

- I. Mycorrhiza in Rhododendrons.
- II. Tremella translucens; a new species on dead
Pine needles.
- III. Note on a Rare Beetle, Cartodere filum Aubé,
eating fungus spores.
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ARRANGEMENT.

	Pages.
I. Mycorrhiza in Rhododendrons.	3 - 56.
Illustrations of I.	follow page 56.
II. <u>Tremella translucens</u> ; a new species on dead Pine needles.	57 - 67.
Illustrations of II.	follow page 67.
III. Note on a Rare Beetle, <u>Cartodere</u> <u>filum</u> Aubé, eating fungus spores.	68 - 79.
Illustrations of III.	follow page 79.

I.

MYCORRHIZA IN RHODODENDRONS.

Contents.	Page.
Introduction	4.
Review of Previous Work	7.
Materials and Methods	12.
The Question of Shoot and Seed Infection	23.
The Course of Normal Infection in Soil	26.
The Effects of Soil and Seed Sterilization	30.
Pure Culture on Agar Media	34.
Pure Culture on Other Media	38.
Conclusions	46.
Discussion	47.
Summary	50.
References	52.
Explanation of Figures	55.
Figures	follow page 56.

Introduction.

It has long been known that the roots of Rhododendrons, in common with those of the rest of the Ericaceae, contain an endophytic fungus, but the genus has received very much less attention from this point of view than several others, notably Calluna and Vaccinium. Yet it is a genus of rather peculiar importance in any consideration of the significance of mycotrophy in this family, for it includes a group of species, mainly from the Yunnan area, which grow naturally on limestone, a very exceptional habitat for Ericaceous plants. Some of these species have been experimentally cultivated on calcareous soils in this country, as on the chalk hills of East Berkshire (Grove, 1916), and have been found to survive and flourish under conditions which would be entirely unsuitable for the majority of Ericaceae. Attempts have been made to explain these facts on the basis of the mycotrophic habit, including the speculative suggestion that, when the plants are growing on lime, the fungus migrates from the roots to the under surface of the leaves, where it occupies a highly favourable place for the fixation of atmospheric nitrogen. This is but one expression

of an idea that has gained some currency, namely that those Rhododendrons owe their tolerance of lime in some way to their mycotrophic habit.

But the type of mycorrhiza found in these lime-tolerating Rhododendrons, at least when cultivated in the usual way on non-calcareous soils in this country, is quite similar to that of the other species of the genus, and of the majority of the other genera in the family. It is of the same type which has been described, in particular, for Calluna and Vaccinium. Some time ago Rayner (1913 and later papers) suggested that there might possibly be a connection between mycotrophy and the calcifuge habit in the Ericaceae. The fact that lime-tolerating and lime-shy members of the family, at least when growing under similar conditions, form the same type of mycorrhiza, emphasizes the need for great caution in relating the opposing peculiarities of either group to a factor which is common to both. Information as to the presence or absence of the endophyte, and its state of development if present, in the roots of lime-tolerating Rhododendrons when actually growing on their native limestone, is greatly to be desired.

Rayner (1915), in her original paper on "Obligate Symbiosis in Calluna vulgaris," included

Rhododendron species amongst those in which she had observed hyphae in the ovary, and to which it was inferred that the distribution of the fungus throughout the shoot, then described for Calluna, would also apply. More recently Freisleben (1935) included four species of Rhododendron amongst a large number of Ericaceae which he investigated, and though his results were by no means in agreement with those of Rayner, he also found that Rhododendron conformed to the general type. That has likewise been the experience of the present writer, so that the normal mycorrhiza of Rhododendron may be quite briefly described.

There is no dense, external sheath of hyphae, as in most examples of tree mycorrhiza, nor do the roots assume a stunted "coralloid" appearance. Rather are they long and thread-like, and the surface, which is devoid of root-hairs, presents a glistening white appearance to the naked eye. While this surface is freely exposed to view, microscopic examination shows that fairly abundant hyphae do nevertheless ramify over it. They are slender, sparsely septate, and give off branches which penetrate the root. These branches may enter the large epidermal cells directly, through their outer walls, or may force a way between these cells and

enter through their lateral walls. Within each cell they form a more or less closely interwoven coil of very slender hyphae, generally almost filling the cell. Hyphae may be observed to pass from one such cell to another, but the fungus does not seem to spread very far in this way from a point of entry; the majority of the epidermal cells show direct connections between the internal and external hyphae, and have probably been independently infected from the superficial mycelium. While these hyphal complexes in the epidermis are by far the most conspicuous evidence of the presence of the fungus, hyphae may also be found, to a less degree, in the underlying cells of the cortex, which is a very much reduced tissue in the roots of the Ericaceae. The typical, naturally developed mycorrhiza described above is very uniform throughout the major part of the family, and previous descriptions are in agreement on its main features.

Review of Previous Work.

In 1915 Rayner, as a result of her work on Calluna, subsequently extended to Vaccinium, put forward a conception of the mycotrophic relations of the Ericaceae which has been a subject of controversy

ever since. In particular the following three opinions expressed by Rayner have been contradicted and re-affirmed:-

1. The endophytic fungus is not confined to the roots, but penetrates to all parts of the plant.
2. (which depends on 1) The fungus enters the ovary and infects the seeds, at least as far as the testa, so that it is dispersed with them and infects the seedlings on germination; in this way infection is assured, and may be described as "cyclic."
3. Infection by the fungus is necessary for the development of the seedlings. In its absence they cease growth after expanding their cotyledons, and in particular are totally unable to form roots.

The first two may be considered together. The presence of hyphae in the sub-aerial parts of the plant and their dispersal with the seed have been denied by Christoph (1921) and Freisleben (1934). The latter carried out very extensive investigations on many genera and species, especially on Vaccinium, the genus for which Rayner (1929 a) had recorded the

most deep-seated seed infection. It is clear, therefore, that there has been a marked divergence of opinion as to the facts of the case; it is not simply a question of discrepancy in the results reported for different genera. As against these denials, a single apparent confirmation is afforded by the papers of Addoms and Mounce (1931,2) working in America on Vaccinium macrocarpon. The present writer must agree with Freisleben (1934) that it is doubtful whether the structures figured do, in fact, represent hyphae. In any case, they are certainly quite different from the inconspicuous shoot infection described by Rayner, and cannot therefore be regarded as a confirmation of her work.

The third controversial tenet, that of "obligate symbiosis", or the inability of the seedlings to form roots in the absence of the fungus, has been contradicted particularly by Knudson (1929), who grew pure-culture seedlings of Calluna on various agar media, and published photographs of such seedlings with well-developed root-systems. The burden of Rayner's reply (1929 b) was that such seedlings probably contained the endophyte which, being very scanty and attenuated under such conditions, had been overlooked. Knudson (1933) met the various criticisms of his previous work, and considerably strengthened his claim that Calluna seedlings can

develop and form roots under sterile conditions.

An interesting series of experiments carried out by Freisleben (1934-5) led him to the conclusion that Ericaceous seedlings fail to form roots in pure culture on ordinary agar media, and also on a medium of peat and sand used by him, so that up to this point he was in agreement with Rayner, but that their further development and the formation of roots can be promoted by any fungus that does not behave as a parasite, so that this favourable influence is not a specific attribute of the endophyte. For example, contamination of the substrate with Penicillium and other common saprophytes was sufficient to ensure the successful development of the seedlings, although the hyphae of these fungi did not penetrate the roots. This is quite a different result from that of Knudson, whose seedlings formed roots in the absence of any micro-organisms whatever.

Further light is shed on these questions by the experiments of Molliard (1934), who devised an ingenious medium consisting of cotton wool soaked with an aqueous extract of peaty soil, the whole being sterilized. In this way the seedlings were supplied with a soil solution suitable for their growth, while the cotton wool was easily penetrated

by their roots, thus avoiding a very probable disadvantage of agar or of sterilized peat, which forms a densely coagulated mass. On this substratum development and root-formation were successful, so long as moisture was adequate, thus confirming the opinion of Knudson, but in the higher and drier parts of the same tubes the seedlings failed to form roots, and displayed similar symptoms of arrested development to those recorded in Rayner's pure cultures. This strongly suggests that such cases of inhibition are to be attributed to cultural conditions unfavourable for growth, and not to the absence of the endophyte or even of fungi generally. It is worthy of mention, in passing, that McLennan (1935) reached similar conclusions in the case of Epacris impressa, a representative of the Epacridaceae, which take the place of Ericaceae in Australia, and normally possess a mycorrhiza of the same type.

The object of the present work has been to apply similar enquiries to Rhododendron, and to offer answers, in the case of this genus, to these controversial questions in the mycotrophy of the Ericaceae.

Materials and Methods.

Some of the methods used are necessarily incorporated in the accounts of the particular experiments to which they apply. It will be convenient, however, to group here a few routine methods which were used repeatedly, and to which reference will then be made briefly in the later descriptions.

Aseptic extraction of seeds. In investigating the question of seed infection within the parent ovary, it is of prime importance to obtain seeds from such ovaries without exposing them to any subsequent contamination, and without disinfection or other treatment which might destroy any fungus already present. This is done by superficially disinfecting the capsules, dissecting them, and removing the seeds with aseptic precautions to a sterile culture vessel of some kind. The capsules must be carefully examined under a lens to ensure that no slight dehiscence has occurred, which might have afforded entry to micro-organisms.

Several methods of sterilizing the surface of the capsule were tried. The easiest and most rapid consisted of immersing the capsule for a moment

in methylated spirit, which was then set alight; with the relatively large and thick-walled capsules of many *Rhododendrons* this can be done without injury to the seeds, but it was found less satisfactory in eliminating contamination than those methods in which mercuric chloride is the sterilizing agent.

One such method is that described by Norton and Chen (1920) for the superficial sterilization of seeds. The seeds, or in this case capsules, are first thoroughly soaked in water (10 - 12 hours). The object of this is to protect the internal tissues by preventing excessive penetration by the sterilizer, which might occur if the seeds or capsules were dry. They are then shaken for 3-5 minutes in a sterilized test-tube with alcoholic corrosive sublimate (2gms. to 1000 c.c. of 50% alcohol). This is followed by rinsing in 93% alcohol, and in three changes of sterile water. This method was found to be satisfactory, but not necessary, for the present purpose.

In most cases the capsules were simply immersed in mercuric chloride solution, in which they were vigorously shaken to remove air bubbles and ensure thorough wetting, and then rinsed in three changes of sterile water. Concentrations from 0.1% to 1.0% of mercuric chloride were used, and it was

found that in the case of Rhododendron capsules there is a large degree of latitude, and sterilization may be made amply adequate without danger to the seeds, as the interior of the capsule is well protected by the hard inner pericarp.

After any such treatment the capsule was quickly dissected with flamed instruments, and the seeds removed with a flamed inoculating loop to the sterilized medium on which they were to germinate. In most cases this was 2.5% malt agar, which readily revealed the occasional contaminations which, not unnaturally, occurred. In some cases, seeds extracted in this manner were sown on other media, to which reference will be made in the appropriate parts of the text.

Disinfection of seeds. To study the growth of "pure culture" seedlings, i.e. seedlings grown in the absence of the endophyte as well as of other micro-organisms, without prejudging the question of seed infection within the parent ovary, it is necessary to subject seeds to a disinfecting or sterilizing process to kill any fungus that may be present on or in them. Such treatment was also necessary in all cases where the seeds had not been aseptically extracted from the ovary - for instance, where they had been collected on dehiscence of the capsules and stored in seed-packets.

Immersion of seeds in 1% mercuric chloride for three minutes, the treatment used by Rayner (1915, 1922) for Calluna, was sufficient to kill the Rhododendron seeds; Rayner states that the margin of safety with this method is a very narrow one. More satisfactory results were obtained in the present work by using a 0.1% solution of mercuric chloride; immersion in this solution for 3-6 minutes, followed by rinsing in three changes of sterile water, was sufficient to sterilize the seeds without preventing germination.

It has been found, however, in common with the experience of most other workers in this field, that calcium hypochlorite is a more suitable sterilizing agent for regular use than mercuric chloride. It is completely effective, but free from the danger of killing the seeds, and the absence of any necessity of rinsing reduces the risk of contamination after sterilization. In some of the earlier experiments controlled concentrations of hypochlorite solution were used, but this was found to be unnecessary as a saturated solution was perfectly safe. The solution was always prepared fresh as required by shaking distilled water in a test-tube with an excess of calcium hypochlorite for at least five minutes, and filtering.

As Rhododendron seeds tend to float on the sterilizing solution, and so remain dry on their upper surface, they were first of all soaked in water, in which their buoyancy was overcome by shaking, centrifuging or air-pumping, or by a combination of these methods. This is most essential where it is required that the sterilizer should act for a stated short time, as when using mercuric chloride, but even with the greater latitude permitted by calcium hypochlorite it is desirable, as ensuring that no part of the seed is protected by air bubbles from the action of the sterilizer.

Seeds soaked in this way were then covered with a saturated solution of calcium hypochlorite, prepared as described above, for widely varying periods of time, and transferred directly, without any rinsing, to the medium on which they were to germinate, generally malt agar, the transference being carried out with a flamed inoculating loop. A little of the hypochlorite solution is, of course, transferred with the seeds, and indeed they are permeated with it. This greatly assists in the preservation of sterility, the chlorine being gradually dissipated afterwards. It follows that the exact duration of immersion in the sterilizing solution before transference can have but little

significance, as the seed is still subject to the action of the sterilizer after transference, and this influence ceases gradually, not at a precise instant. It is not therefore surprising to find that immersion for so short a time as 4 minutes before transference was sufficient to ensure sterility, while periods up to 80 minutes were used without any sign of harm to the seeds or the resulting seedlings. After these preliminary trials the immersion was generally kept between half-an-hour and one hour. Within half-an-hour the seeds had been thoroughly permeated by the sterilizing solution; they had been bleached almost pure white and were semi-transparent. Thus any internal fungus was certainly fully exposed to the action of a sterilizing solution which proved most effective in preventing any fungal growth on the malt agar slopes to which the majority of such seeds were first transferred.

This method proved quite as effective as mercuric chloride treatment in the production of pure culture seedlings; it was much more effective than the latter agent in preserving the purity of the cultures, several hundreds of seeds being germinated on dozens of malt agar slopes without any contaminations occurring; it was much less liable than mercuric chloride to damage the embryo. But

still another advantage of calcium hypochlorite falls to be recorded; it had a most marked beneficial effect on the germination of the seeds, resulting both in acceleration and in an increased percentage of germination. The latter effect is illustrated by the results of an experiment in which a large number of seeds of Rhododendron decorum treated with calcium hypochlorite for about half-an-hour, and a comparable batch which had simply been soaked and washed in sterile water were germinated on filter paper moistened with sterile water. The cultures containing unsterilized seeds became contaminated, but the development of the contaminant fungi on the filter paper was sufficiently slight to permit of the following count being made after 11 weeks. As no new seedlings had appeared for several weeks prior to this date it seems improbable that the remaining seeds would have germinated at any later date.

	Number of seeds sown on Sept. 20, 1935	Results on Dec. 8, 1935		Percentage germination
		Germinated	Not germinated	
Unsterilized seeds	71	46	25	65%
Seeds sterilized with CaClOCl	84	78	6	93%

Freisleben (1934) recorded a similar effect of calcium hypochlorite on the germination of Vaccinium seeds, though he did not observe it in the case of other genera of the Ericaceae.

Media. The majority of the seeds used for pure culture experiments after disinfection, as well as of those aseptically extracted from the capsules, were germinated on 2.5% malt extract agar, which is a very favourable medium for the growth of most fungi, and a useful one for checking sterility. It was used also for verifying the sterility of those media, such as sand, in which fungi might readily grow without becoming apparent. A little of the sand, or other friable substratum, was transferred with a flamed inoculating loop to malt agar slopes, on which, if a fungus were present, it developed freely.

For continued growth of some seedlings the standard malt extract agar was diluted with approximately its own volume of sterile water, and again sterilized. This medium, in which the concentration of malt extract was approximately 1.25%, was allowed to set with the tubes vertical, so that a deep column of agar with the minimum surface was obtained. Such cultures did not readily dry out, and

the seedlings grew much more successfully in this diluted agar than in the original 2.5% medium.

Several agar media were used in a few cases each, but the only other which was used in considerable quantity was Knudson's Solution B. The formula is as follows (Knudson 1933, p 118) :-

$\text{Ca}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	1.0	gram.
$(\text{NH}_4)_2\text{SO}_4$	0.5	"
KH_2PO_4	0.25	"
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25	"
FePO_4	0.002	"
Agar	17.50	"
Distilled water	1	litre.

A medium such as this aims at supplying a full nutrient solution of salts suitable for the growth of green plants, instead of the organic nutriment required in media intended for the culture of micro-organisms.

All these media are subject to the disadvantage that the slender roots of Ericaceous seedlings do not readily penetrate solidified agar. They proved capable of growing through the diluted but still solid malt agar mentioned above, but the rather unfavourable physical character of such

substrata was emphasized by the frequency with which roots remained above the surface of the medium, sometimes becoming arched in their growth as though prevented by the smooth, unbroken surface from extending apically in the normal manner. To obviate this difficulty a number of seedlings were grown in clean sand irrigated with Knudson's Solution B, without agar. The sand was distributed in conical flasks, and after the solution had been added they were plugged with cotton wool and autoclaved. Such a medium offered good physical conditions for rooting, combined with the suitable chemical composition of the culture solution.

Somewhat similar in principle is the cotton wool medium devised by Molliard (1934), briefly referred to in the survey of previous work. A pad of absorbent cotton wool was arranged on one side of a large test-tube. The liquid phase in this case was an aqueous extract of a peaty potting soil suitable for the growth of Rhododendrons. Equal volumes of soil and distilled water were mixed and allowed to stand for three days; then the liquid was decanted, filtered and distributed in the tubes so that the cotton wool was saturated, and a reservoir of free liquid remained in the bottom of the tube. The tubes were then plugged and autoclaved.

Finally, use was made of the peat-sand mixture described by Freisleben (1933-4-5). A mixture of powdered peat and clean sand was distributed to a depth of about 2 cm. in small Erlenmeyer flasks, mostly of 125 c.c. capacity, and rather oversaturated with distilled water. The flasks were then plugged and sterilized by steaming at 100°C for 45 minutes on each of three consecutive days. The peat swelled up and absorbed the excess water, once experience had shown the correct proportions to use, so that a thoroughly moist but porous medium resulted.

Staining. The hyphae of the endophyte are most readily observed by the use of some form of the Cotton Blue staining method; this has been the experience also of previous workers in this field. The hyphae in infected roots could mostly be identified without staining, but in all cases where their presence was in doubt the roots or other members were stained with Cotton Blue. Thus it is to be understood that whenever a plant or member is recorded as free from infection this result is based on careful examination after staining in Cotton Blue, as well as, in most cases, a preliminary examination in the unstained condition. Best results were

obtained by immersing the member in lactic acid or lactophenol until thoroughly cleared, then over-staining in a concentrated solution of Cotton Blue in lactic acid and differentiating to the required degree in lactophenol, in which examination was carried out. Preparations permanently mounted in Canada Balsam were never so satisfactory as those mounted in lactic acid or lactophenol.

The Question of Shoot and Seed Infection.

In all normally grown Rhododendrons examined, both adult plants and seedlings which have been cultivated in potting soil during these experiments, the hyphal complexes in the root cells were easily recognized without staining, but staining in Cotton Blue made the details of infection still more evident. Small seedlings were frequently stained and mounted entire in lactic acid or lactophenol; examined in this way the hyphae in the root were extremely obvious, yet none could at any time be recognized in the hypocotyl or higher parts of the young shoot. In the case of adult plants too, sections were cut from stems, leaves; pedicels, ovaries and ripening capsules, while ripe and unripe seeds were sectioned and also stained and mounted entire. But though the

hyphae in the roots could always be seen with ease, consistently negative results were obtained by detailed examination of the other parts.

An attempt was made to isolate in culture any fungus that might be present in the tissues of the shoot, despite the above negative results. The material was obtained from a pot of Rhododendron ambiguum seedlings which had been grown in ordinary potting soil to study the course of normal infection. This isolation experiment was carried out in April, 1936, when the seedlings were almost two years old. The roots showed copious and typical mycorrhiza, and this was verified in the case of the seedlings raised for use. Small pieces of the various shoot members were thoroughly washed in water and then superficially sterilized, some in 1% mercuric chloride for $1\frac{1}{2}$ - $2\frac{1}{2}$ minutes, others in calcium hypochlorite for 15 minutes. In both cases the pieces were then thoroughly rinsed in sterile water and quickly cut up with flamed instruments. In the case of cylindrical parts - stem, petioles and leaf midrib - both ends were cut off, and a thin layer of tissue removed from the surface, while pieces of leaf lamina were trimmed all round, the object being to expose fresh surfaces where living hyphae might be present. The pieces were then transferred to malt

and oat agar media, but no fungus grew from any of them. Such an experiment is not in itself sufficient proof of the absence of living hyphae from these parts of the plant, for it is notorious that attempts to isolate Ericaceous endophytes even from roots known to be heavily infected have only succeeded in a very few cases. It is also possible that any internal hyphae had been killed by the sterilizers, but it seems improbable that these had penetrated throughout the tissues before they were removed with sterile water. It would be inadmissible to draw a conclusion from this experiment alone, but it is in accord with that obtained by microscopic examination, namely, that the endophyte of Rhododendron is confined to the roots.

Evidence on the question of seed infection must be sought by germinating seeds aseptically removed from the capsules by the method already described. Applying this method to Calluna and Vaccinium, Rayner (1915, 1929 a) reported that fine hyphae grew out from the testa and infected the primary root. No such hyphae have been observed in the present work on Rhododendron. Aseptically extracted seeds were germinated on filter paper moistened with sterile water, and examined both

stained and unstained at various stages of growth, without revealing any sign of the presence of hyphae, nor did any appear when such seeds were germinated on malt agar. It seems, therefore, that the endophyte is not seed-borne, but must infect seedlings afresh from the soil under normal conditions.

The Course of Normal Infection in Soil.

If infection normally takes place from the soil after germination, it becomes a matter of some interest to follow the course of this infection, and to find what relation, if any, it bears to root development. Accordingly, in the early summer of 1934, a pot of ordinary potting soil was sown with seeds of Rhododendron ambiguum (1933 crop) from a seed-packet. Neither soil nor seed was subjected to any process of sterilization; the pot was left in the seed-pit and its treatment was similar in all respects to that of other pots of Rhododendron seedlings being grown in the course of regular horticultural practice. At intervals from germination onwards typical seedlings were removed and examined for the beginnings of infection. The seedlings were washed thoroughly and mounted entire in water, then stained with Cotton Blue and the examination continued, still

keeping the seedlings entire. The results of examination at various dates are shown in tabular form below.

Infection of R. ambiguum in potting soil, 1934.

May 31	June 21	June 29	July 16	Aug. 15	Sept. 28	Oct. 26
seed sown	—	—	—	+	+ —	+ +

As in all such pot cultures the seedlings showed considerable differences inter se in their respective growth rates. On September 28, more than a month after mycorrhiza had first been recognized, 4 seedlings were selected, ranging from one of the largest in the pot to one of the smallest. The root-systems of the two largest were copiously branched, and normal mycorrhiza was observed. The root-system of the third seedling was elongated, but rather sparingly branched, and no infection was present. The smallest seedling had a proportionately small root-system, though growth and branching had taken place; here again the cells were quite uninfected. Infection, then, had not been by any means simultaneous throughout the pot; large seedlings seemed to be infected first and, if there is a causal

connection between the two, then the size, or stage of development, must have conditioned infection, not vice versa, for the individual differences in development amongst the seedlings were present before infection could be recognized in any of them. On October 26, 5 seedlings were similarly selected, ranging from one of the largest to one of the smallest, but in this case mycorrhiza had been formed even by the smallest seedling examined, though infected cells were still much fewer than in the larger seedlings.

The most interesting, and rather unexpected result of this investigation is the complete absence of infection for more than six weeks after sowing. By June 21 root growth was visible; by July 16 several of the seedlings examined had well-developed, branched systems of slender white roots, and had produced their first epicotylar leaf, but neither in root nor shoot were any internal hyphae to be seen. The seedlings examined on August 15 and later dates revealed the early stages of mycorrhiza development; Infected cells appeared sporadically in the roots, sometimes singly, sometimes in little groups. Several such infected regions might occur along a single root, separated by regions where the cells were still uninfected, showing that infection was taking place from the soil more or less simultaneously

at many points and was not spreading from a single centre, nor from main root to laterals as these emerged. It was noted here, as in the roots of older plants, and as mentioned in the earlier description, that almost every infected epidermal cell showed an independent connection with the superficial mycelium.

In all cases, in this experiment with R. ambiguum and in pot cultures of several other species including R. decorum and R. ferrugineum, infection was confined to mature, vacuolated cells. Rayner (1915) has described hyphae from the testa of Calluna as infecting the tip of the root, as well as other parts. In all the roots examined in the present investigation, whether of seedlings or adult plants, the meristematic cells were conspicuously free from the fungus; this fact was also observed for Vaccinium by Freisleben (1934), and may be partly responsible for the delay in infection of seedlings in untreated soil. First the hypocotyl elongates and the cotyledons expand. Then there is a slight pause in development - the critical stage at which it has been claimed that uninfected seedlings are permanently arrested. Then root growth begins, and only after it has progressed far enough to leave a zone of mature vacuolated cells behind the meristem can infection take place. So far from infection being a

pre-requisite condition of root-formation, it is actually the other way about; the formation and growth of the roots are pre-requisite conditions for infection.

The Effects of Soil and Seed Sterilization.

The endophytic fungus appears to be of universal occurrence in potting soil, but it seemed worth while to try the effect of its removal by initial sterilization of the soil. Accordingly two small pots of peaty potting soil were autoclaved, and on September 17, 1935, one was sown with seeds of Rhododendron decorum which had been disinfected with calcium hypochlorite, while the other was sown with seeds that had been soaked in water only. On the following day a pot of unsterilized potting soil was sown with seeds of the same species disinfected with calcium hypochlorite. A preliminary trial had shown that, without sterilization of soil or seed, infection followed a similar course in R. decorum to that already described for R. ambiguum, and infected cells had been clearly seen some two months after sowing - in a pot sown on July 5, 1935, mycorrhiza-formation was found to have commenced in seedlings examined on September 12.

The three pots sown on September 17 and 18, as just described, afforded the three possible combinations of soil and seed sterilization, the object being to ascertain whether the occurrence of infection showed any correlation with the treatment of soil or seed. The pots were left in the seed-pit and the experiment continued for a period of 7 months, the photograph (fig. 1) being taken on April 17, 1936, at which date examination yielded the following results:-

Rhododendron decorum in peaty soil.

Result of examination on April 17, 1936,
7 months after sowing.

Soil	Seed	Root formation	Endophytic infection	Progress of seedlings
sterilized	sterilized	+	—	good
sterilized	unsterilized	+	—	good
unsterilized	sterilized	+	+	good

In the unsterilized soil (centre, fig. 1) the roots had developed typical mycorrhiza, just as in normal pot culture. The seedlings had been infected from the soil, and the seed sterilization had made no difference.

In the other two pots moulds grew very freely at first, as always happens when autoclaved soil is exposed to the air; a number of seedlings were overgrown at an early stage, and died. This fact, together with sampling operations, accounts for the relatively smaller numbers of seedlings in these two pots than in the other, as shown in the photograph; such differences in numbers are not significant in the result of the experiment.

When the initial exuberant growth of moulds died down the seedlings in these two pots grew very similarly to those in the third, i.e. in untreated soil. Yet up to 7 months from sowing, as recorded in the table, the roots were still free from endophytic infection, though by this time they were filling the pots and the seedlings had reached the stage of development shown in the photograph. Saprophytic hyphae were, of course, abundant in the soil, but it may be that the endophyte is not readily spread by air, being present in soil only as a vegetative mycelium; at any rate, it had not yet appeared and entered the roots.

In all pots the seedlings are recorded as having made good progress. The left-hand pot in the photograph has a much less dense mass of foliage than

the other two, mainly because of the difference in numbers already mentioned, but comparison of the seedlings themselves gave no valid grounds for discrimination. There were much greater variations in size amongst the seedlings in any one pot than between comparable seedlings in different pots; in all three pots the leaves had a good green colour, and the seedlings appeared extremely vigorous. On the average, the seedlings in the centre pot (untreated soil) were slightly taller than those in the other two; this is not surprising, as autoclaved soil, especially during the early phase of uncontrolled mould growth, is a much less favourable environment than untreated potting soil. The seedlings in the latter had established a lead before a state of relative biological equilibrium was re-established in the autoclaved soil. But this difference was small, and it was apparent that the seedlings in the autoclaved pots had not suffered any significant hindrance to their growth, as compared with those in the other pot.

The results of this pot culture experiment are in agreement with those obtained by Christoph (1921) in a similar experiment with Calluna, which was criticised by Rayner (1922) in view of the

incomplete control of sterility. It is recognized that this experiment has not the same value as those in which sterility is maintained (and which will also be reported), but taken in conjunction with them it does point strongly to two conclusions. Firstly, since the endophyte develops equally well in untreated soil whether the seed has been disinfected or not, and since it failed to appear - over a period of 7 months - in sterilized soil, also irrespective of seed disinfection, it is apparent that infection proceeds from the soil, not from the seed. Secondly, the absence of the endophyte imposes no significant handicap on the growth of the seedlings.

Pure Culture on Agar Media.

The next step (though it had, in fact, been taken before that just reported) is to exclude soil infection by growing seedlings under aseptic conditions. Seeds were aseptically extracted, by the method already described, from the capsules of a considerable number of species, including Rhododendron pholidotum, R. Nobleanum, R. disquamatum, R. ambiguum, R. decorum, R. ferrugineum, and several unnamed garden hybrids. The non-appearance of hyphae in the early stages has already been recorded in the section

dealing with shoot and seed infection. As the standard laboratory agar media did not seem to be very favourable to their continued growth, a number of seedlings were transferred singly to deep tubes of diluted (1.25%) malt agar. Figs. 2-4 are successive dated photographs of one seedling of an unnamed hybrid. The seed was extracted and sown on oat agar on February 16, 1934, and the seedling transferred to 1.25% malt agar on March 19. Several others in this experiment were germinated on the more usual 2.5% malt agar, and transferred to the diluted medium on the same date; their subsequent development was similar to that of the example shown. Fig. 2, taken on May 20, shows this seedling approximately 3 months after sowing and 2 months after its transference. As compared with soil culture, growth had been very slow; the seedling had reached the critical stage of development, and the curled cotyledons and practical absence of roots might suggest that it was incapable of further growth in pure culture. However, this seedling, and others of which it is typical, successfully passed that critical stage, and fig. 3 is a photograph of the same seedling some six weeks later. It had now formed roots and new shoot growth, and had entered on a more vigorous phase of development. On September 21, about 6 months after its transference to this tube, the same seedling had reached the condition

shown in fig. 4, with a good green shoot, and a well-developed, branched root-system. These roots were embedded in malt agar, a medium on which Ericaceous root fungi have been isolated (Freisleben, 1933-34), but during these six months of growth no hyphae had appeared in the medium, and when the seedling was subsequently removed and its roots stained and examined, no fungus was found in them; the same applies to a number of similar seedlings.

Since these plants were raised from seeds which had not been disinfected, but simply aseptically removed from the capsules, they confirm the opinion that the seeds are not infected within the parent ovary, and their subsequent development shows that Rhododendron can form roots in the absence of the endophyte, or of any other micro-organisms, just as Knudson claimed in the case of Calluna.

It has been stated that 2.5% malt agar is not very favourable to the growth of the seedlings, and this is illustrated in fig. 5. The photograph shows two tubes containing seedlings of Rhododendron eritimum, grown from seed which had been disinfected with calcium hypochlorite; no micro-organisms appeared. These two tubes, and a number of others which yielded similar results, were sown on July 20, 1934, and the

photograph was taken on December 19, when the seedlings were 5 months old. The tube on the left contained 2.5% malt agar, that on the right Knudson's Solution B agar, and the better development of roots on the latter is very obvious. In the malt agar culture small roots can be seen, particularly on the highest and lowest seedlings in the tube, but though root growth has not been entirely prevented it has evidently been greatly depressed. This result supports the idea that such inhibition of growth is due to unfavourable substrata, and at any rate not to the absence of the endophyte or of any other micro-organisms.

Again, fig. 6 shows root formation by Rhododendron decorum on potato agar. The seeds were sterilized with calcium hypochlorite and planted on the agar slopes on September 12, 1935; the photograph was taken on December 16, so that the seedlings were 3 months old. They were still at an early stage in the rather slow development usual in such cultures, and only one root had so far appeared, but it shows the healthy, glistening white appearance of roots produced in pure culture on suitable media.

Pure Culture on Other Media.

Reference has been made in the section on media to the use of sterile sand irrigated with Knudson's Solution B. Aseptically extracted seeds of Rhododendron ambiguum were sown on this medium on December 6, 1935, and the flask left in an unheated greenhouse. Fig. 7 is a photograph of 4 of these seedlings raised on August 6, 1936, 8 months after sowing. They seem surprisingly small, but this is in part explained by the fact that the seeds lay dormant all winter; with rising temperature in early summer they germinated, and the photograph really shows the result of a few months' growth in the summer of 1936. At this stage the seedlings were small but healthy, with leaves of a good green colour. They had developed roots in which careful examination revealed no fungus. The sterility of the medium was simultaneously verified by transferring samples to 2.5% malt agar, on which no growth occurred.

These seedlings, being grown from aseptically extracted seed, without sterilization, confirm the opinion that infection is not seed-borne, as well as the ability of the seedlings to form roots and establish themselves in pure culture.

A similar experiment was carried out with Rhododendron decorum, except that the seeds in this case were obtained from a seed-packet, and were therefore disinfected with calcium hypochlorite and sown, in the first instance, on 2.5% malt agar. This was done on November 27, 1935, and the seeds were left for a week on the agar plate at room temperature to confirm the absence of any contaminants. On December 5 the seeds, which had remained quite clean, were transferred to a flask of sand and Knudson's Solution B, which was left in the unheated greenhouse till August 6, 1936, when the four seedlings shown in fig. 8 were raised from it for examination. These seedlings were larger than those of R. ambiguum raised from sand at the same time. This is in part a specific difference, R. decorum being a more free-growing species than R. ambiguum; in addition the seeds of R. decorum did not remain dormant throughout the winter, though their growth during this period was slow. Their earlier germination may have been due to the accelerating effect of calcium hypochlorite.

Here again no fungus could be found in the roots, nor in the sand by transferring samples to malt agar. Since the seed had been disinfected this experiment has no bearing on the question of seed

infection within the ovary, but it confirms again the ability of fungus-free seedlings to establish themselves and form roots.

Rhododendron decorum was also used for an experiment with Molliard's cotton wool medium. The seeds, from a seed-packet, were disinfected with calcium hypochlorite and sown directly on the cotton wool on January 10, 1936. Fig. 9 is a photograph of this tube taken on June 17, some 5 months after sowing, during which time the tube, and similar cultures of R. eritimum, R. ferrugineum and R. Davidsonianum, had been kept at room temperature. Access of light was provided by keeping the tubes near a window, but their position was chosen and varied to prevent much direct sunlight reaching them. Under these conditions the seedlings had formed vigorous shoots of a good green colour, while their roots had freely penetrated the cotton wool. The well-developed roots of one seedling lay mainly between the cotton and the wall of the tube, and could therefore be photographed, as seen in fig. 10, which was taken on August 11, 1936. Two days later (Aug. 13) and thus some seven months after sowing, sample seedlings were removed and their roots, including those seen in fig. 10, were stained and

examined. No infection could be seen. Samples of the cotton wool were transferred to malt agar, and remained sterile.

This, then, is a further example of the successful growth of pure culture seedlings.

When previous workers have recorded similar results, mainly for Calluna and Vaccinium, it has been suggested by Rayner (1922-25-29 b) that under artificial cultural conditions the fungus is present in an extremely attenuated condition, permitting the development of the seedlings, which could not succeed in its total absence, but not forming typical mycorrhiza, and that it has been overlooked. It is extremely unlikely that this can explain all the negative results that have been recorded, but in any case there is now available a controllable medium in which it is known that typical mycorrhiza does form. This is the peat and sand mixture already referred to in the section on media. Freisleben (1933-4-5) used this medium for experimental synthesis of mycorrhiza, by transplanting to it pure culture seedlings of various Ericaceae, including Rhododendron species, and simultaneously inoculating the substratum with the endophytic fungi which he had isolated from Vaccinium roots. He records the fact, borne out by photographs, that

infection by the endophytes in this medium took the form of a copious mycorrhiza-formation, indistinguishable from that occurring in nature. It may therefore be maintained that negative results obtained with this medium are not subject to the criticism that the fungus might be so attenuated as to escape the most careful search.

It may be recorded at once that the results obtained in the present investigation by the use of this peat and sand medium have been in complete agreement with those already discussed. Seedlings can, and do, form roots and grow successfully in the absence of the endophyte, verified by careful examination of the roots, and in the absence of any other fungi, verified by transferring samples of the substratum to malt agar. This is of interest, because Freisleben, using this medium, was disposed to agree with Rayner as to the failure of pure culture seedlings to form roots, though he found that this condition was relieved by any non-parasitic fungi, and not only by Ericaceous root fungi. In the present investigation roots have been formed quite successfully when no fungi were present at all.

It is quite true that under some conditions fungi appear to be beneficial to the seedlings, even

though they are not indispensable, and in this medium they certainly do no harm. The largest seedling produced in any of these cultures was one of Rhododendron decorum which was grown alone in a 250 c.c. flask, in which the peat-sand substratum had been contaminated with Penicillium. The seed was sterilized with calcium hypochlorite and planted on potato agar on September 12, 1935. On January 17, 1936, 4 months after sowing, this particular seedling was transferred to the flask culture, which became contaminated, as occasionally happened while introducing seedlings, with Penicillium. Such flasks were not discarded, but kept for observation of any effect which the fungus might produce on the growth of the seedlings, Penicillium being one of the fungi recorded by Freisleben (1934) as relieving the inhibition of growth which he found in pure culture seedlings.

At the time of its transference this seedling had but one short lateral root, and no visible epicotylar growth. Fig. 11 is a photograph of the same seedling, still in its flask, some 7 months later, namely on August 5, 1936. On August 11 it was removed from the flask and found to have an extremely well-developed root-system (fig. 12); the leaves were of a fine dark green. Penicillium was known to be

present, and was recovered in apparently pure culture on transference of samples of the medium to malt agar. On microscopic examination of roots the hyphae and spores of the saprophyte were found on the surface, but the root cells were clean and empty, without any sign of mycorrhiza-formation.

This seedling, then, had been conspicuously successful in the absence of the endophyte, but in the presence of Penicillium.

But in this medium it does not appear that seedlings grown in the presence of fungi have any real advantage over pure culture seedlings. On January 17, 1936, when the seedling described above was transferred from potato agar, two other cultures were set up in which seeds of Rhododendron decorum were sown directly on peat-sand mixture in two 250 c.c. flasks. In one were sown about a dozen seeds which had been soaked in water, sterilized by immersion in calcium hypochlorite for about 40 minutes, and transferred directly without rinsing; in the other were sown a similar number of seeds which had been soaked in water, and then rinsed in several changes of sterile water, without sterilization. These seeds were taken from a seed-packet, and the washing of the

second set proved insufficient to prevent contamination. Penicillium developed in the substratum, and was recovered in apparently pure culture when the flasks were opened on June 24, 1936, i.e. 5 months after sowing. It is, however, possible that other saprophytic organisms were present, but were masked by the Penicillium.

On the same date (June 24) sample seedlings were raised and photographed (fig. 13). The central seedling is an average representative of the flask containing Penicillium; the other two are a large and a small seedling from the pure culture flask, in which the average size did not differ appreciably from that in the contaminated flask. After photographing, these seedlings were stained and carefully examined. The roots of that from the infected flask bore hyphae superficially, but not internally; no fungus could be found on or in the roots from the other flask, nor was any revealed by transferring samples of the substrate to malt agar.

Now the seeds sown in the infected flask had not been sterilized, so that mycorrhiza should have developed if the endophyte were seed-borne. Also comparison of the seedlings shown in fig. 13, and the recorded similarity in the average development

of these two sets of seedlings of equal age, show that in this medium the presence of fungi of any kind is not only unnecessary; it does not even confer any apparent advantage. And this experiment was carried out with a medium in which the presence of the endophyte results in typical mycorrhiza-formation, and is therefore not liable to be overlooked.

Conclusions.

The three controversial aspects of Ericaceous mycotrophy mentioned at the beginning can now be answered in the case of Rhododendron:-

1. The endophyte is confined to the roots, and does not penetrate the sub-aerial parts of the plant. This has been determined mainly by microscopic examination, supported by the failure of attempted isolations, and by the absence of seed infection (see below).
2. As implied by 1, the endophyte is not seed-borne. This has been shown by the fact that the endophyte did not appear in any of the cultures from aseptically extracted seed, nor in any culture where the substratum itself had

been sterilized whilst the seed had not, and this remained true even when the substratum was one in which the endophyte is known to form typical mycorrhiza if present.

3. Infection by the endophyte is not an obligate condition of development of the higher plant, which in fact can form roots and establish itself in the total absence of any micro-organisms. This is demonstrated by the success recorded for pure culture seedlings on a considerable variety of substrata.

Discussion.

What, then, is the significance of the mycotrophic habit in these and related plants? In Rhododendron, if the above conclusions are sound, it is plainly not of the vital character envisaged by Rayner for the Ericaceae in general, and for Calluna in particular.

A recent paper by Burges (1936) supports the belief that mycorrhizal fungi are essentially parasites, and that no mutualistic relation is involved. In the same paper it is suggested that

soil fungi generally, including perhaps the mycorrhizal fungi, break down organic matter to a level at which some of it is water-soluble, and can be absorbed directly by the roots of some of the higher plants.

The conclusion that mycorrhizal fungi are essentially parasites has been reached by other workers in this field, and it is the one which seems to accord with the results now presented. This is not inconsistent with the accepted fact that the presence of fungi is sometimes beneficial to the higher plant. It is known that plants do not generally grow well in completely sterilized soil, and that applies to all the higher plants, not only to those which form mycorrhiza. The metabolism of micro-organisms plays an important part in soil fertility. If this role, in the case of soil fungi, consists of breaking down organic matter to a level at which some of it can be absorbed, in accordance with the suggestion just quoted, then it could be understood how, on some substrata, if the available nutriment is scanty, the presence of an endophytic or other fungus might permit the development of the green plant by making available food materials which could not otherwise be used, and this might apply to some of the substrata that have been used in

mycorrhiza research, especially agar media. On the other hand, if the substratum already contains adequate nutriment in an available form, as where soil water or a suitable solution of salts has been incorporated in the medium, then it would be expected that the presence of a fungus would be quite unnecessary, and would even confer no advantage, as found in the present investigation.

Poor growth in some media must be due to a positively inimical factor, and not merely to lack of available food material; this applies, for example, to 2.5% malt agar, where the inhibitory effect was considerably relieved by dilution of the medium, but the availability of nutriment may be the key to understanding of the otherwise anomalous records (especially by Rayner, 1915 and other papers) of improved growth on one and the same medium on the introduction of a fungus.

It appears, then, that the root fungi have no specific importance to the higher plant. The feature which distinguishes them from other soil fungi is simply a certain mild degree of parasitic activity towards these plants, whereby they are able to invade mature root cells and, though held in check by the host plant from further spread or damage, to survive

as parasites within those cells, where they produce the coiled masses of hyphae which are the most characteristic feature of this type of mycorrhiza.

Summary.

In nature the roots of Rhododendron species regularly contain an endophytic fungus; the mycorrhiza is of the same type as in Calluna, Vaccinium and the majority of the Ericaceae.

There are three questions in particular in the mycotrophy of the Ericaceae on which opinion has been divided. These questions are investigated by microscopic and cultural methods, and evidence is presented to justify the following answers in the case of Rhododendron:-

1. The endophyte is confined to the roots, and does not penetrate the sub-aerial parts of the plant.
2. As implied by 1, the endophyte is not seed-borne.
3. Infection by the endophyte is not an obligate condition of development of the higher plant,

which in fact can form roots and establish itself in the total absence of any micro-organisms.

The significance of the mycotrophic habit is briefly considered in the light of these results, which lead the writer to support the view that the endophyte is a relatively feeble parasite, and is of no specific importance to the higher plant.



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Explanation of Figures.

1. Rhododendron decorum, 7 months old.
left: sterilized seed; sterilized soil.
centre: sterilized seed; unsterilized soil.
right: unsterilized seed; sterilized soil.

2. Rhododendron hybrid No 1, growing in 1.25% malt agar;
sown, February 16, 1934; transferred to tube
shown, March 19, 1934.
Photograph taken, May 20, 1934.

3. The same seedling; July 3, 1934.

4. The same seedling; September 21, 1934.

5. R. eritimum, 5 months old.
left: on 2.5% malt agar.
right: on Knudson's Solution B agar.

6. R. decorum, 3 months old.
Root-formation on potato agar. (r=root).

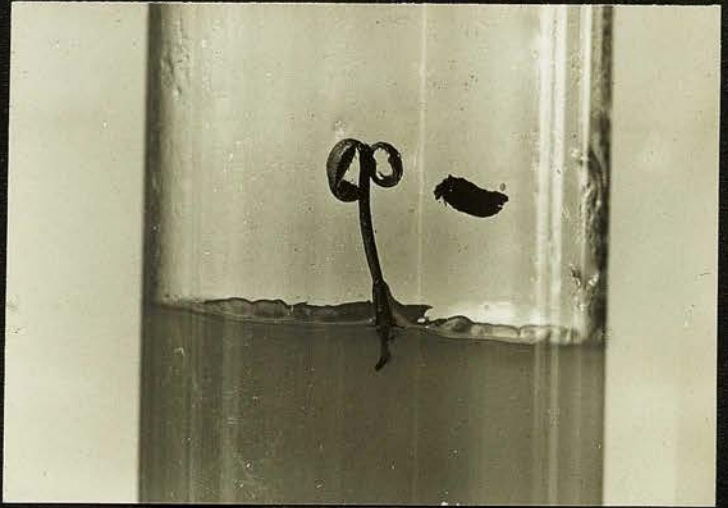
7. R. ambiguum, 8 months after sowing.
Seedlings rooted in aseptic sand culture with
Knudson's Solution B.

8. R. decorum, 8 months after sowing.
Seedlings rooted in aseptic sand culture with
Knudson's Solution B.

9. R. decorum, 5 months old.
Aseptic culture on Molliard's cotton wool medium.
 10. Closer view of same tube 2 months later, showing roots.
 11. R. decorum, 11 months old, in peat and sand infected with Penicillium.
 12. Same seedling removed from flask to show root-system.
 13. R. decorum, 5 months old, grown in peat and sand.
centre: from culture infected with Penicillium.
left and right: from aseptic culture.
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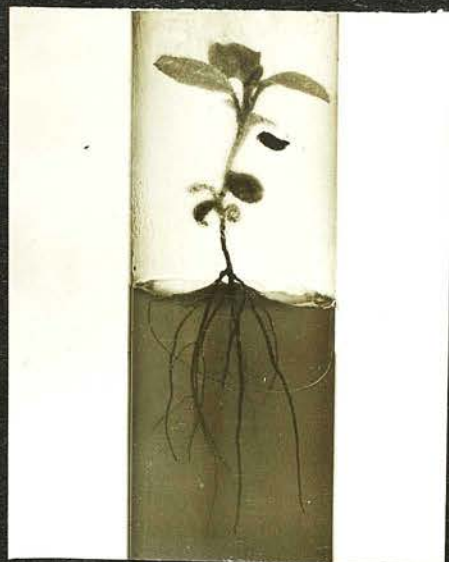
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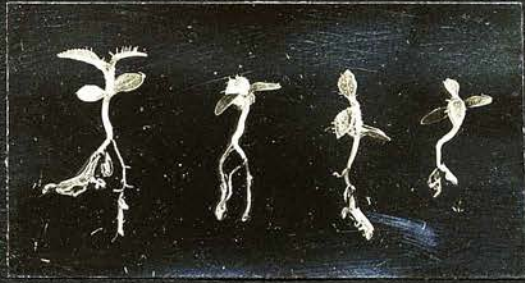
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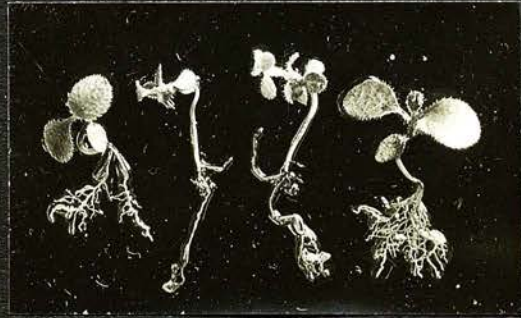
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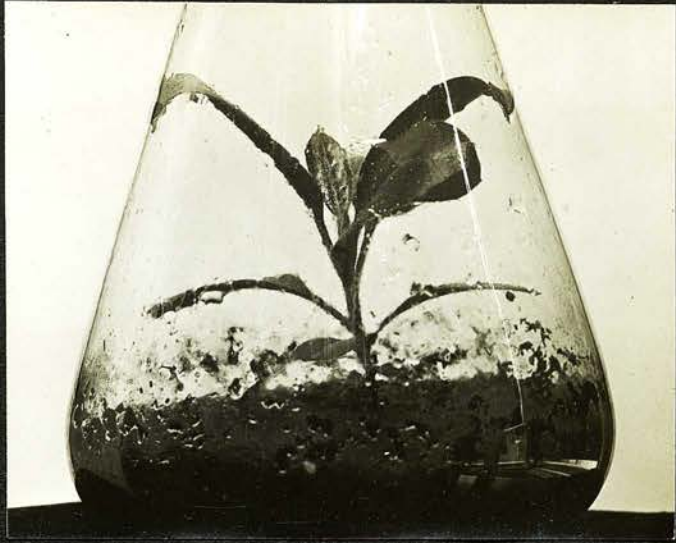
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II.

TREMELLA TRANSLUCENS

A NEW SPECIES ON DEAD PINE NEEDLES.

The fungus forming the subject of this note occurs in comparative abundance along with a number of other fungi on dead leaves of Pinus sylvestris in a wood near Peebles, in southern Scotland. It is most readily recognized by soaking the needles in water, when the fructifications appear as hyaline, greyish-white globules, 0.4 - 3 mm. in diameter, the majority measuring 1 - 2 mm. (fig. 1).

On drying, the fructification contracts to such a degree that it is recognized only by careful examination under a lens. It also assumes a dark colour, generally very dark brown or almost black. By reason of its inconspicuous appearance when dry it is probable that the fungus frequently escapes notice,

and it may well prove to be comparatively widespread.

A transverse section of the leaf reveals abundant hyphae throughout the mesophyll, becoming densely aggregated under the surface where the fructification breaks through. There is a short and relatively thick stalk-like portion, at first fairly distinct, but later becoming merged in the expanding fructification, which is then sessile and centrally attached. The leaf is blackened over a small area surrounding the insertion of each fructification.

The hyphae in the basal region of the fructification are closely arranged and more or less parallel, with comparatively little gelatinous matrix. As they diverge the amount of jelly increases until the hymenium is reached, where the basidia, in consequence of their greater diameter, form a dense layer; outside this again is a layer of jelly through which the epibasidia eventually grow. Thus, as would be expected, the texture of the fructification is denser towards the centre, softer and more gelatinous farther out, but there is no suggestion of the firm, hard nucleus characteristic of the old genus Naematelia of Fries. (fig. 2). The basidia are embedded near the surface of the jelly, and occupy the whole superficial area of the fructification; there is

no differentiation into fertile and sterile surfaces.

The hyphae in the fructification have a diameter of 2.8 - 4.0 μ , while individual cells may be further enlarged, forming irregularly dispersed, vesicular swellings. Clamp connections are frequent.

The basidia are spherical or slightly oval, the majority lying within the limits of 10 - 14 μ in length and 9.5 - 13.5 μ in breadth. The length does not usually exceed the breadth by more than 1 μ ; a similar excess of breadth over length is less frequent, but by no means rare. In isolated cases a marked increase in one or more dimensions may occur, one example measuring 23.9 x 14.6 μ . The basidia are of the usual Tremellaceous type, and the four cells frequently separate right to the base at a late stage of development, a feature which has been recorded for other members of this group of fungi. This separation was most noticeable in fructifications that had been kept very moist.

When dry fructifications are brought into the laboratory and soaked in water they are generally found to bear an abundant crop of mature basidia, cruciately divided into four cells, but without epibasidia or spores. If such fructifications are then transferred to damp filter-paper and kept under

cover, further development takes place after a few hours. The epibasidia vary in diameter from 1.25μ to 4.0μ , so that the average is less than that of the normal hyphae, though the maximum is the same for both. A number of still thicker epibasidia have been observed, bearing two sterigmata and spores (fig. 3). The length of the mature epibasidia is so variable that no average figure can usefully be given; measurements as short as 12μ and as long as 100μ have been obtained, the latter under very moist conditions. The epibasidium sometimes projects freely and to a variable distance above the surface of the jelly; at other times it just reaches this surface, immediately above which the spore is consequently borne. The slender, tapering sterigma is usually $2 - 3 \mu$ long, but its basal limit is not sharply defined from the epibasidium.

The basidiospores are oval, $7.1 - 11.8 \mu \times 3.7 - 6.2 \mu$, with an average about $9 \times 5 \mu$. According to the direction of view both sides of the spore may appear convex, or one may be almost straight, but scarcely ever does one side present a concave appearance, which would result in the curved spore shape characteristic of Exidia and certain other genera of the Tremellaceae. The spore bears a small oblique apiculus at its base, immediately adjacent to

the point of attachment to the sterigma (figs. 3 & 4).

The spores germinate readily in the presence of moisture. Three types of germination may be distinguished, though they grade into one another to some extent; all three types were recorded for Tremella species by Brefeld (1888).

- (i) By repetition; the germ-tube rather resembles the sterigma, and bears a secondary spore more or less similar to the original basidiospore in size and shape. This is generally regarded as the normal mode of germination in Tremella. (fig. 6a).
- (ii) By budding; a number of small yeast-like conidia or sporidiola are produced directly from the basidiospore. (fig. 6b).
- (iii) The germ-tube grows out as a simple or branched mycelium from which, sooner or later, yeast-like conidia are irregularly abstracted. (figs. 6c and 7).

In cases (i) and (iii) the germ-tube may arise from any part of the spore, laterally, sub-terminally or terminally from either end. (fig. 5).

The first method, by repetition, was found to be the most frequent among spores germinating on the moist surface of the fructification, and is probably the commonest in nature. The second method, by budding, was also fairly frequent in this situation.

Germination in hanging drops of water generally followed the second method, large numbers of yeast-like conidia being formed; the first and third methods also occurred, but were less frequent. (fig. 6).

Germination in a nutrient solution, such as dilute malt extract, resulted in greater luxuriance of growth, and germination by repetition was not observed (fig. 7). Many spores followed the second method, while many others produced a germ-tube, or more than one, which sometimes formed a freely budding sprout mycelium immediately, and in other cases attained a considerable length and complexity of branching before bearing conidia. The ultimate result was a luxuriant sprout mycelium, from which yeast-like conidia were irregularly abstricted.

This fungus does not seem to conform to any published description. Perhaps the nearest resemblance is offered by Tremella glacialis Bourd. et Galz., in spite of the different type of substratum on

which that species has been recorded, and a difference in habit which might be connected therewith. The present species, however, is somewhat larger, both in macroscopic and microscopic measurements, and particularly in the length of the spores, which have a more elongated oval form than those of T. glacialis. Neuhoﬀ (1931) regards T. glacialis and the very similar or identical T. Grilletii Boud. as species of Exidia, on account of their spore shape. Whelden (1934) disagrees with this, and figures spores of T. Grilletii which certainly suggest that it should be retained in the genus Tremella. However that may be, the present species would appear to be correctly referred to Tremella: the hymenium covers the entire exposed surface of the fructification, leaving no sterile surface, and is perfectly smooth, without the ridges or papillae common in Exidia; the thick-walled, superficial hyphae recorded by Whelden (1934-5) in dry fructifications of Exidia, but not of Tremella, have not been observed in this case; the spores are oval, not curved, and their germination is characteristic of Tremella; the septation of the spore, and the curved sporidiola characteristic of Exidia, do not occur.

Within the genus Tremella, this species finds its place in the section Tuberculiformes (Nanotremella), which have small smooth fructifications without lobes or furrows. Only three such species are listed as European by Neuhoff (1931), two of which are distinguished by their colour, as well as other characters. The third, T. fusispora Bourd. et Galz., is hyaline to milk-white, but is smaller than the present species, and is distinguished particularly by its spores, which are long and fusiform. Tremella translucens is therefore to be regarded as an additional member of this group.

Tremella translucens sp. nov.

Erumpent, 0.4 - 3 mm. when moist, hyaline, greyish white, spherical or slightly flattened, of soft gelatinous consistency; when dry minute, hard, dark brown or black, with a central depression; basidia spherical or slightly oval, 10 - 14 x 9.5 - 13.5 μ ; spores hyaline, oval, with an oblique basal apiculus, 7.1 - 11.8 x 3.7 - 6.2 μ , average 9 x 5 μ .

On dead leaves of Pinus sylvestris.
Peebles, in southern Scotland.

Tremella translucens sp. nov.

Erumpens, uda hyalina, albo cinerea, gelatinosa, subglobosa, 0.4 - 3 mm. lata; sicca minuta, dura, fusca vel atra, centro depressa; basidiis globosis vel late ellipsoideis, 10 - 14 x 9.5 - 13.5 μ ; sporis hyalinis, ellipsoideis, basi oblique apiculatis, 7.1 - 11.8 x 3.7 - 6.2 μ , plerumque 9 x 5 μ .

Hab. in foliis emortuis Fini sylvestris, Peebles, Scotiae australis.

The type material is deposited in the herbarium at the Royal Botanic Garden, Edinburgh, and material has also been sent to the herbaria of the British Museum (Natural History) and the Royal Botanic Gardens, Kew.

The fungus described in this note was first observed by Dr Malcolm Wilson, to whom the writer is indebted for making material available, and for his help and criticism in the preparation of this description.

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 (1934) I Tremella.
 Mycologia xxvi, 5, 415-435.
 (1935) II Exidia.
 Mycologia xxvii, 1, 41-57.

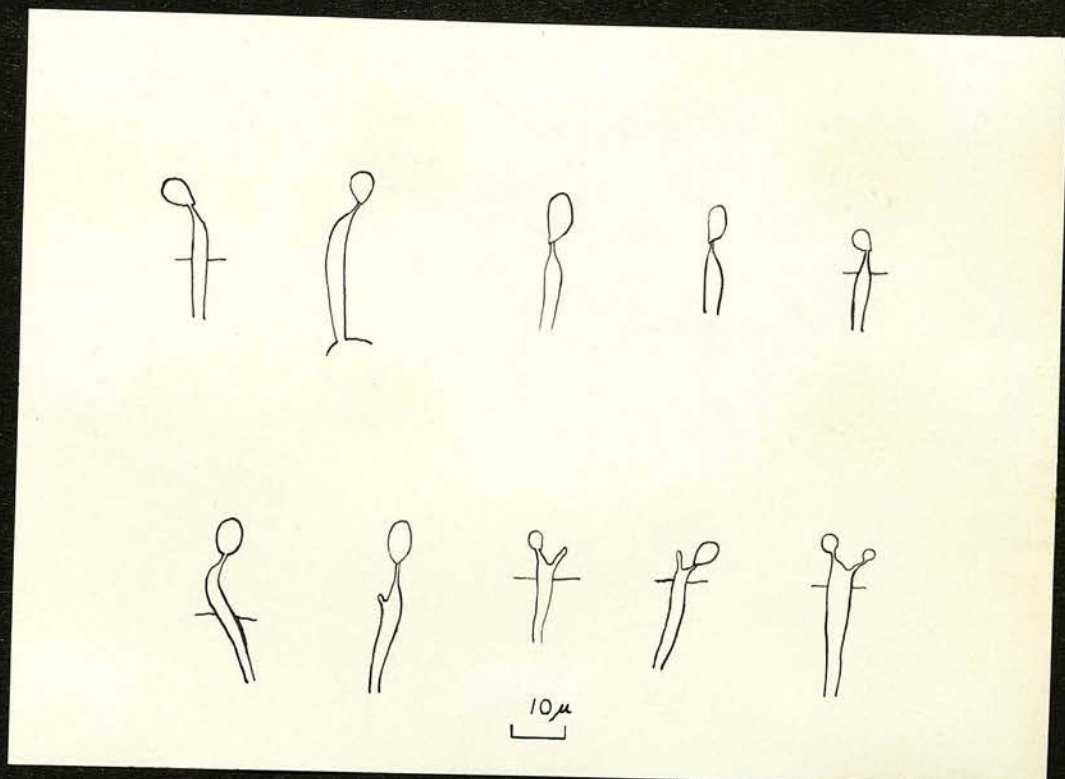
Explanation of Figures.Tremella translucens sp. nov.

1. Fructifications on leaves of Pinus sylvestris, slightly magnified.
2. Thick hand section through a leaf of Pinus sylvestris bearing a fructification, to show the general structure of the latter, x 50. At either side of the fructification some of the small, spherical basidia have been displaced by pressure.

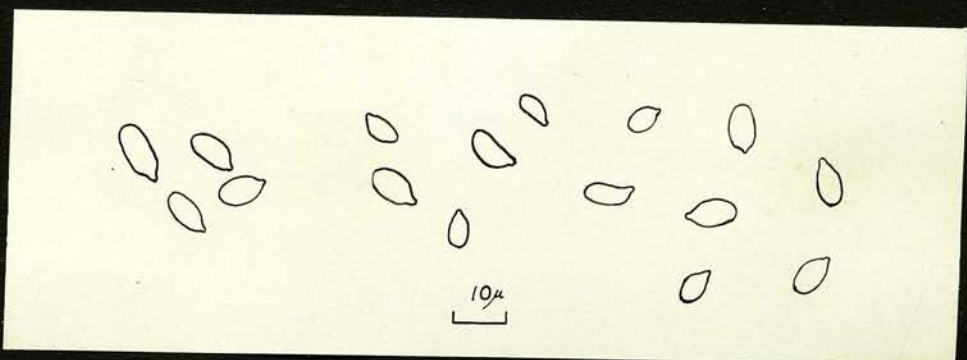
3. Epibasidia with spores. The surface of the jelly, where clearly defined, is indicated by a horizontal line.
 4. Basidiospores.
 5. Basidiospores germinating in water; early stage.
 6. Basidiospores germinating in water,
 - (a) by repetition,
 - (b) by budding,
 - (c) by a simple hypha.
 7. Basidiospores germinating in dilute malt extract solution,
 - (a) after 24 hours,
 - (b) after 45 hours.
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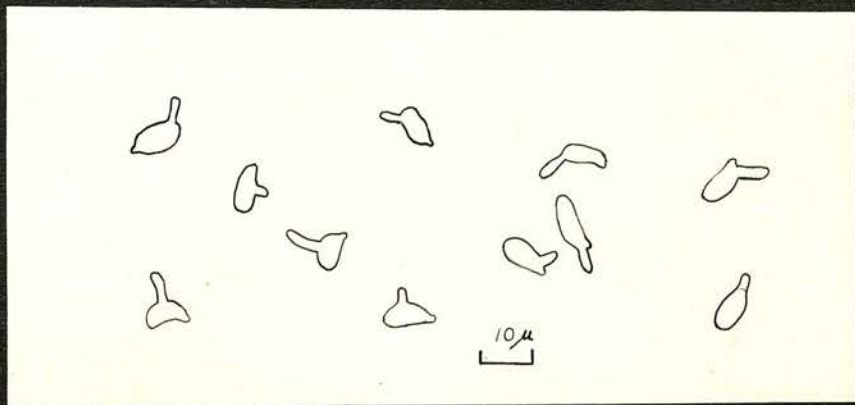
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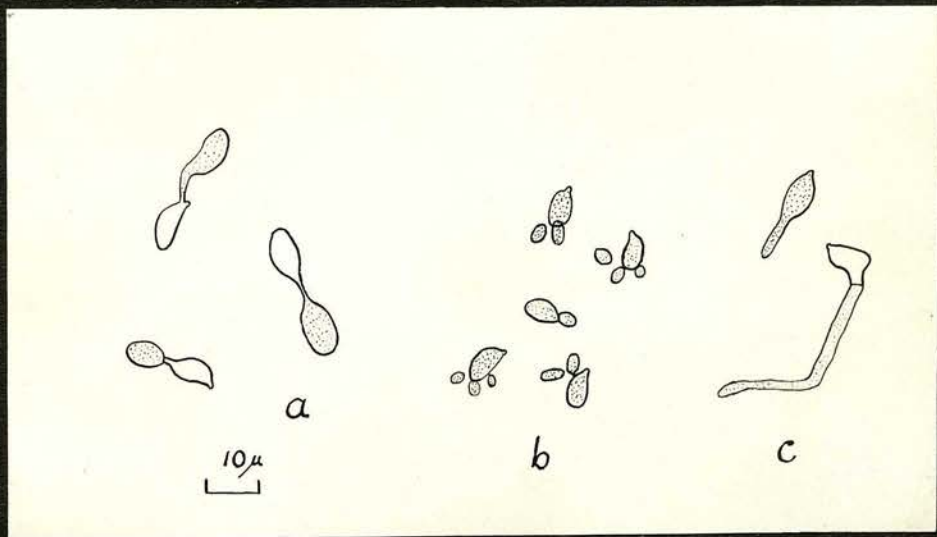
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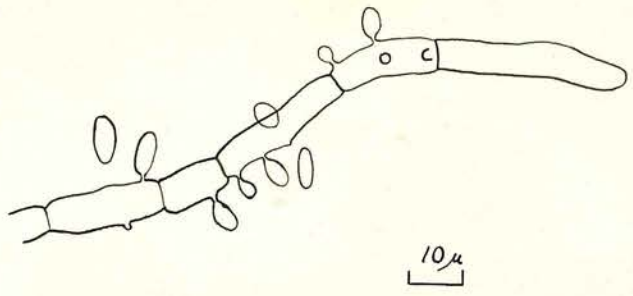
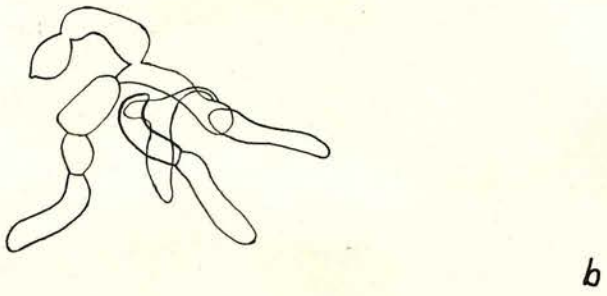
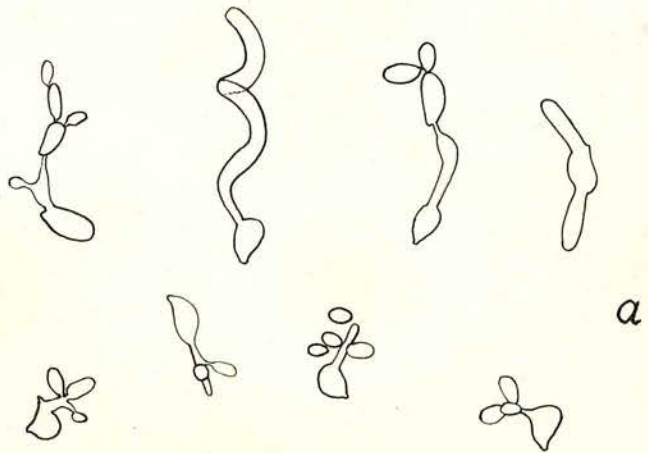
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III.

Note on a Rare Beetle, Cartodere filum Aubé,

eating fungus spores.

For a number of years it has been noticed that the spores of certain fungi kept in a dry condition in the laboratory of the Royal Botanic Garden, Edinburgh, become compacted, after a time, into little cylindrical masses, which strongly suggest that the spores have been ingested by some small animal, and egested in the form of faecal pellets. The fungi attacked in this way are those having dry, powdery spores, e.g. species of Lycoperdon, Ustilago and Tilletia.

The faecal pellets (fig. 1) are roughly cylindrical in form, straight or slightly curved, generally appearing under the microscope as oblong

masses the ends of which may be clean-cut, or more or less irregular. The diameter varies from 23 to 37 μ , but much wider variations occur in the length of the masses, measurements from 40 to 200 μ having been obtained; this is readily understandable, as the diameter must be conditioned by the dimensions of the alimentary canal of the animal depositing them, whereas the length of the individual fragments breaking off on deposition is largely fortuitous. These pellets seem to be made up entirely of closely compacted spores, which retain their normal appearance. Presumably they bear a thin coating of mucid substance, to which their cohesion is due, but no interstitial matter is discernable under the microscope. The number of spores which may be counted in the transverse diameter of a pellet, e.g. at one end, depends on the spore size of the species concerned, as illustrated in the following tabulated observations on the three species most noticeably affected in the laboratory.

Species	Spore size		Number of spores across pellet		Average size
	Range	Average	Usual range	Approx. average	\bar{X} Average number
<u>Lycoperdon pyriforme</u>	3.4-5.0 μ	3.9 μ	5-8	7	27.3 μ
<u>Ustilago Avenae</u>	5.0-7.1 μ	5.7 μ	4-6	5	28.5 μ
<u>Tilletia Tritici</u>	16.4-20.7 μ	18.6 μ	2	2	37.2 μ

The figures for number of spores across the pellet must be taken as approximate only, for the spores are not found arranged in definite rows; being roughly spherical bodies they naturally tend to alternate with each-other. The relatively large spores of Tilletia Tritici are never arranged more than two deep (fig. 2) and actually they are usually a little out of direct alignment with one-another, except in the case of spores under average size, so that the diameter of the pellets is somewhat less than the product shown in the final column of the table. Making allowance for this, it will be noticed that the figures in this column fall within the range already quoted for the diameter of the pellets. It is evident that this somewhat elastic limit is independent of the spore size, and is imposed by the agent responsible for the formation of the pellets, as we should expect if this is the alimentary canal of a small animal.

For some time no insect or other animal which might be responsible for this transformation of the spores was noticed, and its presence was merely inferred from the observed effects. Recently the appearance of faecal pellets in the spores of some smutted oats which had been in store only a few months afforded the opportunity for a systematic

search, and a small brown beetle was found to be present in considerable numbers. Further examination proved that this was responsible for the damage, as will be shown below.

Specimens of the beetle were submitted to Dr A. E. Cameron, of the Edinburgh University Department of Entomology, who identified the beetle as Cartodere filum Aube', of the family Lathridiidae (Clavicornia), and kindly supplied some further information about it. Fowler (1889) comments on this species as follows:

"Very rare; it appears to be chiefly confined to herbaria, although it occasionally occurs in fungi in other countries. Burton-on-Trent (Mr Mason's herbarium, in some small numbers); Scotland, Edinburgh (found by Professor McNab in the herbarium of the Royal Botanic Gardens)."

The beetle has evidently found, amongst the dried specimens of various fungi, conditions of life and food material which suit it admirably, with the result that it has survived for many years, and now appears to be plentiful and flourishing.

Specimens of Ustilago Avenae and U. Hordei collected in Wales in August, 1935 and stored in the

affected cupboards showed no sign of damage in October of the same year, but when these specimens were again being used in February, 1936, faecal pellets were found to be abundant in the smutted ears. It was amongst these specimens that the systematic search referred to above was carried out, and many living mature beetles were found, as well as a fair number of larvae and a few pupae. Under observation, the beetle was seen to deposit the pellets quite rapidly.

A few beetles were killed, dehydrated in absolute alcohol, cleared in clove oil, and mounted in Canada Balsam. This treatment made the animals transparent, and the contents of the alimentary canal could be clearly seen, apparently consisting entirely of smut spores which, in the hinder gut, were compacted together into a solid mass (fig. 3). In the gut this cylindrical column of spores is continuous, breaking up on emergence into the varying lengths which form the characteristic pellets.

The larvae were observed to deposit similar, but generally somewhat more slender pellets, and when a few larvae were similarly cleared and mounted the spores could be seen in the gut just as in the mature beetle (fig. 4). They were also present in the gut

of the pupae, though these, being quiescent, did not deposit pellets. Fowler (1889) says that "the larvae (of Lathridiidae generally) probably feed on cryptogamic substances, the excrement and skin of various insects, etc." In the present case larvae and adults alike were apparently subsisting on the same pure diet of one cryptogamic substance, namely smut spores.

The unaffected spores of Ustilago Avenae and U. Hordei (collected August, 1935) were still viable in February, 1936, germination beginning after a few hours in water. As the spores constituting the faecal pellets were not visibly altered except as to their arrangement, these were tested for viability. To ensure that the spores used had actually passed through the alimentary canal, a living beetle was kept under observation in a clean hollow-ground slide, where it was seen to deposit pellets of spores. These were then transferred to a hanging drop of water.

The majority of the spores failed to germinate, but a few produced their promycelia and sporidia within 18 hours, when the photograph (fig. 5) was taken; by this time the bacteria present in the faeces had multiplied very considerably, as seen in the photograph. The relatively long period of 18 hours should not be stressed too heavily; the spores

had germinated overnight within this time, and even in experiments with unaffected spores some always took a good deal longer than others to germinate. But one evident difference was the very small number of spores which had germinated even after 18 hours. In drops where both unaffected spores and pellets were present the promycelia of the former were generally so abundant as to hamper observation of the latter, and frequently when a promycelium appeared to proceed from one of the pellets the possibility remained that it might belong to an unaffected spore adhering to the mass. But in the present case, where fresh pellets only were introduced into the drop, it was very noticeable that the majority of the spores showed no signs of germination.

From this it appears that, while spores may survive and germinate after passage through the alimentary canal of the beetle, they are nevertheless subject to some adverse influence to which, in the present case, most of them succumbed. This lethal effect was probably caused by the digestive juices of the beetle.

The exact composition of the spore wall of Ustilago appears to be unknown, but it may be taken

that it consists mainly or entirely of some form of cellulose. It can be shown by microchemical means that it is not pure cellulose as found in the cell walls of the higher plants generally and of a few fungi, such as Peronospora. For example, spores of Ustilago and Peronospora were mounted together and tested with sulphuric acid and iodine, when the spore wall of Peronospora gave the blue colour reaction characteristic of cellulose, while that of Ustilago simply assumed a yellowish brown tint. It has been customary to refer to the substance of such walls as "fungus cellulose", as it is believed to consist essentially of cellulose, though in a slightly different form from that occurring in the majority of plants.

If this be so, it would appear from the reduced germination capacity of the egested spores that these must have been acted upon by an enzyme capable of dissolving, at least in part, their protective covering, that is to say, by a cellulase.

According to Mansour and Mansour-Bek (1934) no evidence of the presence of cellulase in insects had been obtained prior to 1919, and any digestion of cellulose which might take place was believed to be due to the presence of micro-organisms in the

intestinal tract. Subsequently to 1919, however, the presence of cellulase has been recorded in a number of insects, including wood-eating beetles. It is therefore quite possible that Cartodere filum may secrete cellulase, especially as the herbarium material on which it generally lives must contain a high percentage of cellulose, but no experimental information on this point is known to the writer.

Reference may be made here to the question of the digestion of spores of Tilletia Tritici by animals. These spores have been found in the faeces of several vertebrates (man, dog, rabbit, guinea pig, etc.) and appear to be affected but little if at all by their passage through the digestive tract (Dobson, 1926). Conflicting results have been reported as to their capacity for germination when obtained from the faeces. In vertebrates the breakdown of cellulose is generally admitted not to be the work of a cytase, but due mainly to the action of intestinal bacteria. It is possible that the conflicting results just referred to may be related to variations in the composition of the bacterial flora.

It was not possible to test the viability of egested Tilletia spores in the present case, as this material was old, and even the unaffected spores were

not capable of germination. Even the spores of Ustilago on which the observations of germination were made were already six months old; with fresher spores it is possible that the decrease in germination capacity would have been less marked.

No attempt has been made here to deal in detail with the entomological aspect of the problem. The purpose of this note is rather to record the agent responsible in the present case for the appearance of these characteristic pellets amongst dry fungus spores, the persistence in the laboratory of this rare beetle, and the fact that spores are capable of germinating after passage through the alimentary canal of the beetle, but do suffer a reduction in their capacity for germination.

References.

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- Mansour, K, and Mansour-Bek, J. J. (1934)
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Explanation of Figures.

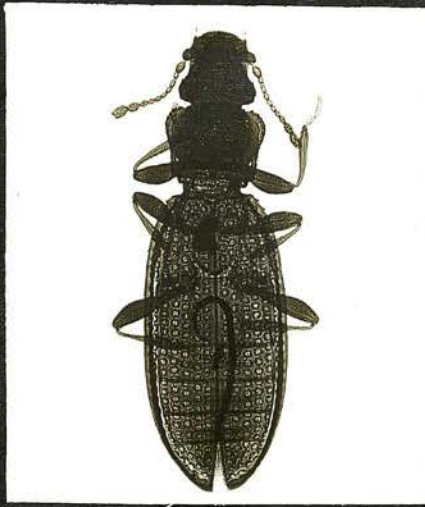
1. Basidiospores of Lycooperdon pyriforme, egested by Cartodere filum, as seen under a low power.
 2. Chlamydospores of Tilletia Tritici, egested by Cartodere filum.
 3. Cartodere filum Aubé; the mature beetle cleared to show the dark mass of spores of Ustilago Avenae in the gut.
 4. Cartodere filum; larva with spores of Ustilago Avenae in the gut.
 5. Chlamydospores of Ustilago Avenae, egested by Cartodere filum: photographed after 18 hours in water; a few spores in the smaller mass have germinated.
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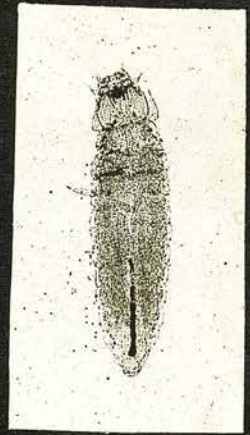
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