STUDIES IN BACTERIOSIS II[1].

A BROWN BLOTCH DISEASE OF CULTIVATED MUSHROOMS.

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INTRODUCTION.

During the spring of 1918 a disease of mushrooms was observed in the beds of a large nursery in Brentford, Middlesex. Mushrooms have been cultivated there for upwards of forty years, and although the disease was not entirely new to the grower there would seem to have been no outbreak in the past of comparable severity. Previous occurrences had been of little importance and had been attributed to the effect of draughts. The disease was first noticed in April, and threatened to assume serious proportions. On examination of one of the houses in the first week of May about one-third of the crop in certain parts of the house was found to be affected[2]. The financial loss at this time when the wholesale price of mushrooms was at its maximum must have been considerable, affected mushrooms being so disfigured as to be unsaleable. Later on in the month, however, the disease became less prevalent, and towards the end of June had become insignificant in amount. The reason for this rapid falling off will be discussed later.

The Symptoms of the Disease.

Circular or irregular spots of a chestnut brown colour appear on the surface of the cap of the mushroom when this has attained a diameter of an inch or so; they spread rapidly and coalesce to form large patches which occasionally cover the entire surface of the pileus. (See Fig. 1.) The stalks also may show the disease, but it is characteristically a disease

[1] The first of this series of studies appears in *The Journal of Agricultural Science*, vol. VIII, 1917.

[2] The areas most affected were those within ten feet of either door; this seemed to indicate that draughts were in some way connected with the spread of the disease.

of the cap. The patches become slightly depressed, dry up and crack radially as the mushroom grows. They arise at or near the margin of the mushroom or at the point of contact of one with another, *i.e.* places at which water is likely to remain for some time after the sprinkling of the bed. At the outset it seemed likely that some micro-organism finds entrance at these spots after multiplying in such standing water. The disease spreads rapidly from one diseased head to others in the same

Fig. 1. Mushrooms naturally infected.

cluster in a way which supports the above view as to the mode of dissemination of the disease, the organism presumably being carried by insects or by splashings from infected drops of water during watering.

The disease is very superficial; on peeling off the "skin" the underlying tissue is found to be affected to a depth of, at most, one to three millimetres (the diagrammatic sketch, Fig. 3, shows clearly the slight extent of penetration of the tissue by bacteria, in this case less than half a millimetre), and very frequently is found to be perfectly white and healthy. The affected underlying tissue has a rather water-soaked appearance and a mouse grey or yellowish grey colour.

Isolation of the Causal Organism.

Microscopical examination of a section of the grey tissue underlying the brown spot shows that the hyphae are invaded by crowds of bacteria. The preparation from which Figs. 3 and 4 are taken shows the pileus completely disorganised and penetration of bacteria to a depth of about half a millimetre; the organisms are mainly within the cells of the hyphae as is shown in Fig. 4.

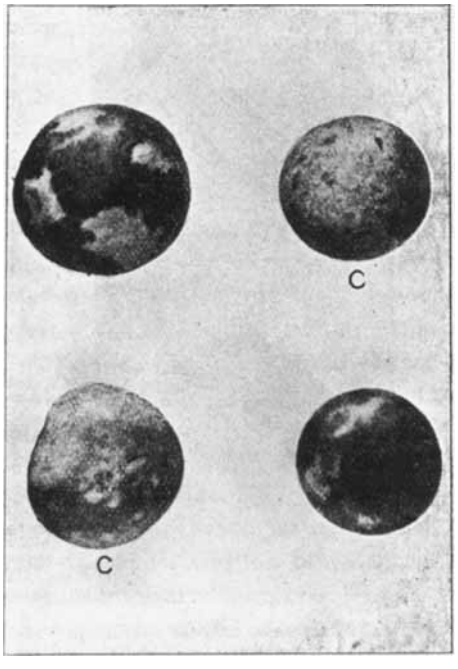

Fig. 2. Mushrooms artificially infected. *cc* controls.

Removal with aseptic precautions of some of the bacterial tissue and inoculation into mushroom-extract or other nutrient media yielded, without the slightest difficulty, pure cultures of an organism which was identical in every case, leaving no doubt that the causal organism had been obtained. The organism was plated several times on mushroom-extract agar and on bouillon-agar in order to ensure purity and from the first the colonies were all of one type.

INFECTION EXPERIMENTS.

Numerous infection experiments have been made upon young mushrooms growing in the beds at Brentford and upon mushrooms removed from the bed and kept in fresh condition with their stalks embedded in moist soil covered with bell-jars. A suspension in sterilised mushroom-

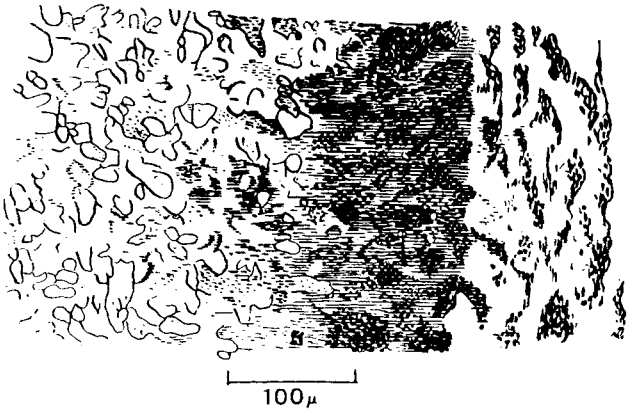

Fig. 3. Camera drawing from a 3μ radial section through the cap of a diseased mushroom, showing complete disorganisation of the outer hyphae and penetration of the tissue by bacteria to a depth of not more than half a millimetre.

Fig. 4. Camera drawing from same section as Fig. 3, showing hyphae penetrated by bacteria.

extract of the organism from an agar slope was painted over the surface of the mushroom with a sterilised camel-hair brush, numerous control experiments with the uninoculated extract being made at the same time. In every case typical brown streaks corresponding closely to and spreading irregularly from the margins of the pattern painted resulted from such inoculations. (See Fig. 2.) The controls showed either no sign at all, or a slight indentation caused by the mechanical injury of the soft tissue of the mushroom. The infections at Brentford were examined only after three days, but in those made in the laboratory the pattern was well developed overnight and did not extend far afterwards. In some of these the browning was distinctly visible in as short a time as five hours; this led to the suspicion that ammonia might be the direct cause of the browning, and that the organism invading the hyphae was one of the common ammonifiers of the soil which had entered dead tissue resulting from the lethal action of ammonia upon the cells, a mode of entry of a saprophyte into a living plant which the work of Jensen (4) has shown to be possible.

To test this hypothesis control experiments were made with mushroom-broth containing additions of ammonia; when these additions were quite small, sufficient only to render the broth just alkaline to litmus, no coloration whatever was produced, but with addition sufficient to make the liquid smell distinctly of ammonia a brown coloration was obtained but of a dull tint easily distinguishable from the warm chestnut shade characteristic of the disease. Further control experiments were made with suspensions of *Bacillus fluorescens liquefaciens*, *Bacillus Proteus*, and *Bacillus mesentericus*, all of which are powerful ammonifying organisms of the soil, and, although repeated on many occasions with cultures of various ages, no browning of the tissue resulted.

The organism was, then, to be regarded as a parasite and this was later definitely established by the loss of virulence in some agar cultures of two months' standing, and in sub-cultures of 24 hours' growth made from these.

DESCRIPTION OF THE ORGANISM.

I. Morphological Characters.

Form and Size. The organism is a short rod with rounded ends. In common with most others it varies considerably in size, according to the rate of growth and the medium employed. Measurements are held by the author to be of little significance, but for the purpose of comparison, measurements were made upon a 24 hours' growth on bouillon-

agar, the sixth transfer from the original, incubated at 22° C. The colony had attained a diameter of 4 mm. The film was fixed by ten minutes' immersion in 4 per cent. formalin, stained for ten minutes in aqueous methyl violet, and examined in oil. The length of the organism varied from 0·9 μ to 1·7 μ and the breadth from 0·4 μ to 0·5 μ. The growth was very viscous and suggested the presence of a capsule; when a suspension of the organism in normal saline was examined by dark-ground illumination, however, no sign of a capsule was discovered.

Motility and Flagella. The organism is actively motile in young cultures in broth and upon agar, but it comes to rest early, frequently after 24 hours at air temperature on solid media. The movement is of a free swimming type without other specific characteristic. The flagella have been stained by Stevens' modification of Van Ermengen's stain and by a new method devised by the late Professor H. G. Plimmer[1]. The flagella are two to four times the length of the organism, one or two in number, occasionally three and more rarely four or five, arranged at one pole. (See Fig. 5.) The organism is therefore a *Pseudomonas*.

Fig. 5. Camera drawing from a preparation stained by the method of Plimmer.

Staining. The organism stains well with carbol fuchsin, gentian violet and methyl violet, does not stain by Gram's method, is not acid fast, and gives negative results with the usual capsule stains.

[1] I hope with Dr R. H. A. Plimmer's permission to publish a description of this staining method if the details can be worked out from the data left by Professor Plimmer.

II. Cultural Characters.

The organism grows equally well in mushroom-extract and in beef-extract (Jardox) bouillon + 10, and on solid media from these. It also grows luxuriantly upon potato-mush agar.

Bouillon + 10. Turbidity was strongly marked after 24 hours at 22° C. A strong pellicle and faint fluorescence were formed in cultures made during the first two weeks after isolation, but both these characters disappeared in later cultures. The pellicle was easily broken by shaking and did not re-form. A strong pellicle also formed on mushroom-extract. In all cases a slight ring was formed, and after a week or so the liquid was very viscous and mucus-like. It had become strongly alkaline to litmus and smelt of ammonia.

Bouillon-gelatine Stab. After 48 hours at air temperature liquefaction had commenced, the top 3 mm. being completely liquid with a funnel-shaped depression extending to a depth of some 10 mm. The bottom of the funnel was occupied by a yellowish white granular mass. Growth along the stab was barely perceptible. Complete liquefaction occurred in about 10 days, at air temperature.

Bouillon-gelatine Plate. After 24 hours at 22° C. the colonies had a diameter of 5 or 6 mm. and liquefaction was basin-shaped with a granular deposit at the bottom. In three days the gelatine was completely liquid with a strong ammoniacal odour, resembling somewhat that of stale urine.

Bouillon-agar Stab. After 24 hours at 22° C. surface growth was yellowish white and about 4 mm. in diameter. Growth along the stab was perceptible only in the top half centimetre. After 72 hours growth could still be seen only towards the top of the stab. The tube was left in the incubator from June 5th till October 2nd, and growth to the bottom of the stab could then be observed, but only by the use of a strong light. The organism was still viable after four months' incubation at 22° C.

Bouillon-agar Slope. The streak was well developed after 20 hours at 22° C. as a dirty bluish-white wet-shining streak 2 mm. wide, with slightly raised flat contour. When collected in mass by a wire the colour was faintly yellow tinted. The surrounding medium was bluish-green but without fluorescence.

Bouillon-agar Slope under Anaërobic Conditions. Growth was slight but distinctly perceptible. The organism is a facultative anaërobe with a very marked preference for aërobic conditions.

Bouillon-agar Plate. Growth was very rapid at air temperature. After 20 hours colonies had a diameter of 1 mm., and after 49 hours of 4 or 5 mm. The colonies were round, raised, wet-shining, and of a dirty greenish-white colour, the margins later became lobed and spreading. There was no fluorescence but a greenish precipitate was formed in the gel surrounding the colonies. Colonies in the depth of the medium were broadly lenticular.

Optimum Temperature for Growth. Tubes of bouillon were inoculated each with one loopful from a broth culture and incubated at 6°, 13°, 18°, 20°, 22°, 25°, and 30° C. After two days' incubation clouding was most dense at 25° C.; growth at 6° C. was not apparent until the fifth day.

Thermal Death Point. Tubes of bouillon were seeded each with 1 cc. of a 24 hours' culture at 25° C. They were immersed in a bath at the usual range of temperatures, the temperature of the tubes being controlled by a thermometer immersed in a control tube of broth. Ten minutes heating was allowed at each of the temperatures given below and the tubes were then incubated at 25° C. for seven days.

Temperature of bath	Temperature of control tube	Result
46°	45° rising to 46°	Living
48°	46° ,, 48°	,,
49°	47° ,, 49°	,,
49·5°	48° ,, 49·5°	,,
51°	50° ,, 51°	Dead
52°	51° ,, 52°	,,

III. PHYSIOLOGICAL CHARACTERS.

The culture used for these experiments was the fifth transfer from the original and had been on no other medium than bouillon-agar + 10. A tube of bouillon was heavily inoculated and a loopful from this used for each test. The temperature of incubation was 22° C.

10 *per cent. Witte Peptone* + 1 *per cent. Glucose.* Acid on the second day, no gas formed in Durham tube; no change after 15 days.

10 *per cent. Witte Peptone* + 1 *per cent. Lactose.* No acid and no gas; a thin film with an oily appearance, a slight ring formed and liquid became slightly turbid.

10 *per cent. Witte Peptone* + 1 *per cent. Saccharose.* No acid and no gas; growth as above.

Bouillon + 2 *per cent. Glucose.* Acid after 24 hours, no gas, no pellicle, no ring; no further change up to 15 days.

Bouillon + 2 per cent. Lactose. No acid, no gas, slight pellicle, no ring.

Bouillon + 2 per cent. Saccharose. No acid, no gas, slight pellicle, no ring.

Bouillon + 2 per cent. Mannite. No acid, no gas, no pellicle, no ring; slightly bleached at bottom of tube after 15 days.

Uschinsky's Solution. Alkaline on second day, no gas, loose pellicle, no fluorescence. After 15 days: completely bleached, no gas, no fluorescence, thin pellicle and strong ring. After two months: blue above, bleached below, no fluorescence, very viscous deposit. After four months: the ultramarine colour had completely returned.

Litmus Milk. Curd separated after the fourth day, the reaction was neutral but became slightly alkaline later. On the seventh day the curd occupied only one-third of the volume of liquid, the litmus was bleached and a strong green fluorescence developed in the whey. Most of the curd was digested but some remained at the bottom of the tube after six weeks.

Milk. The curd formed and was partially digested as above, and the whey became strongly fluorescent.

Potato Plug. After two days the streak was visible as a white, wet-shining, raised mass; after five days the colour had assumed a faint yellow tinge; the potato was not discoloured. Pulped in water after three weeks, the iodine test showed slight diastatic action.

Dunham's Solution. Became slightly turbid on the second day; no pellicle and no ring formed. After 30 days a trace of indol was present.

Nitrate Bouillon. A loose pellicle formed after 24 hours, the liquid became very viscous and of a yellowish-green colour. Nitrate was not produced, but ammonia was formed. This was probably a product of the breakdown of protein since nitrate was still present after two months' incubation. The presence of nitrate was not due to the death of the organism previous to destruction of all the nitrate since the culture was found to be still viable at the end of two months. No gas was formed.

GENERAL DISCUSSION, COMPARISON OF THE ORGANISM WITH ALLIED STRAINS.

In searching for the mode of entry of the organism to the sheds the possible sources to be considered were: (1) the manure used in making the beds; (2) the spawn; (3) the mould used for casing; (4) the straw litter for covering the beds after spawning; (5) the water used for sprinkling; and (6) the air. Suspicion of the manure was removed by

the fact that during fermentation the temperature usually rises to 120° or 130° F., a moist temperature sufficiently near to the thermal death point of the organism to eliminate this source. The other materials used on the bed, though not free from suspicion, were hardly likely to have introduced the organism, since, as stated in the Introduction, the disease did not appear uniformly distributed over the beds. The water used for sprinkling the beds was deemed to be a likely source. This was taken from the town main and from an old well. Both of these supplies were sampled on two occasions and platings made upon mushroom-extract agar, many colonies of ammonifying organisms developed on the plates and most of these were tested by inoculation upon growing mushrooms, but in no case was there any production of the disease symptoms. Early suspicion fell upon the air as the carrier of the organism since, as stated in the footnote on p. 206, the disease, originating near the doors and windows of the sheds, seemed to be associated with draughts. The litter when removed from the beds was dumped down outside the sheds and some was packed at the bottom of the doors to exclude draught as much as possible; dust from this litter would be carried into the shed each time the door was opened. A search for the organism upon this material, however, proved abortive.

The disease commenced to decrease in early June, and towards the end of June had practically ceased to exist. This may have been, and probably was, in large measure due to the rapid growth of mushrooms during this warm period, the organism, though a tremendously vigorous grower at air temperature, presumably not being able to keep pace with its active host. Another interpretation is, however, possible; the onset of the disease in March and its fall in June, coupled with the fact that the sheds were surrounded by fruit trees in a large orchard, drew the author's attention to the synchronising of the disease with the period of fruit-blossoming, and suggested that the cause of the disease might be identical with that of the "Pear-blossom Blight," investigated by Barker and Grove (1) in this country, and by Doidge (2) in S. Africa.

This disease had not been specially noticed by the grower but practically no fruit set this year and the disease might well have been partially responsible for this failure.

Comparison was therefore made of the morphological and physiological characters of the mushroom organism with the published records of those of the Pear Blight organism (1, 2 and 3). In most of these the two organisms seemed to be identical. Through the courtesy of Professor Barker, a culture of the latter organism was obtained and careful com-

parative experiments were made. The table given below shows how closely the two organisms agree in their physiological behaviour; certain morphological and cultural characters, however, point to differences which although they may not be specific differences at least serve to differentiate the two organisms as separate strains; these characters were constantly observed even after the two had been cultivated simultaneously through a succession of transfers during two months over which the experiments were made. Barker and Grove's organism does not grow so rapidly as the mushroom organism, it forms long thread-like involution-forms on the second day at 25° C. which the latter does not, it has its optimum for growth at 18° C. whereas the latter grows best at 25° C., the thermal death point which may be conceived to be one of the most secure of criteria is two degrees lower than that of the mush-room organism. Finally, a marked difference is found in the viscosity of liquid cultures: Barker's organism produces in bouillon and Uschinsky's solution[1] a gelatinous deposit in old cultures, whereas the whole solution becomes mucus-like in three-day-old cultures of the mushroom organism.

The organism described by Miss Doidge(2) as the cause of Pear-blossom Blight in S. Africa and named *Bacterium nectarophilum* has many characters in common with the mushroom disease organism. It differs only in the length and number of its flagella, both of which may be merely cultural variations, in the possession of a capsule and in its inability to liquefy gelatine. This last is a very striking character, but we have not as yet sufficient evidence to be able to judge in how far it is a constant one. The work of Morse(5) has shown that in one case, namely that of *Bacillus solanisaprus*, the power to liquefy gelatine could be developed under suitable cultural conditions and that this organism was really a strain of a vigorous liquefier, *Bacillus atrosepticus*. The identity of *Bacterium nectarophilum* with the organism described in this paper is then not beyond the region of possibility, and the hypothesis that Pear-blossom Blight and the Mushroom Disease may be produced by one and the same organism may yet prove to have some foundation in fact. Barker's organism was used in inoculation of mushrooms and found to have no effect upon them, but this has little significance since the organism had been in artificial culture for a considerable time and

[1] Barker and Grove's organism was found by Miss Doidge (2, p. 58) to give no growth in Uschinsky's solution. This was not my experience. Slight growth resulted and the liquid became strongly alkaline, then bleached at the bottom and a gelatinous precipitate was formed.

might well have lost its original parasitic properties. Careful observation will therefore be made next spring for signs of either disease, and it is hoped that the question of identity in etiology may then be definitely settled.

A disease of Mushrooms has previously been found by Tolaas[6] in which the symptoms were identical with those here described. His description of the causal organism is rather meagre, but in such reactions as are given, except in two instances, there is complete agreement with those of the organism described in this paper. The exceptions are in the reduction of nitrates and in the action upon starch (see comparative schedule, p. 218). It is not clear from his brief statement "Nitrates are reduced" how Tolaas arrived at this conclusion, and, in fact, it is possible that both in this and in testing the action of the organism upon starch the methods of technique employed by Tolaas were different from those employed by the authors, and have led them to opposite conclusions. Putting aside these two characters the organisms are closely similar, and, since the symptoms of the disease correspond exactly with those described by him, there seems little doubt that we have here the disease discovered by Tolaas. He suggests that the organism may be a parasitic strain of *Pseudomonas fluorescens*. This may indeed be the case, but until it is proved it seems well to give the mushroom disease organism a distinguishing name, and, in spite of the slight disagreement in physiological behaviour, the organism here described is believed to be identical with that of Tolaas and the name *Pseudomonas Tolaasi* is suggested. The organism, however, may yet be shown to be identical with that of Barker and Grove, in which case it will be necessary to re-name it in order to give due priority to these investigators.

Control Measures. Means of controlling the disease have not been investigated for the reason that it subsided and disappeared naturally. Tolaas found an efficient method of prevention of the disease in the fumigation of the beds with sulphur previous to spawning. In this connection it is of interest to note that sulphur fumigation has been the general practice in the houses at Brentford, but was abandoned for the season in which the outbreak occurred.

Comparative Schedule.

	Mushroom Disease.		Pear Blossom Blight.	
	Organism of Tolaas	Organism of Paine	Organism of Barker and Grove	Organism of Doidge
1. Dimensions	1·0μ–1·5μ × 0·5μ	0·9μ–1·7μ × 0·4μ–0·5μ	2μ–4μ × 0·5μ–0·8μ	0·5μ–3μ × 0·45μ–0·7μ
2. Flagella	Polar 1 to 2	Polar 1 to 2, occasionally 3. 4 or 5	Polar 3 to 6	Polar 1 to 5
3. Capsule	—	Absent	Absent	Present
4. Optimum temperature	—	25° C.	18° C.	25° C.
5. Thermal death point	—	49°–50° C.	47°–48° C.	49° C.
6. Colour	Fluorescent	Fluorescent	Fluorescent	Fluorescent
7. Bouillon	Well clouded in 36 hours; pellicle and ring—very thick in some cases	Well clouded in 24 hours; thick pellicle in early cultures; thin pellicle and ring in later cultures; very viscous—mucoid	Slightly clouded in 24 hours; slight rim and pellicle; gelatinous precipitate	Well clouded in 24 hours; tendency to pellicle formation; considerable viscid sediment
8. Gelatine stab	Liquefaction saccate	Liquefaction infundibuliform; granular precipitate; growth in upper part of stab only	Liquefaction crateriform; white precipitate; no liquefaction along stab	No liquefaction; growth in upper part of stab only
9. Agar colonies	Shining greyish-white; greenish pigmentation of the medium	Round, raised, wet-shining; later lobed and spreading; greenish precipitate in medium	Round, raised, wet-shining; later lobed and spreading	Spreading irregular margins
10. Uschinsky's solution	No acid; no gas	Alkaline; no gas; bleached; mucoid deposit	Alkaline; no gas; bleached; very little growth	Heavy viscid deposit
11. Litmus milk	Coagulated; alkaline whey; complete digestion of casein	Coagulated; alkaline whey; partial digestion of casein; fluorescence	No coagulation; alkaline; complete digestion of casein	No coagulation; colour unchanged at first, bleached later; slow digestion of casein
12. Indol formation	Slight	Slight	None	None
13. Nitrate reduction	Reduced	Not reduced	Not reduced	Not reduced
14. Diastatic action	None	Feeble	Feeble	Feeble
15. Group number	221·2333133	221·2332123	221·3332123	222·2332123

SUMMARY.

A disease of cultivated mushrooms is described in which patches of a chestnut brown colour so disfigure the pileus as to render the affected crop quite unmarketable.

The disease is identical with that described in America by Tolaas, but left unnamed by him.

The cause of the disease is a small bacterial parasite which may possibly be a strain of *Pseudomonas fluorescens* and may prove to be identical with the organism which produces Pear-blossom Blight.

Until its identity is established by further experiment it seems well to give it a distinguishing mark, and the name *Pseudomonas Tolaasi* is suggested.

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