The Fertilization of *Pythium de Baryanum*.

BY

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With Plate XXXVI.
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PRINGSHEIM ('58) was the first who observed the phenomena of fertilization in *Pythium*. He found that in *Pythium monospermum*, the antheridium sends out a process into the oogonium, while the contents of the latter separate from the wall and accumulate into a spherical mass, the oosphere, which he calls the 'Befruchtungskugel.' After the fertilization-tube reaches the oosphere, it discharges its contents through the opening at the apex. He believed that very minute spermatozoid-like bodies in active motion were found in the antheridium. Cornu ('72') observed the passage of the antheridial contents into the oogonium in *Pythium gracile* and *P. Cystosiphon*, but he denied the presence of spermatozoid-like bodies in the antheridium. After that De Bary ('81) made more careful studies and found that, in several species of *Pythium*, soon after the formation of the fertilization-tube the protoplasm becomes differentiated in the antheridium; the larger and denser granular portion moves into the centre of the cavity, forming there an irregular and somewhat indistinct strand, the gonoplasm, while a thin layer, the periplasm, remains next the wall of the cell. Then the gonoplasm passes slowly through the fertilization-tube which has

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opened in the meanwhile into the oosphere. His results were confirmed by Ward ('83 *b*), Atkinson ('95), and others.

Fisch ('85) studied for the first time the nuclear phenomena in the fertilization of *Pythium*. He used various solutions of Haematoxylin for the stain, and found that in the young oogonium there are usually from ten to twenty nuclei. At the time of the oosphere-formation, he thought, they fused together into a single large nucleus. In the antheridium he always found a single nucleus, but thinks that this may be due to the fusion of several. The nucleus of the antheridium passes into the oosphere with the gonoplasm and fuses with the egg-nucleus.

Later, Dangeard ('90, '92) made some observations on *Pythium monospermum* and *P. proliferum*, in which he showed that the antheridia and oogonia are multinucleate, but he was unable to follow the fate of the nuclei to the time of fertilization. He also pointed out that a central oleaginous globule appears in the oogonium at a certain stage, and that this was probably mistaken by Fisch for a nucleus, and led him to the conclusion that all the nuclei of the oogonium fused together to form a single sexual nucleus.

These are the only papers concerned with the nuclear phenomena of fertilization in *Pythium*, so far as I know. In the light of recent investigations on the fertilization of Peronosporae, some of Fisch's results seem to be very doubtful, and Dangeard's studies do not seem to give much light on this point.

The present investigation was undertaken in the Botanical Laboratory of Cornell University, under the direction of Professor George F. Atkinson, to whom I wish to express my hearty thanks for his kind advice and suggestions.

MATERIAL AND METHODS.

The species I have studied was *Pythium de Baryanum*, a fungus which is responsible for a large part of the 'damping off' of young seedlings. It is very widely distributed, being common in the soil of gardens and fields. I collected earth from a rather low part of a garden, placed it in pots and boxes

in the greenhouse, and planted in it some cucumber (*Cucumis sativus*, L.) seeds. Within two or three days after germination of the seeds, some of the seedlings began to droop, the tissues softening and yielding near the surface of the soil. The prostrate plants were found to be more or less shrunk and collapsed at this point, so that on pulling, the plant broke easily. The Fungus seems to attack the root and the lower part of the stem first, then to proceed upwards, so that in the course of a few days the entire plant wilts and finally decays. If a portion of the collapsed part of the stem is teased apart on the slide and examined under the microscope we can find the abundant colourless branched mycelium of the Fungus extending in the direction of the axis of the stem, and sometimes young oogonia can be seen here and there, in the part which is much decayed.

I then took the hypocotyl of the diseased seedlings, usually choosing one which was not very much collapsed, and placed it in a Petrie dish on moistened filter-paper. After one or two days sexual organs are abundantly formed, older stages being usually found in the lower part of the hypocotyl which was first attacked by the Fungus. Before fixing, small portions of the different parts of the hypocotyl were examined under the microscope in order to see that a number of sexual organs were present in different stages. The suitable portions were cut into pieces 5 to 7 mm. in length, and fixed.

The fixing fluids used were chrom-osmium acetic acid; chrom-acetic acid; corrosive sublimate in aqueous solution both hot and cold; acetic-sublimate solution in alcohol or water; absolute alcohol; Carnoy's fluid, and Merckel's fluid. The first two gave the best results, and the second one was more extensively used.

The material was usually left in the fixing solution from fifteen to twenty-four hours, then washed in several changes of water for ten or more hours. It was then gradually transferred through the usual grades to absolute alcohol. After dehydrating it was brought very gradually into xylol or chloroform, and transferred with equal care into paraffin, with

a melting-point of 54° , in which it was finally imbedded. Sections, from 2 to 5μ in thickness, were cut with a Minot-Zimmermann revolving microtome, and fastened to the slide with albumen fixatives.

Among several staining combinations used, Flemming's triple stain gave the best results, and it was very extensively used throughout the research. Heidenhain's iron-alum Haematoxylin was sometimes used, and it gave some good results. Hartog's ('95) nigrosin-carmin stain, as used by Wager ('96) and Berlese ('97), was tried on material fixed in sublimate solutions, but the results were not very satisfactory.

DEVELOPMENT OF THE SEXUAL ORGANS.

Nuclei are found irregularly distributed in the protoplasm in all parts of the mycelium. The shape of the nucleus is spherical or more or less oval (Plate XXXVI, Fig. 1). But in some parts of the mycelium the nuclei become considerably elongated and deformed. They contain the granules of chromatin, and no nucleoli seem to be present, so far as I am able to determine by the staining reactions. No haustoria are found in any part of the mycelium.

The oogonia are formed as terminal or intercalary swellings of the hyphae (Figs. 2, 3). Large quantities of protoplasm and numerous nuclei pass into them from the hyphae, and when they have reached their full size they are cut off from the hyphae by a wall, or, if their position is intercalary, by two transverse walls (Figs. 4, 5, 6).

About the same time that the oogonia are cut off one or more antheridia begin to develop close to each oogonium. The antheridia are of two kinds, stalk-antheridia and branch-antheridia. The stalk-antheridium, the simpler of the two, is formed from the part of the hypha next to the oogonium, by cutting off an elongated cell, one end of which is thus in contact with the wall of the oogonium, and its contents are only separated from those of the oogonium by the wall of the latter, as in *Monoblepharis*. The terminal oogonium is therefore placed upon it as on a stalk-cell, which may either

remain straight or have a characteristic curvature (Figs. 7, 13). In the intercalary oogonium, two stalk-antheridia may develop, one on either side of the oogonium.

The branch-antheridium is the one more commonly found. It is a terminal cell of a special branch of the hyphae, arising usually as a lateral branch of the hypha supporting the oogonium, and very often quite near the latter (Figs. 4, 8, 11). But sometimes, though rarely, it develops from a separate hypha near the oogonium. The antheridial branch grows towards the oogonium, and its rounded end finally comes in contact with the wall of the latter (Figs. 7-11). A septum is now formed in the branch, cutting off an elongated curved cell, the antheridium. More than one antheridium may be found in connexion with a single oogonium, frequently two antheridia (Fig. 10) and sometimes three.

A young oogonium, which is about 20 to 25 μ in diameter, contains approximately from ten to fifteen nuclei. The nuclei are more or less spherical, being larger than those of the hyphae. The chromatin granules are more prominent, and no nucleoli are found. It is very difficult to count the number of nuclei in the antheridium, owing to its small size and irregular shape. The number seems to vary from two to six.

The division of the nuclei takes place almost simultaneously in the oogonium and antheridium (Figs. 13-17). In the oogonium, the nuclei, before division, arrange themselves near the periphery. The division of the nucleus is karyokinetic and is very similar to that which takes place in *Cystopus* and *Peronospora* as described by Wager ('96, '00), Stevens ('99), Davis ('00) and others. The several karyokinetic figures are shown in Fig. 25.

The chromatin granules first of all appear to be transformed into a number of chromosomes in the shape of very short rods or oval bodies. By the formation of the spindle they arrange themselves in the equatorial plate (Fig. 25 a). The number of chromosomes is very difficult to count, but in several cases I was able to count as many as eight. The nuclear membrane seems to remain intact, at least in

the early stages of the division, as in *Cystopus* observed by Wager, Stevens and Davis. No definite centrosomes are seen, although occasionally granules at the poles of the spindle were observed which might have been taken for such, as is shown in Fig. 25*a*. It is more difficult to study the division in the antheridium. The process does not seem to differ from that which is found in the oogonium. Fig. 25*c* shows one of the karyokinetic figures found in the antheridium.

This division of the nuclei in the oogonia and antheridia previous to fertilization is exactly similar to that which occurs in *Cystopus* and *Peronospora*, and according to Trow ('95, '99) in *Saprolegnia* and *Achlya* also. A similar phenomenon has been described by Eidam ('86), Chmielewsky ('88) and Fairchild ('97) in *Basidiobolus*. The significance of this division is not yet very clear. Hartog ('91) suggests that 'we can regard the nuclear divisions in oogonium and antheridium as phylogenetic reminiscences of the formation of gametes by cell-division.' Wager ('96) in the brief discussion of this subject in his paper on *Cystopus candidus* says that 'the readiest explanation of the phenomenon would of course be that it is a reducing division, but this does not appear probable in the light of recent investigations on the reduction of the chromosomes in plants and animals, and also from the fact that, so far as I was able to observe in *Cystopus*, the number of chromosomes in the dividing nuclei in the oogonium is less than the number in the dividing nuclei of the fully formed oospore.' In his recent paper on *Peronospora* Wager ('00) is inclined to regard it as a reducing division, since he states: 'but it appears to me, from the few observations which I have been able to make, that a reduction in the number of chromosomes takes place during the mitosis in the oogonium and probably in the antheridium.' Berlese ('97) believes that the reduction does not take place in the division of the sexual nuclei, but that it occurs, at least in *Cystopus Portulacae*, which he has actually observed, on the germination of the oospore. But Stevens ('99) says that in *Cystopus (Albugo) Bliti* 'there is some evidence that makes it appear that there

is a reduction in the number of chromosomes during the first mitosis.' It is evident that further investigations on the division of sexual as well as vegetative nuclei are necessary, before any definite explanation can be given as to the significance of the nuclear division in the gametangia.

FORMATION OF THE OOSPHERE AND FERTILIZATION.

While the nuclei arrange themselves in the peripheral part of the oogonium, the central part of the protoplasm becomes more or less vacuolate. After the division of the nuclei, the entire protoplasmic contents differentiate into ooplasm and periplasm (Fig. 17). In *Cystopus* and *Peronospora* this differentiation of the protoplasm into two parts takes place a little earlier than in *Pythium*; it occurs just about the time of nuclear division, or a little before. I have failed to observe any differentiated mass of protoplasm in the centre of the ooplasm, as found in all the species of *Peronosporae* so far studied, and named by Stevens the coenocentrum.

A single nucleus enters into the ooplasm and remains usually at the centre, while all the other nuclei are left in the periplasm and finally degenerate (Fig. 18). Now the oosphere with its nucleus is formed, but no wall seems yet to be formed around it.

It is interesting to note here that in *Cystopus Bliti*, according to Stevens ('99), the oosphere is multinucleate, and the nuclei divide once more before they fuse with the nuclei from the antheridium; the same state of things was found, by the recent investigations of Stevens ('01), in *Cystopus Portulacae*, whose oosphere was described by Berlese ('97) as uninucleate. Stevens ('01) also found that in *Cystopus Tragopogonis*, the oosphere is multinucleate and the nuclei divide again previous to fertilization, but only one of them becomes the functional nucleus of the oosphere, while all the other nuclei degenerate. This species is considered to be a connecting link between the above-mentioned two species of *Cystopus* and *C. candidus* whose oosphere is uninucleate from the beginning. Stevens' recent researches on *Cystopus candidus* differ from those of

Wager ('96) and Davis ('00) in one essential point. Stevens ('01) found that in *Cystopus candidus* the nucleus of the oosphere was derived from one of the nuclei which remained in the ooplasm from the time of its differentiation, instead of coming to the non-nucleated centre of the ooplasm from the periphery. Thus he states that at no time of its development is the central region of the oosphere entirely free from a nucleus, and in this respect *Cystopus candidus* differs from the other species of the genus.

In the antheridium, after division, all the nuclei degenerate except one which becomes enlarged (Fig. 18). While the oosphere is differentiating, the antheridium puts out a fertilization-tube from the point of attachment into the oogonium, piercing through the wall of the latter and pushing its way through the periplasm into the ooplasm (Figs. 19-22). The fertilization-tube is a thin-walled cylindrical tube, often somewhat narrowed at the end, instead of being enlarged as in *Peronospora* and *Cystopus*. The tube does not seem to reach deep into the centre of the oosphere, as in many species of *Peronosporae*, but it appears to open near the periphery of the oosphere. I am not, however, very certain on this point, as in a few preparations something like the fertilization-tube was seen to reach the centre of the oosphere from the periplasm.

The greater part of the antheridial contents with the male nucleus passes gradually into the oosphere through the fertilization-tube (Fig. 19). The nucleus of the antheridium, after entering the oosphere, comes in contact with the female nucleus in the centre, and then they fuse together to form the nucleus of the oospore (Figs. 20-22).

It would be interesting to study the behaviour of the nuclei during fertilization in the oogonium with more than one antheridium. De Bary ('84) mentions that, if there is more than one antheridium, they all usually, but not always, empty their gonoplasm one after another into the oosphere. My present investigation has so far failed to make this point clear.

MATURATION OF THE OOSPORE.

Soon after the discharge of the antheridial contents into the oosphere, a thin membrane is formed around the latter (Figs. 20-22). This is the beginning of the exospore. After fertilization it gradually thickens until it reaches about the thickness of the oogonium-wall. Figs. 23 and 24 show the oospore in which the formation of the exospore is nearly completed. In this stage only a very small quantity of protoplasm remains in the periplasm. It is very likely that the greater part of the periplasm has been used up in the formation of the exospore.

Later the endospore begins to be formed inside the exospore. Fig. 25 shows an exospore in which the endospore is about half way thickened, and in Fig. 27 we can see the endospore is nearly completed. The exospore is more deeply stained than the endospore.

The ripe oospore is uninucleate, and in the nucleus several deeply-stained chromosome-like bodies are clearly visible (Figs. 26, 27). The protoplasm is vacuolate in structure, showing a regular net-work in the section, and it takes the stain more readily. The wall of the oogonium which thickens in the course of the development of the latter, but very slightly or scarcely at all after fertilization, persists as an additional protection of the oospore (Fig. 27, &c.).

From the foregoing statements we can see that, in the mode of sexual reproduction, *Pythium* has more resemblance to the Peronosporae than to the Saprolegniae. In some species of Saprolegniae, the oospore is formed parthenogenetically, the antheridia being very rare or never developed. In others, which have well-developed antheridia, the existence of fertilization is still in doubt. De Bary ('81) first expressed the doubt, and after careful observations came to the conclusion that the Saprolegniae as a group are distinctly parthenogenetic. His conclusion was based on the failure to observe any passage of protoplasm from the fertilization-tube to the oosphere, or even any opening in the tube. Pringsheim ('82, '83)

opposed this view, and maintained that in certain species he actually observed the fertilization taking place by the amoeboid swimmers—'Spermamoebae'—developed in the fertilization-tubes and set free from them. His observations were objected to by Zopf ('82, '83), De Bary ('83), Ward ('83 *a*) and others.

Hartog ('89, '91, '95), Dangeard ('90, '91), Humphrey ('92), and Trow ('95, '99) studied the nuclear phenomena of this group. Hartog and Humphrey agree in the absence of fertilization, Dangeard leaves this question undecided, while Trow maintains that in certain species at least true fertilization takes place.

In the Peronosporae, on the other hand, the true process of fertilization, i. e. the fusion of sexual nuclei, takes place in all the species so far studied. The results of De Bary's ('81) classical researches, confirmed by the recent cytological investigations of Wager ('89, '96, '00), Stevens ('99, '01), Davis ('00) and others leave no room for doubt on the existence of fertilization in this group.

The question naturally arises as to which of these two groups of Fungi *Pythium* is most closely related. Pringsheim ('58), who established the genus *Pythium*, placed it among the Saprolegnieae. Cornu ('72) in his monographic studies of Saprolegnieae, included *Pythium* in that group. Later, De Bary ('81) placed this genus under the Peronosporae, and this arrangement has been accepted by later students of Fungi. More recently, Schröter ('93), in Engler and Prantl's 'Pflanzenfamilien,' classified *Pythium* as a member of Saprolegnieae, placing it under the separate family Pythiaceae. The reason for adopting the new classification is given by him as follows: 'Die Pythiaceae zeigen eine sehr nahe Verwandtschaft zu den Peronosporineae, besonders in Hinsicht auf die Befruchtung und die Oosporenbildung. Sie sind darum auch von De Bary mit den Peronosporineae vereinigt worden. Habituell stehen sie den Saprolegniineae näher, und es erscheint in einem System, welches nicht mehr einsichtig auf ein einzelnes Merkmal (die Sexualität), sondern auf die

Berücksichtigung aller Merkmale begründet ist, passender, sie im Zusammenhang mit den *S.* zu besprechen, als besondere Familie, welche in der Mitte zwischen beiden steht.'

As remarked by Schröter, it is incorrect to base the classification solely on the mode of sexual reproduction. But the sexual organs are usually regarded as the most important characters used in classification, since the vegetative parts of the plant-body are more liable to modification according to the conditions of environment to which the plants are subjected, while the reproductive organs undergo less change. The present studies show more definitely the close relationship of the Peronosporae and *Pythium* in the mode of sexual reproduction.

It appears therefore that its natural position would be in the order Peronosporae rather than in the Saprolegnieae, although it occupies an intermediate position between the two.

SUMMARY.

1. The mycelium of *Pythium de Baryanum* contains numerous nuclei irregularly distributed in the protoplasm.

2. The young oogonium, which is formed as either a terminal or an intercalary swelling on the hypha, contains about 10 to 15 nuclei. The nuclei are larger than those of the hyphae, and the chromatin granules are prominent in stained preparations.

3. The antheridium contains about 2 to 6 nuclei. The structure of the nuclei does not differ from that of the nuclei in the oogonium.

4. The nuclei of the oogonium and antheridium undergo division previous to fertilization. The division is karyokinetic, and similar to that which takes place in *Cystopus* and *Peronospora*. The formation of the karyokinetic spindle is intranuclear, and the nuclear membrane persists at least in the early stages of the division.

5. After the division of the nuclei which were previously arranged near the periphery of the oogonium, the whole

contents of the oogonium differentiate into the ooplasm and periplasm. Then one nucleus from the periphery enters into the ooplasm to become the functional female nucleus of the oosphere, while all the other nuclei in the periplasm degenerate there.

6. The nuclei of the antheridium after division degenerate, except one which remains to become the functional male nucleus. While the formation of the oosphere is taking place in the oogonium, the antheridium sends out a fertilization-tube. The tube reaches to the oosphere, piercing through the wall of the oogonium and periplasm.

7. The greater part of the contents of the antheridium, including the male nucleus, passes gradually into the oosphere, through the fertilization-tube. The male nucleus finally comes in contact with the female nucleus, and they fuse together to form the nucleus of the oospore.

8. After the discharge of the antheridial contents into the oosphere, the exospore begins to form around the latter. The greater part of the periplasm seems to be used in the formation of the exospore which has about the same thickness as the oogonium-wall, when completed. A much thicker endospore develops later inside the exospore. The ripe oospore is uni-nucleate, and in the stained section several chromosome-like granules are visible in the nucleus.

CORNELL UNIVERSITY,
June, 1901.

After this was written, Dr. Trow's paper, 'Observation on the Biology and Cytology of *Pythium ultimum*, n.sp.,' appeared in *Annals of Botany* (June, 1901). His results agree with mine in essential points, but differ somewhat in matters of less importance.

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EXPLANATION OF FIGURES IN PLATE XXXVI.

Illustrating Mr. Miyake's paper on the Fertilization of *Pythium*.

All figures were sketched with the aid of the camera lucida, using Bausch and Lomb's $\frac{1}{2}$ in. oil immersion objective with ocular 1 ($\times 1300$), except Fig. 28, which was drawn with Zeiss' apochromatic 1.5 mm., 1.30 apert., homog. immersion objective, compensation ocular 12 ($\times 2700$).

- Fig. 1. Portions of mycelium showing several nuclei.
- Fig. 2. The formation of an intercalary oogonium.
- Fig. 3. The formation of a terminal oogonium.
- Fig. 4. Intercalary oogonium with an antheridium attached in one side.
- Figs. 5-6. Terminal oogonia.
- Fig. 7. Young oogonia with a stalk-antheridium.
- Figs. 8-9. Young oogonia with branch-antheridia.
- Fig. 10. An oogonium with two antheridia.
- Fig. 11. An oogonium with a branch-antheridium.
- Fig. 12. An oogonium with the nuclei dividing near the periphery.
- Fig. 13. An oogonium with a stalk-antheridium, the nuclei of both are dividing or just about to divide.
- Fig. 14. An oogonium with a branch-antheridium, the nuclei of both are just dividing.
- Fig. 15. An antheridium, showing a dividing nucleus. Only a part of oogonium is shown.
- Fig. 16. An oogonium with a branch-antheridium, the stage a little later than Fig. 14.
- Fig. 17. The contents of an oogonium as just differentiating into the ooplasm and periplasm.
- Fig. 18. Later stage: the oosphere is formed, showing the degenerating nuclei in the periplasm. The antheridium with one functional nucleus and several degenerating nuclei is shown in one side.
- Fig. 19. The antheridium discharging the contents: the male nucleus just entered the oosphere.
- Fig. 20. The male nucleus is approaching the female nucleus. The thin wall is formed around the oosphere.
- Fig. 21. Two sexual nuclei are just in contact.
- Fig. 22. A stage same as Fig. 20, with a stalk-antheridium.
- Figs. 23-24. Young oospores each with a single nucleus, and the exospore are nearly complete.
- Fig. 25. An oospore with the endospore about half-way thickened.
- Fig. 26. An oospore in a stage older than Fig. 25.
- Fig. 27. Nearly ripe oospore with oogonial wall, emptied antheridium still attached.
- Fig. 28. Several karyokinetic figures. *a, b, d, e* in oogonium, *c* in antheridium.

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