THE MYCORRHIZAL RELATIONS OF LARCH

III. Mycorrhiza-formation in Nature

by

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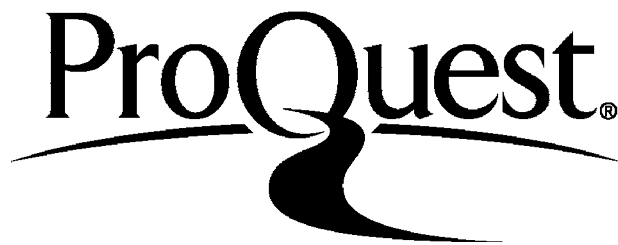

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THESIS SUBMITTED FOR Ph.D. DEGREE BY

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THE MYCORRHIZAL RELATIONS OF LARCH

ABSTRACT

The thesis consists of four papers of which the first two have already been published.

Paper I entitled "A study of Boletus elegans Schum. in pure culture", deals with the morphological characters and the physiological behaviour of this fungus in pure culture.

Paper II entitled "The role of the Larch root in the nutrition of Boletus elegans Schum.", is concerned with the results of adding excised roots to fungal cultures; these indicate that a water-soluble substance capable of stimulating the growth of the fungus is present in the roots of European and Japanese Larch, but not in those of Scots Pine.

Paper III entitled "Mycorrhiza-formation in nature", contains an account of the isolation of three endophytes, detailed descriptions of the structure of seven types of infection and a general survey of mycorrhiza-formation in mature Larch trees.

Paper IV entitled "Mycorrhiza-formation in experimental culture", is concerned with four sets of experimental cultures with Larch seedlings in which various methods of inoculation were tested.

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I. INTRODUCTION

European Larch (*Larix decidua* Mill.) is planted in considerable quantity in the British Isles; although it is not native to these islands it has long been established in some localities. Nevertheless it may be anticipated that many of the fungi with which it forms mycorrhizal associations in its native habitat will not be available in the soils of this country. *Boletus elegans* which is known to be a mycorrhiza-former with Larch does occur in plantations in this country but is probably absent from many sites. Now there is abundant evidence from various parts of the world that exotic trees may fail to establish themselves in otherwise suitable habitats, owing to the absence of their normal endophytes (Hatch 1936); often no success is possible until the soil has been inoculated with the appropriate fungi. In other cases though mycorrhizal associations are formed no balanced condition is reached and the health of the trees is imperilled. This aspect of tree culture has been emphasised by Rayner (1939) who shows how important it is for forestry practice to have some knowledge of the range of mycorrhizal structure found in such exotic conifers and some estimate of what constitutes a balanced condition. The present paper attempts to supply this need in the case of Larch raised in the British Isles.

There are no recent detailed descriptions of the mycorrhizal conditions of mature European Larch trees in their native habitats. McDougal (1914) published a description of root infection in mature Larch in the U.S.A.; Melin (1922) described the conditions in six year old trees in a forest nursery in Sweden; but in neither case do the conditions described correspond to anything observed by the present writer. Laing (1923, 1932) considers that normal Larch mycorrhizas from British habitats can be classified into three types, the ectotrophic, semi-endotrophic and endotrophic. But in the absence of any information as to soil conditions, age of the trees, detailed structure of the mycorrhizas or evidence of the specific nature of the endophytes concerned, these observations are of small value for comparative purposes. Moreover, it is possible that both the semi-endotrophic and endotrophic types result from various abnormal conditions.

II. GENERAL FEATURES OF THE ROOT SYSTEM

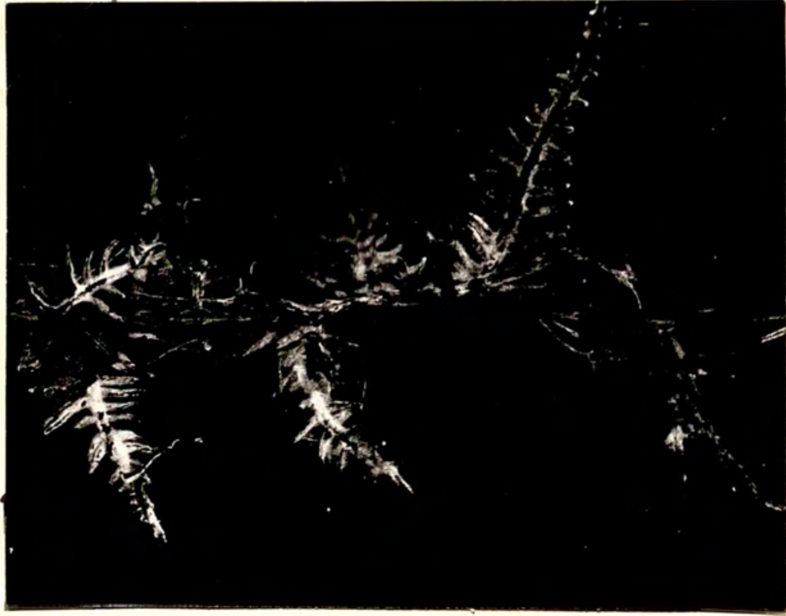
A number of conifers, notably species of Pine, have been shown to have two types of root, polyarch long roots and diarch or monarch short roots, alternatively termed laterals and sublaterals; it is the sublaterals which normally become mycorrhizas. Aldrich-Blake (1930) working with Corsican Pine roots

measured the diameters of circles drawn through the protoxylems in transverse sections taken at the point of emergence of the root, and he found that the values obtained could be grouped into two classes. The so-called long roots had protoxylem diameters above a certain value, the short roots had diameters below this value; so it seemed clear that the two types of root had differed from the beginning. Hatch and Doak (1933) found in pure culture seedlings of Pinus Strobus that sub-laterals were produced which remained short though uninfected and they concluded that mycorrhizas remained short because they were a morphological entity and not because the enwrapping fungus prevented further growth, as was believed at one time (McDougal 1914, Masui 1927).

Casual observation of the root system of Larch does not reveal any clear differences into long and short roots, since the mycorrhizal branch systems often reach considerable length, and all roots except the monarch ultimate branches of the mycorrhizas are diarch whatever their size. However it seemed worthwhile to apply Aldrich-Blake's method to this species. Slender roots which showed no evidence of becoming infected and others which were already mycorrhizas were sectioned at the point of emergence from the mother-root and the protoxylem diameters were measured. Two classes were clearly distinguishable. The mean protoxylem diameter of 16 infected roots was 117 $\pm$ 23.3μ, of 16 uninfected roots 243.2 $\pm$ 50μ; the

probability of significance of the difference between these two measurements was over a 100 to 1. There is no doubt that the uninfected roots were true long roots and the infected ones were short roots. Occasionally infected roots showing monopodial branching are found to be long roots as determined by protoxylem size, but the structure of the mycorrhiza thus formed is different from that in infected short roots and it only serves to emphasise the difference between the two root types (see section V). It is these short roots which normally form mycorrhizas. They are easily distinguished macroscopically when fully developed; for infection results in the repeated formation of rows of branches at right angles to the main axis of the short root to give a flat monopodial branched system (see Plate I). While in the writer's knowledge this typical branch system never occurs apart from infection, infection does not always result in monopodial branching. Where infection occurs without monopodial branching the internal structure always exhibits other non-typical features, such as intracellular infection and absence of a tannin layer, which may be regarded as marks of pseudomycorrhizal infection (Rayner 1934). The fact that, in general, repeated monopodial branching follows infection, disposes finally of the suggestion that the presence of the fungal mantle exerts a restraining influence on the growth in length of the sublaterals thus giving rise to short roots. While superficially that might appear to be the case

PLATE I



Mycorrhizas of Larch, endophyte unknown. x 2.

in Pine, where the infected roots are short, in Larch mycorrhizas they are often 1.5 cms. long and have evidently grown and branched vigorously since first infected. In view of these conditions in Larch the term sublaterals rather than short root will be used throughout the remainder of this paper.

III. FUNGAL CONSTITUENTS AND THEIR ISOLATION

Melin (1925 a and b), Hammerlund (1923), Peyronnel (1920, 1921, 1922 a and b, 1929) and Laing (1932) all produce evidence that Larch can form mycorrhizal associations with a number of different species of fungi. In the present enquiry evidence of infection by a great many different species, most of which remain unidentified, has been obtained from the examination of the hyphal strands and fragments of hyphae attached to the mantle in infected roots. In three cases successful isolations from roots have established infection by Boletus elegans, Paxillus involutus and a black mycelium (see below). In addition three or four distinct hyaline clamp-mycelia have been observed to form well-balanced mycorrhizas and a few yellow and yellow-brown hymenomycete mycelia can also infect the roots, though the latter are probably deleterious in their effects; a number of the types observed may be casual infections, but some occur with great regularity in many different habitats and with

essentially similar morphological effects on the root. In any given locality a number of different infections can usually be found within a very small area, so that any one tree is in association with more than one fungus. Naturally sown seedlings in which it has been possible to examine all the roots have shown three or more different infections on one plant, as if the area inhabited by any one fungus is small and the roots spreading through the soil become infected by many different fungi living in pockets in the soil. This haphazard nature of the infection is very marked in some localities.

Melin (1922) attempted unsuccessfully to isolate B. elegans from Larch mycorrhizas which were assumed to be infected by this fungus; he used the mercuric chloride method of sterilisation which had proved successful with Pinus sylvestris and Picea Abies. He regarded his failure to isolate the fungus as due not to excessive sterilisation but to the loss of the power of independent growth on the part of the endophyte. The present writer's experience, however, proves conclusively that B. elegans does not become incapable of growth after entry into the root; it must be concluded that Melin's sterilisation was in fact too severe.

Isolation of Boletus elegans Schum.

Over a four year period repeated attempts were made to isolate B. elegans from Larch mycorrhizas using sterilisation by mercuric chloride. Eventually success was obtained with

this and another method. The first method used was that of Ternetz (1907); mycorrhizas collected from the vicinity of sporophores of B. elegans were washed repeatedly with sterilised distilled water, placed in 1% hydrochloric acid for 60 seconds and after washing once more in sterilised distilled water were plated on Melin's glucose. All the cultures were considerably contaminated but three cultures also gave rise after four days to a slow-growing, densely tufted, white mycelium which rapidly turned yellow and then orange brown. This appearance, combined with the fact that papillation of the hyphae and 'paarige' branching were observed during microscopic examination left little doubt that the mycelium was that of B. elegans. Further material similar to that used above was sterilised by washing in 0.1% mercuric chloride for less than 20 seconds and then repeatedly in sterilised distilled water, it was then plated on Melin's glucose. Twenty-three cultures remained sterile; one culture however, had a slight bacterial contamination and twenty-one days after inoculation developed a white, densely tufted mycelium which turned yellow and then brown. It proved possible to subculture this without contamination. Presumably this one root escaped the full effects of the sterilising agent as shown by the bacterial contamination and in consequence the endophyte remained alive. It was a matter of chance that on this occasion success was obtained with the mercuric chloride method which is normally too drastic

for the isolation of B. elegans.

The evidence that the mycelium isolated on these two occasions is B. elegans can be summarised as follows:-

(i). For over two years cultures of the endophyte have been grown side by side with cultures of B. elegans obtained from sporophore tissue. The two fungi are indistinguishable macroscopically and behave similarly on all media. The rate of growth of the endophyte has increased slowly during this time and now is only slightly less than that of the cultures isolated from sporophore tissue.

(ii). Microscopic examination reveals that the two mycelia differ in no respect. Both have papillated hyphae 2 - 3μ wide, 'paarige' branching, brown hyphae and compound hyphal strands (see How 1940 for detailed description of the mycelium of B. elegans).

The evidence that the fungus isolated was the true endophyte is as follows:-

(i). When the mantles of the roots actually giving rise to the endophyte were compared with others from the same material, they were found to be similar in structure and from the latter papillated hyphae radiated.

(ii). The hyphal strands in the mycelium around these mycorrhizas were compound and similar in structure to those of B. elegans in pure culture, therefore this species was undoubtedly present in the vicinity of the root.

(iii). This type of mantle structure has been observed many times in many localities and always sporophores have been present in the vicinity.

While it is realised that the only absolute proof of identification would be the production of sporophores of *B. elegans* by the endophyte in pure culture and the development of mycorrhizas similar to those from which it was isolated as a result of the inoculation of pure culture seedlings; nevertheless, familiarity with the fungus in pure culture leaves no doubt in this case that the endophyte is *B. elegans*.

Isolation of *Paxillus involutus* Fr.

Larch mycorrhizas were sterilised for 30 or 60 seconds with 0.1% mercuric chloride, washed repeatedly with sterilised distilled water and plated on beerwort agar. Most of the cultures remained sterile, two however, developed red brown colonies of a mycelium bearing clamp connections. Examination of mycorrhizas exactly similar to those used in the isolation showed that the mantle was formed by hyphae with straw-coloured walls and clamp connections. The mycelium was kept in culture for some time before its identity was established. A culture of *Paxillus panuoides* Fr. supplied by the Forest Products Research Laboratory, Princes Risborough, Bucks., somewhat resembled the brown endophyte. It was therefore thought possible that the fungus might be a terrestrial species of *Paxillus*. The only species of this genus known to occur in the plantation

from which the roots had been collected was P. involutus, which was found abundantly. Accordingly tissue cultures were made from young sporophores of this species. These were brown in colour, though paler than the endophyte; in general appearance and cultural behaviour the two fungi were similar, particularly in their preference for an acid culture medium (see Table 1). Microscopic examination revealed that both fungi had hyphae with straw-coloured walls, 1.5 - 4.5μ wide, and very frequent clamp connections. Again complete proof is lacking, but there is a very high probability that this endophyte is P. involutus.

Table 1

Comparison of the Reactions of P. involutus and an Endophyte to Acidity

	Substrate	P. involutus	Endophyte
(1)	2% malt agar	Heavy browning of substrate. Growth not so good as (2).	Heavy browning of substrate. Growth not so good as (2).
(2)	2% malt agar plus 0.5% malic acid	No colour excreted, growth very good.	No colour excreted, growth very good.
(3)	2% malt agar plus 1% malic acid	No colour excreted, growth similar to (1).	No colour excreted, growth very slow.

Isolation of a 'black' mycelium.

Some dark brown roots obtained from Scotland were sterilised by the Ternetz method. They gave rise to pure cultures, which

were green-grey in colour at first, later greenish black and finally dark brownish black; at this stage the mycelium forms a hard black crust over the surface of the substrate, no pigment is excreted into the medium. The hyphae are 1.5 - 3.5μ wide, hyaline in colour when young, later becoming dark brown. The walls of some hyphae have granular excretions giving a papillose appearance, which however, is quite distinct from that shown by hyphae of Boletus spp.; there are no clamp connections. The apices of some of the aerial hyphae become coiled into a ring. Thin mycelial strands occur 7 - 9μ in width. Strings of spore-like bodies are formed by the substrate hyphae and these hyphae are often terminated by a long tapering filament. Sclerotia are formed on poor media such as distilled water agar.

This fungus is clearly of the same type as Melin's Mycelium radicis atrovirens, isolated from roots of Pinus sylvestris (1923), since it differs in no way from the recorded description of that mycelium. It has since been isolated again from Larch roots collected in South-east England; these cultures are identical with the Scotch cultures. Moreover its behaviour in pure culture, e.g. it attacks filter paper cellulose, leaves no doubt that physiologically it is a member of the 'atrovirens' group of soil fungi and like them parasitic in its attack upon the roots.

IV. THE STRUCTURAL FEATURES OF SOME INFECTIONS

It has been possible to distinguish over a dozen types of infection by using the characters of the mantle supplemented by any information as to the nature of the fungus which may be deduced from the fragments of the hyphae available. The following seven types have been chosen for description either because the nature of the endophyte has been more or less definitely established or because the type occurs very frequently and is probably therefore not a casual infection.

All the material examined was first fixed in Doak's solution (How 1941) and then cut by hand. The descriptions record the appearance of sublateral roots in transverse section.

1. Endophyte - *Boletus elegans* Schum. (Plate II, fig. 1)

This is a widely distributed form in England. The mycorrhizas here described are similar to those from which *B. elegans* was isolated. The mycorrhizas are pinkish-brown in colour and monopodially branched; the branches are irregularly spaced a small distance apart and the apices are pointed. The hyphae radiating from the mantle are numerous, hyaline, thin-walled, papillose and 1.5 - 3.0μ wide; there are no clamp connections. The mantle has a rough margin and is 15 - 60μ in width; if wider than 20μ it consists of two layers, an outer layer of hyphae woven loosely in a direction transverse to the root axis and an inner layer enclosing 0 to 3 layers of dead cortical

filaments with the hyphae tightly woven in a longitudinal direction; below 20μ in width only the inner layer is present; in no case are the hyphae swollen nor has a pseudoparenchymatous effect been observed. The tannin layer is one cell deep, composed of tannin containing cells, granular cells and unmodified cortical cells. The Hartig net extends over the entire outer cortex, the cortical cells are oblong in shape, the walls between them are expanded by the fungus to 2.5μ in width. No intracellular infection has been observed.

There is no evidence from pot cultures or from growth in the field as to whether this type of mycorrhizal infection is accompanied by good growth on the part of the tree. In the absence, however, of any signs of the histological features known to indicate the presence of deleterious conditions in Pine, it may be assumed that a structure of this type is the expression of a balanced mycorrhizal relationship.

2. Endophyte - B. viscidus Linn ?

This type has only been collected from one locality in the vicinity of the sporophores of B. viscidus, a species reputed to grow exclusively in Larch plantations. The identity of the endophyte as B. viscidus is by no means certain but it is rendered probable by the fact that the mycelium below the sporophores is pinkish-white in colour and so are the mycorrhizas; also the hyphae below the sporophores have 'paarige' branching and papillation as do the hyphae in the vicinity of the

mycorrhizas and those radiating from the mantle are also papillated; lastly the structure of the mycorrhizas is reminiscent of the B. elegans type though different, as might be expected if another species of Boletus was concerned.

The mycorrhizas are monopodially branched, though the branching is somewhat irregular and the apices are pointed. The hyphae radiating from the mantle are few in number, hyaline, papillose and 2 - 4μ in width. The mantle has a very rough margin thickly covered with large refractive granules; it varies from 12 to 80μ in width and consists of a single homogeneous layer of transversely woven hyphae. The tannin layer is one cell deep, composed of tannin containing cells and unmodified cells; the next cell layer consists of granular cells. The Hartig net extends across the entire outer cortex, many of the outer cortical cells are oblong in shape and the walls between them are expanded by the fungus to a width of 1.5-4.5μ. No intracellular infection has been observed.

There would be no reason for assuming that this type was anything but a balanced association were it not for the large quantities of granules on the outside of the mantle. This condition may indicate the existence of some abnormal physiological state similar to that which produces the 'messy' type in Pine (Rayner 1939); but more experience would be necessary to confirm this. It is possible that the presence of granules is a constant feature of infection by this species, which

normally forms a balanced relationship with the tree.

3. Endophyte - Paxillus involutus Fr. (Plate II, fig. 2)

This type has only been observed once with certainty. The mycorrhizas here described are similar to those from which P. involutus was isolated. The mycorrhizas are dull brown in colour, with monopodial branching at infrequent intervals and pointed apices. The hyphae radiating from the mantle are numerous, pale straw-coloured with rather thick walls; they are 1.5 - 3.5 μ in width and have frequent clamp connections. The mantle which has a rough margin is 14 - 36 μ in width, pale brown in colour and composed of loosely woven hyphae; these are woven in all directions with a tendency to run in a transverse direction near the outer margin. The tannin layer is one cell deep, with a few non-tannin cells; no granular cells have been observed. The Hartig net extends across the entire outer cortex, many of the cells are oblong and the walls between them are swollen by the hyphae to a width of 3 - 6 μ. There are traces of intracellular infection of the haustorial type. In old mycorrhizas the walls between the cortical cells are even more swollen and contain more than one strand of hyphae, so that the cells appear to be isolated in a pseudoparenchymatous tissue. This and the intracellular infection are the only histological indications that infection by P. involutus may not give rise to a balanced relationship.

4. Endophyte - unknown.

This is a widely distributed form, and as such is of interest even though there is no clue as to the endophyte concerned. The mycorrhizas are pinkish-brown in colour, either unbranched or irregularly branched; the apices are very rounded. The mantle is completely smooth on the outside, hyaline in colour and therefore formed by a hyaline fungus; it is 25 - 65μ in width and parenchymatous in structure, with an outer layer of wide brick-shaped elements and an inner layer of tightly woven narrow elements. The tannin layer is only one cell deep and composed of flattened tannin cells with a few granular cells. The Hartig net extends across the outer cortex irregularly, often it does not reach the inner cortex. The cells are isodiametric and the walls between them when penetrated by the net are 1.5 - 3.5 μ wide. There is no intracellular infection.

The absence of true monopodial branching, the very thick mantle and poorly developed Hartig net are all features which suggest that this type of infection is of doubtful value.

5. Endophyte - A yellow hymenomycete mycelium. (Plate II, fig. 3)

This is another very common infection. Usually the infected roots are brown in colour, a trifle swollen at the tip and unbranched. They are obviously attacked by a hymenomycete mycelium as shown by the clamp connections on the encircling hyphae; these are greenish-yellow in colour and 1.4 - 2.2μ wide. No proper mantle is formed, the hyphae are loosely

grouped round the root. There is no true tannin layer but only one or two layers of flattened cortical filaments. The Hartig net is well developed, extending across the outer cortex, the cortical cells are isodiametric and the walls between them are 1.5-3.0μ in width. There is no intracellular infection.

The absence of mantle and tannin layer suggests that this association is of doubtful value, though the Hartig net formation is normal.

6. Endophyte - Mycelium radicis atrovirens. (Plate II, fig. 4)

This is the type from which M. r. atrovirens was isolated; roots infected by this fungus have not been found frequently, though the mycelium appears to be present on many soils. The infected roots are black in colour and monopodially branched at infrequent intervals. The hyphae which are occasionally found attached to the mantle are 1.5 - 4.0μ in width; they are green brown in colour and have rigid walls without clamp connections. The mantle is 34 - 40μ in width; the outer layer is dark brown due to the deposition of a brown pigment in the walls of the hyphae which are closely woven and run in a transverse plane; the inner layer close to the tannin cells is straw coloured and the hyphae run in a longitudinal direction. The tannin layer is one cell in depth, with an occasional granular cell. The Hartig net is very uneven in depth, sometimes extending to the inner cortex, often only involving one or two layers of the outer cortex. The cortical cells are mainly isodiametric, the

PLATE II

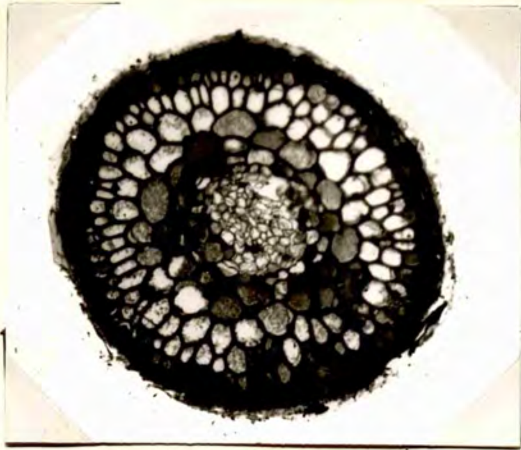


Fig.1. T.S. Larch Root showing Type 1 Infection, Endophyte - B.elegans. x 87.

Fig.2. T.S. Larch Root showing Type 3 Infection, Endophyte - P.involutus. x 87.

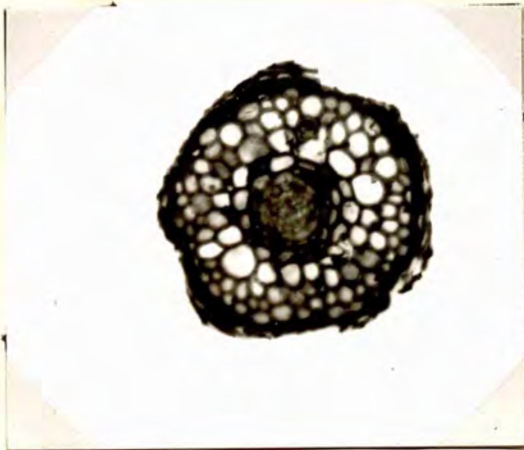


Fig.3. T.S. Larch Root showing Type 5 Infection, Endophyte - Yellow hymenomycete mycelium. x 87.

Fig.4. T.S. Larch Root showing Type 6 Infection, Endophyte - M.R.atrovirens. x 87.

walls between them being 2.2 - 6.5 μ wide and pale brown in colour due to the intercellular hyphae. Intracellular infection of the haustorial type occurs frequently and often hyphae are found to be growing straight through the cells.

Although the condition of the tannin layer and Hartig net bear some resemblance to that found in normal associations the following considerations leave no doubt that this is a deleterious type of infection. Firstly none of the mycelia of 'atrovirens' type grown by Dr I. Levisohn in this laboratory has ever been observed to be anything but a pseudomycorrhiza-former with Pine and Spruce. Secondly there is no evidence in the literature that M. r. atrovirens is capable of forming a balanced association with any species. Thirdly the parasitic tendencies of the strain of M. r. atrovirens isolated from Larch are shown by some observations which were made on pure culture Larch seedlings inoculated with either B. elegans or this strain of M. r. atrovirens (for the method of culture see Paper IV of this series). After 2½ months the seedlings inoculated with B. elegans showed a slight intercellular penetration of the cortex but those inoculated with M. r. atrovirens were subject to a strong infection of the intracellular type. It can therefore be inferred that M. r. atrovirens is very unlikely to form a beneficial association with Larch roots.

7. Endophyte - a 'black' mycelium.

This type has been chosen because it is an example of the

undoubted pseudo-mycorrhizal condition in Larch. The roots are unbranched with black swollen tips, from which radiate stiff dark brown hyphae; these are 1.5 - 2.0μ wide and have no clamp connections. The mantle varies from 15 - 35μ in width; it is composed of a single layer of dark brown hyphae woven tightly together and running in all directions. The tannin layer is poorly developed and there are no granular cells. The Hartig net occurs only sporadically and then it is but one cell deep; the cortical cells are isodiametric with very thin walls, 0.75 - 1.5μ in width. There is no intracellular infection.

V. GENERAL FEATURES OF MYCORRHIZA-FORMATION

In addition to the seven types described in detail above, many other types of mycorrhizal infection have also been examined. It is therefore possible to give a general account of the variations in histological structure resulting from the infection of Larch roots in trees raised in the British Isles. In this general description no account has been taken of mycorrhizal structure in seedlings, since there is evidence that this differs somewhat from that occurring in mature trees; but there is not sufficient information available to determine the exact extent of the difference.

Mantle.

The structure of the mantle depends entirely on the fungus composing it, the type of weaving being a constant feature for any one fungus. The outer margin is usually smooth with occasional hyphae radiating from it; however, in one attack by an unidentified fungus, the outside of the mantle was covered by a single layer of flasked-shaped fungal cells, reminiscent of cystidia. Peyronnel (1921) reported a similar condition in Larch mycorrhizas in Italy, and attributed it to infection by a species of Russula. The width of the mantle may be dependent on the age of the mycorrhiza or the edaphic conditions. In general the mantle increases in width towards the base of a mycorrhiza; most fungal constituents form mantles between 10μ and 70μ in width.

Tannin layer.

This layer, situated where mantle and cortex meet, is usually at least one cell in depth in typical mycorrhizas; the only variations occur in the infection of a lateral root by a true mycorrhizal fungus or the pseudomycorrhizal infection of a sublateral; in these cases the tannin layer is often absent. Transverse sections of normal mycorrhizas often show further layers of tannin cells outside the tannin cells proper; these are flattened and surrounded by the mantle which appears to have partially absorbed the cells in a parasitic manner. Laing (1932) actually stated that the fungus destroys the cell

and thereby eats into the root; but there is no evidence for the occurrence of such parasitism. The examination of serial transverse sections of roots from the apex backwards indicates that these flattened cells are often the remains of cortical filaments which serve as root hairs (see Melin 1922 for a detailed description of these structures). In uninfected roots the cells of these filaments which become stained yellow brown are sloughed off; in infected roots they become surrounded by the mantle before they can be torn off and in consequence become incorporated in it. In some lateral root infections where the mantle does not cover the tip, the layers of flattened cortical cells are missing from the mantle, since these had been sloughed off before the mantle enveloped the outside of the root. The examination of longitudinal sections of mycorrhizas tends to confirm this view of the origin of these outer cell layers in the mantle; the position of the tannin layer proper in relation to the other cortical cell layers does not alter throughout the length of the root but remains fixed so that the tannin layer is continuous with the suberin sheathe over the meristem itself; moreover, in sections stained with Delafield's haematoxylin the tannin cells proper are dark purple in colour; the flattened cortical filaments are golden brown. It has often been supposed that the production of a tannin layer is the response of the cortical cells to their isolation by the encroaching fungus; if this is so, it is difficult to see why the cortical cells of

the Hartig net which are also completely surrounded by fungal hyphae should not respond in the same manner. Probably the tannin layer corresponds merely to the outer protective layer of dead cortical cells surrounding uninfected roots and has no particular relation to fungal infection. The true tannin layer usually consists of cells filled with tannin and empty cells with thickened brown walls; occasionally unmodified cells are included. In pine mycorrhizas Melin (1923) noted two or three layers of cells filled with granules of varying size inside the tannin layer. In larch mycorrhizas only one layer of granular cells is present at most, and often only two or three such cells are visible in a transverse section, where they occur either incorporated in the tannin layer or just inside it.

Hartig Net.

Within the tannin layer the cortex of a Larch root is clearly distinguishable into two parts, an outer two or three layers of cells with unmodified cellulose walls and an inner two layers of cells with cellulose walls much thickened and yellow brown in colour; the exact nature of this modification is not known; the walls are certainly neither suberised nor lignified. The entry of the fungus between the root cells to form a Hartig net never procedes into this inner cortex, it is therefore probable that the modification of the walls acts as a barrier. Usually all the cells of the outer cortex are involved in the Hartig net and are thereby at least partly

isolated from one another by one layer of hyphae lying between the cell walls. Occasionally more than one layer of hyphae penetrates between the cells, which are then completely isolated and appear as islands in a pseudoparenchymatous tissue. This occurs most frequently in old mycorrhizas and is doubtless a sign either of weakness on the part of the root or of the high virulence of the fungus.

In some of the types of infection examined, notably the mycorrhizas formed by B. elegans, the cells of the outer cortex in transverse section in the sublateral roots are roughly oblong in shape, the radial walls being longer than the tangential; this gives a very characteristic appearance in transverse section. If, however, uninfected sublaterals are examined no such appearance is observed, nor is it found in laterals which have become infected; the differential behaviour of infected laterals and sublaterals being particularly marked.

There is no doubt that the uninfected root typically has outer cortical cells which are roughly isodiametric in the transverse plane. There are three possible ways in which this shape could be altered to give a cell of greater width in the radial rather than the tangential plane. Either the square cells could divide by formation of radial walls, so that the tangential walls were bisected, or the presence of the fungus might cause the cells to be compressed radially or both factors might operate together. An attempt was made to determine

in which of these ways the oblong cells are actually formed.

The evidence that it is radial division rather than compression which determines the shape of the cells is as follows:-
(i). Statistical[1] comparisons were made of the number of cells in the fourth cortical row outside the endodermis in various types of sublateral root. This particular cell row was chosen because it was half-way between the thickened inner cortex and the somewhat irregular tannin layer. Unfortunately uninfected sublateral roots are rarely found in nature so that it was difficult to obtain enough examples for comparison.

Table 3

Values of 't'

Types of infection compared	
B. elegans and no infection	12.44 (P = 0.01)
B. elegans and yellow hymenomycete mycelium	8.88 (P = 0.01)
B. elegans and *Mycelium radicis atrovirens* ...	5.66 (P = 0.01)
B. elegans and 2nd black mycelium	9.88 (P = 0.01)

But sublateral roots infected by yellow or black mycelia (i.e. Types 5, 6, or 7), are more common and these were also compared

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[1] The 't' test of Fisher (1930, section 24.1) was used for the determinations of significance.

Table 2

Relation of Cortical Cell Number to Type of Root Infection

Type of Infection	Not Infected	*B. elegans*	Yellow hymenomycete mycelium	*Mycelium radicis atrovirens*	2nd Black mycelium
		Type 1	Type 5	Type 6	Type 7
Number of cortical cells	43	68	45	49	38
	42	59	38	37	32
	41	65	37	44	36
	42	56	36	54	42
	43	96	38	36	49
	43	61	51	47	51
	38	58	39	33	30
	37	63	51	58	34
	37	60	37	50	38
	45	58	42	36	37
Mean[1]	41.1 ± 2.81	61.7 ± 4.43	41.4 ± 5.72	44.4 ± 8.48	38.7 ± 5.89

[1] The standard error of the mean is shown in this and subsequent tables.

with sublaterals known to be infected by B. elegans (Table 2). There is considerable variation in the numbers within any type since hand sections cannot be cut at exactly similar levels and the roots vary somewhat. However, the results clearly indicate that the sublateral roots infected with B. elegans have more cells in the fourth cortical row than any other type examined and that this difference is highly significant (Table 3). The values for the uninfected roots are probably somewhat too high, owing to the impossibility of being certain that every root is a true sublateral; the lateral roots compared with the sublateral roots have a greater number of cells in each cortical layer, and the inclusion of any such roots would raise the mean values. The importance of the low values obtained from Types 5, 6, and 7 lies in the fact that these attacks are extremely unlikely to be followed by a diminution in the number of cortical cells laid down by the growing point since the protoxylem diameters remained the same; therefore the number of cortical cells which would have been obtained had the same roots remained uninfected cannot be larger than the actual number counted in roots attacked by yellow or black fungi, and it might well be smaller if division occurred in these roots also. The fact that counts from such roots give similar numbers to those obtained from uninfected roots suggests that in all probability no increase in cell number takes place as the result of attack by such fungi. But there is no doubt that counts of root

cells support the contention that radial division of cortical cells occurs in roots infected with B. elegans.

(ii). Two samples of material were collected from different localities; each contained some uninfected sublateral roots and some infected by B. elegans. After embedding in paraffin wax transverse sections 10μ thick were cut by microtome from each type of root; the number of cells in the fourth cortical row was counted in certain of the sections taken at frequent intervals from the apex to the base of each root. The variation of cell number with distance from the apex is shown in Figs 1 and 2. It is important when comparing the cell numbers of single roots rather than the mean values of many roots to use roots taken from the same small area since variation in soil etc. may lead to marked differences in the potential number of cortical cells; for example, sample 1 tended to have fewer cortical cells than sample 2. But in each sample the uninfected roots show a tendency for the number of cells to increase up to a certain distance from the apex, after which the number remains steady and may finally decrease. This is a reflection of the life history of the root, in which a certain amount of radial division of the cortical cells laid down by the meristem occurs just behind the apex and then no further division occurs; the decrease in the number of cortical cells in the basal portion results from the slight increase in the size of the growing point after emergence from the parent root. In the infected

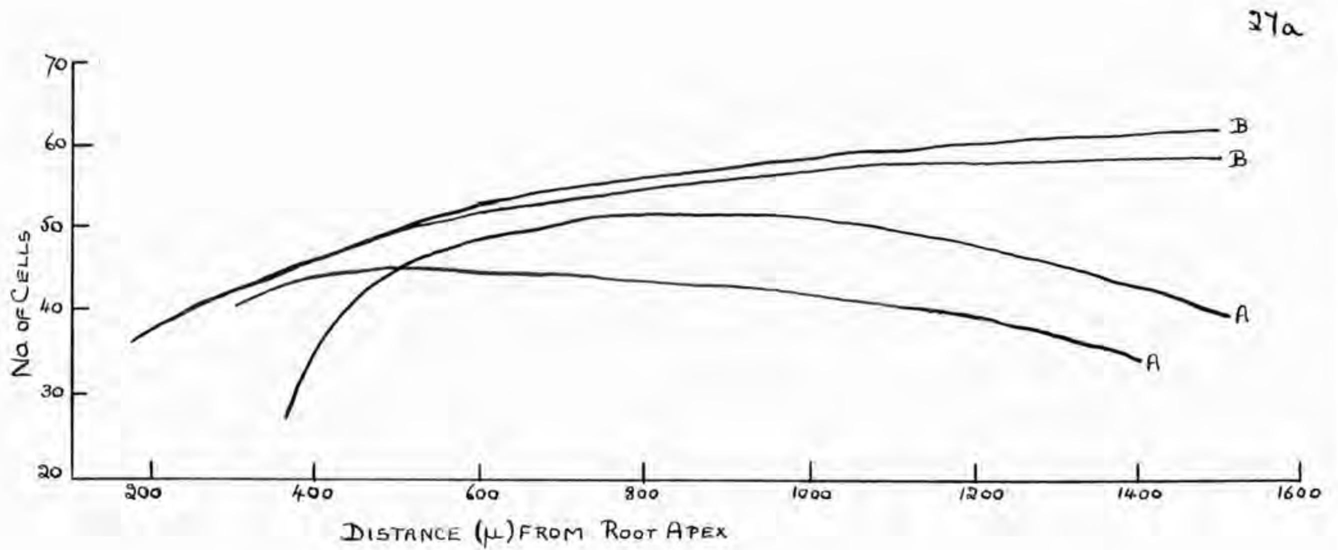


Fig.1. Variation in cortical cell number with distance from the root apex in sample 1; A, uninfected sublateral roots; B, sublateral roots infected with B.elegans.

Fig.2. Variation in cortical cell number with distance from the root apex in sample 2; A, uninfected sublateral roots; B, sublateral roots infected with B.elegans.

root however, the number of cells increases to a much higher level behind the growing point than in uninfected roots, and it remains at that level showing no decrease at the base. Since only roots with similar protoxylem diameters were compared it is legitimate to assume that the potential number of cortical cells was similar in every root, in which case it is clear that more divisions occurred immediately behind the growing point in the infected than in the uninfected roots. The fact that in the former there is no falling off in cell number towards the base further emphasises the much greater activity of cell division, which appears to continue for a greater distance behind the meristem and completely to mask any trace of the smaller number of cortical cells laid down on the first emergence of the root. Had more root material been available the effect of individual variations could have been lessened by the use of mean values; nevertheless this method gives further proof that increased cell division occurs in infected roots.

(iii). It was noted that the diameter of infected roots, excluding the mantle, was not markedly greater than that of uninfected roots; any cell division which occurs in the radial plane must therefore divide the existing cells so that the tangential walls are halved and the radial walls remain the same. If the oblong shape of the cells in the infected roots is due to such division rather than to compression the increase in the number of oblong cells in the infected roots compared

with the uninfected will be twice the difference between the total number of cells in the two types of root. If the difference is more than twice then the shape of some oblong cells is determined by compression rather than division.

Camera lucida drawings were made of the fourth cortical cell row in one section (1600 μ from the apex), from each of the serial sections used previously. The radial and tangential axes of each cell were measured and the number of oblong, isodiametric and tangentially elongated cells was estimated. An 'oblong' cell was defined as one having the radial axis at least 2.5μ longer than the tangential and an 'elongated' cell as one having the radial axis at least 2.5μ shorter than the tangential. In order to reduce the effect of individual variations the cell numbers of four roots are considered together in each case. Table 4 shows that the ratio of the differences between the total number of cells of all types and the number of 'oblong' cells is less than two, viz. 1 : 1.9. Thus division of cells would account for the number of oblong cells without assuming that compression of the cells had also occurred.

(iv). However, further evidence was sought that cell compression played no part. Presumably if cell compression occurred it would act equally in both radial and tangential directions since each cortical cell is wholly surrounded by hyphae.

The average lengths of the radial and tangential walls in

Table 4

Numbers of 'Oblong' Cortical Cells in 4 Uninfected and 4 Infected Sublateral Roots of Larch

	Uninfected root	Infected root	Difference	Ratio of differences
Total number of cells	33	68		
	27	62		
	58	78		
	67	81		
	185	289	104	1.0
Number of oblong cells	9	64		
	4	61		
	30	67		
	31	81		
	74	273	199	1.9

32 cells picked at random from the fourth cortical row in each uninfected root were compared with those in the corresponding infected roots, see Table 5. No significant difference was found between the mean lengths of the radial walls, but the differences between the mean lengths of the tangential walls were found to be highly significant. It is evident therefore, that little or no compression takes place in the tangential direction; it follows that it is unlikely to occur to any extent in the radial direction. The large differences in the length of the tangential walls must be due mainly to division

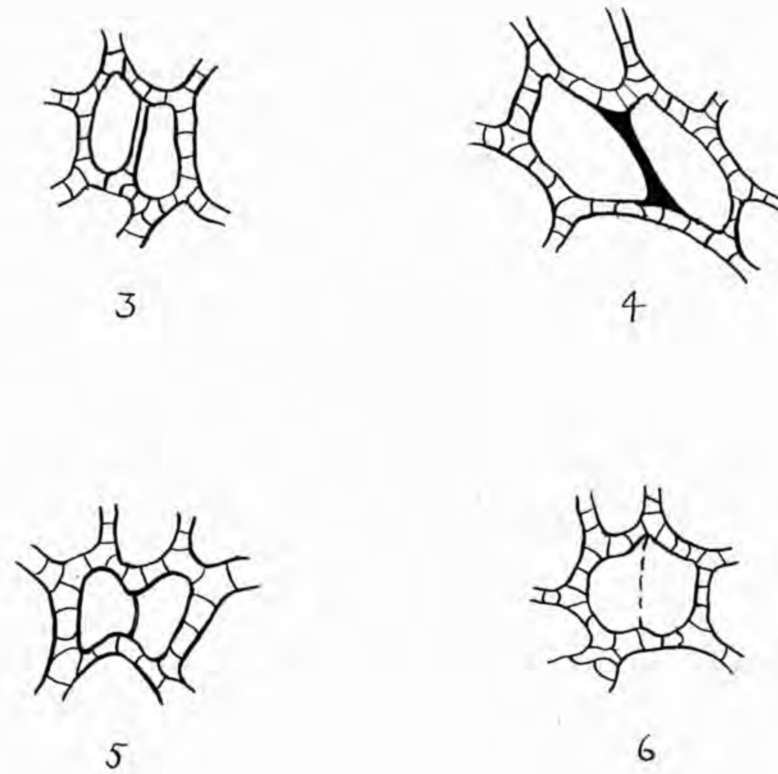
Table 5

Mean Lengths of the walls of 32 Cortical Cells in 4 Uninfected and 4 Infected Sublateral Roots of Larch

	Uninfected Sublateral μ	Infected Sublateral μ	Value of 't'
Radial Walls	33.5 ± 10.1	30.7 ± 8.6	1.66 (P = 0.1)
	38.5 ± 11.9	36.5 ± 11.9	0.95 (P = 0.4)
	26.9 ± 7.9	26.0 ± 6.5	0.68 (P = 0.5)
	23.25 ± 6.0	23.4 ± 12.9	0.05 (P = 0.9)
Tangential Walls	40.3 ± 12.3	15.7 ± 5.5	14.35 (P = 0.01)
	41.4 ± 13.7	18.7 ± 6.1	12.22 (P = 0.01)
	22.6 ± 7.5	15.4 ± 5.3	6.25 (P = 0.01)
	19.65 ± 5.5	11.9 ± 3.6	8.92 (P = 0.01)

of cells. This is further emphasised by the fact that the average length of the tangential walls in the four uninfected roots is almost twice that in four infected roots; the ratio is 39.0 : 20.5.

The four lines of enquiry set out above, tend to confirm the hypothesis that the oblong cell shape in the transverse plane in sublateral roots infected by B. elegans is the result of the division of the cells. Nevertheless it must be noted that no undoubted example of cell division, nor any dividing nuclei have been seen during the examination of many hundreds of sections. Figs 3, 4 and 5 show the only cell structures which could possibly be interpreted as indicating the occurrence of cell division; a pair of cells was divided by a wall in which no hyphae were present, possibly due to the recent



Figs.3-6. Cortical cells of Larch mycorrhizas infected by B.elegans. T.S. ×680.

formation of the wall into which the fungus had not had time to penetrate. Fig. 6 shows a cell in which the protoplasm appears to be divided but no actual cell wall is visible; none of these structures however is conclusive. Furthermore, although division still occurs at 1600 μ from the apex to judge by cell counts, the cortical cells cease to be meristematic at a distance of 300 μ from the apex, from this point onwards they are elongated and somewhat thick walled, so that it seems highly improbable that they can undergo cell division. However the weight of the evidence so far available, is in favour of cell division as the prime factor determining the oblong shape of the cortical cells in Larch mycorrhizas. While this phenomenon has been investigated in detail in roots infected by B. elegans, it has also been observed on mycorrhizas formed by P. involutus and other unidentified mycelia where a balanced relationship has been established.

To sum up, for any particular root the number and shape of the cortical cells depends on the type of root and the type of infection. Thus an uninfected sublateral root will usually have from 30 to 40 elongated or isodiametric cells, a sublateral root infected by a black or yellow mycelium will be similar, but a sublateral root infected by B. elegans or other mycorrhiza-former will have 60 to 70 cortical cells, mostly oblong in shape. A lateral root, however, which has been attacked by B. elegans will have 80 or more cells, depending on

its diameter; all the cells will be isodiametric in shape, since in lateral roots infection usually takes place well behind the apex, when the cells are definitely too old to divide. According to Hatch and Doak (1933) long roots in Pine are not infected because of their greater growth rate; this may be the case in Larch since infection of lateral roots is usually only observed in material collected towards the end of the autumn season when presumably, the rate of growth of the lateral roots has begun to slacken. Even then the tip is never infected, probably because the hyphae cease growth before the rate of growth of the lateral roots has fallen to that of the sublaterals. In view of this it is advisable when investigating a mycorrhizal association in Larch to examine the branches of a monopodial system rather than the axis, for it is possible that the axis is not a sublateral root but an infected lateral in which case the features of mycorrhizal formation observed in it will not be typical of the particular fungal association as a whole.

Some authors have recorded hypertrophy of the cortical cells as one result of mycorrhizal infection in Pine. In particular Hatch and Doak (1933) compared cortical cells in infected and uninfected short roots of pure culture seedlings and found that the cells were significantly larger in the infected roots. No such hypertrophy of the cells has been observed in Larch roots by the present writer, rather the cells

in infected roots tend to be smaller than in uninfected roots as a result of division. Further investigation of the cell reactions of both Pine and Larch is needed before final conclusions can be drawn, but there are indications that here we have two fundamentally different responses to mycorrhizal infection in the ectotrophic mycorrhizas of conifers. *Pinus* spp. with dichotomously branched mycorrhizas respond by enlargement of cells, *Larix* spp. with a monopodially branched system respond by division of cells; it would be interesting to examine another monopodial form such as *Picea* in this connection. If such reactions on the part of the root are an indication of a pathological condition as has been suggested by Burges (1936), it is difficult to understand why the attacks by black fungi in Larch do not result in any increase in cell division, even though these fungi are undoubtedly parasitic in action; similarly in Pine it has been noted by Rayner (1934) that in general pseudomycorrhizas show none of the hypertrophy of the cells which is thought to be a constant feature of true mycorrhizas. It is therefore unsafe to conclude that cell reactions which are known in some cases to accompany pathological infection in plants are inevitably symptoms of disease. The reactions of the root as shown by the histology are not infallible guides to the total effect of the endophyte on the health and vigour of the host.

Intracellular Infection.

Only two types of intracellular infection have so far been observed in Larch mycorrhizas. In the first which is only found occasionally, the hyphae grow straight through the cells in a parasitic manner; these hyphae always belong to the 'yellow' or 'black' mycelia. In the second type which is more frequent and will be referred to as the haustorial type, bladder-like swellings are observed attached to the cell walls and projecting into the cells; they are usually single but sometimes they are found in clumps. A similar condition has been noted in Pine (Young 1940). There is never any indication of the digestion of these hyphae such as occurs in endotrophic mycorrhizas. Infections by a number of different fungi have been found on occasion to exhibit this feature which would appear to be dependent for its development more on environmental factors than on the presence of any particular endophyte. Possibly it was intracellular infection of this kind which led Laing (1923, 1932) to describe some of the normal mycorrhizas of Larch as semi-endotrophic. The use of such a term however, is unfortunate since it implies the presence of features similar to those found in the true endotrophic mycorrhizas (Rayner 1927). Actually there is only one feature common to both Laing's semi-endotrophic and true endotrophic mycorrhizas, viz. the invasion of cells by hyphae. Moreover in view of the relative infrequency of this

haustorial intracellular condition compared with that of the purely intercellular type the conclusion that Larch mycorrhizas are normally semi-endotrophic is certainly not justified.

Melin (1922) also held that intracellular infection was a normal feature of Larch mycorrhizas and moreover that it was the primary condition, the intracellular net being the result of the 'squeezing out' of the mycelium from inside the cells as the resistance of the plant increased. Evidence from many young mycorrhizas examined during the present work does not support this view. Infection always begins by the formation of a thin mantle, which is followed by the development of a Hartig net from the outside inwards by the penetration of the hyphae into the middle lamellae; no intracellular infection has ever been observed at this stage. Furthermore Melin's conclusions were based on nursery material and abnormal features such as intracellular infection are often prevalent under these conditions; thus a totally erroneous impression of the importance of this type of attack would be gained.

Cell Inclusions.

Frequently in the cortical cells one comes across shiny yellowish granules forming masses reminiscent of budding yeast cells. These masses are attached to the walls and may occur in both outer and inner cortical cells, in the endodermal passage cells and in the tannin cells in the vascular bundle. They have been found in at least seven different infections,

some of these infections being truly mycorrhizal, others showing pseudomycorrhizal features. The presence of the granules would appear to be correlated with infection in as much as they do not occur in uninfected roots. But their occurrence in many of the inner tissues of the root which are never penetrated by the mycelium precludes their being the direct result of the presence of the fungus in the cells; they cannot for example be the product of the digestion of intracellular hyphae.

An unsuccessful attempt was made to determine the composition of the granules. They are insoluble in alcohol, ether or concentrated sulphuric acid, but soluble in Eau de Javelle and boiling 3% caustic potash. Osmic acid stains them dark brown; suggesting that they may be fatty or proteinaceous in character; however all specific tests for oil or protein were negative. There is some evidence that this phenomenon occurs most frequently in moribund roots during the winter or early spring; but there is as yet, no clue as to their function or significance.

VI. DISCUSSION

Any judgements concerning the significance of the observations which have been set out in this paper must of necessity be provisional. No final decisions can be reached until the various phenomena recorded have been studied in experimental culture under controlled conditions. It has not so far been possible to do this. Nevertheless certain facts have emerged which call for some evaluation, even though at this stage it is possible to draw only tentative conclusions.

In both Pine and Larch the root systems show a differentiation into two morphological units, lateral and sublateral roots, alternatively named long and short roots; the differentiation apparently corresponds to the formation of long and short shoots in the stem. This development of two types of root is important for mycorrhiza-formation, since each type behaves differently towards infection; the lateral roots normally remain uninfected, the sublateral roots rarely escape infection by one fungus or another. Reference has already been made to the suggestion of Hatch and Doak (1933) that the different behaviour of the two root types in Pine is correlated with their different rates of growth, the fast growing long roots remaining free from infection, the slower growing short roots easily becoming attacked by certain soil fungi. There is some evidence that differential growth rates are similarly

responsible in Larch for the differences in the incidence of fungal infection in the lateral and sublateral roots. Although there is no explanation available as to why a fast growing root should be less easily attacked than a slow growing one, it is clear that if confirmation of this is obtained it has great importance for the growth of the plant in various soils. For soil factors by influencing the rate of growth of lateral and sublateral roots might increase or decrease the number of mycorrhizas formed. Plants grown in a fertile soil in which the sublateral roots have a relatively high rate of growth might tend to have fewer mycorrhizas than plants grown in infertile soil where the rate of growth of even some of the laterals is too low for the roots to remain uninfected. Hatch (1937) did in fact find that fewer mycorrhizas were formed in fertile than in infertile soil; he attributed this to the high internal salt concentration of the plants which had been grown on a rich substrate, but it may well be that a high rate of growth in the sublateral roots is also a factor.

Throughout this paper it has been assumed that a knowledge of the range of structural features found in the mycorrhizas of Larch might enable one to determine by the criterion of structure alone the value of any given infection to the plant. It is realised however that a knowledge of mycorrhiza-formation in native habitats together with the experimental testing of the effect of various endophytes on the health and

vigour of the plant will be necessary before the value of a mycorrhizal association can be estimated solely on grounds of structure. In the course of this investigation it became evident that there is at least one structural feature which can be used to distinguish between the balanced association of a mycorrhiza and the unbalanced association of a pseudo-mycorrhiza. Such a criterion is supplied by the presence in mycorrhizas and the absence in pseudomycorrhizas of oblong-shaped cells in the outer cortex, due to the division of the cells by radial walls. When the presence of oblong cells in the cortex of infected Larch roots is used to classify an association as balanced it is found that the other structural features which accompany this phenomenon are similar to those which occur in Pine mycorrhizas known from experimental cultures to be balanced; furthermore the deviations from this condition sometimes found in infected Larch roots are of the same type as the deviations in structure observed in Pine roots attacked by deleterious fungi. It is on the assumption that a fundamental similarity exists between the mycorrhizal relations of the two genera that the value of each of the seven types of infected root described in Section IV has been estimated.

Accordingly until there is evidence to the contrary a mycorrhizal association in Larch may be said to be 'balanced' when its structure is as follows. The mantle, which varies

from 10 - 60 μ in width according to the endophyte, consists of hyphae closely woven to form a definite structure depending on the particular fungus concerned; the mantle encloses two or three layers of dead cortical filaments, which are adjacent to a single tannin layer containing mainly squarish tannin cells with a few granular cells. The Hartig net extends across the outer cortex to the inner cortex and the cell walls are not swollen and contain only one strand of hyphae; the outer cortical cells are oblong in shape and at least 60 in number; intracellular infection of any kind is absent. The greater majority of the deviations from this structure are clearly due to the encroachment of the fungus at the expense of the root, viz. very wide fungal mantles containing isolated cortical cells, very swollen cortical walls containing two or more strands of hyphae, intracellular infection. Occasionally the reverse condition is found in which the fungus apparently finds difficulty in establishing itself as shown by the almost complete absence of mantle and Hartig net.

In theory, under natural conditions a sublateral root may develop in four possible ways. Firstly, it may remain uninfected, an infrequent occurrence; in this case it remains unbranched. Secondly, it may become infected by B. elegans, or many other mycelia so far unidentified and form a balanced association. Thirdly it may become infected with one of these mycorrhizal fungi but form an unbalanced association; possibly

types 2 and 3 are produced in this way. Finally it may be attacked by a fungus such as M. r. atrovirens which can form only pseudomycorrhizal associations. In practice unless the identity of the endophyte is known it is impossible to distinguish between the third and fourth of these possibilities on grounds of structure alone, since an unbalanced association is produced in each case. It may be found that an unbalanced association with a potential mycorrhiza-former will nevertheless show division of the outer cortical cells, in which case it should be possible to distinguish this condition from the true pseudomycorrhizal state where no such division has been found to occur. Before this can be done with certainty however, considerably more experience will be required of the possible variations in the structure of Larch mycorrhizas.

Which of these four possibilities is realised in any particular root will depend on what species of fungi are actually present in the soil immediately surrounding the root and on internal factors in the root itself, the latter are as yet unknown, apart from the two already mentioned, viz. high internal salt concentration and rate of root growth. Both the external factors such as the fungi and the internal factors will be almost wholly determined by soil conditions. Edaphic factors will largely determine on the one hand, the species and vigour of the fungi available for mycorrhiza-formation, and on the other the growth rate of the roots and their power

of resistance to infection. The examination of all the roots of naturally regenerated seedlings reveals that frequently the mycorrhizas of any one seedling have been formed by a number of different fungi; it therefore appears probable that many habitats are a mosaic of small areas in each of which the soil conditions vary more or less widely from the others. The general result of mycorrhiza-formation for any individual will then depend on the proportion of beneficial and deleterious associations present in its roots. Thus the discovery of pseudomycorrhizas in a plantation is only of significance if they occur frequently. Nor does the presence of hyphae of a known pseudomycorrhiza-former in the soil necessarily imply that pseudomycorrhizas will be formed. Hyphae of 'atrovirens' type have been observed in many soils in which no pseudomycorrhizas could be found. It follows that in appraising the part played by the various soil factors in mycorrhiza-formation, more weight should be placed on those that affect the health and vigour of the roots than on those which affect the presence or absence of the fungus. Hatch (1936) considered that the prime necessity in the establishment of exotic conifers is the inoculation of the soil by suitable mycorrhiza-formers; this is undoubtedly the case in many countries. But in the British Isles where many potential mycorrhiza-formers with Larch exist in most soils attention should be directed more to the study of edaphic factors and their bearing

on the formation of balanced associations in Larch than to the possibilities of adding known mycorrhiza-formers to the soil.

VII. SUMMARY

1. The roots of European Larch can be grouped into two classes, lateral and sublateral roots, corresponding to the long and short roots of Pine. The majority of the mycorrhizas are normally formed by the infection of the sub-lateral roots, a few by the infection of lateral roots.
2. *Boletus elegans* and *Paxillus involutus* have been isolated from Larch mycorrhizas. *Mycelium radicis atrovirens* from pseudomycorrhizas.
3. Evidence is produced that infection of the sublateral roots by a mycorrhiza-former such as *B. elegans* results in an increase in the number of cortical cells in the root as compared with either uninfected sublateral roots or pseudomycorrhizas.
4. Detailed descriptions are given of the structural features resulting from infection by *Boletus elegans*, *B. viscidus*, *Paxillus involutus*, a yellow hymenomycete mycelium, *Mycelium radicis atrovirens* and two unidentified mycelia.
5. The general features of mycorrhiza-formation in Larch are discussed.

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THE MYCORRHIZAL RELATIONS OF LARCH

IV. Mycorrhiza-formation in Experimental Culture

by

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I. INTRODUCTION

It is a relatively easy matter to obtain typical mycorrhizas of Pinus sylvestris in experimental culture; but the production of Larch mycorrhizas has not proved so simple. Early pure culture work was not successful owing to the sensitivity of Larch to environmental conditions, in particular to excessive humidity and a badly aerated substrate; while soil cultures, though providing good conditions for the higher plant, seem in many cases to inhibit the growth of the fungus.

There have been two previous attempts to obtain Larch mycorrhizas experimentally. First, Melin (1922) used pure culture methods with Larch seedlings grown in sand in double flasks and inoculated with pure cultures of Boletus elegans. The mycorrhizas obtained were of an abnormal type due no doubt to the unfavourable conditions for the seedlings; for the lateral roots rather than the sublateral roots were concerned in the association and the infection was largely intracellular. Even the main root was infected intracellularly, suggesting that the fungus had attacked the seedlings parasitically. The photograph published by Melin shows a similar condition in the root to that found in excised root cultures attacked by fungus (How 1941), where the balance is known to be definitely in favour of the fungus. The second attempt was that of Hammerlund (1923); he used soil cultures and again mycorrhizas

were obtained but these had a heavy intracellular infection. It is doubtful whether B. elegans was concerned in these latter experiments since the experimental methods were open to question, but it is clear that no typical ectotrophic mycorrhizas were formed.

No attempt was made to obtain mycorrhiza formation in pure culture during the present investigation since the fact that B. elegans is a mycorrhiza-former with Larch was taken to be fully established (see Melin l.c. and Part III of the present series). Furthermore, although Hatch (1934) has greatly improved the technique of pure culture work it was considered necessary to obtain preliminary data as to time of formation and stages of development of typical mycorrhizas in pot culture before using his complicated apparatus.

II. FACTORS CONCERNED IN MYCORRHIZA FORMATION

With a view to determining which are the factors of importance in the development of mycorrhizas in Larch, four separate sets of cultures were set up. These are reported on in detail in section III. Although satisfactory mycorrhiza formation was obtained in none of them with either of the two experimental fungi, viz. B. elegans or Paxillus involutus (see Part III for evidence that P. involutus is a mycorrhiza-

former with Larch), an analysis of the causes of failure indicates some of the factors which may be concerned in the development of the mycorrhizal association.

It was expected that in Experiment 1, the raising of European Larch seedlings in soil known to contain a mycorrhiza-former together with inoculation from agar cultures of B. elegans and P. involutus would result in mycorrhizal infection by one or other of these three fungi. This did not occur; infection was present, but the endophyte was a brown-walled mycelium which formed an intercellular and intracellular attack of a pseudomycorrhizal character. The most obvious explanation of the failure of the three fungi to infect the seedlings would be to attribute it to the absence of living hyphae in the soil at the period when the seedlings were capable of mycorrhiza formation. In the case of the mycorrhiza-former present in the soil, probably the mycelium had died during the transference of the soil from the plantation to the pots, but in the case of the two species introduced by agar inocula, the inocula had presumably failed to establish themselves in the soil. The probability that the inocula had in fact died is strengthened by direct observation of similar inocula in this particular soil; no inocula have ever been found to germinate when placed in soil in pots or in flasks under laboratory conditions. It is suggested that the inocula failed to grow either because the soil conditions were inhibiting or because

the agar of the parent culture was too low in nutrients to enable the mycelium to live during the transition period from agar culture to soil.

The soil factors will be considered first. B. elegans and P. involutus will only grow in pure culture on acid media, i.e. below a pH value of 6.2; and the pH value of the experimental soil was 6.6 - 6.8; so that the low hydrogen ion concentration might appear to be the inhibiting factor. However in the three subsequent experiments the pH values of the soils ranged from 5.4 - 6.2, yet in none of these cultures did either B. elegans or P. involutus appear to be capable of growth. Furthermore B. elegans has been observed to occur in soils of pH value 6.0 under natural conditions so that clearly the hydrogen ion concentration of the soil cannot be the inhibiting factor in the more acid soils; it is therefore probable that there is some other factor which is primarily responsible for the failure of the inocula to germinate.

Attention was next directed to soil aeration as the soil used in Experiment 1 was heavy in texture. In experiments 2 and 3 the soil was well aerated, particularly in experiment 3 but the inocula again failed to grow out into the soil. Possibly the soil contained inadequate nutritive material such as humus to act as a substrate for the fungus. While this would account for the failure of mycorrhizas to develop in experiment 2 it would not do so in experiment 3 where 40% of

the soil mixture consisted of an organic compost. Actually in laboratory culture there is no evidence that B. elegans is capable of breaking down complex humus products; it is therefore unlikely that the addition of organic matter to the soil will improve it for growth of this fungus. Since single soil factors do not seem to be responsible for the inhibition to growth the alternative explanation must be considered, viz. that a nutrient agar inoculum may be too easily exhausted to supply a good initial substrate for the mycelium in its normally slow growth in the soil.

Experiment 2 in which seedlings were raised in glass-sided boxes was carried out with this in view. The substrates employed for the fungus were either autoclaved wheat or oat seed on which both B. elegans or P. involutus can grow for three or four months, or such materials as compost and steamed soil which might be thought to aid a transition to the experimental soil mixtures. Nevertheless no mycorrhizas were formed by either of the fungi and frequent observation through the glass panels indicated that although the mycelium sprouted at the margins of the various types of inocula it did not enter the soil. Moreover, if proximity to living tree roots was also a necessary factor, success should certainly have been attained in experiment 2 since all the inocula were placed against young roots by removing the glass panels. Clearly the presence of a reservoir of food material and contact

with living roots did not overcome the inhibition to mycorrhiza formation in this experiment.

Therefore a further possibility was suggested, namely that a fungus such as B. elegans was dependent for its nutrition in the soil on an intimate association with living tree roots. Accordingly a new method of inoculation was devised on the principle that once the fungus had become established in the roots it would continue to infect the new roots as they were formed. Seedlings can easily be infected under pure culture conditions on a good substrate, for then the balance is heavily weighted in favour of the fungus which may even become parasitic (cf. Melin's experiments). Accordingly pure cultures of seedlings infected with B. elegans were set up in experiment 3; when the mycelium had penetrated the cortex of the hypocotyl the whole culture was transferred to an artificial soil rich in humus. However in spite of such very favourable conditions for the fungus B. elegans failed to form any mycorrhizas with the roots, nor were the characteristic hyphae ever observed.

In view of the failure of these repeated experiments to establish mycorrhiza-formation with either B. elegans or P. involutus it seemed possible that the inhibiting factors should be sought in the soil conditions as they affected the host rather than in the soil conditions as they affected the fungus. This seemed the more probable since Hatch (1937) found that seedlings of Pinus sp. failed to produce mycorrhizas

in a very fertile soil and he attributed this to the development by the plant of a high internal salt concentration. Whether some such condition was operating in the case of the Larch seedlings was examined in experiment 4. The boxes were filled with a steam-sterilised heath soil poor in nutrients; the seedlings were inoculated with B. elegans under pure culture conditions as before and transferred later. In some cases when seedlings were transplanted pads of infected Sorbex were placed below the radicle, since it might be thought that the previous failure with the inoculation method was due to the entrance of the fungus into the hypocotyl rather than into the root. In spite of this extra precaution when the seedlings were examined at the end of the first growing season the roots were found to be entirely free from the mycelium of B. elegans.

In experiment 4 where a very poor soil had been used, internal factors produced in the Larch seedlings by growth on a highly nutritive substrate could not possibly be operative. It is probable that such factors do not exist for Larch, to judge by the results of the humus inoculation of plants growing in a nursery bed containing soil of high nutritive content (experiment 3). In this very fertile soil mycorrhizas were formed in the first season by an unidentified endophyte, though the relationship did not appear to be fully established. While it is clear that Larch seedlings are capable of forming incipient mycorrhizas with some fungi; ~~but~~ the apparent

immaturity of the associations so formed suggests that some age factor exerts an influence in mycorrhiza formation. It has been noticed repeatedly that first year seedlings even when infected show no monopodial branching and only incipient features of mycorrhiza-formation. There is no mantle, often no tannin layer and rarely does the Hartig net extend evenly over the whole outer cortex. Second year seedlings show a greater development of these features, but still nothing comparable to the mycorrhizas of mature trees. It is possible that very strong mycorrhiza-formers such as the endophyte in the nursery bed can form mycorrhizal associations with first year plants, whereas weaker mycelia, e.g. B. elegans or P. involutus, are incapable of infecting the roots till after the second year.

Apart from this tentative suggestion these four experiments have failed to reveal any single factor as essential if mycorrhizas are to be formed in Larch seedlings by B. elegans or P. involutus. Manifestly the influence of the soil complex taken as a whole on both host and endophyte is of greater importance than that of any single factor taken by itself.

III. EXPERIMENTAL CULTURES

Experiment 1. This was a preliminary series of cultures. In March the top 1½ ins. of soil was transported from a Larch

and Beech plantation in which well balanced mycorrhizas had been formed the previous autumn. The humus covering was very thin so that the main constituents of the soil used for potting was a heavy clay loam with a pH value of 6.6 - 6.8. There were about 15 European larch seedlings in each pot. 12 pots were left as controls, 12 pots were inoculated 2½ months after sowing by chopping each plate culture with a sterilised scalpel and mixing thoroughly with the surface soil round each seedling.

6 pots inoculated with glucose agar cultures of *Boletus elegans*.

6 pots inoculated with glucose agar cultures of *Paxillus involutus*.

At the time of inoculation the seedlings had a small root system with a few short lateral roots. There was no evidence of any infection. After 6 months seedlings from one pot in each series were examined. All the seedlings had well branched root systems with many sublateral roots; there was none of the monopodial branching associated with mycorrhiza-formation. Microscopic examination revealed that some sublateral roots in both control and inoculated seedlings showed infection by a broad septate mycelium which had pale brown walls. No mantle was formed, the tannin layer was well developed, the Hartig net was irregular in development, and intracellular infection was very heavy, many of the cortical

cells being completely filled with hyphae. The mycelium concerned was not the same as that which had formed well balanced mycorrhizas in the plantation, neither was it B. elegans or P. involutus; these fungi had failed to infect the seedlings. The soil was therefore considered to be unsuitable for inoculation experiments and the remaining seedlings were shaken free from soil and repotted in December; a sandy heath soil from a New Forest Larch plantation was chosen for this purpose; the pH value was 4.8. Reinoculation was then carried out as before using glucose agar cultures of the two fungi.

The following autumn when the seedlings were 18 months old one pot from each series was examined, and again a year later when the seedlings were 2½ years old. External examination showed that all the 18 month old seedlings had well branched root systems, similar in character to the 6 month old seedlings; a few of the 2½ year old seedlings had sublateral roots showing the beginnings of monopodial branching. The infection of the roots at both the later ages was similar in type to that described in the 6 month old seedlings. In the 2½ year old plants however, the Hartig net was better developed and the intracellular infection was slight. A few of the lateral roots were also attacked but the majority of the main roots were unaffected.

Experiment 2. Young (1936) had successfully grown Pine seedlings in boxes with glass panels in which it had been

possible to observe the formation of mycorrhizas in situ. A similar method was used for Larch. Boxes were constructed of ½" teak with two sloping glass panels which could be moved up and down in grooves and were protected by zinc sheets. The internal measurements were 11½ x 5½ ins. at the bottom, 11½ x 8 ins. at the top and 12 ins. deep; the base was raised 1 ins. above the ground and was perforated at intervals for drainage (Plate I, fig. 1).

In order to lower the risk of the formation of mycorrhizal complexes by fungi other than the species to be investigated and at the same time avoid the use of sterilised soil, an artificial substrate was used of the following composition:-

Meadow loam	50%	by vol.
Sand	12½%	" "
Sandstone chips	12½%	" "
Sawdust compost C6a (Rayner 1936)	25%	" "

pH value 5.4.

It was thought unlikely that the meadow loam contained any forest soil Hymenomycete capable of infecting Larch roots. Microscopic examination of the soil revealed only the presence of a black thick-walled mycelium and hyphal fragments of apparently Phycomycete type.

European Larch seeds obtained from Johannes Rafn & Son, Copenhagen, were handpicked, scraped free of wings and centrifuged in distilled water for five minutes; after sterilising

in 0.1% mercuric chloride for two minutes they were repeatedly washed in sterilised distilled water and sown directly in the artificial soil. In each box a dozen seeds were evenly spaced in a row about a quarter of an inch from each of the glass panels. The plants were watered with autoclaved rainwater throughout the first growing season. The following treatments were used.

Series L - 3 boxes uninoculated.

Series L B I - 3 boxes - Each sterilised seed placed at the time of sowing in contact with an inoculum 0.5 cms. square taken from an actively growing culture of B. elegans on beerwort agar.

Series L B II - When the seedlings had a main root and at least two lateral roots each was inoculated by placing the inocula infected with B. elegans in contact with the root tips after removing the glass panel.

1 box - inoculated with steamed soil[1] culture 6 weeks after sowing.

1 box - inoculated with oat seed culture 10 weeks after sowing. Re-inoculated a month later with further oat seed cultures.

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[1] A Bagshot heath soil from Wareham was steamed for twenty minutes and used as a substrate for these cultures.

Series L B II (cont.) - 1 box - inoculated with cultures grown on autoclaved sawdust compost 10 weeks after sowing.

Series L B III - 1 box - inoculated with ripe sporophores of B. elegans 6 weeks after sowing. The pileus was cut into 1 cms. squares and placed hymenium downwards beside the roots so that inoculation from germinating spores might occur.

Series L P I - 3 boxes - inoculated at the time of sowing from beerwort agar cultures of P. involutus.

Series L P II - 1 box - inoculated with steamed soil[1] culture of P. involutus 6 weeks after sowing.
1 box - inoculated from oat seed culture 10 weeks after sowing. Reinoculated a month later with further wheat seed cultures.

All the inocula consisting of mycelium growing on an artificial substrate, could be observed sprouting but no visual evidence was obtained that either fungus could grow out into the soil. In series L B III the spores remaining in the soil after the decay of the sporophore tissue showed no sign of germination after 9 months.

Representative seedlings were examined 6½ months after

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[1] A Bagshot heath soil from Wareham was steamed for twenty minutes and used as a substrate for these cultures.

sowing. At this stage the root systems consisted usually of a main root with about a dozen lateral roots bearing frequent short lateral roots. All the roots were pale brown in colour and there was no sign of monopodial branching. Microscopic examination revealed that neither B. elegans or P. involutus had infected the roots. These, however, were subject to two types of attack which were fairly widespread. In the first type the mantle was either absent or very loosely woven, often the tannin layer was absent, sometimes the Hartig net was irregular in depth and the cell walls unevenly swollen; intracellular infection was very frequent. This somewhat unbalanced mycorrhizal structure resulted apparently from attacks by various hyaline fungi, including one probable Basidiomycete. The second attack was caused by a black mycelium, similar to an 'atrovirens', and was clearly pseudomycorrhizal in character.

At the end of the second growing season there was still no sign of monopodial branching, though a small number of roots were infected ectotrophically. These mycorrhizas had a very narrow mantle, no tannin layer and a Hartig net which reached to the inner cortex; the cortical walls were very greatly swollen by the presence of wide hyphae; a trace of intracellular infection occurred. The general impression was that of an unbalanced relationship between root and fungus; possibly this is a more mature stage of infection by one of

the hyaline fungi found the previous year. No other type of attack was observed.

Experiment 3. A new technique for inoculation was tested in this series of cultures in glass sided boxes in an attempt to overcome the difficulties of establishing either B. elegans or P. involutus in the soil. Japanese Larch seedlings were used in addition to those of European Larch. The composition of the substrate was as follows:-

Meadow loam	20% by vol.
Sand + Sandstone chips ...	30% " "
Charcoal	10% " "
Hop waste Compost C5 (Rayner 1936) ...	40% " "

A much higher percentage of compost was used in order to provide a humus substrate for the fungus, a greater proportion of sandstone chips to give a better aerated soil. Since the disadvantages of casual infection were thought to outweigh those of soil sterilisation, the components of the artificial soil were steam-sterilised before mixing. The pH value of the final product was 6.0 - 6.2.

Instead of sowing directly in the boxes as formerly, some of the seedlings were raised in pure culture conditions and infected with the fungus, only later being transferred to the soil in boxes. These seedlings were raised in glass containers ($1\frac{1}{2}$ ins. in diameter x 4 ins.) which had been filled to a depth

of $1\frac{1}{2}$ ins. with one or other of the following materials.

1. Osmunda fibre.

2. Sorbex 7 parts, charcoal 1 part, sand 1 part.

These materials were well moistened with the following nutrient solution:

Glucose	... ...	0.5 gm.
KH_2PO_4	... ...	0.2 gm.
NH_4Cl	... ...	0.1 gm.
$MgSO_4 7H_2O$	... ...	0.01 gm.
Distilled water	...	100 cc.

The Osmunda fibre cultures were sterilised by autoclaving at 15 lbs. pressure for 30 minutes, the Sorbex cultures by steaming for 30 minutes on three successive days.

After centrifuging for five minutes in distilled water, the seeds were sterilised in calcium hypochlorite (Wilson 1915) and put to germinate on Melin's glucose agar so that any contamination could be easily detected. When the testas had split the seeds were transferred singly under aseptic conditions, to the containers which had been inoculated six days previously with B. elegans. Controls were set up without fungal inoculation. The seedlings developed well and were very vigorous, the presence of the fungus in no way impaired their vitality (Plate I, fig. 2). About ten days after planting in the containers the whole contents of the latter were transferred to the boxes so that the roots were undisturbed. No true estimate

PLATE I

Fig.1. Plant Box for the Observation of Mycorrhiza-formation in situ; the Glass Panels have been removed. $\times \frac{1}{6}$.

Fig.2. European Larch Seedling in Osmunda-fibre Culture inoculated with B.elegans. Seedlings 3 weeks old and ready for transplanting. $\times \frac{3}{4}$.

of the mortality rate of the seedlings can be given since a number of the transplants were accidentally damaged by birds. However the majority of the undamaged seedlings raised in the Sorbex cultures appeared to thrive; but there was a high death-rate with the Osmunda fibre cultures, due no doubt, to the open nature of the substrate leading to drying out of the roots where these did not come into intimate contact with the soil. Examination of the inoculated transplants at the time of transference showed that no parasitism had occurred although the fungal hyphae had wound themselves thickly round the hypocotyl. The outer cortical cells had been pushed aside by the fungus in some cases but there was no entry into the cells and the appearances seemed to be due purely to mechanical pressure.

Each box held a dozen seedlings, six European Larch seedlings on one side and six Japanese Larch seedlings on the other. The following series were set up:-

Series A - 2 boxes - Seeds placed directly on pads of sterilised Sorbex in the boxes.

Series B - 2 boxes - Seeds raised in containers of Sorbex before transference.

Series C - 2 boxes - Seeds placed directly into the boxes on pads of Sorbex previously infected with B. elegans.

Series D - 2 boxes - Seeds raised before transference in containers of Sorbex previously infected with B. elegans.

Series E - 2 boxes - Seeds placed directly into the boxes on pads of Osmunda fibre previously infected with B. elegans.

Series F - 3 boxes - Seeds raised before transference in containers of Osmunda fibre previously infected with B. elegans.

Series G - 2 boxes - Seeds sown directly and inoculated 5 weeks later by placing pads of Sorbex or Osmunda fibre inoculated with B. elegans against the roots.

Series H - 2 boxes - Seeds sown directly and inoculated at the time of sowing with soil from a Larch stand.

Series J - 1 box - Filled with unsterilised ~~sand~~ soil from a Larch stand and sown directly. The soil was a sandy loam of pH 5.2.

All the boxes were watered with autoclaved rainwater and kept in a roof greenhouse.

A nursery bed (2' 6" x 3' 6") was also prepared outside for comparison. This was filled with the artificial soil mixture to a depth of a foot. Half the bed was sown with unsterilised European Larch seed and half with Japanese Larch seed. Between the rows of seed small pellets of soil from the Larch stand were introduced five days after sowing.

The seedlings were lifted from the boxes when five months old. At this stage the development of both species in series A - H was very good, and that of the nursery-bed seedlings was outstandingly so; heights of 29 cms. were recorded for the latter, these compare very favourably with a height of 14 cms. attained by naturally regenerated seedlings from Scotland. The root systems were well developed, the sublateral roots being numerous, particularly in the Sorbex transplants. Sorbex clearly produces a better rooted plant than Osmunda fibre. No monopodial branching was observed. In series A - G there was no infection either in the control or the inoculated seedlings. In series H the roots had been attacked very slightly by a fine hyaline mycelium without clamp connections; however, no mantle or tannin layer had been formed, there were traces of a Hartig net and some intracellular infection. The seedlings which had been grown in the Larch soil in series J were less well developed than those in the artificial soil, the root systems being smaller in extent and less well branched. There was infection of an intracellular type and no typical mycorrhizas were found.

Unfortunately it was impossible to continue with the box cultures for a second year. It is clear that the new method of inoculation had not been successful in establishing mycorrhiza formation by B. elegans.

Some of the nursery bed seedlings were examined when six

months old. Infected roots were found on both the European and Japanese Larch seedlings; there was no mantle, the Hartig net was fairly well developed over the entire outer cortex and the walls between the cells were thin; in some cells there was a heavy intracellular infection by wide swollen hyphae. At the end of the second growing season, a few monopodially-branched roots were to be seen, but in the main the sublateral roots were unbranched. The type of infection was similar to that of the previous year, the only difference being the feeble development of a tannin layer in some of the roots.

Experiment 4. This was a repetition of the previous experiment using soil from Wareham, Dorset; this was a sandy heath soil poor in nutrients. The soil was steam-sterilised, and mixed with sandstone chips. The pH value was 5.6 - 5.8. Only European Larch seeds were used. The treatment of the seeds was the same as before with two modifications. Firstly, fine sterilised sand was poured into the Osmunda fibre cultures before transplanting to prevent dessication of the roots. Secondly, in the case of some of the Sorbex cultures a pad of Sorbex infected with B. elegans was placed at the bottom of each hole made for the reception of each transplant so that the radicle would grow down into it. By this practice, together with placing the original fungal inoculum towards the base of the container it was hoped to effect the penetration of the radicle rather than the hypocotyl.

Four boxes were set up as follows:-

Box 1 - 6 Sorbex culture transplants on one side and 6 Osmunda fibre transplants on the other. No inoculation.

Box 2 - 10 Sorbex culture transplants previously inoculated with B. elegans.

Box 3 - 10 Sorbex culture transplants previously inoculated with B. elegans with a pad of Sorbex placed below each seedling.

Box 4 - Osmunda fibre transplants previously inoculated with B. elegans.

For the first two months of growth the boxes were watered with boiled rainwater, later with unsterilised rainwater.

Certain of the seedlings were examined after six months growth. The root systems were poor, there being few sublateral roots, due to the poverty of the soil in nutrients. In only one case was infection observed; this root had no mantle or tannin layer but exhibited a Hartig net with very fine walls and some intracellular infection. A free hypha connected to the root indicated that a hyaline mycelium without clamp connections was probably responsible, but there is no evidence that this mycelium was that of B. elegans; in fact the infection was almost certainly due to some fungus present in Wareham soil which had escaped the steam sterilisation. Clearly mycorrhiza formation by B. elegans is not facilitated in a

the Hartig net which are also completely surrounded by fungal hyphae should not respond in the same manner. Probably the tannin layer corresponds merely to the outer protective layer of dead cortical cells surrounding uninfected roots and has no particular relation to fungal infection. The true tannin layer usually consists of cells filled with tannin and empty cells with thickened brown walls; occasionally unmodified cells are included. In pine mycorrhizas Melin (1923) noted two or three layers of cells filled with granules of varying size inside the tannin layer. In larch mycorrhizas only one layer of granular cells is present at most, and often only two or three such cells are visible in a transverse section, where they occur either incorporated in the tannin layer or just inside it.

<u>Hartig Net</u>.

Within the tannin layer the cortex of a Larch root is clearly distinguishable into two parts, an outer two or three layers of cells with unmodified cellulose walls and an inner two layers of cells with cellulose walls much thickened and yellow brown in colour; the exact nature of this modification is not known; the walls are certainly neither suberised nor lignified. The entry of the fungus between the root cells to form a Hartig net never procedes into this inner cortex, it is therefore probable that the modification of the walls acts as a barrier. Usually all the cells of the outer cortex are involved in the Hartig net and are thereby at least partly

its existence under natural conditions. There are two possibilities, both of which have been explored in the present paper; either the fungus lives on nutrients obtained from the humus in the soil or it draws its nutrients from the Larch roots. The inability of the hyphae to spread out from the inocula would suggest the first alternative, that the soil lacked essential nutrients; this conclusion cannot be disproved while there is evidence that B. elegans is unable to utilise any but the simplest organic compounds in pure culture (How 1940). Moreover, when large quantities of humus in the form of organic compost are added to the soil, there is no improvement in the soil as a substrate for B. elegans; this is only to be expected if B. elegans is incapable of breaking down lignin complexes.

Apparently this inability to effect inoculation of the soil either by pure culture or spores has also been found in the case of the root disease fungi of Tea and Cotton, Tunstall (1930), Taubenhaus et al. (1929); in these cases inoculation with infected roots is usually successful. Gadd (1936) decided that a 'food base' is probably essential for fungi such as these which cannot utilise the humus. As B. elegans is similar in its behaviour it seems most probable that for it too the root is the chief source of nutrients in its growth in the soil. In this connection it is interesting to note that some growth promoting substances have already been shown

to pass from Larch roots to *B. elegans* but so far no passage of nutrients has been demonstrated (How 1941).

The new inoculation technique of transplanting seedlings infected with *B. elegans* into the experimental soil was based on the assumption that this fungus did rely on the plant for its nutrients and that the living plant would constitute a 'food base' from which the mycelium would spread through the soil. The fact that mycorrhiza-formation was not obtained does not necessarily imply that this hypothesis is incorrect, for soil conditions may have neutralised the beneficial effects of this method which would have succeeded in other soils; more probably internal conditions in the host were also of importance in the establishment of mycorrhizas. Although it is impossible to decide with certainty in the present state of our knowledge of this fungus, the balance of the evidence suggests that *B. elegans* normally uses the mycorrhizas from which to spread through the soil to the uninfected roots, probably in addition, obtaining traces of simple food materials from the soil. How it maintains itself from season to season is a mystery, but the mycelium is most likely to remain dormant in the winter in moribund mycorrhizas from which it grows out in the spring. Garrett (1938) has suggested that many active root parasites spread through the soil in this manner, since they are virtually incapable of living independently and have to exist from season to season in infected root fragments.

Allusion has been made above to internal factors in the Larch seedlings which may affect the formation of mycorrhizas; these must now be considered. According to Hatch (1937) mycorrhiza-formation in Pine tends to be suppressed in fertile soils owing to the high internal salt concentration of the cells. In experiments 3 and 4 however, mycorrhiza-formation with B. elegans failed in both fertile and infertile soils, so that the internal salt concentration was clearly not decisive in these cases. A more probable explanation of the inability of B. elegans to continue to infect the seedlings after transplanting is to be sought in the times of formation of the lateral and sublateral roots in Larch seedlings and their differential rates of growth. In paper III of this series the importance of the rate of root growth for mycorrhiza-formation was emphasised; slow growing sublateral roots are easily converted into mycorrhizas, faster growing lateral roots are not so transformed until the late autumn when a reduced growth rate renders them liable to attack. It has been noted repeatedly that Larch seedlings do not produce sublateral roots till late in their first season so that for the first four months there are no sublateral roots which can be converted into mycorrhizas by B. elegans. In experiments 3 and 4 by the time that such roots were available the mycelium had died out being unable to support itself independently in the soil. On the other hand the soil fungus introduced by humus

inoculation was capable of living an independent existence in the soil and when the sublateral roots had developed, mycorrhizal associations could be formed. It is also possible that mycelia may differ in their ability to convert roots into mycorrhizas, some being able to infect even fast growing lateral roots, while others such as B. elegans are incapable of attacking any but sublateral or slow growing lateral roots. Although these are as yet little more than hypotheses the above suggestions may be useful in emphasising the importance of the subtle interaction of organism and fungus which underlies the phenomenon of mycorrhiza-formation. Thus the presence of living mycelium is not the only necessity, it must be available when the sublateral roots are developed and these in their turn must be growing at a rate which permits of infection. Whether or not these conditions are fulfilled depends on the edaphic factors which influence mycorrhiza-formation at every stage of the process. In the case of the formation of mycorrhizas by B. elegans with Larch the exact mode of operation of these factors has still to be determined.

Consideration must now be given to the second main problem raised by these experiments, namely the general features of mycorrhizal structure in Larch seedlings. There is reason for believing that these may differ in two respects from those found in the roots of mature trees (see paper III for description of mycorrhiza-formation in mature trees). The first

difference is the marked lack of monopodial branching in the mycorrhizas of seedlings. While in some cases this may merely be an indication of an unbalanced association the impression is left that the sublateral roots of first and second year Larch seedlings are incapable of branching monopodially and that it is not till the third season that the majority of the sublateral roots respond to infection by the formation of the characteristic monopodial systems. Possibly this points to some general inertia towards mycorrhiza-formation on the part of the plant in the early stages of growth; in which case this would be an additional factor giving rise to the inability of B. elegans to form mycorrhizas with seedlings.

The second difference is the very frequent intracellular infection which accompanies the Hartig net in the mycorrhizas of seedlings; as in the case of mature trees no digestion stages have ever been observed in infected cells. This type of infection would appear at first sight to be due either to the species of fungus involved being unsuitable or to the soil conditions leading to an unbalanced association; both are likely possibilities when it is realised that intracellular infection is heaviest in seedlings grown in artificial soils containing meadow loam which almost certainly contains unsuitable fungi and may even be an unsuitable soil. However this is probably not the complete explanation for it is noticeable that the older the seedlings become, the smaller

in extent is the intracellular attack. It would appear therefore that there is a tendency for the development of intracellular infection in the roots of young seedlings, a condition which later becomes less possible. Melin (1922) held that infection began intracellularly in a seedling root and later became intercellular by a 'squeezing out' process. There is no evidence for this in the present work where intercellular infection always precedes intracellular penetration in all but parasitic attacks by 'atrovirens' type fungi. Nevertheless the heavy intracellular attacks reported by Melin may have been due not only to bad cultural conditions but also to the general tendency for intracellular infection in the roots of first year Larch seedlings. Further experimental work will be necessary to determine whether some such age factor for mycorrhiza-formation exists in Larch; the slight development of mantle, tannin-layer and Hartig net in otherwise well balanced associations in seedlings all lend support to the view that age is of importance. It remains to be seen how far this is significant when compared with edaphic factors which determine the 'balance' or otherwise of an association. In conclusion it is clear from these four sets of experiments that the solution to controlled mycorrhiza-formation in Larch will be found to lie not in the isolation of some single factor but in the understanding of the interaction of the many factors, all of which are essential for the establishment of a proper balance between tree and fungus.

V. SUMMARY

1. The results of four sets of experimental cultures with Larch seedlings are described. Various methods of inoculation were employed, including the transplanting of seedlings previously infected with Boletus elegans under pure culture conditions.

2. The importance of various factors which affect the formation of mycorrhizas are discussed, including the source of nutrients in the soil for B. elegans, the time of formation and rate of growth of the roots and the age of the seedlings.

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THE MYCORRHIZAL RELATIONS OF LARCH

I. A STUDY OF BOLETUS ELEGANS SCHUM. IN PURE CULTURE

Reprinted from Annals of Botany
New Series Vol. IV No. 13 January 1940
Pages 135–150

Printed in Great Britain

The experimental basis for this paper was derived from part of the M.Sc. thesis; new data were included in section IV, (p. 142-3). The presentation of the material was considerably modified and a new introduction and discussion were added, in order that the paper might be suitable for publication as one of series on the " Mycorrhizal Relations of Larch".

The Mycorrhizal Relations of Larch

I. A Study of *Boletus elegans* Schum. in Pure Culture

BY

J. EASTOE HOW

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With sixteen Figures in the Text

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I. Introduction

THE regular occurrence of many soil Hymenomycetes in the vicinity of certain species of trees is well known to field mycologists. It is now realized that in many cases this is the outcome of a mycorrhizal relationship between tree and fungus. By far the most constant of these associations recorded for Boletus is that between *B. elegans* and the larch, a fact commented upon many times in the last 100 years (cf. Melin, 1922, for detailed bibliography); in this particular instance the mycorrhizal basis has been established. It seemed likely that an investigation of such a specialized relationship would throw light both on fungal physiology and the role of mycorrhiza in trees. With this in view Melin (1922) published a preliminary investigation into the physiology of *B. elegans*; a study which was never completed, however, as larch seedlings proved difficult subjects for pure culture work. The present investigation set out to enlarge Melin's observations and to discover, if possible, the cause of this specialized relationship by means of a morphological and physiological study of *B. elegans* in pure culture.

[Annals of Botany, N.S. Vol. IV, No. 13, January 1940.]

II. Isolation of the Fungus

Tissue culture is the only reliable method for the isolation of fungi such as *B. elegans* the spores of which have rarely, if ever, been germinated. Repeated isolations have been made from young sporophores, which were either flamed or washed in 0·1 per cent. mercuric chloride for one minute and repeatedly washed in sterilized distilled water. After sterilization pieces of tissue were cut from the pileus above the stipe and were transferred to the following nutrient agar medium (Melin, 1922):

Glucose	20·0 gm.
$MgSO_4 7H_2O$	0·1 gm.
NH_4Cl	0·5 gm.
KH_2PO_4	1·0 gm.
Agar-agar	20·0 gm.
Distilled water	1,000 c.c.

Stock cultures were at first grown alternately on glucose and beerwort agar, but later mainly beerwort agar was used, with an occasional transference to glucose agar, 2 per cent. malt or potato dextrose agar; a continuous diet of any one medium leads to a lowering of the growth rate. Melin's observations (1925) of the necessity of subculturing at least once a month were confirmed; there is then no loss of vigour even after three or four years.

III. Mycelial Characters

The main points in the description of the Swedish strain of *B. elegans* given by Melin (1922) are as follows: The cultures are white at first with rust-yellow patches appearing in old colonies and a dark brown pigment diffusing out under certain conditions. The hyphae are septate, 2–3 μ wide and frequently branched; the branches emerge singly at an angle of 45° immediately below a transverse wall to give the so-called 'simple branching' (Figs. 1 and 2). Hyphal strands occur in old cultures, hyphal fusions are also found, but clamp connexions do not occur. The aerial hyphae are covered with granular secretions giving a papillated appearance; no true spores or conidia are formed.

Examination of the English strain of *B. elegans* isolated by the writer has revealed a few points in the macroscopic appearance of the cultures to be added to the description of the Swedish strain given above. In the English strain the rust-yellow patches are a feature of glucose media only, brown patches being developed on oat or beerwort media. In glucose agar cultures a bright yellow substance diffuses out under certain conditions not determined; this yellow substance seems to be distinct from the brown substance mentioned by Melin which normally occurs in all nutrient agar cultures. Drops of a brown, oily fluid appear on the surface of the cultures when growth is strong, and occasionally brown crystals form on the surface of the agar beyond the margin.

Microscopic examination has shown that the two strains differ in a number

of features which are of importance in a comparison of this species of *Boletus* with others; these are considered in detail below.

Branching.

Melin (1923) describes two types of branching in the genus, simple branching as described above and 'paarige' branching in which a pair of branches is given off immediately below a transverse wall. According to Melin, while many species exhibit both types, *B. elegans* shows only simple branching. In the English strain of the fungus, however, 'paarige' branching occurs frequently on both substrate and air hyphae (Figs. 1 and 2). Nor after four years' growth in culture is there any evidence that 'paarige' branching is becoming less frequent in occurrence or that it is confined only to the vigorous cultures as Melin found was the case for *Mycelium radicis sylvestris* α and *Boletus* spp.

Structures resembling clamp connexions have been observed only twice in this investigation, it is therefore probable that the English and Swedish strains are similar in this respect. No reliance can be placed on the reported occurrence of clamp connexions in basidiospore cultures of *B. elegans* by Hammerlund (1923), since no figures are given and his soil media were inadequately sterilized. Examination of the mycelium below the sporophores has also failed to establish the occurrence of such connexions in natural soil conditions.

From Melin's work it is possible to classify the Boleti into two groups as follows:

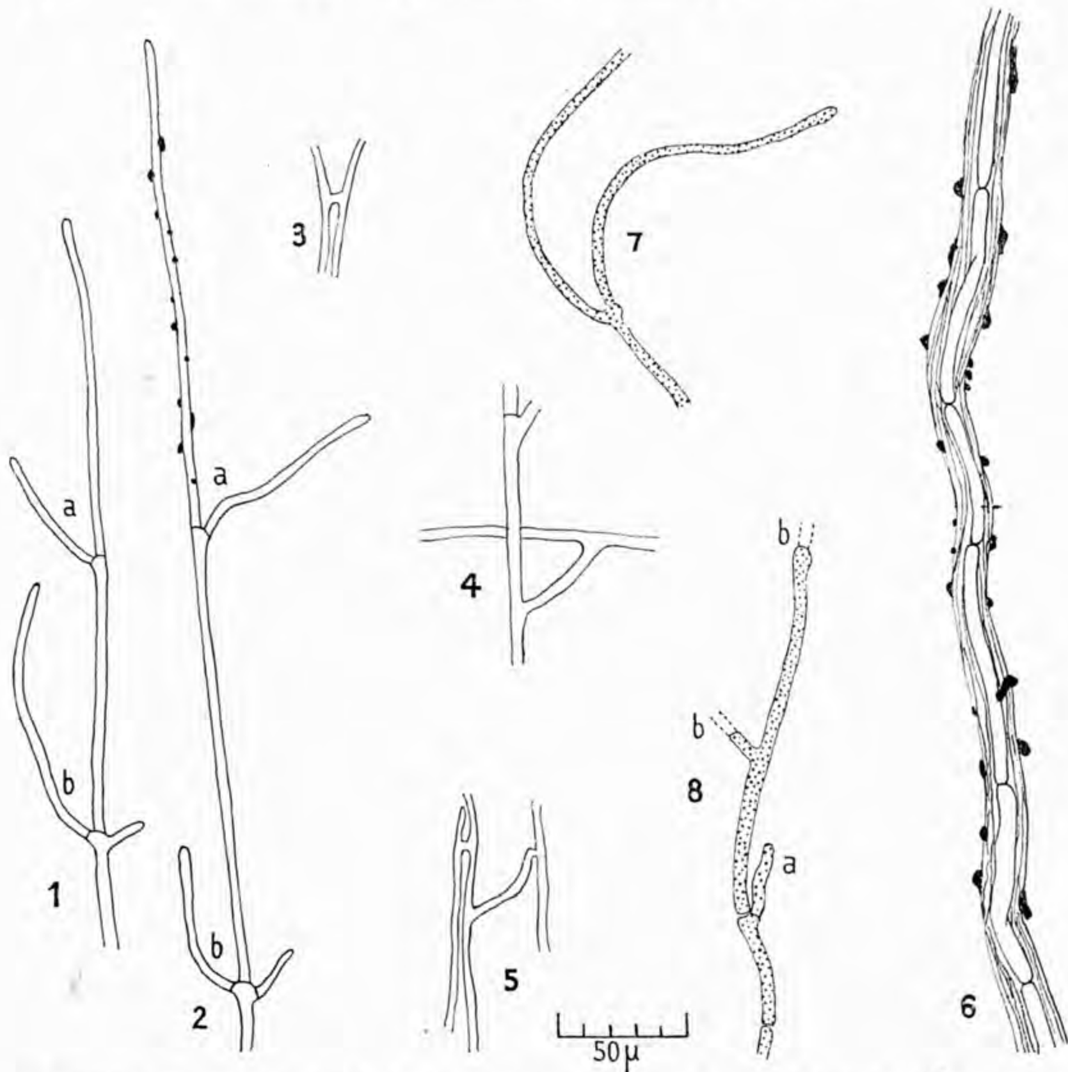
1. Mycelium with frequent 'paarige' branching and numerous clamp connexions, e.g. *B. luteus* and *B. variegatus.*
2. Mycelium with infrequent 'paarige' branching and few if any clamp connexions, e.g. *B. granulatus* and *B. badius.*

While the Swedish strain of *B. elegans* is to be placed in group 2, the English strain exhibits features of both groups, supporting the view that this species does not differ seriously from other members of the genus already described.

Hyphal fusions and hyphal strands.

In contrast to the Swedish strain of *B. elegans* the English strain frequently develops bridging hyphae, especially among the hyphae on the surface of the substrate (Figs. 3, 4, and 5).

Hyphal strands 20 μ in diameter are commonly found on beerwort or oat agar cultures after three weeks' growth. Occasionally the English cultures have produced hyphal strands 45–6 μ thick, which compare favourably with the strands figured by Melin (1922) from cultures in symbiosis with seedlings of larch; these were 40 μ in diameter. Many of the strands examined consist of a central axis of wide hyphae containing glycogen and surrounded by a sheath of narrow hyphae (Fig. 6). These strands show an organization similar to that found in hyphal strands of other Hymenomycetes, e.g. mushroom (Hein, 1930), though the degree of differentiation is less, only two types of hyphal element being involved.



FIGS. 1–8. Figs. 1 and 2. Aerial hyphae from 4-day-old glucose gelatin culture: *a*, simple branching; *b*, 'paarige' branching (drawn from living material). Figs. 3, 4, and 5. Substrate hyphae showing hyphal fusions from a 6-day-old glucose gelatin culture. Fig. 6. Hyphal strand from a 2-months-old beerwort agar culture. Fig. 7. Part of branched 'brown' hypha from 7-weeks-old oat agar culture. Fig. 8. Immature 'brown' hypha from 2-months-old beerwort agar culture: *a*, hyaline branch beginning to change colour; *b*, unchanged parts of original hyaline hypha. All cultures of a strain 1½ years old.

Brown aerial hyphae.

Examination of the brown patches which appear after ten days' growth on sugar or starch media reveals the presence of numerous disconnected hyphal threads with yellow-brown walls (Fig. 7). These hyphae are mostly unbranched and usually terminate abruptly with blunt end-walls forming relatively short threads; occasionally hyphal bridges are observed between two such hyphae. These brown threads always occur on the outer surface of the mycelial mat; varying degrees in the intensity of the yellow-brown colour are found, depending on the age of the culture, the older the culture the darker the colour.

The brown hyphae appear to arise through a modification of the walls of segments of normal, hyaline, aerial hyphae. Occasionally it is possible to find a brown hypha continued at both ends into a normal hyaline hypha (Fig. 8), but in most cases the unmodified portions of the hyphae have shrivelled, leaving the typical isolated brown fragments. The increasing depth of colour with age suggests the gradual deposition of the yellow-brown substance in the walls. Melin (1923) describes and figures hyphae in *Mycelium radicis abietis* which appear to be of a similar nature and are stated to be rich in glycogen; however, there were only traces of glycogen in the brown hyphae of *B. elegans*. Hyphae similar to those in *B. elegans* have been observed in large numbers in cultures of *B. bovinus*; it is probable that they are a general feature of *Boletus* spp. in culture, waste products of metabolism being deposited in this manner.

Secretions.

A very constant feature of the mycelium of *B. elegans*, both in culture and in the soil, is the presence to a greater or less degree of masses of granules on the walls of the hyphae giving them a papillated appearance. Melin noted that the granules vary in their degree of attachment to the hyphae, the small ones not being removed by washing with water or alcohol, the larger easily separating. In the present investigation it was found that the small granules stain deeply with aqueous methylene blue; the larger stain less easily, are partly oily in nature and brown in colour, giving rise with the brown hyphae to the rust-yellow or brown patches on old cultures. These large granules are probably older stages of the small granules which have become impregnated with oily substances and brown excretory pigment. Melin (1923) held that the ability of the mycelium of *Boletus* spp. to produce granules decreased with the length of time in culture and was only restored in symbiosis with conifer seedlings. No such decline with age has been noted in *B. elegans*; but a definite dependence on nutritive conditions has been observed. Papillation is slight in cultures on media with no carbohydrate, very few of the large brown granules being found; it increases in amount with increasing carbohydrate content, but at no time is it entirely absent.

The re-examination of the mycelium of *B. elegans* has disclosed the existence of a greater similarity between the mycelia of this and other species of Boletus than had been suggested by Melin. All the species so far grown in culture show three features in common, simple branching, 'paarige' branching, and papillated hyphae; many also exhibit clamp connexions and brown aerial hyphae—*B. elegans* is no exception.

IV. Physiological Characters

General experimental procedure.

Certain difficulties connected with the standardization of inocula had to be overcome before trustworthy data for growth could be obtained. Not only are spore inocula unavailable, but hyphae alone will not grow, so that a

certain amount of substrate has to be included in each inoculum. Also the previous history of the mother culture seriously affects the rate of growth of the inoculum; at least two generations before the experimental one must be similar in every respect to ensure comparable results (see Appendix).

In the majority of the experiments the inocula were derived from oat agar cultures once removed from stock culture; on this medium (Bonar, 1924)

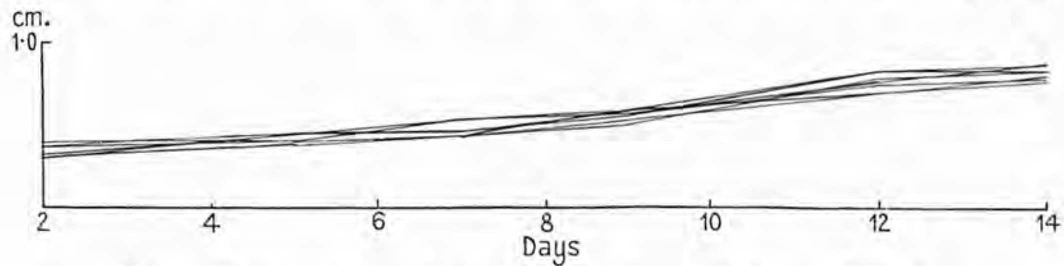


FIG. 9. Variation in growth rate of eight cultures on glucose silica gel.

growth was vigorous. Disc inocula, 0·4 cm. in diameter, were punched out from the growing margins of colonies about four weeks old. The average growth rate of 6–10 cultures of exactly similar origin was used as the basis of comparison in all experiments.

The estimation of dry weight was not a satisfactory method for measuring growth rate owing to the sensitiveness of the fungus to the poor aeration in liquid media. This necessitates a very shallow medium, and in consequence small cultures; matters were further complicated by the appreciable weight of the agar inoculum. On the other hand, the disadvantages of linear measurement as described by Brown (1922, 1923) did not hold in the case of *B. elegans* where the cultures were of very similar mycelial density on all the media used, hence this method was finally adopted. However, the irregularities in the shape of the cultures combined with their small size in the early stages rendered the direct measurement of increase in diameter somewhat inaccurate. In place of this a drawing was made of the image of the culture projected by an epidiascope and enlarged $2\frac{1}{2}$ times. The area of the image was determined by a planimeter and the mean diameter was calculated from this value. The amount of variation obtained after the use of these methods can be gauged from Fig. 9, where are shown the individual mean diameters of a typical set of cultures when grown under exactly similar conditions.

The values of pH were determined colorimetrically; if 10 c.c. or more of a medium was available a Lovibond Comparator was used, if less a B.D.H. Capillator. Unless otherwise stated the experimental cultures were kept in an incubator with the temperature controlled at 20°–21° C.

Concentration of the substrate.

Styer (1930*a*) in his work on the mushroom found that the concentration of the nutrient solution influenced the rate of growth; this was retarded in solutions above 0·2 M. While no experiments have been carried out with the

express object of determining the effect of concentration on the growth of *B. elegans*, it became apparent that there is an upper limit of concentration above which growth is affected adversely. The exact position of this value was not determined.

The influence of concentration on growth rate was shown in two experiments among others. In the first experiment two 10 per cent. gelatin media of pH 5·4 were compared; their composition is shown in Table I.

TABLE I

Concentrations (Molar) of Media in Experiment 1

Nutrients	Medium A	Medium B
Glucose	0·1	0·1
$MgSO_4 7H_2O$	0·0004	—
K_2SO_4	—	0·0006
KH_2PO_4	0·007	—
Na_2HPO_4	—	0·087
NH_4Cl	0·009	0·018
Citric acid	—	0·031
Total concentration	0·1164	0·2366

The average growth rates of the cultures on the two media are shown in Fig. 10; it is clear that growth was much better on medium A than on medium B. Since neither Mg-ions nor citric acid exert any effect on the growth of *B. elegans*, the presence or absence of these cannot be responsible for the result. The conclusion must be drawn that the lower concentration of medium A outweighs its poorer nutritive qualities, viz. less nitrogen and phosphate.

In the second experiment three glucose gelatin media were buffered at pH 5·0 with a citric acid-phosphate buffer (McIlvaine, 1921), added in three strengths, viz. full, three-quarter, and half strength. The resulting concentrations are shown in Table II.

TABLE II

Concentrations (Molar) in Experiment 2

Medium	Nutrients	Phosphates	Citric acid	Total Concentration
I	0·12	0·086	0·037	0·243
II	0·12	0·064	0·028	0·212
III	0·12	0·043	0·018	0·181

From Fig. 11 it is seen that medium III with the lowest concentration gave the best growth. As far as nutritive qualities are concerned all solutions contained an excess of nutrients. Therefore one again concludes that good growth can only be obtained on media of low concentration.

These results, together with the knowledge that a concentration of glucose above 0·2 M always yields poor growth, leads to the conclusion that there is a limiting value for the concentration of the nutrient solution above which the growth of *B. elegans* is affected adversely. It is very probable that this

value lies between 0·18 M and 0·21 M, in which case it is of similar magnitude to that given by Styer for the mushroom. Whether this will be found to be a general characteristic of Hymenomycetes in pure culture remains to be determined; if so they are sharply distinguished from such fungi as *Aspergillus*

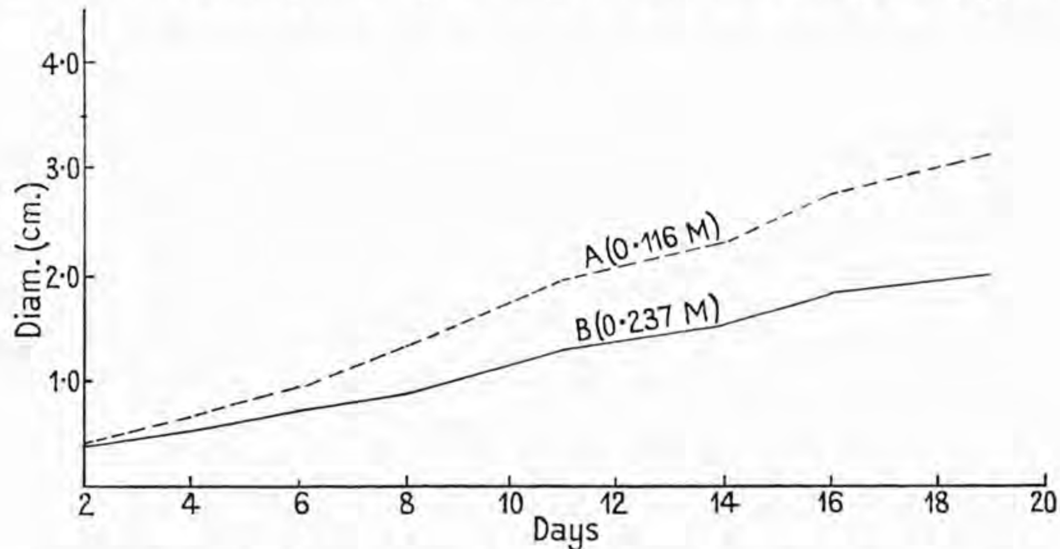


FIG. 10. Influence of concentration on growth rate—average diameters of nine cultures in A and of eight cultures in B (see Table I for detailed composition of media).

niger whose optimum concentration is above 1·0 M (Pringsheim, 1914). Presumably the explanation of this sensitivity to high concentration is to be sought in the osmotic requirements of the fungus; this problem, however, has not been pursued farther. For practical purposes it is sufficient to emphasize the need for considering the concentration of the medium when conducting experiments with soil Hymenomycetes in pure culture.

Nutrient constituents of the substrate.

Melin (1925) states with regard to possible carbon sources that the species of Boletus, including *B. elegans*, only grow well on glucose media. Although no exhaustive study of the carbon nutrition of the fungus was undertaken in the present investigation, certain classes of organic compounds were tested. It was found that while glucose is the best source of carbon for the English strain of *B. elegans*, nevertheless a number of relatively simple organic substances can also be utilized. Sugars such as xylose, fructose, sucrose, and maltose all yield good growth, as does also mannite. The starch of oatmeal is attacked with ease. Organic acids, however, such as tartaric, citric, and malic acids are valueless; nor can *B. elegans* use organic nitrogen compounds such as asparagine, peptone, gelatin, or nucleic acid as sources of carbon.

Since *B. elegans* is a forest soil organism it is important to know its reaction to substances such as pectin, cellulose, and lignin, which occur in varying quantities in the litter and the upper humus layers of the soil. Accordingly

the following materials were tested: cellulose in the form of filter-paper, xylinum cellulose (product of *Bacterium xylinum*), ligno-cellulose materials such as larch litter of various ages and dead larch roots. In no case was growth possible, nor did penetration of the material take place.

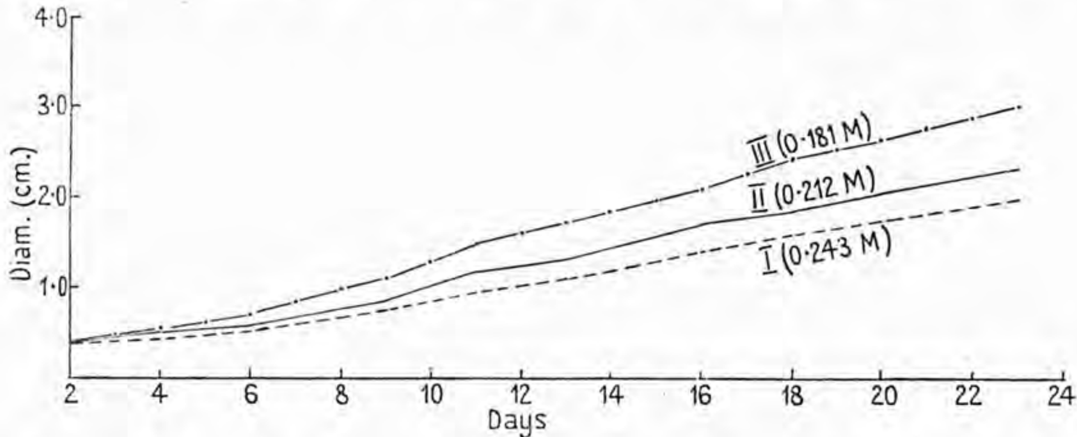


FIG. 11. Influence of concentration on growth rate. The diameter is that of ten cultures (see Table II for detailed composition of media).

When pectin, however, was supplied a very marked positive reaction was obtained. Melin's medium was used (see section II), the glucose being replaced by 1 per cent. soluble citrus pectin which had been precipitated with alcohol twice to remove all traces of sugar. The total dry weights of 10 cultures after 21 days were as follows: 1 per cent. glucose medium, 57·4 mg.; 1 per cent. pectin medium, 23·6 mg. Although growth was slower with pectin, it was very uniform and the cultures appeared particularly healthy, there being no evidence of the staling products which are always produced in unfavourable media. Tests for the presence of pentoses were positive, showing that hydrolysis of the pectin had occurred and that a pectinase was present. Since the insoluble protopectin also found in cell-walls cannot be extracted without hydrolysis to soluble pectin (Norman, 1937), it is impossible to determine whether the fungus can utilize pectin in this form.

The significance of the power of this fungus to utilize pectin is evident when one considers its natural nutritive conditions. There are two sources of pectic compounds in the soil, the litter and the living tree roots. The first is valueless to *B. elegans* since the pectic compounds in the litter are rapidly attacked by fast-growing bacteria and moulds (Waksman, 1927); the amount available in the humus for the higher fungi is therefore negligible. Preliminary investigations of young larch roots have shown, however, that pectin and pectic compounds are present in the middle lamella; these would certainly constitute one source of energy for a fungus such as *B. elegans* which forms a mycorrhizal association of the ectotrophic type. It may even be that the presence of pectic compounds in the young root is a help to its penetration by the fungus in mycorrhiza formation.

A short investigation of the nitrogen nutrition confirmed Melin's observations that ammonium compounds are by far the best nitrogen source for *B. elegans*. A comparison of the growth rates on NH_4Cl, KNO_3, and asparagine in a silica-gel medium is shown in Fig. 12. The preference for

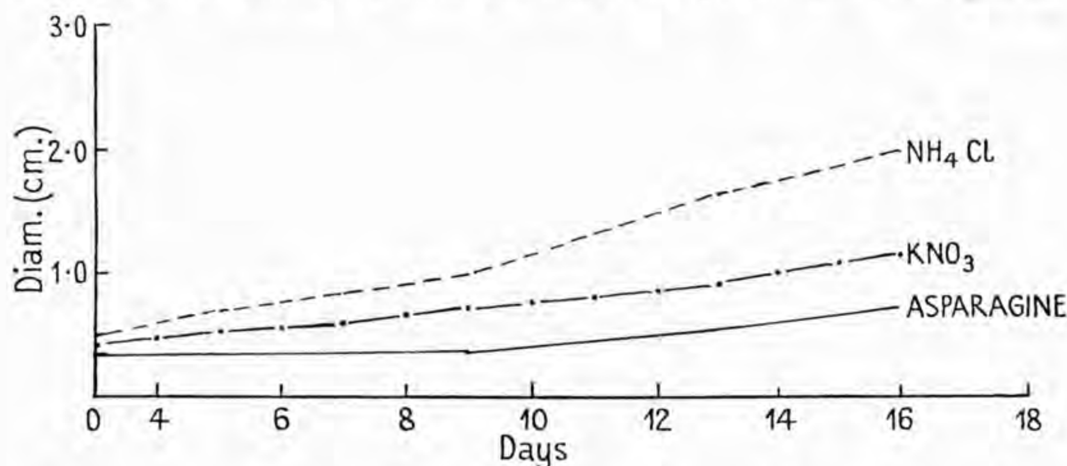


FIG. 12. The relative value of NH_4Cl, KNO_3, and asparagine as nitrogen sources. (The diameter is the average of eleven cultures on each medium.)

ammonia is further emphasized by the rapid increase in acidity resulting from growth on NH_4NO_3. In addition *B. elegans* can liquefy gelatin and utilize peptone and nucleic acid derived from yeast. Melin (1925) stated that all true mycorrhizal fungi were limited to the ammonium salts of inorganic acids and certain protein compounds for their supply of nitrogen. *B. elegans* appears to be typical in this respect, growing most vigorously in pure culture in the presence of ammonium compounds.

As far as nutrition in pure culture is concerned, *B. elegans* prefers the simpler carbon and nitrogen sources, its preference for pectin being the only indication of its specialized habit; presumably, however, it shares this characteristic with other mycorrhiza formers among the species of Boletus.

Range of pH on artificial media.

The pH range of soils carrying larch in the Alps varies from acid to slightly alkaline values (pH 8·0); it is not known whether *B. elegans* occurs throughout this range in its native habitat. In this country, however, the writer has not observed the occurrence of *B. elegans* in soils above pH 6·0. It therefore seemed important to know something of the behaviour of this fungus with regard to pH value.

The desirability of using a solid medium led to the use of silica gels, since neither gelatin nor agar are possible with very acid values. In the preparation of the gels the method outlined by Styer (1930) was used as a basis. A suitable potassium silicate solution of molecular ratio $K_2O : SiO_2 = 1{\cdot}36$ was obtained from British Drug Houses Ltd. and, after dilution to three times the original

volume with distilled water, it was added to a 0·2 M phosphoric acid solution in which the nutrients had been dissolved. The correct proportions of silicate and phosphoric acid which give a gel setting in about twenty minutes were determined by trial. After placing 10 c.c. of gel in each dish and allowing it to set for six hours, a given pH was obtained by leaving 10 c.c. of a citric

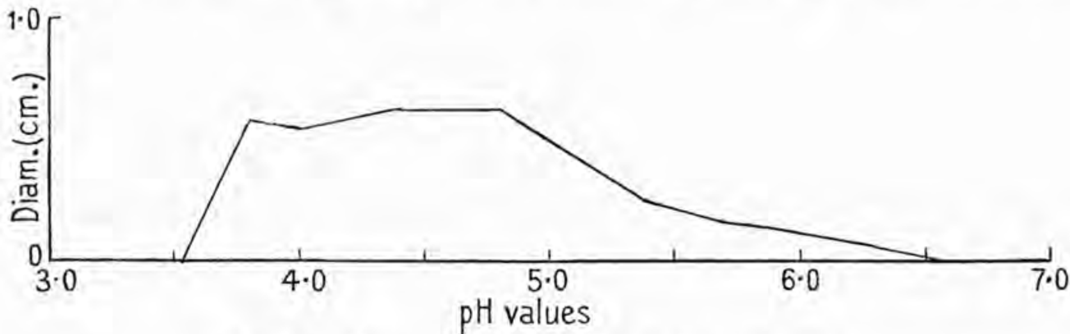


FIG. 13. pH range with NH_4NO_3 as nitrogen source.

acid solution of the proper concentration in contact with a silica-gel plate for twelve to eighteen hours to attain equilibrium. After determining the pH value the supernatant liquid was poured away. The plates were sterilized by autoclaving for twenty minutes at 15 lb. pressure; the formation of bubbles in the plates during sterilization is prevented by the use of boiled distilled water for all solutions.

Two series of experiments were carried out, using NH_4NO_3 and KNO_3 respectively. The compositions of the media were as follows:

	Concentrations (molar)
Glucose	0·1
NH_4NO_3 or KNO_3	0·004
$MgSO_4 7H_2O$	0·0004
Potassium phosphates	0·037
Citric acid	0·04–0·005 (varies with the pH value)
SiO_2 (hydrated)	2% approx.

The pH values of the medium at the margin of certain cultures were determined at intervals during growth. On the NH_4NO_3 media the removal of the NH_3 resulted in an increasing acidity which was appreciable after nine days; the average diameters of the cultures on the ninth day were therefore used as the basis of comparison. From Fig. 13 it is seen that growth with this nitrogen source is possible between pH 3·2–3·4 and 6·4; the region of optimum growth lies between pH 3·8 and 4·8. On the KNO_3 medium the average diameter after fifteen days gave the truest comparison, since there was no appreciable change in pH value during that time and the growth-rate was very slow on such a poor nitrogen source as nitrate. In this series there were no cultures with a pH value below 3·2; at this value, however, growth is

extremely slow, so that the minimum value must be only just below it. The pH range on the KNO_3 media is therefore pH 3·0–3·2 to 5·6–6·2 (Fig. 14).

The greater toleration of alkalinity on the ammonium medium is easily explained on the basis of a quick change towards more acid values enabling the fungus to tolerate a higher pH at the start; the reverse effect occurs at

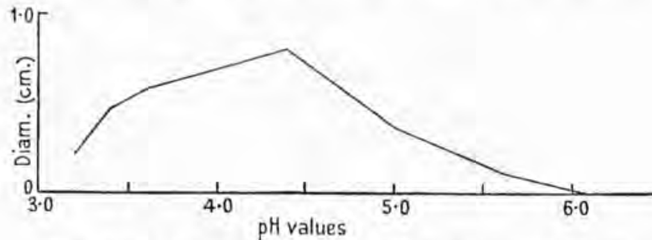


FIG. 14. pH range with KNO_3 as nitrogen source.

the acid end of the range, where a tendency towards increasing alkalinity enables the inoculum to germinate on a slightly more acid substrate.

Considering the two series together one concludes that the maximum range of pH values possible for growth in media containing glucose, salts, and inorganic nitrogen compounds is pH 3·0–3·2 to 6·4. On the alkaline side this range is distinctly less than that found by Melin (1925) for *B. luteus*, *B. variegatus*, and *B. badius*, and the optimum at below pH 4·8 is more acid. Nevertheless, there is no evidence that *B. elegans* differs fundamentally from other Boletus species in its reaction to pH value.

V. DISCUSSION

The present laboratory study of *B. elegans* in pure culture has furnished no clue to the factors concerned in its peculiarly specialized relation to the larch. Thus the examination of the mycelium has shown it to be morphologically very similar to that of other species of Boletus previously described by Melin. Furthermore, physiological investigation has not so far revealed any striking peculiarities; all the species of Boletus observed in pure culture have an acid range, with optimum growth on glucose and ammonium salts—*B. elegans* is no exception. With regard to the deleterious effect of high concentrations, the behaviour of other species of Boletus is not known, but it is very likely that they would exhibit a similar reaction. One is forced to the conclusion that further detailed studies of this nature will not prove of much value in elucidating the problems of specialization.

It remains to consider the physiological characters of *B. elegans* in relation to its habitat as a soil fungus; this raises the whole question of the validity of drawing conclusions from pure culture work and applying them to conditions in the field. Winogradsky (1935) has shown how misleading this can be in the case of Azotobacter. Thus observations of *B. elegans* in pure culture lead to the conclusion that it can only live in an acid soil, upon pectin and

simple sugars such as glucose, and with ammonium salts as nitrogen source. But in association with other soil micro-organisms the fungus may be able to tolerate a much wider range of conditions. For example, it is very unlikely that *B. elegans* has a wider pH range in the soil than that found in pure culture when optimum growth conditions are available; but that does not rule out the occurrence of *B. elegans* in soils whose pH value is outside this range, since the protective action of other organisms is possible.

Again, in a forest soil the amounts of pectin, starch, and sugars available for a slow-growing organism such as *B. elegans* in competition with bacteria must be practically negligible, so that it cannot be upon these that the fungus lives. For while it is safe to infer from pure culture work that *B. elegans* can use simple carbon compounds if available in the soil, it is not proper to conclude that the inability to utilize more complex substances in pure culture will also show itself in the soil. The fact that no evidence has been obtained of the breakdown of cellulose or lignin under laboratory conditions does not exclude these substances as possible carbon sources in the soil, though it renders it unlikely. It is more probable that *B. elegans* uses some degradation products of these materials released by other organisms. But until such substances are available for pure culture work no test of this view is possible.

The following considerations suggest that *B. elegans* normally lives in a state of semi-starvation upon traces of food materials. Firstly, growth in once-steamed soil is similar to that in natural conditions, with very fine hyphae ramifying between the particles; but the addition of even a trace of glucose results in an immense increase of growth which can be further increased up to a level of 0·007 gm. of glucose per gram of soil; still larger quantities of glucose, however, produce no further increase in growth. Secondly, in the soil the hyphae are only sparsely papillated with brown granules; increased quantities of available carbohydrate, however, both in soil and agar cultures always result in a marked increase in the papillation of the hyphae. It is also of significance in this connexion that the hyphae of *B. elegans* on the outside of the mycorrhizal mantle are often covered with similar brown granules, suggesting the presence of an addition to the carbon nutrition of the fungus when in association with the root. It is tentatively suggested that this may be the role of the pectic compounds in the cell-walls of the young root. The evidence for this will be considered in a later paper.

The conclusion is drawn that a laboratory study of this type, while an essential preliminary, cannot produce a solution to the problem of the behaviour of a soil fungus such as *B. elegans*; that will only be achieved after a study of the organism in relation to other members of the soil flora and the higher plant.

VI. Summary

1. The mycelium of *B. elegans* is described and compared with previous descriptions. There is no evidence that the mycelium differs in any important respect from that of other species of Boletus.

2. The growth of *B. elegans* is affected adversely by concentrations greater than about 0·2 M.

3. In pure culture *B. elegans* can use sugars, starch, and pectin, but not cellulose or lignin.

4. While nitrate, asparagine, peptone, and gelatin can serve as nitrogen sources, optimum growth is only obtained on inorganic ammonium salts.

5. The maximum pH range on media containing glucose, salts, and inorganic nitrogen compounds lies between pH 3·0–3·2 and 6·4.

6. It is concluded that the method of pure culture is only of limited value in the study of a soil fungus such as *B. elegans*.

I wish to express my indebtedness to Dr. M. C. Rayner for suggesting this problem, to Professor W. Neilson Jones under whose direction it was carried out, and to both of them for valuable advice throughout the progress of the work.

Appendix

Melin (1925) notes that the growth rate of *B. elegans* is affected by the age of the cultures when used for inoculation. Two experiments show that this effect may extend over more than one generation and that in consequence its importance in pure culture work is even greater than Melin suggested.

Expt. I. Dishes of Melin's glucose agar were inoculated with disc inocula whose past history is shown below. The period given is the age of the culture when it supplied the inoculum for the next.

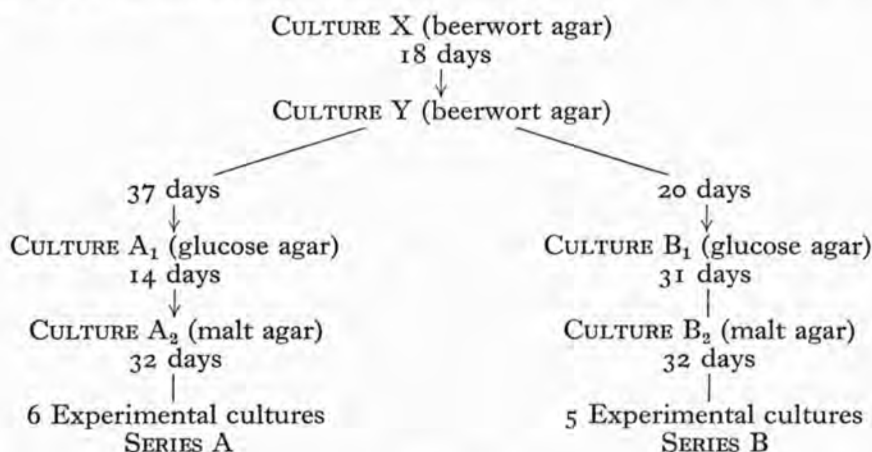


Fig. 15 shows that the growth-rate of series A was greater than that of series B. It is probable that the inocula from culture A_1 used to start culture A_2 contained less staling products than the corresponding inocula from culture B_1 used to inoculate culture B_2. Apparently the initial greater quantity of staling products in B_2 resulted in less vigorous growth with a corresponding decrease in the growth rate of inocula derived from B_2.

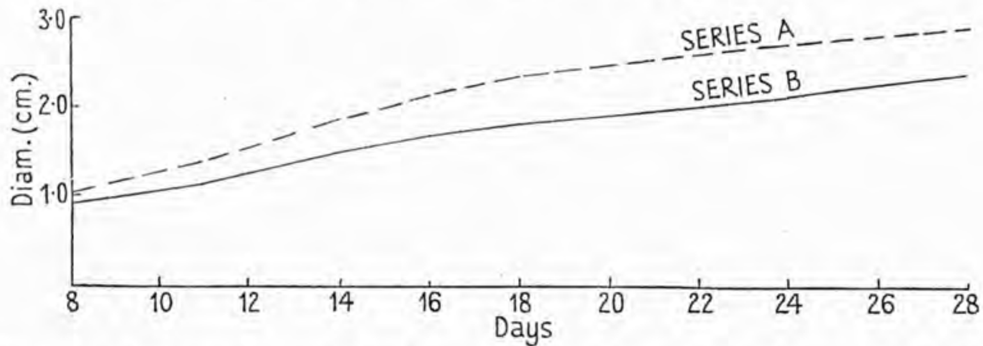


FIG. 15. The effect on growth of the past history of the mother cultures. For details see p. 148.

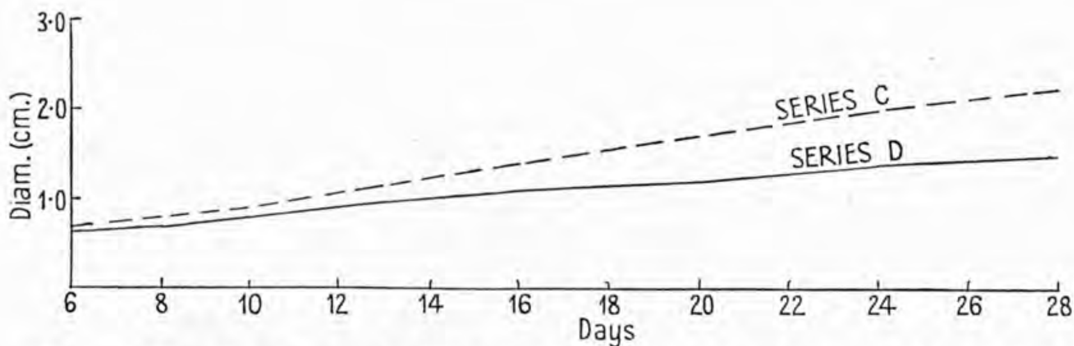


FIG. 16. The effect on growth of the past history of the mother cultures. For details see below.

Expt. II. The medium was similar to that used above, but the glucose was replaced by levulose. The history of the cultures was as follows:

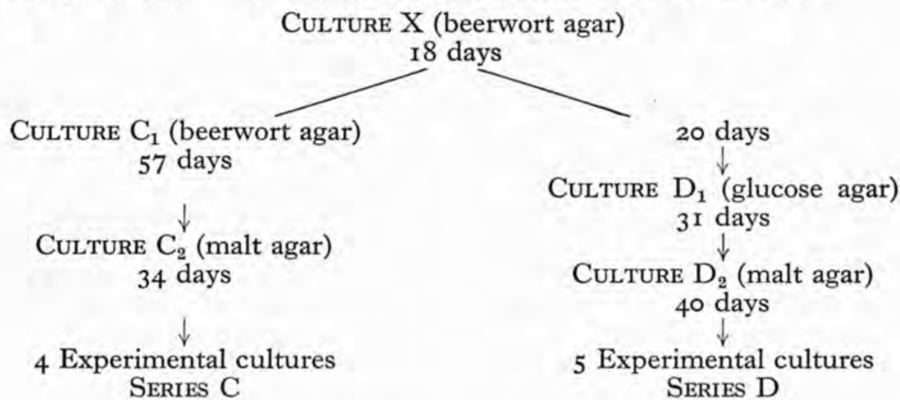


Fig. 16 reveals the marked difference between the two sets of cultures: series C grew more vigorously than series D, although culture C_1 was 57 days old when used and culture D_1 was only 31 days old. It appeared to be of more importance that culture C_2 was only 34 days old when used while culture D_2 was 40 days old. In this experiment the deciding factor appears to have been the smaller quantity of staling products in the younger culture which

masked the effects of its previous history. It should, however, be noted that culture D_1 was on glucose agar, while C_1 was on beerwort agar, which is generally a better medium for growth than glucose agar. It is therefore probable that the deleterious effect of the greater age of C_1 was partially overcome by the more vigorous growth possible on beerwort agar.

Considering the two series together, it is clear that at least two generations prior to the experimental one must be similar if comparable results are to be obtained. That this effect is largely attributable to staling products is evident from the fact that a greatly increased growth rate results from inocula which have been leached in sterile water. In conclusion it should be emphasized that it is not possible to explain these results completely, without assuming that the influence of staling products extends not only to the first but also to later generations.

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PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, OXFORD
BY JOHN JOHNSON, PRINTER TO THE UNIVERSITY

HOW, J. EASTOE

THE MYCORRHIZAL RELATIONS OF LARCH

II. THE ROLE OF THE LARCH ROOT IN THE NUTRITION OF BOLETUS ELEGANS SCHUM

Reprinted from Annals of Botany
New Series Vol. V No. 17 January 1941
Pages 121–132

Printed in Great Britain

The Mycorrhizal Relations of Larch

II. The Role of the Larch Root in the Nutrition of Boletus elegans Schum.

BY

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I. Introduction

IN the previous paper of this series (How, 1940) the writer called attention to the problem presented by the nutrition of a fungus such as *Boletus elegans* which, in pure culture at least, is incapable of using any but the simplest organic compounds; these are present only in traces in the soil and are probably immediately utilized on formation by more rapidly growing soil fungi. The only other nutrient source would appear to be the root with which the fungus is in association. To test this possibility experiments have now been carried out in which excised root-tips of conifer seedlings have been added to pure cultures of the fungus. The present paper records the results, which throw light not only upon the importance of the root as a nutrient source but also on the probable mechanism operating to produce the specificity of *B. elegans* for larch.

II. Experimental Procedure

Conifer seeds were hand picked, scraped clean of wings, and centrifugalized for five minutes in water to wet them thoroughly. After sterilizing in calcium hypochlorite for fifteen minutes, the seeds were washed in sterilized distilled water and germinated in Petri-dishes on damp filter-paper at room temperature (Wilson, 1915). Primary root-tips, varying in length from 2 to 8 mm., were cut off with a sterilized scalpel as required.

Standard culture methods were used throughout, except in experiment 1.

The nutrient medium was as follows: glucose 0·5 per cent., NH_4Cl 0·05 per cent., KH_2PO_4 0·1 per cent., $MgSO_4.7H_2O$ 0·01 per cent., agar-agar (water-washed) 2 per cent. The medium was sterilized by autoclaving at 15 lb. pressure for fifteen minutes. A substrate containing a low concentration of nutrients was used in order to avoid the deleterious effects of high concentrations (How, 1940); preliminary investigations indicated, however, that the concentrations employed were well above the maximum nutritional requirements of the fungus.

Small Petri-dishes (5·5 cm. in diam.) containing 10 c.c. of the medium were inoculated by disc inocula from beerwort agar cultures, the inocula being leached in sterilized distilled water to remove all traces of nutrients and staling products, since it was found that leaching greatly increased the growth rate (Pl. III, Fig. 1, cf. A and C with B and D). In cultures containing root-tips one inoculum was placed in each dish in close contact with an excised root.

The projection method previously described (How, 1940) was used to measure the areas of the cultures in all but experiment 1; the values thus obtained were analysed statistically, using the 't' test of Fisher (1930, section 24.1). It should be realized that the measurement of surface area takes no account of the marked differences in density of the cultures resulting from stimulation; however, any method involving the weighing of mycelium is ruled out by the necessity of using solid media and the presence in many cultures of root-tips of unknown weight.

III. Growth-promoting Qualities of Larch Roots

Experiment 1. This was a preliminary experiment in which only three dishes were used in each set.

Series I. Plain agar medium.

A. Unleached inocula
B. Leached ,,
C. Unleached ,, +root-tip of European larch
D. Leached ,, + ,, ,, ,, ,,

Series II. A similar set in which the nutrient agar was used.

In series I germination of the inocula was obtained but no further growth. Evidently with no other external supply of food material the fungus was unable to utilize whatever nutrients were contained in the root, probably because of its inability to enter the root before the traces of nutrients available to it were exhausted. In series II, however, the presence of a root-tip gave a marked stimulus to growth, the average diameters of the cultures being nearly doubled (Pl. III, Fig. 1). It is impossible that in these cultures the increased growth-rate was due to additional carbon or nitrogen sources available to the fungus on entry into the root, since the nutrients provided in the agar were known to be more than sufficient for growth. It is concluded, therefore, that the stimulant is in the nature of a growth-promoting substance rather than a nutrient. If this is so, it becomes of interest to discover whether the growth

promoter is specific to European larch. The following experiments indicate that it may be.

Experiment 2. Single excised root-tips of European larch, Japanese larch, and Scots pine seedlings were tested on inocula growing on the standard medium; there were six cultures in each set. Table I shows clearly that a stimulus to growth is produced by roots of both European and Japanese larch, but that only a slight stimulus occurs with Scots pine. Statistical analysis of the areas of the cultures after thirteen days' growth indicates that while the mean of the Scots pine cultures differs only just significantly from that of the controls which contained no roots, those of the European and Japanese larch cultures show a highly significant difference. (Table II.)

TABLE I

Areas (sq. cm.) of Cultures 13 Days after Inoculation

	Control Fungus alone.	Fungus + European larch root.	Fungus + Japanese larch root.	Fungus + Scots pine root.
	5·22	7·14	7·58	5·83
	4·53	6·72	7·33	5·06
	4·22	6·55	6·64	5·00
	3·89	6·05	5·94	5·00
	3·64	5·44	5·86	4·94
	3·05	4·78	4·89	4·11
Mean[1]	4·09±0·75	6·11±0·87	6·37±1·0	4·99±0·54

TABLE II

Value of 't'

Treatments compared.	
Control and European larch root . .	4·33 (P = 0·01)
,, ,, Japanese ,, ,, . .	4·45 (P = 0·01)
,, ,, Scots pine root . . .	2·37 (P = 0·05)
European larch and Scots pine roots .	2·66 (P = 0·05)

Experiment 3. The previous experiment was repeated using European larch and Scots pine roots only. A marked stimulus was again obtained with European larch, but only a negligible stimulus with Scots pine (see Tables III and IV). Statistically the mean area of the European larch cultures after fourteen days showed a highly significant difference from that of the controls, while the mean of the Scots pine cultures was not significantly different. However, the Scots pine cultures showed considerable variation among themselves; three of the cultures reached values as high as those of the poorest European larch cultures, giving an impression of definite stimulation.

Experiment 4. This was designed to discover whether the observed results

[1] The standard error of the mean is shown in this and subsequent tables.

were due to differences in the quality of the growth promoter in Larch and Pine roots or only in the quantity. Four series of cultures were set up containing no roots, one European larch root, four European larch roots, and four Scots pine roots, respectively. It is clear from Table V that the increase in

TABLE III

Areas (sq. cm.) of Cultures 14 Days after Inoculation

	Control Fungus alone.	Fungus + European larch root.	Fungus + Scots pine root.
	4·33	6·33	5·5
	3·97	6·05	4·92
	3·64	5·72	4·69
	3·55	5·61	3·55
	3·22	5·22	3·50
	3·17	5·08	3·39
	3·06	5·00	3·22
	2·94	4·83	3·08
	2·44	4·77	2·89
	2·22	4·44	2·72
Mean	3·25±0·65	5·3±0·61	3·75±0·95

TABLE IV

Values of 't'

Treatments compared.	
Control and European larch root .	. 7·32 (P = 0·01)
,, ,, Scots pine root .	. 1·38 (P = 0·2)
European larch and Scots pine roots	. 4·38 (P = 0·01)

TABLE V

Areas (sq. cm.) of Cultures 14 Days after Inoculation

	Control Fungus alone.	Fungus + one root of larch.	Fungus + four roots of larch.	Fungus + four roots of pine.
	3·53	4·28	5·22	3·00
	3·28	3·67	4·72	2·72
	3·05	3·39	3·61	2·72
	2·67	2·89	3·47	2·61
	2·22	2·39	3·39	2·22
Mean	2·95±0·52	3·32±0·73	4·08±0·83	2·65±0·28

the number of Larch roots results in increased growth of the fungus, but a similar increase in the number of pine roots does not increase the growth but tends rather to depress it. Unfortunately it was only possible to set up a small number of cultures in each series, thus considerably reducing the likelihood that significant differences would be found between the mean values. However, the mean of the areas of the 'four-root' Larch cultures was significantly greater than that of the controls, although the mean of the 'one-root' cultures was not; moreover, the difference between the 'four-root' larch cultures and the 'four-root' pine cultures was highly significant (see Table VI).

TABLE VI

Values of 't'

Treatments compared.	
Control and one-root larch	0·92 (P = 0·4)
,, ,, four-root ,,	2·58 (P = 0·05)
,, ,, ,, pine	0·54 (P = 0·7)
Four-root larch and four-root pine	3·63 (P = 0·01)

It is thus evident that the differences in growth cannot be attributed to differing amounts of one growth substance but are due to a difference in quality in the substances offered to the fungus by the root. It is therefore concluded that a substance which promotes the growth of *B. elegans* is present in the primary roots of European and Japanese larch but not in those of Scots pine. There may be other growth-promoters which are occasionally present in sufficient quantity in pine roots to stimulate the growth of the fungus as was observed in experiments 2 and 3, but it is clear that no sign of the existence of such substances appeared in experiment 4, where their effect might be expected to be four times as strong. This discrepancy points, no doubt, to the reaction of the fungus being due to the influence of a number of substances in the root, some stimulating and some inhibiting; but the technique so far employed is too crude to detect this with certainty. The evidence at present available only warrants the conclusion that a growth-promoting complex is present in larch roots but not in Scots pine roots. The term growth-promoter, therefore, is used in this paper merely to cover the phenomena recorded in the experiments, and with no suggestion that one is necessarily concerned with a single substance.

IV. NATURE OF THE GROWTH-PROMOTER

Experiment 5. Five excised roots were crushed with a sterilized glass rod under aseptic conditions, and each was left overnight in 60 c.c. of sterilized distilled water; five other roots were crushed similarly but were not washed. The growth rates obtained from cultures to which these treated roots had been added were compared with others containing one untreated root and no root (Tables VII and VIII).

TABLE VII

Areas (sq. cm.) of Cultures 19 Days after Inoculation

	Control Fungus alone.	Fungus + Uncrushed root.	Fungus + Crushed root.	Fungus + Crushed and washed root.
	3·39	8·28	5·39	4·5
	3·00	5·33	5·22	3·61
	2·58	4·78	4·94	3·22
	2·39	4·72	4·33	2·72
	1·69	3·83	3·55	2·5
Mean	2·61±0·64	5·39±1·7	4·69±0·75	3·31±0·79

TABLE VIII

Values of 't'

Treatments compared.	
Crushed root and uncrushed root . .	0·84 (P = 0·5)
,, ,, ,, crushed and washed root	2·82 (P = 0·05)
Control ,, ,, ,, ,,	1·53 (P = 0·2)

Experiment 6. This was a repetition of the previous experiment with four cultures in each series. The results are recorded in Tables IX and X.

TABLE IX

Areas (sq. cm.) of Cultures 14 Days after Inoculation

	Control Fungus alone.	Fungus + Uncrushed root.	Fungus + Crushed root.	Fungus + Crushed and washed root.
	4·33	6·33	6·17	4·44
	3·55	5·61	6·03	4·28
	3·06	5·00	4·67	3·61
	2·22	4·44	4·61	2·66
Mean	3·29±0·88	5·34±0·94	5·37±0·84	3·75±0·81

TABLE X

Values of 't'

Treatments compared.	
Crushed root and uncrushed root . .	0·05 (P = 0·9)
,, ,, ,, crushed and washed root	2·77 (P = 0·05)
Control ,, ,, ,, ,,	1·74 (P = 0·2)

In both experiments the mean areas of the crushed root cultures do not differ significantly from those with untreated roots; but crushing followed by washing in water results in the complete loss of the power to stimulate growth. Microscopic examination of the roots after washing in water shows that many of the cells remain intact, so that the washing probably removed only the water-soluble substances and not the entire protoplasm.

Experiment 7. Since a number of growth-promoters are known to be soluble in chloroform it was decided to test whether any part of the growth-promoting complex in larch roots could be removed with liquid chloroform. Some excised roots were subjected to chloroform vapour for twenty-four hours and others to liquid chloroform. After the evaporation of the chloroform under aseptic conditions the roots were used in the standard way. From Table XI it is seen that treatment with chloroform vapour does not affect the growth-promoting power of the root, but that treatment with liquid chloroform partially removes it. This is only partial, however, since the mean areas of root cultures treated with liquid chloroform differs from that of the controls

highly significantly, but from that of the untreated root cultures only significantly (Table XII). It can therefore be concluded that treatment with liquid chloroform only slightly impairs the effect of the growth-promoter.

TABLE XI

Areas (sq. cm.) of Cultures 19 Days after Inoculation

	Control Fungus alone.	Fungus + untreated root	Fungus + chloroform vapour root.	Fungus + liquid chloroform root.
	3·39	8·28	6·17	5·33
	3·05	5·39	6·06	4·39
	2·78	5·33	5·61	3·82
	2·58	4·78	5·33	3·72
	2·44	4·72	5·11	3·5
	2·39	4·39	4·5	3·5
	1·69	3·83	4·17	3·17
Mean	2·62±0·54	5·25±1·4	5·28±0·75	3·92±0·64

TABLE XII

Values of 't'

Treatments compared.	
Untreated root and chloroform vapour root	0·05 (P = 0·9)
,, ,, ,, ,, liquid ,,	2·28 (P = 0·05)
Control ,, ,, ,, ,,	4·11 (P = 0·01)

Experiment 8. The previous experiment had suggested that complete extraction of the growth-promoter might only be possible if washing in liquid chloroform or absolute alcohol was combined with leaching in water. Accordingly sets of roots were subjected to one of three treatments, either chloroform vapour to kill without extraction, or liquid chloroform or absolute alcohol to extract the fractions soluble in these; all roots were then washed for twenty-four hours in sterilized distilled water before adding to the cultures. When the mean areas of the cultures are compared after fourteen days with the mean area of the controls it is seen that they do not differ significantly from the latter (see Tables XIII and XIV); it is therefore evident that the chief constituents of the growth-promoter can be removed by washing dead roots in water, as well as by crushing and washing. It will also be observed that the results of this experiment do not confirm the suggestion that either liquid chloroform or absolute alcohol remove a further part of the growth-promoter, since the roots treated with liquid chloroform before washing in water give as good growth as those treated with the vapour, in fact slightly better. It should also be noted that unsuccessful attempts were made to demonstrate the exudation of a water-soluble growth-promoter from the roots of larch seedlings grown in culture. Further attempts would have to be made, however,

before this evidence could be used to disprove the solubility of the growth-promoter in water.

TABLE XIII

Areas (sq. cm.) of Cultures 14 Days after Inoculation
(All roots washed in water after treatment)

	Control Fungus alone.	Fungus + chloroform vapour root.	Fungus + chloroform liquid root.	Fungus + absolute alcohol root.
	3·53	3·55	3·58	3·39
	3·28	2·83	3·5	3·00
	2·67	2·69	2·83	2·72
	2·22	1·78	2·72	2·17
Mean	2·92±0·59	2·71±0·73	3·16±0·44	2·82±0·51

TABLE XIV

Values of 't'

Treatments compared.	
Control and chloroform vapour root	0·45 (P = 0·7)
,, ,, ,, liquid ,,	0·65 (P = 0·6)
,, ,, absolute alcohol ,,	0·26 (P = 0·9)

V. MICROSCOPIC EXAMINATION OF THE ROOT-FUNGUS CULTURES

Living cultures of both infected and uninfected root-tips were examined. For the first two days the root-tips continued to grow, as shown by the development of geotropic curvature and for about ten days the root cells appeared to be living, there being no sign of plasmolysis; after this period many of the cells showed signs of disorganization. There were no indications that infection by *B. elegans* either hastened or delayed the death of the root cells, except where these were invaded by mycelium.

It was noted that hyphal strands were particularly numerous in root cultures while they were never present in the controls; this confirms Melin's observation (1922) of a marked stimulus to the production of hyphal strands in mycorrhizal synthesis experiments with larch seedlings.

For detailed investigation of the penetration of the root by the fungus the cultures were fixed in Doak's fixative: 50 per cent. alcohol 90 c.c., 40 per cent. formalin 8·5 c.c., glacial acetic acid 1·5 c.c.

When hand sections of infected larch roots were examined it was found that the fungal mycelium rapidly formed a thick mantle round the root; comparison showed, however, that the hyphae were more loosely woven than in normal larch mycorrhiza formed by *B. elegans* in the soil. Scattered irregularly through the mantle were isolated cells of the cortical filaments and outer cortex (see Melin, 1922, for the structure of the cortex in larch roots). Directly within the mantle the cells were often filled with a dense hyphal

tissue and in some cases the cells so filled had burst, fragments of their cell walls lying embedded in the mantle. It was not possible to determine how far penetration into the root could go, since staling of the cultures intervened even before the inner cortical cells were reached. Pressure from the growing hyphae would appear to be the main cause of the disintegration of the root; for so many isolated and empty cortical cells remain in the mantle that penetration by means of mechanical weaknesses in the walls seems more probable than absorption of the cell membranes (Pl. III, Fig. 2).

A structure similar to the above is exhibited by Scots pine roots infected by *B. elegans*, but penetration is less deep; only a few scattered cells of the outer cortex become absorbed into the mantle, and entry of cells by hyphae is rare. Scots pine roots appear to resist the fungus better than larch roots, possibly because the outer cortical cells in Scots pine have somewhat rigid walls. It was interesting to observe that although larch roots infected with *Penicillium* sp. showed less disintegration than those infected with *B. elegans*, the typical appearance of the root was essentially similar; there was a mantle enclosing isolated cortical cells and the broken walls of others. It seems probable, therefore, that the phenomena observed in all the excised roots infected with fungi is the result mainly of pressure produced by growing mycelium on a relatively soft tissue.

In conclusion, there is no indication that the morphology of excised larch roots infected by *B. elegans* corresponds in any way to that found in true mycorrhiza formation; it is clearly a case of quasi-parasitic invasion by the fungus. This being so, it is noteworthy that the description and the photomicrograph of roots of larch seedlings grown in pure culture and infected with *B. elegans* as given by Melin (1922) are very similar to those recorded in the present experiments; there is, for example, no tannin layer present in either case. It is probable that his seedlings were parasitized by the fungus and that no true mycorrhiza formation took place.

VI. Discussion

It has been established in the present paper that excised roots of Japanese and European larch seedlings are capable of stimulating the growth of *B. elegans*; and that no such power of stimulation is possessed by roots of Scots pine. This at once suggests an indication of the mechanism which brings about the specificity of *B. elegans* for larch. Thus in the soil the growth-promoter stimulates the fungus to greater growth in the immediate vicinity of larch roots and mycorrhiza formation results. Without such increased growth activity penetration is presumably very difficult; so that mycorrhiza formation does not occur with roots, such as Scots pine, which possess no growth-promoter. This hypothesis is dependent, however, on the future demonstration that the growth-promoter normally exudes from the root; so far there is no evidence for this, though the fact that the growth-promoter is soluble in water renders it possible. The further consideration must not be

overlooked that the entry of the mycelium into the cells in the root-fungus cultures may render substances available to the fungus which are not available in the natural mycorrhizal relationship. If the hypothesis were to be rejected on this ground, an explanation would still be required for the remarkable parallel between the presence of a growth-promoter in the roots of larch, the only genus known to be capable of forming mycorrhiza with *B. elegans*, and the absence of such a substance from the roots of Scots pine in which mycorrhiza formation by *B. elegans* is unknown. Therefore, until definite evidence is forthcoming to the contrary, it may be concluded that one factor bringing about the specificity of *B. elegans* for larch is the presence in the roots of a growth-promoter. If this is so, then it follows that, whatever benefits may or may not accrue to the tree from the mycorrhizal association, the fungus at least can obtain growth-promoting substances.

It remains to consider the nature of the growth-promoter. That this will eventually prove to be a complex of substances rather than a single substance has already been suggested in section III. Apart from the fact that the main constituents of the growth-promoter are soluble in water, the present investigation provides no further information. It is of interest to note that Melin (1922) recorded the stimulation of the growth of *B. elegans* immediately contact had been established with the hypocotyl of larch, spruce, and pine seedlings; but he gave no suggestion as to the cause of the stimulation. There are, however, in the literature two further references to substances capable of stimulating the growth of various species of Boletus known to be mycorrhiza formers. Melin (1925) showed that exudations from pine and spruce seeds had a growth-promoting power; it was suggested that this was due to the presence of phosphatides which Hansteen-Cranner had previously reported to be exuded by coniferous seeds and roots (see footnote, Melin, 1925). Steward (1928) failed to find any evidence for the diffusion of phosphatides from living plant cells as Hansteen-Cranner had claimed, so that the nature of the growth-promoter found by Melin is uncertain. Later, Melin and Lindberg (1939) showed that *B. elegans* is stimulated by yeast extract and vitamin B_1; it is probable that traces of vitamin B_1 are present in larch roots, from analogy with excised tomato roots (Bonner and Greene, 1938), and stimulation might therefore be expected from the addition of such roots to fungal cultures. But neither the stimulator found by Melin in conifer seeds nor vitamin B_1 can possibly be responsible for the phenomenon recorded in this paper, since these stimulators are not specific to larch but occur in many other plant species. Any clue to the identity of the growth-promoter is therefore dependent on its extraction from larch roots and its subsequent investigation.

The bearing of these experiments on the wider issues of the mycorrhizal problem must be mentioned. Melin held that the so-called virulence of the fungus was largely responsible for the type of association which developed between any two partners in a mycorrhizal relationship, strongly virulent fungi became parasitic, less virulent ones formed well-balanced mycorrhizas.

The present experiments serve to emphasize how important a factor is the physiological condition of the host, since weakness of the root due to severance from the parent plant results in the extreme case of unbalanced association described in this paper.

VII. Summary

The effects resulting from the addition of excised primary roots of conifer seedlings to cultures of *B. elegans* have established that a water-soluble substance capable of stimulating the growth of the fungus is present in the roots of European and Japanese larch, but not in those of Scots pine. During the course of the experiments the roots became subject to a quasi-parasitic attack by the fungus, which penetrated by means of the mechanical pressure exerted by the growing mycelium.

I wish to express my grateful thanks to Professor W. Neilson Jones and Dr. M. C. Rayner for criticism and advice in the preparation of this paper.

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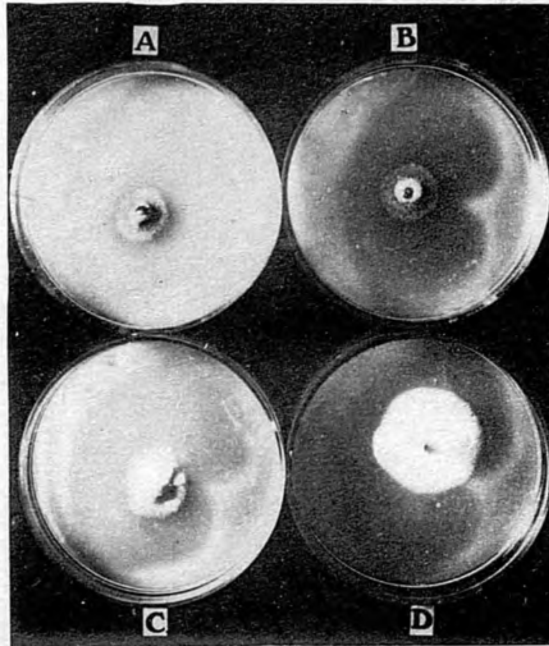
EXPLANATION OF PLATE III

Illustrating Miss J. E. How's paper on 'The Mycorrhizal Relations of Larch. II. The Role of the Larch Root in the Nutrition of *Boletus elegans* Schum.'

Fig. 1. Cultures of *B. elegans* from experiment 1; 13 days old ($\times \frac{1}{2}$ approx.) A. unleached inoculum, no root; B. leached inoculum, no root; C. unleached inoculum + root-tip of European larch; D. leached inoculum + root-tip of European larch.

Fig. 2. Transverse section of an excised primary root of European larch, 15 days after inoculation with *B. elegans*. *a*, mantle of loosely woven hyphae; *b*, group of cortical cells filled with mycelium; *c*, outer empty cortical cells separated from the root by the mantle. ($\times 75$.)



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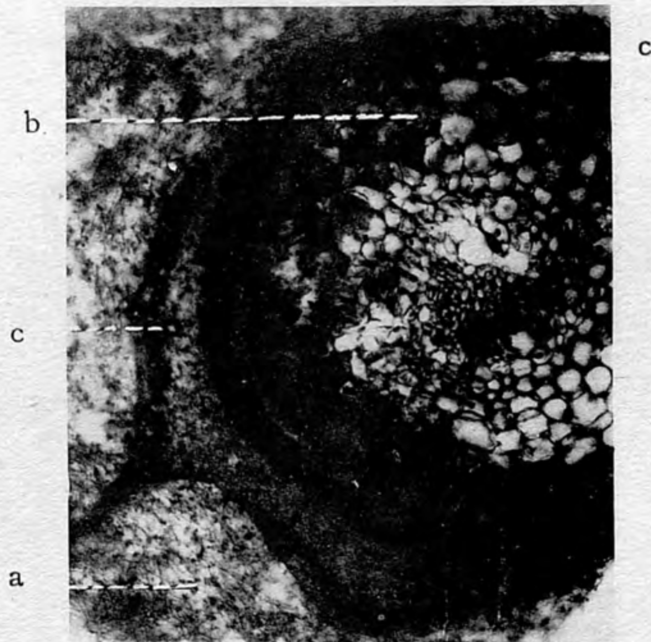

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HOW — MYCORRHIZAL RELATIONS OF LARCH.

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