

COMPOSITION OF FUNGUS HYPHAE IV: PHYTOPHTHORA¹

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The genus *Phytophthora* was established by de Bary (2). De Bary realized that there was a close relationship between the genera *Phytophthora* and *Pythium*, yet he considered that the difference in the germination of the sporangia of the species, known at that time, justified the separation.

In the germination of *Pythium*, it was observed that the contents of a sporangium first passed into a thin-walled vesicle, which later burst, freeing the spores. This was thought to be a specific characteristic of *Pythium*. As new species of *Phytophthora* were discovered, it soon became apparent that some of them displayed similar sporangial germination. Thus, the real differences between the two genera in morphology and cytology have become meager.

More recently, Fitzpatrick (3) has stated that there is no adequate reason for distinguishing *Phytophthora* from *Pythium* and that, in time, the two genera may be merged under the older name, *Pythium*, unless a more valid basis of differentiation can be established.

After the writer had completed a study of the composition of the hyphae of representative species of *Pythium* (8), attention was next directed to a similar investigation of a number of species which have been assigned to the genus *Phytophthora*. It was thought that such a comparative study might furnish further evidence either for merging *Phytophthora* and *Pythium* into one group, as suggested by Fitzpatrick, or for continuing them as two distinct genera of Phycomycetes.

In Dastur's (1) description of the species *Phy. parasitica*, an account of the composition of the mycelium is also included. He reports the presence of pectic-like material and cellulose. Tests for callose were negative, and chitin was not detected. Methods employed and viewpoints of analysis were considerably different 30 years ago from what they are now. In endeavoring to compare the reports of Dastur on *Phytophthora* with the more recent analysis of *Pythium*, by the writer, no adequate idea of similarities or differences of the two genera, can be obtained.

SPECIES INVESTIGATED

For the purpose of this investigation, the following species of *Phytophthora* were secured: *Phy. cactorum* (Leb. & Cohn) Schröt., *Phy. parasitica* Dastur, *Phy. infestans* (Mont.) de Bary, *Phy. capsici* Leon., *Phy. hydrophyla* Curzi, *Phy. pini* Leon., *Phy. cinnamoni* Rand, *Phy. melongenea* Swada, and *Phy. citrophthora* (Smith & Smith) Leon.

PLAN OF STUDY AND RESULTS

The species of *Phytophthora* used in this study, with the exception of *Phy. infestans*, grew well in potato broth. For this species, lima bean broth was substituted. In contrast to the *Pythiums*, the *Phytophthoras* made much slower growth. All of them formed mats more or less compactly interwoven, except *Phy. cinnamoni*, which developed a discrete colony type of growth without much interlacing. These colonies were fragile and could be broken apart readily. The

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same type of growth was displayed by *Phy. melongenea* during the first two or three weeks. Later the colonies grew together, forming a mat.

The same plan of analysis which had been found to be useful in determining the composition of other groups of fungi (6, 7, 8) was followed. Microchemical tests were used to direct the course of macrochemical procedure. Only qualitative methods were considered, and attention was directed entirely to the mycelium.

Some of the species of *Phytophthora* produced spores in potato broth; while others did not. Cultures varying in age from one week to three months were included in the study.

No difference could be detected between young and old cultures when the mycelia were observed in water mount in polarized light. Young cultures, three to four days old, and old cultures, three to four months old, were very dimly anisotropic, a condition which indicated the presence of callose or cellulose. When the hyphae were treated with a weak aqueous solution of ruthenium oxychloride, the dye was retained and could not be removed by prolonged washing in water. This characteristic of the mycelium, together with the absence of double refraction in polarized light, indicated the presence of pectic material on the outside.

Hyphae treated with Lugol's iodine solution followed by 70 per cent H_2SO_4 developed a blue color, which revealed the presence of cellulose. The presence of cellulose was confirmed with the aid of other cellulose reagents, such as $CaCl_2$ -iodine, H_3PO_4 -iodine, and $LiCl$ -iodine (4). With some species of *Phytophthora* the blue color developed by the cellulose reagents appeared quickly; while in others the reaction was slow. In no case with fresh hyphae was there any swelling of the cellulose layer.

Information gained in these preliminary tests served as a guide for the procedure of analysis which was later formulated. It consisted in resolving the hyphae into component parts and identifying them.

Several fungus mats were cut into strips three to four millimeters wide, washed thoroughly to remove culture solution, then extracted for one hour in hot 0.5 per cent ammonium oxalate. The liquid was filtered off, and an equal volume of 95 per cent alcohol added. A finely divided precipitate appeared, which after a time settled to the bottom of the flask. A second extraction removed a smaller amount of material and a third none. The precipitates from the ammonium oxalate extractions were collected on a fritted glass filter, washed with alcohol, then taken up in a small quantity of water. When the Molisch test was made, a violet color appeared, indicating that a condensation product between alpha-naphthol and furfural was formed. A faint pink to brownish color developed when the orcinol-HCl reaction was applied. When a drop of $FeCl_3$ solution was added, a brilliant green color promptly resulted. The green color could be entirely removed by shaking with amylic alcohol. Pectic compounds could not be demonstrated as pectic acid by the addition of concentrated HCl or as calcium pectate with $CaCl_2$. When hydrolyzed with H_2SO_4 the product reduced Fehling's solution. The material removed with the ammonium oxalate extraction was not pectic compounds but was probably a mixture of pentose and hexose anhydride, as indicated by the orcinol HCl- $FeCl_3$ reaction.

With the outside covering removed, the mycelium did not permanently retain the ruthenium oxychloride dye, but it did stain with methylene blue. Extraction was next made with ammoniacal cupric hydrate sufficiently strong to dissolve cellulose promptly. When the liquid was filtered off and neutralized with HCl, a copious precipitate appeared. This was found to be isotropic when examined in polarized light, yet it gave a positive reaction when tested with cellulose reagents.

Further extraction with ammoniacal cupric hydrate removed nothing. The mycelium was still dimly anisotropic as at first and did not fix dyes. This inert character of the hyphae was suspected to be due to the presence of fatty acids.

Refluxing in 2 per cent alcoholic potash saponified the fatty acids in the outer portion of the hyphae with a result that the blue color appeared more quickly when the cellulose reagents were applied, and when the mycelium was again extracted with the cellulose solvent more material went into solution. It was necessary to repeat the process of saponification of fatty acids followed by extraction with ammoniacal cupric hydrate at least once, and in some instances, twice, before all the cellulose was removed. All the cellulose of *Phy. cinnamoni* could be removed without saponification of fatty acids.

The cellulose precipitates were collected on a glass filter, thoroughly washed, dried, and then suspended in 10 cc. of 17.5 per cent NaOH. The hydrate was allowed to act for 30 minutes with occasional stirring. The solution was then diluted with an equal volume of water and filtered through a glass filter. Part of the cellulose dissolved in the alkali and part did not. The residue was washed with 20 cc. of 8 per cent NaOH, then with water, followed with 1 per cent acetic acid, and finally with water until all free acid was removed. The portion which did not dissolve was found to be doubly refractive in polarized light, whereas the portion which was recovered from the extract by neutralizing the alkali was not doubly refractive. Both precipitates readily gave a blue color with cellulose reagents, and upon hydrolysis yielded reducing sugars. Tests for proteins were negative. It was concluded that two forms of cellulose were present: the alpha (5) form, which did not dissolve in the alkali, and another form, which did dissolve and could be recovered.

At this stage of the analysis, the walls of the hyphae were still intact. When viewed in polarized light they were isotropic, developed a brown color with cellulose reagents, and did not fix aniline dyes. The residue was proved to be chitin by a positive chitosan reaction. No callose or structural proteins were detected.

COMPARISON OF MYCELIUM OF PHYTOPHTHORA AND PYTHIUM

Very young *Pythium* hyphae are isotropic when observed with a polarizing microscope. This condition is due to a layer of pectic compounds on the outside. When this layer is removed, the anisotropic cellulose becomes visible. The pectic material is used up and disappears after five to seven days as the hyphae reach maturity, leaving the cellulose the outside layer.

Phytophthora hyphae present a dimly anisotropic appearance at all stages of growth. There is no true pectic layer on the outside. When the covering is removed, the mycelium presents the same appearance as at first, and at no time can the cellulose be made brightly anisotropic. Furthermore, two forms of cellulose were detected in *Phytophthora*.

In view of the biochemical differences which exist between these two supposedly closely related genera of fungi, it becomes evident that in the location of a new species, consideration might properly be given to the structure of the hypha to determine whether it conforms to the general pattern of the genus to which the species appears to belong. Previous studies by the writer have shown that there is a close conformity in mycelial structure among species of a genus.

SUMMARY

An investigation of nine different species of *Phytophthora* has been made in order to determine the composition and structural pattern of the mycelium. A basic skeleton of chitin was found to have superimposed upon it a mixture of two forms of cellulose, one of which was doubly refractive in polarized light and the other was not. These were impregnated with fatty acids. It was necessary to saponify the fatty acids with alcoholic potash before the cellulose could be removed by solution in ammoniacal cupric hydrate.

The outside layer was found to be a carbohydrate mixture which gave the

orcinol-HCl test for pentoses. The presence of a hexose was also indicated. No pectic compounds were present.

When viewed in polarized light, the same appearance is presented by young and old cultures of typical species of *Phytophthora*. In this respect there is a marked contrast between the two groups of fungi, *Phytophthora* and *Pythium*.

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