A Fat-Destroying Fungus.

BY

R. H. BIFFEN,

*Research Student of Emmanuel College, and Demonstrator of Botany
in the University of Cambridge.*

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With Plate XIX.
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AMONG some specimens of germinating Coco-nuts brought from Ceylon in January, 1898, by Mr. H. H. W. Pearson, one was found half full of a thick white flock of the mycelium of a Fungus growing from the basal end of the nut. On keeping the nut moist under a bell-jar, the mycelium increased considerably, and soon reduced the endosperm to a slimy, brownish-grey pulp which gave off a pleasant ethereal odour something like that of amyl butyrate. It seemed probable that one of the effects of the action of the Fungus was to destroy the coco-nut oil contained in the endosperm, so I began an investigation into the biology of the Fungus in the hope of obtaining some results in connexion with the destruction of fats. A detailed examination of the original nut could not be made owing to its being soon over-run by a *Coremium*-form of *Penicillium*, and later by *Eurotium*.

The Fungus was, however, easily obtained in pure culture from sowings of its conidia in plates made up with 1 per cent.

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asparagin, 1 per cent. cane-sugar and gelatine, or with yeast-extract and gelatine. White patches of mycelium appeared in a few days, and the jelly surrounding them turned an olive-green colour. The mycelium was then lifted out with a sterilized needle and transferred to sterilized blocks of coco-nut endosperm, kept moist in plugged test-tubes by means of wads of cotton-wool saturated with water. The sterilization was effected by boiling in a steam-sterilizer for an hour on three successive days. Attempts were made to obtain pure cultures without this heating, and consequent change of the endosperm-contents, by sawing off a piece of the shell and a thin layer of endosperm, singeing the cut surface and cutting out blocks of endosperm with a sterilized cork-cutter, but without success. On infecting these blocks the Fungus grew more rapidly than on those sterilized by boiling, but a crop of *Penicillium* invariably grew at the same time.

A series of cultures were also made on sterilized Brazil-nuts. The Fungus grew equally well on these, and gave throughout the same results as obtained with cultures on coco-nut endosperm.

In a week's time the greater part of the infected block was covered with a coarse snow-like mass of mycelium, whether the soft endosperm or the hard brown testa was infected. This mycelium increased so rapidly that in a few weeks it half filled the tubes. During its growth large quantities of water were secreted. After about a month or six weeks the growth of the mycelium appeared to have ceased, except that at irregular intervals of time close spherical tufts of mycelium, from a quarter to half an inch in diameter, were formed. Meanwhile the shape of the blocks of coco-nut endosperm and of the Brazil-nuts altered considerably, owing to the breaking down of the cells of the interior into a pulp, which gradually disappeared, leaving only a thin contorted shell. The pleasant ethereal odour already mentioned was very noticeable in the tubes.

For anatomical investigation the cultures were taken up at weekly or fortnightly intervals, placed in Rath's solution for

three or four hours, washed for twenty-four hours in running water, and gradually dehydrated in alcohol.

Before my investigations had gone very far, it became evident that the Fungus in question was one of the Hypocreales¹, probably belonging to the group of the Nectrieae; but on this latter point I cannot at present speak with certainty. The evidence for this is given in detail below.

The conidia are elliptical in shape, with thin, smooth walls (Fig. 1 *a*); each contains a single nucleus, one or more highly refringent bodies, and on staining with an alcoholic extract of alkanet-root the presence of small quantities of oil can be detected. Single conidia placed in hanging drops of nutrient gelatine began to germinate in twenty-four hours at a temperature of 16° C. The mycelium grew rapidly, and the refringent bodies passed out into it, and in a short time showed signs of diminution in size. The first septum appeared when the mycelium was about six times as long as the spore. The first-formed cells could always be distinguished from those formed later, even in an old mycelium, on account of their being shorter, more swollen, and full of vacuoles. Each has, as a rule, one well-marked nucleus. The mycelium branches vigorously in all directions, and after about six days many of its hyphae turn abruptly from their original course in order to grow alongside other hyphae, rarely fusing with them however (Fig. 1 *b*). In this way strands, containing a dozen or more hyphae, are formed which give the masses of mycelium a characteristic coarse appearance. After a time the ends of these strands fray out, and the hyphae forming them abstrict conidia terminally and singly (Fig. 1 *b*). The formation of these conidia takes place at a great pace; in one case eight were formed in sixteen hours from the apex of a hypha.

On staining the mycelium with Delafield's haematoxylin at this stage, it appeared to be crowded with nuclei, there being often as many as twenty or thirty in a single young segment (Fig. 2). In the older parts of the mycelium, however,

¹ See Lindau, in Engler's *Pflanzenfamilien*, I, 1, p. 343 et seq.

they were few in number, usually one, but sometimes two, in each segment. At the points of branching a large single one, often surrounded by much smaller ones, was almost invariably to be found (Fig. 2 *b*), while the ends of hyphae which had finished abstricting conidia rarely contained any.

The variability of their size and their number did not seem to agree with the supposition that they were nuclei, so cultures were specially grown to determine, if possible, their nature. For this purpose conidia were sown in flasks of sterilized coco-nut milk, and when a healthy mycelium was visible to the naked eye it was lifted out and fixed in the usual manner in Rath's solution. The nucleus-like bodies were then found in great abundance. They stained deeply with haematoxylin, fuchsin, methyl-green, and with safranin-gentian-violet orange G. They gave no reactions when tested for glycogen and oil. On treating the mycelium with a solution of gastric juice at a temperature of 25° C. for twelve hours, and again staining, they were found to have disappeared, while the true nuclei could still be easily distinguished. From this fact and from their action towards staining agents it seems clear that the bodies are proteid in constitution. Too much importance must not, however, be assigned to these micro-chemical reactions. The bodies have been observed in many Fungi and described as nuclei. One of the most careful accounts of them is given in Guégen's study of the biology of *Penicillium glaucum*¹, and he hesitates in calling them true nuclei. In this case, however, their size and their difference in reaction when treated with gastric juice distinguish them from nuclei, and as they pass out into the conidia they may possibly serve as reserve stores for use during germination.

Here and there the nuclei showed signs of division by karyokinesis, but not definitely enough to make out any details. The most noticeable feature about them was the frequency with which they were found in close connexion with the vacuoles. In most cases they were to be found on

¹ Guégen, Bull. de la Soc. Myc., t. xv, p. 23; for literature also.

the margin of a vacuole; while sometimes, where a nucleus appeared to have just divided, the daughter-nuclei were separated by a large vacuole (Fig. 3).

In hanging-drop and plate cultures, chains of chlamydospores were developed from the mycelium which produced microconidia, especially as the gelatine began to dry. The chlamydospores are spherical, or slightly elliptical, in shape, with thick brown walls, and contain many of the proteid reserve bodies already mentioned (Fig. 4). On germination they gave rise to a mycelium which again produced microconidia.

These were the only forms of spores produced in the gelatine-cultures, but macroconidia were formed in the cultures on coco-nut endosperm and Brazil-nuts. Like the microconidia, they are abstricted terminally and singly from the hyphae. At first the young macroconidia cannot be distinguished from microconidia, but they soon become sickle-shaped; and when they have reached their full size they divide by transverse walls, so that ultimately each is composed of three or four cells each with its own nucleus (Fig. 5). Like the microconidia, they contain considerable quantities of oil.

Germination may occur from any one, or from all three or four cells. The mycelium formed is similar to that formed by the microconidia and the chlamydospores; but it is characterized by having many of its hyphae united into a network by the fusion of short lateral processes, very like those put out by conjugating *Spirogyra* (Fig. 6).

In cultures four weeks old the mycelium in the endosperm had given rise to a stroma formed from strands of hyphae running parallel with one another, and united on the surface of the blocks by branches running at right angles (Fig. 7). These branches interlace to such an extent that the mode of development is soon lost sight of, and the stroma appears to be composed of hyphae running in every direction. When mature it is chocolate-brown in colour and fleshy in texture. Its thickness is from one to two mms.

Even in this early stage rudimentary perithecia can be

distinguished in the stroma itself, and later they are formed in great abundance on its surface, where they stand out in warty brown masses two or more mms. high. They have been produced continually for the last nine months. Unfortunately they have proved barren in every case. They consist merely of a flask-shaped shell of thickened dark-brown hyphae, woven into a very close layer, enclosing a plexus of colourless hyphae often of considerable size (Fig. 8). Keeping the cultures at a temperature of 20° C. and 25° C. for a month, and altering the hygrometric conditions by partially drying with calcium chloride, has had no effect, and after making many attempts to force them to produce ascospores I have had to abandon the problem. Owing to this the further identification of the Fungus has to be postponed.

Yet another form of reproduction was met with, but once only, namely, a pycnidium with pycnidiospores. This occurred in a stroma on a culture four weeks old. The pycnidium was flask-shaped, with a narrow ostiole. Its walls were composed of smaller, less thickened hyphae, which were not so closely woven as those of the walls of the perithecia. The spore-abstricting hyphae were branched and directed to the ostiole. Under these circumstances the germination of the pycnidiospores could not be observed.

As the perithecia or ascocarps are open at the apex, the Fungus evidently belongs to the Pyrenomycetes, which may be subdivided into three groups, the Hypocreaceae, the Sphaeriaceae, and the Dothideaceae, the texture of the stroma and the position of the perithecia serving as distinguishing characteristics. The perithecia of the Sphaeriaceae are, as a rule, embedded in the stroma, and have hard, dark-coloured walls, while those of the Dothideaceae are so embedded that they have no distinct wall of their own. The Hypocreaceae, on the other hand, have soft, coloured perithecia, usually placed in groups on a fleshy stroma¹. We may class the Fungus in question then among the Hypocreaceae, a group which contains several parasitic genera, some species

¹ Brefeld, *Unters. aus d. Gesamtgeb. d. Mykol.*, Heft x, p. 162.

of which are only known at present in their conidial stages (e.g. *Fusarium*). For its further identification we require to know the colour of the ascospores, and of this we have, as yet, no evidence. If they are dark-coloured it belongs to the Melanosporeae; if hyaline, yellow or red, to the Nectriaceae¹.

In this connexion we may notice that Brefeld has shown that various species of *Nectria* reproduce by forms very similar to those already described. *Nectria coccinea* (Pers.) may serve as one example². It forms microconidia and macroconidia, similar to, and in the same manner as, those of the Fungus described, while chlamydospores and pycnoconidia occur in *N. sinopica* (Fries) and other species.

Sections through blocks of coco-nut endosperm infected a fortnight previously show that the mycelium has penetrated the tissues considerably, and that in a very characteristic way. In longitudinal sections the tissue appears to be broken up into alternating bands of a white and grey colour (Fig. 9). The white bands consist of unattacked tissue, the grey of attacked tissue. In transverse section the attacked portion appears as grey patches surrounded by the unattacked white portion.

On staining with Delafield's haematoxylin, the mycelium can easily be distinguished in the cells, especially if the sections are previously treated with chloroform to remove the large quantities of oil they contain. Each cell of the attacked portion is then seen to be crowded with the luxuriantly growing mycelium, which here and there forms dense knots owing to its having turned on itself on reaching the transverse wall of the cell (Fig. 10). The growth in a longitudinal direction is singularly constant for the first three weeks; then, however, numerous branches grow out at right angles and pierce the longitudinal cell-walls, sometimes in as many as twenty places in a single wall. In this way the first-formed bands of hyphae are united and a dense meshwork of mycelium is formed in the tissue.

¹ Lindau, l. c.

² Brefeld, l. c., p. 173, and Taf. iv, Figs. 23 and 24.

The piercing of the walls is evidently brought about by the secretion of a cellulose-dissolving enzyme, for they swell strongly, and the hyphae push their way directly through the gelatinized walls without showing any signs of constriction. The hyphae, on reaching the walls to be pierced, either swell directly into a bulbous ending, or give out two or three short branches, which then dilate and apply themselves to the wall. These endings each contain a large nucleus which stains deeply with haematoxylin, numerous small vacuoles, and oil-drops (Fig. 11). It is probable that they secrete the cellulose-enzyme, which presumably gelatinizes the cell-walls, just as the haptera of the Lily-disease *Botrytis* do¹. The walls are pierced and dissolved to such an extent that in cultures six weeks old it is difficult to find a trace of the endosperm cell-walls, the whole mass being reduced to a slimy pulp, through which the mycelium runs in all directions. The hard brown testa undergoes little change, and that mostly mechanical, owing to the mycelium breaking through it and carrying out fragments into the stroma. No action on its thickened cell-walls could be detected. Meanwhile some very obvious changes have been brought about in the oil-contents of the endosperm; for, instead of large masses of fat being present in the attacked cells, the quantity has become exceedingly small, being in many cases only represented by a small quantity of a fine emulsion.

This is especially well seen in longitudinal sections of cultures two weeks old stained for six hours in an alcoholic extract of alkanet-root. They appear to the naked eye to be composed of alternating bands of deep and pale red tissue (Fig. 9). Under the microscope the unattacked cells are found to be full of the deeply stained oil, while in the attacked cells the oil is chiefly to be found in the mycelium. So abundant is it there that it is an easy matter to trace the course of the hyphae through the cell-walls by means of the dark red drops.

In cultures in which the stroma has been formed, the dis-

¹ Marshall Ward, *Ann. of Bot.*, vol. ii, p. 339.

tribution of the oil is even more interesting. Sections stained in the same way, or, better still, stained with osmic acid by allowing the culture-blocks to remain in Rath's solution for twelve hours, show a plentiful supply of oil in the mycelium within the endosperm, and in the lower portions of the stroma. The upper portion of the stroma, however, rarely shows a trace of oil, though drops can be distinguished in the conidia standing away from it. The absorption of oil by the mycelium and its disappearance in the stroma points to its utilization as a food-stuff, and, as oils are indiffusible substances, to its conversion into diffusible bodies by fermentation.

So far little has been done to establish the existence of fat-splitting enzymes, though they must be of very general occurrence in the vegetable kingdom, judging from the number of seeds grown solely for the purpose of extracting the oil they contain. Sachs¹ appears to have been the first to observe that the oil gradually disappears on the germination of oil-containing seeds, just as starch does in the case of farinaceous seeds. From this he was able to argue that oil was a reserve-substance in many cases. Fleury² went a step further, and from an examination of germinating seeds of Almond, Castor-oil, and Rape was able to show that a fatty non-volatile acid was formed at the same time. These results were confirmed by Müntz³ in 1871, who suggested that the decomposition was brought about by means of an enzyme which split the fat into a free fatty acid and glycerine. He was, however, unable to detect the glycerine. This view was generally accepted, but it was not until 1889 that the enzyme was isolated by Green⁴ from germinating seeds of *Ricinus*. He succeeded in obtaining extracts of the seeds with which an emulsion of castor-oil was decomposed into a fatty acid and glycerine. The glycerine could not be detected

¹ Sachs, Bot. Zeit., 1859, p. 178. Physiology, Eng. ed. p. 347.

² Fleury, Ann. de chimie, Sér. 4, t. 4, p. 38, 1865.

³ Müntz, ibid., t. 22, 1871.

⁴ Green, Proc. Roy. Soc., vol. xlviii, p. 370.

in the young plant, on account of its having undergone further changes, probably into sugars.

There is a great probability that this enzyme occurs as frequently in Fungi as in Flowering-plants. Thus fats are found in considerable quantities in many forms of spores, e. g. in the oospores and zoospores of the Chytridiaceae and Saprolegniaceae, in uredospores, &c., where they are without doubt a reserve store for the germinating spores to draw upon. Many Fungi contain large quantities of fat in the mycelium; *Lactarius deliciosus*, e. g., contains 5.86 per cent., while those typical reserve-stores, the sclerotia, as a rule contain very considerable quantities, amounting in the case of *Claviceps purpurea* to 35 per cent.¹

In many cases a large fat-content is abnormal, and due, as shown by Loew and Naegeli², to fatty degeneration, but this can hardly apply to the cases mentioned.

Then again we are acquainted with Fungi which grow exclusively on fat-containing bodies, such as *Empusa*³ and *Cordyceps* spp. on animal remains, and *Cyclonium oleaginum*⁴ (Cast) and *Inzengaea asterosperma*⁵ (Borzi) on olive fruits. Others, such as *Penicillium* and *Eurotium* spp., though not necessarily restricted to growing on oil-containing substances, may often be found doing so. One frequently finds them, for instance, growing in the layer of sweet-oil placed over bottled fruits to prevent decomposition. These Moulds also attack cotton, rape, and other cakes made from the waste left after crushing oleaginous seeds for the oil they contain. Ritthausen and Baumann⁶ have shown that a great loss of oil occurs when cakes are attacked in this way; e. g. two samples of rape-seed cake containing 10.53 per cent. and 8.5 per cent. of oil contained after two years only 1.98 per cent. and 1.87 per cent. respectively when overrun by these Fungi.

¹ Zopf, Die Pilze, p. 138.

² Naegeli, Unters. über niedere Pilze, 1882. Zopf, *ibid.*

³ Brefeld.

⁴ Brizi, Bot. Centrbl., Bd. lxii, p. 81. (Abstract.)

⁵ Borzi, Bot. Centrbl., Bd. xxiv, p. 14. (Abstract.)

⁶ Ritthausen and Baumann, Versuchs-Stat., 1896, Bd. xlvii, p. 389. Reitmar, Versuchs-Stat., 1887, Bd. xxxviii, p. 373.

Penicillium is of especial interest in this connexion, for it has been shown to contain, among other enzymes, a lipase, which was successfully extracted by Gérard¹, and shown to be capable of splitting monobutyryne into butyric acid and glycerine.

Under these circumstances there can be little doubt that the disappearance of the oil from rape-seed cake, &c., is due to the Moulds being able to utilize it as a food-material.

Among the Bacteria several forms are known which are capable of splitting fats. An example of this is afforded by the *Bacillus fluorescens non liquefaciens* of Krueger², isolated from butter which had become 'cheesy.' Several pathogenic forms, such as *Bacillus typhi abdominalis*, *B. pyocyaneus*, and *Vibrio cholerae asiaticae*, have also been shown by von Sommaruga³ to possess this power.

In order to obtain the mycelium in quantity to test for the presence of this supposed fat-splitting enzyme, large cultures were grown in flasks of sterilized coco-nut milk kept at a temperature of 20° C. In from eight to ten days the surface of the liquid was covered with a thick growth of mycelium and the liquid itself had become acid to litmus paper. In one series of flasks the milk was coloured with neutral litmus and infected with the conidia of the Fungus on Nov. 2. On Nov. 7 the purple colour had changed to a bright red, and a small quantity of mycelium was visible floating on the surface. The growth of the mycelium had therefore given rise to the formation of a free acid. Whether this was from the fat or from other constituents, such as sugar, remained to be proved.

The mass of mycelium was then taken out of the flasks, washed rapidly in distilled water, and ground to a thin paste with kieselguhr and water in a Bacterium-mill, or with clean sand and water in mortar. On filtering this paste under

¹ Gérard, Bull. d. la Soc. Myc. d. France, t xiii, p. 182.

² Centrbl. f. Bakt. vii, 1890, No. 14-16, p. 87.

³ Zeitschr. f. Hygiene, Bd. xviii, 1894, p. 441. Lafar, Technical Mycology, Engl. ed., p. 199.

pressure through several thicknesses of filter-paper, a faintly brown opalescent fluid was obtained which gave a slight acid reaction. It showed no signs of oiliness. The extract was then neutralized with sodium carbonate and mixed with 2 per cent. potassium cyanide solution, to inhibit the action of the Bacteria which had entered during the process of extraction, and then placed in quantities of about 5 c.c. in sterile plugged tubes for further experiments.

In the first place thick sections of coco-nut endosperm were placed in three tubes containing the fresh extract, and in three containing the extract previously boiled and cooled to serve as a control. The tubes were kept at a temperature of 24° C. for a day, and the sections then taken out and stained over night in alkanet-extract.

In the control experiment the sections showed no signs of change; but in the sections from the tubes of fresh extract the oil had either completely disappeared or was reduced to a fine emulsion.

A further series of tubes were then prepared as before; but this time, instead of sections of endosperm, cover-slips coated with a thin layer of coco-nut oil were used. This substance is of special value in testing for lipases, as it is a pure fat free from acid, and contains, according to the analysis of Fresenius, 99.979 per cent. of fat¹. The layer soon became liquid and formed an emulsion which floated on the surface of the extract and gradually diminished in quantity. Testing with litmus-paper showed that the liquid was acid, and on neutralizing with sodium carbonate solution the fatty acid formed floated to the surface². The liquid had the same pleasant ethereal odour as the original nut.

To another series of tubes of this extract a 2 per cent.

¹ Kew Bulletin, 1890, Art. clxxiii, p. 230.

² This acid was not investigated further. I may note here that the literature on the question of the constitution of coco-nut oil is confused owing to the unfortunate habit of spelling coco-nut 'cocoa-nut.' As pointed out by Balfour (Ann. Bot., vol. i, p. 184), this is a mistake. Thus in Watt's Dict. of Chemistry, vol. ii, p. 231, we find 'cocoa-nut-oil or cocoa-butter,' cocoa-butter being the product of *Theobroma Cacao*!

mixture of monobutyryne and water was added¹. Twelve hours later the mixture smelt strongly of butyric acid and gave an acid reaction with litmus.

These experiments leave little doubt that a fat-splitting enzyme is present in the Fungus.

Like other enzymes, it proved to be precipitated by the addition of an excess of absolute alcohol, as a flocculent precipitate. On drying this precipitate over calcium chloride, a whitish-grey powder was obtained which was readily soluble in cold water. A solution of the enzyme obtained in this way gave the same results, when tested with coco-nut oil and monobutyryne, as the original extract.

The course of action of the enzyme is first to emulsify the fat, and then to split it into a fatty acid and glycerine, which substance, as has often been shown, can be utilized by plants as a food-material and converted into sugars. Whether the fatty acid can be utilized in a similar way is doubtful, for the coco-nut milk becomes more and more acid as the mycelium increases. The presence of oil in the mycelium itself offers the same difficulty as the presence of oil in the cotyledons of germinating *Ricinus* did to Sachs. Whether the oil can pass directly through the walls, as Sachs supposed was the case, or whether it is re-formed from the products of its decomposition, cannot at present be determined². Certainly the fatty acid cannot diffuse as a soap, as is the case with the fatty acids formed by the steapsin of the pancreatic juice, for the reaction of the infected coco-nut endosperm and milk is invariably acid.

The disappearance of the oil from the endosperm when attacked by this Fungus suggests the interesting question whether the coco-nut embryo on germination can utilize the oil in a similar manner by the formation of a lipase.

¹ Gérard, l. c.

² Cf. Green, l. c.

EXPLANATION OF FIGURES IN PLATE XIX.

Illustrating Mr. Biffen's paper on a Fat-Destroying Fungus.

- Fig. 1. (a). Microconidia germinating.
(b). A strand fraying out into conidia-bearing hyphae.
- Fig. 2. (a). Apex of a hypha which has not yet produced conidia.
(b). A hypha branching, showing the 'proteid reserves.'
- Fig. 3. Daughter-nuclei separated by a vacuole.
- Fig. 4. A chain of chlamydospores, from the hanging-drop-culture from which Fig. 1 was drawn fifteen days previously.
- Fig. 5. (a). Young macroconidia on hyphae standing away from a stroma.
(b). Stages in development.
(c). Stages in germination after twenty-four hours at 16° C. in yeast-extract and gelatine, and after forty-eight hours.
- Fig. 6. A portion of the mycelium formed by 5 c. seven days later.
- Fig. 7. The development of the stroma from a culture on coco-nut endosperm, three weeks old.
- Fig. 8. Median longitudinal section through one of the sterile perithecia, showing the parenchymatous wall and the plexus of hyphae within it.
- Fig. 9. Section through a block of coco-nut endosperm, showing the alternating bands of infected and uninfected tissue. The infected tissue is shaded to represent the grey colour of the original. The stroma is beginning to form on the upper surface. The dark line on the lower surface represents the brown testa.
- Fig. 10. Longitudinal section through a fortnight-old culture on coco-nut endosperm to show the distribution of the mycelium. On the right of the figure the growth at right angles to the original direction has commenced.
- Fig. 11. The branched and swollen hyphal endings about to grow through gelatinized transverse walls.

Fig. 4.

Fig. 5.

Fig. II.

Fig. 10.

Biffen, Roland H. 1899. "A fat-destroying fungus." *Annals of botany* 13, 363–376. <https://doi.org/10.1093/oxfordjournals.aob.a088737>.

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