On *Trichosphaeria Sacchari*, Massee; a Fungus causing a Disease of the Sugar-cane known as 'Rind Fungus.'

BY

ALBERT HOWARD, B.A., A.R.C.S.

Foundation Scholar of St. John's College, Cambridge; Mycologist to the Imperial Department of Agriculture for the West Indies.

THE life-history of *Trichosphaeria Sacchari* was first investigated by Mr. George Massee at the Royal Gardens, Kew, and his results are summed up in a paper contributed to the 'Annals of Botany' for December, 1893 (vol. vii, p. 515). I was requested by the Imperial Commissioner of Agriculture for the West Indies to repeat Mr. Massee's work under tropical conditions with a view of ascertaining how far local conditions affect the life-history of the Fungus. The present paper gives an account of the first portion of the work and deals with the *Melanconium*, *micro*- and *macroconidial* stages of the Fungus in so far as the present investigation supplements Mr. Massee's work.

MELANCONIUM STAGE.

A casual visitor to any cane-field or estate-yard in Barbados during crop time (March to June) is bound to be struck by the large number of diseased canes, locally known as 'rotten-canes,' to be met with. These diseased canes are brown in colour, more or less shrivelled, and in nearly all cases are

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studded with black curly hair-like structures, from a quarter of an inch to two inches or more in length, which are formed under the rind and grow out into the air by rupturing the surface. These hair-like structures, on microscopic examination, are found to be immense numbers of spores of the *Melanconium*-stage of the 'Rind Fungus' loosely cemented together. Extensive cultures of these spores were made under widely different circumstances, the results of which are described below.

Pure Petri-dish cultures of these spores, obtained from a diseased cane, were made in a food-material consisting of raisin-extract containing eleven per cent. of gelatine. The spores developed a white septate branched mycelium which covered the plates as a dense white glistening felt which, as the substratum became exhausted, gradually turned brown, and in fourteen days was found to have developed brown chlamydospores in the hyphae. The nature of these bodies was investigated later and will be described below. Nothing further of interest was observed in this series.

On account of the high temperature of the laboratory, which in the middle of the day sometimes reaches 32°C , it was found that gelatine solutions containing at least fifteen per cent. of that substance are necessary for Petri-dish and hanging-drop cultures. When required for immediate use I find eighteen per cent. gelatine solutions are necessary; when required in a few days' time it is better to prepare the more dilute solution, viz. fifteen per cent., so as to allow for drying up.

Pure plate-cultures were next prepared, using fifteen per cent. saccharose-gelatine containing Klebs' solution:

{	Potassium nitrate	2 grams per litre.
	Magnesium sulphate	1 „ „
	Potassium phosphate	2 „ „

In preparing this series some of the old mycelium, containing chlamydospores from the above cultures, well beaten up in sterile water, was used for infection. A dense white-felted mycelium was developed as before, in which well-marked

chlamydospores were noted seven days after infection. Similar flask-cultures made at the same time gave similar results. Evidently under these conditions the *Melanconium*-mycelium does not develop in the manner described by Massee in his Kew experiments.

Meanwhile hanging-drop cultures of *Melanconium*-spores were made according to Professor Marshall Ward's method of drop culture¹ in fifteen per cent. saccharose-gelatine containing Klebs' solution. Germination took place twenty-two hours after sowing, and a colourless branched septate mycelium was developed. The day temperature of the laboratory during the development of this series of drop-cultures varied between 26° C. and 29° C. After germination the mycelium developed rapidly, and in two days the drops were filled with mycelium which in many cases showed fusion of the hyphae as described by Marshall Ward². Stages in this process were observed later and will be described below. In three days the mycelium showed extensive vacuolation, and after thirteen days the protoplasm of the mycelium of a single spore-culture showed a tendency to aggregate in certain parts of the hyphae, leaving the rest empty. This proved to be the commencement of chlamydospore formation, stages in which were observed. In twenty-eight days in the drop in question the hyphae were noted to have knotted up together in two areas nearly in contact forming dense stromata. In thirty-four days the edges of the stromata were found to contain *Melanconium*-spores. The stromata were roughly elliptical in shape and measured .59 × .24 mm. and .3 × .24 mm. respectively. The *Melanconium*-spores obtained therefrom varied from 14.7 μ to 9.2 μ in length and measured 3.7 μ in breadth. It was unfortunate that the early stages in the formation of these *Melanconium*-stromata were not observed. It was not until after many failures that a hanging-drop culture containing a single *Melanconium*-spore was

¹ The Ginger Beer Plant and the organisms composing it. Phil. Trans., vol. 183 (1892), pp. 130-132.

² A Lily Disease. Ann. Bot., vol. ii, p. 329 (1888).

obtained and stages were observed in the formation of a stroma. Working under tropical conditions I have experienced great difficulty in preventing hanging-drops being washed off the cover-glass by condensation of water thereon. This usually happens during the first two days, after which the danger from this source is small.

Next hanging-drop cultures were made in a food-material composed of raisin-extract containing eighteen per cent. of gelatine. Germination took place in one instance in twenty-five hours, in other cases not till forty-eight hours after sowing. Chlamydospores were noted in the hyphae in twelve days, but no stroma was observed in any hanging-drops when raisin-extract was used as a food-material. In flask-cultures, however, where this substance was employed, *Melanconium*-stromata were observed on the surface of the culture in twenty-nine days.

Hanging-drop cultures were next obtained in sugar-cane extract containing eighteen per cent. of gelatine. The cane extract was obtained by boiling small pieces of cane in distilled water for half an hour, filtering the extract and sterilizing in the usual way. When pieces of cane were allowed to soak in water for more than a day it was found that the development of Bacteria rendered the juice so acid that neither *Melanconium*-spores nor vigorous mycelium from other cultures would grow in such a substratum. A hanging-drop containing a single spore, which germinated in thirty-two hours, was obtained. A long colourless hypha was developed which at length branched. In three days the mycelium began coiling up at the point where this branching took place, and from the coil long unseptate hyphae, full of brilliant fine-grained homogeneous protoplasm, radiated in all directions and after a time branched profusely. Subsequent observations showed that these long hyphae were feeders for the coil. The coil rapidly increased in complexity and in five days a stroma was formed. At this point a second smaller stroma was noted in the elliptically-shaped drop, the two being placed approximately at

the foci of the ellipse. In seven days *Melanconium*-conidia were noted at the edges of the stromata. The stromata were elliptical in shape, the larger one measured $.59 \times .52$ mm. the smaller one $.54 \times .4$ mm. on the surface of the cover-glass, and both extended about one millimetre downwards. Evidently there is a great tendency on the part of the mycelium developed from a *Melanconium*-conidium to reproduce similar spores. Except as regards time, a natural and an artificial substratum appear to yield the same result, viz. the formation of a stroma on the exhaustion of the substratum from which *Melanconium*-conidia are developed. In this and the other drop-cultures fusion of the hyphae was extremely abundant. Working at Kew with a similar food-material Massee states that he observed no trace of fusion of hyphae in any of the numerous cultures examined when grown in sugar-cane solution¹. This result is no doubt due to the different method of working adopted and to the different behaviour of the Fungus under Kew conditions. Massee seems to have used hanging-drops only for germination purposes and to have relied solely on flask-cultures for further development. In the host plant, on which *Melanconium*-stromata were abundant, I have frequently observed fusion of the hyphae in the cells near the epidermis, which in many cases are lined with a basket-shaped meshwork of fused hyphae. I have frequently observed similar structures in hanging-drop cultures of *Melanconium*-spores. Perhaps they are the beginnings of stromata which have ceased to grow on account of the exhaustion of the substratum.

A series of plate-cultures with a similar food-material were made at the same time, and here again the formation of *Melanconium*-stromata was observed in great numbers, which after ten days were found to contain *Melanconium*-spores.

Next flask-cultures were made of chlamydospores from previous plate-cultures in fifteen per cent. saccharose gelatine and Klebs' solution, and also in raisin-extract containing

¹ On *Trichosphaeria Sacchari*. Ann. Bot., vol. vii, p. 128 (1893).

fifteen per cent. of gelatine. In sixteen hours a delicate white mycelium was observed with the naked eye at the points of infection and, after a copious development of white mycelium, chlamydospores and *Melanconium*-spores were formed. Evidently the chlamydospores enable the Fungus to tide over an unfavourable period, and explain the remarkable vitality of the hyphae in old specimens of diseased cane observed by Massee.

Up to this point cultures of *Melanconium*-conidia and chlamydospores had reproduced one or both of these spores. Attempts were next made to reproduce Massee's *macro*- and *microconidia* from *Melanconium*-spores grown in cane-juice of varying concentration.

The first series of cultures was made in dilute cane-juice, obtained by boiling slices of ripe cane in distilled water for fifteen minutes, filtering and sterilizing by intermittent boiling, using spores obtained from a pure culture. In three days the flasks were filled with white septate branched hyphae, and in five days dark spots were noted on the matted surface of the culture round the margin. In ten days they were found covering the surface, and microscopic examination showed them to be stromata containing *Melanconium*-spores. The mycelium was found to be very much knotted and to be full of oil drops. I have very frequently noted these oil drops in the mycelium of the Fungus in the host plant, especially near a stroma. No *macro*- or *microconidia* were found in any of this series of cultures after the most careful examination. The submerged mycelium, however, was in many cases found to have divided into free chlamydospores.

The second series of flask-cultures was made in sterilized cane-juice obtained from the clarifiers of a sugar factory. The juice had been heated with lime and gave a slightly alkaline reaction with phenol-phthalein. In seventeen days *Melanconium*-stromata were abundant, but there was no trace of *macro*- or *microconidia*.

Lastly a series of flask-cultures was made in a food-material prepared by boiling slices of very young cane-shoots in dis-

tilled water. In nine days the familiar *Melanconium*-spores were abundant on the surface of the cultures, and the submerged hyphae were found to have divided up into free chlamydospores by the disappearance of the walls of the empty connexions.

Cultures were now made in sterilized cane-slabs by infection with spores from a pure culture. A white mycelium spread out from the point of infection and in six days the mycelium was found to have knotted up in several places forming stromata, which in nine days were found to contain *Melanconium*-spores. At the edges of the cane-slabs the internal mycelium had formed stromata underneath the rind, which grew out into hairs in an exactly similar manner to that noted in naturally infected canes. Similar cultures made on blocks of sterilized oak sap-wood gave similar results. In eleven days the *Melanconium*-spores were abundant on the infected surface.

All attempts to produce the micro- and macroconidial phases of the Fungus in sterilized media had failed.

An extended search in cane fields where 'Rind Fungus' was present and among heaps of rotten canes in estate yards was now made in order to obtain specimens of macro- and microconidia. At length a cane was found, which, on being split open, showed blackening and disorganization of the tissues. The dark colour was found to be due to a copious development of Fungus-spores, which from their size and mode of origin agreed with the micro- and macroconidia described by Masee. Except for the abnormal development of roots at the nodes the cane in question appeared to be healthy till split open. Afterwards similar diseased specimens were obtained from the Marienberg Plantation, Surinam, through the kindness of the manager, Mr. James Mavor. These canes also showed a copious development of roots at the nodes. I have also frequently noted this abnormal root-development in canes which have been badly attacked by the 'moth-borer' (*Diatraea saccharalis*, Fabr.) and by the mycelium of the *Melanconium*-phase of the 'Rind Fungus.' Except in

cases where it is due to rain-water lodging between the leaf sheaths and the stem, it may be regarded as a sure sign of disease. The above specimens were kept in a moist chamber for some time, when they developed *Melanconium*-spores which ruptured the rind.

In view of the occasional development of micro- and macroconidia in the interior of a cane, a further attempt was made to reproduce these phases from the *Melanconium* condition. Two dozen healthy cane pieces were selected about two feet in length, and the cut ends were immediately dipped into hot melted paraffin to prevent the entry of Fungi and Bacteria. The rind of the canes was carefully cleaned and rubbed over with an alcoholic solution of corrosive sublimate. Two series of experiments were made as follows:—

1. Six of the canes were partially split with a sterilized knife and in three of the slits *Melanconium*-spores from a pure culture were sown, while in the other three growing mycelium from a culture of *Melanconium*-spores in sugar-cane extract was introduced. The canes were then lightly bound up with tape and the split end was again dipped into melted paraffin. Six other canes were at the same time treated in an exactly similar manner, except that no spores or mycelium were introduced, to serve as a control experiment.

2. Six canes were split as before and a cavity was made in the cane by cutting out a portion of the interior with a sterilized knife. In three of the cavities spores were sown, while in the other three mycelium was introduced as before. Six other canes were used as a control.

In five days all the canes which had been inoculated with spores were found to exhibit a considerable development of macro- and microconidia near the point of infection. In ten days all the canes which had been inoculated with mycelium gave a similar result. In no case were these spores found in the control canes. In nearly all the canes the split surface was reddish in colour. In nineteen days several of the inoculated canes showed *Melanconium*-pustules on the exterior and blackening under the rind due to the develop-

ment of macroconidia. These two series of experiments confirm Masee's observation that the micro- and macroconidial phases of the Fungus can be obtained from the *Melanconium* condition.

An attempt was now made to obtain a similar result in sterilized cane-slab cultures. Two series of experiments were made, using cane-slabs in the interior of which cavities had been made by splitting the slabs and cutting out a portion of the inside. The two halves were bound up with cotton which had been boiled with water to remove any mineral impurities. In some of these slabs a groove was cut in one of the halves to form a connexion between the cavity and the air. After sterilization, *Melanconium*-spores from a pure culture were sown on the ends of the slabs. In seven days the cane pieces were covered with small *Melanconium*-stromata, and similar structures were found lining those cavities which communicated with the air by means of a groove. In the cavities which had no connexion with the air no spore-formation was noted, although the tissues forming the walls of the cavity were filled with mycelium. In both series no *macro-* or *microconidia* were observed. The day temperature varied from 26° C. to 31° C. during the development of these cultures. In these and other *Melanconium*-cultures on sterilized cane-slabs the red colouration seen in the vascular bundles of living canes attacked by the Rind Fungus was not observed.

Inoculation experiments were performed on healthy canes with *Melanconium*-spores obtained from a pure culture. The spores were placed on the fresh wounds produced by tearing off leaves near the upper part of the cane, and in the small perforations made with a sterilized needle on the internodes of a cane where the rind had been washed and sterilized with an alcoholic solution of corrosive sublimate. After infection the wounds were covered with sterilized vaseline. Other canes were treated in an exactly similar manner, except that no spores were sown on the wounds, to serve as a control. In five days the tissues round the points of infection were

discoloured, and microscopic examination of the tissues at the edges of the discoloured areas showed the presence of hyphae agreeing in all respects with the mycelium of the Rind Fungus. Portions of this tissue taken just under the rind and about half an inch from the point of infection were cultivated in sterile cane-extract. In ten days the mycelium developed therefrom produced characteristic *Melanconium*-stromata. No infection was noted in the control canes. The parasitic character of the *Melanconium*-spores is therefore placed beyond doubt. So far all attempts to inoculate a healthy cane with *Melanconium*-spores without previously wounding the plant have failed.

The result of the above culture experiments with this phase of the Fungus is in close accord with its behaviour in a natural condition. Practically the only spores of the Fungus met with in Barbados are those of the *Melanconium* condition. These are carried by the high trade winds which sweep across the Island during the period when the canes are ripening and when the Fungus is naturally abundant, and bombard the old and young crops. Unfortunately the tunnels of the larvae of the prolific 'Moth-Borer' (*Diatræa saccharalis*) provide the means of entry of these spores right into the heart of the cane. Infection speedily takes place, and when the mycelium has exhausted and killed the host, stromata are formed under the rind from which numberless spores are produced which again spread the disease. The formation of these spores is evidently of more use to the Fungus than the micro- and macroconidia which are formed inside the tissues of the cane, and which therefore would not escape until complete disorganization had taken place.

At the present time little attempt is made in Barbados to keep the Fungus under control. During crop time large piles of 'rotten cane' covered with countless millions of spores are to be found in most of the estate yards of the Island. In many cases these are made into stacks and left for many months, sometimes till the next year. In other cases they are collected by the estate workmen and stored for fuel. In

consequence the yearly loss suffered by the planters from 'Rind Fungus' is very large, and it is obvious that these losses will be incurred in the future unless universal action is taken to destroy the spore-laden rotten canes as quickly as possible by burning.

MACROCONIDIAL STAGE.

All cultures of *micro-* and *macroconidia* in liquid media were made in sugar-cane extract which was in some cases stiffened with from fifteen to eighteen per cent. of gelatine.

Preliminary cultures of the mixed *micro-* and *macroconidia* found in the interior of a cane were made on sterilized cane-slabs, in order to ascertain if only one Fungus was present. In twenty-four hours the slabs were covered with a white mycelium which in three days turned black through the development of large numbers of *microconidia* and a few *macroconidia*. Careful examination of these cultures did not disclose the presence of any other Fungus. To test this supposition six plate-cultures were made, using these spores for infection. The plates were speedily covered with a white mycelium, which in two days developed *micro-* and *macroconidia*. The plates were kept under observation till the substratum dried up, but no other Fungus was observed. These plates were used for the following cultures.

A series of flask-cultures was now made, using spores from the above plates. In twenty-four hours the flasks showed a considerable development of a submerged colourless mycelium, which in forty-eight hours darkened on account of the formation of large numbers of *micro-* and *macroconidia*. On account of this extremely rapid spore-formation it appeared desirable to grow the *macroconidium* in hanging-drops and to study the development of this stage of the Fungus starting from a single *macroconidium*.

On account of the simultaneous development of both *macro-* and *microconidia* in all the cultures obtained, some difficulty was experienced in obtaining a drop containing a single *macroconidium* unaccompanied by *microconidia*.

The required culture was at length obtained by beating up some mycelium from a flask-culture, in which micro- and macroconidia were developed in about equal numbers, with sterile water, pouring off the upper portion and using some of the remainder to infect a gelatine-tube. The macroconidia being larger and heavier, subsided more quickly than the lighter microconidia, and therefore the lower layers of water were richer in the former bodies.

The spore germinated in five hours after sowing, and in eleven hours the hypha commenced to branch. In eighteen hours the drop was filled with a branched septate colourless mycelium which exhibited very rapid growth. In twenty-four hours some of the hyphae commenced to grow down into the air, especially round the margin of the drop. This behaviour soon became general all over the drop. The aerial mycelium appeared olive in colour and grew with great rapidity. One of these aerial hyphae was fixed and stages in its development were obtained under the high power. Three hours after leaving the drop the hypha commenced to bend and the contents showed segmentation. Soon after this appearance the hypha became top-heavy and fell back on to the surface of the drop. This was the signal for the rapid liberation of a chain of greyish rectangular conidia, averaging $7 \times 10 \mu$, from the distal end of the aerial hypha. This behaviour of the aerial hyphae was found to be general, and except round the edges, the surface of the drop was speedily covered with ejected spores. The aerial hyphae proved to be microconidiophores and the spores microconidia. When first extruded from the hyphae the conidia are greyish in colour, rectangular in shape and filled with granular protoplasm. In an hour after extrusion from the conidiophore the protoplasm of the conidium became vacuolated and the conidia became rounder and larger. After this they gradually turned brown and in twenty-four hours became reddish-brown with a darker coloured central portion, and measured 15 to $10 \mu \times 9$ to 7μ . The conidiophores measured 300μ or more in length.

During the above developments the submerged hyphae were observed to form short branches from which chains of conidia, larger and darker in colour than the microconidia, were produced. Several likely portions of mycelium were fixed, and stages in the formation of these, which proved to be macroconidia, were observed. The short clavate hyphae soon showed the formation of a clear band near the apex which divided off the protoplasm of the globose end from the remainder. Five minutes later the cell-wall at the apex of the hypha disappeared and a spherical mass of granular protoplasm was extruded. A distinct cell-wall was evident forty minutes after, and the protoplasm was now more coarsely granular and showed vacuolation. After this more conidia were formed in basipetal succession in a chain. Their protoplasm became vacuolated and the walls gradually darkened. When first extruded the protoplasm is finely granular, about thirty minutes afterwards several small vacuoles appear which gradually approach the centre and coalesce. After the formation of the central vacuole the wall begins to darken, and in twelve hours becomes sooty-black in colour, when the central vacuole can no longer be observed. They measure on the average $22 \times 15 \mu$.

In many cases in this drop submerged macroconidiophores and collapsed aerial microconidiophores were seen to be developed from the same hypha, thus bearing out Massee's statement that micro- and macroconidia are developed from the same mycelium. The drop in question was freely exposed to the diffused light of the laboratory, but not to direct sunlight, consequently darkness is not necessary for the formation of macroconidia. A similar development was observed in several other hanging-drops containing macroconidia only.

Inoculation experiments were performed on healthy canes, using a mixture of micro- and macroconidia obtained from a pure culture. These were placed on a fresh wound made by tearing off a growing leaf, and also in wounds made in the cane with a sterilized needle. Neighbouring canes were treated in an exactly similar way, to serve as a control. In

all cases the wounds were covered with sterilized vaseline to prevent the entry of other spores. In eight days the tissues of the cane for some distance round the point of infection were much discoloured, and microscopic examination showed that the cells were filled with mycelium which passed from cell to cell by means of pits in the walls after the manner described by Massee. Cultures made of portions of this discoloured tissue showed the copious development of micro- and macroconidia in two days. The control experiments showed no infection.

MICROCONIDIAL STAGE.

It now became necessary to obtain a single spore-culture of this phase of the Fungus and follow out the development as in the case of the macroconidia above.

A hanging-drop culture was obtained containing a single microconidium. The spore germinated six hours after sowing and sent out a colourless septate hypha which soon branched. The mycelium quickly extended right through the drop, and its subsequent development was similar in all respects to that described above in the case of the macroconidia. Aerial microconidiophores were formed as before which ejected chains of microconidia, while the submerged hyphae formed chains of macroconidia inside the drop.

The number of microconidia formed by one conidiophore is frequently very large, as many as ninety being observed.

The development of the micro- and macroconidia is therefore practically identical; had the drops not been labelled it would have been impossible to have distinguished between them.

In several cases the microconidia in this and other drops were noted, when a few days old, to germinate in the drop in a curious manner. A narrow hypha was produced $180\ \mu$ or more in length, into which the brown contents of the microconidium passed, leaving the conidium empty and colourless.

The darkening of the conidia is therefore due to a change in colour in the spore-contents and not in the spore-wall. Possibly the change in colour noted in the macroconidia is due to the same cause. The hypha now proceeded to divide up by transverse walls commencing at the distal end, and the segments which were from 10 to 15 μ in length and 2 to 3 μ in breadth now proceeded to round themselves off into a chain of spores. No further changes resulted.

In many of the drops the old brown mycelium was seen to enter on a second phase of growth by sending out very narrow, colourless unseptate hyphae which rarely branched. After a time the growing end passed down into the air as a conidiophore which formed microspores in the ordinary way.

ASCIGEROUS STAGE.

Extended search on diseased canes and in old cultures of macroconidia has up to the present not been rewarded by the discovery of perithecia resembling those described by Masee. I am preparing a series of cultures of macroconidia in large flasks with the hope of obtaining perithecia and following out the development of the ascospores.

Howard, Albert. 1900. "On Trichosphaeria sacchari, Masee; a fungus causing a disease of the sugar-cane known as 'rind fungus'." *Annals of botany* 14, 617–631. <https://doi.org/10.1093/oxfordjournals.aob.a088795>.

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