SPORE GERMINATION AND THE PRE-INFECTION PHASE IN ECTOMYCORGAL FUNGI

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by

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SUMMARY

Spore germination of seven species of ectomycorrhizal fungi was studied under different conditions.

Spores of most species tested did not germinate on laboratory media without stimulators. Various stimulators were tested and the greatest activation was that by Pseudomonas stutzeri which was obtained from fruitbodies of Hebeloma crustuliniforme and stimulated about 21% of spores of that fungus.

Effects of plant roots on spore germination were studied in the laboratory and in soil. In mineral salts medium, spores of Paxillus involutus were stimulated by five of seven tree species and one of four non-tree species tested. Spores of Laccaria laccata and H. crustuliniforme were stimulated by birch and pine only, while those of Lactarius turpis and Amanita fulva were only slightly stimulated by birch roots. Birch was more stimulatory and the highest germination (30%) was that of H. crustuliniforme at 0-1 mm from the root edge.

In soil, spores of P. involutus only, of the seven species tested, were stimulated to germinate near roots of birch and spruce in steamed and untreated soils. Birch stimulated up to 96% of spores to germinate in untreated birch wood soil. Spruce had much less effect on germination in either steamed or untreated soil. The majority of germ tubes grew towards the roots.

Exudate from roots of seedlings of birch previously grown in soil was active in stimulating germination of H. crustuliniforme, but not of the other six species tested. Activity of the root exudate was associated with the ninhydrin-positive fraction.

The role of the basidiospores of the test fungi in initiating mycorrhizas in soil was also studied. Spores of P. involutus and L. laccata established mycorrhizas with birch, but not spruce or pine roots. Spores of the other five species tested did not establish mycorrhizas with any of the plant tested.
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Chapter 1: LITERATURE SURVEY

Mycorrhiza (fungus root): is a symbiotic relationship between the susceptible host root and the mycelium of the fungus.

The mycorrhizal phenomenon was first described by Unger in 1840. He illustrated the mantle but did not recognize its fungal nature. At the same time Hartig (1840) described the mantle and the subsequently termed "Hartig net" without recognizing their fungal nature. The history of mycorrhizas begins with Frank (1885) when the term "Mycorrhiza" (with one r) was proposed for the first time. He emphasised that the mycorrhizal rootlets are covered by a mantle of fungal tissue from which hyphae pass inwards between the cells and into the soil. The term mycorrhiza has since been adopted for many other mutualistic associations between soil fungi and below-ground plant organs, some of which, notably the protocorms of orchids, are not even roots.

1.1 Classification of mycorrhizas

Mycorrhizal associations are usually divided into four main groups: 1). ectomycorrhizas or sheathing mycorrhizas; 2). vesicular-arbuscular (VA) mycorrhizas; 3). mycorrhizas of the Ericales, and 4). mycorrhizas of the Orchidaceae (Lewis, 1973; 1975; 1976; and Read, 1982).

The last three groups were called endomycorrhizas by Peyronel et al. (1969), and they used the term ectendomycorrhizas to describe a group of mycorrhizas which share features characteristic of both sheathing mycorrhizas and endomycorrhizas (Hofsten 1969; Laiho 1965; Wilcox 1971).

1.1.1 Mycorrhizas of Orchidaceae

This kind of mycorrhiza is associated with Basidiomycetes (e.g. genus Rhizoctonia) of various affinity which are able to break down cellulose and sometimes lignin. Orchids comprise a range of types, from species containing abundant chlorophyll at maturity to
completely achlorophyllous and presumably saprophytic forms. The tissues of the host plant are penetrated by septate hyphae of the causal fungus that often bear clamp connections. The hyphae form intracellular loops and coils known as pelotons. A characteristic feature of the orchid mycorrhiza is degradation of the intracellular fungal structures by the host.

1.1.2 Mycorrhizas of Ericales

Mycorrhizas of Ericales are of two types: (1) ericoid type, which is formed by a penetration of the root cortex by septate hyphae (usually of Ascomycetes eg. *Pezizella ericae*) which form intracellular coils (Harley, 1959 and 1969); (2) arbutoid type which resemble the ectendo type, in which there is an organized fungal sheath as well as inter- and intracellular penetration of the cortex (Lutz and Sjolund, 1973).

1.1.3 Vesicular-Arbuscular Mycorrhizas (VAM)

In this kind of mycorrhiza the primary cortex of the host root is invaded both inter- and intracellularly by aseptate hyphae of fungi belonging to the family Endogonaceae (irregular septa may occur occasionally). The fungi form branched haustoria (arbuscules) in the cells and swollen vesicles inside and outside the host tissues. Many form spores on mycelia or in sporocarps of complex structure. VAM occur in a wide range of hosts which are economically important, eg. members of the Rosaceae, Gramineae, and Leguminoseae. These include all grain crops and the majority of temperate fruit trees and shrubs. Crops such as citrus, coffee, tea, rubber, and sugar cane also have VA mycorrhizas.

1.1.4 Ectomycorrhizas

The majority of fungi which form ectomycorrhizas with forest trees are Basidiomycetes. Ascomycetes contain few genera only which form ectomycorrhizas, and most of them have hypogeous sporophores,
eg. the truffle-forming fungi *Tuber*, *Terfezia* and *Tirmania*. The imperfect fungus *Cenococcum graniforme* and E-strain fungi belong to the Ascomycetes and also form ectomycorrhizas.

In the ectomycorrhizas the roots are surrounded by a more or less well-developed fungal sheath or mantle. From this sheath hyphae or hyphal cords may extend into the soil and into the base of fruitbodies of the fungi. Hyphae also penetrate intercellularly into the host cortex forming a complex network of hyphae, called the Hartig net, which may completely replace the middle lamella between the cortical cells. Usually there is little or no hyphal penetration into the cells of the host of young mycorrhizas, but in the senescent parts of a mycorrhizal axis, the cortex becomes colonized by hyphae within the cells (Atkinson, 1975). The hyphae never penetrate the endodermal tissues and the stele.

Generally, ectomycorrhizas are formed only on roots with primary tissues. Secondarily thickened roots remain uninfected. Mycorrhizal tree roots are often divided into long roots that grow continuously, and short roots that branch repeatedly and grow little. The majority of ectomycorrhizas are formed on the short roots. Sometimes, a loose sheath and no Hartig net may be formed on the long roots (Harley, 1969). Whether formation of short roots precedes, or is a result of infection is still controversial.

Ectomycorrhizas occur chiefly in temperate tree species. All members of the gymnosperm family Pinaceae eg. *Pinus*, *Picea*, *Pseudotsuga*, *Larix*, *Tsuga*, etc., as well as certain angiosperms, eg. *Juglandaceae* (*Juglans*, *Carya*); *Fagaceae* (*Quercus*, *Nothofagus*); *Betulaceae* (*Betula*); and *Myrtaceae* (*Eucalyptus*, whose species are important components of forest and bush in temperate and sub-tropical Australasia) are ectomycorrhizal. Also, some tree species of the family Dipterocarpaceae (belonging to the tropical regions) have been proved to be ectomycorrhizal.
1.2 Identification of ectomycorrhizal fungi:

Fungi associated with ectomycorrhizas in nature may be characterized by morphological or anatomical features of the mycorrhizal structure to which they give rise (Dominik, 1969 and Zak, 1971a), such as sheath colour, the shape and size of the mycorrhizas, the presence of surrounding mycelium and rhizomorphs, sheath texture and thickness, chemical colour reactions and fluorescence, odour and taste, clamp connections, if present, and hyphal form.

As the above characteristics in some fungi may change with age and environmental conditions; the following methods are also useful for identification of some ectomycorrhizal fungi.

1). Tracing the cords and hyphae from a known sporocarp to the underlying mycorrhizas. This method is applicable only to those mycorrhizal fungi which form sporocarps (Chilvers, 1968; Schramm, 1966; Woodroof, 1933; Zak, 1969; Zak and Marx, 1964).

2). Comparison of the fungus isolated from the mycorrhiza with known cultures derived from sporocarps (Lamb and Richards, 1970). This method is of course limited to those fungi that will grow on laboratory media.

3). Mating studies between compatible monoporous mycelia or monokaryons are also useful for identifying mycorrhizal fungi; hyphal fusions, or anastomoses between two compatible monokaryons will lead to dikaryotic hyphae.

Fries and Mueller (1984) examined the utility of employing mating studies to determine the degree of intra- and interspecific genetic isolation in Laccaria. In their study, no clamps were found in pairings between monokaryons from different species or groups.

Mueller and Fries (1985) were able to identify 74 isolates (representing ten Laccaria species) to species or species group by cultural and mating studies. In their study, monokaryotic mycelium was formed by dedikaryotization of dikaryotic mycelium (Kemp, 1974;
and Leal-Lara and Eger-Hummel, 1982).

4). Pure culture synthesis of mycorrhiza;

With the culturable mycorrhizal fungi, it is possible to re-form the mycorrhizas on seedlings in pure culture synthesis (Hacskaylo, 1953; Marx and Zak, 1965; Melin, 1921; Pachlewski and Pachlewska, 1968), and compare the resulting mycorrhizas with those found in nature. Under artificial conditions the synthesized mycorrhizas may differ in their morphology from those occurring under natural conditions (Zak, 1971b); this could be due to the effect of the composition of the growth medium on the synthesized mycorrhizas (Thomas and Jackson, 1979). Production of sporocarps by synthesized mycorrhizas will permit a positive identification of the fungus.

5). Another technique successfully used for identification of ectomycorrhizal species is immunofluorescence (Schmidt et al., 1974). By this technique protein antisera were prepared against two mycorrhizal fungi (Thelephora terrestris and Pisolithus tinctorius) by injection of the antigens (washed, homogenized mycelia of the test fungi in saline) in rabbits. One week later the blood was collected by cardiac puncture, and the antiserum fractionated and labelled with fluorescein isothiocyanate (Bohlool and Schmidt, 1970; Schmidt et al., 1968).

Schmidt et al. (1974) found that the fluorescent antibodies of each mycorrhizal fungus when tested on mycelial smears reacted strongly with their corresponding fungus antigen.

6). Recently an unambiguous method was used to identify ectomycorrhizal fungi, using dot-blot DNA hybridization, in which the DNA was isolated from repeatedly washed mycelium and/or fruitbodies. By this method Straatsma et al. (1985) proved that the different Cantharellus cibarius cultures, they obtained by different methods, were genetically identical to fruitbodies of C. cibarius found in nature.
1.3 Process of infection:

Ectomycorrhizal infections are assumed to be initiated from propagules in soil such as basidiospores; chlamydospores; sclerotia; or from vegetative mycelia from nearby ectomycorrhizal roots.

The means by which the hyphae of mycorrhizal fungi penetrate the host tissue is unclear. However, there is no visible degradation or alteration of cells in ectomycorrhizal root formation (Foster and Marks, 1966 and 1967; Marks and Foster 1973).

The process of intercellular penetration in ectomycorrhizal formation is predominantly mechanical; the walls between the cortical cells of the host root are separated or loosened at the middle lamella due to the effect of the fungal hyphae. The hyphae then wedge themselves into the fissures so formed and advance further by mechanical pressure (Foster and Marks, 1967). Whether enzyme production is also involved is not known; but Ramstedt and Soderhall (1983) indicated that protease and phenoloxidase produced by the fungus may have a role in the penetration process, whereas pectinase activity has never been shown to be significant in mycorrhizal fungi.

Harley and Smith (1983) hypothesized the role of the fungal enzymes in the penetration process as follows:

1). The fungal enzymes at the hyphal apex or on the hyphal walls might inhibit or complex with host polymerizing enzymes (which are involved in wall polymer building, Vanderplank, 1978) resulting in deactivation of polymerizing enzymes and alteration of wall formation, thus soluble carbohydrates will be available to the fungus.

2). Alternatively, Harley and Smith (1983) suggested that all ectomycorrhizal fungi possess enzymes on their walls capable of hydrolysing the wall material of their hosts.

Evidence to confirm these hypotheses has not yet been obtained.

After the penetration process is achieved; the hyphae spread longitudinally between the cells of the long root cortex and branch
to infect newly emerging short roots. The infection can also spread externally from the sheath by hyphal cords from one short root to another (Robertson, 1954).

1.3.1 Factors affecting infection:

The process of infection is influenced by soil factors; the properties of the host root and the interactions between these factors and the environment:

1). Soil nutrient:-

Many studies in the literature reported that the intensity of infection is greater under conditions of low or unbalanced nutrient supply (Hatch, 1936; Marx et al., 1977; Schmidt, 1947; Stahl, 1900); starvation levels of essential nutrients decrease the intensity (number of roots infected) of mycorrhizas (Hatch, 1936 and 1937). Bjorkman (1942) reported that very severe deficiencies of P or N have an adverse effect on mycorrhizal formation. However, when these nutrients are present at low levels the mycorrhizal intensity is increased.

2). Soil pH:-

Ectomycorrhizas are present over a wide pH range, but more abundant in raw humus soils (pH 3.5 - 4.0) than in mull soils (pH 5.0 - 5.5) (Mosse et al., 1982). The effect of soil pH on ectomycorrhiza formation is difficult to evaluate; changes in soil pH lead to changes in the chemical properties of the soil such as the release of soluble Al, Mn, and Fe under low soil pH (Nye and Ramzan, 1979). Thus the effects on mycorrhiza formation may be due to these metals rather than pH.

3). Soil organic matter:-

Although no direct chemical effects of soil organic matter have been reported; the organic matter affects mycorrhiza formation through its effect on soil structure, water holding capacity, nutrient mineralization, etc. or due to the presence of substances which stimulate the growth of both fungus and host (Melin, 1953).
4). Soil moisture and aeration:-

Soil with excessive water may affect mycorrhiza formation because it reduces the availability of O₂ (Miller and Laursen, 1978), since this gas diffuses slowly through water, but often soil water contains sufficient dissolved O₂ to prevent anaerobiosis (Scott-Russell, 1977).

The lack of O₂ affects mycorrhizas, which are strongly aerobic (Harley, 1969), only at the pre-infection phase; since O₂ is transported down air-filled spaces within the plant from shoot to root (Greenwood, 1967). Also Read and Armstrong (1972) reported that ectomycorrhizal infection may be initially stimulated by O₂ diffusing from host roots.

Soil with low water affects root growth and accelerates suberization, which sometimes leads to changes in the fungal partner (Marks and Foster, 1967; Worley and Hacskaylo, 1959), e.g. the fungus Cenococcum graniforme replaced unidentified white fungi on Pinus virginiana under dry conditions (Meyer, 1964).

5). Temperature:-

In general ectomycorrhizal fungi grow best around 20°C, but mycorrhizal development has been shown to be strongly temperature dependent (Hacskaylo and Vozzo, 1965; Marx et al., 1970a; Melin, 1925; Mikola, 1948; Moser, 1956). Temperature may affect mycorrhiza formation through its effect on root growth and physiology (Barney, 1951; Lyr and Hoffmann, 1967).

6). Light:-

Mycorrhiza formation is strongly affected by light intensity; at high light intensity large numbers of short roots are colonized with mycorrhizal fungi (Bjorkman, 1942; Hatch, 1937).

The light received by the shoot has indirect effects on root development; reduction in light intensity reduces shoot growth (Anonymous, 1953; Brown, 1955; Daft and El-Ghiahmi, 1978; Elison, 1968; Hayman, 1974; Richardson, 1956) and the number of new roots produced (Hoffmann, 1967). These studies provide strong evidence that root growth is stimulated by substances photosynthesized in
the shoot. This interaction between light, shoot, and root may affect mycorrhizal colonization.

7). Growth hormones:

Growth hormones such as auxins, cytokinins, gibberellins produced by the mycorrhizal fungi (Fortin, 1967 and 1970; Slankis, 1973), may exert a regulatory influence on mycorrhiza formation. There is not enough information about the mechanism through which the fungal hormones influence mycorrhizal formation; but they may have a role in mobilization of carbohydrates in plants (Meyer, 1966) or as found from a study in general plant physiology, fungal hormones (cytokinins) may cause accumulation of amino acids, phosphates, and various other substances in the localized areas to which these hormones are applied (Gunning and Barkley, 1963; Mothes, 1960). Slankis (1971) has described experiments on the relationship between auxin production by the fungus, soil nitrogen level and light level, and their combined effects on the plant root. He found that high nitrogen levels or low light levels inhibited mycorrhizal formation, but from additional studies with auxin he speculated that high nitrogen inhibits the synthesis of auxin by the fungus and at low light intensity some factor interferes with fungal auxin. He interpreted this factor as being either the prevention of a required interaction between fungal auxin and some root metabolite, or possibly the formation of auxin inhibitors in the root.
1.4 Host-fungus specificity

In mycorrhizal associations the degree of specificity between fungi and their hosts is widely variable. One species of mycorrhizal fungus may form mycorrhizal relationships with more than one species of host, e.g. the fungus *Cenococcum geophilum* (graniforme) forms ectomycorrhizas with the species of more than 20 genera of vascular plant (Harley and Smith, 1983), and the fungus *Pisolithus tinctorius* infects many hosts (Marx, 1977). A few fungi are reported to be very restricted in their hosts, for example *Suillus grevillei* (*Boletus elegans*) which forms mycorrhizas with species of *Larix* only (Meyer, 1973). Also any one species of host plant may form mycorrhizas with many species of fungi, e.g. Douglas fir (*Pseudotsuga menziesii*) can probably form mycorrhizas with 2000 species (Trappe, 1977), while a host accepting only one species is unknown. Meyer (1973) reported that under natural conditions, species of *Abies*, *Larix*, *Picea*, *Pinus*, *Fagus* and *Quercus* are obligate ectomycorrhizal trees. Whereas others such as *Cupressus*, *Salix*, *Betula*, *Alnus* and *Eucalyptus* are more facultative. On the other hand, most ectomycorrhizal fungi are ecologically obligate-parasites (Garrett, 1960).

From field observations, Molina and Trappe (1982a) recognized what they called "sporocarp-specific host associations". In these a fungus is only known to fruit either in association with a single genus, a single species, or with a wider taxon, e.g. *Pinaceae*. They recognized that the mycelium of a fungal species may exist ecologically in other associations than those in which the fruitbodies are found. This phenomenon has been described by Harley and Smith (1983) as "ecological specificity".
1.5 Importance of the mycorrhizal association between plants and fungi

The mycorrhizal association is the most widespread symbiosis and very common under natural soil conditions. There are usually outgrowths of hyphae from the surface of mycorrhizas into the soil, sometimes extending several centimetres from the root surface which, in some ectomycorrhizas, form aggregates, hyphal cords or highly specialized rhizomorphs (Skinner and Bowen, 1974a,b; Trappe and Fogel, 1977). This may help in binding soil particles and thus increase soil stabilization and perhaps erosion control (Smith, 1980). The ability of fungal mycelia to bind sand and soil particles had been observed by Koske et al. (1975) and confirmed by Tisdall and Oades (1979). They reported that hyphae have an important function in increasing stable soil aggregates greater than 2.0mm.

The mycorrhizal relationship is usually beneficial to both partners (fungus and plant). In most ectomycorrhizas, the fungi appear to be dependent on their hosts for carbon and energy sources (Harley, 1978; Last et al., 1979; Melin, 1921; Rommell, 1938). Melin and Nilsson (1957) detected 14C-labelled photosynthate in the fungal sheath of Pinus sylvestris mycorrhizas. Also, Lewis and Harley (1965) showed that 14C-labelled sucrose fed to cut stumps of Fagus sylvatica L. was translocated towards the tips of mycorrhizas and converted into trehalose, mannitol and glycogen which cannot be reabsorbed by the host root. From this study and others (Hacskaylo, 1973; Reid and Woods, 1969) it can be deduced that the mycorrhiza acts as a sink in which the carbohydrates are accumulated.

In general, the most important function of the mycorrhizal association is its ability to improve the growth and physiological characteristics of the host plant:

Mycorrhizas improve the host growth by providing an efficient nutrient and water absorbing root system (Hatch, 1937), especially in soils of low or imbalanced nutrient status (Harr et al., 1977; Voigt, 1971).

Mycorrhizas are more efficient than non-mycorrhizal roots in the absorption of soluble soil phosphate. Different "in vitro"
systems have been successfully used to demonstrate the fungal pathway of phosphate uptake in different mycorrhizal associations (Harley and Smith, 1983). The extramatrical hyphae absorb phosphate and translocate it to the host root; in this way they act as an extension of the root and as a bypass for phosphate through the depleted zone which develops around roots. Finlay and Read (1986) reported that labelled phosphorus fed to the distal part of the fungal mycelium from infected roots, moves freely through the strands over distances in excess of 40cm within 84 hours.

Nothing is known of the absorbing properties of the external mycelium of mycorrhizas, for instance, where hyphae absorb or whether they have high affinity absorption sites for phosphate. However, much of the detailed experimental work on phosphate absorption by hyphae of ectomycorrhizal fungi has been done with excised mycorrhizas where most of the extramatrical hyphae and also the shoots have been eliminated. Thus the results of these experiments may show less phosphate absorption than that which occurs in the intact mycorrhizas, or in nature. As could be expected, uptake by fungal sheaths is an active, metabolically dependent process; being temperature sensitive and requiring oxygen (Harley et al., 1958). The results obtained with excised mycelial strands indicate that the uptake by external hyphae is also an active, metabolically dependent process (Skinner and Bowen, 1974).

Hyphae of mycorrhizal fungi can accumulate and store reserve phosphorus as polyphosphate in their vacuoles (Ashford et al., 1975; Chilvers and Harley, 1980). This sequestration of phosphate into the vacuoles no doubt permits the fungi to accumulate large amounts of phosphorus which would otherwise interfere with cell metabolism (Bowen, 1970).

In mycorrhiza, polyphosphate has been implicated in both translocation and storage of phosphorus within fungal hyphae. As far as translocation is concerned, most studies have been made with the VA mycorrhizal fungus Glomus mosseae. High phosphorus flux rates in the fungal hyphae show that this fungus transports phosphate rapidly (Cooper and Tinker, 1978; Pearson and Tinker, 1975). Cooper and Tinker (1981) reported that the transport process
is metabolically dependent; as the translocation rate is reduced by low temperature, and requires oxygen. Also they reported that cytoplasmic streaming is the mechanism which would most likely account for the high phosphorus flux rates in hyphae of mycorrhizal fungi; since translocation of phosphate was stopped by "cytochalasin B" an inhibitor of cytoplasmic streaming. Rapid cytoplasmic current would carry the phosphate down a concentration gradient in the cytoplasm; this concentration gradient would depend on phosphate uptake rates in the external hyphae and phosphate removal rates from the internal hyphae to the host cells, as well as the efficiency of the stirring by the cytoplasmic streaming along the pathway of translocation.

The other possible mechanism for phosphate translocation could be by the movement of the granule-containing vacuoles in the streaming cytoplasm and, most likely by continuous loading and unloading of their contents to maintain the concentration of phosphate in the cytoplasm and so ensure its rapid displacement in the cytoplasmic currents (Tinker, 1975).

Polyphosphate formation is usually considered more as a system for storing large amounts of phosphate than as a transport system for phosphorous.

When cytoplasmic orthophosphate levels fall, the polyphosphate is hydrolysed. This has been demonstrated in a Hebeloma species by Martin et al. (1985), also Harley and Brierley (1954) had shown that under these conditions phosphate moves out from the fungus into the host tissues in ectomycorrhizas. This ability of fungal sheaths in ectomycorrhizas to accumulate large amounts of phosphorus as polyphosphate and rapidly mobilise it when required (Bowen, 1973), makes them a potentially important storage organ for the host plant.

Ectomycorrhizal fungi have also been shown to be active in solubilising organic (insoluble) phosphate such as phytate, in pure culture experiments. Theodorou (1968, 1971a) demonstrated that mycorrhizal fungi such as strains of *Rhizopogon luteolus*, *Bolus luteus* and *Cenococcum graniforme* (geophilum) are able to hydrolyse phytates (inositol hexaphosphate and lower phosphate esters of
myo-inositol) and to use the soluble phosphate for growth. Similarly Ho and Zak (1979) showed that strains of six mycorrhizal fungi, *Hebeloma crustuliniforme*, *Laccaria laccata*, *Amanita muscaria*, *Thelephora terrestris*, *Piloderma bicolor* (*Corticium coroceum*), *Rhizopogon vinicolor*, hydrolyse p-nitrophenyl phosphate by surface phytases. Bousquet et al. (1986) reported that *Pisolithus tinctorius* can hydrolyse para-nitrophenyl phosphate and Na phytate by surface phosphatase and phytase. Phosphatase activity was found to be increased in conditions of phosphate deficiency (Alexander and Hardy, 1981; Calleja et al., 1980). These findings may explain why, in certain soils, ectomycorrhizal plants sometimes respond more than non-mycorrhizal plants to rock phosphate applications.

Mycorrhizal roots have advantages over non-mycorrhizal roots: they are more resistant to infection by root pathogens (Levisohn, 1954 and 1957; Marx and Bryan, 1969; Marx and Davey, 1969a,b). Mycorrhizas protect host roots from attack by pathogens by mechanical and chemical means (Zak, 1964). In most ectomycorrhizas the thick fungal mantle (sheath) provides a mechanical barrier against the pathogens. Protection may also be afforded by antibiotic production. Marx (1969a,b) reported that some mycorrhizal fungi (*Laccaria laccata*; *Lactarius deliciosus*; *Leucoxaxillus cerealis*; *Pisolithus tinctorius*; and *Suillus luteus*) produce antibiotics in agar culture which inhibit growth of 44% of the root pathogens tested (eg. *Phytophthora cinnamomi* and *Pythium spp.*). Mycorrhizal associations may result in production of metabolites which inhibit growth of root pathogens (Marx, 1972).

Besides protection against pathogens, mycorrhizal associations make the host root more tolerant to frost, high soil temperatures (Cromer, 1935; Harley, 1940; Marx and Bryan, 1971; Marx et al., 1970a), drought (Muttiah, 1972; Worley and Hacskaylo, 1959), soil phytotoxins (Benson et al., 1980; Bowen, 1973; Zak, 1971a) and certain biocides (Volgt, 1954). Mycorrhizas formed by *Pisolithus tinctorius* could partly inactivate high concentrations of toxic metals, such as Zn, resulting from application of sewage sludge (Berry and Marx, 1976).
1.6 Interaction between mycorrhizas and other microorganisms

Antagonism, competition or synergism between microorganisms in the soil and the rhizoplane are probably the most important, but the least understood, phenomena in rhizosphere biology. These phenomena are extremely difficult to study under natural conditions, especially in mycorrhizas because of their complex ecosystems. However, investigators often extrapolate from laboratory experiments on mycorrhizas to the field situations.

The microbial flora in the mycorrhizosphere (zone in the soil surrounding mycorrhizal roots) often differs from that in the rhizosphere of non-mycorrhizal roots, for instance, the pine mycorrhizosphere was reported to contain 9-10 times larger populations of fungi (Tribunskaya, 1955), and that of the yellow birch about a ten times larger bacterial population (Katznelson et al., 1963) than the rhizosphere of non-mycorrhizal roots.

Katznelson et al. (1962) showed that non mycorrhizal birch roots harbour mostly bacteria with simple mineral nutrient requirements, whereas those associated with mycorrizal roots have more complex amino acid requirements and a lower proportion of rapidly growing phosphate-solubilizing bacteria. Also, Foster and Marks (1967) found that different types of microbial populations are associated with different types of mycorrhizas on the same host plant.

Neal et al. (1964) examined the rhizosphere microfloras of three morphologically different mycorrhizas of a Douglas-fir, and compared them with the microflora surrounding adjacent suberized roots and in non-rhizosphere soil. They found that the total population of bacteria surrounding mycorrhizal roots and suberized roots is significantly greater than those found in non-rhizosphere soil. Rhizospheres of yellow and grey mycorrhizas have higher populations of bacteria than the rhizosphere of suberized roots. However, the latter contain more bacteria than the rhizosphere of white mycorrhizas. In all the rhizospheres with the exception of the rhizosphere of the white mycorrhiza, the number of streptomyces is significantly higher than in the non-rhizosphere
soil. Mould counts are lower in mycorrhizal rhizospheres than in the non-rhizosphere soil. In all rhizospheres, the predominating moulds are members of the genus *Penicillium*.

From all these studies, it can be concluded that the microflora surrounding the mycorrhizal roots is influenced quantitatively and qualitatively by the type of mycorrhizal fungi present. This influence may be attributed, in part, to the availability or supply of inorganic and organic nutrients which could favour selective development of specific groups or species of microorganisms around the mycorrhizal roots.

Mycorrhizal establishment may be inhibited or stimulated by the presence of other microorganisms:

Rayner and Neilson-Jones (1944), demonstrated that pine and some other conifer seedlings planted in heathland soil failed to establish ectomycorrhizas even though the soil harboured the ectomycorrhizal fungus *Suillus (Boletus) bovinus*. This inhibition has been attributed to gliotoxin produced by certain *Penicillium* species (Brian et al., 1945), and to the antagonistic action of the *Calluna* endophyte (Handley, 1963).

Levisohn (1957), found that *Alternaria tenuis*, which is common in arable soil, inhibited growth of *Rhizopogon luteolus* and four *Boletus* species in laboratory media, and that in the field only *Leccinum scabrum* was established, but not the other mycorrhizal fungi that were more susceptible to *A. tenuis* in laboratory media. Theodorou (1967) succeeded in establishing symbiosis between *Pinus radiata* and *Rhizopogon luteolus* in nursery soil only after elimination of antagonists by partial soil sterilization.

Inoculation with *Trichoderma* and *Azotobacter* promoted mycorrhiza formation with oak and pine seedlings (Malyshkin, 1955; Tribunskaya, 1955; Vedenyapina, 1955). *Trichoderma lignorum* was more stimulatory than *Azotobacter* on pine. Greatest development of oak mycorrhiza was achieved with the above organisms combined with fluorescent bacteria (Shenakhanova, 1962). In nature, *Azotobacter chroococcum*, *Trichoderma lignorum* and *Pseudomonas fluorescens* were found closely associated with oak roots throughout the year (Malyshkin, 1955).
Bowen and Theodorou (1979) tested the interactions between eight bacteria isolated from different sources and eight ectomycorrhizal fungi on laboratory media and in the rhizoplane of Pinus radiata. They found that different bacteria could depress, have no effect, or even stimulate root colonization by mycorrhizal fungi and that such effects did not necessarily correspond to their "in vitro" effects on fungal growth. Some bacteria gave protection against the depressive effects of others. Depressive effects may be based on antibiosis or competition for nutrients. The most interesting finding by those authors was that the strain of Bacillus that stimulated growth of Rhizopogon luteolus was isolated from washed mycorrhizas of Pinus radiata roots with R. luteolus.

Meyer and Lindemann (1986) in their experiments on the inoculation of clover (Trifolium subterraneum) with VAM fungi and a plant growth-promoting bacterium PGPR (Pseudomonas putida), found that the VAM fungus infection in the roots was increased significantly from 7 to 23% by the presence of PGPR at six weeks, also they observed significant increase in root and shoot dry weight when both the PGPR and VAM fungus were present compared to that in the presence of PGPR alone, VAM fungus alone or uninoculated controls.

Although the presence of Pseudomonas spp or other bacteria is not essential for VAM establishment (Mosse and Hepper, 1975), infection by VAM fungi in nature may be aided by the better infection conditions created at or near the root surface by these common rhizosphere bacteria. However, it should be realized that pseudomonads in the rhizosphere are a diverse group of bacteria and their interactions with the plant and with mycorrhizal fungi will differ between strains of Pseudomonas. For example, Bowen and Theodorou (1979) observed a fluorescent pseudomonad markedly suppressing the development of several ectomycorrhizal fungal species in pine roots.

Chakraborty et al. (1985), tested the effect of three mycophagous amoebae on the colonization of Pinus radiata roots by the ectomycorrhizal fungus Rhizopogon luteolus. They found that two
mycophagous amoebae (Saccamoebae sp. and Gephyraamoeba sp.), reduced
the colonization of pine roots in soil by the ectomycorrhizal
fungus *R. luteolus* when added at the same time as the fungus or two
weeks later. The occurrence of mycophagous amoebae as frequent
colonizers of the rhizosphere in several countries (Chakraborty,
1983; Old and Patrick, 1979) and their ability to feed on a wide
range of fungal species, including vesicular-arbuscular and
ectomycorrhizal fungi, has been recorded (Old and Patrick, 1979).
1.7 Basidiospores

1.7.1 Production

In most hymenomycetous ectomycorrhizal fungi, basidiocarps are built up by complicated interwoven hyphae of the filamentous mycelium. The caps of these basidiocarps bear gills or tubes on their lower surfaces. The gills or tubes are also composed of interwoven hyphae. These hyphae produce a series of specialized club-shaped cells, the basidia in a layer termed the hymenium. On the apex of these basidia, basidiospores develop on stalks (sterigmata) from which, as they mature, the spores are discharged in a violent manner. Buller (1958) reported that discharging of spores in hymenomycetous fungi occurs continuously at a fairly consistent rate. They are never all discharged simultaneously or set free in intermittent showers. The process of spore-discharge often requires a considerable period of time. This may be conveniently called "the spore-fall period" (Buller, 1958). So far no report is published about the spore-fall period in ectomycorrhizal fungi.

1.7.2 Genetics of basidiospores

There is little information about the cytology and nuclear behaviour of basidiospores of ectomycorrhizal basidiomycetes, but they conform to the usual pattern of nuclear behaviour in higher basidiomycetes (Harley and Smith, 1983). Generally, in hymenomycetes (eg. Agarics) each hymenial cell, when first formed, contains two nuclei (Buller, 1958; Olah et al., 1977). In cells destined to become basidia, the nuclei fuse to form a single diploid nucleus (Karyogamy). By means of two successive meiosis divisions the fusion nucleus then divides into four haploid nuclei, whereupon the sterigmata and spores begin their development. When the spores have attained a certain size, the four nuclei each simultaneously approach the four sterigmata, creep through them, and pass into the spores, each of which thus becomes provided with
a single haploid nucleus. The spores upon germination, produce a primary mycelium, the cells of which are uninucleate. When compatible pairs of such mycelia are grown together, Plasmogamous fusions occur between certain compatible cells, but nuclear union is delayed until basidia are formed. This plasmogamy initiates the secondary mycelium, with binucleate \((n+n)\) cells from which the basidiocarp is formed.

In the ectomycorrhiza fungus *Hebeloma spp.*, Bruchet et al. (1986) reported that, as in most members of the Agaricales studied, spores receive one of the four haploid nuclei arising from the nuclear divisions which occur in the basidium, and this haploid nucleus divides mitotically in the spore so that the spores are binucleate. Also they reported that the spores germinate to form a primary (or homokaryotic) mycelium composed of cells all of which are uninucleate except for certain species (*Hebeloma sinapizans*) in which the growing cells remain pluri- to multi-nucleate. Plasmogamy between two compatible homokaryotic mycelia will initiate the secondary mycelium which is always made up of binucleate \((n+n)\) cells, the two compatible nuclei forming a dikaryon. Secondary mycelia often display clamp connections.

1.7.3 Spore function

While mycelia of ectomycorrhizal fungi can survive successfully after inoculation into sterile soils (Theodorou, 1967) and in natural soils under ideal conditions, they frequently fail or establish poorly under other field conditions, eg. where soil dries out or where biological antagonisms occur (Theodorou, 1971b). However, Lamb and Richards (1974) have shown that basidiospores of ectomycorrhizal fungi can survive relatively high soil temperatures (c.40°C) and low relative humidity (c.30%) which are not suitable for the hyphae. This has great ecological importance in natural wind-borne dispersion and also has practical importance on inoculation practices (Bowen and Theodorou, 1973).

Basidiospore function in mycorrhizal initiation is little understood. However, it is apparent that the initiation of the
symbiosis is dependent upon the availability of susceptible host root and the presence of viable infective propagules of the fungal symbiont. Although mycelium is known to grow internally along with the growing mother roots, thereby infecting newly emerging lateral roots as they move through the cortex (Clowes, 1951; Robertson, 1954; Wilcox, 1968), the initial infection of the root system must be derived from external sources eg. vegetative mycelium from nearby ectomycorrhizal roots or from basidiospores, or other propagules. The frequent occurrence of ectomycorrhizal infection on trees grown in fumigated nursery beds (Trappe and Strand, 1969) and ectomycorrhizal synthesis on trees grown in containers of sterile soil (Marx et al., 1970b; Robertson, 1954) have been reported. These studies suggested that the active propagules which initiate infections under these conditions are air-borne basidiospores.

Direct documentation on the role of basidiospores in initiation of infection is limited for most ectomycorrhizal fungi. Theodorou (1971b) presents evidence that spores of Rhizopogon luteolus can initiate ectomycorrhizas on Pinus radiata. But the inclusion of some mycelium in the spore inoculum diminishes the clarity of this finding. Using spores as inoculum, Thapar et al. (1967) found that Scleroderma verrucosum formed ectomycorrhizas with Eucalyptus grandis but reisolation to verify the fungal symbiont was not attempted. Marx and co-workers have shown that basidiospores of Pisolithus tinctorius (Marx 1976) and Thelephora terrestris (Marx and Ross, 1970) are effective inoculum for the synthesis of ectomycorrhizas with loblolly pine. Ivory (1983) reported that inoculation with basidiospores of Pisolithus tinctorius, Rhizopogon nigrescens or Scleroderma texense led to the formation of abundant distinctive mycorrhizas at four months on Pinus caribaea.

Fox (1983), in her experiments on inoculation of birch seedlings with basidiospores of some ectomycorrhizal fungi, reported that "early-stage" mycorrhizal fungi (Paxillus involutus, Hebeloma sacchariolens, H. leucosa, Inocybe lacera and I. lanuginella) can establish mycorrhizas on seedlings from spores in soil, but that "late-stage" mycorrhizal fungi (Lactarius pubescens
and *Leccinum roseofracta* cannot do so. Evidence from these studies indicates that spores can serve as functional inoculum.

Because of the difficulty in using pure cultures of ectomycorrhizal mycelium as inoculum (Mikola 1973, Theodorou 1967), Lamb and Richards (1974) recommended basidiospores as an alternative. Also Theodorou (1971b) reported that an easy and effective way of introducing mycorrhizal fungi into soil is by seed inoculation with fresh spores rather than by mixing fruiting bodies, litter or fungal mycelia within the soil. However, the sudden appearance and brief duration of the sporophores of most ectomycorrhizal fungi and the lack of information on the germinability and viability of spores and their survival during storage (Molina and Trappe, 1982b), have hindered the potential of spores as a source of inoculum in commercial practice.

### 1.7.4 Spore germination

The *Hymenomycetes* comprise a broad group including coprophilous, lignicolous, litter-decomposing and ectomycorrhizal fungi.

Spores of non-mycorrhizal *hymenomycetes*, in general, germinate readily on artificial media; many will germinate freely in plain water (Fries, 1966). In contrast, spore germination of the ectomycorrhizal *hymenomycetes* is very difficult to achieve under laboratory conditions (Benedict *et al.*, 1967; Brefeld, 1908; Fries, 1966, 1978, 1984, 1985; Kneebon, 1950; Marx and Ross, 1970; Romell, 1921; Stack *et al.*, 1975). Germination is often dependent upon, or stimulated by, the presence of some activating factor, usually of biological origin.

Fries (1941 and 1943), succeeded for the first time in germinating spores of some species of *Boletus*, *Amanita* and *Tricholoma*, when a living yeast (i.e. *Torulopsis sanguinea* or *Rhodotorula glutinis*) was used as stimulator. Lamb and Richards (1974) obtained germination of basidiospores of *Pisolithus tinctorius* (Lycoperdales), *Rhizopogon roseolus* and *Suillus*
granulatus only in the presence of the yeast Rhodotorula glutinis. However, many mycorrhizal fungi which were tested did not respond at all to the influence of Rhodotorula.

Since some studies (Fries, 1976 and 1978) indicated the presence of inhibitory factors in the nutrient agar media employed, steps were taken to identify and remove these inhibitors. First it was found in experiments with species of Suillus that germination is very sensitive to the content of ammonium ions in the substrate. A radical reduction of the ammonium concentration considerably improved both rate and percentage of germination in Suillus (Fries, 1976). Later it was discovered that germination in various ectomycorrhizal fungi was prevented by an inhibitor in agar which is formed from agarose during autoclaving. Activated charcoal has been used to remove this inhibitor (Fries, 1978). This inhibitor has been identified as a weak organic acid, chiefly active on mycorrhizal-hymenomycetes (Bjurnan, 1984). By using activated charcoal in combination with Rhodotorula glutinis as an inducing organism, germination could be induced in additional ectomycorrhizal species, eg. Laccaria laccata (Fries, 1977), Lactarius helvus, Paxillus involutus, Leccinium scabrum (Fries, 1978) and Cantharellus cibarius (Fries, 1979a).

Living mycelium of a mycorrhizal fungus, either alone or in combination with Rhodotorula glutinis and activated charcoal was found to be active in stimulating germination of spores of the same fungus (Birraux and Fries, 1981; Fries, 1978, 1981a and b, 1983a, b; Straatsma et al., 1985)

Even in the presence of such activators, germination percentages of less than 1% during incubation periods of one to four weeks are not uncommon for these ectomycorrhizal fungi. However, by the use of such activators, Fries (1943, 1966) obtained germination of Suillus luteus in about one week. Without the presence of Rhodotorula, at least three to four weeks were necessary for germination to begin. Other boletes, such as Boletus granulatus, B. variecatus, B. bovinus, B. elegans and B. viscidus, could be germinated by co-culture with Rhodotorula or a growing
Boletus mycelium for one week. Some of these species would begin germination on synthetic media if incubation was extended to one month or more, but no germination was observed on a malt extract medium. Fries also noted that Amanita rubescens (Fr.) Gray, would germinate without an activator, but the requisite incubation time in the presence of Rhodotorula was reduced to only five to seven days.

The nature of the stimulant produced by the yeast or mycelium is still unknown; several compounds may be involved. Volatile compounds have been found able to induce spore germination in some fungi, for example isovaleric acid which induces spore germination of Agaricus bisporus (Losel, 1964; Rast and Stauble, 1970). Fries (1978) tested the effect of isovaleric acid on germination because he suspected that the effects of the associated Rhodotorula or living mycelium were due to a volatile agent, isovaleric acid was without effect on any of the spores of mycorrhizal fungi tested. Oort (1974) found that some species of Lactarius germinated when exposed to volatile exudations from Ceratocystis fagacearum, a fungus which already has been mentioned as an inducer of germination in two xylphilous Polyporus species.

Bjurman and Fries (1984) reported that culture filtrates from Leccinum aurantiacum could substitute for a living mycelium in triggering spore germination. This indicated the existence of a germination inducing factor (GIF). Although this factor has not yet been identified, it was characterized as: non-volatile; diffusible; soluble in water, methanol and in ethanol; resistant to heat at 100°C for fifteen minutes; stable after lyophilization if preserved at -20°C and it has a low molecular weight (<8000 dalton).

Melin (1959, 1962) observed that excised pine roots stimulated spore germination in species of Russula, Suillus, Amanita, Paxillus, Cortinarius, and Lactarius. These studies were conducted in Melin's "maximum nutrient medium", a substrate containing glucose, various mineral salts, ten B-vitamins and a mixture of nineteen amino acids. The excised roots of Pinus sylvestris used in Melin's studies were grown aseptically in nutrient solution in flask culture for approximately five months.
prior to use.

In earlier work by Melin and co-workers, it was demonstrated that roots of herbaceous plants (e.g. Lycopersicon esculentum; Medicago sativa; Cannabis sativa; Triticum aestivum; and Lepidium sativum) also affected the growth of tree mycorrhizal fungi (Melin, 1954; Melin and Das, 1954). These herbaceous roots were not tested for their ability to stimulate spore germination, but their ability to stimulate vegetative growth parallels, for the most parts, that obtained with pine roots. In later studies, Melin (1963) used filter- or autoclaved-sterilised exudates obtained from Scots pine seedlings grown in pots. The culture-grown roots were used either directly in the test media or, as with the pot-grown seedlings, the exudates were obtained by diffusion into re-distilled water at 4°C for six days. Pot-grown and aseptically-cultured roots were similar in their effects upon growth of Boletus variegatus. Also, Melin reported that increased dosages of exudate can exert an inhibitory effect on mycelial growth. This has led to the belief that the exudate contains, in addition to the stimulatory factor, one or more inhibitory principles that can be effective at higher concentration.

By using agar media, seeded with homogenized mycelium of mycorrhizal fungi, usually Boletus (Suillus) variegatus, Melin was able to determine the sites of the release of the stimulatory factor and the inhibitor from the root system of Pinus seedlings which had been raised in aseptic conditions. The growth of the fungus was reduced or inhibited in the neighbourhood of secondarily thickened roots and greatly stimulated around primary rootlets.

Since Melin's (1963) studies, the effects of plant roots on germination of spores of ectomycorrhizal fungi have been studied only briefly:

Marx and Ross (1970) were unsuccessful in their attempts to germinate basidiospores of Thelephora terrestris (Aphyllophorales) by employing extracts or detached roots of loblolly pine in the media, but the spores germinated and successfully formed ectomycorrhizas with intact roots of aseptic seedlings. Heinemann and Gaie (1979) observed about 1% germination of Russula versicolor.
spores after seven days when a cover glass bearing spores was
placed 1 cm deep in a medium of perlite mixed with ground sphagnum
in which a seedling of Betula pendula was planted.

Fries and Birraux (1980) found that laying living roots of
Pinus sylvestris on agar media seeded with spores of Hebeloma spp.
(H. crustuliniforme, H. ingratum and H. mesophaeum) increased
germination to 1%, whereas in the absence of the roots germination
was about 0.1%. In a further study, Birraux and Fries (1981) found
an indication of specificity in response of spores to roots of
different plant species. They also found that basidiospores of
Thalephora terrestris were stimulated by the growing roots of very
young seedlings of Pinus sylvestris, Picea abies, Betula verrucosa
and Alnus glutinosa as they grew on agar near the spores. Seedlings
of ten species not forming ectomycorrhizas (Pisum sativum,
Trigonella foenumgraecum, Medicago sativa, Trifolium repens,
Phaseolus aureus, Lycopersicon esculentum, Raphanus sativus,
Lepidium sativum, Triticum vulgare and Zea mays) were inactive, and
one (Lupinus regalis) very slightly effective in this regard. Also,
Bowen and Theodorou (1985) reported that roots of pine (Pinus
radiata) stimulated spore germination of Rhizopogon to 60%, whereas
roots of Eucalyptus globulus, Medicago truncatula, Trifolium
subterraneum and Lolium perenne were inactive. This study was made
by dipping roots of sterile seedlings of the test plants in 1%
water agar previously seeded with spores of the test fungus. Then
the roots were drained and placed in sterile vermiculite at 25/15°C
day/night temperature. Fries and Swedjemark (1986) tested the
effect of roots of 5 species of trees and 16 species of herbs and
grasses on germination of spores of Hebeloma mesophaeum on
synthetic medium N6:5. All five tree species exerted a strong
effect on the spores, inducing germination within a week. Seedlings
of carrot (Daucus carota) among the sixteen non-tree seedlings
tested exerted an effect on the spores similar to that of the tree
seedlings. Very little germination was noticed with seedlings of
blueberry (Vaccinium myrtillus), white clover (Trifolium repens) or
radish (Raphanus sativus) after more than two weeks incubation. The
effect of non-tree seedlings in inducing germination was uncertain;
since \textit{H. mesophaeum} is known to start germination spontaneously after two or three weeks on agar plates (Bruchet 1973, Fries and Birraux 1980).

The results of Melin (1959, 1963), Fries and Birraux (1980), Birraux and Fries (1981), and of Bowen and Theodorou (1985) would indicate that prior to infection the host can significantly affect the mycelial growth and spore germination of the fungal symbiont in the region external to the root by the release of certain host-produced metabolites.

Melin (1963) has referred to this or these metabolites as "M-factor". Conclusive evidence to the nature of Melin's "M-factor" is not available despite efforts directed towards that goal by Melin and his associates. The search has been fraught with difficulties due to the negative effect of inhibitors and the complexity of root exudate (Melin 1963, Rovira 1969). Melin (1963) reported that H. Nilsson had concluded that the M-factor could be replaced in its effect on hyphal growth by nicotinamide adenine dinucleotide (NAD) which is, of course, a hydrogen acceptor essential to many dehydrogenase reactions. No further details have been forthcoming on this report. However, Benedict et al. (1967) tested the effects of exogenous NAD and the exudate from tomato roots on the mycelial growth of eight species, and spore germination in thirteen additional species of ectomycorrhizal Agaricales. Germination did not occur in any of the thirteen species tested during the incubation period (45-60 days). Only one (Leucopaxillus (Clitocybe) anarum f. roseibrunneus sf. majusculus) of the eight fungi tested for mycelial growth was stimulated by NAD. The remaining seven did not respond or were inhibited. Therefore it was concluded that some unidentified component(s) (other than NAD) of pine root exudate was responsible for the stimulatory effects noted by Melin.

Gogola (1970) identified cytokinin activity in the extract of the roots and germinating seeds of \textit{Pinus sylvestris}. He compared their effect on the growth of Boletus edulis var. pinicola with pure kinetin, tryptophane, gibberellic acid (GA3) and indoleacetic.
acid. Kinetin and the extracted cytokinins stimulated growth at low concentrations whereas the other compounds uniformly inhibited growth between concentrations of $10^{-6}$ and $10^{-3}$ g/l. Both extracted kinins and kinetin stimulated growth between $10^{-6}$ and $10^{-1}$ g/l but at higher concentrations sharply inhibited it. Gogola was of the opinion that the M-factor of Melin might be a cytokinin and that both its stimulatory and its inhibitory action at high concentrations might therefore be explained.

Information about the nature of inhibitors noted by Melin (1963) is not available, but Krupa and co-workers in several studies (Krupa and Fries, 1971; Krupa and Nylund, 1972; Krupa et al., 1974; Melin and Krupa, 1971) have demonstrated that roots of host plants (*Pinus* spp) produce volatiles (e.g. terpenes) and non-volatiles (e.g. phenols) which are effective inhibitors of both mycorrhizal fungi and root pathogens. They postulated that these host-produced inhibitors, at least in part, regulate the symbiosis.

1.7.5 Spore dormancy

Sussman (1965) defined dormancy as "any rest period or reversible interruption of the phenotypic development of the organism".

In general there are two types of dormancy: constitutional dormancy and exogenous or environmental dormancy. Spores in environmental dormancy are inactive because of unfavourable conditions of their environment but will resume development at once if transferred to a favourable situation. Constitutional dormancy is a condition in which a propagule possesses some innate property such as a wall with a barrier to the penetration of nutrients; a metabolic block, or the production of self-inhibitors. Where such self-inhibitors of germination are present, their effects are evidenced by reduced germinations in larger populations of spores. Their effects can be overcome either by washing the substances out of the spores, or by adding to the spores some substance which will counteract the inhibitor. A clear distinction between these two types of dormancy is often impossible to make.
Dormancy of spores, sporocarps or sclerotia has been recorded for fungi from all major taxonomic groups. Many of them are biotrophs (Turian and Hohl, 1981; Weber and Hess, 1976) but little is known about the spores of ectomycorrhizal fungi in this respect. However, the inability of ectomycorrhizal spores to germinate under conditions which otherwise would be favourable for growth, and their requirement for special stimulants to germinate, tend to indicate that ectomycorrhizal spores are in a state of constitutional dormancy (Sussman 1966, Taber and Taber 1982).

Dodd and McCracken (1972) have hypothesized that short chain amylose molecules in the spore wall of some Russula species prevent oxygen uptake and hence preserve dormancy. They indicated that removal of the starch by washing with water would allow germination to proceed. Although permeability has been implicated as a deciding factor in preserving dormancy in many fungi (Allen, 1965; Merrill, 1970), Blakeslee (1974) provided evidence that permeability is not a limiting factor with the ectomycorrhizal species he studied, as ready penetration of the spore wall by the rather large molecule of nitro-blue tetrazolium chloride (NBT) would indicate that an impenetrable spore wall is not involved. Also, Blakeslee (1974) failed to demonstrate the existence of self-inhibitors in five species of ectomycorrhizal fungi (Amanita citrina; Amanita rubescens; Strobilomyces floccopus; Suillus cothurnatus, and Suillus hirtellus), in experiments in which he inoculated agar plates with varying quantities of spores, but no concentration-related effect could be detected in any instances. Therefore he concluded that the inability of the test basidiospores to germinate was not due to the presence of an impermeable spore wall or some self-inhibitor, but rather due to a deficiency in supply of some stimulant; since these spores are stimulated in their germination by co-culture with a pink yeast (Rhodotorula sp.) or living mycelium of the same species as the spores and also in the presence of living roots of pine seedlings.

Fries (1978) interpreted the poor germination or inability to germinate of most ectomycorrhizal fungi on laboratory media in various ways, eg. as an expression of dormancy, as a need for a
germination-inducing factor, or as the effect of an endo- or exogenous inhibitor. Some evidence in favour of the two latter possibilities has been obtained in experiments with Boletus (Suillus) species, where amino acids stimulate and ammonium ions inhibit germination (Fries 1976).

1.7.6 Spore longevity

Length of viability of different spores varies tremendously even under nearly optimum conditions (Fries 1943). Information about the longevity of ectomycorrhizal basidiospores in nature is lacking. Under laboratory conditions some information is available for a few ectomycorrhizal fungi; Fries (1966) indicated that spores of Suillus luteus can germinate after six months of storage at low temperature (-10°C). Basidiospores of Thelophora terrestris could produce ectomycorrhizas following storage for one month at 5°C (Marx and Ross, 1970) and for 15 months at 4°C (Birraux and Fries, 1981). Stack et al. (1975) obtained ectomycorrhizal synthesis from spores of Laccaria laccata, either fresh or rehydrated after lyophilization (in liquid nitrogen) and subsequent storage at room temperature or at -10°C for up to seven months. Attempts to germinate these spores on laboratory media were unsuccessful. Basidiospores of Pisolithus tinctorius, kept dry in darkness at 5°C, have been successfully used in ectomycorrhizal synthesis often from one week to 34 months storage (Marx, 1976). Theodorou and Bowen (1973) demonstrated that basidiospores of the fungus Rhizopogon luteolus (mycorrhizal with Pinus radiata) could be used successfully as seed inoculum after freeze-drying and storage for three months at 22°C, provided that inoculum level was increased 100-fold by comparison with fresh spores, and that spores inoculated onto seed could be held dry for at least two days before planting and produce as much infection as fresh spores, provided inoculum was increased ten-fold. Also they reported that drying spores in soil over ten weeks and leaving them for a further two months at 24°C day temperature and 16°C night temperature did not affect their viability. Lamb and Richards (1974) examined the
effect of some environmental conditions on the storage and
germination of various types of propagules of seven mycorrhizal
g fungi. Chlamydospores of three highly effective unidentified
mycorrhizal symbionts, the oidia of Xerocomus subtomentosus and the
basidiospores of Rhizopogon roseolus, Suillus granulatus and
Pisolithus tinctorius were tested. They found that chlamydospores
had low heat tolerance and lost viability in storage even under
optimal conditions of humidity. For germination they showed narrow
ranges of tolerance to temperature and pH. Chlamydospores thus
appear to have little survival value. Oidia and basidiospores
showed high heat tolerance (50-60°C) for 48 hours and germinated
well after storage for 60 days at relative humidities near 50%.
They also germinated over wider ranges of temperature (10-40°C) and
substrate pH(4.0-7.5) than chlamydospores. Basidiospores of P.
tinctorius showed particularly broad tolerances to environmental
conditions during both storage and germination. Thus they concluded
that basidiospores could be recommended as seed inoculants for
direct sowing programs. Fox (1983) reported that storage of soil
that had been supplemented with basidiospores of ectomycorrhizal
fungi (Inocybe spp. and Hebeloma spp.) for ten months in outdoor
conditions or in a growth room at 18°C, did not alter the ability of
basidiospores to produce mycorrhizas.
1.8 The aims of this study

From the literature survey it can be concluded that the germination of basidiospores of ectomycorrhizal fungi has been a rather neglected field of study. In most previous work, basidiospore germination has been studied either in simple solutions, or on synthetic media in the presence of growth substances or stimulatory factors such as growing mycelia of filamentous fungi, red yeast or plant roots. Direct observations on basidiospore germination in nature, and their role in the initiation of infection are still lacking.

In the present study attempts are being made to:

1). Determine conditions for germination of basidiospores of selected ectomycorrhizal fungi.

2). Study the effect of other microorganisms in stimulating germination of basidiospores of ectomycorrhizal fungi.

3). Determine whether basidiospores are stimulated by host roots and whether growth from basidiospores is directed to host roots.

4). Determine whether there is any specificity in response of basidiospores to roots.

5). Study basidiospore germination under conditions similar to those in nature, that is in the presence of roots and soil.

6). Determine the longevity of basidiospores under different storage conditions.

* The fungal species of ectomycorrhizal fungi studied in this project were selected according to their availability in the studied area and also to include groups of fungi which are different ecologically i.e. those occurring early and late in the mycorrhizal succession.
2.1 Laboratory media

2.1.1 Fries agar (Fries, 1978)

The medium contained:

- Glucose 4.00g
- Ammonium tartrate 1.00g
- KH2PO4 0.20g
- MgSO4.7H2O 0.10g
- NaCl 20mg
- CaCl2.2H2O 26mg
- ZnSO4.7H2O 0.80mg
- MnSO4.4H2O 0.81mg
- FeCl3.6H2O 0.80mg
- Malt extract (Difco) 1.00g
- Agar (Difco) 15.00g
- Distilled water 1000ml

And the following vitamins: thiamin 100ug, pyridoxine 100ug, riboflavin 100ug, biotin 25ug, nicotinamide 100ug, P-amino-benzoic acid 100ug, pantothenic acid 100ug, and inositol 10,000ug, were also added to 1 litre of the above medium. Autoclaved at 120°C for 10 minutes.

In all the germination tests this medium was used with the above ingredients except where otherwise stated.
2.1.2 Modified Melin Norkrans medium (MMN) (Marx, 1969a)

CaCl$_2$ 0.05g  
NaCl 0.025g  
KH$_2$PO$_4$ 0.50g  
(NH$_4$)$_2$HPO$_4$ 0.25g  
MgSO$_4$.7H$_2$O 0.15g  
FeCl$_3$(1% solution) 1.2ml  
Thiamine (HCl) 100ug  
Malt extract 3.0g  
Glucose 10.0g  
Bacto-agar 15.0g  
Distilled water 1000.0ml

2.1.3 Mineral salts nutrient medium (Ingestad, 1962)

NH$_4$NO$_3$ 0.40g  
NaH$_2$PO$_4$.2H$_2$O 0.50g  
KCl 0.33g  
KH$_2$PO$_4$ 0.43g  
CaCl$_2$.6H$_2$O 0.65g  
MgSO$_4$.7H$_2$O 0.49g  
MgCl$_2$.6H$_2$O 0.40g  
Na$_2$SO$_4$.10H$_2$O 0.64g  
FeCl$_3$.6H$_2$O 0.014g  
MnCl$_2$.4H$_2$O 1.8mg  
H$_3$BO$_3$ 2.9mg  
ZnCl$_2$ 0.12mg  
CuCl$_2$ 0.15mg  
Na$_2$MoO$_4$.2H$_2$O 0.022mg  
Distilled water 1000.0ml

Autoclaved at 120°C for 10 minutes.  
Quarter strength of the above medium was used.
Our tests confirmed Ingestad's (1962) observation that full strength solution was unsuitable for young (c. 3 week-old) seedlings. 1% (w/v) agar was added when solid medium was required.

2.1.4 Nutrient agar

28g/litre Oxoid nutrient agar (CM3).

2.1.5 Potato dextrose agar (PDA)

39g/litre Oxoid PDA (CM139).

2.1.6 Pseudomonas Agar Base CM 559 (Oxoid Ltd.)

24.2g of the agar base, CM559, was suspended in 500ml of distilled water, then 5ml of glycerol was added, boiled to dissolve completely, and autoclaved at 121 °C for 15 minutes. To 500ml of agar base cooled to 50 °C, the contents of 1 vial of Pseudomonas C-F-C supplement SR103 (rehydrated with 2ml of sterile distilled water) was added, the medium was mixed and poured into sterile Petri dishes.

The antibiotic C-F-C (SR103) contains 0.005g Cetrimide and 0.005g Fucidin, both equivalent to 10mg per litre of medium, and 0.025g Cephaloridine equivalent to 50mg per litre.
2.2 Melzer’s Reagent

The reagent was prepared by dissolving 1.5g iodine, 5g potassium iodide and 100g chloralhydrate in 100ml warm distilled water.

2.3 SV, Sulpho-Vanillin

Prepared by dissolving a few crystals of vanilla in 2ml concentrated sulphuric acid + 2ml distilled water to give a yellow solution.

2.4 Formalin-acetic-alcohol

Consists of
- Formaldehyde (40%) = 5ml
- Glacial acetic acid = 5ml
- Alcohol (70%) = 90ml

2.5 Acetic-analine blue stain (Jones and Mollison, 1948)

15ml of 5.0% (v/v) aqueous solution of phenol, 1ml of 1.0% (w/v) aqueous solution of water-soluble aniline blue and 4ml of glacial acetic acid, were mixed and filtered about one hour after preparation.

2.6 Preparation of saturated salt solution (Winston and Bates, 1960)

The solution was prepared by dissolving solid CaCl2.6H2O in boiling water to saturation. This solution was then allowed to stand and cool for a few days to ensure saturation. Then the
saturated solution was shaken and distributed in 1 litre jars (100ml/jar) and a wire screen (of c. 5 x 5mm mesh) was placed over the solution and supported by bending its corners downwards to make feet (Plate 1). Plates containing spore prints were placed on the screen and the jars were closed and stored at c. 5°C, this maintained a relative humidity of c. 40% inside the jars.
Plate 1  Spores in Petri dishes stored over saturated CaCl₂ 6H₂O in closed jar
2.7 Root exudate

Root exudate was collected by a modification of the procedure of Ratnayake et al. (1978). Seedlings of birch (Betula pubescens) grown for six weeks in untreated birch wood soil, were carefully lifted and washed to remove sand and debris from the roots. They were then placed in beakers (5 seedlings/beaker) with their root systems completely covered with (200ml) distilled water, aerated by an aquarium aerater for 18-20 hours in a growth chamber under continuous light at 20 °C. The contents of the beakers was collected, passed through Whatman No.1 filter paper, and the volume of the root exudates then reduced by freeze drying (Chemlab Instruments Ltd. SB3 Freeze Dryer) to 4ml/seedling (pH 5.8) before being filter-sterilized by passage through a Millipore filters (HAWP 025, pore size 0.45um) and stored at -20°C. The roots were dried at 65 °C until constant weight, and their dry weights were recorded.

Mean dry weight was 82.0mg/root.
2.8 Modified Fahraeus cells

The modified Fahraeus cell is made from a microscope slide and a coverslip separated by coverslip spacing pieces (Nutman, 1970).

To make coverslip spacing pieces; a large coverslip (no. 1.5, 24 x 50mm) was stuck to gummed paper, 2mm coverslip squares were cut using a diamond pencil. Then the gummed paper with the coverslip squares was placed in water. The small glass squares floated free and were washed in diluted hydrochloric acid, distilled water (several times), acetone and dried.

To make the cell, a standard microscope slide was placed on a sheet of paper on which the positions of spacers and coverslip were marked. Small amounts of freshly made Araldite mixture (epoxy resin) were placed on the slide at the four spacer positions. Then a coverslip square was added at each position and pressed flat. Two, three or four squares of No. 1.5 coverslip were used to give cell depths of 1-2mm. Then, the large coverslip was fixed to the spacer squares with Araldite. The cells were baked for one hour at 100 °C to harden the Araldite. They were then acid washed, followed by several washings in distilled water and placed individually in Petri dishes. They were then sterilized in an oven at 180 °C for two hours.
2.9 Procedures for Scanning Electron Microscope (S.E.M.) study

Fixation

Small pieces of birch roots bearing mycorrhizas with *Paxillus involutus* or *Laccaria laccata* were fixed in 4% (v/v) glutaraldehyde in 0.2M sodium cacodylate (pH 7.2), at 4°C for 3 hours, then washed with the same buffer to remove the glutaraldehyde and post fixed in 1% (w/v) osmium tetroxide in 0.2M sodium cacodylate at 4°C for 20 hours.

Dehydration

After washing with the same buffer, the specimens were dehydrated at 4 °C through a graded acetone series (20, 40, 60, 80, and 100% v/v in distilled water), being held for 30 minutes at each stage, with three changes for one hour in 100% acetone.

Critical point drying

Specimens in acetone were critical-point dried, for one hour using a Polaron bomb with carbon dioxide as the transitional fluid (Anderson, 1951, 1966). They were then mounted on aluminium stubs using double sided Sellotape, sputter coated with gold using a Nanotech unit and examined with a Cambridge S100 scanning electron microscope fitted with a Nikon 35mm camera.
2.10 Seeds

The seed-lots of tree species: Birch [Betula pubescens (Ehrh.)] 80 (43); Corsican pine [Pinus nigra (Hoess, Badoux)] 82 (4026); Sitka spruce [Picea sitchensis (Bong.) Carr.] 83 (1012); European larch [Larix decidua (Mill.)] 82 (2006); Douglas fir [Pseudotsuga menziesii (Mirb.) Franco] 80 (7974); Western hemlock [Tsuga heterophylla (Raf.) Sarg.] 79 (7972); and Eucalyptus [Eucalyptus nitens Maiden] were supplied by the Forestry Commission (Alice Holt).

Seeds of the non-tree species: lettuce (Lactuca sativa L.) Unrivalled; tomato (Lycopersicon esculentum Mill.) Money-maker; onion (Allium cepa L.) Ailsa Craig; cabbage (Brassica oleracea L.) Winnigstadt; and white clover (Trifolium repens L.), (commercial vegetable seeds).

2.10.1 Sterilization of seeds

Seeds were surface sterilized by soaking in 30% (v/v) hydrogen peroxide (30 minutes for the tree seeds and 15 minutes for the other seeds), followed by one wash in sterile distilled water.

2.10.2 Germination of seeds

a - germination on agar

For laboratory experiments, the surface-sterilized seeds were aseptically transferred to water agar (1.0% w/v) plates and incubated at 20°C under lights.

b - germination on peat

For soil experiments, the surface-sterilized seeds were stored in sterile distilled water for 24 hours at 4°C to allow imbibition. They were sown in moistened peat in propagation trays, and incubated in the greenhouse (temperature between 12 and 20°C), in the dark until c. 50% germination had occurred.
2.11 Soil

The soils used in this study were of three different types;

a - Birch wood soil, from an area around birch trees at Whitmoor Common.

b - Spruce forest soil, from an area around spruce trees, Alice Holt Forest, Hampshire.

c - Arable soil, University of Surrey.

(See Table 1 for soil descriptions)

2.11.1 Soil analysis

Soil samples were analysed by Reading Soil Services within The Department of Soil Science, The University of Reading.

(See Table 1 for soil analysis)

2.11.2 Collection

The soils were collected from the top 30cm of the soil profile, after removing surface debris and the litter-layer. The soils were air-dried, passed through a 3mm sieve and stored in plastic bags, until used.

2.11.3 Steaming

The soils were steam-treated for three hours on two successive days.
### Table 1. Soil Analysis

<table>
<thead>
<tr>
<th>Soil</th>
<th>Soil Descriptions</th>
<th>Textural Class</th>
<th>pH</th>
<th>Lime Requirement t ha/l</th>
<th>Extractable Phosphorus mg/l</th>
<th>Extractable Potassium mg/l</th>
<th>Extractable Magnesium mg/l</th>
<th>Readily Oxidisable Carbon %</th>
<th>Total Nitrogen mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birch Wood</td>
<td>Holidays Hill No.3 Association (Stagnogley-Podzol)</td>
<td>Humose Sand</td>
<td>4.05</td>
<td>27.6</td>
<td>13</td>
<td>60</td>
<td>33</td>
<td>5.8</td>
<td>3.44</td>
</tr>
<tr>
<td>Spruce Forest</td>
<td>Essendon Association (Poleo-argillic Stagnogley)</td>
<td>Humose Sandy-Loam</td>
<td>4.30</td>
<td>26.2</td>
<td>5</td>
<td>41</td>
<td>33</td>
<td>9.0</td>
<td>3.17</td>
</tr>
<tr>
<td>Arable</td>
<td>Wickham Association (Stagnogley over clay)</td>
<td>Sand</td>
<td>7.40</td>
<td>-</td>
<td>44</td>
<td>84</td>
<td>120</td>
<td>3.2</td>
<td>1.80</td>
</tr>
</tbody>
</table>

**P**: was extracted in NaHCO₃ and determined colorimetrically on the EEL Colorimeter (filter No. 608).

**K**: was extracted in NH₄NO₃ and measured by a flame photometer.

**Mg**: was extracted in NH₄NO₃ and determined by atomic absorption spectrophotometer.

**Easily Oxidisable Carbon**: Soil sample was digested with dichromate mixture and concentrated H₂SO₄ and the absorbance of the solutions was determined at 620nm.

**Total Nitrogen**: Soil sample was digested in concentrated H₂SO₄. Nitrogen content was determined by distillation of ammonium followed by titration with 0.01M HCl.
2.11.4 Fumigation of soils

The soils were fumigated using a chemical sterilant Dazomet (Basimid). The chemical compound breaks down in soil to a dithiocarbomate which then converts to a volatile toxic compound, methyl isothiocyanate. This was considered to be effective in eliminating mycorrhizal fungal propagules (Iyer and Wojahn, 1976). Dazomet was added to the soil at the rate of 30gm Dazomet to 70 litres of soil, and thoroughly mixed in with a fork. The soil was stored in a sealed plastic bag for three weeks, after that the soil was emptied out into trays in a closed bay of the greenhouse and left for three weeks to allow toxic volatiles to evaporate off.

Cress seeds were used to test the residual phytotoxicity in the soil; cress seeds were sown onto treated soil in Petri dishes. The soil was moistened, the dishes were sealed with P.V.C. tape and the seeds left to germinate for 1-3 days. Successful germination of seeds in comparison to those on control unsterilized peat indicated that the soil was free of significant toxicity.
As an initial step in the present study, collection and identification of mycorrhizal basidiocarps was made on a regular basis (weekly), during the periods June to December in 1983 and 1984 around birch trees at Whitmoor Common in Surrey.

A number of complete specimens of each fungus were collected in cardboard boxes, and field observations were recorded. The specimens were quickly brought to the laboratory, quantitative and qualitative, macroscopic and microscopic characteristics were recorded for each fungus.

Identification was made according to the following characteristics:

1. Size, shape, colour and texture of the cap.

2. Height, width and colour of the stem, also the presence of a ring, volva, root or basal bulb.

3. Colour and texture of the flesh, the exudation of milk, the smell and taste.

4. Colour, shape and the attachment of gills to the stem.

5. Colour and shape of the spores, and presence or absence of warts (spine).

Chemical characterizations were also made when needed such as the production of a distinctive colour on the stem of Russula species with SV (sulpho-vanilin) or FeSO4, and the formation of blue-black colour (amyloid reaction), by spores of certain species in Melzer's reagent (see materials, 2.2 and 2.3).
The following references were used in the identification of
the basidiocarps:

Smith et al. (1981); Moser (1983); Phillips (1983), (see
reference list); and personal assistance was given by R. Jackson; M.
Moss; and D. Reid.

Basidiocarps of 28 species of ectomycorrhizal fungi were
identified and their identities and taxonomic position are
summarised in Table 2. Of the listed ectomycorrhizal species, seven
(Hymenomycetes) were chosen and their basidiospores were used for
this study. The occurrence and abundancy of the chosen species are
shown in Figure 1.

Basidiospores of one species (Scleroderma citrinum) of
Gastromycetous ectomycorrhizal fungi were also studied (see appendix
1).
Table 2  Species of ectomycorrhizal fungi collected and identified during the periods (June-December) in 1983 and 1984 beneath birch trees at Whitmoor Common, Surrey.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Family</th>
<th>Order</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amanita citrina  (Schaeff) S.F.Gray</td>
<td>Amanitaceae</td>
<td>Agaricales</td>
</tr>
<tr>
<td>Amanita fulva  (Schaeff) Seer</td>
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<tr>
<td>Amanita muscaria (L.ex Fr.) Hooker</td>
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<tr>
<td>Amanita rubescens ([Pers.]Fr.) S.F.Gray</td>
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<tr>
<td>Boletus badius Fr.</td>
<td>Boletaceae</td>
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<tr>
<td>Boletus chrysenteron</td>
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<tr>
<td>(Bull.ex.St.Amans)</td>
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<tr>
<td>Boletus impolitus Fr.</td>
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<tr>
<td>Hebeloma crustuliniforme</td>
<td>Cortinariaceae</td>
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<tr>
<td>(Bull.ex.St.Amans) Quel.</td>
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<tr>
<td>Laccaria amethystea</td>
<td>Tricholomotaceae</td>
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<tr>
<td>(Bull.ex Merat) Murr.</td>
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<tr>
<td>Laccaria laccata (Scop.ex.Fr.) Cke.</td>
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<tr>
<td>Laccaria proxima (Boud) Pat</td>
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<tr>
<td>Lactarius rufus (Scop.ex Fr.) Fr.</td>
<td>Russulaceae</td>
<td>Russulales</td>
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<tr>
<td>Lactarius quietus (Fr.) Fr.</td>
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<td>Lactarius turpis (Weinm.) Fr.</td>
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<td>Lactarius vietus (Fr.) Fr.</td>
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<td>Leccinum scabrum (Fr.)S.F.Gray</td>
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<td>Leccinum versipelle (Fr. &amp; Hok) Snell.</td>
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<td>Paxillus involutus (Fr.) Fr.</td>
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<td>Russula betularum Hora</td>
<td>Russulaceae</td>
<td>Russulales</td>
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<tr>
<td>Russula cyanoxantha</td>
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<tr>
<td>(Schaeff.ex.Secr) Fr.</td>
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<td>Russula claroflava Grove</td>
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<tr>
<td>Russula nitida (Pers.ex Fr.) Fr.</td>
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<tr>
<td>Russula ochroleuca (Pers.ex.Secr) Fr.</td>
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<tr>
<td>Russula sororia (Fr.) Romell</td>
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<tr>
<td>Russula velenovskyi Melzer &amp; Zvara</td>
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<tr>
<td>Russula violeipes Quel.</td>
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<tr>
<td>Scleroderma citrinum Pers.</td>
<td>Sclerodermataceae Sclerodermatales</td>
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<tr>
<td>Thelephora terrestris (Ehrh.) Fr.</td>
<td>Thelephoraceae</td>
<td>Aphylophorales</td>
</tr>
</tbody>
</table>

48
Figure 1  Occurrence and abundancy of the test fungi during the season of 1983 and 1984 around birch trees in Whitmoor Common, Surrey.

Amanita fulva
Amanita rubescens
Hebeloma crustuliniforme*
Laccaria laccata
Lactarius turpis
Paxillus involutus
Russula nitida

: Total observed season of basidiocarp production.

: Period of greatest abundance

* : Basidiocarps of H. crustuliniforme were collected around willow (Salix spp) trees at the University of Surrey.
2.12.2 Collection of spores

Clean and mature basidiocarps were selected, the stems were cut off and the caps were surface-sterilized by wiping with tissue soaked in ethanol, and were supported over sterile plastic Petri dishes on two cocktail sticks. Paper towels soaked with water were placed underneath the plates to maintain humidity. The plates were covered with a plastic tray and left (from 1-20 hours) until spore prints were seen in the plates. Then the spores were either used directly or for further study they were stored under different conditions.

2.12.3 Methods of storing spores

1. Plates with spore prints were placed in polythene bags and stored at 5 or -20°C (under 95 and 45% relative humidity respectively).

2. Plates with spore prints were stored at 5°C and c. 40% relative humidity over a saturated calcium chloride solution in closed containers (see method 2.6).

3. Gills from basidiocarps of the test fungi were wrapped with Kleenex tissues, freeze-dried for 24 hours and then stored over anhydrous calcium chloride under vacuum (in a desiccator) at room temperature.
2.12.4 Isolation of ectomycorrhizal fungi from fruitbodies

The caps of young and healthy, freshly collected fruitbodies of the test mycorrhizal fungi were surface-sterilized by wiping with tissue soaked in ethanol, broken open under aseptic conditions, and small portions of the vegetative tissue were dissected out and placed on plates of modified Melin-Norkrans agar (MMN) containing 15 ug/ml aureomycin (chlorotetracycline), and incubated at 20°C. When hyphae grew out of the fruitbody fragments to about 5-10mm, discs (5mm diameters) were cut with sterile cork-borer from the margin of the young mycelium and transferred to plates of fresh MMN and maintained on this medium.

Five species of the test fungi were obtained in pure culture by this method, and the other two (Amanita fulva and Russula nitida) did not produce mycelial cultures from fruitbodies.

2.12.5 Isolation of ectomycorrhizal fungi from mycorrhizas

A modification of the procedures described by Chu-Chou (1979) and Chu-Chou and Grace (1981) was used. Freshly collected mycorrhizal roots were washed under running tap water to remove adhering soil particles. Segments of these roots, 5-10 mm long and bearing mycorrhizal tips were transferred to universal bottles containing 15-20ml of Tween 80 (0.003% v/v) and agitated on a Gallenkamp wrist action shaker for 15 minutes to remove soil debris, followed by agitation in three changes of distilled water to remove the Tween 80 residue. The roots were then surface-sterilized by immersion in 30% (v/v) hydrogen peroxide for 10-30 seconds and washed again by agitation for 10 minutes in sterile distilled water. Individual mycorrhizas were then cut off and plated out onto plates of MMN agar containing 15ug/ml aureomycin, and incubated at 20°C.
Chapter 3

EXPERIMENTS

3.1 Germination without stimulants

Germination tests were made on a synthetic basic medium of Fries (Fries, 1978) which was shown to be suitable for spore germination of most ectomycorrhizal fungi tested, (Fries, 1978; 1981a; 1983a).

A series of tests was also made in order to find out whether the major components of the basic medium are in concentrations optimal for spore germination of the test fungi; glucose, ammonium ions and malt extract were tested at different concentrations either singly or in combination with other components of the basic medium. Twelve combinations were used as shown in Table 3. The rest of the components were added as described by Fries (1978) (see materials, 2.1.1). The media were then autoclaved at 120°C for 10 minutes. These combinations were used as liquid, or in a solidified medium made by adding 15g/l Difco agar before autoclaving.

Other changes were also made as follows:

1. Glucose in some treatments was filter-sterilized separately and added to the autoclaved medium, this is because glucose was shown, occasionally, to be inhibitory to bacteria and fungi, and this was attributed mostly to break-down products of glucose in heat sterilizing processes (Stanier, 1942).

2. Since agar media may contain growth-inhibitory substances, (Fries, 1978; Bjurman, 1984), activated charcoal was added because of its capacity to absorb and thus remove inhibitory compounds from the culture medium (Butler and Bolkan, 1973; Day and Anagnostakis, 1971; Fries, 1978).

3. For the reason stated above, pure agarose (Electrophoresis Grade - BRL) was used instead of Difco agar to solidify the original medium.
Table 3. Concentration of glucose, malt extract and ammonium tartrate in Fries medium and in different combinations tested.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration of the tested components gram/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium 1</td>
</tr>
<tr>
<td></td>
<td>Original</td>
</tr>
<tr>
<td>Glucose</td>
<td>4</td>
</tr>
<tr>
<td>Malt Extract</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium Tartrate</td>
<td>1</td>
</tr>
</tbody>
</table>

- : Nil
3.1.1 Germination on solid media

Spore prints of the test ectomycorrhizal fungi were removed from the plastic Petri dishes (immediately after collection) by dispersing them in sterile distilled water (mixtures of spores from 3-5 fruitbodies of the same species were used). 0.1ml of this suspension (containing c. 500,000 spores) was spread over the surface of the agar plate of 9.5cm diameter (containing 20ml of the test media). Three plates were prepared for each treatment. The plates were left overnight to allow absorption of water by the agar. Half of the agar plate was covered with (c. 7mg/plate) activated charcoal (Darco G60). Then the plates were sealed with P.V.C. tape, incubated in darkness at 20°C, and examined daily under the light microscope for spore germination.

3.1.2 Germination in liquid solution

A piece (2 x 3cm) of sterile cellulose film (boiled in distilled water for 10 minutes before autoclaving to remove any additive material) was placed on a filter pad in a sterile plastic Petri dish of 4.5cm diameter. The filter pad was moistened with the test solution or with sterile distilled water as a control. 0.05ml of the same spore suspension used in (3.1.1) was spread over the cellulose film. Three plates were made for each treatment. The plates were then taped and incubated as above. Examination of the films was made under the light microscope by removing the cellulose films carefully and placing them into another plate without a filter pad. After a quick examination, the films were returned to the plates with filter pads and reincubated. This was repeated every three days, and sterile distilled water was added when needed to avoid drying of the cellulose films.

3.1.3 Results

Spores of Paxillus involutus, Hebeloma crustuliniforme, Amanita fulva, Lactarius turpis and Russula nitida did not
Spores of *Amanita rubescens* germinated in all media tested, except when cellulose films were placed on a filter pad moistened with distilled water, but the germination never exceeded 0.1%, and usually occurred when spores were in clusters. Germination started after two weeks incubation, and macrocolonies were produced on agar plates from germinating spores within 3-4 weeks.

In *Laccaria laccata*, germination was obtained only when pure agarose was used instead of Difco agar. Germination (<0.1%) occurred after five weeks incubation and macrocolonies appeared on the surface of the agar plate a week later. Activated charcoal showed no effect in any of the tests.

Incubation was continued for up to four months when solid media were used, but when cellulose film was used the experiment was terminated after three weeks, as all the plates became contaminated due to the frequent opening of the plates for examination.
3.2 Germination on laboratory medium in the presence of stimulatory factors

3.2.1 Germination in the presence of living mycelium of the same fungus as the spores

Mycelial discs (5mm diameter) obtained from the margin of an actively growing mycelial colony of the test fungi on MMN agar (see method 2.12.4) were transferred to fresh plates of Fries agar, (Fries, 1978, see 2.1.1) previously seeded with spores (as in 3.1.1) of the same fungus.

Because of failure to get mycelial cultures from basidiocarps of Amanita fulva and Russula nitida; mycelium of related fungi, for example, Amanita rubescens and Lactarius turpis were used with spores of A. fulva and R. nitida respectively. Six plates were made for each fungus, three of them were covered with activated charcoal. The plates were then sealed with P.V.C. tape, incubated in darkness at 20 °C, and examined daily under the light microscope for spore germination.

3.2.2 Germination in the presence of fragments of basidiocarps of the same species as the spores

Caps of clean and healthy basidiocarps of the test ectomycorrhizal fungi were surface-sterilized by wiping with tissue soaked in ethanol. Fragments from the inside tissues were placed on the surface of Fries agar plates previously seeded with spores of the same fungus. Six plates were made for each fungus, three of them were covered with activated charcoal. The plates were then sealed with P.V.C. tape, incubated in darkness at 20 °C and examined daily for spore germination.
3.2.3 Germination in the presence of volatile compounds from vegetative mycelia or basidiocarps of the same species as the spores

The inverted dish method (Brown and Merrill, 1973) was used to test for the occurrence of volatile compounds active in stimulating germination of spores of the test fungi.

Plates of Fries agar seeded with spores of the test fungi were taped to plates of growing mycelia on Fries agar or plates containing fragments of clean basidiocarps of the same fungus as the spores. Some of the plates containing the spores were covered with charcoal. Six plates were made for each fungus. Incubation was in darkness at 20°C.

3.2.4 Results

Germination of spores of A. fulva, L. turpis, and R. nitida was not stimulated by living mycelium or activated charcoal (Table 4) even after a prolonged incubation period of more than four months. Living mycelium of Paxillus involutus in combination with activated charcoal induced germination of spores of the same fungus (5-7 spores out of 500,000) after two weeks incubation, but the germ tubes did not grow longer than three times the spore diameter even after four months incubation. In Hebeloma crustuliniforme, germination (up to 6 spores out of 100,000) started after about four weeks incubation near the mycelial colony and only in the part of the agar plate covered with charcoal. Germinating spores produced mycelial colonies a week later.

Spores of Laccaria laccata germinated after ten days incubation, but only within 1 cm of the growing mycelium and within the area dusted with charcoal. Germinating spores produced mycelial colonies within two weeks. Few spores surrounding the germinating spores were stimulated to germinate. However, germination did not exceed 1% before the growing mycelium covered the whole surface of the plate.
Table 4  Germination of basidiospores on Fries agar with or without living mycelium and activated charcoal.

<table>
<thead>
<tr>
<th>Basidiospores</th>
<th>Supplements</th>
<th>No Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mycelium</td>
<td>mycelium</td>
</tr>
<tr>
<td></td>
<td>+ charcoal</td>
<td></td>
</tr>
<tr>
<td>Paxillus involutus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hebeloma crustuliniforme</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Laccaria laccata</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Amanita rubescens</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Amanita fulva</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactarius turpis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Russula nitida</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- : no germination

+ : <0.01% (5 - 7 spores/plate)

++ : 0.01 - 0.1%

+++ : 0.1 - 1%
Germination of *A. rubescens* spores occurred in the presence and absence of growing mycelium and activated charcoal, but the time required for the germination to occur was reduced from two weeks to 4-5 days in the presence of its own mycelium. Mycelial colonies were produced from the germinating spores within seven days after germination. No self stimulation was seen.

In all these fungi one germ tube always developed from one of the end points of the spore.

Spores of the test fungi were not stimulated when fragments from sporophores of the same species were used as stimulator. Also, no active volatile compound was proved to be produced by growing mycelia or sporophores of the test mycorrhizal fungi.
3.3 Testing the effect of other microorganisms on spore germination of the test fungi

Germination of basidiospores of the test ectomycorrhizal fungi was studied in the presence of different isolates of bacteria and fungi obtained from different sources:

a - bacteria and fungi obtained from birch wood soil.
b - bacteria and fungi associated with spores or sporophores of the test ectomycorrhizal fungi.
c - bacteria and fungi associated with the mycorrhizal sheath of the test ectomycorrhizal fungi.
d - bacteria and fungi appearing among the spores, as natural contaminants on laboratory media.

The number and sources of the isolates of the first three groups are shown in Table 5.

3.3.1 Isolation of bacteria and fungi from soil

The dilution plating method was used: fresh soil samples were collected, from the top 30 cm of the soil profile in July of 1985 from twelve different places beneath birch trees at Whitmoor Common, Surrey. The samples were passed through a 3 mm sieve and four composite samples were made. 10 grams of each sample were suspended in 90 ml sterile distilled water, shaken in a Gallenkamp wrist-action shaker for 15 minutes, and allowed to stand for five minutes. From this suspension serial dilutions (up to $10^{-4}$) were made, and 1 ml of each dilution was plated (by the poured plate method) with nutrient agar for bacteria, and with potato dextrose agar (PDA) for fungi, three plates were made for each dilution and incubated at 20°C for seven days.

Forty bacterial isolates and thirty fungal isolates were selected and screened for their effect in stimulating germination of spores of the test ectomycorrhizal fungi.
Table 5  Number and sources of isolates of bacteria and fungi that have been tested for stimulating germination.

<table>
<thead>
<tr>
<th></th>
<th>Paxillus involutus</th>
<th>Laccaria lacca</th>
<th>Hebeloma crustuliniforme</th>
<th>Amanita fulva</th>
<th>Amanita rubescens</th>
<th>Lactarius turpis</th>
<th>Russula nitida</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolates associated with sporophore</td>
<td>Bacteria</td>
<td>5</td>
<td>6</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>Isolates associated with mycorrhizal roots *</td>
<td>Bacteria</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>Soil Isolates</td>
<td>Bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30</td>
</tr>
</tbody>
</table>

* - All isolates were obtained from mycorrhizal short roots of birch attached to sporophores of the test fungi except in H. crustuliniforme mycorrhizal roots of willow (Salix spp) were used.
3.3.2 Isolation of bacteria and fungi associated with spores or sporophores of ectomycorrhizal fungi

Caps of clean and healthy sporophores of the test ectomycorrhizal fungi were surface-sterilized by wiping with tissue soaked in ethanol, peeled, broken open under aseptic conditions, and small pieces of the inside tissue were placed onto MMN agar (5 pieces per plate). Several plates were made for each fungus and incubated at 20 °C for seven days. Colonies with different morphological characteristics (42 of bacteria and 23 of fungi) were selected.

3.3.3 Isolation of bacteria and fungi associated with mycorrhizal sheaths of the test fungi

Mycorrhizal roots of birch (Betula sp.) or willow (Salix spp.) collected beneath sporophores of the test mycorrhizal fungi, and those having the characteristic morphology of the species concerned were chosen and treated as in method 2.12.5. Individual mycorrhizal tips (c. 40/species) were then plated out onto MMN agar, 5-8 in a plate, and incubated at 20 °C. 36 bacterial colonies and 24 fungal colonies with different morphological characteristics were selected.

All the bacterial isolates were transferred to fresh plates of nutrient agar and maintained on slopes of the same medium. Fungal isolates were transferred to fresh plates of PDA and maintained on the same medium.

To study the effect of these isolates on spore germination of the test mycorrhizal fungi; a disc of fungal mycelium or streak (c. 4cm long) of bacterial inoculum was placed on the surface of Fries agar plate previously seeded with spores of the test mycorrhizal fungi. Six plates were made for each treatment, three of them covered with activated charcoal. Some of the plates seeded with spores were left uninoculated with any of the test microorganisms as controls. The plates were then sealed with P.V.C. tape and incubated in darkness at 20 °C. Examination was daily under the light microscope.
3.3.4 Results

Table 6 shows the total number of the isolates tested and the number of isolates stimulating spore germination of ectomycorrhizal fungi.

Of 42 bacterial isolates associated with sporophores of mycorrhizal fungi, seven were active in stimulating spore germination of mycorrhizal fungi. All these isolates were obtained from sporophores of *Hebeloma crustuliniforme*, and stimulated spore germination of that fungus (Table 7), one isolate only stimulated spore germination of *Paxillus involutus* as well as *H. crustuliniforme*.

Also, two bacterial isolates, out of 36 (associated with the mycorrhizal sheath) were active in stimulating spore germination (Table 6). These isolates were obtained from mycorrhizas of *Salix* collected beneath sporophores of *H. crustuliniforme* and stimulated spores of the same fungus and of *P. involutus* (Table 7).

Two bacterial isolates out of 40 (obtained from birch wood soil) were active in stimulating spore germination; one of them, *Pseudomonas* sp. (S30) stimulated germination of *H. crustuliniforme* spores and the other, (an unidentified Gram+ coccus S19), stimulated spore germination of *H. crustuliniforme*, *P. involutus*, and *Laccaria laccata* (Table 7).

One fungus (*Tritirachium roseum*) and one bacterium (*Micrococcus roseus*), which appeared on the surface of agar plates, among spores of *H. crustuliniforme*, as a natural contaminant (Table 7) stimulated spores of *H. crustuliniforme* around their colonies.

None of the fungal isolates, obtained from sporophores, mycorrhizal sheaths or from soil was active in stimulating spore germination of any of the test ectomycorrhizal fungi up to 15 days incubation; observation for longer was not possible, as the fungal mycelium grew and covered the whole surface of the plate within 15 days.

*Pseudomonas stutzeri* was the most active bacterium in stimulating germination of *H. crustuliniforme* spores, germination started within three days and increased until it was c. 21% up to 63
Table 6  Total number of isolates tested and number of isolates stimulating germination of ectomycorrhizal fungi.

<table>
<thead>
<tr>
<th>Origin of the isolates</th>
<th>Isolates</th>
<th>Total number of isolates tested</th>
<th>Number of isolates stimulating germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sporophore of mycorrhizal fungi</td>
<td>Bacteria</td>
<td>42</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>23</td>
<td>None</td>
</tr>
<tr>
<td>2. Mycorrhizal roots</td>
<td>Bacteria</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>24</td>
<td>None</td>
</tr>
<tr>
<td>Natural contaminant</td>
<td>Bacteria</td>
<td>(-) *</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>(-)</td>
<td>1</td>
</tr>
<tr>
<td>Soil</td>
<td>Bacteria</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>30</td>
<td>None</td>
</tr>
</tbody>
</table>

* = (-) uncounted
Table 7  Identity, source, and stimulating activity * of the test bacterial and fungal isolates.

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Identity of the active isolates</th>
<th>Sources</th>
<th>Spores stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pseudomonas stutzeri (Lehmann and Neumann).</td>
<td>Sporophores of H. crustuliniforme</td>
<td>H. crustuliniforme</td>
</tr>
<tr>
<td>2</td>
<td>Pseudomonas sp. (no.1)</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>Pseudomonas sp. (no.2)</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>Pseudomonas sp. (no.3)</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>5</td>
<td>Pseudomonas sp. (no.4)</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>6</td>
<td>Corynebacterium sp. (no.1) (Lehmann and Neumann).</td>
<td>&quot;</td>
<td>H. crustuliniforme and P. involutus</td>
</tr>
<tr>
<td>7</td>
<td>Isolate of Enterobacteriaceae</td>
<td>&quot;</td>
<td>H. crustuliniforme</td>
</tr>
<tr>
<td>8</td>
<td>Micrococcus roseus (Flugg)</td>
<td>Natural contaminant on spores of H. crustuliniforme</td>
<td>&quot;</td>
</tr>
<tr>
<td>9</td>
<td>Tritirachium roseum</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>10</td>
<td>Arthrobacter sp. Conn and Dimmick).</td>
<td>Mycorrhizas of Salix sp. with H. crustuliniforme</td>
<td>H. crustuliniforme</td>
</tr>
<tr>
<td>11</td>
<td>Corynebacterium sp. (6a) (Lehmann and Neumann).</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>12</td>
<td>Pseudomonas sp. (S30)</td>
<td>Birch wood soil</td>
<td>H. crustuliniforme</td>
</tr>
<tr>
<td>13</td>
<td>Unidentified bacterium (S19)</td>
<td>&quot;</td>
<td>H. crustuliniforme, P. involutus, and L. laccata</td>
</tr>
</tbody>
</table>

*: For the details of identification of these isolates, see appendix No. 2.
1cm from the bacterial colony after ten days incubation (Table 8) (spores were counted in ten microscopic fields, chosen randomly in each of three replicate plates). 1-2cm from the bacterial colony germination was c. 1.7%. Numerous spores also germinated underneath the bacterial growth.

Plate No. 2 and 3 shows the germinating spores of *H. crustuliniforme* on Fries agar plate near a colony of *P. stutzeri* and *M. roseus* (respectively).

The effect of *P. stutzeri* was tested in the presence of activated charcoal and growing mycelium of *H. crustuliniforme* either singly or in combination with each other (Table 8). Charcoal slightly increased germination in the presence of mycelium but not the bacterium. Mycelium had no effect on germination either alone or in combination with the bacterium.

Spores of *H. crustuliniforme* were stimulated to germinate by the two isolates of *Corynebacterium* after two weeks incubation, and after five weeks by *M. roseus* and *T. roseum*. The exact percentage of germination could not be counted with these isolates, but it was <1% very close to the activator colonies and in the presence and absence of charcoal. Also slight stimulation (<0.1% germination) occurred in the presence of other active bacteria (*Pseudomonas* No. 1, 2, 3 and 4; *Arthrobacter* sp. and an isolate belonging to the Enterobacteriaceae).

Spores of *P. involutus* were stimulated by the two isolates of *Corynebacterium* sp., *Arthrobacter*, and the unidentified soil bacterium (S19) (Table 7) but in the presence of activated charcoal only. The percentage of germination (<0.1%) was not counted, as most of the spores germinated either very close or underneath the bacterial growth.

Spores of *L. laccata* were stimulated by the unidentified soil bacterium (S19) in the presence of activated charcoal. The percentage of germination was very low (<0.01).

In all cases, germinating spores produced mycelial colonies which were then transferred to fresh plates of MMN agar to produce mycelial cultures.
Table 8  Spore germination of H. crustuliniforme on Fries agar with different supplements.

<table>
<thead>
<tr>
<th>Distance from the bacterial colony</th>
<th>No supplement</th>
<th>Bacterium (1)</th>
<th>Bacterium + charcoal</th>
<th>Bacterium + mycelium</th>
<th>Bacterium + mycelium + charcoal</th>
<th>Mycelium (2)</th>
<th>Mycelium + charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1cm</td>
<td>Nil</td>
<td>21.2</td>
<td>20.7</td>
<td>21.3</td>
<td>21.6</td>
<td>Nil</td>
<td>&lt;0.01 *</td>
</tr>
<tr>
<td>1-2cm</td>
<td>Nil</td>
<td>1.7</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1) : Bacterium = P. stutzeri  
(2) : Mycelium = mycelium of H. crustuliniforme  
*: after 30 days incubation  
(-): uncounted
Plate 2  Germinated spores of *H. crustuliniforme* close to a colony of *P. stutzeri* (x250)
Plate 3  Germinated spores of *H. crustuliniforme* close to a colony of *Micrococcus roseus* (x250)
3.4 Screening a wide range of Pseudomonas isolates for stimulatory activity on spore germination of Hebeloma crustuliniforme

From the previous experiment, it was found that all the active isolates of Pseudomonas, irrespective of their origin, stimulated germination of spores of H. crustuliniforme but not spores of any of the other mycorrhizal fungi tested. Therefore it was necessary to test the ability of a wide range of Pseudomonas isolates in stimulating germination of spores of H. crustuliniforme.

Seventy isolates obtained from soil at the University of Surrey were tested.

3.4.1 Isolation

Fresh soil samples were collected around willow (Salix spp.) trees at the University of Surrey, and treated as in (3.3.1). One composite sample was made, and 10 grams were suspended in 90ml sterile distilled water, shaken in a Gallenkamp wrist-action shaker for 15 minutes, and allowed to stand for 5 minutes. From this suspension, serial dilutions, (up to \(10^{-5}\)) were made, and 0.1ml of each dilution was used to inoculate the surface of Pseudomonas Agar Base supplemented with antibiotic C-F-C SR103 (see materials, 2.1.6). Three replicas were made for each dilution, and incubated at 20°C for seven days.

3.4.2 Germination test

All the colonies (70 colonies) were used to inoculate plates of Fries agar previously seeded with spores of H. crustuliniforme. The plates were then sealed with P.V.C. tape and incubated at 20°C.
3.4.3 Results

Of 70 isolates, two were active and stimulated germination of spores of the test fungus. Germination was less than 1% close to the bacterial colony.
3.5 Study of the nature of stimulatory compounds produced by Pseudomonas stutzeri

From the previous experiment (3.3) it has been found that stimulation of spore germination occurred not only very close, but also more than 2cm away from the bacterial colony. This suggests the production of a diffusible compound or compounds by the bacterium causing stimulation.

To learn more of the nature of this or these compounds the following possibilities have been tested.

3.5.1 Test for volatility

The possibility that the active compound produced by the bacterium is volatile was tested by the "inverted dish method" (Brown and Merrill, 1973): Plates of Fries agar were seeded with spores of H. crustuliniforme (as in 3.1.1) and left overnight to allow absorption of water by the agar. Then these plates were taped to plates of two day-old cultures of P. stutzeri on Fries agar. Four replicates were made and incubated at 20°C with the plate containing spores on the top, and examined daily under the light microscope for spore germination.

3.5.2 Stable diffusible compounds

To determine whether the stimulation was caused by stable compounds diffusing into the agar medium; P. stutzeri was grown on Fries agar for 3, 7, or 14 days. Three plates were used for each period. Then the growth was washed, the agar from plates of each treatment was collected separately in 100ml Duran bottles, autoclaved at 120°C for 10 minutes and re-poured in sterile Petri dishes. The surface of these plates was seeded with spores of H. crustuliniforme. The plates were then taped, incubated at 20°C, and examined daily under the light microscope for spore germination.
3.5.3 Extracellular compounds

Cell-free culture filtrate was used for this test: 200ml of Fries medium in each of four conical flasks was inoculated with 1ml from the same culture of P. stutzeri (48 hour-old culture) and incubated at 20 °C with continuous shaking using MKV orbital shaker, amplitude 5.

The content of one flask at 1, 2, 3, and 7 days incubation (with bacterial count of 2.0, 3.8, 3.8, 3.7 x 10^8 respectively) were centrifuged (MSE-Chilspin) at 4500 rpm and 4 °C for 40 minutes. Then the supernatants were collected and half the volume of each supernatant was concentrated 10x by freeze-drying. Both the concentrated and unconcentrated filtrates were sterilized by passing through Millipore filters (HAWP 025, pore size 0.45um), and used directly or, for further study stored at -20 °C.

The effect of cell-free culture filtrate on spore germination was tested as follows:

a - 50ul of the concentrated and several dilutions (1, 2.5, 5, 10, 50, 100% v/v in sterile distilled water) of the unconcentrated filtrate were introduced into 3mm diameter holes in Fries agar plates (4.5cm diameter) previously seeded with spores of the test fungus (H. crustuliniforme). Four holes were made in a plate and four plates used for each treatment. The plates were then taped and incubated at 20 °C.

b - Filtrate was added to molten and cooled Fries agar in concentrations of 1, 5, 10, and 15% v/v (filtrate/agar) of the unconcentrated filtrate and 1, 2, 4, 8 and 10% v/v (filtrate/agar) of the concentrated filtrate. Small Petri dishes (4.5cm diameter were used for this test).

The surface of these plates was then seeded with spores of the test fungus, the plates sealed with P.V.C. tape and incubated at 20 °C. Four plates were made for each concentration. Examination was daily under the light microscope.
3.5.4 Intracellular compounds

Cell-free extracts were used for this test: These were prepared according to the method used by Garcia-Rodriguez et al. (1984) with some modifications. The cells harvested during the preparation of cell-free culture filtrate (Exp. 3.5.3) were washed twice with 0.1M sodium phosphate buffer at pH 6.8. They were then re-suspended in 5ml of the same buffer and sonicated on ice for three minutes at amplitude 3 using an MES sonicator. The disrupted cell suspension was centrifuged (MSE High Speed 18) at 1500 rpm and 4 °C for 30 minutes. The supernatant was collected and the volume adjusted to 10 ml with distilled water. Then the resulting sample was sterilized by passing through a Millipore filter (HAWP 025, pore size 0.45um) and used directly or, for further study, stored at -20°C.

50ul of the undiluted and 1, 2.5, 5, 10, 20, 50% (v/v in sterile distilled water) dilutions of the prepared cell extract were introduced into 3mm diameter holes in Fries agar plates (4.5cm diameter) previously seeded with spores of the test fungus. Four holes were made in a plate and four plates for each treatment. The plates were then taped and incubated at 20°C.

3.5.5 Iron-chelating compounds

Under conditions of iron deficiency many bacteria produce specific high-affinity iron-chelating compounds, termed siderophores (Lankford, 1973). A species of Pseudomonas (UV3), isolated from leaves of red beetroot by Blakeman and Parbery (1977), produced a siderophore in iron-deficient medium that was shown to be highly stimulatory to the germination of Colletotrichum musae (McCacken and Swinburne, 1979, 1980). Also Slade et al. (1986) found that the addition of a suspension of washed cells of Pseudomonas sp. (isolate UV3) to conidial suspension of Colletotrichum acutatum stimulated germination and appressorium formation; filtrate from iron-deficient cultures of the Pseudomonas were themselves highly stimulatory. Also they found that a
siderophore (SA), purified from low-iron culture of the bacterium, stimulated germination and appressorium formation to a significantly greater extent than the bacterial cells alone.

To test the possibility of production of a siderophore-like compound by Pseudomonas stutzeri; the bacterium was grown in media with no iron and with low and high concentrations of iron. Fries medium was used for this as follows:

a - without iron.
b - with 0.8mg/1 FeCl3.6H2O (representing a normal concentration).
c - with 8 mg/1 FeCl3.6H2O.

Another set of the above three media was made, but omitting the vitamin mixture.

100ml of each of the above media was inoculated with 1ml from the same culture of P. stutzeri (48 hour-old culture) and incubated for seven days at 20°C with continuous shaking [orbital shaker (MKV)] at amplitude 5.

Living bacteria and filtrates from the above cultures were tested for their effect on germination of spores of H. crustuliniforme.

The filtrates were prepared as in experiment 3.5.3 and incorporated into molten and cooled agar of the same media (with and without iron or vitamins) in concentrations of 0.1, 0.5, 1, 2, 4, 8, and 10% v/v (filtrate/agar) of the 10X concentrated filtrate. Small Petri dishes (4.5cm diameter) containing 5ml agar were used in this test. Four plates were made for each concentration, and after the agar had set the surface was seeded with spores of H. crustuliniforme.

Germination was also tested on plates of the same media but without filtrate, some of them were inoculated with bacteria from broth of the same media, by making a thick streak (c. 2cm long) of the bacterial inoculum on the centre of the plate; some of the plates were left uninoculated as controls.
3.5.6 Results

Testing all the possibilities mentioned in this experiment; no germination had occurred in the absence of living growth of *P. stutzeri*.

In the presence of the vitamin mixture, only living bacteria from media containing 0.8mg/l and 8mg/l FeCl₃.6H₂O stimulated spore germination on the same media (Table 9), whereas in the absence of the vitamins, stimulation occurred in medium containing 8mg/l but not 0.8mg/l FeCl₃.6H₂O, bacteria from iron or vitamin deficient media did not stimulate spore germination on the same media. This indicates that, for spore germination the role of vitamins can be replaced by increasing the amount of iron in the medium.

Growth of *P. stutzeri* (on the basis of cell number) was affected by the amount of iron used in the growth medium, and also by the presence and absence of vitamins (Table 10). In the presence of vitamins, the best growth was obtained in a medium containing 0.8mg/l FeCl₃.6H₂O in which the total bacterial count was twice that in the medium containing 8mg/l and more than that in medium without iron.
Table 9  Effect of iron and vitamins in its growth medium on the stimulatory activity of P. stutzeri.

<table>
<thead>
<tr>
<th>Concentration of iron in the growth medium</th>
<th>+ vitamin mixture</th>
<th>- vitamin mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ 8mg/l FeCl3.6H2O</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+ 0.8mg/l FeCl3.6H2O</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>without iron</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ : stimulation
- : no stimulation
Table 10  Effect of iron and vitamins in the growth medium on the total numbers of P. stutzeri.

<table>
<thead>
<tr>
<th>Concentration of iron in the growth medium</th>
<th>Total count of P. stutzeri x 10^6 (Haemocytometer)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ vitamin mixture</td>
</tr>
<tr>
<td>+ 8mg/l FeCl3.6H2O</td>
<td>9.24</td>
</tr>
<tr>
<td>+ 0.8mg/l FeCl3.6H2O</td>
<td>18.03</td>
</tr>
<tr>
<td>without iron</td>
<td>7.81</td>
</tr>
</tbody>
</table>
3.6 Germination near plant roots in mineral salts medium

Plants

Seedlings of tree and non-tree species mentioned in the materials section (2.10) were used.

Procedures

A modification of the Fahraeus slide technique (Nutman, 1970) was used for this study. The modified Fahraeus slide is made from a microscope slide and coverslip separated by glass spacing pieces (see method 2.8).

Basidiospores of the test mycorrhizal fungi were removed from the plastic Petri dishes (immediately after collection) by dispersing them in sterile distilled water (a mixture of spores from 3-5 fruitbodies was used).

Quarter-strength Ingestad's mineral salts agar (Ingestad, 1962 - see materials, 2.1.3), cooled to c. 40°C after autoclaving, was seeded with the spore suspension, to give a final concentration of c.5 x 10^5 spores/ml agar. This seeded agar was then used to fill the space between the slide and the coverslip (c. 1ml/cell). The cells were planted (immediately after filling with agar medium) with 5 day-old seedlings (from aseptically germinated seeds on water agar) of the test plants, by pushing the radicle gently underneath the coverslip, one seedling per cell, control slides had no seedlings. Three replicates were used for each treatment. Each cell was then placed into a boiling tube (32 x 200mm) containing 10ml of quarter strength Ingestad's liquid medium (Plate 4). The plant culture tubes were held in racks and the roots were shaded with black polythene. Incubation was in growth cabinets, the temperature range was 12-20 °C and the light period was 18 hours. Illumination (1000 lux) was by fluorescent lights placed c. 80cm above the seedlings.

Slides were examined weekly by light microscopy. Spores germinating within 1mm from the root edge, and germ tubes growing
Plate 4 A tube containing Ingestad mineral solution, and Fahraeus cell filled with Ingestad mineral agar and planted with one birch seedling
towards the roots were counted in 10 microscopic fields (in each of the three replicates) under 250x magnification, photographs were taken of the germinating spores near the roots. Also the maximum distance from the root where stimulation occurred was measured.

3.6.1 Results

Roots of five of seven tree species tested and of white clover (Trifolium repens) stimulated germination of Paxillus involutus spores. Spores of Laccaria laccata and Hebeloma crustuliniforme were stimulated by birch (Betula pubescens) and pine (Pinus nigra) only, while those of Lactarius turpis and Amanita fulva were slightly stimulated by birch roots. In all treatments, irrespective of the presence of roots, a few spores (only when they were in clusters), of Amanita rubescens germinated. Spores of Russula nitida did not respond to roots of any of the plants tested (Table 11).

Birch had the greatest effect of the plants tested and germination of spores of P. involutus, L. laccata and H. crustuliniforme near its roots started during the second week of incubation. By the end of the second week, growth of germ tubes of these fungi was too dense to permit counting within 1mm of the proximal 5mm of the roots.

The highest countable germination (30%) was that of H. crustuliniforme near the mid-point of the roots. Germination of the three fungi was <0.1% close to root tips and did not occur further than 5mm from root tips. Spores of H. crustuliniforme germinated up to 8mm from the root edge, L. laccata 5mm and P. involutus 3mm.

Within c.1mm from the birch root edge 80% of the germ tubes produced by P. involutus basidiospores, 82% of L. laccata, and 76% of H. crustuliniforme grew towards the roots.

The growth of germ tubes of L. laccata towards birch roots is shown in Plate 5.
Table 11  Germination of basidiospores near *roots* of seedlings of different tree and crop species in mineral salts medium (up to eight weeks incubation)

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>P. involutus</th>
<th>L. laccata</th>
<th>H. crustuliniforme</th>
<th>A. rubescens</th>
<th>A. fulva</th>
<th>L. turpis</th>
<th>R. nitida</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betula pubescens</td>
<td>9(1)</td>
<td>13(2)</td>
<td>30(2)</td>
<td>&lt;1</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>Pinus nigra</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Picea sitchensis</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Larix decidua</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eucalyptus nitens</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudotsuga menziesii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tsuga heterophylla</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactuca sativa</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Allium cepa</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lycopersicon esculentum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brassica oleracea</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trifolium repens</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control (without plant)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(1) : root surface to 1mm
(2) : at the end of the second week. These figures are significantly different from one another at p = 0.001. (See appendix 3, A.1.2).
(+): up to six spores out of 100,000
- : no germination
Plate 5 Germinated spores of *L. laccata* and growth of germ tubes towards root of birch seedling in mineral salts medium in Fahraeus cell (x500)
3.7 Germination near plant roots in soil

Plants

1 - Birch (Betula pubescens)
2 - Spruce (Picea sitchensis)

Soils

1 - Birch wood soil
2 - Spruce forest soil
3 - Arable soil

Procedures

2.0% (w/v) water agar molten and cooled (c. 40°C after autoclaving) was seeded with spores of the test fungi (as in 3.6). Microscope slides were dipped in alcohol, flamed, cooled, and coated with the seeded agar c. 1mm thick (immediately after seeding with spores). After the agar had set, the slides were buried obliquely in 200cm³ pots of steamed or untreated soils. Birch and spruce seedlings (one week-old from seeds germinated on peat), were planted over the slides so that the roots were likely to make contact with the slides and grow over them, one seedling per pot. Control pots had no seedlings. All the pots were watered to maintain c. 60% water holding capacity. Incubation was in growth cabinets, the temperature was in the range 12-20°C, and the light (1000 lux) was from fluorescent tubes placed c. 80cm above the seedlings, for 18 hours/day.

Three slides from each treatment were carefully removed (the pots were unwatered four days before slide recovering) at 2, 4, 6, and 8 weeks after burial. They were air dried and most of the soil was gently brushed off them. Also larger root pieces were carefully removed with the tip of a sharp scalpel. The slides were stained by immersion for 1 hour in acetic aniline blue stain (Jones and Mollison, 1948), (see materials and methods 2.5), then they were
rapidly washed with water and dehydrated in 95% ethanol for five minutes. Permanent preparations were made by mounting in Euparal (G.B.I. Laboratories Ltd.)

The following observations were made on each slide:

1 - Number of spores germinated, and the direction of germ tubes or hyphae produced by the germinating spores at different distances from the root edge were recorded. Counts were made in 10 microscopic fields (in each of the three replicates) under 250X magnification.

2 - The length of 40 germ tubes of each treatment was measured under the same magnification using an eyepiece micrometer.

3 - Photographs were taken to show the germinating spores and direction of germ tubes with a 35mm camera.

4 - Other observations were also recorded.

Results

3.7.1 The influence of roots on basidiospore germination in birch wood soil

Only *P. involutus* spores of the seven species tested were stimulated by birch and spruce roots in steamed and untreated soil throughout the duration of the experiment (8 weeks). Birch roots (within 1mm from the edge) stimulated up to 96% of spores to germinate in untreated soil, which is significantly more than in steamed soil (88%) (Table 12).

Germination at 0-1mm was greater than that at 2-4mm from the root edge, and the majority of germ tubes grew towards the root.

Spruce had much less effect on spore germination in either steamed or untreated soil (Table 12) and germination did not increase near primary roots up to 8 weeks.

Germ tubes grew longer near birch than near spruce roots or in the control slides (Table 13).
### Table 12  Germination of Paxillus involutus basidiospores and growth of germ tubes near birch and spruce roots in birch wood soil after two weeks incubation

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Distance from root edge mm</th>
<th>Steamed Soil</th>
<th>Untreated soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germination %</td>
<td>Germ tubes growing towards root %</td>
<td>Germination %</td>
</tr>
<tr>
<td>birch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>88</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>2-4</td>
<td>5</td>
<td>40</td>
<td>19</td>
</tr>
<tr>
<td>&gt;4</td>
<td>&lt;1</td>
<td>-</td>
<td>&lt;1</td>
</tr>
<tr>
<td>spruce</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>2.4</td>
<td>31</td>
<td>2.8</td>
</tr>
<tr>
<td>2-4</td>
<td>&lt;1</td>
<td>-</td>
<td>&lt;1</td>
</tr>
<tr>
<td>&gt;4</td>
<td>&lt;0.1</td>
<td>-</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.01</td>
<td></td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

- : uncounted

Differences between numbers of germinated spores near birch roots (0-1 and 2-4mm) in steamed and untreated soil are significant at $p = 0.001$

Differences between numbers of spores germinated near birch and those near spruce roots are significant at $p = 0.001$ in all cases.

Differences between number of spores with germ tubes growing towards birch roots at 0-1 and 2-4mm are significant at $p = 0.001$ in steamed and untreated soil.

Differences between number of spores with germ tubes growing towards birch and those growing towards spruce are significant at $p = 0.001$ (see appendix 3, A.2.2.).
Table 13  Lengths of germ tubes (um)* produced by Paxillus involutus basidiospores within 1mm of the root edge after two weeks being buried in birch wood soil.

<table>
<thead>
<tr>
<th>Seedling root</th>
<th>Steamed soil</th>
<th>Untreated soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>birch</td>
<td>65.0 a</td>
<td>72.8 a</td>
</tr>
<tr>
<td>spruce</td>
<td>48.2 b</td>
<td>47.8 b</td>
</tr>
<tr>
<td>Control</td>
<td>21.1 c</td>
<td>19.7 c</td>
</tr>
</tbody>
</table>

* : Mean lengths of 40 germ tubes

(1) : Figures followed by different letters are significantly different from one another at p = 0.001 (see appendix 3, B.2.).
One germ tube only developed from one of the end points of the spore, and usually grew towards the root without branching (Plate 6).

Approximately 9% of germ tubes branched in two immediately after emerging from the spore, one branch grew and progressed whereas the other remained very short (c. 5 μm) and adjacent to the spore (Plate 7).

The tips of some germ tubes branched and formed fan-shaped appressorium-like structures (Plate 8). This was frequently observed near or on the surface of the roots or root hairs of birch (Plate 9), and it may be analogous to the beginning of infection or fungal sheath formation on the roots.

When the incubation period was prolonged to 8 weeks, germ tubes grew longer, a net of hyphae was formed around the root, and short roots when present became mycorrhizas (Plates 10). Unfortunately, the number of mycorrhizas could not be estimated here; as most of the larger pieces of the attached roots were removed and discarded before mounting the slides. Whether monokaryon or dikaryon hyphae caused the infection is unknown, but no clamp formations were seen in the hyphae around the mycorrhizas.

Germ tubes of P. involutus were shown sometimes to be attracted by spores (usually germinated spore). The germ tube either grew directly towards the spore and formed a fan-shaped structure before apparently becoming attached to the spore, (Plate 11), or encircle the spore in a spiral manner (Plate 12). Contact between spore and germ tube was not clear.

3.7.2 The influence of roots on basidiospore germination in spruce forest soil and arable soil

Birch seedlings in spruce forest and arable soils grew poorly and had little or no effect on the germination of P. involutus basidiospores (Table 14), in contrast to birch wood soil.

Roots of spruce in spruce forest soil had a similar effect to that in birch wood soil, but spruce seedlings died in arable soil.
Plate 6 Germinated spores of *P. involutus* and growth of germ tubes towards root of birch seedling (R) on a slide buried in birch wood soil (x300)
Plate 7 Spores of *P. involutus* showing germ tubes with two branches, on a slide buried in birch wood soil (x1200)
Plate 8 Fan-shaped appressorium-like structure produced by germinated spore of *P. involutus* on a slide buried in birch wood soil (x1200)
Plate 9 Fan-shaped appressorium-like structures produced by germinated spores of *P. involutus*, A: on the surface of a detached cortical cell (CC) (x400), B: on the surface of a root hair (RH) (x1200). On slides buried in birch wood soil.
Plate 10 Mycorrhiza of birch produced by germinated spores of *P. involutus* on a slide buried in birch wood soil for eight weeks (x135)
Plate 11  Germ tubes from spores of *P. involutus* growing towards spores of the same species on a slide buried in birch wood soil (x400).
Plate 12 Germ tubes from several spores of *P. involutus* growing and encircling the spores of the same species (x400)
Table 14 Germination % of Paxillus involutus basidiospores near roots of birch and spruce in two different soils. (up to 1mm from the root edge).

<table>
<thead>
<tr>
<th>Seedling Root</th>
<th>Spruce Forest Soil **</th>
<th>Arable Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steamed</td>
<td>Untreated</td>
</tr>
<tr>
<td>birch</td>
<td>2.3</td>
<td>2.6</td>
</tr>
<tr>
<td>spruce</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*: Spruce seedlings died

**: Differences in number of germinated spores near birch and spruce roots in steamed and untreated spruce forest soil are not significant at p = 0.10 (see appendix 3, A.3.2.).
3.8 Response of Paxillus involutus basidiospores to roots of host and non-host tree seedlings in soil

Basidiospores

Basidiospores of *P. involutus* were obtained from sporophores collected beneath trees of two different species (pine and birch) from two different areas (Witley and Whitmoor Common, Surrey). The spores were stored for a month at 5°C and relative humidity c. 40% before use.

Soil

Untreated birch wood soil was used for this test.

Plants

Seedlings of birch (*Betula pubescens*) and pine (*Pinus nigra*).

Germination test

Germination of spores from both collections was tested with seedlings of pine and birch using the buried slide technique, similar to that used in experiment 3.7 and under the same conditions of temperature and light.

Three slides from each treatment were recovered after four weeks incubation, air-dried and most of the soil and large root pieces gently removed (as in 3.7). The slides were then stained and mounted in Euparal (as in 3.7).

Numbers of spore germinated and the direction of germ tubes produced by the germinating spores were estimated in 10 microscopic fields (under 250X magnification) near the root (0-1mm from the edge) in each of the three replicates.
Results

The results in Table 15 show no specificity in response of spores of *P. involutus* to seedling roots of the host plant. The number of spores germinated near roots of the non-host seedlings is slightly higher than that near roots of host seedlings. On the other hand the number of germ tubes growing towards roots of birch, when the origin of spores was birch, was higher than when the spores originated from pine. However, the numbers of germ tubes growing towards pine were nearly the same whether the spores originated from pine or birch.

Roots of birch had a greater effect on spore germination and attraction of germ tubes than those of pine in all cases.
Table 15 Effect of roots of host and non-host tree seedlings on germination of spores of *P. involutus* in soil.

<table>
<thead>
<tr>
<th>Origin of P. involutus</th>
<th>Germination test seedlings</th>
<th>Germination % (within 1mm from the root edge)</th>
<th>Germ tubes directed towards root %</th>
</tr>
</thead>
<tbody>
<tr>
<td>birch</td>
<td>birch</td>
<td>(1) 84 a</td>
<td>(1) 98 a</td>
</tr>
<tr>
<td>pine</td>
<td>birch</td>
<td>86 a</td>
<td>94 b</td>
</tr>
<tr>
<td>birch</td>
<td>pine</td>
<td>20 b</td>
<td>75 c</td>
</tr>
<tr>
<td>pine</td>
<td>pine</td>
<td>18 b</td>
<td>74 c</td>
</tr>
<tr>
<td>birch</td>
<td>control (no seedling)</td>
<td>&lt;0.01</td>
<td>-</td>
</tr>
<tr>
<td>pine</td>
<td>control (no seedling)</td>
<td>&lt;0.01</td>
<td>-</td>
</tr>
</tbody>
</table>

(1) : Differences between figures followed by the same letter are significant at p = 0.05 but not at p = 0.01, and those followed by different letters are significant at p = 0.001.

(2) : Differences between figures followed by different letters are significant at p = 0.001, and those followed by the same letters are not significant at p = 0.10 (see appendix 3, A.4.2.).
Germination in the presence of root exudate "in vitro"

Exudations collected in distilled water for 18-20 hours from roots of birch seedlings, previously grown for 6 weeks in birch wood soil (see method 2.7) were tested for their effect on spore germination of the test fungi.

Various methods were tested as follows:

1 - 50ul of the undiluted and dilutions (10, 5 and 2.5% v/v in deionized water) of the root exudate preparation were introduced into 3mm diameter holes (4 holes/plate) in Fries agar plates (4.5cm diameter) previously seeded with spores of the test fungi (c. 2.0 x 10^6 spores/plate).

2 - Undiluted exudate was incorporated into molten and cooled Fries agar in the proportion of 1, 5, 10, and 20% (v/v) exudate/agar. The agar surface was then seeded with spores of the test fungi.

3 - Spores of the test fungi were soaked in root exudate (undiluted and 10% (v/v) in deionized water) for one hour and 24 hours at 5°C. Then 0.1ml of the soaked spores was spread over the surface of the Fries agar.

Six plates were made for each concentration using the above methods; in three of them the surface was dusted with activated charcoal. The plates were then sealed with P.V.C. tape and incubated in darkness at 20°C.

4 - Spores of the test fungi were suspended in root exudate (undiluted and 10% v/v in deionized water) 2mm deep in glass rings cemented to glass slides, in sterile Petri dishes, and incubated at 20°C.

Results

Root exudates did not stimulate germination of Paxillus involutus, Laccaria laccata, Amanita fulva, Amanita rubescens,
Lactarius turpis, or Russula nitida spores in any concentration used up to three months incubation. Spores of Hebeloma crustuliniforme were stimulated after three days incubation by root exudate introduced in holes or incorporated in Fries agar in concentrations of 5, 10, and 20% (v/v) but not 1% (Plate 13), either in the presence or absence of activated charcoal. Also, spores germinated when they were incubated directly in root exudate (undiluted and 10% (v/v) in deionized water).
Plate 13 Mycelial colonies from spores of *H. crustuliniforme* germinated on Fries agar plates containing, A: 1%, B: 5%, and C: 10% root exudate/agar
3.10 Relationship between number of spores and their germinability

In order to find out whether there is any relationship between numbers of spores and their germinability, different densities of spores of *Hebeloma crustuliniforme* were tested on Fries agar plates containing 10% root exudate (see exp. 3.9).

Three different spore concentrations between $1.0 \times 10^3$ to $1.0 \times 10^5$ spores/plates were used. Spores used in this test were stored (for three months before use) at 5°C and relative humidity c. 40% (see method 2.12.3).

The results in Table 16 show the number of spores used and the number of spores germinated after 10 days incubation at 20°C.

From this table it can be seen that the number of spores germinated is approximately proportional to the number of spores used.

Table 16 Relationship between spore densities and number of spores germinated in *H. crustuliniforme* on Fries agar with 10% root exudate.

<table>
<thead>
<tr>
<th>Total number of spores/plate</th>
<th>Number of spores * germinated</th>
<th>Germination %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1.23 \times 10^5$</td>
<td>2820</td>
<td>2.3</td>
</tr>
<tr>
<td>$1.23 \times 10^4$</td>
<td>200</td>
<td>1.6</td>
</tr>
<tr>
<td>$1.23 \times 10^3$</td>
<td>15</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* : mean of two replicates
3.11 Characterization and identification of the active compounds in the root exudate

For characterization and identification of the active compound(s) in the root exudate, the following tests were made:

3.11.1 Volatility test

This test was made by the inverted dish method (Brown and Merrill, 1973). Plates of Fries agar seeded with spores of H. crustuliniforme were taped to plates containing root exudate or filter paper soaked with root exudate.

The plates were incubated at 20°C in darkness.

Result

No active volatile compounds were demonstrated to be present in the root exudate, since no germinating spores were seen up to three months incubation.

3.11.2 Ion exchange experiment

Cationic, anionic and neutral compounds of the root exudate were separated using cation- and anion-exchange resins (see flow diagram).

Cation-exchange resin Dowex 50 x 8, hydrogen form, mesh 100-200, and anion-exchange resin Dowex 1 x 8, chloride form, mesh 100-200 (Dow chemical USA), were packed (separately) in columns (3cm by 15cm), and washed with deionized water until the pH of the washing water became c. pH 7. 40ml of the root exudate preparation were passed slowly through the cation-exchange column which was then washed by passing about 500ml deionized distilled water through it. The resulting sample (which contains the neutral and the anionic compounds), was passed through the anion-exchange column and washed with about 500ml deionized distilled water. This final effluent was presumed to contain the neutral substances e.g. sugars.
Flow diagram: Fractionation of root exudates

Root exudates in distilled water

↓

Freeze-dried

↓

Made up to 4ml/root with H2O (pH5.8)

↓

40ml (of the above)

↓

Cation-exchange column

500ml H2O

↓

Absorbed

↓

1L. 4N. NH4OH

↓

Eluate

↓

Freeze-dried

↓

Filter sterilized

↓

Bioassay *

↓

Absorbed

↓

Anion-exchange column

↓

Eluate

↓

500ml H2O

↓

Absorbed

↓

1L. 5N. HCOOH

↓

Freeze-dried

↓

Made up to 20ml with H2O (pH5) and adjusted to pH5.8 with 10N.KOH

↓

Filter sterilized

↓

Bioassay *

↓

Bioassays were made by incorporating fractions to be tested into molten and cooled Fries agar in the proportions of 5, 10, 20% v/v, in small Petri dishes (4.5cm diameter). The surface of these plates was seeded with spores of the test fungus.
The cationic substances were eluted from the cation-exchange column with 1 litre 4N.\text{NH}_4\text{OH}, and the anionic substances with 1 litre 5N.\text{HCO}_2\text{OH} from the anion-exchange column.

The three fractions were evaporated to dryness (freeze-drying) and dissolved in 20ml deionized water.

The pH of the three fractions was respectively 5.6, 5.0, and 4.0 and all were adjusted with 10N.KOH to pH 5.8, that of the original root exudate. The fractions were sterilized by passing through a Millipore filter (HAWP 025, pore size 0.45um) and stored at -20°C. These fractions were incorporated into molten and cooled Fries agar (at c. 40°C after autoclaving) in the proportions of 5, 10, and 20% (v/v). Small Petri dishes (4.5cm diameter) were used. After the agar had set the surface was seeded with spores of \textit{H. crustuliniforme} (c. 200,000 spores/plate). Four plates were made for each concentration, sealed with P.V.C. tape, and incubated at 20°C in darkness.

Results

Spores of \textit{H. crustuliniforme} germinated on plates containing the fraction of the root exudate eluted from the cation-exchange column, at all concentrations tested, after three days incubation. The other fractions were inactive.

Stimulation by the active fraction was about half that of the unfractionated root exudate, this may be because some of the active compound was still held on the resin particles either because the volume of the eluent (\text{NH}_4\text{OH}) was not enough to extract all the active compounds from the resin, or because of the presence of some other compounds which could not be eluted by \text{NH}_4\text{OH}.

3.11.3 Ninhydrin test

About 0.1ml of the unfractionated exudate and the active fraction was spotted on strips of filter paper, air dried, sprayed with ninhydrin solution (0.2% in acetone); air-dried again and heated over a hot plate for two minutes.
Results

Spots of both the unfractionated and active fraction of the root exudate gave a positive reaction to ninhydrin (purple colour).

3.11.4 Paper chromatography

a - Separation of the active compound by descending paper chromatography.

Samples:
1 - crude root exudate (unfractionated).
2 - active fraction of the root exudate (eluted from cation-exchange column).

These samples were used either in water (i.e. without further treatment) or 10ml of each sample was freeze-dried and the powder dissolved in 1ml diethyl ether.

Standard amino acids:
Fourteen amino acids were used as standards. These were:
Glycine(L), Alanine(L), Valine(L), Leucine(L), Isoleucine, Aspartate, Glutamate(L), Asparagine, Glutamine, Lysine(L), Arginine(L), Histidine(L), Serine, and Threonine(L).

10mM solutions of each of these amino acids were prepared in 20% ethanol in water.

Solvent system:
Ethanol: water: ammonia soln. 0.880.
(80 : 10 : 10 by volume)

Procedure:
On Whatman No. 1 paper (56 x 46cm) a pencil line was drawn 10cm from one of the narrow ends. The origins of the samples were marked on this line.
Different amounts (20, 30, 50ul) of the crude root exudate
and the active fraction in water or in diethyl ether were spotted on the marked places (using a 10ul capillary tube). One sheet was used for the sample in water and one for the sample in diethyl ether.

20ul of each of the 14 standard amino acids were spotted between the samples on each of the two sheets. The spots were dried rapidly with a hair dryer. Then the prepared papers were hung in the tank with the end closest to the origin of the samples sitting in the trough. The solvent was poured carefully into the trough and into the bottom of the tank an hour before hanging the paper to allow saturation of the environment with the solvent.

After eight hours running (solvent moved c. 30cm), the chromatograms were lifted from the solvent, air-dried and sprayed with ninhydrin solution (0.2% in acetone).

Results

The spots of the standard amino acids gave positive reactions with ninhydrin, and each amino acid moved to a distance different from that of the other amino acids.

Samples did not show any spots reacting positively with ninhydrin at any concentration tested, but a streak of fluorescent substances was observed when the paper was examined under UV light. When the crude exudate was used the fluorescent streak extended 2cm from the origin, whereas the fluorescent streak of the active fraction was 12cm long but the brightest part of it was between 0.5 and 1.5cm from the origin.

b - Bioassay of the chromatogram

For bioassay; 50 and 100ul of the crude exudate or the active fraction (in water or in diethyl ether) were spotted on preparative (Whatman 3MM) filter paper (56 x 46cm). Two sheets were used for the sample in water and two for the sample in diethyl ether.

Spots on one of the two sheets were dried with hot air,
whereas spots on the other one were left to dry at room temperature. Four spots, on each sheet were made for each quantity. Then the chromatograms were run in the same solvent for the same time as in the previous experiment (a).

At the end of the running period (eight hours) the chromatograms were lifted from the solvent, air-dried under aseptic conditions and cut into strips (1 x 2cm) from the origin to the solvent front.

The bioassay test was performed as follows:

1 - The strips were eluted by placing them in 5ml of cooled and molten Fries agar in small Petri dishes (one strip/plate), and after the agar had set the surface was seeded with spores of H. crustuliniforme.

2 - The strips were placed on the surface of Fries agar plates (one strip/plate) previously seeded with spores of the test fungus.

3 - The strips were placed on sterile slides (one strip/slide), in Petri dishes and covered with about 1ml of Fries agar previously seeded with spores of the test fungus.

4 - The strips were placed in small Petri dishes (one strip/plate), moistened with sterile distilled water and covered with sterile dialysis membrane. The spores of the test fungus were placed on the dialysis membrane.

All the plates were sealed with P.V.C. tape and incubated at 20°C.

Results

No germinating spores were seen in any of the above tests up to three months incubation compared with more than 1% germination on plates containing 10% root exudate. This might be due to the low concentration of the active compounds on the strips tested.
c - Testing the effect of synthetic amino acids on spore germination of H. crustuliniforme

The fourteen amino acids which were used as standard samples in the paper chromatography, were tested for their effect on spore germination of the test fungus, either singly or in combinations of "three" (chosen randomly) as follows:

Glycine + Alanine + Valine
Glycine + Leucine + Aspartate
Glycine + Isoleucine + Glutamate
Glycine + Serine + Threonine
Glycine + Isoleucine + Histidine
Alanine + Leucine + Glutamate
Alanine + Histidine + Lysine
Valine + Isoleucine + Asparagine
Leucine + Arginine + Aspartate
Aspartate + Serine + Lysine
Asparagine + Arginine + Threonine
Glutamate + Asparagine + Histidine
Glutamine + Lysine + Serine
Lysine + Arginine + Histidine

and Casamino acids (acid hydrolysed casein) Oxoid code LA1

20mM solutions in water were prepared from each of the above amino acids and from casamino acids. To test the effect of these amino acids on spore germination; two different methods were used; firstly: 50ul of each amino acid or casamino acids were introduced into 3mm diameter holes (3 holes) in Fries agar plate, the surface of which had previously been seeded with spores of the test fungus (spores were stored for 4 months at 5°C and c. 40% relative humidity, and a spore suspension was made in sterile distilled water), and ; secondly: each amino acid was incorporated into the agar medium in a concentration of 10% (v/v) amino acid solution/agar.

When a combination of three amino acids was tested; 50ul of each of them was introduced into one of the three holes in the same
plate. Also equal amounts of the three amino acids were mixed, and the mixture was incorporated into Fries agar in a concentration of 10% (v/v) mixture/agar, and after the agar had set the surface was seeded with spores of the test fungus (as above).

Three plates were made for each treatment, taped, and incubated at 20°C in darkness.

Results

In all plates containing amino acids, either singly or in combination of three, and also casamino acids, few spores (5-10 out of 200,000 spores) germinated after 10 days incubation, and produced mycelial colonies a week later, compared with no germination at all in the control plates (without amino acids).

It is possible that an increase in the concentrations tested, and the use of combinations other than those used here could lead to better germination.

Fries (1976) reported that spores of Boletus luteus were stimulated to germinate when a mixture of 18 amino acids (AAM) (with a composition resembling that of hydrolysed casein) was added to the nutrient medium (Fries agar) in a concentration of about 1% (10mM). The effect of AAM increased when the nutrients of the agar medium were diluted to a quarter of the original concentration. Also, Fries (1976) reported that glutamic acid at a concentration of about 10mM in nutrient medium exhibited a stronger germination inducing activity than any of the other amino acids tested.

3.11.5 Molecular weight determination

3.11.5.1 By ultrafiltration

A microconcentrator (centricon-10, Amicon, Figure 2), with membrane of 10,000MW cut off was used.

The principle of this method is, that the compound with molecular weight (< 10,000) smaller than the membrane pores emerges as filtrate and is collected in the filtrate cup. At the same time, macromolecular compounds larger than the membrane pores are
retained and become concentrated in the sample reservoir.

**Procedure**

2 ml of the root exudate preparation were placed in the sample reservoir, and centrifuged (MSE-High Speed 18) at 5000 rpm and 4 °C for 90 minutes or until c. 50 ul of the sample remained in the sample reservoir. Then 1 ml of distilled water was added to the remaining 50 ul and recentrifuged for 20 minutes. The volume of the concentrated sample was adjusted to that of the filtrate to avoid the concentration effect.

These two fractions were incorporated within Fries agar at a concentration of 10% v/v, and after the agar had set, the surface was seeded with spores of *H. crustuliniforme*, sealed with P.V.C. tape and incubated at 20 °C in darkness.

**Results**

Spores germinated in plates containing the fraction with molecular weight <10,000 but not in plates containing compounds with molecular weight larger than 10,000.
Figure 2  Centriicon-10, in the concentration mode
3.11.5.2 By Dialysis membrane

Dialysis tubing (Visking) was used for this test. This tube is permeable to water and the average pore radius is 24 angstrom units. Thus the tubing will permit the diffusion of low molecular weight (12-14,000) compounds in aqueous solution through its walls, but not allow that of the higher molecular weight compounds.

This tube is made from a regenerated cellulose and contains glycerine and about 0.1% sulphur. Strips were cut from this tube, boiled for ten minutes in water to remove glycerine and sulphur, and autoclaved for 10 minutes at 121°C. Then these strips were placed on the surface of Fries agar plates containing 10% v/v root exudate or on strips of filter paper soaked with root exudate. Spores of *H. crustuliniforme* suspended in sterile distilled water were spread over the dialysis membrane strips. The plates were taped and incubated at 20°C in darkness.

Results

Spores on the dialysis membrane were stimulated to germinate, this indicates that the active compounds of the root exudate passed through this membrane and stimulated spore germination.

3.11.6 Testing the thermo-stability of the active compound

Eppendorf tubes containing 1ml of the unfractionated and of the active fraction of root exudate were placed in boiling water for 20 minutes. Then the treated root exudates were incorporated in Fries agar in a concentration of 10% v/v, and after the agar had set the surface was seeded with spores of *H. crustuliniforme*. Control plates containing unboiled exudate were also made. The plates were then taped and incubated at 20°C.
Result:

Germination on plates containing boiled and unboiled exudate was similar, this indicates that boiling has no effect on the activity of the exudate.
3.12 Effect of storage conditions on viability and germinability of basidiospores

Storage conditions can affect the viability and germinability of fungal spores. Optimum storage conditions for basidiospores of ectomycorrhizal fungi are not known. However, in this study, basidiospores of the test ectomycorrhizal fungi were stored under different humidities at different temperatures (see methods of storing spores, 2.12.3).

3.12.1 Viability Test

To test the viability of fungal spores and hyphae; biological assays, eg. germination or growth, are commonly used. Such assays are suitable for cells that germinate or grow rapidly. Spores of many fungi (eg. mycorrhizal fungi), however, are difficult to germinate in a reasonable period of time (Dewey and Tyler, 1958; Fries, 1978; Meiners and Waldher, 1959), and although viable, they may require months to germinate. Therefore rapid viability tests are needed.

Blakeslee (1974) used the ability of spores, of some mycorrhizal fungi he studied to reduce nitro-blue tetrazolium chloride as a quick test for viable and physiologically active spores. This test was adapted from that generally used in the testing of seed viability (Moore, 1962).

In the present study, fluorescein diacetate (FDA) stain was used as a vital stain for spores of the test fungi.

FDA is a fluorogenic substrate, which is non-fluorescent until it is enzymatically hydrolysed. This stain was used by Soderstrom (1977) for the vital staining of fungi in pure cultures and in soil. He obtained a good correlation between relative staining efficiency, growth rate, and respiration.

Staining Procedure:

A stock solution of fluorescein diacetate (Koch-Light 116
Laboratories Ltd, Colnbrook, England) containing 2mg.mL$^{-1}$ acetone was prepared and kept at -20°C. This solution was diluted to 10ug/ml in phosphate buffer (60mM, pH 7.4) shortly before use.

Basidiospores to be tested were suspended in sterile distilled water. As controls, spores were autoclaved for ten minutes at 120°C to kill them.

One or two drops from the prepared spore suspensions were added to 2ml of the diluted stain solution and left for five minutes. Then the spores were collected by filtration through a non-fluorescent membrane (Millipore MF Black filter 0.8um) in a Pyrex microanalysis filter holder. The filter was gently sucked off and placed on a glass slide. A drop of non-fluorescent immersion oil was added to the topside of the filter, which was then covered with a coverglass.

The preparation thus obtained was immediately (within one hour) studied microscopically. The result was recorded as very bright-green fluorescence, bright green, faint and non-fluorescent.

The microscope used was a Leitz Dialux 20, fitted with a Ploenpak Epifluorescence illumination system (Mercury lamp, HBO 50N). Filter Block for F.I.T.C. Objectives used were NP2 Fluotar2 40/0.70, and 100/1.32 oil. Microphotographs were taken with an Olympus OM2.

Results

By this test; dead spores (autoclaved) of all fungi tested gave negative results (non-fluorescent) with FDA. Freeze-dried spores of the test fungi showed a very low percentage of viability. Less than 0.1% of the spores gave bright green fluorescence, and the remainder were either very faint or non-fluorescent, this observation was seen with unstored and stored spores for up to 20 months.

The majority of untreated spores of the test fungi were viable for up to 20 months storage under different humidities at different temperatures. More than 90% of spores of Paxillus involutus and Hebeloma crustuliniforme (plates 14 and 15), gave
very bright-green fluorescence when they were fresh or stored for up to 20 months. Spores of *Laccaria laccata*, *Amanita fulva*, *Amanita rubescens*, *Lactarius turpis* and *Russula nitida* were not tested for their viability (with FDA) when they were fresh, but after 20 months storage, most of the spores when aggregated in clusters and less than 1% of spores not in clusters were very bright-green fluorescent.

Exact percentages could not be counted; as the stained spores fade quickly (within seconds) when exposed to the fluorescent light during examination.
Plate 14 Fluorescein spores of *P. involutus* stained with fluorescein diacetate (FDA) (x1200)
Plate 15 Fluorescein spores of *H. crustuliniforme* stained with fluorescein diacetate (FDA) (x1200)
3.12.2 Germinability test

One must distinguish between viability and germinability; not all viable spores will germinate, especially those of ectomycorrhizal fungi, and this may be attributed to the fact that the optimum conditions for germination have not yet been found.

In the present study the ability of spores to germinate differed with different methods of testing.

Basidiospores of *R. nitida* did not germinate (either fresh or stored for up to 20 months under different conditions) in any germination tests. Those of *L. turpis* and *A. fulva* germinated very poorly when they were fresh (within 24 hours of collection) near birch roots grown in mineral salts medium but not in any other conditions tested. Spores of *A. rubescens* germinated on laboratory media when they were fresh or stored for up to one month at different humidities and temperatures. However, freeze-dried spores could not be tested on laboratory media because of fungal and bacterial contamination which covered the surface of the agar plates within a few days. This was because the spore inoculum was prepared by maceration of freeze-dried gills which normally contained many contaminants.

Fresh spores of *L. laccata* germinated near birch and pine roots grown in mineral salts medium, and on the surface of Fries agar solidified with pure agarose or in the presence of the active bacteria (*S19*). Stored spores did not germinate under these conditions.

Germinability of spores of *H. crustuliniforme* was tested monthly for up to 18 months storage under different conditions.

Two methods were used:

1 - On the surface of Fries agar plates supplemented with birch root exudate (10% v/v exudate/agar): Germination of fresh and stored spores started within three days incubation, and increased until the surface of the agar plates were covered with mycelial colonies produced by the germinating spores.
2 - Germination on Fries agar plates inoculated with a stimulatory bacterium (Pseudomonas stutzeri):

Germination of stored spores differed from that of fresh spores; the time required for germination to occur increased from 3 days for fresh spores to 15 days for spores stored for up to 8 months, and 25 days for up to 18 months under any of the conditions studied. Also the germination percentage decreased with the age of the spores until it became <0.01 very close to the bacterial colony after 18 months storage.

Stored spores of _P. involutus_ did not germinate on laboratory media, even in the presence of growing mycelium of the same species, or in the presence of the active bacteria (Corynebacterium _spp._, Arthrobacter _sp._ or S19), which stimulated fresh spores to germinate.

Stored spores of _P. involutus_ were stimulated to germinate near birch roots grown in soil, but not in mineral salts medium following 20 months storage.

The buried slide technique and birch wood soil were used for this test.

After four weeks burial the slides were recovered, air-dried, and permanent preparations were made (as in experiment 3.7). Spores germinating near roots (0-1mm from the edge) were counted in 10 microscope fields in three replicates (slides) of each treatment.

Table 17 shows the germination percentages of _P. involutus_ spores following different storage times under different conditions. Germination percentage of spores stored for 10 months at 5 °C and a relative humidity of c. 40% was higher than that of spores stored under other conditions for the same period of time. Whereas after 20 months storage all spores stored under different humidities at 5 °C, and at -20 °C gave similar percentages of germination (c. 40%).

Freeze-dried spores germinated very poorly (<1%) following 10 or 20 months storage at room temperature. Also (see Table 17), spores of _P. involutus_ lost a third of their germinability after 10
months storage at 5 °C and c. 40% relative humidity compared with 96% germination of fresh spores (experiment 3.7) and more than half following storage at 5 and -20 °C under 95% and 45% relative humidities (respectively) for the same period of time.
Table 17  Germination of spores of *P. involutus* following different periods of storage under different conditions.

<table>
<thead>
<tr>
<th>Storage Conditions</th>
<th>Temperature (°C)</th>
<th>Relative Humidity (%)</th>
<th>period of storage (months)</th>
<th>Germination (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>40</td>
<td>10 *</td>
<td>67 ***</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>40</td>
<td>20 **</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>95</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>95</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>45</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>45</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Freeze-dried</td>
<td>over anhydrous CaCl2</td>
<td>10</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>spores at room</td>
<td>under vacuum</td>
<td>20</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

(1): near birch root (0-1mm from the root edge), in all cases germination in the control slide (without root) was less than 0.01%.

* : Number of spores germinated after 10 months storage (except freeze-dried spores) are significantly different (p = 0.001) from those stored for 20 months under the same conditions.

** : After 20 months storage under different conditions, numbers of germinated spores are not different from one another at p = 0.10.

*** : Number of germinated spores after 10 months storage at 5°C and 40% r.h. is significantly different (p = 0.001) from those stored under other conditions for the same period (10 months) (see appendix 3, A.5.2.).
3.13 Testing the role of basidiospores in initiating ectomycorrhizas

Basidiospores

Basidiospores of *Paxillus involutus*, *Laccaria laccata*, *Hebeloma crustuliniforme*, *Amanita fulva*, *Amanita rubescens*, *Lactarius turpis* and *Russula nitida*.

Plants

Seedlings of birch (*Betula pubescens*), spruce (*Picea sitchensis*) and pine (*Pinus nigra*).

Soils

1 - Birch wood soil.
2 - Spruce forest soil.

Procedure

Three week-old seedlings (from sterile seeds germinated on peat) were sown in 500cm³ pots containing soil (previously fumigated with Dazomet, see method 2.11.4); birch seedlings were sown in pots of birch wood soil, and those of spruce and pine in pots of spruce forest soil. One seedling was planted in each pot. All the pots were watered with sterile tap water, to maintain c. 60% of water holding capacity. One week later basidiospores of the test fungi were added to the pots in the following way; the stipes were partly removed and the caps were surface-sterilized with ethanol and suspended close to the surface of the soil (cocktail sticks were used to support the caps). Four fruitbodies of *L. laccata* and only one of the other fungi were used for each pot. After 24 hours, the fruitbodies were removed and discarded, a small volume of water (30ml) was added to each pot to wash the spores below the surface. Control pots had no added spores.
Three pots were used for each treatment and distributed randomly in a growth cabinet. The temperature ranged from 12-20°C and the light period was 18 hours/day. Illumination (1000 lux) was from fluorescent lights placed c. 80cm above the seedlings. Watering was with sterile tap water from below the pots (the pots were stood in saucers).

Seedlings from all the pots were harvested after three months growth, and the roots carefully removed from the soil.

For assessment of mycorrhizal development, the root systems of three seedlings were washed free of soil, random samples (about a third of the root size) were taken and the total number of short roots and those with mycorrhizas were counted under the binocular microscope.

To isolate the causal fungus, short roots with mycorrhizas were collected, washed, sterilized, and plated out on MMN agar containing 15ug/ml aureomycin (as in method 2.12.5).

Short roots with mycorrhizas were fixed in formalin-acetic alcohol (see 2.4), and the freezing microtome was used to cut 6um thick transverse sections, then these sections were mounted with lactophenol, and examined under the light microscope to observe fungal sheath and Hartig net formations.

For scanning electron microscope study, the mycorrhizal short roots were fixed and prepared as in method (2.9) and photographed.

Results

Basidiospores of H. crustuliniforme, A. fulva, A. rubescens, L. turpis and R. nitida did not produce mycorrhizas with roots of any of the test plants. Also no mycorrhizas were formed on seedling roots in the control pots (unsupplemented with spores).

Spores of P. involutus and L. laccata produced mycorrhizas with roots of birch, but not spruce or pine roots.

Mycorrhizas were established in all the three seedlings of birch inoculated with spores of L. laccata, and only in one out of three inoculated with P. involutus (Table 18), and nearly half the
Table 18  Development of mycorrhizas on seedlings of Betula pubescens grown in birch wood soil supplemented with fresh spores of two mycorrhizal fungi.

<table>
<thead>
<tr>
<th>Basidiospore inoculum</th>
<th>P. involutus</th>
<th>L. laccata</th>
<th>None - Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of seedlings</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>inoculated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of seedlings</td>
<td>1</td>
<td>3</td>
<td>Nil</td>
</tr>
<tr>
<td>with mycorrhizas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of root tips</td>
<td>1292</td>
<td>2813</td>
<td>-</td>
</tr>
<tr>
<td>counted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of root tips</td>
<td>587</td>
<td>1224</td>
<td>-</td>
</tr>
<tr>
<td>with mycorrhizas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of root tips</td>
<td>45.4</td>
<td>43.5</td>
<td>-</td>
</tr>
<tr>
<td>with mycorrhizas</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
number of the root tips was infected with the inoculum spores.

Attempts to isolate the causal fungus from the sheath were unsuccessful; because the mycorrhizas were either contaminated because of insufficient sterilization, or killed when treated with hydrogen peroxide for 10-30 seconds. But microscopical examination confirmed the presence of fungal sheath and Hartig net in the transverse sections of the short roots. Also photographs by SEM show the hyphae of mycorrhizal fungi with clamp connections (Plates 16 and 17).
**Plate 16** Mycorrhiza of birch with *L. laccata* in birch wood soil
(scanning electron microscope)

**Plate 17** Enlargement of the above, showing a clamp connection.
Chapter 4  

DISCUSSION

4.1 Spore germination

Sussman (1966) defined germination as the first irreversible stage which is recognizably different from the dormant organism. For this discussion germination will be considered in three distinct though relatively arbitrary phases viz.

1). The physiological and morphological changes which must occur within the spore before germ tube emergence can take place.

2). The act of protrusion of the germ tube from the spore wall. This constitutes the major visible criterion of germination although from a metabolic standpoint germination is well advanced by this time.

3). The elongation of the germ tube and the establishment of polar growth giving rise to the characteristic growth form of the fungus.

The biology of spore germination within the ectomycorrhizal Hymenomycetes is distinct from that of non-mycorrhizal Hymenomycetes. In general, spores of non-mycorrhizal fungi germinate readily on artificial media; or in plain water (Fries, 1966). In contrast, spore germination of the ectomycorrhizal fungi is very difficult to achieve under laboratory conditions, and often dependent upon, or stimulated by the presence of some activation factor, usually of biological origin.

The failure of basidiospores of ectomycorrhizal fungi to germinate or poor germination may be due, at least in part, to a type of dormancy (Fries, 1978; Merrill, 1970; Taber and Taber, 1982).

For most ectomycorrhizal fungi studied, a period of rest (or a maturation period) is not necessary before a spore can germinate (Kneebon, 1950). Buller (1909 - 1931) in his studies on sporulation in the Agarics, reported that a discharged spore is probably mature; such spores were used in the present study.
So far, no condition among ectomycorrhizal fungi has been found which could be considered analogous to morphological immaturity in seeds. That is, no species are known in which the spores require a period of dormancy to attain morphological maturity.

Germination of spores of the test ectomycorrhizal fungi did not improve following storage under cold conditions for periods up to twenty months.

Endogenous or self-inhibitors could possibly be a cause of the inability of ectomycorrhizal spores to germinate on laboratory media (Fries, 1978). In the present study there is no indication that endogenous or self-inhibitors are produced by spores of the fungi studied within the limits of the spore densities tested. In the literature, there are no reported studies of these fungi in this respect, except that of Blakeslee (1974) who tested the possibility of production of self-inhibitors by the spores of *Amanita rubescens* and four other species of ectomycorrhizal fungi (*Amanita citrina*; *Strobilomyces floccopus*; *Suillus conthurnatus* and *Suillus hirtellus*), by inoculation of MHM (modified Hagem medium) with varying quantities of spores, but no concentration-related effect was detected in any instances.

Spores of the test species were not shown to have an impermeable wall. The rapid uptake (three to five minutes) of fluorescein diacetate by spores of all the fungi tested indicates the permeability of the spore wall.

However, germination of spores of all the fungi tested except one (*Russula nitida*) were stimulated at least by one treatment as shown in the following sections.

These observations might support the idea that spores of ectomycorrhizal fungi are in a state of constitutional dormancy (Sussman, 1966), in which they are unable to germinate under conditions that normally allow vegetative growth to proceed and required an external activator to germinate. This may be due to an innate property of the spore itself which is not yet known.
The mechanism by which the external activator stimulates dormant spores of ectomycorrhizal fungi is still unknown. In general spores contain reserve foods, sufficient for the germination process, but they are unable to break down these reserve foods to utilizable respiratory substrates (Kneebon, 1950; Rast and Stauble, 1970; Taber and Taber, 1982) until activation occurs. Therefore, the role of the activator could be to provide a suitable stimulus which might activate the dormant enzymes of the spores and increase the respiratory rate of the spores.
4.2 Germination without stimulants

Germination tests were performed on Fries medium which was shown to be suitable for spore germination of most ectomycorrhizal fungi (Fries, 1978, 1983b). Also several modifications of this medium were tested in order to find out whether the components of the basic medium were present in concentrations optimal for spore germination of the test fungi, and whether they have any inhibitory or stimulatory effect on germination.

With all modifications tested, spores of Amanita rubescens were induced to germinate within two weeks without addition of any stimulatory factors, but the germination never exceeded 0.1%. Germination of spores of Laccaria laccata was obtained only when pure agarose was used instead of Difco agar. This suggests the presence of compounds in Difco agar which inhibited spore germination of L. laccata, and the absence or small concentrations of such inhibitors in pure agarose. Spores of Paxillus involutus, Hebeloma crustuliniforme, Amanita fulva, Lactarius turpis and Russula nitida did not germinate in any of the media tested without activators.

Only very few ectomycorrhizal-Hymenomycetes have been found which are capable of germinating on synthetic nutrient media without any activator organisms, or without any special organic supplements, eg. Tricholoma sp; Amanita rubescens (Fries, 1943, 1966) and Hebeloma mesophaeum (Bruchet, 1973; Fries and Birraux, 1980).
4.3 Germination in the presence of stimulatory factors

Spores of all ectomycorrhizal fungi tested, except those of *Russula nitida*, were stimulated by at least one method used during this study.

4.3.1 Stimulation by growing mycelium of the same fungus as the spores.

Spores of *Paxillus involutus*, *Laccaria laccata* and *Meleloma crustuliniforme* were slightly stimulated by growing mycelium of the same species as the spores (Table 4) and in the presence of activated charcoal only. Germination of *Amanita rubescens* spores was accelerated in the presence of its own mycelium; as the time required for germination to occur decreased from two weeks in the absence of the mycelium to 4-5 days with the mycelium. For this fungus the same thing has been observed by Fries (1966) and Blakeslee (1974).

The response of spores to living mycelium has been reported in a number of mycorrhizal fungi, including *P. involutus*, *Leccinum spp.* and *L. laccata* (Fries, 1979a, 1979b, 1981b, 1983b). The reaction between spores and vegetative hyphae which leads to spore germination is called an "inductor reaction", and it is mediated by substance or substances exuded from the hyphae which induce the spores to germinate.

The nature of the substance or substances by which the mycelium stimulate spore germination was not determined in the present study, but they are most likely to be non-volatile compounds, as no active volatile compound was proved to be produced by growing mycelia of the test fungi.

Rast and Stauble (1970), demonstrated that the triggering agent for spore germination in *Agaricus bisporus* is isovaleric acid which is produced not only by mycelium of this species, but also by several other fungi, including yeast, and may be of relatively widespread occurrence. However, when Fries (1978) tested the effect of isovaleric acid on germination, it was without effect on the
spores of any of the mycorrhizal fungi tested. Bjurman and Fries (1984) found that culture filtrates from Leccinum aurantiacum were active in stimulating germination of spores of the same fungus as was the growing mycelium. The active compound has not been identified, but it is non-volatile; diffusible; soluble in water; methanol and in ethanol; resistant to heat at 100°C for fifteen minutes; stable after lyophilization if preserved at -20°C and has a low molecular weight (<8000 dalton).

4.3.2 Stimulation by other microorganisms

In the present study seven isolates (out of 42) of bacteria obtained from sporophores of ectomycorrhizal fungi, two (out of 36) obtained from mycorrhizal roots and two (out of 40) obtained from soil (Table 6) were found to be active in stimulating germination of certain mycorrhizal fungi. None of the fungal isolates obtained from the same sources was active in stimulating germination of any of the test fungi.

One fungus (Tritirachium roseum) and one bacterium (Micrococcus roseus) which appeared among the spores of H. crustuliniforme on the surface of agar plates as natural contaminants were also active in stimulating germination of spores near their colonies. The most active isolate was that of Pseudomonas stutzeri which was obtained from sporophores of H. crustuliniforme and induced c.21% of spores of the same fungus to germinate (Table 8) within 1cm of its colony. This bacterium and all the active isolates of Pseudomonas spp. which were obtained from sporophores of H. crustuliniforme (four isolates) or from soil (one isolate) were specific in that they stimulated spores of H. crustuliniforme but not those of any of the other six fungi tested (Table 7).

Isolates of Corynebacterium spp. obtained from sporophores of H. crustuliniforme, and mycorrhizal roots of willow (Salix spp.) with H. crustuliniforme were active in stimulating spores of H. Crustuliniforme and of Paxillus involutus. Spores of Laccaria laccata were stimulated by one isolate (unidentified soil bacterium
S19) only. This isolate also stimulated spores of *P. involutus* and *H. crustuliniforme*.

Spores of *Amanita fulva*, *Amanita rubescens*, *Lactarius turpis* and *Russula nitida* did not respond to any of the bacterial and fungal isolates tested in this study.

These findings indicate that only certain species of microorganisms can stimulate spore germination of certain species of ectomycorrhizal fungi.

Information about the effect of other microorganisms on spore germination of ectomycorrhizal fungi is very limited; for example, the fungus *Ceratocystis fagacearum* was used to germinate spores of *Lactarius* species by Oort (1974). Also red yeasts (i.e. *Torulopsis sanguinea* and *Rhodotorula glutinis*) obtained from sporophores of ectomycorrhizal fungi were reported to be active in stimulating spore germination of some ectomycorrhizal fungi (Fries, 1941, 1943, 1966; Lamb and Richards, 1974). However, the germination induced by the red yeast was usually characterized by being slow and sparse, the percentage seldom exceeding 1%. Fries (unpublished data, cited by Fries, 1984) claimed that no new germination inducers more powerful than the rather inefficient *Rhodotorula* were discovered during his extensive testings of various sorts of microorganisms isolated from forest soil.

So far, no named species of bacteria has been mentioned to be effective in stimulating spore germination of ectomycorrhizal fungi. However, Bowen and Theodorou (1979) studied the interactions between eight bacteria isolated from different sources and eight ectomycorrhizal fungi on laboratory media, and in the rhizoplane of *Pinus radiata*. They found that only one bacterium "*Bacillus sp. WR1*" (out of two isolated from washed mycorrhizas of *P. radiata* roots with *Rhizopogon luteolus*) significantly increased colonisation of pine roots by four of the mycorrhizal fungi tested, and also stimulated growth of *R. luteolus* on agar media. Whereas other bacteria, isolated from nursery soil, either showed no effect or inhibited growth of the mycorrhizal fungi tested.
Azcon et al. (1986) tested the effect of a number of soil microorganisms (bacteria and fungi) on spore germination and development of the VAM fungus Glomus mosseae. They found that the germination rate of surface-sterilized chlamydospores and development of hyphae was significantly hastened by the presence of un-named free-living fungi tested. Daniels and Trappe (1980) noted that spore germination of the VAM fungus Glomus epigaeus was inhibited in the absence of microorganisms. Meyer and Linderman (1986), in their experiments on the inoculation of clover (Trifolium subterraneum) with VAM fungi and a plant growth-promoting bacterium PGPR (Pseudomonas putida) found that VAM fungus infection in the roots was increased significantly from 7 to 23% by the presence of the PGPR. Also they observed significant increase in root and shoot dry weight when both the PGPR and VAM fungus were present compared to that in the presence of PGPR alone, VAM fungus alone, or uninoculated controls.

The active substances by which microorganisms stimulate spore germination of mycorrhizal fungi is still unknown. However, Fries (1966 and 1976) reported that yeast could stimulate germination by production of diffusible substance(s) (eg. amino acids), and volatile compounds (unknown), or by removing inhibitory compounds (eg. NH4⁺) from the medium.

In the case of Pseudomonas stutzeri reported in this study; volatile compounds, cell-free culture-filtrate and cell extracts were inactive in stimulating germination of the test ectomycorrhizal fungus (H. crustuliniforme). Also, germination did not occur on agar plates on which P. stutzeri was allowed to grow for sometime before the growth was washed off and the agar autoclaved, poured in Petri dishes and re-used for germination tests without the bacterium. Germination occurred only in the presence of growing bacteria close to the spores. This indicates that the stimulatory compound(s) produced by P. stutzeri are chemically labile and inactivated immediately after exudation into the medium. Fries (1966) reached the same conclusion.
4.3.3 Stimulation by plant roots

There is experimental evidence that the basidiospores of some species of mycorrhizal fungi do have the potential to initiate mycorrhizas (Fox, 1983; Ivory, 1983; Marx, 1976). However, little is known of the details of this process.

The fact that few or no basidiospores of most ectomycorrhizal species tested germinate on common laboratory media suggests that in nature special conditions occur, presumably near roots, that result in germination. Such conditions and their effects constitute pre-infection interactions which appear to regulate the initial infection, and hence the occurrence of mycorrhizas, and they differ from post-infection interactions which appear to be responsible for such phenomena as development, abundance, and maintenance of the mycorrhiza (Blakeslee, 1974).

The pre-infection stages, as described by Barea (1986) and Nylund and Unestam (1982), consist of four phases; 1 - activation of mycorrhizal propagules in soil until germination is initiated; 2 - hyphal growth through soil; 3 - fungal growth in the rhizosphere; and 4 - establishment of hyphal envelope on the root from which the intercellular penetration will occur.

In the pre-infection phase, the prevailing direction of the influence (especially when basidiospores are used as inoculum) is from host root to the fungus (Nylund and Unestam, 1982), since the interaction is between active (host roots) and dormant (basidiospores) organisms.

The exudation of various organic and inorganic substances is the primary mechanism through which plant roots can exert an influence on their external environment.

In fact, the effects of plant roots in inducing spore germination of ectomycorrhizal fungi have been observed by several authors. Melin (1959) reported observation of germinating spores of species of Suillus, Amanita, Lactarius and Paxillus when exposed to exudates from pine and tomato roots. Also germination in Hebeloma spp. was strongly stimulated by pine roots (Fries and Birraux, 1980), and germination in Thelephora terrestris by pine and birch.

All these experiments, and others, were performed under laboratory conditions, either in solution or on agar plates. The artificial conditions of these experiments have little relation to the natural conditions to which basidiospores are subjected in the rhizosphere in soil. However, in the present study, germination of basidiospores and their behaviour were studied near plant roots in the laboratory situation (in mineral solution) and under semi-natural conditions; that is in the presence of roots and soil.

Spores of the test fungi, except those of *Russula nitida*, were stimulated at least near roots of one species of the test plants grown in mineral solution (Table 11), but only *Paxillus involutus* spores of the seven species tested were stimulated near roots of the test plants in soil.

The best germination rate (96% of *P. involutus* spores within 1mm of the root edge) was observed in (untreated) birch wood soil close to birch roots (Table 12), and this was about ten times greater than that near birch roots in mineral salt medium. This is probably related to the interaction between roots, soil, and rhizosphere microorganisms which might modify and increase root exudation as shown by Barber and Martin (1976). They demonstrated that exudation from roots grown in soil is much greater than had been estimated previously from solution culture studies, and they related this, at least in part, to the presence of microorganisms; since soil microorganisms are able to produce compounds which increase root cell-permeability. Microorganisms also influence plant growth by increasing the availability of nutrients.

Ayers and Thornton (1968) had demonstrated that wheat roots growing in sand consistently exuded greater amounts of ninyhydrin-reacting compounds than did roots growing in nutrient solution. They attributed the difference to abrasive injury caused by the sand particles.
Steamed soil is not sterile, depending on the duration of treatment and temperature reached. Most fungi and vegetative cells of bacteria are likely to be killed, while bacterial spores will survive. Following steaming, bacteria multiply rapidly, usually to numbers exceeding those in untreated soil; this may be due to decomposition and release of the nutrients from the dead cells (Warcup, 1957). The poorer germination of spores of *P. involutus* near birch roots in steamed rather than in untreated soil, might not be only related to the effect of microorganisms, but also to some other factors caused by steaming. Soil steaming may result in the release of substances which may be toxic to the fungal spores and plants. Also, steaming alters the physical properties of soil, for example, its water holding capacity (Warcup, 1957).

The germination percentage of *P. involutus* spores near birch roots in birch wood soil was higher than that in spruce and arable soil. This is most likely attributed to the effect of soil components on growth and root exudation of birch seedlings, and also possibly on the spore germinability. Also, these soils probably contain different populations of microorganisms which might compete differently with spores of ectomycorrhizal fungi.

Spores of *P. involutus* respond differently to roots of different plant species grown in mineral salt medium or in soil; birch was usually most stimulatory. However, comparison between the effects of different plants is difficult, particularly because the size and quantity of roots of different species were not equivalent.

In contrast to spores of *P. involutus*, those of *Laccaria laccata* and *Hebeloma crustuliniforme* were stimulated by birch roots in mineral salts medium but not in soil. This suggests different requirements for germination, or differences in competitiveness.

The failure of basidiospores of *Amanita fulva*, *Amanita rubescens*, *Lactarius turpis* and *Russula nitida* to germinate in soil near roots, and lack of germination (*R. nitida*) or poor germination (*A. fulva*, *A. rubescens*, *L. turpis*) in mineral salts medium near
roots may be understandable if these are regarded as "late-stage" mycorrhizal fungi. Fox (1983) suggested that "early-stage" mycorrhizal fungi of birch (P. involutus, Hebeloma sacchariolens, H. leucosa, Inocybe lacera and I. lanninella) can establish mycorrhizas on seedlings from spores in soil, but that "late-stage" fungi (Lactarius rubescens and Leccinum roseofracta) are unable to do so. "Late-stage" fungi appear to depend on a continuing supply of photosynthate from a mature tree to colonize seedling roots (Fleming, 1984).

4.3.3.1 Growth of germ tubes

Growth of Germ tubes towards roots, most apparent in the response of P. involutus spores to birch roots, probably represents a chemotropic response to a gradient of one or more components of the root exudate, not necessarily those stimulating germination (Tommerup and Kidby, 1980). It is analogous to the observed behaviour of other fungi, including pathogens, in soil near roots (Jackson, 1957, 1960), and of obvious advantage to the fungus particularly if its spores remain viable in soil for quite long periods (Fox, 1983).

Such responses have not been seen in VAM fungi. Studies by Mosse and Hepper (1975) showed that the initial direction of the germ tubes produced by germinating VA spores was not influenced by the presence of host roots. The hyphae appear to spread out radially, and the mycelia, with low hyphal density, tend to occupy a spherical region surrounding the spore (Sanders and Sheikh, 1983). As these authors pointed out, the rate of this radial spread of the hyphae can be related to the probability of a hypha encountering a suitable rhizosphere. Obviously, the possibility of chance contact between VA mycelia and roots must exist. The risk of failure to make contact with the root appears to be minimized in Gigaspora species, which produce aerial germ-tubes able to locate the roots. This could be a response to a volatile attractant released by the plant (Koske, 1982). Also, Powell (1976) showed that the hyphae from Endogone spores on slides buried in sterilized
soil did not grow towards the root until about 1.6mm from them, where the main hypha (diameter 20-30um) then gave rise to characteristic fan-shaped complexes of septate lateral branches (hyphal diameter 2-7 um) and infection of the root usually occurred from these narrow lateral hyphae (pre-infection fans).

In the literature, there are no reports of the production of pre-infection structures by hyphae or basidiospores of ectomycorrhizal fungi; but observations reported in this study demonstrated the formation of fan-shaped structures by germ tubes from spores of *P. involutus*, (Plate 8). This may be analogous to the beginning of the infection or sheath formation; since some of the fans were formed very close to or on the surface of the roots or root hairs.

Mycorrhizas were formed from hyphae produced by germinating spores of *P. involutus*, but it was not clear whether the casual hyphae were monokaryon or dikaryon.

Another sort of chemo-tropism was occasionally observed between germ tubes and spores (usually germinated spores) of *P. involutus* in soil. This is an indication of the production of substances by spores which attract hyphae nearby to grow towards the spore. This mode of reaction was first observed by Morton and French (1970) in *Polyporus dryophilus* and by Bitis (1970) in *Clitocybe trunciola*. Similar reactions were observed between oidia and hyphae in many species of *Coprinus* (Kemp, 1970, 1975, 1977). Kemp described the reaction as "homing". Fusion between oidia and hyphae was also observed, and this is followed by a lethal or non-lethal cytoplasmic reaction depending on the taxonomic relationship of the partners. Homing reaction also occurs among ectomycorrhizal-Hymenomycetes, such as *Laccaria*, (Fries, 1983b), and *Leccinum*, (Fries, 1981b). In *Laccaria* homing takes place between germinated spores and hyphae of monosporous or tissue culture mycelium. In *Leccinum*, spores usually produce vesicles when induced to germinate by mycelium of the same fungus (Fries, 1978). Such spores (with vesicles), were attractive to the hyphae of the inducer mycelium. Ungerminated spores (without vesicles), were never attractive. The response involved growing of the hypha
towards a vesicle and attachment to it by the hyphal tip (Fries, 1981b), Fries called this hypha "responsive hypha".

Although no evidence is available that homing reactions lead to fusion between hyphal tips and spores of ectomycorrhizal fungi, some observations by Fries (1981b) may support this idea. Fries observed that homing reactions between hyphae of *Leccinum aurantiacum* and spore vesicles of *Leccinum versipelle* or *Leccinum vulpinum* lead to the death of the attached hyphal tip cell and probably also of the vesicle, whereas, when mycelia and spores both belonged to *L. aurantiacum*, no lethal reaction was observed. Fries, discussing this further, stated that if the spore and the hypha belong to the same species and no lethal reaction occurs, a transfer of nuclei from the spore vesicle to the hypha could take place. This will lead to dikaryotization if the mycelium is homokaryotic and, in heterothallic species, is of a compatible mating type.

All responsive mycelia of *Leccinum* species were obtained via spore germination, and thus presumed to be monosporous, except one mycelium (no.668 *L. aurantiacum*), which was obtained as a heterokaryotic basidiocarp tissue culture.

From his results, Fries (1981b) speculated that the occurrence of neutral mycelia of *Leccinum* sp., which do not respond to the spore vesicles of the same species, may be a consequence of senescence. However, when he (Fries, 1983c) tested new tissue cultures for homing capacity immediately after isolation from basidiocarps, no case of homing was observed. On the other hand, when further monosporous mycelia of *L. aurantiacum* and *L. versipelle* were isolated, most of them turned out to be responsive. However, if homokaryotic, but not heterokaryotic, mycelia are responsive to the chemotropically active substances produced from the spore vesicles, one would expect all monosporous (homokaryotic) mycelia to be responsive, and the tissue culture (heterokaryotic) mycelia non-responsive. Fries (1983c) attributed the negative results with some monosporous mycelia probably to the technical shortcomings of the isolation procedure. That is, the presence of potentially slow-germinating spores among the hyphae of developing
monosporous mycelium could lead to undetected matings, which would change the mycelium from a homo- to a heterokaryotic one, i.e. from responsive to non-responsive. Also, he attributed the responsiveness of the heterokaryotic tissue culture of the strain no.668, *L. aurantiacum*, to the probability of a spontaneous dedikaryotization.

All these are speculative interpretations, and more investigation is needed to explain the genetic and physiological mechanisms which control these different modes of reaction.

Homing reactions between spores and hyphae of *P. involutus* have not been reported before. However, observations in this study showed that homing reactions occurred only between some spores and hyphae, but not between others (possibly because of incompatibility). More experimental work is needed to confirm this finding.

4.3.4 Stimulation by root exudate "in vitro"

Evidence published by several authors (eg. Birraux and Fries, 1981; Bowen and Theodorou, 1985; Fries and Birraux, 1980; Fries and Swedjemark, 1986; Melin, 1959) shows that one or more compounds in root exudates have the ability to promote germination of spores of ectomycorrhizal fungi.

In the present study, exudate from birch roots was active in stimulating spore germination of *Hebeloma crustuliniforme*. Although several methods were used to identify the active substances in the exudate, exact identification could not be made. However, the active substances are non-volatile; diffusible; soluble in water; resistant to heat treatment at 100 °C for at least 20 minutes, stable at -20°C. Also, the active substances are ninhydrin positive and seemed to possess ionizable groups because of retention on cation-exchange resin (Dowex 50). From this resin the active substances were eluted with 4N NH4OH (pH>14). The active substances have a relatively low molecular weight; they passed through a membrane with 10,000MW cut off, and diffused through a dialysis
membrane of 12-14 x 10^8 MW cut off. Attempts to separate the active substances and to determine the Rf using paper chromatography were unsuccessful. More attempts should be made with different quantities of sample, different solvent systems and different location reagents.

Although no specific stimulant of spore germination in root exudates has been identified so far, there are some indications in the literatures of the nature of compounds having the ability to stimulate germination, for example, Nilsson (cited by Melin, 1963) reported that the stimulatory effect of the M-factor on hyphal growth could be replaced by nicotinamide adenine dinucleotide (NAD), but the results of Benedict et al. (1967) do not fully agree with this. They tested the effect of exogenous NAD on mycelial growth of eight species, and spore germination in thirteen additional species of ectomycorrhizal Agaricales. Germination was not obtained in any of the thirteen species tested. Only one of the eight fungi tested for mycelial growth, was stimulated by NAD, the remaining seven were without response, or showed inhibition. Therefore, it was concluded that some unidentified component(s) (other than NAD) of pine roots was responsible for the stimulatory effects noted by Melin. Also, Gogala's (1970) results disagreed with those of Nilsson. He identified cytokinin activity in an extract of the roots and germinating seeds of Pinus sylvestris, and compared their activity on the growth of Boletus edulis with pure kinetin, tryptophane, gibberllic acid (GA3), and indoleacetic acid. Kinetin and the extracted cytokinins stimulated growth at low concentrations, whereas the other compounds uniformly inhibited growth between concentrations of 10^-6 and 10^-3g1^-1. Both extracted kinins and kinetin stimulated growth between 10^-6 and 10^-1g1^-1, but at higher concentrations they inhibited it. Gogala was of the opinion that the M-factor of Melin might be a cytokinin and that both its stimulatory and its inhibitory action at high concentration might therefore be explained.

Blakeslee (1974) reported that basidiospore germination of Amanita rubescens was stimulated by exogenously supplied
cytokinins (kinetin). In his study, he interpreted the effect of *Pinus taeda* short roots in stimulating germination as being partially due to the production of cytokinin-like compounds. His attempt to test the ability of the cytokinin-active fraction of *P. taeda* root extract to stimulate germination of *A. rubescens* was unsuccessful for unknown reasons.

Fries *et al.* (1985) found that the vegetative growth of two ectomycorrhizal fungi was stimulated by lipids from root exudate of *Pinus sylvestris*. Recently, Fries and Swedjemark (1986) reported that the lipidic phase but not the water phase from pine root extract was active in stimulating germination of spores of *Hebeloma mesophaeum*. Also, they reported that the lipidic phase was still active, even when the free fatty acids had been removed by alkali treatment.
Numerous experiments on the growth of young coniferous and broadleaved trees in fumigated nursery beds and in containers of sterile soil have shown that they become mycorrhizal by apparently air-borne sources of infection, eg. basidiospores (Marx et al., 1970b; Robertson, 1954; Trappe and Strand, 1969).

Direct documentation on the role of basidiospores in initiation of infection is available only for a limited number of ectomycorrhizal fungi: Theodorou (1971b) presents evidence that spores of *Rhizopogon luteolus* can initiate ectomycorrhizas on *Pinus radiata*. But the inclusion of some mycelium in the spore inoculum diminishes the clarity of this finding. Using spores as inoculum, Thapar et al. (1967) found that *Scleroderma verrucosum* formed ectomycorrhizas with *Eucalyptus grandis*, but reisolation to verify the fungal symbiont was not attempted. Also basidiospores of *Pisolithus tinctorius* (Marx, 1976) and *Thelephora terrestris* (Marx and Ross, 1970) were shown to be effective inoculum for the synthesis of ectomycorrhizas with loblolly pine. Ivory (1983) reported that inoculation with basidiospores of *Pisolithus tinctorius*, *Rhizopogon nigrescens* or *Scleroderma texense* led to the formation of abundant distinctive mycorrhizas on *Pinus caribaea*.

However, there is little, if any, information in this respect about the ectomycorrhizal species tested in this study.

In the greenhouse pot experiment using soil fumigated with *Dazomet*, basidiospores of *Hebeloma crustuliniforme*, *Amanita fulva*, *Amanita rubescens*, *Lactarius turpis* and *Russula nitida* did not produce mycorrhizas with roots of any of the test plants (birch - grown in birch wood soil; pine and spruce - grown in spruce forest soil). Spores of *Paxillus involutus* and *Laccaria laccata* produced mycorrhizas with roots of birch, but not of spruce or pine.

Species of *Amanita*, *Lactarius* and *Russula* have been shown or presumed to be "late-stage", and those of *Paxillus*, *Hebeloma* and *Laccaria* have been shown to be "early-stage" mycorrhizal fungi (Deacon et al., 1983).

These authors established that a broad distinction can be
made between early- and late-stage fungi (i.e., those occurring early and late in the mycorrhizal succession). Early-stage fungi readily infect seedlings from resident or introduced inocula in sterile soil whereas late-stage fungi cannot (Danielson et al., 1984; Deacon et al., 1983; Last et al., 1985). Late-stage fungi have also been shown to be apparently dependent on a continuing supply of photosynthate from a mature tree to colonize seedling roots (Fleming, 1984).

The factors responsible for the observed differences in the distributions of mycorrhizal fungi with respect to age of the host are not known, but presumably changes in the rhizosphere induced by the host are of major importance. Smith (1970) reported that in sugar maple, root exudates of young (three-week-old) seedlings contain a diverse range of carbohydrates, whereas mature trees, (fifty-five year-old) predominantly exude amino acids, amides and organic acids. It may be that "early-stage" and "late-stage fungi differ in their nutrient requirements during the initial phases of root colonisation, host age thus directly influencing their capacity to establish mycorrhizas; alternatively, competitive interactions involving rhizosphere organisms may prevent some mycorrhizal fungi from establishing infections. The failure of basidiospores of A. fulva, A. rubescens, L. turpis and R. nitida to establish mycorrhizas in this study could be explained by the above finding if these fungi are regarded as "late-stage" mycorrhizal fungi.

The reason for the failure of basidiospores of H. crustuliniforme (which is presumed to be an "early-stage" fungus) in establishing mycorrhizas is unknown, but it might be related to unsuitability of the soil that was used in this study.

Birch wood and spruce forest soils (with pH of 4.05 and 4.3 respectively) differ from the soil from which fruitbodies of Hebeloma were collected (pH 7.5). In a study by Last et al. (1985) the colonization of birch roots by Hebeloma sacchariolens was tested in four different soils (two mineral soils pH 5.4 and 5.1; Sedge peat pH 4.7; and Sphagnum peat pH 3.4). They found that the persistence of H. sacchariolens was very strongly related to soil
type. In the first season inoculated seedlings growing in Sphagnum peat had few (25%) mycorrhizas attributable to *H. sacchariolens* compared with 100% in the other three soils. This fungus continued to predominate during the second season on seedlings growing in three of the four soils tested, but it failed in the most acid, viz. Sphagnum peat. Therefore they suggested that *H. sacchariolens* is able to colonise birch roots growing in unsterile substrates with a pH of 4.7 but not in more acidic (pH 3.4) conditions.

Basidiospores of *P. involutus* and *L. laccata* failed to produce mycorrhizas on roots of pine and spruce during the three months duration of the experiment. This may be because, during this time, pine or spruce did not produce enough root material which would cause significant stimulation of spore germination.
4.5 Conclusion

In the present study, investigations were made into the role of basidiospores in initiating mycorrhizas. In soil, most of Paxillus involutus basidiospores germinated very close to the root, and the majority of germ tubes grew towards it, this resulted in the mycorrhizal formation. 4 mm beyond the roots, the majority of the spores did not germinate (<0.1% germinated). This would be of selective advantage to both partners (fungus and root) if the population of spores in soil could lie in wait for new roots to grow and reach them.

It is not known for how long the spores of ectomycorrhizal fungi will survive in soil, but under controlled laboratory conditions tested in this study, spores remained viable for up to twenty months, and they might survive longer in soil. Fox (1983) reported that spores of Inocybe spp. and Hebeloma spp. remained infective for at least ten months in soil stored at 18°C.

However, the function of basidiospores as mycorrhizal initiators is not understood if the failure of some ectomycorrhizal fungi to produce fruitbodies is considered.

In their field work, R. Jackson and D. Rogers (personal communication) observed the occurrence of mycorrhizas of some species of ectomycorrhizal fungi, but without production of fruitbodies. Also, Molina and Trappe (1982a) reported that some species of ectomycorrhizal fungi which are known to produce fruitbodies with some species of the host plants, do not do so with others. They called this as "sporocarp-specific host associations", in which the mycelium may exist ecologically in other associations than those in which the fruitbodies are found. In such cases infection of newly formed roots will be by hyphae from the infected ones. But it is possible that primary infection may happen by spores carried in air or by any other means.

Basidiospores may have a further function in addition to that of dispersal and initiation of mycorrhizas. The homing reaction observed by Fries (1981b) between hyphae and spores might provide a potential mechanism of variation; for new dikaryotic
combinations might arise when basidiospores germinate in the vicinity of existing dikaryons.

However, in view of both the ecological and the economic importance of spores as a source of mycorrhizal infection of seedlings, more knowledge of the physiology of those of mycorrhizal fungi is needed.
ACKNOWLEDGEMENTS

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REFERENCES


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APPENDIX 1

Germination of *Scleroderma citrinum* basidiospores.

Basidiocarps of *Scleroderma citrinum* were collected beneath birch trees at Whitmoor Common, Surrey.

Mature basidiocarps were surface sterilized by wiping with tissue soaked in ethanol, broken open under aseptic conditions and the spores were placed in a sterile plastic Petri dish and kept at 5°C and 40% relative humidity.

Spore germination on the surface of agar plates and near plant roots in mineral salts medium (using the Fahraeus slide technique) was unsuccessful because of heavy contamination of the spores with bacteria and fungi which covered the surface of the agar plates and killed the seedlings of the test plants within a few days. In birch wood soil, using the buried slide technique similar to that used in experiment 3.7 and under the same conditions a few spores (c. 1%) germinated. Two germ tubes were produced by most of the germinated spores, as shown in the photograph below.

Plant roots (birch) had no significant effect on the percentage of germination but germ tubes grew towards the root when germination occurred near the root surface.

![A spore of *Scleroderma citrinum* with two germ tubes on a slide buried in birch wood soil (x1500).](image)
**APPENDIX 2**

**Identification of isolates stimulating spore germination.**

1. **Bacterial Isolates**

Initially the isolates were identified to genus using the criterion shown in the following table.

**Table 1. Initial characteristics of the isolates**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of isolates as listed in Table 7 on Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 10 11 12 13</td>
</tr>
<tr>
<td>Shape</td>
<td>R R R R R R S S/R R R S</td>
</tr>
<tr>
<td>Gram stain</td>
<td>- - - - + + + + + - +</td>
</tr>
<tr>
<td>Spore formation</td>
<td>- - - - - - - - - - -</td>
</tr>
<tr>
<td>Motility</td>
<td>+ + + + + + - - - + -</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+ + + + + + + + + +</td>
</tr>
<tr>
<td>Growth unaerobically</td>
<td>- - - - - (+) (+) - - (+) - -</td>
</tr>
<tr>
<td>Catalase</td>
<td>+ + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+ + + + + - - - - - + +</td>
</tr>
<tr>
<td>Glucose (acid)</td>
<td>+ + + + + - - - - - + -</td>
</tr>
<tr>
<td>Carbohydrates O/F/-</td>
<td>O O O O O O - F - - - O -</td>
</tr>
<tr>
<td>Temperature 5°C</td>
<td>- - - - - - - - - - - -</td>
</tr>
<tr>
<td>20°C</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>25°C</td>
<td>+ + + + + + + + + + + +</td>
</tr>
<tr>
<td>30°C</td>
<td>(+) (+) (+) (+) (+) (+) + + + (+) (+) (+)</td>
</tr>
<tr>
<td>37°C</td>
<td>- - - - - - - - - - -</td>
</tr>
</tbody>
</table>

R = Rod-shaped
S = Sphere
S/R = Variable
+ = Positive reaction
(-) = Very slightly positive
= Negative reaction
O = Oxidation
F = Fermentation
The isolates could then be ascribed to the following genera:

1). *Pseudomonas*: isolates No. 1, 2, 3, 4, 5 and 12.

2). *Corynebacterium*: isolates No. 6 and 11.


4). Isolate No. 7 belongs to the family Enterobacteriaceae.

5). Isolate No. 13 could not be identified.

6). Isolate No. 10 was very close to the genus Arthrobacter: this bacterium as described by Skerman (1967), when grown in a complex medium, undergoes a marked change in form during the growth cycle. Older cultures (2-7 days) are composed entirely or largely of coccoid cells. In some strains the coccoid cells are uniform in size, and spherical, and resemble micrococci. In others they are spherical to ovoid or slightly elongate. Gram-positive, however the rods may be readily decolorized and may show only Gram-positive granules in otherwise Gram-negative cells. Coccoid cells are Gram-positive but may be weakly so.

Chemoorgantrophs, metabolism respiratory never fermentative, little or no acid is produced from glucose in peptone medium, catalase positive, strict aerobes, optimum temperature 20-30°C; most strains grow at 10°C but usually not at 37°C.

The second stage is to identify these isolates to species.

Isolate No.1 (the most active isolate of the genus *Pseudomonas*), isolates belonging to the genus *Corynebacterium* (No. 6 and 11), *Micrococcus* (No. 8) and isolates belonging to the family Enterobacteriaceae (No. 7) were studied further in order to identify them to species.

Several tests were made as shown in Table 2. According to this
table, isolate No. 1 was identified as \textit{Pseudomonas stutzeri}, isolates No. 6 and 11 of the genus \textit{Corynebacterium} could not be identified to the species but they are very close to the \textit{Corynebacterium hofmannii}, isolate No. 8 was identified to \textit{Micrococcus roseus}. Isolate No. 7 of Enterobacteriaceae could not be identified to species but it is very close to \textit{Cedecea lapagei}.

The following references were used in the identification:


Table 2  Further characterization of the isolates

<table>
<thead>
<tr>
<th>Tests</th>
<th>Isolate No.1 Pseudomonas</th>
<th>Isolate No.6 Corynebacterium</th>
<th>Isolate No.11 Micrococcus</th>
<th>Isolate No.8 Enterobacteriaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigmentation</td>
<td>-</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Fluorescence in U.V. light</td>
<td>-</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Growth on MacConkey</td>
<td>+</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PHB (Poly-B-hydroxybutyrate)</td>
<td>-</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Nitrate reduced to nitrite</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of citrate</td>
<td>+</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Phenyl-alanine</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Arginine Hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H2S production</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N.T.</td>
</tr>
<tr>
<td>MR</td>
<td>N.T.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VD</td>
<td>N.T.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>N.T.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>N.T.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sacrose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = Positive reaction.  (+) = Slightly positive  - = Negative reaction  N.T. = Not tested.
2. Fungal Isolate

The active fungus (isolate No. 9, Table 7) was identified as *Tritirachium roseum* VAN-BEYMA according to the characteristic features described by Hughes (1953)\(^{(1)}\) and Van-Beyma (1942)\(^{(2)}\) as follows:

- Slow growing, colonies 5-10 mm diameter, cushion-like, pink-violet coloured, no odour, agar not stained.
- Conidia are small, sub-spherical to cylindrical, mainly 1.74-2.32 x 2.32-2.9 um, produced regularly to the left and to the right and the fertile region of the sporogenous cell resembles the rachis of a wheat ear and may be conspicuously zig-zag (Fig. 1).
- The sporogenous cells are verticillate branches of the main conidiophore.

---


Figure 1. *Tritirachium roseum*, conidiophore and conidia from five weeks culture on Fries agar.
APPENDIX 3

Statistical Analyses

A - The chi-squared ($X^2$) test (Clarke, 1982) was used to analyse the data in tables 11, 12, 14, 15 and 17. In all these tables, comparison of the treatments with the control was not made because of very low (<0.01%) germination percentages in the control.

In each table, for the comparison between replicates (3 replicates) the level of significance was calculated according to the chi-square 3x2 contingency table (the calculations were made by computer) and for the comparison between treatments (2 treatments) the 2x2 contingency table was used as shown below:

<table>
<thead>
<tr>
<th>Observed numbers</th>
<th>With characteristic (i.e. germinated)</th>
<th>Without characteristic (i.e. non-germinated)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>a</td>
<td>b</td>
<td>(a + b)</td>
</tr>
<tr>
<td>Sample 2</td>
<td>$\frac{c}{a + c}$</td>
<td>$\frac{d}{b + d}$</td>
<td>(c + d)</td>
</tr>
</tbody>
</table>

$$N = a + b + c + d$$

$$X^2 = \frac{N(ad-bc)^2}{(a + b) (a + c) (b + d) (c + d)}$$

Abbreviations and levels of significance used in this test

Abbreviations:

- g: Number of spores germinated
- ng: Number of spores non-germinated
- g%: Germination percentages
- d: Number of spores with germ tubes directed towards roots
- nd: Number of spores with germ tubes non-directed towards roots
- d%: Percentage of germ tubes directed towards roots

Levels of significance:

- *: significant at $p = 0.05$
- **: significant at $p = 0.005$
- ***: significant at $p = 0.001$

Figures without asterisk non-significant at $p = 0.10$

---

A.1 Contingency tables and chi-squares for the results presented in Table 11, page 82)

In this table, statistical analyses were made for spore germination and direction of germ tubes (not shown in this table) of *Paxillus involutus*, *Laccaria laccata* and *Hebeloma crustuliniforme* near birch roots. Other data were not analysed because of very low (<1%) or no germination at all.

A.1.1. Comparison between replicates.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Germination of spores</th>
<th>Direction of germ tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicate no.</td>
<td>g</td>
</tr>
<tr>
<td><em>P. involutus</em></td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>84</td>
</tr>
<tr>
<td><em>L. laccata</em></td>
<td>1</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>215</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>205</td>
</tr>
<tr>
<td><em>H. crustuliniforme</em></td>
<td>1</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>117</td>
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</tbody>
</table>
A.1.2 Comparison between fungi

<table>
<thead>
<tr>
<th>Fungi to be compared</th>
<th>Germination of spores</th>
<th>Direction of germ tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>ng</td>
</tr>
<tr>
<td><strong>P. involutus</strong></td>
<td>166</td>
<td>1610</td>
</tr>
<tr>
<td><strong>L. laccata</strong></td>
<td>554</td>
<td>3617</td>
</tr>
<tr>
<td><strong>P. involutus</strong></td>
<td>166</td>
<td>1610</td>
</tr>
<tr>
<td><strong>H. crustuliniforme</strong></td>
<td>475</td>
<td>1101</td>
</tr>
<tr>
<td><strong>L. laccata</strong></td>
<td>554</td>
<td>3617</td>
</tr>
<tr>
<td><strong>H. crustuliniforme</strong></td>
<td>475</td>
<td>1101</td>
</tr>
</tbody>
</table>
A.2 Contingency tables and chi-squares for the results presented in Table 12, page 86

Spore germination and direction of germ tubes at 2-4 mm from the edge of spruce roots, and also more than 4 mm from birch and spruce roots were not analysed because of very low (<1%) germination percentages.

A.2.1. Comparison between replicates.

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Soil treatment</th>
<th>Plant</th>
<th>Distance from root edge (mm)</th>
<th>Germination of Spores</th>
<th>Direction of germ tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Replicate number</td>
<td>Replicate number</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>g  ng  g%  X²</td>
<td>d  nd  d%  X²</td>
</tr>
<tr>
<td>1</td>
<td>Steamed</td>
<td>birch</td>
<td>0-1</td>
<td>1  2084 280 88 3.20</td>
<td>1  2042 42 98 1.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2  1647 259 86</td>
<td>2  1605 42 97</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3  1868 259 88</td>
<td>3  1824 44 98</td>
</tr>
<tr>
<td>2</td>
<td>Steamed</td>
<td>birch</td>
<td>2-4</td>
<td>1  95 1827 5 6.18*</td>
<td>1  36 57 40 0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2  118 1835 6</td>
<td>2  47 71 40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3  82 1824 4</td>
<td>3  33 49 40</td>
</tr>
<tr>
<td>3</td>
<td>Untreated</td>
<td>birch</td>
<td>0-1</td>
<td>1  2150 64 97 25.96***</td>
<td>1  2106 44 98 2.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2  1972 122 94</td>
<td>2  1940 32 98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3  1964 100 95</td>
<td>3  1937 27 98</td>
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<tr>
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<td>Untreated</td>
<td>birch</td>
<td>2-4</td>
<td>1  390 1622 19 5.45</td>
<td>1  141 249 36 0.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2  377 1712 18</td>
<td>2  129 248 34</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>3  450 1705 20</td>
<td>3  165 285 37</td>
</tr>
<tr>
<td>5</td>
<td>Steamed</td>
<td>spruce</td>
<td>0-1</td>
<td>1  44 1986 2.2 2.12</td>
<td>1  15 29 34 0.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2  49 2039 2.3</td>
<td>2  13 36 26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3  60 2051 2.8</td>
<td>3  19 41 31</td>
</tr>
<tr>
<td>6</td>
<td>Untreated</td>
<td>spruce</td>
<td>0-1</td>
<td>1  48 2032 2.3 6.85*</td>
<td>1  13 35 27 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2  77 2088 3.6</td>
<td>2  21 56 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3  54 2062 2.5</td>
<td>3  14 40 26</td>
</tr>
</tbody>
</table>
### Comparison between treatments

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Germination of spores</th>
<th>Direction of germ tubes</th>
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<tbody>
<tr>
<td></td>
<td>g</td>
<td>ng</td>
</tr>
<tr>
<td>1</td>
<td>5599</td>
<td>798</td>
</tr>
<tr>
<td>2</td>
<td>295</td>
<td>5486</td>
</tr>
<tr>
<td>3</td>
<td>6090</td>
<td>282</td>
</tr>
<tr>
<td>4</td>
<td>6090</td>
<td>282</td>
</tr>
<tr>
<td>5</td>
<td>153</td>
<td>6076</td>
</tr>
<tr>
<td>6</td>
<td>179</td>
<td>6182</td>
</tr>
<tr>
<td>7</td>
<td>153</td>
<td>6076</td>
</tr>
<tr>
<td>8</td>
<td>179</td>
<td>6182</td>
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</table>
A.3. Contingency tables and chi-squares for the results presented in Table 14, page 96)

The analysis was made only for the results in spruce forest soil; results in arable soil were not analysed because of very low (<0.01%) germination percentages in all cases.

A.3.1. Comparison between replicates.

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Soil treatment</th>
<th>Plant</th>
<th>Replicate number</th>
<th>g</th>
<th>ng</th>
<th>g%</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Steamed</td>
<td>birch</td>
<td>1</td>
<td>47</td>
<td>2045</td>
<td>2.2</td>
<td>0.23</td>
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<tr>
<td></td>
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<td>2</td>
<td>50</td>
<td>1985</td>
<td>2.5</td>
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</tr>
<tr>
<td></td>
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<td>3</td>
<td>47</td>
<td>2015</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
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<td>untreated</td>
<td>birch</td>
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<td>62</td>
<td>2060</td>
<td>2.9</td>
<td>1.22</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>48</td>
<td>1965</td>
<td>2.4</td>
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<td></td>
<td></td>
<td></td>
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<td>52</td>
<td>1978</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
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<td>steamed</td>
<td>spruce</td>
<td>1</td>
<td>52</td>
<td>2019</td>
<td>2.5</td>
<td>2.15</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>2</td>
<td>37</td>
<td>1965</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>43</td>
<td>1994</td>
<td>2.1</td>
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</tr>
<tr>
<td>4</td>
<td>untreated</td>
<td>spruce</td>
<td>1</td>
<td>42</td>
<td>1933</td>
<td>2.1</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>54</td>
<td>2077</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td>3</td>
<td>59</td>
<td>2033</td>
<td>2.8</td>
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</tr>
</tbody>
</table>
### A.3.2 Comparison between treatments

<table>
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<tr>
<th>Treatment number</th>
<th>Germination of spores</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
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<td>4</td>
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</tr>
<tr>
<td>4</td>
<td>155</td>
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</tbody>
</table>
A.4 Contingency tables and chi-squares for the results presented in Table 15, page 99

A.4.1 Comparison between replicates.

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Origin of P. involutus</th>
<th>Germination test plant</th>
<th>Germination of Spores</th>
<th>Direction of Germ Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Replicate number</td>
<td>g</td>
</tr>
<tr>
<td>1</td>
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<td>1033</td>
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<td>1102</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>3</td>
<td>1020</td>
</tr>
<tr>
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<td>pine</td>
<td>birch</td>
<td>1</td>
<td>1000</td>
</tr>
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<td></td>
<td></td>
<td>2</td>
<td>1050</td>
</tr>
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<td></td>
<td></td>
<td>3</td>
<td>978</td>
</tr>
<tr>
<td>3</td>
<td>birch</td>
<td>pine</td>
<td>1</td>
<td>265</td>
</tr>
<tr>
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<td>270</td>
</tr>
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<td></td>
<td></td>
<td>3</td>
<td>255</td>
</tr>
<tr>
<td>4</td>
<td>pine</td>
<td>pine</td>
<td>1</td>
<td>226</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>2</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>220</td>
</tr>
</tbody>
</table>
### A.4.2 Comparison between treatments

| Treatment number | Germination of spores | | Direction of germ tubes | | |
|------------------|-----------------------|----------------------|----------------------|----------------------|
|                  | g  | ng | g% | $X^2$ | d  | nd | d% | $X^2$ |
| 1                | 3155 | 608 | 84 | 3.92 | 3077 | 78 | 98 | 55.37 |
| 2                | 3028 | 513 | 86 |       | 2836 | 192 | 94 |       |
| 1                | 3155 | 608 | 84 | 3071 | 3077 | 78 | 98 | 503  |
| 3                | 790  | 3077| 20 |       | 590  | 200| 75 |       |
| 2                | 3028 | 513 | 86 | 3349 | 2836 | 192| 94 | 237  |
| 4                | 654  | 3066| 18 |       | 483  | 171| 74 |       |
|                  | 790  | 3077| 20 | 9.98  | 590  | 200| 75 | 0.129 |
| 4                | 654  | 3066| 18 |       | 483  | 171| 74 |       |
A.5 Contingency Tables and chi-squares for the results represented in Table 17, (Page 124)

A.5.1. Comparison between replicates

<table>
<thead>
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<th>Treatment number</th>
<th>Storage conditions (1)</th>
<th>Germination of spores</th>
</tr>
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<td>Temperature (°C)</td>
<td>Relative humidity (%)</td>
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<tr>
<td></td>
<td></td>
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</tr>
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<tr>
<td>2</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-20</td>
<td>45</td>
</tr>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-20</td>
<td>45</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1): Data for the freeze-dried spores was not analysed because of very low (<1%) germination percentage.
### A.5.2 Comparison between treatments

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Germination of spores</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>ng</td>
<td>g%</td>
<td>X</td>
</tr>
<tr>
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<td>3350</td>
<td>1616</td>
<td>67</td>
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<tr>
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<td>2145</td>
<td>3216</td>
<td>40</td>
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<tr>
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<td>3350</td>
<td>1616</td>
<td>67</td>
<td>580***</td>
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<td>3</td>
<td>2800</td>
<td>3468</td>
<td>45</td>
<td></td>
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<tr>
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<td>2145</td>
<td>3216</td>
<td>40</td>
<td>2.16</td>
</tr>
<tr>
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<td>2545</td>
<td>4033</td>
<td>39</td>
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<td>2145</td>
<td>3216</td>
<td>40</td>
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<td>2832</td>
<td>4281</td>
<td>40</td>
<td></td>
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<tr>
<td>3</td>
<td>2800</td>
<td>3468</td>
<td>45</td>
<td>47.27***</td>
</tr>
<tr>
<td>4</td>
<td>2545</td>
<td>4033</td>
<td>39</td>
<td></td>
</tr>
<tr>
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<td>2545</td>
<td>4033</td>
<td>39</td>
<td>1.81</td>
</tr>
<tr>
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<td>2832</td>
<td>4281</td>
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<td>2972</td>
<td>43</td>
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<tr>
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<td>2832</td>
<td>4281</td>
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</table>
B. The level of significance for the data in Table 13 (page 87) was calculated according to Student's t-test as follows:

\[
t = \frac{\text{Difference between means}}{\sqrt{\text{Standard error of differences}}}
\]

B.1: Statistical analysis of the lengths of germ tubes produced by *P. involutus* near birch and spruce roots in steamed and untreated soil.

<table>
<thead>
<tr>
<th>Treatment Number</th>
<th>Soil treatment</th>
<th>Plant</th>
<th>Length of germ tubes (mean of 40) μm</th>
<th>SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>steamed birch</td>
<td>65.0</td>
<td>22.9</td>
<td>3.62</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>untreated birch</td>
<td>72.8</td>
<td>24.63</td>
<td>3.89</td>
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</tr>
<tr>
<td>3</td>
<td>steamed spruce</td>
<td>48.2</td>
<td>21.99</td>
<td>3.50</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>untreated spruce</td>
<td>47.8</td>
<td>23.60</td>
<td>3.74</td>
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</tr>
<tr>
<td>5</td>
<td>steamed control</td>
<td>21.1</td>
<td>5.53</td>
<td>0.87</td>
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</tr>
<tr>
<td>6</td>
<td>untreated control</td>
<td>19.7</td>
<td>4.03</td>
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</table>

SD: Standard deviation  
SE: Standard error of differences

B.2 Comparison between treatments

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<th>t (df = 78)</th>
</tr>
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<tbody>
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<td>1 and 2</td>
<td>1.50</td>
</tr>
<tr>
<td>1 and 3</td>
<td>3.34 *</td>
</tr>
<tr>
<td>1 and 5</td>
<td>11.50 **</td>
</tr>
<tr>
<td>2 and 4</td>
<td>4.60 **</td>
</tr>
<tr>
<td>2 and 6</td>
<td>13.21 **</td>
</tr>
<tr>
<td>3 and 4</td>
<td>0.078</td>
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<tr>
<td>4 and 6</td>
<td>7.41 **</td>
</tr>
<tr>
<td>5 and 6</td>
<td>1.26</td>
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</table>

*: significant at p = 0.002  
**: significant at p = 0.001  
figures without asterisk non-significant at p = 0.10