SOURCES OF INOCULUM OF SHEATHING MYCORRHIZAL FUNGI OF BIRCH (Betula spp.)

by

F.M. Fox

Ph.D. Thesis
Edinburgh University
1983
To my parents
DECLARATION

I declare that the work in this thesis is my own, that the thesis has been composed by myself and that none of the material contained herein has been submitted for any other degree or professional qualification. Part of the work has been published in a scientific paper which is appended.

F.M. Fox
ACKNOWLEDGEMENTS

I am especially grateful to Dr. J.W. Deacon and Professor F.T. Last for their supervision of this project and guidance in the preparation of this manuscript.

I thank also Dr. P. Jefferies and Dr. A. Crossley for assistance in electron microscopy, Dr. P.A. Mason, Dr. J. Wilson and Mr. L.V. Fleming for invaluable discussion, Mr. K. Ingleby and Mr. R. Munro for technical assistance and Mrs. Frances Anderson for the typing of this thesis.

I appreciate the use of facilities in the Department of Microbiology, Edinburgh University and the Institute of Terrestrial Ecology, Bush Estate, Penicuik, Midlothian, and the provision of a studentship by the S.E.R.C.

Finally, I would like to thank Dr. and Mrs. R.W. Tonkin and my sister, Alison Fox, for their support and encouragement during the preparation of this thesis.
SUMMARY

Investigations were made into the role of basidiospores and sclerotia as inocula of sheathing mycorrhizal fungi of *Betula* spp., and the ultrastructure of both sclerotia and mycelial strands of some mycorrhizal fungi was investigated by transmission and scanning electron microscopy.

Birch seedlings were grown for 12-16 weeks in a growth room, in pots of unsterile soil supplemented with spores of sheathing mycorrhizal fungi, from freshly collected fruitbodies. All tested species (8) of *Hebeloma*, *Inocybe* and *Laccaria* established mycorrhizas from spores, to the exclusion of mycorrhizal fungi naturally occurring in, or contaminating, the unsterile soil. But several species (15), including those of *Cortinarius*, *Lactarius*, *Leccinum* and *Russula*, did not establish mycorrhizas from spores in these conditions. *Hebeloma crustuliniforme* established mycorrhizas on *Pinus sylvestris* as well as birch from spore inocula in unsterile soil whereas *Suillus luteus* did not infect either host. The results confirm a distinction between "early-stage" (e.g. *Hebeloma*, *Inocybe*, *Laccaria*) and "late-stage" (e.g. *Cortinarius*, *Lactarius*, *Leccinum* and *Russula*) sheathing mycorrhizal fungi: only the "early-stage" fungi established mycorrhizas from spores added to unsterile soil. This distinction was usually independent of substrate, the same patterns being seen in brown earth, vermiculite-peat and coal spoil; but *Paxillus involutus* formed mycorrhizas in coal spoil but not in brown earth. Moist spore-supplemented soils could be stored outdoors or at 18°C for up to 9 months without loss of infectivity of the spore supplements. Spores also remained viable and infective after storage dry at room temperature or moist at 5°C in laboratory conditions.
Successful establishment of *Laccaria* from spore supplements did not affect seedling growth, whereas plant growth was significantly decreased by *Inocybe* spore supplements and increased by *Lactarius* even though the latter did not establish mycorrhizas.

Sclerotium-like bodies of *Hebeloma sacchariolens* remained ultrastructurally intact and infective to seedlings after 9 months burial in moist soil, whereas sclerotia of *P. involutus* degenerated over this time. Ultrastructurally, the sclerotia of *P. involutus* and *Cenococcum geophilum* were similar to thos of other root-infecting fungi, and were formed on mycelial strands; sclerotium-like bodies of *H. sacchariolens* were unusual in their ultrastructure and in being intimately associated with the mycorrhizal sheath. Their role as possible temporary survival structures is discussed.

Mycelial strands of *Lactarius rufus*, *Leccinum scabrum* and *P. involutus* were ultrastructurally similar to mycelial strands of other fungi except that they lacked thick-walled fibre hyphae; from ultrastructural evidence, stages in the differentiation of these mycelial strands are described.
CONTENTS

Declaration iii
Acknowledgements iv
Summary v

SECTION I: GENERAL INTRODUCTION
1.1 Definition and Classification of Mycorrhizas 1
1.2 The Role of Sheathing Mycorrhizas 2
1.3 Potential for Use of Mycorrhizas by Manipulation 5
1.4 Basidiospores as a Source of Inoculum 7
1.5 Survival of Sheathing Mycorrhizal Fungi: Production of Asexual Survival Structures 13
1.6 Successions 15
1.7 Aims and Objectives of the Work in this Thesis 20

SECTION II: MATERIALS AND METHODS
2.1 Materials
2.1.1 Fungi 21
2.1.2 Growth media 21
2.1.3 Isolation of sheathing mycorrhizal fungi
2.1.3.1 Isolation of fungi from fruit-bodies 25
2.1.3.2 Isolation of fungi from mycorrhizas 25
2.1.4 Collection and preparation of seed 26
2.1.5 Source and preparation of soil 27
2.1.6 Description of sites from which fungal material was collected
2.1.6.1 Coal spoil tip at Newtongrange, Midlothian 28
2.1.6.2 Experimental birch plot, Institute of Terrestrial Ecology (I.T.E.), Bush Estate, Penicuik, Midlothian 31
2.1.6.3 Experimental birch plot, Glencorse, Midlothian 31
2.1.6.4 Birch screen, Scottish Crop Research Institute (S.C.R.I.), Pentlandfield, Midlothian 32
2.1.6.5 Loch Inver, Sutherland 32
SECTION III: INFECTION OF ROOTS OF BIRCH SEEDLINGS FROM BASIDIOSPORES AND OTHER SOURCES OF INOCULUM OF SHEATHING MYCORRHIZAL FUNGI

3.1 Introduction 44
3.2 Materials and Methods 46
3.3 Experimental: Investigation of the infectivity of freshly collected basidiospores of sheathing mycorrhizal fungi in unsterile brown earth. 49
3.4 Investigation of the infectivity of freshly collected basidiospores of sheathing mycorrhizal fungi in unsterile vermiculite-peat 62
3.5 Analysis of the effects of spore supplementation on seedling growth and mycorrhizal development in experiments reported in Sections 3.3 and 3.4 67
3.6 Investigation of the infectivity of fresh basidiospore inocula of *Paxillus involutus*, *Lactarius turpis* and *Leccinum scabrum* added to unsterile brown earth and coal spoil 70
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7</td>
<td>Development of mycorrhizas on seedlings of birch, spruce and pine from basidiospore inoculum added to unsterile brown earth</td>
<td>73</td>
</tr>
<tr>
<td>3.8</td>
<td>Effects of single and dual supplements with basidiospores on mycorrhizal development on birch in unsterile soil</td>
<td>76</td>
</tr>
<tr>
<td>3.9</td>
<td>Investigation of the infectivity of stored basidiospore inocula of sheathing mycorrhizal fungi on birch in unsterile brown earth</td>
<td>79</td>
</tr>
<tr>
<td>3.10</td>
<td>Development of mycorrhizas from basidiocarp tissue added as inoculum to unsterile brown earth</td>
<td>93</td>
</tr>
<tr>
<td>3.11</td>
<td>Establishment of sheathing mycorrhizal fungi on birch from dispersed mycelial inoculum added to unsterile brown earth</td>
<td>97</td>
</tr>
<tr>
<td>3.12</td>
<td>Establishment of mycorrhizas on birch seedlings grown in samples of coal spoil taken from beneath fruitbodies of sheathing mycorrhizal fungi</td>
<td>101</td>
</tr>
<tr>
<td>3.13</td>
<td>Discussion</td>
<td>110</td>
</tr>
</tbody>
</table>

**SECTION IV: THE STRUCTURE AND ROLE OF SCLEROTIA OF SHEATHING MYCORRHIZAL FUNGI**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>127</td>
</tr>
<tr>
<td>4.2</td>
<td>Materials and Methods</td>
<td>129</td>
</tr>
<tr>
<td>4.3</td>
<td>Distribution of sclerotia and mycorrhizas of <em>Hebeloma sacchariolens</em> on 16-week old birch seedlings grown under glasshouse conditions</td>
<td>132</td>
</tr>
<tr>
<td>4.4</td>
<td>Histochemical analysis of the sclerotia of some sheathing mycorrhizal fungi</td>
<td>135</td>
</tr>
<tr>
<td>4.5</td>
<td>Structure of sclerotia associated with sheathing mycorrhizas of <em>Hebeloma sacchariolens</em> on birch</td>
<td>144</td>
</tr>
<tr>
<td>4.6</td>
<td>Structure of sclerotia associated with sheathing mycorrhizas of <em>Paxillus involutus</em> on birch</td>
<td>150</td>
</tr>
<tr>
<td>4.7</td>
<td>Structure of sclerotia associated with sheathing mycorrhizas of <em>Cenococcum geophilum</em> on pine.</td>
<td>155</td>
</tr>
</tbody>
</table>
4.8 Survival of sclerotia in soil

4.8.1 Roles of sclerotia of Hebeloma sacchariolens and Paxillus involutus in establishing mycorrhizas on birch seedlings

4.8.2 Ultrastructural changes in detached sclerotia of Hebeloma sacchariolens after burial in unsterile soil

4.8.3 Ultrastructural changes in detached sclerotia of Paxillus involutus after burial in unsterile soil

4.8.4 Microorganisms associated with sclerotia of some sheathing mycorrhizal fungi of birch

4.9 Discussion

SECTION V: STRUCTURE OF MYCELIAL STRANDS FORMED BY SOME SHEATHING MYCORRHIZAL FUNGI ON BIRCH

5.1 Introduction

5.2 Materials and Methods

5.3 Structure of mycelial strands associated with sheathing mycorrhizas of Paxillus involutus on birch

5.4 Structure of mycelial strands associated with sheathing mycorrhizas of Leccinum scabrum on birch

5.5 Structure of mycelial strands associated with sheathing mycorrhizas of Lactarius rufus on birch

5.6 Discussion

SECTION VI: CONCLUDING DISCUSSION

REFERENCES
SECTION I

General Introduction
1.1 Definition and Classification of Mycorrhizas

The term mycorrhiza (derived from the Greek mykes = fungus, rhiza = a root) was first coined by Frank (1885) to describe a "composite organ formed by the association between a plant root and a fungus". Mycorrhiza has since been used to describe a heterogeneous assemblage of several distinct symbioses formed between fungi and the fine roots of vascular plants. Although taxonomically and physiologically diverse, mycorrhizas share several features in common. They are mutualistic, non-pathogenic associations in which the host generally responds by improved growth. The fungus enters the root but the interface between the fungus and plant cell remains intact and undamaged for most, or all of the duration of the symbiosis (Harley, 1978; Smith, 1980). Ninety-five per cent of the world's species of vascular plants are characteristically mycorrhizal; i.e. "mycorrhizas have developed as the norm in terrestrial plants and not the exception" (Trappe, 1962).

Traditionally, mycorrhizas have been divided into two morphological groups (Melin, 1921; Trappe, 1962; Harley, 1969). (1) Sheathing mycorrhizas (ectotrophic mycorrhizas, ectomycorrhizas) are intercellular infections; hyphae extend between the cortical cells of the host to form a "Hartig net" but the majority of fungal development is external to the root. (2) Endomycorrhizas (endotrophic mycorrhizas) are predominantly intracellular symbioses; they are further categorised according to the host species and mode of intracellular penetration which may be in the form of vesicles, coils, arbuscules or haustoria (Melin, 1923). The distinction between the two groups is not always well defined, however, and intermediate ectendomycorrhizal types, which share features characteristic of both sheathing mycorrhizas and endomycorrhizas, have been reported (Laiho, 1965; Hofsten, 1969; Wilcox, 1971).
Whereas most endomycorrhizas are associations formed between members of the family Endogonaceae and herbaceous plants, sheathing mycorrhizas are characteristically formed between the higher fungi - Ascomycetes and Basidiomycetes - and woody perennials. The importance of mycorrhizas in the successful establishment of commercial forests has been the subject of intensive research over the last 40 years (Marks and Foster, 1973; Mikola, 1973).

1.2 The Role of Sheathing Mycorrhizas

Most sheathing mycorrhizal fungi appear to be dependent on their hosts for carbon and other metabolites for normal metabolic processes (Melin, 1921; Harley, 1978) and for completion of the life cycle (Rommell, 1938; Last et al., 1979). Lewis and Harley (1965) showed that 50-75% of $^{14}$C-sucrose fed to cut stumps of Fagus sylvatica L. was translocated towards the tips of mycorrhizas and converted into trehalose, mannitol and glycogen, none of which can be effectively reabsorbed by the host. These and other studies (Reid and Woods, 1969; Hacskaylo, 1973) indicate that the fungal symbiont creates a significant drain on current host photosynthate. Despite this sink effect, the sheathing mycorrhizal association is a ubiquitous and necessary requisite for healthy development of most woody perennials so there must be selective advantages for the host in the relationship. Some of the ways by which sheathing mycorrhizas benefit their hosts are briefly reviewed below.

Improved host nutrient uptake: the role of mycelial strands

Mycorrhizal development is associated with improved host growth (Hatch, 1937), an increase in the concentration of mineral nutrients within the host tissues and a redistribution of dry matter between root
and shoot (Bowen, 1973; Smith, 1980). This is attributable to a more efficient nutrient - and water - absorbing root system provided by the symbiont. This role has been demonstrated by Hall, Scott and Johnstone (1977) who showed that differences in growth between mycorrhizal and non-mycorrhizal plants are greatest in soils of low or imbalanced nutrient status. Differences decrease as soil nutrient levels are increased, i.e. if soil nutrients impose no limitation on direct root absorption, then the contribution of the fungal associate is unnecessary.

There is good evidence for metabolically mediated pathways by which the fungal symbiont can take up and translocate essential mineral nutrients - most notably phosphorus - more efficiently than do non-mycorrhizal roots (Bowen and Theodorou, 1973; Smith, 1980). The greater capacity for absorption, coupled with the storage capacity of the hyphal sheath (Harley, 1978), and the relative permanence of the mycorrhizas compared to root hairs (Grime, 1977) comprise a system of exploiting temporary flushes of nutrients. In addition, sheathing mycorrhizas possess a larger physiologically active surface area for mineral nutrient absorption compared with that of non-mycorrhizal roots, due to extensive extra-matrical growth of hyphae and hyphal aggregates which ramify in the soil (Skinner and Bowen, 1974a,b). Hyphal aggregates (mycelial strands) may play a major role in the absorption of water and nutrients. Skinner and Bowen (1974a) demonstrated that $^{32}$P-orthophosphate can be absorbed by cut ends of strands of *Rhizopogon luteolus* Fr. and translocated 12 cm in field soil to mycorrhizal roots of *Pinus radiata* D. Don. Trappe and Fogel (1977) traced a hypha of *Cenococcum geophilum* Fr. 2 metres from its mycorrhiza and found that it had more than 120 lateral branches or fusions formed with other hyphae. This extensive network significantly increases the
potential for exploitation by the roots. It may overcome the phosphate-depleted zones created by actively elongating roots (Smith, 1980) and it may also improve soil structure by binding soil particles (Smith, 1980).

The development of extra-matrical hyphae varies considerably between different symbionts and Bowen (1973) stressed that the selection of mycorrhizal fungi for inoculation programmes should be based on the capacity of the fungus to produce mycelial strands under a wide range of environmental conditions. Reid and Woods (1969) found that $^{14}$C supplied originally in glucose or sucrose to the foliage of *Pinus taeda* L. seedlings was present in mycelial strands 12 cm from mycorrhizas formed by *Thelephora terrestris* (Ehrh.) Fr., but only slight movement occurred in hyphae of mycorrhizas formed by *Pisolithus tinctorius* (Mich.exPers.) Coker & Couch which did not produce strands under the experimental conditions.

Protection from disease

The mycorrhizal association may confer considerable protection against root pathogens by mechanical and chemical means (Zak, 1964). Marx (1969a,b) demonstrated, in agar plate tests, that antibiotic production by a number of mycorrhizal fungi (*Laccaria laccata* [Scop. ex Fr.] Cke., *Lactarius deliciosus* [L. ex Fr.] S.F. Gray, *Leucopaxillus cerealis* var. *piceina* [Peck] ined., *P. tinctorius* and *Suillus luteus* [Fr.] S.F. Gray) inhibited zoospore or mycelial growth of 44% of the root pathogens tested, including *Phytophthora cinnamomi* Rands and *Pythium* spp. Despite a failure to establish mycorrhizas, *Laccaria laccata*, in the form of basidiospores or mycelia, had a protective influence against *Fusarium oxysporum* Schlect. when inoculated onto roots of *Pseudotsuga menziesii* (Mirb.) Franco (Stack and Sinclair, 1975; Sylvia and Sinclair, 1983a).
1.3 Potential for Use of Mycorrhizas by Manipulation

Artificial inoculation of trees with sheathing mycorrhizal fungi has long been recognised as a necessary requisite for the successful establishment of forests in areas where suitable indigenous inocula are limited or absent, e.g. in the establishment of exotic pine plantations (Bowen, 1965; Mikola, 1973), afforestation of formerly treeless areas (White, 1941), reclamation of disturbed land (Schramm, 1966; Marx, 1975), on land subject to continuous agricultural or nursery practice (Trappe and Strand, 1969) and in nurseries after soil fumigation (Molina and Trappe, 1982). Traditionally, inoculum has been provided in the form of soil or litter from existing plantations or as interplanted mycorrhizal "mother" seedlings (Mikola, 1970), but these methods are non-selective, create problems with bulk transport, and risk the introduction of weeds and pathogens (Mikola, 1973; Trappe, 1977).

Selective inoculation programmes

With the recent availability of improved sources of inocula such as pure culture vegetative mycelium (Marx and Bryan, 1975; Marx, Bryan and Cordell, 1976; Marx, Morris and Mexal, 1978; Marx, 1980), crushed sporocarps (Mikola, 1973; Theodorou and Bowen, 1973; Mullette, 1976), and basidiospores (Marx and Ross, 1970; Theodorou, 1971; Lamb and Richards, 1974a,b; Marx, 1976a; Ruehle, 1980), the potential for manipulation of specific mycorrhizal types has become possible. Criteria to be considered in selection programmes include the ease with which the inoculum can be obtained and grown on a commercial scale, improved seedling yield at the nursery stage, the persistence of the mycorrhizal type after outplanting, and ecological adaptability of the symbiont. Different fungi (and different isolates within the same
species) differ significantly in their capacity to improve host growth although the reasons are poorly understood. Bowen (1968) showed with $^{32}$P tracers, large differences in the uptake of phosphate by different types of excised and intact mycorrhizas of P. radiata; the poorest performers in terms of seedling dry matter production tended to be the mycorrhizal types indigenous to that area. Some of the differences were ascribed to the extent of sheath and extra-matrical hyphal - and mycelial strand development - and differences in uptake at the cellular level.

Marx and Bryan (1971b) demonstrated that under four temperature regimes with diurnal fluctuations between 19°C and 47°C, mycelial inoculum of Pisolithus tinctorius stimulated twice as much dry matter production as in seedlings mycorrhizal with naturally occurring fungi, including Thelephora terrestris. Further, more than other fungi, Laccaria laccata is reported to stimulate the shoot growth of seedlings of Picea sitchensis (Bong.) Carr, Pinus ponderosa Dougl. and Pseudotsuga menziesii (Sinclair, Sylvia and Larsen, 1982; Mason et al., 1983a).

Research has tended to concentrate on the improvement of host growth by sheathing mycorrhizal inoculation at the nursery stage, but adaptability to extreme environments and the capacity of the inoculated fungi to compete against indigenous mycorrhizal fungi and persist after outplanting may, under some circumstances, be of more benefit to the long term growth of the host. Inoculation of five species of Pinus with P. tinctorius significantly increased the survival and total growth response of the seedlings after outplanting over a two-year period and although indigenous mycorrhizas also became established, nevertheless, P. tinctorius persisted on the roots especially at the poorer sites (Marx, et al., 1976).
Certain sheathing mycorrhizal fungi have been shown to be specifically adapted to some extreme environments and selection programmes may enable plantations to survive on sites which are otherwise difficult to reafforestate (Molina and Trappe, 1982). *P. tinctorius* survives well on sites suffering from drought or high temperature extremes (Trappe, cited in Molina and Trappe, 1982). *Cenococcum geophilum* appears to be drought-tolerant (Pigott, 1982) and succeeds other mycorrhizal fungi under conditions of low soil moisture availability. It may be a valuable symbiont for the reafforestation of arid zones although it is inefficient in promoting seedling growth in the nursery (Mexal and Reid, 1973).

*P. tinctorius* is now being grown in pure culture on a commercial scale to inoculate containerised seedlings in the south-eastern United States (Marx et al., 1982). However, to gain maximum benefit from mycorrhizal inoculation practices there needs to be more extensive research into the selective use of symbionts for specific purposes, such as maximum host response at the seedling stage or improved rate of survival after outplanting.

### 1.4 Basidiospores as a Source of Inoculum

Although it is generally assumed that air-borne spores are the primary agents for disseminating effective inoculum of most sheathing mycorrhizal fungi, there is only limited evidence to support this view.

Robertson (1954) observed that 30% of seedlings of *Pinus sylvestris* L. became mycorrhizal when grown in open bottles containing sterile soil and placed near a pine plantation, but there was no mycorrhizal development on seedlings grown in closed bottles. Bowen (1963) found that sheathing mycorrhizas of *P. radiata*, introduced into native eucalypt areas in Australia, were invariably found in soils up to two
miles from established pine plantations but were absent beyond this; he concluded that this distance reflected the limit for air-borne dispersal in this area.

By filtering air entering a growth room, Marx and Bryan (1969) eliminated all casual mycorrhizal infection on seedlings of *P. echinata* Mill., although 90% of seedling roots grown in fumigated uninoculated soil outside the growth room became mycorrhizal.

The difficulty of germination of basidiospores of mycorrhizal fungi in pure culture has hindered the potential of spores as a source of inoculum in commercial practice. Spores of genera such as *Amanita*, *Boletus*, *Cortinarius*, *Lactarius* and *Russula* have rarely, if ever, been germinated at high percentages under experimental conditions (Kneebone, 1950; Fries, 1966). When germination does occur, it is generally at a very low percentage (less than 1%). But this may be increased in the presence of activated charcoal, which removes inhibitory compounds formed during the autoclaving process (Fries, 1979a), and by "activating factors" provided by the yeast *Rhodotorula glutinis* (Fres.) Harr., (Fries, 1966, 1979b), or by living mycelium of the mycorrhizal fungal species itself (Lösel, 1964). In the case of *Leccinum* spp. the stimulating effect is species – and species group specific (Fries, 1979a, 1981). Living host roots also stimulate germination of basidiospores (Melin, 1959; Marx and Ross, 1970; Fries and Birraux, 1980; Birraux and Fries, 1981), and the activating factors produced by fungi have been likened to the "M" factor (Melin, 1963) which is present in living root exudates and stimulates the vegetative growth of sheathing mycorrhizal fungi around roots.

The failure of spores to germinate in pure culture does not appear to be attributable to nutritional deficiency or unfavourable environmental
conditions (Kneebone, 1950); it is due to a state of constitutive
dormancy in which the spores survive at a very low metabolic rate
(Kneebone, 1950; Rast and Stäuble, 1970). Kneebone (1950) and
Taber and Taber (1982) have suggested that the respiratory rate in
dormant spores is limited by a failure in the degradation of reserve
nutrients to utilisable substrates. The correct activating factor, which
may differ between species (Fries, 1981), will provide the necessary
stimulus to bridge the gap in the metabolic pathway and thus increase
the respiratory rate.

The failure to induce germination of mycorrhizal spores in pure
culture may be partly attributable to previous drying and rapid re-
wetting of the spores, resulting in disorganisation of the cell membranes
and rapid leakage of essential metabolites (Simon, 1974). Blakeslee
(1974) emphasised that gradual rehydration of spores is essential for
successful germination under bixenic conditions.

Spores of some sheathing mycorrhizal fungi will readily germinate
in the presence of host seedlings. Thus, although basidiospores of
T. terrestris (Marx and Ross, 1970; Birraux and Fries, 1981),
L. laccata (Stack, Sinclair and Larsen, 1975) and Hebeloma spp. (Fries
and Birraux, 1980) germinated at a rate of less than one per cent on
various agar media, they established mycorrhizas on seedlings of Pinus
taeda, Pseudotsuga menziesii and Pinus sylvestris, respectively,
within 4½ months after an aqueous suspension of spores was added to
sterile soil. In the case of L. laccata, 46% of spore-inoculated seedlings
developed mycorrhizas attributable to Laccaria, whereas the uninoculated
controls remained uncolonised. Under similar conditions, Blakeslee
(1974) established mycorrhizas on seedlings of P. taeda from basidio-
spores of two Suillus spp. and two Amanita spp. which had been stored
at 3°C and slowly rehydrated in a moist chamber before inoculation. He provided evidence that cytokinins in host root exudates may be major factors responsible for the induction of spore germination.

Establishment of mycorrhizas from spores has also been reported in unsterile pot culture. Theodorou (1971) successfully inoculated seed of *P. radiata* with spores of *Rhizopogon luteolus* in fumigated and unsterile soil although seedling yield, percentage mycorrhizal infection and colonisation of the soil by mycelial strands was significantly greater in the sterilised soil. He believed that the reduced microbial activity in the latter increased the availability of nutrients; inoculation into the sterile soil further improved host growth by enhancing the capacity of the host to absorb nutrients.

A similar response to soil fumigation was shown by seedlings of *P. radiata* when inoculated with basidiospores of *Rhizopogon roseolus* (Corda.) T.M. Fr., *Suillus granulatus* (Fr.). O. Kuntze and chlamydospores of three unidentified symbionts of *Pinus* spp. (Lamb and Richards, 1974a). Fumigation stimulated the rate of mycorrhizal establishment and enhanced the host growth response; this effect was greater with the chlamydospore inoculum than with basidiospore inoculum and may have been due to differential sensitivities to microbial antagonists.

Host growth response and intensity of mycorrhizal colonisation appear to be closely related to the initial inoculum density (Theodorou and Bowen, 1973; Lamb and Richards, 1974a,b). Thus, Theodorou and Bowen produced a dose - response curve when basidiospores were applied to soil: 100 spores/290 cm³ pot were sufficient for the establishment of mycorrhizas of *R. luteolus* in sterile soil although colonisation increased with increasing spore density to a maximum of 10⁵ spores per pot. In non-sterile soil, Lamb and Richards (1974a) showed that by
increasing the inoculum density of spores of *R. roseolus*, *S. granulatus* and *P. tinctorius*, seedling dry matter production and the proportion of roots that became mycorrhizal were increased significantly; this effect was greatest on the more fertile of the two soils tested.

Although it was previously assumed that mycorrhizal fungi become established in the long root of the host and that short roots become colonised on emergence through the long root cortex (Robertson, 1954; Clowes, 1951), yet, Theodorou and Bowen (1973) observed that 9-month old seedlings of *P. radiata* mycorrhizal with *R. roseolus* lacked infection of the long roots and the acropetal pattern of mycorrhizas normally associated with it. They concluded that, especially in soils of low phosphorus status, mycorrhizal development from ectotrophic hyphal spread is secondary in importance to separate lateral colonisation from the soil; this would provide an explanation for the observed dose - response curve.

A delay in supplementation of unsterile soil with spores after seed-sowing caused a reduction in the proportion of roots that became mycorrhizal (Moser, 1959); this effect could be reduced if soil fertility was increased (Lamb and Richards, 1974a,b). This may be attributable to early suberisation of roots in poor soils - a process that reduces the capacity of germinating spores to become established.

The use of spores as inocula for nursery seedlings on a commercial scale would eliminate many of the difficulties associated with the present use of soils from existing plantations and pure culture vegetative mycelium; the most notable difficulties in these cases concern the period of vegetative growth required to obtain sufficient bulk of mycelial inoculum and consequent transport costs (Mikola, 1973; Molina and Trappe, 1982). However, practical application of spore inoculum is limited because of the short and unreliable season for collecting sporo-
carps in quantity, and the lack of information on the germinability and viability of spores and their survival during storage (Molina and Trappe, 1982). Moreover, Marx et al. (1976) showed for P. tinctorius that basidiospores are less effective than vegetative mycelium as a source of inoculum on three Pinus species. This was attributable to the delay in colonisation of seedling roots from basidiospores; mycorrhizas formed by P. tinctorius were evident 6 weeks after inoculation with mycelia but only 3 months after inoculation with basidiospores. In the meantime, other mycorrhizal types had become established and host growth response was not significantly increased by the addition of P. tinctorius.

Of the mycorrhizal fungi tested on a large scale, some gasteromyces, for example, P. tinctorius and Rhizopogon spp., appear to be most promising in terms of successful inoculation with spores. In terms of the numbers of outplantable seedlings produced, Marx et al. (1979) have demonstrated that the most successful method of spore inoculation on a nursery scale is to mix the spores with a "hydromulch" (commercial compost mixed in water) which is then incorporated into the soil.

Theodorou and Bowen (1973) successfully inoculated seed of P. radiata with spores of R. luteolus in pot culture but the inoculum density had to be increased 100-fold after 3 months' storage at 22°C. Inoculated seed could be held dry for at least 2 days prior to planting, provided that the inoculum was increased 10-fold.
1.5 Survival of Sheathing Mycorrhizal Fungi: Production of Asexual Survival Structures

The mode of survival of sheathing mycorrhizal fungi in soil is largely unknown (Harley, 1969). The possibility that certain sheathing mycorrhizal fungi may have a means for survival when not in active association with the host arises from (1) the demonstration that several symbionts can be isolated from a single mycorrhizal root (Zak and Bryan, 1963; Zak and Marx, 1964) and (2) the observed seasonal fluxes in the mycoflora populations on root of *Pinus* sp. (Lamb, 1979). If these fungi, many of which are unidentified, represent a group of basidiomycetes which do not fruit, then it seems likely that they are adapted to survive in soil either by saprophytic growth or by means of specialised resting structures.

It is generally assumed that most sheathing mycorrhizal fungi are obligate symbionts; they lack the enzymes necessary for litter decomposition, they are wholly dependent on the host for a continuous carbon supply and certain unidentified growth factors (Melin, 1923, 1959, 1963), and they will not fruit in the absence of active host roots (Rommell, 1938; Last *et al.*, 1979). Some of the Boletaceae, however, appear to be facultative symbionts with a capacity to produce polyphenol oxidases for litter decomposition when not in active association with the host (Melin, 1959; Harley, 1969). Davis and Jong (1976) obtained an isolate of *L. laccata* which, under a diurnal light regime, produced fruitbodies on various agar media... Giltrap (1981) reported the production of primordia and immature fruitbodies of *Boletus* spp. and *Suillus piperatus* (Bull. ex Fr.) O. Kuntze on agar. Watling (1981) proposed that grades of adaptation to the mycorrhizal habit among the Boletaceae may be reflected in the ease with which formation of fruitbodies occurs in pure culture.
Under laboratory conditions, in soil which had been previously sterilised, *R. luteolus*, a mycorrhizal symbiont of *P. radiata*, showed some capacity to utilise the root exudates of non-host plants (Theodorou and Bowen, 1971). Although the mycelium did not colonise the root systems, growth of *R. luteolus* was significantly greater in the rhizospheres of living roots of *Eucalyptus* sp. and grasses, *Lolium perenne*, *Phalaris tuberosa* and *Trifolium subterraneum*, than in the presence of control glass fibres. It was concluded that some sheathing mycorrhizal fungi may be able to survive as independent vegetative structures in the vicinity of non-hosts when suitable hosts are absent.

Harvey, Larsen and Jurgensen (1980) investigated the survival capacity of sheathing mycorrhizal fungi on residual root systems after clear cutting of a stand of mixed conifers. Low numbers of active mycorrhizal roots were found 10 months after clear-cutting but there was no evidence of any activity after one year. Thus, it appears that root systems quickly lose the ability to support mycorrhizas after removal of the shoots.

Sheathing mycorrhizal fungi with no saprophytic ability in soil may produce specialised resting structures. Rayner and Neilson-Jones (1944) reported widespread occurrence of mycelial strands of *Suillus bovinus* (Fr.) O.Kuntze in heath soils in the absence of host trees, an observation confirmed for two other fungal species, *Leccinum scabrum* (Fr.) S.F. Gray and *Cenococcum geophilum*, by Levisohn (1956). Lamb and Richards (1974a, b, c) isolated chlamydospores of three mycorrhizal symbionts and oidia of *Xerocomus* sp., from roots of *Pinus* sp., both types of asexual structure could be produced on agar culture.

The production of sclerotia has been reported in only a few sheathing mycorrhizal fungi, notably *Cenococcum geophilum*, *Pisolithus*
tinctorius and Paxillus involutus (Fr.)Fr. Kropp (1981) and Shaw and Sidle (1982) have provided evidence that sclerotia of C. geophilum survived 5 years in soil in a clear-cut forestry site in Oregon and then provided sufficient inoculum to effectively colonise newly planted seedlings. Sclerotia of P. involutus are produced in large quantities (Laiho, 1970) and provide a potentially valuable source of inoculum for commercial practices. The production of sclerotia by sheathing mycorrhizal fungi and information on their physiology and ecology is discussed further in Section 4.

1.6 Successions

Successions, in which groups of organisms are replaced by others in a well-ordered sequence, are known to occur amongst a wide range of soil-inhabiting fungi; they have been reported, inter alia, for coprophilous fungi (Harper and Webster, 1964), saprophytic decomposers of plant remains (Garrett, 1951) and fairy rings of grasslands (Gregory, 1982).

The distribution of fruitbodies of known sheathing mycorrhizal fungi have been mapped during a 10-year period on an experimental mixed birch plot at the Institute of Terrestrial Ecology, Bush Estate, Penicuik, Midlothian; the results have shown that fruitbodies develop in a highly characteristic temporal and spatial pattern (Mason et al., 1982, 1983a).

Within 2 years after planting of the trees, fruitbodies of Hebeloma crustuliniforme (Bull. ex St. Amans) Quél. appeared in concentric rings around the bases of the trees at a mean distance of 22 cm and by the sixth year their positions had moved outwards to a mean radius of 73 cm. Thelephora terrestris appeared in the third year and Inocybe lanuginella
(Schroed.) Konrad & Maublanc and *Lactarius pubescens* (Fr. ex Krombh.) Fr. were first reported in the fourth year. *L. pubescens*, like *H. crustuliniforme*, appeared in a ring initially close to the tree (mean radius 40 cm) and moved progressively outwards to a mean radius of 69 cm in the sixth year. Other *Hebeloma* spp. appeared in the fifth year after planting, *Cortinarius* spp. and *Leccinum* spp. (mean radius 27 cm) in the sixth year and *Russula* spp. in the tenth year.

Unlike other fruitbody types, *Laccaria* sp., which first appeared within the first 2 years did not occur in rings, but in clumps, apparently along the lines of secondarily thickened roots. It seems, therefore, that fruitbodies of these mycorrhizal fungi develop in a succession, different species appearing first in different years, initially close to the base of the tree and progressively moving outwards (in increasing numbers) over successive years (Ford et al., 1980; Mason et al., 1983a).

Rings of known mycorrhizal fungi around their host trees have been reported elsewhere (Horn, 1933; Becker, 1956; Tominaga, 1975). Becker (1956) observed around a solitary tree of *Pinus sylvestris*, three concentric zones of fruitbodies – an inner zone of species of *Hygrophorus*, *Boletus* and *Tricholoma*, a middle zone of *Lactarius deliciosus* (L. ex Fr.) S.F. Gray, and an outer zone of *Boletus granulatus* (*Suillus granulatus* [Fr.] O. Kuntze) and *Comphidiis* sp. *Inocybe lacerà* (Fr.) Kummer has frequently been recorded in rings around nursery-grown *Pseudotsuga menziesii* (Trappe and Strand, 1969). In addition to these reports there appears to be a close correlation between the distribution of fruitbodies and the age of the host. The appearance of fruitbodies of *H. crustuliniforme*, *L. laccata* and *T. terrestris* on young nursery seedlings has been reported for *Pinus* spp. by Chu-Chou (1979), Lamb (1979) and Trappe and Strand (1969); other *Hebeloma* spp. are known to be early colonisers of *Tilia*. 
cordata Mill and Picea sitchensis, while L. laccata has similarly been reported to be an early colonist of Quercus sp. (Watling, 1981). Watling has suggested that L. laccata and Hebeloma spp. are pioneer fungi of young planted and naturally regenerating woodland trees, with the capacity to adapt to diverse environments where appropriate hosts are present. Similarly, T. terrestris is frequently associated with pioneer communities on terminal moraines and anthracite wastes (Trappe and Strand, 1969) and it is a common symbiont of nursery seedlings in North America.

In contrast, species of Russula, Amanita, Lactarius and Leccinum are associated only with older trees. A. muscaria (L. ex Fr.) Hooker first appeared in Pinus plantations after 12 years (Chu-Chou, 1979; Chu-Chou and Grace, 1981). Last et al. (1981) reported a significant correlation between the number of fruitbodies of A. muscaria and the age of Pinus patula Schl. & Cham. plantations; the mean number of fruitbodies per tree increased from 50 at 6 years to more than 100 on 15-year old trees. Although the trees are now 12 years old, A. muscaria has not yet appeared on the experimental plot at I.T.E. Edinburgh.

Harper and Webster (1964) emphasised that an understanding of succession should not be based solely on the production of fruitbodies. In an analysis of mycorrhizal successions, correlation of above-ground fruitbody production with the associated mycorrhizal symbionts is complicated by the fact that many mycorrhizal fungi do not fruit readily, and that several symbionts may be isolated from a single mycorrhizal root (Melin, 1923; Zak and Marx, 1964).

Despite these difficulties, there does appear to be a close relationship between the presence and position of fruitbodies and their associated mycorrhizas (Laiho, 1970; Tominaga, 1975; Chu-Chou, 1979;
Chu-Chou and Grace, 1981, 1982). Warcup (cited in Mason et al., 1983a) reported that most mycorrhizal fungi could be isolated from beneath their fruitbodies; Tominaga (1975) observed that mycorrhizas of *Tricholoma matsutake* (S. Ito et Imai) Sing were most abundant directly beneath or just beyond the rings of their fruitbodies. Deacon *et al.* (1983) showed, from an analysis of soil cores taken at different distances around an 8-year old birch tree on the experimental plot at I.T.E., Bush Estate, that the distribution of mycorrhizal types broadly reflected the distribution of fruitbodies. The question, first posed for coprophilous fungi by Harper and Webster (1964), is whether all fungi are present on the substrate initially, the observed sequence thus being attributable to the time needed for different fungi to produce a fruitbody, or whether different fungi arrive and colonise at different times, depending on such factors as mineral nutrient status, availability of root exudates and presence of microbial antagonists.

Chu-Chou (1979) and Chu-Chou and Grace (1981, 1982) isolated the symbiont *H. crustuliniforme* from mycorrhizal roots of nursery seedlings, whereas *A. muscaria* could be isolated only from roots of trees older than 13 years. This suggests that fungi in the succession are not all initially present on the substrate. Deacon *et al.* (1983) analysed by dissection, and seedling assay, soil cores from beneath fruitbodies associated with *B. pubescens*. Beneath *Hebeloma*, *Lactarius* and *Leccinum* fruitbodies there was a predominance of their respective mycorrhizal types but only seedlings grown in cores taken from beneath *Hebeloma* sp. became infected by mycorrhizas of the fruitbody species; *Hebeloma*-type and four unknown mycorrhizal types developed on seedlings grown in cores from beneath *Lactarius* and *Leccinum* fruitbodies. A mixture of mycorrhizal types were found beneath fruitbodies of
Inocybe and Laccaria; the fruitbody types were never dominant, yet seedlings grown in these soil cores always developed mycorrhizas typical of the fruiting species.

Similar results were obtained when mycorrhizas were established on birch seedlings from a pure culture source (vermiculite-peat), added to unsterile soil (Deacon et al., 1983). Seedlings planted into natural soils supplemented with H. sacchariolens, H. crustuliniforme and Laccaria sp. formed Hebeloma and Laccaria-type mycorrhizas, respectively, and partially excluded any indigenous fungi. In contrast, L. pubescens only partially colonised the seedlings and only after an extended incubation period; inoculum of Leccinum roseofracta Watling and A. muscaria failed to colonise.

These findings suggest that the succession of fruitbodies around the host is controlled by different physiological mechanisms existing in two broad categories of fungi. Thus a concept of "early-stage" and "late-stage" fungi has been developed in which, under natural conditions, "early-stage" fungi are those capable of colonising young seedlings and, as observed in H. crustuliniforme, may disappear from parts of the root systems as these hosts mature (Mason et al., 1983a). In contrast, "late-stage" fungi appear much later in the succession, on older trees, and, although capable of establishing mycorrhizas on young seedlings under bixenic conditions, they fail to do so in unsterile soil.

Other workers have reported failures in the establishment of what can be considered "late-stage" fungi under unsterile conditions. Thus, Amanita sp. and Lactarius sp. did not form mycorrhizas on Pinus (Marx, 1973). Similarly, Amanita pantherina (DC. ex Fr.) Secr. and Astraeus pteridus (Shear) Zeller failed to colonise Picea sitchensis, although L. laccata and C. geophilum did become established (Shaw and Molina,
1980). These results, however, are not conclusive; Shaw, Molina and Walden (1982) successfully inoculated *A. muscaria* onto *P. sitchensis* in unsterile vermiculite-peat, whereas Marx (1973) failed to establish *L. laccata* on seedlings of *Pinus* sp.

The evidence that sheathing mycorrhizal fungi possess fundamental physiological differences in their capacity to colonise roots of their hosts has important practical implications in the exploitation of mycorrhizal fungi as inocula for the successful establishment of forest plantations. However, extensive information is still needed on the various environmental and inherent factors involved in the epidemiology of the symbiotic relationship.

1.7 Aims and Objectives of the Work in this Thesis

The work in this thesis was designed to investigate sources of inoculum of fungi mycorrhizal with birch, with particular regard to the concept of mycorrhizal succession developed by workers at Edinburgh (Mason *et al.*, 1982, 1983a; Last *et al.*, 1983; Deacon *et al.*, 1983; Fleming, 1983a,b), and elsewhere (Chu-Chou, 1979; Chu-Chou and Grace, 1981, 1982). Especial attention was given to the potential role of basidiospores as inoculum, and to the role of sclerotia as dormant survival structures. Further, an ultrastructural approach was used to study the mycelial strands of selected sheathing mycorrhizal fungi, as mycelial strands are suggested to be important in the symbiotic relationship (Skinner and Bowen, 1974a,b; Read and Malibari, 1978; Duddridge, Malibari and Read, 1980; Brownlee *et al.*, 1983), but have received very little attention to date.
SECTION II

Materials and Methods
2.1 Materials

2.1.1 Fungi

The sheathing mycorrhizal fungi used in this study are listed in Table 2.1. All of the fungi listed were used as basidiospores from freshly collected fruitbodies; those marked with an asterisk were also maintained in culture and originated from fruitbodies.

2.1.2 Growth Media

Various agar media were used for the maintenance of fungal isolates and for some pure-culture studies. Those supplied commercially are as follows.

Davis agar (Gelatine Ltd, Leamington Spa, U.K.)
Malt extract agar, L39 (Oxoid Ltd)
Potato dextrose agar, CM139 (Oxoid Ltd)
Tryptone-soy agar, CM131 (Oxoid Ltd)

In addition, the following growth media were prepared as required.

Chitin Agar (Lingappa and Lockwood, 1962) g/litre distilled water

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colloidal chitin</td>
<td>2.0</td>
</tr>
<tr>
<td>Davis agar</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Thornton's Standardised Agar (cited in Johnson and Curl, 1972)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.1</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>Trace</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>0.5</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.5</td>
</tr>
<tr>
<td>Mannitol</td>
<td>1.0</td>
</tr>
<tr>
<td>Davis agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>
Hagem's medium (Hagem, 1910)

<table>
<thead>
<tr>
<th>Component</th>
<th>g/litre distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>FeCl₃ (1% solution)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0</td>
</tr>
<tr>
<td>Malt extract</td>
<td>5.0</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>50.0 µg</td>
</tr>
<tr>
<td>Davis agar</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Modified Melin Norkrans medium (MMN)
(Marx, 1969a)

<table>
<thead>
<tr>
<th>Component</th>
<th>g/litre distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>0.05</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.025</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>(NH₄)H₂PO₄</td>
<td>0.25</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.15</td>
</tr>
<tr>
<td>FeCl₃ (1% solution)</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>100.0 µg</td>
</tr>
<tr>
<td>Davis agar (10.0 g)</td>
<td>for solid media</td>
</tr>
</tbody>
</table>

Palmer's Basic Medium (PBM)
(cited in Johnson and Curl, 1972)

<table>
<thead>
<tr>
<th>Component</th>
<th>g/litre distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>5.0 µg</td>
</tr>
<tr>
<td>Microelement solution</td>
<td>10.0 ml (see below)</td>
</tr>
</tbody>
</table>

Microelement solution:
- FeNO₃·9H₂O 723.5 mg in 700 ml distilled water,
- MnSO₄·4H₂O 203.0 mg acidified with H₂SO₄ until the solution clears.
- ZnSO₄·7H₂O 439.8 mg

Davis agar (10.0 g) was added to PBM for solid media.
TABLE 2.1: Taxonomic positions of sheathing mycorrhizal fungi of birch used for experimental purposes.

1. Basidiomycotina

A. Boletales

1. Boletaceae
   a Leccinum
      i *L. scabrum* (Fr.) S.F. Gray*
      ii *L. roseofracta* Watling
   b Suillus
      i *S. luteus* (Fr.) S.F. Gray
   c Paxillus
      i *P. involutus* (Fr.) Fr.*

B. Agaricales

1. Cortinariaceae
   a Cortinarius
      i *C. bulbosus* Fr. s. Rick.
      ii *C. delibitus* Fr.
   b Hebeloma
      i *H. crustuliniforme* (Bull. ex St. Amans) Quélt*
      ii *H. leucosarx* P.D. Orton
      iii *H. sacchariolens* Quélt*
   c Inocybe
      i *I. geophylla* (Sow. ex Fr.) Kummer
      ii *I. lacera* (Fr.) Kummer
      iii *I. lanuginella* (Schroet.) Konrad & Maublanc
2. Tricholomataceae
   a Laccaria
      i L. laccata (Scop. ex Fr.) Cke.
      ii L. proxima (Boud.) Pat.*
      iii L. tortilis ([Bol.] S.F. Gray) Cke.

C. Russulales

1. Russulaceae
   a Lactarius
      i L. blennius (Fr. ex Fr.) Fr.
      ii L. pubescens (Fr. ex Krombh.) Fr.*
      iii L. rufus (Scop. ex Fr.) Fr.
      iv L. spinosulus Quél
      v L. turpis (Weinm.) Fr.
      vi L. vietus (Fr.) Fr.

   b Russula
      i R. cyanoxantha (Schaeff. ex Secr.) Fr.
      ii R. grisea (Pers. ex Secr.) Fr.

D. Aphyllophorales

1. Thelephoraceae
   a Thelephora
      i T. terrestris (Ehrh.) Fr.*

E. Sclerodermatales

1. Sclerodermataceae
   a Scleroderma
      i S. citrinum Pers.*

2. Ascomycotina

A. Tuberales

1. Elaphomycetaceae
   a Elaphomyces
      i E. muricatus Fr.
2.1.3 Isolation of Sheathing Mycorrhizal Fungi

2.1.3.1 Isolation of fungi from fruitbodies

The caps of young, freshly collected fruitbodies were broken open under aseptic conditions, and small portions of the vegetative tissue were dissected out and placed onto Hagem's agar or potato dextrose agar at half or full-strength concentrations (Section 2.1.2). With the notable exception of Inocybe spp., most sheathing mycorrhizal fungi used in the present study were readily obtained in pure culture by this method.

2.1.3.2 Isolation of fungi from mycorrhizas

In some experiments, accurate identification of mycorrhizal types was checked by reisolation of the symbiont from mycorrhizal roots, and in these cases the procedure described by Chu-Chou (1979) and Chu-Chou and Grace (1981), a modification from that of Harley and Waid (1955), was used. Freshly collected segments of roots, 3–5 cm long and bearing mycorrhizal tips, were washed under running tap water for 10 minutes to remove adhering soil particles. They were then transferred to McCartney bottles containing 20–30 ml sterile distilled water and agitated on a Gallenkamp wrist-action shaker for 2 minutes. They were then transferred aseptically to fresh bottles of sterile distilled water and shaken for 2 minutes; this process was repeated ten times. The washed roots were surface-sterilised for 10 minutes in 20 ml of a solution (0.7%) of calcium hypochlorite and then rinsed in three changes of sterile distilled water. The roots were then transferred to sterile empty Petri dishes and the mycorrhizal tips were removed aseptically and then blotted dry on sterile filter papers. Mycorrhizal tips were then plated onto either half-strength potato dextrose agar with strepto-
mycin, or Hagem's agar with streptomycin (Section 2.1.2). Five to ten mycorrhizal tips were spaced apart on each plate; the plates were incubated at 20°C and examined microscopically every 2-3 days.

2.1.4 Collection and Preparation of Seed

Seed of Betula pendula Roth. was collected during the autumn of 1980 and 1981 from one tree (seed lot 5, clone b; origin Dinnet, Scotland) which had been planted in 1975 at the Institute of Terrestrial Ecology (I.T.E.), Bush Estate, Penicuik, Midlothian.

After collection, the catkins were dried at room temperature for 7-14 days and then rubbed over an 8 mesh (2 mm) B.S. sieve to remove wings and chaff; the seeds were then stored in sealed brown envelopes at 5°C. As required, seed samples were immersed in industrial methylated spirits and allowed to settle. All floating material, representing non-viable seed (Baldwin, 1932), was decanted off, and that which had settled was blotted dry on filter paper. Viable seeds were transferred, in lots of 50, to McCartney bottles containing 3 ml hydrogen peroxide (30% w/v), in which they were surface-sterilised for 20 minutes by shaking on a Gallenkamp wrist-action shaker. Working under aseptic conditions, the seed was floated to the top of the McCartney bottles with sterilised tap water; individual seeds were transferred with a sterilised needle to 1% tap water agar, 20-30 seeds per plate (Mason, 1980). Each Petri dish was sealed with strips of "cling-film" and then incubated in aluminium foil-lined cabinets under lights; the Gro-lux fluorescent light strips (40 W) provided a light intensity of 10 Wm² at plant level, as measured with a Kipp’s solarimeter. Seeds were incubated for 14-21 days or until the radicles of the germinated seeds were 5-8 mm long. Checks for contamination were made at 3 day intervals and any contaminated seeds were aseptically removed with the underlying agar.
2.1.5 Source and Preparation of Soil

Unless otherwise stated, the soil used for all pot-culture studies was a brown earth collected from a tree-less site at Castlelaw, south of Edinburgh (N.G.R. NT 228638). After collection, it was air-dried for at least 21 days and then stored in strong plastic sacks. Prior to use, the soil was sieved <2 cm and mixed with grit (3:1 v/v) which had been washed and then autoclaved for 30 minutes at 121°C, to give a loose, aerated soil. The soil-grit mixture was transferred to plant pots and watered to 40% saturation (Keen and Raczkowski, 1921); it was left for 4 days before use. Chemical analysis of untreated soil (Table 2.2) was done at I.T.E., Merlewood, Cumbria. In one experiment (Section 3.6), coal spoil, collected from a disused coal spoil tip at Newtongrange (Section 2.1.6.1), was used as a growth medium for birch seedling assay. It was prepared in the same way as the brown earth described above, the only difference being that it was watered to its original water content, approximately 23% saturation, prior to use.

TABLE 2.2: Chemical analysis of brown earth collected from Castlelaw, south of Edinburgh; the results represent the values of two samples, all results being expressed on a dry weight basis, mg/100 g.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td>K</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Ca</td>
<td>330</td>
<td>300</td>
</tr>
<tr>
<td>Mg</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Fe</td>
<td>35</td>
<td>290</td>
</tr>
<tr>
<td>P</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>P%</td>
<td>0.14</td>
<td>0.17</td>
</tr>
<tr>
<td>extractable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>extractable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₂ + NO₃</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>NH₄</td>
<td>7.1</td>
<td>0.27</td>
</tr>
</tbody>
</table>
2.1.6 Description of Sites from which Fungal Material was Collected

Fungal material, namely fruitbodies, mycorrhizas, mycelial strands and sclerotia of sheathing mycorrhizal fungi, was collected from a number of sites within a 10 mile radius of I.T.E., Bush Estate, Penicuik, Midlothian, and also from Loch Inver, Sutherland. The main features of each site are described below; the major sources of the mycorrhizal species used in the present study are summarised in Table 2.5.

2.1.6.1 Coal spoil tip at Newtongrange, Midlothian

The coal spoil tip at Newtongrange (N.G.R. NT 330649), Midlothian, has been disused since 1950 but is still subject to "after-burning". It comprises a central burning area on top of the tip which is devoid of plant life. Surrounding this, however, extensive colonisation by naturally regenerating birch has occurred; the oldest of these trees has been estimated to be approximately 15-20 years old (K. Ingleby, pers. comm.). Large numbers of fruitbodies of a restricted range of species known or suspected to form sheathing mycorrhizas with birch (Trappe, 1962) occurred during the autumn of 1981 and 1982. Their distribution was not uniform; rather, the fruitbodies of different species were usually found on different parts of the site. Table 2.3 shows such contrasting distributions of the fungi in two sample areas, the most marked difference, which was confirmed by examination of the mycorrhizas on the roots, being that Paxillus involutus was found almost exclusively on the periphery of the burning area. Of interest, Scleroderma citrinum was most abundant on the hotter parts of the tip, and its fruitbodies were often found up to 3 metres from the nearest tree. In contrast to the periphery of the burning area, the south-facing slope had ground cover vegetation and, in some places,
a thin layer of soil, high in organic matter. Fruitbodies of *Lactarius* spp., absent from the top of the site, were abundant here. Those of *L. turpis* and *Cortinarius* spp. were invariably associated with run-off channels in which large quantities of partially decomposed leaf litter had collected. Chemical analysis (Table 2.4) of the coal waste was

**TABLE 2.4:** Chemical analysis of samples of coal waste taken at 28 points along transects on a disused coal spoil tip at Newtonrange; mean values for two sample depths (data provided by K. Ingleby).

<table>
<thead>
<tr>
<th>Mean value of 14 samples for each depth</th>
<th>Depth (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-15</td>
</tr>
<tr>
<td>pH</td>
<td>4.31</td>
</tr>
<tr>
<td>Total N (%)</td>
<td>0.47</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>K</td>
<td>0.74</td>
</tr>
<tr>
<td>Ca</td>
<td>16.9</td>
</tr>
<tr>
<td>Al</td>
<td>267.14</td>
</tr>
<tr>
<td>Mn</td>
<td>0.085</td>
</tr>
</tbody>
</table>

done on 28 separate samples collected at depths of either 10-15 cm or 15-30 cm along two transects (K. Ingleby, pers. comm.). All coal spoil samples were acidic, ranging from pH 3.9–4.9; the available levels of all major nutrients and especially of phosphorus were low. Levels of nitrogen were generally high, but this element was probably present in organic matter; potentially, phytotoxic levels of aluminium (>200 mg/100 g) were found at several points along the transects.
TABLE 2.3: The occurrence of fruitbodies of sheathing mycorrhizal fungi recorded on four dates during September and October 1982, on a disused coal spoil tip at Newtongrange.

<table>
<thead>
<tr>
<th>Fruitbody species</th>
<th>South-facing slope</th>
<th>Periphery of burning area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14, 29 September</td>
<td>10, 30 October</td>
</tr>
<tr>
<td></td>
<td>14, 29 September</td>
<td>10, 30 October</td>
</tr>
<tr>
<td>Amanita muscaria</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cortinarius spp.</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Hebeloma crustuliniforme</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Inocybe lacera</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Lactarius rufus</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Lactarius turpis</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Leccinum roseofracta</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Leccinum scabrum</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Paxillus involutus</td>
<td>nil</td>
<td>+</td>
</tr>
<tr>
<td>Scleroderma citrinum</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

nil, absent; +, occasional; ++, frequent; +++ , abundant.
2.1.6.2 Experimental birch plot, Institute of Terrestrial Ecology (I.T.E.), Bush Estate, Penicuik, Midlothian

A plot of 60 birch trees, representing twelve seed collections of *Betula pendula* and nine of *B. pubescens* Ehrh., was established in 1971 at I.T.E., Bush Estate (N.G.R. NT 246638). Although initially planted as an arboretum, with 3 metre spacing between and within rows, the plot has subsequently been used for experimental purposes. The soil is an agricultural brown earth of pH 6.4; the site had previously supported potatoes and soft fruit for at least 30 years. Since 1975, fruitbodies occurring in association with the birch trees have been mapped using a permanently marked grid (Mason *et al.*, 1982); in addition the distribution of sheathing mycorrhizal types has been studied (Deacon *et al.*, 1983). As a consequence, the development of the mycorrhizal associations on many of the individual trees is known.

2.1.6.3 Experimental birch plot, Glencorse, Midlothian

Fruitbodies of *Hebeloma crustuliniforme*, *Laccaria proxima* and *Laccaria tortilis* were collected from an experimental plot of *Betula pendula* and *B. pubescens* at Glencorse (N.G.R. NT 245633), Midlothian. The trees were 2 years old, 1-1½ metres in height, and represent a collection of 196 seed lots taken from sites representing the 13 major land classes in Scotland. The soil is a well-drained agricultural brown earth; the site was formerly used for barley culture, and a predominately ryegrass sward was established as ground cover under the trees at planting.
2.1.6.4 Birch screen, Scottish Crop Research Institute (S.C.R.I.), Pentlandfield, Midlothian

Fruitbodies of Leccinum spp., Inocybe geophylla and Cortinarius spp. were collected from a screen of about 60 birch trees established originally as part of a birch provenance trial set up at Bush Estate in 1957. The birch screen at S.C.R.I. (N.G.R. NT 246638) represented 13 different provenances of Betula pendula and B. pubescens collected from Britain, Sweden and Norway. The soil is a well-drained agricultural brown earth; the site previously supported potatoes and soft fruit for at least 15 years.

2.1.6.5 Loch Inver, Sutherland

Fruitbodies of a number of sheathing mycorrhizal fungi (Table 2.5), not found near Edinburgh, were collected from natural birch woodland in the vicinity of Loch Inver, Sutherland (N.G.R. NT 236098). The soil is generally peaty and high in organic matter.

2.2 General Experimental Methods

The following general methods were used throughout the study; further details of specific experimental methods are given in the appropriate experimental sections.

2.2.1 Production of Inoculum of Sheathing Mycorrhizal Fungi

For general purposes, sheathing mycorrhizal fungi were cultured in vermiculite-peat medium moistened with Modified Melin Norkrans (MMN) nutrient solution (Marx, 1969a).

Horticultural vermiculite was sieved with an 8 mesh (2 mm) B.S. sieve to remove all the fine dust; the fraction retained by the sieve was mixed with similarly sieved peat of pH 3.5 (9:1 v/v); batches of
TABLE 2.5: Summary of the main sources of fruitbodies of sheathing mycorrhizal fungi used for experimental purposes.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Site</th>
<th>Soil type</th>
<th>Approx. age of host (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Laccaria tortilis</em></td>
<td>Glencorse</td>
<td>mineral</td>
<td>2</td>
</tr>
<tr>
<td><em>Laccaria proxima</em></td>
<td>Glencorse</td>
<td>mineral</td>
<td>2</td>
</tr>
<tr>
<td><em>Hebeloma sacchariolens</em></td>
<td>Bush Estate</td>
<td>mineral</td>
<td>12</td>
</tr>
<tr>
<td><em>Hebeloma leucosarx</em></td>
<td>Bush Estate</td>
<td>mineral</td>
<td>12</td>
</tr>
<tr>
<td><em>Hebeloma crustuliniforme</em></td>
<td>Glencorse</td>
<td>mineral</td>
<td>2</td>
</tr>
<tr>
<td><em>Inocybe lacera</em></td>
<td>Newtonrange</td>
<td>coal spoil</td>
<td>15-20</td>
</tr>
<tr>
<td><em>Inocybe lanuginella</em></td>
<td>Bush Estate</td>
<td>mineral</td>
<td>12</td>
</tr>
<tr>
<td><em>Inocybe geophylla</em></td>
<td>S.C.R.I.</td>
<td>mineral</td>
<td>25</td>
</tr>
<tr>
<td><em>Paxillus involutus</em></td>
<td>Newtonrange</td>
<td>coal spoil</td>
<td>15-20</td>
</tr>
<tr>
<td><em>Lactarius pubescens</em></td>
<td>Bush Estate</td>
<td>mineral</td>
<td>12</td>
</tr>
<tr>
<td><em>Lactarius rufus</em></td>
<td>Loch Inver</td>
<td>mineral</td>
<td>mature</td>
</tr>
<tr>
<td><em>Lactarius blennius</em></td>
<td>Newtonrange</td>
<td>coal spoil</td>
<td>15-20</td>
</tr>
<tr>
<td><em>Lactarius vietus</em></td>
<td>Loch Inver</td>
<td>mineral</td>
<td>mature</td>
</tr>
<tr>
<td><em>Lactarius spinosulus</em></td>
<td>S.C.R.I.</td>
<td>mineral</td>
<td>25</td>
</tr>
<tr>
<td><em>Russula grisea</em></td>
<td>Loch Inver</td>
<td>mineral</td>
<td>mature</td>
</tr>
<tr>
<td><em>Russula cyanoxantha</em></td>
<td>Loch Inver</td>
<td>mineral</td>
<td>mature</td>
</tr>
<tr>
<td><em>Cortinarius delibitus</em></td>
<td>Newtonrange</td>
<td>coal spoil</td>
<td>15-20</td>
</tr>
<tr>
<td><em>Cortinarius bulbosus</em></td>
<td>Loch Inver</td>
<td>mineral</td>
<td>mature</td>
</tr>
<tr>
<td><em>Leccinum scabrum</em></td>
<td>Newtonrange</td>
<td>coal spoil</td>
<td>15-20</td>
</tr>
<tr>
<td><em>Leccinum roseofracta</em></td>
<td>S.C.R.I.</td>
<td>mineral</td>
<td>25</td>
</tr>
<tr>
<td><em>Scleroderma citrinum</em></td>
<td>Newtonrange</td>
<td>coal spoil</td>
<td>15-20</td>
</tr>
<tr>
<td><em>Elaphomyces muricatus</em></td>
<td>Struan Wood*</td>
<td>mineral</td>
<td>mature</td>
</tr>
</tbody>
</table>

*Calvine, Perthshire.
250 ml of the mixture were then added to 500 ml capacity Erlenmeyer flasks. Each batch of medium was moistened with 180 ml MMN nutrient solution (Section 2.1.2), the flasks were plugged with cotton-wool which was covered with aluminium foil and the flasks were then autoclaved for 30 minutes at 121°C; the final pH of the medium after autoclaving was 4.8 - 5.2. The flasks were inoculated with usually two or three agar blocks cut from just behind the margins of 4-8 week old colonies. The inoculated flasks were incubated in darkness at 20°C for 2-3 months or until the vegetative mycelium had colonised the medium.

2.2.2 Bixenic Synthesis of Sheathing Mycorrhizal Fungi

Seedlings bearing mycorrhizas of selected sheathing mycorrhizal fungi were required for some experiments and they were obtained using the sterile closed system developed by Mason (1980) and Mason et al. (1983b). Horticultural seed trays, 220 mm long, 165 mm wide and 55 mm deep, without drainage holes, were fitted with transparent propagator lids (Stewart Plastics Ltd, Croydon, Surrey). The trays were fitted with 140-150 grey plastic tubes (Figure 2.1) as supplied to the Forestry Commission by Telcon Plastics, Orpington, Kent. Aeration holes in the propagator lids were fitted with plugs of cotton-wool, the lids were secured to the bases with autoclave tape and the containers were then sealed individually in polythene bags, together with a seed tray, the whole constituting a "unit" which was then sterilised by gamma radiation (2.5 Mrad).

Vermiculite-peat medium was prepared in Erlenmeyer flasks as described in Section 2.2.1, but supplemented with only 150 ml MMN nutrient solution per 250 ml vermiculite-peat. The following procedures, requiring two people, were carried out on laminair flow cabinets to ensure
FIGURE 2.2: Closed system for establishing sheathing mycorrhizas during propagation (reproduced by kind permission of Dr. P.A. Mason)
sterility. The spare seed-tray of each "unit" was used for mixing the 250 ml vermiculite-peat medium, prepared as above, with 750 ml inoculated medium, prepared as described in Section 2.2.1. Using a long-handled spoon, the medium was transferred to the tubes in the seed-tray, the tubes being gently tapped to ensure adequate packing. Thereafter, one seedling, aseptically germinated as described in Section 2.1.4 was transferred to each tube. The propagator lid was then replaced and sealed to the tray with adhesive tape; the sealed propagator units were incubated in open laboratory growth cabinets at 20°C under Gro-lux fluorescent strips supplying a light intensity of 10 W/m² at plant height as measured with a Kipp's solarimeter. After 8 weeks' incubation, when mycorrhizas had developed on the roots, the cotton-wool plugs were removed from the propagator lids; this served both to break sterility and to adjust the seedlings to more normal conditions. Subsequently, the lids were removed for increasing lengths of time each day before the open trays were transferred to a glasshouse or a growth room. During the 2 weeks of "hardening-off", seedlings were sprayed daily with sterile water.

2.2.3 Environmental Conditions in Growth Room and Glasshouse

Unless otherwise stated, seedlings grown in plant pots were incubated either in a heated glasshouse or in a growth room which provided stricter control of temperature and illumination; the seedlings were watered daily with sterile or unsterile tap water.

2.2.3.1 Heated glasshouse

Pot-grown seedlings were maintained on steel mesh benches beneath 400 W mercury vapour lamps supplying light intensities ranging from 3 W/m² to 10 W/m² at plant height depending on the position of the
bench; the lamps provided a 16 h light/8 h dark cycle but were switched off for 4 h each day during the winter and for 7 h each day during the summer, to take account of natural daylight. The mean temperature ranges for each month during 1982 were recorded (Table 2.6).

### Table 2.6: Mean minimum and maximum temperatures (°C) recorded during 1982 in a heated glasshouse in which pot-grown seedlings were incubated.

<table>
<thead>
<tr>
<th></th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>10.2</td>
<td>15.9</td>
</tr>
<tr>
<td>February</td>
<td>14.2</td>
<td>18.0</td>
</tr>
<tr>
<td>March</td>
<td>13.8</td>
<td>19.8</td>
</tr>
<tr>
<td>April</td>
<td>14.4</td>
<td>22.0</td>
</tr>
<tr>
<td>May</td>
<td>14.7</td>
<td>23.8</td>
</tr>
<tr>
<td>June</td>
<td>14.9</td>
<td>23.6</td>
</tr>
<tr>
<td>July</td>
<td>14.4</td>
<td>28.2</td>
</tr>
<tr>
<td>August</td>
<td>15.0</td>
<td>24.9</td>
</tr>
<tr>
<td>September</td>
<td>14.6</td>
<td>22.5</td>
</tr>
<tr>
<td>October</td>
<td>14.3</td>
<td>18.7</td>
</tr>
<tr>
<td>November</td>
<td>14.1</td>
<td>17.4</td>
</tr>
<tr>
<td>December</td>
<td>14.0</td>
<td>16.8</td>
</tr>
</tbody>
</table>

**2.2.3.2 Growth room**

Pot-grown seedlings were maintained on a steel mesh frame placed 30 cm above ground level and beneath four mercury vapour lamps (400 W) supplying light intensities ranging from 16 W/m² to 31 W/m² at plant height in a 16 h light/8 h dark cycle. The temperature recorded between October 1981 and April 1983 ranged from a minimum of about 15°C to a maximum of about 18°C, with little variation over the whole 18-month period.
2.2.4 Procedure for the Assessment of Mycorrhizal Establishment on Roots of Pot-Grown Seedlings

Unless otherwise stated, the establishment of sheathing mycorrhizas on the root systems of pot-grown seedlings was assessed using the following procedure. Each seedling was removed from its pot and the root system was washed over an 8 mesh (2 mm) B.S. sieve. The shoot system was cut from the root system at the root/hypocotyl junction, and the stem height and total number of leaves, including cotyledons if still present, were recorded. The shoots were then placed individually in small brown envelopes and dried to constant weight at 80°C.

The extent of the root system was measured and the roots of each seedling were then aligned on a grid and cut into 1 cm lengths. One-centimetre portions were then sampled at alternate 1 cm intervals, each portion being placed in water and examined under a Wild stereo-scan microscope. The total numbers of mycorrhizal and non-mycorrhizal root tips were recorded for each 1 cm portion. Mycorrhizas were typed according to gross morphological characters using reference material that had been collected from the field or formed in bixenic culture with isolates derived from fruitbodies. Samples of different mycorrhizal types were preserved in 2% glutaraldehyde at 5°C.

2.2.5 Transmission Electron Microscopy

The transmission electron microscope (T.E.M.) was used to investigate the structure of sclerotia and sclerotium-like bodies of several sheathing mycorrhizal fungi on birch, most notably *Hebeloma sacchariolens* and *Paxillus involutus*. In addition, preliminary studies were done on the structure of mycelial strands of *Leccinum scabrum* and *P. involutus* obtained from the root zone of naturally regenerating birch at Newtongrange (Section 2.1.6.1) and
from the experimental birch plot at I.T.E., Bush Estate, Penicuik, Midlothian (Section 2.1.6.2).

2.2.5.1 Preparation procedures for T.E.M. studies

Fixation:

Sclerotial material and mycelial strands collected from the field, and from laboratory syntheses, were washed under running tap water and adhering soil particles were removed with forceps and a camel hair brush. The sclerotia were cut in half and the mycelial strands were cut into 1 mm lengths while immersed in fixative (see below). Unless otherwise stated, the following procedures were done at 4°C.

Sclerotia and mycelial strands were fixed for 4 h in a solution of 2.5% glutaraldehyde and 1% formaldehyde in 0.1M sodium cacodylate buffer. They were then rinsed in three, 15 minute changes of 0.1M sodium cacodylate buffer at pH 7.2, post-fixed for 8 h in 1% (w/v) osmium tetroxide in 0.1M sodium cacodylate buffer and finally rinsed in three, 15 minute changes of 0.1M sodium cacodylate buffer at pH 7.2.

Dehydration:

All the specimens were dehydrated through a graded ethanol series (25, 50, 75, 85 and 100% v/v) in distilled water at room temperature, being held for 15 minutes at each stage, and for 20 minutes, with one change, in 100% ethanol. Finally, the specimens were transferred through two changes (20 minutes) of 100% anhydrous ethanol.

Embedding:

The specimens were infiltrated with a mixture of Spurr's resin (supplied by TAAB Laboratories Ltd, Croydon, Surrey) and anhydrous ethanol, prepared as described in Spurr (1969). The proportion of the resin in the mixture was progressively increased as follows:
Sclerotial material and mycelial strands were embedded in 100% Spurr's resin in the flat tops of TAAB polythene embedding capsules. The resin was then polymerised at 80°C for 24 h.

**Sectioning:**

Sections with gold or silver-grey interference colours, i.e. 50-60 nm thick, were cut with glass knives on a Reichart ultratome. The sections were picked up on copper-paladium grids (G300, 3.05 diameter; Polaron Equipment Ltd) and then stained for 30 minutes at 60°C in 5% uranyl acetate in 1% acetic acid, followed by Reynolds lead citrate (Reynolds, 1963) for 10 minutes. They were then washed in 10 changes of distilled water and allowed to dry on filter papers placed in the base of Petri dishes. The sections were examined on a transmission electron microscope, model A.E.I. 801A. Thicker sections with blue-green interference colours, i.e. of 60-70 nm thickness, were cut for light microscopical examination; these were stained in 1% toluidene blue in 1% borax in distilled water.

2. 2. 6 **Scanning Electron Microscopy**

The scanning electron microscope (S.E.M.) was used to further investigate the structure of sclerotium-like bodies and sclerotia of several sheathing mycorrhizal fungi. Preliminary studies were also done on the structure of mycelial strands of *Lactarius rufus*, *Leccinum scabrum* and *Paxillus involutus* harvested either from the root zone of birch at the experimental plot, I.T.E., Bush Estate (Section 2.1.6.2).
or from beneath fruitbodies of these species associated with naturally regenerating birch at a disused coal spoil tip at Newtongrange (Section 2.1.6.1).

2.2.6.1 Preparation procedures for S.E.M. studies

Mycelial strands cut into short lengths, and sclerotial material, were fixed, after minimal washing in running tap water, in a mixture of equal parts of 3.6% aqueous glutaraldehyde, 0.6M sodium cacodylate buffer and 1500 ppm ruthenium red (Luft, 1971), the ruthenium red being used primarily to detect the presence of mucilaginous substances. A minimum fixation period of 24 h was found necessary in order to achieve effective penetration of thick-walled material, particularly of sclerotia. After fixation, the specimens were washed in three, 20 minute changes of 0.6M sodium cacodylate buffer.

Dehydration:

The specimens were dehydrated at 4°C through a graded acetone series (25, 50, 75, 85, 100% v/v) in distilled water, being held for 15 minutes at each stage, with two changes for 1 h in 100% acetone.

Critical point drying:

Various methods of fixation and dehydration, including air-drying, have been used for the preparation of woody root material for the S.E.M. (Campbell and Rovira, 1973; Seviour, Hamilton and Chilvers, 1978), but the superiority of critical point drying in that it causes minimum distortion is now widely accepted (Gusnard and Kirschner, 1977). Critical point drying was done according to the methods described by Anderson (1951, 1966) and involved, (1) the substitution of the acetone in dehydrated specimens by carbon dioxide; (2) heating to super-critical temperature under pressure; and (3) pressure release.
Fungal material was dried using a Polaron E300 Critical Point Drying apparatus.

**Mounting and coating:**

Immediately after critical point drying, the specimens were mounted on S.E.M. metal stubs using double sided "Sellotape"; they were then cracked open with a safety razor blade under a stereoscan microscope, coated with gold using a "Nanotech S.E.M. prep 2" sputter coater and examined on a Cambridge Stereoscan microscope, operating at 30 KV.

2.2.7 **Light Microscopy**

Replicate samples of mycelial strands and sclerotial material, prepared for transmission electron microscopy, were examined by light microscopy. A range of histochemical staining techniques were used to provide information on the major storage reserves within sclerotium-like bodies and sclerotia; they are described fully in Section 4.4. For general purposes, root and fungal material was stained in 1% cotton blue in lactophenol.

2.2.8 **Statistical Analysis**

Unless otherwise indicated, the experimental data were subjected to Analysis of Variance, and further analysed if the variance ratio test was significant; because most experiments had uneven numbers of replicates, differences between means were estimated using the Student-Newman-Keuls multiple range test (Sokal and Rohlf, 1969).

An underlying assumption of Analysis of Variance is that the sample variances are equal, but this requirement was seldom met in the collected results because variance was often proportional to the mean; therefore, statistical analysis was done on log₁₀ transformations of the
results, in order to make the variance of a sample independent of the mean. As the numerical results occasionally included zeros, the transformation, log(x+1), was used throughout. Results obtained as percentages or proportions were transformed to arcsines for statistical analysis (Gregory, 1948). In tabulating the results, the transformed logarithmic means are presented in parentheses beneath the arithmetic means. However, percentages are presented in the tables in their original forms only, the significant differences (calculated as transformed data) being indicated by superscripts.
SECTION III

Infection of Roots of Birch Seedlings from Basidiospores and Other Sources of Inoculum of Sheathing Mycorrhizal Fungi
3.1 Introduction

Several workers (Shemakhanova, 1962; Azvedo, 1970, cited in Marx, 1980; Trappe, 1977; Marx, 1980) have attempted to use basidiospores of sheathing mycorrhizal fungi for the inoculation of nursery and containerised tree seedlings, but the results have been equivocal. To date, there have been few reports of successful inoculation of tree roots using spore inocula in untreated soil (Mullette, 1976). Yet, Robertson (1954) concluded, from indirect evidence, that basidiospores of at least some sheathing mycorrhizal fungi must act as infective units. He demonstrated that, when placed near a pine plantation, seedlings of *Pinus sylvestris* developed mycorrhizas when grown in autoclaved soil in open containers but not in identical conditions in closed containers. A similar finding was reported by Marx and Bryan (1969) who eliminated all "casual" mycorrhizal infection seedlings of *Pinus echinata* by filtering air entering a growth room; in contrast, seedlings grown in the same fumigated, non-inoculated soil conditions but outside the growth room developed a high proportion of mycorrhizal roots.

A major obstacle in experimental work on this topic is the difficulty of germinating spores of sheathing mycorrhizal fungi in pure culture (Fries, 1966, 1978), so the factors responsible for the success or failure of spore inocula remain largely unknown. A modification of the method used for testing the viability of higher plant seeds (Moore, 1962) has been used for the assessment of spore viability in mycorrhizal fungi (Blakeslee, 1974); nevertheless, Marx (1980) has expressed the opinion that the only reliable means of determining spore viability is by seedling assay. Unfortunately, this method makes accurate quantification difficult and is of little use for fungi that do not readily infect tree roots from spore inocula.
The most extensive studies on the infectivity of spores of sheathing mycorrhizal fungi have been done by Marx and co-workers using *Pisolithus tinctorius* (Marx and Bryan, 1975; Marx, 1976a; Marx, Bryan and Cordell, 1976; Marx, Morris and Mexal, 1978; Marx, Mexal and Morris, 1979). Interest in this fungus stems mainly from its widespread occurrence on coal wastes where it can tolerate extremes of drought and temperature (Schramm, 1966). In some soil environments it is a characteristic mycorrhizal symbiont of mature pine, but it rarely occurs naturally on nursery seedlings (Marx, 1980). The addition of spores of *P. tinctorius* to "hydromulch" (a mixture of commercial compost and water) incorporated into fumigated nursery soil was shown to be the most effective method of spore inoculation (Marx et al., 1979). Thus, after 6 months' growth, 75% of the inoculated seedlings of *Pinus taeda* had developed mycorrhizas of *P. tinctorius*; the treatment provided a 15% increase in the number of seedlings that could be outplanted as well as giving a 25% increase in overall seedling growth (Marx et al., 1979). The time of spore inoculation is reported to be a critical factor affecting the successful establishment of mycorrhizas (Lamb and Richards, 1974a,b). Seedlings inoculated with *P. tinctorius* 8 weeks after germination developed fewer mycorrhizas than did seedlings inoculated 2 weeks after germination and incubated for a similar time; this difference was attributable to the early establishment of mycorrhizas of *Thelephora terrestris*, an aggressive colonist of roots and the predominant mycorrhizal fungus occurring in nurseries in the south-eastern United States.

As discussed in Section 1.6, research at Edinburgh (Mason et al., 1982, 1983a; Deacon et al., 1983) has shown that a succession of sheathing mycorrhizal fungi occurs on birch trees as they age, and that
a broad distinction can be made between "early-stage" and "late-stage" mycorrhizal fungi. As defined, "late-stage" fungi are those that can establish infections on seedlings under bixenic conditions, indicative of compatibility with seedling roots, yet fail to establish from vermiculite-peat cultures containing vegetative mycelium, or from other dispersed sources of inoculum in unsterile soil. In contrast, "early-stage" fungi will readily establish infections from mycelial inoculum sources dispersed in unsterile soil.

Experiments in this section were designed to test the role of basidiospores as inocula of mycorrhizal fungi for infection of birch seedlings in unsterile soil. This was done with particular regard to the distinction, noted above, between "early-stage" and "late-stage" mycorrhizal fungi. In addition, attention was paid to the influence of soil type on the efficacy of spore inocula, and to the effects of storage of spores under different conditions on their subsequent infectivity.

3.2 Materials and Methods

Fruitbodies of twenty-four basidiomycetes and one ascomycete, known to form sheathing mycorrhizas with birch species (Trappe, 1962; Mason et al., 1982) were collected from beneath birch during the autumn of 1981 and 1982; details of the sites are given in Section 2.1.6. The stipes were partly removed and the caps were then suspended close to the surface of brown earth soil, or, in some experiments, coal spoil, contained in 5 cm³ plant pots; one fruitbody was used for each pot. After 24 h in dark, humid conditions, the fruitbodies were removed and discarded, and the spores that had been deposited were lightly mixed into the top 1 cm of soil in each pot. No attempt was made to count the number of spores released from each fruitbody, but in each case, the
corner of a glass coverslip was placed beneath part of the cap to ensure that spores had been deposited in large numbers. In most experiments a brown earth collected from a tree-less site at Castlelaw (Section 2.1.5) was used, but in one experiment a brown earth collected from S.C.R.I. (Section 2.1.6.4) was substituted for it and in another experiment a coal spoil which had been collected from Newtonrange (Section 2.1.6.1) was used. In all cases, the soils and coal spoil were unsterile; they had been air-dried for 21 days, rewetted to 40% saturation (Keen and Raczkowski, 1921) or, in the case of the coal spoil, to 23% saturation, and kept in plant pots at this level of saturation for 4 days before use.

Variable numbers of replicates were prepared, depending on the availability of fruitbodies. In most cases, the soil was sown to birch immediately after addition of spores, but in some experiments the basidiospore-supplemented soils were stored to test survival of the added spore inocula. To test the infectivity of spores, the following birch assay procedure was used. Seeds of *Betula pendula* were surface-sterilised in H₂O₂ and germinated on 1% tap water agar (Section 2.1.4). After 21 days' growth, or when the radicles of the seedlings were about 10 mm long, two seedlings were planted into each pot. In the first six experiments (Sections 3.3, 3.4), in which the infectivity of fresh spore inocula was tested, the pots were arranged in pairs with the different treatment pairs being spaced 20 cm apart in a randomised layout in a growth room. The growth room provided a controlled 16 h light/8 h dark cycle and a mean temperature range of 15-20°C. In the other experiments (Sections 3.5, 3.6, 3.7, 3.8, 3.9, 3.10, 3.11, 3.12), the pots were individually spaced 20 cm apart in a randomised layout under the same growth room conditions. The pots were watered daily with sterilised tap water. The seedlings were thinned to one per pot after
6 weeks and, unless otherwise stated, all the experiments were harvested after 12-16 weeks' growth.

For assessment of mycorrhizal development, the root systems were washed free of soil, placed linearly on a grid and cut into 1 cm portions (Section 2.2.4). Alternate portions were scored for numbers of root tips and numbers of different mycorrhizal types, these being compared with reference material which had been either collected from beneath fruitbodies in the field or harvested from pot-culture syntheses. In all experiments, pots of unsupplemented soil were used for comparison with the treatment series. Further details of soil preparation, growth room conditions and methods of assessment of the root systems are given in Section 2.

Explanatory notes for tables and text:

In each table, mean numbers of root tips on the seedlings and shoot oven-dry weights for each treatment are given; the respective geometric means (log x+1) were used for statistical analysis (Section 2.2.8) and are given in parentheses beneath the arithmetic means. In addition, the proportions of root tips uncolonised or colonised by different mycorrhizal types are given in the form of percentages, the significant differences (calculated as arcsin-transformed percentages) being indicated by superscripts.

Mycorrhizal fungi that became established from resident sources of inoculum in soil or from air-borne or water-borne spores are usually referred to as "resident" or "resident-type" mycorrhizas in the tables or text. Mycorrhizas attributable to the fungus used for inoculation are referred to as "inoculant" or "inoculant-type" mycorrhizas.
3.3 Experimental: Investigation on the Infectivity of Freshly Collected Basidiospores of Sheathing Mycorrhizal Fungi in Unsterile Brown Earth

3.3.1

In the initial experiment of this series, the infectivity of freshly collected spore inocula of seven fungi known to form sheathing mycorrhizas on birch was tested by birch seedling assay. Fruitbodies were collected during the autumn of 1981 from three sites near Edinburgh - Bush Estate, S.C.R.I. and Newtonrange (Section 2.1.6); the caps were suspended over pots of unsterile brown earth as previously described (Section 3.2) and birch assay seedlings were grown in the pots for 16 weeks in a growth room (Section 2.2.3.2).

In the control (unsupplemented) series, the seedlings developed two mycorrhizal types, attributable to *Laccaria* and *Hebeloma* on three and two seedlings, respectively; two of the seedlings remained non-mycorrhizal (Table 3.1).

Supplementation of soil with spores of some mycorrhizal fungi markedly altered the pattern of establishment of mycorrhizas on seedlings. Thus, seedlings grown in soil supplemented with spores of *Inocybe lacera*, *I. lanuginella*, *Hebeloma sacchariolens*, *H. leucosarx* and *Paxillus involutus* developed "inoculant-type" mycorrhizas, the percentage infection ranging from 20% to 59%. In these cases, there was a corresponding reduction in both the variety of "resident" mycorrhizal types present, and in the proportion of root tips infected by these types compared with in controls. The term "resident" is used here for convenience, to distinguish naturally occurring mycorrhizas from those attributable to the added inoculum. As the soil used in this experiment had no history of tree growth, the "resident" mycorrhizal
types must have developed from contaminating basidiospore inoculum in the growth room or originally present in the soil. The term "resident" seems preferable to "contaminant" because the latter suggests undesirable characteristics.

Supplementation of soil with spores of other mycorrhizal fungi, namely *Lactarius pubescens* and *Leccinum roseofracta*, did not lead to the establishment of mycorrhizas attributable to these species. Although the total number of root tips on seedlings in these treatments did not differ significantly from that in controls, there was, nevertheless, an increase in the proportion of root tips that were mycorrhizal, and this was attributable to the development of a variety of resident mycorrhizal types. There was, in general, a tendency for these resident mycorrhizal types to predominate singly on seedlings to the exclusion of other mycorrhizal types, possibly because the mycorrhizas on any single seedling developed from only one or a few spores.

Although mycorrhizas attributable to *Hebeloma* occurred on 8% of the root tips of the control seedlings, yet this mycorrhizal type occurred in significantly larger (*p = 0.001*) numbers (44% to 59% of root tips) on seedlings grown in soil supplemented with spores of *Hebeloma* spp. This enhancement of infection by *Hebeloma* is most readily attributable to increased inoculum density resulting from the soil supplement rather than to a secondary effect of spore supplementation in enhancing infection from resident inoculum. This view is supported by the fact that the apparent enhancement of infection by resident mycorrhizal fungi in the soil supplemented with spores of *L. pubescens* and *L. roseofracta* did not involve any enhancement of infection by *Hebeloma*. *Inocybe* mycorrhizas, also, were found on one of the seedlings grown in soil supplemented with spores of *P. involutus*, but the proportion of root
tips infected by *Inocybe* in this instance was significantly lower (p = 0.001) than in the treatment involving spore inoculum of *Inocybe* spp. (Table 3.1). The correct identification of mycorrhizas formed by *Inocybe* was confirmed by the appearance of a fruitbody of *I. lacera* in one of the pots containing soil supplemented with spores of this species (Plate 3.1). However, attempts to reisolate the symbiont from mycorrhizal apices (Section 2.1.3.2) were not successful, although *Laccaria* and *Hebeloma* were successfully reisolated.

Whereas the results of spore-supplementation with *Inocybe* and *Hebeloma* spp. are relatively clear-cut, i.e. there was greatly enhanced infection by these mycorrhizal fungi compared with in controls, and whereas, similarly, the results of supplementation with *L. pubescens* and *L. roseofracta* were clear-cut - an enhancement of mycorrhizal development from resident sources of inoculum - the results for supplementation of soil with spores of *P. involutus* were equivocal. *Paxillus* colonised only four of the six seedlings grown in soil supplemented with its spores, whereas no colonisation by *Paxillus* was seen in the controls. Nevertheless, the number of mycorrhizas attributable to *Paxillus* in the spore-supplemented soil was consistently low, and it did not differ significantly from the number seen in some other treatments - for example, in soils supplemented with *L. pubescens*, *H. sacchariolens* and *I. lanuginella*. Moreover, *Paxillus* did not have an effect in restricting colonisation of roots by resident mycorrhizal types, as did the most successful inoculant fungi, *Inocybe* and *Hebeloma* spp.

The establishment of mycorrhizas from the introduced spore inocula did not lead to an increased seedling yield in terms of shoot dry weight or number of root tips produced. Interestingly, the highest recorded
Table 3.1: Development of mycorrhizas on seedlings of Betula pendula grown in unsterile brown earth supplemented with fresh spores of seven sheathing mycorrhizal fungi; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised mycorrhizal</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident</th>
<th>Proportion of seedlings with different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inocybe lacera</td>
<td>$\frac{6}{6}$</td>
<td>265</td>
<td>46.4</td>
<td>53.6</td>
<td>52.1 $^{a}$</td>
<td>$^{T4, \frac{4}{6}}$</td>
</tr>
<tr>
<td>Inocybe lanuginella</td>
<td>$\frac{6}{6}$</td>
<td>411</td>
<td>69.7</td>
<td>30.3 $^{b}$</td>
<td>29.7 $^{a}$</td>
<td>$^{P, \frac{4}{6}}$</td>
</tr>
<tr>
<td>Hebeloma sacchariolen</td>
<td>$\frac{5}{6}$</td>
<td>527</td>
<td>50.7</td>
<td>49.3</td>
<td>43.6 $^{a}$</td>
<td>$^{P, \frac{4}{6}}$</td>
</tr>
<tr>
<td>Hebeloma leucosarx</td>
<td>$\frac{6}{6}$</td>
<td>622</td>
<td>33.8</td>
<td>66.2 $^{a}$</td>
<td>58.6 $^{a}$</td>
<td>$^{L, \frac{4}{6}}$</td>
</tr>
<tr>
<td>Paxillus involutus</td>
<td>$\frac{6}{6}$</td>
<td>346</td>
<td>36.5</td>
<td>63.8 $^{a}$</td>
<td>20.0 $^{b}$</td>
<td>$^{I, \frac{4}{6}}; L, \frac{4}{6}$</td>
</tr>
<tr>
<td>Lactarius pubescens</td>
<td>$\frac{6}{6}$</td>
<td>683</td>
<td>44.5</td>
<td>55.5</td>
<td>nil $^{b}$</td>
<td>$^{L, \frac{4}{6}}; P, \frac{4}{6} ; T, \frac{4}{6}$</td>
</tr>
<tr>
<td>Leccinum roseofracta</td>
<td>$\frac{6}{6}$</td>
<td>470</td>
<td>34.5</td>
<td>65.9 $^{a}$</td>
<td>nil $^{b}$</td>
<td>$^{L, \frac{4}{6}}; T, \frac{4}{6} ; T4, \frac{4}{6}$</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>$\frac{-}{6}$</td>
<td>380</td>
<td>61.5</td>
<td>38.5</td>
<td>$^{38.5}$ $^{b}$</td>
<td>$^{H, \frac{6}{6}}; L, \frac{4}{6}$</td>
</tr>
</tbody>
</table>

1 Source of inoculum unknown.
2 Within columns, figures followed by different letters are significantly different from one another, $p = 0.05$.
3 $^{H}$, Hebeloma; $^{I}$, Inocybe; $^{L}$, Laccaria; $^{P}$, Paxillus; $^{T3}$, "Type 3"; $^{T4}$, "Type 4"
seedling yield was associated with the treatment in which the greatest variety of resident mycorrhizal types became established, i.e. on seedlings grown in soil supplemented with spores of *L. pubescens*. In this treatment, mycorrhizas attributable to *Thelephora terrestris*, *Laccaria* and *Paxillus* individually dominated five of the six replicate seedlings, the proportion of root tips mycorrhizal with these individual fungi ranging from 30% to 70%. Of the treatments in which introduced spore inocula were successful, *Hebeloma* spp. tended slightly to increase seedling yield, whereas *Inocybe* spp., if anything, tended to reduce yield.

In summary, the results of this experiment show two main points. (1) Spores of some mycorrhizal fungi, namely *Inocybe* spp. and *Hebeloma* spp., can initiate infection of seedling roots in unsterile soil and, in so doing, apparently suppress mycorrhizal establishment from naturally occurring resident sources of inoculum in the soil. (2) In contrast, spores of some other fungi, namely *L. pubescens* and *L. roseofracta*, cannot initiate infection in these conditions but, nevertheless, spore-supplementation with these fungi, seems to enhance the degree of colonisation of seedling roots from resident sources of inoculum.

### 3.3.2

An extended range of mycorrhizal fungi were next tested by the same method as before, in order to investigate the infectivity of basidiospores of fungi that have not, to date, been categorised as either "early-stage" or "late-stage" (Section 3.1), but which nevertheless are commonly found associated with birch. In addition, ascospores of *Elaphomyces muricatus* were tested in parallel with the other fungi, some fruitbodies of this species having been collected from Struan Wood, Calvine, Perthshire.
Fruitbodies of Laccaria proxima, Lactarius vietus, Lactarius blennius and Cortinarius delibitus were collected from birch woodland near Loch Inver, Sutherland (Section 2.1.6.5), during the autumn of 1982, and brown earth soil was supplemented with spores of these species in the standard way (Section 3.2). In addition, spores of E. muricatus were obtained by crushing two fruitbodies in a sterile polyethylene bag and distributing the contents between eight replicate pots; in this instance the inoculum therefore included fragments of ascocarp tissue in addition to the spores. The birch seedlings used for assay were grown in the spore-supplemented soils for 12 weeks in a growth room.

At harvest, all of the seedlings in the control (unsupplemented) series were mycorrhizal with Thelephora, Hebeloma or Laccaria which, as before, tended to be mutually exclusive (Table 3.2). Of all the spore supplements, only that of L. proxima resulted in the establishment of mycorrhizas; in this instance 67% of the total root tips were mycorrhizal with this species. Also, the establishment of Laccaria on the seedling roots apparently suppressed development of mycorrhizas from resident sources of inoculum. In fact, Laccaria mycorrhizas were found on only 3 of the 30 seedlings in the other treatments in this experiment, and then always in low numbers, so the development of this mycorrhizal type in soil supplemented with spores of L. proxima can confidently be ascribed to a direct role of the supplement.

In contrast to the results for L. proxima, none of the other introduced species developed mycorrhizas on the seedling roots. The seedlings in these treatments became mycorrhizal with a range of fungi, especially Thelephora, Hebeloma, Laccaria and Paxillus; of these, Thelephora and Hebeloma were most numerous in terms of both the
number of seedlings infected and the number of inoculant mycorrhizas on the individual seedlings. In this experiment, there were no significant between-treatment differences in the proportion of the root systems that were mycorrhizal; however, as before (Section 3.3.1), supplementation of soil with spores of some fungi that did not, themselves, establish mycorrhizas tended to increase the amount of mycorrhizal development on the roots.

There was no significant difference between treatments in either the shoot dry weight or the total number of root tips produced, because of the large degree of variation in plant growth between replicate pots. It is notable, however, that shoot growth was neither consistently nor markedly increased in the soil-supplemented treatments, even in instances where this led to the development of mycorrhizas; rather, the treatment involving spores of *L. proxima* was associated with the smallest plants overall.

In summary, the results of this experiment which was done in a different year support the observations made in the earlier experiment of this type (Section 3.3.1). Thus, spores of *L. proxima*, a reported or presumed "early-stage" mycorrhizal fungus (P.A. Mason, pers. comm.), successfully initiated mycorrhizal development in unsterile soil, to the virtual exclusion of development from resident sources of inoculum. In contrast, spores of fungi normally associated with mature birch trees and which might therefore be considered as potentially "late-stage" mycorrhizal fungi (*sensu* Deacon *et al.*, 1983) (Section 1.6) did not initiate infection in unsterile soil and in some instances, tended rather, to enhance mycorrhizal development from resident sources of inoculum.
TABLE 3.2: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth supplemented with fresh spores of five sheathing mycorrhizal fungi; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % root tips mycorrhized</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Proportion of seedlings with different resident mycorrhizal types&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Laccaria proxima</em></td>
<td>6/6</td>
<td>232 (2.340)</td>
<td>30.4</td>
<td>69.6</td>
<td>67.4 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T3, 3/6</td>
<td>64 (1.789)</td>
</tr>
<tr>
<td><em>Lactarius biennius</em></td>
<td>7/7</td>
<td>275 (2.421)</td>
<td>53.9</td>
<td>46.1</td>
<td>nil 46.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H, 3/7; P, 3/7; T, 3/7; T3, 3/7</td>
<td>147 (2.095)</td>
</tr>
<tr>
<td><em>Lactarius vietus</em></td>
<td>7/7</td>
<td>327 (2.419)</td>
<td>55.0</td>
<td>45.0</td>
<td>nil 45.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>L, 3/7; P, 3/7; T, 3/7</td>
<td>108 (1.948)</td>
</tr>
<tr>
<td><em>Cortinarius delibutus</em></td>
<td>7/4</td>
<td>345 (2.508)</td>
<td>28.9</td>
<td>73.1</td>
<td>nil 73.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H, 3/4; P, 3/4; T, 3/4</td>
<td>147 (2.074)</td>
</tr>
<tr>
<td><em>Elaphomyces muricatus</em></td>
<td>7/8</td>
<td>183 (2.202)</td>
<td>26.1</td>
<td>73.9</td>
<td>nil 73.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H, 3/8; T, 3/8</td>
<td>64 (1.729)</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>7/4</td>
<td>143 (2.145)</td>
<td>58.4</td>
<td>41.6</td>
<td>– 41.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H, 3/4; L, 3/4; T, 3/4</td>
<td>126 (2.059)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Source of inoculum unknown.

<sup>2</sup> Within columns, figures followed by different letters are significantly different from one another, p = 0.05.

A third experiment, set up in an identical way to those described in Sections 3.3.1 and 3.3.2, was designed to test the infectivity of spores collected from an unusually early autumnal flush of fruitbodies in August 1982. Pots of unsterile brown earth were supplemented with freshly collected spores of seven fungal species collected from beneath birch at four sites – Bush Estate, Glencorse, S.C.R.I. and Newtongrange (Section 2.1.6). The birch assay seedlings were grown for 12 weeks in a growth room.

Irrespective of treatment, the degree of mycorrhizal development on the seedlings was uniformly low (Table 3.3), as was the degree of seedling growth. Seedlings grown in unsupplemented soil developed mycorrhizas of two types, attributable to *Hebeloma* or, in one case, *Laccaria*, and one seedling remained non-mycorrhizal. In the other treatments, *Laccaria proxima*, *Laccaria tortilis* and *Inocybe geophylla* established mycorrhizas from spore inocula on some of the replicate seedlings, but always to a low degree, whereas *Leccinum roseofracta*, *Russula grisea* and *Lactarius rufus* did not establish mycorrhizas from spore inocula. *Hebeloma sacchariolens* similarly did not establish mycorrhizas from spore inocula, though it did so in a previous experiment (Section 3.3.1). Of interest, the number of seedlings that developed resident-type mycorrhizas was proportionally lower in all of the spore-supplemented treatments than in the control (unsupplemented) series (Table 3.3).

Application of the Student-Newman-Keuls test (Section 2.2.8) showed that there were significant differences in seedling growth in terms of both shoot dry weight and the number of root tips produced: the yield and root number were decreased by supplementation of soil
with spores of *I. geophylla*, *H. sacchariolens* and *L. roseofracta*, compared with yield and root number in unsupplemented soil and in soil supplemented with spores of *R. grisea*. Indeed, spore-supplementation with *H. sacchariolens* completely suppressed development of mycorrhizas on the seedlings even though the introduced spore inoculum was not, itself, effective; similarly, *L. tortilis* apparently suppressed mycorrhizal development from resident sources of inoculum on the four seedlings that it did not, itself, colonise. A highly significant correlation (*r*=0.841) was observed between the number of root tips and the shoot dry weight of seedlings in the experiment as a whole.

3.3.4

Spores of the gasteromycetes, *Rhizopogon roseolus*, *Rhizopogon luteolus* and *Pisolithus tinctorius* have been shown to be effective as sources of inoculum for the establishment of mycorrhizas on pine seedlings (Lamb and Richards, 1974a; Marx, 1980). In the present study, *Scieroderma citrinum* was found to be ubiquitous beneath naturally regenerating birch on a disused coal spoil tip at Newtongrange (Section 2.1.6.1), especially on the hotter parts still subject to "after-burning". In the final experiment of this series in which unsterile brown earth soil was used as a growth medium, the role of spores of *S. citrinum* as a source of inoculum for mycorrhizal development on birch was investigated. At the time when fruitbodies of *S. citrinum* were obtained, fruiting by *Lactarius pubescens* was abundant beneath birch at Bush Estate, so this species was used for comparison with *S. citrinum*. The experiment served also to check the results obtained earlier for *L. pubescens* (Section 3.3.1), when spore-supplementation with *L. pubescens* was found to enhance the degree of establishment of mycorrhizas from
TABLE 3.3: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth supplemented with fresh spores of seven sheathing mycorrhizal fungi; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas (%)</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonized and mycorrhizal (%)</th>
<th>Mean % mycorrhizal root tips attributable to different types of inoculant and resident$^1$ (%)</th>
<th>Proportion of seedlings with different resident mycorrhizal types$^2$</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Laccaria proxima</em></td>
<td>$^{\frac{3}{4}}$</td>
<td>250 (2.386)</td>
<td>63.7$^b$</td>
<td>36.3</td>
<td>P, $^{\frac{3}{4}}$</td>
<td>76 (1.649)</td>
</tr>
<tr>
<td><em>Laccaria tortilis</em></td>
<td>$^{\frac{7}{6}}$</td>
<td>169 (2.165)</td>
<td>89.0</td>
<td>11.0</td>
<td>nil</td>
<td>43 (1.573)</td>
</tr>
<tr>
<td><em>Inocybe geophylla</em></td>
<td>$^{\frac{1}{6}}$</td>
<td>117$^b$ (1.926)</td>
<td>76.8</td>
<td>23.2</td>
<td>nil</td>
<td>42$^b$ (1.166)</td>
</tr>
<tr>
<td><em>Hebeloma sacchariolens</em></td>
<td>$^{\frac{3}{6}}$</td>
<td>85$^b$ (1.855)</td>
<td>100.0$^a$</td>
<td>nil</td>
<td>nil</td>
<td>18$^b$ (1.148)</td>
</tr>
<tr>
<td><em>Leccinum roseofracta</em></td>
<td>$^{\frac{3}{6}}$</td>
<td>107$^b$ (1.976)</td>
<td>92.7</td>
<td>7.3</td>
<td>nil</td>
<td>29$^b$ (1.387)</td>
</tr>
<tr>
<td><em>Lactarius rufus</em></td>
<td>$^{\frac{3}{6}}$</td>
<td>259 (2.352)</td>
<td>94.5</td>
<td>5.5</td>
<td>T2, $^{\frac{3}{6}}$</td>
<td>59 (1.700)</td>
</tr>
<tr>
<td><em>Russula grisea</em></td>
<td>$^{\frac{3}{6}}$</td>
<td>313$^a$ (2.492)</td>
<td>83.6</td>
<td>16.4</td>
<td>nil</td>
<td>81$^a$ (1.904)</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>$^{\frac{7}{6}}$</td>
<td>323$^a$ (2.484)</td>
<td>77.9</td>
<td>22.1</td>
<td>H, $^{\frac{3}{6}}$; L, $^{\frac{3}{6}}$</td>
<td>91$^a$ (1.777)</td>
</tr>
</tbody>
</table>

$^1$Source of inoculum unknown.

$^2$Within columns, figures followed by different letters are significantly different from one another, p = 0.05.

$^3$H, Hebeloma; L, Laccaria; P, Paxillus; T2, "Type 2".
resident sources of inoculum. The method used was as before, seedlings being incubated for 12 weeks in a growth room.

Seedlings grown in unsupplemented soil developed mycorrhizas of two types, attributable to *Hebeloma* and *Thelephora*; two seedlings were colonised by a type designated "Type 2". Supplementation of soil with spores of either *S. citrinum* or *L. pubescens* did not appreciably alter the pattern of mycorrhizal development. Thus, neither of the inoculant fungi established mycorrhizas on the roots, and neither seemed to affect the spectrum of resident types that became established. There was a slight, but not significant, increase in the total degree of mycorrhizal development in soil supplemented with *L. pubescens* compared with in the control; there was no significant difference between treatments in shoot growth. As observed in the previous experiment (Section 3.3.3), a significant correlation (r = 0.641) was observed between shoot dry weight and the total number of root tips on seedlings.
TABLE 3.4: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth supplemented with fresh spores of two sheathing mycorrhizal fungi; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % mycorrhizal root tips attributable to different types of inoculant resident¹</th>
<th>Proportion of seedlings with different resident mycorrhizal types²</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Scleroderma citrinum</em></td>
<td>%</td>
<td>402 (2.491)</td>
<td>52.8</td>
<td>47.2-nil</td>
<td>H, ½⁺; L, ½⁻; P, ½⁻; T, ½⁻</td>
<td>152 (1.937)</td>
</tr>
<tr>
<td><em>Lactarius pubescens</em></td>
<td>%</td>
<td>210 (2.303)</td>
<td>49.0</td>
<td>51.0-nil</td>
<td>H, ½⁻; T, ½⁻</td>
<td>79 (1.885)</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>%</td>
<td>200 (2.295)</td>
<td>63.2</td>
<td>36.8-nil</td>
<td>H, ½⁻; T, ½⁻; T2, ½⁻</td>
<td>106 (1.980)</td>
</tr>
</tbody>
</table>

¹Source of inoculum unknown.
²H, Hebeloma; L, Laccaria; P, Paxillus; T, Thelephora; T2, "Type 2".
3.4 Investigation on the Infectivity of Freshly Collected Basidiospores of Sheathing Mycorrhizal Fungi in Unsterile Vermiculite-Peat

3.4.1

In this and the following experiment, the infectivity of basidiospores of fungi forming sheathing mycorrhizas on birch was tested in unsterile vermiculite-peat medium moistened with MMN nutrient solution without glucose (Section 2.1.2), for comparison with the results of previous experiments using unsterile brown earth (Section 3.3). The vermiculite-peat medium, when sterile, was known to be suitable for mycelial growth by both "early-stage" and "late-stage" fungi (Deacon et al., 1983), and when unsterile it presumably differed from unsterile brown earth in having an initially lower and more restricted resident microbial population. The vermiculite-peat medium was prepared as described in Section 2.2.1, but the concentration of MMN solution given in Section 2.2.1 was reduced 5-fold. The experimental method was exactly as before (Section 3.2), the birch assay seedlings being grown for 12 weeks in a growth room (Section 2.2.3.2).

In all important respects, the results (Table 3.5) were similar to those in the previous experiments involving unsterile brown earth (Tables 3.1, 3.2, 3.3, 3.4). Seedlings developed mycorrhizas attributable to Laccaria, Inocybe or Paxillus in the control (unsupplemented) vermiculite-peat, the different mycorrhizal types being mutually exclusive on individual seedlings. Both Laccaria proxima and Inocybe lacera established large numbers of mycorrhizas on all replicate seedlings grown in soil supplemented with their respective spores, and there was a corresponding marked reduction in establishment from resident inoculum sources. Nevertheless, neither shoot growth nor the total number of root tips on the seedlings was affected by spore-supplementation.
TABLE 3.5: Development of mycorrhizas on seedlings of Betula pendula grown in unsterile vermiculite-peat supplemented with spores of two sheathing mycorrhizal fungi; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonized</th>
<th>Mean % mycorrhizal root tips attributable to different types of inoculant</th>
<th>Proportion of seedlings with different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccaria proxima</td>
<td>$\frac{8}{9}$</td>
<td>438 (2.603)</td>
<td>32.0</td>
<td>68.0$^a$</td>
<td>68.0 nil nil</td>
<td>121 (2.029)</td>
</tr>
<tr>
<td>Inocybe lacera</td>
<td>$\frac{8}{9}$</td>
<td>487 (2.581)</td>
<td>41.6</td>
<td>58.4$^b$</td>
<td>53.0 5.6$^b$ P, $\frac{3}{8}$</td>
<td>118 (1.924)</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>$\frac{7}{8}$</td>
<td>520 (2.595)</td>
<td>58.9</td>
<td>41.1$^b$</td>
<td>- 41.1$^a$ I, $\frac{5}{8}$; L, $\frac{2}{8}$; P, $\frac{7}{8}$</td>
<td>145 (1.935)</td>
</tr>
</tbody>
</table>

1Source of inoculum unknown.

2Within columns, figures followed by different letters are significantly different from one another, p = 0.05.

3I, Inocybe; L, Laccaria; P, Paxillus.
Despite the presence of mycorrhizas attributable to *Laccaria* and *Inocybe* in the control pots, the levels of establishment by these fungi were significantly greater (p = 0.01) in the respective treatment series than in the controls.

3.4.2

In the second experiment of this series, spores of a wider range of sheathing mycorrhizal fungi were added to vermiculite-peat and their infectivity was assessed in the standard way (Section 3.2) by birch seedling assay. The seedlings were grown for 12 weeks in a growth room.

All of the seedlings grown in the control (unsupplemented) series developed mycorrhizas attributable to a mixture of four types, namely *Thelephora, Paxillus, Hebeloma* and a type designated "Type 3" (Table 3.6). *Inocybe lacera* and *I. geophylla* successfully established mycorrhizas from spore inocula on most of the replicate seedlings though the level of infection was low. Spore inocula from both species of *Inocybe* apparently suppressed development by resident mycorrhizal fungi, at least on the seedlings that developed inoculant mycorrhizas. In contrast to the *Inocybe* spp., *Lactarius spinosulus, Cortinarius bulbosus* and *Russula cyanoxantha* did not establish mycorrhizas from their respective spore inocula; instead, a mixture of mycorrhizas developed from resident sources, to a degree similar to that in the controls. In the absence of successful inoculants, mycorrhizas of *Thelephora* predominated on the root systems of the seedlings. Application of a Student-Newmans-Keuls test showed significant between-treatment differences in seedling yield. Thus, in terms of shoot dry weight and number of root tips on seedlings, spore inoculum of *L. spinosulus* apparently enhanced seedling growth.
although this fungus itself did not effectively colonise seedlings. In contrast, spore inocula of *Inocybe* spp. reduced shoot dry weight of seedlings compared with in controls; differences in root numbers, however, were not significant.
TABLE 3.6: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile vermiculite-peat supplemented with fresh spores of five sheathing mycorrhizal fungi; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant</th>
<th>Mean % mycorrhizal root tips attributable to different types resident^1</th>
<th>Proportion of seedlings with different resident mycorrhizal types^3</th>
<th>Mean % root tips attributable to different types inoculant</th>
<th>Mean % root tips attributable to different types resident^1</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Inocybe lacera</em></td>
<td>%</td>
<td>(62^{b2}) (1.778)</td>
<td>84.4</td>
<td>15.6^b</td>
<td>11.6</td>
<td>4.0^a</td>
<td>P, %</td>
<td></td>
<td>9^b (0.985)</td>
</tr>
<tr>
<td><em>Inocybe geophylla</em></td>
<td>%</td>
<td>(82^{b}) (1.904)</td>
<td>51.4</td>
<td>48.6</td>
<td>26.3</td>
<td>22.3^a</td>
<td>P, %; T %</td>
<td></td>
<td>13^b (1.079)</td>
</tr>
<tr>
<td><em>Lactarius spinosulus</em></td>
<td>%</td>
<td>(220^{a}) (2.328)</td>
<td>34.2</td>
<td>65.8^a</td>
<td>nil</td>
<td>65.8^b</td>
<td>H, %; P, %; T, %</td>
<td></td>
<td>37^a (1.558)</td>
</tr>
<tr>
<td><em>Cortinarius bulbosus</em></td>
<td>%</td>
<td>(120^{b}) (2.014)</td>
<td>43.2</td>
<td>56.8^a</td>
<td>nil</td>
<td>56.8^b</td>
<td>H, %; I, %; P, %; T, %</td>
<td></td>
<td>19^c (1.171)</td>
</tr>
<tr>
<td><em>Russula cyanoxantha</em></td>
<td>%</td>
<td>(102^{b}) (1.959)</td>
<td>58.0</td>
<td>42.0</td>
<td>nil</td>
<td>42.0^{bc}</td>
<td>I, %; P, %; T, %</td>
<td></td>
<td>16^c (1.130)</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>%</td>
<td>(113^{b}) (2.038)</td>
<td>37.9</td>
<td>62.1^a</td>
<td>–</td>
<td>62.1^b</td>
<td>H, %; P, %; T, %</td>
<td></td>
<td>15^c (1.150)</td>
</tr>
</tbody>
</table>

^1 Source of inoculum unknown.

^2 Within columns, figures followed by different letters are significantly different from one another, p = 0.05.

^3 H, Hebeloma; I, Inocybe; L, Laccaria; P, Paxillus; T, Thelephora; T3, "Type 3".
3.5 Analysis of the Effects of Spore Supplementation on Seedling Growth and Mycorrhizal Development in Experiments Reported in Sections 3.3 and 3.4

Although supplementation of soil with basidiospores of sheathing mycorrhizal fungi had a variable effect on seedling growth and mycorrhizal development in individual experiments (Sections 3.3, 3.4), yet there was evidence of trends in the results overall, so multiple Analysis of Variance was done to establish the significance of the trends concerning seedling yield, reflected in shoot oven-dry weight and numbers of root tips, and, in addition, the effects of spore inocula on the development of resident mycorrhizal fungi.

As shown in Table 3.7, seedlings grown in unsterile brown earth or unsterile vermiculite-peat supplemented with freshly collected spores of *Inocybe* spp. were significantly smaller in terms of both shoot dry-weight and numbers of root tips produced than were seedlings in the controls. There was some evidence from the individual experiments that spore inocula of *Lactarius* spp., especially *L. spinosulus* (Section 3.4.2), enhanced seedling growth compared to that in unsupplemented media, but the effects of *Lactarius* spp. in this respect were not significant (Table 3.7).

Spores of mycorrhizal fungi that effectively colonised seedlings, namely, *Inocybe* spp., *Laccaria* spp. and *Hebeloma* spp., evidently suppressed the development of naturally occurring mycorrhizal fungi. In contrast, the spores of mycorrhizal fungi that failed effectively to colonise seedling root systems - for example, *Cortinarius* spp., *Lactarius* spp. and *Russula* spp. - did not suppress the development of resident mycorrhizal fungi; indeed, in many cases both the variety of resident fungi and the proportion of root tips infected by these fungi appeared
to be enhanced compared with in controls. Analysis of Variance showed that the spore-supplementation treatments involving *Inocybe* spp., *Laccaria* spp. and *Lactarius* spp. significantly affected the proportion of root tips colonised by resident fungi. Thus, although there was no significant difference in the proportion of uninfected root tips between treatments (Table 3.7), seedlings grown in soil or vermiculite-peat supplemented with spores of *Inocybe* spp. and *Laccaria* spp. had significantly fewer (p=0.001) root tips colonised by resident types compared to with in the controls. In contrast, seedlings grown in media supplemented with *Lactarius* spp. had a significantly greater (p = 0.05) proportion of root tips colonised by resident mycorrhizal fungi than did seedlings in the controls.
TABLE 3.7: Effects of fresh basidiospore inocula added to unsterile soil or unsterile vermiculite-peat on birch seedling growth and the establishment of "resident-type" mycorrhizas: combined results from a between-experiment Analysis of Variance (Sections 3.3.1, 3.3.2, 3.3.3, 3.3.4, 3.4.1, 3.4.2).

<table>
<thead>
<tr>
<th></th>
<th>Control (unsupplemented)</th>
<th>Inocybe</th>
<th>Laccaria</th>
<th>Lactarius</th>
<th>Least significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p=0.05</td>
</tr>
<tr>
<td>Mean number of root tips (log x+1)</td>
<td>2.344</td>
<td>2.129</td>
<td>2.356</td>
<td>2.502</td>
<td>0.164</td>
</tr>
<tr>
<td>Mean shoot dry weight (log x+1)</td>
<td>1.800</td>
<td>1.529</td>
<td>1.729</td>
<td>1.912</td>
<td>0.193</td>
</tr>
<tr>
<td>Mean per cent uninfected root tips (arcsin)</td>
<td>49.9</td>
<td>51.8</td>
<td>40.8</td>
<td>49.5</td>
<td>NSD†</td>
</tr>
<tr>
<td>Mean per cent root tips colonised by &quot;resident-type&quot; mycorrhizas (arcsin)</td>
<td>38.1</td>
<td>3.1</td>
<td>2.4</td>
<td>55.1</td>
<td>16.5</td>
</tr>
</tbody>
</table>

† NSD = No significant difference. Where p=0.05, the standard error of the difference between means = 5.27 (n=6).
3.6 Investigation of the Infectivity of Fresh Basidiospore Inocula of *Paxillus involutus*, *Lactarius turpis*; and *Leccinum scabrum* Added to Unsterile Brown Earth and Coal Spoil

In a previous experiment (Section 3.3.1) spores of *Paxillus involutus* did not initiate mycorrhizal development on birch grown in unsterile brown earth. *P. involutus* occurs most commonly on birch on coal spoil in the Edinburgh area, so its poor establishment from spore inoculum in brown earth might be attributable to unsuitable soil conditions. An experiment was now done to test this possibility.

Coal spoil was collected from beyond the root zone of birch at Newtongrange (Section 2.1.6.1); it was air-dried for 21 days, transferred to 5 cm³ plant pots and then watered to its original moisture content (approximately 23% m.h.c.). A parallel series of plant pots containing unsterile brown earth, as used in some previous experiments, were prepared for comparison. Spores of *P. involutus*, *Lactarius turpis* and *Leccinum scabrum* were added to the soil and coal spoil in the standard way (Section 3.2) from fruitbodies that had been collected from Newtongrange, and birch assay seedlings were then grown for 16 weeks in a growth room.

In the control (unsupplemented) series, seedlings grown in coal spoil developed two mycorrhizal types, of which *Thelephora* predominated (Table 3.8), whereas in the controls in the brown earth mycorrhizas of *Hebeloma* predominated. A significantly higher proportion of root tips developed mycorrhizas in the control coal spoil than in the control brown earth series.

Supplementary inoculum of *P. involutus* was successful in establishing mycorrhizas in coal spoil but not in brown earth and, in so doing, it suppressed mycorrhizal development by *Thelephora* which predominated
as a natural colonist of seedling roots in several of the treatments. In contrast, *L. turpis* and *L. scabrum* did not establish mycorrhizas from spore inocula in either coal spoil or brown earth. However, in brown earth supplementation with spores of these fungi enhanced colonisation by resident mycorrhizal fungi, the high proportion of infected root tips in these treatments being attributable mainly to *Thelephora* but also, on some individual seedlings, to *Hebeloma* or other mycorrhizal fungi.

Seedling growth was generally poorer in coal spoil than in brown earth (Table 3.8) and, again in general, seedling growth was not enhanced by spore supplementation. The sole exception to this was a significant increase in shoot dry weight and numbers of root tips on seedlings grown in coal spoil supplemented with spore inoculum of *L. turpis*; it is notable that the 'L. turpis' treatment gave comparatively larger seedling yields than did spore-supplementation with other fungi in both rooting media.

The results of this experiment suggest that *P. involutus* more readily initiates mycorrhizal development from spore inoculum in coal spoil than in mineral soil. These results raise the interesting matter, discussed later (Section 3.13), of how "early-stage" fungi should be defined, i.e. with reference to soil and other abiotic and biotic factors.
TABLE 3.8: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth and coal spoil supplemented with fresh spores of three sheathing mycorrhizal fungi; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips colonised</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paxillus involutus</td>
<td>⅓</td>
<td>190&lt;sup&gt;a&lt;/sup&gt; (2.264)</td>
<td>28.6</td>
<td>71.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>H, ⅓; T, ⅔</td>
</tr>
<tr>
<td>Lactarius turpis</td>
<td>%</td>
<td>273&lt;sup&gt;a&lt;/sup&gt; (2.428)</td>
<td>23.0</td>
<td>77.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>H, ⅓; I, ⅔; T, ⅔</td>
</tr>
<tr>
<td>Leccinum scabrum</td>
<td>⅔</td>
<td>217&lt;sup&gt;a&lt;/sup&gt; (2.318)</td>
<td>32.8</td>
<td>67.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>H, ⅓; T3, ⅔</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>-⅓</td>
<td>184&lt;sup&gt;a&lt;/sup&gt; (2.261)</td>
<td>57.4</td>
<td>42.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H, ⅓; P, ⅔</td>
</tr>
</tbody>
</table>

Brown earth

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips colonised</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paxillus involutus</td>
<td>⅓</td>
<td>225&lt;sup&gt;a&lt;/sup&gt; (2.321)</td>
<td>40.0</td>
<td>60.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T, ⅓</td>
</tr>
<tr>
<td>Lactarius turpis</td>
<td>%</td>
<td>192&lt;sup&gt;a&lt;/sup&gt; (2.279)</td>
<td>40.5</td>
<td>59.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T, ⅔</td>
</tr>
<tr>
<td>Leccinum scabrum</td>
<td>⅔</td>
<td>117&lt;sup&gt;b&lt;/sup&gt; (2.038)</td>
<td>19.2</td>
<td>80.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>I, ⅓; T3, ⅔</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>-⅓</td>
<td>93&lt;sup&gt;b&lt;/sup&gt; (1.954)</td>
<td>26.7</td>
<td>73.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T, ⅔; T3, ⅔</td>
</tr>
</tbody>
</table>

Coal spoil

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips colonised</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paxillus involutus</td>
<td>⅓</td>
<td>225&lt;sup&gt;a&lt;/sup&gt; (2.321)</td>
<td>40.0</td>
<td>60.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T, ⅓</td>
</tr>
<tr>
<td>Lactarius turpis</td>
<td>%</td>
<td>192&lt;sup&gt;a&lt;/sup&gt; (2.279)</td>
<td>40.5</td>
<td>59.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T, ⅔</td>
</tr>
<tr>
<td>Leccinum scabrum</td>
<td>⅔</td>
<td>117&lt;sup&gt;b&lt;/sup&gt; (2.038)</td>
<td>19.2</td>
<td>80.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>I, ⅓; T3, ⅔</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>-⅓</td>
<td>93&lt;sup&gt;b&lt;/sup&gt; (1.954)</td>
<td>26.7</td>
<td>73.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>T, ⅔; T3, ⅔</td>
</tr>
</tbody>
</table>

<sup>1</sup>Source of inoculum unknown.

<sup>2</sup>Within columns, figures followed by different letters are significantly different from one another, p = 0.05.

<sup>3</sup>H, Hebeloma; I, Inocybe; P, Paxillus; T, Thelephora; T3, "Type 3".
3.7 Development of Mycorrhizas on Seedlings of Birch, Spruce and Pine from Basidiospore Inoculum Added to Unsterile Brown Earth

In order to extend the findings of previous experiments, pots of unsterile brown earth were supplemented in the standard way (Section 3.2) with basidiospores of *Hebeloma crustuliniforme*, collected from beneath birch, and with spores of *Suillus luteus*, collected from beneath pine. Three-week old, aseptically pre-germinated seedlings of *Betula pendula*, *Pinus sylvestris* and *Picea sitchensis* were planted into replicate pots and the assay seedlings were grown for 22 weeks in a growth room.

*H. crustuliniforme* established mycorrhizas on all of the replicate birch seedlings and on three of the five pine seedlings, but on only one of the four spruce seedlings (Table 3.8). Mycorrhizal development by this species was extensive on both pine and birch and this was visually apparent when the seedlings were removed from the pots. In contrast, *S. luteus* did not form mycorrhizas on any of the seedling types from the spore inoculum.

Comparison of the treatments for individual tree species showed interesting patterns. For birch, soil supplementation with spore inoculum of *H. crustuliniforme* did not markedly affect the proportion of the total root system that developed mycorrhizas, whereas, for pine, inoculation with *Hebeloma* seemed to enhance total mycorrhizal development. For spruce, the addition of spores of either *H. crustuliniforme* or *S. luteus* seemed to reduce overall mycorrhizal development, and this result was statistically significant for supplementation with spores of *H. crustuliniforme*. For all three tree species, supplementation of soil with spores of *H. crustuliniforme* apparently suppressed development of mycorrhizas from resident sources of inoculum; this suppressive effect occurred even in cases such as on some replicate spruce seedlings, in which mycorrhizas of *Hebeloma* did not develop.
Supplementation of soil with spores of *H. crustuliniforme* and *S. luteus* did not affect seedling growth in terms of the number of root tips on pine or spruce, although it markedly increased the number of root tips on birch compared with in controls; also, supplementation of soil with spores of *H. crustuliniforme* markedly increased shoot dry weight of birch over that of seedlings grown in soil supplemented with *S. luteus*.

In summary, the experiment shows that *H. crustuliniforme* can develop mycorrhizas on seedlings of all three tree species from spore inoculum in unsterile soil, whereas *S. luteus*, like the other members of the Boletaceae tested in previous experiments (Sections 3.3.1, 3.3.3), apparently cannot readily develop from spore inoculum in these conditions. It is notable in this regard that *S. luteus* is a common and characteristic mycorrhizal symbiont of pine; Chu-Chou (1979) has reported that fruitbodies of *Suillus* spp. are most frequently seen in stands of *Pinus radiata* more than 5 years old, yet the symbiont was isolated only from roots of trees younger than 5 years.
TABLE 3.9: Development of mycorrhizas on seedlings of *Betula pendula*, *Pinus sylvestris* and *Picea sitchensis* grown in unsterile brown earth supplemented with fresh spores of two sheathing mycorrhizal fungi.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % root tips mycorrhizal</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident</th>
<th>Proportion of seedlings with different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B. pendula</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hebeloma crustuliniforme</td>
<td>5/5</td>
<td>329a2 (2.511)</td>
<td>25.0</td>
<td>75.0a</td>
<td>75.0</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Suillus luteus</td>
<td>0/4</td>
<td>329 (2.503)</td>
<td>22.0</td>
<td>78.0</td>
<td>nil</td>
<td>78.0</td>
<td>H, 3/4; I, 1/4; T, 2/4; T3, 2/4</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>-/4</td>
<td>141 (2.053)</td>
<td>34.9</td>
<td>65.1</td>
<td>-</td>
<td>65.1</td>
<td>I, 1/4; P, 2/4; T, 3/4</td>
</tr>
<tr>
<td><strong>P. sylvestris</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hebeloma crustuliniforme</td>
<td>3/5</td>
<td>86b (1.919)</td>
<td>32.4</td>
<td>67.6a</td>
<td>48.6</td>
<td>19.0</td>
<td>P1, 1/5</td>
</tr>
<tr>
<td>Suillus luteus</td>
<td>0/5</td>
<td>70 (1.832)</td>
<td>47.0</td>
<td>53.0</td>
<td>nil</td>
<td>53.0</td>
<td>P3, 1/5; P4, 2/5; P5, 1/5</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>-/4</td>
<td>106 (2.025)</td>
<td>51.4</td>
<td>48.6</td>
<td>-</td>
<td>48.6</td>
<td>H, 3/4; P2, 4/4; P3, 1/4</td>
</tr>
<tr>
<td><strong>P. sitchensis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hebeloma crustuliniforme</td>
<td>1/4</td>
<td>48b (1.667)</td>
<td>75.6</td>
<td>24.4b</td>
<td>5.4</td>
<td>19.0</td>
<td>S1, 1/4; S2, 1/4</td>
</tr>
<tr>
<td>Suillus luteus</td>
<td>0/5</td>
<td>61 (1.724)</td>
<td>68.3</td>
<td>31.7</td>
<td>nil</td>
<td>31.7</td>
<td>S1, 1/5; S3, 3/5</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>-/4</td>
<td>48 (1.675)</td>
<td>51.2</td>
<td>48.8</td>
<td>-</td>
<td>48.8</td>
<td>S1, 1/4; S2, 1/4</td>
</tr>
</tbody>
</table>

1 Source of inoculum unknown.

2 Within columns, and for each mycorrhizal fungus, figures followed by different letters are significantly different from one another, p=0.05.

3 H, Hebeloma; I, Inocybe; P, Paxillus; T, Thelephora; P1, "Type 1" (pine); P2, "Type 2" (pine); P3, "Type 3" (pine); P4 "Type 4" (pine); S1, "Type 1" (spruce); S2, "Type 2" (spruce); S3, "Type 3" (spruce).
3.8 Effects of Single and Dual Supplements with Basidiospores on Mycorrhizal Development on Birch in Unsterile Soil

During the autumn of 1982, a large number of fruitbodies of *Laccaria proxima* and *Hebeloma crustuliniforme* occurred in close proximity to each other around 2-year old birch trees at the Glencorse site (Section 2.1.6.3). The competitive interaction between these fungi was now studied using spore inocula; both fungi are definable as "early-stage" mycorrhizal fungi *sensu* Deacon *et al.* (1983), and therefore might be expected to compete with one another for colonisation of seedling roots. The experimental method was much as before; the caps of fruitbodies being suspended over brown earth contained in 5 cm$^3$ plant pots. Soil was supplemented with spore inoculum either of *H. crustuliniforme* alone or of *L. proxima* alone; in these treatments, spores from two fruitbodies were deposited onto the soil, one in the first 24 h and one in the second 24 h, the surface 1 cm depth of soil being mixed after each fruitbody was removed. Soil in a third series of pots was supplemented first with spores of *H. crustuliniforme* in the first 24 h, and then with spores of *L. proxima* in the second 24 h, again with mixing after each spore supplementation. A birch assay seedling was planted in each pot and sampled after 12 weeks growth.

All seedlings grown in soil supplemented with only *H. crustuliniforme* or only *L. proxima* developed mycorrhizas attributable to the respective species (Table 3.10); these results are of interest because this is the first experiment in which *H. crustuliniforme* has been used as spore inoculum.

Of the seedlings grown in soil supplemented with spore inocula of both species, two developed only *Hebeloma* mycorrhizas, two developed only *Laccaria*, and two developed mycorrhizas of both types, suggesting
a degree of competitive interaction. *Laccaria* developed poorly in the dual inoculum treatment compared with in the single inoculum treatment, whereas *Hebeloma* did not show such a marked difference between these treatments. The result is especially interesting because in the single inoculation treatments, *L. proxima* is seen to have been the more effective mycorrhizal colonist (Table 3.10). The result is unlikely to be explicable in terms of the total number of spores of this species added to soil, because spores were deposited in very large numbers from even a single fruitbody, and in previous experiments (3.3.2, 3.4.1) *L. proxima* was found to colonise a high proportion of root tips from a single dose of inoculum.

Seedling yield was not significantly affected by single or dual inoculum doses, nor were there any differences in shoot dry-weight or the number of root tips when seedlings colonised by *H. crustuliniforme* only (from both dual and single inoculum treatments) were compared with seedlings colonised by *L. proxima* only (similarly, from both treatments).
TABLE 3.10: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth supplemented with single or combined fresh spore inocula of *Hebeloma crustuliniforme* and *Laccaria proxima*; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement 1st</th>
<th>Proportion of seedlings with mycorrhizas attributable to inoculant types</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % root tips mycorrhizal</th>
<th>Mean % root tips attributable to inoculant or different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. crustuliniforme</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. crustuliniforme</em></td>
<td>5% 5% 5% 134 (1.871)</td>
<td></td>
<td>54.7 45.3 b2</td>
<td></td>
<td>45.3 nil nil</td>
<td>34.5 (1.426)</td>
</tr>
<tr>
<td><em>L. proxima</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. proxima</em></td>
<td>5% 5% 5% 74 (1.665)</td>
<td></td>
<td>17.6 82.4 a</td>
<td></td>
<td>nil 82.4 a nil</td>
<td>11.6 (1.015)</td>
</tr>
<tr>
<td><em>H. crustuliniforme</em> <em>L. proxima</em></td>
<td>5% 5% 5% 134 (1.999)</td>
<td></td>
<td>43.6 56.4 b</td>
<td>35.2 21.1 b</td>
<td>nil</td>
<td>18.8 (0.864)</td>
</tr>
</tbody>
</table>

1 Source of inoculum unknown.

2 Within columns, figures followed by different letters are significantly different from one another, p = 0.05.
3.9 Investigation of the Infectivity of Stored Basidiospore Inocula of Sheathing Mycorrhizal Fungi on Birch in Unsterile Brown Earth

3.9.1

Fruitbody production by most sheathing mycorrhizal basidiomycetes occurs in the autumn prior to leaf fall and host dormancy; it is generally assumed that, under natural conditions, basidiospores then persist over winter and establish mycorrhizas on elongating roots in the following spring. A major difficulty hindering the use of spores for selective mycorrhizal inoculation programmes is the lack of available information on appropriate methods of storage of spore collections to minimise loss of viability. By using seedling assay as a method to test spore viability, it has been shown that basidiospores of *Pisolithus tinctorius* are still effective as inocula after 2 years' storage at room temperature (Marx, 1980). However, Theodorou and Bowen (1973) have demonstrated that the inoculum density of freeze-dried or air-dried spores of *Rhizopogon luteolus* must be increased up to 100-fold when spores have been stored for 3 months at 22°C in order to obtain a level of infection on *Pinus radiata* equivalent to that using fresh spore inoculum. Basidiospores of *Laccaria laccata* can be germinated on charcoal-dusted agar containing peptone and colonised by living mycelium of this species (Fries, 1983). When preserved at 4°C, most spore collections maintained their viability for 2 to 3 months; at lower temperatures (-18°C), viability was prolonged compared with this by at least 1 month.

The following experiments concerned the effect of storage on the viability of spores of some sheathing mycorrhizal fungi of birch. In the first experiment of this series, spores of "early-stage" and "late-stage" mycorrhizal fungi - *Inocybe lacera*, *Hebeloma leucosarx*, *Lactarius pubescens* and *Paxillus involutus* - had been added to pots of unsterile
brown earth as an additional part of the experiment reported in Section 3.3.1. But instead of being sown to birch immediately, the pots were randomly arranged in trays and the trays were then wrapped in polyethylene and covered with silver foil and then nylon mesh. Some trays were stored at constant 18°C in a growth room; other trays were stored outside over the winter of 1981, during which time temperatures dropped to a minimum of -21°C. Replicate pots were sampled after 6 months and 10 months, and the infectivity of the spore-supplemented soil was tested by growing birch seedlings for 12 weeks (Tables 3.11, 3.12).

The results reflect a complex situation and they must be interpreted with caution because inevitably the seedling assays were done at different times. So it is possible that slightly different growth conditions for the seedlings, or different sources of contaminant inoculum in the growth room at different times, could have influenced the results obtained. Nevertheless, several general features are apparent, and some of them are best seen by abstracting the relevant results from Table 3.11 and Table 3.12, together with those from Table 3.1, referring to the original seedling assay done in the soils before storage. The relevant information is summarised in Table 3.13.

In control (unsupplemented) soil, the seedlings developed mycorrhizas of several types, including types attributable to Laccaria and Hebeloma and a type designated "Type 2"; most of the control seedlings became mycorrhizal.

The degree of establishment of mycorrhizas from spore inocula was variable both between fungal species and between times of sampling. Paxillus involutus colonised, in all, 20 of the 32 seedlings in soil supplemented with its spores, compared with only 1 of 30 seedlings in the control soil. It formed only few mycorrhizas on the seedlings that it
did infect, except in soil stored for 10 months at either 18°C or outdoors, when its level of establishment was greatest. *H. leucosarx* and *I. lacera* established well from their respective spore inocula, both in terms of the number of seedlings infected and in terms of the degree of development on individual seedlings; for both fungi, storage of the soils at 18°C (the only treatment used) had little effect on the subsequent establishment on seedlings. The spores of these fungi thus remained viable in soil for several months.

The degree of establishment from 'resident' sources of inoculum in control pots was variable, but from Table 3.13 it seems that establishment of mycorrhizas was reduced after storage of soils. Of especial interest, establishment from these resident sources of inoculum was markedly reduced on seedlings that developed mycorrhizas from the added spore inocula, the only exception to this being in the case of soil supplemented with *P. involutus* and stored for 6 months outdoors; in this case, few mycorrhizas developed on the seedlings even though the *Paxillus* inoculum was, itself, ineffective.

Table 3.13 shows one other interesting point, namely that storage of control soil outdoors resulted in more mycorrhizal development than did storage at 18°C; this was true at both the 6 and 10-month samplings and it indicates that resident sources of inoculum may have lost viability more rapidly at the higher temperature associated with indoor conditions.

3.9.2

In a second experiment, run concurrently with that described in Section 3.9.1, pots of unsterile brown earth containing spore inoculum of *Paxillus involutus* and stored for 10 months at 18°C were sown to birch in the standard way (Section 3.2); for comparison, pots of similar
TABLE 3.11: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth that had been supplemented with spores of sheathing mycorrhizal fungi and stored for 6 months at 18°C or in outdoor conditions over winter; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % root tips mycorrhizal</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident</th>
<th>Proportion of seedlings with different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paxillus involutus</em></td>
<td>3/6</td>
<td>209a&lt;sup&gt;2&lt;/sup&gt; (2.273)</td>
<td>93.9</td>
<td>6.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.7 4.4</td>
<td>H, 3/6</td>
<td>68&lt;sup&gt;a&lt;/sup&gt; (1.759)</td>
</tr>
<tr>
<td><em>Lactarius pubescens</em></td>
<td>3/6</td>
<td>193a&lt;sup&gt;2&lt;/sup&gt; (2.235)</td>
<td>78.7</td>
<td>21.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nil 21.3</td>
<td>H, 3/6; L, 3/6</td>
<td>44&lt;sup&gt;a&lt;/sup&gt; (1.596)</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>-6</td>
<td>193a&lt;sup&gt;2&lt;/sup&gt; (2.276)</td>
<td>63.6</td>
<td>36.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>- 36.4</td>
<td>H, 3/6; L, 3/6</td>
<td>79&lt;sup&gt;a&lt;/sup&gt; (1.850)</td>
</tr>
<tr>
<td><em>Paxillus involutus</em></td>
<td>3/6</td>
<td>168 (2.194)</td>
<td>66.8</td>
<td>38.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3 35.9</td>
<td>I, 3/6</td>
<td>34&lt;sup&gt;a&lt;/sup&gt; (1.488)</td>
</tr>
<tr>
<td><em>Lactarius pubescens</em></td>
<td>3/6</td>
<td>169 (2.159)</td>
<td>77.1</td>
<td>22.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nil 22.9</td>
<td>H, 3/6; I, 3/6</td>
<td>38&lt;sup&gt;a&lt;/sup&gt; (1.494)</td>
</tr>
<tr>
<td><em>Inocybe lacera</em></td>
<td>3/5</td>
<td>65&lt;sup&gt;b&lt;/sup&gt; (1.744)</td>
<td>63.7</td>
<td>36.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.3 nil</td>
<td>nil</td>
<td>9&lt;sup&gt;b&lt;/sup&gt; (0.910)</td>
</tr>
<tr>
<td><em>Hebeloma leucosarx</em></td>
<td>3/5</td>
<td>148 (2.007)</td>
<td>46.5</td>
<td>53.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.9 0.6</td>
<td>L, 3/6</td>
<td>32 (1.396)</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>-6</td>
<td>182&lt;sup&gt;a&lt;/sup&gt; (2.251)</td>
<td>91.4</td>
<td>8.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>- 8.6</td>
<td>L, 3/6</td>
<td>118&lt;sup&gt;a&lt;/sup&gt; (1.687)</td>
</tr>
</tbody>
</table>

1 Source of inoculum unknown.

2 Within columns, figures followed by different letters are significantly different from one another, p = 0.05.

3 H, Hebeloma; I, Inocybe; L, Laccaria.
<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % mycorrhizal root tips attributable to different inoculant resident types</th>
<th>Proportion of seedlings with different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paxillus involutus</td>
<td>%6</td>
<td>218</td>
<td>45.4</td>
<td>54.6a</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Lactarius pubescens</td>
<td>%6</td>
<td>260</td>
<td>51.8</td>
<td>48.2</td>
<td>nil</td>
<td>48.2</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>%7</td>
<td>291a (2.457)</td>
<td>75.2</td>
<td>24.8</td>
<td>-</td>
<td>24.8</td>
</tr>
<tr>
<td>Paxillus involutus</td>
<td>%6</td>
<td>156</td>
<td>63.1</td>
<td>36.9</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Lactarius pubescens</td>
<td>%6</td>
<td>145</td>
<td>70.6</td>
<td>29.4</td>
<td>nil</td>
<td>29.4</td>
</tr>
<tr>
<td>Hebeloma leucosorx</td>
<td>%6</td>
<td>185</td>
<td>55.1</td>
<td>44.9</td>
<td>44.0</td>
<td>0.9</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>%7</td>
<td>115b (1.982)</td>
<td>86.4</td>
<td>13.6</td>
<td>-</td>
<td>13.6</td>
</tr>
</tbody>
</table>

1 Source of inoculum unknown.

2 Within columns, figures followed by different letters are significantly different from one another, p=0.05.

3 HS, Hebeloma sacchariolens; I, Inocybe; P, Paxillus; T, Thelaphora; T2, "Type 2".
TABLE 3.13: Summary of the development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth supplemented with spores of sheathing mycorrhizal fungi and sown to birch immediately or after 6 and 10 months storage at 18°C or outdoors.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Period (months)</th>
<th>None (un-supplemented soil)</th>
<th>Paxillus involutus</th>
<th>Hebeloma sacchariolens</th>
<th>Inocybe lacera</th>
</tr>
</thead>
<tbody>
<tr>
<td>outdoors</td>
<td>0</td>
<td>$\frac{5}{9}$ (39)</td>
<td>$\frac{5}{6}$ (64)</td>
<td>$\frac{6}{6}$ (66)</td>
<td>$\frac{6}{6}$ (54)</td>
</tr>
<tr>
<td>18°C</td>
<td>6</td>
<td>$\frac{6}{6}$ (36)</td>
<td>$\frac{4}{6}$ (6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>outdoors</td>
<td>10</td>
<td>$\frac{5}{6}$ (25)</td>
<td>$\frac{6}{6}$ (55)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18°C</td>
<td>10</td>
<td>$\frac{3}{5}$ (14)</td>
<td>$\frac{5}{6}$ (37)</td>
<td>$\frac{6}{6}$ (45)</td>
<td>-</td>
</tr>
</tbody>
</table>

Proportion of seedlings bearing mycorrhizas of any type (mean % root tips with mycorrhizas in parentheses)  
- outdoors 6 months: $\frac{6}{6}$ (36)  
- 18°C 6 months: $\frac{3}{6}$ (9)  
- outdoors 10 months: $\frac{6}{6}$ (25)  
- 18°C 10 months: $\frac{3}{5}$ (14)

Proportion of seedlings with "Inoculant-type" mycorrhizas (mean % root tips with "Inoculant-type" mycorrhizas in parentheses)  
- outdoors 6 months: -  
- 18°C 6 months: $\frac{2}{6}$ (2)  
- outdoors 10 months: $\frac{6}{6}$ (55)  
- 18°C 10 months: $\frac{5}{6}$ (37)  

Proportion of seedlings with "Resident-type" mycorrhizas (mean % root tips with "Resident-type" mycorrhizas in parentheses)  
- outdoors 6 months: $\frac{6}{6}$ (36)  
- 18°C 6 months: $\frac{3}{6}$ (9)  
- outdoors 10 months: $\frac{6}{6}$ (25)  
- 18°C 10 months: $\frac{3}{5}$ (14)
soil were supplemented with spores from freshly collected fruitbodies of \textit{P. involutus} from the same site as before (Newtonrange) and sown to birch. The birch assay seedlings were grown for 12 weeks in a growth room.

Seedlings grown in the 'fresh' unsupplemented control soil developed four mycorrhizal types, the types occurring separately on individual seedlings; one seedling of the six remained non-mycorrhizal (Table 3.14). A similar proportion of root tips were colonised by two resident mycorrhizal fungi in supplemented soil stored for 10 months; of these mycorrhizal types, \textit{Thelephora} was predominant whereas it did not occur in the 'fresh' control series.

Seedlings grown in soil supplemented with freshly collected spore inoculum of \textit{P. involutus}, and those grown in soil that had been supplemented with this fungus and stored, developed mycorrhizas attributable to \textit{P. involutus} (Table 3.14); but a significantly lower proportion of the root tips were mycorrhizal with \textit{Paxillus} in the stored than in the 'fresh' supplementation treatment (Table 3.14). In both the stored and non-stored supplemented soils, the establishment of mycorrhizas by \textit{Paxillus} occurred to the virtual exclusion of other mycorrhizal types, as was found in many of the previous experiments.

Of interest, seedling growth was significantly better in the 'fresh' (unstored) soil than in the stored soil, irrespective of inoculum supplement, and despite the fact that the batches of soil had been taken from the same site and treated in the same way before use. This comparison is complicated by the fact that the design of the experiment precluded use of a single batch of soil, but the results are supported by those for seedling growth in Tables 3.11 and 3.12, in which soil stored at 18°C supported less seedling growth than did exactly the same batch of soil.
TABLE 3.14: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth supplemented with freshly collected spores of *Paxillus involutus* or in spore-supplemented soil that had been stored for 10 months at 18°C; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % root tips mycorrhizal</th>
<th>Mean % root tips attributable to different types of inoculant</th>
<th>Proportion of seedlings with different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (unsupplemented soil)</td>
<td>7/6 (1.902)</td>
<td>47.0 (1.287)</td>
<td>53.0 (1.902)</td>
<td>50.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0</td>
<td>T, 7/6</td>
</tr>
<tr>
<td>Paxillus involutus</td>
<td>7/6 (2.098)</td>
<td>59.0 (1.779)</td>
<td>41.0 (1.779)</td>
<td>41.0</td>
<td>37&lt;sup&gt;a&lt;/sup&gt; (1.498)</td>
<td></td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>7/6 (1.767)</td>
<td>54.4 (1.767)</td>
<td>45.6 (1.767)</td>
<td>45.6</td>
<td>8&lt;sup&gt;b&lt;/sup&gt; (0.896)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Source of inoculum unknown.

<sup>2</sup> Within columns, figures followed by different letters are significantly different from one another, p = 0.05.

<sup>3</sup> H, Hebeloma; I, Inocybe; L, Laccaria; P, Paxillus; T, Thelephora.
stored outdoors over winter. In other words, it seems that storage of the soil at 18°C renders it less suitable for seedling development than does storage at lower temperatures or, indeed, lack of storage.

3.9.3

In the third experiment of this series, spores of two sheathing mycorrhizal fungi were stored for 6 months in different laboratory conditions in the absence of soil before their infectivity was investigated. Spore prints of two "early-stage" mycorrhizal fungi - *Inocybe lacera* and *Hebeloma leucosarx* - were obtained by suspending the caps of mature fruitbodies over dry, sterilised filter papers for 24 h in humid conditions. Spore prints of individual fruitbodies were then transferred to opaque screw-top jars and incubated at 5°C or at 20°C for 6 months. In addition, some filter papers bearing spore prints were immersed in 5 ml sterile distilled water in screw-top vials and maintained at 5°C for 6 months. After this time, the spore prints that had been stored dry were mixed with 7 ml sterile distilled water and the resulting spore suspension was pipetted uniformly over the surface of unsterile brown earth contained in a 5 cm³ plant pot; this procedure was repeated for several replicate pots. Spores stored in water were directly pipetted over soil contained in a further set of replicate pots and each bottle was rinsed out with a further 2 ml sterile distilled water, which was then added to the soil. The spores were lightly mixed into the top 1 cm of soil in each pot, and the pots were immediately sown to birch in the standard way (Section 3.2); six-fold replication was used for each treatment, and six further pots of unsupplemented soil were also included.

After growth for 12 weeks in a growth room, four of the six seedlings grown in control (unsupplemented) soil had developed mycor-
rhizas attributable to *Thelephora* and two remained non-mycorrhizal (Table 3.15). *H. leucosarx* successfully established mycorrhizas on seedlings in all treatments involving spore supplements of this species, the proportion of root tips colonised by this fungus stored dry at 5°C being similar to that recorded in a previous experiment (Section 3.3.1) in which freshly collected spores from the same source of fruit-bodies were used. In contrast, no colonisation of seedlings from spores of *I. lacera* occurred in the "5°C dry" storage treatment.

Comparison of the different storage treatments (Table 3.15) shows that *H. leucosarx* was less successful in initiating mycorrhizal development after storage either dry at 20°C or "wet" at 5°C than dry at 5°C. Nevertheless, mycorrhizas of this species did develop on most or all of the seedlings in these treatments, to the complete exclusion of mycorrhizas from resident sources of inoculum. *I. lacera* developed only very few mycorrhizas in the experiment as a whole, most of this development being in soil supplemented with spores stored moist at 5°C. The significance of this low degree of development compared with virtual absence of development from dry-stored spore inoculum of this species is unknown.

3.9.4

In the final experiment of this series investigating the effects of storage on infectivity, basidiospores were subjected to more severe drying conditions than before. Spore prints of *Hebeloma crustuliniforme*, an "early-stage" mycorrhizal fungus *sensu* Deacon *et al.* (1983), were obtained by suspending the caps of mature fruitbodies over large glass slides in humid conditions for 24 h. The spore prints were then transferred to airtight plastic boxes containing either a thin layer of silica gel
TABLE 3.15: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth supplemented with spores of *Hebeloma leucosarx* and *Inocybe lacera* which had been stored for 6 months in different conditions before addition to soil; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident</th>
<th>Mean % mycorrhizal root tips attributable to different resident mycorrhizal types</th>
<th>Proportion of seedlings with different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry at 5°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. leucosarx</em></td>
<td>¾</td>
<td>191</td>
<td>54.3</td>
<td>45.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nil</td>
<td>nil</td>
<td>42&lt;sup&gt;a&lt;/sup&gt; (1.602)</td>
</tr>
<tr>
<td><em>I. lacera</em></td>
<td>¾</td>
<td>103</td>
<td>100.0</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>10&lt;sup&gt;b&lt;/sup&gt; (1.009)</td>
</tr>
<tr>
<td>Dry at 20°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. leucosarx</em></td>
<td>¾</td>
<td>159</td>
<td>75.2</td>
<td>24.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nil</td>
<td>nil</td>
<td>26&lt;sup&gt;a&lt;/sup&gt; (1.341)</td>
</tr>
<tr>
<td><em>I. lacera</em></td>
<td>¾</td>
<td>139</td>
<td>98.5</td>
<td>1.5</td>
<td>nil</td>
<td>nil</td>
<td>19&lt;sup&gt;a&lt;/sup&gt; (1.197)</td>
</tr>
<tr>
<td>In water at 5°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. leucosarx</em></td>
<td>¾</td>
<td>230</td>
<td>61.9</td>
<td>38.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nil</td>
<td>nil</td>
<td>48&lt;sup&gt;a&lt;/sup&gt; (1.537)</td>
</tr>
<tr>
<td><em>I. lacera</em></td>
<td>¾</td>
<td>176</td>
<td>85.3</td>
<td>14.7</td>
<td>12.8</td>
<td>1.9</td>
<td>T3, ¾&lt;sup&gt;a&lt;/sup&gt; (1.192)</td>
</tr>
<tr>
<td>Control</td>
<td>¾</td>
<td>207</td>
<td>79.7</td>
<td>20.3</td>
<td>20.3</td>
<td>T, ¾&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32&lt;sup&gt;a&lt;/sup&gt; (1.408)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Source of inoculum unknown.

<sup>2</sup> Within columns, figures followed by different letters are significantly different from one another, p = 0.05.

<sup>3</sup> T, Thelephora; T3, "Type 3".
to remove atmospheric moisture or a salt solution (Table 3.16) providing a relative humidity of 75%. After 24 h, half of the replicate spore prints from the silica gel series were gradually rehydrated, during 24 h, over a series of solutions providing relative humidities of 30, 55, 85.5 and 93% in the vapour phase (Table 3.16).

TABLE 3.16: Relative humidity in the vapour phase over solutions at 25°C (from Stokes and Robinson, 1949).

<table>
<thead>
<tr>
<th>Concentration in water</th>
<th>Relative humidity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>44.36%</td>
</tr>
<tr>
<td>Glucose</td>
<td>saturated</td>
</tr>
<tr>
<td>Potassium tartrate</td>
<td>saturated</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O₂</td>
<td>saturated</td>
</tr>
<tr>
<td>NH₄HPO₄</td>
<td>saturated</td>
</tr>
</tbody>
</table>

Spore prints from the three treatments, i.e. stored at 75% RH for 48 h, stored over silica gel for 48 h, and stored over silica gel for 24 h and then rehydrated for 24 h, were added to pots of unsterile brown earth with seven-fold replication; spores from one glass slide representing one fruitbody were used for each pot. The infectivity of the spores added to soil was tested in the standard way (Section 3.2) by birch seedling assay; the seedlings were harvested after 12 weeks' growth in a growth room.

Mycorrhizas attributable to *Hebeloma* developed on all seedlings irrespective of the spore drying treatment (Table 3.17). However, significantly fewer root tips attributable to *Hebeloma* developed from spore inoculum that had been dried over silica gel for 48 h and transferred to moist soil without an intervening rehydration period than in the other treatments, though this did not affect seedling yield in terms of shoot dry weight or the number of root tips on seedlings.
Unfortunately, an unsupplemented soil treatment was not included as a control in this experiment; nevertheless, the high proportion of root tips infected by *Hebeloma* in all treatments was not observed in any control series of other experiments using this soil and run concurrently with this experiment (Tables 3.1 and 3.3). Thus establishment of *Hebeloma* can be ascribed confidently to the spore inocula. The results show that spores of *H. crustuliniforme* are more effective as inocula when stored at 75% RH for 48 h than when stored over silica gel for the same time; in addition, the spores remained effective after 24 h dehydration over silica gel provided that they were rehydrated gradually. The time period of storage was not of sufficient duration to ascribe any significance to the effect of dehydration on spore viability, and further experiments in which basidiospores are subjected to a drying period of several days are needed to further investigate the phenomenon.
TABLE 3.17: Development of mycorrhizas on seedlings of *Betula pendula* grown in unsterile brown earth supplemented with variously dried spores of *Hebeloma crustuliniforme*; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Spore storage treatment</th>
<th>Proportion of seedlings with inoculant mycorrhizas (and mean of log(x+1))</th>
<th>Mean no. root tips uncolonised</th>
<th>Mean % root tips mycorrhizal</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident$^1$</th>
<th>Proportion of seedlings with different resident mycorrhizal types$^1$</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried for 48 h over silica gel</td>
<td>$\frac{7}{7}$</td>
<td>245</td>
<td>42.8</td>
<td>$\frac{57.2}{b^a}$</td>
<td>$\frac{41.1}{b}$</td>
<td>$\frac{16.1}{7}$</td>
</tr>
<tr>
<td>Dried for 24 h over silica gel then gradually rehydrated for 24 h</td>
<td>$\frac{7}{7}$</td>
<td>189</td>
<td>25.5</td>
<td>$\frac{74.5}{a}$</td>
<td>$\frac{70.7}{a}$</td>
<td>$\frac{3.8}{T, \frac{7}{7}}$</td>
</tr>
<tr>
<td>75% RH for 48 h</td>
<td>$\frac{5}{5}$</td>
<td>155</td>
<td>17.1</td>
<td>$\frac{82.9}{a}$</td>
<td>$\frac{76.1}{a}$</td>
<td>$\frac{6.2}{I, \frac{5}{5}; T, \frac{3}{5}}$</td>
</tr>
</tbody>
</table>

$^1$Source of inoculum unknown.

$^2$Within columns, figures followed by different letters are significantly different from one another, p=0.05.

$^3$I, Inocybe; T, Thelephora; T3, "Type 3".
Crushed fruitbodies have been used as inocula of mycorrhizal fungi since the eighteenth century, when sporophores of *Tuber* spp. were added to the soil around oak seedlings to enhance truffle development (Trappe, 1977). More recently, dried basidiocarps of *Pisolithus tinctorius* and *Rhizopogon luteolus* have been used successfully to establish mycorrhizas on nursery and containerised seedlings (Azvedo, 1970, cited in Marx, 1980; Donald, 1975). In experimental studies, Mullette (1976) reported that 72-90% of roots of pine seedlings became infected by *Pisolithus tinctorius* when seedlings were grown in sterile quartz or unsterile soil derived from sandstone and supplemented with fruitbodies which had previously been dried and crushed; storage at room temperature for 2 years did not significantly affect viability of this inoculum. Although the use of fresh fruitbodies involves the addition of mycelium with the spores, Marx (1980) expressed the opinion that the vegetative matrix should be disregarded as part of the inoculum because of the rapidity of its decomposition.

In previous experiments, spore inocula of *Lactarius* spp. failed to establish mycorrhizas on seedlings in unsterile soil or unsterile vermiculite-peat (Sections 3.3, 3.4). An experiment was therefore done to see if supplementation of unsterile soil with basidiocarp tissue, thus providing an inoculum of mycelium as well as basidiospores, despite Marx's (1980) reservations, could lead to the establishment of *Lactarius* on young birch seedlings. For this purpose, fruitbodies of *L. spinosulus* were collected from beneath birch at S.C.R.I. (Section 2.1.6.4) during the autumn of 1982, and fruitbodies of *Inocybe geophylla*, previously shown to establish mycorrhizas from spore inocula
(Sections 3.3.3, 3.4.2), were collected from the same site and used for comparison. Seven fresh fruitbodies of each species were crushed in sterile polyethylene bags and the contents in each case were distributed between seven, 5 cm$^3$ plant pots containing an unsterile brown earth. The soil was collected from S.C.R.I. just beyond the root zone of the trees, and was air-dried for 21 days and then rewetted to 40% saturation (Section 2.1.5) before use. The infectivity of the inocula was tested in the standard way (Section 3.2), the birch assay seedlings being grown for 16 weeks (Table 3.18).

Three of the six seedlings in control (unsupplemented) soil developed mycorrhizas, these being attributable to either *Hebeloma* or *Thelephora*; three replicates were non-mycorrhizal (Table 3.18).

*I. geophylla* established mycorrhizas on six of the seven seedlings grown in soil supplemented with crushed fruitbodies of this species, and the development of resident mycorrhizal fungi was correspondingly reduced in this treatment compared with in controls. The proportion of root tips mycorrhizal with *Inocybe* (23.1%) was similar in this experiment to that recorded in previous experiments in which fresh spore inoculum was added to unsterile soil (23.2%; Table 3.3) or to unsterile vermiculite-peat (26.3%; Table 3.6). In contrast to the case with *I. geophylla*, basidiocarp inoculum of *L. spinosulus* failed to establish mycorrhizas; instead, two of the replicates were non-mycorrhizal and four developed mycorrhizas attributable to one or other of *Hebeloma, Inocybe* and *Paxillus*, which apparently established from resident inoculum sources. The presence of *Inocybe* mycorrhizas on one of the seedlings in this treatment does not invalidate the suggestion that *Inocybe* could develop mycorrhizas from added inoculum in the appropriate supplementation treatment, because mycorrhizas of *Inocybe* occurred on only 2.4% of the
root tips of the one replicate in the treatment involving spore inoculum of *L. spinosulus*, compared with 23% on seedlings grown in soil supplemented with spore inoculum of *L. geophylla*. This enhancement of infection by *Inocybe* is most readily attributable to increased inoculum density resulting from the soil supplement rather than to a secondary effect of spore supplementation in enhancing infection from resident inoculum.

The different inoculum treatments had no effect on seedling yield in terms of shoot dry weight or the numbers of root tips, although there was, as in many previous experiments, an indication that the addition of spore inoculum of *Inocybe* suppressed seedling development (Table 3.18).
<table>
<thead>
<tr>
<th>Basidiocarp supplement</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident</th>
<th>Proportion of seedlings with different resident mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Inocybe geophylla</em></td>
<td>$\frac{6}{7}$</td>
<td>144 (2.094)</td>
<td>75.7</td>
<td>74.3</td>
<td>$\frac{1}{7}$</td>
<td>74 (1.730)</td>
</tr>
<tr>
<td><em>Lactarius spinosulus</em></td>
<td>$\frac{5}{6}$</td>
<td>282 (2.367)</td>
<td>92.8</td>
<td>7.2</td>
<td>$\frac{1}{6}$; $\frac{1}{6}$</td>
<td>121 (1.971)</td>
</tr>
<tr>
<td>None (unsupplemented soil)</td>
<td>$\frac{7}{6}$</td>
<td>288 (2.430)</td>
<td>91.2</td>
<td>8.8</td>
<td>$\frac{7}{6}$; $\frac{1}{6}$</td>
<td>121 (2.076)</td>
</tr>
</tbody>
</table>

1 Source of inoculum unknown.

2 H, Hebeloma; I, Inocybe; P, Paxillus; T, Thelephora.
3.11 Establishment of Sheathing Mycorrhizal Fungi on Birch from Dispersed Mycelial Inoculum Added to Unsterile Brown Earth

In previous experiments all of the mycorrhizal fungi that are reported, or can be assumed, to be "late-stage" by the criteria of Deacon et al. (1983) failed to establish from spore inocula on birch seedlings, and one such fungus, *Lactarius spinosulus*, also failed to establish from crushed basidiocarp inoculum (Section 3.10). It was recognised, however, that the basidiocarp tissue, as such, would probably senesce and perhaps autolyse rapidly thereby contributing little to the amount of effective inoculum in soil (Marx, 1980). So a further experiment was done, in which vegetative mycelium of two "late-stage" fungi, *Lactarius pubescens* and *Leccinum roseofracta*, in the form of colonised vermiculite-peat, was added to unsterile soil. It was hoped that the mycelium would not lyse as rapidly as would basidiocarp tissue.

Vermiculite-peat inocula of *Hebeloma sacchariolens*, *Lactarius pubescens* and *Leccinum roseofracta* as well as uninoculated vermiculite-peat were prepared as described in Section 2.2.1, and mixed separately (1:4, v/v) into unsterile brown earth as used in most previous experiments (Sections 3.3, 3.6, 3.7, 3.8, 3.9). The variously supplemented soils were transferred to 5 cm³ plant pots and the infectivity of the inocula was tested by birch seedling assay in the standard way (Section 3.2). Samples of seedlings for each treatment were harvested after 11, 20 and 30 weeks' growth, each root system being assessed for mycorrhizal development as described previously (Section 3.2).

After 11 weeks' growth, few of the seedlings in the control (unsupplemented) series or in the soils supplemented with *L. pubescens* or *L. roseofracta* had developed mycorrhizas; where they occurred all of the mycorrhizas were attributable to *Thelephora terrestris* (Table 3.19).
In contrast, all of the seedlings grown in soil containing mycelial inoculum of *H. sacchariolens* developed mycorrhizas attributable to this species within 11 weeks, and the degree of mycorrhizal development in this treatment was thus significantly greater than in all other treatments (Table 3.19). Moreover, in this treatment, mycorrhizas of *Hebeloma* developed to the exclusion of *Thelephora*.

By 20 weeks, most if not all of the seedlings in all of the treatments had become mycorrhizal. Yet, still, *Thelephora* predominated in all except the soil supplemented with *H. sacchariolens*; mycorrhizas attributable to *Lactarius* and *Leccinum* had not developed from the added inocula. Of interest, *H. sacchariolens* had maintained its dominance on the root systems in the appropriate treatment, and, as at 30 weeks, it occurred largely to the exclusion of *Thelephora*. But at 20 weeks, as at 30 weeks, the proportion of the total root system that was mycorrhizal did not differ between treatments. Thus, mycorrhizas had developed from resident sources of inoculum to more or less the same degree in all treatments except that involving *H. sacchariolens*, which maintained its dominance throughout the experiment. The results at 30 weeks again showed no development of mycorrhizas attributable to *L. pubescens* or *L. roseofracta*. The mycelial inoculum of these species was thus ineffective, though it may be noted that *Thelephora terrestris* is considered to be an "aggressive" colonist and therefore, once established, it might have excluded development by *L. pubescens* and *L. roseofracta*.

The experiment as a whole showed a clear trend in the progressive increase of seedling growth with time of incubation; nevertheless, even at the earliest sampling time, when differences in total mycorrhizal establishment were most pronounced, there was no measurable difference in seedling development between treatments. In this, as in most of the
other experiments in this section, the shoot dry weight of seedlings was positively and closely correlated \((r = 0.881)\) with the total number of root tips, irrespective of treatment differences.
TABLE 3.19: Development of mycorrhizas on seedlings of *Betula pendula* grown for different times in unsterile brown earth supplemented with vermiculite-peat colonised by *Hebeloma sacchariolens*, *Lactarius pubescens* or *Leccinum roseofracta*; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Incubation period (weeks)</th>
<th>Inoculum</th>
<th>Proportion of seedlings with inoculant mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % root tips mycorrhizal</th>
<th>Mean % mycorrhizal root tips attributable to different types inoculant resident(^1)</th>
<th>Proportion of seedlings with different resident mycorrhizal types(^2)</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td></td>
<td>%</td>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>11</td>
<td><em>H. sacchariolens</em></td>
<td>6%</td>
<td>(119^{a,b}) (2.073)</td>
<td>44.8</td>
<td>55.2</td>
<td>nil</td>
<td>nil</td>
<td>36 (1.558)</td>
</tr>
<tr>
<td></td>
<td><em>L. pubescens</em></td>
<td>6%</td>
<td>(161^b) (2.156)</td>
<td>95.8</td>
<td>4.2</td>
<td>nil</td>
<td>4.2 ((T, %))</td>
<td>45 (1.555)</td>
</tr>
<tr>
<td></td>
<td><em>L. roseofracta</em></td>
<td>6%</td>
<td>(151^a) (2.164)</td>
<td>98.1</td>
<td>1.9</td>
<td>nil</td>
<td>1.9 ((T, %))</td>
<td>37 (1.560)</td>
</tr>
<tr>
<td></td>
<td>None (unsupplemented soil)</td>
<td>6%</td>
<td>(86^b) (1.916)</td>
<td>94.0</td>
<td>6.0</td>
<td>-</td>
<td>6.0 ((T, %))</td>
<td>31 (1.451)</td>
</tr>
<tr>
<td>20</td>
<td><em>H. sacchariolens</em></td>
<td>5%</td>
<td>(319^b) (2.491)</td>
<td>40.5</td>
<td>59.5</td>
<td>57.8</td>
<td>1.7 ((T, %))</td>
<td>130 (2.057)</td>
</tr>
<tr>
<td></td>
<td><em>L. pubescens</em></td>
<td>5%</td>
<td>(349^b) (2.535)</td>
<td>37.9</td>
<td>62.1</td>
<td>nil</td>
<td>62.1 ((H, %; (T, %))</td>
<td>129 (2.151)</td>
</tr>
<tr>
<td></td>
<td><em>L. roseofracta</em></td>
<td>5%</td>
<td>(493^a) (2.633)</td>
<td>20.1</td>
<td>79.9</td>
<td>nil</td>
<td>79.9 ((T, %; (T4, %))</td>
<td>118 (2.027)</td>
</tr>
<tr>
<td></td>
<td>None (unsupplemented soil)</td>
<td>5%</td>
<td>(341^b) (2.532)</td>
<td>57.3</td>
<td>42.7</td>
<td>-</td>
<td>42.7 ((H, %; (T, %))</td>
<td>131 (2.120)</td>
</tr>
<tr>
<td>30</td>
<td><em>H. sacchariolens</em></td>
<td>4%</td>
<td>(464) (2.628)</td>
<td>29.2</td>
<td>70.8</td>
<td>61.7</td>
<td>9.1 ((T, %))</td>
<td>216 (2.310)</td>
</tr>
<tr>
<td></td>
<td><em>L. pubescens</em></td>
<td>4%</td>
<td>(681) (2.830)</td>
<td>14.8</td>
<td>85.2</td>
<td>nil</td>
<td>85.2 ((T, %))</td>
<td>231 (2.370)</td>
</tr>
<tr>
<td></td>
<td><em>L. roseofracta</em></td>
<td>4%</td>
<td>(545) (2.719)</td>
<td>30.9</td>
<td>69.1</td>
<td>nil</td>
<td>69.1 ((T, %))</td>
<td>124 (2.062)</td>
</tr>
<tr>
<td></td>
<td>None (unsupplemented soil)</td>
<td>4%</td>
<td>(562) (2.743)</td>
<td>47.7</td>
<td>52.3</td>
<td>-</td>
<td>52.3 ((T, %))</td>
<td>252 (2.361)</td>
</tr>
</tbody>
</table>

\(^1\) Source of inoculum unknown.

\(^2\) Within columns, and for each sampling time, figures followed by different letters are significantly different from one another, \(p = 0.05\).

\(^3\) H, Hebeloma; T, Thelephora; T4, "Type 4".
3.12 Establishment of Mycorrhizas on Birch Seedlings Grown in Samples of Coal Spoil Taken from Beneath Fruitbodies of Sheathing Mycorrhizal Fungi

Analysis, by dissection and by seedling assay, of soil cores taken from beneath fruitbodies on the experimental birch plot at I.T.E., Bush Estate (Section 2.1.6.2) showed that a distinction could be made between different mycorrhizal fungi in relation to (a) the predominant mycorrhizal types that were present beneath the fruitbodies in "dissection-cores", and (b) the mycorrhizal types that became established on birch assay seedlings grown in replicate cores (Deacon et al., 1983). Soil cores taken from beneath fruitbodies of some mycorrhizal fungi, namely Inocybe sp. and Laccaria sp., contained a mixture of mycorrhizal types. Although the mycorrhizal types attributable to the fruiting species were never dominant in these cores, yet seedlings grown in the cores always developed mycorrhizas attributable to the fruiting species. In contrast, mycorrhizas attributable to Lactarius and Leccinum occurred in large numbers in soil beneath fruitbodies of Lactarius sp. and Leccinum sp., respectively, yet seedlings grown in these soil cores never developed mycorrhizas corresponding to these species.

The following experiment was done to investigate if a similar pattern existed in samples taken from beneath fruitbodies associated with naturally regenerating birch on a disused coal spoil tip at Newtongrange (Section 2.1.6.1). Chemical analysis of the coal waste had shown it to be acidic (pH 3.95 - 4.85), with very low levels of phosphorus, calcium and potassium but high levels of aluminium and organic nitrogen. These factors, combined with high soil temperatures and tendency to drying (K. Ingleby, pers. comm.), were thought to constitute an extreme environment which might influence the pattern of establishment of mycorrhizas on seedlings from "resident" sources of inoculum present in the spoil.
Intact samples of coal spoil were taken from directly beneath fruit-bodies associated with birch at Newtongrange during the autumn of 1981, details of the local sites from which the fruitbodies were collected being given in Table 3.20. Twelve to 14 replicate samples, taken from beneath fruitbodies of *Inocybe lacera*, *Paxillus involutus*, *Scleroderma citrinum*, *Leccinum roseofracta* and *Amanita muscaria* (Table 3.20) were individually transferred immediately to 5 cm³ plant pots to minimise damage to structure during transit; for comparison, similar 'control' samples were taken from the periphery of the sampling area, 9 m for the nearest clump of mature birch. After being returned to the laboratory, half of the pots were planted with 3-week old aseptically pre-germinated seedlings of *Betula pendula* and the pots were distributed, in a randomised layout, in a growth room. The seedlings were grown at a mean temperature of 18°C for 16 weeks, the pots being sprayed daily with sterilised water. In order to maintain the approximate original moisture content of the coal spoil, each pot was weighed at the start of the experiment and returned to its original weight after 6 and 12 weeks. The seedlings were thinned to one per pot after 6 weeks. At harvest, mycorrhizal development on the seedlings was assessed as described in Section 3.2. The remaining replicate spoil samples were washed separately under running water over a 2 mm mesh sieve and all root material contained within them was examined microscopically, the different mycorrhizal types being categorised using reference material collected previously (Section 2.2.4).

A number of mycorrhizal types were evident in the samples taken from beneath fruitbodies and analysed by dissection (Table 3.21). Numbers of root fragments contained within the control cores were very low and there was little evidence of active mycorrhizas; the few
root pieces present were probably derived from young (<1 year old) naturally regenerating seedlings in the vicinity. One-quarter to three-quarters of the mycorrhizas in all of the cores were attributable to *Paxillus*, irrespective of the species of fruitbody from beneath which each core was taken. Mycorrhizas attributable to *Inocybe* were recorded beneath fruitbodies of *I. lacera* but they were present at a lower percentage than were mycorrhizas of *Paxillus*; mycelial strands attributable to *Leccinum* spp. were also recorded in cores beneath fruitbodies of *I. lacera* although Leccinum mycorrhizas were absent. Mycorrhizas of *Leccinum, Scleroderma* and *Amanita* were recorded in cores taken from beneath fruitbodies of these species, but of these mycorrhizas, only those of *Scleroderma* were present in large numbers.

A total of five resident mycorrhizal fungi were seen on seedlings grown in the control cores taken from beyond the root zone of birch trees (Table 3.22). Mycorrhizas attributable to *Inocybe* were predominant in terms of both the number of replicate seedlings on which they occurred and the proportion of root tips infected; mycorrhizas of *Paxillus* were present on two of the eight seedlings, and three other mycorrhizal types occurred at a low frequency on individual seedlings. Mycorrhizas attributable to *Paxillus* and *Inocybe* were dominant on seedlings grown in spoil samples taken from beneath fruitbodies of *P. involutus* and *I. lacera* respectively, and these mycorrhizal types were significantly more common than on seedlings grown in control spoil samples. Associated with the successful establishment by these inoculant types, there was a corresponding suppression or exclusion of resident fungi. Seedlings grown in spoil samples taken from beneath fruitbodies of *L. roseofracta, S. citrinum* and *A. muscaria* failed to develop inoculant-type mycorrhizas, although mycorrhizas corresponding to these species were
present on the parent tree roots in spoil samples analysed by dissection; instead, in these treatments a mixture of resident fungi became established, of which *Paxillus* was especially common. The proportion of root tips colonised by *Paxillus* in these treatments was, however, significantly lower (p = 0.01) than the proportion on seedlings grown in coal spoil samples taken from beneath fruitbodies of *P. involutus* (Table 3.22). Interestingly, mycorrhizas of *Thelephora* were present on all seedlings grown in samples taken from beneath *A. muscaria*, but were absent from, or only poorly represented in, the other treatments.

A Student-Newman-Keuls test (Section 2.2.8) showed significant between-treatment differences in seedling yield in terms of the shoot oven-dry weights and the numbers of root tips on seedlings (Table 3.22). Seedlings grown in spoil samples taken from beneath fruitbodies of *S. citrinum* were larger than in all other treatments. These seedlings were also associated with a large variety of resident mycorrhizal fungi, but the overall proportion of root tips that were colonised by mycorrhizas was significantly lower than in any of the other treatments. Control seedlings and seedlings grown in cores taken from beneath fruitbodies of *I. lacera* were significantly smaller than seedlings grown in all other treatments; the low yield of seedlings grown in the presence of inoculum of *Inocybe* spp. has been reported in previous experiments (Section 3.5). As mentioned earlier, dissection of the control coal spoil samples revealed little evidence of active mycorrhizas (Table 3.21), yet seedlings grown in these samples developed a wide variety of mycorrhizal types. This suggests that inoculum in these cases must have been in a form other than on mycorrhizal roots.

In summary, the results show that inoculum present in coal spoil samples taken from beneath fruitbodies of *P. involutus* and *I. lacera*
effectively established mycorrhizas on seedlings, whereas *L. roseofracta*, *S. citrinum* and *A. muscaria* failed to establish mycorrhizas on seedlings grown in spoil samples taken from beneath their fruitbodies. These findings, in part, support the results of Deacon *et al.* (1983) who showed different patterns of infection of seedlings grown in soil cores taken from beneath fruitbodies of a range of mycorrhizal fungi associated with birch on the experimental plot, I.T.E., Bush Estate (Section 2.1.6.2). In other words, the evidence from coal spoil (Table 3.22) supports the separation of "early-stage" from "late-stage" mycorrhizal types by the criterion of Deacon *et al.* (1983). Soil cores and coal spoil taken from beneath *Inocybe* spp. contained mycorrhizas attributable to *Inocybe* but other mycorrhizal types were also present (*Paxillus*, in the case of coal spoil); in this respect, again, the results parallel those for soil presented by Deacon *et al.* (1983). However, whereas in soil, the mycorrhizas present beneath fruitbodies of *Lactarius* sp. and *Leccinum* sp. were almost exclusively attributable to the respective fruitbody species (Deacon *et al.*, 1983), yet in coal spoil (Table 3.21) mycorrhizas attributable to the fruiting species were never dominant in samples taken from beneath fruitbodies of *L. roseofracta*, *S. citrinum* and *A. muscaria*. In these instances, *Paxillus* mycorrhizas invariably occurred at a similar or at a greater frequency than did mycorrhizas of the fruitbody types.

Large numbers of fruitbodies of *P. involutus* occurred in the sampling area during the autumn of 1981 and 1982 and, as expected, mycorrhizas, mycelial strands and associated sclerotia of *P. involutus* were also ubiquitous; indeed, mycorrhizas attributable to *Paxillus* were present in all samples analysed by dissection, irrespective of the fruiting species beneath which the samples were collected. In terms of the
frequency of fruitbodies in the sampling area at Newtongrange, 
*S. citrinum* was co-dominant with *P. involutus*. It is therefore perhaps 
surprising that mycorrhizas attributable to *Scleroderma* were not re-
corded with a similar frequency to those of *P. involutus* in the dissection 
cores, and, in addition, that although mycorrhizas of *S. citrinum* were 
commonly found on young naturally regenerating seedlings in the vicinity 
of fruitbodies on the site, yet these mycorrhizas never became estab-
lished on seedlings grown in coal spoil samples taken from beneath fruit-
bodies of *S. citrinum*.
TABLE 3.20: Details of local sites where coal spoil samples were collected beneath fruitbodies of different mycorrhizal fungi at Newtongrange.

<table>
<thead>
<tr>
<th>Fruitbody type</th>
<th>Spoil sample</th>
<th>Other relevant details</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Inocybe lacera</em></td>
<td>Light stony soil; very little coal waste evident.</td>
<td>Cores taken from beneath 3 clusters of fruitbodies (total 46) of mean cap diameter 26 mm. One immature fruitbody of <em>S. citrinum</em> (mean diameter 20 mm) was present within 1 m.</td>
</tr>
<tr>
<td><em>Paxillus involutus</em></td>
<td>As above.</td>
<td>Cores taken from beneath 12 fruitbodies of <em>P. involutus</em> of mean cap diameter 88 mm. No other fruitbodies were seen within 1 m.</td>
</tr>
<tr>
<td><em>Scleroderma citrinum</em></td>
<td>Stony; coal waste evident; low in organic matter.</td>
<td>Cores taken from beneath 8 separate clusters of fruitbodies of <em>S. citrinum</em> of mean diameter 94 mm, some of which were mature. One small fruitbody of <em>P. involutus</em> of mean diameter 24 mm was present within 1 m.</td>
</tr>
<tr>
<td><em>Leccinum roseofracta</em></td>
<td>Stony but high in undecomposed organic matter.</td>
<td>Cores taken from beneath 10 spaced fruitbodies of <em>L. roseofracta</em> of mean diameter 60 mm. Two fruitbodies of <em>I. lacera</em> of mean diameter 18 mm were seen within 1 m.</td>
</tr>
<tr>
<td><em>Amanita muscaria</em></td>
<td>Light friable soil, high in decomposed organic matter; no coal waste evident.</td>
<td>Cores taken from beneath 10 spaced fruitbodies of <em>A. muscaria</em> of mean cap diameter 83 mm; 11 fruitbodies of <em>I. lacera</em> of mean diameter 16 mm were seen within 1 m.</td>
</tr>
</tbody>
</table>
TABLE 3.21: Qualitative and semi-quantitative estimates of mycorrhizal types recorded in coal spoil samples taken from directly beneath fruitbodies associated with birch at a disused coal spoil tip at Newtongrange.

<table>
<thead>
<tr>
<th>Fruitbodies beneath which samples were taken</th>
<th>Mycorrhizal types present in coal spoil samples&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Paxillus</th>
<th>Inocybe</th>
<th>Leccinum</th>
<th>Scleroderma</th>
<th>Amanita</th>
<th>Type 6</th>
<th>moribund</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paxillus involutus</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Inocybe lacera</td>
<td>++</td>
<td>+</td>
<td>0&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leccinum roseofracta</td>
<td>++</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Scleroderma citrinum</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amanita muscaria</td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None (control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>1</sup> 0, no occurrence; +, <1-24% of mycorrhizal root tips; ++, 25-49%; +++, 50-74%; ++++, 75-100%.

<sup>2</sup> Mycelial strands attributable to *Leccinum* sp. recorded.
**TABLE 3.22:** Development of mycorrhizas on seedlings grown in samples of coal spoil taken from beneath fruitbodies of different sheathing mycorrhizal fungi at Newtonrange; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Species of fruitbody beneath which samples were taken</th>
<th>Proportion of seedlings with &quot;inoculant&quot; mycorrhizas</th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % mycorrhizal root tips attributable to different types &quot;inoculant&quot; &quot;resident&quot;</th>
<th>Proportion of seedlings with different &quot;resident&quot; mycorrhizal types</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inocybe lacera</td>
<td>9/6</td>
<td>180&lt;sup&gt;a&lt;/sup&gt; (2.224)</td>
<td>24.7</td>
<td>75.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>P, 9/6</td>
<td>47&lt;sup&gt;c&lt;/sup&gt; (1.655)</td>
</tr>
<tr>
<td>Paxillus involutus</td>
<td>6/6</td>
<td>243&lt;sup&gt;b&lt;/sup&gt; (2.358)</td>
<td>36.5</td>
<td>63.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Leccinum roseofracta</td>
<td>9/6</td>
<td>262&lt;sup&gt;b&lt;/sup&gt; (2.330)</td>
<td>23.1</td>
<td>76.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nil</td>
<td>60&lt;sup&gt;b&lt;/sup&gt; (1.801)</td>
</tr>
<tr>
<td>Scleroderma citrinum</td>
<td>9/7</td>
<td>390&lt;sup&gt;a&lt;/sup&gt; (2.521)</td>
<td>65.7</td>
<td>34.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H, 9/7; I, 9/7; P, 9/7; T, 9/7; T3, 9/7; T5, 9/7</td>
<td>164&lt;sup&gt;a&lt;/sup&gt; (2.199)</td>
</tr>
<tr>
<td>Amanita muscaria</td>
<td>9/8</td>
<td>210&lt;sup&gt;b&lt;/sup&gt; (2.314)</td>
<td>28.1</td>
<td>71.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>P, 9/8; T, 9/8</td>
<td>66&lt;sup&gt;b&lt;/sup&gt; (1.822)</td>
</tr>
<tr>
<td>None (control coal spoil)</td>
<td>7/8</td>
<td>125&lt;sup&gt;c&lt;/sup&gt; (2.016)</td>
<td>32.3</td>
<td>67.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>H, 9/8; I, 9/8; P, 9/8; T, 9/8; T5, 9/8; T6, 9/8</td>
<td>28&lt;sup&gt;c&lt;/sup&gt; (1.353)</td>
</tr>
</tbody>
</table>

1. The terms "inoculant" and "resident" are used here for consistency though all types were "resident" in the coal spoil; "inoculant" in this table therefore refers to mycorrhizas attributable to the fruiting species.

2. Source of inoculum unknown.

3. H, Hebeloma; I, Inocybe; P, Paxillus; T, Thelephora; T5, "Type 5"; T6, "Type 6".

4. "Within columns, figures followed by different letters are significantly different from one another, p = 0.05."
3.13 Discussion

The results in this section, on the role of basidiospores as inocula of sheathing mycorrhizal fungi, have revealed two clear patterns of behaviour. Spores of some fungi readily established mycorrhizas on seedling root systems in unsterile soil whereas the spores of other fungi failed to do so. The only exception to this clear distinction concerns *Paxillus involutus*, which will be considered separately later. My results relate closely to the concept of mycorrhizal successions (Ford *et al.*, 1980; Deacon *et al.*, 1983; Mason *et al.*, 1983a) and particularly to the distinction that has been drawn between "early-stage" and "late-stage" mycorrhizal fungi. These terms were originally coined to denote the early or late appearance of the fruitbodies as the trees aged, but Deacon *et al.* (1983) further distinguished these two categories of fungi based on their behaviour in unsterile soil: whereas both "early-stage" and "late-stage" fungi can establish mycorrhizas from mycelial inoculum on birch seedlings in bixenic conditions, only "early-stage" fungi readily establish mycorrhizas under unsterile conditions.

Several species in the three genera, *Hebeloma*, *Inocybe* and *Laccaria*, have been shown in my work to establish mycorrhizas from spore inocula, as summarised in Table 3.23. These fungi have been shown or presumed to be "early-stage" in the sense of Deacon *et al.* (1983). In contrast, species of the genera, *Cortinarius*, *Lactarius*, *Leccinum* and *Russula*, as well as several individual species in other genera (Table 3.23), did not establish mycorrhizas from spore inocula in my work, and such fungi have been shown or presumed to be "late-stage". Thus, the work in this section has shown a major and seemingly absolute distinction between these behavioural groups of mycorrhizal fungi, though doubtless there will be found to be differences of behaviour within the groups.
The proposed distinction between "early-stage" and "late-stage" mycorrhizal fungi is supported further by the results for coal spoil samples in Section 3.12. Samples taken from beneath the fruitbodies of different sheathing mycorrhizal fungi always contained some mycorrhizas attributable to the fruiting species; however, seedlings grown in the spoil samples did not develop mycorrhizas of *Amanita muscaria*, *Leccinum roseofracta* or *Scleroderma citrinum* but only of *Inocybe lacera* and *Paxillus involutus*. Exactly similar findings had been made by Deacon *et al.* (1983) but for a somewhat different range of fungi in brown earth at Bush Estate. It can be suggested either that mycorrhizal fungi develop from basidiospores in soil in such circumstances (in which case the results merely confirm those for soils purposefully supplemented with spores), or that mycelial inoculum in the soil can initiate infection.

In one experiment (Section 3.11), mycelial inoculum was purposefully used, to test the general validity of the "early-stage"/"late-stage" distinction. It was shown that the "late-stage" fungi, *Lactarius pubescens* and *Leccinum roseofracta*, did not develop mycorrhizas even when vermiculite-peat inoculum was mixed into soil. So clearly the distinction is not based solely on spore infectivity or differences in germinability of spores.

In the initial series of experiments in which unsterile brown earth or unsterile vermiculite-peat was supplemented with fresh basidiospore inocula (Sections 3.3 and 3.4), only two of the 36 seedlings grown in control (unsupplemented) soil remained non-mycorrhizal. Mycorrhizal development on these seedlings was attributable to a number of mycorrhizal fungi (Section 3.3.1), the mycorrhizas having been termed "resident" to distinguish them from "inoculant" mycorrhizas that developed
from spore-supplements. The source of inoculum in these control treatments is not known. Some inocula may have been spores originally present in the soil (Marx and Ross, 1970; Marx, 1980) but this is unlikely to have been so in the unsterile vermiculite-peat. It is more likely that air-borne propagules arrived during the course of the experiments or that, despite careful handling, there was some splash contamination between plant pots. The layout of some of the experiments, in which pots were randomly distributed in treatment pairs spaced 20 cm apart, was designed to minimise cross-contamination. The "resident" fungi that became established were of a consistent but restricted range of species during the experimental period. Thus, on the control seedlings in the first six experiments with unsterile brown earth and unsterile vermiculite-peat (Sections 3.3 and 3.4), Hebeloma, Laccaria and Inocybe occurred most commonly and with similar frequency. Although variable, there was a tendency for Thelephora terrestris to occur on seedlings grown in soil supplemented with "late-stage" fungi; where it did occur, it was often dominant and colonised a high proportion of root tips. Marx and Ross (1970) reported T. terrestris to be the primary coloniser of autoclaved and fumigated soil, and in the southern United States, in particular, it is the major naturally occurring mycorrhizal colonist in nurseries (Marx, Bryan and Grand, 1970; Marx, 1980) and on containerised seedlings (Marx and Barnett, 1974). Its rapid colonisation of seedling roots can preclude colonisation by other mycorrhizal types and, for this reason, it has been reported to hinder the successful establishment of mycorrhizal fungi used for selective inoculation programmes.

The successful establishment of mycorrhizas from basidiospore inocula was consistently associated with a marked suppression or exclusion
of "resident" mycorrhizal fungi, both with respect to the variety of resident fungi that became established and with respect to the proportion of root tips infected by them. Application of Analysis of Variance (Section 3.5) showed that effective colonisation of seedlings by *Inocybe* spp. and *Laccaria* spp. from spore-supplements significantly reduced the proportion of root tips infected by the "resident" fungi compared with in controls, even though many root tips remained uninfected and thus potentially available for colonisation. Similar findings have been reported by Mason *et al.* (1983a) on birch seedlings propagated under bixenic conditions with *H. sacchariolens* and *P. involutus* and later transferred to unsterile mineral soil; "inoculant" mycorrhizas developed to the virtual exclusion of naturally occurring types during the first season's growth although this effect was not necessarily evident in subsequent years. Several sheathing mycorrhizal fungi are known to produce antibiotics effective against bacteria and fungal root pathogens (Zak, 1964; Marx, 1969a,b, 1972) and basidiospores of *Laccaria laccata* have been shown significantly to reduce the incidence of root rot caused by *Fusarium oxysporum* on Douglas-fir seedlings even in the absence of mycorrhizal establishment (Stack and Sinclair, 1975). *In vitro* studies have shown that *L. laccata* produces antibiotic substances inhibitory to *F. oxysporum*; however, recent evidence (Sylvia and Sinclair, 1983a,b) has shown that antibiosis may be less important than the deposition of phenolic osmiophilic materials induced in the host cortical tissue by the presence of *L. laccata* and thus conferring a degree of host resistance against pathogenic attack. Mycorrhizal fungi apparently differ in their ability to elicit host induction of phenolic materials (Sylvia and Sinclair, 1983b) and it is possible that a similar mechanism operates in the suppression of "resident" fungi by spore inocula. Spores of "early-stage"
fungi may induce phenolic production in the host, thus rendering roots unsuitable for colonisation by "resident" mycorrhizal fungi, though the "inoculant" fungus can, itself, continue to develop. An interesting feature, in this respect, was the observation (Section 3.3.3) that "early stage" spore inocula of H. sacchariolens suppressed development of "resident" fungi even when the inoculum in the experiment did not establish mycorrhizas.

Spore inocula of "late-stage" fungi did not usually suppress development of naturally occurring fungi; indeed, where spore-supplements failed to establish mycorrhizas on seedling root systems, colonisation by "resident" fungi was often enhanced both in terms of the variety of mycorrhizal types that established and in the proportion of total root tips infected. This finding is difficult to interpret. Nutrient leakage from the spore-supplements might have been sufficient directly to promote mycorrhizal development from "resident" sources, or indirectly to encourage this by promoting root growth and thus providing an increased availability of new roots for colonisation. It is likely, however, that leaked nutrients would be rapidly utilised by other rhizosphere organisms, rendering the nutrients unavailable for stimulation of the growth of mycorrhizal fungi. Alternatively, spore leachates may include specific metabolites and growth factors conducive to establishment of mycorrhizal fungi. The successful establishment from spore inocula of Hebeloma spp., Laccaria spp. and Inocybe spp. and the corresponding suppression or exclusion of naturally occurring fungi has important practical implications in the choice of suitable mycorrhizal symbionts for selective inoculation programmes.

Supplementation of soil with fresh basidiospore inocula resulted in variable seedling yield in terms of shoot oven-dry weight and the
numbers of root tips formed on seedlings. The successful establishment of mycorrhizas from spore inocula did not generally result in increased seedling yield; rather there was a tendency for spore-supplements of *Laccaria* spp., *Inocybe* spp. and *Hebeloma* spp. to reduce seedling yield. This suppression of host growth was most notable in treatments involving *Inocybe*, as summarised in Section 3.5. Mycorrhizal development is generally associated with improved host growth (Section 1.2) and Mason et al. (1983a) reported marked increases in the stem height of *Picea sitchensis* during the first season's growth, in response to mycorrhizal development by *Laccaria* sp.; however, suppression of seedling growth by mycorrhizal fungi has also been reported (Nielson and Griffith, 1978; Chet et al., 1979; Sylvia and Sinclair, 1983a) and this influence may be associated with colonisation of very young seedlings that are unable to limit the fungal symbiont and provide sufficient carbohydrate for normal development of both partners. The possible production of hormones, toxins and antibiotic substances by "early-stage" mycorrhizal fungi, while suppressing naturally occurring fungi, may also restrict seedling growth in the early stages of host development. Sylvia and Sinclair (1983a) reported that substances produced by *L. laccata* were inhibitory to root growth of *Pseudotsuga menziesii* in soil-free systems, although the inhibitory effect was removed in soil, possibly due to adsorption of the active compounds onto soil colloids, or to less luxuriant growth of the fungal symbiont. Further studies are needed to determine the reasons for suppression of seedling growth by mycorrhizal fungi such as *Inocybe* spp.

In contrast, seedlings grown in soil supplemented with spore inocula of some "late-stage" fungi, most notably *Lactarius* spp., slightly
enhanced seedling growth compared with in controls. This observa-
tion is perhaps attributable to the high levels of infection by resident 
fungi in these instances and it is of interest that, in several cases, 
the highest seedling yield was associated with the greatest variety of 
mycorrhizal fungi established on the root systems. This was observed 
in seedlings grown in soil supplemented with *Lactarius pubescens* 
(Section 3.3.1), *L. biennius* (Section 3.3.2) and *Scleroderma citrinum* 
(Section 3.3.4) and in these cases *Thelephora terrestris* was the major 
colonist. The selection of mycorrhizal fungi for maximum host growth 
should not exclude the possibility that combined inocula of mycorrhizal 
fungi may confer greater advantages than do single inoculations.

In general, the pattern of mycorrhizal development from spore 
inocula was not significantly altered by soil conditions. "Late-stage" 
fungi failed to establish in any growth medium tested. *Lactarius turpis* 
and *Leccinum scabrum*, two common symbionts on birch on the coal spoil 
tip at Newtongrange, failed to become established from spore-supplements 
in coal spoil, indicating that even in conditions conducive for normal 
mycorrhizal development by these species, spores were ineffective as 
inocula. Basidiospores of *P. involutus*, however, were more effective 
as inocula in coal spoil than in brown earth, and in coal spoil this species 
behaved typically as an "early-stage" mycorrhizal fungus in that it 
colonised roots from spores and, in so doing, it suppressed the develop-
ment of "resident" mycorrhizal fungi. *P. involutus* is a characteristic 
mycorrhizal symbiont of birch on coal spoil in the Edinburgh area but 
only rarely occurs on mineral soil. Mycorrhizas of *Paxillus* developed 
on seedlings in most experiments in which fresh or stored spore sup-
plements of this species were used, but the proportion of root tips that 
became infected in media other than coal spoil was low and variable.
To my knowledge, the only other reported attempt to synthesise mycorrhizas of *P. involutus* from spores was made by Laiho (1970); in this case, spore inocula in autoclaved soil failed to establish mycorrhizas on birch or pine seedlings. The status of *P. involutus* as on "early-stage" or "late-stage" symbiont thus seems subject to the qualification that the soil type should be specified. Of possible significance in this respect, *P. involutus* is regarded as a facultative mycorrhizal symbiont (Laiho, 1970), i.e. one that has saprophytic ability in soil and is therefore not wholly dependent on the host.

The establishment of "early-stage" fungi and the failure of "late-stage" fungi to colonise seedling root systems from spore inocula was also observed on different host species (Section 3.7). *Suillus luteus* failed to establish from spore supplements on birch, spruce and pine and thus seems to behave as a "late-stage" mycorrhizal fungus, as do the other members of the Boletaceae that were tested, i.e. *Leccinum* spp. (Sections 3.3.1, 3.3.3, 3.6, 3.11, 3.12), even though *S. luteus* is a common symbiont of young pine under natural conditions. In contrast, *Hebeloma crustuliniforme* successfully established mycorrhizas on birch and perhaps on pine, though it only poorly colonised spruce. Chu-Chou and Grace (1981) reported the frequent occurrence of *H. crustuliniforme* on *Pseudotsuga menziesii*, *Pinus radiata* and *Eucalyptus* spp. growing in nursery conditions; it therefore seems to have a wide host range, but appears to be confined to seedlings and is seldom recorded on trees older than 4-5 years (Chu-Chou, 1979; Chu-Chou and Grace, 1981, 1982; Mason et al., 1983a). As with *P. involutus*, *Hebeloma* spp. are strongly influenced by soil type; Mason *et al.* (1983a) reported the failure of mycorrhizas of *H. sacchariolens* to persist on birch during the second growing season in
a peaty soil, though this species remained well established in mineral soils. Interestingly, in my work, mycorrhizas of *Hebeloma* were seen to develop from "resident" or "contaminant" sources of inoculum only in mineral soil.

Because of the predominance of fruitbodies of *Scleroderma citrinum* in the coal spoil at Newtongrange (Sections 2.1.6.1 and 3.12), it seemed likely that this fungus, like *P. involutus*, would establish mycorrhizas on birch seedlings in spore-supplemented spoil. This, however, was not so: *S. citrinum* did not establish mycorrhizas on seedlings in either spoil (Section 3.12) or brown earth (Section 3.3.4) and therefore appears to behave as a "late-stage" mycorrhizal symbiont. Yet, Chu-Chou and Grace (1981) reported the widespread occurrence of *Scleroderma* sp. on conifer and eucalypt seedlings in nursery conditions. Moreover, basidiospores and mycelia of this genus and, indeed, of other gasteromycetes, have frequently been reported to be effective as inocula (Theodorou and Bowen, 1970, 1973; Marx, 1980) though it may be significant that these reports are based on experiments using fumigated or otherwise partially sterilised soils. *P. involutus* and *S. citrinum* may be mycorrhizal fungi which are intermediate with respect to the definition of "early-stage" and "late-stage" fungi, and their capacity to establish mycorrhizas may be dependent on the interactions between soil conditions and competition from other mycorrhizal symbionts. Indeed, the spores of *Scleroderma*, and of *Elaphomyces*, which was also ineffective in my work (Section 3.3.2), may need to pass through the gut of a herbivore before their dormancy is broken (R. Watling, pers. comm.) and this might account for their failure as inocula in the present studies.
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 (3-week old) seedlings comprise a diverse range of carbohydrates whereas mature trees (55 years 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. In this respect, *P. involutus* and *P. tinctorius* (Theodorou and Bowen, 1973) seem to be mycorrhizal fungi confined to extreme environments such as coal spoil where there is likely to be less competition from rhizosphere antagonists or potentially competitive mycorrhizal fungi.

The effect of storage on basidiospore inocula was investigated firstly to determine the time span over which spores of "early-stage" mycorrhizal fungi remain infective and, secondly, to see if the failure of "late-stage" spore inocula was attributable to constitutive dormancy that could be broken by extended incubation of the spores in different conditions. The results have indicated that spores of *Inocybe* spp. and *Hebeloma* spp. remain infective for at least 10 months in soil stored at 18°C; storage did not affect the subsequent level of infection on birch assay seedlings (Section 3.9.1) in comparison with that from fresh spore inocula (Section 3.3). Soil-free storage treatments adversely affected spores of *Inocybe* spp. (Section 3.9.3) but not those of *Hebeloma* spp. when subsequently tested for the ability to establish mycorrhizas.
However, there was a marked reduction in the efficacy of spore inocula of *H. crustuliniforme* when spores were held dry for 48 hours and then transferred to moist soil without an intervening rehydration period (Section 3.9.4). Although the results of this experiment were inconclusive, Simon (1974) has described a process of "rehydration injury" which occurs in higher plant seeds, fungal spores and other propagules. Drying causes a disorganisation of the phospholipid in cell membranes, resulting in a greater permeability to water and solutes. If the propagules are then placed directly into a moist environment, reorientation of the membrane components does not proceed sufficiently rapidly to prevent rapid water uptake accompanied by a marked leakage of nutrients. Reduced viability is then attributable to loss of essential metabolites and increased microbial competition in the presence of released nutrients. Because these potential problems were recognised, all comparative studies between different mycorrhizal fungi were done with spores that had been purposefully kept moist, without an opportunity to become air-dried.

Spores of most mycorrhizal fungi are produced and dispersed in the autumn and presumably lie dormant until new root growth commences in the following spring. Spore germination studies on agar have shown that basidiospores of most agarics, including *Hebeloma* spp. (Fries and Birraux, 1980), *Thelephora terrestris* (Birraux and Fries, 1981) and *Laccaria laccata* (Fries, 1983), are in a state of constitutive dormancy which can be broken by various "triggers" provided, in many cases, by living host roots and/or living mycelium of the same or different fungal species. The basidiospore studies of this section suggest that "early-stage" mycorrhizal fungi will germinate and colonise seedling birch roots, but living host roots evidently do not induce germination of spores of "late-stage" fungi.
The triggering of spore germination by host roots - whether it is a specific activation or merely an annulment of fungistasis - has clear ecological significance. The response of spores to living mycelium, however, is more difficult to interpret, but is known to occur in a number of mycorrhizal fungi including *P. involutus*, *Leccinum* spp. and *Laccaria laccata* (Fries, 1979a,b, 1981, 1983). Rast and Stäuble (1970) demonstrated that the triggering agent for spore germination in *Agaricus bisporus* is isovaleric acid which is produced by living mycelium of this species; isovaleric acid becomes incorporated into the basidiospores prior to germination and acts by increasing the reaction rate in a metabolic chain to a level sufficiently high for activation and subsequent germination. Isovaleric acid is produced not only by mycelium of this species, but also by several other fungi, including yeasts, and may be of relatively widespread occurrence. In contrast, Fries (1979, 1981) has demonstrated that the germination-inducing factor in *Leccinum* spp. is species- and species group-specific; moreover, a distinct "homing" reaction occurs between compatible hyphae and germ tubes. In order to explain why spores should be induced to germinate in habitats where the species, or indeed, species-group is already present, Fries (1981) proposed that fusion of hyphae with germ tubes from spores could lead to transfer of nuclei and dikaryotisation of the developing spore mycelium. There is also evidence that transfer of nuclei from spores to already dikaryotic mycelia may occur; incorporation of new nuclei would enable the fungi to vary their degree of heterokaryosis thereby increasing adaptability to the environment (Fries, 1981). It is not known if mycorrhizal fungi in general colonise seedlings from homokaryotic spore mycelia or from dikaryotic spore mycelia resulting from fused hyphae or germ tubes of spores. Of
interest in this respect, Rayner and Todd (1977) reported that the invasive power of monokaryotic mycelia from spores of some wood decaying fungi is considerably less than that of dikaryotic mycelia. It is thus possible that spores of "late-stage" sheathing mycorrhizal fungi such as Leccinum spp. may germinate at too low a percentage for adjacent germ tubes to anastomose, if dikaryotisation is a prerequisite for mycorrhizal development. Attempts at pure-culture syntheses of mycorrhizas from spore inocula are needed to investigate more fully the behaviour of spores.

Explanations of why "late-stage" mycorrhizal fungi fail to establish on birch seedlings are complicated by the lack of information on the initial viability of the spore inocula. Fries (1983) emphasised the marked variability in the germination requirements and viability of different spore collections within species such as L. laccata and Suillus variegatus; it is improbable, however, that non-viability was responsible for the consistent failure of "late-stage" inocula in my work because similar failure occurred in experiments involving mycelial inocula produced in pure culture and added to soil (Section 3.11) and when seedlings were grown in soil samples collected from beneath fruitbodies of "late-stage" mycorrhizal fungi (Section 3.12). Nevertheless, it is notable that in the experiment involving basidiospore inocula from an unusually early autumnal flush of fruitbodies in 1982 (Section 3.3.3), the levels of infection by both "early-stage" and "resident" mycorrhizal fungi were exceptionally low; this may have been related to low spore viability and, in the case of "resident" fungi, to low levels of air-borne or soil-borne inocula.

In summary of the work described in this section, Table 3.22 records the outcome of studies involving spore inocula. Clearly, only
a restricted range of species - those in the four genera, *Hebeloma*, *Inocybe*, *Laccaria* and *Paxillus* - were able readily to initiate mycorrhizal development from spores, and in one of these cases, namely *P. involutus*, there was strong evidence that this was soil-dependent. Spores of several other fungi - for example, species of *Lactarius*, *Leccinum* and *Russula* - did not establish mycorrhizas from spores even if the seedlings were incubated for extended times, up to 30 weeks, and even if the spores had been stored in soil at 18°C or in outdoor conditions for up to 10 months.

It is suggested that the simple methodology used in this study could have important implications for studying the ecology and behaviour of sheathing mycorrhizal fungi. For example, *Inocybe* spp. have not yet been grown in axenic culture (Chu-Chou, 1979; P.A. Mason, pers. comm.), but spore-supplementation of soil or vermiculite-peat may now provide a useful means of establishing these fungi for experimental purposes, especially as successful establishment of *Inocybe* spp. seems to preclude the development of mycorrhizas of other types. More generally, the work on supplementation of soils has shown a clear distinction between mycorrhizal fungi, in parallel with the distinction between "early-stage" and "late-stage" types as proposed by Deacon *et al.* (1983), and further evidence in support of this distinction was provided by the experiment in Section 3.12, in which seedlings were grown in samples of coal spoil taken from directly beneath fruitbodies.

The fact that "late-stage" fungi do not establish mycorrhizas in seedlings from either spores or mycorrhizal inoculum added to soil, and yet these fungi can readily establish mycorrhizas on seedlings in bixenic conditions, suggests that soil microorganisms limit their development on seedling roots. In this respect there is a clear
distinction between "early-stage" and "late-stage" mycorrhizal fungi - in establishment from dispersed inoculum in soil. It may, nevertheless, be expected that "late-stage" fungi will eventually infect - perhaps by chance or because of an age-related change in the host or the rooting environment. Such eventual establishment would, perhaps, limit the further development of "early-stage" fungi on the root systems, either by totally excluding "early-stage" types or by confining these to an outer, peripheral zone of the root system, as has been observed in the successional studies at Edinburgh (Ford et al., 1980; Deacon et al., 1983; Mason et al., 1983a).

Part of the work in this section has been published as a scientific paper which is appended.
TABLE 3.23: Summary of results from experiments in which birch seedlings were grown in unsterile rooting media supplemented with spores or mycelial inocula of sheathing mycorrhizal fungi; observations made after 12-16 weeks incubation in a growth room at 20°C.

<table>
<thead>
<tr>
<th>Fungi able to form sheathing mycorrhizas</th>
<th>Fungi unable to form sheathing mycorrhizas</th>
</tr>
</thead>
<tbody>
<tr>
<td>a* Hebeloma crustuliniforme</td>
<td>f* Amanita muscaria</td>
</tr>
<tr>
<td>a H. leucosarx</td>
<td>b Cortinarius bulbosus</td>
</tr>
<tr>
<td>ae H. sacchariolens</td>
<td>a C. delibutus</td>
</tr>
<tr>
<td>abd Inocybe geophylla</td>
<td>a Elaphomyces muricatus</td>
</tr>
<tr>
<td>abf I. lacera</td>
<td>a Lactarius blennius</td>
</tr>
<tr>
<td>a I. lanuginella</td>
<td>ae L. pubescens</td>
</tr>
<tr>
<td>ab Laccaria proxima</td>
<td>a L. rufus</td>
</tr>
<tr>
<td>a L. tortilis</td>
<td>bd L. spinulosus</td>
</tr>
<tr>
<td>cf Paxillus involutus</td>
<td>ac L. turpis</td>
</tr>
<tr>
<td></td>
<td>a L. vietus</td>
</tr>
<tr>
<td></td>
<td>aef Leccinum roseofracta</td>
</tr>
<tr>
<td></td>
<td>ac L. scabrum</td>
</tr>
<tr>
<td></td>
<td>b Russula cyanoxantha</td>
</tr>
<tr>
<td></td>
<td>a R. grisea</td>
</tr>
<tr>
<td></td>
<td>af Scleroderma citrinum</td>
</tr>
<tr>
<td></td>
<td>a Suillus luteus</td>
</tr>
</tbody>
</table>

* Conditions of experiments:

a, spores in brown earth;  
d, crushed fruitbodies in brown earth;  
b, spores in vermiculite-peat;  
e, mycelial inoculum in brown earth;  
c, spores in coal spoil;  
f, coal spoil samples from beneath fruitbodies.
PLATES 3.1 - 3.2: Establishment of sheathing mycorrhizas on birch seedlings from basidiospore inoculum

PLATE 3.1: Seedling of *Betula pendula* (14 weeks old) supporting a fruitbody of *Inocybe lacera* in unsterile brown earth supplemented with freshly collected spores of *I. lacera*.

PLATE 3.2: Part of a root system of *B. pendula* (14 weeks old) mycorrhizal with *Inocybe* sp.
SECTION IV

The Structure and Role of Sclerotia of Sheathing Mycorrhizal Fungi
4.1 Introduction

Sclerotia of root-inhabiting fungi are aggregates of modified vegetative hyphae that function either as temporary resting structures or as resistant survival propagules and which eventually germinate, normally by renewed hyphal growth, to initiate infection of new hosts (Garrett, 1970; Willetts, 1972). Three main types have been distinguished according to their ontogeny and the structure of mature sclerotia (Townsend and Willetts, 1954). *Rhizoctonia solani* Kuhn, the cause of damping off of seedlings and black scurf of potatoes, forms loosely aggregated sclerotia through the localised irregular branching and increased septation of adjacent cells; the component hyphae remain filamentous and do not differentiate an outer rind layer. In a second type, shown, for example, by *Sclerotinia gladioli* Drayt and *Phymatotrichopsis omnivorum* (Duggar) Henneb., sclerotium initials form by the production of numerous side branches along one or more parallel hyphae; multiple septation and adhesion follow and the tissue becomes differentiated into two or more layers of pseudoparenchyma and prosenchyma. Characteristic of these "lateral-type" sclerotia, growth continues after differentiation of a narrow outer rind and consequently the cells of the outer layers become stretched tangentially. "Terminal-type" sclerotia, e.g. of *Botrytis allii* Munn and *Sclerotium cepivorum* Berk, develop through a well defined pattern of dichotomous branching at tips of hyphae; growth of the internal tissue layers stops when a thickened rind becomes differentiated.

Nutrients are translocated to, and stored in, cells of developing sclerotia (Willetts, 1972). Major storage compounds are mainly insoluble and soluble carbohydrates, of which glycogen, mannitol and trehalose are of widespread occurrence; lipids may also provide important reserve materials (Coley-Smith and Cooke, 1971).
For sheathing mycorrhizal fungi which fruit rarely, if at all, and have limited saprophytic ability in soil (Marks and Foster, 1967; Lamb and Richards, 1974a,b,c), specialised survival structures such as sclerotia may play a significant role as sources of inoculum and as a means of survival in the absence of suitable hosts. However, little is known of the role of survival propagules of mycorrhizal fungi and even the production of sclerotia by sheathing mycorrhizal fungi has been reported for only a restricted range of species, namely *Cenococcum geophilum* (Mikola, 1948; Trappe, 1962, 1964, 1969; Shaw and Sidle, 1982), *Boletus porosporus* (Imler) Watling (Giltrap, 1979), *Paxillus involutus* (Laiho, 1970; Giltrap, 1979; Molina, 1981; Piché and Fortin, 1982), *Pisolithus tinctorius* (Dennis, 1980; Janerette, 1981; Piché and Fortin, 1982) and *Hebeloma crustuliniforme* (Zak, 1973; Froidevaux and Källin, 1981).

Mycorrhizas of *Hebeloma sacchariolens* formed artificially, or occurring naturally on birch in the Edinburgh area are frequently associated with globose sclerotium-like bodies; to my knowledge, these structures have not been reported elsewhere in the literature, although J. Garbaye (pers. comm.) has found "gall-like" aggregates of mycelium associated with mycorrhizas of *H. sacchariolens* on *Quercus* spp.

The present study was done to compare the sclerotium-like bodies of *H. sacchariolens* on birch with sclerotia produced in association with sheathing mycorrhizas by *P. involutus* and *C. geophilum*. In this section, the term "sclerotia" has been applied to the sclerotium-like bodies of *H. sacchariolens*; this has been done for convenience although, as discussed in Section 4.9, they differ in some respects from the sclerotia of some other root-infecting fungi described by Townsend and Willetts (1954), and only preliminary studies have been done to assess their survival in soil and their role as infective propagules.
4.2 Materials and Methods

Sheathing mycorrhizas of *Hebeloma sacchariolens* were synthesised on *Betula pendula* under bixenic conditions (Section 2.2.2) and, after 8 weeks growth, seedlings with good mycorrhizal development were transferred to 7 cm³ plant pots containing an unsterile brown earth collected from Castlelaw, a tree-less site, south of Edinburgh (Section 2.1.5). After 4 weeks growth under glasshouse conditions (Section 2.2.3.1), large numbers of sclerotium-like bodies were associated with the mycorrhizas of *H. sacchariolens*; they persisted on the roots for the following 12 months and, unless otherwise stated, were used for all investigations.

Using the same method, sclerotia associated with sheathing mycorrhizas of *Paxillus involutus* were produced on birch seedlings though in relatively smaller quantities; however, in the brown earth used, *P. involutus* was largely replaced by other mycorrhizal fungi over a 6-month growth period, so sclerotia from this source were supplemented with sclerotia produced in association with seedlings grown in soil supplemented with basidiospores of *P. involutus* (Section 3.2) and, also, with sclerotia harvested from the root zone of naturally regenerating birch at Newtongrange, a disused coal spoil tip, south of Edinburgh (Section 2.1.6.1).

Sclerotia of *Cenococcum geophilum* were collected from mycorrhizal roots of mature *Pinus* spp. at Glentress forest, Peebleshire but, because of a shortage of readily available material, sclerotia of this species were studied solely by scanning electron microscopy.

Sclerotia of *H. sacchariolens* and *P. involutus* were dissected from host roots and treated in the following ways; in most cases, freshly harvested sclerotia were compared with material that had previously been air-dried for 24 h.
1. Sclerotia of *H. sacchariolens* and *P. involutus* were freshly harvested from mature trees or from pot-culture syntheses and prepared for light microscopy, scanning electron microscopy and transmission electron microscopy; details of the procedures are given in Sections 2.2.5 and 2.2.6.

2. Samples of sclerotia of *H. sacchariolens* and *P. involutus* were transferred to 4 cm² squares of nylon gauze which were tied into sacs and then buried in 5 cm³ plant pots containing a moist unsterile brown earth, previously collected from Castlelaw (Section 2.1.5). Replicate pots containing freshly harvested or air-dried sclerotia were distributed, in a randomised layout, in a growth room and incubated at 18°C for a maximum of 10 months, each pot being watered lightly with tap water at 5 day intervals. Sclerotia of *P. involutus* were retrieved from soil after 16 weeks, and those of *H. sacchariolens* after 16 and 40 weeks incubation. Some of the samples were prepared for light microscopy or electron microscopy as in (1) above; the remainder were treated as in (3) below.

3. Sclerotia treated as in (2) above were retrieved from replicate pots and pooled; they were then transferred, in approximately equal numbers, to 2 x 1 cm plastic tubes (Section 2.2.2) which had been inserted into the centre of 5 cm³ plant pots containing the same unsterile brown earth as that used previously. The tubes, slit along one side, had an internal diameter of 13 mm. The sclerotia were placed on the soil surface in the tubes approximately 1.5 cm beneath the soil surface in the pots; further soil was then added to bring the soils to the same level and then two, 3-week old
aseptically pre-germinated seedlings of *B. pendula* were planted into the centre of each tube. The seedlings were thinned to one per pot after 6 weeks growth. The pots were randomised with an unsupplemented control series in a growth room; after 12-16 weeks growth at 18°C, the seedlings were removed from the tubes and assessed for mycorrhizal development as described previously (Section 2.2.4).
4.3 Distribution of Sclerotia and Mycorrhizas of *Hebeloma sacchariolens* on 16-week old Birch Seedlings Grown Under Glasshouse Conditions

After 16 weeks growth in brown earth (Section 4.2) seedling roots of *Betula pendula* mycorrhizal with *Hebeloma sacchariolens* were associated with large numbers of white sclerotium-like bodies (Plates 4.1 - 4.3), unique amongst *Hebeloma* species and, indeed, amongst all sheathing mycorrhizal fungi. These "sclerotia" were usually pure white or occasionally yellowish, occurring either as clusters on mycorrhizas or individually on subtending secondarily thickened roots (Plates 4.2, 4.3). They ranged in size from 100 μm to 400 μm diameter, sometimes forming irregularly shaped aggregates up to 800 μm diameter. They were readily discernible with the naked eye because of their usually white, glistening appearance, but in some instances the clusters of sclerotia were obscured by adherence of soil particles to nearby wefts of radiating hyphae.

In order to determine the distributional pattern of sclerotia in relation to that of mycorrhizas of *H. sacchariolens*, six replicate birch seedlings were harvested from pots of soil in which mycorrhizas of *H. sacchariolens* had been established 8 weeks earlier (Section 4.2). Root systems of each seedling were washed free of adhering soil; they were then placed linearly onto a grid and cut into 1 cm lengths. Total numbers of mycorrhizal and uninfected roots and the numbers of sclerotia were recorded in alternate 1 cm portions.

The greatest concentration of sclerotia, as of *Hebeloma* mycorrhizas, occurred in or near the oldest part of the root system, 4-5 cm from the root/hypocotyl junction (Figure 4.1). In fact, in this region and at 7-8 cm down the root systems, the mean number of sclerotia was almost identical to the mean number of *Hebeloma* mycorrhizas, though
examination of the roots showed that there was not a close one-to-one relationship of these structures - in other words the sclerotia were not uniformly distributed, one per mycorrhizal root tip. In contrast to the results for the 4-5 cm and 7-8 cm regions, the number of sclerotia fell rapidly with distance from the root/hypocotyl junction, so that they were virtually absent from the regions 13-14 cm and 16-17 cm down the roots. Yet, mycorrhizas attributable to *Hebeloma* still accounted for 35% of the total root tips in the younger regions, no other mycorrhizal types being present.

From these results it is clear that the sclerotia of *H. sacchariolens* are formed as recognisable structures only after the mycorrhizas themselves have developed; nevertheless, the *Hebeloma* mycorrhizas in the regions of maximum sclerotial numbers still appeared healthy and active.

The mycorrhizas of *H. crustuliniforme* on birch are markedly different in appearance compared to those of *H. sacchariolens* and typically have dense wefts of radiating hyphae covering their outer surfaces in which angular structures are often embedded (Plate 4.4). These angular bodies were originally regarded as sclerotia by Zak (1973) but more recently, Froidevaux and Kälin (1981) have shown them to be crystals of calcium oxalate. It seems, therefore, that *H. sacchariolens* is the only species in this genus to form sclerotium-like structures.
Distance down roots from which 1 cm lengths were taken

FIGURE 4.1: Mean numbers of sclerotia and percentage of root tips mycorrhizal with *Hebeloma sacchariolens* in 1 cm root lengths of seedlings of *Betula pendula* grown in pots of unsterile soil: histograms represent mean per cent mycorrhizal root tips (the mean numbers of *Hebeloma* mycorrhizas also being stated); line graph shows mean number of sclerotia per 1 cm root length.
4.4 Histochemical Analysis of the Sclerotia of Some Sheathing Mycorrhizal Fungi

When sclerotia of *Hebeloma sacchariolens* were crushed in water they released globules of an oily material, so histochemical tests were done in order to investigate this and other potential energy storage compounds in the sclerotia. As discussed by Bullock *et al.* (1980), considerable energy reserves are utilised during the germination of sclerotia of most root-inhabiting fungi, the energy storage reserves being either insoluble or soluble carbohydrates or lipids (Coley-Smith and Cooke, 1971). For comparison with *H. sacchariolens* in the present work, sclerotia of *Paxillus involutus* and of the plant pathogens *Rhizoctonia solani, Sclerotinia sclerotiorum* (Lib.) de Bary and *Claviceps purpurea* (Fr.) Tul. were used.

**Sources of material**

Sclerotia of two isolates of *R. solani* and one of *S. sclerotiorum* were harvested from 30 to 40-day old colonies grown on potato dextrose agar. Ergots of *C. purpurea* were removed from the flowering heads of *Dactylis glomerata* L. Sclerotia of *P. involutus* were obtained from roots of 18-week old birch seedlings grown in unsterile brown earth under glasshouse conditions (Section 4.2) and, also, from roots of mature birch growing on coal spoil at Newtongrange (Section 4.2). In addition, some sclerotia of *P. involutus* were retrieved from nylon gauze sacs that had been buried in unsterile soil for 16 weeks (Section 4.2); these buried sclerotia had not been air-dried prior to burial. Sclerotia of *H. sacchariolens* were dissected from 18-week old and 18-month old birch grown under the same glasshouse conditions as were the seedlings mycorrhizal with *P. involutus*. In addition, samples of sclerotia of *H. sacchariolens* were retrieved from nylon gauze sacs that had been
buried in unsterile soil for 4 and 16 weeks, some of these sclerotia having been air-dried for 24 h prior to burial.

Staining procedures

Lugol’s iodine (Gurr, 1956). Sclerotia harvested from different sources were either crushed on glass microscope slides or hand-cut into sections in 70% ethanol and then stained in Lugol’s iodine (1 g iodine and 2 g potassium iodide in 100 ml water). A mahogany-red reaction is obtained when large quantities of glycogen are present; traces of glycogen stain pale brown.

Sudan Black B and Sudan IV (Jensen, 1962). Fresh samples were crushed on glass microscope slides in 50% ethanol and left for 2–3 minutes. They were then stained for 5–10 minutes in either a saturated solution of Sudan Black B in 70% ethanol or a saturated solution of Sudan IV in 70% ethanol and then differentiated in 50% ethanol for 1 minute. Sudan IV stains neutral lipids (fats, oils and waxes); in addition to these, Sudan Black B stains phospholipids and unsaturated fatty acids.

Nile blue (Jensen, 1962; Jennings and Watkinson, 1982). Freshly harvested sclerotia or samples of sclerotia that had been buried in unsterile soil were crushed on glass microscope slides in 1% aqueous nile blue. The oxazone dye component of the stain reacts with neutral lipids which stain red.

Osmium tetroxide (Jensen, 1962). Freshly harvested sclerotia and sclerotia retrieved after burial in unsterile soil were immersed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer and the colour changes of the solution were recorded at one-hourly intervals over a
period of 5 h. A black compound of osmium is formed by the oxidation of double bonds present in unsaturated fatty acids; consequently osmium tetroxide reacts with lipid materials, such as phospholipids that have a relatively large number of double bonds. Because osmium tetroxide reacts with other cell constituents, some sclerotia were first treated with pyridine to remove all lipids before staining and these sclerotia were used as controls. For lipid extraction (Jensen, 1962), fresh sclerotia were placed for 30 minutes in pyridine at room temperature; they were then transferred to fresh pyridine for 24 h at 60°C and finally washed for 2 h in running water.

*Peracetic acid – Schiff's reagent* (Jensen, 1962). Sections from freshly harvested sclerotia were placed in 40% peracetic acid for 2-5 minutes, washed in running water for 5 minutes and then placed in Schiff's reagent for 30 minutes. They were finally washed in running water for 10 minutes. Peracetic acid oxidises double bonds present in unsaturated fatty acids, resulting in the formation of an aldehyde which stains with the Schiff's reagent. Biomine, which blocks the double bonds rendering the peracetic acid ineffective, was also added to some sclerotia. For this, fresh sections were placed in bromine (1 ml bromine in 39 ml carbon tetrachloride) for 1 h and then stained as described above.

*Staining reactions observed*

With the exception of *C. purpurea*, the sclerotia of all the fungi tested reacted positively with Lugol's iodine thus indicating the presence of glycogen; the reaction was intense in freshly collected sclerotia of *P. involutus, S. sclerotiorum* and both isolates of *R. solani*, but only a slight reaction was obtained with crushed sclerotia of *H. sacchariolens*
Differences in the intensity of the staining reaction were apparent in both sclerotia and mycorrhizas of *P. involutus* taken from different parts of the root system of 18-week old birch seedlings. In the root zone 1-2 cm from the root/hypocotyl junction, mycorrhizas attributable to *Paxillus* appeared moribund and had become replaced by other mycorrhizal types; however, sclerotia of *P. involutus* were still present in this region and they reacted strongly with Lugol's iodine. In thin, hand-cut sections through sclerotia of *P. involutus*, the glycogen deposits were seen to be localised, tending to be concentrated in groups of cells in the inner zone of the sclerotia but absent from the outer layers. In the 7-8 cm zone down the root systems, sclerotia stained intensely though the mycorrhizas that were present did not react with the stain. At 13-14 cm down the roots, the mycorrhizas appeared more healthy than in the 7-8 cm root zone and they also stained more intensely; similarly, sclerotia from the 13-14 cm root zone stained intensely. Sclerotia buried for 16 weeks reacted weakly with the stain and, in some cases, failed to react; this suggests that only traces of glycogen were still present. Samples of sclerotia of *P. involutus* harvested from roots of naturally regenerating birch at Newtonrange (Section 2.1.6.1) in January 1982 varied in their staining reactions; some appeared to contain large quantities of glycogen, as evidenced by a positive reaction with Lugol's iodine, whereas others showed no reaction.

Hand-cut sections of sclerotia of *R. solani* and *S. sclerotiorum* showed moderate to strong reactions with Lugol's iodine (Table 4.1), but, as with sclerotia of *P. involutus* harvested from 18-week old birch seedlings, glycogen deposits apparently were concentrated in some parts of the internal tissue but were notably absent from the peripheral outer layers.
Sclerotia of *H. sacchariolens* freshly harvested from 18-week old and 18-month old birch reacted weakly with Lugol's iodine (Table 4.1) indicating the presence of traces of glycogen only, though sclerotia taken from different parts of the root systems differed in the intensity of the staining reaction. Thus, sclerotia of *H. sacchariolens* did not react with Lugol's iodine in the root zone 1-2 cm from the root/hypocotyl junction; the staining reaction was intense 7-8 cm down the roots, but it was weak in the youngest part of the root system, 10-11 cm down the roots. Mycorrhizas from all root regions failed to react with the stain.

Hand-cut sections through sclerotia of *H. sacchariolens* taken from 7-8 cm down the roots of 18-week old and 18-month old birch again showed a scattered distribution of glycogen deposits, but, in contrast to sclerotia of *P. involutus*, *R. solani* and *S. sclerotiorum*, the glycogen deposits occurred in a region midway between the centre and the periphery of the sclerotia. Traces of glycogen were still present in sclerotia that had been buried in unsterile soil for 4 to 16 weeks.

Sclerotia of *P. involutus* and *S. sclerotiorum* did not react with any of the lipid stains used (Table 4.2) and there was only a very weak reaction with one of the lipid stains in the case of sclerotia of *R. solani* (Table 4.2). In contrast, lipids were evidently abundant in sclerotia of *H. sacchariolens* and *C. purpurea*; neutral lipids seemed predominant although the positive reaction of sclerotia of *H. sacchariolens* with Peracetic acid - Schiff's reagent indicated that other lipid materials were also present. Osmium tetroxide solution turned purple-black within 2 to 3 h on the addition of either freshly harvested sclerotia of *H. sacchariolens* or samples of these sclerotia previously buried for 4 to 16 weeks without prior air-drying (Table 4.2);
however, there was no colour change on the addition of sclerotial material that had been air-dried prior to burial for 16 weeks. The addition of freshly harvested or buried sclerotia of *P. involutus* did not result in a colour change of the osmium-tetroxide solution; interestingly, however, a moderate colour change (pink-purple was observed when mycelial strands were left attached to the sclerotia of *P. involutus* (Table 4.2). The reaction with osmium tetroxide suggests that phospholipids or other unsaturated fatty acids were present in sclerotia of *H. sacchariolens*, and possibly in mycelial strands (but not in sclerotia) of *P. involutus*. However, despite the negative reaction with osmium tetroxide in the control series (Table 4.2) in which sclerotia of *H. sacchariolens* were treated with pyridine prior to staining, the results need to be interpreted with caution because, with time, osmium tetroxide may also react with other cell constituents (Jensen, 1962).

In summary, a distinction can be made between the different sclerotia tested, based on the nature of the main storage compounds: whereas glycogen appears to be predominant in sclerotia of *P. involutus*, *R. solani* and *S. sclerotiorum*, lipid compounds are the major constituents in sclerotia of *H. sacchariolens* and *C. purpurea* (Table 4.2). The concentration of glycogen in sclerotia of *P. involutus* was reduced after burial for 16 weeks whereas the same burial treatment apparently did not affect the content level of lipid contained within sclerotia of *H. sacchariolens* except in those samples that had been air-dried for 24 h prior to burial (Table 4.2). Glycogen deposits in sclerotia of *P. involutus*, *R. solani* and *S. sclerotiorum* were apparently concentrated in some parts of the internal tissue and were notably absent from the peripheral layers; in contrast, only traces of glycogen were seen in
sclerotia of *H. sacchariolens*, where they were localised near the periphery and appeared to be absent from the central regions.
TABLE 4.1: Histochemical analysis of the main energy storage materials present in freshly harvested sclerotia associated with some sheathing mycorrhizal fungi and in sclerotia of some plant pathogens.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Specificity of stain</th>
<th>Rhizoctonia solani</th>
<th>Sclerotinia sclerotiorum</th>
<th>Claviceps purpurea</th>
<th>Hebeloma sacchariolens</th>
<th>Paxillus involutus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sudan Black B</td>
<td>All classes of lipid</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Sudan IV</td>
<td>Neutral lipids</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Nile blue</td>
<td>Neutral lipids</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Peracetic acid-Schiff's reagent</td>
<td>Unsaturated fatty acids</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Lugol's iodine</td>
<td>Glycogen</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

* 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction; 4, intense reaction; nt, not tested.
TABLE 4.2: Colour changes in a solution of osmium tetroxide in 0.1M sodium cacodylate buffer after the addition of sclerotia of *Hebeloma sacchariolens* and *Paxillus involutus*.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Approximate age of birch from which sclerotia were harvested</th>
<th>Treatment of sclerotia before staining</th>
<th>Time of testing (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. sacchariolens</em></td>
<td>Young&lt;sup&gt;1&lt;/sup&gt;</td>
<td>None (freshly harvested)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>nil</td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>Buried for 4 weeks</td>
<td>nil</td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>Buried for 16 weeks</td>
<td>nil</td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>Air-dried for 24 h and then buried for 16 weeks</td>
<td>nil</td>
</tr>
<tr>
<td><em>P. involutus</em></td>
<td>Young</td>
<td>None (freshly harvested)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>nil</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>None (freshly harvested)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>nil</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>None (freshly harvested) with attached mycelial strands</td>
<td>nil</td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>Buried for 16 weeks</td>
<td>nil</td>
</tr>
</tbody>
</table>

<sup>1</sup> Young, 18-week old birch; Mature, naturally regenerating birch approximately 15-20 years old growing on coal spoil at Newtongrange.

<sup>2</sup> No colour changes were observed after the addition of sclerotia treated with pyridine.
4.5 Structure of Sclerotia Associated with Sheathing Mycorrhizas of *Hebeloma sacchariolens* on Birch

Transverse sections through frozen or resin-embedded sclerotia of *Hebeloma sacchariolens* freshly harvested from 18-week old birch seedlings and examined with the light microscope indicated that, structurally, the sclerotia are almost exclusively composed of tightly packed pseudoparenchyma (Plate 4.5) surrounded by a narrow zone, 6 to 7 layers deep, composed of radially flattened cells (Plates, 4.5, 4.6). The cells comprising the narrow outer zone were of similar dimensions to vegetative hyphae of *H. sacchariolens* (1.5 - 2.0 μm) and appeared to be without contents. In contrast, the cells of the pseudoparenchyma were large and often angular in shape with distinctive cell walls; these cells were evidently filled with polygonal bodies just discernible by light microscopy (Plate 4.6).

The ultrastructure of sclerotia of *H. sacchariolens*, as seen with the S.E.M. and T.E.M., corresponded closely with the structure of sclerotia of this species observed with the light microscope. Sclerotia freshly harvested from 18-week old and 18-month old birch and cracked open after critical point drying showed, under S.E.M., the differentiation into an inner and an outer zone (Plate 4.7); in some specimens the external surface of the sclerotia appeared smooth as seen, for example, in Plate 4.7, arrow, whereas in others the sclerotia were covered with radiating hyphae (Plate 4.8).

The central zone was conspicuous, being composed of large, irregularly shaped cells with highly thickened cell-walls and filled with osmiophilic material in the form of discrete, polygonal bodies embedded in an electron-dense granular matrix (Plate 4.9); this osmiophilic material resembles lipid as described in other ultrastructural studies.
(Bullock et al., 1980; Nemec, 1981). Histochemical stains (Section 4.4) also suggest that the sclerotia of *H. sacchariolens* are filled with neutral lipids, although chromatographical analysis is needed to confirm the exact nature of the lipid material. In the absence of tilting facilities on the T.E.M., it could not be determined if the lipid bodies were membrane-bound, though in some instances (Plate 4.10, arrows), the junctions of the lipid bodies appeared to have a different staining property from that internally.

Examination of freshly harvested sclerotia by S.E.M. again showed lipid-like globular structures in most of the cells of the inner zone (Plates 4.11, 4.12, 4.13). In some cells the lipid material was apparently fused into a single large mass (Plate 4.12, arrow), whereas in other cells the globules were individual discrete bodies (Plate 4.11) or appeared to be held together in chains (Plate 4.13). In some samples of sclerotia, lipid material was not seen; instead, the intracellular contents resembled more normal cytoplasmic contents (Plate 4.14).

To further examine the nature of the osmiophilic bodies, some sclerotia were treated with pyridine prior to fixation, in order to extract any lipids present (Section 4.4). Except in the most centrally positioned cells, the osmiophilic material was removed by this treatment (Plate 4.15). After extraction, the cells appeared empty or with only traces of residual cytoplasm (Plate 4.15); in some cells, however, and especially in the peripheral layers of the outer zone, electron-dense rosettes resembling glycogen (Kellenberger and Ryter, 1964) were evident.

As seen in cross-section, there was evidence of a radial gradient of cell size (Plate 4.17), the peripheral layers of the pseudoparenchyma having a more clearly marked tangential arrangement and being smaller in diameter than those in the centre which, in many sclerotia, appeared
more randomly arranged (Plate 4.7); representative measurements of the cell sizes are given in Table 4.3.

The cell walls of the pseudoparenchyma were fibrillar (Plate 4.16) and highly thickened compared to the walls of the outer zone (Table 4.3). Frequently, incomplete cross walls were observed (Plate 4.15, arrow 1); the reasons for these incomplete walls are not known but, as shown in Plate 4.15, their rounded edges are not indicative of damage caused during preparation of the sclerotial material.

In contrast to the cells of the pseudoparenchyma, the cells of the outer zone had thin, undifferentiated walls resembling those of normal vegetative hyphae (Plates 4.18, 4.19). A small transitional region was found between the outer and inner zones. The cells in the transitional zone seemingly lacked lipid bodies (Plate 4.19) but had somewhat thickened walls and there was extensive intercellular material (Plate 4.19, arrows); these cells also had electron-dense contents, in contrast to the cells or hyphae of the outer zone (Plate 4.19).

The outer zone itself showed a transition from apparently normal vegetative hyphae at the outer face, seen in cross-section in Plate 4.20, arrow, and in longitudinal section in Plate 4.21, arrow 1, dolipore septa occasionally being seen (Plate 4.21, arrow 2, 4.22), to a prosenchyma or apparently crushed pseudoparenchyma towards the inside. Some of the cells of the outer zone contained glycogen deposits (Plate 4.21) but most cells appeared empty in contrast to those of the pseudoparenchymatous region of the sclerotia (Plate 4.24).

The outer surface of the sclerotia varied in appearance. A closely arranged network of hyphae bearing numerous clamp connections (Plate 4.23, arrow) was evident in some samples; in other samples collapsed hyphae on the sclerotium surface seemed to lie beneath sheets of
membranous material, though this appearance may possibly have been caused by drying (Plate 4.24).

Sclerotia of *H. sacchariolens* are easily detached from host roots, yet they have never been seen to occur independently in the surrounding soil. Sections through sclerotia and mycorrhizas observed with the light microscope indicate that the outer zone of each sclerotium is intimately associated with cells and hyphae of the sheath and Hartig net (Plate 4.25). Interestingly, the outer cortical cell layers of the root were often seen to be absent from the sites of attachment of sclerotia (Plate 4.25). It would have been interesting to examine the areas of apposition of sclerotia and secondarily thickened roots, but unfortunately suitable specimens for this purpose could not be obtained. In summary of the above findings, the proposed structure of the sclerotia of *H. sacchariolens* is shown in Figure 4.2.
TABLE 4.3: Dimensions of the structural components of sclerotia of *Hebeloma sacchariolens*; figures represent the mean dimensions from sections cut from 30 freshly harvested sclerotia from 18-week old birch seedlings examined by S.E.M.

<table>
<thead>
<tr>
<th>Component</th>
<th>Diameter</th>
<th>Thickness/Cell Diameter</th>
<th>Wall Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of sclerotia</td>
<td>360 μm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer zone (flattened cells):</td>
<td></td>
<td>31-40 μm</td>
<td>0.2 μm</td>
</tr>
<tr>
<td>thickness</td>
<td></td>
<td>3-4 μm</td>
<td></td>
</tr>
<tr>
<td>cell diameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner zone (pseudoparenchyma):</td>
<td></td>
<td>240-280 μm</td>
<td></td>
</tr>
<tr>
<td>radius</td>
<td></td>
<td>11 μm</td>
<td>2.0 μm</td>
</tr>
<tr>
<td>cell diameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell diameter (outermost cells)</td>
<td></td>
<td>8-9 μm</td>
<td>1.7-1.8 μm</td>
</tr>
</tbody>
</table>
FIGURE 4.2: Diagrammatic interpretation of the structure of sclerotia of *Hebeloma sacchariolens*, based on studies by light and electron microscopy.

1. Inner zone  Pseudoparenchyma with radial differentiation, comprising cells with thick walls and containing lipid globules.

2. Outer zone  Flattened collapsed pseudoparenchyma overlaid by hyphae of normal appearance.

   (A small transitional zone occurs between (1) and (2) above; it seems to lack lipid globules but has partially thickened walls and contains glycogen.)

3. Zone of attachment to root  The outer sclerotial zone is intimately associated with, and undistinguishable from, the cells comprising the Hartig net.
4.6 Structure of Sclerotia Associated with Sheathing Mycorrhizas of *Paxillus involutus* on Birch

Sheathing mycorrhizas of *Paxillus involutus* on birch are frequently associated with sclerotia that are borne laterally, and seemingly at random, along well differentiated mycelial strands; sometimes the sclerotia occur attached to mycelial strands several centimetres away from the host roots. Unlike the sclerotia of *H. sacchariolens*, those of *P. involutus* are rarely, if ever, seen attached directly to mycorrhizas.

The small (100-300 μm diameter), pale buff to golden-brown sclerotia are produced during the early stages of mycorrhizal formation on 8 to 9-week old birch seedlings inoculated with *P. involutus*; larger (200-800 μm diameter), deeply pigmented sclerotia were frequently associated with mycorrhizas of *P. involutus* on naturally regenerating birch on coal spoil at Newtongtranget (Section 2.1.6.1). Nevertheless, sclerotia were not consistently produced by *P. involutus*, because at some sampling points at Newtonrange there was an abundance of *Paxillus* mycorrhizas that were not associated with sclerotia. No obvious reason for this variation could be seen.

Although no detailed study was made of the developmental morphology of sclerotia on pot-grown seedlings, the early stages of sclerotium production were seen as spherical, hollow "shell-like" aggregates of hyphae originating from localised regions of mycelial strands and evidently involving a proliferation of some of the outer investing hyphae of the strands. In later stages of sclerotium formation, the hyphal aggregates become solid, with apparent infilling of the central region and tight interweaving of the hyphae; such structures when crushed in water, released a milky fluid but there was no evidence of lipid globules as in the case of *H. sacchariolens* (Section 4.5).
Fully differentiated sclerotia varied in size; the degree of pigmentation and the loss of external radiating hyphae seemed to be more indicative of age and maturation than was size as such. Thus, young sclerotia bore, on their surface, a radiating mass of hyphae of about the same diameter (2.4 μm) as those comprising the outer layers of the mycelial strands (Plates 4.26, 4.30). Later, the surface of the sclerotium was seen to have a consolidated labyrinthine appearance, the surface hyphae being contorted and irregularly swollen (Plates 4.27, 4.28). This seemed to represent an intermediate stage of development, because in mature sclerotia (evidenced by a deep brown colour) the surface patterns seen in Plate 4.28, were obscured; the surface then appeared smooth but encrusted with hyphal debris and squamulose material (Plate 4.29).

Young sclerotia of *P. involutus* had, in some respects, a similar structural differentiation of tissue to that of sclerotia of *H. sacchariolens*, being composed almost exclusively of pseudoparenchyma surrounded by a narrow but clearly demarcated outer zone (Plates 4.30, 4.31). In section, the outer zone had an obviously hyphal origin (Plate 4.32) but the hyphae and tissue that they comprised seemed empty of contents (Plate 4.32). In mature sclerotia, the outer zone was clearly compacted and consisted of crushed and partially fused cells (Plates 4.33, 4.34), the cells of which were sometimes seen as parallel lamellae (Plate 4.33, arrow). The crushed hyphae comprising this zone were, in some cases, continuous with radiating hyphae (Plates 4.33, 4.34), some of which bore clamp connections (Plate 4.33). The evidently crushed and collapsed appearance of the outer zone is consistent with the fact that sclerotia of *P. involutus* are of the "lateral-type" in the sense of Townsend and Willetts (1954), because a developmental feature of this sclerotial type is its continued expansion after the outer "rind" has been differentiated (Willetts, 1972).
The internal pseudoparenchymatous zone comprised wide, irregularly shaped cells of about 7-8 μm diameter (Plates 4.35, 4.36); the cell walls were apparently unthickened and the convoluted outline of many of the cells suggested that the walls were not structurally rigid (Plate 4.36). The cell contents occasionally appeared amorphous (Plate 4.35, arrow), but more frequently granular with a distinctive aereolate appearance (Plates 4.31, 4.35, 4.36). Abundant spherical bodies, 0.2-0.3 μm diameter, resembling oil globules (Plate 4.37) were released from the cytoplasm of cells of young sclerotia harvested from 18-week old seedlings but were absent from cells of older sclerotia. Of interest, the contents of some cells of the pseudoparenchyma bore neck-like protuberances (Plate 4.35, arrow), perhaps indicating that the cells bore, in some cases, extended arms. If so, then the cells might have been tightly packed together, with few, if any, intercellular spaces. As seen with the T.E.M. (Plate 4.38), the cells of the pseudoparenchyma seemed often to lack normal cytoplasmic contents but had, instead, electron-dense deposits resembling glycogen; this is consistent with histochemical tests (Section 4.4).

The cell walls of mature sclerotia of *P. involutus* (Plates 4.36, 4.39) were relatively unthickened in comparison to the walls of the pseudoparenchyma in sclerotia of *H. sacchariolens* (Plate 4.16) but they were lamellate in appearance and adjacent cells were joined to one another with some deposits of electron-dense material that sometimes appeared fibrillar (Plate 4.39). Within the pseudoparenchymatous zone, there was no discernible radial gradation of cell size; large cells (7-8 μm diameter) seemed to be interspersed throughout with much smaller cells (3.0-3.5 μm diameter) as seen in Plates 4.31, 4.35, 4.36, but the smaller "cells" might represent extended arms of some of the larger cells.
(Plate 4.35). In contrast, in *H. sacchariolens* a central region of large cells was surrounded by progressively smaller cells towards the periphery (Plates 4.7, 4.17).

In summary, the proposed structure of sclerotia of *P. involutus* is shown in Figure 4.3.
FIGURE 4.3: Diagrammatic interpretation of the structure of sclerotia of *Paxillus involutus*, based on studies by light and electron microscopy.

1. **Mycelial strand**
   Aggregation of longitudinally orientated hyphae.

2. **Inner zone**
   Pseudoparenchyma with little or no radial differentiation and without obvious lipid content.

3. **Outer zone**
   Prosenchyma of obvious hyphal origin comprising hyphae that lack contents and which, in older sclerotia, are crushed leaving only discernible wall lamellae.

There is little to no evidence of wall thickening in any part of the sclerotium, though some intercellular matrix is evident.
4.7 Structure of Sclerotia Associated with Sheathing Mycorrhizas of *Cenococcum geophilum* on Pine

Sclerotia of *Cenococcum geophilum* were collected from the root zone of mature *Pinus* spp. and examined with the S.E.M. Mycorrhizas and associated sclerotia of this species were not synthesised in pot culture and, because of the unknown ages of samples collected from the field, no attempt was made to examine the ultrastructural changes associated with burial of sclerotia in unsterile soil, as is described later for *Hebeloma sacchariolens* and *Paxillus involutus* (Sections 4.8.2, 4.8.3).

Sclerotia of *C. geophilum* vary considerably in size, ranging from 0.05 to 4.0 mm diameter, and are generally larger than the sclerotia of *H. sacchariolens* and *P. involutus*. They develop laterally on mycelial strands and thus fit the "lateral-type" category of sclerotial development described by Townsend and Willetts (1954), but they are frequently found independently in the soil without any discernible connection with host roots.

Structurally, mature sclerotia are composed almost entirely of pseudoparenchyma and have a discrete solid appearance (Plate 4.40) with few to no hyphae radiating from their outer surface. Of the twenty sclerotia examined, only four had appreciable cellular contents which appeared dense and granular, in some respects, similar to the cytoplasm seen in the pseudoparenchyma of sclerotia of *P. involutus* (Plate 4.35), but always seemingly in a degenerate condition. Trappe (1969) reported that sclerotia of *C. geophilum* are filled with an ethanol-soluble oil though there was no evidence for this in the samples examined.

The sclerotia had a clearly defined rind (Plates 4.41, 4.42) similar to that reported for sclerotia of other root-infecting fungi by Townsend
and Willetts (1954), but internally they showed little further differentiation, all of the internal pseudoparenchyma being equivalent to thick-walled "cortex" as reported for other sclerotia (Townsend and Willetts, 1954).

The component cells of the pseudoparenchyma had a clearly defined radial arrangement (Plates 4.40, 4.41). The cell walls were amorphous in appearance (Plate 4.43) with seeming continuity between cell wall material as such and intercellular matrix material. The rind also, was composed of pseudoparenchyma but with constituent cells of smaller diameter (4-5 μm) than those of the inner zone; in many instances, the lumina of the rind cells were occluded to form a highly consolidated outer tissue (Plate 4.42).

The external surface of the sclerotia was generally rugose in appearance (Plate 4.44) and clearly of hyphal origin, though the course of individual hyphae could not be followed over long distances and the hyphal surfaces seemed to be roughened with squamulose material (Plate 4.44); in some sclerotia, the surface had a honeycomb appearance (Plate 4.45) not dissimilar to the external surface of some sclerotia of *P. involutus* (Plate 4.28).

In summary, the proposed structure of sclerotia of *C. geophilum* is shown in Figure 4.4.
FIGURE 4.4: Diagrammatic interpretation of the structure of sclerotia of Cenococcum geophilum based on studies by light and electron microscopy.

1. Inner zone  Pseudoparenchyma with obvious radial arrangement, comprising cells with apparently thickened walls.

2. Outer zone  Pseudoparenchyma comprising cells with highly thickened walls and occluded lumina, overlaid by narrow zone of prosenchyma and hyphae of normal appearance.
4.8 **Survival of Sclerotia in Soil**

In order to study the survival in soil of sclerotia of some sheathing mycorrhizal fungi, it was desirable to obtain a readily available source of sclerotia of known age and produced under identical conditions. Various attempts were therefore made to induce sclerotium production by *Hebeloma sacchariolens* and *Paxillus involutus* in pure culture. Many environmental and nutritional factors are reported to be involved in the initiation and maturation of sclerotia of root-infecting fungi (Willetts, 1972) and, of these, the sources of carbon and nitrogen and the C:N ratio in the medium may be of critical importance. In a range of preliminary experiments, outlined here, the effects of nitrogen source, and also of mechanical barriers and staled medium, on sclerotium production by *H. sacchariolens* and *Paxillus involutus* were investigated in liquid and on agar culture. Palmer's basic medium (Johnson and Curl, 1972) containing glucose as carbon source and NH₄Cl as nitrogen source (Section 2.1.2) was prepared either as liquid or agar and, in addition, the following modifications to it were used.

1. Glucose was substituted with 5 g malt extract.

2. NH₄Cl was substituted with 0.69 g NaNO₃ to give an equivalent elemental nitrogen content.

3. NH₄Cl was substituted with 1.06 g bacteriological peptone to give an equivalent elemental nitrogen content.

These media, with 2% malt extract (as liquid or agar) and potato dextrose agar (PDA) for comparison, were autoclaved in 250 ml amounts in 500 ml glass bottles at 121°C for 15 minutes. The liquid medium was transferred in 30 ml amounts to 150 ml capacity sterile, straight-sided
plastic jars (Sterilin Ltd); the agar media were transferred, in approximately 10 ml amounts, to 30 ml capacity sterile plastic McCartney bottles and allowed to solidify as slants. The agars and liquid media were inoculated with agar discs of *H. sacchariolens* or *P. involutus* cut from behind the advancing margins of 30-day old colonies on PDA. The inoculated containers were then distributed in a randomised layout, with six-fold replication, and incubated at 20°C for 16 weeks, being examined at weekly intervals. The inoculated liquid cultures were shaken regularly.

There was no evidence of sclerotium production either by *H. sacchariolens* or *P. involutus* in any of the treatments. Growth was extensive in liquid culture and on agar culture when organic nitrogen was supplied, and, after 10 weeks, *H. sacchariolens* had formed small (0.5–1.0 μm) compact hyphal aggregates in the liquid culture. Similar aggregates had previously been observed in some isolates of *H. sacchariolens* grown in sterile vermiculite-peat medium moistened with MMN nutrient solution (Section 2.1.2); however, in both liquid culture and in vermiculite-peat these bodies were composed of loose aggregates of undifferentiated vegetative hyphae resembling the pellet growth typical of many fungi growing in static liquid culture and they were not regarded as sclerotium initials.

With the exception of media containing NaNO₃, in which growth by both fungi was poor, the agar slants were fully colonised after 4–6 weeks growth, and after 14 weeks the agar showed signs of shrinkage. Sclerotium production by other fungi has been found to be induced as a response to nutrient-starvation (Christias and Lockwood, 1972), but sclerotium initials in this experiment did not form either on ageing agar cultures or when mycelial growth was physically restricted.
by the size of the containers. Similarly, two isolates of *Cenococcum geophilum* harvested from mycorrhizal roots of *Picea sitchensis* and *Pinus* sp. did not form sclerotium initials on PDA or malt extract agar even in ageing (25 weeks) cultures in which the underlying agar had shrunk. This is in contrast to reports by Mikola (1948) and Trappe (1978) that sclerotia of *C. geophilum* form in ageing agar cultures; it seems that specific conditions, not provided in this experiment, are needed for sclerotium production or that different isolates may vary in their capacity to produce sclerotia in pure culture.

No further studies were done on sclerotium production in pure culture; instead, sclerotia used in the following experiments were obtained either from the field or from pot-culture syntheses.

4.8.1 Roles of Sclerotia of *Hebeloma sacchariolens* and *Paxillus involutus* in Establishing Mycorrhizas on Birch Seedlings

As described in Section 1.5, sclerotia of *Cenococcum geophilum* have been reported to be effective as a source of inoculum for the establishment of mycorrhizas on *Tsuga heterophylla* after dormant survival in soil for 5 years (Kropp, 1971). The present experiment was done to investigate if sclerotia produced by other sheathing mycorrhizal fungi, namely *Hebeloma sacchariolens* and *Paxillus involutus*, are similarly effective as inocula on birch seedlings in unsterile soil.

Mycorrhizas of *H. sacchariolens* and *P. involutus* were synthesised on seedlings of *Betula pendula* in bixenic conditions (Section 2.2.2); after 8 weeks growth, the seedlings were transplanted into an unsterile brown earth (Section 4.2) and grown under glasshouse conditions for 5 months. After this time, sclerotia of *H. sacchariolens* and *P. involutus* were dissected from the root systems, some of them were air-dried for
24 h and then the two sets were transferred to squares of nylon gauze which were tied into sacs. The sacs were buried in 5 cm³ pots containing moist, unsterile brown earth and the pots were incubated at 20°C (Section 4.2). After 16 and 40 weeks burial, samples of sclerotia of *H. sacchariolens* were retrieved, the samples from replicate pots were pooled and they were then divided into eight lots of 30; in a similar way, sclerotia of *P. involutus* were retrieved after 16 weeks and divided into lots of 30. The infectivity of the samples was tested by birch seedling assay (Section 4.2), the birch seedlings being grown for 12 weeks in a growth room. Some samples of sclerotia from the non-dried and air-dried treatments at both sampling times were retained for electron microscopical studies.

Sclerotia of *H. sacchariolens* were retrieved without difficulty after burial in soil for 16 and 40 weeks; the retention of their white, glistening appearance made them highly conspicuous. In contrast, the sclerotia of *P. involutus* were much more difficult to retrieve after only 16 weeks burial because of their fragile and shrivelled condition; it was not possible to retrieve sufficient numbers after 40 weeks burial to test their infectivity by birch seedling assay.

In the part of the experiment involving *H. sacchariolens* (Table 4.4), six of the thirteen seedlings grown in the control (unsupplemented) soil developed mycorrhizas, these being attributable to *Inocybe*, *Thelephora* or a type designated "Type 3". Sclerotial inoculum of *H. sacchariolens* previously buried for 16 weeks was effective in establishing mycorrhizas on 5 of 7 test seedlings, 29% of the root tips being colonised by *Hebeloma* to the exclusion of other mycorrhizal types. A higher proportion of root tips was colonised by *Hebeloma*, on all six of the replicate seedlings, when inoculated with sclerotia that had been
buried for 40 weeks, but the results from the two sampling times are not directly comparable because the growth conditions may have differed slightly at the different times. Again, mycorrhizas of *Hebeloma* developed to the exclusion of other mycorrhizal types at the 40-week sampling.

The infectivity of scierotia that had been air-dried prior to burial was not tested at the 16-week sampling time. But after 40 weeks burial, the initial air-drying treatment had apparently reduced the ability of scierotia to function as inoculum – both the number of seedlings that developed mycorrhizas of *Hebeloma* and the proportion of root tips bearing these mycorrhizas were lower than in the case of non-dried scierotia (Table 4.4). It is noteworthy that mycorrhizas attributable to *Hebeloma* were not recorded on any seedlings in the control (unsupplemented) series in these experiments and thus the development of *Hebeloma* mycorrhizas in the treatment series can confidently be ascribed to the scierotial inoculum. On two of the seedlings inoculated with scierotia of *H. sacchariolens* after 16 weeks burial, a greater number of scierotia were found attached to the seedling roots than were originally added to the soil, so scierotia must have been produced on the seedling roots during the 16 week period of host growth.

The infectivity of scierotia of *P. involutus* that had been either freshly harvested or previously buried for 16 weeks was tested in a separate experiment, though it was run concurrently with the experiment involving scierotial inoculum of *H. sacchariolens*. A high proportion of seedling roots in the control (unsupplemented) series remained non-mycorrhizal (Table 4.5). In contrast, all of the seedlings grown in soil supplemented with freshly harvested scierotia of *P. involutus* developed mycorrhizas attributable to this species, and a
high proportion of root tips on individual seedlings became mycorrhizal with *Paxillus*, to the exclusion of other, "naturally occurring" mycorrhizal fungi. Sclerotia previously buried for 16 weeks were markedly less effective as inoculum, only 2 of the 6 seedlings becoming mycorrhizal with *Paxillus* (Table 4.5). Freshly harvested inoculum of *P. involutus* apparently improved seedling growth compared with that in controls.

In summary, the results in Tables 4.4 and 4.5 indicate that sclerotia of *H. sacchariolens* and *P. involutus* are at least partly effective as inocula on birch after a period of burial in unsterile soil in the absence of living host roots. These preliminary studies suggest, however, that the survival of the sclerotium-like bodies of *H. sacchariolens* may be greater than that of sclerotia of *P. involutus*, as evidenced by their efficacy as inocula after a period of burial and by their appearance when retrieved from soil: the sclerotia of *P. involutus* were in a considerably more degenerate condition after 16 weeks burial than were the sclerotium-like bodies of *H. sacchariolens*. A potentially relevant point in this respect is that the soil in the experiments had been air-dried prior to use (but was remoistened for burial of the sclerotia), and air-drying considerably reduces the activities of the soil microfauna. It therefore remains to be seen if sclerotia of the mycorrhizal fungi could act as effective survival propagules in normal soil conditions. With respect to the survival of sclerotia of *H. sacchariolens* and their potential role as infective propagules for the establishment of mycorrhizas on new hosts or on newly elongating roots of already established hosts, it is of interest that apparently healthy sclerotia were frequently found attached to moribund mycorrhizas although they were never seen to occur independently in the soil.
### TABLE 4.4: Development of mycorrhizas on seedlings of *Betula pendula* grown in tubes of unsterile brown earth with or without sclerotia of *Hebeloma sacchariolens* previously buried in unsterile soil; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proportion of seedlings with mycorrhizas attributable to <em>Hebeloma</em></th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % root tips mycorrhizal</th>
<th>Mean % mycorrhizas attributable to different mycorrhizal types</th>
<th>Proportion of seedlings with other mycorrhizal types</th>
<th>Mean no. sclerotia attached to seedling roots</th>
<th>Mean shoot oven-dry weight (mg) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>No sclerotia</td>
<td>0/7</td>
<td>188&lt;sup&gt;a&lt;/sup&gt; (2.268)</td>
<td>76.8</td>
<td>23.2</td>
<td>nil</td>
<td>23.2</td>
<td>T3, 2/7</td>
<td>nil</td>
</tr>
<tr>
<td>+ sclerotia buried 16 weeks</td>
<td>5/7</td>
<td>71&lt;sup&gt;b&lt;/sup&gt; (1.773)</td>
<td>70.8</td>
<td>29.2</td>
<td>29.2</td>
<td>nil</td>
<td>nil</td>
<td>19</td>
</tr>
<tr>
<td>No sclerotia</td>
<td>0/6</td>
<td>134 (2.034)</td>
<td>58.8</td>
<td>41.2</td>
<td>nil</td>
<td>41.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1, 1/6; T, 2/6</td>
<td>nil</td>
</tr>
<tr>
<td>+ sclerotia buried 40 weeks</td>
<td>6/6</td>
<td>84 (1.898)</td>
<td>40.6</td>
<td>59.4</td>
<td>59.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nil</td>
<td>nil</td>
<td>6.7 (1.018)</td>
</tr>
<tr>
<td>+ air-dried sclerotia buried 40 weeks</td>
<td>2/6</td>
<td>58 (1.742)</td>
<td>79.8</td>
<td>20.2</td>
<td>10.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>T, 1/6</td>
<td>nil</td>
</tr>
</tbody>
</table>

1 Within columns, and for each sampling time figures followed by different letters are significantly different from one another, p<0.05.
2 Source of inoculum unknown.
3 I, Inocybe; T, Thelephora; T3, Type 3.
TABLE 4.5: Development of mycorrhizas on seedlings of *Betula pendula* grown in tubes of unsterile brown earth with or without sclerotia of *Paxillus involutus* previously buried in unsterile soil; means of log(x+1) in parentheses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proportion of seedlings with mycorrhizas attributable to <em>Paxillus</em></th>
<th>Mean no. root tips (and mean of log(x+1))</th>
<th>Mean % root tips uncolonised</th>
<th>Mean % mycorrhizas attributable to different mycorrhizal types <em>Paxillus</em></th>
<th>Proportion of seedlings with other mycorrhizal types</th>
<th>Mean stem height (cm) (and mean of log(x+1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>No sclerotia</td>
<td>0/6</td>
<td>43&lt;sup&gt;b1&lt;/sup&gt; (1.607)</td>
<td>96.9</td>
<td>3.1&lt;sup&gt;b&lt;/sup&gt; nil</td>
<td>3.1</td>
<td>H, 2/6 (1.348)</td>
</tr>
<tr>
<td>+ sclerotia freshly harvested</td>
<td>4/4</td>
<td>89&lt;sup&gt;a&lt;/sup&gt; (1.899)</td>
<td>27.0</td>
<td>73.0&lt;sup&gt;a&lt;/sup&gt; nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>+ sclerotia buried 16 weeks</td>
<td>2/6</td>
<td>41&lt;sup&gt;b&lt;/sup&gt; (1.599)</td>
<td>71.4</td>
<td>28.6&lt;sup&gt;b&lt;/sup&gt; 12.0&lt;sup&gt;b&lt;/sup&gt; 16.6</td>
<td>I, 2/6</td>
<td>21 (1.336)</td>
</tr>
</tbody>
</table>

1 Within columns, figures followed by different letters are significantly different from one another, p=0.05.

2 Source of inoculum unknown.

3 H, Hebeloma; I, Inocybe.
4.8.2 *Ultrastructural Changes in Detached Sclerotia of Hebeloma sacchariolens After Burial in Unsterile Soil*

Preliminary experiments (Section 4.8.1) on the infectivity of detached sclerotia of *Hebeloma sacchariolens* after periods of burial in unsterile soil indicated that these structures can remain effective as inoculum for at least 9 months.

Upon retrieval after 16 and 40 weeks burial, the sclerotia were structurally softer in texture than originally; nevertheless, they were still conspicuously white in appearance and, as indicated by S.E.M. and T.E.M., they had retained their gross structural integrity and, perhaps surprisingly, showed little sign of degeneration (Plates 4.46, 4.47, 4.49). In most samples, the internal pseudoparenchyma appeared to be without contents (Plate 4.47) though in some sclerotia isolated cells still containing some lipid were evident (Plate 4.49, arrow); however, the general absence of lipid materials or other cytoplasmic contents cannot be ascribed solely to burial in the absence of host roots, as some sclerotia freshly harvested in the spring from roots of 18-month old birch grown under glasshouse conditions, or from the root zone of mature birch in the vicinity of the previous year’s fruitbodies of this species (F. Gibson, pers. comm.), also appeared to be without contents. As evidenced by S.E.M., the walls of the pseudoparenchyma of buried sclerotia were roughened (Plate 4.47) in comparison to those of freshly harvested sclerotia (Plate 4.48), though the rough appearance was less apparent in buried sclerotia that still contained some lipid (Plate 4.49).

The few ultrastructural changes associated with burial of sclerotia, as seen with the T.E.M., were characteristic of sclerotia retrieved after both 16 weeks and 40 weeks burial. At both sampling times the lipid material, where present, appeared to have coalesced and was less
Conspicuous (Plates 4.50, 4.51, 4.52) than originally (Plate 4.9); the lipid material was also absent from many of the cells but some residual cytoplasm was evident (Plate 4.53). With the exception of the hyphal layers of the outer zone which appeared degenerate (Plates 4.50, arrow, 4.51), the cell walls were intact and intercellular material was still extensive (Plates 4.52, 4.53). Bacterial colonies were evident (Plate 4.51, arrow), though microbial attack and extensive degradation seemed to be confined to the outer zone of the sclerotia (Plate 4.50). However, observations made on buried sclerotia of *H. sacchariolens* with the S.E.M. indicated that a range of microorganisms were intimately associated in both the outer zone and inner pseudoparenchyma; this was especially apparent in sclerotia that had been air-dried for 24 h prior to burial (Section 4.2). Some of the microorganisms most consistently seen are described in Section 4.8.4.

4.8.3 *Ultrastructural Changes in Detached Sclerotia of Paxillus involutus After Burial in Unsterile Soil*

Even initially, three quarters of the sclerotia of *Paxillus involutus* collected from the root zone of mature birch at Newtongrange (Section 2.1.6.1) appeared moribund irrespective of the time of year when they were collected. Eighteen of the 20 sclerotia prepared for S.E.M. after 16 weeks burial in pots of unsterile brown earth (Section 4.2) were degenerate with a collapsed shrivelled appearance. Compression and collapse of the outer layers seen initially in sclerotia of this species (Plate 4.30) had extended throughout buried sclerotia (Plates 4.54, 4.55) so that the interior appeared to be composed entirely of compacted wall material and intercellular matrix. As indicated by histochemistry (Section 4.4), the glycogen content of buried sclerotia was negligible. After 40 weeks burial, very few sclerotia of *P. involutus* were still
retained within the gauze sacs and those that were still present were in too degenerative a condition to prepare for electron microscopy.

4.8.4 Microorganisms Associated with Sclerotia of Some Sheathing Mycorrhizal Fungi of Birch

As seen by S.E.M., sclerotia of Cenococcum geophilum and Paxillus involutus freshly harvested from the root zone of naturally regenerating birch at Newtonrange (Section 2.1.6.1), and sclerotia of P. involutus and Hebeloma sacchariolens retrieved after burial for 16 or 40 weeks in pots of unsterile, moist brown earth (Section 4.8.2) were frequently and consistently associated with a restricted range of microorganisms. An attempt was made to combine S.E.M. observations with isolation methods in order to identify some of the microorganisms that were intimately associated with these sclerotia.

Methods

By means of the S.E.M., the main characteristics and relative frequencies of some of the microorganisms were recorded for sub-samples of sclerotia from various sources; further sub-samples were subjected to the flotation method of Trappe (1969) and some of these were prepared for isolation of associated microorganisms. For the latter purpose, some sclerotia were washed in five changes of sterile distilled water for 10 minutes each time, on a Gallenkamp wrist-action shaker. They were then blotted dry on sterile filter papers and surface sterilised for 3-5 minutes in either 30% (v/v) hydrogen peroxide or 7% (w/v) calcium hypochlorite solution before washing, once again, in sterile water. Other sclerotia were washed as above but they were not surface sterilised. Sclerotia from each treatment were transferred, 8-12 per plate, to the following agar media (Section 2.1.2): chitin agar for
detection of actinomycetes; Thornton's standard agar for detection of fungi and bacteria and Tryptone-soy agar (TSA), containing streptomycin and aureomycin, for isolation of fungi. In addition, some sclerotia were placed just behind the advancing margins of 14 to 21-day old colonies of *Phialophora radicicola* Cain on PDA, these precolonised agar plates being reportedly specific for mycoparasitic fungi (Deacon and Henry, 1980). Most of this work was done with sclerotia of *P. involutus* but some attempts were made to isolate microorganisms associated with sclerotia of *H. sacchariolens* and, for this, samples of sclerotia were dissected from different distances down the root systems of 2-year old, glasshouse-grown birch saplings originally inoculated with *H. sacchariolens* under bixenic conditions (Section 2.2.2).

**Results from scanning electron microscopy**

Examination by S.E.M. showed a marked variation in the numbers and types of microorganisms associated with sclerotia of *C. geophilum*, *H. sacchariolens* and *P. involutus*; however, it is emphasised that only gross morphological features could be distinguished by S.E.M. and the categories of microorganisms detected may not necessarily represent single species (Table 4.6).

*Cenococcum geophilum*

Two distinct hyphal types were frequently seen coursing through the empty pseudoparenchyma of sclerotia of *C. geophilum*; bacterial colonies, however, were generally confined to the outer rind layers (Plate 4.56) being rarely seen either in the pseudoparenchyma or on the outer surface of the rind. Hyphae of wide diameter (Type e, Table 4.6) shown in Plates 4.57, 4.58, which were sometimes associated with bacterial rods (Plate 4.57, arrow), penetrated the cell walls by
means of narrow pores (1.0 μm diameter). The smooth, sometimes concave appearance of the pits around these pores (Plate 4.59, arrows) suggested that enzymatic rather than purely mechanical processes were involved in their formation; moreover, the absence of any appreciable apposition of wall material, for example, papillae, in their vicinity (Plates 4.58, arrow, 4.59, arrows) indicated that they may have been formed after death of the host cells. Hyphae of narrow diameter (Type f, Table 4.6) and bearing abundant spore-like bodies (0.6 - 0.7 μm diameter) (Plates 4.60, 4.61) were abundant throughout the pseudo-parenchyma and were commonly seen in or near cells containing the wider hyphae (Plate 4.61). These narrow hyphae were less obviously associated with pits in the walls though narrow pores were present in their vicinity (Plate 4.60, arrows, 4.61, arrow). Examination of the rind of the sclerotia showed no obvious sites of entry of the hyphae.

*Hebeloma sacchariolens*

Whereas there was little evidence of microbial colonies on the surface of sclerotia of *H. sacchariolens* freshly harvested from 2-year old birch (Plate 4.62), sclerotia that had been air-dried for 24 h and then buried in unsterile soil for 16 weeks bore numerous microorganisms associated with the external surface (Plates 4.63, 4.64). Sclerotia of *H. sacchariolens* that had not been air-dried prior to burial (Plate 4.65) had many fewer organisms on their external surfaces.

The organisms present on the surfaces of previously air-dried sclerotia of *H. sacchariolens* (Plate 4.63) included round or elliptical spore-like bodies (Type a, Table 4.6) as shown in Plate 4.63, arrow a and Plate 4.64, dense colonies of short bacterial rods (Type b, Table 4.6) (Plate 4.63, arrow b) and large cells with flagellate-like appendages at one of their poles (Type c, Table 4.6) (Plate 4.63, arrow c). Where
cytoplasm was still evident in the interior parts of sclerotia, it was generally densely colonised by short bacterial rods (Type b, Table 4.6) or by filaments (Plate 4.66) of variable length and width (2.9 - 3.2 x 0.16 - 0.2 μm). Bacterial rods of variable length (Type d, Table 4.6) were ubiquitous throughout the pseudoparenchymatous zone (Plate 4.67, arrow), especially in cells without cytoplasmic contents.

Hollow spaces were seen in the pseudoparenchymatous zone of two of the ten sclerotia of H. sacchariolens previously air-dried and then buried in unsterile soil for 16 weeks. In these hollows were seen hyphae with clamp connections (Plates 4.68, arrow, 4.69), and much narrower hyphae invested with spore-like bodies (Plate 4.69, arrow), these are recorded as Type F in Table 4.6. Other narrow hyphae had few or no spores attached to them (Plates 4.70, 4.71, 4.72, 4.73). These narrow hyphae, especially those invested with spore-like bodies, closely resembled the narrow hyphae seen in sclerotia of C. geophilum (Plates 4.60, 4.61). In some cells, narrow hyphae were seen that appeared to have fragmented (Plate 4.71). Based on size (<1.0 μm diameter) and gross morphology, and based also on the arrangement of the spore-like bodies, all of these narrow hyphae are suggested to represent filaments of actinomycetes. Sometimes these actinomycetous filaments were associated with pores in the cell walls (Plate 4.73, arrow).

Paxillus involutus

Considering their degenerative condition after 16 weeks burial in unsterile brown earth (Section 4.8.2), it was perhaps surprising that sclerotia of P. involutus were associated with very few microorganisms as seen by S.E.M. (Table 4.6). However, a peculiar ultrastructural appearance was found in one sclerotia of the many examined (Plates 4.74 to 4.79). The sclerotium (307 x 361 μm diameter) was filled with a number
of distinctive round to oval structures with an average size of 63 x 48 μm, and these were embedded in a mass of torulose hyphae (Plates 4.74, 4.76, 4.77). These structures had a smooth, amorphous surface appearance (Plates 4.74, 4.76) and in section they were seen to be composed of a rind-like "shell", 3.5 - 4.0 μm thick, enclosing a distinctly cellular tissue (Plates 4.77, 4.78). The component cells were mostly empty but some were filled with an amorphous, apparently cytoplasmic material (Plate 4.78). One of the structures (Plate 4.75, arrow, 4.79) seemed to be in a state of degeneration, its appearance (Plate 4.79) closely resembled that of some collapsed, buried sclerotia of P. involutus (Plate 4.55). The origin and nature of these structures is not known; the outer zone of the sclerotium that contained them appeared to be intact (Plates 4.74, 4.75) and there was no evidence of penetration of the outer sclerotium layers by invading organisms. The component cells of these structures (mean diameter 8.3 μm) were of similar size to the component cells of fresh sclerotia of P. involutus (mean diameter 8.6 μm; Plate 4.35), and the general organisation of the small structures was also similar to that of whole freshly collected sclerotia of P. involutus. These facts, coupled with the similar degenerative appearance of one of the small structures (Plate 4.79) and of whole sclerotia of P. involutus (Plate 4.54), suggest that the small structures were "secondary sclerotia" formed within a larger degenerating sclerotium. Secondary sclerotia are known to be produced inside the sclerotia of some root-infecting plant pathogens, for example, Phymatotrichopsis omnivorum (King, Loomis and Hope, 1931) and Sclerotinia sclerotiorum (Williams and Western, 1965). In P. omnivorum, fresh secondary sclerotia develop as an excrecent growth from old sclerotial structures and King et al. (1931) reported renewed growth even in cultures that had
TABLE 4.6: Frequency of microorganisms associated with sclerotia of *Cenococcum geophilum* collected from mature pine roots, and with sclerotia of *Hebeloma sacchariolens* and *Paxillus involutus* retrieved after 16 weeks burial in unsterile soil. Data based on 10 sclerotia for each species examined by S.E.M.

<table>
<thead>
<tr>
<th>Type of microorganism(^1) observed by S.E.M.</th>
<th>Frequency of occurrence(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. geophilum</em></td>
</tr>
<tr>
<td></td>
<td>external tissue</td>
</tr>
<tr>
<td>a, spore-like bodies</td>
<td>0</td>
</tr>
<tr>
<td>b, bacterial colonies</td>
<td>0</td>
</tr>
<tr>
<td>c, polar flagellate cells</td>
<td>0</td>
</tr>
<tr>
<td>d, bacterial colonies</td>
<td>+</td>
</tr>
<tr>
<td>e, wide hyphae</td>
<td>0</td>
</tr>
<tr>
<td>f, narrow hyphae</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\) 0, absent; +, occasional; ++, frequent.

\(^2\) a, Round or elliptical spore-like bodies (1.2 - 1.4 μm) covered with wart-like protrusions (Plate 4.63, arrow a, 4.54).

b, Bacterial rods (0.33 x 0.29 μm) generally seen as dense colonies (Plate 4.63, arrow b).

c, Curved cells of variable length but uniform diameter (1.5 - 1.9 x 0.5 μm), each with a flagellum-like appendage (Plate 4.63, arrow c).

d, Bacterial rods of variable length (1.3 - 1.5 x 0.3 - 0.4 μm) (Plate 4.67, arrow).

e, Hyphae of wide diameter (4.2 - 4.9 μm) occasionally associated with rounded pits in the cell walls (Plates 4.57, 4.58, 4.59).

f, Hyphae of narrow diameter (<1.0 μm) often associated with spherical spore-like bodies along their lengths (Plates 4.60, 4.61, 4.69, arrow, 4.70, 4.72, 4.73); occasionally fragmented (Plate 4.71).
apparently ceased growing for several months. Similar renewed growth of old sclerotia to form secondary sclerotia occurs in *S. sclerotiorum* and because sclerotia of this species are not associated with mycelial growth in the surrounding soil, the energy materials required for new growth must occur by transfer of materials from the parent sclerotia or by direct absorption from the surrounding soil.

Unfortunately, only one of the many sclerotia of *P. involutus* that were examined contained the structures shown in Plates 4.74 - 4.79, and the viability of this sclerotium had, of course, been lost as a result of the S.E.M. preparation procedures, so it was not possible to investigate the phenomenon further.

**Results from assessments of viability and from attempted isolations**

Few of the sclerotia of *Paxillus involutus* freshly collected from Newtongrange were found to sink in water, and none of the sclerotia buried for 16 weeks did so. According to Trappe (1969) these observations suggested that most of the sclerotia from this source were non-viable initially and all had lost viability after 16 weeks burial in unsterile soil. For attempted isolation of microorganisms, the sclerotia that floated in water and that sank in water were treated as separate sub-samples (Table 4.7) except for the buried sclerotia that could not be divided in this way. None of the sclerotia subjected to surface-sterilisation in either hydrogen peroxide or calcium hypochlorite yielded any colonies on any of the agar media. Colonies did develop, however, from sclerotia washed in several changes of sterilised distilled water but otherwise untreated prior to plating (Table 4.7), and the majority of sclerotia yielded microbial colonies on all of the agars except chitin agar.

It was seldom possible to relate directly the observations made by S.E.M. with the pure-culture studies. Nevertheless, 50-70% of the
sclerotia of *P. involutus* (fresh and buried) plated onto agar precolonised by *P. radicicola* had given rise to colonies of actinomycetes after 14 days incubation (Table 4.7) and these actinomycetes are thought to be equivalent to the hyphae of narrow diameter (Type F, Table 4.6) that were invested with spore-like bodies and shown, for example, in Plates 4.60, 4.61 and 4.69 (arrow) within the pseudoparenchyma of *Cenococcum geophilum* and *Hebeloma sacchariolens*.

*Gliricladium roseum* was also frequently isolated from sclerotia plated onto precolonised plates, while *Penicillium* sp. and *Pythium* sp. were isolated less frequently in this way (Table 4.7). *Trichoderma* sp. and *Penicillium* sp. were the predominant microorganisms isolated from seemingly viable sclerotia on Thornton's agar and TSA, as also from non-viable sclerotia though the latter also yielded colonies of, for example, *Scopulariopsis brevicaulis*, *Mortierella* sp. and *Verticillium cinabarinum* (Plates 4.80 - 4.85).

A similar range of microorganisms to those from sclerotia of *P. involutus* were isolated from sclerotia of *H. sacchariolens* obtained at different distances from the root systems of 2-year old birch saplings (Table 4.8). A high proportion (89%) of the sclerotia of *H. sacchariolens* yielded microbial colonies which is surprising in view of the fact that the sclerotia appeared white and "healthy" and had been freshly detached from active mycorrhizas. However, the sampling was done in the spring and the sclerotial material may have represented sclerotia that had been formed in the previous year. It has already been stated that the white colour of the sclerotia of *H. sacchariolens* does not necessarily indicate juvenility.

In general, the range of isolates from sclerotia of *H. sacchariolens* (Table 4.8) was similar to that from sclerotia of *P. involutus* (Table 4.7).
The major differences concerned the isolation of *Absidia* sp. from *H. sacchariolens* but not from *P. involutus*, and the absence of isolates of *Gliocladium* sp. and actinomycetes from *H. sacchariolens* whereas these were frequently obtained from *P. involutus*. S.E.M. observations indicated the presence of an actinomycete within the pseudoparenchyma of sclerotia of *H. sacchariolens*, yet no actinomycete was seen to grow from the sclerotia plated onto agar. The likely explanation of this is that the agar was unsuitable for isolation of actinomycetes especially in competition with faster growing fungi; indeed, in the case of *P. involutus*, actinomycetes were isolated only where sclerotia were incubated on plates precolonised with *P. radicicola* (Table 4.7) - a method not used for sclerotia of *H. sacchariolens*. 
<table>
<thead>
<tr>
<th>Medium</th>
<th>Proportion of all scierotia yielding colonies after 14 days</th>
<th>Apparently viable scierotia</th>
<th>Apparently non-viable scierotia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin agar</td>
<td></td>
<td>Bacteria*</td>
<td>Bacteria*</td>
</tr>
<tr>
<td>Thoron's agar</td>
<td></td>
<td>Penicillium sp.</td>
<td>Penicillium sp.</td>
</tr>
<tr>
<td>Tryptone-soy agar</td>
<td></td>
<td>Penicillium sp.</td>
<td>Penicillium sp.</td>
</tr>
<tr>
<td>+ streptomycin + aureomycin</td>
<td></td>
<td>Trichoderma sp.</td>
<td>Scopulariopsis brevicaulis</td>
</tr>
<tr>
<td>Precolonised plates of</td>
<td></td>
<td>Penicillium sp.</td>
<td>Penicillium spp.</td>
</tr>
<tr>
<td>Phialophora radicicola</td>
<td></td>
<td>Trichoderma sp.</td>
<td>Mortierella sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unknown</td>
<td>Pythium sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Verticillium cinabirinum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Scopulariopsis brevicaulis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cylindrocarpon sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unknown sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillium sp.</td>
<td>Penicillium sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Actinomycete sp.</td>
<td>Pythium sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gliocladium roseum</td>
<td>Actinomycete sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gliocladium roseum</td>
</tr>
</tbody>
</table>

* gram negative.
† proportion of scierotia yielding microbial colonies.
TABLE 4.8: Microorganisms isolated on Tryptone-soy agar from sclerotia of *Hebeloma sacchariolens* harvested from different distances down the root systems of 2-year old saplings grown in brown earth under glasshouse conditions.

<table>
<thead>
<tr>
<th>Distance down root system (cm)</th>
<th>Proportion of sclerotia yielding colonies</th>
<th>Colony types</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>(9/10)</td>
<td><em>Absidia</em> sp. 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacteria (gram negative) 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Penicillium</em> sp. 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unknown sp. 1</td>
</tr>
<tr>
<td>4-5</td>
<td>(6/10)</td>
<td><em>Absidia</em> sp. 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Penicillium</em> sp. 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Scopulariopsis brevicaulis</em> 1</td>
</tr>
<tr>
<td>7-8</td>
<td>(10/10)</td>
<td><em>Absidia</em> sp. 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacteria (gram negative) 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Penicillium</em> sp. 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Trichoderma</em> sp. 5</td>
</tr>
<tr>
<td>10-11</td>
<td>(9/10)</td>
<td><em>Absidia</em> sp. 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacteria (gram negative) 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Mortierella</em> sp. 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Penicillium</em> sp. 2</td>
</tr>
</tbody>
</table>
4.9 Discussion

Introduction

Investigations in this section of the thesis were done primarily to determine if the globose bodies found attached to sheathing mycorrhizas of *Hebeloma saccharioliens* on birch in the Edinburgh area can be considered as sclerotia in terms of their structural similarity to sclerotia of other root-infecting fungi and in terms of a role in dormant survival. The sclerotia of *Paxillus involutus* and *Cenococcum geophilum* were studied less intensively, for comparison.

Sclerotia of root-infecting fungi function either as temporary resting structures or as long-term survival propagules; they generally contain large reserves of energy storage materials and they maintain a low metabolic rate during dormancy (Coley-Smith and Cooke, 1971). Several root pathogens produce sclerotia that can survive in soil for considerable periods of time and that can resist adverse environmental conditions, these features usually being associated with (a) an outer rind composed of thick-walled cells, and (b) dark pigmentation (melanisation) that is thought to confer resistance to microbial attack (Townsend and Willetts, 1954; Willetts, 1972; Coley-Smith, 1979).

Structure

The sclerotia of *C. geophilum* are similar to those of other root-infecting fungi and fit into the "lateral-type" category of sclerotium development described by Townsend and Willetts (1954): they are borne laterally along well differentiated mycelial strands and comprise a firm central zone of pseudoparenchyma surrounded by a rigid pseudoparenchymatous and prosenchymatous outer rind which is narrow but deeply pigmented and composed of small thick-walled, densely packed...
cells often with occluded lumina. The entire pseudoparenchymatous region seems equivalent to "cortex" as described in other sclerotia (Townsend and Willetts, 1954) and there is no further differentiation into "medullary" as opposed to "cortical" tissues as is seen in some other sclerotia, e.g. of *Phymatotrichopsis omnivorum* (Townsend and Willetts, 1954). The pseudoparenchyma shows marked radial differentiation, suggesting growth from a central region, and because the rind becomes differentiated and delimiting before growth has ceased (Willetts, 1972), the outer layers of the pseudoparenchyma become distorted tangentially. Although no work was done on survival of the sclerotia of *C. geophilum*, Kropp (1971) has suggested that they can survive for at least 5 years. All the available evidence, therefore, suggests that these structures are "true" sclerotia, equivalent to those of root-infecting plant pathogens.

Structurally, the sclerotia of *H. sacchariolens* only superficially resemble sclerotia of *C. geophilum* or of other fungi. They lack a distinct rind; instead they have only a narrow outer zone of crushed pseudoparenchyma overlaid by normal vegetative hyphae. Moreover, they lack dark pigmentation, even with age, unlike the sclerotia or other specialised survival structures of root-infecting fungi and of *C. geophilum* and *P. involutus*. Internally, they contained an innermost zone of thick-walled pseudoparenchyma, and the wall thickness increased progressively from the outside of the structure inwards, in sharp contrast to the organisation of "true" sclerotia which tend to have least wall thickening in the internal regions. Another interesting feature, contrasting with that of sclerotia of *C. geophilum*, of *P. involutus* or of other root-infecting fungi, is that the sclerotia of *H. sacchariolens* were very closely associated with the mycorrhizal sheath on the plant roots. Indeed, the sclerotia of *H. sacchariolens* seemed to represent an
extension of the sheath, insofar as they were partly immersed in the sheath and their outer layers were apparently continuous with the tissue comprising the sheath and Hartig net. A further difference from the sclerotia of other fungi is that the sclerotia of *H. sacchariolens* contained predominantly lipid storage reserves, whereas sclerotia normally contain glycogen or other polysaccharide materials as main energy storage compounds. Nevertheless, this is not an absolute distinction because the ergots of *Claviceps* spp., for example, contain a predominance of lipids.

The sclerotia of *P. involutus* seem to be intermediate in several respects between those of *C. geophilum* and *H. sacchariolens*. Like those of *H. sacchariolens*, they lack an outer rind and are composed predominantly of pseudoparenchyma. But, like those of *C. geophilum*, they are formed on mycelial strands, are darkly pigmented and, as in sclerotia of several root pathogens, they contain glycogen. Unlike the sclerotia of *C. geophilum* and *H. sacchariolens*, those of *P. involutus* have little, if any, wall thickening of their component cells and they lack extensive deposits of intercellular material. Evidently, the sclerotia or sclerotium-like bodies of sheathing mycorrhizal fungi are at least as variable as are those of other root-infecting fungi (Townsend and Willetts, 1954). As yet, only those of *C. geophilum* and possibly those of *P. involutus* can be accommodated in the categories of sclerotia recognised by Townsend and Willetts (1954).

**Survival**

Sclerotia are generally recognised to be specialised survival structures, able to remain dormant in soil and to germinate either by producing fruiting bodies (carpogenic), by producing vegetative hyphae (myceliogenic) or by producing asexual spores (sporogenic). Ironically,
however, Ainsworth (1961) does not include dormant survival as a criterion of sclerotia; instead, a sclerotium is defined by these workers merely as "a firm, frequently rounded, mass of hyphae with or without the addition of host tissue or soil, normally having no spores in or on it."

In my work, the sclerotium-like structures of *H. sacchariolens* were found to persist in soil for 9 months at 18°C. After this time they were structurally intact, contained subcellular organelles and some lipid (Plates 4.52, 4.53) and had the capacity to initiate mycorrhizal development on seedlings of *Betula pendula*. Moreover, they still had a pure white or "glistening" appearance, indicating that they had undergone little decomposition or attack by microorganisms. Ultrastructurally, they were seen to contain relatively few microorganisms after burial for 9 months and such microbial invasion was seen predominantly in sclerotia that had been air-dried before burial. Smith (1972) has shown, for sclerotia of *Sclerotium rolfsii*, that air-drying prior to burial greatly increases the degree of microbial attack on sclerotia, this being associated with damage to the rind caused by air-drying. However, Coley-Smith (1979) has found that air-drying of sclerotia of a range of *Sclerotium* spp., *Rhizoctonia tuliparum* and *Sclerotinia gladioli* did not necessarily cause enhanced rates of nutrient leakage (with possibly concomitant effects on their survival in soil) that might be expected to result from damage to the rind; leakage levels varied greatly between species and were not necessarily related to size of the sclerotia. Anyhow, the prolonged survival of both air-dried and non-dried sclerotia of *H. sacchariolens* in my work indicates that these structures may have a dormant survival capacity and that this is not necessarily related to either dark pigmentation or presence of a discrete rind.
Sclerotia of *P. involutus* had only a poor capacity to survive burial in soil when detached from host roots: only a small proportion of birch seedlings developed Paxillus-type mycorrhizas when inoculated with sclerotia previously buried for 3 months, and the sclerotia buried for 9 months had nearly all disintegrated, making it impossible to test their infectivity. Ultrastructural evidence showed almost complete or complete collapse and compaction of the component cells of the sclerotia of *P. involutus* after 3 months' burial. Few microorganisms could be seen in the collapsed tissues, though many microorganisms were isolated from these buried sclerotia (Section 4.9). It is perhaps significant that the sclerotia of *P. involutus* did not contain cells with markedly thickened walls; this feature may have contributed to the collapse and eventual disintegration of these sclerotia, unlike those of *H. sacchariolens*. Yet wall thickening as such is not necessarily important in survival, because the component hyphae of sclerotia of *Rhizoctonia solani* do not have markedly thickened walls; rather, the dense cytoplasmic contents of the sclerotia of *R. solani* are suggested to help confer resistance against dessication (Willetts, 1972).

As stated previously, Coley-Smith and Cooke (1971) have suggested that sclerotia can function as either short-term resting bodies or long-term survival propagules. In the case of *H. sacchariolens*, it is possible that the sclerotium-like bodies function not as independent dormant propagules but rather as temporary storage bodies able either to initiate infection of roots after winter dormancy or to supply developing fruit-bodies with the necessary carbon nutrients. The distribution of these bodies is consistent with either of these proposals. Thus, the sclerotium-like bodies are seen frequently to be attached to moribund mycorrhizas on roots in the field and they are most numerous in the zone of fruitbody
production by *H. sacchariolens* around tree bases though, of course, the mycorrhizas of *H. sacchariolens* are also most numerous in this zone. A storage function for fruitbody production may be thought to be necessary for a mycorrhizal symbiont, especially as fruiting occurs towards the end of the host's growing season whereas maximal flow of carbohydrates to the roots is reported to occur early in the growing season (Krueger and Trappe, 1967). Moreover, Wessels and Sietsma (1979) have shown, for *Schizophyllum commune*, that fruitbody production entails depletion of the soluble carbohydrates in the fungal mycelium and then breakdown of some of the wall glucans to supply further soluble carbohydrates. At this stage, an energy reserve in sclerotium-like bodies could be utilised. In a different context, some appressoria of leaf-infecting fungal pathogens are thought to act as temporary survival structures (Emmett and Parbery, 1975), enabling the nutrients in the germinating spores to be conserved in the appressoria until the fungus is able to initiate infection when host resistance declines. Thus, there is a well-defined role for temporary survival structures in other fungal systems and this role is somewhat different from that of structures involved in long-term survival in the absence of a host. The mycorrhizal fungi that do not form sclerotia may possibly have other mechanisms of temporary survival - for example, energy storage reserves may be deposited in the sheath rather than in separate structures (Smith, 1980).

It is notable that relatively few sheathing mycorrhizal fungi produce sclerotia or sclerotium-like structures, whereas all of these fungi must need to overcome the same difficulties, namely persistance in the absence of a host or during the host dormant period and to supply nutrients to developing fruitbodies (if this is a function of the sclerotium-like bodies of *H. sacchariolens*). It is notable, also, that all of the sheathing mycorrhizal fungi reported to form sclerotia fall into the
"early-stage" category of Deacon et al. (1983), Last et al. (1983) and Mason et al. (1983a) or have behaviour consistent with that of "early-stage" sheathing mycorrhizal fungi. These "early-stage" fungi, as shown in Section 3, can initiate infection from fresh basidiospores in soil whereas "late-stage" fungi cannot do so. Possibly, therefore, the basidiospores of "late-stage" fungi have greater survival capacities than do those of "early-stage" fungi, though this cannot as yet be tested because of the difficulties of assessing survival by means of host assays. But the size of sclerotia is not, itself, important in survival, as evidenced by the fact that single-celled chlamydospores can survive in soil for many years (Garrett, 1970). Rather, large size has been interpreted as being important in subsequent germination and growth. In carpogenic species, the large size and energy reserves of sclerotia are important in supporting fruiting in the absence of external nutrients (Coley-Smith and Cooke, 1971). In myceliogenic species, the large size and energy reserves are thought to confer high inoculum potential on the fungus, enabling it to initiate regrowth and infection of a resisting plant organ. Punja and Grogan (1982a,b), for example, found that "eruptive" germination of the sclerotia of S. rolfsii enabled this fungus to infect roots of susceptible hosts whereas germination by means of hyphae originating from only the peripheral sclerotium tissues led to lesser ability to infect roots.

Fleming (1983a,b) has shown that "late-stage" mycorrhizal fungi like Lactarius pubescens can initiate infection of seedling roots from mycelial strands that remain attached to parent trees and thus can draw on a supply of nutrients for infection. Possibly the sclerotia of some "early-stage" fungi have a similar role as structures with a high inoculum potential for infection.
PLATES 4.1 - 4.4: Sclerotia of *Hebeloma sacchariolens* on birch seedlings grown under glasshouse conditions

PLATE 4.1: Light micrograph of seedling roots of *Betula pendula* (16 weeks old) associated with white sclerotium-like bodies.

PLATES 4.2-4.3: Detail of Plate 4.1, showing clusters of sclerotia attached to roots and mycorrhizas of *Hebeloma*.

PLATE 4.4: Light micrograph of mycorrhizas of *Hebeloma crustuliniforme*, showing angular structures associated with the wefts of hyphae radiating from the mycorrhizas.
PLATES 4.5 - 4.9: Structure of freshly harvested sclerotia of *Hebeloma sacchariolens*

PLATE 4.5: Light micrograph of a sclerotium, showing differentiation into an inner zone of pseudoparenchyma and a narrow outer zone of thin-walled hyphae resembling vegetative hyphae; the sclerotium had been fixed in osmium tetroxide, stained in uranyl acetate and lead citrate and, after sectioning, stained in toluidene blue.

PLATE 4.6: Detail of Plate 4.5, showing the outer zone (oz) comprising 6-7 layers of radially flattened cells; the inner zone (iz) of pseudoparenchyma has densely staining walls and is filled with osmiophilic bodies, just discernible with the light microscope.

PLATE 4.7: S.E.M. A sclerotium cracked open after critical point-drying, showing narrow outer zone (oz) and an inner zone of pseudoparenchyma, the cells of which appear randomly arranged in the centre but radially arranged towards the periphery; the outer surface of the sclerotium appears smooth (arrow).

PLATE 4.8: S.E.M. Two sclerotia attached to a small secondarily thickened root; the sclerotia are covered with radiating hyphae.

PLATE 4.9: T.E.M. Cross-section through part of the inner pseudoparenchyma, showing angular-shaped cells with highly thickened walls and embedded in an intercellular matrix; the cells are filled with osmiophilic material resembling lipid (1).
PLATES 4.10 - 4.14: Structure of freshly harvested sclerotia of *Hebeloma sacchariolens*

PLATE 4.10: T.E.M. Cross-section through part of the inner pseudoparenchyma, showing cells filled with polygonal lipid bodies (1); the junctions of some of the lipid bodies (arrows) have a different staining property from that internally.

PLATE 4.11-4.13: S.E.M. Part of the inner pseudoparenchyma, showing cells filled with lipid-like material; the lipid may appear as individual discrete bodies (Plate 4.11) or sometimes fused into a large mass almost filling individual cells (Plate 4.12, arrow); some lipid bodies seem to be joined to one another in chains (Plate 4.13).

PLATE 4.14: S.E.M. Psuedoparenchyma of a sclerotium harvested from 18-month old birch, showing cytoplasmic contents apparently without lipid.
PLATES 4.15 - 4.16: Structure of freshly harvested sclerotia of *Hebeloma sacchariolens*

PLATE 4.15: T.E.M. Cross-section through the central part of the pseudoparenchyma after pyridine treatment; lipid (1) from all except the most central cell has been extracted; incomplete cross walls with rounded ends are evident (arrow 1) as is a dolipore septum (arrow 2).

PLATE 4.16: T.E.M. Detail of the walls (cw) of the pseudoparenchyma which are thickened and fibrillar, adjacent walls being separated by granular intercellular material (im).
PLATES 4.17 - 4.19: Structure of freshly harvested sclerotia of *Hebeloma sacchariolens*

PLATE 4.17: S.E.M. Part of a sclerotium treated with pyridine and cracked open after critical point drying, showing radial gradient of cell size, cells near the centre of the sclerotium being large and angular whereas those near the periphery are smaller.

PLATE 4.18: T.E.M. Transverse-section through part of the outer zone (oz), showing radially flattened cells with thin undifferentiated walls; the cells appear empty.

PLATE 4.19: T.E.M. Transverse section through part of the outer zone (oz) and inner zone (iz) of a sclerotium; a narrow transitional zone (tz) is evident, comprising cells of intermediate size and without lipid but having somewhat thickened walls; intercellular material (arrows) is extensive in this region.
PLATES 4.20 - 4.25: Structure of freshly harvested sclerotia of *Hebeloma sacchariolens*

**PLATES 4.20-4.21:** T.E.M. Outer zone of a sclerotium, showing outermost layers of undifferentiated vegetative hyphae in cross-section (Plate 4.20, arrow) and longitudinal section (Plate 4.21, arrow 1), and inner layers of prosenchyma; dolipore septa (Plate 4.21, arrow 2) are evident and some cells are filled with glycogen (g).

**PLATE 4.22:** T.E.M. Detail of a dolipore septum in an hypha of the outer zone.

**PLATE 4.23:** S.E.M. Outer surface of a sclerotium, showing a closely arranged network of hyphae with clamp connections (arrow).

**PLATE 4.24:** S.E.M. Cut face of a sclerotium, showing outer zone (oz) of empty prosenchyma overlaid with flattened hyphae apparently covered in a membranous material; the cells of the inner zone (iz) have contents.

**PLATE 4.25:** Light micrograph, showing a cross-section through a sclerotium in the region of attachment to a mycorrhiza; the outer zone of the sclerotium is intimately associated with the cells of the Hartig net (hn) and sheath (sh) and the host cortical cells (hc) are absent from the site of attachment.
PLATES 4.26 - 4.29: Structure of freshly harvested sclerotia of *Paxillus involutus*

PLATE 4.26: S.E.M. Immature sclerotium comprising a globose aggregate of closely interwoven hyphae and subtended by a mycelial strand.

PLATE 4.27: S.E.M. Zone of attachment between a sclerotium (s) and mycelial strand (ms); the outer surface of the sclerotium is continuous with hyphal branches of the mycelial strand which is composed of closely packed, longitudinally orientated hyphae with numerous clamp connections (c).

PLATE 4.28: S.E.M. Detail of Plate 4.27, showing labyrinthine appearance of the outer surface of the sclerotium.

PLATE 4.29: S.E.M. Outer surface of a mature, deeply pigmented sclerotium, showing increased compaction of the originally labyrinthine surface (Plate 4.28) overlaid with encrusted debris and squamulose material.
PLATES 4.30 - 4.33: Structure of freshly harvested sclerotia of
Paxillus involutus

PLATE 4.30: S.E.M. A sclerotium cracked open after critical point
drying, showing differentiation of tissue into an internal
pseudoparenchyma and narrow outer zone which is
continuous with radiating hyphae.

PLATE 4.31: S.E.M. Detail of Plate 4.30, showing part of the inner
zone (iz) of pseudoparenchyma and narrow outer zone
(oz); the pseudoparenchyma comprises a seemingly
random arrangement of large cells interspersed with
much smaller cells, the larger cells having granular
or amorphous contents.

PLATE 4.32: S.E.M. Detail of Plate 4.31, showing part of the outer
zone (oz) which is clearly hyphal in origin though most
of the component hyphae appear to be without contents.

PLATE 4.33: S.E.M. Detail of Plate 4.30, showing early stages in the
compaction of the outer zone in which crushed cells
appear as parallel lamellae (arrow); the crushed cells
are continuous with hyphae on which clamp connections
(c) are sometimes evident.
PLATES 4.34 - 4.39: Structure of freshly harvested sclerotia of *Paxillus involutus*

PLATE 4.34: S.E.M. Cut face of a sclerotium, showing compaction of outer zone (oz) which appears to be composed of crushed cells of the inner zone (iz) of pseudoparenchyma and, also, of crushed hyphae radiating from the outer surface.

PLATE 4.35: S.E.M. Detail of the inner zone, showing pseudoparenchyma with thin walls and granular, or sometimes amorphous cell contents; some of the cell contents have extended arms (arrow).

PLATE 4.36: S.E.M. Detail of pseudoparenchyma showing large cells interspersed with much smaller cells; the walls are unthickened and appear convoluted.

PLATE 4.37: S.E.M. Detail of the cellular contents of the pseudoparenchyma of an immature sclerotium, showing release of small oil-like globules from the cytoplasm.

PLATE 4.38: T.E.M. Detail of the cellular contents of the pseudoparenchyma, showing cells filled with electron-dense deposits resembling glycogen (g); the cells are highly irregular in shape.

PLATE 4.39: T.E.M. Detail of the cell walls (cw) of the pseudoparenchyma, showing lamellate appearance; fibrillar intercellular material (im) is evident - it has similar staining properties to that of the cell walls.
PLATES 4.40 - 4.45: Structure of freshly harvested sclerotia of *Cenococcum geophilum*

PLATE 4.40: S.E.M. Sclerotium cracked open after critical point drying, showing differentiation of tissue into an inner zone of pseudoparenchyma and a narrow but closely defined outer "rind"; the cells of the pseudoparenchyma have a marked radial arrangement.

PLATE 4.41: S.E.M. Detail of Plate 4.40, showing radial arrangement of the cells of the inner zone (iz); the cells lack contents; the rind (r) is consolidated and has few to no hyphae radiating from its outer surface.

PLATE 4.42: S.E.M. Detail of Plate 4.41, showing rind (r) composed of small cells with occluded lumina.

PLATE 4.43: S.E.M. Detail of Plate 4.41, showing the walls of the pseudoparenchyma; these appear amorphous and are indistinguishable from intercellular material.

PLATE 4.44: S.E.M. Part of the outer surface of a sclerotium, showing rugose appearance, composed of interwoven hyphae encrusted with verrucose material.

PLATE 4.45: S.E.M. Part of the outer surface of a sclerotium, showing uneven "honeycomb" appearance.
PLATES 4.46 - 4.51: Structure of detached sclerotia of *Hebeloma sacchariolens* after burial in unsterile soil.

PLATE 4.46: S.E.M. Cut face of part of a sclerotium retrieved after burial and cracked open after critical point drying, showing an intact, though partially compressed, outer zone (oz); the walls of the inner zone (iz) are similarly intact though the cells lack contents.

PLATES 4.47 - 4.48: S.E.M. Detail of pseudoparenchyma of the inner zone of sclerotia after burial (4.47) and before burial (4.48); the cell walls of buried sclerotia are roughened in appearance, in contrast to the walls of freshly harvested sclerotia.

PLATE 4.49: S.E.M. Detail of pseudoparenchyma of a sclerotium after burial; the walls have remained intact and lipid (arrow) is still evident.

PLATES 4.50 - 4.51: T.E.M. Cross-sections through the outer regions of sclerotia retrieved after burial, showing degradation of the outer zone (Plate 4.50, arrow), microbial colonies being evident (Plate 4.51, arrow); the inner zone of pseudoparenchyma, in which coalesced lipid material (1) is still evident, has remained largely intact (Plate 4.50).
PLATES 4.52 - 4.55: Structure of detached sclerotia of *Hebeloma sacchariolens* and *Paxillus involutus* after burial in unsterile soil

PLATES 4.52 - 4.53: T.E.M. Detail of the pseudoparenchyma of buried sclerotia of *H. sacchariolens*, showing coalesced lipid (l) and residual cytoplasm (cy) in which organelles such as mitochondria (m) are apparent; the walls of the pseudoparenchyma are intact, and extensive intercellular material (im) is evident.

PLATES 4.54 - 4.55: S.E.M. Detail of buried sclerotia of *P. involutus*, showing extensive collapse and consolidation of the component cells; the compaction of the outer zone (oz) seen in mature, freshly harvested sclerotia (Plate 4.34) now extends throughout the inner zone (iz).
PLATES 4.56 - 4.61: Microorganisms associated with sclerotia of *Cenococcum geophilum*

PLATE 4.56: Cut face of a sclerotium is an apparent state of degeneration; the walls of the pseudoparenchyma are roughened as is also seen in sclerotia of *Hebeloma sacchariolens* that had been buried in unsterile soil (Plate 4.47).

PLATES 4.57-4.58: S.E.M. Pseudoparenchymatous region of a sclerotium, showing hyphae of wide diameter "coursing" through cells by means of pits (4.58, arrow); rods resembling bacteria are associated with the hyphae (4.57, arrow).

PLATE 4.59: S.E.M. Pseudoparenchymatous region of a sclerotium, showing narrow pores in the walls which have a smooth appearance and no evidence of an apposition of wall material in their vicinity.

PLATES 4.60-4.61: S.E.M. Pseudoparenchymatous region of a sclerotium showing hyphae of narrow diameter, frequently seen with attached spore-like bodies and often found near hyphae of wide diameter (Plate 4.61); small pores in the walls (arrows) are seen in the vicinity of the hyphae of narrow diameter.
PLATES 4.62 - 4.67: Microorganisms associated with sclerotia of *Hebeloma sacchariolens*

**PLATE 4.62**: S.E.M. Outer surface of a freshly harvested sclerotium on which few, if any, microorganisms are evident.

**PLATE 4.63**: S.E.M. Outer surface of a sclerotium air-dried for 24 h and then buried for 16 weeks in unsterile soil, showing a number of associated microorganisms including round or elliptical spore-like bodies with wart-like protrusions (arrow a), dense colonies of short bacterial rods (arrow b) and large cells with flagellum-like appendages (arrow c).

**PLATE 4.64**: S.E.M. Outer surface of a sclerotium treated like that shown in Plate 4.63, showing an abundance of spore-like bodies with wart-like protrusions.

**PLATE 4.65**: S.E.M. Outer surface of a sclerotium that had been buried for 16 weeks in unsterile soil, but not previously air-dried, showing relatively few associated microorganisms; those that are evident appear to be bacterial rods (arrow).

**PLATES 4.65 - 4.67**: S.E.M. Detail of the pseudoparenchyma of sclerotia that had been air-dried for 24 h and then buried for 16 weeks in unsterile soil, showing dense colonies of long filaments associated with cellular contents (Plate 4.66) and bacterial rods of variable length often associated with empty cells (Plate 4.67, arrow).
PLATES 4.68 - 4.73: Microorganisms associated with sclerotia of *Hebeloma sacchariolens*

PLATES 4.68 - 4.69: S.E.M. Part of the inner zone of a sclerotium that had been air-dried for 24 h and then buried in unsterile soil for 16 weeks, showing a cavity containing a hypha of wide diameter with a clamp connection (Plate 4.68, arrow, 4.69) and a hypha of narrow diameter with attached spore-like bodies (Plate 4.69, arrow).

PLATES 4.70 - 4.73: S.E.M. Detail of inner zone of sclerotia treated like those shown in Plates 4.68 and 4.69, showing hyphae of narrow diameter traversing empty cells; these hyphae occasionally had attached spore-like bodies (Plate 4.70, arrow), appeared fragmented (Plate 4.71), or were frequently branched (Plate 4.72); they were associated with narrow pores in the cell walls (Plate 4.73, arrow).
Unidentified structures found within a sclerotium of *Paxillus involutus* that had been buried for 16 weeks in unsterile soil

PLATE 4.74: S.E.M. Cut face of part of a sclerotium cracked open after critical point drying, showing presence of internal oval structures; the outer zone of the sclerotium appears intact.

PLATE 4.75: S.E.M. The opposite half of the sclerotium shown in Plate 4.74, showing a number of structures with distinct cellular composition and including one which appears to be in a state of degeneration (arrow).

PLATE 4.76: S.E.M. Detail of the outer surface of one of the structures shown in Plate 4.74, showing smooth amorphous external surface closely associated with torulose hyphae.

PLATE 4.77: S.E.M. Detail of Plate 4.75, showing the internal cellular composition of one of the structures; the component cells appear to be without contents.

PLATE 4.78: S.E.M. Detail of Plate 4.75, showing the consolidated outer region of an oval structure; some of the cells appear to contain cytoplasmic contents (arrow).

PLATE 4.79: S.E.M. Detail of Plate 4.75 (arrow) showing an oval structure apparently in a state of degeneration.
PLATES 4.80 - 4.85:

Range of microorganisms isolated from sclerotia of *Paxillus involutus* harvested from the root zone of naturally regenerating birch at Newtongrange and plated onto TSA; the micrographs illustrate the appearance of colonies after 14 days incubation at 20°C.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td><em>Trichoderma</em> sp.</td>
</tr>
<tr>
<td>b</td>
<td><em>Penicillium</em> sp.</td>
</tr>
<tr>
<td>c</td>
<td><em>Penicillium vermiculatum</em></td>
</tr>
<tr>
<td>d</td>
<td><em>Verticillium cinabrinum</em></td>
</tr>
<tr>
<td>e</td>
<td><em>Cylindrocarpon</em> sp.</td>
</tr>
<tr>
<td>f</td>
<td><em>Pythium</em> sp.</td>
</tr>
<tr>
<td>g</td>
<td><em>Mortierella</em></td>
</tr>
<tr>
<td>h</td>
<td>Sterile mycelia, unknown</td>
</tr>
<tr>
<td>i</td>
<td>Sterile mycelia, unknown</td>
</tr>
</tbody>
</table>
SECTION V

Structure of Mycelial Strands Formed by

Some Sheathing Mycorrhizal Fungi on Birch
5.1 Introduction

Except in the case of *Hebeloma sacchariolens*, sclerotia formed by sheathing mycorrhizal fungi are produced laterally along mycelial strands, which, as defined by Townsend (1954), are linear aggregations of modified vegetative hyphae. Because of their association with sclerotia, and because of their potentially more general role in the mycorrhizal symbiosis (Skinner and Bowen, 1974a,b; Reid and Malibari, 1978; Duddridge, Malibari and Reid, 1980; Brownlee et al., 1983) it was of interest to investigate the structure of mycelial strands associated with some sheathing mycorrhizal fungi.

In a review by Watkinson (1979), it was suggested that the formation of mycelial strands, sclerotia, rhizomorphs and coremia produced by a wide variety of fungi involves, in all cases, a fundamental departure from the normal pattern of vegetative growth. Hyphae cease to behave as individuals; instead they merge into aggregated structures which then differentiate into a distinct form. Similar basic processes seem to be involved in the development of sclerotia and mycelial strands (Willetts, 1972; Watkinson, 1979); in both cases, initials develop by localised aggregation of hyphae and, for this to occur, the factors that lead to the normal divergence of vegetative hyphae, one from another, must be overcome. Thus, in vegetative mycelium growing in nutritive environments, there is a tendency for hyphae to grow up a diffusion gradient of nutrients and/or away from the staling products that accumulate around other hyphae during active metabolism (Garrett, 1960). However, in nutrient-poor or non-nutritive environments, there is a reported leakage of translocated material from hyphae (Watkinson, 1971a,b, 1975); such leakage provides localised regions of relatively high nutrient concentration, which are suggested to promote localised growth of
hyphae towards one another. This hypothesis (Garrett, 1960; Watkinson, 1971a) is supported by the frequent observation that sclerotia and mycelial strands characteristically develop in aged or staled media in which conditions are no longer conducive to normal vegetative growth. With respect to the initiation of sclerotia, Willetts (1972) has further suggested that secretion of mucilaginous polysaccharide gels, which eventually form intercellular deposits between hyphal aggregates, may, apart from serving to bind the hyphae together, exert a nullifying effect on the forces that normally "inhibit hyphal association and contact". After the initial aggregation of hyphae, subsequent development of both sclerotia and mycelial strands involves increased branching and septation, hyphal fusion, deposition of intercellular matrices and differentiation of tissue.

Much research has been done on the mycelial strands of *Serpula lacrimans* (Fr.) Karst, a commercially important fungus causing dry rot of timber (Watkinson, 1971a,b, 1975, 1979; Coggins, Jennings and Clark, 1980; Brownlee and Jennings, 1981; Jennings and Watkinson, 1982). Structurally, the strands of this fungus represent an intermediate type between the simple, relatively undifferentiated mycelial strands produced by, for example, *Helicobasidium annosum* Pat. (Garrett, 1946) and the highly organised rhizomorphs of *Armillaria mellea* (Vahl ex Fr.) Kummer which grow from an apical zone that has been likened to the apical meristem of higher plants (Townsend, 1954).

As described by Butler (1958), mycelial strands of *S. lacrimans* develop by the gradual accretion, from the base, of narrow branch hyphae around older, wide parent hyphae; the strands are thus seen in cross-section to be composed of a core of wide "vessel" hyphae that appear aseptate, thin-walled and without contents, surrounded by
several layers of "tendril" or "structural" hyphae which are narrow, thin-walled and contain cytoplasm. At a later stage of development, narrow, highly thickened "fibre" hyphae develop; in their lamellate wall structure and almost occluded lumina they resemble the "fibre" hyphae of *A. mellea* (Townsend, 1954) and the skeletal hyphae of fruitbodies of, for example, *Polystictus xanthopus* (Corner, 1932).

Although there is incontrovertible evidence that mycelial strands of *S. lacrimans* (Watkinson, 1971b, 1975) and some sheathing mycorrhizal fungi (Skinner and Bowen, 1974a; Reid and Malibari, 1978; Duddridge *et al.*, 1980) can absorb and translocate water and nutrients, it has not been demonstrated that the aggregation of hyphae makes them more efficient in translocation than are undifferentiated hyphae (Butler, 1966; Watkinson, 1979). The vessel hyphae of *S. lacrimans*, as described by Butler (1958), are wide and aseptate and therefore may function as water conduits, yet they are also thin-walled and seem unlikely to be able to withstand the internal hydrostatic pressure postulated by Jennings *et al.* (1974) as the driving force for translocation. More recent ultrastructural studies (Jennings and Watkins, 1982), however, have indicated that mature strands of *S. lacrimans* are structurally different from the developing strands described by Butler (1958), and have an organisation of tissue consistent with their proposed role as organs involved in translocation. Thus, at maturity, outer layers of extremely hard, thick-walled "fibre" hyphae surround a core of longitudinal, hollow channels without obvious walls but which are embedded in an extensive extrahyphal matrix composed of the original intercellular material and the cell walls of collapsed, dead hyphae. It was not determined whether the central channels represent the original lumina of hyphae that have undergone inflation, or if the complete lysis
of the vessel hyphae leaves spaces that are then effectively extra-hyphal. Nevertheless, Jennings and Watkinson (1982) have proposed that the inextensible extrahyphal matrix can probably confer sufficient structural rigidity for the hollow channels to withstand pressure-driven mass flow of water and nutrients as postulated by Jennings et al. (1974).

Garrett (1960) has proposed that the main role of mycelial strands may be, not so much as efficient translocatory organs, but rather as organs with sufficient inoculum potential, provided by the aggregated hyphal apices, for infection or colonisation of new hosts or substrates. The potential importance of this role of mycelial strands of sheathing mycorrhizal fungi with respect to the observed succession of mycorrhizal fungi on host roots (Section 1.6) has been investigated by Fleming (1983a,b) with respect to the distinction between "early-stage" and "late-stage" fungi in the sense of Deacon et al. (1983) but is still not fully understood.

5.2 Materials and Methods

Mycelial strands associated with some sheathing mycorrhizal fungi, namely *Lactarius rufus*, *Leccinum scabrum* and *Paxillus involutus*, were obtained from several sources during 1982 and 1983 and prepared for examination with the scanning electron microscope (S.E.M.) and transmission electron microscope (T.E.M.) as described in Sections 2.2.5 and 2.2.6. Mycelial strands of *L. rufus* and *P. involutus* were collected during the autumn of 1982 from beneath fruitbodies of these species around naturally regenerating birch on a disused coal spoil tip at Newtongrange (Section 2.1.6.1) and examined with the light microscope and scanning electron microscope. Mycelial strands of *L. scabrum*
were similarly collected from beneath fruitbodies of this species at Newtongrange, and, in addition, samples were harvested, during the spring of 1983, from mycorrhizal roots in the vicinity of the previous year's fruitbodies at the experimental birch plot, I.T.E., Bush Estate (Section 2.1.6.2); a detailed study of samples from both sources was made with the light microscope, S.E.M. and T.E.M.

5.3 Structure of Mycelial Strands Associated with Sheathing Mycorrhizas of *Paxillus involutus* on Birch

The main distinguishing features of sheathing mycorrhizas of *Paxillus involutus* on birch (Plate 5.1) are their pale buff to golden brown pigmentation and their association with globose sclerotia (Section 4.5) subtended by mycelial strands (Plates 5.1, 5.2, 5.3).

The mycelial strands comprise loose aggregates of longitudinally orientated hyphae, groups of which frequently radiate and then converge to form a dense interconnecting network between mycorrhizal roots; the hyphal composition is always evident because of the abundance of radiating hyphae and this feature distinguishes the mycelial strands of *P. involutus* from those of some other sheathing mycorrhizal fungi (Sections 5.4, 5.5) which, when fully differentiated, generally lack radiating hyphae over most of their length and generally have an amorphous surface appearance.

Small hyphal aggregates, which are presumed to represent immature stages of strand development, were harvested from 18-week old pot-grown birch seedlings and stained in 1% aqueous nile blue (Jennings and Watkinson, 1982). They were composed of interwoven hyphae of two types though the distinction was not always clear. The majority of the hyphae stained unevenly; they were frequently
branched, several branches arising at localised points in the vicinity of clamp connections. The branches so formed ran closely adpressed to the parent hypha and to one another, with shorter lateral branches frequently encircling groups of three to five parallel hyphae and seeming to bind them together as seen, for example, in Plate 5.4 (arrow). Hyphae of wide diameter, which tended to occur near the centre of the aggregates, did not react with the stain. Both immature mycelial strands and more highly differentiated, presumably mature, strands failed to react with Lugol's iodine (Section 4.4); this was in contrast to sclerotia, and, also, in contrast to mycorrhizas, which reacted strongly with the stain thus indicating the presence of glycogen.

Many of the mycelial strands harvested during the autumn of 1982 from the root zone of birch at Newtongrange, appeared moribund. They were composed mainly of residual wall material of collapsed, apparently dead hyphae (Plate 5.5) but the central region of the strands usually contained two or three apparently hollow channels that seemed to have been formed by collapse of the walls of wide hyphae. Where sections were taken through mycelial strands attached to sclerotia (Plates 5.6, 5.7), the hyphae of the strands and of the sclerotia in the zone of contact were seen to be of similar shape and size and had cellular contents, but the rest of the sclerotium was composed of larger, apparently empty cells whereas the mycelial strands were composed predominantly of collapsed wall material. The outer prosenchymatous zone of the sclerotium was continuous with the surface hyphae of the mycelial strand (Plate 5.7).

In other samples, however, the mycelial strands of P. involutus showed more obvious differentiation: they comprised several layers of narrow hyphae surrounding, and interspersed with, 7-12 longitudinally
running hyphae of intermediate to wide diameter (Plates 5.8 - 5.12). The narrow hyphae comprising the outer regions of the strands were loosely interwoven and bore outwardly radiating branches (Plate 5.8); they were of usually 3.5 µm diameter but frequently appeared irregularly swollen. In cross-section, the narrow hyphae appeared empty and their cell walls were smooth and apparently unthickened (Plates 5.9, 5.10); they frequently appeared compressed (Plate 5.11) and their walls seemed to be intimately associated with those of wider hyphae where they were contiguous (Plate 5.12, arrow).

The wider hyphae were also empty (Plate 5.9) but differed from the narrower ones in having more thickened (Plate 5.11, arrow), but still apparently non-lamellate, walls; thus the walls of the wider hyphae were approximately 0.5 µm thick, compared with 0.24 µm for the narrower hyphae. The component hyphae of the mycelial strands were separated by intercellular spaces (Plates 5.10, arrow, 5.11), though in some regions there was evidence for an amorphous intercellular matrix (Plate 5.10).

The differentiation of tissue in the mycelial strands of *P. involutus* as seen with the S.E.M., was, in some respects, similar to that described in *S. lacrimans* (Butler, 1958) in which several layers of narrow hyphae surround a core of wider hyphae. Hereafter, the terms "structural" and "vessel" hyphae, as used by other workers (Butler, 1958; Foster, 1981; Jennings and Watkinson, 1982), will be used to distinguish the narrow and wide hyphae respectively.
5.4 Structure of Mycelial Strands Associated with Sheathing Mycorrhizas of *Leccinum scabrum* on Birch

Mycelial strands associated with sheathing mycorrhizas of *Leccinum scabrum* were harvested from two sources: (1) during the autumn of 1981 from beneath fruitbodies of this species associated with naturally regenerating birch on coal spoil at Newtongrange, and (2) during the spring of 1983 from the vicinity of the previous year's fruitbodies of *L. scabrum* around birch at the experimental plot, I.T.E., Bush Estate. Preliminary studies with the light microscope and scanning electron microscope were done using material harvested from the experimental birch plot; further investigations were made with the transmission electron microscope using material harvested from Newtongrange.

*Light microscopy*

Mycorrhizas of *L. scabrum* (Plate 5.13) are generally associated with distinctive mycelial strands which are mottled, cream-to-yellow pigmented structures, with a diameter normally ranging from 0.1 to 0.2 mm, though strands attached to the base of fruitbodies are more irregular, often tangentially flattened and may be up to 1.5 mm diameter. Mycelial strands traverse considerable distances from mycorrhizal roots in soil, and leading hyphal aggregates give rise to many narrower aggregates which arise obliquely or at right angles; the surfaces of the narrower aggregates bear numerous individual radiating hyphae (Plates 5.14, 5.15). At high magnifications the strands are seen to be discrete structures comprising tightly interwoven, longitudinally orientated hyphae (Plate 5.16). The component hyphae, which are difficult to tease apart without prior treatment in 10% KOH, are of two types.
(Plates 5.17, 5.18). The outer part of the strand comprises many layers of closely packed hyphae of narrow diameter surrounding, and interspersed with, hyphae of wide diameter in the central regions of the strands. The eccentric position of two wide hyphae in Plate 5.18 may represent a branch arising from the parent strand.

In longitudinal section as seen by the light microscope, the narrow "structural" hyphae were seen to have a diameter of approximately 40 μm; they were frequently septate with septa occurring at 24-26 μm intervals and clamp connections occurring on every 2 to 4 septa. Several branches arose from the vicinity of clamp connections and the branches so formed remained closely adpressed to the parent hyphae. The reaction of these hyphae to Lugol's iodine (Section 4.4), used to detect glycogen, was positive in some regions along the hyphae but not in others. The wide "vessel" hyphae often had thicker cell walls than those of the "structural hyphae". In the youngest regions of the strands, where the apex had a swollen bulbous appearance, the wide hyphae had very short interseptal distances. Behind the apical zone, however, septa were less frequent, occurring at approximately 70 μm intervals, and they appeared to be absent from some hyphae. Glycogen was detected with Lugol's iodine in some parts of the wide hyphae, especially near the tips of the strands.

**Scanning electron microscopy**

For S.E.M. studies, mycelial strands of *L. scabrum* were harvested during the spring of 1983 from the vicinity of the previous year's fruitbodies of this species at the experimental birch plot, I.T.E., Bush Estate. They showed a marked zonation of differentiated tissue, the inner regions containing wide "vessel" hyphae interspersed with narrow "structural" hyphae, and the outer regions being composed only
of small structural hyphae; this confirms preliminary observations
made with the light microscope (Plates 5.17, 5.18).

Most samples appeared to have a discrete but undifferentiated
peripheral zone composed of tightly packed, longitudinally orientated
hyphae (Plates 5.19, 5.20, 5.26, 5.28), giving the external surface a
markedly ridged appearance (Plates 5.16, 5.28, 5.29) from which a
few branched hyphae radiated. Although the basic structure was similar
in all of the 30 mycelial strands examined, the mycelial strands never-
theless varied with respect to their cell contents, and three categories
of strands from within the one sample at I.T.E., Bush Estate, are
treated separately below.

1. In some mycelial strands (Plates 5.19 - 5.25), 10-12 layers of
narrow, evenly-sized hyphae of approximately 3-8 µm diameter and
rounded in cross-section surrounded a zone containing 12-14 hyphae
of much wider (20 µm) diameter (Plates 5.19, 5.20). In several
instances - for example, Plates 5.18, 5.19 - hyphae of wide diameter
were eccentrically positioned, perhaps representing the origin of
a branch of the mycelial strand. The "structural" hyphae of narrow
diameter had distinctive cell walls, varying in width from 0.3 to
0.6 µm, and were filled with dense cytoplasmic contents which
appeared either granular, with numerous wart-like or papillate
protrusions (Plates 5.22, arrow 1, 5.25), or less dense and reticulate
(Plate 5.22,arrow 2); a few hyphae appeared to be empty (Plate
5.22, arrow 3). Groups of individual structural hyphae appeared
to be separated by intercellular spaces although intercellular
deposits were also evident, adjacent hyphae frequently being
embedded in an amorphous matrix (Plate 5.22, arrow 4). Occasion-
ally, spherical bodies were seen attached to the external surfaces of
individual hyphae (Plate 5.22, arrow 5).
The wide "vessel" hyphae were distinctive in that the cell walls were highly thickened, approximately 1.2 μm diameter, and had a lamellate appearance (Plates 5.23, 5.24); in some instances the wall layers of these hyphae were seen to have separated, one from another (Plate 5.21, arrow 1).

The vessel hyphae, like the structural hyphae, varied in internal composition. Some (Plates 5.21, arrow 2, 5.24) appeared mostly empty apart from large spherical bodies and other, apparently collapsed, material lining the walls. But other vessel hyphae, particularly those of intermediate diameter (Plate 5.23), were filled with a reticulum as seen in some of the structural hyphae. This material resembles that described by Foster (1981) and may represent a stage in the dissolution of the cytoplasm. The formation of vessel hyphae appeared to occur through the progressive breakdown of cell walls between adjacent structural hyphae and vessel hyphae of intermediate diameter (Plates 5.23, 5.25), the contents of which then apparently degenerated.

In some of the mycelial strands, all of the structural hyphae were filled with the same reticulate material (Plates 5.26, 5.27) as that seen in some structural hyphae and vessel hyphae of the first type described above (Plates 5.22, arrow 2, 5.23) though a few appeared to be empty (Plate 5.27, arrow 1). The structural hyphae were round in cross-section, evenly-sized and of approximately the same diameter (3.7 μm) as those described earlier; however, they appeared to be more loosely arranged and apparently separated by intercellular spaces (Plate 5.27, arrow 2). Some intercellular deposits were evident (Plate 5.27, arrow 3), although they were not extensive.
The vessel hyphae were distinctive, 15-16 μm diameter, and lay closely associated with one another (Plate 5.27); their walls showed no evidence of lamellae as were seen in the first type of mycelial strand described above. An interesting feature of these strands, as shown in Plate 5.27, arrow 4, was the presence of some hyphae intermediate in size between the large vessel hyphae and the small structural hyphae, and similarly intermediate in wall thickness; such "intermediate" hyphae were usually present near the centre of the strands and had reticulate contents. They may represent a stage in the development of wider vessel hyphae.

3. A third type of mycelial strand (Plates 5.28 - 5.31) was composed of fewer structural hyphae and these had a slightly narrower mean diameter (3.0 μm) than those described previously; in addition, the central "core" of vessel hyphae was indistinct, being composed of hyphae of only slightly wider diameter (5.6 μm) than the structural hyphae (Plate 5.28). A striking feature of these mycelial strands was the abundance of wart-like protrusions over the surface of the component hyphae (Plates 5.28, 5.29); interestingly, these structures did not occur, or were much less conspicuous, on the hyphal branches radiating from the strands (Plate 5.28, arrow). Possibly equivalent irregularly sized bodies lined the internal walls of the structural hyphae, where they frequently appeared to be fused with one another (Plate 5.30). In some instances, conglomerations of apparently similar material filled the structural hyphae (Plate 5.31).
Transmission electron microscopy

Mycelial strands of *L. scabrum* were harvested from beneath fruitbodies of this species associated with birch at Newtongrange and were examined with the T.E.M. In all respects they were similar in structure to material harvested in the spring from roots mycorrhizal with *L. scabrum* occurring in the vicinity of the previous year's fruitbodies at the experimental birch plot, I.T.E., Bush Estate.

Observations with the T.E.M., on transverse sections cut through mycelial strands of *L. scabrum*, confirmed the complex organisation of tissue comprising the strands, as seen with the light microscope and S.E.M. Thus, differentiated strands were composed of two distinct types of hyphae: thin-walled 'structural' hyphae of narrow diameter surrounded, and were interspersed with, thick-walled, apparently empty hyphae of much wider diameter (Plates 5.32, 5.33, 5.34).

Six to seven layers of structural hyphae with a diameter of approximately 3.6 μm formed the outer part of each mycelial strand (Plate 5.32), but at the surface of the strands there was no evidence of a membranous or gelatinous sheath (Plate 5.32, arrow). In the peripheral two to five layers of hyphae, the spaces between adjacent hyphae were often filled with an electron-dense, amorphous material but this was not extensive and some intercellular spaces were seen in all parts of this region (Plates 5.32, 5.35). This outer zone was characterised by the presence of small electron-dense bodies on the outsides of the hyphae (Plates 5.32, 5.35, 5.37); similar bodies were seen within the hyphae but they were more electron-dense (Plates 5.35, 5.36). The extrahyphal bodies were hemispherical to globose in shape and of approximately 0.4 μm diameter, and in places they appeared to be continuous with the outer layer of the hyphal walls (Plates 5.37, 5.39);
sometimes they were stalked (Plate 5.39, arrow). In a few instances two such bodies seemed to fuse with one another in the intercellular spaces between adjacent hyphae (Plate 5.38, arrow). In some cases — for example, Plate 5.38 — the bodies appeared to have discrete outer edges, whereas in others — for example, Plate 5.39 — the outer edge was diffuse. The intracellular bodies varied in size but were generally larger than the extracellular bodies; they seemed most often to line the cell vacuoles (Plates 5.36, 5.41, 5.42) and even when they appeared to be adpressed to the wall, higher magnifications (Plates 5.41, 5.42) revealed a narrow zone of cytoplasm between the intracellular bodies and the walls. Frequently, individual bodies seemed to fuse to form a large electron-dense mass ranging from 0.8 to 1.0 μm diameter (Plates 5.36, arrow, 5.42, arrow 2).

Towards the centre of the mycelial strands, the structural hyphae became more closely packed, the intercellular spaces were seen to be filled with an amorphous material (Plate 5.36), and the intercellular bodies appeared to have fused with the intercellular material (Plates 5.40, 5.42, arrow 1), eventually becoming indistinguishable from it (Plates 5.41, 5.42, 5.43). The ultrastructural evidence strongly suggests that the extracellular bodies seen on the outside of the mycelial strands represent an early stage in the development of the intercellular matrix.

The structural hyphae varied with respect to their intercellular contents (Plate 5.34, arrows 4,5). Some appeared to be metabolically active as evidenced by the presence of ribosomes (Plate 5.42), organelles resembling mitochondria (Plate 5.41) and nuclei (Plate 5.41). Other hyphae appeared highly vacuolate (Plate 5.34, arrow 4), sometimes with conspicuous plasmalemmasomes (Plate 5.47, arrow 6). But almost all, if not all, of the hyphae contained some cytoplasm.
The central region of the mycelial strands was occupied by a number of hyphae of intermediate to large diameter, ranging from 17 to 24 μm (Plates 5.33, 5.34, 5.46). These "vessel" hyphae generally appeared empty (Plate 5.46), though in some of them highly condensed residual cytoplasm was evident (Plates 5.33, 5.34); its appearance is indicative of a more rapid cell death than is seen in the residual cell contents of some of the structural hyphae (P. Jeffries, pers. comm.). The walls of the vessel hyphae were considerably thickened and lamellate in appearance (Plates 5.33, 5.34, 5.46), the wall thickness (1.6 - 2.4 μm diameter) representing a five to eight-fold increase over that of the structural hyphae. Such wall thickening of the vessel hyphae could be important with regard to the potential role of mycelial strands as translocatory structures. There was extensive deposition of intercellular material in the centre of the strands; this matrix generally appeared amorphous (Plates 5.34, 5.44, 5.46) and closely associated with the hyphal wall layers (Plates 5.43, 5.44, arrow 1), but in other regions the matrix appeared reticulate (Plate 5.48). In one instance (Plates 5.34, arrow 1, 5.48), a large plasmalemmasome-like body composed of bilamellate structures arranged in short thick fascicles and in whorls was seen in the intercellular matrix. Its occurrence is interesting because plasmalemmasomes are normally, if not exclusively, intracellular structures (P. Jeffries, pers. comm.).

The association between the narrow structural hyphae and the intermediate sized and wide vessel hyphae appeared to be extremely close in some parts of the mycelial strands (Plates 5.44, arrow 3, 5.45, arrow). Of interest, some of the narrow hyphae in these regions were densely cytoplasmic, and these apparently metabolically active hyphae were much less regular in shape than were the other, highly vacuolate,
narrow hyphae (Plates 5.44, arrow 2, 5.47, arrow 1): they frequently appeared triangular, diamond-shaped or elongate in section (Plate 5.44, arrow 2). In some instances these hyphae had similar dimensions where they were adjacent to one another (Plates 5.44, arrow 5, 5.47, arrow 3). The simplest explanation of these observations is that these hyphae were not orientated parallel to the axis of the mycelial strand but, rather, had been sectioned obliquely or longitudinally in some instances, suggesting that they were "coursing" their way through the intercellular spaces or matrix between the longitudinal running hyphae; if so, then structures like those marked in Plates 5.34, arrows 2 and 3, and shown at higher magnification in Plates 5.46, arrows 2 and 3, might represent septa. It may thus be suggested that these were younger hyphae than the majority (or else they had retained their juvenility), and had developed secondarily after the main part of the mycelial strand had formed. Of especial interest, in several instances these "coursing" hyphae seemed to have fused with empty hyphae of intermediate size as shown in, for example, Plates 5.45, arrow, 5.44, arrow 4, the cell walls being absent from the points of contact. Possibly, these instances represent stages in the development of wide vessel hyphae. The occurrence of the "coursing" hyphae, and the ultra-structural evidence for their eventual degeneration, may explain why plasmalemmasome-like bodies (Plate 5.48) and other apparent cytoplasmic debris (Plate 5.47, arrows 4 and 5) were found in the intercellular matrix.
5.5 Structure of Mycelial Strands Associated with Sheathing Mycorrhizas of *Lactarius rufus* on Birch

Mycelial strands associated with sheathing mycorrhizas of *Lactarius rufus* were harvested, during the autumn of 1982, from beneath fruitbodies of this species occurring around birch at Newtonrange. Only a few samples were investigated; however, the strands, which were evidently degenerating, showed several interesting features and they have therefore been included in this section.

Mycelial strands of *L. rufus* were of notably uniform width (0.1 mm) along their length and branched frequently; they ramified extensively between mycorrhizal roots and extended for considerable distances into the surrounding soil. They were white, in contrast to the rust-brown mycorrhizas, and had a ribbed appearance (Plate 5.49) with only occasional radiating hyphae.

As seen with the S.E.M., the mycelial strands were composed of two to four layers of 'structural' hyphae surrounding one or more 'vessel' hyphae of intermediate to wide diameter and surrounding, also, some large irregularly shaped lacunae in which there was evidence of the breakdown of hyphal walls (Plate 5.51). A continuous membranous covering was seen over the outer surface of the strands (Plates 5.50, 5.51, 5.53). The structural hyphae of approximately 11.0 µm diameter were round or angular in cross-section and they appeared empty. The central vessel hyphae were of similar general appearance but were wider, approximately 25-40 µm diameter (Plate 5.51); their wall thickness could not be determined exactly from scanning electron micrographs, because parts of the walls of adjacent, disintegrated structural hyphae remained attached to the vessel hyphae, increasing their apparent wall
thickness (Plate 5.53, arrow). Although none of the component hyphae of the mycelial strands showed evidence of cell contents, yet a number of narrow hyphae of approximately 3.5 - 4.0 \( \mu \text{m} \) diameter seemed to run longitudinally through the hollow spaces of the strands (Plate 5.50, arrow) and also, in some parts, through the lumina of the structural hyphae (Plates 5.51, arrow 1, 5.52, arrow). These narrow hyphae may have been hyphae of \textit{L. rufus} growing "intrusively" in the strands, as was described for \textit{S. lacrimans} by Jennings and Watkinson (1982); alternatively, they may have been invading hyphae of other fungi in the moribund mycelial strands.
5.6 Discussion

The light microscopical and electron microscopical studies in this section of the thesis have shown a basic organisation of the mycelial strands of *Lactarius rufus*, *Leccinum scabrum* and *Paxillus involutus* similar to that described for mycelial strands of other fungi. Most notably, the mycelial strands of the sheathing mycorrhizal fungi studied here contained (1) centrally positioned hyphae of wide diameter with little or no cytoplasmic content, equivalent to the 'vessel hyphae' described by Butler (1958, 1966), Watkinson (1971a, 1979) and Jennings and Watkinson (1982) for *Serpula lacrimans*, and by Foster (1981) for mycelial strands associated with an unidentified sheathing mycorrhizal fungus on *Pinus radiata*, and (2) narrower hyphae, some of which had cytoplasmic contents, equivalent to the "structural hyphae" in strands of *S. lacrimans* (Butler, 1958; Jennings and Watkinson, 1982) and an unidentified mycorrhizal fungus on pine (Foster, 1981). The structural hyphae were always seen in a zone around the vessel hyphae but were also interspersed with these. The most notable difference between the mycelial strands studied here and those of other fungi is the absence of any recognisable or distinct "fibre" hyphae from the mycelial strands of *L. rufus*, *L. scabrum* and *P. involutus*. Such fibre hyphae are described for mycelial strands of *S. lacrimans* (Butler, 1958; Jennings and Watkinson, 1982), an unidentified mycorrhizal fungus of *P. radiata* (Foster, 1981) and rhizomorphs of *Armillaria mellea* (Garrett, 1960); they are thought to be equivalent to the skeletal hyphae in fruitbodies of *Polystictus xanthopus* (Corner, 1932). In mycelial strands of *S. lacrimans* they are found to develop only upon ageing; nevertheless, some of the material used in my work was of a similar age to that used by other workers (Butler, 1958; Watkinson, 1971a, 1979), so the absence of fibre hyphae cannot be ascribed solely to juvenility of my material.
Mycelial strands are generally believed to function as translocatory structures and experimental evidence in support of this view has been obtained for translocation of water (Reid and Malibari, 1978; Duddridge et al., 1980; Brownlee et al., 1983), carbohydrates (Brownlee and Jennings (1981) and mineral nutrients, most notably nitrogen (Watkinson, 1975, 1979) and phosphorus (Watkinson, 1971b; Skinner and Bowen, 1974a,b), though there is no evidence that mycelial strands are more efficient in this respect than is undifferentiated mycelium. It is also generally assumed that most of the translocation of water and nutrients occurs in the wide vessel hyphae. The mechanism of translocation in strands of *S. lacrimans* has been suggested to be by a pressure-driven mass flow (Jennings et al., 1974) and this is assumed to require structural rigidity of the strand such that it can withstand internal hydrostatic pressures. Early observations (Hartig and von Tubeuf, 1902, Faick, 1912) suggested that the vessel hyphae have "beam" and "ring" thickenings, analogous to those in sclerenchyma and xylem vessels in higher plants, but this has not been substantiated. Instead, the structural rigidity of mycelial strands and thus their capacity as translocatory organs is suggested to be conferred by (a) an outer zone of extremely thick-walled fibre hyphae, (b) close packing of the internal structural and vessel hyphae, and (c) a large degree of infilling by intercellular matrix material. Jennings and Watkinson (1982) suggest that the latter is most important in helping to restrain the vessel hyphae so that these thin-walled hyphae can withstand the forces associated with pressure-driven mass flow. In *L. scabrum* there is a large degree of such infilling by intercellular matrix material (Section 5.4), consistent with reports for *S. lacrimans* (Jennings and Watkinson, 1982) and also for an unidentified mycorrhizal symbiont.
of *P. radiata* (Foster, 1981). However, the vessel hyphae of *L. scabrum* also have noticeably thicker walls than do the adjoining and associated structural hyphae - a feature not reported for *S. lacrimans* but seen also in some of the micrographs of mycelial strands of the mycorrhizal fungus studied by Foster (1981). Plates 5.34 and 5.46, in this thesis, show clearly the lamellate thickened nature of the walls of the vessel hyphae of *L. scabrum*.

The origin of the intercellular matrix material cannot be determined with certainty, but in the youngest hyphae (at the periphery) of mycelial strands of *L. scabrum* an extrahyphal material was seen as discrete electron-dense bodies on the hyphal walls; towards the centre of the strands these bodies appeared to merge imperceptibly with a general matrix material with similar staining properties. It thus seems that the matrix is deposited first in more or less discrete localised regions on the hyphal surface and later these regions fuse or merge with one another. Foster (1981) reported that a polysaccharide was released from structural hyphae on the surfaces of the mycelial strands of an unidentified mycorrhizal fungus, and this material, as seen in some of his electron micrographs, is similar to that seen on hyphae of *L. scabrum* (Plates 5.28 and 5.29). In a few instances, plasmalemmasome-like bodies or apparently degenerate accumulations of cytoplasm were seen in the interhyphal spaces or embedded in the matrix (Plates 5.47, 5.48). This is indicative of hyphal lysis or disruption, so it can be suggested that some of the matrix material may arise not merely by secretion from hyphae but rather from hyphal disintegration. Jennings and Watkinson (1982) have stated that the intercellular matrix in mycelial strands of *S. lacrimans* is largely composed of hyphal walls in different stages of compression and collapse. My work with *L. scabrum* suggests that such
collapse provides only a minor component of the intercellular matrix material. However, most of the mycelial strands of *P. involutus* that were examined contained an abundance of collapsed hyphae (Plate 5.5), so in this respect *P. involutus* may be similar to *S. lacrimans*.

**Origin of vessel hyphae**

The origin and role of development of vessel hyphae is one of the most problematical aspects of strand morphogenesis. In *L. scabrum* (Section 5.4), as in *S. lacrimans* (Jennings and Watkinson, 1982) and in mycelial strands of an unidentified mycorrhizal fungus of *P. radiata* (Foster, 1981), the vessel hyphae are closely associated with smaller structural hyphae and may show indentations in the regions of association with structural hyphae (Plate 5.44, arrow 3). Moreover, sometimes the walls of vessel hyphae and structural hyphae seem to fuse to form a common wall (Plate 5.47, arrow 2) while in some instances the evidence suggests that the walls break down to give cytoplasmic continuity (Plate 5.45, arrow). It is thus possible that some of the vessel hyphae develop and enlarge by lateral fusion with adjacent structural hyphae. Of especial interest in this respect, many of the structural hyphae closely associated with the vessel hyphae have dense and apparently functional cytoplasmic contents (Plates 5.44, arrow 4, 5.47), in contrast to many of the structural hyphae comprising the bulk of the mycelial strand. Either the apparently metabolically active structural hyphae have retained their contents longer than have the majority, or the metabolically active hyphae are younger. The latter is possibly consistent with reports of "intrusive growth" of hyphae in mycelial strands of *S. lacrimans* (Jennings and Watkinson, 1982). An interesting feature seen here, but not previously reported, is that several of the metabolically active structural hyphae appear to have been sectioned
longitudinally or para-longitudinally in transverse-sections of mycelial strands of *L. scabrum*; indeed in a few instances their dolipore septa were seen in transverse section (Plate 5.46, arrows 2 and 3). It thus appears that these hyphae run in all directions within the "body" of the strand, in the intercellular spaces and matrix. In preliminary studies on mycelial strands of *L. rufus*, some intrusively growing hyphae were seen in the lacunae formed apparently by the breakdown of other hyphae. In *S. lacrimans*, Jennings and Watkinson (1982) have reported that intrusive growth occurs within fibre hyphae as well as in the interhyphal matrix, and the subsequent degeneration of the intrusive hyphae contributes to the wall thickening of the fibre hyphae.

In summary of this section, it is clear that the mycelial strands of the sheathing mycorrhizal fungi show several parallels with those of *S. lacrimans*, the strand-forming basidiomycete studied most intensively to date. But, as in the case of *S. lacrimans*, there is still a need for a complete developmental study involving all sequential stages of strand development and maturation, because the precise origins of some of the component parts of the strands can as yet only be determined by inference.
PLATES 5.1 - 5.2: Structure of mycelial strands of *Paxillus involutus*

PLATE 5.1: Light micrograph of sheathing mycorrhizas with associated mycelial strands (ms), and a sclerotium (s).

PLATE 5.2: S.E.M. Part of a sclerotium (s) subtended by a differentiated mycelial strand (ms) composed of longitudinally orientated, interwoven hyphae with frequent radiating hyphal branches.
PLATES 5.3 - 5.8: Structure of mycelial strands of *Paxillus involutus*

PLATE 5.3: S.E.M. Zone of attachment of sclerotium (s) to mycelial strand (ms), showing close interweaving of the hyphae of the strand; branches radiating from the strand bear frequent clamp connections (arrow).

PLATE 5.4: S.E.M. Mycelial strand traversing a secondarily thickened root of birch; small groups of parallel hyphae with clamp connections (c) are bound together by short lateral branches (arrow).

PLATE 5.5: Light micrograph of a cross-section through a moribund mycelial strand showing central hollow channels in which collapsed walls of wide hyphae are evident; the channels are embedded in a dense extrahyphal matrix composed of collapsed walls of apparently dead hyphae.

PLATE 5.6: Light micrograph of a cross-section through a sclerotium (s) and mycelial strand (ms); the sclerotium is composed of irregularly shaped, closely packed cells whereas the mycelial strand is composed mainly of densely staining collapsed wall material.

PLATE 5.7: Detail of Plate 5.6 showing zone of attachment in which the cells comprising the sclerotium and the mycelial strand are irregularly shaped but of similar size, closely packed and appear to have contents.

PLATE 5.8: S.E.M. Surface appearance of differentiated mycelial strand in which the outer layers of hyphae appear loosely interwoven and bear many short radiating branches; a broken end of the strand is visible at the extreme right of the micrograph.
PLATES 5.9 - 5.10: Structure of mycelial strands of *Paxillus involutus*

PLATE 5.9: S.E.M. Cross-section through a mycelial strand, showing wide, apparently empty hyphae interspersed with narrow hyphae.

PLATE 5.10: S.E.M. Detail of Plate 5.9, showing narrow hyphae, some of which appear compressed, separated by intercellular spaces (arrow) and some intercellular material (im); the walls of the narrow hyphae are amorphous and apparently unthickened.
PLATES 5.11 - 5.12: Structure of mycelial strands of *Paxillus involutus*

PLATE 5.11: S.E.M. Detail of Plate 5.10 showing compressed narrow hyphae; the walls of the wide hyphae (arrow) are twice the thickness of those of the narrow hyphae.

PLATE 5.12: S.E.M. Detail of Plate 5.9; the walls of the narrow hyphae appear, in places (arrow), to be intimately associated with those of adjacent hyphae of wide diameter.
PLATES 5.13 - 5.18: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.13: Light micrograph of sheathing mycorrhizas of *L. scabrum* on birch, with attached mycelial strands.

PLATES
5.14 - 5.15: S.E.M. External appearance of mycelial strands, showing main wide hyphal aggregates with narrower, branch aggregates that arise obliquely or at right angles and that have more radiating hyphae than do the main aggregates; clamp connections and irregular swellings (Plate 5.15, arrow) are seen.

PLATE 5.16: Detail of Plate 5.15, showing that the strands are composed of closely packed, longitudinally orientated hyphae without any obvious modifications to the outer layers of hyphae.

PLATES
5.17 - 5.18: Light micrographs of cross-sections through mycelial strands, showing differentiation into wide, irregularly shaped hyphae surrounded and interspersed with narrow, closely packed hyphae; the eccentric arrangement of some wide hyphae in Plate 5.18 may represent a branch of the mycelial strand.
PLATES 5.19 - 5.21: Structure of mycelial strands of *Leccinum scabrum*

PLATES 5.19 - 5.20: S.E.M. Cross-sections through two mycelial strands, showing differentiation of tissue into narrow "structural" hyphae surrounding, and interspersed with, "vessel" hyphae of wide diameter, some of which are eccentrically positioned.

PLATE 5.21: S.E.M. Detail of Plate 5.20; the narrow structural hyphae (sh) form a closely packed outer zone surrounding wide vessel hyphae (vh), the wall layers of which, in some cases, appear to have separated from one another (arrow 1); in other vessel hyphae spherical bodies line the walls (arrow 2).
PLATES 5.22 - 5.23: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.22: S.E.M. Detail of structural hyphae of a mycelial strand, showing granular cytoplasmic contents with papillate protrusions (arrow 1), reticulate contents (arrow 2), empty hyphae (arrow 3), intercellular deposits (arrow 4) and extrahyphal bodies in the intercellular spaces (arrow 5).

PLATE 5.23: S.E.M. Detail of a vessel hypha (vh) of intermediate diameter, as seen in Plate 5.21; it contains a reticulum and has thickened, lamellate walls.
PLATES 5.24 - 5.25: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.24: S.E.M. Detail of central region of a mycelial strand illustrated in Plate 5.21 (arrow 2), showing a vessel hypha (vh) that is apparently empty but with spherical bodies and other collapsed material lining the walls.

PLATE 5.25: S.E.M. Detail of central region of a mycelial strand illustrated in Plate 5.23; the walls of adjacent structural hyphae containing cytoplasm with papillate protrusions have disintegrated; the remaining, intact, walls are thickened and lamellate.
PLATES 5.26 - 5.27: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.26: S.E.M. Cross-section through a mycelial strand, showing outer layers of loosely packed, narrow structural hyphae (sh) and central region of wider, empty vessel hyphae (vh); the outer edge of the strand is discrete but without a membranous covering.

PLATE 5.27: S.E.M. Detail of Plate 26, showing structural hyphae filled with a reticulum, though some (arrow 1) appear empty; the hyphae are separated by intercellular spaces (arrow 2) and some intercellular material (arrow 3); the wide, empty vessel hyphae are closely associated with one another; hyphae of intermediate diameter (arrow 4) are filled with a reticulum.
PLATES 5.28 - 5.29: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.28: S.E.M. Cross-section through a mycelial strand, showing loosely arranged structural hyphae (sh), and vessel hyphae (vh) of only slightly larger diameter than the structural hyphae; the outermost hyphae are covered with wart-like protrusions which are less conspicuous on some radiating hyphal branches (arrow).

PLATE 5.29: S.E.M. Detail of extrahyphal protrusions, many of which appear to merge with the wall material of the hyphae.
PLATES 5.30 - 5.31: Structure of mycelial strands of *Leccinum scabrum*

**PLATE 5.30:** S.E.M. Detail of cellular contents of structural hyphae, showing large, often fused bodies lining the internal walls of the hyphae; the intrahyphal bodies appear more granular than do the extrahyphal bodies.

**PLATE 5.31:** S.E.M. Detail of cellular contents of structural hyphae, showing conglomerations of fused bodies filling the cells; this material may be equivalent to that lining the hyphal walls, as shown in Plate 5.30.
PLATES 5.32 - 5.34: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.32: T.E.M. Cross-section through part of a mycelial strand, showing outer layers of structural hyphae (sh) and part of inner region containing wider vessel hyphae (vh); the structural hyphae are separated by intercellular spaces and covered on their outer surfaces with electron-dense bodies; there is no evidence of a membranous outer sheath on the surface of the strand (arrow).

PLATE 5.33: T.E.M. Cross-section through part of the central region of a mycelial strand, showing a wide vessel hypha (vh) with thickened lamellate walls; highly condensed, electron-dense residual cytoplasm is evident within the hypha.

PLATE 5.34: T.E.M. Cross-section through the central region of a mycelial strand, showing wide vessel hyphae (vh) interspersed with structural hyphae (sh) and embedded in intercellular material (im); an intercellular plasma-lemmasome-like body is seen (arrow 1), as are apparent dolipore septa between adjacent structural hyphae (arrows 2 and 3); some structural hyphae are vacuolate (arrow 4) whereas others are filled with electron-dense material (arrow 5).
PLATES 5.35 - 5.36: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.35: T.E.M. Outer layers of structural hyphae, covered with electron-dense bodies and separated by intercellular spaces and some intercellular material (im).

PLATE 5.36: T.E.M. Cross-section through middle layers of structural hyphae of a mycelial strand, showing structural hyphae which are more closely packed than at the outer edge of the strand, embedded in more extensive intercellular material (im) containing apparently degenerating cell contents and electron-dense bodies, some of which are fused together (arrow).
PLATES 5.37 - 5.42: Structure of mycelial strands of *Leccinum scabrum*

PLATES 5.37 - 5.39: T.E.M. Detail of extrahyphal bodies shown in Plates 5.32 and 5.35. The bodies have the same, or similar staining properties to those of the hyphal walls and appear to have a discrete (Plate 5.38) or diffuse (Plates 5.37 and 5.39) appearance; they sometimes appear fused together (Plate 5.38, arrow), sometimes stalked (Plate 5.39, arrow). A mitochondrion (m) is evident in Plate 5.37.

PLATES 5.40 - 5.42: T.E.M. Detail of middle layers of structural hyphae of a mycelial strand. The extrahyphal bodies seem to become compressed where the structural hyphae are more closely packed towards the centre of the strand (Plates 5.40 and 5.42, arrow 1); they are continuous with intercellular material (im) as seen in Plate 5.40, and in the innermost regions of the strand (Plate 5.41) they are indistinguishable from the intercellular material. Some of the structural hyphae appear to be metabolically active, containing mitochondria (m), nuclei (n) (Plate 5.41) and ribosomes (rb) (Plate 5.42); the intracellular electron-dense bodies are often fused into a large mass (Plate 5.42, arrow 2) and are always contained within the cell vacuoles, being separated from the cell walls by a thin layer of cytoplasm; an intracellular plasmalemmasome-like body (pl) is evident in Plate 5.42.
PLATES 5.43 - 5.45: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.43: T.E.M. Detail of structural hypha in middle layers of a mycelial strand, containing electron-dense contents and sub-cellular organelles; the hypha is embedded in an intercellular matrix, which appears to be continuous with the hyphal walls; compressed extrahyphal bodies are evident (arrow).

PLATE 5.44: T.E.M. Central region of a mycelial strand, showing structural hyphae (sh) and vessel hyphae (vh) embedded in an amorphous intercellular material which often appears to be continuous with the walls of the vessel hyphae (arrow 1); some of the structural hyphae with electron-dense contents are irregular in shape (arrow 2) and often appear closely associated (arrow 3) or fused (arrow 4) with hyphae of intermediate diameter; some structural hyphae have similar dimensions, one to another, where they are adjacent to each other (arrow 5).

PLATE 5.45: T.E.M. Detail of part of Plate 5.44, showing structural hypha fused with hyphae of wide diameter (arrow).
PLATES 5.46 - 5.48: Structure of mycelial strands of *Leccinum scabrum*

PLATE 5.46: T.E.M. Central region of a mycelial strand, showing wide empty vessel hyphae (vh) with thickened walls, narrow structural hyphae (sh) with thin walls and hyphae of intermediate diameter with thickened walls (arrow 1); structures resembling dolipore septa (arrows 2 and 3) are evident between some structural hyphae.

PLATE 5.47: T.E.M. Detail of Plate 5.44, showing structural hyphae with dense contents (arrow 1) fused with other structural hyphae and also hyphae of wide diameter (arrow 2); the structural hyphae often have similar dimensions where they lie adjacent to one another (arrow 3); some condensed cytoplasmic debris (arrows 4 and 5) is evident in the intercellular matrix, and plasmalemmasomes are evident (arrow 6) in some vacuolate structural hyphae.

PLATE 5.48: T.E.M. Detail of an intercellular plasmalemmasome-like body shown (arrow 1) in Plate 5.34.
PLATES 5.49 - 5.51: Structure of mycelial strands of *Lactarius rufus*

PLATE 5.49: S.E.M. Detail of the external surface of a mycelial strand, showing the ribbed appearance in which the longitudinal orientation of hyphae is discernible; the individual hyphae appear smooth and this is attributable to a membranous covering over the surface shown in cross-section in Plate 5.50.

PLATES 5.50 - 5.51: S.E.M. Cross-sections through mycelial strands, showing outer membranous covering, central vessel hyphae (vh), structural hyphae (sh), lacunae (l) apparently formed by the breakdown of adjacent structural hyphae, and hyphae of narrow diameter (arrows) running longitudinally through the lacunae (Plate 5.50) and through some structural hyphae (Plate 5.51).
PLATES 5.52 - 5.53: Structure of mycelial strands of *Lactarius rufus*

PLATE 5.52: S.E.M. Cross-section through part of a mycelial strand, showing hyphae of narrow diameter running longitudinally through some structural hyphae (arrow).

PLATE 5.53: S.E.M. Cross-section through part of the outer region of a mycelial strand, showing lacunae (l) formed by the disintegration of adjacent structural hyphae; the hyphal walls appear thickened and lamellate (arrow) formed by the adpression of walls of adjacent structural hyphae that have disintegrated.
SECTION VI

Concluding Discussion
All of the work in this thesis has involved study of the properties and behaviour of inoculum sources of sheathing mycorrhizal fungi. Basidiospores have been shown to be effective inoculum sources of some ("early-stage") but not other ("late-stage") sheathing mycorrhizal fungi on seedling roots in unsterile soil (Section 3). Sclerotia or sclerotium-like bodies of *Hebeloma sacchariolens* have similarly been shown to function as inoculum, capable of dormant survival for at least 9 months in soil (Section 4). Although my work, as such, has not concerned the role of mycelial strands as inocula, Fleming (1983a,b) has obtained evidence that mycelial strands of "late-stage" fungi can initiate infection of seedling roots provided that the strands remain attached to a "food base" - a parent tree.

The separate aspects of the work in this thesis have already been discussed in detail in sections 3.13, 4.9 and 5.6, so it is perhaps most appropriate here to discuss some general aspects. Particular attention will be focused on the potential roles of different sources of inoculum of sheathing mycorrhizal fungi in commercial practice.

The aims of mycorrhizal inoculation programmes may, at first sight, appear to be straightforward, but in practice much depends on the specific objectives. For example, in some cases it is desirable to obtain rapid early seedling growth in nursery beds (Theodorou and Bowen, 1970; Mason *et al.*, 1983a), in which case specific mycorrhizal fungi can relatively easily be selected for this purpose. In other instances, the seedlings may be destined for outplanting to "extreme" sites, adverse for plant growth, in which case a mycorrhizal symbiont specifically suited to such conditions may be needed (Schramm, 1966; Marx and Bryan, 1971b; Marx, 1976b, 1980; Trappe, 1977); for this purpose, fungi may be selected that do not necessarily improve host
growth under nursery conditions (Marx, 1975). In yet other instances, it may be desirable to select symbionts for sustained high growth rate or yield of the tree crop (Marx et al., 1977) irrespective of early plant performance. In addition to these points, good mycorrhizal establishment may confer on seedlings a degree of resistance to drying and other adverse conditions associated with the operation of outplanting to the field. M. Coutts (pers. comm.), for example, has stated that a major economic loss associated with commercial forestry is the degree of early plant death after outplanting.

There are several much-quoted early examples of the need for mycorrhizal inoculation programmes in Africa (Kessel, 1927), America (Hatch, 1937; White, 1941) and other parts of the world (Shemakhanova, 1962) where the incorporation of soil or litter from existing plantations had the effect of overcoming large-scale loss and poor performance of exotic pines on formerly tree-less sites; the introduced soil provided the inoculum of mycorrhizal fungi necessary for tree growth and survival. Subsequent work on mycorrhizal inoculation programmes was done by Levisohn (1953, 1956, 1960) but despite Levisohn's reports of successful inoculation and enhanced plant growth with some mycorrhizal symbionts, this approach was largely discontinued, and even today there is no purposeful inoculation of commercial forestry planting stock in Britain. Marx (1976a,b, 1977a,b) and co-workers (Marx and Barnett, 1974; Marx and Bryan, 1975; Marx et al., 1976, 1978, 1979) were largely responsible for the current, renewed interest in the exploitation of sheathing mycorrhizal fungi. Today, in America, Australia and elsewhere, there are several developing inoculation programmes and attention is being directed to the selection of mycorrhizal symbionts with the greatest growth promoting effects on the host, both in the
short-term (in the nursery) and in the long-term, after outplanting (Marx et al., 1977, 1979). The inoculum is most often applied as colonised vermiculite-peat, mixed into nursery soil or plant containers, though some recent reports refer to the use of mycelium produced in liquid culture and incorporated into a gel-like suspending medium (Garbaye, 1983). The advantages of these types of inoculum are their relative ease of production in bulk and their ability to promote rapid establishment of mycorrhizas. Moreover, in contrast to the use of natural soil as inoculum, they entail minimal risk of introducing and encouraging the activities of root-infecting pathogens, pests and weeds.

Spores have been relatively little used for inoculation programmes, perhaps mainly because of the inconsistent establishment of mycorrhizas from spore inocula of different symbionts and the low levels of germination under laboratory conditions (Fries, 1966, 1978, 1979a,b). Yet, Robertson's (1954) early work left little doubt that spores are responsible for naturally occurring infection of seedling roots of pine, and since then spores have also been shown successfully to initiate mycorrhizal development on other trees, for example *Pisolithus tinctorius* on *Pinus* spp. (Marx et al., 1979), *Rhizopogon luteolus* on *Pinus radiata* (Theodorou, 1971; Theodorou and Bowen, 1973) and *Rhizopogon roseolus* and *Suillus granulatus* on *P. radiata* (Lamb and Richards, 1974a,b,c). Indeed, spores have been used in pilot inoculation programmes by Theodorou and Bowen (1973) and Marx and co-workers (1976, 1978, 1979), usually in soils fumigated or otherwise partially sterilised as a part of normal forestry practice. My work has now shown the feasibility of using spores of several mycorrhizal fungi for inoculation of birch seedling roots, but it is clear that only some fungi can easily be established in this way in unsterile soil - notably species in the genera
Hebeloma, Inocybe and Laccaria. These, in any case, are the fungi that are most easily established from mycelial inoculum sources (Mason, 1980; Deacon et al., 1983), so spores are no less useful in this respect than are mycelial inocula. Ivory and Munga (1983) have also shown that spores of some sheathing mycorrhizal fungi, namely Pisolithus tinctorius, Rhizopogon spp., Scleroderma spp. and Thelephora terrestris, can be used successfully for the inoculation of containerised pine seedlings.

Marx (1980) has raised the objection that development of mycorrhizas from spore inocula is slow in comparison with that from mycelial inoculum sources, with the consequence that other, non-selected mycorrhizal fungi can become established before the inoculant; a major "natural" colonist in this respect was reported to be Thelephora terrestris. But my work shows that spores of appropriate fungi can establish mycorrhizas rapidly in soil, to the exclusion of mycorrhizas, including those of Thelephora terrestris, that develop from "contaminating" sources. A possible explanation of these discrepant findings is that the work of Marx et al. (1976) was done in different conditions - in fumigated nursery soil and with different sheathing mycorrhizal fungi. The argument of Marx (1980), that mycorrhizal development is delayed when spore inoculum is used, is partly refuted, however, because the main naturally developing colonist, T. terrestris, in his experiments probably developed from spores.

A major, and to some extent, insurmountable disadvantage of spore inocula concerns the availability of material. Fruiting by mycorrhizal basidiomycetes occurs only during a relatively short time in the autumn, and is weather-dependent; indeed, in some countries, like South Africa (J.W. Deacon, pers. comm.), fruitbodies of sheathing
mycorrhizal fungi are relatively seldom seen in many years. Thus, it would be difficult to obtain sufficient bulk of spore inoculum for a mycorrhizal inoculation programme unless the plants are raised, at least initially, in small volumes of soil, as in the tube-method used by the Forestry Commission (Low, 1975), or unless bare-rooted plants are dipped into an inoculum source at the time of planting. In small-scale inoculation programmes, Theodorou and Bowen (1973) have successfully used spore-encapsulation of seed as a method of inoculation, using *R. luteolus* as the inoculant. Such encapsulated seed could be stored for at least 2 days without serious loss of infectivity. My work on storage of basidiospores suggests that storage of spores over much longer periods than this is feasible, either when spores are incorporated into moist soil or when they are stored moist at 5°C.

It will, of course, be impractical to use mycelial strands as inoculum sources in commercial practice, except by incorporating mother plants into nursery beds as was advocated by Mikola (1970, 1973). Fleming (1983b) has discussed in detail the role of mycorrhizal mother plants in this respect, as also in the establishment of mycorrhizas on naturally regenerating seedlings in woodland environments.

Sclerotia or sclerotium-like bodies, however, may prove useful for inoculation programmes, particularly, perhaps, in augmenting the use of mycelial inoculum. Sclerotia of some sheathing mycorrhizal fungi, most notably *Hebeloma sacchariolens*, have the advantage of longevity in soil (Section 4.8); this could be particularly advantageous for the supplementation of soil with inoculum before seeds are sown in nursery conditions, especially if, for any reason, the seeds take a long time to germinate or if early seedling growth is delayed. However, before sclerotia can be considered as plausible sources of inoculum, more information is needed on the conditions required for their production in culture.
REFERENCES


JANERETTE, C.A. (1981). In vitro development of sclerotia by Pisolithus tinctorius. USDA Forest Service, Northeastern Forest Experiment Station, Forest Physiology Laboratory, ARC-West, Beltsville, Maryland 20705.


Role of basidiospores as inocula of mycorrhizal fungi of birch

FRANCES M. FOX
Microbiology Department, School of Agriculture, West Mains Road, Edinburgh, EH9 3JG, Scotland
and Institute of Terrestrial Ecology, Bush Estate, Penicuik, Midlothian, EH26 0QB, Scotland

Key words Basidiospore Betula Ectomycorrhiza Mycorrhiza Succession

Summary Fruitbodies of sheathing mycorrhizal fungi collected under birch (Betula pendula and B. pubescens) were suspended over pots of soil and the resulting spore-supplemented soils were planted with seedlings of B. pendula. Inocybe lacera, I. lanuginella, Hebeloma sacchariolens and H. leucosarx formed mycorrhizas readily. Lactarius pubescens and Leccinum roseofracta did not form mycorrhizas from basidiospore inocula, even after prolonged periods of seedling growth. Paxillus involutus gave equivocal results, perhaps because the soil was unsuitable for this species. Storage of the basidiospore-supplemented soils for 6 months in outdoor conditions or in a growth room at 18°C did not materially alter the results.

The results are discussed in relation to the concept of mycorrhizal succession.

Introduction

Basidiospores are generally assumed to be the main means by which ectomycorrhizal fungi are dispersed and initiate infections, but there is only limited experimental evidence to support this view. A general difficulty in this respect concerns the low degree of germinability of the spores, though this can be overcome to some extent by adding activated charcoal or the yeast Rhodotorula glutinis or germinating in the presence of living roots or mycelium of the appropriate type.

A succession of mycorrhizal types has been demonstrated on roots of birch (Betula pendula Roth. and B. pubescens Ehrh.) in collaborative studies at Edinburgh, and a general distinction has been made in this respect between 'early stage' and 'late stage' mycorrhizal fungi. The late-stage types do not readily infect seedling roots in unsterile soil, though they can do so in aseptic conditions. As part of this programme the infectivity of basidiospores of early- and late-stage mycorrhizal fungi was investigated.

Materials and methods

Fruitbodies of seven sheathing mycorrhizal basidiomycetes were collected beneath birch trees at three sites in autumn 1981. The stipes were partly removed and the caps were then suspended individually over 50 cm³ plant pots containing brown earth soil, by pressing the remaining part of the stipe into the soil. After 24 h in dark humid conditions the fruitbodies were removed and discarded, and the spores that had fallen onto the soil were buried by lightly mixing the top 1 cm of soil in each pot. No attempt was made to count the number of spores released from each fruitbody, but in each...
case the corner of a glass coverslip was placed beneath part of the cap to ensure that spores had been released in large numbers. The soil had been taken from a tree-less site south of Edinburgh and had been stored air-dry for 21 days. It was sieved < 2 cm, mixed with autoclaved grit (3:1, v/v), watered to 40% saturation and left in pots for 4 days before use. The soil was unsterile.

Variable numbers of replicates were prepared, depending on availability of fruitbodies. In all treatments at least twelve replicate pots were prepared and these were sown to birch immediately as described below. In some instances further replicates were available; these pots were placed in plastic plant propagators which were sealed in polyethylene bags and then wrapped in aluminium foil. The propagators were stored for 6 months either in a growth room at constant 18°C or in outdoor conditions where they were exposed to winter temperatures (which dropped as low as −20°C).

To test the infectivity of spores in soil, seeds of *B. pendula* were surface-sterilized in H₂O₂ and placed on tap-water agar, on which the resulting seedlings were allowed to grow for 20 days at room temperature. Then two seedlings were transferred to each pot and the pots were incubated in a growth room at 18°C, with a 16 h light/8 h dark cycle. The pots were arranged in pairs with the different pairs being spaced in a randomized layout. They were sprayed daily with water. The seedlings were thinned to one per pot after 6 weeks and harvested at 14 to 16 weeks, though in many cases a few pots were incubated for longer to see if the pattern of results changed on prolonged incubation of seedlings. For assessment, the root systems were washed free from soil, placed on a grid and cut into 1 cm sections. Alternate sections were scored for numbers of root tips and numbers of tips with different mycorrhizal types, these being compared with reference material. Throughout the experiment, pots of unsupplemented soil were used for comparison with the treatment series.

### Results

Results of the main part of the experiment, involving the immediate planting of seedlings, are shown in Table 1. Of the 6 control seedlings growing in unsterile soil and assessed after 16 weeks, two had Hebeloma-type mycorrhizas, three had Laccaria-type mycorrhizas and two had Inocybe-type; it is notable that each mycorrhizal type occurred almost to the exclusion of other types on

<table>
<thead>
<tr>
<th>Basidiospore inoculum*</th>
<th>None</th>
<th>I. lac</th>
<th>I. lan</th>
<th>H. sac</th>
<th>H. leu</th>
<th>P. inv</th>
<th>L. pub</th>
<th>L. ros</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. seedlings examined</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>No. with inoculant-type mycorrhizas</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mean no. root tips</td>
<td>379</td>
<td>264</td>
<td>410</td>
<td>526</td>
<td>622</td>
<td>346</td>
<td>684</td>
<td>635</td>
</tr>
<tr>
<td>Mean % root tips:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) uninfected</td>
<td>63</td>
<td>46</td>
<td>70</td>
<td>48</td>
<td>34</td>
<td>37</td>
<td>44</td>
<td>34</td>
</tr>
<tr>
<td>(b) with inoculant type mycorrhizas</td>
<td>52</td>
<td>30</td>
<td>49</td>
<td>59</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Establishment of mycorrhizas on seedlings of *Betula pendula* planted in soil supplemented with basidiospore inocula of different fungi and stored for 6 months

<table>
<thead>
<tr>
<th>Basidiospore inoculum* and conditions of storage</th>
<th>Growth room (18°C)</th>
<th>Outdoor (winter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>I. lac</td>
<td>H. leu</td>
</tr>
<tr>
<td>No. seedlings examined</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>No. with inoculant-type mycorrhizas</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>Mean no. root tips</td>
<td>182</td>
<td>65</td>
</tr>
<tr>
<td>Mean % root tips: (a) uninfected</td>
<td>87</td>
<td>70</td>
</tr>
<tr>
<td>(b) with inoculant-type mycorrhizas</td>
<td>–</td>
<td>30</td>
</tr>
</tbody>
</table>

* See Table 1.

individual seedlings. Treatments involving spores of *Inocybe lacera* (Fr.) Quel., *I. lanuginella* (Schroet.) Konrad & Maublanc, *Hebeloma sacchariolens* Quelet and *H. leucosarx* Orton gave successful mycorrhizal establishment: the inoculant types developed on most or all seedlings and in relatively large numbers on those seedlings. It is notable that these fungi are in the early-stage category of Deacon et al. In contrast, *Lactarius pubescens* Fr. and *Leccinum roseofracta* Watling did not establish on seedling roots from spore inocula; instead, the seedlings bore Laccaria-type or Thelephora-type or, less commonly, Paxillus-type mycorrhizas. The inoculant fungi in these cases are late-stage. The results for *Paxillus involutus* (Fr.) Karsten spore inocula were equivocal: some seedlings were infected but few mycorrhizas were attributable to *P. involutus*, the predominant type on most replicate seedlings being Laccaria (Table 1). Indeed the overall mean of 20% root tips attributable to Paxillus in this treatment was similar to that in the treatment involving *Lactarius pubescens* (results not presented in detail in Table 1).

Some of the remaining seedlings in replicate pots from this part of the experiment were incubated for 26 weeks because it was thought that mycorrhizas of Lactarius or Leccinum might develop after longer incubation periods. But mycorrhizas of these fungi were still not present after 26 weeks.

When soils that had been stored for 6 months were planted, the development of mycorrhizas was generally similar to that described above (Table 2). Mycorrhizas of Inocybe and Hebeloma developed in pots supplemented with spores of these fungi; Paxillus gave few mycorrhizas and Lactarius gave none.
Discussion

Results in this paper suggest that early-stage mycorrhizal fungi of birch can establish mycorrhizas on seedlings from spores in soil but that late-stage mycorrhizal fungi cannot do so. The failure of some spores to initiate infections does not seem to alter during 6 months’ storage in soil – even over winter – but the effects of prolonged storage are being studied further. It is notable that the spores of early-stage mycorrhizal fungi remained infective for at least 6 months at 18°C. This seems to be ecologically relevant because spores released from fruitbodies in the autumn in Britain would presumably need to persist in soil until the following growing season.

The results for Paxillus are difficult to interpret. This fungus is commonly found as a mycorrhizal symbiont on very poor soils, including coal-Spoil heaps, but it has not been found in the experimental plot of birches at Bush Estate, where the soil is richer*. Possibly the spores of *P. involutus* would initiate infections in coal spoil, and this is currently under study. The fungus has been found to produce mycorrhizas when vermiculite-peat inocula are added to coal spoil in glasshouse conditions (P A Mason, personal communication).

My work has helped to reinforce the view that a broad distinction is possible between early- and late-stage mycorrhizal fungi of birch, and it has identified one of the important differences in this respect, *i.e.* that of infectivity of spores in soil. The failure of some previous attempts to use spore inocula might now be explained by the choice of fungi used. But it is possible also that the success of this work was due to the fact that spores were not allowed to dry: they were released into a humid environment and immediately mixed into moist soil. Simon¹⁶ has reviewed the effects of drying and re-wetting fungal spores and other plant propagules, a consistent finding being that substantial loss of nutrients occurs if rehydration takes place too rapidly. Perhaps any such loss of nutrients significantly reduces the germinability or infectivity of spores⁴ or predisposes them to microbial antagonism⁶.

Acknowledgements I am very grateful to Dr J W Deacon and Professor F T Last for suggesting and supervising this work, and to the Science Research Council of Great Britain for the award of a Research Studentship. I also thank Dr P A Mason for valuable help and discussion.

References

BASIDIOSPORES AS INOCULA


