Studies in the Physiology of Parasitism.¹

III. On the Relation between the 'Infection Drop' and the underlying Host Tissue.

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IN the first of these studies, when the general action of the extract of *Botrytis cinerea* was examined, it was found that the cuticle appeared to offer a great obstacle to the action of the extract, so that in order to obtain rapid results it was necessary to inject the extract into the tissues. The object of the present paper is to examine the relation of the extract to cuticle, and more generally to determine the relation of the 'infection drop'² to the underlying tissue of the host plant. This problem is of considerable biological interest, as on its solution will depend to a large extent our conception of the physiology of the early stages of parasitic attack by fungi of the type of *Botrytis*.

The only work which deals directly with this question is that of de Bary and of Nordhausen. De Bary³ examined the early stages of the attack by *Sclerotinia Libertiana* on the stems of broad bean. Placing the bean stems a short distance in front of the advancing hyphae, he was able to arrange that the latter came in contact with the surface of the stem after a short passage through the air; the stem was thus in contact with the aerial portion only of the mycelium. Under these circumstances, the hyphae did not immediately enter the tissue but proceeded to form attachment organs. While these were being formed de Bary noted that the underlying cells collapsed and became blackened; and this at a time when, as he states, the fungus had not yet penetrated the cuticle of the plant. As a consequence of the death of the underlying cells an exosmosis of nutrient material took place on to the surface. The fungus,

¹ For No. I of this series see Ann. Bot., vol. xxix, 1915, p. 313; for No. II, see Ann. Bot., vol. xxx, 1916, p. 389.

² By this term is meant the drop of fluid in which the spores lie upon the surface of the plant.

³ de Bary, A. . Ueber einige Sclerotinien und Sclerotienkrankheiten. Bot. Zeit., 1886.

now invigorated, commenced an active growth, sending out hyphae in all directions, and in particular through the cuticle of the bean stem into the internal tissue. When the fungus was applied to the bean stem in a drop of nutrient material, de Bary found a similar sequence of phenomena, with the exception that the formation of attachment organs was omitted. De Bary's argument on this subject is as follows: 'The cause of the omission of attachment organ formation (in the latter case) can hardly be other than that the hyphae which are directly surrounded with nutrient secrete the toxin necessary for the softening (*Erweichung*) of the substrate more rapidly than those which have grown through the air, and to which nourishment must first be drawn from the distant assimilating mycelium; that therefore the softening of the host tissue which makes penetration possible takes place immediately after contact of the fungus with the epidermis, so that the resistance which constitutes the stimulus for the formation of attachment organs is not forthcoming.'

On the basis of these observations, de Bary postulates a toxic substance which is capable of diffusing through the cuticle. In examining his extracts from this point of view he was unable to arrive at any definite conclusion. In some cases he found that the fungal extract when placed on the epidermis had no action whatever on the underlying tissue; in others he found that some action did take place. Action of the extract through the cuticle was as a rule more definite with the liquid obtained from sclerotia than with the extracts obtained from ordinary mycelium. The failure to obtain positive results in all cases was ascribed by de Bary to the extract losing its activity before it could get through the cuticle.

Nordhausen¹ accepted de Bary's conclusions as a working hypothesis in his experiments on *Botrytis*. He states that while it is difficult to obtain infection when the spores are sown in large drops of liquid, infection is readily produced when the drops are small. Failure of infection when the drops are large he considers to be the result of undue dilution of the toxic substance, which therefore fails to reach a sufficiently high concentration to be effective. This effective concentration, on the other hand, is readily attained when sowings are made in small drops. As a result the underlying cells are killed and infection is thereby assured. Two experiments of Nordhausen may be specially mentioned:

1. In order to see if the fungus in process of penetrating a membrane excreted any acid substance (such as oxalic acid), he stained pieces of epidermis of *Allium* with Congo red, and allowed the fungus to grow through these. Any excretion of acid would be shown by formation of the blue acid dye in the neighbourhood of the penetrating hyphae. No such effect was however observed.

¹ Nordhausen, M.: Beiträge zur Biologie parasitärer Pilze. Jahrb. f. wiss. Bot., vol. xxxiii, 1899.

2. In order to determine if the parasitic action of the germinating spores was diminished by removing the toxic substances formed in the infection drop, Nordhausen experimented as follows: A strip of filter paper was placed in the infection drop and a current of water made to flow through the paper. Such an arrangement might be expected to carry away a large amount of the toxic substances formed by the drop. Nevertheless the entrance of the fungus was not in any way prejudiced. From this experiment Nordhausen concluded that the toxic substance must be very active even in a state of extreme dilution.

It may be observed, in passing, that the conclusions drawn from the experiment just described are in direct contradiction with those drawn from his observations on the relative efficiency of large and small infection drops.

In the light of the above work it was important to determine whether the extract employed throughout the present investigation was able to penetrate from the outside into the tissue of the host plant. In carrying out this series of experiments, two methods of applying the active extract were adopted:

1. In one series the extract was applied in considerable quantities contained in a chamber formed by a glass ring which was attached by vaseline to the surface of the leaf; the chamber so formed had a capacity of 1–1.5 c.c. This method lent itself readily to the investigation of the activity of the extract at the end of the experiment, so that it could be seen how far failure of the extract to penetrate the cuticle could be set down to its loss of activity by lapse of time, as suggested by de Bary.

2. As the amount of available air might conceivably be concerned in the process, a series of experiments were carried out in which the extract was applied to the leaf in the form of drops comparable in size with the average infection drop (about 30 drops to the c.c.).

The extract was tested on wounded as well as on intact leaves, the wound being produced by shaving off a small piece of epidermis. Also the active extract was compared with extract which had been de-activated by heating to 65° C. The tests were repeated many times and on the leaves and flowers of a large number of plants.

In the case of the wounded petals and of the great majority of wounded leaves a distinct action on the part of the extract spreading outwards from the region of the wound could be seen (in a few hours in the case of petals, overnight in the case of leaves). Where no action could readily be demonstrated under these conditions, the experiments on the corresponding uninjured leaves were rejected. It was certain therefore that all the unwounded leaves and petals under consideration would show a readily noticeable reaction to the presence of the extract if any passage of the latter through the cuticle took place.

In the case of the majority of plants examined, passage of the active

principle of the extract through the cuticle was not observed to take place. Such a negative result was invariably found in experiments with leaves and petals (both surfaces) of *Viola*, *Petunia*, *Dahlia*, leaves of *Vicia Faba*, *Begonia heraclaeifolia*, &c. In a number of cases a certain amount of variation in the results obtained was noticed. Thus, with some specimens, no entrance whatever took place; with others of the same species a certain amount of action was observed. This took the form of discoloured spots, varying in number and extent, but in no case did anything approximating to a general discoloration spreading over the surface of contact make its appearance. Such discoloured spots were occasionally obtained in tests on petals of *Tropaeolum* and *Geranium*; and more frequently in the case of petals of *Rosa* and *Fuchsia*. Nevertheless, even in rose petals which showed many discoloured spots after twenty-four hours' treatment with the extract, portions could be found which were quite unaltered.

Bearing in mind the possibility of the existence of previously undetected injuries of the cuticle (such as insect bites, &c.)¹ as well as the possibility of accidental contamination with living spores which is not always readily detected, we may state that there is no action on the part of the fungal extract when applied to the cuticle; in other words the cuticle, apart from accidents which may occur in cases with some frequency, constitutes an impassable barrier to the passage of the active principle of the fungus. The experiments with petals of *Viola*, which are readily attacked by the fungus, show, moreover, that the germ tubes can easily infect a tissue the cuticle of which is completely impervious to the fungal extract.

In the preceding experiments the extracts employed were such that on injection into the various tissues the latter were disintegrated in the striking manner described in the first paper of this series. Furthermore, there was no question of the extract having lost its activity during the period of the experiment. One particular experiment may be cited in illustration of this point.

A fungal extract when laid on a rose petal for twenty-four hours produced no change whatever. After this lapse of time, injection of the same liquid into the same petal produced complete disintegration within half an hour.

Finally, in a parallel series of experiments with infection drops consisting of a dilute suspension of spores in a dilute nutrient, discoloration was found to take place in 12–24 hours from the time of sowing. An investigation shortly to be published has shown that at no time during the period from sowing to discoloration can the concentration of active

¹ Such injuries may occur very frequently, even in apparently healthy leaves. See in this connexion a paper by Barker and Gimingham, *Annals of Applied Biology*, vol. i, 1914, p. 13.

principle in the infection drop at all approximate to its concentration in the extracts employed in these experiments.

The above results are contrary to the view of de Bary according to which killing takes place previous to penetration of the cuticle by the fungus. The discrepancy can only be explained by assuming either that the conclusions of de Bary do not hold for *Botrytis cinerea*, or that the method of extraction here employed has failed to obtain some toxic substance which plays an important part in the early phases of natural infection. All question of the presence of a cuticle-dissolving enzyme may be put on one side, as no evidence has yet been obtained that any fungus produces such an enzyme; and certainly no such enzyme is present in the extract in question.

For the application of de Bary's result to the case of *Botrytis* attack, there is no direct evidence apart from Nordhausen's statement that browning and killing of the cells of a moss leaf took place before the hypha had penetrated and even before the spore had germinated. This statement has been criticized in another place,¹ and it is very doubtful if any weight can be attached to it in this matter. Again, the experiment with stained epidermis of *Allium* discounts the excretion by the fungus of any acid substance, at any rate of such a comparatively strong acid as oxalic acid. The experiment in which the toxic products of the fungus were removed or considerably diluted by means of a stream of water can only be readily interpreted according to the view that there is in the infection drop no toxin which is capable of passing through the cuticle. The incompatibility with one another of certain of Nordhausen's conclusions renders further criticism of his work unnecessary.

The hypothesis of killing in advance of penetration of the cuticle necessitates a crystalloidal toxin. The maximum concentration of this toxin would occur in the neighbourhood of the hypha; but owing to the high diffusibility which must be postulated of the toxic substance, it is impossible to believe that any considerable concentration gradient of toxin could subsist within the limits of the infection drop. A toxic substance capable of diffusing through the cuticle on the one side would freely diffuse into the spore-free region of the infection drop on the other. The action of such a toxin should therefore not be confined to the immediate neighbourhood of the hyphae, and the existence of such a substance should also be demonstrable in the general liquid of the infection drop.

The remainder of this paper is devoted to the examination of the infection drop from the point of view of the presence of a crystalloidal toxin and in particular of a soluble oxalate.

¹ No. I of this series. Ann. Bot., vol. xxix, 1915. pp. 329, 330.

A. *On the question of the presence of a crystalloidal toxin in the infection drop.*

The following observations have been made :

1. In many cases the first signs of discoloration of the tissue underlying the infection drop appear along the margins of the latter. This effect is best seen when the drops do not spread over a large surface of the leaf. Microscopic observation shows this effect to be correlated with more rapid germination of the spores along the margin. The fact that such a variation in the time of attack can be obtained within the limits of a drop so small as 0.03 of a c.c. cannot be explained on the basis of the de Bary hypothesis.

2. If a drop containing spores be placed on the leaf, the spores allowed to settle into contact with the epidermis (a process which requires about one hour), and the drop be now slightly displaced so as to include a new portion of the leaf surface, the discoloration as it first appears, and for a considerable time afterwards, is seen to outline strictly the area originally occupied by the drop ; in other words, the discoloration effect is strictly localized to the immediate neighbourhood of the germinating spores. This result is again directly contrary to the hypothesis of de Bary.

3. With the same number of spores, the size of the drop can be varied within wide limits without appreciably affecting the time of appearance of discoloration ; and similarly with drops of the same medium and of the same size the number of spores can be varied within wide limits without producing any marked variation in the time of appearance of discoloration. Thus sowings on rose petals in which the concentrations of spore suspension were in the ratio 10 : 1 gave 10–12 hours and 12–14 hours respectively as the times at which discoloration could be first detected by the naked eye. If the discoloration effect were due to the accumulation of a toxic substance in the drop, the effective concentration should be reached much more rapidly in the case of the denser sowing (the concentration of nutrient in the drop being sufficient to allow of vigorous germination), and the time of appearance of discoloration should therefore be correspondingly accelerated. Nevertheless we find that the shortening of the period between sowing and the appearance of discoloration with increasing concentration of spores is very slight. Bearing in mind the fact that discoloration in its earliest stages is not apparent to the naked eye, and further that when once started, the degree of discoloration would be proportional roughly to the number of spores present, we can readily conceive that the slight time difference above noted is explicable in this manner.

4. When infection drops, in which discoloration was just becoming visible, were collected, cleared of spores by centrifuging, and tested on the surface of the most sensitive petals, no toxic action whatever could be demonstrated.

In all the preceding experiments, the spores were sown in a medium of sufficient strength to enable germination of all the spores present to take place. It may be thought that in such a case there might be no killing antecedent to penetration, there being sufficient nutriment already present in the infection drop; but that the formation of the toxin might be called forth by a condition of starvation in the infection drop. This hypothesis is *a priori* improbable; the following experiments were made to settle this point:

Suspensions of spores of equal density were made in water and in a series of media of strength m , $\frac{m}{2}$, $\frac{m}{4}$, $\frac{m}{8}$, $\frac{m}{16}$, $\frac{m}{32}$, $\frac{m}{64}$, where m represents a glucose-peptone medium (glucose 1 per cent., peptone 0.2 per cent.). Within this range of strength of medium all variations were obtained from very poor to fairly vigorous germination. Drops of these suspensions were placed on petals of rose and on leaves of broad bean. In all cases discoloration was produced, but in the case of sowings in water on bean leaf only very slowly. When discoloration was established over the surface of contact of the original drop with the leaf, the drop was slightly displaced so as to include a small portion of the adjacent surface. In no case, however, was any discoloration of the newly included surface shown.

The above experiments show conclusively that there is no accumulation in the infection drop of a substance which can diffuse through the cuticle and bring about death of the underlying cells.

B. *On the presence of a soluble oxalate in the infection drop.*

The evidence already brought forward under (A) applies also to this special case. As considerable importance has been attached by various writers¹ to the part played by oxalates in conditioning parasitic attack, it may not be considered superfluous to bring forward certain additional evidence on this point.

In the infection drop, as has already been stated, the first appearance of discoloration of the underlying tissue (rose petal, bean leaf) was observable in about twelve hours from sowing. Now in parallel experiments it was determined that, in the case of both plants, a concentration $\frac{n}{40}$ of oxalic acid when placed in the form of drops on the surface produced no noticeable action within a period of twelve hours. The same statement applies to a concentration $\frac{n}{20}$ of potassium oxalate. If oxalic acid or oxalate be the toxic substance concerned, the concentration of the same in the infection drop must at least equal $\frac{n}{40}$ or $\frac{n}{20}$ in the respective cases. In

¹ See especially R. E. Smith: Bot. Gaz., vol. xxxiii, 1910, p. 385.

the infection drops employed there was however no possibility of such high concentrations, either of acid or of salt. In the experiments in this connexion a glucose-peptone medium was employed. This medium contained a trace of a calcium salt (derived from the commercial peptone preparation), which in the medium as made up was determined by titration to be of strength

$\frac{n}{800} - \frac{n}{1,000}$. Sowings were made in this medium in the usual way, and as

soon as discoloration was observed the drops were collected and cleared of spores by centrifuging. The addition of a drop of potassium oxalate to this liquid gave a distinct precipitate, thus showing that a soluble calcium salt was still present in the infection drop. It is plain therefore that the

figure $\frac{m}{800}$ represents an upper limit to the amount of oxalate excreted by

the fungus into the infection drop. The actual amount of oxalate formation, if any, is certainly much below this figure. Thus in plate sowings of two days' age in which the same glucose-peptone medium was employed, the excretion of soluble oxalate had not gone so far as to precipitate all the calcium from solution.

It appears therefore that the formation of soluble oxalate in young fresh cultures of *Botrytis*, if it take place at all, is negligible in amount, and that vigorous attack by the fungus can take place under conditions where the presence of a soluble oxalate is quite excluded.

CONCLUSIONS.

The infecting germ tubes of *Botrytis cinerea* are unable to affect chemically the cuticle of the host, nor do they secrete any toxic substance which can pass through the cuticle and bring about the death of the underlying cells. The fungus is unable to affect the underlying tissue until the obstacle afforded by the cuticle has been overcome. Chemical action being excluded, penetration of the cuticle must take place in a purely mechanical way. Once penetration of the cuticle has been accomplished, the underlying tissue is attacked after the manner described in the first number of this series.

The results of this purely physiological study are in exact agreement with the results of a microscopical study by Blackman and Welsford¹ of infection of the bean leaf by *Botrytis cinerea*.

¹ No. II of these studies. Ann. Bot., vol. xxx, 1916, p. 389.

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