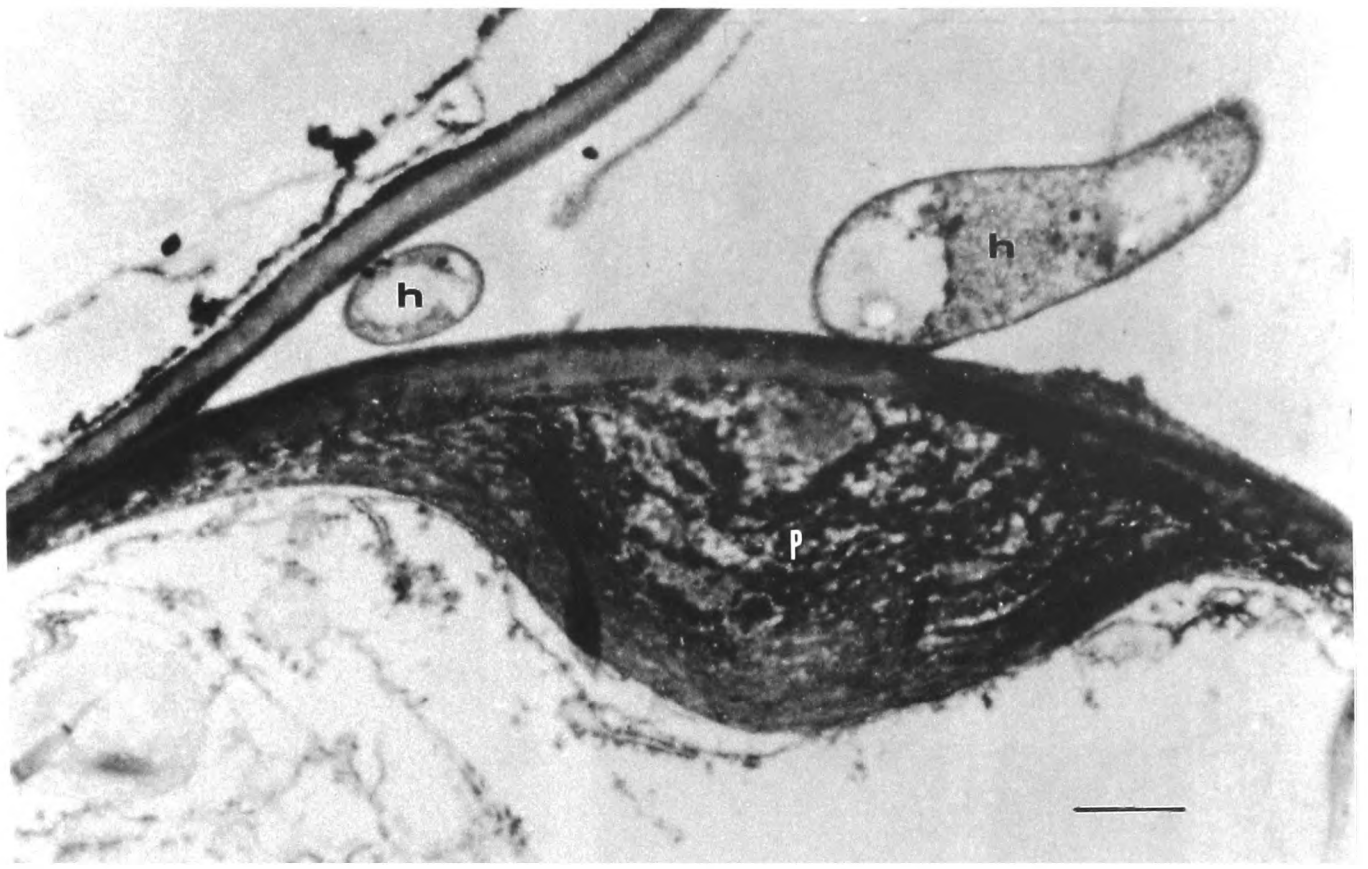


Figure 3.6. A TEM micrograph showing the lamellar structure observed in the majority of *P. sylvestris* papillae (p). h = hypha. Scale bar = 1μm.

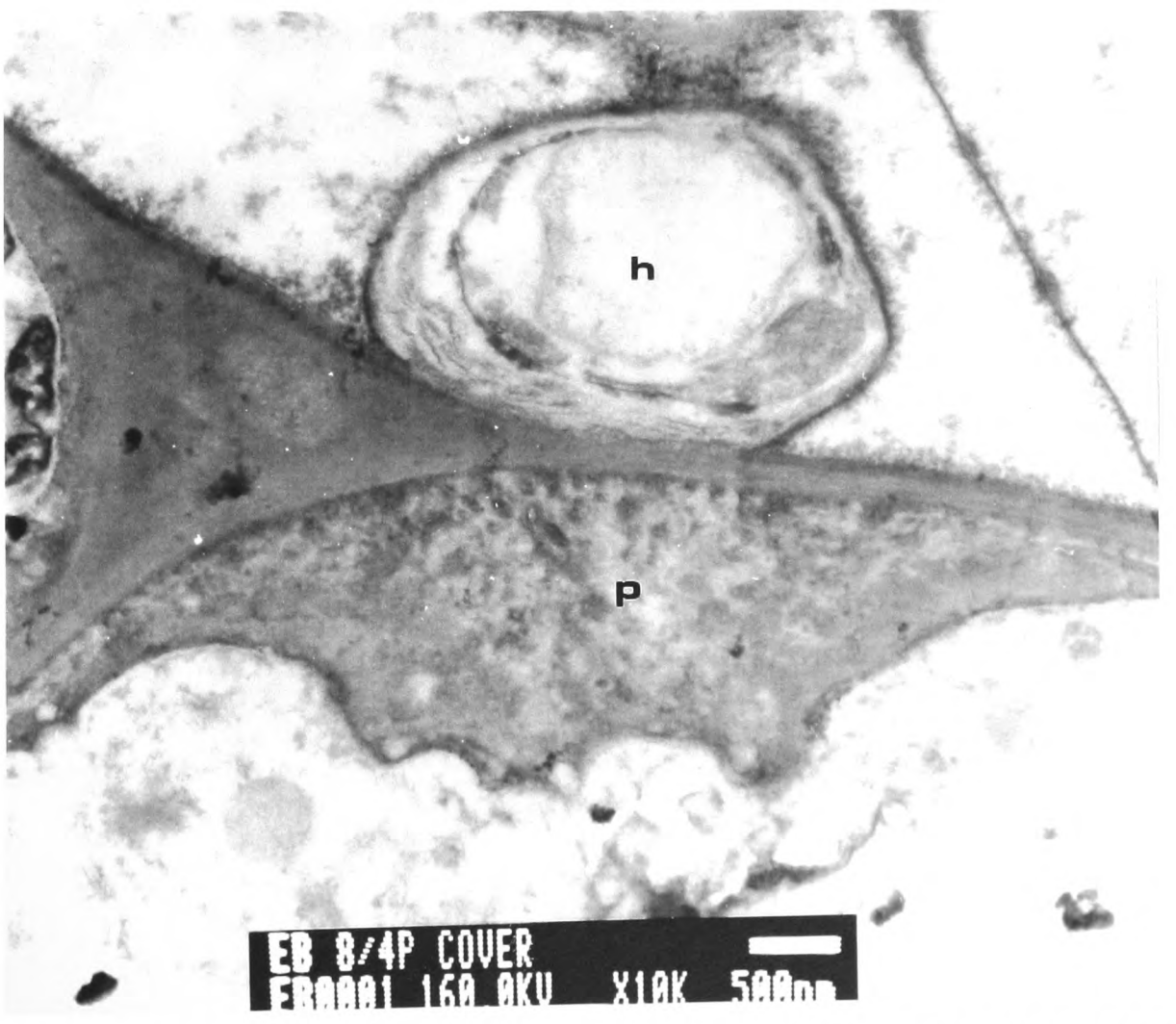


Figure 3.7. Amorphous appearance of a presumed young papilla (p). The host cytoplasm is still accumulated around the wall apposition. Densely stained bodies are embedded in the matrix. h = hypha.

3.3.1.3 Histochemistry of papillae

The results obtained from a wide range of histochemical tests for plant cell wall materials are summarized in Tables 3.2, 3.3, 3.4.

Pine papillae reacted positively, both in sections and whole-mounts, with tests indicative of pectic substances. A positive response was obtained with ruthenium red (Fig. 3.8), widely regarded as the best stain for acidic polysaccharides, although it is not entirely specific since it stains other acidic substances, like nucleic acids (O'Brien & McCully, 1981). The positive molybdenum blue reaction (Fig. 3.9), and the magenta colour observed in a few papillae (presumably those with no bound phenolics) by toluidine blue staining (Fig. 3.2) support this interpretation, although no staining was obtained with the hydroxylamine-ferric chloride test. Cell wall areas adjacent to intercellular hyphae also reacted positively with ruthenium red.

All fluorescence and histochemical tests performed for the ß-1,3-glucan callose, both on sections and on whole-mounts, proved negative.

In sections, the classical histochemical reagents for cellulose, zinc-chlor-iodine and IKI-H_2SO_4, did not react at all with any cell wall. Cell walls in the stele reacted quite strongly with Calcofluor White M2R New and were birefringent when observed in polarized light.

Table 3.2

Histochemical tests and reactions for cellulose and acid polysaccharides

	Sections			Whole-Mounts
	Papillae	Cortical-cell walls	Stele-cell walls	Papillae
cellulose:				
Calcofluor White M2R New-induced fluorescence (UG1 + k430) (Jensen, 1962; O'Brien & McCully, 1981)	?	+-	+++	-
Zn-Cl-I (O'Brien & McCully, 1981)	-	-	-	?
IKI-H_2SO_4 (O'Brien & McCully, 1981)	-	-	-	?
polarized light-induced birefringence (O'Brien & McCully, 1981)	-	+-	++(especially tracheids)	
acid polysaccharides:				
ruthenium red (Jensen, 1962; O'Brien & McCully, 1981)	++	+	+++	++
toluidine blue (pH 6.5) (magenta staining) (Jensen, 1962; O'Brien & McCully, 1981)	+-	+-	++	?
molybdenum blue (Locquin & Langeron, 1983)	+++	+	+	?
hydroxylamine-ferric chloride (1) (Jensen, 1962)	-	-	-	-

See Table 3.4 for captions

Table 3.3

Histochemical tests and reactions for phenolics and lignins

	Sections			Whole-Mounts
	Papillae	Cortical-cell walls	Stele-cell walls	Papillae
phenolics and lignins: autofluorescence (UG1 + k430) (O'Brien & McCully, 1981)	++(bluish-white; yellowish)	+-(brown-orange)	+++(parench.: deep green) (tracheids: bluish-white bluish-green)	++(bluish white)
toluidine blue (pH 6.5) (O'Brien & McCully, 1981)	++	+	++(tracheids)	+
phloroglucinol HCl [24,32] (Jensen, 1962; O'Brien & McCully, 1981)	+-	-	+(tracheids)	+-
Maule test (Jensen, 1962; O'Brien & McCully, 1981)	-	-	?	*
lignin pink-chlorazol black (Gurr, 1965)	-	-	-	*
Schiff's reagent (O'Brien & McCully, 1981)	+-	-	?	++

See Table 3.4 for captions

Table 3.4

Histochemical tests and reactions for callose, lipids, and proteins

	Sections			Whole-Mounts
	Papillae	Cortical-cell walls	Stele-cell walls	Papillae
callose:				
lacmoid (Jensen, 1962; O'Brien & McCully, 1981)	-	-	-	-
aniline blue-induced fluorescence (BG12 + k530) (Jensen, 1962; O'Brien & McCully, 1981)	-	-	-	-
chlorantine fast green BLL (Pearce, 1986)	-	-	-	-
lipid-like materials:				
Sudan black, Sudan IV (O'Brien & McCully, 1981)	?	?	?	?
proteins:				
Coomassie brilliant blue R-250 (Smart *et al.*, 1986)	++	++	+	*

(-) tissues were not processed for methylation of pectic acids
+, ++, +++, visually estimated response intensities
- negative reaction
+- variable outcome
? interpretation unclear
* test not performed

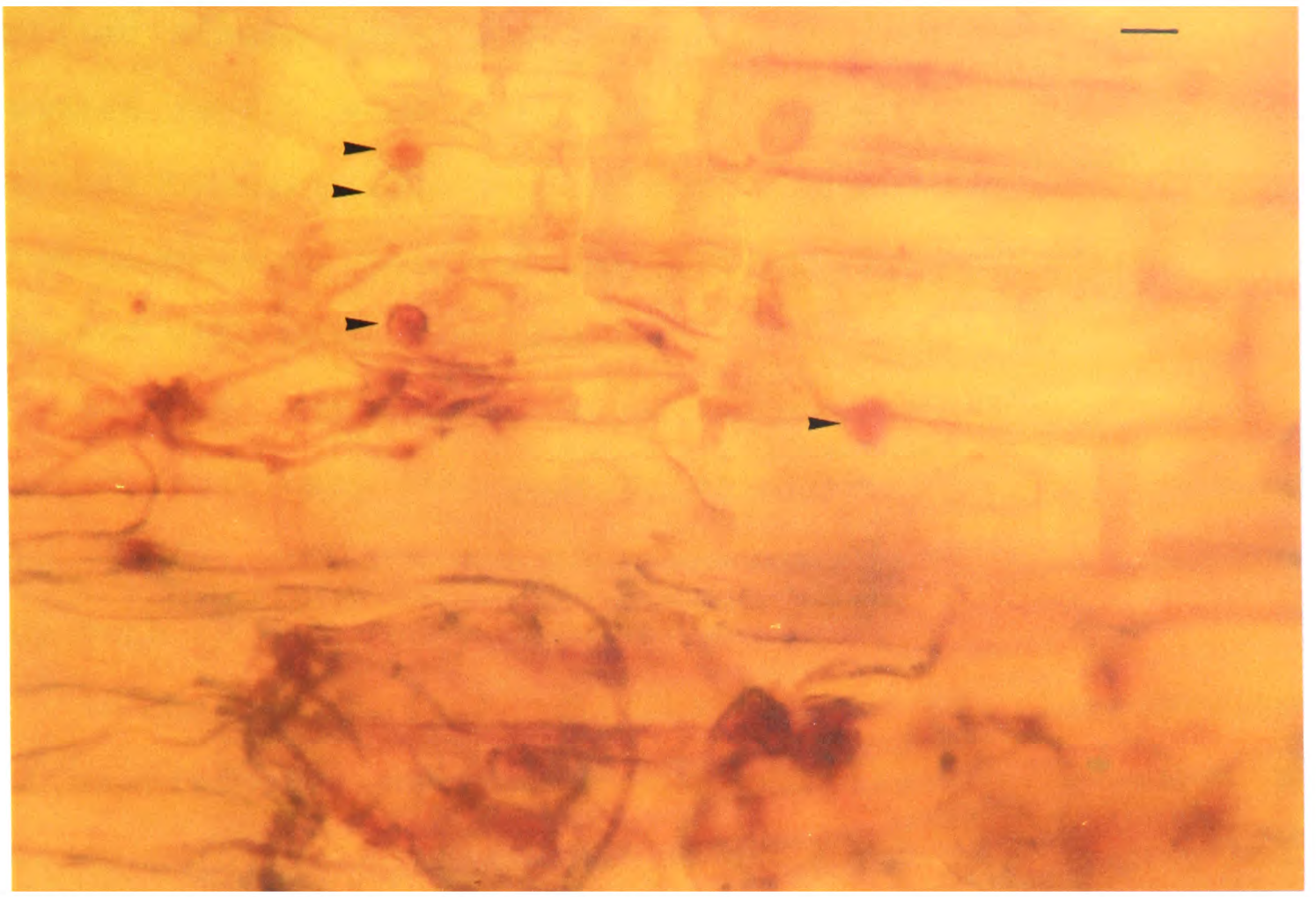


Figure 3.8. A fresh whole-mount of an infected root stained with ruthenium red. Pine papillae (arrows) reacted positively to this stain for acidic polysaccharides (pectic materials). Scale bar: 50µm.

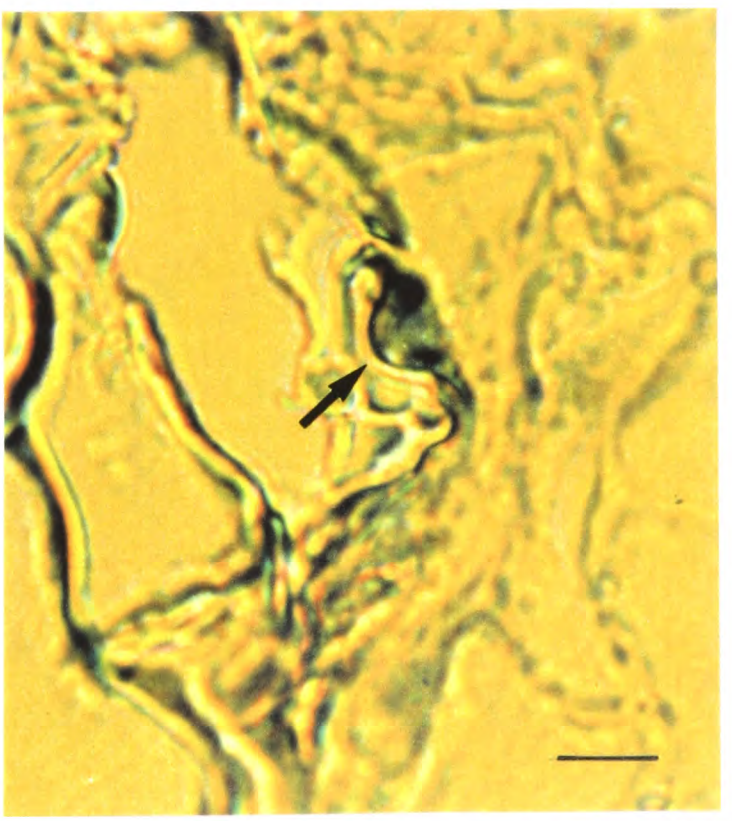


Figure 3.9. A papilla (arrow) positively stained with molybdenum blue, suggestive of the presence of pectins. Transverse section. Scale bar: 5µm.

Papillae always gave negative reactions for cellulose with standard tests, were never birefringent, and did not give reliably interpretable reactions with Calcofluor because their autofluorescence was closely similar to the fluorescence obtained with this fluorochrome.

Besides exhibiting autofluorescence, most papillae reacted positively with phloroglucinol-HCl, Schiff's reagent (Fig. 3.10), and stained green or blue-green with toluidine blue both in sections and in whole-mounts.

In challenged roots, unthickened walls sometimes appeared to be infused with phenolic materials (revealed by phloroglucinol-HCl staining and autofluorescence), in particular at sites adjacent to intercellular hyphae (Fig. 3.11).

Evidence from light microscopy for the infusion of papillae with polyphenolic materials was corroborated by ultracytochemical studies. Wall appositions stained with alkaline bismuth (Fig. 3.12), which is, chemically, the fine structural equivalent of Schiff's reagent (Lewis & Knight, 1977) [being specific for aldehyde groups, Schiff's reagent is traditionally used on its own to detect lignin-like substances (O'Brien & McCully, 1981)]. However, the colloidal-iron test for acidic polysaccharides, employed to support the histochemical evidence that papillae contain much pectic material, did not provide a clear result: the iron particles were essentially evenly distributed throughout the sections.

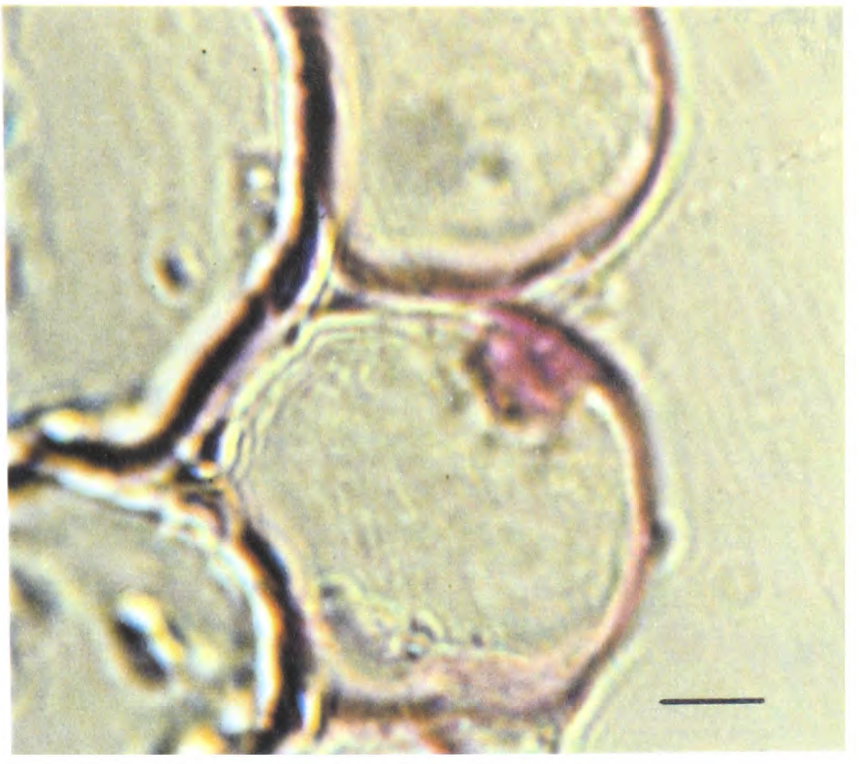


Figure 3.10. A papilla in a rhizodermal cell, stained with Schiff's reagent, which indicates the presence of polyphenolic compounds. Transverse section. Scale bar: 5µm.

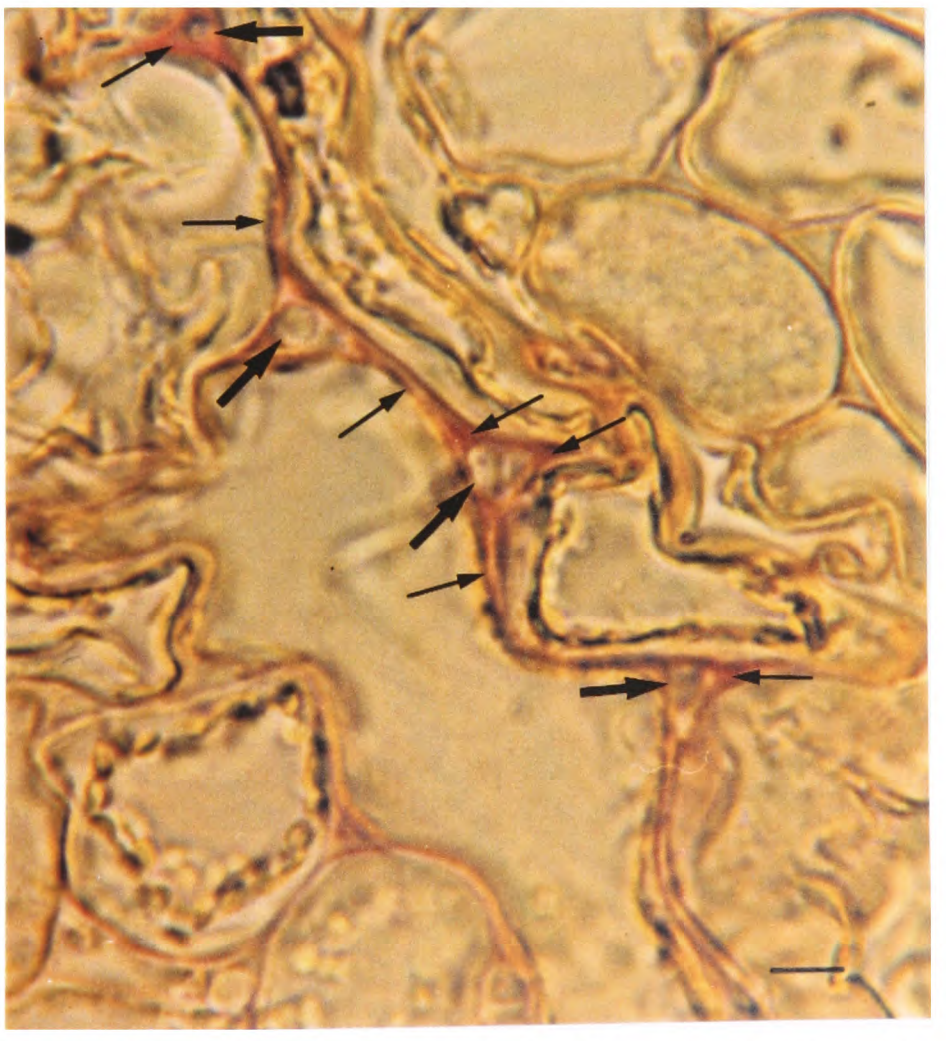


Figure 3.11. Positive staining (small arrows) of cortical cells with phloroglucinol-HCl in areas of intercellular fungal growth (large arrows). This is suggestive of the presence of lignin-like materials. Transverse section. Scale bar: 5µm.

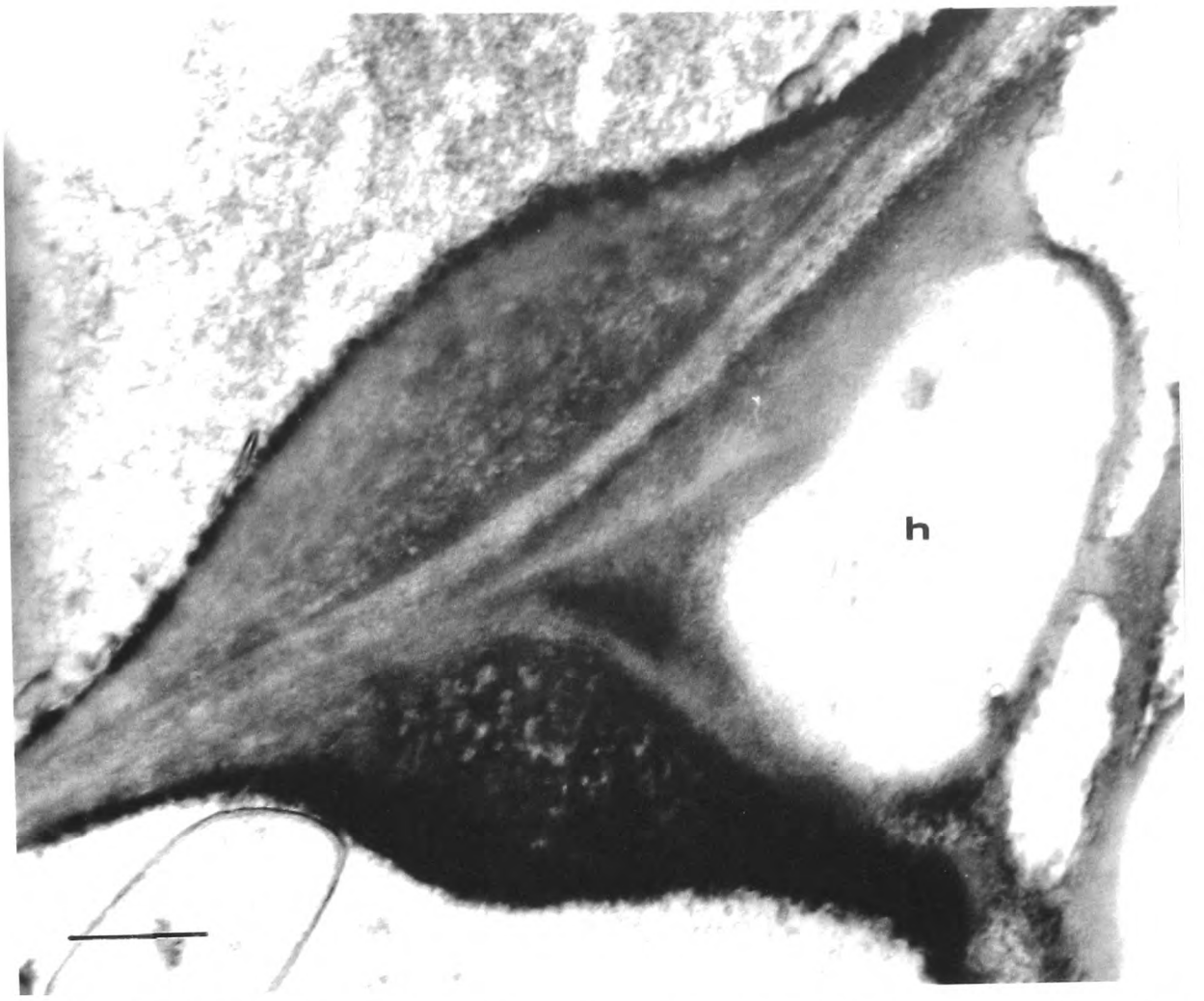


Figure 3.12. A TEM micrograph of pine papillae stained with alkaline-bismuth, a further indication that these structures contain polyphenolic compounds (dark staining). h = hypha. Scale bar: 500nm.

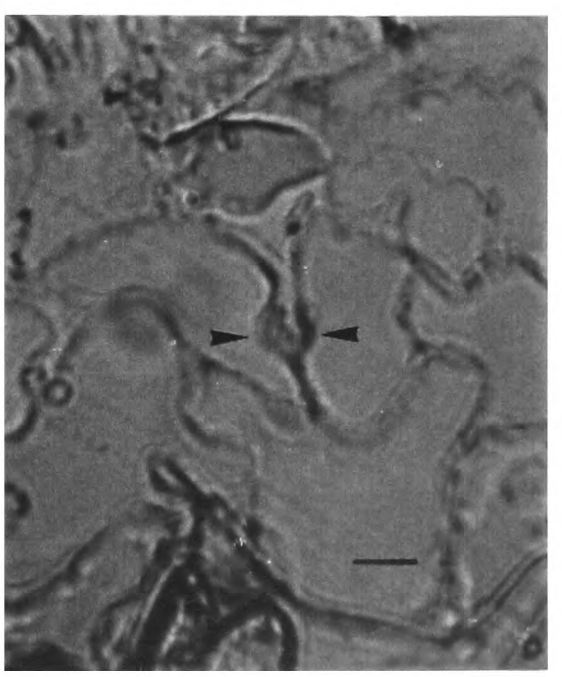


Figure 3.13. Detection of proteins in papillae (arrows) and cortical walls of *P. sylvestris* by Coomassie brilliant blue R250 staining. Transverse section. Scale bar: 10µm.

Staining lipid-like substances in plastic sections was not feasible, because the Sudan dyes partitioned into the resin, making interpretation impossible, and also the ethanol rinsing stage deformed the sections. The thickness of materials present in whole-mounts resulted in a generally confused image, but there was no apparent preferential accumulation of the Sudan dyes in the papillae.

Proteins, detected by staining with Coomassie blue R250, were present in the papillae (Fig. 3.13).

3.3.1.4 Chemical aspects of the infection process

Although impossible to quantify visually, there appeared to be a general accumulation of phenolics (autofluorescence, toluidine blue and phloroglucinol-HCl staining) in the cortical cell walls of challenged roots. However, this phenomenon was not observed consistently. In order to quantify such diffuse lignification, the ionization difference spectrum method was used. The results are represented in Fig. 3.14. According to Ride (1975), a peak in the region of 245nm identifies simple phenolic groups, while more complex phenolic polymers (e.g. lignins) should be represented by a peak in the 350nm region. In the present experiment there was practically no difference in OD at 350nm between control and infected seedlings. However, in the infected material, the peak at 245nm increased markedly (+38%, measured as change in OD at peak wavelength), indicating that aromatic compounds were present largely as simple phenolics.

Assay results for total soluble phenols, calculated using

the standard curve for the reference phenol gallic acid given in Fig. 3.15, are presented in table 3.3. A decrease in the mean concentration of total soluble phenols was recorded between control and infected seedlings (-18.1%). However, this difference was not statistically significant (Student-t test).

Table 3.3

Total soluble phenols as mg (gallic acid equivalent) g^{-1} dry weight, in primary roots of *P. sylvestris*.

	Control	Infected
mean (+-SEM)	14.9 (+-0.52)	12.2 (+-1.12)
Difference of means non significant (Student-t test) (3 replicates per treatment)		

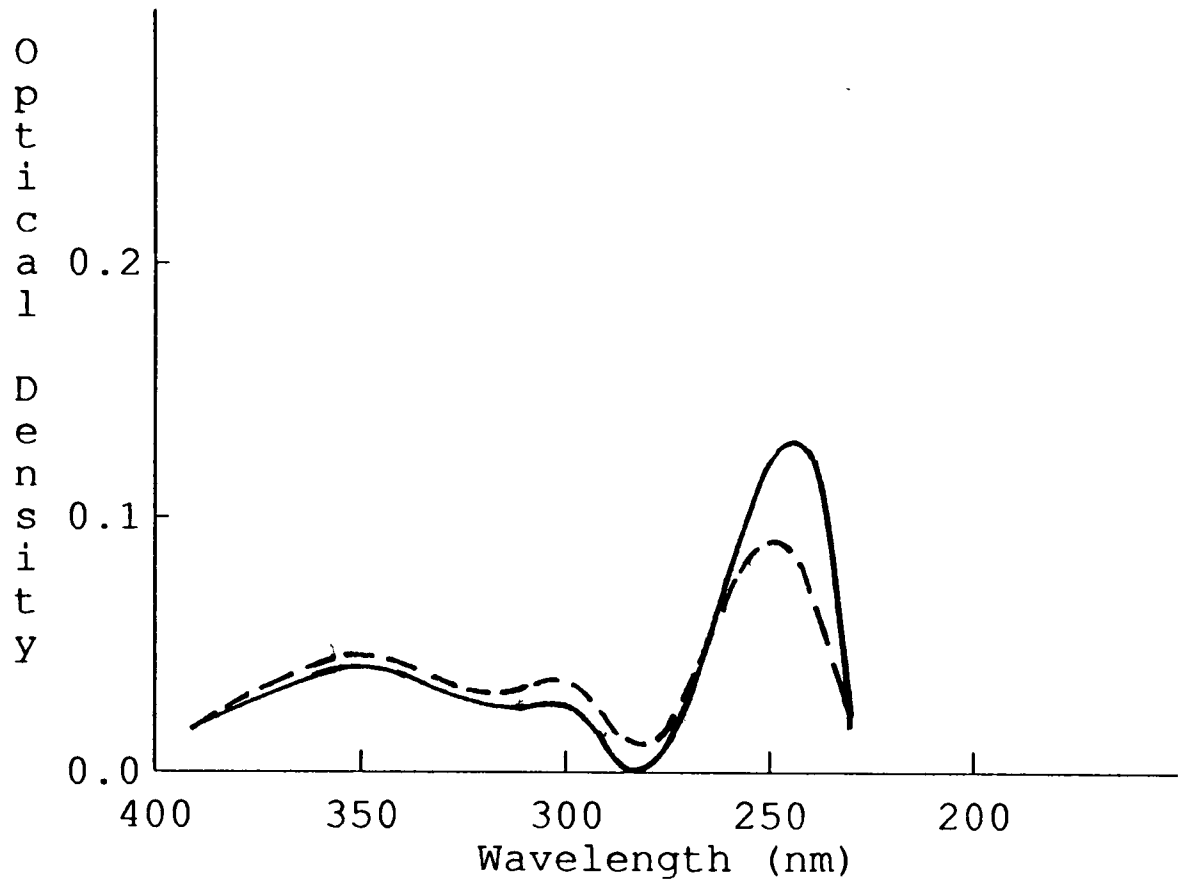


Figure 3.14. Estimation of lignification: ionization difference spectra of root extracts of *P. sylvestris* seedlings (control --- and infected —— with *C. destructans*). Infected roots show a higher content of simple phenols esterified to the cell wall (peak in the 245nm region).

Standard curve for gallic acid

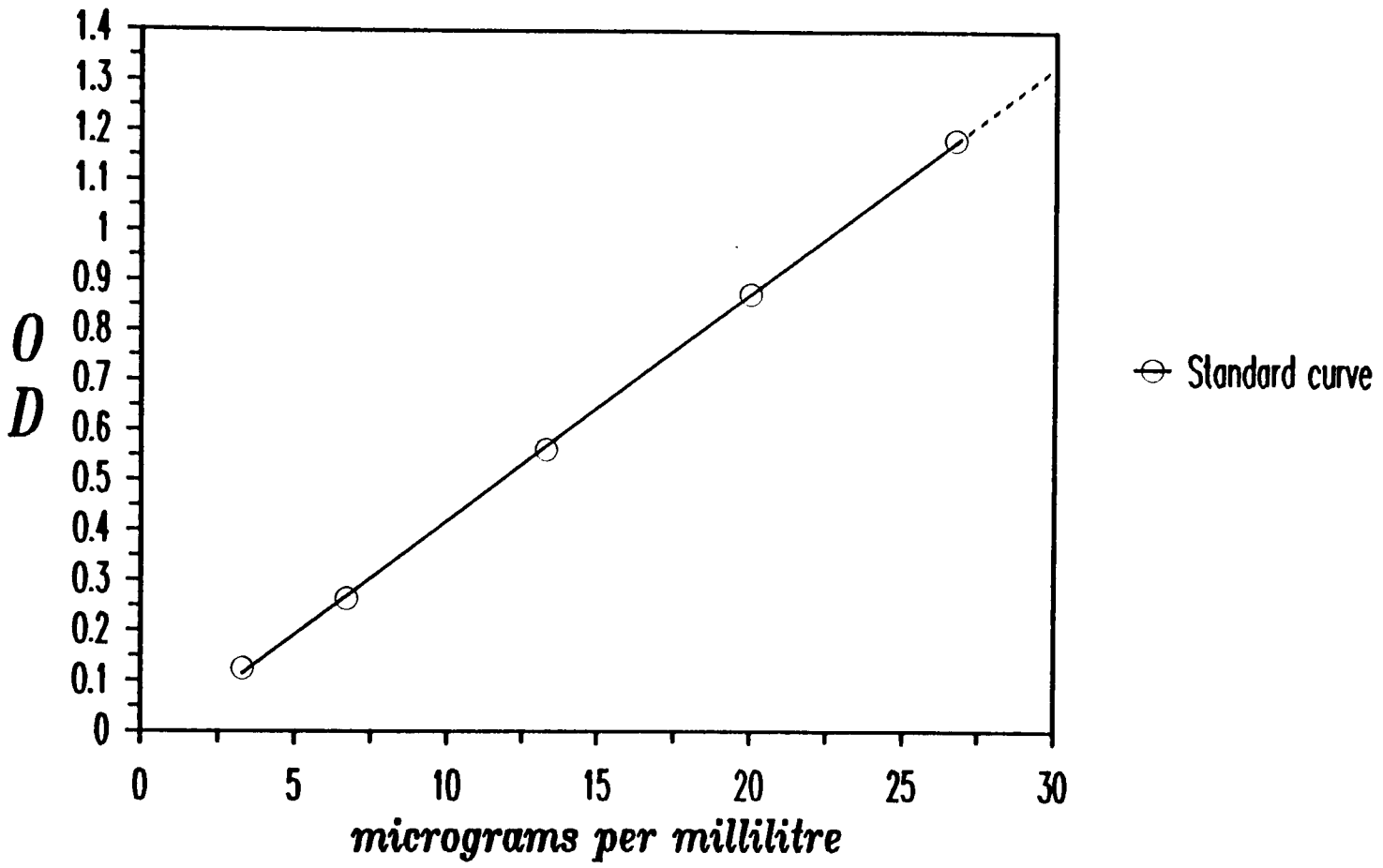


Figure 3.15

3.3.1.5 PIXE microanalysis of papillae

Spatial distribution images were obtained by PIXE for the elements Al, Ca, Cl, Cr, Cu, Fe, Mg, Mn, Ni, P, S, Si, and by Rutherford backscatter for C, N, O, although images in C and O were of little consequence, being dominated by signals from the embedding resin. A representative papilla is shown as an autofluorescent (light) image in Fig. 3.16, together with the corresponding elemental maps for Ca, P, and Si. An outline of the cellular structure analysed, which can be recognized as the same as in the autofluorescent image in Fig. 3.16a, is evident in the map for calcium (Fig. 3.16b), with an enhancement of this element in the papilla relative to surrounding normal cell walls. Phosphorus (Fig. 3.16c) appears to have accumulated in two main areas, possibly identifiable as cell nuclei, while the map for silicon (Fig. 3.16d) shows a uniform distribution of this element on the section, which includes the resin background.

Absolute concentrations of elements were obtained from point analyses from normal cell walls and from papillae. A representative X-ray emission spectrum is shown in Fig. 3.17. The absolute concentrations of Ca and Si are reported in Table 3.5, while Table 3.6 summarizes representative data for a range of detectable elements from roots of seedlings grown in Si-enriched culture. In Table 3.6, the ratios between the concentrations, in papillae and normal walls, of Ca and Si (5.26 and 0.99 respectively), indicate an enhancement in Ca levels in the wall appositions. If the means of all point determinations for Ca are taken into account, including data

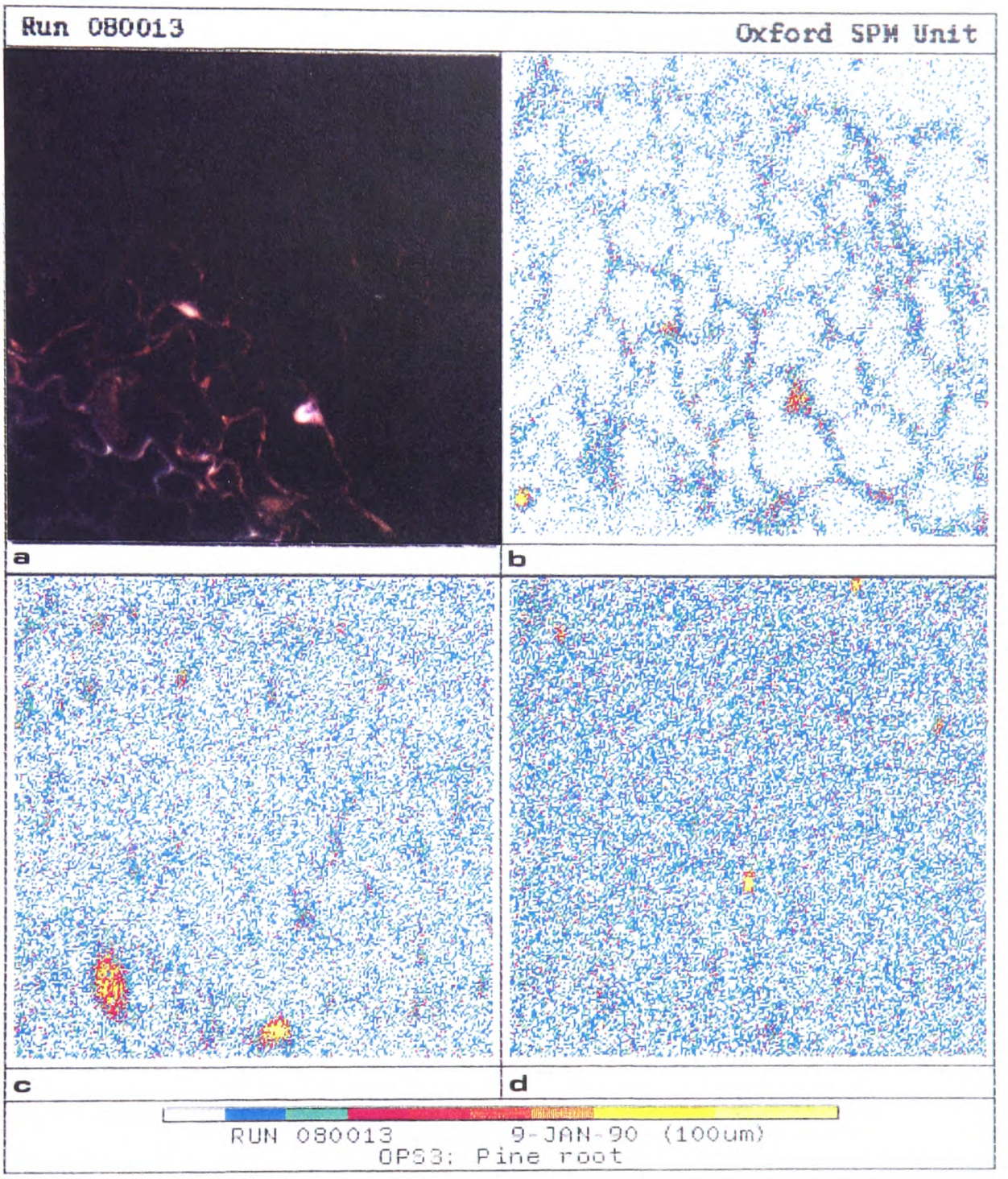


Figure 3.16. Elemental mapping by PIXE. a) An autofluorescent papilla (UG1 exciter filter + k430 barrier filter) in a plastic section. b), c), and d) corresponding spatial distributions of Ca, P and Si, respectively, from the same field. The colour scale is only indicative of the relative concentrations of each element on the section, with yellow being the highest. For precise quantitations see Tables 3.**5** and 3.**6**. Field size: 100 X 100 µm.

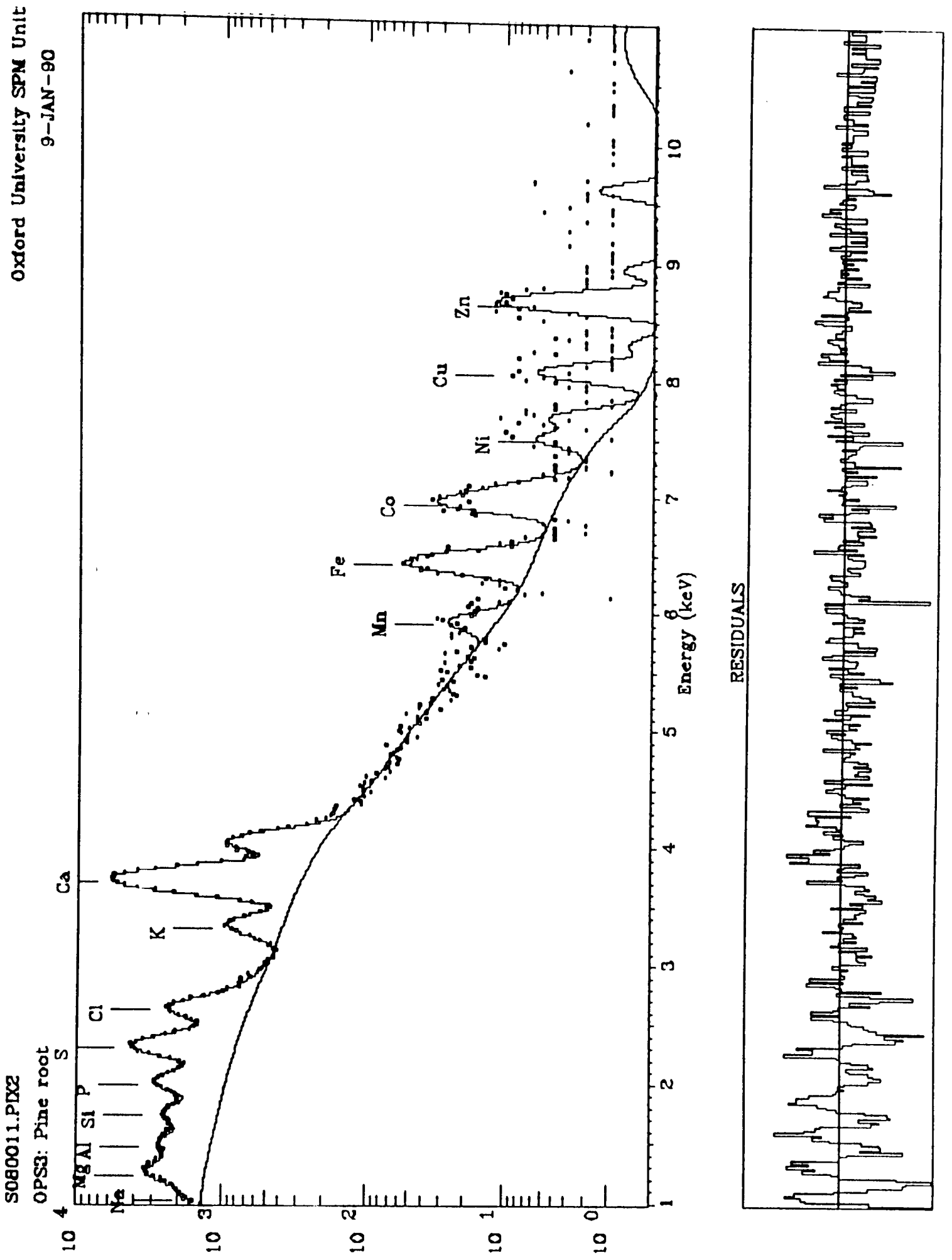


Figure 3.17. X-ray emission spectrum for a papilla taken from a primary root of a *P. sylvestris* seedling grown in Si-enriched culture. Notice the prominence of the peak for calcium.

Table 3.5

Concentrations of Ca and Si (mM) in papillae and normal cell walls of challenged and unchallenged *P. sylvestris* *seedlings*

		Challenged roots		Unchallenged roots	
Element	Structure	Si-enriched DWA[a]	Non-Si-enriched DWA[a]	Si-enriched DWA[a]	Non-Si-enriched DWA[a]
Calcium	[papilla]	215.0	177.0		
	[normal wall]	40.9	69.3	31.1	14.2
Silicon	[papilla]	20.0	17.5		
	[normal wall]	20.2	16.3	47.5	23.2

Each value is a mean from two roots

[a] Deionized Water Agar

Table 3.6

Absolute concentrations (mM), and ratios between the concentration of the elements detected in papillae and normal cell walls ([papilla]/[wall]) of challenged and unchallenged seedlings grown on Si-enriched deionized water agar, and in the embedding resin.

Element	Challenged					Unchallenged		[Resin]	MME[a]
	Absolute concentrations				Ratio				
	[papilla]	MME[a]	[wall]	MME[a]	[pap.]/[wall]	[wall]	MME[a]		
Aluminium	22.35	2.1	17.85	2.3	1.25	18.55	7.2	27.60	2.8
Calcium	215.00	0.5	40.95	1.1	5.26	31.15	4.4	4.43	17.8
Chlorine	33.50	1.6	9.96	2.8	3.36	5.86	14.1	8.58	9.1
Cobalt	3.47	9.9	0.46	26.0	7.5	--	--	--	--
Iron	6.03	6.8	0.76	20.9	7.97	0.73	53.4	0.44	43.7
Magnesium	35.00	1.5	23.95	2.0	1.46	22.50	5.8	18.65	5.3
Manganese	0.72	19.8	--	--	--	--	--	--	--
Phosphorus	23.30	1.6	13.50	4.6	1.72	14.64	14.3	8.57	9.1
Sulphur	44.95	0.9	15.90	2.6	2.83	14.95	7.3	10.25	6.7
Silicon	20.00	2.0	20.20	2.0	0.99	47.50	3.1	23.70	3.4

Values are from means of two roots
-- under detectable limits
[a] Maximum Methodological Error: see text for explanations (discussion section)

from both Si-enriched and non Si-enriched cultures (i.e., a total of 4 papillae and 4 normal cell walls), a ratio of 3.56 is obtained between papillae and normal walls. In the case of Si, if the same calculations are performed, a value close to unity (1.03) is obtained.

3.3.1.6 Relationship between papilla response and resistance

Mortality rates of *P. sylvestris* seedlings challenged *in vitro* with *P. oligandrum* are given in Fig. 3.18, expressed as total and partial mortality curves. The time of sampling for histological examination is also indicated.

There was no apparent correlation between the number of papillae in primary roots and resistance to infection with *Pythium oligandrum* in this *in vitro* system. The number, position and size of papillae in sections appeared to vary rather randomly both within and between susceptible and resistant seedlings. Attempts to use whole mounts for the detection of the most peripheral papillae in the cortex proved fruitless because of the extensive coverage of the root by fungal hyphae. No statistical analysis of the counts was therefore carried out. The only indication of any difference between resistant and susceptible individuals was that, generally, the largest and most vigorous seedlings appeared to be the most resistant.

Percentage mortality of Scots pine seedlings infected with <u>Pythium oligandrum</u>

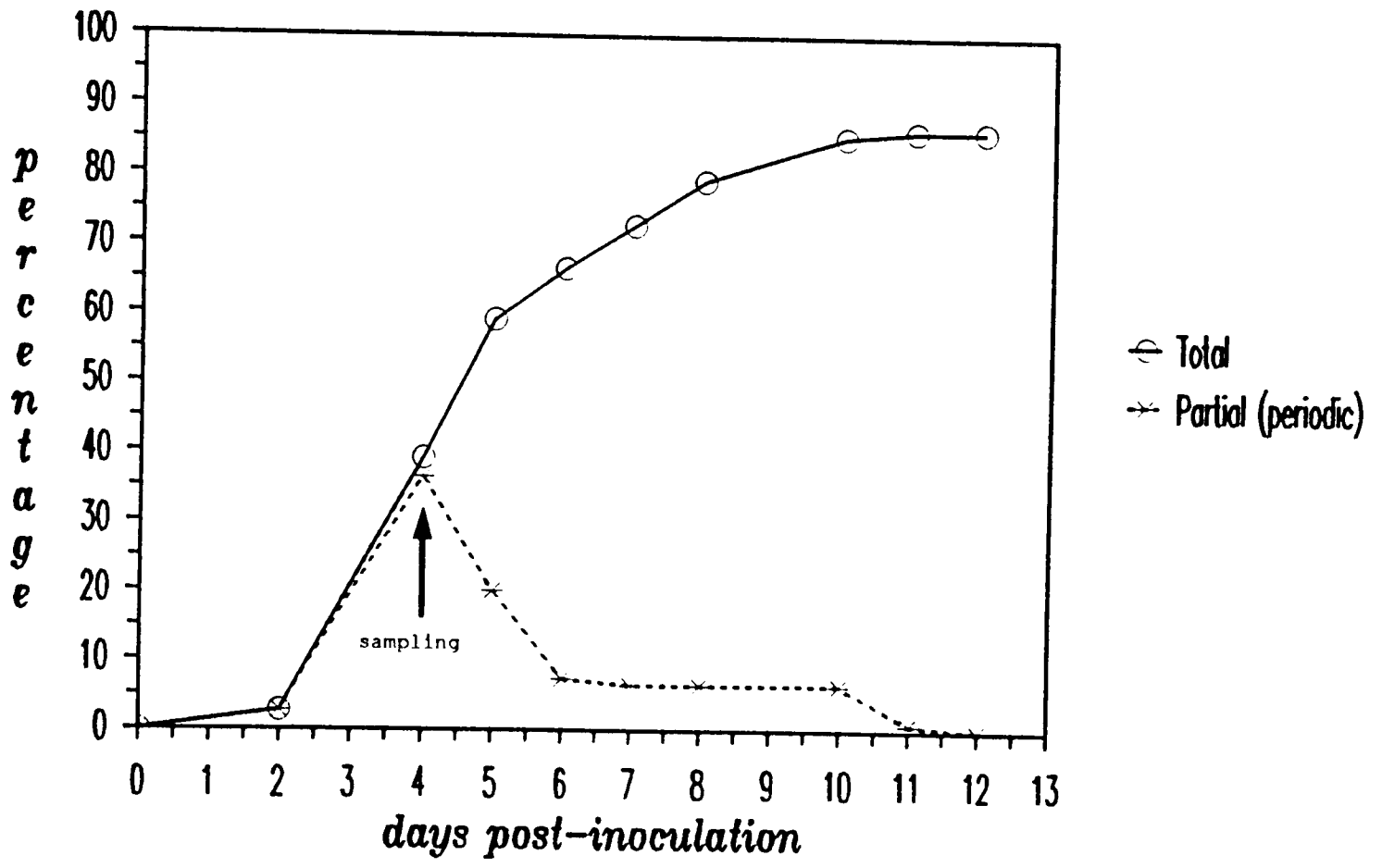


Figure 3.18. Mortality curves used for the experiment on the relationship between extent of the papilla response in the cortical cells and disease resistance. Seedlings were collected for histological examination at the indicated time.

3.3.2 Biochemical defences

3.3.2.1 Detection of antifungal compounds

TLC and bioassay of the A phase from extracts of control roots did not yield any antifungal activity, but two spots (Rf's 0.73 and 0.64) of constitutive antifungal activity were revealed in the O phase from the same material. These were termed R1 and R2 respectively, with R2 appearing visibly stronger than R1 (Fig. 3.19).

No unequivocal evidence for new compounds (or groups of compounds) with antifungal activity (phytoalexins) was obtained from the infected roots: only the constitutive antifungal compounds described above appeared in the O phase, and no antifungal activity in the A phase. A third spot of antifungal activity (termed R3, Rf=0.23 - Fig. 3.19) seemed to appear only in infected seedlings. But while R1 and R2 appeared consistently, R3 proved rather elusive, appearing only in some preparations. Considering that R1 had a much weaker activity than R2, that R3 was very elusive, and because of time constraints, it was decided to concentrate the attention on R2 only.

Under UV light, R2 had a very pronounced quenching effect on self-indicating TLC plates at 254nm, and showed no autofluorescence at 365nm.

No antifungal compounds were ever detected in *C. destructans* culture filtrate extracts, either in the O or in the A phases.

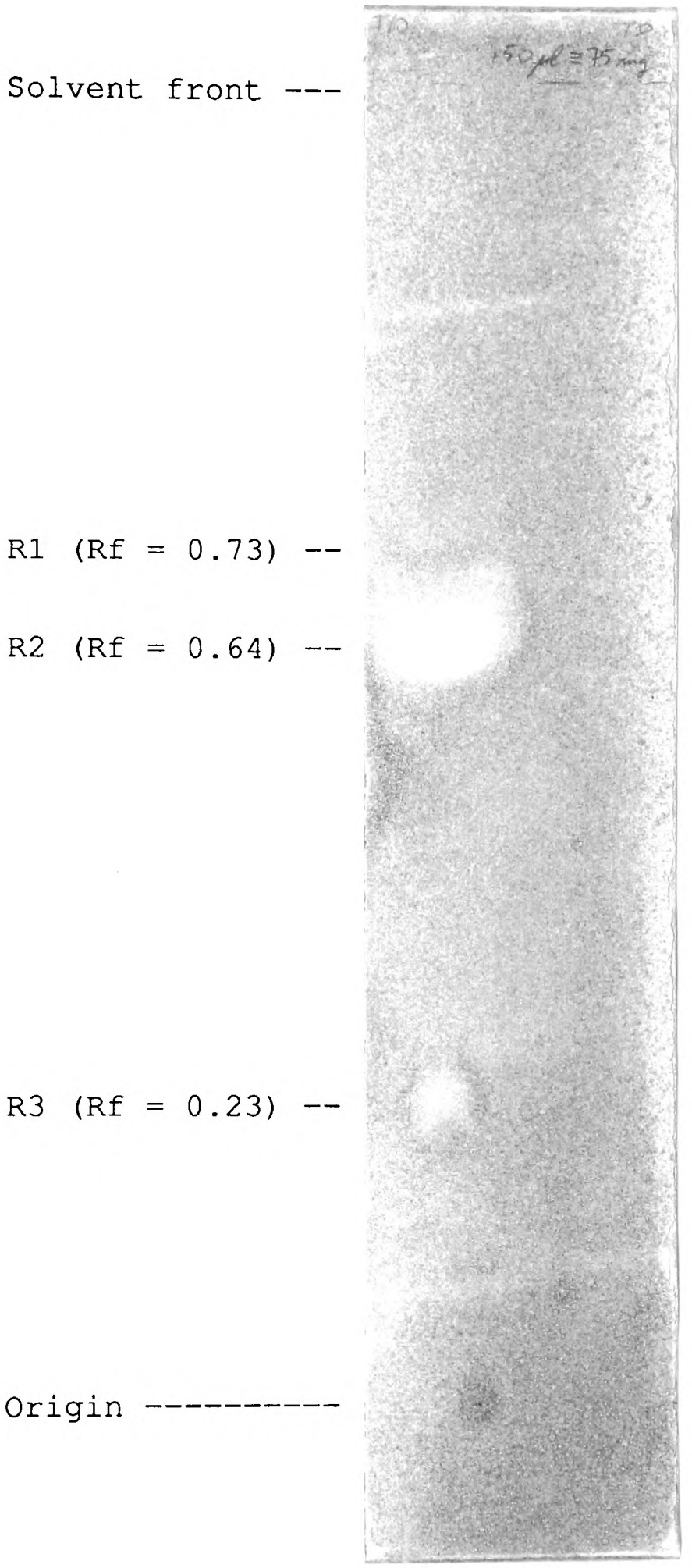


Figure 3.19. TLC bioassay with *Cladosporium cucumerinum* of the organic phase of extracts from infected primary roots of *P. sylvestris*.

3.3.2.2 Isolation of R2

After elution from the TLC plate, R2 was separated by HPLC. A single, very prominent peak eluted with a retention time (Rt) of approx. 15min (Fig. 3.20a). This peak was not present in HPLC profiles of *C. destructans* culture filtrates.

3.3.2.3 Characterization of R2

GC and MS of R2 indicated that it was a mixture of very closely related compounds belonging to the diterpene resin acid group. In particular, it appeared to be a mixture of substituted isomers of abietic acid, of which a relatively minor component appeared to be abieta-8,11,13-trien-18-oic acid (dehydroabietic acid, MW = 300). Five unidentifiable dienoic acids (MW = 302) represented the large part of the plant extract fraction. The identification of dehydroabietic acid in R2 was not straightforward. It was evident, from the GC profiles, that compound 3 of R2 (peak 3 in Fig. 3.21a) was, in reality, a mixture of two compounds, specifically a trienoic acid (M/E of TMS derivative = 372) and a dienoic acid (M/E of TMS derivative = 374) (peaks 3_a and 3_b in Fig. 3.22). Comparison of peak 3 in the GC profile of R2 with that of AA/R2 (Fig. 3.21) proved essential for the identification of the trienoic compound. This compound, corresponding to peak 3 of AA/R2 (Fig. 3.21b), was identified by mass spectrometry, beyond reasonable doubt, as dehydroabietic acid: the major peaks at M/E = 73, 239, 357 and 372 (Fig. 3.23a) matched very well those of library data for dehydroabietic acid (Fig. 3.23b).

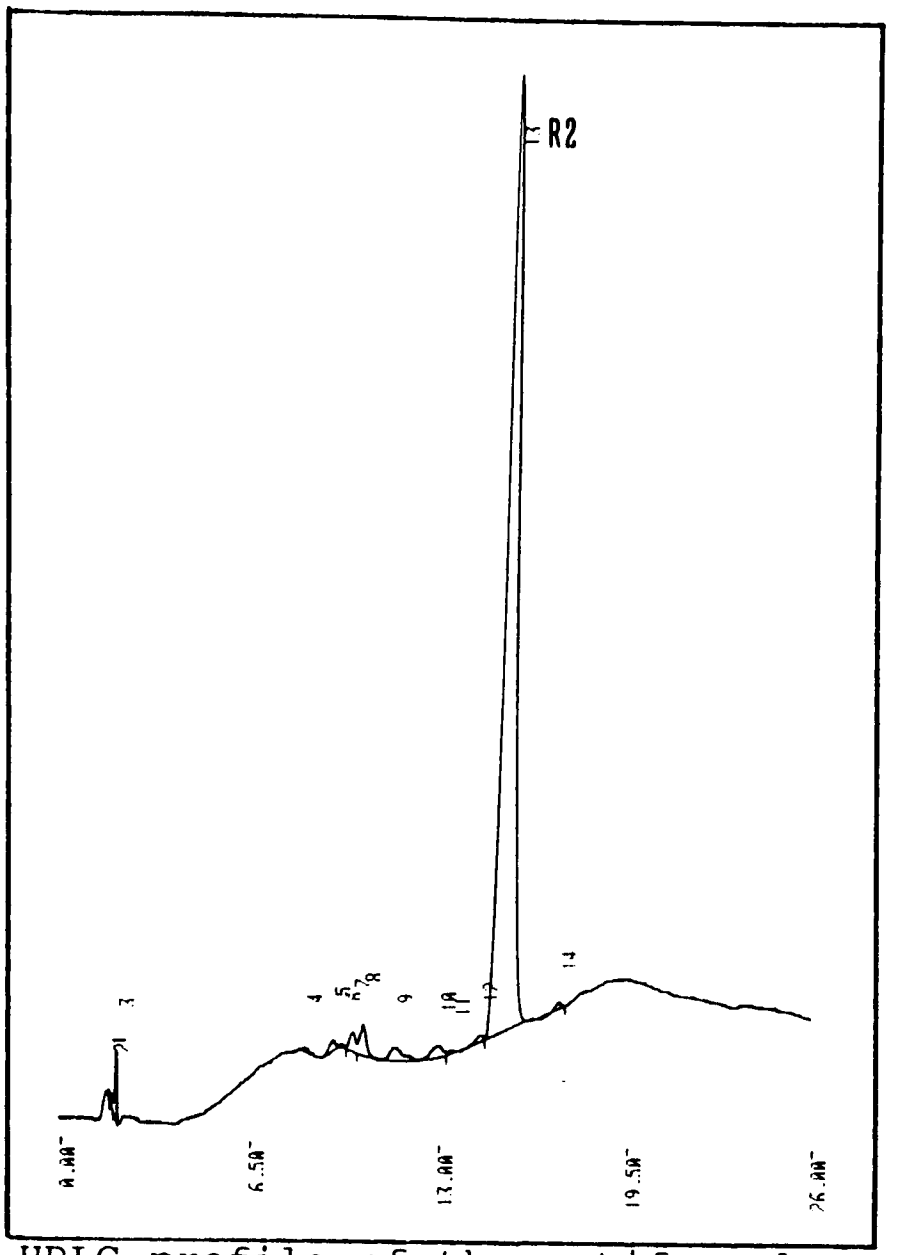


Figure 3.20a. HPLC profile of the antifungal compound(s) R2 eluted from a TLC plate (*cf.* Fig. 3.19). The single prominent peak R2 had a Retention time of approx. 15 min. See Table 3.1 for the gradient used.

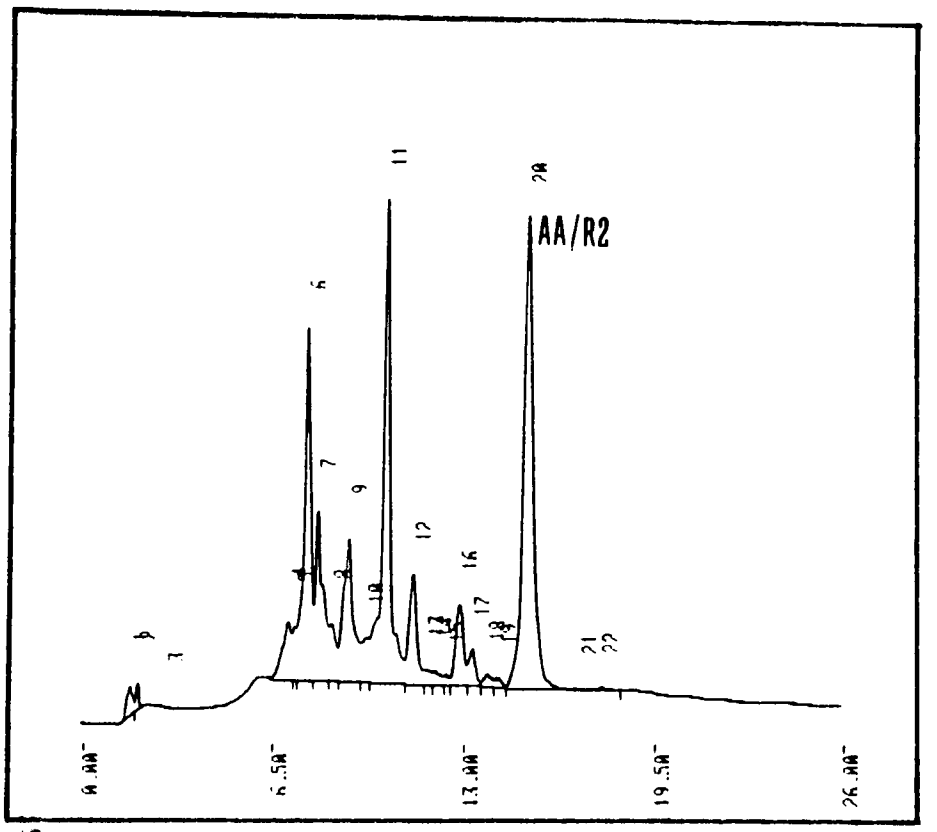


Figure 3.20b. HPLC profile of a commercial preparation of abietic acid (AA) (Sigma). The peak called AA/R2 had a Retention time of approx. 15 min, which corresponds to R2 in Fig. 3.20a. See Table 3.1 for the gradient used.

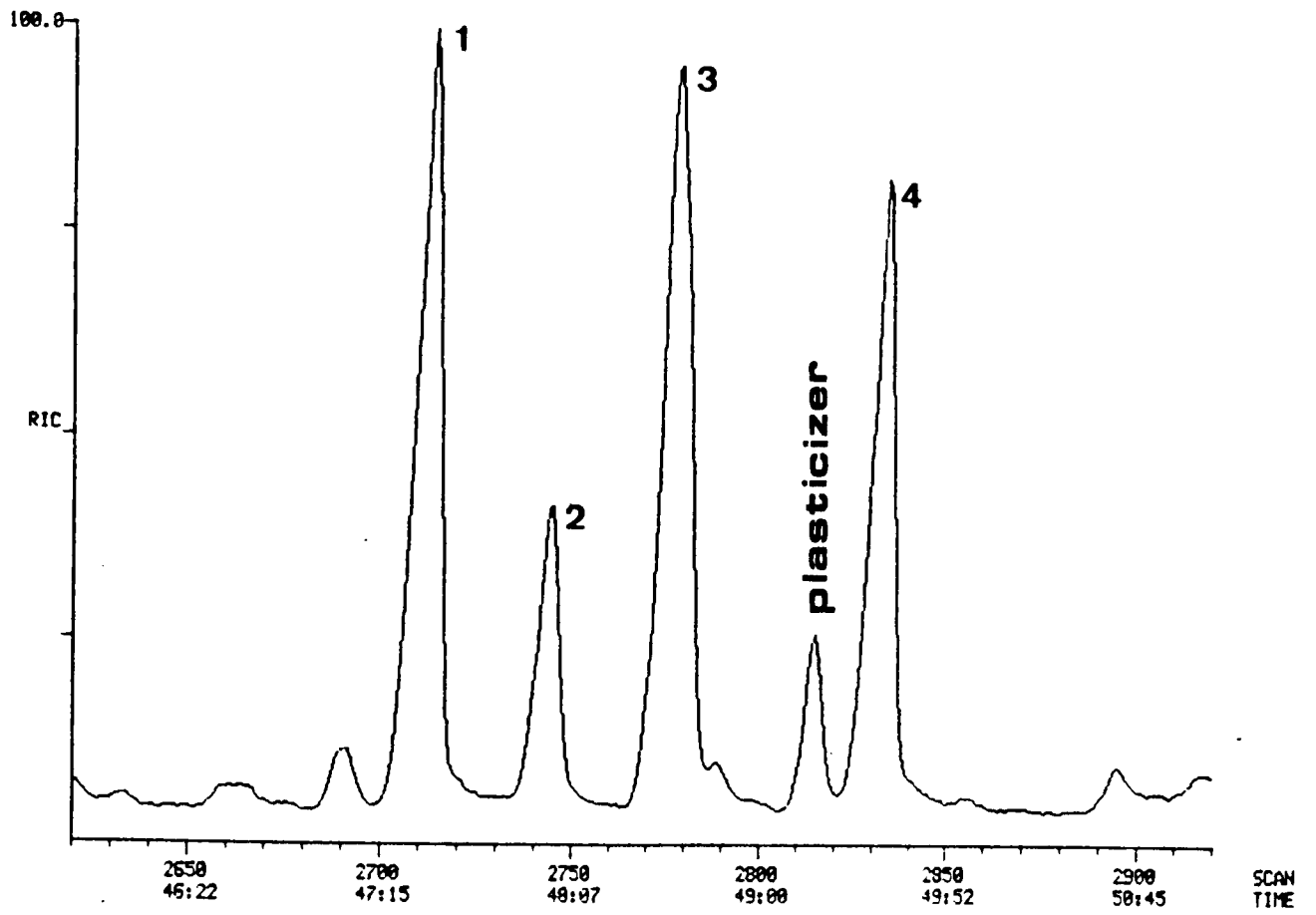


Figure 3.21a. Gas chromatography profile of the TMS-derivatives of R2. Peaks 1, 2, 3 and 4 correspond to peaks 1, 2, 3, and 4 in Fig. 3.21b. For the set-up parameters of the combined GC-MS machine see text.

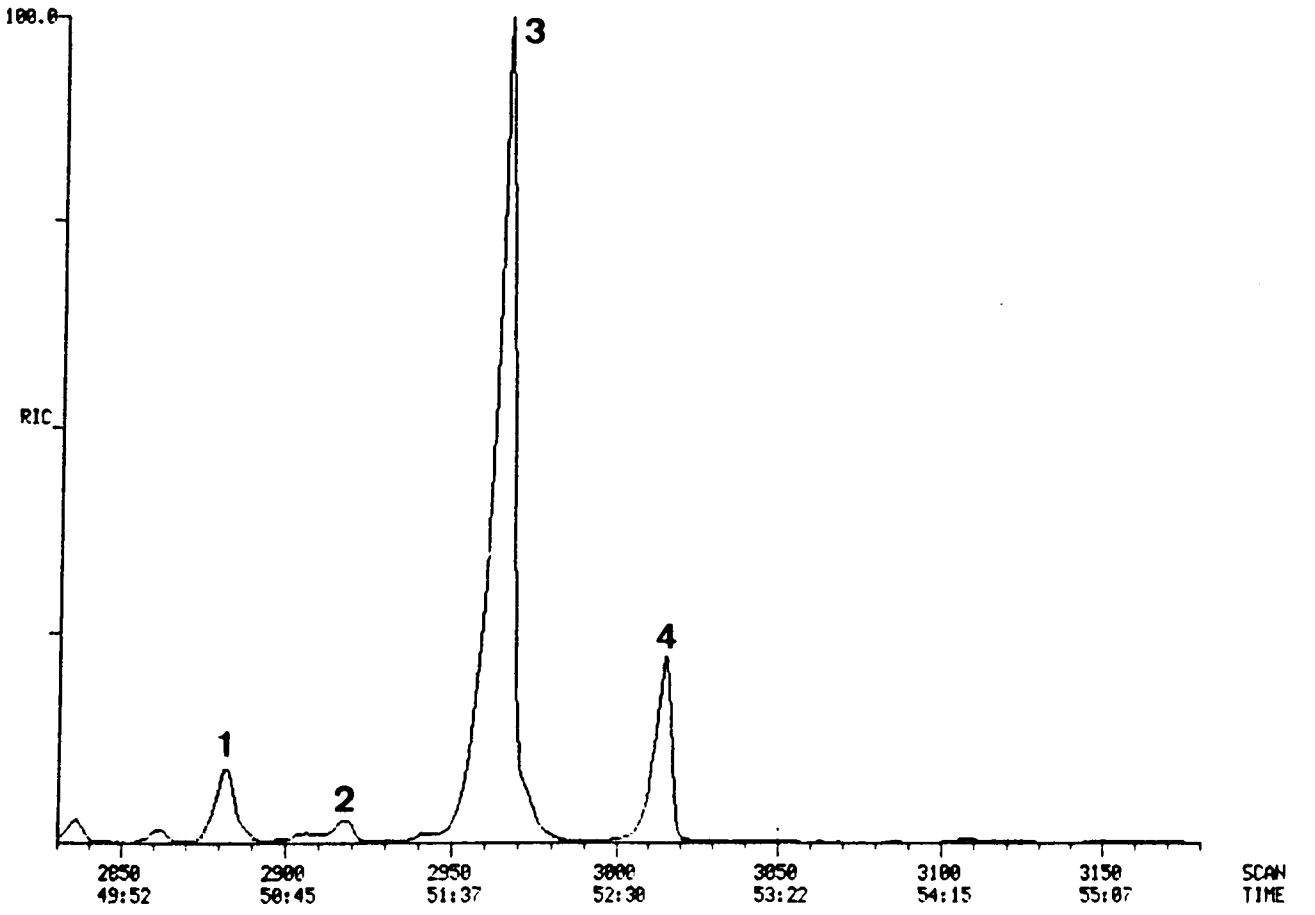


Figure 3.21b. Gas chromatography profile of the TMS-derivatives of AA/R2. Peak 3 has been identified by mass spectrometry as dehydroabietic acid (see Fig. 3.23).

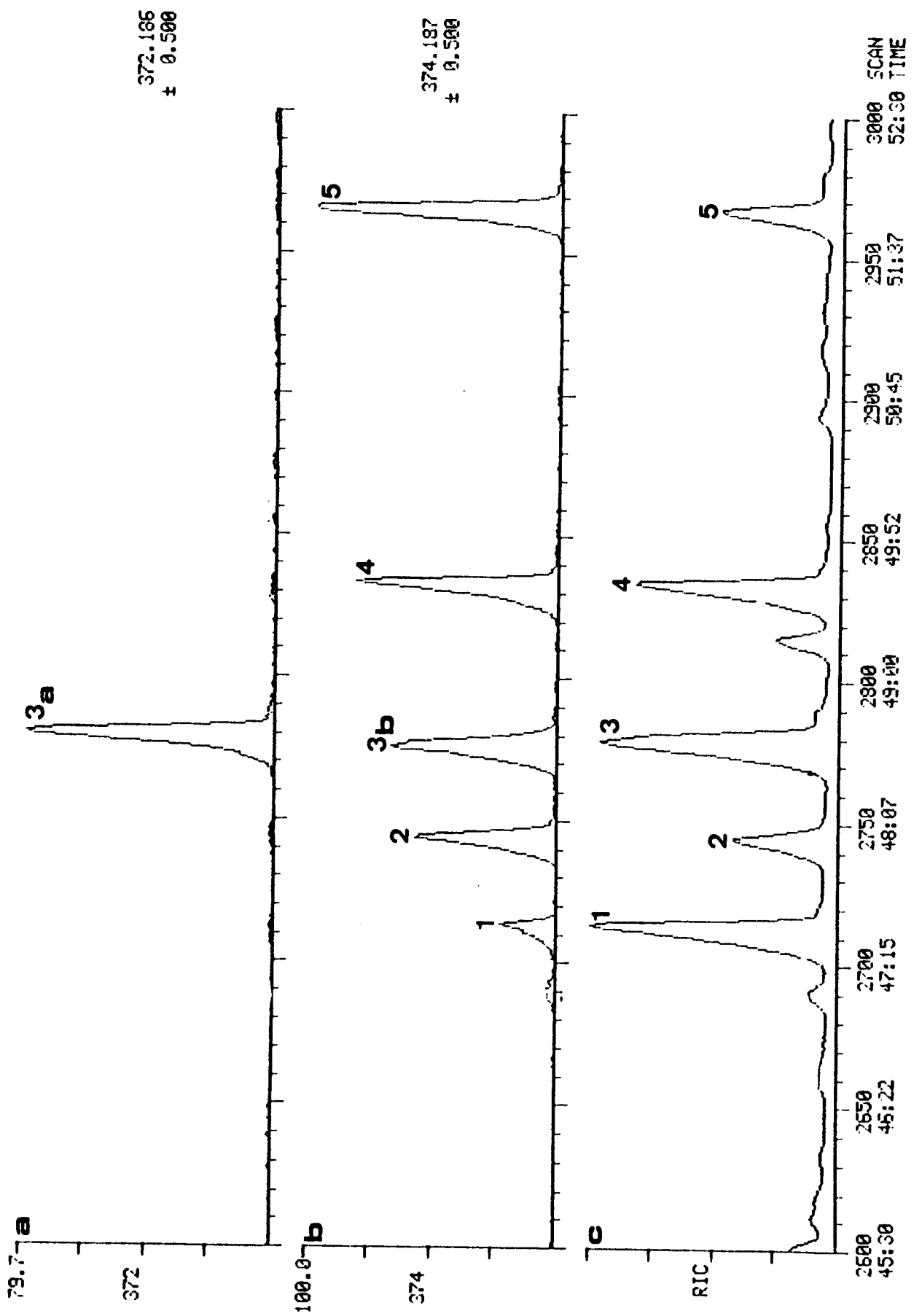


Figure 3.22. Gas chromatography profiles of the TMS-derivatives of R2. The 3 profiles indicate, respectively: a) trienoic acids, with 1 peak (3_a) (M/E of TMS-derivatives = 372); b) dienoic acids, with 5 peaks (1, 2, 3_b, 4, 5) (M/E of TMS-derivatives = 374); c) total profile (as in Fig. 3.21a), with peak 3 as the sum of peaks 3_a and 3_b.

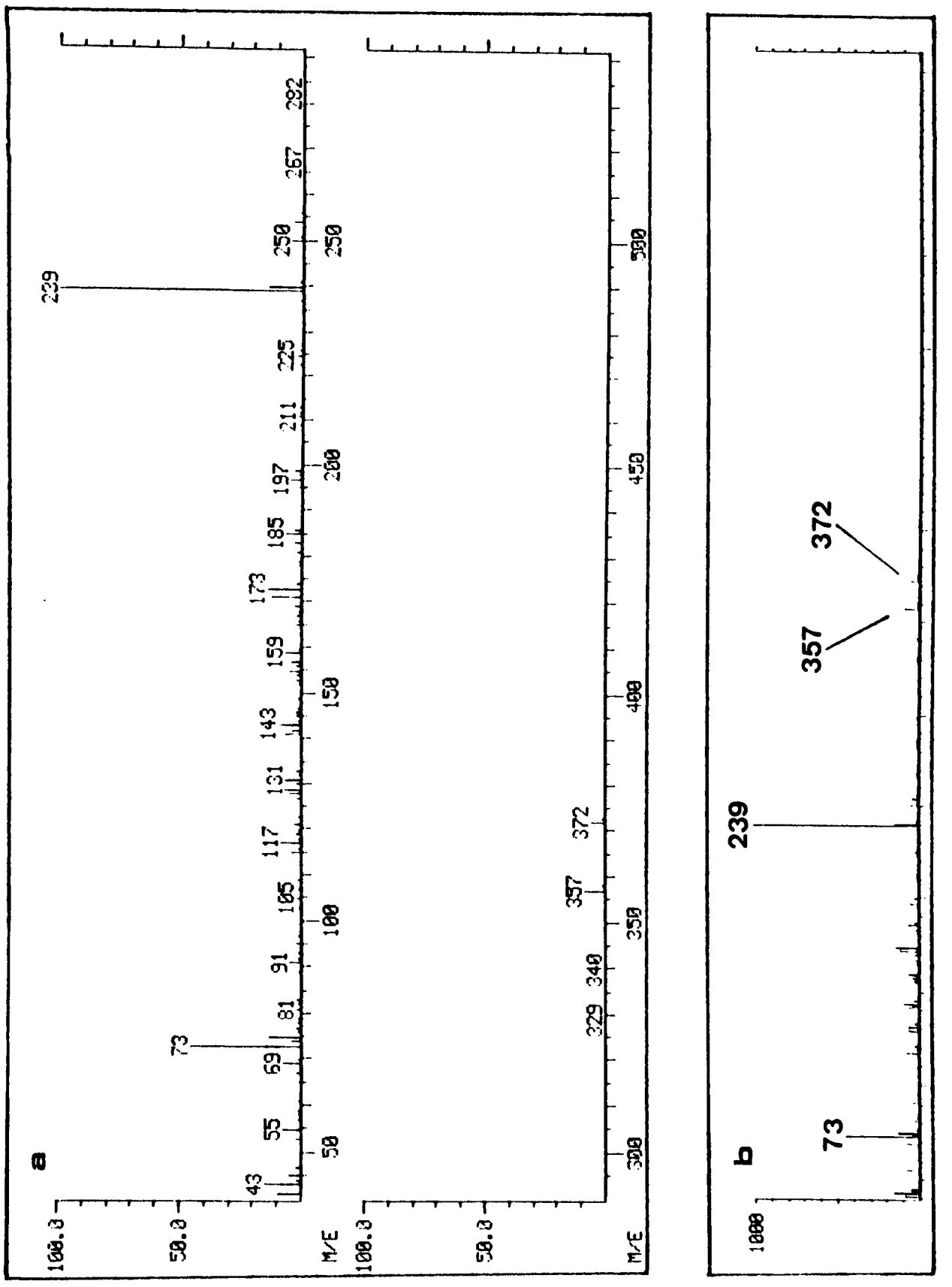


Figure 3.23. Mass spectra of: a) compound 3 in Fig. 3.21b (from the TMS-derivatives of AA/R2); b) TMS-derivative of dehydroabietic acid (abieta-8,11,13-trien-18-oic mono-TMS) (from library data). The correspondence of the main peaks (at M/E = 73, 239, 357, and 372) is sufficiently good to identify compound 3 of AA/R2 as dehydroabietic acid. N.B.: scale of a) is twice that of b).

In one determination, dehydroabietic acid was found to be mixed, in peak 3 of R2 (Fig. 3.22c), with a dienoic acid, in a ratio of 51.6% of that peak. Further calculations showed that this trienoic acid constituted 13.9% of the total of the "abietic acids" in R2 (represented by peaks 1, 2, 3_a, 3_b, 4, and 5 in Figs. 3.22a and 3.22b).

3.3.2.4 Identification of terpenes on TLC plates

The terpenic nature of the mixture was confirmed by treatment of a TLC plate with antimony pentachloride (Stahl, 1969), which gives an orange-brown reaction colour for this class of compounds.

3.3.2.5 HPLC analysis of R2

Following gas chromatography and mass spectrometry, a comparison using HPLC was made between R2 and a commercial preparation of abietic acid (AA) (Sigma) dissolved in MeOH. This sample appeared quite impure (a fact already evident from the TLC preparation of AA/R2), but one of the major components, with a retention time of approx. 15min (Fig. 3.20b), corresponded very well to the prominent peak obtained with R2.

The fraction corresponding to that peak of AA was then partially purified by preparative HPLC, and was shown to correspond to the AA/R2 fraction analysed previously by GC-MS, TLC, and spectrophotometry.

3.3.2.6 Spectrophotometry of R2 and AA/R2

Confirmation that R2 was a diterpene resin acid was also sought by spectrophotometric analysis. The UV spectra of R2 and AA/R2 were very similar, and when the peaks and shoulders were compared directly, it appeared that they corresponded very well, with two peaks at 237nm and 241nm (at 237nm AA/R2 only shows a shoulder), and a major shoulder at 251nm (Fig. 3.24). A peak at 241nm has been reported to identify true abietic acid (Fries *et al.*, 1987).

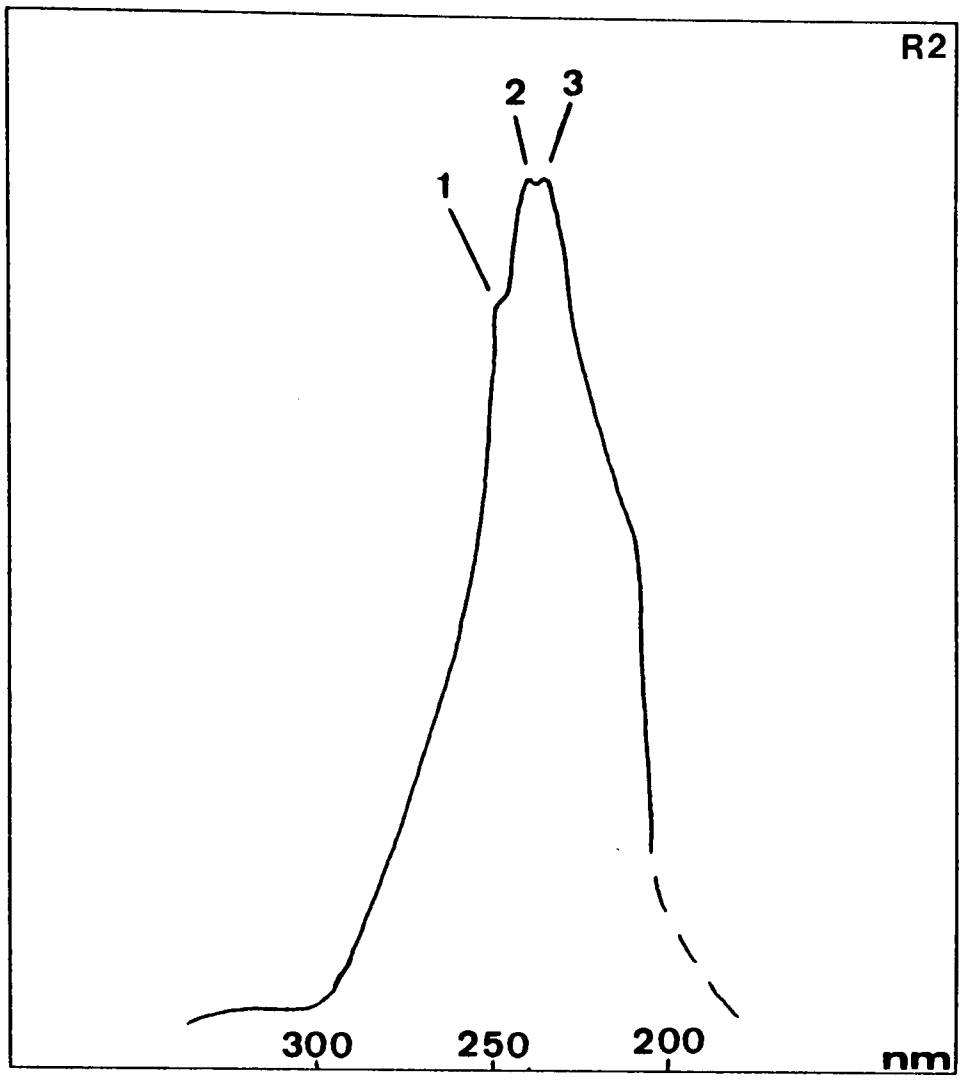
3.3.2.7 Quantification of R2 in infected and control seedlings

The results obtained by HPLC are summarized in table 3.7, with the standard curve for abietic acid (AA/R2) given in Fig. 3.25. The mean concentration of R2 in control and infected roots, as mg equivalent of AA/R2 g^{-1} dry weight (roots contained approx. 90% water), is shown, together with the SEM's, and the percentage change in concentration after infection. Statistical analysis of the difference of the means (Student-t test) showed that it was not significant.

Table 3.7

Quantification (and percentage change) of abietic acid *sensu lato* (R2) in methanol extracts of primary roots of Scots pine seedlings (mg AA/R2 g^{-1} dry weight)

	Control	Infected
Mean (+-SEM)	5.2 (+-0.9)	9.7 (+-2.3)
Change from Control to Infected = +85.2%		
Difference of means non significant (Student-t test) (4 replicates per treatment)		



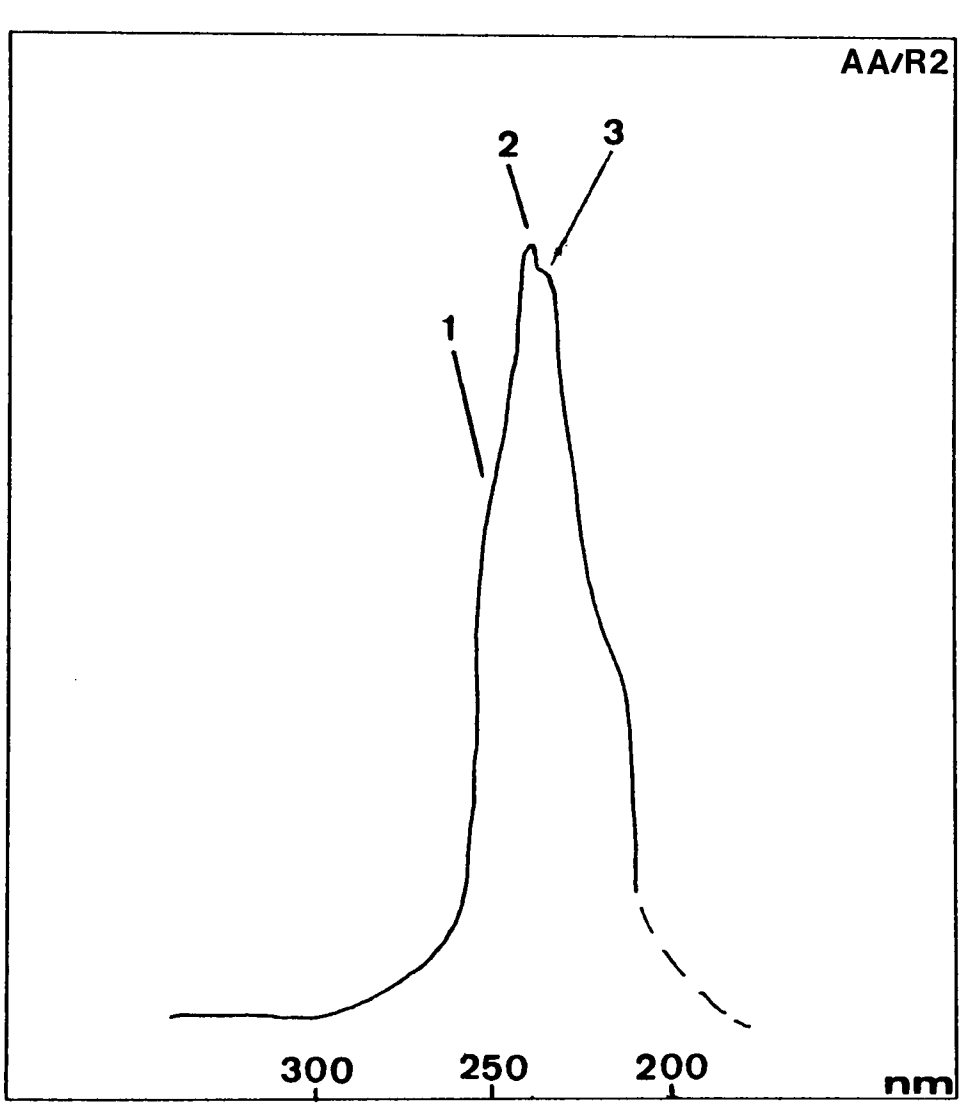


Figure 3.24. Spectrophotometric comparison between R2 and AA/R2. Notice the correspondence between the peaks and shoulders labelled 1, 2, 3 (251, 241, 237nm, respectively).

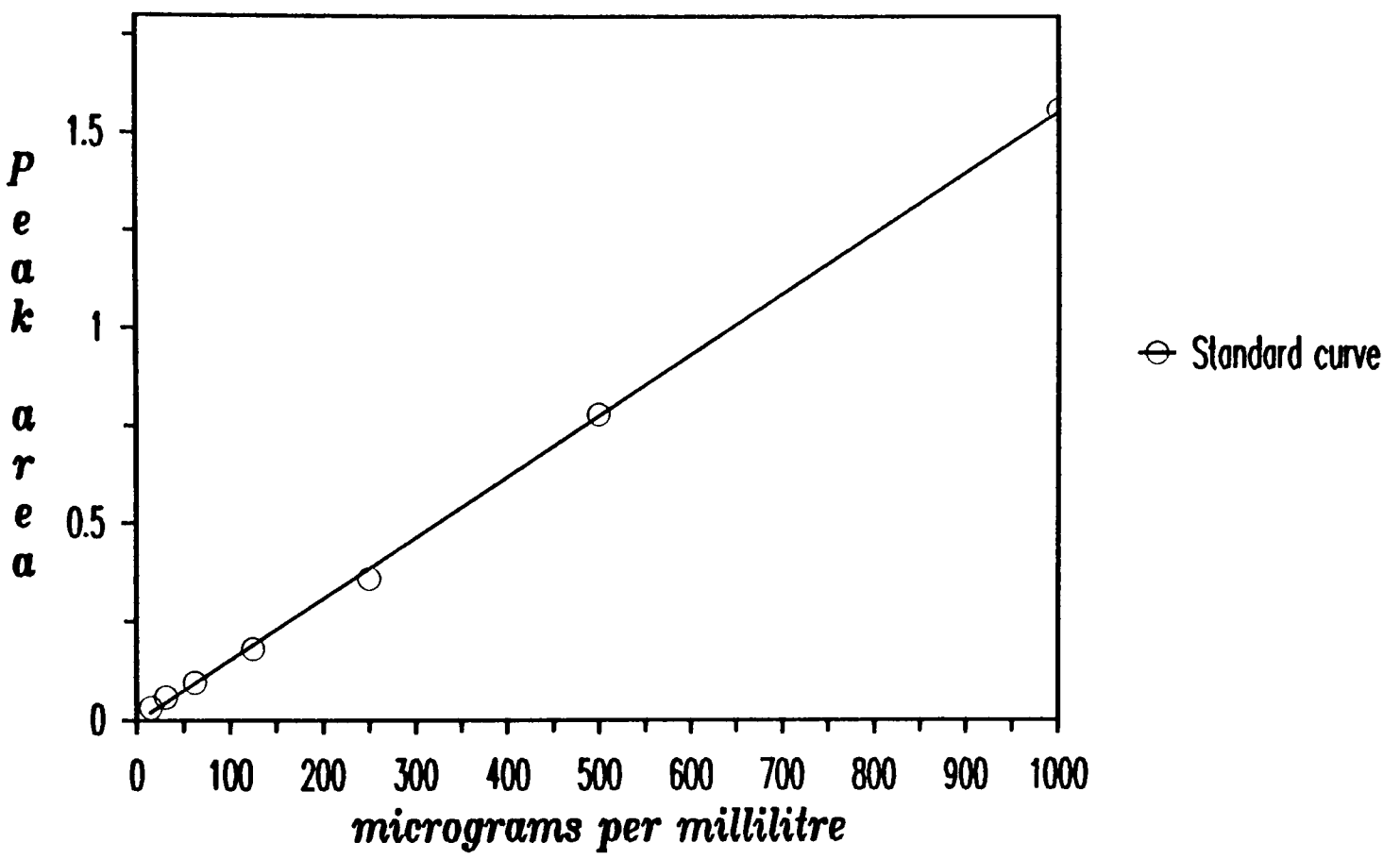

Standard curve for abietic acid (AA/R2)
(peak area X10E-6)
peak area
1.5
1
0.5
0
0
100
200
300
400
500
600
700
800
900
1000
micrograms per millilitre
Standard curve

Figure 3.25

3.3.2.8 Estimation of the antifungal activity of abietic acid *in vitro*

The commercial preparation of AA (Sigma) was not active, in a *Cylindrocarpon destructans* spore germination inhibition assay, up to a concentration of 2mg ml^{-1} in the final test mixture. Only at the highest concentration used (4mg ml^{-1}) did AA have a slight inhibitory effect, lowering the germination rate from 95.3% in the corresponding control to 90.3% (for each treatment, 100 spores were counted in each of 3 replicates per concentration) (Fig. 3.26). However, a gross effect on the morphology of conidia, and on germ tube length was observed down to 1mg ml^{-1} (Fig. 3.27). Conidia appeared swollen and irregularly shaped, while germ tube growth was depressed by up to 69.1% (at 4mg AA ml^{-1}) relative to controls (as determined by measuring 5 randomly chosen germ tubes in each micrograph in Fig. 3.27).

Effect of abietic acid on the germination percentage of Cylindrocarpon destructans spores

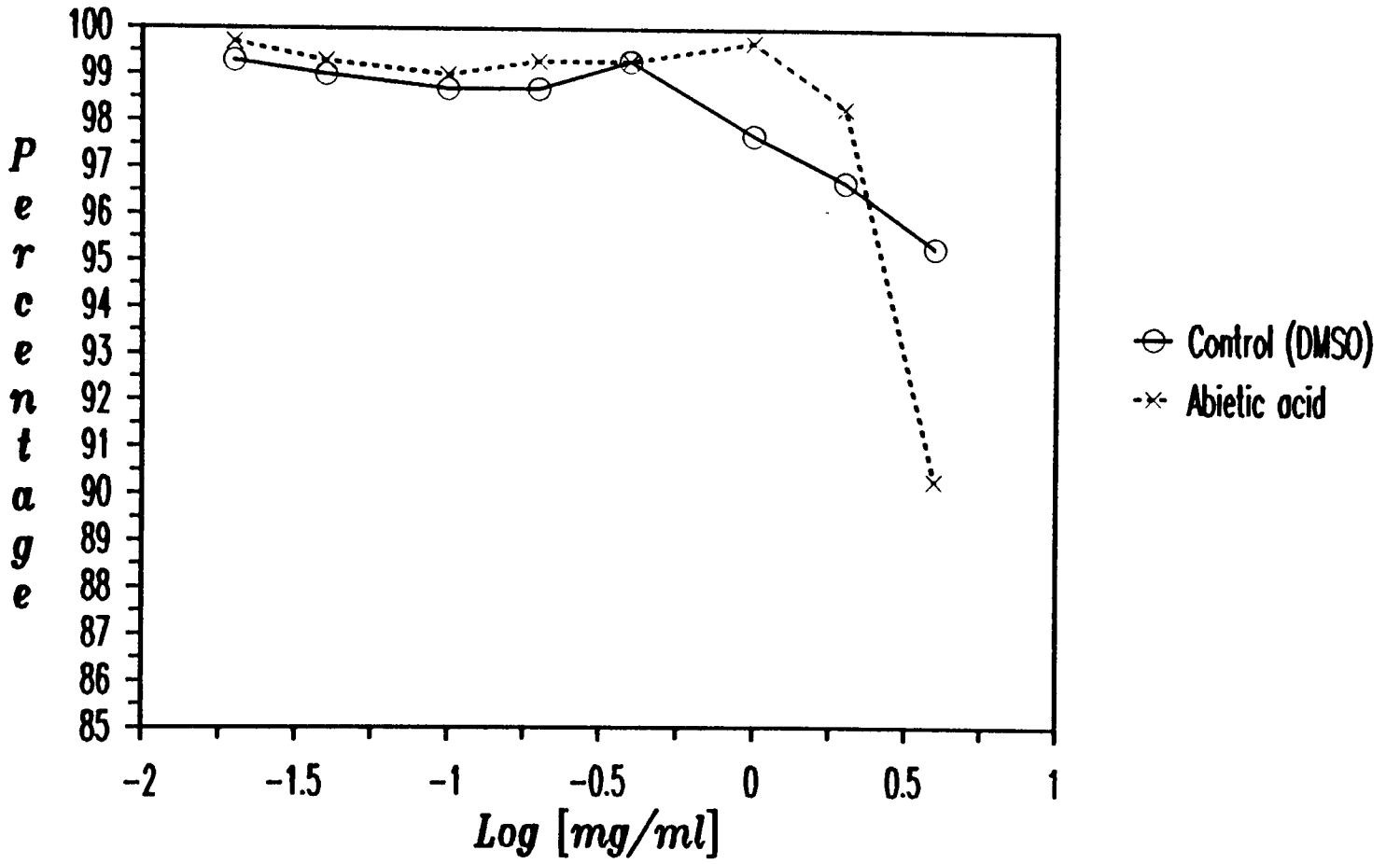


Figure 3.26. The concentrations of abietic acid (Sigma) used ranged from 0.02 to 4 mg ml^{-1}. DMSO: dimethylsulphoxide.

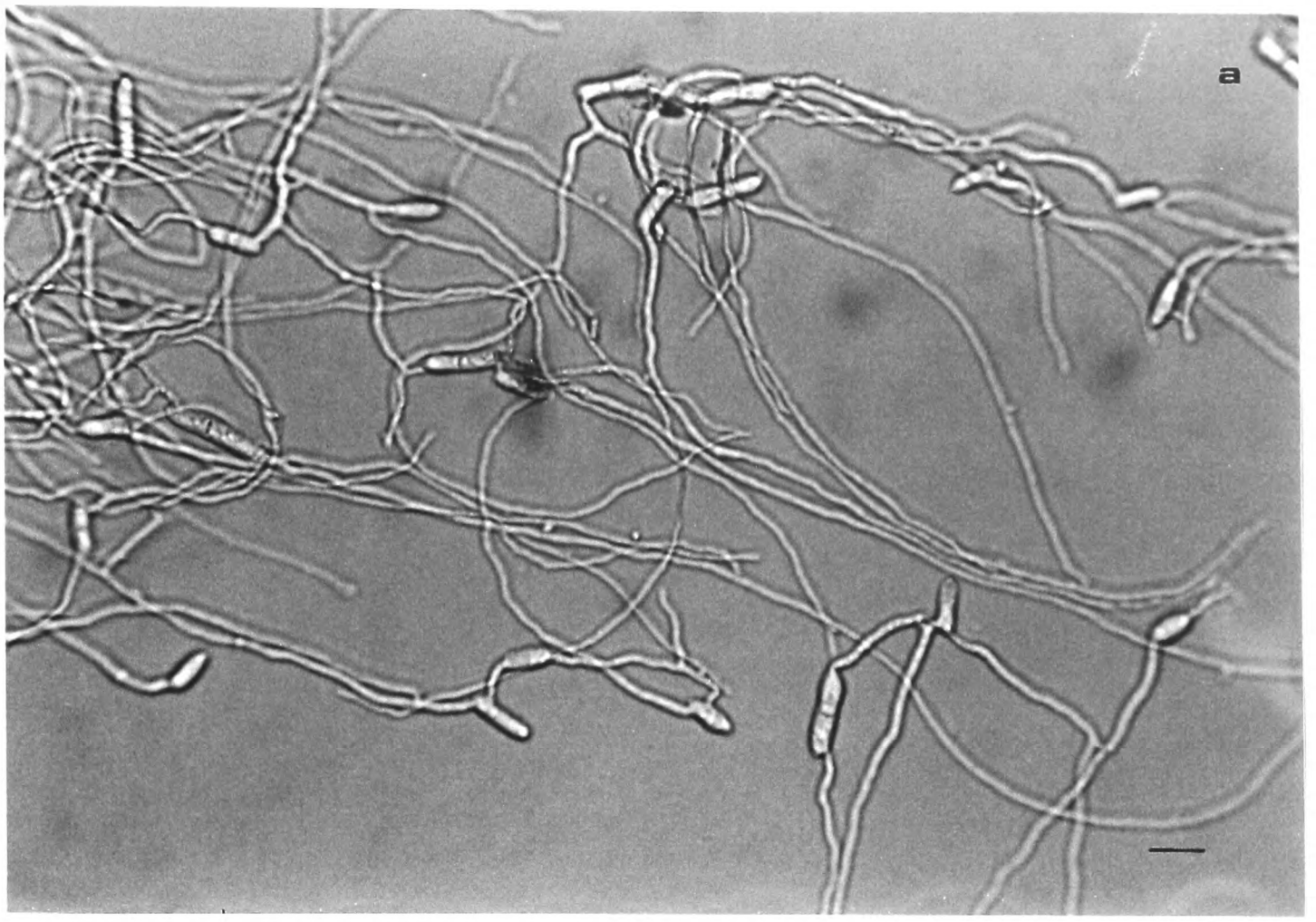


Figure 3.27. Effects of abietic acid (Sigma) on the morphology and germ tube growth of *Cylindrocarpon destructans* spores. a) Control (4% DMSO); b) Conidia appear swollen and abnormally shaped, while germ tube length is considerably reduced (approx. 70%), when germinating in a solution (4% DMSO) containing 4mg abietic acid ml^{-1}. Scale bar = 10µm.

3.4 **Discussion**

3.4.1 Structural defences

Cell wall alterations, including papilla formation, represent the only known structural defence mechanism operating at the cellular level (Aist, 1976; 1983; Beckman, 1980; Ride, 1983). Such wall alterations, usually occurring by the apposition of new cell wall material at sites of attempted fungal penetration, are known from the aerial parts of a range of plants, including woody species (Beckman, 1980; Edwards & Ayres, 1981). There is, however, much less information regarding their occurrence in primary root tissues. Reports in herbaceous species include *Gaeumannomyces graminis*-infected wheat (Fellows, 1928); hop roots infected with *Verticillium albo-atrum* (Talboys, 1958); *Phytophthora cinnamomi*-infected maize (Hinch & Clarke, 1982; Whetherbee *et al.*, 1985); endomycorrhizal *Allium porrum* roots (Codignola *et al.*, 1989), and ectomycorrhizal *Dryas integrifolia* (Melville *et al.*, 1987), whilst in woody angiosperms their occurrence has been reported in *Acacia pulchella* infected with *Phytophthora cinnamomi* (Tippett & Malaiczuk, 1979). In gymnosperms, no reports of cell wall appositions as responses to pathogen attack in non-mycorrhizal roots are known, although reports of wall appositions in mycorrhizal roots exist (Duddridge, 1986[a,b]; Duddridge & Read, 1984[a,b]; Melville *et al.*, 1987; Nylund *et al.*, 1982). Their nature and function in such mutualistic associations remain, however, uncertain.

This chapter presents evidence of an inducible papilla response in non-mycorrhizal primary roots of *Pinus sylvestris* challenged with a fungal pathogen.

Structurally, the papillae described from pine primary roots broadly resembled those reported from other plants (*cf.* Aist, 1976; 1983), comprising variously shaped deposits of new materials onto the cell wall. Some heterogeneity of fine structure was apparent. Use of a wide range of histochemical tests indicated that pectic materials were a major polymeric component of these wall appositions. Although the sample preparation procedure used (embedding in LR White plastic) appeared to interfere with tests for cellulose (zinc-chlor-iodine and IKI-H_2SO_4) and lipids (Sudan dyes), the use of these tests on unembedded, fresh (whole-mounts) material gave confused, or similarly negative, results.

Of the tests for pectic materials, only the hydroxylamine-ferric chloride test was negative. This result may have been due to a low level of methylation in the native pectic acids (Jensen, 1962). The increased concentration of insoluble calcium in the papillae may reflect an abundance of pectic acids with free carboxylic groups rather than highly esterified pectins. Pectic materials have been reported as one of the major components of papillae (Aist, 1976).

Lignification and the binding of phenolic compounds to plant cell walls are important general responses to microbial invasion (Aist, 1983; Vance *et al.*, 1980). These compounds make walls and papillae mechanically tougher (Aist, 1983;

Vance *et al.*, 1980), and their presence has been correlated with the effective operation of papillae as a plant defence (Edwards & Ayres, 1981; Ride & Pearce, 1979). In pine roots only a small proportion of papillae were not autofluorescent or gave negative responses to the relevant histochemical stains for lignin-like or phenolic materials, indicating an association of wall-bound phenolic compounds with these structures.

Light microscopy hinted that one of the responses of pine roots to pathogenic infection may be a generalized accumulation of phenolic materials in the cortical cell walls. This appears to be confirmed by the quantification of diffuse phenolic accumulation. Although the total amount of lignin did not increase after challenge, an increase in the total amount of simple phenolics esterified to the cell walls was noted. Ride (1975) warns that at least part of this apparent simple phenolic fraction may in fact be unrecognized tyrosine residues of protein. Experimental constraints prevented the author from pursuing this matter further. Nevertheless, the interpretation that there was a higher amount of phenolics esterified to the cell walls was supported by the observation that there was a contemporaneous diminution in the total amount of soluble phenols in infected seedlings. All this is suggestive of a movement of phenols from the vacuoles to the cell walls, to which they become esterified. On the basis of these results it is not possible to say whether this is an active or passive type of response by the plant: this transfer of simple phenolics may just be a consequence of vacuolar

disruption caused by fungal invasion.

The observed variation in the infusion of papillae with phenolic compounds may reflect different developmental stages in papilla formation encountered at the time of observation. The fact that the great majority of papillae gave positive histochemical reactions for bound phenolic compounds is suggestive that infusion with phenolics is a relatively early event in papilla development. This result is closely comparable to what has been observed in other plants (Ride & Pearce, 1979; Sherwood & Vance, 1976; Sherwood & Vance, 1980).

In resistant seedlings the pathogen was halted within the cortical tissues, never reaching the stele. This resistance appeared to be essential for the survival of the plant, as invasion of the stele caused severe tissue disruption (Fig. 3.1), with consequent wilting and death of the seedling.

Successful penetration of papillae by *C. destructans* appeared to be a rare event. The resistance of papillae to this fungus may be partially explained by the fact that *C. destructans* possesses only a limited pectolytic activity (Dahm & Strzelczyk, 1987).

Silicon has been reported to accumulate in the papillae of various plants, particularly cereals (Heath, 1981[b]; Kunoh & Ishizaki, 1975; Sargent & Gay, 1977), where it has been suggested it may contribute to the resistance of these structures. No such enhanced accumulation of silicon occurred in the pine root papillae investigated in this study, either

in plants grown in normal, or silicon-enriched culture (to ensure availability of this element to the seedlings). As those species with silicified papillae often have a high silicon content normally [e.g., maize, rice (Bidwell, 1978)], the absence of silicon accumulation in pine root papillae is not, perhaps, surprising.

PIXE microanalysis and elemental mapping provides a powerful tool for the study of elemental changes in plant tissues. It indicated that the papillae in pine roots were not enhanced in Si. Indeed, it appeared that the source of the silicon detected, with comparable values in normal cell walls, papillae and resin (Table 3.6), was the resin itself, perhaps due to contamination by the glass knives during sectioning (which could also explain the higher concentration in the unchallenged material). A similar conclusion might be drawn from the uniform silicon distribution shown in Fig. 3.16d.

PIXE microanalysis has also provided evidence for elemental differences between the wall appositions and the cell walls. The most significant and consistent of these is an accumulation of insoluble calcium, with mean values (4 determinations from challenged seedlings) increasing from 55.1mM in normal cell walls to 196mM in apposition material. This elevated concentration of calcium appears real, and is unlikely to be attributable to overlap of the beam spot (diameter < 1μm) beyond the thickness of unaltered cell walls [> 1μm at points chosen for analysis (double wall of adjacent cells)]. This is indicated by the high Ca levels in papillae, compared with normal cell walls, recorded on the elemental

maps (Fig. 3.16b). The precision attainable with the beam targeting also supports this interpretation.

Calcium accumulation has been reported in papillae in other species, where it has been indirectly associated with the resistance of these structures to fungal penetration (Aist & Israel, 1986[a,b]; Bayles & Aist, 1987; Kunoh *et al.*, 1986).

In this case it seems likely that the insoluble calcium is a component of the pectic materials present, increasing the strength of the bonds between adjacent polymer chains by cross-linking these through their acidic groups. The hardening of plant primary walls by calcium cross-linking of pectic polymers (Goldberg *et al.*, 1989; Rinaudo, 1989) has been reported to confer increased resistance to pathogen attacks (Akai & Fukutomi, 1980; Beckman, 1980). Furthermore, accumulation of calcium and other polyvalent cations appears to be associated with some inhibition of polygalacturonase activity (Bateman, 1964). Thus, although the papillae formed in response to fungal challenge in pine roots generally resembled those described from angiosperms, their composition may differ, primarily in the apparent absence of callose (the most characteristic component of appositions in other species), and the seemingly high level of pectic materials present.

Although calcium is a mediator in polysaccharide synthesis (Aist, 1983; Kohle *et al.*, 1985) this is unlikely to be its sole function in the papilla area, as under these conditions it would be subject to leaching during sample preparation, as a consequence of its ionic nature.

For such elements as Co, Mn and Fe (*cf.* Table 3.6), a role as co-factors in enzyme processes associated with the papilla response is possible; others such as Cr [detected only locally at relatively high concentrations in maps (data not shown)] may be artifacts due to contamination of the sample, e.g. from the loop used to pick up the sections from the microtome trough.

Further determinations would be desirable to increase the precision of the quantitative data of both Ca and, especially, the other elements detected by PIXE, for which the inherent methodological errors (MME, Table 3.6) were proportionally much greater than for calcium, due to their low concentrations in the sample. These errors reflect the precision of the photon counts for each element peak, relative to background radiation: the higher the concentration of an element, the sharper the peak, resulting in a smaller error. However, due to external constraints on the application of this new technique to the study of this experimental system, further replication to improve the quantitative precision of these elemental data was not possible in the present study.

As a result of uncertainties in the timing of the establishment of the interaction between the fungus and the host root, consequent upon the nature of the experimental system used, and the fact that the papillae were formed internally within the cortical tissues of the roots, no attempt was made to follow the development of these structures with time. Such studies have been performed with more amenable

herbaceous plant systems (e.g., barley, wheat, kohlrabi) (Aist, 1983; Aist & Israel, 1986[a,b]; Bayles & Aist, 1987; Kunoh *et al.*, 1986; Ride & Pearce, 1979), indicating that papillae are deposited quickly after the onset of fungal penetration (sometimes in a matter of only few hours - Aist, 1983), and that they often become lignified as they mature (Ride & Pearce, 1979).

It was however possible to assess that, in resistant pine seedlings, the pathogen was halted within the cortical tissues (where the papilla response was expressed), never reaching the stele. This resistance appeared to be essential for the survival of the plant, as invasion of the stele in susceptible individuals caused very severe tissue disruption (Fig. 3.1), with consequent wilting and death of the seedling.

Attempts to correlate papilla induction to resistance by using a more virulent pathogen, *Pythium oligandrum,* were marred by various practical problems. For such a study, it is very important to know the relative timing of disease development events. However, it was not possible to know which seedlings would be susceptible to infection until they were actually dead. This means that if a susceptible seedling is harvested before it dies it will be wrongly recognized as a resistant one. It was therefore decided to sample seedlings at the point of maximum wilting rate, estimated by the position of the slope maximum on the mortality curve (maximum partial mortality). The experiment seemed to indicate that papilla formation had little or no relevance in this particular host-

pathogen system, because no correlation was evident between the extent of the papilla response and resistance. Methodological and time constraints dictated the choice of the *Pythium*-pine combination as opposed to the original *P. sylvestris-C. destructans*. The author believes that this choice was justified on the assumption that papilla induction is a general type of response (Aist, 1983), independent of the particular pathogen, but is also aware that caution is due in interpreting these results. The two model systems, *P. sylvestris-C. destructans/P. oligandrum*, may not be completely interchangeable, as different pathogens may behave quite differently on the same host, particularly in relation to the timing of the establishment of the parasitic relationship, and in the relative significance of particular host defences. This is a factor of the utmost importance for the outcome of the interaction, i.e. whether the host is resistant or not. *P. oligandrum*, the most virulent of the two pathogens considered, is characterized by much higher growth and infection rates than *C. destructans*.

It is of note that papilla induction in pine roots does not appear to be as quick as it is in other host-pathogen interactions, notably in cereals (Aist, 1976). This may well be a decisive factor in the effectiveness of this response in pine. In any case, it is known that single defence mechanisms very rarely play a central role in defence: resistance usually results from the expression of a number of different defence mechanisms. The results with *P. oligandrum* indicate that host properties other than papilla apposition (e.g., the general

vigour of the seedlings) may be crucial for the outcome of this plant-pathogen interaction.

Although papillae appear to confer no particular advantage to the defence of Scots pine primary roots from *P. oligandrum*, the evidence suggests that they may play a more fundamental role in the interaction with *C. destructans*.

3.4.2 Biochemical defences

True phytoalexin production in pine is poorly documented. Franich *et al.* (1986) demonstrated convincingly that benzoic acid was synthesized *de novo* in the needles of *P. radiata* subjected to dothistromin treatment, and that it had a strong fungistatic effect against the *P. radiata* leaf pathogen *Dothistroma pini*. The classic work by Shain on reaction zones in pines (1967, 1971) also hinted that pinosylvin and its monomethyl ether, which were present in the reaction zone but not in the healthy sapwood (but are commonly found in pine heartwood - Shain, 1967), behaved like phytoalexins in loblolly pine (*Pinus taeda*) attacked by *Heterobasidion annosum*. The author is aware of only one other claim of phytoalexin production in pines (Hare, 1972). Although Hare reported phytoalexin synthesis in both susceptible (slash) and resistant (shortleaf) pines, it could not be related to resistance to *Cronartium fusiforme*, and the results of his experiments were obviously confused by the contrasting effects of different concentrations of this putative phytoalexin, and by the alleged presence of a fungal germination promoter produced by the susceptible slash pine. Most importantly, no

clue on the identity of this putative phytoalexin was given.

In the present work, although compound R3 (Rf = 0.23 on TLC plates) seemed to appear only in infected material (while at the same time no compounds with the same Rf, or activity, could be detected in *C. destructans* extracts), it was decided not to pursue its identification because its detection proved to be rather elusive. No reliably detectable phytoalexins could therefore be described in primary roots of *P. sylvestris* infected *in vitro* with *C. destructans*. This provides a further demonstration that, generally, gymnosperms appear to lack a well-developed phytoalexin response to infection, which is a common component of infection responses in the angiosperms.

A number of constitutive antifungal compounds have been described in various organs of *P. sylvestris*, including the stilbene pinosylvin and its derivatives (see chapter 1). An increase in their concentration is often associated with microbial attack (Kemp & Burden, 1986), but a causal relation to resistance has never been unequivocally demonstrated by *in vivo* experiments. This applies also to defences in the large majority of plant species studied (Mansfield, 1983).

In the present study two groups of constitutive antifungal compounds were detected in primary roots of *Pinus sylvestris*. On TLC plates, R1 corresponded to the position of the pigments, including chlorophylls and yellow carotenoids. Therefore, R1's antifungal activity may have been only apparent, resulting from the unwettability of the spot caused by the presence of low polarity, lipophilic molecules, i.e.

the pigments. This unwettability is likely to prevent the fungal spores used for bioassay from establishing good growth on the TLC plate. For this, and other reasons mentioned in the results section, it was decided to leave R1 aside and focus attention on R2.

R2 was shown to belong to the group of diterpene resin acids, and in this context will be defined as abietic acid *sensu lato*. In conifers, diterpenes constitute a large proportion of the resin acids, and several reports show an increase on wounding (Kemp & Burden, 1986). For example, a large increase in the content of diterpene resin acids (in which dehydroabietic acid predominated) occurred following mechanical wounding of *Picea glauca* (Hart *et al.*, 1975).

Diterpene resin acids have been shown to inhibit the growth of fungal pathogens (Shain, 1971; Henriks *et al.*, 1979), and their presence in tree tissues suggests that they may have a role in protecting trees from attacks by parasitic fungi, although it has also been demonstrated that abietic acid *sensu stricto* stimulates the germination of the basidiospores of 4 mycorrhizogenic *Suillus* spp.
(**Fries *et al.*, 1987**).

The mean concentration of abietic acid in infected roots was shown to be approx. 10mg g^{-1} dry weight: since the root material contained about 90% water, it can be reckoned that the concentration of abietic acid in fresh infected tissues was around 1mg ml^{-1} (if a density of 1g ml^{-1} is assumed for fresh root tissues).

Bioassay of a commercial preparation of abietic acid

(Sigma) showed that this class of compounds has a relatively limited inhibitory activity against germination of *Cylindrocarpon destructans* spores. Only at a concentration four times higher than in the root tissues (4mg ml^{-1}) did abietic acid begin to lower the germination rate. However, gross morphological changes of the conidia, and, most importantly, a depression of germ tube growth were observed down to a concentration of 1mg ml^{-1}, corresponding to the calculated concentration of abietic acid in fresh, infected roots. A crude commercial preparation of abietic acid was used instead of R2 in this study because of difficulties in obtaining sufficient root tissue for bulk preparations from pine. A further reason was the failure of a preliminary attempt using AA/R2 (the purified fraction from the commercial abietic acid used elsewhere in this study, which is most similar to the plant-derived fraction), due to instantaneous precipitation of the purified compound following dilution of the DMSO solution with water. This problem did not occur with the crude commercial preparation. Although these different solubilities may have implications on the nature of the plant-derived compounds relative to the commercial preparation, the interpretation of these implications was not pursued in the present work.

In this study the mean concentration of abietic acid was shown to increase by 85.2% in the roots of infected seedlings. Although this difference was not statistically significant, such a change could represent an average, with much greater local levels of accumulation, at sites of attempted fungal

penetration. This pattern of uneven distribution and accumulation of antifungal compounds in plant tissues has been described previously, e.g. in herbaceous plants, with localized accumulation of phytoalexins during the expression of the hypersensitive response (Deverall, 1976; Cruikshank, 1980). In the case of the present study such aspect could have a much greater effect on fungal development than the gross assay results would indicate, especially if the findings relative to depression of germ tube growth are taken into account.

The very close molecular similarity among the class of abietic acids, in which often the only difference between compounds is the position of a double bond, or is due to stereoisomerism, makes it very difficult to differentiate among them. Nevertheless, a comparison of both ME and TMS derivatives, a thorough search in computer libraries for both types of derivatives, and a comparison with published data (Zinkel *et al.*, 1971) indicated that the trienoic diterpene resin acid, dehydroabietic acid, and five dienoic diterpene resin acids were present in R2. However, the only quantifiable compound was dehydroabietic acid, precisely because only its peak could be unequivocally recognized (as the only trienoic acid) in the GC spectra. A definitive and conclusive identification of the dienoic acids, among which abietic acid *sensu stricto* was likely to be present (as it has been reported in *P. sylvestris* primary roots - Fries *et al.*, 1987), would only be possible if the compounds were available in pure form,

so that they could be co-chromatographed and analysed using combined GC-MS. As these compounds were not readily available, the time-consuming process of characterizing all compounds of this fraction was not pursued. In the context of this work, which dealt with the detection of molecules with a potential defensive role, the identification of the R2 inhibitory compound as one or more diterpene resin acids ('Abietic acid') was considered sufficient.

CHAPTER 4

Defence responses to pathogenic infection in mycorrhizal primary roots of *Pinus sylvestris*

4.1 Introduction

Apart from enhancing the nutritional status of their hosts (*cf.* chapter 1), mycorrhizae are reported to improve disease resistance to soil-borne pathogens. This last aspect has been investigated in a number of cases, particularly in relation to ectomycorrhizae (e.g. Chakravarty & Unestam, 1987[a,b]; Chakravarty *et al.* 1990; Duchesne *et al.*, 1987).

Although improved disease resistance appears to be a feature of mycorrhization (Read, 1986) the physiological mechanisms by which this is effected are poorly understood, although it has been suggested that both structural (Marx & Davey, 1969[a,b]; Marx, 1970) and biochemical (e.g. Duchesne *et al.*, 1987, 1989) barriers (*cf.* chapter 1) may be involved.

Ectomycorrhization also appears to decrease the infectivity of the soil, sometimes quite substantially. In the case of *Pinus sylvestris* the inoculum density and the infective potential of *Pythium* spp. has been shown to be lower in soils in which the mycorrhizal fungus *H. crustuliniforme* was present (Perrin & Noveau, 1986). The work reported by Sinclair *et al.* (1982), and Sylvia and Sinclair (1983[a]), is another example of disease suppression, in this case against *Fusarium oxysporum,* induced by *Laccaria laccata* on Douglas fir (*Pseudotsuga menziesii)* seedlings.

Among the putative mechanisms of disease protection for the host afforded by ectomycorrhization, the production by the ectomycobionts of antibiotics, and particularly of low molecular weight antifungal compounds, has received much attention (Zak, 1964; Marx, 1973). Zak (1964) suggested, on the basis of his own experience and also of others, that antibiotic secretion may be one way for mycorrhizal fungi to afford protection to the root. Krywolap *et al.* (1964) demonstrated that *Cenococcum graniforme,* an ectomycobiont, produced an antibiotic in pure culture, and that this substance was present in the roots, but was also translocated to the needles, of various conifers (including *Picea abies)* mycorrhizal with that fungus. Marx (1969[b]) showed that *Leucopaxillus cerealis* var. *piceina* produces a non-volatile antifungal and antibacterial compound identifiable as diatretyne nitrile, which was very active against *Phytophthora cinnamomi*. Although no mention was made of their biological activity, three triterpenes were isolated from the peridium of *Astraeus hygrometricus,* a mycorrhizal basidiomycete, by Takaishi *et al.* (1987). Toyota & Hostettmann (1990) described the isolation and identification of 4 new diterpenic esters (cavipetins) extracted from fruitbodies of *Boletinus cavipes* showing antifungal activity against *Cladosporium cucumerinum,* while Duchesne *et al.* (1989) implied that a less complex organic compound like oxalic acid may have a role in the disease suppression induced by *Paxillus involutus* against *Fusarium oxysporum* f.sp. *pini* on *Pinus resinosa* seedlings. More recently, Kope & Fortin (1990) have reported the

production of an antifungal compound (as yet unidentified) by *Pisolithus tinctorius,* which was active against a range of root pathogenic fungi.

Biochemical analysis of whole ectomycorrhizae, especially in relation to aromatic substances, has been previously reported. Krupa & Nylund (1972) demonstrated the antifungal activity, against *Phytophthora cinnamomi* and *Heterobasidion annosum,* of volatile mono- and sesquiterpenes produced by ectomycorrhizal root systems of Scots pine. More recently, Munzenberger *et al.* (1990), and Weiss *et al.* (unpublished data), described changes in the content of antifungal phenolics in primary roots of Norway spruce (*Picea abies)* following ectomycorrhization, even in relation to root age and tissue type, but did not test for the possible relationships between these changes and disease resistance.

The paucity of studies on direct mycorrhiza-pathogen interactions is striking. Marx and Davey (1969[a,b]) demonstrated that artificially inoculated and semi-natural mycorrhizal roots of shortleaf (*Pinus echinata)* and loblolly (*P. taeda)* pine were resistant to attack by *Phytophthora cinnamomi,* and that the mantle was the primary source of (physical) protection. Sylvia and Sinclair (1983[b]) showed that *Laccaria laccata* induced disease protection against *Fusarium oxysporum* (another important damping-off agent) on Douglas fir (*Pseudotsuga menziesii)* seedlings. In this case the defence mechanism involved appeared to be biochemical: the

intracellular accumulation of phenolics induced by mycorrhizal infection was inversely proportional to the extent of infection by the root pathogen.

The aim of this chapter was to make a contribution to the understanding of the expression of disease resistance in ectomycorrhizal roots of Scots pine seedlings. The work was subdivided in 3 sections. The first included a microscopical investigation of the ectomycorrhizal associations between *P. sylvestris* and three common ectomycorrhizal fungi: *Pisolithus tinctorius, Suillus bovinus,* and *Hebeloma crustuliniforme.* The hypothesis from which this work originated was that mycorrhizal infection, in its varying degrees of compatibility (*cf.* chapter 1), may elicit host responses which could be instrumental in the host's ability to withstand subsequent pathogenic attacks. Specifically, the expression of the structural and histochemically detectable defences described in chapter 3 were sought in ectomycorrhizae synthesized *in vitro*. Mycobionts that, by the characteristics illustrated in chapter 1, appeared less compatible with *P. sylvestris,* may induce a higher degree of protection against subsequent attack by *C. destructans* and *P. oligandrum.*

The second section of the work attempted to detect non-volatile, low molecular weight antifungal compounds produced by the 3 selected ectomycobionts, and to determine the level of biological activity shown by the ectomycobionts against different root pathogenic fungi *in vitro*.

The third section aimed to examine the mycorrhiza-pathogen interaction, rather than the separate host-pathogen and mycobiont-pathogen interactions, in an attempt to bring together the concepts mentioned previously. Here the study involved only one of the three mycobionts selected initially: *Hebeloma crustuliniforme.* Ectomycorrhizae synthesized *in vitro* were challenged with *C. destructans* and *P. oligandrum.* After establishing the degree of protection against *C. destructans* and *P. oligandrum,* an attempt was made to evaluate the role of the physical barrier to infection represented by the mantle and the Hartig net, and also to see whether any low molecular weight antifungal compounds were produced *de novo* by the mycorrhiza.

4.2 **Materials and methods**

4.2.1 Microscopy and histochemistry of the mycorrhizal infection process

Pine seedlings were grown, set up in petri dish systems, and inoculated with plugs of mycelial cultures of *P. tinctorius, S. bovinus,* and *H. crustuliniforme* as described in 2.3.2. The age of the cultures at inoculation ranged from approx. one month (*P. tinctorius)* to approx. 3 months (*H. crustuliniforme*), depending on the growth rate of the fungus. The mycelial plugs for inoculation were generally taken from the margin of the colony.

Ectomycorrhizal rootlets were collected at different times after inoculation to obtain a developmental series, ranging from incipient colonization to fully sheathed

ectomycorrhizae. Older mycorrhizae were obtained from plants grown in a peat/vermiculite filled petri dish synthesis system (see 2.3.2).

Ectomycorrhizae from these petri dish systems were collected 5 months after inoculation. Controls consisted of uninoculated main and lateral roots.

Excised rootlets were processed for light microscopy as described in 3.2.1.2. The histochemical tests listed in table 4.1 were used:

Table 4.1

Histochemical tests employed for microscopical observation of the ectomycorrhizal infective process

Lignin pink-Chlorazol black E: lignin (Gurr, 1965)
Phloroglucinol-HCl: lignin (Jensen, 1962; O'Brien & McCully, 1981)
Schiff's reagent: lignin (O'Brien & McCully, 1981)
Lacmoid (0.25% in water): callose (ß-1,3-linked glucans) (Jensen, 1962; O'Brien & McCully, 1981)
Ruthenium red: acid polysaccharides, pectins (Jensen, 1962; O'Brien & McCully, 1981)
Sudan Black: lipidic materials (O'Brien & McCully, 1981)
Toluidine blue: polyphenolic materials and general morphology (O'Brien & McCully, 1981; Smart *et al.*, 1986)
Zinc-Chlor-Iodine: cellulose (O'Brien & McCully, 1981)

4.2.2 Biochemical analysis of secondary metabolites of ectomycorrhizal fungi and assessment of their antifungal activity *in vitro*

4.2.2.1 TLC analysis and bioassay

Liquid cultures of *P. tinctorius*, *S. bovinus*, and *H. crustuliniforme* were grown in 250ml conical flasks containing 100ml of 2% malt extract broth prepared as described in 3.2.2.9. 5 flasks were inoculated with each fungus as described in 3.2.2.9 and incubated for 2.5 months at 25°C in the dark. A similar set of 5 uninoculated flasks were used as controls. Aliquots (2ml) of crude culture filtrate from each flask where saved and stored at approx. -20°C for later analysis by HPLC. The remaining liquid was pooled for each species, and processed as described in 3.2.2.9, except that it was concentrated 10X by freeze drying, instead of 20X. 2ml of this concentrated culture filtrate were then extracted as described in 3.2.2.9. The organic and aqueous phases were redissolved in 2ml of redistilled ethyl acetate and MeOH respectively.

0.4ml of extracts (corresponding to 4ml of the original culture filtrate) were loaded onto analytical silica gel TLC plates (as described in 3.2.2.2), and developed with a $CHCl_3$:MeOH (4:1) solvent system. The developed plates were bioassayed with 2 pathogens: *Cladosporium cucumerinum* and *Cylindrocarpon destructans*. Fresh spore suspensions were prepared and sprayed on the TLC plates as described in 2.5.

4.2.2.2 Inhibition tests in agar medium

A dialysis membrane method was used to test the biological activity of diffusates (extracellular metabolites) in agar cultures of *P. tinctorius*, *S. bovinus* and *H. crustuliniforme* against *Pythium oligandrum*, *Phytophthora citricola* and *Cylindrocarpon destructans*. The dialysis membrane (Cuprophan 150, nominal molecular weight cut-off = 10,000 - Medicell International Ltd., London, U.K.) was cut in discs of approximately the same size as the standard petri dish (diametre of the membrane discs = 8.5cm). The discs were then boiled twice in deionized water for 10 min each time to eliminate the plasticizer present in the membrane (glycerine), and autoclaved in deionized water. The discs were laid on the surface of MMN agar medium, which was modified so that the concentration of KH_2PO_4 and $(NH_4)_2HPO_4$ was 0.1 M (*cf.* MMN recipe in 2.4), and these salts were present in the appropriate proportions (KH_2PO_4 : $(NH_4)_2HPO_4$ approx. 42 : 8, with the diammonium salt used in place of the disodium salt) to act as a phosphate buffer of pH 6 (before addition of the agar). "Noble" agar (Difco) was used because of its higher purity. The petri dishes were inoculated at the centre of the membrane disc with a 5mm plug taken from the margin of an actively growing agar culture of the test fungus, and incubated in the dark at 25°C. Once the mycelium of the mycorrhizal fungus had nearly reached the limit of the membrane disc (confirmed by microscopic examination) the disc was peeled off the agar surface. A 5mm mycelial plug taken from the margin of an actively growing colony of each pathogenic fungus was then

placed directly on the agar at the centre of the petri dish.

Prior to this inoculation the pH of the medium was checked. The agar from one plate from each series of mycorrhizal cultures was cut in small pieces and forced through a 10ml syringe fitted with a needle to produce a slurry. An extracting unit was assembled by inserting a 10ml polypropylene syringe body (no needle) into a McCartney bottle. Internally, the syringe outlet was covered with a disc of double thickness Whatman No. 1 filter paper. The agar slurry was put into the syringe body, which was then spun (approx. 1500g) in an MSE benchtop centrifuge for 45 min. Approx. 2ml of liquid were recovered from each agar disc, enough for pH determination.

The growth rate of the pathogens was then measured daily, for each mycorrhizal fungus and for the controls, which consisted of plates of the same medium incubated under the same conditions, but without inoculation with mycorrhizal fungi.

4.2.2.3 pH effects on the growth of pathogens in agar medium

An experiment was also carried out to test the effect of different pH's on the growth rates of the 3 pathogenic fungi. The media used were MMN acidified with H_3PO_4 to pH 3.1, 4 and 5, and deionized water agar (1.5% agar as the MMN medium). 4 replicates per treatment were used.

4.2.3 Mycorrhiza-pathogen interactions

4.2.3.1 Mycorrhizal protection

Scots pine seedlings mycorrhizal with *Hebeloma crustuliniforme* were obtained *via* the *in vitro* system described in 2.3.2, except that 0.8% deionized water agar was used instead of MMN. Seedlings were inoculated with *H. crustuliniforme* 2 weeks after transfer to the petri dishes. After one month of incubation with the mycobiont the root systems were examined for percentage mycorrhization, by counting the mycorrhizal short roots identifiable with the aid of a stereomicroscope. After 2 more weeks the seedlings were transferred to new petri dish systems containing 0.8% deionized water agar, taking particular care in placing the mycorrhizal root systems on the agar surface. Each plate was inoculated with 6-10 5mm plugs each of either *C. destructans* or *P. oligandrum*. Controls were non-mycorrhizal seedlings grown identically.

Death of seedlings was monitored over a 38 day period and final mortality was analysed statistically (Chi-squared method).

4.2.3.2 Microscopy and histochemistry of the mycorrhiza-pathogen interaction

The root systems of 4-8 weeks old Scots pine seedlings grown in glass jars as described in chapter 2 were laid directly on the surface of pure cultures of *Hebeloma crustuliniforme*, with the aerial portion of the seedlings protruding out of the petri dishes as described in 2.3.2. The

seedlings were incubated on a window sill for approximately one week, until the first mycorrhizal feeder roots appeared. The infected seedlings were transferred to new petri dish systems containing 0.8% deionized water agar, and inoculated after 10 days with *C. destructans* as described previously in 2.3.1.2. Roots were collected for fixation, resin embedding, and sectioning (as described in 3.2.1.2) after 2 further weeks of incubation.

4.2.3.3 Biochemical analysis of mycorrhizal extracts

Mycorrhizal root systems were synthesized in 0.8% deionized water agar, and harvested after one and a half months. Many lateral short roots had a mantle; many others, embedded in the agar, did not have a mantle but were club-shaped (early mycorrhization stages). Approx. 0.5g mycorrhizal roots (fresh weight) were extracted overnight in MeOH (10ml) and processed as described in 3.2.2.2. 300µl each of organic and aqueous phases were separated and bioassayed on TLC plates as described in 3.2.2.2.

4.3 **Results**

4.3.1 Microscopy and histochemistry of the mycorrhizal infection process

The inoculation method in agar medium described in 2.3.2 worked very well with both *P. tinctorius* and *H. crustuliniforme,* less well with *S. bovinus.* In the best cases, root infection (Fig. 4.1) had commenced a week after inoculation, with the first fully sheathed ectomycorrhizae

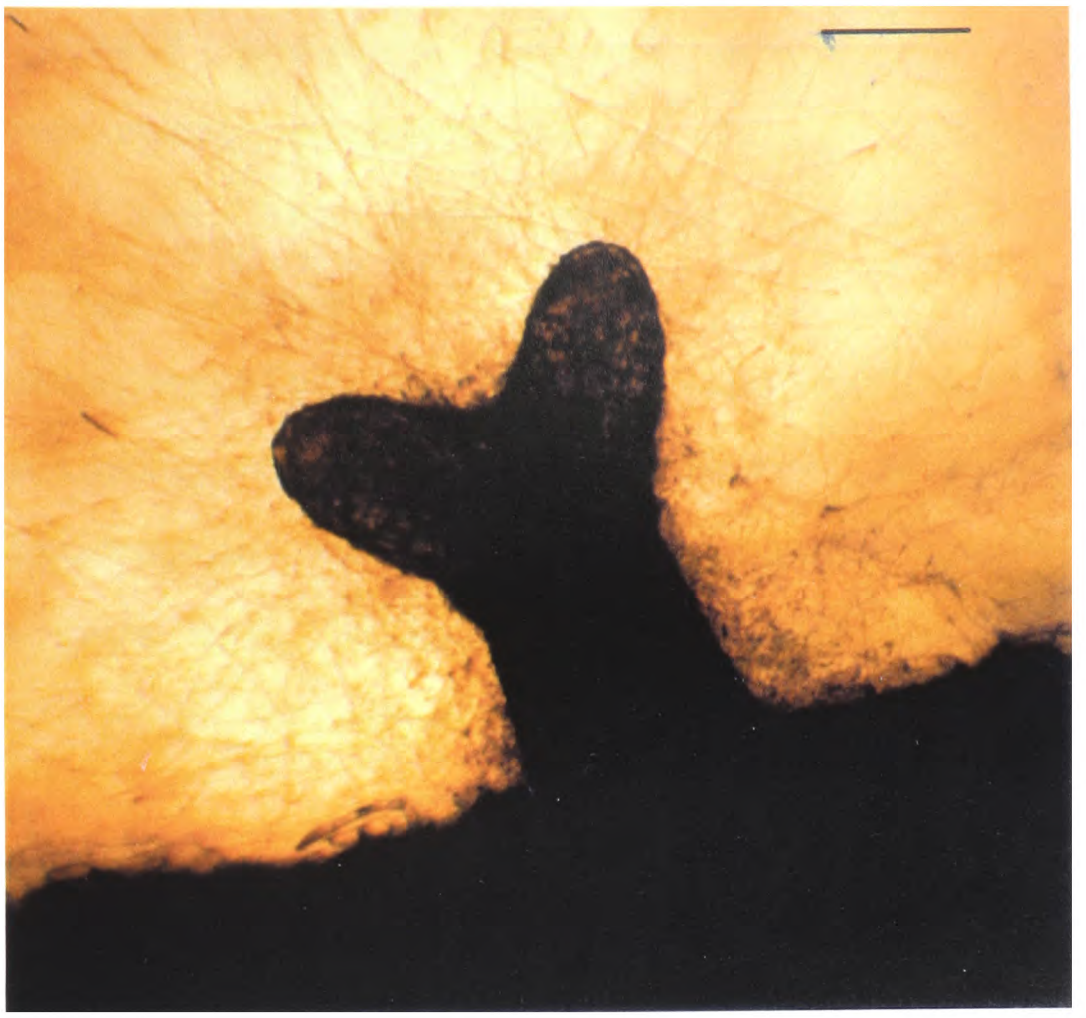


Figure 4.1. A dichotomously branching short lateral root of a *Pinus sylvestris* seedling being infected by *Pisolithus tinctorius* in agar medium. The root is embedded in the agar. The chemotropism of the hyphae towards the root is evident. Unstained specimen viewed through the petri dish with a compound microscope. Scale bar = 250µm.

protruding from the agar surface after 3 weeks.

Infection by *S. bovinus* occurred much more easily than with the other two fungi in the P-V systems (Fig. 4.2), but the system itself presents various drawbacks, including laborious and messy preparation, a higher frequency of contamination, and the impossibility of following the infection process closely.

All 3 ectomycorrhizal associations behaved similarly with regard to the timing of infection, the pattern of penetration, and the elicitation of plant responses. Morphological observations were made on toluidine blue stained sections, integrated with other histochemical tests whenever appropriate or necessary.

In view of this close similarity, the following description is based on the infection process of *P. tinctorius*. Differences with the other 2 ectomycobionts will be emphasized as appropriate.

The mycorrhization of lateral roots followed closely what has been described in the literature for the ontogeny of conifer ectomycorrhizae (Harley & Smith, 1983). The root cap was usually very lightly infected, showing a very thin sheath of unorganized, loose hyphae, with the amount of infected tissues, including an organized Hartig net and mantle, increasing in basipetal direction. However, in more organized dichotomously branching rootlets a well formed mantle was visible even on the root tip. Fig. 4.3 shows a longitudinal section of an ectomycorrhizal root in which a thin mantle, the early phases of Hartig net formation, and the initial

Figure 4.2. A first order lateral root of a *P. sylvestris* seedling ectomycorrhizal with *Suillus bovinus*. The white mantle and extramatrical mycelium are well developed. Synthesis in peat-vermiculite medium (*cf.* Fig. 2.2). Scale bar = 2mm.

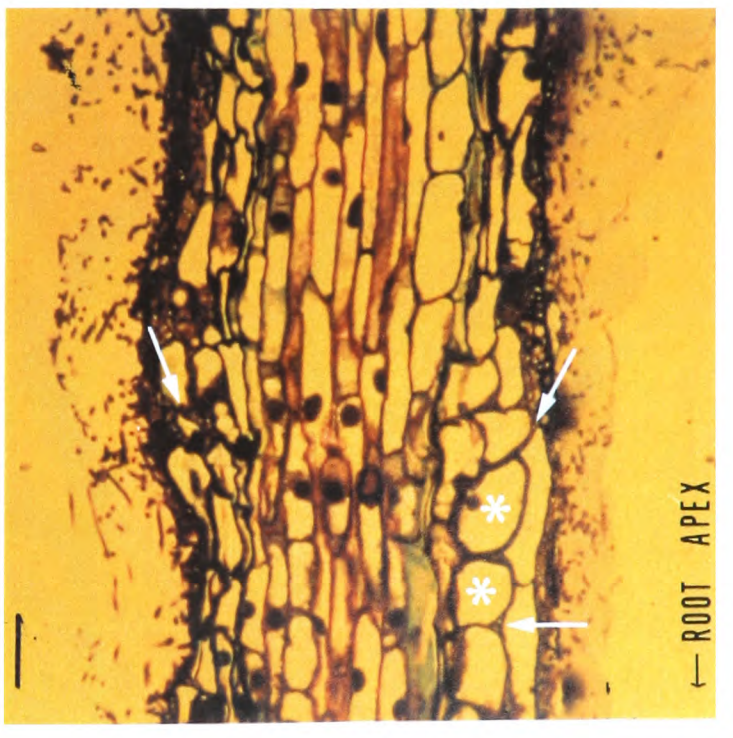


Figure 4.3. A longitudinal section of a long, first order lateral root of a *P. sylvestris* seedling ectomycorrhizal with *Pisolithus tinctorius*. Synthesis in agar medium. A thin mantle is visible, together with the incipient formation of the Hartig net (arrows) and deformation in radial direction of some cortical cells (asterisks). Stained with toluidine blue. Scale bar = 50µm.

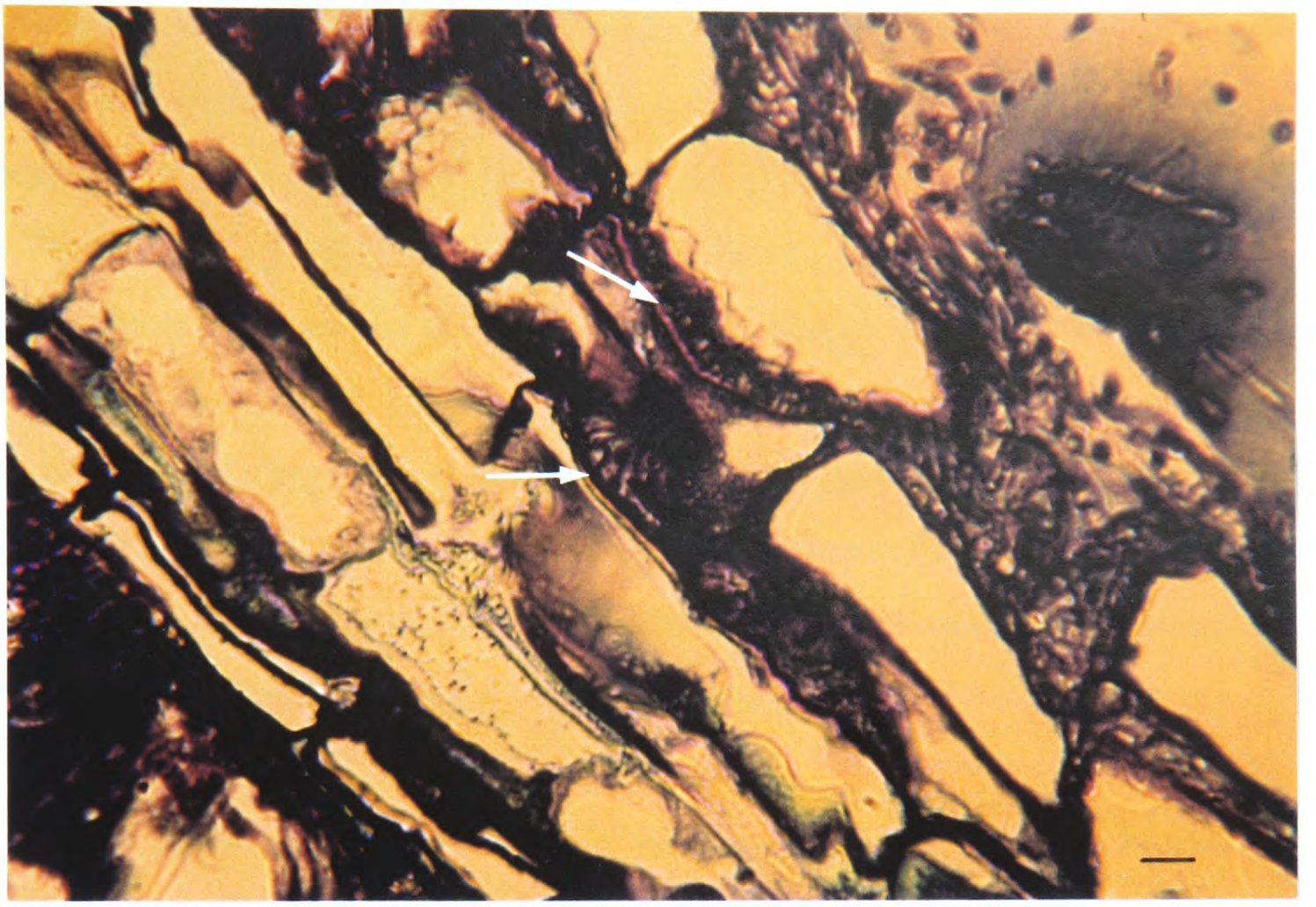


Figure 4.4. An example of labyrinthic pseudotissue (*cf.* Nylund and Unestam, 1982) (arrows), a special characteristic of the Hartig net, from a *Pinus sylvestris-Pisolithus tinctorius* ectomycorrhiza synthesized in agar medium. Longitudinal section stained with toluidine blue. Scale bar = 10µm.

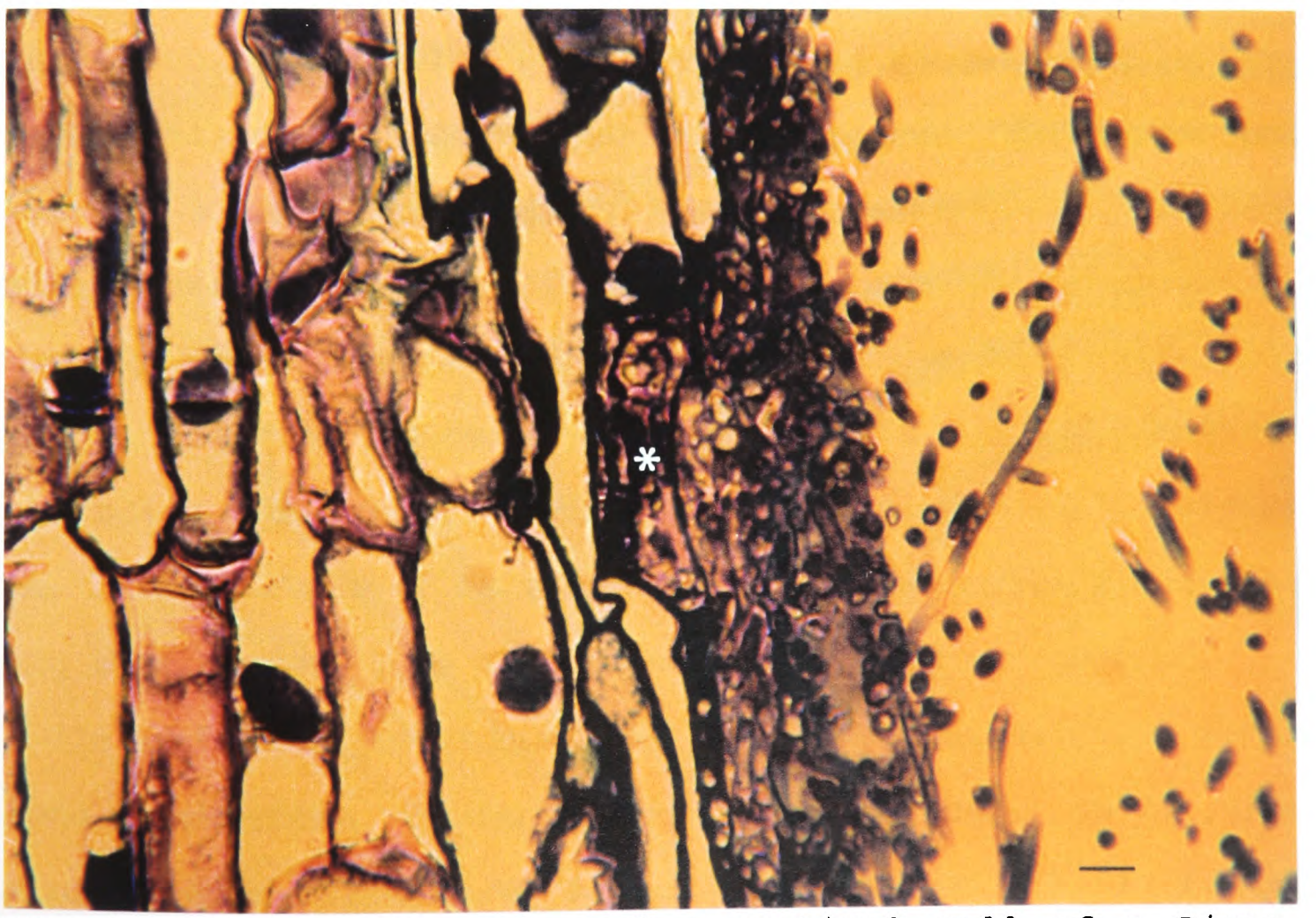


Figure 4.5. Massive invasion of a cortical cell of a *Pinus sylvestris-Pisolithus tinctorius* ectomycorrhiza by the ectomycobiont (asterisk). Longitudinal section stained with toluidine blue. Scale bar = 10µm.

stages of deformation of the outer cortical cells are visible. Fig. 4.4 shows an example of labyrinthic pseudotissue, a peculiarity of fungal morphology associated with the formation of the Hartig net (Nylund & Unestam, 1982). Only with *P. tinctorius* has massive invasion of single cells been observed (Fig. 4.5), albeit rarely, while a limited number of instances of intracellular penetration has been seen with all 3 fungi. With toluidine blue staining, a magenta-red coloured matrix was often visible at the interface between mantle hyphae and rhizodermal cell walls.

No cell wall appositions could be identified definitely, with any of the 3 fungi. By staining with toluidine blue, what looked like a generalized accumulation of phenolics, particularly in the cytoplasm of the root tip cells (uniform aqua-green staining), and in the walls of cortical cells (aqua-green to navy-blue staining), could be detected in both mycorrhizal and uninoculated short roots. This was not observed, or at least not to the same extent, in the long (main) roots. Attempts to confirm this phenolic accumulation by using other stains and autofluorescence have generally failed. In some cases, cortical cells of *S. bovinus* mycorrhizae appeared to contain, on staining with toluidine blue, large numbers of bright-green coloured droplets adsorbed along what looked like the tonoplast of a large vacuole.

In old ectomycorrhizae the most striking feature was a strong blue-green colouring of cortical cell walls upon staining with toluidine blue, common to all 3 fungi.

Typically, *H. crustuliniforme* ectomycorrhizae had a very thin sheath when compared to the other 2 species of mycobionts.

Apart from toluidine blue, all other histochemical stains failed to give any unequivocal indications of the presence of their target compounds on the sections examined.

4.3.2 Biochemical analysis of secondary metabolites of ectomycorrhizal fungi and assessment of their antifungal activity *in vitro*

4.3.2.1 TLC analysis and bioassay

The aqueous phases of the mycorrhizal culture filtrates contained large amounts of substances insoluble in methanol (presumably carbohydrates). This caused problems similar to those encountered with the preparation of the aqueous phase of culture filtrates of *C. destructans* (*cf.* 3.2.2.**9**). Attention was therefore focussed on the organic phase. In this fraction, no antifungal compounds could be detected with *C. cucumerinum* in any of the fungal culture filtrates, but a plate sprayed with a *C. destructans* spore suspension revealed a weak inhibition area, with an approx. Rf = 0.5, in the *H. crustuliniforme* filtrate. However, this finding was not confirmed in a repeat experiment.

4.3.2.2 Inhibition tests and pH effects on the growth of pathogens in agar medium

Virtually all *H. crustuliniforme* subcultures, and a few subcultures of the other two ectomycobionts, had the capacity to digest the cellulose membrane and pass into the agar medium, where they started growing as in regular culture. This major problem became evident a few days after inoculation with these fungi, and because of this, testing of *H. crustuliniforme* was abandoned.

By the time the colonies had neared the edge of the membranes, the pH of *P. tinctorius* and *S. bovinus* agar discs was 4.5 and 2.9 respectively, measured as values from single plates. In retrospect, the choice of buffering salts might have been a poor one, as the fungi should have been expected to utilize, at least partially, the phosphates. This is the reason why a subsequent test on pH effects on the growth rate of root pathogens was set up.

The mean radii of the mycorrhizal colonies at the time of peeling off the membrane, calculated from two orthogonal diametres, including the original plug of inoculum, are shown in table 4.2:

Table 4.2

Dialysis membrane experiment. Mean radii (cm) (from 6 replicates) of the colonies of mycorrhizal fungi at time of membrane removal

	Pisolithus tinctorius	*Suillus bovinus*
mean (+-SEM)	3.1 (+-0.09)	3.9 (+-0.09)

The results from this experiment and from the culturing of root pathogens at different pH's are shown in Figs. 4.6, 4.7, and 4.8. *Pisolithus tinctorius* and *Suillus bovinus* had a strong inhibitory effect against *Pythium oligandrum,* which could not be explained directly in terms of pH changes, as this pathogen appears to be able to grow vigorously even at low pH (Fig. 4.6). On the other hand, such clear-cut evidence was not *obtained with Phytophthora citricola* and Cylindrocarpon *destructans.*

In 5 out of 6 replicates with *Pisolithus tinctorius,* and in all replicates with *S. bovinus,* growth of *Pythium oligandrum* was depressed virtually completely, while comparable inhibition occurred only in 1 replicate, with both *P. tinctorius* and *S. bovinus,* for *Cylindrocarpon destructans.* The strongest inhibition of *Phytophthora citricola* was induced by *S. bovinus,* while with *P. tinctorius* the mean growth rate of *P. citricola* was very similar to the control. Even in this case though, one plate showed almost complete inhibition.

It appeared that the darker the medium had turned as a consequence of the growth of the mycorrhizal fungus (a feature of both *P. tinctorius* and *S. bovinus* cultures), the stronger the inhibitory activity against *P. oligandrum,* which also showed a more sparse mycelium than in the control.

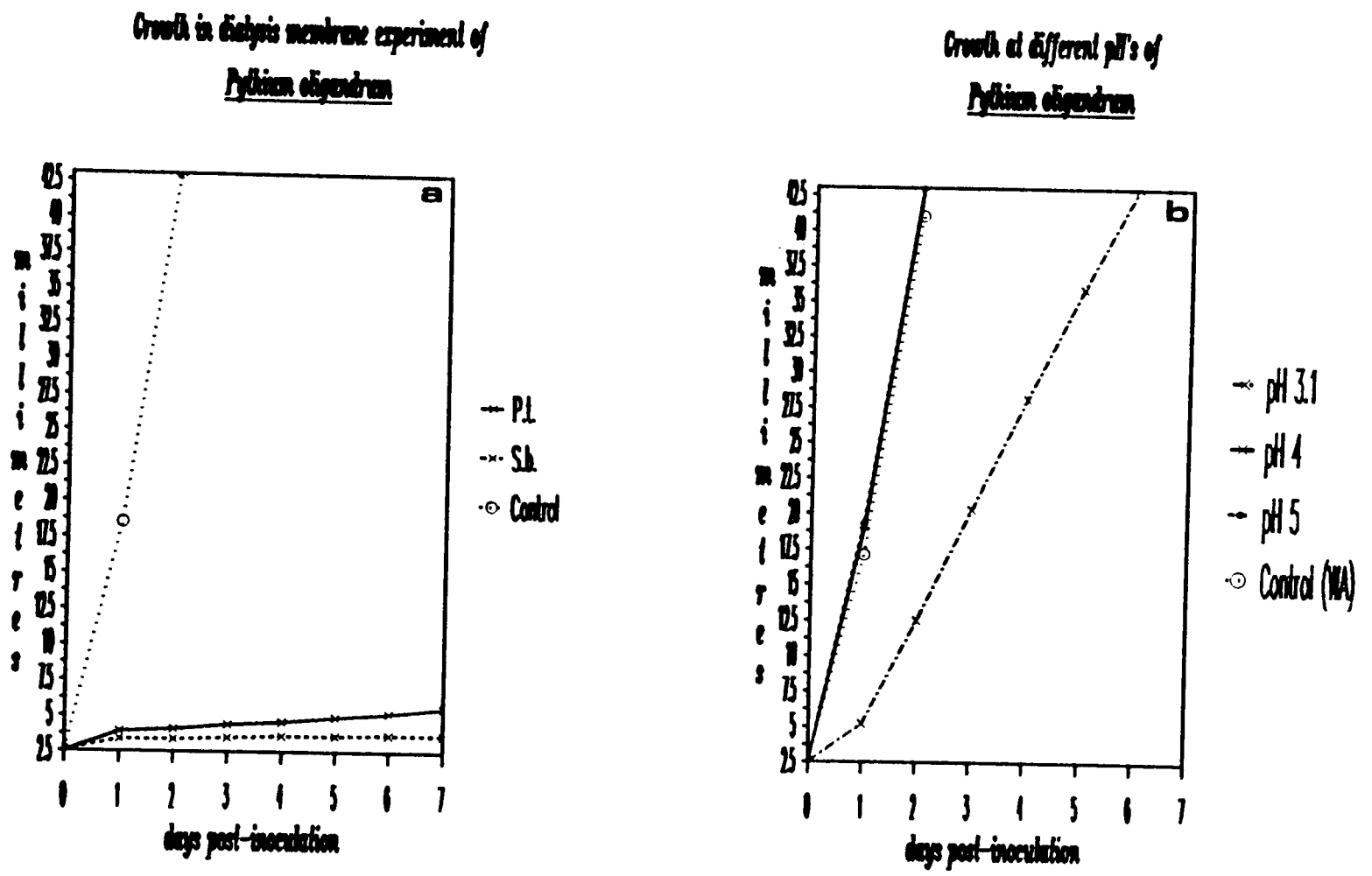


Figure 4.6. a) Antagonistic effects of *Pisolithus tinctorius* and *Suillus bovinus* on *Pythium oligandrum*. Growth of the pathogen is strongly depressed by both ectomycobionts. b) pH effects on *Pythium oligandrum*. Only pH 3.1 inhibits growth of the pathogen relative to control (WA: water agar). Each value in a) and b) is the mean of 6 and 4 replicates, respectively.

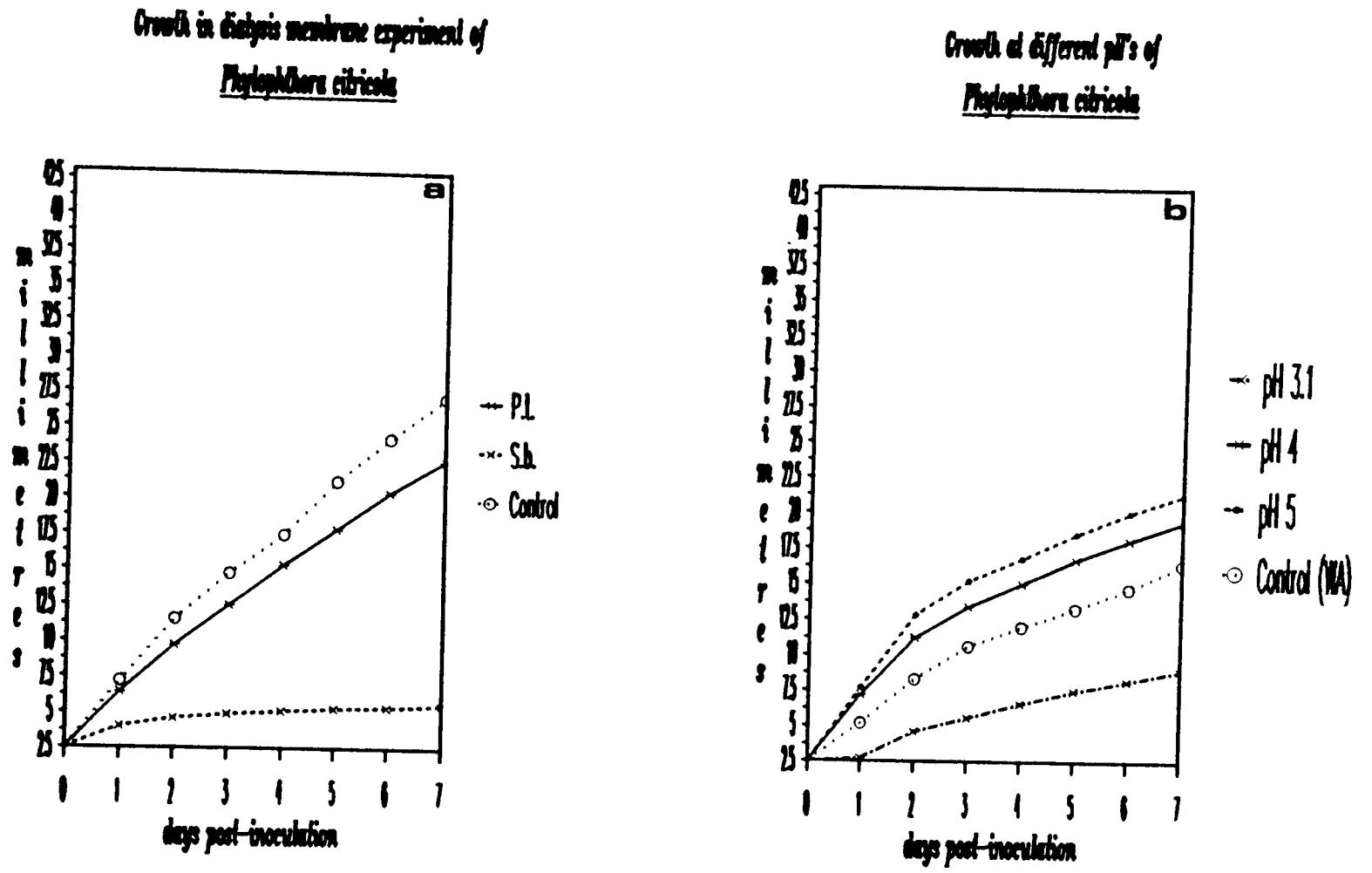


Figure 4.7. a) Antagonistic effects of *Pisolithus tinctorius* and *Suillus bovinus* on *Phytophthora citricola*. Growth of the pathogen is strongly depressed only by *Suillus bovinus*. b) pH effects on *Phytophthora citricola*. The lowest pH (3.1) inhibits growth of the pathogen only moderately relative to control (WA: water agar), while pH's 4.0 and 5.0 appear to stimulate growth. Each value in a) and b) is the mean of 6 and 4 replicates, respectively.

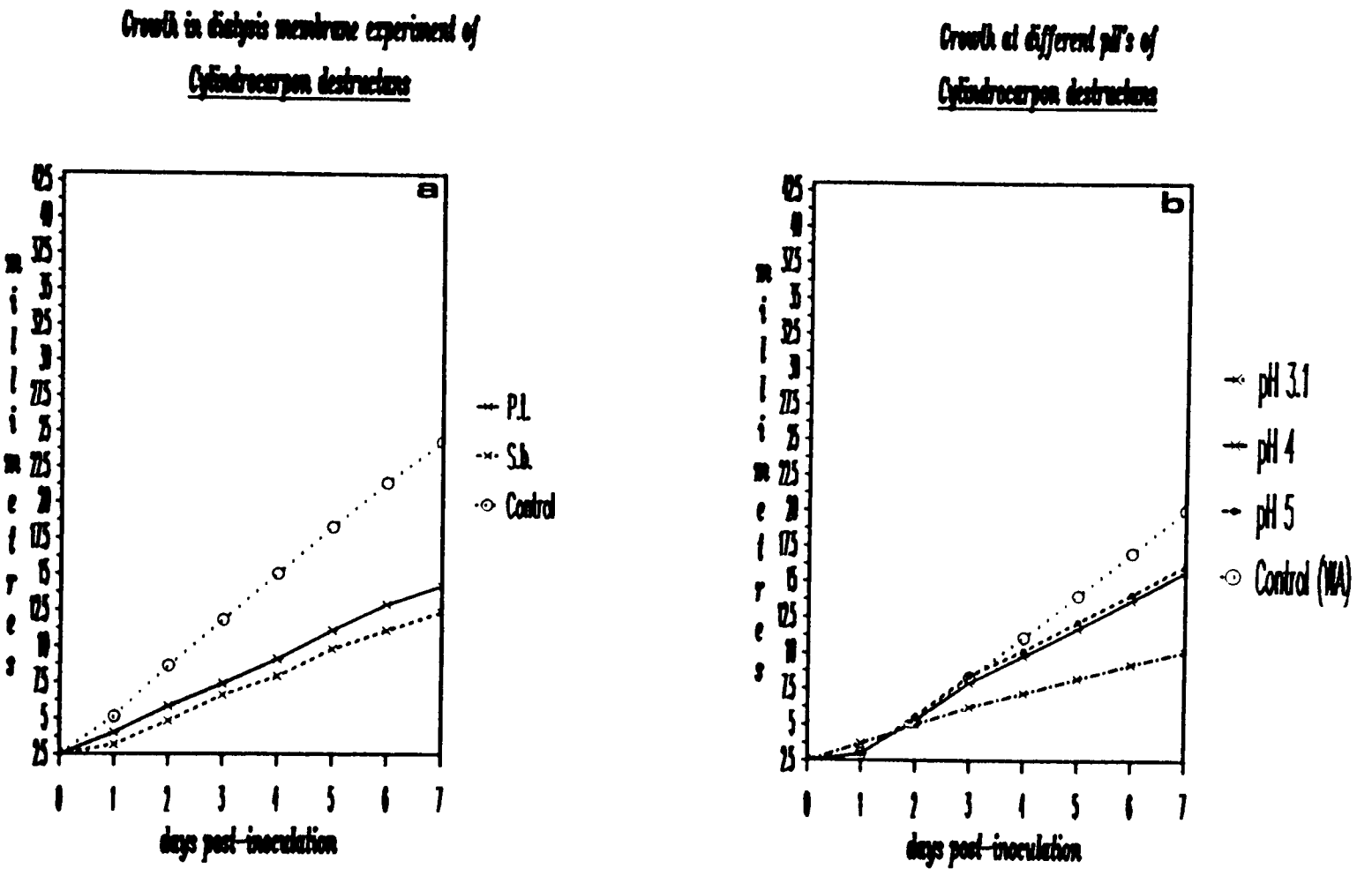


Figure 4.8. a) Antagonistic effects of *Pisolithus tinctorius* and *Suillus bovinus* on *Cylindrocarpon destructans*. Growth of the pathogen is depressed moderately by both ectomycobionts. b) pH effects on *Cylindrocarpon destructans*. Only pH 3.1 has a slightly inhibitory effect on the growth of the pathogen relative to control (WA: water agar). Each value in a) and b) is the mean of 6 and 4 replicates, respectively.

4.3.3 Mycorrhiza-pathogen interactions

4.3.3.1 Mycorrhizal protection

Before pathogenic challenge the *H. crustuliniforme* infected Scots pine seedlings were examined for percentage mycorrhizal infection of first and second order laterals. Out of a sample of 15 seedlings, mean percentage mycorrhization was 73.83 +- 3.33 (SEM).

Incubation with *C. destructans* and *P. oligandrum* was followed up to 38 days. No seedlings (out of 23 per treatment) succumbed to *C. destructans* in that period, while *P. oligandrum* killed 26.1% of the mycorrhizal and 47.8% of the non-mycorrhizal Scots pine seedlings by the end of the experiment (Fig. 4.9). The difference between the death rates of mycorrhizal and non-mycorrhizal Scots pine seedlings after 38 days incubation with *P. oligandrum* was not statistically significant (Chi-squared method).

4.3.3.2 Microscopy and histochemistry of the mycorrhiza-pathogen interaction

Direct interaction between *Hebeloma crustuliniforme* Scots pine mycorrhizae and *C. destructans* showed that the hyphae of the two fungi intermingled freely, both in the mantle (Fig. 4.10) and in the Hartig net. Instances of direct cell penetration through the mantle and the Hartig net by *C. destructans* were observed (Fig. 4.11). No papilla formation was recorded, while intracellular penetration by *C. destructans* was observed in the cortex, to a larger extent

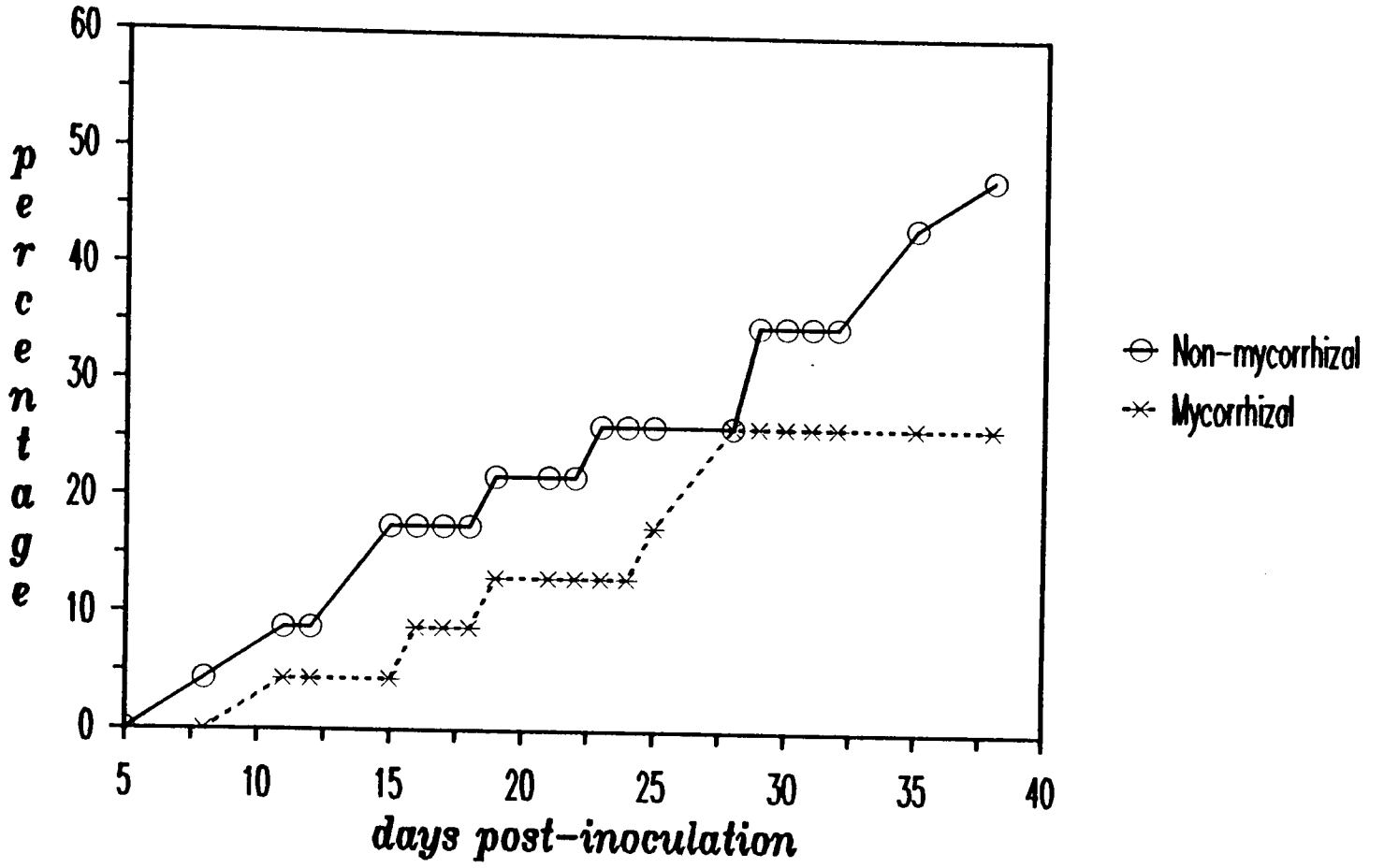


Figure 4.9. Disease protection against *Pythium oligandrum* by mycorrhization of *Pinus sylvestris* seedlings with *Hebeloma crustuliniforme*. Although the difference between the numbers of surviving and dead seedlings was not statistically significant after 38 days (Chi-squared test), a trend in protection can be recognized, particularly in the first 25 days after inoculation. 23 seedlings were used in each treatment.

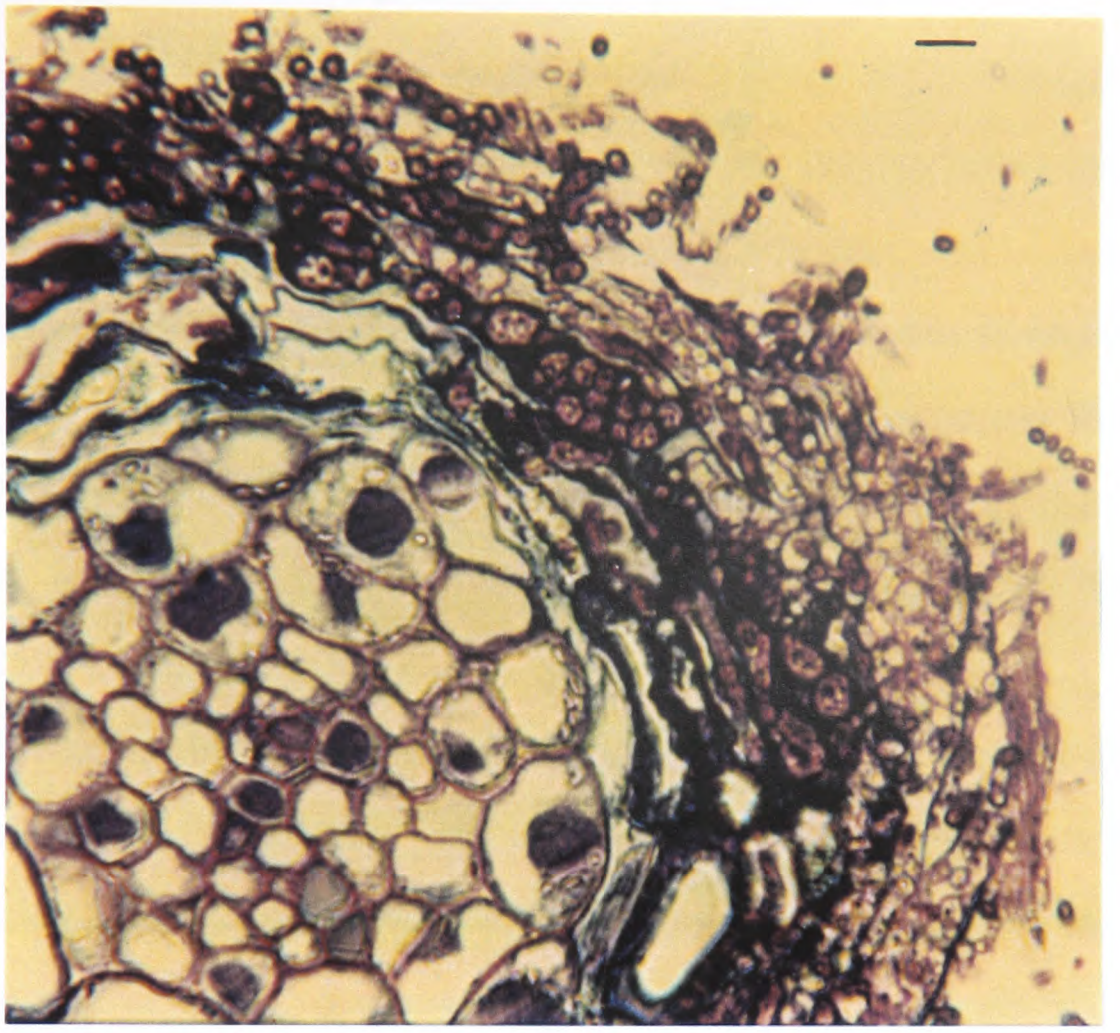


Figure 4.10. Free intermingling of hyphae of *Cylindrocarpon destructans* with mantle pseudotissue in a *Pinus sylvestris-Hebeloma crustuliniforme* ectomycorrhiza synthesized in agar medium. The pathogen's hyphae are recognized by their comparatively darker colouring (magenta-brown). Transversal section stained with toluidine blue. Scale bar = 10µm.

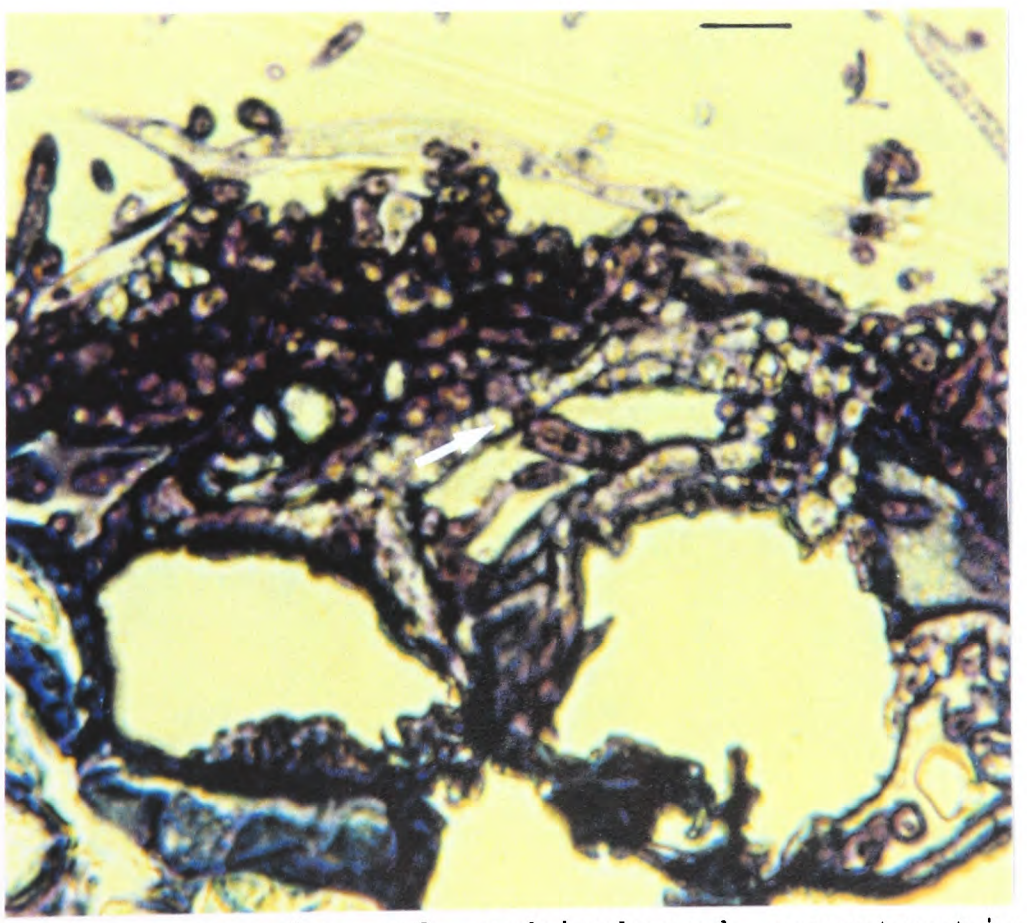


Figure 4.11. An example of unhindered penetration of a cortical cell through the mantle of a *Pinus sylvestris-Hebeloma crustuliniforme* ectomycorrhiza by a hypha of *Cylindrocarpon destructans* (arrow). Longitudinal section of an ectomycorrhiza synthesized in agar medium. Stained with toluidine blue. Scale bar = 10µm.

than in the resistant roots described in chapter 3, particularly towards the base of the mycorrhizal rootlets where the mantle was thinner or absent. The large cortical cells were often disrupted and at times presented very thick cell walls, which stained dark-blue/black with toluidine blue. It was not possible to ascertain, using the methodology described, whether this cell wall swelling was real or apparent due to darkly stained peripheral cytoplasm.

Autofluorescence studies did not show any unequivocal accumulation of phenols in the cortex. Only a reddish autofluorescence was visible in the outer cortex, never resembling the autofluorescence of tracheids in older roots, and often appearing to be coming from cytoplasmic materials. Staining of sections with Phloroglucinol-HCl and Schiff's reagent for the detection of lignin-like compounds also gave negative results.

4.3.3.3 Biochemical analysis of mycorrhizal extracts

No antifungal activity was detected in the aqueous phase, while no antifungal activity different from the previously described R1 and R2 inhibitory zones (see chapter 3) was detected in TLC bioassay of the organic phase.

4.4 **Discussion**

4.4.1 Microscopy and histochemistry of the mycorrhizal infection process

The inoculation method in agar medium described here has proved to have a number of desirable features for the purpose of microscopical and histochemical studies on the establishment of ectomycorrhizae. The major advantages are that (a) - the identity of the mycorrhizal fungus on the host is certain; (b) - it is relatively easy to keep the systems free from contamination, and in any case it is possible to detect contaminants very early; (c) - at sampling it is not necessary to clean and wash roots of any substrate debris; (d) - it is very easy to follow the infection process *in situ* by microscopical examination, either through a stereo microscope or through a compound microscope fitted with a low magnification lens (4X or 10X) (Fig. 4.1). Even the simple deionized water, 0.8% agar medium used in 4.2.3.1 gave very satisfactory results. Other methods described in the literature, including the P-V system (Duddridge, 1986[a]) used in one part of this study, the plastic pouch method (Fortin *et al.*, 1980), and many other approaches (Fortin *et al.*, 1983), have many disadvantages for the stated purpose, including special nutrient and buffering requirements, bulkiness of apparatus, watering problems, physiological stress, etc. (Fortin *et al.*, 1983). The major disadvantage of the agar medium method is that it can be labour intensive at the setting-up stage. However, providing very large numbers of seedlings are not required, the many positive features

described above amply compensate for this disadvantage.

Possible water saturation effects on the root system in agar medium, like ethylene accumulation with possible consequences on root anatomy (e.g. hypertrophy, cell separation) (Garza, unpublished), impaired oxygen exchange, and increased leakage of root metabolites through an imperfectly developed rhizodermis, do not appear to affect the mycorrhization process *per se* in any substantial way, nor in any way which could be prejudicial to the purpose of the experiment. The mantle may develop imperfectly on rootlets embedded in agar, and this may seem a drawback for studies on mycorrhiza-pathogen interactions. However, once the mycorrhizal root systems are transferred to the surface of fresh water agar for subsequent pathogen inoculation, a macroscopically normal mantle develops promptly. Furthermore, in similar studies, it has been established that the method does not alter mycorrhizal ontogeny significantly (Garza, personal communication). However, the author is aware that the system described is highly artificial, and may not fully represent events taking place under more natural conditions.

Although the mycorrhizal association is almost invariably considered to be of a mutualistic nature, it cannot be doubted that it may also be viewed as a special case of parasitism (Wilcox, 1983). In this respect, ectomycorrhizae can be differentiated into compatible and incompatible interactions, just as with any plant-microbe association.

Various plant structural defence mechanisms can be considered of a general nature: cell wall appositions, and cell wall alterations, e.g. by accumulation of polyphenolic and lignin-like materials, have already been described in chapter 3. These have been reported also in incompatible mycorrhizal associations, and indeed they are often used to differentiate between compatibility and incompatibility. For example, cell wall appositions and phenolic accumulation have been described by Duddridge (1986[a,b]; 1987) in *Suillus grevillei-P. sylvestris* and *S. grevillei-Pseudotsuga menziesii* mycorrhizae synthesized *in vitro*, and by Nylund *et al.* (1982) in *Picea abies-Piloderma croceum/Pisolithus tinctorius* combinations. Lignification, or more generally deposition of phenolics, has been reported in *Pseudotsuga menziesii-Laccaria laccata* (Sylvia & Sinclair, 1983[b]), in *Pinus strobus-Pisolithus tinctorius* (Piche' *et al.*, 1981), and in possibly incompatible associations of various potentially ectomycorrhizal fungi with *Pinus radiata* (Malajczuk *et al.*, 1982) and with *Eucalyptus* spp. (Malajczuk *et al.*, 1984). Hypersensitive responses have been seen in the same *Eucalyptus* spp. associations (Malajczuk *et al.*, 1984). Furthermore, cell wall-bound phenols have been described in endomycorrhizae (Codignola *et al.*, 1989).

Peroxidase, an enzyme which, along with phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO), is associated with lignification processes, has been localized at the interface between incompatible but not between compatible symbionts (Duddridge, 1987), and in cell walls of

endomycorrhizae (Spanu & Bonfante-Fasolo, 1988). Conversely, no significant difference in the activity of PAL and PO was found in short roots of mycorrhizal (with compatible *Laccaria laccata)* and non-mycorrhizal *P. sylvestris* (Ronald & Soderhall, 1985).

Even in what are usually considered as compatible associations external factors, such as high carbohydrate content in the synthesis medium, can induce signs of incompatibility, for instance between *P. sylvestris* and *S. bovinus* (Duddridge & Read, 1984[c]), one of the associations used also in this study.

The associations described here appear to be compatible because no wall appositions, no consistent accumulation of phenolics on the cell walls, and no hypersensitive responses were detected. Moreover, there were no obvious differences in the distribution of intracytoplasmic phenolics between ectomycorrhizal and uninoculated short roots (similar to previously described cases - Piche' *et al.*, 1981), apart from the association with *S. bovinus,* in which droplets apparently of phenolic compounds, appeared adsorbed on the tonoplast of cortical cells. This is also similar to a previous description, involving the *Pinus strobus-Pisolithus tinctorius* interaction (Piche' *et al.*, 1983).

The magenta colour reaction of the amorphous matrix between mantle and rhizodermal cell walls, shown by toluidine blue staining, suggests the presence of acid polysaccharides

(O'Brien & McCully, 1980), although staining with ruthenium red was not tried for confirmation in this particular case. These mucilage-type compounds, either produced by the host, by the fungus or synergistically, may have a cementing action, an important feature of the positive plant-fungus recognition process (Anderson, 1985; Duddridge, 1987).

On the basis of the data gathered from the experiment, and from the existing literature, it could be hypothesized that failure to elicit (or capacity to suppress) plant responses against the mycobiont in compatible ectomycorrhizae may hinder the capacity of ectomycorrhizae to improve disease resistance. In a way, the "more compatible" the relationship, the fewer the chances that the plant defences would be alerted and prepared to ward off subsequent pathogenic infections. These aspects will be discussed in more detail in paragraph 4.4.3.

4.4.2 Biochemical analysis of secondary metabolites of ectomycorrhizal fungi and assessment of their antifungal activity *in vitro*

In 1973 Marx published a list of mycorrhizal fungi, derived from his own experience and from published works up to that time, that were reported to produce antibiotics either in basidiocarps or in pure culture. As is evident from that list, quite a substantial amount of work had been carried out on that subject, but there is little subsequent literature. In the list, *Suillus bovinus* was reported to produce antifungal

compounds, *Hebeloma crustuliniforme* was tested only for, and exhibited, antibacterial activity, and no mention was made of *Pisolithus tinctorius,* the third ectomycobiont used in the present study.

Rather equivocally, the only ectomycorrhizal fungus in which some antifungal activity could be detected by TLC plate bioassay against the root pathogen *Cylindrocarpon destructans* was *H. crustuliniforme.* However, because this result could not be confirmed by a repetition of the experiment, and in view of the fact that neither of the other two ectomycobionts produced any detectable amounts of antifungal compounds extractable into the organic phase from pure culture filtrates, no further work was carried out on this aspect.

Although pure culture studies must always be interpreted very cautiously, more interesting results were obtained with the dialysis membrane experiments. Low molecular weight diffusates from both *P. tinctorius* and *S. bovinus* seemed to strongly inhibit the growth of *Pythium oligandrum,* while only *S. bovinus* appeared to show any inhibitory effect against *Phytophthora citricola.* Further studies on the effect of pH on the growth rate of the root pathogens suggested that, in the case of *Pythium oligandrum,* factors other than a pH change must play a role in its inhibition by *P. tinctorius* and *S. bovinus.*

On the other hand, the low growth rate of *Phytophthora citricola* at pH 3.1 strongly suggested that a pH effect may be among the sources of its inhibition by *S. bovinus,* which

lowered the pH of the medium to 2.9. This would seem to discount true antibiosis as the sole reason for the inhibition of *P. citricola* observed in the dialysis membrane experiment. *P. citricola* was actually stimulated by *P. tinctorius* when compared with its growth rate at pH's (pH 4 and 5) similar to that induced by *P. tinctorius* (pH 4.5).

In the case of *Cylindrocarpon destructans* no clear effect could be detected with either of the two mycorrhizal fungi. This suggested that *C. destructans* may not be the best choice for TLC bioassay of culture filtrates from *P. tinctorius* and *S. bovinus*. Apparently, therefore, *Pythium oligandrum* could be a more sensitive test organism. However, a preliminary attempt to perform a TLC bioassay with comminuted hyphae of *P. oligandrum*, according to the method described by Woodward and Pearce (1985), failed, probably as a consequence of the non-septate nature of *P. oligandrum* hyphae. Presumably the fungus lost its viability during fragmentation, thus it could not grow on the TLC plate. It may nevertheless be possible to induce sporulation of this organism in pure culture (chlamydospores, zoospores), in which case a spore suspension may be usable for the test.

The visual correlation between darkness of the medium and activity against *Pythium oligandrum* is rather intriguing. Pigments, along with antibiotics, are often produced by fungi under stress conditions (Rayner & Boddy, 1988), which may exist in the culture systems used in this study. The dark colour of the growth media of both *P. tinctorius* and *S.*

bovinus may therefore be attributed to the presence of pigments, and in darker media, with a greater concentration of pigments, the production of antibiotics may be concomitantly increased also.

4.4.3 Mycorrhiza-pathogen interactions

Mycorrhization of Scots pine roots with *Hebeloma crustuliniforme* appeared to induce a degree of protection against infection with *Pythium oligandrum in vitro*. Although the difference in death rate between mycorrhizal and non-mycorrhizal seedlings was not particularly remarkable (indeed, it was not statistically significant), a trend in protection could be identified in Fig. 4.9. This relatively small difference may be due to the host not being inherently susceptible to the pathogens used, including *Cylindrocarpon destructans*, because of its vegetative status at inoculation time. In order to obtain a suitable degree of mycorrhization (with mantle development) the host seedlings had to be incubated for one and one half months prior to pathogen challenge. By this age Scots pine seedlings may not be very susceptible to attack by pre- and post-emergence root pathogens like *C. destructans* and *P. oligandrum*, irrespective of whether the seedlings are mycorrhizal or not. This indeed appeared to be the case. No seedlings were killed by *C. destructans* within a period of 38 days, and only 47.8% of the non-mycorrhizal seedlings were killed by *P. oligandrum* in the same period, a percentage substantially lower than that reached in the experiment described in 3.3.1.7, when 86.4% of

all seedlings died within 11 days from inoculation. In retrospect, *C. destructans* appears to have been a poor choice for detecting mycorrhizal protection because of its inherently low pathogenicity on Scots pine, but it was used for consistency between studies on defence mechanisms in mycorrhizal and non-mycorrhizal roots. Perhaps a more virulent root pathogen, adapted also to attack relatively older plants, such as *Rhizoctonia solani*, would be preferable for this type of study.

Microscopical investigations on the interaction between *C. destructans* and *H. crustuliniforme* mycorrhizae on Scots pine revealed that the pathogen was not particularly hindered in its penetration of the root tissues by the presence of the mantle. Indeed, it appeared that its hyphae could freely intermingle with the mantle pseudotissue. The Hartig net also appeared to be a penetrable barrier, contrary to what Marx and Davey (1969[a,b]) and Marx (1970) described in their model systems. It is another example of the risks involved in generalizing defence concepts when considering mycorrhizal interactions.

Mycorrhizal roots did not appear to produce any wall appositions, even in response to direct intracellular penetration by *C. destructans, which appeared more extensive than in the non-mycorrhizal roots of the resistant seedlings described in chapter 3.* A plausible interpretation of this outcome is that mycorrhization by *H. crustuliniforme,* a compatible mycobiont on Scots pine seedling roots (*cf.* 4.4.1), may entail suppression of host structural defence responses.

This in turn implies that *C. destructans* may be able to penetrate host cells unobstructed, which has in fact been observed. At the same time, no detrimental effect on the host vegetative status should have been expected, since this pathogen did not damage or kill non-mycorrhizal seedlings of comparable age. Perhaps at that age Scots pine seedlings can accept and withstand limited cell invasion by *C. destructans* with no mortality ensuing. Production of wall appositions may therefore be irrelevant for the survival of the plant at that stage.

No significant histochemical differences were seen in pathogen-challenged mycorrhizal roots compared with normal mycorrhiza: cortical cell walls (which at times appeared thickened) often stained very dark blue with toluidine blue. Furthermore, the phenolic nature of the substrate could not be confirmed by autofluorescence, the most sensitive and reliable test for aromatic compounds. Infection of mycorrhizal roots by *C. destructans* did not appear to induce the generalized accumulation of phenolics observed in the cortex of non-mycorrhizal roots, described in 3.3.1.4 . This may be another facet of suppression of host responses brought about by the compatible mycorrhizal infection. These hypotheses seem to contrast with what was suggested by Nylund *et al.* (1982), who put forward the idea that control of the mycorrhizal association resides mainly with the host. It is likely that there is a combination of factors, of both host and mycobiont origin, which ultimately regulate this interaction and, consequently, the mycorrhiza-pathogen

interaction. However, the information available at the moment is too scanty to be able to substantiate either hypothesis.

Attempts to detect low molecular weight antifungal compounds characteristic of the mycorrhizal association have failed. Although the constitutive antifungal compounds of host origin described in chapter 3 were detected in mycorrhizal roots, their quantification was not attempted, because of severe limitations to obtaining sufficient biomass (mycorrhizal roots *in vitro*) to work with. Coupled with the absence of detectable antifungal compounds in pure cultures of the mycobiont, it appears likely that antibiosis would not have any role in this particular mycorrhiza-pathogen interaction.

High molecular weight compounds, i.e. proteins, specific to mycorrhizae (called ectomycorrhizins, which are not present in the separate host and mycobiont protein extracts) have been reported previously (Hilbert & Martin, 1988) in *Eucalyptus* spp.-*Pisolithus tinctorius* associations, but no mention was made of their possible involvement in disease protection. The author is aware of only one study on ectomycorrhizae in which accumulation of chitinase, a potentially defensive enzyme (Boller, 1987), was induced in Norway spruce infected with *Amanita muscaria* (Sauter & Hager, 1989), but that enzyme was almost certainly of host origin, i.e. not specific to the mycorrhizal associations. Examples of chitinase accumulation in endomycorrhizae also exist (Spanu *et al.*, 1989).

Overall it appears that *H. crustuliniforme* mycorrhizae on

Scots pine do not confer any particular advantage to the host in terms of disease protection, at least *in vitro* in the model systems employed. This is consistent with the findings of Chakravarty and Unestam (1986), who stated that in their model systems, which included *P. sylvestris* and *H. crustuliniforme,* among other mycobionts, antibiosis and the physical barrier represented by the mantle were not responsible for disease protection. They suggested that phenolic accumulation induced by mycorrhization may be the primary defence mechanism involved in their systems. This does not seem to be the case for the experiments described in this work, in which no unambiguous phenolic accumulation in mycorrhizal roots could be described, although the evidence is rather circumstantial, as it is based only on histochemical observations rather than on chemical analysis.

The rather small difference in death rates between mycorrhizal and non-mycorrhizal seedlings challenged with *P oligandrum* may perhaps be explained in terms of improved vegetative status induced by mycorrhization: vigorously growing non-mycorrhizal seedlings also appeared more resistant to *P. oligandrum* (*cf.* 3.3.1.7) than less vigorous individuals.

CHAPTER 5

Pathogenesis-related (PR) proteins in non-mycorrhizal Scots pine seedlings

5.1 Introduction

The nature and significance of pathogenesis related proteins (PR-proteins) in plant infections have already been described in chapter 1. Most PR-proteins have been detected and characterized in the angiosperms, where they have generally been localized in the intercellular spaces (apoplast) of leaves.

No reports of PR-proteins *sensu stricto* (van Loon, 1985) in conifers are known, but examples of inducible hydrolytic enzymes with potential antifungal activity, i.e. chitinases and ß-1,3-glucanases, exist in the literature: these enzymes have been found to accumulate in roots of Norway spruce and Scots pine seedlings, following treatment with fungal preparations and infection with *Heterobasidion annosum* respectively (Sauter & Hager, 1989; Nsolomo & Woodward, unpublished). Some proteins also appeared *de novo* in needle intercellular fluids of ozone treated Scots pine, but they were not characterized and were more aptly named "stress proteins" (Sandermann *et al.*, 1989).

No reports of polyacrylamide gel electrophoresis (PAGE) analysis of conifer root protein extracts are known. The aim of this chapter was therefore to determine whether *P. sylvestris* seedlings infected with *C. destructans* produce any PR-proteins detectable by PAGE. These were sought in

intercellular fluids of both primary roots and primary needles of root challenged seedlings, because the apoplastic localization of these proteins appears to be the norm in the plants examined hitherto. Furthermore, it was attempted to determine whether there is a pathogenesis-induced accumulation, or *de novo* synthesis, of chitinase and ß-1,3-glucanase, and whether any PR-proteins detected correspond to these hydrolytic enzymes. To this end, ENACTS (ENzyme ACTivity Staining) methods were used to obtain PAGE profiles of the isoforms of these two hydrolases. Conventional enzyme assays were not utilized because of difficulties with obtaining sufficient materials.

5.2 **Materials and methods**

5.2.1 Total protein profiles

P. sylvestris seedlings were grown and inoculated in glass jars as described in 2.1.2.1 and 2.3.1.1. Seedlings, 1-4 months old at the time of inoculation, were incubated with *C. destructans* for 3 weeks before protein extraction. A method derived from that described by Parent & Asselin (1984) was used for extraction of apoplastic fluid proteins, with minor differences in the extracting assembly: the body of a 2ml polypropylene syringe was placed on top of a graduated Eppendorf tube, containing approx. 0.1ml of polyvinylpyrrolidone (PVP) (to remove phenolic substances), and the whole unit placed in a 50ml, polyallomer ultracentrifuge tube (Oak Ridge tubes, Nalgene, USA) (see diagrammatic representation in Fig. 5.1).

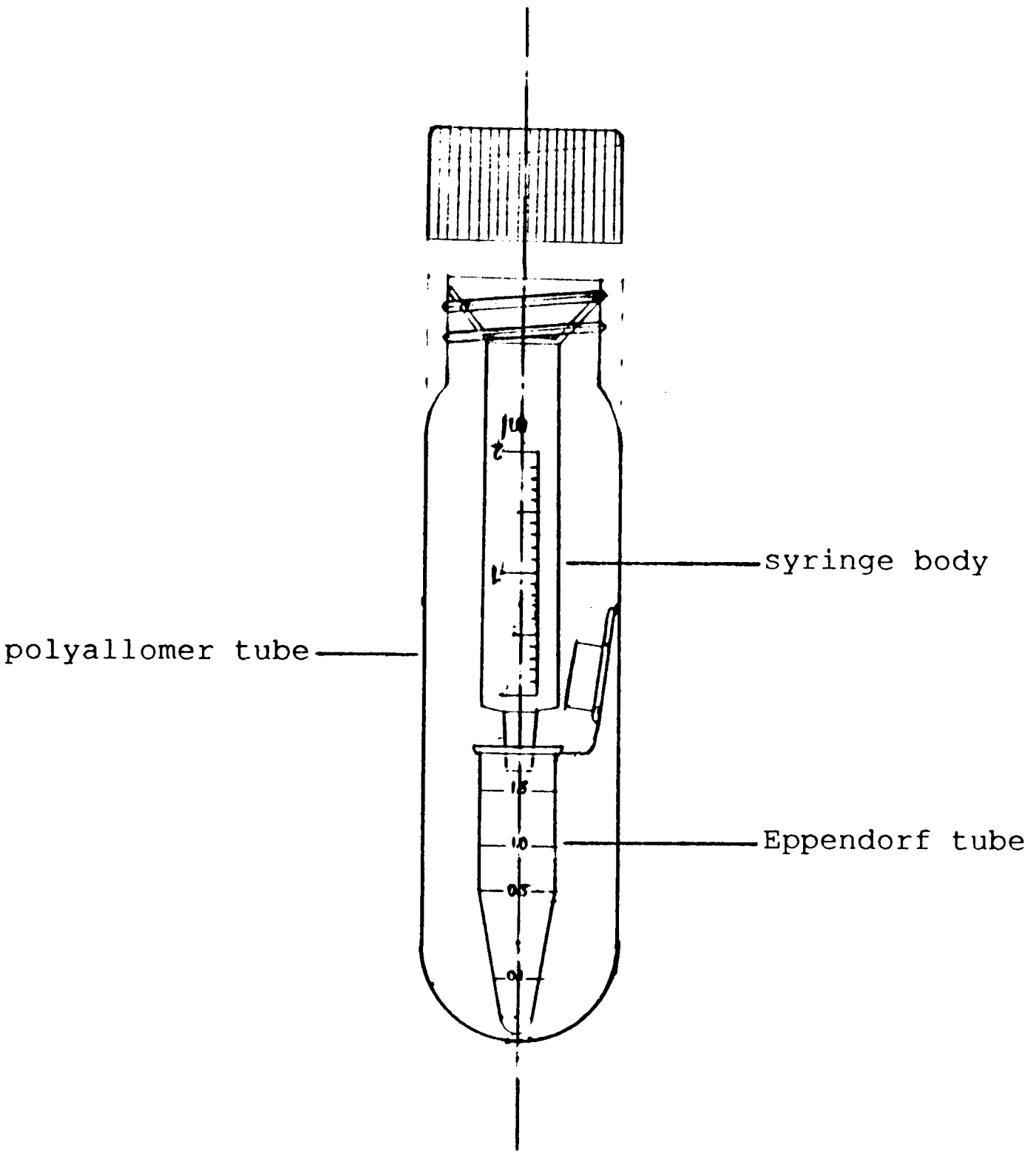


Figure 5.1. Assembly for protein extraction.

The needles and rootlets were infiltrated with cold (4°C) buffer (Table 5.1) *in vacuo* for 10min, in a desiccator, on top of ice.

Table 5.1

Extraction buffer used for infiltration of plant tissues. 1 part of stock A was mixed with 1 part of stock B to obtain the extraction buffer.

-Stock A: 50 mM Tris-HCl (pH 7.8); -Stock B: 1 M Sucrose; 20 mM $MgCl_2$; 20 mM $CaCl_2$; 1 mM PMSF 10 mM 2-mercaptoethanol
Tris: tris(hydroxymethyl)-methylamine (BDH) PMSF: phenylmethylsulfonyl fluoride (a protease inhibitor) (Sigma)

The desiccator was gently shaken occasionally during the infiltration period to aid the release of air bubbles originating from the tissue. The needles and rootlets were drained and gently blotted as dry as possible with tissue paper, and inserted in the syringes of the extracting units. These were spun in an ultracentrifuge at 4°C, at approx. 10000g for 15 min. The yield was represented by a yellowish solution which was frozen at -20°C until use. Total protein determination in the plant extracts (diluted 5X, because of paucity of material) was performed using the Bradford microprotein Coomassie blue assay as described in Dunbar (1987). Briefly, 20mg of Coomassie Brilliant Blue G-250 (Sigma) were diluted to 10ml with 90% ethanol. This solution was stirred for 4h with 20ml of 85% orthophosphoric acid

(H_3PO_4) and 170ml of distilled water (total volume = 200ml), and filtered with Whatman filter paper. 1ml of this stock solution was added to each of 100µl sample dilutions. A reference standard curve was obtained with 50, 100, 150, 200, 250µg BSA ml^{-1} distilled water.

Polyacrylamide gel electrophoresis was carried out under native protein conditions. The discontinuous, non-dissociating gels were derived from Maizel (1971) and Hames and Rickwood (1981). The stock solutions and the protocols for the preparation of 0.75mm stacking and resolving slab gels are shown in Table 5.2. The electrode buffer stock solution was diluted 2X before use.

After total protein determination, all samples were diluted with extraction buffer to the same final protein concentration. Concentrated sample loading buffer (SLB) was added to these protein solutions at the ratio indicated in table 5.3. 100µl samples were loaded into 180µl wells (in the stacking gel).

Vertical gel electrophoresis was carried out using a Bio-Rad Protean-II cell connected to a LKB (model 2197) power pack. Gel run conditions were as follows:

Stacking gel: 15mA for 70min

Resolving gel: 25mA for 120min

Tot.: 190min

Table 5.2

Stock solutions used for preparation of slab gels for discontinuous, non-dissociating electrophoresis of native proteins

1) 30% acrylamide-bisacrylamide (dilution of the 40% Sigma preparation)

2) TEMED (N,N,N′,N′-tetramethylethylenediamine)

3) Ammonium persulphate (APS) (BDH, U.K.) (10%): 1g in 9ml of water

4) 0.1% (w:v) Riboflavin (BDH, U.K.) (stable in fridge, but light sensitive)

5) Resolving gel buffer: 48ml of 1N HCl and 36.3g of tris up to 100ml of solution with water, adjusted to pH 8.9 stable)

6) Stacking gel buffer: 25.6ml of 1M orthophosphoric acid and 5.7g of tris up to 100ml of solution with water, adjusted to pH 6.7 (stable)

7) Electrode (tank) buffer: 6g of tris and 28.8g of glycine up to 1 L with water, adjusted to pH 8.3 (stable)

Protocols for gel preparation (1 slab). Non dissociating high pH, discontinuous buffer system

Mix the following:

-for 10ml of 4% stacking gel:
- 1.25ml of 1
- 0.008ml of 2
- 0.100ml of 4
- 1.25ml of 6
- 7.4ml of water

-for 20ml of 12.5% resolving gel:
- 8.34ml of 1
- 0.010ml of 2
- 0.15ml of 3
- 2.5ml of 5
- 9.0ml of water

Table 5.3

Protocol for preparation of 10ml of concentrated sample loading buffer (SLB) [from Parent & Asselin (1984), Hames and Rickwood (1981), modified]

Mix the following:
- 0.43ml of concentrated (14.74M) phosphoric acid - 1.45g of Tris - 0.02g of bromophenol blue - water up to 10ml of solution
The samples to be loaded are prepared by mixing 100µl of sample with 6µl of SLB

Protein bands were fixed and stained using, and according to the directions in, the Sigma silver stain kit. Silver staining was used instead of the more classic Coomassie blue staining because of its reported greater sensitivity (up to 100 times more sensitive, according to Ochs *et al.* (1981). Photographs were taken on a visible-light transilluminator.

5.2.2 ENACTS for chitinase detection

Chitinase detection was carried out using a modification of the gel overlay method described by Trudel & Asselin (1989). The substrate, colloidal glycol chitin, was prepared as described in that paper, by acetylation of glycol chitosan (Sigma). A stock suspension of 1% colloidal glycol chitin containing 0.02% sodium azide as a preservative was used in the protocol described in table 5.4 for the preparation of the substrate-containing gel.

Table 5.4

Protocol for the preparation of a 0.75mm glycol chitin-containing slab gel

Mix the following:	
-	5.000 ml of 30% acrylamide-bisacrylamide
-	0.010 ml of N,N,N′,N′-tetramethylethylenediamine (TEMED)
-	0.150 ml of ammonium persulphate (APS)
-	0.200 ml of 1% Glycol Chitin
-	14.650 ml of 100mM Acetate buffer, pH 5.0
	tot. vol.= 20ml

The glycol chitin gel (GCG), prepared on the morning of the analysis, was stored in acetate buffer, of the same molarity and pH as in the gel, until use.

After the total protein gel (TPG) was run, it was conditioned in 150mM acetate buffer, pH 5.0, for 5min. The buffer was then removed and the TPG was overlaid with the GCG, making sure that no air bubbles were trapped between the two gels. The overlay gel was trimmed to fit the TPG exactly. The gels were then incubated in a moist box at 35°C for 1h. Following incubation the GCG was removed and stained for 5min in freshly prepared 0.01% (w:v) Calcofluor white M2R New (Cyanamid) solution in 100mM Tris-HCl (pH 8.9). The Calcofluor solution was removed and the gel incubated (destained) for 1h at room temperature in distilled water. The method works on the principle that the brightener stains glycol chitin but not the lysis products. Lytic zones were therefore visualized as dark bands on a fluorescent background by placing the gels on a Vilbert Lourmat UV transilluminator (model TF-20L), emitting

at 365nm. Photographs were taken using a yellow filter [Hoya Y(K2)] to eliminate background radiation.

5.2.3 ENACTS for ß-1,3-glucanase detection

The following overlay method was developed. This was partially deduced from a list of ENACTS methods compiled by Vallejos (1983). The principle on which this method is based is illustrated in table 5.5.

Table 5.5

Detection of ß-1,3-glucanase activity on polyacrylamide gels

Laminarin	ß-1,3-glucanase -->	Glucose
Glucose + NADP	Glucose Dehydrogenase (GDH) >	Gluconolactone + NADPH
NADPH + PMS(ox)	----->	NADP + PMS(red)
PMS(red) + INT	----->	PMS(ox) + Formazan (insoluble)

NADP: nicotinamide-adenine dinucleotide phosphate
PMS : phenazine methosulphate
INT : p-iodonitrotetrazolium violet

Laminarin, NADP, GDH, PMS (a compound with the capacity to be easily oxidized and reduced) and INT (a tetrazolium salt with high reducing potential) were obtained from Sigma. The overlay agar gel was prepared as shown in table 5.6. Two different pH's (pH 6.0 and 7.0) were used.

Table 5.6

Protocol for the preparation of 50ml overlay agar gels for ß-1,3-glucanase detection

Sol.1)		
Tris-maleate buffer 0.1M (pH 6.0 or 7.0)	25	ml
Agar	0.375	g
Sol.2)		
Tris-maleate buffer 0.1M (pH 6.0 or 7.0)	25	ml
Laminarin*	50	mg
INT	10	mg
PMS	2	mg
NADP	15	mg
GDH	15	units

* Substrate, more easily dissolved in warm buffer. To be cooled before the addition of the other ingredients.

Solution 2 was added, while stirring continuously on a heated magnetic stirrer, to solution 1 after the agar had been dissolved in a boiling water bath, and only after solution 1 was cooled down to approx. 45°C. This mixture was immediately poured on a TPG, left to set, and incubated in a moist box at 35°C for approx. 2h. Enzymatic activity bands, due to hydrolysis of laminarin by ß-1,3-glucanase, are visualized by precipitation of the tetrazolium salt (INT) as an insoluble formazan, which is violet in colour.

For testing this method a commercial laminarinase preparation (Sigma) was used as a reference enzyme. Approx. 0.5 units of this enzyme preparation were loaded into a well in parallel with plant extracts, after being dissolved in extraction buffer to which SLB was added as described in table 5.3.

Photographs were taken using a green filter [Hoya G(X1)]

to enhance the contrast of the violet stain on black and white film.

5.3 **Results**

5.3.1 Total protein profiles

The production of workable biomass for protein extraction in the system described constitutes a major experimental constraint. This is evident from the data in appendix , in which the fresh weights, the approx. volumes (total and per g fresh weight) of the extracts, and the protein concentrations obtained are reported.

Before PAGE was carried out, the protein concentrations of all extracts (determined from the standard curve given in Fig. 5.2) were normalized to the protein concentration of needles from uninoculated seedlings, i.e. the controls (NC) (250μg ml^{-1}). Nominally, therefore, each well contained 25μg of protein. The gel was run until the dye started to bleed from the gel into the electrode buffer.

Very poor results were obtained with the root material. Bad lane streaking, following silver staining, prevented any reasonable reading of the total protein profiles. This effect, present in both control and challenged roots, was probably caused by the presence of phenolic compounds in the extracts, which PVP may have failed to remove. However, the needle extracts electrophoresed much better, with comparatively lighter background development following silver staining.

Standard curve for protein determination
[microassay from Bradford, according to Dunbar (1987)]

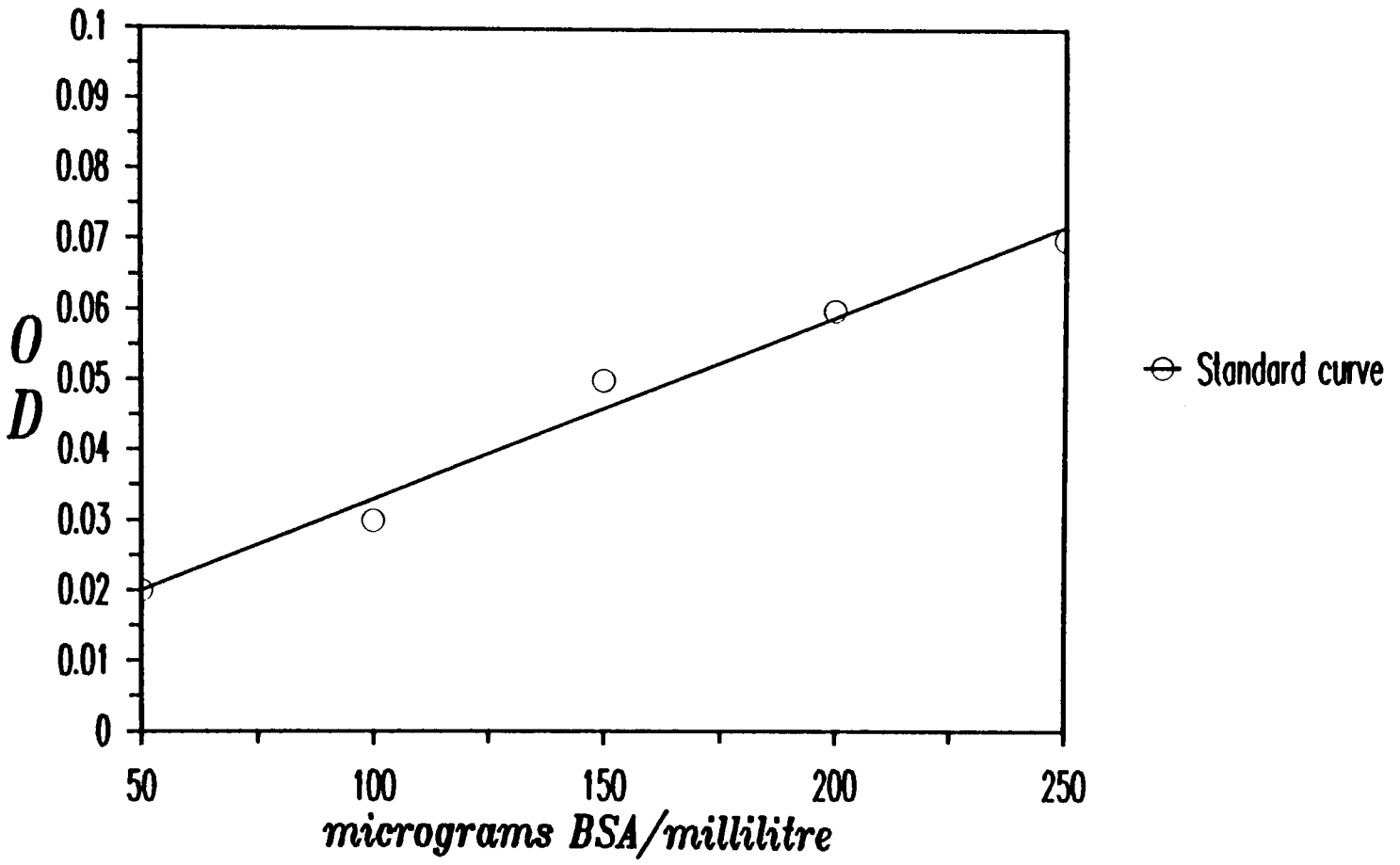


Figure 5.2

Several bands of constitutive proteins appeared more intensely stained in needle extracts of root challenged than in needle extracts of control seedlings. Some other bands of constitutive proteins seemed to be less intense in needle extracts of challenged seedlings (Fig. 5.3a). Conversely, no proteins produced *de novo* were detected in these profiles.

5.3.2 ENACTS for chitinase detection

Several protein bands exhibiting chitinolytic activity could be detected, in both needle and root extracts (Fig. 5.3b). Although no new chitinase bands were evident, the intensity of constitutive chitinase bands increased in the extracts of both needles and roots of root-challenged seedlings (Fig. 5.3b). The bands which, by their position, could be identified in the total protein profiles of fig. 5.3a, have been marked with an asterisk.

5.3.3 ENACTS for ß-1,3-glucanase detection

Before the actual method was developed to its present form, preliminary work to examine the feasibility of some of the redox reactions which are at its foundation was carried out. In particular, the activity of glucose dehydrogenase (GDH) was tested at different pH's. Activity was estimated from the conversion of NADP to NADPH, detectable by spectrophotometry, in the presence of glucose: the reduced form of this coenzyme can be detected at 340nm (Dixon & Webb, 1964). Of the 3 pH's that were tested (pH 5.4, 6.4, 7.6), GDH worked better at pH 5.4 (table 5.7), but its activity was

acceptable at all 3 values. Coincidentally, this was very similar to the pH optimum (pH 5) suggested by Sigma for their laminarinase preparation. However, to avoid inhibition of reduction of the tetrazolium salt at low pH (Vallejos, 1983), the actual test for ß-1,3-glucanase activity in plant extracts was conducted at pH 6.0 and 7.0.

The method described was able to detect the activity of the laminarinase preparation (Sigma), particularly at pH 6.0, but was unsatisfactory with the plant extracts (Fig. 5.4). The whole gel developed a uniform background, on which some apparent activity inhibition zones developed. The larger of these bands seem to be positioned roughly at the same Rf as the major triplet of chitinase bands (*cf.* Fig. 5.3b).

Table 5.7

Activity of glucose dehydrogenase (GDH) at different pH's, indicated as initial linear rate (slope) of conversion of NADP into NADPH in the presence of glucose.
NADPH detected at 340nm.

	pH		
	5.4	**6.4**	**7.6**
$\frac{\Delta OD}{\Delta t}$	0.069	0.043	0.046
OD: optical density t : time (min)			

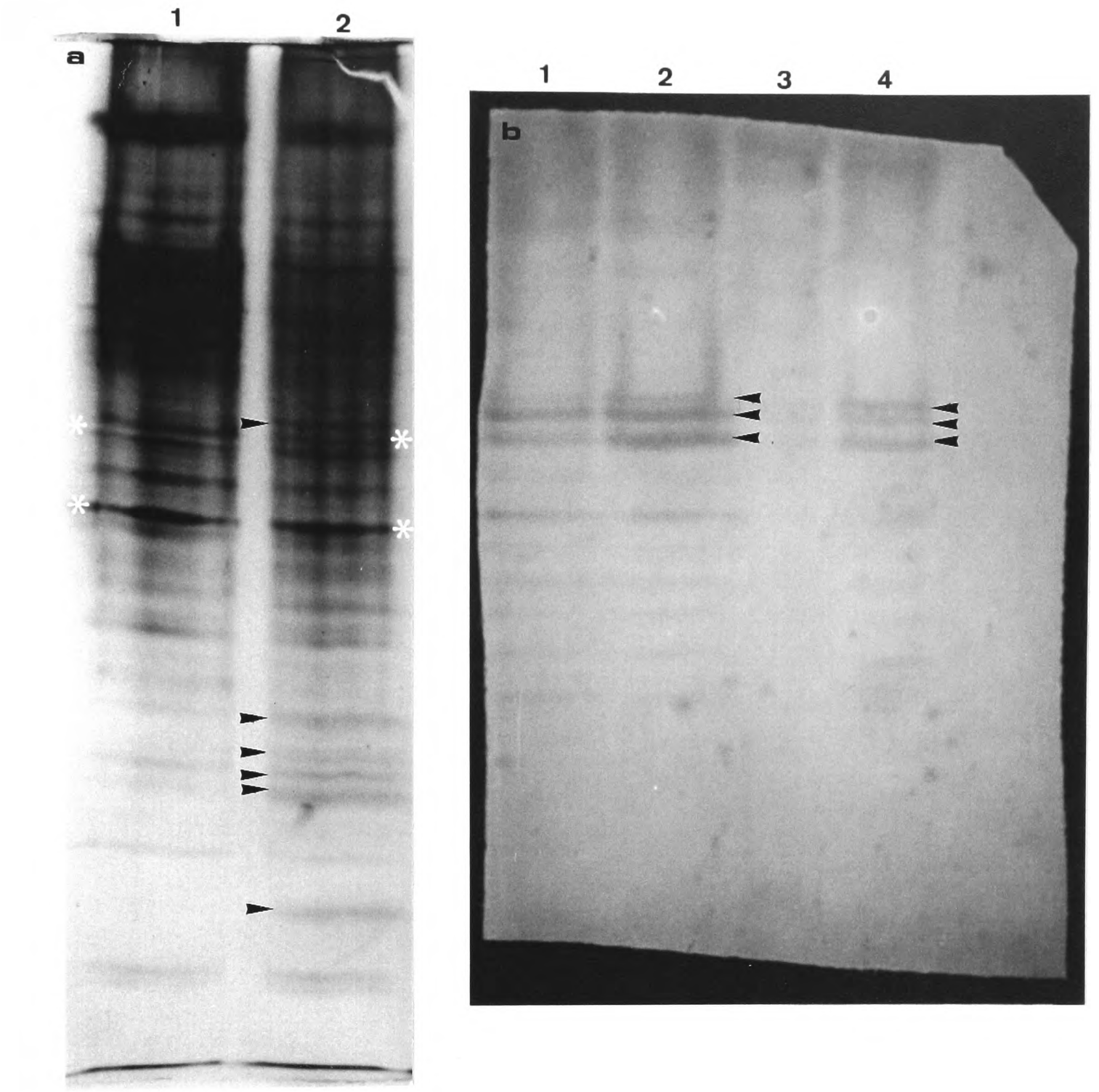


Figure 5.3. a) PAGE profile (silver staining) of needle intercellular, native proteins of *P. sylvestris* seedlings. Lanes 1 and 2 are extracts from control and seedlings infected with *C. destructans*, respectively. Several bands appear enhanced in the challenged seedlings (arrows). b) Enzyme activity staining (ENACTS) of a gel similar to that described in Fig. 5.3a for the detection of chitinase. Lanes 1 and 2 are as in Fig. 5.3a; lanes 3 and 4 are root extracts, from control and seedlings infected with *C. destructans*, respectively. Several bands appear enhanced in the challenged seedlings (arrows). The bands that can be identified as chitinases have been marked with asterisks in Fig. 5.3a.

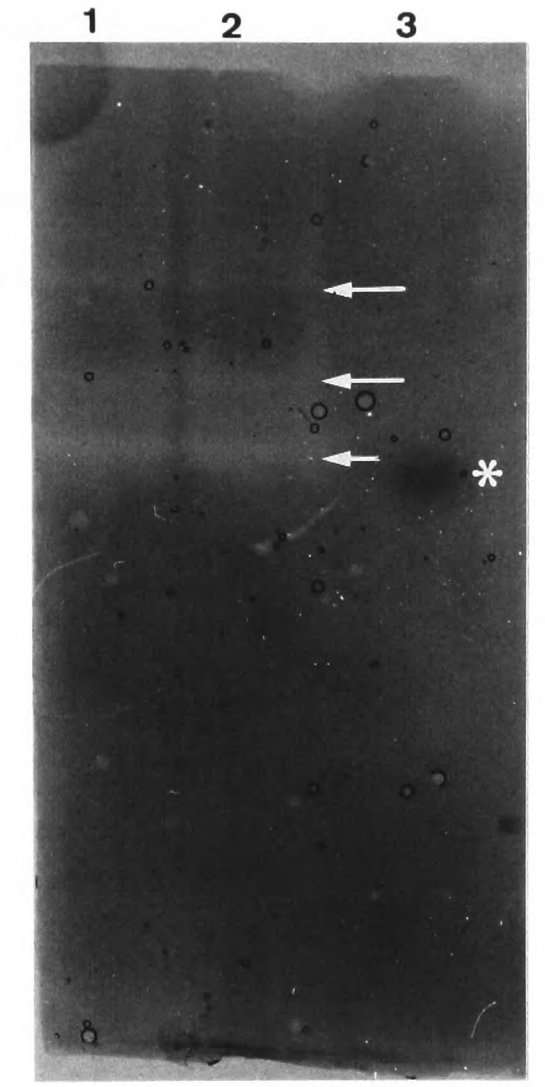


Figure 5.4. Enzyme activity staining of a gel similar to that in Fig. 5.3a, for the detection of ß-1,3-glucanase. Lanes 1 and 2 are needle extracts from control and seedlings infected with *C. destructans*, respectively; lane 3 is a laminarinase preparation (Sigma). The development of a strong, fairly uniform background does not allow for any meaningful interpretation. Only the commercial preparation of laminarinase (asterisk) can be identified. Several areas of inhibition can be seen in the plant extracts, corresponding to some of the chitinase bands in Fig. 5.3b (arrows).

5.4 **Discussion**

According to a widely accepted definition, changes in the pattern of soluble protein makeup in plants undergoing pathogenic processes, constitute evidence for the existence of PR-proteins (Redolfi, 1983). A more strict definition would regard as PR-proteins only proteins appearing *de novo* following infection (van Loon, 1985). In this perspective, the looser definition reported by Redolfi would seem to apply more appropriately to the system described in this work.

In fact, no *de novo* production of any proteins has been detected. Nevertheless, several apoplastic proteins seemed to accumulate, both locally and systemically, in Scots pine seedlings challenged with the root pathogen *C. destructans*. Thus, under Redolfi's definition, it appears that the PR-protein phenomenon occurs in *Pinus sylvestris*. However, if van Loon's concepts are accepted, perhaps a better designation for these proteins in Scots pine could be "pathogenesis-enhanced" (PE) proteins. This definition will be used throughout the following discussion.

The recognition of localized accumulation of PE-proteins in roots has been hindered, in silver stained gels, by the interference of unknown substances. Despite careful handling of roots and needles, and the application of relatively low speed centrifugation, to minimize cellular disruption during the tissue extraction process, these hindrances were likely to be due to the presence of soluble, non-protein-bound phenols interfering with the silver stain only, because the chitinase profiles looked acceptable even in the root extracts. This

suggests that the phenols had not reacted with the proteins to form phenol-protein conjugates, which would alter the chitinase profile as well as the total protein profile. If this is so, the use of PVP in the collecting Eppendorf tube had only limited effectiveness in eliminating interference from phenols. The use of some other method, like dissolving PVP directly (Kephart, 1990) (which is rather difficult), and/or the use of some phenol-oxidation inhibitor (e.g. reducing agents like ascorbic acid - Kephart, 1990) in the extraction buffer, may provide better results.

Despite these problems with the root extracts, accumulation of several proteins appeared to occur, qualitatively, in needle extracts of root challenged seedlings [which, incidentally, suggests the operation of some mechanism of systemic induction (*cf.* chapter 6)]. Comparison of the total protein profiles with the chitinase profiles confirmed that at least 3 of the detected PE-proteins are chitinase isozymes. Induction (including systemic induction) of chitosanolytic and chitinolytic activity in microbially challenged plants is a widespread phenomenon in the angiosperms (Boller & Metraux, 1988; Grenier & Asselin, 1990; Legrand *et al.*, 1987; Metraux *et al.*, 1988; Garcia Breijo *et al.*, 1990; Pierpoint *et al.*, 1990; Nasser *et al.*, 1990), but has only recently been observed also in conifers. Chitinase activity has been shown to accumulate locally in Norway spruce seedling roots treated with cell wall preparations of *Amanita muscaria,* an ectomycorrhizal fungus (Sauter & Hager, 1989). More relevantly, localized stimulation of chitinase activity

has also been noted in seedling roots of Scots pine following challenge and infection with *Heterobasidion annosum* (Nsolomo & Woodward, unpublished). The results from the present study have partly confirmed this localized induction, but have also shown that chitinase is induced systemically and is present in Scots pine seedlings as a range of isozymes: at least 9 bands could be identified in the profiles of needle extracts of both control and root challenged seedlings, although not all of them appeared induced. Similar multiplicity of chitinolytic isozymes has also been reported in the angiosperms (*cf.* Garcia Breijo *et al.*, 1990; Jacobsen *et al.*, 1990; Legrand *et al.*, 1987).

The author is aware that, besides the lack of quantitative data, a major limitation of this work lies in the fact that no test for intracellular chitinase was carried out (both shortcomings were due to the great difficulties in obtaining sufficient material to work with). However, at least some Scots pine chitinases appear to be located in the intercellular spaces, which means outside the protoplast and probably in the cell wall. This is analogous to other observations in the angiosperms (Redolfi, 1983), and may reflect the basically antifungal nature of this response, in this particular case against the invading, initially intercellular, hyphae of *C. destructans* (*cf.* Boller & Metraux, 1988). The results also showed that, because no new proteins were detected, this type of plant response in Scots pine is probably not pathogen specific, quite similarly to what has been proposed for the angiosperms (van Loon, 1985).

Nevertheless, a lack of new proteins is only a necessary, but not sufficient, condition to exclude pathogen-specific, protein-type responses.

No analysis of protein extracts from the pathogen, *C. destructans,* was included. This was deemed unnecessary, because the proteins present in the challenged tissue were already constitutively present in the unchallenged tissue.

In addition to chitinase, which can be considered as the main antifungal hydrolytic enzyme produced by the host, because of its strong inhibitory activity against fungal pathogens *in vitro* (Schlumbaum *et al.*, 1986), and because it has no known function in the host plant metabolism (Boller, 1987), ß-1,3-glucanases are considered to play an antifungal role, although they are also important in the turnover of ß-1,3-glucans of plant origin, e.g. callose (Boller, 1987). Again, several PR-proteins in the angiosperms have been identified as ß-1,3-glucanases (Kauffmann *et al.*, 1987; Pierpoint *et al.*, 1990; Nasser *et al.*, 1990; Ye *et al.*, 1990). For conifers, information on the subject is almost completely lacking, although stimulation of localized ß-1,3-glucanase activity by infection with *Heterobasidion annosum* has been observed in seedling roots of Scots pine (Nsolomo & Woodward, unpublished).

In the present study, a method developed for the detection of ß-1,3-glucanase in polyacrylamide gels worked acceptably only with a commercial enzyme preparation (laminarinase), but gave very poor results with plant extracts. The bands of apparent ß-1,3-glucanase inhibition

observed in plant extracts may correspond to the positions of some chitinases. However, the possibility of an artifact is likely, as the apparent inhibitory activity may simply be due to the inhibition of the glucose dehydrogenase, or interference with any of the coupled redox reactions used in the method itself.

This method obviously remains to be perfected, although it shows promise. The author believes that there is scope for further development, improvement by the selection of an appropriate pH, a different tetrazolium salt with a lower reduction potential to limit background staining, and perhaps in the use of a polyacrylamide overlay gel as opposed to an agarized medium.

In conclusion, no PR-proteins were detected in intercellular fluids of *Pinus sylvestris* seedlings challenged with a root pathogen. However, several electrophoretic protein bands were apparently enhanced following fungal infection. Among these, chitinase isoenzymes could be recognized. Nevertheless, because of experimental constraints, the extent of this putative accumulation was not quantified. To do this, purification of the enzyme (ideally of each isoenzyme) would be necessary.

An overlay method for the direct detection of ß-1,3-glucanase bands in PAGE gels of plant extracts, presented here for the first time, seems promising, but needs considerable further development and refinement.

CHAPTER 6

Systemic induced resistance in mycorrhizal seedlings

6.1 Introduction

General aspects of systemic induced resistance (SIR) have already been outlined in 1.2. SIR has been studied rather extensively in crop plants like cucumber and tobacco. It generally consists of an increased level of resistance, putatively acquired all over the organism, induced by a primary infection of some plant organ (usually a leaf) with an attenuated pathogen, or a weak strain of a pathogen (Kuc, 1983; 1987). This results in decreased severity of symptoms in subsequent infections, expressed throughout the plant, with reductions in diseased areas of over 90% in certain cases (Dean & Kuc, 1987).

The great majority of information on SIR relates to induction of resistance from one aerial organ to another aerial organ (e.g. Hammerschmidt & Kuc, 1982; Hammerschmidt *et al.*, 1982; Stumm & Gessler, 1986; Ye *et al.*, 1990). However, it is possible that, as with any other plant-microbe interaction, mycorrhizal infection may elicit a continuous, low level stress, of the sort that generally appears instrumental in the expression of SIR (Kuc, 1983). Although mycorrhization is usually beneficial to the plant in terms of general metabolism, mycorrhizal fungi can be regarded as one extreme in the continuum of biotrophic parasitism, and some stress related to this cannot be excluded.

Very little is known about possible systemic effects on disease induced by mycorrhizal associations. In one such case (Schoenbeck & Dehne, 1979) it appeared that, although endotrophic mycorrhization improved disease resistance to root pathogens, foliar diseases became more serious than in non-mycorrhizal plants. This study appears, therefore, to oppose the idea of mycorrhiza-induced SIR.

The aims of this work were to assess whether *Hebeloma crustuliniforme*, an ectomycobiont, can induce (systemically) resistance to the air-borne pathogen *Botrytis cinerea* in Scots pine seedlings, i.e. whether ectomycorrhizal infection can induce needle protection.

6.2 **Materials and methods**

6.2.1 Plant material and inoculation procedures

The mycobiont *Hebeloma crustuliniforme* was used in this experiment. This fungus was grown in sterilized, 500ml glass jars (Kilner, Ravenhead, U.K.) containing approx. 100ml of 0.8% deionized water agar. The agar was inoculated with 10-15 5mm plugs taken from the margins of actively growing mycelial cultures. The jars were incubated at 25°C in the dark. After an incubation of 1.5 months, during which *H. crustuliniforme* grew throughout the medium, Scots pine seedlings were transferred to the jars, as described in 2.1.2.1, 2-3 weeks after the seeds were plated out for germination. 30 seedlings were transferred to each jar containing *H. crustuliniforme* and to each uninoculated jar (control). The jars were incubated in

the cabinet described in 2.1.2 for 1.5 months, to allow for thorough infection by *H. crustuliniforme*.

During this long incubation period, aerial contamination of some seedlings became evident, in the form of a whitish, apparently non-sporulating, floccose mycelium. Because, from previous attempts, this contamination appeared to be virtually unavoidable (despite all the possible aseptic measures taken during handling of the material), it was decided to continue the experiment after elimination of the contaminated seedlings. This was done immediately prior to inoculation of the aerial parts of the seedlings with *Botrytis cinerea*. 12 contaminated seedlings from a *H. crustuliniforme* jar and 4 from a control jar were thus discarded.

A suspension of *B. cinerea* macroconidia in deionized water ($7.5X10^5$ spores/ml, prepared as described in 2.5) was sprayed on the seedlings' needles. 5 sprayings (approx. 2s each) of spores were directed orthogonally to the neck of each open jar (approx. 10cm from seedlings), turning the jar 90° between sprayings to maximize uniformity of spore deposition. The jars were then recapped and maintained on a window ledge in the laboratory for 2 weeks at a room temperature of approx. 25°C (summertime), after which surviving seedlings were counted. Seedlings were assessed as infected when their needles appeared flaccid to completely withered, when the whole seedling had died.

In a parallel experiment, the capacity of *Botrytis cinerea* to infect needles of hardened seedlings was tested. 20 Scots pine seedlings growing in petri dish systems (as

described in 2.1.2.2 - roots unchallenged) were liberally sprayed with a suspension of *Botrytis cinerea* spores similar to that indicated above, after which they were placed in a propagator. To maintain high humidity levels, a few bungs of plastic foam drenched in water were placed in the bottom tray of the propagator, and the lid was sealed all around with polythene tape. The seedlings were checked periodically for disease symtoms.

6.2.2 Microscopical examination of needle surface

In order to avoid washing spores off, the needles were cleared by a method using chlorine gas, derived from that of Janes (1962). 4 or 5 needles were made to adhere vertically, by water tension, to the inner wall, and in proximity to the rim, of a McCartney bottle containing approx. 1.5g of calcium hypochlorite. 5ml of concentrated HCl were then added to the calcium hypochlorite to produce free chlorine, and the bottle capped. After approx. 1h the needles had cleared. Cleared needles were transferred to a microscope slide and stained with methyl green for spore detection, by mounting in a drop of stain prior to examination.

6.2.3 Effect of *Hebeloma crustuliniforme*-produced volatiles on germination of *Botrytis cinerea* spores

Because the main experiment was conducted *in vitro*, i.e. in an enclosed environment, where atmospheric saturation with volatiles was likely, an experiment to test for active volatiles produced by *H. crustuliniforme* was conducted.

A suspension of *B. cinerea* macroconidia was prepared as described in 2.5, with the difference that filtered, 0.2% malt extract broth was used instead of deionized water, to a final concentration of $2.5X10^6$ spores/ml. Pairs of hanging droplets (25µl each, measured with a micropipette) were set up as follows. One drop was pipetted out at the centre of a coverslip, on which 4 blobs of soft, white paraffin had already been deposited at its corners. Glass slides were then positioned on pairs of coverslips so that the blobs of paraffin would touch them. The whole units, each comprising the slide and the 2 coverslips, were then carefully flipped over, and placed on the surface of actively growing, 5 months old colonies of *H. crustuliniforme,* and of uninoculated, half strength MMN agar (control). The petri dishes were sealed with a double layer of parafilm and incubated at 25°C in the dark. Percentage spore germination was calculated after 24h and 48h.

6.3 **Results**

6.3.1 Systemic induced resistance

Although the data from the 2 control jars were statistically homogeneous (cumulative data could therefore have been used), the results from the 2 jars in the *Hebeloma crustuliniforme* treatment were not statistically homogeneous, i.e. the data from the two jars were statistically different [$p < 0.05$ (Parker, 1984)]. Therefore, cumulative frequencies could not be used for statistical analysis, so the means were used instead (Chi-squared method). The difference in survival

rate between *H. crustuliniforme* treated seedlings and non-mycorrhizal control seedlings was statistically significant ($p < 0.05$) (table 6.1).

Table 6.1

Percentage of control and mycorrhizal (with *Hebeloma crustuliniforme*) Scots pine seedlings surviving 2 weeks after infection of their needles with *Botrytis cinerea*

	Control	Mycorrhizal
mean (+-SEM)	7.1 (+-0.5)	37.5 (*) (+-7.8)
*: significant difference (Chi-squared method, $p < 0.05$)		

In a parallel test, *Botrytis cinerea* macroconidia failed to produce any disease symptoms on the needles of hardened Scots pine seedlings. The experiment was terminated after 2 months.

6.3.2 Microscopy of needle surface

While extensive hyphal development was observed on infected needles, only very few, usually ungerminated, shrivelled spores of *B. cinerea* could be detected on needles of surviving seedlings [mean of 4 spores per needle surface, calculated from 6 needles taken from 6 different resistant seedlings (1 needle per seedling)].

6.3.3 Effect of *Hebeloma crustuliniforme*-produced volatiles on germination of *Botrytis cinerea* spores

Percentage germination was determined from 4 replicates (4 droplets on 2 slides, in 2 petri dishes) for both *H. crustuliniforme* plates and the uninoculated control. The mean germination percentages after 24h of incubation are reported in table 6.2. No data are presented for 48h because the percentage germination for the control remained unchanged, while it was impossible to determine percentage germination in the *H. crustuliniforme* treatment because hyphal development was too extensive.

Since the data from the 4 replicates at 24h were homogeneous (Parker, 1984), the Chi-squared test was applied to the aggregate results. The difference in mean percentage germination between control and *H. crustuliniforme* treatment was very highly significant ($p < 0.001$).

Table 6.2

Effect of *Hebeloma crustuliniforme*-produced volatiles on the germination of *Botrytis cinerea* macroconidia. Percentage germination at 24h.

	Control	*Hebeloma crustuliniforme*
mean (+-SEM)	2.25 (+-0.48)	13.75 (***) (+-1.55)

***: significant difference (Chi-squared method, $p < 0.001$)

6.4 **Discussion**

Systemic induced resistance to fungal pathogens is a well documented phenomenon in angiosperms (Kuc, 1983), particularly in crop species like cucurbits (e.g. Caruso & Kuc, 1977[a,b]). In nearly all known cases, induction of systemic foliar protection was effected by a primary infection, usually with an attenuated pathogen, of some part of the plant's aerial organs, generally the first true leaves, or cotyledons (Caruso & Kuc, 1977[a,b]; Dean & Kuc, 1986; Hammerschmidt & Kuc, 1982; Hammerschmidt *et al.*, 1982). However, examples exist of systemic induced protection elicited in the foliage but acting also on the root system of cucumber seedlings. Primary infection with *Colletotrichum lagenarium* appeared to protect the root system from infection with *Fusarium oxysporum* f. sp. *cucumerinum*. (Gessler & Kuc, 1982).

Induced localized protection of plant roots subsequent to infection with weak strains of root pathogens has been previously described, particularly in wilt diseases (Kuc, 1983). However, perhaps the best known examples of this type of protection come from mycorrhizal studies (see chapter 4), including endomycorrhizae (Schonbeck & Dehne, 1979).

The cases so far described pertain to elicitation and expression of SIR in the following systems: leaf to leaf, leaf to root, and root to root. Schonbeck & Dehne (1979) worked on a root to leaf system, with barley, french bean, cucumber, lettuce and tobacco plants endomycorrhizal with *Glomus mossae*, and demonstrated that these plants were more susceptible to aerial pathogens than non-mycorrhizal plants. No other work on

root-to-leaf induction systems is known. In any case the work of Schonbeck and Dehne was not discussed in terms of accepted SIR theories (Kuc, 1983).

This chapter describes a preliminary investigation of the possibility that systemic resistance may be induced in the primary needles of Scots pine seedlings by mycorrhization with *Hebeloma crustuliniforme*. The experiments were conducted *in vitro*, which entailed some drawbacks due to imperfect root development, possible atmospheric saturation with volatiles, and imperfectly developed needle cuticles. However, this last weakness actually proved very useful, because it allowed the strain of *Botrytis cinerea* adopted here to infect the pine needles. This would not normally occur in the laboratory with fully hardened seedlings, as shown by a parallel experiment. It was clear that resistance to the foliar pathogen *B. cinerea*, a fairly common pathogen of conifer seedlings in nurseries (causing pre- and post-emergence damping off - Pawsey, 1964; Peace, 1962; Phillips & Burdekin, 1982; Smith *et al.*, 1988) increased substantially after mycorrhization, contrary to the findings of Schonbeck and Dehne (1979). This result was strengthened further by the observation that germination of *B. cinerea* macroconidia was not inhibited by volatiles produced by *H. crustuliniforme*. On the contrary, germination of *B. cinerea* spores was greatly enhanced by *H. crustuliniforme*-produced volatiles. It appears, therefore, that this type of inhibition of spore germination is unlikely to be the cause of lower infection rates.

Various physiological mechanisms have been suggested for the operation of systemic induced resistance. It is widely believed that this phenomenon is expressed through a quicker and more intense deployment of host defence mechanisms. These include both structural and biochemical barriers, like cell wall alterations (wall appositions - Stumm & Gessler, 1986; lignification - Hammerschmidt & Kuc, 1982;), production of low molecular weight antifungal compounds (phytoalexins -Kuc, 1987), and of hydrolytic enzymes displaying antifungal activity, like chitinase and ß-1,3-glucanase (Kuc, 1987; Ye *et al.*, 1990). Peroxidase, an important enzyme in the metabolism of simple and complex phenolic substances (e.g. lignin), has also been shown to increase several-fold in systemically protected plants (Hammerschmidt *et al.*, 1982; Ye *et al.*, 1990).

Because of time constraints, no time course studies and no detailed examination of the possible physiological mechanims involved in the apparent systemic protection of Scots pine seedlings were attempted. It cannot be excluded that the mechanisms mentioned above may be operating in this model system. However, microscopical observations of the inoculated seedlings revealed that, by the end of the experiment, protected needles from both mycorrhizal and non-mycorrhizal seedlings, were almost devoid of *B. cinerea* macroconidia, and the few left were ungerminated and shrivelled. Moreover, there was no evidence of mycelial growth on the needle surface, and no leaf penetration attempts by the

pathogen could be detected. Thus, the available evidence suggests that this systemic protection may just be a potentiation of constitutive defences, and may not act within the plant, as described in other reports of SIR in angiosperms. Rather, some form of direct inhibition of spore germination could be the main mechanism of the protection observed here.

In this respect, it is known that several ectomycobionts produce ethylene in pure culture (Garza, unpublished). However, it is unlikely that this gaseous, highly active phytohormone would be directly responsible for inhibition of spore germination, even if produced by *H. crustuliniforme* in pure culture (which was not tested), because *H. crustuliniforme*-produced volatiles showed exactly the opposite effect, i.e. enhanced spore germination and development.

Ethylene is known to affect a large number of plant metabolic processes (Arrigoni, 1980; Bidwell, 1978), including plant defence mechanisms, e.g. induction of accumulation of chitinase (Boller *et al.*, 1983) and phenolics (Baldwin & Schultz, 1983) in angiosperms. Although it obviously remains to be demonstrated, ethylene could be a mediator in the inhibition of spore germination in the model system used, by inducing the production of volatile terpenes. These compounds have been shown to accumulate (locally) in the ectomycorrhizal roots of several conifers, and to exhibit various degrees of inhibitory activity against a range of plant fungal pathogens (Krupa & Fries, 1971; Krupa & Nylund, 1972; Krupa *et al.*, 1973).

The results also hint that, in this particular experimental system, protection is not expressed as a generalized reduced severity of disease symptoms on individual plants (smaller and fewer lesions), which may then survive, as described in other cases (e.g. Caruso & Kuc, 1977[a,b]; Kuc & Richmond, 1977). It appears, instead, that mycorrhization with *H. crustuliniforme* enhances the expression of total protection to *B. cinerea* in the Scots pine seedlings population as a whole. In fact, resistant Scots pine seedlings did not show any readily detectable disease symtoms at all, neither in the mycorrhizal nor in the control treatments.

In short, a gross expression of elevated resistance in the mycorrhizal plants, remote from the mycorrhizae themselves, was observed: however, the data obtained in this study were insufficient to indicate whether true SIR, mediated *via* some internal messenger in the host plant, was involved, or whether some other mechanisms, resulting in similar gross effects, had operated. Such effects could be mediated by an external influence or 'messenger', e.g. ethylene produced by the fungus altering the plant surface properties to confer resistance, or the possibility (albeit unlikely under the present experimental conditions) that an improved nutritional status of the mycorrhizal plants could enhance resistance non-specifically. Alternatively, the mycorrhizae could act as a sink for excess photosynthate, reducing available nutrients (carbohydrates) in the needles. *Botrytis cinerea* has often been shown to be dependant on external sources of nutrients

for successful infection of hosts to occur (Coley-Smith *et al.*, 1980).

Although this work is clearly incomplete, it does however open a new line of investigation in the field of mycorrhizal protection of conifer seedlings against pathogens. It is hoped that this study will stimulate further research aimed at understanding the physiology of this phenomenon in the hitherto neglected field of conifers, particularly studies aiming to characterize the defence mechanisms involved.

CHAPTER 7

General discussion and conclusions

The study of plant-pathogen interactions is a major sector of plant pathology. Within this sector, morphological and structural studies have always played a notable role, but in the last 20-25 years, thanks mainly to the development of new techniques and experimental approaches (Callow, 1983), research on the biochemical aspects of plant defence responses has also gained prominence (Friend & Threlfall, 1976; Horsfall & Cowling, 1980; Callow, 1983; Pegg & Ayres, 1987).

Very little is known of plant-pathogen interactions in forest species, in particular in Gymnosperms, especially at the biochemical level, whereas a substantial amount of information related to agricultural species (Angiosperms) is available. The work presented here aimed to contribute to rectifying the deficiency of knowledge on tree species by attempting to answer a few of the key questions relating to the disease resistance mechanisms expressed in conifers, more precisely in (primary) roots of Scots pine seedlings. Moreover, this work brought together two aspects of fungal parasitism which are traditionally treated separately: pathogenic infections and mycorrhization. Pegg and Ayres (1987) recognized the limitations of this artificial separation, in that these two groups of associations share, more often than not, common grounds in many aspects of nutritional relationships, recognition processes, host

specificity, and elicitation of plant defence responses.

In the present study fungal elicitation of papilla formation has been demonstrated, for the first time, in the non-mycorrhizal primary roots of a Gymnosperm, in the model system *Pinus sylvestris-Cylindrocarpon destructans*. Apposition of cell wall material induced by fungal infection was also observed, albeit more rarely, in other model systems, involving the same host species, during the preliminary screening for suitable root pathogens (*Pythium oligandrum* and *Phytophthora citricola)*. These physical defence structures appeared to be composed largely of pectic substances, often reinforced by infusion with phenolic materials and enrichment with calcium, both factors known to increase impenetrability of cell walls and cell wall appositions to fungal parasites (Aist, 1983; Aist & Israel, 1986; Bayles & Aist, 1987; Edwards & Ayres, 1981; Kunoh *et al.*, 1986; Ride & Pearce, 1979; Vance *et al.*, 1980). However, a specific role for wall appositions in the expression of disease resistance of Scots pine seedlings could not be proven with *Pythium oligandrum*. Although this undoutedly confuses somewhat the interpretation of the results mentioned above, the hypothesis that in Gymnosperms wall appositions could contribute to resistance can certainly be advanced by analogy with more completely documented cases from the Angiosperms.

Papillae were never detected in the ectomycorrhizal systems used, but this is to be expected in the compatible

interactions (*cf.* Duddridge, 1987) which result from the association between Scots pine primary roots and *Pisolithus tinctorius, Suillus bovinus* or *Hebeloma crustuliniforme*, which were used in this work. Furthermore, no papillae were detected in the ectomycorrhizal roots of the system *Pinus sylvestris-Hebeloma crustuliniforme* when these were challenged with the pathogen *C. destructans*. This evidence strongly suggests that one outcome of primary root infection with compatible ectomycobionts may be the suppression of the papilla response, one of the main types of known structural defence mechanisms (Aist, 1983; Ride, 1983). Concominantly, a higher degree of cortical intracellular invasion was observed with *C. destructans* than in the non-mycorrhizal roots of resistant seedlings mentioned above. Theoretically, then, this could make seedlings infected with compatible ectomycobionts more susceptible to root pathogens than non-mycorrhizal plants. However, as often reported (e.g. Callow, 1983), and as will be evident from the remainder of this discussion, no single defence mechanism can be deemed to be the only responsible factor in the balance of disease resistance as a whole. In fact, the trend towards enhanced protection of Scots pine seedlings against the more virulent root pathogen *Pythium oligandrum* brought about by mycorrhization (Fig. 4.9), appears to confirm that even if papilla induction is suppressed, mycorrhizal seedlings may still be better off than non-mycorrhizal ones in terms of disease resistance.

Although more detailed studies on the timing and extent of the papilla response are undoubtedly needed, the evidence

available from the present research seems to suggest that structural defence mechanisms may not be primary determinants in the resistance of Scots pine seedlings to diseases of the primary root system.

Perhaps more significant findings came from biochemical studies. Both low and high molecular weight antifungal compounds were examined, although the latter could not be analysed very extensively.

In non-mycorrhizal roots no phytoalexins could be detected, but the mean concentration of a group of constitutive antifungal compounds, recognized as belonging to the array of known abietic acids (diterpene resin acids), increased after infection with *C. destructans*. Although the increase averaged approx. 85%, the difference between control and infected roots was not statistically significant. However, the antifungal activity of a commercial preparation of abietic acid *in vitro*, at concentrations similar to live infected tissues, seemed to indicate that some inhibition of fungal growth could occur *in vivo*, especially if the distribution of abietic acid is not uniform in pine root tissues. Localized accumulation of these diterpenes, for instance at sites of attempted fungal penetration, may have a much more important role in resistance than a uniformly raised average level (detected by the HPLC assay) may indicate. Constitutive phenolics are known to be unevenly distributed in tissues of other conifers, e.g. in the bark of Sitka spruce (Toscano-Underwood & Pearce, 1991).

No low molecular weight antifungal compounds specific to the ectomycorrhizal association *P. sylvestris-H. crustuliniforme* were detected, nor could such antifungal compounds be detected in the chloroform-soluble (organic) fractions from culture filtrates of this ectomycobiont. No antifungal compounds were detected in similar fractions of the other two ectomycobionts either. Nevertheless, the presence of the abietic acids mentioned above was apparent in the extracts from the *H. crustuliniforme* ectomycorrhizae. Whether mycorrhization with *H. crustuliniforme*, besides affording a better nutritional status to the host (which in itself can enhance disease resistance), induces accumulation of these abietic acids in the host to levels inhibitory to *P. oligandrum* is not known.

Levels of the hydrolytic enzyme chitinase, a high molecular weight antifungal compound which appeared to be located in the apoplastic spaces of primary roots and needles as a multiplicity of isoenzymes, seemed to increase after challenge with *C. destructans*, both locally and systemically. However, this increase could only be gauged qualitatively from PAGE profiles, because quantitative enzyme assays could not be performed. Nevertheless, studies conducted on similar systems have demonstrated that levels of chitinase activity do indeed increase (at least locally) in Scots pine primary roots challenged with *Heterobasidion annosum* (Nsolomo & Woodward, unpublished). Chitinolytic activity may therefore be another line of defence mobilized against pathogenic attack, both in

non-mycorrhizal and mycorrhizal roots. This last aspect was not investigated, but examples exist in the literature of chitinase induction in the host by both ecto- (Sauter & Hager, 1989) and endomycobionts (Spanu *et al.*, 1989).

Studies on the possible involvement of ß-1,3-glucanase in defence were marred by various problems and did not lead to definitive results, but it cannot be excluded that this hydrolytic enzyme may also have a role in the defence of Scots pine seedlings. In fact, such role has already been indicated by the quantitative data obtained by Nsolomo and Wooward (unpublished) on the accumulation of this enzyme in the primary roots of Scots pine, following infection with *H. annosum*.

It is tempting to speculate that these hydrolases may act in concert with the abietic acids, and in some cases with papilla formation and accumulation of phenols on the cell walls, to confer at least partial protection against root pathogens of Scots pine seedlings. Suppression of some of these mechanisms (papilla deposition), and tolerance to others (accumulation of abietic acids and hydrolases) may explain the impunity demonstrated by mycorrhizal fungi during root invasion (*cf.* Anderson, 1985). In fact, abietic acid may even have a stimulatory effect on some ectomycobionts (Fries *et al.*, 1987), and chitinase is known to be induced upon mycorrhizal infection, but its activity tends to decrease to lower-than-control levels after the association is fully established (Spanu *et al.*, 1989).

Some form of systemic protection, expressed as substantially increased survival of Scots pine seedlings to the aerial pathogen *Botrytis cinerea*, which appears to be induced by mycorrhizal infection, is reported here for the first time in a forest species. Any or all the mechanisms mentioned above might have a role to play in this systemic effect, but it was not possible to verify this in the course of the present study. However, the available data indicate that this protection may be mediated *via* the inhibition of germination of the spores of the aerial pathogen on the needle surface. This was not caused by any direct inhibitory effect of volatiles produced by the ectomycobiont in the enclosed environment used in the experiments. Other interpretations must therefore be sought. The most tempting of these is perhaps the idea that mycorrhization may induce accumulation of ethylene in the host, produced by either the ectomycobiont (Garza, unpublished), the host, or both. This hormone plays a number of different and influential roles, at a systemic level, in the general metabolism of plants (Arrigoni, 1980; Bidwell, 1978), but in this context its effect on host defence mechanisms is the most relevant. It is known that ethylene accumulation is involved in the induction of chitinase in angiosperms (Boller *et al.*, 1983), but a more likely explanation for the lack of spore germination on needles of protected seedlings may be that ethylene may induce production of volatile terpenes by the host, although this was not tested in this study. Whether or not ethylene is involved, it is known that mycorrhization induces local accumulation of mono-

and sesquiterpenoids in primary roots of conifers, and that these volatiles show inhibitory activity against a range of fungi (Krupa & Fries, 1971; Krupa & Nylund, 1972; Krupa *et al.*, 1973).

In vitro studies may not represent a natural situation faithfully. However, they are certainly among the best experimental approaches available to examine previously unexplored problems, which may involve large numbers of variables. At initial stages, simplified microcosms are essential to study plant-microbe interactions, especially at the root level. The situation in a soil, which would be closer to reality, or even in a simplified soil substitute like a peat-vermiculite mixture, is still far too complex to interpret easily, particularly if biochemical responses are considered. For instance, extracts from roots in soil always run a risk of being contaminated by other biological material present on the rhizoplane, and this would considerably complicate the interpretation of the plant responses, especially if compounds produced *de novo* by the host (phytoalexins) are being sought. 'Systemic' induction of plant defence mechanisms by rhizoplane (but also by phylloplane) microorganisms cannot be excluded either. For example, only if the host plant is grown in axenic culture can the investigator be confident that the host is not responding systemically to an infection: in all other cases in which a potentially inducing microflora is present, such defence responses may already be expressed, at least to a partial level. The

detection of these mechanisms following a test infection would therefore be impaired.

However, it is hoped that this work, besides providing some insights in a previously largely unexplored field of plant pathology, can be a starting point for further research in the hitherto little investigated field of forest physiopathology. Also, in the relatively short term, it may be possible to apply these and subsequent findings to more practical uses, like the control of damping-off diseases in forest nurseries. Nowadays this problem is predominantly approached by focusing attention either on measures involving the soil, e.g. by the use of physico-chemical methods for the elimination of soil-borne pathogens before planting (like heat-sterilization and fungicidal fumigation of the soil - Gradi, 1980), or on therapeutic means, with the use of generally protective fungicides (Blatchford, 1979; Hamel, 1983).

However, 'environmentally friendly' disease prevention (i.e., without, or with more limited, use of chemicals) is certainly more desirable. The most logical way to do this would be by emphasizing the potential for disease resistance. Early screening of forest material for disease resistance could be envisaged by searching for some particular biochemical markers, like optimal levels of low molecular weight antifungal compounds or the activity of hydrolases like chitinase and ß-1,3-glucanase. To do this routinely, simplification and optimization of methodology would certainly be required, but these should not be unsurmountable obstacles,

given the current and foreseeable technical developments in biochemical analysis.

In the long run, biotechnology may also come into the picture: the use of genetic engineering to steer the plant metabolism towards either a higher level of synthesis of the above mentioned substances or *de novo* synthesis of other antifungal compounds (not necessarily of host origin) is nowadays not so implausible.

Appendix

Fresh weights, volumes of apoplastic fluids (total and per g fresh weight), and total protein concentrations recovered for PAGE analysis of protein extracts of primary needles and roots of Scots pine seedlings:

	NC	NI	RC	RI
fresh weights (g)	4.08	4.85	2.46	2.11
ml extract	1.7	1.9	0.65	0.7
ml extract g^{-1} fresh weight	0.42	0.39	0.26	0.33
protein [$\mu g\ ml^{-1}$]	250	300	300	400

notes: NC, NI: needles of control, needles of root infected seedlings

RC, RI: roots of control, roots of root infected seedlings

The samples were constituted by the pooled harvest of 10 jars of seedlings each for control and challenged material, each jar containing 20-30 seedlings. Total protein concentration was determined using the method of Bradford, as described in Dunbar (1987) (see chapter 5).

REFERENCES

Aguamah, G.E., Langcake, P., Leworthy, D.P., Page, J.A., Pryce, R.J., Stranger,R.N. (1981). Two novel stilbene phytoalexins from *Arachis hypogea*. *Phytochemistry* **20**, 1381-1383.

Aist, J.R. (1976). Papillae and related wound plugs of plant cells. *Annual Review of Phytopathology* **14**, 145-163.

Aist, J.R. (1983). Structural responses as resistance mechanisms. In *The Dynamics of Host Defence*. Ed. by J.A. Bailey & B.J. Deverall, Academic Press, Sydney, pp. 33-70.

Aist, J.R. and Israel, H.W. (1986). Autofluorescent and UV-absorbing components in cell walls and papillae of barley *Hordeum vulgare* cultivar 'Proctor' coleoptyles and their relationship to disease resistance. *Canadian Journal of Botany* **64**, 266-272.

Aist, J.R. and Israel, H.W. (1986). Relationship of autofluorescent and UV-absorbing components in cell walls and wall appositions to disease resistance in kohlrabi roots. *Canadian Journal of Botany* **64**, 273-275.

Aist, J.R., Gold, R.E., Bayles, C.J., Morrison, G.H., Chandra, S. and Israel, H.W. (1988). Evidence that molecular components of papillae may be involved in ml-o resistance to barley powdery mildew. *Physiological and Molecular Plant Pathology* **33**, 17-32.

Akai, S. and Fukutomi, M. (1980). Preformed internal physical barriers. In *Plant Disease - an Advanced Treatise. Volume V. How Plants Defend Themselves*. Ed. by J.G. Horsfall and E.B. Cowling. Academic Press, New York, 139-159.

Anderson, A. (1985). The problems of living with a plant root: factors involved in root colonization. In *Proceedings of the 6th North American Conference on Mycorrhizae*. Ed. by R. Molina. Cornvallis: Oregon State University Press, 175-178.

Antoniw, J.F., Ritter, C.E., Pierpoint, W.S. and van Loon, L.C. (1980). Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV. *Journal of General Virology* **47**, 79-87.

Antoniw, J.F. and White, R.F (1983). Biochemical properties of the pathogenesis-related proteins from tobacco. *Netherlands Journal of Plant Pathology* **89**, 255-264.

Arrigoni, O. (1980). *Elementi di Biologia Vegetale - Botanica Generale*. Casa Editrice Ambrosiana, Milano, pp. 541 (Italian).

Baldwin, I.T. and Schultz, J.C. (1983). Rapid changes in tree leaf chemistry induced by damage: evidence for communication between plants. *Science* **221**, 277-279.

Bateman, D.F. (1964). An induced mechanism of tissue resistance to polygalacturonase in *Rhizoctonia*-infected hypocotyls of beans. *Phytopathology* **54**, 438-445.

Bayles, C.J. and Aist, J.R. (1987). Apparent calcium mediation of resistance of an ml-o barley mutant to powdery mildew. *Physiological and Molecular Plant Pathology* **30**, 337-345.

Beckman, R.H. (1980). Defences triggered by the invader: physical defences. In *Plant Disease - an Advanced Treatise. Volume V. How Plants Defend Themselves*. Ed. by J.G. Horsfall and E.B. Cowling. Academic Press, New York, 139-159.

Bidwell, R.G.S. (1978). *Fisiologia Vegetale*. Piccin Editore, Padova, pp. 721 (Italian).

Biggs, A.R., Merrill, W. and Davis, D.D. (1984) Discussion: response of bark tissues to injury and infection. *Canadian Journal of Forest Research* **14**, 351-356.

Blatchford, O.N. (ed.) (1979). *The Use of Chemicals in the Forestry Commission*. Forestry Commission, Edinburgh, pp. 98.

Boller, T. (1987). Hydrolytic enzymes in plant-disease resistance. In *Plant-Microbe Interactions. Molecular and Genetic Perspectives*. Ed. by T. Kosuge and E.W. Nester. MacMillan, pp. 385-413.

Boller, T., Gehri, A., Mauch, F. and Voegeli, U. (1983). Ethylene induced chitinase: is it a pathogenesis-related protein? *Netherlands Journal of Plant Pathology* **89**, 318 (communication).

Boller, Th. and Metraux, J.P. (1988). Extracellular localization of chitinase in cucumber. *Physiological and Molecular Plant Pathology* **33**, 11-16.

Brownlee, C., Duddridge, J.A., Malibari, A. and Read, D.J. (1983). The structure and function of mycelial systems of ectomycorrhizal roots with special reference to their role in forming inter-plant connections and providing pathways for assimilate and water transport. In *Tree Root Systems and Their Mycorrhizas*. Ed. by D. Atkinson, K.K.S. Bhat, M.P. Coutts, P.A. Mason and D.J. Read. Martinus Nijhoff/Dr. W. Junk Publishers, 433-443.

Bryngelsson, T. and Green, B. (1989). Characterization of a pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. *Physiological and Molecular Plant Pathology* **35**, 45-52.

Callow, J.A. (ed.) (1983). *Biochemical Plant Pathology*. John Wiley & Sons, Chichester, New York, pp. 484.

Campbell, J.L. and Johanssen, S.A.E. (1988). *PIXE - A Novel Technique for Elemental Analysis*. Wiley, New York.

Caruso, F.L. and Kuc', J. (1977[a]). Protection of watermelon and muskmelon against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. *Phytopathology* **67**, 1285-1289.

Caruso, F.L. and Kuc', J. (1977[b]). Field protection of cucumber, watermelon, and muskmelon against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. *Phytopathology* **67**, 1290-1292.

Chakravarty, P. and Unestam, T. (1986). Role of mycorrhizal fungi in protecting damping-off of *Pinus sylvestris* L. seedlings. In *Proceedings of the 1st European Symposium on Mycorrhizae*, ed. by V. Gianinazzi-Pearson and S. Gianinazzi. INRA, Paris, 811-814.

Chakravarty, P. and Unestam, T. (1987[a]). Mycorrhizal fungi prevent disease in stressed pine seedlings. *Journal of Phytopathology* **118**, 335-340.

Chakravarty, P. and Unestam, T. (1987[b]). Differential influence of ectomycorrhizae on plant growth and disease resistance in *Pinus sylvestris* seedlings. *Journal of Phytopathology* **120**, 104-120.

Chakravarty, P., Peterson, R.L. and Ellis, B.E. (1990). Integrated control of *Fusarium* damping-off in red pine seedlings with the ectomycorrhizal fungus *Paxillus involutus* and fungicides. *Canadian Journal of Forest Research* **20**, 1283-1288.

Christ, U. and Mosinger, E. (1989). Pathogenesis-related proteins of tomato: I. Induction by *Phytophthora infestans* and other biotic and abiotic inducers and correlations with resistance. *Physiological and Molecular Plant Pathology* **35**, 53-65.

Codignola, A., Verotta, L., Spanu, P., Maffei, M., Scannerini, S. and Bonfante-Fasolo, P. (1989). Cell wall bound-phenols in roots of vesicular-arbuscular mycorrhizal plants. *New Phytologist* **112**, 221-228.

Coley-Smith, J.R., Verhoeff, K. and Jarvis, W.R. (eds.) (1980). *The Biology of Botrytis*. Academic Press, London, pp. 318.

Collmer, A. and Keen, N.T. (1986). The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24**, 383-409.

Covassi, M., *et al.* (1975). Osservazioni preliminari sui rapporti tra *Coryneum cardinale* (Wag.) and *Phloeosinus aubei* Perris in Toscana. *Redia* **LXVI**, 159-166.

Cruikshank, A.M. (1980). Defences triggered by the invader: chemical defences. In *Plant Disease - an Advanced Treatise. Volume V. How Plants Defend Themselves*. Ed. by J.G. Horsfall and E.B. Cowling. Academic Press, New York, 247-267.

Dahm, H. and Strzelczyk, E. (1987). Cellulolytic and pectolytic activity of *Cylindrocarpon destructans* (Zins.) Scholt. isolates pathogenic and non-pathogenic to fir (*Abies alba* Mill.) and Pine (*Pinus sylvestris* L.). *Journal of Phytopathology* **118**, 76-83.

Darvill, A.G. and Albersheim, P. (1984). Phytoalexins and their elicitors - a defense against microbial infection in plants. *Annual Review of Plant Physiology* **35**, 243-275.

De Philippis, A. (1957). *Lezioni di selvicoltura speciale*. University of Florence, Italy (Italian), pp. 325.

Dean, R.A. and Kuc', J. (1986). Induced systemic protection in cucumbers: the source of the 'signal'. *Physiological and Molecular Plant Pathology* **28**, 227-233.

Dean, R.A. and Kuc, J.A. (1987). Immunization against disease: the plant fights back. In *Fungal Infection of Plants*. Ed. by G.T. Pegg and P.G. Ayres. Cambridge University Press.

de Bary, A. (1863). Recherches sur le developpement de quelques champignons parasites. *Ann. Sci. Nat. Bot. Biol. Veg.* **20**, 5-148.

Deverall, B.J. (1976). Current perspectives in research on phytoalexins. In *Biochemical Aspects of Plant Parasite Relationships*. Ed. by J. Friend and D.R. Threlfall. Academic Press, London, 208-223.

De Wit, P.J.G.M (1987). Specificity of active resistance mechanisms in plant-fungus interactions. In *Fungal Infection of Plants*. Ed. by G.F. Pegg and P.G. Ayres. Cambridge University Press, 1-24.

De Wit, P.J.G.M. and Bakker, J. (1980). Differential changes in soluble tomato leaf proteins after inoculation with virulent and avirulent races of *Cladosporium fulvum* (syn. *Fulvia fulva)*. Physiological Plant Pathology **17**, 121-130.

Dixon, M. & Webb, E.C. (1964). *Enzymes*. Longmans, London, pp. 950.

Drago-Toscano, C. (1989). Variation in antimicrobial defence mechanisms in Sitka spruce (*Picea sitchensis* (Bong.) (Carr.)) and its relation to disease resistance and susceptibility. D.Phil. thesis, Oxford.

Duchesne, L.C., Peterson, R.L. and Ellis, B.E. (1987). The accumulation of plant produced antimicrobial compounds in response to ectomycorrhizal fungi: a review. *Phytoprotection* **68**, 17-27.

Duchesne, L.C., Ellis, B.E. and Peterson, R.L. (1989). Disease suppression by the ectomycorrhizal fungus *Paxillus involutus*: contribution of oxalic acid. *Canadian Journal of Botany* **67**, 2726-2730.

Duddridge, J.A. (1986[a]). The development and ultrastructure of ectomycorrhizas. III. Compatible and incompatible interactions between *Suillus grevillei* (Klotzsch) Sing. and 11 species of ectomycorrhizal hosts *in vitro* in the absence of exogenous carbohydrate. *New Phytologist* **103**, 457-464.

Duddridge, J.A. (1986[b]). The development and ultrastructure of ectomycorrhizas. IV. Compatible and incompatible interactions between *Suillus grevillei* (Klotzsch) Sing. and a number of ectomycorrhizal hosts *in vitro* in the presence of exogenous carbohydrate. *New Phytologist* **103**, 465-471.

Duddridge, J.A. and Read, D.J. (1984[a]). The development and ultrastructure of ectomycorrhizas. I. Ectomycorrhizal development on Pine in the field. *New Phytologist* **96**, 565-573.

Duddridge, J.A. and Read, D.J. (1984[b]). The development and ultrastructure of ectomycorrhizas. II. Ectomycorrhizal development of Pine *in vitro*. *New Phytologist* **96**, 575-582.

Duddridge, J.A. and Read, D.J. (1984[c]). Modification of the host fungus interface in mycorrhizas synthesized between *Suillus bovinus* (Fr.) O. Kuntz and *Pinus sylvestris*. *New Phytologist* **96**, 583-588.

Duddridge, J.A. (1987). Specificity and recognition in ectomycorrhizal associations. In *Fungal Infection of Plants*. Ed. by G.T. Pegg and P.G. Ayres. Cambridge University Press, 25-44.

Dunbar, B.S. (1987). *Two-dimensional Electrophoresis and Immunological Techniques*. Plenum Press, New York, pp. 372.

Dwinell, L.D. (1969). Reaction of black oak (*Quercus velutina*) to infection by *Cronartium fusiforme* and *C. quercuum*. *Phytopathology* **59**, 113 (Abstr.).

Edwards, M.C. and Ayres, P.G. (1981). Cell death and cell wall papillae in the resistance of oak species to powdery mildew disease. *New Phytologist* **89**, 411-418.

Ehle, H. (1988). *Nectria radicicola* Gerlach & Nilsson. In *European Handbook of Plant Diseases*. Ed. by I.M. Smith, J. Dunez, R.A. Lelliott, D.H. Phillips, and S.A. Archer. Blackwell Scientific Publications. Oxford, London, Edinburgh, p. 286.

Fellows, H. (1928). Some chemical and morphological phenomena attending infection of the wheat plant by *Ophiobolus graminis*. *Journal of Agricultural Research* **37**, 647-661

Fortin, J.A., Piche', Y. and Lalonde, M. (1980). Technique for the observation of early morphological changes during ectomycorrhiza formation. *Canadian Journal of Botany* **58**, 361-365.

Fortin, J.A., Piche', Y. and Godbout, C. (1983). Methods for synthesizing ectomycorrhizas and their effect on mycorrhizal development. In *Tree Root Systems and Their Mycorrhizas*. Ed. by D. Atkinson, K.K.S. Bhat, M.P. Coutts, P.A. Mason and D.J. Read. Martinus Nijhoff/Dr. W. Junk Publishers, pp. 552.

Fox, F. (1983). Role of basidiospores as inocula of mycorrhizal fungi of birch. In *Tree Root Systems and Their Mycorrhizas*. Ed. by D. Atkinson, K.K.S. Bhat, M.P. Coutts, P.A. Mason and D.J. Read. Martinus Nijhoff/Dr. W. Junk Publishers, 269-273.

Franich, R.B., Carson, M.J. and Carson S.D. (1986). Synthesis and accumulation of benzoic acid in *Pinus radiata* needles in response to tissue injury by dothistromin, and correlation with resistance of *P. radiata* families to *Dothistroma pini*. *Physiological and Molecular Plant Pathology* **28**, 267-286.

Friend, J. and Threlfall, D.R. (1976). *Biochemical Aspects of Plant-Parasite Relationships*. Academic Press, London, pp. 354.

Fries, N., Serck-Hanssen, K., Hall Dimberg, L. & Theander, O. (1987). Abietic acid, an activator of basidiospore germination in ectomycorrhizal species of the genus *Suillus* (Boletaceae). *Experimental Mycology* **11**, 360-363.

Garcia Breijo, F.J., Garro, R. and Conejero, V. (1990). C_7(P32) and C_6(P34) PR proteins induced in tomato leaves by citrus exocorticoid viroid infection are chitinases. *Physiological and Molecular Plant Pathology* **36**, 249-260.

Gellini, R. (1973). *Botanica Forestale*. Vol I. Edizioni CLUSF, Firenze, Italy (Italian), pp. 208.

Gessler, C. and Kuc', J. (1982). Induction of resistance to Fusarium wilt in cucumber by root and foliar pathogens. *Phytopathology* **72**, 1439-1441.

Gianinazzi, S., Martin, C. and Vallee, J.C. (1970). Hypersensibilite aux virus, temperature et proteines solubles chez le *Nicotiana* Xanthi nc. Apparition de nouvelles macromolecules lors de la repression de la synthese virale. *C.R. Acad. Sci. Paris* **270D**, 2383-2386.

Gianinazzi, S., Ahl, P., Cornu, A., Scalla, R. and Cassini, R. (1980). First report of host b-protein appearance in response to a fungal infection in tobacco. *Physiological Plant Pathology* **16**, 337-342.

Gibson, F. and Deacon, J.W. (1988). Experimental study of establishment of ectomycorrhizas in different regions of birch root systems. *Transactions of the British mycological Society* **91(2)**, 239-251.

Gindrat, D. & Pilloud, R. (1985). Fungal rot of potato tubers in controlled atmospheres. *Potato research* **28(2)**, 153-160.

Goldberg, R., Devillers, P., Prat, R., Morvan, C. Michon, V. and Herve' du Penchoat, C. (1989). Control of cell wall plasticity. Relationship to pectin properties. In *Plant Cell Wall Polymers - Biogenesis and Biodegradation*, Ed. by N.G. Lewis and M.G. Paice,, pp. 312-323, ACS Symposium Series 399, American Chemical Society, Washington, DC, U.S.A.

Gradi, A. (1980). *Vivaistica Forestale*. Edagricole, Bologna, Italy, pp. 568.

Grasso, S. (1984). Infezioni di *Fusarium oxysporum* e di *Cylindrocarpon destructans* associate a una moria di giovani piante di vite in Sicilia. *Informatore Fitopatologico* **34(1)**, 59-63.

Greenaway, W., English, S. and Whatley, F.R. (1990). Phytochemical affinity of *Populus angustifolia* with Sect. *Aigeiros* poplars established by GC-MS of bud exudate. *Bioch. Syst. Ecol.* **18**, 439-445.

Grenier, J. and Asselin, A. (1990). Some pathogenesis-related proteins are chitosanases with lytic activity against fungal sopres. *Molecular Plant-Microbe Interactions* **3(6)**, 401-407.

Grime, G.W. and Watt, F. (1990). Nuclear microscopy - Elemental Mapping Using High Energy Ion Beam Techniques. *Nuclear Instruments and Methods B50 (1990)*, 197-207.

Grime, G.W., Dawson, M., Marsh, M., McArthur, I.C. and Watt, F. (1990). *The Oxford Sub-Micron Nuclear Microscopy Facility.* Proceedings of the Second International Conference on Nuclear Microprobe Technology and Applications, Melbourne 1990. *Nuclear Instruments and Methods (in press).*

Gurr, E. (1965). *The Rational Use of Dyes in Biology.* Leonard Hill, London, pp. 422.

Hamel, D.R. (1983). *Forest Management Chemicals.* USDA Forest Service, Agriculture Handbook Number 585, pp 645.

Hames, B.D. and Rickwood, D. (eds.) (1981). *Gel Electrophoresis of Proteins - a Practical Approach.* IRL Press Ltd, pp. 290.

Hammerschmidt, R. and Kuc', J. (1982). Lignification as a mechanism for induced systemic resistance in cucumber. *Physiological Plant Pathology* **20**, 61-71.

Hammerschmidt, R., Nuckles, E.M. and Kuc', J. (1982). Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiological Plant Pathology* **20**, 73-82.

Hammond-Kosack, K.E., Atkinson, H.J. and Bowles, D.J. (1989). Systemic accumulation of novel proteins in the apoplast of the leaves of potato plants following root invasion by the cyst-nematode *Globodera rostochiensis*. *Physiological and Molecular Plant Pathology* **35**, 495-506.

Hare, R.C. (1972). Physiology and biochemistry of resistance to pine rusts. In *Biology of Rust Resistance in Forest Trees. Proceedings of a NATO-Iufro Advanced Study Institute.* August 17-24, 1969. U.S. Department of Agriculture and Forest Service. Misc. Publication No. 1221, 465-479.

Harley, J.L. and Harley, E.L. (1987). A checklist of mycorrhiza in the British flora. *New Phytologist* (supplement) **105**, 1-102.

Harley, J.L. and Smith, S.E. (1983). *Mycorrhizal Symbiosis.* Academic Press, London, New York, pp. 483.

Hart, J.H., Wardell, J.F. and Hemingway, R.W. (1975). Formation of oleoresin and lignans in sapwood of White spruce in response to wounding. *Phytopathology* **65**, 412.

Heath, M.C. (1979[a]). Partial characterisation of the electron-opaque deposits formed in the non-host plant, French bean, after cowpea rust infection. *Physiological Plant Pathology* **15**, 141-148.

Heath, M.C. (1979[b]). Effects of heat shock, actinomycin D, cycloheximide and blasticidin S on nonhost interactions with rust fungi. *Physiological Plant Pathology* **15**, 211-218.

Heath, M.C. (1980). Reaction of nonsuscepts to fungal pathogens. *Annual Review of Phytopathology* **18**, 211-236.

Heath, M.C. (1981[a]). A generalized concept of host-parasite specificity. *Phytopathology* **71**, 1121-1123.

Heath, M.C. (1981[b]). Insoluble silicon in necrotic cowpea cells following infection with an incompatible isolate of the cowpea rust fungus. *Physiological Plant Pathology* **19**, 273-276.

Henriks, M.-L., Ekman, R. and von Weissenberg, K. (1979). Bioassay of some resin and fatty acids with *Fomes annosum*. *Acta Acad. Abo. Ser. B.* No. 9, **39**, 1-7.

Hilbert, J.L. and Martin, F. (1988). Regulation of gene expression in ectomycorrhizas. *New Phytologist* **110**, 339-346.

Hinch, J.M. and Clarke, A.E. (1982). Callose formation in *Zea mais* as a response to infection with *Phytophthora cinnamomi*. *Physiological Plant Pathology* **21**, 113-124.

Homans, A.L. and Fuchs, A. (1970). Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. *Journal of Chromatography* **51**, 327-329.

Horn, N.M. (1985). *Effects of air pollution and acid rain on fungal and bacterial diseases of trees*. Dorschkamp Research Institute for Forestry and Landscape Planning, Wageningen, The Netherlands. Uitvoerig verslag Band 20, nr. 1.

Horsfall, J.G. and Cowling, E.B. (1980). *Plant Disease - an Advanced Treatise. Volume V. How Plants Defend Themselves*. Academic Press, New York, pp. 534.

Hutchinson, W.G. (1935). Resistance of *Pinus sylvestris* to a gall-forming peridermium. *Phytopathology* **25**, 819-843.

Ingham, J.L. (1976). 3,5,4′-trihydroxystilbene as a phytoalexin from groundnut (*Arachis hypogea*). *Phytochemistry* **15**, 1791-1793.

Ingram, D.S., Sargent, J.A. and Tommerup, I.C. (1976). Structural aspects of infection by biotrophic fungi. In *Biochemical Aspects of Plant-Parasite Relationships*. Ed. by J. Friend and D.R. Threlfall. Academic Press, London, New York, San Francisco, 43-78.

Ingram, D.S. (1978). Cell death and resistance to biotrophs. *Annals of Applied Biology* **89**, 287-378.

Jacobsen, S., Mikkelsen, J.D. & Hejgaard, J. (1990). Characterization of two antifungal endochitinases from barley grain. *Physiologia Plantarum* **79**, 554-562.

Janes, B.S. (1962). Leaf-clearing technique to assist fungal spore germination counts. *Nature* **193**, 1099-1100.

Janicka, I. (1987). Changes in protein pattern of bean plants systemically infected with tobacco mosaic virus. I. Extraction of pH 7-soluble proteins. *Journal of Plant Disease and Protection* **94(5)**, 520-526.

Jensen, W.A. (1962). *Botanical histochemistry*. W.H. Freeman and Company, San Francisco, London, pp. 408.

Kaewruang, W., Sivasithamparam, K. & Hardy, G.E. (1987). Fungal root rot of Gerbera in Western Australia. *Plant Disease* **71(12)**, 1146.

Katznelson, H., Rouatt, J.W. and Peterson, E.A. (1962). The rhizosphere effect of mycorrhizal and non-mycorrhizal roots of yellow birch ssedlings. *Canadian Journal of Botany* **40**, 257-276.

Kauffmann, S., Legrand, M., Geoffroy, P. and Fritig, B. (1987). Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3-ß-glucanase activity. *EMBO Journal* **6(11)**, 3209- 3212.

Kemp, M.S. and Burden, R.S. (1986). Phytoalexins and strees metabolites in the sapwood of trees. *Phytochemistry* **25(6)**, 1261-1269.

Kephart, S.R. (1990). Starch gel electrophoresis of plant isozymes: a comparative analysis of techniques. *American Journal of Botany* **77(5)**, 693-712.

Kiraly, Z. (1980). Defences triggered by the invader: hypersensitivity. In *Plant Disease - an Advanced Treatise. Volume V. How Plants Defend Themselves*. Ed. by J.G. Horsfall and E.B. Cowling. Academic Press, New York, 201-224.

Kizikelashvili, O.G. (1985). Pathogenicity of fungal pathogens of root rots of *Pinus pithyusa*. *Lesnoe Khozyaistvo* **4**, 65-66.

Knogge, W., Kombrink, E., Schmelzer, E. and Hahlbrock, K. (1987). Occurrence of phytoalexins and other putative defence-related substances in uninfected parsley plants. *Planta* **171**, 279-287.

Kohle, H., Jeblick, W., Poten, F., Blaschek, W. and Kauss, H. (1985). Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+}-dependent process. *Plant Physiology* **77**, 544-551.

Kope, H.H. & Fortin, J.A. (1990). Antifungal activity in culture filtrates of the ectomycorrhizal fungus *Pisolithus tinctorius*. *Canadian Journal of Botany* **68**, 1254-1259.

Krupa, S. and Fries, N. (1971). Studies on ectomycorrhizae of pine. I. Production of volatile organic compounds. *Canadian Journal of Botany* **49**, 1425-1431.

Krupa, S. and Nylund, J.E. (1972). Studies on ectomycorrhizae of pine. III. Growth inhibition of two root pathogenic fungi by volatile organic constituents of ectomycorrhizal root systems of Pinus sylvestris L.. *European Journal of Forest Pathology* **2**, 88-94.

Krupa, S., Andersson, J. and Marx, D.H. (1973). Studies on ectomycorrhizae of pine. IV. Volatile organic compounds in mycorrhizal and non-mycorrhizal root systems of Pinus echinata Mill. *European Journal of Forest Pathology* **3**, 194-200.

Krywolap, G.N., Grand, L.F., and Casida, L.E. Jr. (1964). The natural occurrence of an antibiotic in the mycorrhizal fungus *Cenococcum graniforme*. *Canadian Journal of Microbiology* **10**, 323-328.

Kuc', J. (1972). Phytoalexins. *Annual Review of Phytopathology* **10**, 207-232.

Kuc', J., Shockley, G. and Kearney, K. (1975). Protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. *Physiological Plant Pathology* **7**, 195-199.

Kuc', J. and Richmond, S. (1977). Aspects of the protection of cucumber against Colletotrichum lagenarium by Colletotrichum lagenarium. *Phytopathology* **67**, 533-536.

Kuc', J. (1983). Induced systemic resistance in plants to diseases caused by fungi and bacteria. In *The Dynamics of Host Defence* Ed. by **Bailey** and B.J. Deverall. Academic Press, 191-221.

Kuc', J. (1987). Translocated signals for plant immunization. *Annals of the New York Academy of Sciences* **494**, 221-223.

Kunoh, H., Aist, J.R. and Israel, H.W. (1986). Elemental composition of barley coleoptile papillae in relation to their ability to prevent penetration by *Erysiphe graminis*. *Physiological and Molecular Plant Pathology* **29**, 69-78.

Kunoh, H. & Ishizaki, H. (1975). Silicon levels near penetration sites of fungi on wheat, barley, cucumber and morning glory leaves. *Physiological Plant Pathology* **5**, 283-287.

Langcake, P. and Wickins, S.G.A. (1975). Studies on the action of the dichlorocyclopropanes on the host-parasite relationships in the rice blast disease. *Physiological Plant Pathology* **7**, 113-126.

Langcake, P. and Pryce, R.J. (1976). The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. *Physiological Plant Pathology* **9**, 77-86.

Last, F.T. and Hamley, R.E. (1956). A local-lesion technique for measuring the infectivity of conidia of *Botrytis fabae* Sardina. *Annals of Applied Biology* **44(3)**, 410-418.

Last, F.T., Mason, P.A., Ingleby, K. and Fleming, L.V. (1984). Succession of fruitbodies of sheathing mycorrhizal fungi associated with *Betula pendula*. *For. Ecol. Man.* **9**, 229-234.

Lee, D.H. (1984). Fungi associated with soybean seed, their pathogenicity and seed treatment. *Korean Journal of Mycology* **12(1)**, 27-33.

Legrand, M., Kauffmann, S., Geoffroy, P. and Fritig, B. (1987). Biological function of 'pathogenesis-related' proteins: four tobacco pathogenesis-related proteins are chitinases. *Proceedings of the National Academy of Sciences USA* **84**, 6750-6754.

Lewis, P.R. and Knight, D.P. (1977). Staining methods for sectioned material. In *Practical Methods in Electron Microscopy*, Ed. by A.M. Glauert, North Holland Publishing Company, Amsterdam, New York, Oxford, pp. 311.

Locquin, M. & Langeron, M. (1983). *Handbook of Microscopy*. Butterworth & Co (Publishers), pp. 322.

Loon, L.C. van and Kammen, A. van (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN'. II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology* **40**, 199-211.

Loon, L.C. van, and Antoniw, J.F. (1982). Comparison of the effects of salicylic acid and ethephon with virus-induced hypersensitivity and acquired resistance in tobacco. *Netherlands Journal of Plant Pathology* **88**, 237-256.

Loon, L.C. van (1985). Pathogenesis-related proteins. *Plant Molecular Biology* **4**, 111-116.

Mai, W.F. and Abawi, G.S. (1987). Interactions among root-knot nematodes and Fusarium wilt fungi on host plants. *Annual Review of Phytopathology* **25**, 317-338.

Maizel, J.V. (1971). Polyacrylamide gel electrophoresis of viral proteins. In *Methods in Virology, vol. V*. Ed. by K. Maramorosh and H. Koprowski. Academic Press, New York, London, pp. 179-246.

Malajczuk, N., Molina, R. and Trappe, J.M. (1982). Ectomycorrhiza formation in *Eucalyptus*. I. Pure culture synthesis, host specificity, and mycorrhizal compatibility with *Pinus radiata*. *New Phytologist* **91**, 467-482.

Malajczuk, N., Molina, R. and Trappe, J.M. (1984). Ectomycorrhiza formation in *Eucalyptus*. II. The ultrastructure of compatible and incompatible mycorrhizal fungi and associated roots. *New Phytologist* **96**, 43-53.

Mansfield, J.W. (1983). Antimicrobial compounds. In *Biochemical Plant Pathology*. Ed. by J.A. Callow. John Wiley & Sons, 237-265.

Marx, D.H. (1969[a]). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* **59**, 153-163.

Marx, D.H. (1969[b]). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. II. Production, identification, and biological activity of antibiotics produced by Leucopaxillus cerealis var. piceina. *Phytopathology* **59**, 411-417.

Marx, D.H. and Davey, C.B. (1969[a]). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. III. Resistance of aseptically formed mycorrhizae to infection by *Phytophthora cinnamomi*. *Phytopathology* **59**, 549-558.

Marx, D.H. and Davey, C.B. (1969[b]). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. IV. Resistance of naturally occurring mycorrhizae to infections by Phytophthora cinnamomi. *Phytopathology* **59**, 559-565.

Marx, D.H. (1970). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. V. Resistance of mycorrhizae to infection by vegetative mycelium of *Phytophthora cinnamomi*. *Phytopathology* **60**, 1472-1473.

Marx, D.H. (1972). Ectomycorrhizae as biological deterrents to pathogenic root infections. *Annual Review of Phytopathology* **10**, 429-454.

Marx, D.H. (1973). Mycorrhizae and feeder root diseases. In *Physiological Ecology*. Ed. by G.C. Marks and T.T. Kozlowski. Academic Press, New York, London, pp. 444.

Mason, P.A., Wilson, J., Last, F.T. and Walker, C. (1983). The concept of succession in relation to the spread of sheathing mycorrhizal fungi on inoculated tree seedlings growing in unsterile soils. In *Tree Root Systems and Their Mycorrhizas*. Ed. by D. Atkinson, K.K.S. Bhat, M.P. Coutts, P.A. Mason and D.J. Read. Martinus Nijhoff/Dr. W. Junk Publishers, pp. 552

Matuo, T. & Miyazawa, Y. (1984). Scientific name of *Cylindrocarpon* sp. causing root rot of ginseng. *Annals of the Phytopathological Society of Japan* **50(5)**, 649-652.

Mayama, S. and Shishiyama, J. (1978). Localized accumulation of fluorescent and u.v.-absorbing compounds at penetration sites in barley leaves infected with *Erysiphe* graminis *hordei. Physiological Plant Pathology* **13**, 347-354.

Medeghini Bonatti, P., Stefani, E. and Mazzucchi, U. (1985). Ultrastructural evidence for cellular response in tobacco leaves following infiltration with protein-lipopolysaccharide complexes of *Pseudomonas syringae* pv. *tabaci. Phytopath. Zeitung* **112**, 117-126.

Melville, L.H., Massicotte, H.B. and Peterson, R.L. (1987). Ontogeny of early stages of ectomycorrhizae synthesized between *Dryas integrifolia* and *Hebeloma cylindrosporum. Botanical Gazette* **148(3)**, 332-341.

Metraux, J.P., Streit, L. and Staub, Th. (1988). A pathogenesis-related protein in cucumber is a chitinase. *Physiological and Molecular Plant Pathology* **33**, 1-9.

Miller, T., Cowling, E.B., Powers, H.R. and Blalock, T.E. (1976). Types of resistance and compatibility in slash pine seedlings infected by *Cronartium fusiforme. Phytopathology* **66**, 1229-1235.

Molina, R. (1981). Ectomycorrhizal specificity in the genus *Alnus. Canadian Journal of Botany* **59**, 325-334.

Moser, M. (1978). Rohrlinge und Blatterpilze. 4 Auflage. Band 11b "der kleinen Kryptogamenflora", ed. H. Gams. Stuttgart: Gustav Fisher Verlag.

Mourichon, X. and Salle', G. (1981). Etude ultrastructurale des relations hote-parasite au cours de l'infection des pommes par le *Phytophthora cactorum. Canadian Journal of*

Botany **59**, 248-264.

Mullick, D.B. (1977). The non-specific nature of defence in bark and wood during wounding, insect and pathogen attack. *Recent Advances in Phytochemistry* **11**, 395-441.

Munzenberger, B., Heilemann, J., Strack, D., Kottke, I. and Oberwinkler, F. (1990). Phenolics of mycorrhizas and non-mycorrhizal roots of Norway spruce. *Planta* **182**, 142-148.

Nasser, W., de Tapia, M. and Burkard, G. (1990). Maize pathogenesis-related proteins: characterization and cellular distribution of 1,3-ß-glucanases and chitinases induced by brome mosaic virus infection or mercuric chloride treatment. *Physiological and Molecular Plant Pathology* **36**, 1-14.

Nylund, J. E., Kasimir, A., and Strandberg Arveby, A. (1982). Cell wall penetration and papilla formation in senescent cortical cells during ectomycorrhiza synthesis *in vitro*. *Physiological Plant Pathology* **21**, 71-73.

Nylund, J.E. and Unestam, T. (1982). Structure and physiology of ectomycorrhizae. I. The process of mycorrhiza formation in Norway spruce *in vitro*. *New Phytologist* **91**, 63-79.

O'Brien, T.P. and McCully, M.E. (1981). *The Study of Plant Structure - Principles and Selected Methods*. Termarcarphi Pty. Ltd., Melbourne Australia.

Ochs, D.C., McConkey, E.H. and Sammons, D.W. (1981). Silver stains for proteins in polyacrylamide gels. A comparison of 6 methods. *Electrophoresis* **2(5-6)**, 304-307.

Parent, J. and Asselin, A. (1984). Detection of pathogenesis-related proteins (PR or b) and of other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. *Canadian Journal of Botany* **62**, 564-569.

Parker, R.E. (1984). *Introduzione alla Statistica per la Biologia*. Edagricole, Bologna, pp. 159.

Pawsey, R.G. (1964). *Grey mould in forest nurseries (Botrytis cinerea)*. Forestry Commission leaflet 50.

Peace, T.R. (1962). *Pathology of trees and shrubs*. Clarendon Press, Oxford, pp.753.

Pearce, R.B. (1986). Chlorantine Fast Green BLL as a stain for callose in oak phloem. *Stain Technology* **61(1)**, 47-50.

Pearce, R.B. and Holloway, P.J. (1984). Suberin in the sapwood of oak (*Quercus robur* L.): its composition from a compartimentalized barrier and its occurrence in tyloses in undecayed wood. *Physiological Plant Pathology* **24**, 71-81.

Pearce, R.B. and Rutherford, J. (1981). A wound-associated suberized barrier to the spread of decay in the sapwood of oak. *Physiological Plant Pathology* **19**, 359-369.

Pegg, G.F. and Ayres, P.G., eds. (1987). *Fungal infection of plants*. Cambridge University Press, pp. 428.

Pegg, G.F. and Young, D.H. (1981). Changes in glycosidase activity and their relationship to fungal colonization during infection of tomato by *Verticillium albo-atrum*. *Physiological Plant Pathology* **19**, 371-382.

Perrin, R. and Garbaye, J. (1983). Influence of ectomycorrhizae on infectivity of Pythium-infested soils and substrates. In *Tree Root Systems and Their Mycorrhizas*. Ed. by D. Atkinson, K.K.S. Bhat, M.P. Coutts, P.A. Mason and D.J. Read. Martinus Nijhoff/Dr. W. Junk Publishers, 354-351.

Perrin, R. and Noveau, M. (1986). L'association mycorhizienne de *Pinus sylvestris* avec *Hebeloma crustuliniforme* et *Laccaria laccata* et les maladies causees par *Pythium* spp. In *Proceedings of the 1st European Symposium on Mycorrhizae*, ed. by V. Gianinazzi-Pearson and S. Gianinazzi. INRA, Paris, 793-798.

Phillips, D.H. and Burdekin, D.A. (1982). *Diseases of forest and ornamental trees*. The Macmillan Press Ltd., London, pp. 435.

Piche', Y., Fortin, J.A. and LaFontaine, J.G. (1981). Cytoplasmic phenols and polysaccharides in ectomycorrhizal and non-mycorrhizal short roots of pine. *New Phytologist* **88**, 695-703.

Piche', Y., Peterson, R.L., Howarth, M.J. and Fortin, J.A. (1983). A structural study of the interaction between the ectomycorrhizal fungus *Pisolithus tinctorius* and *Pinus strobus* roots. *Canadian Journal of Botany* **61**, 1185-1193.

Pierpoint, W.S., Jackson, P.J. and Evans, R.M. (1990). The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*). *Physiological and Molecular Plant Pathology* **36**, 325-338.

Ragazzi, A., Dellavalle Fedi, I. & Mesturino, L. (1989). The oak decline: a new problem in Italy. *European Journal of Forest Pathology* **19(2)**, 105-110.

Ralton, J.E., Smart, M.G. and Clarke, A.E. (1987). Recognition and infection processes in plant pathogen interactions. In *Plant-Microbe Interactions. Molecular and Genetic Perspectives*. Ed. by T. Kosuge and E.W. Nester. MacMillan, pp. 217-252.

Rayner, A.D.M. and Boddy, L. (1988). *Fungal decomposition of wood*. John Wiley & Sons, Chichester, New York, pp. 587.

Redolfi, P. (1983). Occurrence of pathogenesis-related (b) and similar proteins in different plant species. *Netherlands Journal of Plant Pathology* **89**, 245-254.

Read, D.J. (1986). Non-nutritional effects of mycorrhizal infections. In *Proceedings of the 1st European Symposium on Mycorrhizae*, ed. by V. Gianinazzi-Pearson and S. Gianinazzi. INRA, Paris, 169-176.

Ride, J.P. (1975). Lignification in wounded wheat leaves in response to fungi and its possible role in resistance. *Physiological Plant Pathology* **5**, 125-134.

Ride, J.P. (1983). Cell walls and other structural barriers in defence. In *Biochemical Plant Pathology*. Ed. by J.A. Callow. John Wiley & Sons, 215-236.

Ride, J.P. and Pearce, R.B. (1979). Lignification and papilla formation at sites of attempted penetration of wheat leaves by non-pathogenic fungi. *Physiological Plant Pathology* **15**, 79-92.

Rinaudo, M. (1989). Effect of chemical structure of pectins on their interaction with calcium. In *Plant Cell Wall Polymers - Biogenesis and Biodegradation*, Ed. by N.G. Lewis and M.G. Paice,, pp. 312-323, ACS Symposium Series 399, American Chemical Society, Washington, DC, U.S.A.

Ronald, P. and Soderhall, K. (1985). Phenylalanine ammonia lyase and peroxidase activity in mycorrhizal and nonmycorrhizal short roots of Scots pine, *Pinus sylvestris*. *New Phytologist* **101**, 487-494.

Samuels, G.J. and Brayford, D. (1990). Variation in *Nectria radicicola* and its anamorph, *Cylindrocarpon destructans*. *Mycological Research* **94**(4), 433-442.

Sandermann, H. Jr., Schmitt, R., Heller, W., Rosemann, D. and Langebartels, C. (1989). Ozone-induced early biochemical reaction in conifers. In *Acid Deposition: Sources, Effects and Controls*. Ed. by J.W.S. Longhurst, British Library, London, 1989.

Sargent, C. & Gay, J.L. (1977). Barley epidermal apoplast structure and modification by powdery mildew contact. *Physiological Plant Pathology* **11**, 195-205.

Sauter, M. and Hager, A. (1989). The mycorrhizal fungus *Amanita muscaria* induces chitinase activity in roots and in suspension-cultured cells of its host *Picea abies*. *Planta* **179**, 61-66.

Schlosser, E.W. (1980). Preformed internal chemical defences. In *Plant Disease - an Advanced Treatise. Volume V. How Plants Defend Themselves*. Ed. by J.G. Horsfall and E.B. Cowling. Academic Press, New York, 161-177.

Schlumbaum, A., Mauch, F., Vogeli, U. and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**, 365-367.

Schonbeck, F. and Dehne, H.-W. (1979). The influence of endotrophic mycorrhiza on plant disease. 4. Fungal parasites on aerial plant parts, Olpidium brassicae, TMV. *Journal of Plant Diseases and Protection* **86(2)**, 103-112.

Shain, L. (1967). Resistance of sapwood in stems of loblolly pine to infection by *Fomes annosus*. *Phytopathology* **57**, 1034-45.

Shain, L. (1971). The response of sapwood of Norway spruce to infection by *Fomes annosus*. *Phytopathology* **61**, 301-307.

Shain, L. (1979). Dynamic responses of differentiated sapwood to injury and infection. *Phytopathology* **69**, 1143-7.

Sherwood, R.T. and Vance, C.P. (1976). Histochemistry of papillae formed in reed canarygrass leaves in response to noninfecting pathogenic fungi. *Phytopathology* **66**, 503-510.

Sherwood, R.T. and Vance, C.P. (1980). Resistance to fungal penetration in Gramineae. *Phytopathology* **70**, 273-279.

Shigo, A.L. and Marx, H.G. (1977). *Compartmentalization of decay in trees*. U.S.D.A. Forest Service, Agricultural Information Bulletin No. 405.

Sinclair, W.A., Sylvia, D.M. and Larsen, A.O. (1982). Disease suppression and growth promotion in Douglas-fir seedlings by the ectomycorrhizal fungus *Laccaria laccata*. *Forest Science* **28**, 191-201.

Skipp, R.A. and Bailey, J.A. (1977). The fungitoxicity of isoflavanoid phytoalexins measured using different types of bioassay. *Physiological Plant Pathology* **11**, 101-112.

Smart, M.G., Aist, J.R. and Israel, H.W. (1986). Structure and function of wall appositions. 1. General histochemistry of papillae in barley *Hordeum vulgare* coleoptyles attacked by *Erysiphe graminis* f.sp. *hordei*. *Canadian Journal of Botany* **64**, 793-801.

Smith, I.M., Dunez, J., Lelliott, R.A., Phillips, D.H. and Archer, S.A. (1988). *European Handbook of Plant Diseases*. Blackwell Scientific Publications, Oxford, London, Edinburgh, pp. 583.

Spanu, P. and Bonfante-Fasolo, P. (1988). Cell-wall-bound peroxidase activity in roots of mycorrhizal *Allium porrum*. *New Phytologist* **109**, 119-124.

Spanu, P., Boller, T., Ludwig, A., Wiemken, A., Faccio, A. and Bonfante-Fasolo (1989). Chitinase in roots of mycorrhizal *Allium porrum*: regulation and localization. *Planta* **177**, 447-445.

Stahl, E. (1969). *Thin Layer Chromatography - A Laboratory Handbook*. Springer-Verlag, Berlin, pp. 1041.

Stenstrom, E. and Ek, M. (1990). Field growth of *Pinus sylvestris* following nursery inoculation with mycorrhizal fungi. *Canadian Journal of Forest Research* **20**, 914-918.

Stumm, D. and Gessler, C. (1986). Role of papillae in the induced systemic resistance of cucumbers against *Colletotrichum lagenarium*. *Physiological and Molecular Plant Pathology* **29**, 405-410.

Sylvia, D.M. and Sinclair, W.A. (1983[a]). Suppressive influence of *Laccaria laccata* on *Fusarium oxysporum* and on Douglas-fir seedlings. *Phytopathology* **73**, 384-389.

Sylvia, D.M. and Sinclair, W.A. (1983[b]). Phenolic compounds and resistance to fungal pathogens induced in primary roots of Douglas-fir seedlings by the ectomycorrhizal fungus *Laccaria laccata*. *Phytopathology* **73**, 390-397.

Takaishi, Y., Murakami, Y., Ohashi, T., Nakano, K., Murakami, K. and Tomimatsu, T. (1987). Three triterpenes from *Astraeus hygrometricus*. *Phytochemistry* **26(8)**, 2341-2344.

Takasugi, M., Nagao, T., Masamune, T., Shirata, A. and Takahashi, K. (1978). Structure of moracin A and B, new phytoalexins from diseased mulberry. *Tetrahedron Letters*, 797-798.

Talboys, P.W. (1958). Some mechanisms contributing to Verticillium resistance in hop roots. *Transactions of the British Mycological Society* **41**, 227-241.

Tippett, J. and Malaiczuk, N. (1979). Interaction of *Phytophthora cinnamomi* and a resistance host, *Acacia pulchella*. *Phytopathology* **69**, 764-772.

Tippett, J.T. and Shigo, A.L. (1980). Barrier zone anatomy in red pine roots invaded by *Heterobasidion annosum*. *Canadian Journal of Forest Research* **10**, 224-232.

Toscano-Underwood, C. and Pearce, R.B. (1991). Astringin and Isorhapontin distribution in Sitka spruce trees. *Phytochemistry* (in press).

Toyota, M. & Hostettmann, K. (1990). Antifungal diterpenic esters from the mushroom *Boletinus cavipes*. *Phytochemistry* **29(5)**, 1485-1489.

Trudel, J. and Asselin, A. (1989). Detection of chitinase activity after polyacrylamide gel electrophoresis. *Analytical Biochemistry* **178**, 362-366.

Vallejos, C.D. (1983). Enzyme activity staining. In *Isozymes in Plant Genetics and Breeding, Part A*. Ed. by S.D. Tanksley and T.J. Orton. Elsevier Science Publishers B.V., Amsterdam.

Vance, C.P. and Sherwood, R.T. (1976). Cycloheximide treatment implicate papilla formation in resistance of reed canarygrass to fungi. *Phytopathology* **66**, 498-502.

Vance, C.P., Kirk, T.K. and Sherwood, R.T. (1980). Lignification as a mechanism of disease resistance. *Annual Review of Phytopathology* **18**, 259-288.

Ward, H.M. (1902). On the relations between host and parasite in the bromes and their brown rust, *Puccinia dispersa* (Erikss.). *Annals of Botany* **16**, 233-315.

Watt, F. and Grime, G.W. (eds.) (1987). *Principles and Applications of High Energy Ion Microbeams*. Adam Hilger, Bristol.

Wetherbee, R., Hinch, J.M., Bonig, I. and Clarke, A.E. (1985). Responses of *Zea mais* roots to infection with *Phytophthora cinnamomi*. II. The cortex and stele. *Protoplasma* **126**, 188-197.

Wilcox, H.E. (1983). Fungal parasitism of woody plant roots from mycorrhizal relationships to plant disease. *Annual Review of Phytopathology* **21**, 221-242.

Wildon, D.C., Doherty, H.M., Eagles, G., Bowles, D.J. and Thain, J.F. (1989). Systemic responses arising from localized heat stimuli in tomato plants. *Annals of Botany* **64**, 691-695.

Wong, W.C. and Preece, T.F. (1978). *Erwinia salicis* in cricket bat willows: phenolic constituents in healthy and diseased wood. *Physiological Plant Pathology* **12**, 349-357.

Woodward, S. and Pearce, R.B. (1985). Methods for visualizing hyaline fungi for the detection of antifungal compounds on thin-layer chromatograms. *Plant Pathology* **34**, 477-480.

Woodward, S. and Pearce, R.B. (1988[a]). The role of stilbenes in resistance Of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) to entry of fungal pathogens. *Physiological and Molecular Plant Pathology* **33**, 127-149

Woodward, S. and Pearce, R.B. (1988[b]). Wound associated responses in Sitka spruce root bark challenged with *Phaeolus schweinitzii*. *Physiological and Molecular Plant Pathology* **33**, 151-162.

Ye, X.S., Pan, S.Q. and Kuc', J. (1989). Pathogenesis-related proteins and systemic resistance to blue mould and tobacco mosaic virus induced by tobacco mosaic virus, *Peronospora tabacina* and aspirin. *Physiological and Molecular Plant Pathology* **35**, 161-175.

Ye, X.S., Pan, S.Q. and Kuc', J. (1990). Association of pathogenesis-related proteins and activities of peroxidase, ß-1,3-glucanase and chitinase with systemic induced resistance to blue mold of tobacco but not to systemic tobacco mosaic virus. *Physiological and Molecular Plant Pathology* **36**, 523-531.

Zak, B. (1964). Role of mycorrhizae in root disease. *Annual Review of Phytopathology* **2**, 377-392.

Zentmyer, G.A. (1980). *Phytophthora cinnamomi* and the diseases it causes. *APS Monograph* **No 10**. American Phytopathological Society, St. Paul, pp. 96.

Zinkel, D.F., Zank, L.C. and Wesolowski, M.F. (1971). *Diterpene Resin Acids - A Compilation of Infrared, Mass, Nuclear Magnetic Resonance, Ultraviolet Spectra and Gas Chromatographic Retention Data*. USDA Forest Service, Forest Products Laboratory, Madison, WI.