STUDIES OF THE LIFE HISTORY OF CERCOSPORA APII FRES.

Thesis for the Degree of M. S.

Ralph W. Lewis

1937

Cercospora
Series

Botany

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INTRODUCTION

These studies of Cercospora apii Fres. are a continuation of work previously reported by Klotz. (7) In this earlier work he proved the pathogenecity of the fungus and demonstrated that it over-winters in celery refuse. Klotz did not develop a method of obtaining copious conidial production in pure culture, nor did he find a perfect stage of the fungus. In the present work a method for the production of conidia is described and a study of sexual potentialities is reported. Although this part of the work was unsuccessful in producing the perfect stage, a great number of interesting observations are described and certain structures observed that are probably closely associated with the sexual development of the fungus.

CONIDIAL PRODUCTION

Klotz reported that conidia were produced on rice, corn meal, carrot plug, nutrient dextrose agar, corn meal agar, celery refuse on muck, and on muck alone. Of all these media conidia were formed in quantities only on celery refuse, carrot plug, and on corn meal agar (when the culture was kept at 15° - 17°C.). On the other media the numbers of conidia were few.

Nagel (9, 10) recommended a short interval system of transfers to maintain the capacity of some Cercosporae to produce conidia. He worked with Cercospora beticola, C. dubia, C. cruenta, C. davisii, C. physalidis, and C. setariae. He was able to maintain these fungi in a sporulating condition by growing them on sugar-beet leaf agar or potato dextrose agar. They continued to sporulate only when transferred regularly at three to seven-day intervals. It was necessary to use a newly isolated culture

at the beginning and to transfer only the spores.

This is an excellent method of keeping these fungi in a sporulating condition, but it necessitates continuous vigilance and care in making transfers. The writer describes a method of stimulating conidial production from any stock culture of Cercospora apii at any time (unless the culture shows deterioration) and possibly this method may be applied to other species of Cercospora.

Jenkins (5) has shown that Cercospora cerasella Sacc. (Mycosphaerella cerasella Aderh.) will produce conidia on several agars (potato, corn meal, bean, etc.) to which have been added fragments of diseased cherry leaves. Conidia are found only after these cultures had been sterilized and replanted with cultures from an ascospore source. How abundantly the conidia were produced was not mentioned.

Wolf (12) in his work with Cercospora rubi Sacc. (Mycosphaerella dubia (Sacc.) Wolf) noted that conidia were produced on newly made cultures grown on agar media. After a week no more conidia are formed. Latham (8) also found that for Cercospora cruenta Sacc. (Mycosphaerella cruenta (Sacc.) Latham) no conidia were present on agar media after five days. Hopkins (5) said that Cercospora medicaginis E. & E. produces spores in abundance upon partial drying out of plate cultures.

In the present work a variety of agar culture media were used with no success in producing conidia of Cercospora apii in abundance. The media tried were: potato dextrose, synthetic, prune, oat, corn meal, and celery decoction agars. The potato dextrose and celery decoction were used with different percentages of agar: 1, 2, 3, 4, and 5%. Celery petioles or leaves embedded in agar were also used. All these media gave good vegetative growth, but conidial production was rare.

Eight tubes were tried following Jenkins' method of producing

conidia. Cultures were grown on potato dextrose agar for twenty-seven days. These were sterilized fifteen minutes at fifteen pounds pressure and replanted with a single spore culture. None of these formed conidia. This cannot be considered as a fair trial of this method because the transfer for the subculture did not come from an ascospore source, nor were enough trials made.

In Klotz's work it appears that conidia were produced abundantly in pure culture when the fungus was grown on celery refuse in contact with muck soil. His description shows that he did not use this method to obtain conidia for innoculations. Field observations by the writer showed that in dry weather conidia were formed only on those diseased leaves which were in contact with the damp soil. From Klotz's work and from this observation an improved method has been developed for the productions of conidia of Cercospora apii in pure culture.

To a 250 c.c. Erlenmeyer flask was added soil enough to fill it 2 cm. deep. Muck, compost and sand have all been used with little difference in results. Water was added so that after the whole had been sterilized (four hours at fifteen pounds) there is a little free water in the soil, but none on the surface. After the soil had been sterilized, six thoroughly washed celery leaflets were placed in contact with the soil. The medium is then sterilized for twenty minutes. Conidia were produced in four to six days after the fungus was planted on the leaflets. They were present as long as there was space for new growth on the leaf surface. The conidia were produced more copiously on the newer fungus growth. More than nine experiments of this kind were carried out successfully with each of the fifteen strains of Cercospora apii on hand. The following table (I) shows the results from such an experiment.

TABLE I

Production of conidia on sterile celery leaves on soil at various time intervals. Cultures were grown at room temperature.

Strains of Cercospora	Number of Conidia			
	After 6 days	After 16 days	After 22 days	After 28 days
1.	*Abundant contaminated	Abundant	***Few	Few
2.	Abundant	Abundant	Many	Few
3.	Abundant	**Many	Many	Few
4.	Abundant	Abundant	Many	Abundant

*Abundant - more than 100 conidia per microscopic field, low power.

**Many - 10 to 100 conidia per microscopic field.

***Few - 1 to 10 conidia per microscopic field.

The conidia were collected from the culture by drawing the end of a flattened needle approximately one centimeter across the surface of the culture. The conidia collected were deposited in a drop of water on a slide, covered, and examined under the low power of the microscope. This method gives only an estimate, but where numbers are recorded as "many" or "abundant" there were present ample conidia for innoculation purposes.

Some of the cultures used had been grown on agar for more than a year and all of the transfers were mycelial.

The conidiophores on which the conidia were formed by this method were not typical of those produced in the field. Mostly the "internodes" (distance between the geniculations) were much longer as compared with the internodes, 10-30 microns, reported by Klotz; these were variable, often being hundreds of microns long. Some conidiophores were more nearly normal, but they grew much longer and produced many conidia. (Pl. I: 1-3).

In sectioning field material it was noticed that the fungus, aside from distorting the tissues, deposited some substance among the collapsed cells. This material stained deeply with safranin and iron alum haematoxylon. Sections of normal leaf have no such substance present. (Pl. I: 4,5). Tissues at the periphery of a lesion which looked normal in structure showed the deep staining spots and always upon further examination revealed the presence of fungus hyphae.

In order to determine whether or not the soil is necessary for the production of conidia an experiment was conducted as follows: In five flasks were put respectively, wooden blocks, glass beads, glass wool, sea sand and absorbent cotton each about 2 cm. deep. Enough water

was added to come almost to the surface of each material. On top of these were placed a few celery leaflets and the whole autoclaved. These were then planted with the fungus. The results are shown in Table II.

TABLE II.

Conidial production on sterilized celery leaves with various materials as a supporting substratum.

Support for Celery leaflets	Numbers of conidia				
	After 6 days	After 8 days	After 10 days	After 16 days	After 26 days
Wood blocks	abundant	abundant	abundant	abundant	abundant
Glass beads	abundant	abundant	abundant	abundant contaminated	
Glass wool	no growth				
Sea sand	abundant	abundant	abundant	abundant	many
Cotton	abundant	abundant	abundant	abundant	many

It is evident that conidia are produced readily with celery leaves as the only source of nutrients.

Conidia have been produced abundantly on materials other than celery leaves when these materials are placed on wet soil or in such an arrangement that there is a large surface for the evaporation of water. Leaves other than celery have been used as a source of nutrients for the fungus. They were treated as described for the celery leaflets on soil. The results in Table III show that spores are quite readily produced under these conditions.

TABLE III.

Production of conidia on various leaves when grown at room temperature before and after exposing to outdoor temperatures from October to April.

Leaves Used	Number of Conidia				
	After 5 days	After 14 days	After 24 days	After 5 months outside	3 days after brought into laboratory
Maple	No growth	----	----	----	----
Willow	few	contaminated	----	----	----
Corn	"	many	many	many	abundant
Soybean	"	few	few	----	"
Basswood	"	"	many	many	many
Sudan	little growth	"	"	----	abundant
Tobacco	few	abundant	"	many	"
Tomato	"	many	few	none	abundant
Raspberry	little growth	"	"	contaminated	----
Helianthus	Few	abundant	abundant	----	many
Celery	"	many	few	contaminated	----

After growing for twenty-four days these flask cultures were placed outside the window where they were subject to winter weather. Five months later, from October to April, they were brought into the laboratory, examined for spores, and watered. Many shrunken conidia were observed on some of the cultures. Three days of laboratory temperature caused many of the cultures to produce spores. This again shows that the fungus may winter-over as mycelium which forms new conidia when warmth and moisture are supplied. See Table III.

Conidia were obtained in abundance by growing the fungus on filter paper cones*. The cone was made from filter paper nine cm. in diameter and put in 75 c.c. flask. About 20 c.c. of potato decoction was added and the whole autoclaved. The fungus was then planted on the side of the cone.

Large numbers of conidia were produced by this method, but usually they were less typical than those produced by the fungus on celery leaves on soil. Mostly the conidia had fewer cells, the number varying from 3-8. (Pl. 1: 6). In some cases there were many one celled conidia. Table IV. shows the results of one experiment of this type.

(*Coons, G. H. Factors involved in the growth and the pycnidium formation of Plenodomus fuscomaculans. Jour. Agr. Res. 5: 721. 1916)

TABLE IV.

Conidia produced by various strains of Cercospora apii when grown on filter paper cones.

Strain of fungus	Number of Conidia		
	After 7 days	After 10 days	After 15 days
1A	culture deteriorated	no conidia	
2A	many	many	many
3A	none	few	few
9A	culture deteriorated	no conidia	
10A	many	many	many
11A	"	"	"
12A	"	few	few
13A	few	"	"
14A	many	many	many
15A	"	"	"
16A	none	none	none
17A	few	few	few

In the above table it is noted that two of the cultures have "deteriorated". The exact nature of the deterioration is not known, but the fungus shows characteristic changes. First, the loss of its olivceous green color on agar slants. The color gradually turns grey and then white. In some cultures white sectors would develop without any intermediate grey stages. Second is the toughening of the mycelial mat. It often becomes so tough that it is difficult to secure a portion to transfer. When in this condition the cultures do not often produce spores.

At first it was thought that the non-production of conidia was a matter of nutrition. This was probably not the reason for sterility on agar media. It has been shown that conidia fail to develop on celery decoction agar and on celery leaves or petioles embedded in agar. Because conidia are produced on sterilized celery leaves under different conditions than that afforded by the agar medium, it points to the fact that it was the physical conditions or the presence of metabolic substances which inhibited conidial production. By growing Cercospora apii on sterilized celery leaves on soil there is procured an adsorption medium and at the same time a very humid atmosphere. These two factors are apparently conducive to the production of conidia even when mycelium is used as a source of transfer. These experiments show that this fungus after growing on agar for months does not lose its potentialities for conidial production. However, the right conditions are necessary to bring out these potentialities.

STUDIES OF SEXUAL REPRODUCTION

Because of the growing importance of early blight of celery, it is of economic interest to know the complete development of this fungus. Klotz demonstrated that it over-wintered in celery refuse. Closely related fungi are known to over-winter in the form of sexual structures.

It is logical to assume that Cercospora apii may also over-winter in this form which could be an important source of initial infection.

Coons and Larmer (1) while studying Cercospora beticola Sacc. found sclerotium-like masses of loosely woven hyphae on submerged mycelium when the fungus was grown on agar plates. Duggar (2) reported that efforts to find the perfect stage of Cercospora apii failed.

Higgins (3) in his studies of Cercospora bolleana (Thüm.) Speg. (Mycosphaerella bolleana (Thüm.) Higgins), of Cercospora viticola (Ces.) Sacc. (Mycosphaerella personata Higgins) and Cercospora liriodendri E. & H. (Mycosphaerella tulipiferae (Schw.) Higgins) describes the young spermagonia, the mature spermagonia, young perithecia with trichogynes, the development of the perithecium, and the production of asci. In studying Cercospora viticola and Cercospora liriodendri he reports: "All attempts to study the life history of the fungus in pure culture on artificial media resulted in failure." In the thousands of sections of material collected from nature he obtained no evidence to prove that spermatia were functional; however, his last work furnishes evidence which is quite convincing that spermatia do function.

In studying Cercospora cerasella Sacc. (Mycosphaerella cerasella Aderh.) Jenkins (5) found in field material, spermagonia bearing spermatia, young and mature perithecia with asci and ascospores. He also reported that mature spermagonia and what were apparently perithecial primordia were produced in pure culture. These developed on sterilized agar cultures which were replanted with the fungus from an ascospore or conidial source.

Jones and Pomeroy (6) reported that no conidia or sexual structures developed in Cercospora concors (Casp.) Sacc. when grown on synthetic media. Klotz (7) makes no mention of structures which might be associated

with sexual reproduction of Cercospora apii.

Latham (8) has described spermagonia, spermatia, perithecia, asci, and ascospores of Cercospora cruenta Sacc. (Mycosphaerella cruenta (Sacc.) Latham) as they were found in nature and reported that no sexual reproduction takes place on agar cultures. Wolf (12) found and described the perfect stage of Cercospora rubi Sacc. (Mycosphaerella dubia Wolf). As in the above works spermagonia, spermatia, perithecia, asci, and ascospores were found only on material from nature.

In these cited reports the function of spermatia was not demonstrated. It is thought by some that they are functionless, but in the light of researches in other Ascomycetes, it seems probable that the spermatia here are functional.

The following is a report of attempts to find the perfect stage of Cercospora apii in field material and to produce the perfect stage in pure culture. Both attempts were unsuccessful. However, interesting observations were made which may be of value in future studies.

Material was collected from celery fields near Kalamazoo, Michigan. Plants were chosen which were known to have been diseased with early blight during the summer. Collections were made on September 9, November 22, December 12, 1936 and on March 17 and April 8, 1937. The material was examined under the binocular microscope. Portions were chosen which showed the old conidiophores on the surface or which had structures present which resembled incipient perithecia. The material was embedded in paraffin in the usual manner and sectioned at six or eight microns. More than twenty different portions were sectioned and stained with iron alum haematoxylon.

In the fresh material as well as in sectioned material it was

very difficult to discriminate between Cercospora apii and other fungi present. Septoria apii is likely to be present on much of the material. However, where Septoria was fruiting it was easily identified by the characteristic spores in pycnidia. Alternaria sp. or Macrosporium sp. were always present on dead celery leaves and petioles which had come in contact with the soil. These fungi along with other rotting organisms and insects so effectively destroy the leaves of celery that only those produced late in the summer are preserved through the winter.

More than one hundred and eighty slides (over 1000 sections) of field material have been studied and have yielded very little information. Many sclerotial masses have been found associated with what appeared to be conidiophores of Cercospora apii. In none of these were there ascogomia or spermatia produced. A few conidiophores could be identified definitely because of typical spores of Cercospora apii associated with them; the rest could not, without doubt, be differentiated from those of Alternaria. Some perithecium-like structures were observed which contained much enlarged ascogomia, the trichogynes of which could not be seen. A mycelial connection could not be traced to conidiophores of Cercospora apii, and no later stages were observed, so these were assumed to have no relation to the fungus in question.

The pure culture studies have yielded more fruitful results. About the middle of November, 1936 small black bodies were noticed growing in a culture on filter paper on sea sand. The nutrients were furnished by potato decoction to which had been added one per cent glucose. Shortly after, the same kind of bodies, designated sclerotia, were noticed in thirty cultures growing on potato decoction agar with one per cent glucose. Among these tubes were ten different isolates, seven from Michigan, one

from Florida, one from New York, and one from Ohio. These cultures were mated on filter paper cones in various combinations. They all grew well, producing many subcolonies. Most of the growth was at the edge of the liquid medium. Many small, floating cultures developed in the flasks from the growth of conidia.

On the filter paper near the liquid or in the floating colonies many sclerotia were produced. Many of these perithecium-like structures were crushed, but none showed development of asci and ascospores. All of these structures contained oil which was easily seen when they were crushed. Many were sectioned and will be described below.

All of these cultures were carried along on sterile celery petioles and in December they also were producing perithecium-like bodies. More transfers were made and sclerotia were produced in the daughter cultures. These were also sectioned and will be described. (Pl. III: 15)

Sclerotia were produced in cultures from mass isolates and in those originating from single spores. Upon crushing these sclerotia it was seen that the outer coating was thick-walled pseudo-parenchymatous tissue and the inner part of thin walled, colorless cells.

It is improbable if spermatia were produced in agar cultures that fertilization would take place unless some agent were present to transport them to the trichogyne. Cercospora apii grows so slowly that all loose water has evaporated before sclerotia are produced. To furnish an agent for transporting spermatia, if produced, the various cultures were mated on filter paper cones as described under conidial production. The flasks were shaken from time to time in order to distribute spermatia if present. Sixteen strains of Cercospora apii were mated. One culture each from New York, Florida, Ohio, and Formosa; three from California

and nine from Michigan. No mature perithecia or spermagonia were induced by this technique.

Celery petioles were prepared on soil as previously described for leaves and subcultures were planted in October. Some were placed outside on the window sill; others left in the laboratory. In April sclerotia were observed on both sets.

Studying sections (45 different portions were sectioned, 260 slides, more than 2000 sections) of the sclerotia from these cultures there appear to be three distinct kinds: (1) those in which there is no differentiation of cells, (2) those which are merely mound-like structures in the mycelial mat and (3) those in which the cortical cells are differentiated from the internal cells (named the perithecial type).

Those sclerotia which belong to group 1 are illustrated in plate II, figure 7. They are produced on all kinds of media often growing on the same culture with the perithecial type. In diameter they vary from 50 - 100 microns. The cells are all uniformly brown in color and the cell walls approximately the same thickness. It appears from studying these structures that they originate from a single hypha. The branches of this hypha intertwine and produce the sclerotial mass. These are quite similar to the perithecial type except for internal cell structure. Staining with iron alum haematoxylon shows the protoplasmic structure to be similar in all cells.

A variation of this type is shown in plate II, figures 8 and 9. Here it appears as though the rounded mass had continued to grow and formed a tall column. These were formed on agar cultures and on celery petioles which had over-wintered outside the laboratory and on similar cultures in the laboratory.

The sclerotia of the second type were formed only on potato dextrose agar and from outward appearance were thought to be perithecial type (Pl. II: 10). The outer layers of cells were thick walled and brown; the inner layers were thin walled and colorless. Running through these inner layers in an irregular manner dark staining mycelia were often observed.

The third type was called "perithecial" type because they resemble undeveloped perithecia. (Pl. II: 11, 12; Pl. III: 13). Plate III, figure 15 shows the surface of a celery petiole culture with the perithecial structures and figure 16 similar structures in a test tube culture.

The perithecial structure has an outer wall two to four cells thick. These cortical cells have thickened, brown walls. The inner cells are colorless with thin walls. The protoplasm is granular and where nuclei could be distinguished, only one occurs in each cell. In these structures, however, only small nuclei were present and it was, in most cases, impossible to determine which of the deep staining particles present were nuclei.

In some of these perithecial structures there were hyphae which took the stain much more readily than the rest of the inner cells. In many sections these resembled an ascogonium, but in none could a trichogyne be seen, nor a dissolving of the inner cells. No structures which might be called ascogenous hyphae were present. (Pl. II: 11,12).

Only in one culture which grew on a celery petiole was there evidence of a spermagonium. Two were sectioned. These contained what appeared to be spermatia. No sperm mother cells could be distinguished from the wall cells. This cannot be accepted until more material is

studied.

DISCUSSION

From these studies of the production of perithecial bodies, it seems safe to assume that there is a sexual potentiality present. The exact causes which prevent complete development of a perithecium with asci and ascospores might be many.

The most plausible explanation is that the fungus is heterothallic and that the opposite sexual phases have not been secured in the same culture under favorable conditions. It appears from the above studies that all the strains collected have been of one sexual phase and therefore sexual development has been incomplete. Larger collections of the fungus from all the world may find the other sexual phase. If the conditions were right for development, it can be assumed that all the cultures used were of one sexual phase. However, the conditions under which the fungus was grown may have prevented full sexual development.

In pure culture studies there are always present a larger number of metabolic products than in the field and these influence the growth and development of the fungus. In future studies of this fungus a method should be developed which will remove metabolic materials as they are carried away in nature. Some mechanical means will be necessary to carry the spermatia to the trichogyne. And then if both sexes and favorable conditions of temperature and moisture are present also, sexual structures will probably complete their development.

SUMMARY

Conidia of Cercospora apii can be produced by growing the fungus on sterile celery leaves on wet sterile soil. Mycelial transfers from old cultures produce thousands of conidia when grown in this manner.

Field material was searched to find the sexual stage of the fungus. It was not found. Pure culture studies produced many perithecium-like bodies, none of which developed into mature perithecia.

EXPLANATION OF PLATES

Plate I.

Figure 1. Conidiophores showing elongated internodes. The internodes shown are approximately 100 microns and 64 microns long.

Figure 2. A fasicle of conidiophores.

Figure 3. A much elongated conidiophore with many geniculations.

Figure 4. Cross section of a normal celery leaf.

Figure 5. Cross section of diseased celery leaf showing conidiophores and dark staining angular spots associated with infected areas.

Figure 6. A typical conidium from filter paper cone culture, 59 microns long.

Plate II.

Figure 7. Section through sclerotial body showing little differentiation of cortical and internal cells. 83 microns in diameter.

Figure 8. Section through elongated sclerotium; 220 microns long.

Figure 9. Enlarged portion of elongated sclerotium of figure 8.

Figure 10. Section through mound-type of sclerotium, 390 microns wide.

Figures 11 and 12. Sections through perithecial type of sclerotia,55 and 100 microns in diameter respectively, showing dark staining internal cells.

Plate III.

Figure 13. Section through perithecial type sclerotium, 110 microns in diameter, with no dark staining hyphae.

Figure 14. Section through culture grown on celery petiole showing many perithecial type bodies varying in diameter from approximately 34 microns to 120 microns.

Figure 15. Surface view of culture on celery petiole showing sclerotia.

Figure 16. Surface view of test tube agar culture showing sclerotia.

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Plate I

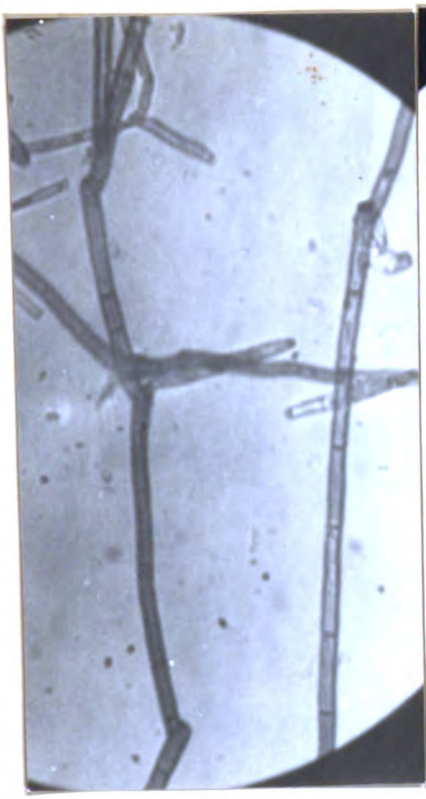
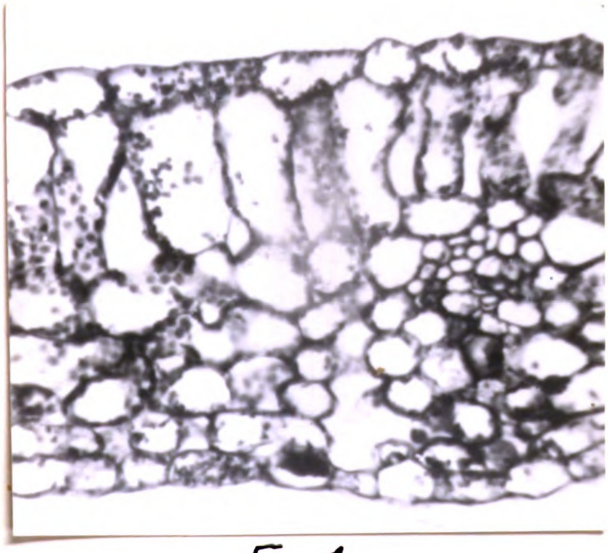


Fig. 1

Fig. 2

Fig. 3



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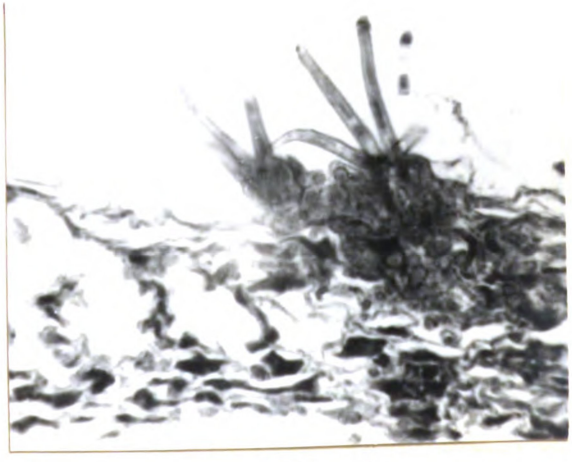

Fig. 5

Fig. 6

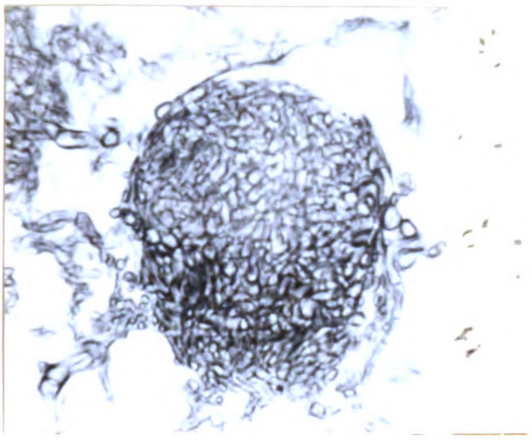

Fig. 7

Fig. 8

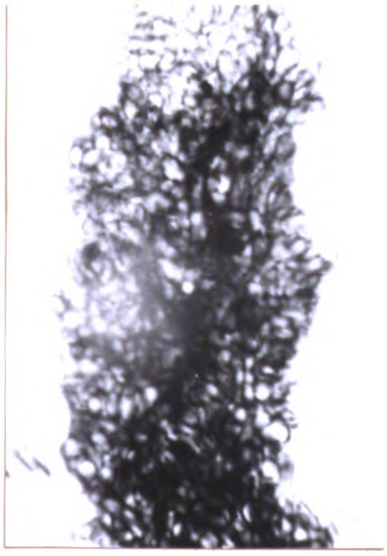

Fig. 9

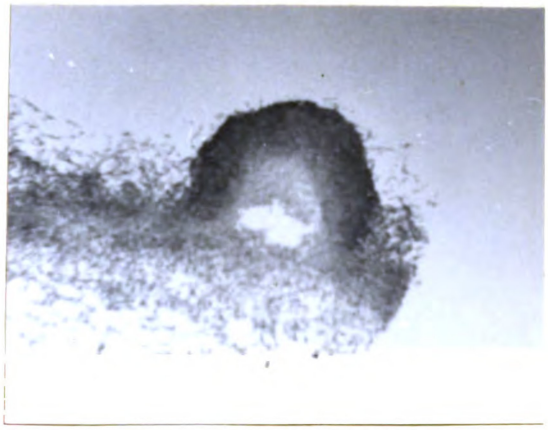

Fig. 10

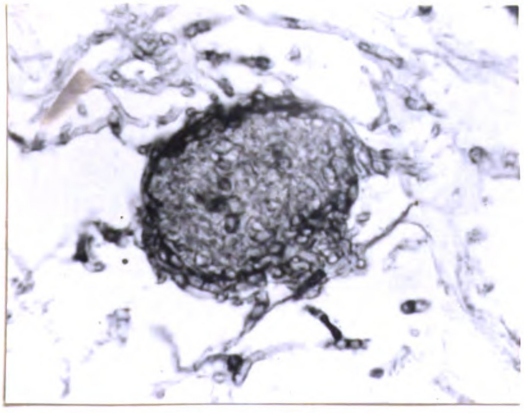

Fig. 11

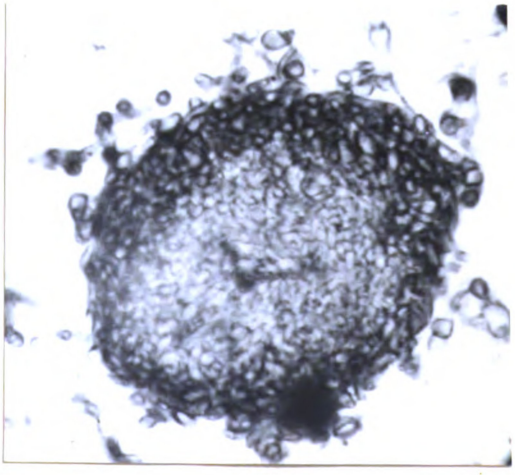

Fig. 12

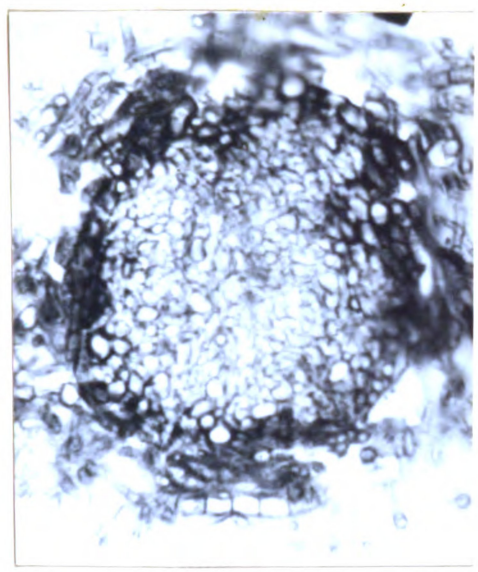

Fig. 13

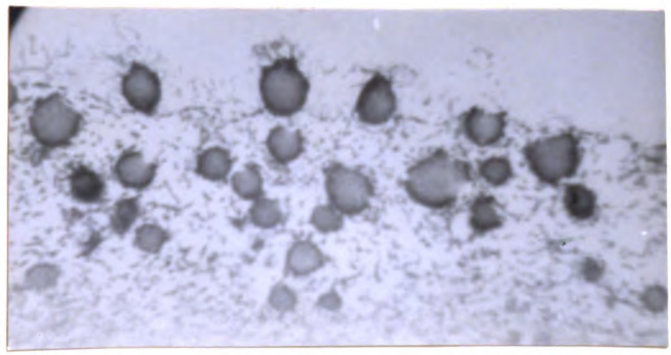

Fig. 14

Fig. 15

Fig. 16