

Studies in the Physiology of Parasitism.

V. Infection by *Colletotrichum Lindemuthianum*.

BY

P. K. DEY, B.Sc.

From the Department of Plant Physiology and Pathology, Imperial College of Science and Technology, London.

With Plate XXI.

IN all previous works on bean anthracnose caused by the well-known parasite *Colletotrichum Lindemuthianum*, no observations of the actual mode of infection have been made. Whetzel (14) gives a diagram of penetration of the germ-tube through the outer surface of the bean-pod but he does not appear to have made any study of the details of penetration. Frank (6) observed that the germ-tubes of *C. Lindemuthianum* produced on the surface of the host dark-brown appressoria from which infection took place, but he neither described nor gave any figures of the process of infection.

In the case of infection by *Botrytis cinerea*, workers like Büsgen (4) and Marshall Ward (12), believed that the germ-tube effected its entrance by softening and dissolving the cuticularized epidermal wall of the host; Voges (11) also speaks of the slime formed by the germ-tube of *Fusicladium* softening the cuticle. But, as pointed out by Blackman and Welsford (1), no critical observations were in any case made. It was Brown (2) who, working with *B. cinerea*, first showed that the cuticle of the host is quite unaffected by the powerful extract which he obtained from young hyphae. When such an extract is placed on a delicate, uninjured rose petal, the cuticle and the underlying tissue remain unchanged; when placed on wounded petals, however, disorganization of the tissue takes place in a very short time. He thus indicated that the germ-tubes of *B. cinerea* have no power of causing a softening of the cuticle, and that the latter is impermeable to the enzymes which dissolve the non-cuticularized walls. It thus seemed probable that penetration of the cuticle by the germ-tube takes place by mechanical means. Blackman and Welsford (1) made careful microscopic study of this question, and showed that the passage of the germ-

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tube of *B. cinerea* into the bean-leaf was effected by a rupture of the cuticle, due to the mechanical pressure exerted by the germ-tube.

The object of the present work was to make a careful microscopic examination of the stages of infection of the bean by *Colletotrichum Lindemuthianum*, and to discover if this fungus, belonging to a quite different group, acts in the same way as *Botrytis cinerea*.

Methods. The cultures were made mostly in a medium of maize-meal agar, also on French bean-pods, autoclaved in test-tubes. The maize-meal agar medium was made in the following way—30 grm. of maize-meal were cooked in 1,000 c.c. water at 100° C. for half an hour. To this 15 grm. of agar were added, and the mixture heated to 110° C., to dissolve the agar. The medium thus prepared was 'tubed' and autoclaved at 120° C. for 20 minutes.

Spores were sown on slopes and Petri dishes. In three to four days small white patches appear. Examination of the young growth shows a felted mass of fine hyphae, with indications here and there of formation of acervuli. The hyphae frequently swell up into vesicles, as described by Stoneman (9), and branch copiously, giving a knotted appearance to the mycelium. After six days these spreading patches were dotted over with very small black areas, which even with a hand lens were seen to consist of very dark spines enclosing a pinkish spore mass. The spores are borne on short erect hyphae, which arise from swollen vesicles, and are abjoined from the tips of the hyphae, two or three in a series. At the same time mucilage is formed which surrounds the spores. After a few more days of growth acervuli become still more numerous, and gradually turn the white background of mycelium into a black mass. The spore masses are now visible to the naked eye as round pinkish globules. The hyphae of the fungus are colourless, but the dark appearance of an old colony is due to the black stiff spines of the numerous acervuli. In cultures a month old or more, the spore masses, instead of remaining as moist pink globules, seem to dry up and shrink, becoming at the same time rather creamy in colour. They also become set with dark-coloured spines, so that old cultures become almost uniformly black and rugged in appearance.

Germination of Spores. Spores from cultures about two weeks old were sown in thin films of sterile tap-water on a clean and flamed slide kept in a Petri dish containing sterile, moist blotting-paper. Under these conditions spores germinate quite readily in 18–24 hours at 20°–25° C.—the optimum temperature for the growth of the fungus, according to Edgerton (5), lying somewhere between 21°–23° C. It was observed that if the drop of water containing the spores had a sharp convex surface, spores lying in the middle of the drop hardly germinated, while most of those lying near the border did; this difference of behaviour is probably due to difference in oxygen supply. During the early stages of germina-

tion the spores swell up and become rather constricted in the middle, and thus have a more or less dumb-bell shaped appearance, as observed by former workers; they sometimes become septate. A germ-tube comes out generally from one end of the spore, but the rare, septate ones produce a germ-tube at each end. In a very few cases more than two germ-tubes were found to develop from a spore. The germ-tube, as it grows, comes in contact with the hard surface of the glass, and as soon as this occurs its tip swells out into a dark-brown, thick-walled, spore-like body, the appressorium (of Fränk) or adhesion organ. Muncie (10) holds that the appressorium is not formed until the germ-tube has reached a certain length. Observation shows, however, that it is formed whenever the germ-tube touches a hard foreign substance; thus it may be produced soon after the germ-tube comes out of the spore, so that it lies almost in contact with the spore (Pl. XXI, Fig. 3 A); on the other hand, the germ-tube may grow to a considerable length before it is produced (Fig. 3). As to the stimulus necessary for its formation, there is no doubt it is the result of contact, as Hasselbring (7) has shown in the case of *Gloeosporium fructigenum*. In this species Hasselbring observed a distinct germ-pore in the appressorium, through which a hypha later grows out. In *C. Lindemuthianum*, however, no such germ-pore is to be found, the hypha always arising at the point of contact of the appressorium with the glass, whatever orientation the appressorium may possess. The young appressorium is sheathed in a mucilaginous coat (Figs. 4, 4 A). This can clearly be demonstrated by staining with very dilute watery gentian violet for 30 seconds, or by mounting the germinated spores in 'collargol' (1). By means of this sheath the appressorium becomes attached to the surface, so that even a fairly strong jet of water does not remove it. In fixed and stained preparations this mucilage appears to be reduced by the dehydrating agents to irregular granules and threads, analogous to the similar structures observed in the germ-tube of *Botrytis cinerea* by Blackman and Welsford (1).

Penetration of the Germ-tube into the Host. In following the details of penetration by the young 'infection hypha', freshly picked; young, juicy pods of French bean (*Phaseolus vulgaris*, vars. Canadian Wonder and Brown Dutch) were used. These two varieties were found to be very susceptible to the disease. The size of the bean-pods varied from 5 to 8 cm. long by about $\frac{1}{2}$ cm. wide. In such young pods the cuticle is thin, and so they were likely to prove suitable for infection studies. Young leaves were first tried, but as their cuticle and the outer walls of their epidermal cells are exceedingly thin, such leaves were found unsuitable for observation of the changes occurring in the cuticle and subcuticular layers during penetration. Spores were taken from a culture on maize-meal agar which had been at 25° C. for about a month; these were stirred thoroughly in 1 c.c. of sterile tap-water till the mucilage holding them together in

masses was removed and a uniform suspension was obtained. Ordinary tap-water was used instead of a nutrient solution, as used by Blackman and Welsford in similar experiments with *B. cinerea*, as infection readily occurs in this, the normal medium, under natural conditions. The density of the suspension was estimated with the naked eye, till a faintly milky appearance was arrived at.

Small drops of this suspension were placed on bean-pods, previously washed thoroughly with sterile water to remove dust and foreign spores, and kept in a moist chamber. Care was taken that the drops spread out in thin films, so that nearly all the spores might be under similar condition of oxygen supply. The pods were then incubated at 25° C. On the second day, that is twenty-four hours after sowing, small bits of the pod under the 'infection drops' (3) were cut out every four hours, and fixed, generally in Carnoy's fluid (absolute alcohol, 6 parts; chloroform, 3 parts; glacial acetic acid, 1 part), but sometimes in Flemming's stronger solution of half strength. The tissue underneath the 'infection drop' does not show any sign of infection during early stages, so that it is not possible to gauge by the eye the stage of infection reached. Now and then small brown spots were visible with the microscope below the 'infection drop'; Frank noticed those spots about twenty-four hours after sowing the spores, and held that they were due to infection. These spots, however, appear even when tap-water is substituted for the 'infection drops'. Thus they are not the result of infection, but may be due to osmotic disturbances.

The fixed material was embedded in paraffin, and in every case sections 4 μ in thickness were cut. Heidenhain's iron-alum haematoxylin and erythrosin were first used for staining, but as erythrosin does not bring out the cuticle, a counterstain, Sudan III, was employed instead. In making up this stain 0.01 grm. of Sudan III (Grübler's) is first made into a paste with absolute alcohol, and then made up to 5 c.c. with 95 % alcohol. It is allowed to stand for a few days, and then 5 c.c. of pure glycerine are added to it. Sections thus stained were mounted in glycerine jelly (10).

Observations. The germ-tube produced by the spore soon forms a brown, thick-walled, more or less spherical appressorium on the surface of the pod (6). The appressorium is pressed closely against the surface, the part in actual contact with the pod becoming somewhat flattened. The appressorium appears to be held on the surface by means of its mucilaginous sheath. The spore is also fixed in some way to the surface, though no mucilaginous envelope has been observed around it; further growth of the germ-tube thus causes the tube to curve up, since it is fixed at both ends (Fig. 5). Some pressure is thus exerted on the surface, and, no doubt as a result of this, a slight indentation of the wall of the epidermal cell is to be observed. This is the first preliminary to penetration.

In material on which spores have been growing for 24-40 hours, nothing beyond this preliminary stage is to be observed. The activities of the young germ-tube during this period are confined to the production of the appressorium, for the development of the infection hypha is never observed earlier than forty-eight hours after sowing, while the appressoria may be formed within twenty-four hours. Muncie held that the incubation period (between sowing and penetration) varied from $3\frac{1}{2}$ to 6 days, according to the amount of moisture present and the temperature. Edgerton was not able to obtain definite signs of infection earlier than four and a half days after sowing (8). These workers, of course, did not observe the very earliest stages of infection investigated here.

The next stage in infection is the development of a slight protuberance on that part of the appressorium in contact with the cuticular surface. This protuberance pushes in the cuticle still farther, so that a distinct hollow is formed on the surface (Fig. 5 A). From this protuberance there now develops a very fine peg-like outgrowth, the 'infection hypha', which stains homogeneously, and in which the distinction of wall and cavity could not be observed.¹ As the result of the development of the 'infection hypha' a rupture is caused in the relatively inelastic cuticle (Fig. 6). A very careful and searching study was made for evidence of disorganization or swelling or any other change in the cuticle at this stage or later, but no such evidence was obtainable. *C. Lindemuthianum* forms then no special substance which is capable of producing softening or chemical change in the cuticle. These facts prove that, as in the case of *B. cinerea*, the 'infection hypha' obtains entrance by breaking or rupturing the cuticle of the host as the result of the pressure exerted by the development of the 'infection hypha' arising from the appressorium. In sections this rupture is indicated by a break in the continuity of the cuticle near the peg (Figs. 7, 8, 10, 14, 15). The perforation to be observed is considerably wider than the 'infection hypha' itself. This may be explained by the fact that the epidermis of the bean-pod is no doubt in a state of tension, owing to the turgescence of the inner tissue.² When the 'infection hypha' enters through the cuticle and brings about softening and disorganization of the subcuticular layers, the cuticle is free to contract at that particular spot, and consequently the aperture is widened.

Once the cuticle is ruptured, the pressure due to growth in length of the germ-tube forces the appressorium a little farther into the surface of the pod; this is well seen in Fig. 15. In the same figure it is to be noted that the penetrating peg, which is at first exceedingly fine, swells into a hypha of normal size soon after it reaches the softer cellulose layers.

¹ The relation of the 'infection hypha' to the inner and outer layers of the appressorium could not be determined.

² That the outer tissues of the fruit are so stretched can be well demonstrated in the cucumber.

The passage of the fungus through the cell-wall is associated with a swelling and dissolution of the cellulose layers. The first sign of this action on the wall is the disappearance of the uniform stratification of the cell-wall, and the appearance of a clearer area round the 'infection hypha'. No such change in the subcuticular layers is to be noticed, however, before the break in the cuticle is effected. The cuticle apparently bars completely the passage into these layers of any enzyme secreted by the 'infection hypha'. The behaviour of *C. Lindemuthianum* is in this respect quite analogous with that of *B. cinerea*.

The 'infection hypha', after growing a short distance into the host, produces at its end a small vesicle (Figs. 9, 10, 11, 12, 13, 14) from which one or more branches emerge and spread through the host tissue. This vesicle may be developed in the swollen subcuticular layers of the wall, or its formation may be delayed till the 'infection hypha' has penetrated far into the cell. This vesicle appears to be similar to that described by Marshall Ward (13) for the infection process in *Uredo dispersa*; the nature of this vesicle is obscure.

The effect of the entrance of the hypha into the host-cell is to be observed in Figs. 14, 15, 16. From these figures it is evident that the collapse of the cell immediately beneath the place of entry takes place only after a hypha is well established in its cavity; the invading hypha may be the original 'infection hypha' (Fig. 15) or a branch from the 'vesicle' (Fig. 16). When the 'infection hypha' enters the cavity of the cell, the protoplasmic contents of the latter apparently flow towards the hypha and collect round it (Figs. 13, 14, 15). Movement of nuclei, similar to that found by Blackman and Welsford in the bean-cell invaded by *B. cinerea*, has never been observed in this case.

The stomata of the host do not seem to afford an easier channel of infection than that through the epidermal cell. Only one instance of the passage of an 'infection hypha' through the stoma was found in all the material examined. Even there infection has taken place from an appressorium in the usual manner, and not directly by the germ-tube (Fig. 17).

In conclusion, the author wishes to express his gratitude to Professor V. H. Blackman, at whose instigation the work was undertaken, and from whom he has throughout received most helpful criticism and advice.

SUMMARY.

The spore of *Colletotrichum Lindemuthianum*, when germinating on the host plant, produces a germ-tube, which directly it comes in contact with the host surface develops at its end a thick-walled, dark-coloured appressorium. The appressorium becomes attached closely to the surface by the help of its mucilaginous envelope.

From the surface of the appressorium in contact with the host, a peg-like 'infection hypha' grows out, which ruptures mechanically the cuticular layer, and then brings about swelling of the subcuticular layers, no doubt by enzymic action.

There is no evidence that the 'infection hypha' can exert any swelling, disorganizing, or other chemical action upon the cuticle.

The mechanism by which the 'infection hypha' of *C. Lindemuthianum* penetrates the host surface is thus in all respects similar to the mechanism employed by the germ-tube of *Botrytis cinerea*.

REFERENCES.

1. BLACKMAN, V. H., and WELSFORD, E. J.: Studies in the Physiology of Parasitism. II. Infection by *Botrytis cinerea*. Ann. Bot., vol. xxx, p. 389, 1916.
2. BROWN, W.: Studies in the Physiology of Parasitism. I. The Action of *Botrytis cinerea*. Ann. Bot., vol. xxix, p. 313, 1915.
3. ———: Studies in the Physiology of Parasitism. III. On the Relation between the 'Infection Drop' and the Underlying Host Tissue. Ann. Bot., vol. xxx, p. 439, 1916.
4. BÜSGEN, M.: Ueber einige Eigenschaften der Keimlinge parasitischer Pilze. Bot. Zeit., vol. i, p. 53, 1893.
5. EDGERTON, C. W.: Effect of Temperature on *Glomerella*. Phytopath., vol. v, p. 247, 1915.
6. FRANK, B.: Ueber einige neue, weniger bekannte Pflanzenkrankheiten. Land. Jahrb., vol. xii, p. 511, 1883.
7. HASSELBRING, H.: The Appressoria of the Anthracnoses. Bot. Gaz., vol. xlii, p. 135, 1906.
8. MUNCIE, J. H.: Experiments on the Control of Bean Blight. Michigan Agri. Coll. Expt. Sta. Technical Bull., 38, 1917.
9. STONEMAN, B.: A Comparative Study of the Development of some Anthracnoses. Bot. Gaz., vol. xxvi, p. 69, 1898.
10. STRASBURGER und KOERNICKE: Botanisches Praktikum, 5th ed., p. 809, 1913.
11. VOGES, E.: Die Bekämpfung des *Fusicladium*. Zeit. f. Pflanzenkrank., vol. xx, p. 385, 1910.
12. WARD, H. M.: A Lily Disease. Ann. Bot., vol. ii, p. 319, 1888.
13. ———: On the Histology of *Uredo dispersa*, Erikss., and the 'Mycoplasm' Hypothesis. Phil. Trans., B, vol. cxcvi, p. 26.
14. WHETZEL, H. H.: Some Diseases of Beans. Cornell Univ. Agri. Expt. Sta. Bull., 239, 1906.
15. ———: Bean Anthracnose. Ibid., 255, 1908.

EXPLANATION OF FIGURES IN PLATE XXI.

Illustrating Mr. Dey's paper on Physiology of Parasitism.

All figures except 1-4 A were drawn with the camera lucida, under Koristka $\frac{1}{15}$ in semi-apochromatic, oil-immersion objective, and No. 8 eyepiece. Figs. 1-4 A were drawn under Leitz $\frac{1}{12}$ in. oil-immersion objective, and No. 4 eyepiece.

The host tissue figured is that of the pod of *Phaseolus vulgaris*.

Fig. 1. Germinating spore. $\times 1050$.

Figs. 2-3 A. Germinating spore, showing the appressorium: drawn from material fixed in picro-nigrosin after twenty-four hours of growth. $\times 1050$.

Figs. 4, 4 A. Germinating spore, showing mucilaginous sheath round the appressorium: drawn from fresh material stained with dilute gentian violet. $\times 1050$.

Fig. 5. Germinating spore attached to the epidermis. The germ-tube is curved and is fixed at both ends. $\times 1500$.

Fig. 5 A. A slight protuberance on the appressorium causing an indentation of the wall of the host. The mucilage round the appressorium appears to be reduced to thread-like structures. $\times 1500$.

Fig. 6. The 'infection hypha' has grown out from the appressorium and penetrated the cuticle. $\times 1500$.

Figs. 7, 8. The 'infection hypha' is growing in the subcuticular layers after effecting its entrance by perforating the cuticle. The hole produced in the cuticle is somewhat wider than the 'infection hypha'. $\times 1500$.

Fig. 9. A somewhat later stage than Fig. 8. Swelling and disorganization of the cell-wall is clearly seen. $\times 1500$.

Figs. 10, 11. The 'infection hypha' is swollen into a vesicle in the disorganized cellulose layers. $\times 1500$.

Fig. 12. A somewhat later stage than Fig. 11. The vesicle has given origin to a branch. $\times 1500$.

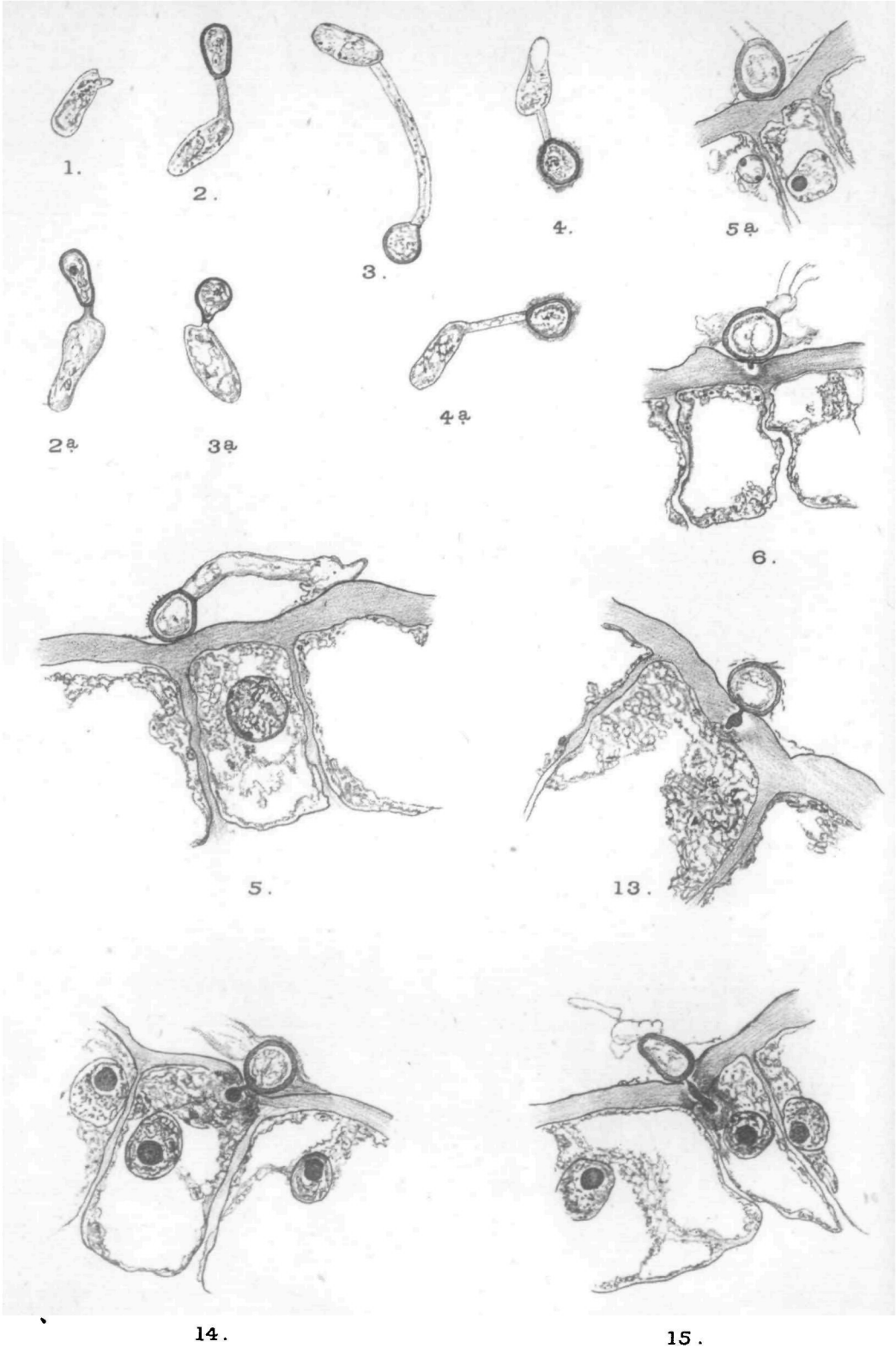
Fig. 13. The vesicle and its outgrowth are visible. Swelling and disorganization of the subcuticular layers have taken place. The protoplasm of the epidermal cell appears to have accumulated in the upper part of the cell, probably as the result of the action of the invading hypha. $\times 1500$.

Fig. 14. The 'infection hypha' has formed the vesicle after passing into the cavity of the cell. Disorganization of the wall and the protoplasm has occurred. $\times 1500$.

Fig. 15. The 'infection hypha' has passed into the cavity of the epidermal cell. Disorganization of the protoplasmic contents is well advanced. The break in the cuticle is now much enlarged and the appressorium is pressed slightly into it. The 'infection hypha', which is very narrow at its base, has swollen into a hypha of normal size in the layers of the cell-wall below the cuticle. The protoplasm of the cell appears to have accumulated round the 'infection hypha'. $\times 1500$.

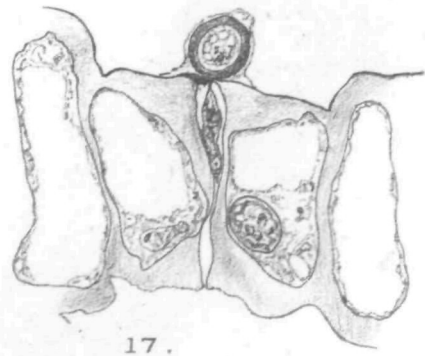
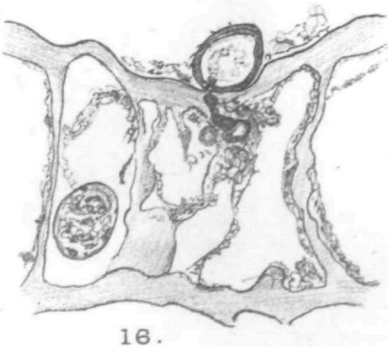
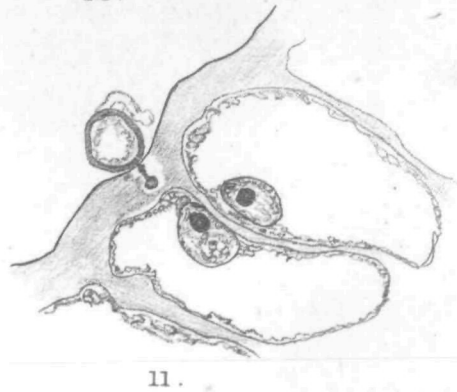
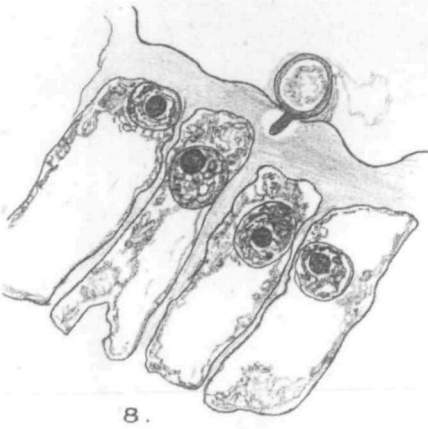
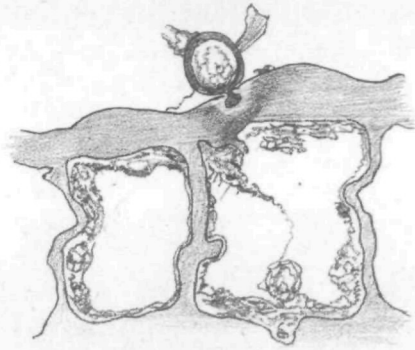
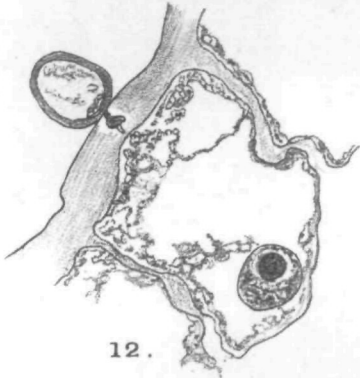
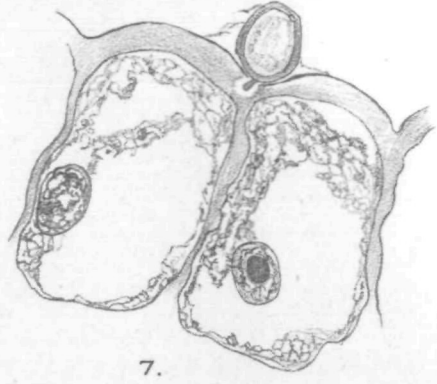
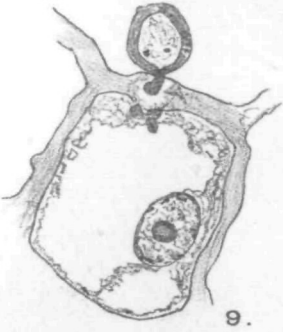
Fig. 16. The 'infection hypha' passing into the cavity of a guard cell has swollen out into a vesicle, and has caused shrinkage and death of the cell. $\times 1500$.

Fig. 17. The appressorium has formed over a stoma. The 'infection hypha' is growing down through the pore of the stoma. $\times 1500$.



P. K.D. del.

DEX-COLLETOTRICHUM.



Huth lith. et imp.

