DIMORPHISM IN CONIOTHYRIUM PIRINUM SHELDON[1]

C. H. Crabill

Several years ago the writer began a study of the Phyllostictas and Coniothyriums associated with the frog-eye leaf spot of apple. In a previous paper[2] the morphology, cultural features, and host relationships of *Coniothyrium pirinum* were briefly dealt with. The following report is the outcome of pure culture studies of *C. pirinum* isolated from apple leaf spots.

The first cultures of this fungus, Strain I, were secured in 1911 from Black Ben Davis leaves at Blacksburg, Va. In 1913 another strain, Strain II, was obtained from the same source. Although the mycelium, pycnidia and spores of the two are in no wise to be distinguished from each other under the microscope, the two strains are readily distinguished when grown in Petri dish culture on artificial media. Starch agar[3] has been used almost exclusively in these studies. Plates of this agar were poured, cooled, and inoculated at the center by the use of a platinum wire. Tubes of this medium were used in making dilution and stock cultures.

Strain I, four years after isolation, exhibits the same characters as when first secured. It may be described as follows:

Petri Dish Culture:[4] Starch Agar

Mycelium pure white or pinkish until about the 10th day, acquiring then in spots, which spread gradually throughout, an olivaceous color. Pycnidia very few, large, multilocular, prolific, irregularly distributed about the point of inoculation, appearing 8 to 30 days after inoculation (Fig. 1).

Strain II when first isolated was described as follows:

[1] Paper No. 40 from the laboratories of Plant Pathology and Bacteriology, Va. Agr. Exp. Sta.

[2] Crabill, C. H. Studies on Phyllosticta and Coniothyrium occurring on apple foliage. Rep. Va. Agr. Exp. Sta. 95–115. 1911–12.

[3] Made according to the following formula: H_2O, 900 cc.; $MgSO_4$, .5 g.; K_2HPO_4, 1.0; $NaNO_3$, 2.0; KCl, .5; $FeSO_4$, .1; Agar agar, 20.0. Cook, filter, and while still hot add the following paste: H_2O (cold), 100 cc.; cornstarch, 10 g.; and sterilize.

[4] Unless otherwise stated, cultures were incubated in moist chamber.

Petri Dish Culture: Starch Agar

Mycelium pure white or pinkish until about the 5th day, when an olivaceous color begins to develop just inside the margin of the colony. Pycnidia multitudinous, small, prolific, uniformly distributed over the colony, appearing 3 to 6 days after inoculation (Fig. 2). As the pycnidia develop, the olivaceous color is absorbed, so that the greenish zone near the margin of the colony is continually advancing.

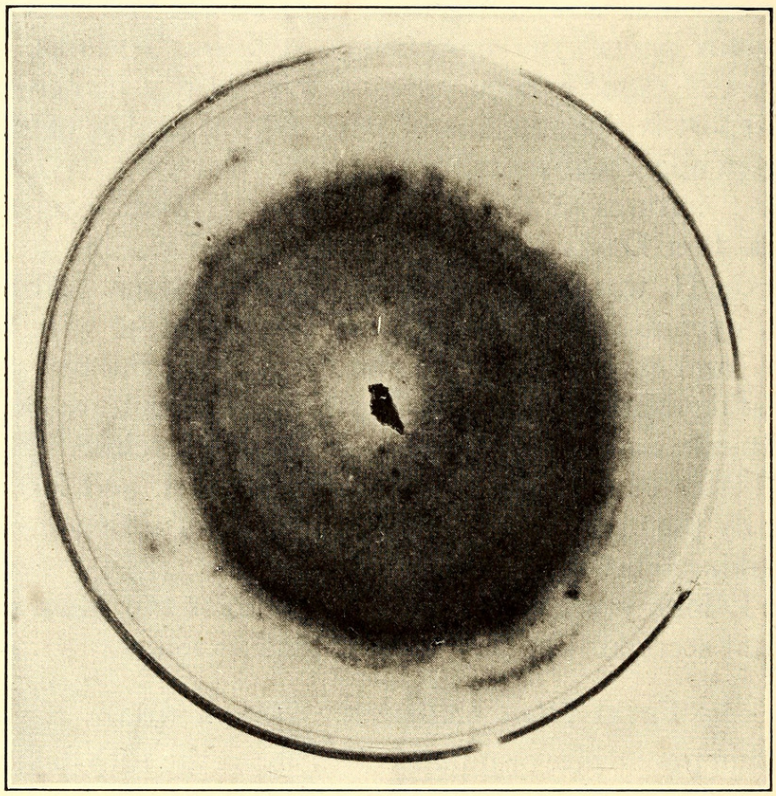


Fig. 1. Strain I in about the 43d generation, 24 days old. The tardy production of relatively few large multilocular pycnidia is characteristic of this strain. A few young pycnidia have just appeared in this culture.

Note: The photographed plates here figured may be located on the chart shown in Fig. 6 by sub-numbers which correspond to the numbers of the figures.

For a year after isolation, Strain II was grown in test-tube cultures and transferred to fresh media every two months. At the end of that time plates of starch agar were center inoculated with it. Mycelium, as well as spores, was transferred. All of the colonies which developed showed fruiting and non-fruiting sectors. The fruiting sectors were in all respects typical of Strain II. The non-

fruiting sectors produced typical mycelium which however never developed either color or fruiting bodies, although they were kept for two months.

Other plates were then inoculated with spores and with mycelium from the fruiting sectors and with mycelium alone from the non-fruiting sectors. These plates were labeled II + and II − respectively. The use of plus and minus to designate different strains of a fungus species is not new. Blakeslee[5] in 1904 applied these terms to

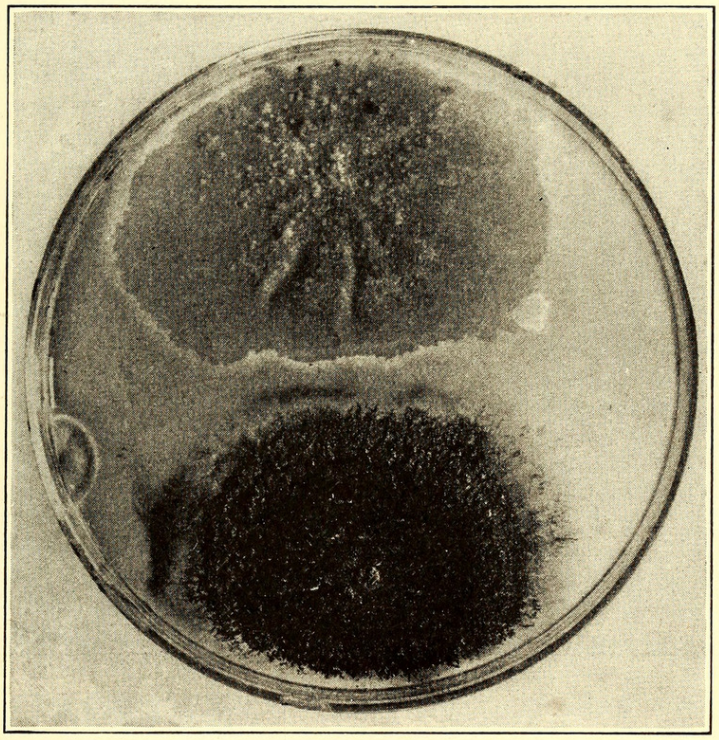


FIG. 2. Plus and minus cultures of Strain II, 7 days old. This plate was inoculated directly from the fruiting and non-fruiting sectors of the plate culture in which the first variation was noticed.

sexual strains of the Mucorineae which when bred together produced zygospores. Edgerton[6] in 1914 followed Blakeslee's example and used plus and minus to differentiate strains of Glomerella which when grown together produced the ascogenous stage in abundance.

The present paper deals with a fungus reproducing asexually. Plus and minus must therefore not be interpreted as representing

[5] Blakeslee, A. F. Proc. Amer. Acad. **40**: 203–321. 1904.

[6] Edgerton, C. W. Plus and minus strains in the genus Glomerella. Amer. Journ. Bot. **1**: 244. 1914.

male and female strains. Plus is used in the present instance to denote a strain fruiting abundantly and minus to denote a relatively poor fruiting strain.

In the II + colonies on the plates referred to above, the growth was typical of Strain II as it appeared when first isolated. The II − colonies grew rapidly but remained white for 12 days. They then began to take on the olive-green color characteristic of the species

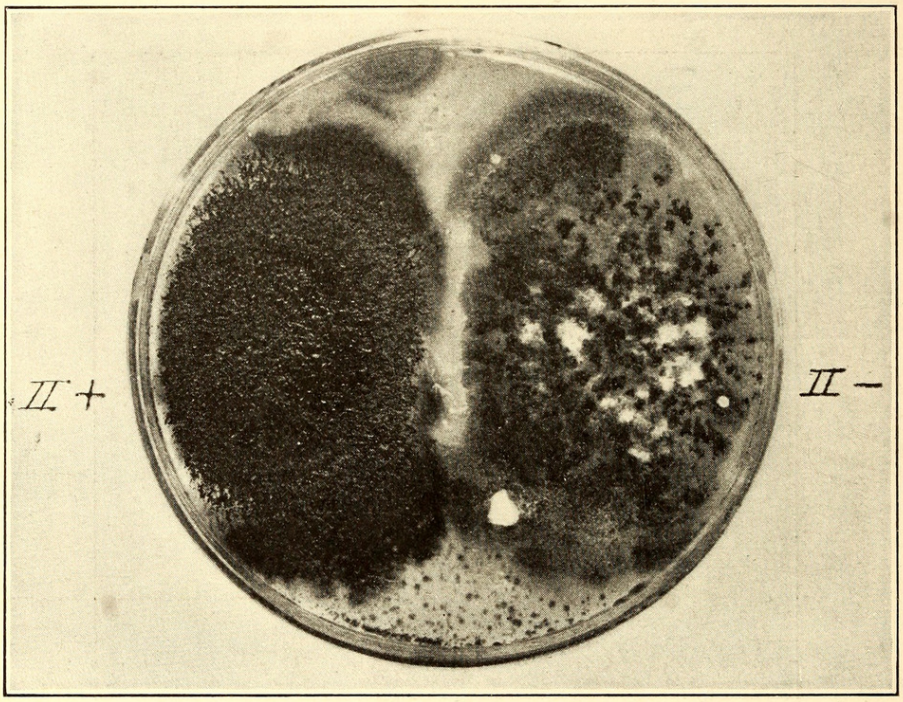


FIG. 3. Same as Fig. 2, 17 days old. Note that II − has produced numerous pycnidia from which the black spore masses are oozing. It was necessary to open the plates when photographing. The contaminations around the edge of the plate got in when the plate was photographed the first time.

and in 20 days from the time of inoculation produced a few large pycnidia about the center of the culture (Figs. 2 and 3). Microscopic examination showed the mycelium, pycnidia and conidia all typical of *C. pirinum*. The − colonies were exactly like Strain I in morphological and cultural features.

It appeared that from Strain II there had arisen a poor fruiting strain identical with Strain I. Poor fruiting is here used in a relative sense. The minus strain produces great multitudes of spores from the few large pycnidia; but, when compared with the enormous numbers

of pycnidia and spores produced by the plus strain, the prolificacy of the minus strain falls into insignificance.

To test out the evidence that a minus strain had split off from a plus parent, dilution plates were poured, using spores from both the plus and minus strains. All the progeny of the plus strain were typically plus. All the progeny of the minus strain were typically minus (Figs. 4 and 5). The former produced abundant pycnidia and

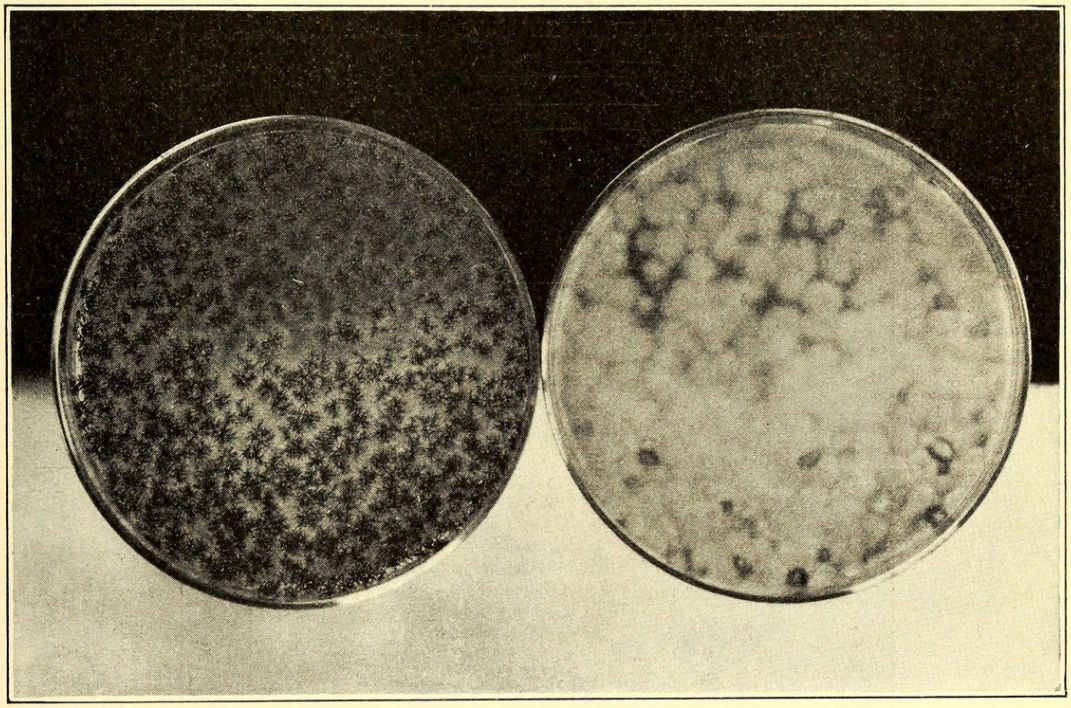


FIG. 4. Second dilution plates poured from the colonies shown in Fig. 3. These plates are 5 days old and of the second generation after the mutation. They therefore have the labels II + 2 and II − 2.

Note: Wherever two or more cultures appear in the same figure they were inoculated at the same time, incubated the same time under the same belljar, and photographed on the same plate.

fruited in 4 days. The latter fruited sparingly only after 14 days. Spores from these dilution cultures were used to pour other plates. Again all progeny of plus parents were typically plus and all progeny of minus parents typically minus. For 12 generations the plus strain remained entirely plus. Then suddenly it gave rise to a minus sector in a petri dish culture. The progeny of this sector were typically minus, whether poured or transferred by wire. The progeny of

the plus sectors of the same colony were typically plus and remained so for 5 generations when a third minus strain developed.

Fig. 6 will show better than words the history of the development of these strains.

On November 5, 1914, a culture of *C. pirinum* was received from Mr. J. W. Roberts, of the Bureau of Plant Industry at Washington. It was labeled III and immediately subjected to test to see if it would also develop plus and minus strains.

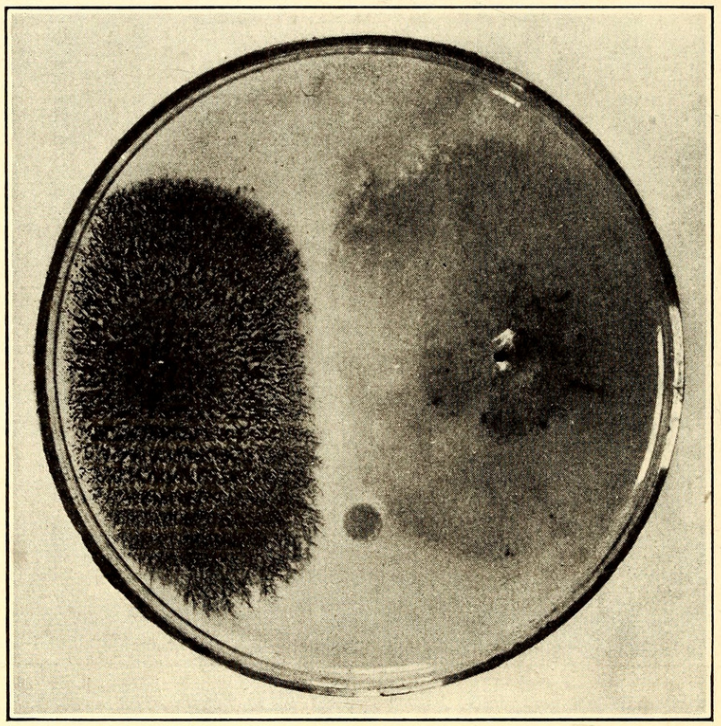


FIG. 5. II + 2 and II − 2 of the same parentage as those in Fig. 4. These cultures are 8 days old. A single large pycnidium has developed at the point of inoculation in the II − 2 colony. When spores alone are transferred the formation of pycnidia begins much later. When mycelium is transferred as in the present case a few pycnidia often develop at an early age due to the fact that young pycnidia are sometimes transferred on the mycelium. These naturally hasten to maturity as soon as food is supplied.

The first set of subcultures was made on Petri dishes of starch agar. Every colony showed fruiting and non-fruiting sectors (Fig. 7). Progeny of the minus sectors have remained constant, for 9 generations or until the present time. Progeny of the plus strain remained constant only 4 generations, when a small minus sector appeared in

one of the plus colonies (Fig. 11). Progeny of this minus sector have bred true until the present time.

These results indicate that the *C. pirinum* from the Bureau of Plant Industry was dimorphic in stock culture when received. The separation of the plus and minus forms was accomplished as in Strain

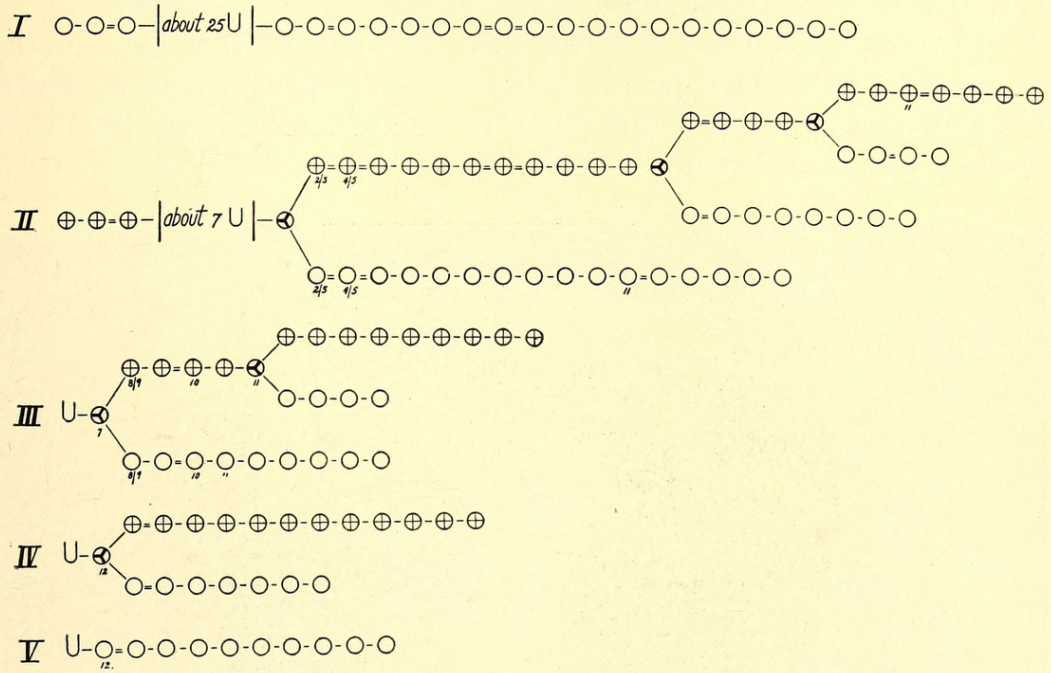


FIG. 6. Chart showing the history of 5 cultures of C. pirinum.

○ Petri dish culture of minus strain.

⊕ " " " " plus "

⊗ " " " " mixed strains. It is in such plates that the plus and minus sectors have appeared.

U Test tube culture.

= Succeeding plates poured from spores of preceding culture.

The plus strains suddenly give rise to minus strains which are subsequently constant generation after generation. It will be noticed that fewer generations of minus strains than plus strains have been grown in nearly every case. This is due to the fact that the plus strains fruit more quickly than the minus strains and more generations can be produced in a given time. I and II were isolated by the writer, III (IV) and V by workers of the Bureau of Plant Industry. Subnumbered plates have been photographed. The numbers correspond with the numbers of figures appended.

II. The minus form remained minus. The plus form threw off another minus form in the following 4th generation.

On January 2, 1915, two other cultures of *C. pirinum*, B. P. I. 227 and B. P. I. 345, were received from Dr. J. S. Cooley and desig-

nated IV and V respectively. The latter, B. P. I. 345, was isolated by Dr. Cooley from a soft rot of apple on December 3, 1914. The behavior of these cultures is shown in the diagram. Very likely IV is of the same parentage as III, received from Mr. Roberts of the same laboratory. The first subcultures showed it to be dimorphic, *i. e.*, producing plus and minus sectors (Fig. 12).

The first subcultures of V (B. P. I. 345) showed it to be a minus strain (Fig. 12), and it has so remained for 10 generations as shown in the diagram. It is identical with Strain I and with all the other minus strains developed in culture.

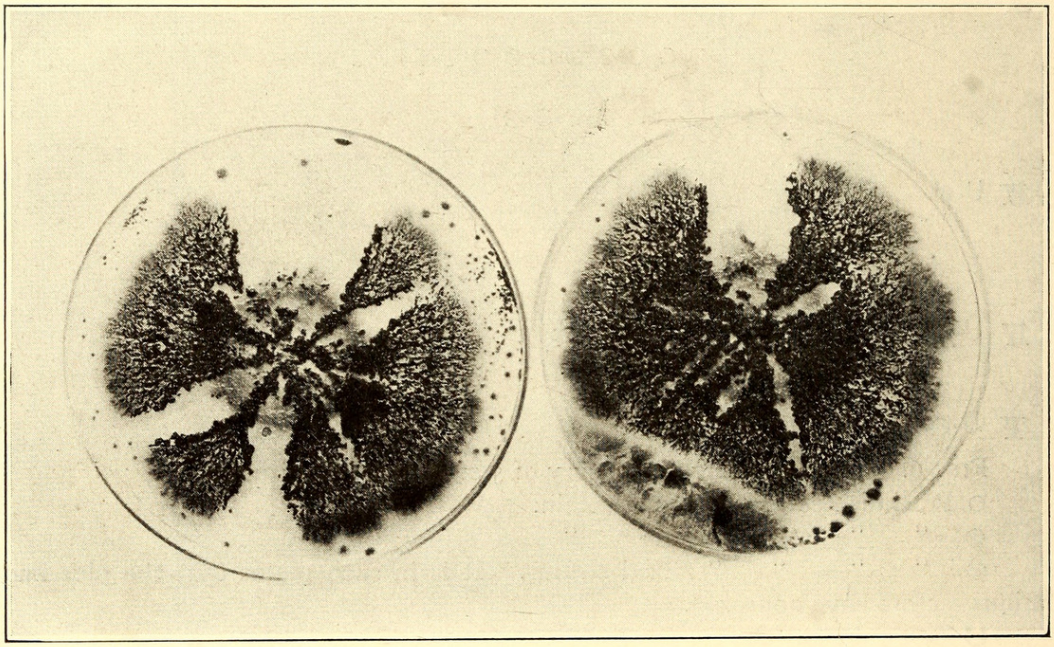


FIG. 7. C. pirinum III. First subculture. 19 days old. Plus and minus sectors were more numerous in these cultures than in any previously or subsequently examined. These plates were opened for examination five days previous to photographing, hence the contaminations around the edges of the cultures.

The plus strains are all identical in appearance and behavior. The fact that the minus strain of *C. pirinum*, viz., I and V, and the plus strain, viz., II and III (IV), have been twice isolated by investigators working separately indicates that plus and minus strains exist in nature.

The cultural studies show that minus strains may arise from plus strains by a sudden sporting or mutation. An objection might

be raised that these cultures were impure, *i. e.*, mixtures of two strains. In anticipation of such an idea it seems desirable to state that frequent pourings of dilution cultures were used to preclude such a possibility. Progeny were then selected only from well-isolated plants, microscopic examination of which showed that each was derived from a single spore. By consulting the chart, the frequency of these pourings may be considered. In each instance the poured plate is preceded by the symbol =. It will be evident from this that the possibility of either of the strains being constantly a mixture is eliminated. Both strains have repeatedly arisen from the progeny of a single

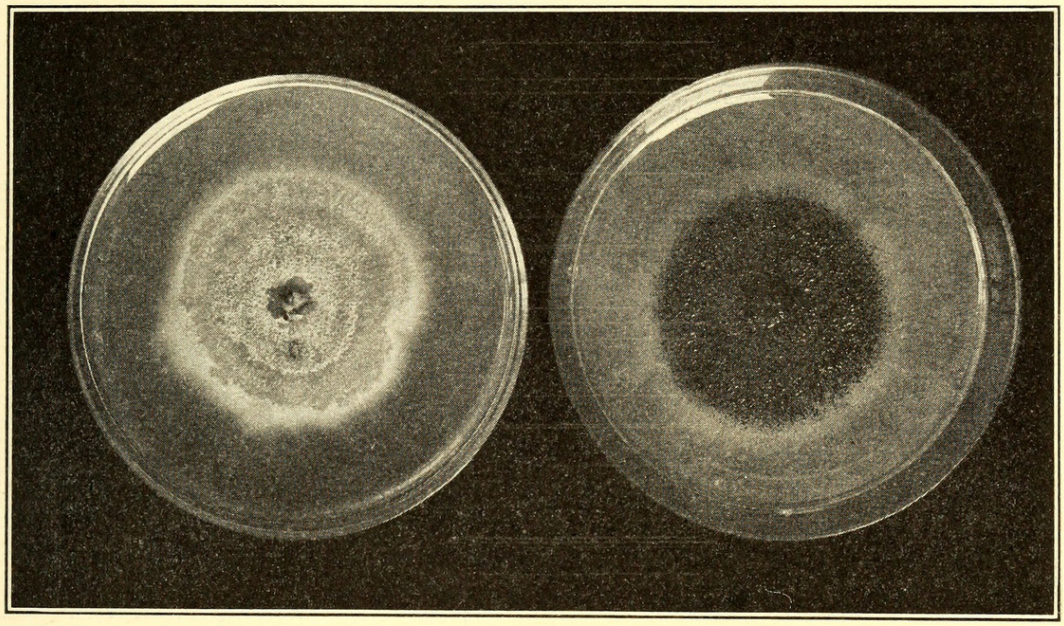


FIG. 8. III + and III −, 8 days old. These plates were inoculated from the plus and minus sectors respectively of the colonies shown in Fig. 7.

plus spore. When once purified the minus strains remain constant from generation to generation. The variation apparently is occurring in only one direction.

Attempts to determine the factor which disturbs the stability of the plus strains and causes the liberation of new minus strains have proved fruitless up to the present time.

Attempts have been made to develop a minus strain from a plus strain by artificial selection alone. The following methods have been employed:

1. Mycelium was continuously selected from the extreme edge of plus colonies where no pycnidia were yet forming.

2. Mycelium was continuously selected from colonies which were subjected to temperatures so low and so high that fruiting was poor.

3. Mycelium was continuously selected from colonies grown in such dry atmosphere that fruiting was poor.

Several generations of such selections gave no promise of the development of a minus strain.

Attempts have also been made to develop a plus strain from a minus strain.

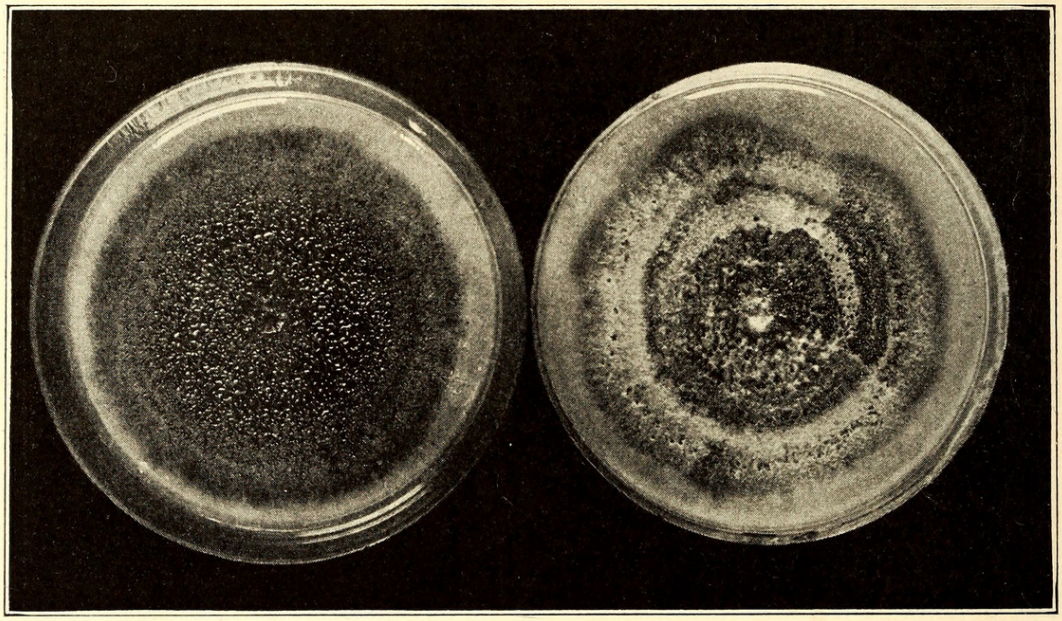


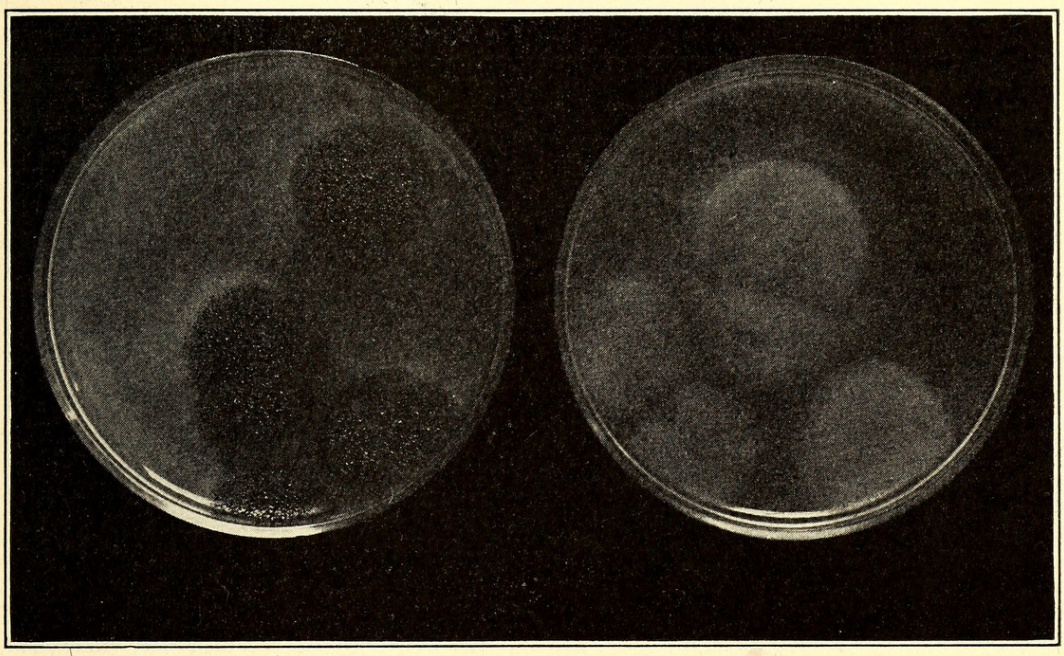
FIG. 9. Same as Fig. 8, 16 days old.

In all the minus strains studied there appear colonies which with age show greater prolificacy in some sectors than in others (Fig. 9). By the transplantation of spores only from the pycnidia of the prolific sectors generation after generation it was thought possible to build up a plus strain. Selection of this sort however has in no way altered the minus strain. Pedigreed cultures after such selection are in no respect different from pedigreed cultures obtained by continuous selection of mycelium from the poorest fruiting sectors of the minus colonies.

These experiments show that selection is not a factor in the origin

of the two strains. Since *C. pirinum* reproduces asexually segregation from heterozygous parents cannot explain the origin of the two strains. The only explanation which remains is that the minus strain is a sport or mutant arising from the plus strain at irregular and unprognosticable intervals. What makes it arise and what are the controlling factors of such mutation are worthy of speculation and experiment.

The writer[7] has reported a somewhat similar mutation in a fungus belonging apparently t the genuso Phyllosticta.



\+ −

FIG. 10. Third dilution plates of III + 3 and III − 3, 6 days old.

DEVELOPMENT OF PLUS AND MINUS SECTORS

A single isolated plant of *Coniothyrium pirinum* like most fungi grows radially. A germinating spore may produce one or two hyphae which, by a continuous dendritic branching, may give rise to large numbers of branches all of which however are nearly equal in diameter for their entire lengths and many of which end at the growing margin. Each hypha with its many branches then covers an area shaped like the sector of a circle. Therefore if something should happen to a hypha which would change its growth characters, its branches would

[7] Crabill, C. H. A mutation in Phyllosticta. Phytopathology 4: 396. 1914.

no doubt also exhibit this change and a sector unlike the rest of the plant would be the result.

Similarly with a colony. Different spores might give rise to hyphae unlike in their growth characters and sectoring of the colony

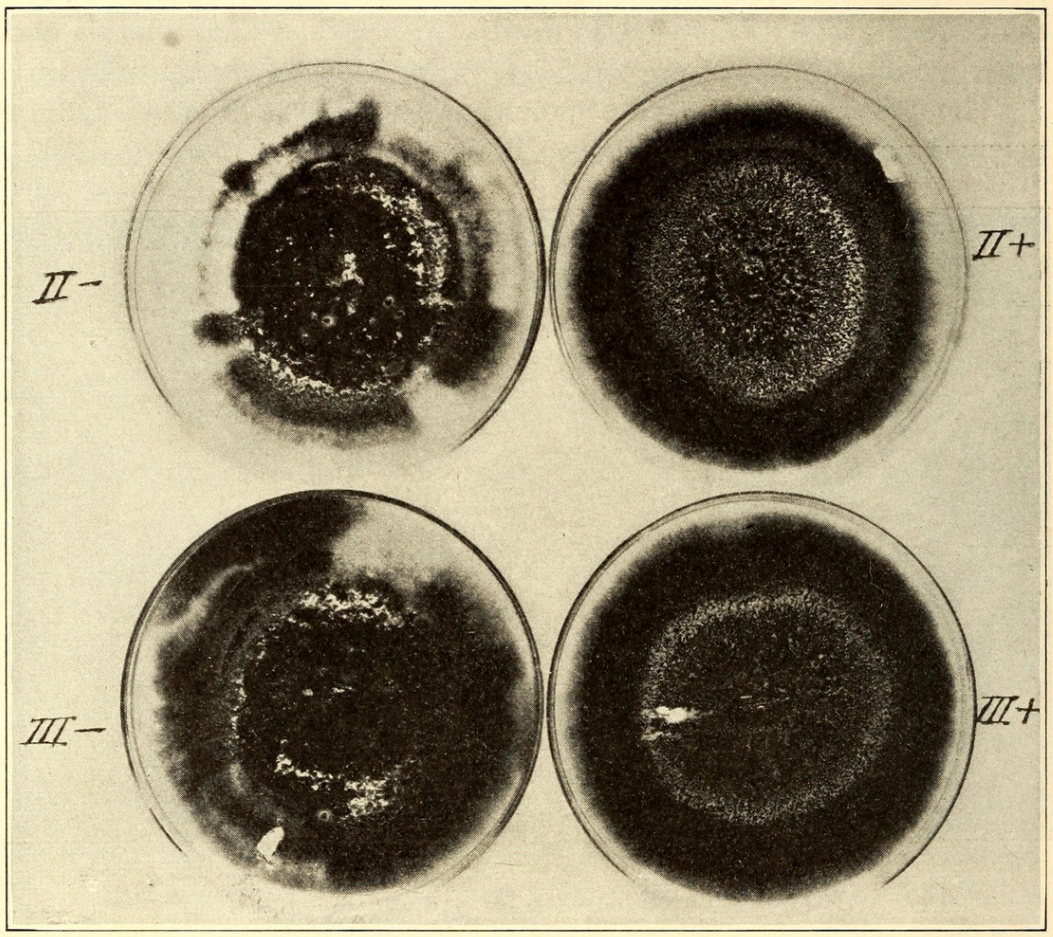


FIG. 11. 24 days old cultures of minus and plus strains of II and III for comparison. II − is in the 11th generation. II + is in the 3d generation after the splitting off of the third minus strain. III − is in the 4th generation. III + is in the 5th generation and shows the small minus sector which gave rise to the 2d minus strain from III. The cultural characters of the two minus and the two plus cultures are identical. In the minus cultures it will be noticed that some sectors produce more pycnidia and more color than others. This fact was taken advantage of in trying to develop a plus strain from a minus strain by selection.

would result. In the present studies sectoring in a single plant has not been observed. The sectoring has so far always occurred in colonies. Although the data are insufficient for positive proof the

evidence points to the conclusion that the variation which gives rise to minus strains occurs in the spore rather than in the mycelium which develops from it.

The study of plants of *C. pirinum* on Petri dishes shows beyond a doubt that the fungus is chemotactic, *i. e.*, growing only in the direction of its food supply or that it produces some toxic substance detrimental to its own growth.

Whichever the case may be, two plants in close proximity never grow entirely together (Fig. 13). The mycelial threads of the two do not interlace. They are to that extent antagonistic.

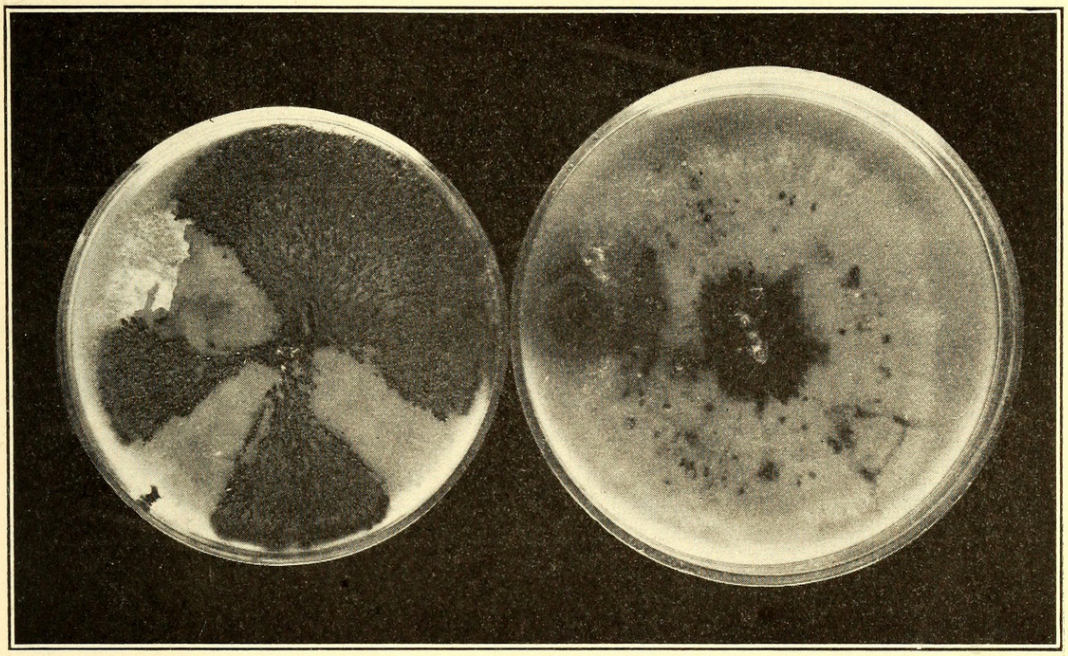


FIG. 12. IV and V, respectively. First sub-cultures, 24 days old.

Some simple tests indicate that this apparent antagonism is not due to excreted toxins. The fungus was grown for two months on a liquid medium containing starch. This medium was then filtered through a sterilized Chamberland filter.

The extract, which was found sterile on plating, was used as follows:

1. A large drop of the plain extract was placed at the margin of a thrifty colony on each of several Petri dishes.

2. Boiled extract was used in a like manner.

3. Plates were poured with plain extract and cooled agar and subsequently inoculated.

4. Boiled extract was used likewise.

In no case was the growth of the fungus hindered by the presence of the extract. It appears then that it is a lack of food rather than a toxic secretion which keeps colonies of *C. pirinum* from growing together.

A microscopic examination of thin Petri dish cultures shows that the mycelial strands exhibit a similar chemotaxis. They tend to

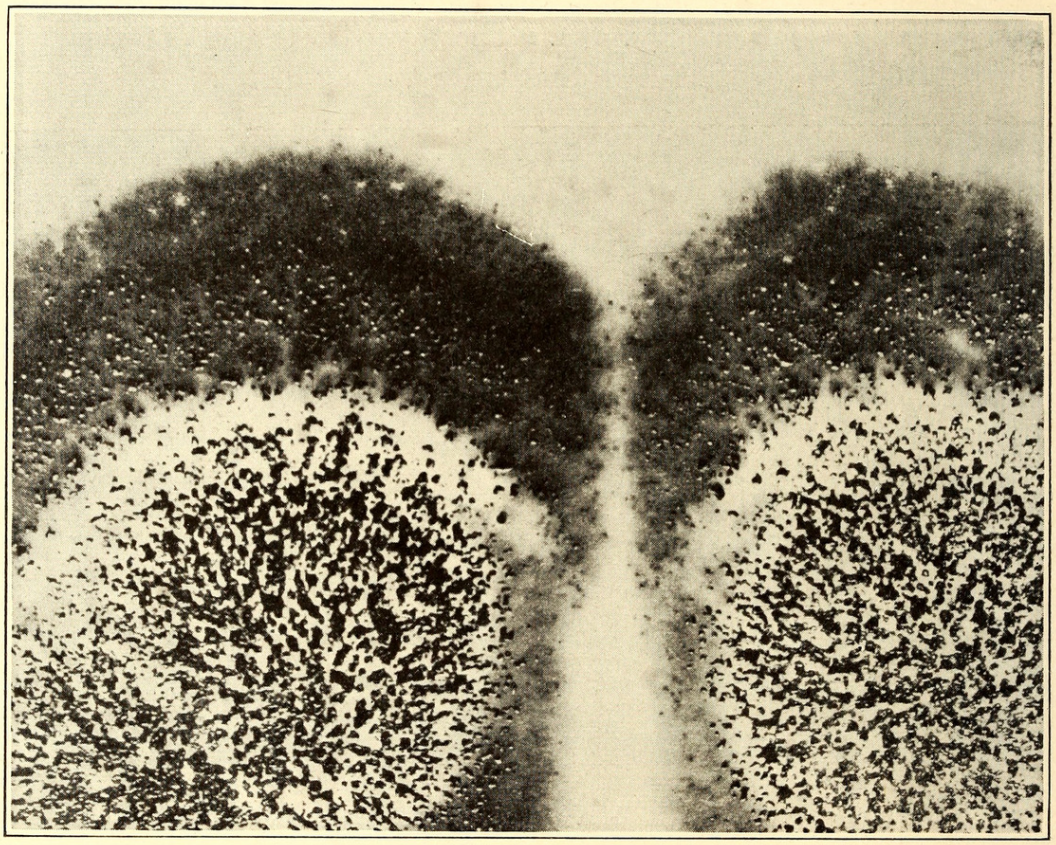


FIG. 13. Two colonies of a plus strain somewhat enlarged. The dark zone is olive green in color and just inside the white advancing margin. Inside of this zone the mycelium is hyaline. The colored oil has been withdrawn from it and deposited in the spores. The two colonies are antagonistic and do not grow together on adjacent sides.

diverge continually. If somewhat crowded they grow parallel but seldom converge or cross. It is doubtful therefore if plus mycelium ever crosses over into minus sectors and vice versa. In this way the integrity of the plus and minus sectors is preserved.

Some experiments were conducted to throw light on this question.

1. Small pieces of agar containing plus and minus mycelium respectively were placed side by side on starch agar. In the resulting

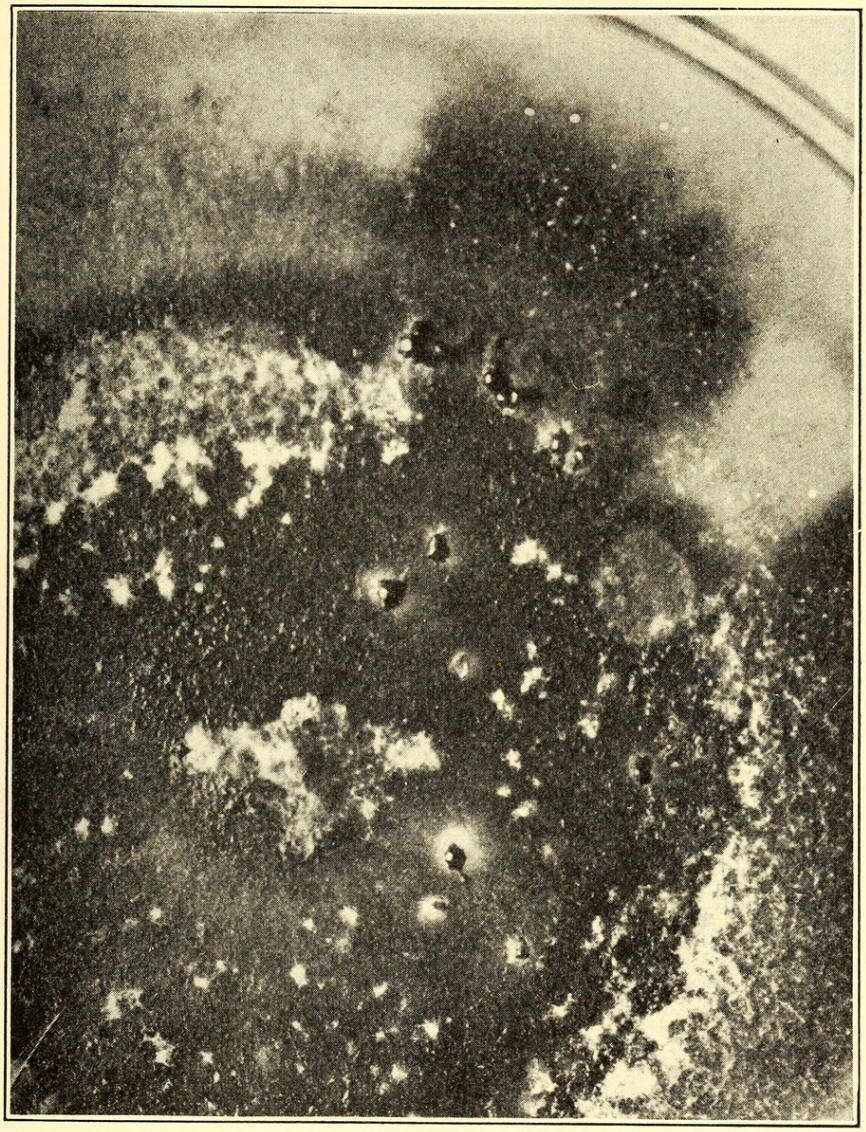


FIG. 14. Culture of a minus strain with the same enlargement as Fig. 13. The mycelium is dark throughout the greater part of the culture. The pycnidia are few, large, producing shining black masses of spores. The greenish color has been removed from the mycelium immediately surrounding the pycnidia. Some white fluffy aerial mycelium is present on the surface.

growth the two sectors were entirely distinct and typical. The minus portion was in all cases much larger than the plus portion.

2. Spores of plus and minus strains were mixed and then used to

inoculate plates. In the resulting colonies sectoring was prominent. The plus sectors were about equal to the minus in number, somewhat less in width and were decidedly distinct as usual.

In some of the photographs appended it is evident that the minus sectors of certain colonies are not quite sector-shaped in outline but narrow somewhat toward the margin (Figs. 7 and 11). As stated

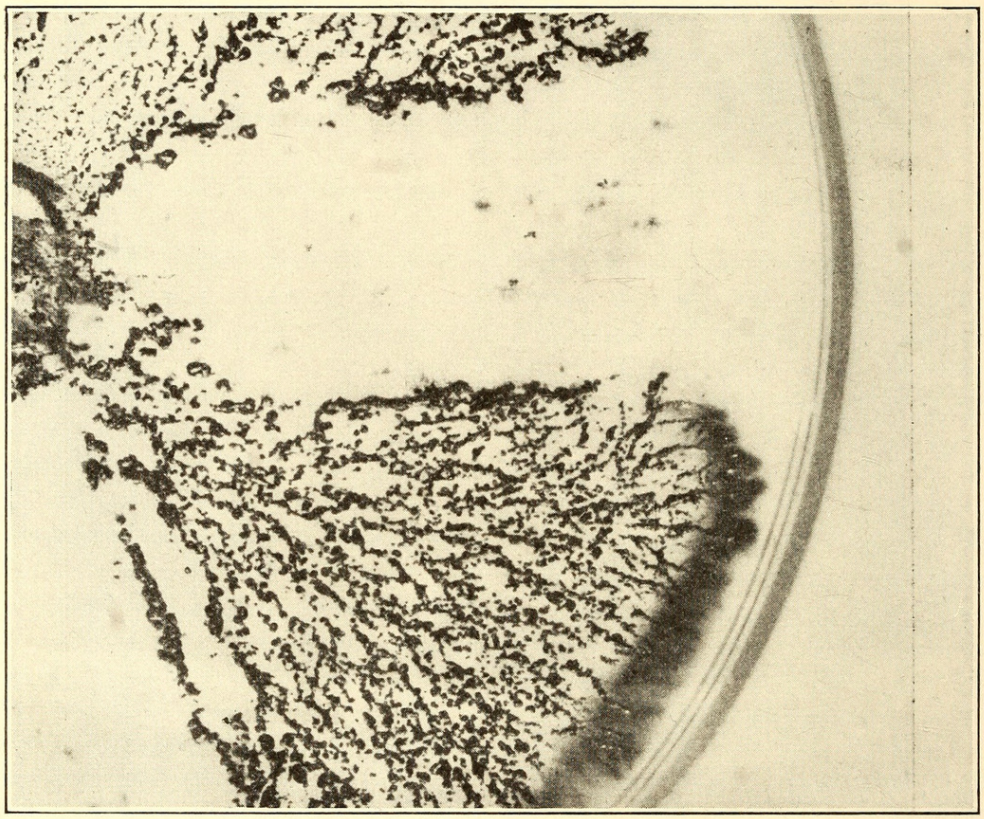


FIG. 15. Enlarged photograph of the IV colony shown in Fig. 12. The mycelium in the minus sector is almost entirely hyaline and fruitless. In the plus sector the color zone which was much broader a few days previous is very narrow and surrounds the outermost ends of the mycelium. Some of the color has diffused out into the agar.

above, when pieces of agar containing mycelium of the two were used to inoculate simultaneously the minus sectors were larger than the plus sectors. These observations together with measurements of the rate of growth in colonies of like age indicate that unless the plus strain has a relatively large amount of inoculating material to start from, the minus strain will predominate in growth volume. This is

accounted for by the fact that the prolific spore production in the plus strain requires food material and energy which would otherwise be used for vegetative growth.

Color Phenomena and Spore Production in Relation to Temperature

The production of an olive green color is characteristic of the mycelium of *C. pirinum*. On some media color production is more pronounced than on others. The present observations have been made on the two strains growing on starch agar in Petri dishes.

An interesting correlation between color production, sporulation and incubation temperature has been studied. It was found by experience that vegetative growth was most rapid at 25° C., and that sporulation was most active at 18–20° C. It was therefore customary to grow the cultures in moist chambers for 3–6 days at 25° C. and then at 18°–20° C. The moist chambers, each consisting of a plate of water and a tall belljar, maintained an atmosphere of absolute humidity and prevented the drying of the culture medium. The dishes were incubated upside down to prevent moisture from running over the surfaces of the cultures. Under the above conditions the following observations have been recorded.

Plus Strain.—Plus colonies grow rapidly but remain white 2–5 days. In three days pycnidia are usually present in abundance. Most of them are pink at this time but a few may be black due to the dark color of mature spores within. About the 5th day the mycelium just back of the growing ends begins to take on an olivaceous color. Later as the pycnidia mature in abundance this color is all absorbed, leaving the mycelium hyaline (Fig. 13). At the same time more color is formed farther out toward the margin of the radiating colony. The result is that an olivaceous zone migrating continuously outward always exists just behind the growing ends of the mycelium.

Microscopic study shows that the olivaceous mycelium is replete with refringent droplets of oil. In the inner zone of spore production the mycelium is hyaline and contains no oil drops. It is evident that the oil supplies the color. It is manufactured and stored temporarily in the mycelium. Later it is withdrawn and passes into the spores as they develop the dark color characteristic of the species. In cultures only 1–3 days old, spore production precedes color formation

No oil is at that time stored in the mycelium but passed directly into the spores.

In some old cultures the greenish color diffuses out into the agar (Fig. 15). This is especially noticeable in colonies which have reached the edge of the Petri dish in which they are growing. In many cases it has been subsequently absorbed on sporulation.

Cultures 20 days old kept at 13°–15° C. for 10 more days become quite black in the outer zones. This temperature is too low for active production of spores but does not materially hinder the growth and manufacture of oil by the mycelium. The oil is therefore not used as rapidly as produced and necessarily accumulates in the mycelium and imparts to it a dark color.

Cultures incubated constantly at 25° C. produce much less color, as well as fewer pycnidia, than those incubated at lower temperatures.

Minus Strain.—Minus colonies remain white 10 to 20 days. The olive green color then appears in minute spots usually around very young immature pycnidia. The color spreads gradually sometimes appearing more pronounced in some sectors than in others. The pycnidia on maturing absorb the color from the mycelium immediately surrounding them but owing to the fact that these are so few in number, the cultures retain most of the olive color which darkens with age until the culture is quite black (Fig. 14). Minus cultures incubated constantly at 25° C. produce very little color. The optimum temperature for spore production is 18°–20° C. Some cultures will scarcely fruit at all at 25° C. but fruit typically at 18°–20° C. Color production and sporulation are both maximum at the latter temperatures.

Mixed Cultures.—In mixed cultures in which plus and minus strains grow in respective sectors, the plus sectors develop and later absorb color as described for pure plus strains. The minus sectors on the other hand may never develop this color or if they do it is in small amounts and very tardy in appearing (Fig. 15). In many cases there is no spore production whatever. Normally a few pycnidia appear with characteristic tardiness.

Inoculations

The plus and minus strains of *Coniothyrium pirinum* have been used to inoculate apple leaves. Holes were seared in the leaves with a hot needle, wetted and smeared with spores. The leaves were then bagged.

In two weeks the spots inoculated with each strain turned whitish and bore several pycnidia from which it was easy to reisolate the strain. The cultures show that both the strains retained their diagnostic characteristics. One month after inoculation no enlargement of the seared spots has taken place. In fact some of the dead tissue bearing pycnidia of the fungus has fallen out producing a "shot hole" effect. This is further evidence that *Coniothyrium pirinum* is a saprophyte. The above experiment was conducted under controlled conditions in the greenhouse.

Conclusions

1. *Coniothyrium pirinum* is sometimes dimorphic in culture and probably also in nature.

2. Two distinct strains have been isolated, viz., a plus strain, which fruits abundantly, and a minus strain, which fruits poorly.

3. The minus strain arises in artificial culture by sudden sporting from the plus strain. This phenomenon has been observed in four separate instances.

4. Minus strains never give rise to plus strains but remain constant, generation after generation.

5. Attempts to develop the strains from each other by continuous selection of extremes have been unsuccessful.

6. Attempts to determine the cause of the sporting have been fruitless.

Virginia Agricultural Experiment Station,
Blacksburg, Va.