Obligate Symbiosis in *Calluna vulgaris*.¹

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With Plate VI and four Figures in the Text.

INTRODUCTORY.

AS an important member of the moorland flora of Northern Europe, *Calluna vulgaris* is commonly associated with soils of a definite type, especially when it occurs as the dominant or sub-dominant species of moorland associations. As such, it is especially characteristic of dry heath soils, deficient in lime and often acid in reaction.

So obviously is this the case, that the current hypothesis to explain the edaphic relations of this and other calcifuge ericaceous species assumes that their local dominance connotes soil conditions inimical to the growth of other plants.

This hypothesis, however, did not adequately explain the success of small *Calluna* communities in the special case selected,² and a close scrutiny of ecological records revealed similar inconsistencies elsewhere.

Experimental evidence has already been brought forward indicating that the 'lime-shy' habit is not a direct reaction to excess of calcium carbonate *as such* in the soil, but is associated with correlated peculiarities of calcareous soils (1). It seemed possible that detailed experimental investigation of a special case might throw light on the significance of the 'calcifuge' habit in general.

A detailed record of the soil conditions found in the special case referred to above has already been published (2).

The observations recorded in this earlier paper, taken in conjunction with the results of water-culture experiments on seedlings of *Calluna* extending over a year, served to strengthen the impression that the soil preferences of the plant are probably intimately connected with the biological relations of the roots with one or more micro-organisms.

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² The investigation of which this paper gives an account was undertaken as the result of an inquiry into the precise ecological conditions—edaphic and biological—which are associated with the presence of small well-defined communities of the Common Ling (*Calluna vulgaris*) in a restricted area of the Wiltshire Downs.

Subsequent experimental work led to the following conclusions, the data in support of which have been recorded (3) :

1. Seeds of *Calluna vulgaris* may be sterilized, and seedlings germinated in a sterile condition, i. e. free from fungal or bacterial infection.

2. In seeds removed with aseptic precautions from unopened fruits, the embryo and endosperm are sterile ; the testa is infected with a Fungus identical with the mycorrhizal Fungus of the root.

3. Germination and the early stages of growth of sterile seedlings are normal, but, in the absence of infection, arrest of root formation occurs, with subsequent inhibition of growth.

4. Infection of the primary root of the seedling takes place subsequent to germination, by a growth of mycelium from the seed-coat, the latter being infected while still in the ovary.

5. Pot cultures in soils, favourable and unfavourable respectively to the growth of the plant in the field, demonstrate that *Calluna* grows *normally* in the former, *abnormally* in the latter. Abnormality of growth is exhibited in :

(a) reduced germination capacity ; (b) retarded germination ; (c) arrest of seedling root and curvatures of growing region ; (d) arrest of shoot ; (e) small size and red coloration of leaves.

6. Intimately connected with these abnormalities is the presence of colonies of Bacteria on the roots, especially round the tips. These Bacteria are to be regarded either as pathogenic agents, or as indicators of soil conditions unfavourable to the Fungus or to the plant. The presence of bacterial colonies is directly correlated with the abnormal growth displayed by the roots, but evidence is not conclusive that they are the immediate *cause* of this condition.

7. The evidence available points therefore to the conclusion that the relation between the plant and its mycorrhizal Fungus is an obligate one. Successful growth is bound up with infection of the roots at an early stage by the Fungus, and with the subsequent healthy growth of the latter. The soil preferences exhibited by the plant depend on the maintenance of a biological balance between the roots and the constituents of the microflora which beset them.

Assuming these conclusions to be valid, it is apparent that before any special case involving the soil preferences of the plant can be attacked directly—before, indeed, such a problem can be clearly formulated—the ground must be cleared by an investigation into the precise relations existing between the plant and the Fungus which plays such an important part in its life-history.

The work recorded in the present paper was commenced with this end in view. Owing to the somewhat unexpected nature of the results, it has been carried out as an independent research.

The facts established supply the data requisite for a direct attack upon the original ecological problem ; they throw further light on the rather vexed question of the significance of mycorrhiza, and they have demonstrated for *Calluna*, and probably for most ericaceous species, an unsuspected symbiotic relation, in some respects unique among flowering plants.

The record of the results of the present investigation, since it is concerned more especially with the isolation and biology of the mycorrhizal Fungus of *Calluna*, is prefaced by a brief historical review of previous work on endotrophic mycorrhiza.

MYCORRHIZA—HISTORICAL.

The peculiar association of the vegetative stage of a Fungus with the roots of the higher plants, known by this name, has been familiar to botanists since the middle of the last century, and although many cases have since been carefully investigated, especially from the cytological point of view, comparatively little is yet known with certainty of the bionomics of the relationship, and still less of the life-histories and systematic position of the Fungi concerned.

Early observers were usually content to record the presence of hyphae in or upon the roots of various plants without attempting to investigate the exact relations between the two organisms.

In the early part of the nineteenth century the presence of mycelium in plant tissues was recorded by Schleiden, and in 1882 Kamienski drew attention to the external investment of hyphae on the roots of *Monotropa Hypopitys* (4).

In 1887 a great impetus was given to research by Frank, who first made use of the term *mycorrhiza* and formulated a definite theory of symbiosis, the possibility of which had already been suggested by Pfeffer, Kamienski, Treub, and Goebel.

Frank's observations were made on a large number of plants, and he distinguished two forms of union: *ectotrophic mycorrhiza*, in which the Fungus forms an external investment on the root but does not penetrate the cells—especially characteristic of many forest trees; and *endotrophic mycorrhiza*, in which the cells of the plant are actually invaded by fungal hyphae.

Based on experimental study of ectotrophic mycorrhiza in forest trees, Frank formulated his well-known theory of the symbiotic rôle of the Fungus ; namely, that root-hairs are frequently absent and are replaced functionally by fungal hyphae, which are responsible for the absorption by the plant of mineral salts or organic nitrogen compounds from the soil—a debt repaid by the transference of carbohydrates from plant to Fungus.

Subsequently, a similar function was claimed for the Fungus of endotrophic mycorrhiza, the absorption of nitrogen compounds from the Fungus involving, in this case, their digestion by the cells of the plant (5).

Frank and his pupil Schlicht drew attention to the widespread occurrence of endotrophic mycorrhiza, especially in the Natural Orders Orchidaceae, Epacridaceae, and Ericaceae.

In the case of Ericaceae, Frank described and figured the very fine hair-like roots of certain heath plants, the cells of which are constantly filled with fungal hyphae (6).

In such roots he emphasizes the complete absence of root-hairs, the disappearance or reduction in amount of cortical tissue, and observed the 'epidermal' layer of large cells filled with 'knot-like' masses of mycelium, branches from which penetrate the external walls.

Frank recognized the possible existence of more specialized relations between Fungus and flowering plant in the case of certain groups, such as the Ericaceae and Orchidaceae, but pointed out that comparative cultures of *infected* and *uninfected* plants of this type were not yet available for discussion.

The conclusions of Frank as to the rôle of the Fungus in ectotrophic mycorrhiza were challenged by Sarauw (7) and by Möller (8), the latter of whom pointed out that ectotrophic mycorrhiza was often well developed in soils poor in humus.

It was also shown subsequently that root-hairs were not uncommonly formed by plants possessing ectotrophic mycorrhiza, and Von Tubeuf (9) demonstrated that the endotrophic type was often characteristically developed in forest trees.

On the whole, since Frank's time, views as to the significance of ectotrophic mycorrhiza have diverged from his theory without entirely abandoning it.

Subsequent to the researches of Frank, the endotrophic forms attracted more attention. Among a number of publications dealing more especially with their cytology and general significance may be mentioned those of Groom (10), Thomas (11), Janse (12), Magnus (13), Shibata (14), and Peklo (15). The first of these authors agreed substantially with the view of Schlicht and recognized a series of transition forms leading from the condition observed in Ericaceae—which, in his view, approached the ectotrophic type present in forest trees—to the highly specialized relations found in *Thismia Aseroë*.

In 1900 Stahl published his well-known work on the comparative biology of autotrophic and mycotrophic plants (16).

He assigned to the fungal partner of mycotrophic plants the rôle of obtaining mineral salts from the soil, and pointed out that such salts are especially valuable to plants which, because they grow in humus, or for other reasons (e.g. slow transpiration), are unable to absorb water with sufficient rapidity to satisfy their requirements for mineral salts.

The experimental work on which Stahl based his conclusions would

seem to be open to criticism in the light of recent researches, and is further discussed at the end of this paper (p. 125).

In recent years the mycorrhiza of the Orchids has attracted attention owing to the abundance of the endophyte in the tissues, and to its very characteristic relations with the cells of the plant.

The researches of Bernard mark an epoch in the knowledge of endotrophic mycorrhiza and of its biological significance. His discoveries were not only of great theoretical interest but are of some potential value to Orchid growers.

It had long been known to horticulturists that the seeds of Orchids—especially of certain genera, e. g. *Odontoglossum* and *Vanda*—are extremely difficult to germinate.

Working with a number of Orchid species, Bernard found that it was impossible to germinate seeds removed under aseptic conditions from sterilized capsules. Some degree of development usually took place, but except in rare cases—e. g. *Bletilla hyacinthina*—the embryo did not reach an advanced stage. In no case, in his cultures, did development of the seedling reach the stage of root formation, unless injection from the substratum took place.

The next step in the investigation was the isolation of the mycorrhizal Fungus; seed cultures were inoculated from a pure culture of the Fungus so obtained, and normal Orchid seedlings were raised successfully by this means.

Various transition stages with regard to the degree of dependence of the plant upon the Fungus were observed by Bernard.

In *Bletilla hyacinthina*, a relatively unspecialized type, uninfected seeds germinate and the seedlings form several leaves, i. e. the plantlet reaches a comparatively advanced stage, but is unable to develop roots without infection. In other Orchids, development of the embryo is arrested at a much earlier stage.

According to Bernard, the degree of specificity between plant and Fungus is variable: thus, using species of *Cattleya* and *Cypripedium*, he found that the Fungus isolated from a species of the one genus could be used successfully for inoculation of the other, but in the case of seeds of species of *Phalaenopsis* and *Odontoglossum*, which under normal conditions are difficult to germinate, the Fungus was found to be specific to the plant. It was determined further that Orchid species differ in respect of their ability to resist invasion by the mycorrhizal Fungus from another species. Thus, in the case of a species of *Phalaenopsis*, infection by the Fungus of *Cattleya* sp. killed the seeds outright—the plant cells made no attempt to resist the attack; in another species, infection took place, followed by digestion of the Fungus by the cells of the embryo and subsequent arrest of development.

In the Orchids, therefore, the symbiotic relation between plant and micro-organism is an obligate one, and has resulted in a high degree of

dependence on the part of the former. The Fungus has been isolated, grown in pure culture outside the plant, and used with success for the reinoculation of sterile seeds (17).

A theory of tuberization, as a general consequence of infection by mycelium, was elaborated by Bernard as a result of his earlier researches (18).

In 1909 Burgeff published a monograph on the root Fungi of the Orchids, recording researches in the course of which he repeated the experiments and confirmed the results of Bernard, and made further contributions to our knowledge of the metabolism and physiology of the endophytes (19).

The conclusions of Burgeff differ somewhat from those of Bernard with respect to the degree of degeneration shown by the Fungus when grown outside the plant, as tested by subsequent inoculation.

According to the latter author, the longer the Fungus is grown as an independent organism outside the plant, the more markedly does it lose its power of causing germination and inducing root formation. Burgeff, on the contrary, claims that one of his Fungi, after growing for twenty-six months on artificial media, had the same capacity for effecting germination as when first isolated from the plant.

For purposes of comparison with the more specialized types, Gallaud (20) investigated a large number of Phanerogams, the roots of which contain an endophytic mycelium, but in which the relations between plant and hyphae are apparently more simple and unspecialized than in the case of the Orchids.

The researches of Gallaud appear to be of special importance in regard to the evolution of the more specialized types of root symbiosis, and some of the facts are discussed from this point of view in a subsequent part of this paper (p. 127).

It is of interest to note that, when selecting endotrophic types for examination, Gallaud rejected the members of Ericaceae for the purpose, as possessing a mycorrhiza more nearly related to the ectotrophic forms.

So far from this being the case, I hope to demonstrate in the following pages that the conditions existing in *Calluna vulgaris* (and probably common to all ericaceous species) connote a wider distribution of the endophyte in the tissues than has been recorded hitherto for any mycorrhizal Fungus, involving in some respects the most highly specialized relation between the two symbionts that has been yet described for a flowering plant and a Fungus.

The Fungi concerned in Endophytic Mycorrhiza. Historical.

Up to the present, the Orchids have provided the only case in which absolute proof of the identity of the endophytic Fungus has been established by the successful inoculation of sterile seeds or seedlings from a pure culture (p. 101).

With this exception, in spite of the large and constantly increasing number of plants which are known to possess an endotrophic Fungus in the root tissues, nothing is known with certainty of the systematic position of the Fungi concerned.

Many workers have endeavoured to isolate the Fungus concerned in endotrophic mycorrhiza, and the history of these efforts is not without interest.

The task of isolating the Fungus from pieces of Orchid root was essayed by Wahrlich (1886) (21), Chodat and Lendner (1898) (22), and Bernard (1903) (23), and in all three cases resulted in the extraction of a species of *Fusarium*, which was believed for a time to be the true endophyte.

Gallaud (1905) (20), in a series of researches extending over several years, worked on thirty-five species of flowering plants of widely separated affinities. From the roots of thirty of these he isolated with great regularity a species of *Fusarium*, which he obtained also but with rather less regularity from the roots of four of the remaining species.

His cultures showed that, in addition to *Fusarium*, a number of other Fungi were constantly present on washed roots; among the more common genera were *Mortierella*, *Trachoderma*, *Cephalospermum*, and *Gliocladium*, with which are constantly associated species of *Alternaria*, *Acrostalagmus*, &c.

Inoculation experiments showed that *Fusarium* and the other species named were not the true endophytes, and established the fact that the species obtained in this way are constituents of the mycelial flora habitually associated with roots growing in ordinary soils.

Gallaud concluded that it was impossible to extract the endophyte directly from the roots, and believed the difficulty to arise in some degree from the alteration or partial digestion undergone by the Fungus in the cells of the plant, and its subsequent inability to grow out of the cells.

Bernard, in 1903 and succeeding years, isolated fungal species from the roots of various Orchids; using a pure culture of the appropriate Fungus, and sterile seeds removed from unopened capsules with aseptic precautions, he was able—for the first time—to effect the synthesis of Orchid plant and Fungus, and thus to induce successful germination of the former. He referred these Fungi provisionally to the genus *Oospora* (24).

In the course of subsequent researches (1905, 1906) (25) *Rhizoctonia* was suggested as the nearest generic ally among free-living Fungi for species isolated from *Phalaenopsis*, *Odontoglossum*, and *Cattleya*.

Burgeff holds that the endophytic Fungi of the Orchids are to be regarded as forming a group morphologically and physiologically distinct, for which he suggests the generic name of *Orcheomyces*, leaving the correct systematic position of the genus an open question (14).

Ternetz (26), working at the root Fungi of Ericaceae, isolated pycnidia-

producing Fungi from the roots of various ericaceous species (of which *Calluna vulgaris* appears to have been one) and referred them to the genus *Phoma*. Complete proof of the identity of these Fungi with the species endophytic in roots of the respective plant-species was unfortunately not possible, since sterile seedlings were not obtained. Incidentally, this author recognized the possibility of seed-coat infection in Ericaceae, but did not investigate it further.

It was claimed that five of these species of *Phoma* in pure culture fixed nitrogen from the air, although in very different degrees; in no case was combined nitrogen found necessary for their development. Appreciable nitrogen fixation from the air has been claimed also by this author for *Aspergillus niger* and for *Penicillium glaucum*, and this result was corroborated by Froelich (27) in 1907 for the same species. On the other hand, a negative result is reported for the same and additional species by Wino-gradski (28), Koch (29), Czapek (30), &c., and more recently by Goddard for a large number of soil Fungi (31).

The fixation of free nitrogen from the air has been claimed also for the endotrophic Fungus of *Podocarpus* (32), and is suggested, but not established, for the Fungus which infests the seed and vegetative parts of *Lolium temulentum* (33). There is no evidence for fixation of atmospheric nitrogen by the Fungi concerned in the root mycorrhiza of Orchids, and attempts to cultivate them on nitrogen-free substrata have not been successful (19).

The whole question of the possibility of fixation of free nitrogen by Fungi growing on nitrogen-free media, as determined by direct estimation of the combined nitrogen present at the end of the experiment, is still in an unsatisfactory condition, and, in spite of the positive results claimed by a number of observers, the balance of evidence appears to be on the negative rather than on the positive side.

The sources of error are obvious. The possibility of working with impure cultures, the difficulty of obtaining a substratum free from traces of nitrogen, and the small quantities of nitrogen fixation reported by most observers who claim a positive result, together with the comparatively large margin of experimental error, demand greater uniformity of results before ability to fix free nitrogen can be claimed as a general property of Fungi in the same or even in less degree than has been finally established and placed on a sure scientific basis for a number of Bacteria.

THE PRESENT INVESTIGATION.

The purpose of the investigation described in the following pages is as follows:

1. To confirm and extend the conclusions already reached with respect to the inability of *Calluna* seedlings to form roots unless infected at an early stage by a specific Fungus identical with that present in the cells of the root,

and to demonstrate infection of the seeds by this Fungus while still enclosed in the fruit (p. 98).

2. To investigate the possibility of replacing the stimulus resulting from fungal infection of the seedling by small amounts of various organic substances supplied to sterile seedlings (grown under aseptic conditions), in addition to the requisite mineral salts.

3. To determine the source of infection of the ovary tissues (p. 98).

4. To isolate the mycorrhizal Fungus and establish its identity by successful inoculation of sterile seedlings.

5. To investigate the life-history of the Fungus when grown in pure culture as an independent organism outside the plant.

1. *Dependence of root formation on infection.* The conclusions already reached with regard to inability of sterile seedlings to form a root-system have been confirmed.

If due precautions are observed, seeds can be sterilized without injury to the embryo by washing in 1 per cent. corrosive sublimate solution. Complete sterilization is not easy to effect, and the margin of safety is a narrow one, owing no doubt to the delicacy of the testa and the fact that infection of the cells of the seed-coat is more extensive and deep-seated than is the case with air-infected seeds.

If seeds were left a few seconds too long in the sterilizing solution, the embryo was killed outright, germination was delayed, or the seedlings which germinated showed complete chlorosis.

After sterilization by this method and repeated washings in distilled water, seeds were sown by means of a sterile pipette on agar plates, and kept under dust-free conditions in a closed germinator.

The agar medium on which they were sown contained dextrose and peptone in addition to mineral salts, in order to facilitate the growth of micro-organisms if present. By this means a number of plates of seedlings were obtained which remained entirely free from infection by micro-organisms.

As an additional test of sterility, seedlings were transferred singly to tubes of glucose broth and other media, and kept under observation for three weeks. All the tubes remained sterile.

The evidence is conclusive therefore that the embryo and endosperm of *Calluna* seeds are free from infection, and that, by adequate sterilization, seedlings can be obtained free from infection by micro-organisms. Sterile seedlings obtained in this way were planted out in sand and in agar, in a series of cultures extending over several years.

The tubes originally used for sterile sand cultures are described and figured elsewhere (3).

In order to eliminate imperfect aeration as a possible factor in the non-production of roots, a special apparatus was designed for use in subsequent

cultures, which allowed air to be drawn through the sand in the tubes periodically (Text-fig. 1).

These tubes proved satisfactory and remained sterile for several months, but gave no better results than the non-aerated sand cultures. In no case was a root-system developed. Sterile seedlings from these sand cultures resembled those from agar cultures (Text-fig. 2).

Unsterilized controls cultivated for several months in these tubes grew slowly but developed a normal root-system (Pl. VI, Fig. 10).

For agar cultures, sterile seedlings were transferred to tubes containing a solution previously used with success for water cultures,² made up with 0.12 per cent. agar. These cultures have an advantage over those grown in sand in that infection of the medium by micro-organisms is at once evident. Such tubes have been maintained in a sterile condition for ten months.

The seedlings usually formed a few leaves, chlorotic or reddish in colour, but did not develop roots, although they remained in a turgid condition and apparently alive for five to six months (Text-fig. 2).

The cultivation of unsterilized controls in agar presents some difficulty owing to the vigorous development of the epiphytic microflora associated with the roots. Otherwise the growth of such seedlings is normal, and comparable with that of those grown in soil, e.g. the shoot-system of such a seedling had reached a height of 5 cm. four months after planting.

TEXT-FIG. 1. Apparatus for growing sterile sand cultures. f_1 - f_3 = Massen filter candles; r_1 - r_6 = rubber corks; a = glass cap for attachment to aspirator; b = glass cap to exclude dust; k = sand; p = cotton-wool plug; s = nutrient solution; x = wax joint.

¹ The water-culture solution known for the sake of brevity as Solution A was one in which unsterilized seedlings grow vigorously (3, p. 60). It formed the basis of all media used in experimental cultures. The composition is as follows:

Potassium nitrate (KNO_3)	1.0	gram.
Magnesium sulphate (MgSO_4)	0.4	gram.
Calcium sulphate (CaSO_4)	0.5	gram.
Calcium monophosphate ($\text{CaH}_4\text{P}_2\text{O}_8$)	0.5	gram.
Sodium chloride (NaCl)	0.5	gram.
Ferric chloride	Trace.	
Water	2,000	c.c.

(Acidity: 0.012 normal.)

That the inability to form roots, shown by the sterile seedlings, is not due either to lack of water or to lack of aeration in the substratum, but to absence of infection by the mycorrhizal Fungus, has been fully demonstrated by subsequent cultures (p. 120).

2. *Effect of organic nutrient on uninfected seedlings.* Uninfected seedlings germinated on blotting-paper or agar, and supplied with distilled water or with an appropriate solution of mineral salts, do not form roots, and show yellowing or discoloration of the leaves at an early stage.

Seedlings infected normally with the Fungus germinated under similar conditions, and supplied with distilled water only, form a well-developed root-system and several leaves (Text-fig. 3).

The failure to form roots, therefore, in the absence of infection, is not due to lack of inorganic food material, since the only differentiating

TEXT-FIG. 2. Sterile seedlings from agar cultures. Five months after sowing; four months after planting (cf. Text-fig. 3). Camera lucida drawings.

TEXT-FIG. 3. Seedling from germinator, infected normally. Five months after sowing; about four months after germination (cf. Text-fig. 2). Camera lucida drawing.

feature in the two cultures at germination consists in the presence or absence of the Fungus in the tissues of the respective seedlings.

Since also a mycelium is developed without a supply of food material from external sources, beyond the traces of impurity present, it seems clear that, at this stage and under the conditions described, the micro-organism must obtain the greater part of its food material from the seedlings, the only other source of supply being the air.

As the latter show no sign of injury, and the leaves remain green, one is tempted to suggest that the Fungus possesses, in some degree, the power of nitrogen fixation.

The remarkable vitality shown by seedlings kept for long periods on blotting-paper moistened with tap-water may possibly have some significance as indirect evidence in support of the same view.

Such seedlings form a well-developed root-system and several leaves,

which retain their green colour for several months; e. g. a large proportion of them were alive and green at the end of *five* months. The starvation symptoms—arrest of roots, yellowing of leaves, &c., characteristic of sterile seedlings shortly after germination—are absent (Text-fig. 3).

It was thought possible, therefore, that the failure to form roots by uninfected seedlings might be correlated with some disturbance of nitrogen metabolism in the plant, assuming interchange of nitrogenous material from Fungus to plant to be a feature of the symbiosis under normal conditions.

Although without *direct* evidence to support this hypothesis, it seemed desirable to ascertain whether a supply of organic nitrogen would induce root formation, or postpone the appearance of starvation symptoms in uninfected seedlings. To test this possibility, two parallel series of sterile sand and agar cultures were carried on during several months.

The solutions used for watering the sand cultures were as follows:

Series *a*. Solution A; 0.1 per cent. dextrose; 0.1 per cent. Witte's peptone.

Series *β*. Solution A; 0.1 per cent. saccharose; 0.032 per cent. asparagin.

Series *γ*. Solution A; 0.1 per cent. dextrose; 0.04 per cent. uric acid.

Series *δ*. Solution A; 0.1 per cent. saccharose; 0.03 per cent. glyocol.

In a parallel series of agar cultures, solutions of 0.3 per cent. total concentration of Solution A were used in each case, made up with the addition of 0.12 per cent. powdered agar.

Since the result of these cultures was in every case negative, the experiments are not recorded in detail. In each series, tubes remained sterile for several months—as judged by absence of cloudiness in the media—e. g. agar tubes planted August 13, 1913, were still sterile on April 30, 1914.

The vitality of the seedlings on the different substrata varied. The only solution which appeared to be actively injurious was that used in Series *δ* to which glyocol had been added, the seedlings planted in which showed rapid discoloration of the leaves, and succumbed after a few weeks.

The greatest vitality was shown in the agar cultures of Series *a* and Series *γ*, more especially in the latter. In both cases the tubes were sterile and the seedlings alive—although moribund—at the end of six months.

The seedlings supplied with uric acid (Series *γ*) showed signs of activity after planting, but the growth initiated in the early stages was not maintained; the hypocotyl elongated, several leaves were formed, and in one case the plant produced a few small abortive rootlets. The medium in this tube

was free from bacterial or fungoid infection, and had not lost an appreciable amount of water.

All these cultures were grown in a small cool greenhouse, under the same conditions as normal seedlings in soil, special precautions being taken to prevent excessive loss of water from the tubes.

It may be inferred from these experiments that the stimulus to root development which follows entry of the Fungus into the tissues cannot be replaced by supplying the seedling with organic nitrogen in the forms mentioned.

3. *The source of infection.* Infection of the seedling root, shortly after germination, has been described in an earlier paper (3, p. 68).

The necessary observations can readily be made on seeds germinating on blotting-paper in closed dishes. Infection takes place by the outgrowth of delicate hyaline hyphae from the cells of the testa, simultaneously with — or shortly after — emergence of the seedling root.

In the majority of seed cultures observed, infection has occurred with great regularity at this stage, and has been followed by the rapid development of a number of fine, transparent roots, arising adventitiously from the base of the hypocotyl. Certain irregularities have been observed nevertheless, of which at present no explanation can be given.

In a few cultures, for instance, sown in the early winter, soon after collection of the seed, infection for some reason seemed to be inhibited or delayed. These seedlings remained in a rootless condition in the seed dishes for weeks, and soon showed marked browning and discoloration of the base of the hypocotyl (Text-fig. 4); removed to another dish and placed in contact with a normally infected seedling, they at once began to develop roots.

In the paper already cited (3) it was stated that infection of the seed-coat takes place while the seeds are still in the ovary, and is independent of the bacterial and fungal infection from the air, to which all seeds are liable after they have been shed.

In order to determine the source of this infection, seeds were removed from unopened capsules for examination, and the unripe fruits containing seed investigated by means of sections cut from fixed material, embedded in paraffin or celloidin.

Thick sections of material prepared in this way make clear the manner in which infection takes place.

TEXT-FIG. 4. Seedlings from germinator; delayed infection accompanied by inhibition of root formation five months after sowing; about four months after germination. Camera lucida drawings.

The ovary of *Calluna* is four-chambered, each loculus containing a number of anatropous ovules pendent from the massive placenta at the stylar end.

When ripe, the ovary wall consists of several layers of strongly cuticularized cells, the outermost of which grows out to form a thick investment of unicellular hairs.

In some sections it is easy to observe mycelium in the fruit chambers, and about the enclosed seeds. Delicate branched hyphae are present in the cells of the wall, in the tissue of the central column, and in the funicles of the seeds. Branches from this mycelium grow across from the cells of the ovary wall to those of the seed-coat, and extend from one seed to another.

The hyphae are septate, colourless, and so transparent in fixed material as to be visible with difficulty in balsam preparations unless stained.

A microphotograph of a longitudinal section of such an unopened fruit is reproduced in Pl. VI, Fig. 1 *a*, and on this scale it is just possible to see the traversing hyphae. Parts of the same section, enlarged to show the details of infection, are shown in Fig. 1 *b*.

The degree of infection apparent in seeds removed from unopened capsules is very variable. In some, a few hyphae project from the cells of the seed-coat; in others, mycelium is difficult to find, and can only be satisfactorily seen after the seeds have been specially treated; in others, hyphae are abundant all over the seed-coat.

More especially is it difficult, as a rule, to observe hyphae on the testa of the resting seed, this difficulty of observation being due in part to the structure of the seed-coat. The latter is composed of a few cuticularized walls and an external layer of large cells which, during the final stages of development, break down, leaving at last only their 'foundations' (consisting of the inner walls and the proximal parts of the lateral walls) to form the regularly sculptured covering of the seed. The presence of the folded membranes of these cells, and of the remains of their contents, increases the difficulty of observing fine hyphae on the surface, after the seeds have become dry.

According to Church, the seeds of *Calluna* are not shed until the following spring. 'The fruits ripen in late autumn (November), and the seeds are shed in the succeeding spring, when the capsules open under desiccation' (34).

The behaviour of fruits in this respect doubtless varies with the locality and the season, but the observation does not appear to be of general application, since some difficulty has been experienced in finding unopened capsules after the middle of November.

This, although a minor point, is worth recording, because it seems not

impossible that such differences may be related with inconsistencies with regard to infection, which, as mentioned above, are sometimes observed in germinating seeds. Further, since fungal infection determines the development of the seedlings, it may also be correlated with observed irregularities in the distribution of the plant, and with the rate at which heather can become re-established on a given area by means of seed.

THE ORIGIN OF OVARIAL INFECTION.

The question at once arises—whence come the hyphae which infect the interior of an unopened fruit?

Two possibilities evidently exist:

1. Local infection of the ovary or young fruit, from air-borne spores which reach the stigma independently, or with the pollen.

2. Infection from the root upwards, involving a distribution of the mycorrhizal Fungus throughout the plant.

The latter hypothesis has proved to be the correct one, and the facts now adduced in support of it involve the existence of a delicately balanced symbiosis of a kind not hitherto observed in any mycorrhizal plant.

The facts to be described may be summarized in advance:

1. When infection by the Fungus takes place at, or soon after germination, all parts of the seedling—root, hypocotyl, and cotyledons—are invaded by fungal hyphae.

2. In older seedlings, and in the mature plant, mycelium of the same Fungus occurs in all parts of the sub-aerial organs, in intimate relation with the tissues of the plant, and of the same nature throughout. Infection of the leaves is characteristic, and suggests a very delicate adjustment of the relations between leaf-cells and Fungus.

Infection of the ovary and other parts of the flower is, therefore, only a special case of a condition common to all the vegetative parts.

The observations on which these facts are based will be stated as briefly as is consistent with clearness, rather than in the form of a detailed cytological study.

METHODS.

Fixation. A number of fixatives were tried with varying success, e. g. absolute-acetic, picric-formol, and chromo-acetic solutions of various strengths. The best results were obtained by the use of Carnoy's fluid, which has been used for the greater part of the work.

The use of a mixture of lactic acid and phenol as a clearing and mounting agent for whole roots, &c., has been found of great service, and is invaluable for the recognition of mycelium in fresh or unstained tissues.

For microtome work, material was embedded in paraffin, and the sections stained on the slide by one of the methods mentioned below. For sectioning ripe fruits enclosing seeds, material was also embedded in celloidin to avoid displacement of the seeds after removal of the paraffin.

Staining methods. The method found most generally useful for the differentiation of mycelium in the tissues was a concentrated solution of cotton blue (Baumwollblau 4 B) in lactic acid. Sections stained for some hours (8–24) in this solution can be differentiated in lactic acid, and examined in this reagent or in glycerine, or they may be dehydrated and mounted in balsam. By the use of whole roots, very satisfactory preparations of the Fungus in the root-cells can be obtained in this way.

The method can also be used for microtome sections if precautions are taken to prevent the sections floating off the slide.

The orseillin-aniline-blue method (Strasburger) for differentiating mycelium, and various modifications of it, were also used; satisfactory preparations of the leaves and shoot were obtained by this means, and likewise by use of iron-alum haematoxylin (Benda), and other stains in common laboratory use.

For rapid identification of mycelium in the tissues, slow maceration in sulphuric acid is useful, as is also treatment by ammoniacal cupric hydrate and chloral hydrate.

A. *The Seedling.*

Infection of the seedling tissues by the Fungus can be readily followed in material germinated on blotting-paper, fixed in Carnoy's fluid, stained and differentiated in 'cotton blue' in lactic acid, and mounted for examination in either pure lactic acid or a mixture of lactic acid and phenol.

Infection may begin at the tip of the root, by hyphae forcing their way between the cells of the apex; more usually it takes place simultaneously at several points, and the mycelium immediately becomes intracellular in distribution.

The hyphae penetrate cell membranes with ease; there is no trace of swelling at the point of entrance, and they ramify in the external tissues of the root as if cell-walls offered no obstruction to their growth. Infection spreads rapidly from cell to cell; some hyphal branches grow out and infect fresh rootlets as they develop; others form a tangled skein of fine hyphae in the superficial cells.

In these early stages, the mycelium is homogeneous throughout, and consists of a system of delicate colourless threads which stain deeply with 'cotton blue', both within and without the cells of the root.

The tissues of the young root, like those of healthy seedlings growing in soil, are quite colourless. In the absence of infection, arrest of growth

occurs, and this is invariably accompanied by browning and discoloration of the cell contents. Subsequent to infection, rapid elongation of the roots takes place.

In stained and cleared preparations of the young seedling, it may be further observed that infection is not confined to the rootlet, but that penetration of the tissues of the hypocotyl and cotyledons by hyphae likewise occurs and it is often possible to determine that the same mycelium is continuous from root to hypocotyl.

In the sub-aerial parts the mycelium does not develop so extensively on the surface of the plant, nor do the hyphae become massed in the superficial cells as in the roots. They are irregularly distributed in the tissues, penetrating the cell-membranes with the same apparent ease as in the root. Hyphae not infrequently grow out to the exterior, and since this may also be observed in seedlings germinated in soil, the tendency to extend outside the plant cannot be entirely due to growth in saturated air.

It is suggested that this frequent outgrowth of hyphae to the outside of the sub-aerial parts of the plant is significant in connexion with the problem of nitrogen fixation by the Fungus (p. 107). [See also Hiltner (33).]

In vertical section, the cotyledons, in contradistinction to the later leaves, show typical dorsiventral structure of the assimilating tissue: the mesophyll shows marked differentiation into palisade and spongy parenchyma; stomata are present on the lower surface, the guard-cells being slightly above the general level of the other epidermal cells.

In young seed-leaves progressive degeneration of cells of the mesophyll, such as is recorded in the mature leaf, is not apparent (p. 117), nor is there an accumulation of crystals or crystal-aggregates of calcium oxalate.

Hyphae, though not abundant, can be identified in the tissues of the cotyledon under high magnifications. They are often extremely attenuated, show a preference for the intercellular spaces, but undoubtedly invade cells of the mesophyll, some of which become filled with a tangled mass of hyphae (Pl. VI, Fig. 2).

B. *The Mature Plant.*

Material for examination of the mature tissues was collected from many sources, and from wild and cultivated plants. In all cases the condition observed was substantially the same, and in no case—so far as my observations go—are the green parts of the plant free from mycelium.

The Root. The distribution and general appearance of the mycelium in the cells of the root of ericaceous plants were described many years ago by Frank for *Andromeda polifolia* (35), and more recently by Coville for *Vaccinium corymbosum* (36). My observations on the younger parts of

the mature roots of *Calluna* agree with these records, and need only be briefly described.

The outside of the young root consists of a single layer of rather large cells; the cortical tissues are much reduced in extent, and there is a slender central vascular strand. Root-hairs are absent, but a papillate outgrowth of cells may occur in the radicles of young seedlings.

The surface of the root is traversed by a network of hyaline, septate hyphae, brownish yellow in colour, and irregularly branched.

These hyphae are closely applied to the outer cell-walls and frequently grow between the cells, forcing them apart; they usually penetrate near the corners, where a cluster of branches is formed, some of which pierce the cell-wall. Within the cells they develop several stout coils, from which, at intervals, crowded clusters of short thick branchlets are given off, completely filling the cell (Pl. VI, Fig. 11). Almost every cell of the younger part of the root is infected in this way, and in surface view such cells present an appearance as if filled with dense coils of closely interwoven hyphae.

In addition to this characteristic infection, a system of finer hyphae is present, the branches of which are often especially abundant around the root-tip, penetrating cells in the same way, and forming coiled masses within them.

With the exception of the characteristic 'clusters' in root-cells described above, the mycelium *within* the plant tissues is distinguished from the same mycelium outside only by the smaller diameter of the hyphae composing it, the two systems of hyphae being continuous. Outside the cells, the mycelium sometimes exhibits the structure shown in Pl. VI, Fig. 3. The hyphae figured are very transparent, of rather large diameter, and show characteristic swellings at intervals. This photograph may be compared with Pl. VI, Fig. 4, which shows hyphae, identical in structure, from a pure culture of the Fungus extracted from the ovary, growing on *Calluna*-extract gelatine (p. 129).

These swellings have not been observed on the hyphae of the inter- or intracellular mycelium. They do not seem to correspond to the 'vesicles' of Gallaud and other observers, but are apparently formed by the vegetative hyphae when growing saprophytically, either in proximity to the root, or on certain artificial media.

The Shoot. Historical. The members of Ericaceae possess anatomical peculiarities—especially with regard to leaf structure—which have led to repeated investigation by plant anatomists.

In particular, the ericoid leaf has attracted attention as a characteristic xerophytic type, and the variations of structure shown by the leaves of the several members of the group have been recorded in great detail, and used as a basis for classification.

A summary of the chief features of general anatomical interest in various genera has been given by Solereder (37).

The details of leaf structure in the members of the sub-order Ericoideae have been the subject of a monograph by Ljungström (38), who used his observations as a basis for subdivision of the group into four classes, each distinguished by characteristic leaf structure; in the fourth of these classes he places *Calluna* together with *Erica dianthifolia*.

The Vaccinioideae, Arbutoideae, and Rhododendroideae have also been monographed from this point of view by other observers (39, 40).

Contributions to the comparative anatomy of the members of the order have been made also by Simon (41).

These observers have drawn attention to various features of interest in the anatomy of the leaf: the characteristic shape, with corresponding anomalies in the distribution of the assimilating tissues; the complicated structure of the cell-walls and cuticle; the distribution and structure of many different types of covering and secretory hairs; and the presence of accumulations of calcium oxalate in the tissues of the stem and leaf, either as single crystals or crystal-aggregates.

There is no record in these papers of any observations dealing with a regular infection of the tissues of the shoot by fungal hyphae, such as is about to be described.

In view of the very full accounts given in the papers cited above, a brief summary of the anatomy of the leaf will suffice.

The various species of *Erica* and its allies have often been described as possessing 'rolled leaves', a misleading title since the characteristic hollowing of the under side does not arise by a *rolling back* of the upper side of the leaf, but is developed secondarily as a groove or furrow (or several such) on the lower side.

The leaves of *Calluna* are decussate in arrangement, of small size, and of the familiar ericoid type. The mature leaf is almost quadrangular in section, the sides tapering gradually to the abaxial surface, of which most of the width is occupied by a single conspicuous groove, the walls of which are beset with hairs.

The adaxial side of the leaf is adpressed to the stem, and owing to this, and to the shape of the leaf, the flanks receive most illumination; correlated with which the bulk of the chlorophyllous tissue is found beneath the epidermis in this region.

Stomata are present on both sides of the leaf.

The assimilating tissue in the mature leaf has lost all trace of the dorsi-ventrality found in the cotyledon, and the arrangement of the assimilating tissue—as in other ericoid types—is closely correlated with the shape and position of the leaf, and its consequent illumination.

Chlorophyllous tissue is practically absent from the upper side of the

leaf; the upper epidermis roofs over an almost continuous air-space, traversed only by the branches of the central vascular strand and the tissues which surround it (Pl. VI, Fig. 5).

In median longitudinal sections parallel to the upper and lower surfaces, the relations of the large air-spaces to the vascular strands and to the assimilating tissue below the epidermis of the lateral face of the leaf are made clear. A few layers of assimilating cells on either side pass internally into spongy mesophyll, consisting of strands of green cells, which bridge over at intervals the space between the assimilating tissue and the parenchyma surrounding the bundles.

Towards the base of the young leaf there is a continuous tissue of thin-walled cells. In the mature leaf this region is filled with the remains of these cells intermingled with a dense accumulation of crystal-aggregates of calcium oxalate.

All stages of degeneration are to be observed in these cells and in those forming the trabeculae. Empty cells may be seen, each filled by a single large crystal-aggregate, or groups of such crystals lie free in the space, entangled among the walls of the cells which originally contained them. The cells are represented often only by a framework of walls, though sometimes traces of the cell-contents are recognizable (Pl. VI, Fig. 6).

The presence and distribution of the fungal hyphae in these tissues will now be described.

THE DISTRIBUTION OF THE FUNGUS.

Longitudinal sections through the young shoots provide abundant evidence of the prevalence, on the outside of the stem and leaves, of a network of fine hyphae, ramifying among the hairs or closely applied to the cuticle of the epidermal walls. These hyphae are often excessively fine and are easily overlooked in unstained preparations.

In fairly thick hand-sections or in serial sections of the shoot, it may be determined that this external mycelium is part of a system which pervades the tissues of the stem and leaves. Hyphae penetrating the epidermal cells can be traced into the deeper-lying tissues of the leaf and into close contact with cells of the mesophyll.

The microphotograph produced in Pl. VI, Fig. 7 shows a tangle of hyphae between the leaves of a young shoot, branches from which penetrate the tissues of the leaves on either side.

The hyphae show no preference for special points of entrance or egress. They penetrate with equal ease the cuticularized cells of the epidermis or the base of a hair, and may be occasionally seen emerging from the large hair which terminates the leaf apex.

In sections which have been specially treated and stained, it is possible to follow the ramifications of these fine hyphae across the air-spaces and into the cells of the mesophyll. They are often of extreme tenuity,

and in the absence of the mass of cumulative evidence available doubt might arise as to their true nature.

The staining reactions of the hyphae in contact with the mesophyll cells become affected and the appearance of many of these cells is consistent with an active disintegration and digestion of the invading mycelium.

The difficulty of finding active mycelium in the leaf seems to be due chiefly to the fact that in the unaltered condition it is present in the intercellular spaces only, these being relatively of such large extent that the contained hyphae are usually torn out or displaced in sections.

It is possible, however, to obtain preparations which provide conclusive evidence of the presence of functional hyphae in the leaf tissues and of their invasion of mesophyll cells.

In the chlorophyllous cells, progressive stages of degeneration may be observed; fragments of mycelium, irregular in outline but still stainable, can be found in close contact with, and penetrating such cells; many of them obviously contain hyphae in various stages of degeneration, while the same process of disintegration is affecting the cells themselves.

Plasmolysis in various degrees, alteration of staining properties, degeneration of the chloroplasts, and finally of the whole contents of the cell, mark progressive stages in the process. By suitable staining methods, strands of very attenuated mycelium can be differentiated, bridging across the spaces, sending branches to the cells, and demonstrably continuous in places with those on the outside of the leaf (Pl. VI, Fig. 12).

Calcium oxalate is present in remarkable abundance in the leaves in the form of large crystal-aggregates.

The crystals accumulate chiefly in the mesophyll tissue towards the base of the leaf, and are conspicuous macroscopically in small pieces of shoot, cleared in cedar-wood oil.

In leaf sections, the crystals are most abundant in the cells of the spongy mesophyll in the lower half of the leaf; single aggregates are present in the empty cells of the trabecular tissue, completely filling them, and occur also in groups intermingled with the cells and cell-remains in the central part of the basal region of the leaf (Pl. VI, Fig. 6).

Hyphae may be recognized in close contact with the crystals; sometimes free in the intercellular spaces; more often in the form of strands or fragments of greenish membrane mingled with the remains of the cell-walls (Pl. VI, Fig. 13).

A network of fine mycelium with looped masses of hyphae may often be found in the cells of this part of the mesophyll, and can be identified after treatment of the section with concentrated sulphuric acid.

There is no doubt that the region of the leaf which contains most abundant evidence of the presence of mycelium is also the region in which the greatest accumulation of calcium oxalate occurs.

Whether this association of hyphae and crystals is accidental, or whether it is one of the links in a chain of events which connects the metabolism of the plant with that of the Fungus, remains an open question; but the presence of such relatively enormous accumulations of a calcium salt in the tissues of a plant believed to be unusually intolerant of lime salts in the soil raises many points of interest.

The oxalic acid may be produced by the plant cells or by the Fungus; if by the former, it may be an indication of interference with normal cell metabolism, induced possibly by the increased demand upon the available oxygen supply.

If the calcium absorbed by the plant from the soil, in the form of calcium salts, is constantly in requisition for the neutralization of oxalic acid, the edaphic preferences of the plant for acid soils and those deficient in lime salts are puzzling.

If, on the other hand, the calcium salt is derived immediately from the Fungus, it must be obtained either from the soil (which raises the same difficulty), or from some part of the plant tissues, e. g. the middle lamella of the cell-walls.

The establishment of the fact that the relation existing between the plant and the Fungus is an obligate one permits the problem of the origin of the calcifuge habit to be stated in a new form, and hence provides a fresh point of departure for experimental research into this important phenomenon.

Assuming that the Fungus forms and excretes oxalic acid—and possibly makes its primary attack upon the plant by this means—it may well be that the amount of calcium salts present in the plant at the moment of infection is a decisive factor in determining whether infection shall or shall not occur.

If the amount is considerable, the plant will resist the attack of the Fungus, and, by its very resistance, cuts off all chance of growth on normal calcareous soils, the presence of the Fungus having become one of the first essentials to development on the part of the seedling.

If, on the other hand, the plant cells are deficient in lime salts, the excess of oxalic acid can act, directly or indirectly, by preparing the way for the entrance of the parasite, symbiosis is established, and, so long as equilibrium is maintained, the plant flourishes.

This working model of the conditions underlying the origin of the 'lime-shy' habit makes no claim to completeness, but, as already stated, it immediately suggests fresh methods by which to attack the problem experimentally.

The excretion of calcium oxalate by Fungi growing in plant tissues or on artificial media is not uncommon (42), and an alteration of the calcium oxalate content of the cells of the host has recently been noted as one of

the indirect results of invasion of leaves by parasitic Fungi (43, 44). The discussion of hypotheses to account for the storage of a calcium salt in the plant tissues may profitably be postponed until more data are available as to the nutritive preferences of the Fungus.

The Stem. The presence of mycelium on the outside of the young stem and the penetration of branches from it into the underlying tissues have already been noted (p. 116). Evidence of the presence of mycelium may be found in most tissues of the stem, e.g. in the parenchyma of the cortex, and in that associated with the vascular tissues; the cells of the pith, also, probably contain hyphae in a vestigial condition.

The tissues of the pith and of the cortex likewise contain quantities of calcium oxalate in the form of large crystals and crystal-aggregates. Untreated sections are often rendered absolutely opaque by the presence in every cell of these crystals. In rare cases small fragments of hyphae which stain deeply with cotton blue or aniline blue can be identified in the neighbourhood of crystals; more frequently the hyphae are present in an extremely attenuated condition, are very transparent, refuse to take up the characteristic stains, and can only be identified with difficulty.

The most satisfactory method of obtaining conclusive proof of the presence of mycelium in such tissues is by slow maceration in sulphuric acid, or by prolonged treatment with clearing agents, such as chloral hydrate, eau de Javelle, or potash, before staining.

4. *The Isolation of the Fungus.*

During the past three years repeated and fruitless attempts have been made to isolate the mycorrhizal Fungus from the root-cells.

The method first adopted was to remove clean transparent young roots from the outside of a ball of soil surrounding the roots of a pot plant. These roots were washed in fast-running water for twenty-four hours, rinsed repeatedly in sterilized distilled water, after which small pieces were planted out on agar plates. (Direct sterilization of the surface of the root, by heat or momentary immersion in weak solutions of mercuric chloride, was found to be impracticable.)

Using this method, a number of fungal species were isolated, none of which, however, satisfied the test of successful inoculation into sterile seedlings.

Among those constantly found were species of *Cladosporium*, *Penicillium*, *Citromyces*, *Fusarium*, and *Alternaria*, all of which are apparently constant or fairly constant members of the epiphytic microflora of the roots. A species of *Cladosporium* was invariably present in my cultures, and is always dominant if unsterilized seedlings are planted in agar.

The hanging-drop method of culture was tried with the same lack of success. Pieces of washed root were transferred to hanging drops of sterile water, and to similar drops of various solutions, which it was

hoped might favour the growth of the endophyte, as compared with that of its more epiphytic competitors; neutral and slightly acid sugar solutions, soil extracts, peat extract, *Calluna* extract, were all used for this purpose.

In many of these drop cultures the endophyte was obviously active, but on transferring to plate cultures, the growth, if present, was always masked by that of one or several of the Fungus species mentioned above.

Attempts were also made, using similar hanging-drop cultures, to extract the Fungus from the seed-coat of the resting seed, and from seedlings soon after infection.

In several cases interesting results were obtained, throwing light on the formation of bacterial colonies on the roots of seedlings, as described in an earlier paper (3), but the results were negative in so far as the isolation of the endophyte was concerned.

The discovery of mycelium within the ovary, and of its distribution throughout the plant, provided a fresh starting-point. Unripe capsules were sterilized by passing them through a flame, or by immersion in 1 per cent. mercuric chloride. The seeds and internal tissues were then removed and transferred with aseptic precautions to agar plates.

Colonies of a non-sporing Fungus developed on several of these plates, both from seeds and from pieces of ovary tissue, and were subcultured on various media. The Fungus isolated in this way was used for the inoculation of sterile seedlings. These seedlings, immediately after infection, developed a root-system and grew vigorously in a perfectly normal manner under aseptic conditions in closed tubes (Pl. VI, Fig. 14).

The agar medium in which they were planted was similar to that used previously, without success, for the cultivation of sterile seedlings (p. 106).

As in former cultures, uninfected controls remained rootless, and in other ways showed more or less complete inhibition of growth (Pl. VI, Fig. 15, *a* and *b*).

Demonstration of an obligate relation between the plant and the Fungus which besets its roots, and of the identity of this Fungus with that isolated from the ovary, is therefore complete.

Uninfected seedlings, grown under strictly aseptic conditions, or subjected to casual infection by Fungi and Bacteria from the air, fail to form roots.

Similar seedlings, infected with a Fungus isolated from the ovary of the flower morphologically identical with that in the cells of the root, develop a root-system, and continue to grow normally under aseptic conditions, the rooting medium being alike in the two cases (cf. Pl. VI, Figs. 14 and 15).

7 Only as an embryo in the resting seed does the heather plant retain an

independent existence; from the moment of germination onwards it is a dual organism, the artificial synthesis of which has now been accomplished. The endosperm and embryo are the last strongholds of independence retained by the plant.

It is interesting to speculate on the course of the evolutionary path which has been traversed, and to inquire if this condition of dependence has been reached as the result of a long series of capitulations on the part of the plant, each marking an extension of the area in which the Fungus is suffered as an endophyte in the tissues.

When first extracted, the Fungus makes a rather weak growth on artificial media, but becomes more vigorous when subcultured.

These differences in vigour are correlated with corresponding differences in the ease with which seedlings can be successfully inoculated.

Thus, seedlings inoculated from a sub-culture growing on *Calluna*-extract gelatine immediately formed roots and continued to grow vigorously (Pl. VI, Fig. 14), while seedlings of the same age, inoculated from a strongly-growing old culture on rice, formed roots, but were at once partly or completely parasitized by the Fungus which became conspicuous externally on the leaves, or, in the case of weak seedlings, killed them outright before a root-system was formed (Pl. VI, Fig. 16).

In the former case the endophyte grew almost entirely below the surface of the rooting medium; in the latter it developed vigorously on the surface.

Further investigations are in progress with regard to these differences of behaviour, which are evidently closely correlated with the nutritive conditions before and after infection.

This was strikingly apparent in the two cultures from which representative seedlings are figured on Pl. VI, Figs. 17, 18, and of which the history was as follows. Sterile seedlings were planted out on filter-paper in sterile tubes, inoculated from the vigorous rice culture mentioned above and supplied with distilled water, with the result that roots were formed in every case, although some of the seedlings showed signs of being too strongly invaded (Pl. VI, Fig. 17).

Seedlings similarly inoculated and planted out on paper, but supplied with a nutrient solution (Solution A (p. 106), 0.15 per cent. total concentration) instead of distilled water, were immediately attacked by the Fungus and destroyed, the mycelium subsequently forming an extremely vigorous growth on the paper (Pl. VI, Fig. 18).

The results of these isolation and inoculation experiments may be summarized as follows:

1. A Fungus species, showing identical morphological characters in each case, has been isolated from the unopened fruit and from seeds removed from the unopened fruits of *Calluna vulgaris*.

2. Sterile seedlings, inoculated with this Fungus (from either source), subsequently produce normal root and shoot systems when grown in closed tubes, under aseptic conditions.

3. Control seedlings remain rootless.

4. The ease with which plant and Fungus can be successfully synthesized depends upon the nutritive and other conditions under which the experiment is conducted, the age of the seedling, the age and vigour of the fungal culture used, and, in all probability, upon the composition of the nutrient material upon which the latter was cultivated outside the plant.

5. The Fungus isolated in this way is morphologically identical with that present in the root mycorrhiza.

THE ENDOPHYTE.

Inside the plant. The vegetative mycelium as it appears in the various organs of the plant has already been fully described (p. 112). With the exception of the characteristic clusters of branches which fill the root-cells (Pl. VI, Fig. 11), and the swellings on hyphae outside the root but continuous with those in the tissues (Pl. VI, Fig. 3), no special organs have been observed. There is no doubt but that the distribution of the endophyte in the artificially synthesized plant is as wide as that described for normal seedlings; there is no reason to believe that the details of development in the tissues differ in any respect from those described for the latter.

The formation of tubercles. Invasion of the roots of a vascular plant by either Fungi or Bacteria is often marked by the formation of tubercles or nodules. The root tubercles of Leguminosae and the nodules on the roots of *Alnus glutinosa* and *Podocarpus* are familiar examples.

So far as I am aware, there is no record of tubercles on the roots of an ericaceous plant, and their formation must, therefore, be relatively rare.

Tubercles of a quite characteristic kind are, however, formed sometimes by healthy plants of *Calluna* growing on typical heath soils.

The tissues of these tubercles contain fungal hyphae and Bacteria. The details of their structure and the relations, if any, between the two micro-organisms is being investigated and may throw light on the nature of the bacterial colonies present on the roots when growing in certain soils.

Outside the plant. Sub-cultures of the Fungus from the original colonies derived from the plant tissues were made on rice and on gelatine-*Calluna* extract.

The behaviour of the organism on these and other media, together with a detailed account of the morphological characters, will be found in the Appendix (p. 128).

Unless otherwise stated, all the cultures described were made from this original sub-culture on rice, and were kept at room temperature.

The morphological characters of the Fungus agree with those of the genus *Phoma*.

They coincide generally with those described for the five pycnidia-forming Fungi, isolated from the roots of five ericaceous species by a previous observer (26), and referred to this genus.¹ In view of the anomalous distribution in the tissues of the plant, and the fact that the members of the genus *Phoma*, as commonly understood, are obligate parasites in all parts of plants *except the leaves*, it is suggested that the species now described should be placed in a new sub-genus *Phyllophoma*.

With regard to the physiology of the Fungus, many problems present themselves.

The reaction of the micro-organism to acid and alkaline media and to various soil extracts; the possible excretion of acid by the Fungus when grown on various substrata; a more detailed knowledge of the nature of the enzymes produced; and the behaviour of the Fungus in pure culture when inoculated with Bacteria present on the roots of plants growing in calcareous soil, all suggest lines of research of special interest from the point of view of the ecology of the plant.

THE DISTRIBUTION OF THE ENDOPHYTE IN OTHER SPECIES OF ERICACEAE.

A number of other ericaceous species have been investigated in order to determine whether the condition described for *Calluna* is common to other members of the order.

In every species examined, mycelium could be identified in the ovary of the unopened flower—in many cases removed from the resting bud for examination—which showed relations with the plant tissues similar to those described for *Calluna*. It has not yet been possible to make a detailed examination of the vegetative tissues in each case, but in some of the species the endophyte is undoubtedly present in the leaves also.

The ease with which mycelium can be demonstrated varies very much. In the members of Ericoideae it is usually extremely reduced and difficult to recognize in the tissues.

Since the list of species appended contains representatives from the Rhododendroideae, Arbutioideae, and Vaccinioideae, as well as from the Ericoideae, it seems reasonable to conclude that the distribution of the endophyte described in detail for *Calluna* is common to all members of Ericaceae. The agreement of members of Vaccinioideae in this respect is of interest

¹ It is doubtful whether any of these species can be identified with certainty with the species of *Phoma* recorded by Rabenhorst from members of Ericaceae (see Ternetz, 26).

for systematic reasons, and favours the view that the members of this sub-order are closely related to the hypogynous forms.

Since ovarial infection is the rule, the seeds of all species will evidently always be liable to infection by the specific Fungus; whether the relation between seedling and Fungus is of the same obligate character as has been demonstrated for *Calluna* can only be determined by experimental investigation of each case. The observation recorded by an earlier investigator, that young seedlings of *Andromeda polifolia* germinating viviparously in the capsule showed fungal infection of the roots, is worthy of notice in this connexion (26).

Experimental investigation is also required in order to determine whether the endophyte is specific to each plant species, or can be used successfully for the inoculation of any member of the group in which an obligate relation can be demonstrated.

Appended is a list of the species in which ovarial infection has been observed, and a similar distribution of the Fungus may probably be inferred.

RHODODENDROIDEAE.

Ledum palustre, *Rhododendron ponticum* (Garden var.), *Rhododendron indicum* (*Azalea indica*, garden var.), *Rhododendron sinense* (*Azalea sinense*, garden var.), *Leiophyllum buxifolium*, *Kalmia angustifolia*.

ARBUTOIDEAE.

Pieris floribunda, *Pieris japonica*, *Gaultheria acutifolia*, *Arctostaphylos Uva-ursi*, *Arbutus Unedo*.

VACCINIOIDEAE.

Vaccinium Vitis-idaea, *Pentaptergyium serpens*.

ERICOIDEAE.

Calluna vulgaris, *Erica carnea*.

SUMMARY.

1. In common with other members of Ericaceae, *Calluna vulgaris* possesses a characteristic root mycorrhiza.

2. Infection by the mycorrhizal Fungus takes place shortly after germination, the source of such infection being the testa of the seed.

3. Infection does not cease with the formation of the characteristic mycorrhiza associated with the roots but affects all parts of the young seedling.

4. In the mature plant, likewise, the Fungus is not confined to the roots or colourless parts, but is present also in the sub-aerial organs—in the tissues of the stem, leaf, flower, and fruit.

5. The ovary—and later the young fruit—contains mycelium in all parts of the internal tissues. This mycelium infects the seed-coats of the developing seeds.

6. The embryo and endosperm of the resting seed are free from infection.

7. By appropriate methods, seeds can be sterilized and seedlings germinated, free from fungal and bacterial infection.

8. Failing infection by the appropriate Fungus, such seedlings do not develop roots; they suffer complete inhibition of growth, remaining alive, but rootless, for several months.

9. The mycorrhizal Fungus has been isolated from unopened fruits and from seeds removed from unopened fruits and has been grown in pure culture; sterile seedlings inoculated from a pure culture of this Fungus develop normally under aseptic conditions. The synthesis of Fungus and plant has thus been accomplished.

10. In morphological characters the Fungus resembles the genus *Phoma*. In view of its distribution in the plant, and the unusual biological relations exhibited, it is proposed that the species described must be placed in a new sub-genus, for which the name *Phyllophoma* is suggested.

11. Ovarial infection has been observed (and a similar distribution of the Fungus in the vegetative parts inferred) for a number of ericaceous species, including members of the Vaccinioideae.

12. It has not been found possible to replace the stimulus to development which follows seedling infection by supplies of various organic nitrogenous substances in the food material.

DISCUSSION OF RESULTS.

In view of the facts described in this paper, the conclusions of Stahl, with regard to the relations between ericaceous plants and their mycorrhizal Fungi, require revision.

As a result of experimental work on *Vaccinium*, &c., germinated and grown in heath soil, sterilized by heat and by ether vapour, Stahl summarizes his conclusions as follows:

‘Während manche obligaten Mycorrhizenpflanzen, wie wir früher gesehen haben, der Anzucht aus Samen und der Kultur grosse Schwierigkeiten bereiten, lassen sich die Ericaceen auch ohne Gegenwart von Wurzelpilzen unschwer kultiviren, und ihre Samen gehen, zwar oft langsam, aber in grossem Procentsatz und sicher ohne Mitwirkung symbiotischer Pilze auf’ (16).

According to Stahl, seedlings of *Vaccinium Myrtillus* sown in May, on soil which had been previously heated or treated with ether vapour, when examined in October of the same year were completely free from fungal infection (‘völlig pilzfrei’).

Was the fungus found in the
tissues of the strong-growing
seedlings?

As a general statement affecting all members of the Natural Order Ericaceae, the conclusions expressed in the passage quoted above can no longer be accepted.

It is true that an obligate relationship with the mycorrhizal Fungus cannot, at present, be predicted with certainty for every member of the group, but, in view of the fact that some species of *Vaccinium* (p. 124) show ovarial infection of the kind described for *Calluna*, a repetition of Stahl's experiments, under conditions in which negative evidence regarding infection of the roots can be more satisfactorily tested than by microscopic examination only, is required.

Recent work by Russell (45), on the sterilization of soil by heat and antiseptics, appears also to have a bearing on the interpretation of the results in these and similar experiments carried out by Stahl.

The condition described for *Calluna* does not seem to have an exact counterpart among mycorrhizal plants.

The dependence of the plant on the fungal symbiont is paralleled, if not exceeded, among the Orchids, in some species of which development of the embryo ceases at an early stage, unless infection occurs. In the Orchids, however, the endophyte is strictly confined to the non-chlorophyllous tissues, and this restricted distribution is not accidental, for Bernard has shown that portions of the stem of some Orchids have a poisonous effect upon the mycorrhizal Fungus of the same plant. In cultures, the fluid diffusing from these tissues killed the hyphae; heated to 55° C., the toxic properties disappeared, from which it was inferred that the poisonous substance was probably of the nature of an enzyme (46).

While also exhibiting complete dependence upon the Fungus at an early stage in the life-history, *Calluna* has an advantage over the Orchids, since the seeds are insured against the risk of non-infection—no small advantage in the case of small, light seeds, distributed by wind. This advantage would seem to be counterbalanced by the presence of mycelium of a facultatively parasitic nature in the tissues of the shoot.

There is no evidence for nitrogen fixation from the air by the Orchid Fungi, but it is usually believed that the Orchid plant obtains nitrogenous food material from the Fungus by the digestion of mycelium in specialized cells ('Verdauungszellen').

It is now suggested that *Calluna* and its allies have solved the nitrogen problem in a different way.

The toleration by the plant of mycelium in the intercellular spaces of the leaves continuous with that on the outside of the shoot, and the ultimate relations of the hyphae with the mesophyll cells, point to the possibility of the fixation of atmospheric nitrogen in some degree by the Fungus.

Indirect evidence favouring the same view has already been cited

(p. 107), while nitrogen fixation has been claimed for five pycnidia-bearing Fungi isolated from members of Ericaceae, growing in pure culture outside the plant (26). The possibility of growing the artificially infected plant of *Calluna* in a nitrogen-free substratum awaits experimental proof.

The only green plant for which has been described a like distribution of mycelium—not obviously pathogenic—in the tissues, combined with ovarial infection of the seeds, is the Darnel grass (*Lolium temulentum*).

In this case, however, the plant does not form mycorrhiza, nor has it been established that the relation with the Fungus is an obligate one (47, 48).

Some degree of symbiosis has been inferred, but the experiments of Hiltner (33) to establish nitrogen fixation for this Fungus are inconclusive.

The case of the saprophytic Orchid *Gastrodia elata*, described recently by Kusano (49), is of interest, since there is dependence of the plant upon fungal infection for a part of the life-history only, coupled with a strictly limited distribution of the Fungus in the tissues.

The higher symbiont in this curious relationship is a rootless saprophytic species of Orchid; the fungal partner is *Armillaria mellea* ('*Rhizomorpha subterranea*'), a well-known facultative parasite. The association is an obligate one for the plant, in so far as the flowering stage is not reached unless 'mycorrhiza' is formed, but the vegetative period of the life-history is independent of infection, which takes place only occasionally in nature. It involves symbiosis, by means of a stem mycorrhiza, on the part of two heterotrophic plants, the fungal partner remaining apparently unmodified by its temporary association with the plant.

The view is put forward by this author, that the first step towards the formation of mycorrhiza can here be recognized, involving a temporary modification of parasitic habit on the part of the Fungus concerned.

It is evident, from a consideration of mycorrhiza in general, that it is still impossible to frame a definition which will include all the known cases. The theories of earlier observers implied a strict symbiosis with reciprocal advantages of an obvious kind. Later workers tend rather to regard the relation as primarily one of *parasitism* on the part of the Fungus, tolerated and often turned to account by the plant, or even become indispensable to it.

Thus Gallaud (20) concludes that there is no 'symbiose harmonique' between plant and endophyte. The latter is only an internal saprophyte of a special kind which the plant cells can keep in check without preventing further development.

Bernard holds similar views with regard to the Orchids:

'A un point de vue théorique il résulte de ces constatations que l'état dit de symbiose est en quelque sorte un état de maladie grave et prolongée, intermédiaire entre l'état des plantes atteintes d'une maladie rapidement

mortelle et celui des plantes qui jouissent d'une immunité complète' (loc. cit.).

A similar interpretation may perhaps be suggested by the condition in Ericaceae, many of the members of which have solved the problem of growth upon the poorest and most unpromising soils, but have solved it at the price of their independence.

A study of these delicately-balanced relations inevitably provokes comparison with phagocytosis and the phenomena of immunity as observed in animals.

The recent work of Fellmer (50), and of Wendelstadt and Fellmer (51), is suggestive in this connexion as indicating how the *specificity* shown in relations between a vascular plant and a Fungus, such as those described above, or possibly in symbiotic associations in general, can be brought into line with the facts of immunity and reaction to immune sera in animals.

These authors have shown (1) that plant extracts ('Eiweissstoff'), when injected into animals, produce *specific* precipitin reactions, with anaphylactic phenomena quite analogous to those produced by the injection of animal sera; and (2) that similar specific reactions are given by animals to extracts of Fungus protoplasm prepared in the same way.

The attempt to use the latter to produce immunity in the higher plants against attacks of Fungi, although theoretically possible, has not yet proved successful in practice.

Only investigation of special cases can demonstrate with certainty the exact relation between the degree of development of the Fungus in the ericaceous plant and the well-being of the latter for any given conditions of growth, or provide a key to the soil preferences of these plants, both in the field and under cultivation.

It is unlikely that observed peculiarities in this respect can always be explained as consequences of the xerophytic habit, with the decreased transpiration current and retarded absorption which this entails.

I am indebted to Dr. R. Stenhouse Williams for testing the sterility of seedlings used in inoculation cultures, and to Professor Keeble for helpful criticism of this paper.

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APPENDIX.

5. *The Endophyte in pure Culture.*

1. *Rice and tap-water.*

Thirty days' culture. Vigorous superficial growth; mycelium snow-white at first, becoming greyish brown. The medium slowly changes colour from yellow to dark brown.

Six weeks' culture (Pl. VI, Fig. 19). As last, but many small dark dots on the

bared parts of the surface, indicating the formation of pycnidia. Older mycelium greyish brown; younger mycelium from germination of the pycnidiospores snow-white. Mycelium resembling that found in the root mycorrhiza of the plant. Larger hyphae hyaline, regularly septate, yellow-brown, diameter 0.007–0.008 mm., giving rise directly or by gradual transition to fine, colourless, branched hyphae; the ultimate branches very attenuated.

Larger hyphae rarely shortly jointed in growth or swelling to form terminal or intercalary swellings; often forming strands of parallel-growing hyphae. The colourless vegetative hyphae become densely interwoven, but there is no formation of a definite stroma.

Pycnidia produced freely on the surface; globose or slightly irregular in shape, with apical papilla; size variable, diameter from 0.2 mm. upwards; colour dark brown (Pl. VI, Fig. 8).

Pycnidiospores oval, 0.003 mm.–0.004 × 0.002–0.003 mm.; wall pale greenish yellow to yellow-brown (Pl. VI, Fig. 9).

2. *Calluna-Extract Gelatine*.¹

Ten days' culture. Colony 4 cm. diameter. Superficial growth of mycelium, well developed and snow-white in colour.

Fifteen days' culture (Pl. VI, Fig. 20). Colony 6.5 cm. diameter. Mycelium snow-white. Gelatine slowly liquefying.

Mycelium vigorous; hyphae branched, septate, colourless to yellow-brown, resembling those from culture on rice, but in older cultures the majority form characteristic swellings—intercalary, terminal, or in chains. Mycelium often forming parallel strands.

No formation of spores or pycnidia observed.

Complete liquefaction of the gelatine in older cultures.

3. *Water-culture solution A* (p. 106) and 0.12 % *Agar*.

Twelve weeks' culture. Medium slightly clouded, colour unchanged. Growth on surface feeble or absent, except for a slight growth of hyphae on the sides of the tube. Mycelium homogeneous, composed of fine, branched, colourless hyphae, frequently anastomosing. The mycelium in this culture was identical with that observed in the early stages of infection of sterile seedlings.

4. *Solution A + 0.1 % Dextrose + 0.1 % Witte's Peptone + 0.12 % Agar.*

Twenty days' culture. Colony 7 cm. diameter.

Thirty „ „ Colony covered plate.

Growth colourless; scanty white mycelium on surface.

In older cultures the medium darkens very slowly, becoming yellow to yellowish brown.

Growth vigorous, chiefly below surface. Hyphae colourless to yellow-brown in old cultures. Older hyphae often closely septate and with thicker walls; no separation of chlamydospores observed. No formation of swellings in cultures up to five months old. Refractive droplets abundant in hyphae and as an excretion in the medium. No formation of spores or pycnidia observed.

¹ 130 gm. of young shoots of *Calluna* extracted in water; extract made up to 1 litre, and 120 gm. sheet gelatine added = dark-brown medium.

5. *Nutrient Agar*,¹ + 10 acidity.

Twenty days' culture. Colony 3 cm. diameter.

Thirty „ „ Colony 3.25 cm. diameter.

Forty „ „ Colony 3.5 cm. diameter.

Colony yellowish, raised, with very scanty growth of mycelium on surface. Colour of the medium unchanged. Mycelium showing a preponderance of the larger hyphae, in which the cross-walls are strongly marked; branches often 'jointed' in structure; no separation of chlamydospores observed (Pl. VI, Fig. 21).

The mycelium shows a vigorous development of large swellings—terminal, intercalary, and in chains.

6. *Nutrient Agar*, —5 alkalinity.

Twenty days' culture. Colony 1.5 cm. diameter.

Thirty „ „ Colony 1.8 cm. diameter.

Forty „ „ Colony 2.0 cm. diameter.

No further growth. Colony similar in appearance to last, but the medium tending to deepen in colour.

Mycelium as last, with very conspicuous development of swellings.

Hydrolysis of arbutin. The glucoside arbutin is widely distributed among the members of Ericaceae.

It occurs abundantly in the tissues of *Calluna*; in a watery extract of the leaves and young shoots made in the cold, the colour, at first yellowish, slowly deepens until the solution becomes dark brown.

This browning of the extract is presumably due to hydrolysis of the glucoside to glucose and hydroquinone, the latter of which oxidizes to form a brown pigment.

The browning of the plant-cells in unhealthy seedlings is possibly due to related causes.

The production of an enzyme by the Fungus able to effect the hydrolysis of arbutin has been determined by transferring mycelium from a pure culture on rice to tubes containing a 0.5 % solution of arbutin. At the end of a week the tubes showed slight browning of the solution, the coloration gradually becoming more marked. Control tubes, containing a 0.5 % solution of arbutin without the Fungus, remain colourless.

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¹ The nutrient agar was made up with Lemco and standardized in the usual way.

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EXPLANATION OF PLATE VI.

Illustrating M. C. Rayner's paper on Obligate Symbiosis in *Calluna vulgaris*.

Figs. 10, 15 *b*, 16, 17, 18, 19, 20 = photographs. Fig. 21 = microphotograph. Figs. 11, 12, 13 = camera lucida drawings. Figs. 14, 15 *a* = drawings.

Fig. 1 *a*. Ovarial infection. Part of a longitudinal section of the unopened fruit, showing seeds still attached to funicles. Mycelium in all parts of the ovary tissues, and traversing the spaces between ovary wall and seeds, &c. *h.* = hyphae. $\times 60$ diam.

Fig. 1 *b*. Part of the section shown in Fig. 1 *a*, more highly magnified. *h.* = hyphae; *o.* = ovary wall; *t.* = seed. $\times 300$ diam.

Fig. 2. Cell from mesophyll (bundle sheath) of the cotyledon filled with hyphae. *h.* = hyphae; *v.* = vessel in median vascular strand; *chl.* = chloroplast. Camera lucida drawing from a section $22\ \mu$ stained cotton blue in lactic acid. Leitz obj. 6; Zeiss compens. oc. 12.

Fig. 3. Mycelium of mycorrhizal Fungus outside root; hyphae continuous with those in cells. *r.* = root; *v.* = 'vesicles'. $\times 330$ diam.

Fig. 4. Mycelium of mycorrhizal Fungus, from pure culture of same on *Calluna*-extract gelatine. *v.* = 'vesicles'. $\times 330$ diam.

Fig. 5. Transverse sections of successive leaves near apex of shoot. *g.* = groove on abaxial side; *s.* = air-space. $\times 100$ diam.

Fig. 6. Longitudinal section of leaf parallel to upper surface. *c.* = crystals of calcium oxalate. $\times 68$ diam.

Fig. 7. Coils of mycelium between adjacent leaves, penetrating the epidermis and mesophyll on either side. *e.* = epidermis; *m.* = mesophyll cell; *h.* = hypha. From longitudinal section of shoot, stained Benda hæmatoxylin. $\times 540$ diam.

Fig. 8. Mycorrhizal Fungus. Hyphae and pycnidia from pure culture on rice. Six weeks. *p.* = pycnidium. $\times 75$ diam.

Fig. 9. Mycorrhizal Fungus. Pycnidium more highly magnified after escape of pycnidiospores. *p.* = pycnidium; *s.* = pycnidiospore. $\times 210$ diam.

Fig. 10. Normally infected seedling from unsterilized sand culture in tube as shown in Text-fig. 1. 282 days after planting.

Fig. 11. Root mycorrhiza. Superficial cell of young root with hyphae. Camera lucida drawing from whole root, stained cotton blue in lactic acid. Zeiss. hom. imm. 3 mm. Compens. oc. 12. $\times 1620$ diam.

Fig. 12. Infection of leaf. Cells of the mesophyll with invading hyphae. *m.* = mesophyll cell of leaf; *h.* = hyphae; *chl.* = chloroplast. Camera lucida drawing from transverse section of shoot (Fig. 5). Zeiss. hom. imm. 3 mm. Compens. oc. 8. $\times 880$ diam.

Fig. 13. Infection of leaf. Mycelium in air-space of leaf, with crystals of calcium oxalate. *C.* = CaO ; *h.* = hyphae. Camera lucida drawing from longitudinal section of a fresh leaf. Leitz obj. 6. Zeiss compens. oc. 12. $\times 880$ diam.

Fig. 14. Sterile seedling growing in nutrient agar and artificially infected from a pure culture of the mycorrhizal Fungus. Ninety-five days from sowing; twenty-seven days after planting and inoculation. *f.* = limit of fungal growth in agar. This seedling subsequently reached a height of 6 cm. $\times 1\frac{1}{2}$.

Fig. 15 *a.* Sterile control seedling in nutrient agar. Ninety-five days from sowing; twenty-seven days after planting. No subsequent growth took place in this or other controls. $\times 1\frac{1}{2}$.

Fig. 15 *b.* Sterile control seedlings on filter-paper. Same age as Fig. 15 *a.*

Fig. 16. Sterile seedling, infected artificially from a 'strong' culture of the mycorrhizal Fungus in nutrient agar. Ninety-five days from sowing, twenty-seven days after planting. *h.* = mycelium growing out from shoot. X

Fig. 17. Sterile seedlings on filter-paper, inoculated from a pure culture of the mycorrhizal Fungus supplied with distilled water only (cf. Fig. 18). Ninety-five days from sowing; twenty-one days after inoculation. *f.* = Fungus.

Fig. 18. Sterile seedlings of same age under similar conditions, but supplied with a dilute nutrient solution extract of distilled water. Seedling completely parasitized and destroyed; Fungus vigorous and forming pycnidia. X

Fig. 19. Mycorrhizal Fungus. Pure culture on rice. Five weeks. Diameter 4.5 cm.

Fig. 20. Mycorrhizal Fungus. Pure culture on *Calluna*-extract gelatine. Fifteen days.

Fig. 21. Mycorrhizal Fungus. Mycelium from five-weeks' culture on nutrient agar. $\times 250$ diam.

1a.

1b.

3.

4.

7.

2.

8.

5.

6.

9.

M.C. R.del.

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