HOST - PARASITE RELATIONSHIPS IN A MYCOPARASITE

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ABSTRACT

A mycoparasite, Piptocephalis virginiana, shows a resemblance to fungal parasites of higher plants in the fine structure of hyphae and haustoria. The morphology and fine structure of host and parasitic fungi have been described. The mode of penetration of the host cell, Choanephora cucurbitarum, probably involves mechanical forces. Although the presence of cell wall degrading enzyme was not detected by conventional techniques, its role in penetration can't be ruled out. A collar around the haustorial neck is formed as an extension of the host cell wall. No papilla was detected although appressorium was seen during penetration. The young haustorium is enclosed in highly invaginating plasmalemma of the host cell and numerous cisternae of endoplasmic reticulum. Appearance of an electron-dense sheath around the mature haustorium seems to coincide with the disappearance of cisternae of endoplasmic reticulum from the host cytoplasm in the vicinity of the haustorium. The role of host cytoplasm particularly of endoplasmic reticulum in the development of the sheath is discussed.

Extensive accumulation of spherosomes-like bodies, containing lipids, is found in haustorium, parasite and host hypha. Electron microscope revealed the parasitic
culture spore has more lipid content than the axenic culture spore of _P. virginiana_. The biochemical and cytochemical tests also support these results.

The mature spore of _C. cucurbitarum_ possesses a thick three-layered cell wall, different from the hyphal wall. Its germination is accompanied by the formation of an elastic thin inner layer which surrounds the emerging germ tube and the growing hypha. High resolution autoradiography showed that $^3$H N-acetyl-glucosamine, a precursor of chitin, was incorporated preferentially in the thin inner layer of the spore wall and also in the cell wall of the growing hypha. When the label was fed to the infected cells, at different intervals after inoculation, grains were observed on the sheath which developed around the haustorium of _P. virginiana_, 30 hours after inoculation. The significance of these results in relation to the origin and composition of the sheath is discussed.
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DEDICATION

To my parents, and to my late Grandfather, whose love and self-sacrifice have made this work possible.
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INTRODUCTION

Mycoparasitism denotes the interrelationships of a fungus parasitic on another fungus. In nature, fungi parasitic on each other are quite common and their combinations are widespread ranging from Phycomycetes, Ascomycetes, Basidiomycetes to Deuteromycetes, e.g. Olpidiopsis vexans is a parasite of Saprolegnia spp., Eudarluca caricis of rust, and Mycogone perniciosa of cultured mushroom (Madelin 1968). Since the mycoparasites have little economic importance, these fungi have been either poorly investigated or completely ignored.

During the last century, man has studied many plant diseases but the basic problems of parasitism are still unsolved. Many recent studies on host-parasite relations at a cellular level pertain to fungal parasites on higher plants (Ehrlich and Ehrlich 1963, Peyton and Bowen 1963, Shaw and Manocha 1965, Mckeen et. al. 1966, Zimmer 1970). Most of these studies have been concerned with the structure of the haustorial apparatus and the interpretation of the interface (Bracker, 1967). Many specialized activities were noticed at the host-parasite interface. In Puccinia graminis, Ehrlich and Ehrlich (1963) observed a continuity of material from the haustorium cytoplasm
through small channel-like areas in the haustorium wall and into the encapsulation. The character of the substance composing the encapsulation is similar in appearance to the cytoplasm of the haustorium. In *Peronospora manshurica*, Peyton and Bowen (1963) have observed numerous vesicles in the region of the host plasma membrane and they have interpreted these as secretory bodies derived from the host cytoplasm and fusing with the host plasma membrane to discharge material into the zone of apposition. The observations of Shaw and Manocha (1965) also agreed with the formation of vesicles and indicated that an extensive development of smooth-surfaced endoplasmic reticulum around the haustorium of *Puccinia graminis* may be associated with the secretion of the encapsulation. Mckeen *et. al.* (1966) stated that the haustorial body and branches of *Erysiphe cichoracearum* contained numerous chondriosomes, vesicles, and large electron-dense bodies. The encapsulation is composed of by-products which result from host-parasite interaction. Armentrout and Wilson (1969) have described that the blebs from the sheath of *P. virginiana* appears to be releasing material from the parasite via the sheath matrix into the cytoplasm of *Mycotypha microspora*. They further reported that spherosomes of the parasite may contribute enzyme to the sheath which also contain acid
phosphatase. However these studies have not provided us with an answer to problems such as the nature of disease resistance and an explanation for obligate parasitism. It is quite likely that the complexity of the host plant has probably obscured the true picture of the interaction between the host and the parasite.

Rarely has a mycoparasite been an object of investigation. For these reasons a mycoparasite of Piptocephalis virginiana on Choanephora cucurbitarum was selected to elucidate the basic principles underlying parasitism. Mycoparasitic systems have certain advantages. They require little time and space for their culture and also the environment of the host can be controlled. Armentrout and Wilson (1969) have described that the relationships of mycoparasite are similar to those fungi parasitic on vascular plants. We believe that further understanding of the mechanism of parasitism in a simple system may throw light on the problem of parasitism in higher plants.

Both the host and parasitic fungi belong to the order Mucorales. C. cucurbitarum is a member of Choanephoraceae and belongs to the class Zygomycetes. It is a weak parasite, attacking squash blossoms and fruit, sometimes causing considerable damage (Alexopoulos, 1966).
P. virginiana is a member of Piptocephalidaceae. The members of this family are obligate parasites of other fungi. Of particular interest is P. virginiana which attacks only the fungi of Mucorales.

The present study is in the main light and electron microscopy which is intended to reveal the general morphology and ultrastructure of host and parasitic fungi with special emphasis on their interface and its relationship to the components of the host cell. Of special interest is the study of the sheath zone, its origin, development and function. High resolution autoradiography has been used to elaborate on this aspect. Another feature encountered in this particular system is that the parasitic culture spores of P. virginiana can germinate to produce one generation in the absence of the host, whereas the axenic culture spores cannot produce another generation. It is expected to find ultrastructural and biochemical differences between these spores.
An obligate fungal parasite is one which, in nature, must infect a living host to complete its life cycle. Fungi can parasitize plants, animals, and other fungi. Those which infect plants are economically important since they cause damage to staple food crops. Consequently, they have been the object of much research. Those which parasitize other fungi are known as myco-parasites. Since these associations have no direct affect on man's economic policies, they have either been poorly investigated or completely ignored. Yet, a similar mechanism of parasitism may exist in both fungal-plant and fungal-fungal associations.

All stages of infection of plants by obligate fungal parasites have been studied at the ultrastructural level. The fungal spore lands on a suitable host plant and germinates. At the tip of the germ tube, an adhesive structure called an appressorium develops which is necessary for host penetration (Yarwood, 1956). In fact, in the cucumber anthracnose fungus, *Colletotrichum lagenarium*, appressorial formation enables penetration directly through the host epidermal cells (Anderson and Walker, 1962). The host cell wall is invaded by means of an infection peg, a structure differentiated from the appressorium. However,
the mechanism of penetration is controversial. It may be purely mechanical, chemical, or a combination of the two. Goodman et. al. (1967) has mentioned that the penetration of fungus through the cell wall is regarded as a mechanical process. On the other hand, Smith (1900) found that when the hyphae of *Erysiphe communis* came in contact with geranium epidermal cells, the host cell wall would stain deeply with safranin. This indicated the possibility of chemical degradation. Mckeen et. al. (1969) in their histochemical and electron microscopy study, reported that both chemical and morphological alterations occur in the epidermal walls of barley, clover, strawberry, and sunflower, around the infection pegs of different powdery mildew fungi. They pointed out that even though chemical alterations did occur in the epidermal walls, this does not rule out the possibility that mechanical pressure is required for penetration. Supporting this, a recent observation on the penetration process during infection of barley by *Erysiphe graminis* has revealed that penetration consists of both the enzymatic digestion of the cellulose portion of the epidermis and the mechanical pushing of the infection peg through the papilla (Edward and Allen, 1970).

Once the host cell wall has been penetrated, the portion of the fungus which expands inside the host cell is
known as the haustorium. This is thought to be an absorption organ which obtains nutrients from the host (Yarwood, 1956). Brown (1936) has suggested that the haustorium is specifically adapted for the uptake of proteins from the interior of the plant cell which cannot otherwise be absorbed by the intercellular mycelium.

Although the host cell wall is penetrated, the host plasmalemma not ruptured, but is merely invaginated and remains intact (Peyton and Bowen, 1963; Berlin and Bowen, 1964; Shaw and Manocha, 1965; Ehrlich and Ehrlich, 1966; Bracker 1968). In the early infection stages, the host plasmalemma is closely appressed to the cell wall of the haustorium. In later stages of disease development the host plasmalemma and the haustorial cell wall become separated by an amorphous layer called the sheath. Once the sheath has developed between the host and parasite, the host plasmalemma is referred to as the sheath membrane. In fact, it has been shown that this membrane around both the haustoria of *Puccinia graminis* (Ehrlich and Ehrlich, 1963) and *Erysiphe graminis* (Bracker, 1968) is thicker than the host plasmalemma in a healthy cell. It may be a specialized portion of the host plasmalemma. However, Van Dyke and Hooker (1969), reported that the sheath membrane in corn smut does not appear to be any thicker than that of
the normal host. In any case, this membrane may be
necessary for the maintenance of a balanced host-parasite
relationship. The vitality of the host cell depends on
the integrity of the sheath membrane (Hirata and Kojima,
1962) since after the membrane is broken, the host cell dies.

The sheath itself between the host and parasite
is a zone of unknown composition and function. Although
authors have used various names to describe this zone;
encapsulatio'n (Ehrlich and Ehrlich, 1963), zone of apposition
(Peyton and Bowen, 1963), and sheath (Smith, 1900), they all
refer to the area between the haustorial cell wall and the
host plasmalemma, in later stages of disease development.
Smith (1900) was the first to identify the presence of a
sheath, describing it as a sack-like covering around the
haustorium of a powdery mildew fungus. There is evidence
that this zone may originate from the fungus, the host, or
be composed of the products of both. Smith (1900) felt that
the sheath represented cellulose debris from the host cell
wall developing around the haustorium. Blackwell (1953)
who found a thickening around the haustorium of Phytophthora
infestans in blighted tubers, and naming it callose, also
presumed that it originated from the host cell wall. On
the other hand, Ehrlich and Ehrlich (1963) felt that the
encapsulation, in wheat rust, originated from the haustorial
protoplast. They observed small membrane bound vesicles in the host cytoplasm around well developed haustoria, and suggested that they might contain encapsulation material taken in from the zone by pinocytosis. Peyton and Bowen (1963) observed secretory bodies in host cytoplasm infected with *Peronospora manshurica* near the haustorium. According to them, these vesicles were discharged through the host plasmalemma into the zone of apposition. Berlin and Bowen (1964) also felt that the encapsulation which lay between the haustorium of *Albugo candida* and the host plasmalemma, was secreted by the host itself. In addition to vesicles, they observed a system of connecting tubules in the host cytoplasm near the host-parasite interface. Shaw and Manocha (1965) also supported the hypothesis that the encapsulation might be secreted by the host. They suggested that in rust infected wheat leaves, the extensive development of smooth endoplasmic reticulum in the host cell close to the encapsulation might be associated with sheath formation since smooth endoplasmic reticulum are not abundant in healthy cells. However, they also stated the possibility that the fungus may contribute some material to the encapsulation. Manocha (1966) pointed out that this smooth endoplasmic reticulum could be secreting host cell wall material into this zone. Mckeen *et. al.* (1966) working with *Erysiphe cichoracearum* reported that the granular encapsulation (sheath)
here, was separated from both the haustorial cell wall and the host plasmalemma by a clear zone of unknown composition. Hence, they believed that the encapsulation was composed of by-products which resulted from host-parasite interaction and served no useful purpose. Also working with *Erysiphe*, Bracker (1968) expressed the view that the sheath was composed of neither fungal nor host protoplasm since he was unable to detect any recognizable cytoplasmic components in the amorphous sheath. He did find that the sheath matrix increased in width with age, a well developed sheath reaching 5 μ in thickness.

The sheath may be composed of a number of distinct layers. Chou (1970) found that this zone of apposition in cabbage cotyledons infected with *Peronospora parasitica* appeared to have a three layered structure. Unlike most of the above mentioned studies, he failed to detect secretory vesicles around the haustorium. Zimmer (1970) has also reported three prominent regions of the sheath; an outer one believed to be primarily liquid in composition, a middle area permeated by electron opaque particles that may reflect physiological activity, and an inner layer which may be deposited by-products of physiological processes. He has observed lomosomes on both haustoria and hyphal cells which may be involved in cell wall deposition.
The sheath may be connected with the mechanism of disease resistance. Ehrlich and Ehrlich (1962) found that haustoria of Puccinia graminis in resistant wheat varieties tended to have fewer mitochondria and less endoplasmic reticulum than those in susceptible varieties. However, contrary to this, Shaw and Manocha (1965) stated that haustoria in resistant varieties were similar to those in susceptible wheat varieties in having as many mitochondria and endoplasmic reticulum. Furthermore, they (Shaw and Manocha, 1965; Manocha, 1966) found that the development of the encapsulation around the rust haustorium in a resistant variety, Khapli, was much faster than in a susceptible host, Little Club. Because of this, many of the haustoria in Khapli wheat became necrotic quickly, whereas, in Little Club, the fungus had undergone sporulation before necrosis set in. The breakdown of the plasmalemma and other subcellular structures in the invaded host cell was also much more rapid in Khapli. Stavely et al. (1969) working with Erysiphe polygoni, found that resistant and susceptible reactions could be differentiated soon after the fungus had penetrated the host cell wall and passed through the collar. In resistant most host cells of Trifolium pratense the plasma and nuclear membranes were rapidly destroyed. The sheath membranes in resistant cells,
never showed the vesicular invaginations found in susceptible infected host cells and consequently, the fungus did not establish a nutritional relationship with the resistant variety. The study of *Puccinia carthami* and the ultrastructural nature of exclusionary seed resistance of Safflower by Zimmer (1970) has indicated that resistance is manifested by cytoplasmic collapse, degradation of the haustorial sheath, and the deprivation of the haustorium of nutrients essential for glycogen synthesis. Glycogen is abundant in haustoria and hyphae of susceptible varieties, but absent in the same region of resistant hosts. Zimmer also believes that crystal-containing microbodies may play an important role in resistance. These microbodies are found in both infected resistant and infected susceptible hosts; however, they disappear more rapidly in resistant host cells.

Mycoparasitism has been recognized for only 100 years. This relationship was first discussed by de Bary in 1865 when he described the mycoparasitic association of *Cicinnobolus cessati* and *Piptocephalis freseniana*. In fact, he was the first to establish the genus *Piptocephalis* in which all known species are obligate parasites of other fungi. Much of the early research on this topic was confined to descriptions of the taxonomy, host range, mode of infection,
and morphological changes of the host and parasite during their relationship.

The first extensive study of the host range of *Piptocephalis* was carried out by Matruchot (1900) who used *P. tieghemiana* to test a wide range of fungal species as potential hosts; a Myxomycete, an Oomycete, 25 Ascomycetes, 9 Basidiomycetes, and a number of Entomophthoraceae. He concluded that this fungus only parasitizes members of the order Mucorales, since only ten species of the Mucoraceae and one species of the Pilobolaceae were infected. However, Dobbs and English (1954) discovered the new species of *Piptocephalis xenophila* which not only attacked non-mucorine hosts, but grew and sporulated more abundantly on members of the Ascomycetes and Fungi Imperfecti. Another species *Piptocephalis virginiana* was tested for host range by Berry and Barnett (1957). They found that only certain species of the mucorales were infected. Of the 20 species tested outside the mucorales, none supported parasitic growth. The most rapid and abundant growth of *P. virginiana* occurred on hosts of Choanephora cucurbitarum, Cunninghamella elegans, Mycotypha microspora, and Mortierella pusilla. On the other hand, growth of the parasite was generally poor on phycomyces blakesleeanus and Rhizopus nigricans. Despite the differences in host range between *P. virginiana* and *P. xenophila*,
a comparison of the study of Dobbs and English (1954) with that of Leadbeater and Mercer (1957) reveals no significant morphological difference between the two. Members of the genus Piptocephalis have also been studied with regard to spore germination. Brefeld (1872) investigated the growth of P. freseniana and found that in the absence of its host, growth of the germ tube ceased. However, he failed to mention the germination factor or the medium used in his experiments. Dobbs and English (1954) discovered that although P. xenophila would swell on a moist medium of malt agar or maize extract agar, no germination took place in the absence of other fungi. The germination of P. virginiana was studied by Berry and Barnett (1957). They noted that the spore failed to germinate either in distilled water or on water agar. In addition, synthetic media which contained mixtures of 16 amino acids, the B vitamins thiamine, biotin, and pyridoxine, vitamin B_{12}, nucleic acids, guanine, uracil, glycylglycine, and glucose-asparagine synthetic media all failed to support germination. However, germination did occur, in the absence of the host, on a natural medium of malt extract-yeast extract agar. Spores of Piptocephalis virginiana also germinated on many synthetic media if certain fungi, not necessarily the hosts, were present. Filtrates
of the host and other fungi such as Aspergillus niger, Botrytis cinerea, and Fusarium oxysporum also supported germination.

The standard host of Piptocephalis virginiana, Choanephora cucurbitarum, can be grown in axenic culture, but occasionally in nature it is a weak facultative parasite on certain flowers and young fruits, causing rapid rot. (Barnett and Lilly, 1950). This fungus produces 2 types of spores under different cultural conditions, either sporangia or conidia. Christenberry (1938) found that the greatest number of conidial heads were formed under 12 hour alternating periods of light and darkness, red-yellow light being the most effective portion of the spectrum. More detailed studies concerning the environmental and nutritional factors influencing the growth of Choanephora cucurbitarum revealed that a relative humidity approaching 100% favoured the production of sporangia whereas, under conditions of low humidity, conidia were formed. As well, conidia did not form under either total light or dark conditions. Other factors such as carbon dioxide and temperature also influenced sporulation (Barnett and Lilly, 1950, 1955).

Wolf (1917) stated that only the conidial stage of C. cucurbitarum has been reported in nature. However, Palm and Jochems (1924) while studying the rot disease of
Amaranthus, found the sporangial stage occasionally. Sinha (1940) also observed sporangia on rotting Capsicum plants.

Choanephora cucurbitarum was first selected as the standard host of Piptocephalis virginiana for the study of host-parasite relations by Berry and Barnett (1957). This was not only because C. cucurbitarum is a highly susceptible host, but also because the spores of P. virginiana can be easily collected free from contamination by the host spores. This can be accomplished by growing the fungi in the dark which inhibits the sporulation of the host but not that of the parasite.

Studies on the physiological aspects of disease dynamics of P. virginiana reveal that the growth of the parasite on different host species is influenced by temperature. Thamnidium elegans is highly susceptible at 25°C but resistant at 15°C and 20°C. On the other hand, Helicostylum spp. is susceptible at 15°C and 20°C but resistant at 25°C. (Berry, 1959). Berry also found that the degree of parasitism of P. virginiana on Helicostylum spp. was affected by the maturity of the host cell wall. The parasite grew as a ring around the outer edge of the host colony where the mycelium was young and immature, but not in the centre where old mycelium was abundant. Karling (1942) described the same phenomenon in Rozella cladochyttri
which infected young hyphae of *Chytrids* to a greater degree than old hyphae. England (1969) made a comprehensive study of the relation of host age to the susceptibility of attack by *Piptocephalis*. Here, *P. virginiana* penetrated young hyphae of *Phycomyces blakesleanus* whereas, the wall of the fully mature host hyphae appeared to be resistant to penetration. Any condition which slowed down the rate of cell wall maturity appeared to result in increased susceptibility. Therefore, in these cases, the immunity of older hosts was primarily due to the failure of *P. virginiana* to penetrate the host cell wall. Thus, host age determined the success or failure of the parasite in establishing a nutritional relationship.

More recent work has dealt with the physiological and nutritional aspects of mycoparasite systems. Berry (1959) stated that changes in host nutrition greatly affect the growth of the parasite, even when growth of the host was only slightly, or not at all affected. He concluded that a high nitrogen content of the medium favoured parasitism. Shigo *et. al.* (1961) also disclosed that the growth of *Piptocephalis* spp. on their host fungi is greatly affected by the concentration of carbon and nitrogen in the medium. The degree of parasitism was directly proportional to the amount of available nitrogen in the medium and the percentage of soluble nitrogen in the host mycelium. However, it was
inversely proportional to the sugar concentration of the medium.

Barnett (1964) has stated that any interest in the physiological aspects of host-parasite relations in mycoparasitic systems, has no doubt been stimulated by plant pathologists studying similar associations between pathogenic microorganisms and their plant hosts. Bracker (1967) in his review of the ultrastructure of fungi has also pointed out that ultrastructural information is scarce with respect to mycoparasitism. In fact, there has only been one major study of the host-parasite relations of mycoparasites at the electron microscope level. Armentrout and Wilson (1969) described the haustorium-host interactions during infection of *Mycotypha microspora* by *Piptocephalis virginiana* at high resolution and found that the haustorium and sheath of mycoparasitic systems are similar to those of fungal-plant disease relationships. For these reasons a mycoparasitic system was chosen for the present study.
MATERIALS AND METHODS

Materials

I Fungi

The cultures of *Choanephora cucurbitarum* (Berk. & Rav.) Thaxter and *Piptocephalis virginiana* Leadbeater and Mercer are the generous gifts from Professor H.L. Barnett, Department of Plant Pathology and Bacteriology, West Virginia University, Morgantown, West Virginia. The *P. virginiana* was collected in a cave near Morgantown, West Virginia in 1953.

II Media

A) Rich medium:

The host fungus with or without the parasite is multiplied on a rich medium of the following composition recommended by Berry and Barnett (1957).

- Malt extract: 20 g.
- Yeast extract: 2 g.
- Agar: 20 g.
- Distilled water: 1 l.

pH 6.2

B) Basal synthetic medium:

It is a modification of Lilly and Barnett's medium (1951) with the following composition.
Carbon source 20 g.
Nitrogen source 5 g.
Potassium dihydrogen phosphate 1.0 g.
Magnesium sulfate 0.5 g.
Sodium hydrogen phosphate 0.3 g.
Distilled water 1 l.
Agar 20 g.

pH 6.2

All the media were autoclaved at 10 lbs. pressure for 15 minutes. The inoculation is carried out by mixing the spores of both host and parasite in a suspension on the sterilized medium. A well mixed spore suspension was obtained by adding sterilized distilled water in an eight days old culture which contained the spores of both host and parasite fungi.

Methods

I Light microscope

A. Measurement of fungi.

All measurements and counts were made from water mounts. The exact size of the spore and sporophore was measured by using an ocular micrometer, which was calibrated against a stage micrometer. More than twenty spores
and sporophores were measured for each fungus.

B. Lactophenol cotton blue staining method.

The observations on general morphology of host and parasitic fungi were made from slides mounted in lactophenol cotton blue. The stain was prepared by mixing 5 ml. of 1% cotton blue in 20 ml. of lactophenol.

C. Test for acid phosphatase (Armentrout and Wilson 1969).

The infected mycelium was immersed in 0.6 g. lead nitrate in 500 ml. of 0.05 M acetate buffer at pH 4.4 to which 50 ml. of 0.1 M sodium glycerophosphate was added. The mixture was incubated at 37°C. for 24 hours.

D. Identification of lipid (Jensen 1962).

Mycelium and spore were fixed in Formalin-Acetic acid-Alcohol and stained with Sudan IV in 70% of alcohol for 20 minutes.

II Electron microscopy

A. Spore:

The spores were prefixed in a mixture of 2% acrolein and 2% glutaraldehyde for 2 hours, washed several times with distilled water, and post-fixed in 4% KMnO₄ for 40 minutes.

B. Mycelium:

Properly washed mycelia and germ tubes were
fixed either in (1) 3% KMnO₄ for 20 minutes, or (2) 3% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8 for 2-3 hours and post-fixed in 2% OsO₄ prepared in the same buffer for 4 hours. The mycelia were washed in distilled water and stained with 0.5% uranyl acetate overnight.

Both the spores and mycelia were dehydrated in enthanol and propylene oxide series and were embedded in Epon mixture (Luft, 1961).

Thin sections were cut by Reichert Ultramicrotome UM 3 using glass knives. Sections were mounted on copper grids and stained with lead citrate solution (Reynolds, 1963) for 5-8 minutes.

To demonstrate lipids in Epon-embedded ultrathin sections method of Eurenius and Jaråsker (1970) was followed.

III Autoradiography

Tritiated N-acetyl-glucosamine (specific activity 243 mCi/mM) was obtained from Amersham/Searle Corporation.

The spores of the host fungus, C. cucurbitarum, were floated on a rich medium labelled with 20 mc/ml of ³H N-acetyl-glucosamine. The germination started in about 5 hours and after feeding for another hour on the labelled medium, the germinated spores were harvested. The infected
host cells, 18, 24 and 30 hours after inoculation, were fed on the labelled medium for two hours. The spores and hyphae of the parasitic fungus P. virginiana, were treated in the same fashion. In controls, tritiated N-acetyl-glucosamine was replaced by non-radioactive compound. All samples, prior to fixation for electron microscopy were washed thoroughly in non-radioactive N-acetyl-glucosamine and distilled water.

The method of fixation and embedding was the same as described under electron microscopy. Thin sections were mounted on carbon coated grids. A gelled mono-granular layer of Ilford L4 emulsion was applied on the sections following the method of Caro and van Tubergen (1962). The grids were exposed for 2-3 weeks at 4°C. in sealed light-proof boxes containing silica gel. The autoradiographs were developed in Microdol X at 20°C. for 5 minutes and fixed in acid fixer for 5 minutes. The sections were stained with lead at high pH (Karnovsky, 1961) for 15 minutes. Numerous micrographs were examined for each treatment and typical representative of each were selected. As a result of short exposure period (2-3 weeks) and extreme meticulous care during preparation procedures, the problem of background was reduced to negligible.
IV Lipid extraction from the parasitic and axenic culture spore

A. Lipid extraction:

The equal amounts of parasitic and axenic culture spores were obtained by use of a colorimeter. The spores were then broken down by grinding with silicon powder in a mortar. The lipid was extracted three times with CHCl₃-methanol (2:1) for one hour at room temperature. The extracts were then evaporated until all the solvent had been removed. The lipid samples were then suspended in the initial solvent (about 0.2 ml) and it is ready to be used in thin layer Chromatography.

B. Thin layer chromatography:

A thin layer of silica gel approximately 250 μ thick was spread on the glass plate. After the plate was dried, a fine line was drawn parallel to 2 cm from one edge of the plate. The lipid samples were put on the line in a spot by a micro pipette until all the samples were placed. The plate was then placed in the developing jar containing the solvent mixture, Chloroform-methanol-water (35:15:2). It was air dried and sprayed lightly with iodine solution.
RESULTS AND OBSERVATIONS

I. The morphological aspect of host and parasitic fungi.

A. The morphology of *Choanephora cucurbitarum*

*Choanephora cucurbitarum* produces both sporangia and conidia under normal conditions of growth. The conidiophore usually arises from the mycelium earlier than the sporangiophore. The young conidiophore bears 5-8 spherical head cells. Each spherical head cell forms numerous sterigmata which in turn produce numerous conidia (Fig. 1). A globose sporangium develops at the tip of an unbranched sporangiophore. Each sporangium contains a large number of spores (Fig. 2). The sporangia are not uniform in size, hence the number of spores is also variable. The conidium is elliptical in shape with an average size of 18 \( \mu \times 9 \mu \) (Fig. 3). The spore is oval in shape with an average size of 15 \( \times 11 \mu \) (Fig. 4). Both the conidium and spore are brown in color.

B. The fine structure of *C. cucurbitarum*

1. Spore

The spore shows three distinct layers in the wall (Fig. 5). The outer layer, the perisporium, is thin and less electron-dense layer which covers the surface with relatively smooth appearance. The middle layer, episporium, consists
of electron-dense ridges along its whole surface. The inner layer, endosporium, is electron transparent. Three fairly large nuclei each bounded by a double membrane interrupted by several nuclear pores are located near the centre of the spore (Fig. 5). Inside the nucleus is the chromatin material. Many mitochondria and small particles of glycogen (Revel 1964) are scattered all over the cytoplasm. A few cisternae of smooth-surfaced endoplasmic reticulum can be observed in the area between the nuclei and mitochondria (Fig. 5). Numerous vesicles or small vacuoles with electron transparent areas are present.

2. Germinating spore

The spore cytoplasm shows distinct changes during germination. A new inner wall is formed during the spore swelling. The original three-layered spore wall is ruptured and the emerging germ tube is enveloped by the newly formed extensible inner wall (Fig. 6). The glycogen particles tend to disappear as germination proceeds and big vacuoles appear. The mitochondria become ovoid in shape.

3. Germ tube

A narrow and smooth cell wall surrounds the germ tube. Closely adpressed to the cell wall is a plasmalemma enclosing cytoplasm (Fig. 7). The cytoplasm contains numerous ribosomes and mitochondria, the latter with
characteristic double membranes and well developed cristae. Two well defined nuclei, each enclosed in a double membrane interrupted by pores and with a distinct nucleolus, are located at the centre of the cell. A few cisternae of endoplasmic reticulum with small vesicles are also observed in the cytoplasm (Fig. 7).

4. Hypha

As the hypha matures, many vacuoles develop and contain densely stained bodies (Fig. 8) probably lipid in nature. Figure 9 is a mature hypha stained with Sudan IV showing numerous dark lipid globules under light microscope. Thin sections of glutaraldehyde-osmium fixed and epon-embedded hypha reveal the absence of dense bodies after treatment with a lipid solvent (Figs. 10 and 11).

As the hypha grows older, the cytoplasm become highly vacuolated and the vacuoles may contain multi vesicular structures (Fig. 12). The cell wall is distinctly differentiated into two layers with different degree of electron density (Fig. 12). Plasmalemma also shows invaginations at certain points probably due to the growth of the inner layer of the cell wall. Endoplasmic reticulum is sparse and rarely visible in the hypha.
C. The morphology of *Piptocephalis virginiana*

The sporophore arises erectly from the host mycelium. The sporophores are 2 to 6 times dichotomously branched, with each dichotomy being shorter or narrower than the last (Fig. 13). The terminal branch gets enlarged to become a head cell. Each head cell develops 15-25 cylindrical rod like spores. The spores are smooth with an average size of 6 x 2.4 µ (Figs. 14-16).

D. The fine structure of *P. virginiana*

1. Spore

The fine structure of a mature spore reveals a rough cell wall which appears rather smooth under the light microscope (Compare Figures 17-18 with Figs. 14-16). The spore cell wall shows 2 layers, the outer layer is electron dense with numerous spines protruding out (Figs. 17, 18) and the inner layer is electron transparent. A large nucleus is present at the centre enclosed by a double membrane which is interrupted by pores (Figs. 17, 18). Examination of many micrographs has revealed only one nucleus per spore. Two prominent terminal lipid bodies have a smooth outline appearing transparent with KMnO₄ fixation (Fig. 17). Several mitochondria and a few cisternae of endoplasmic reticulum
are located around the nucleus and no vacuoles or vesicles are observed (Figs. 17, 18).

2. Germinating spore

As the spore germinates, it swells up two to three times its original size. The inner layer becomes electron dense (Figs. 19, 20). The endoplasmic reticulum consists of parallel electron-dense strands separated by an electron-transparent lumen. The strands may lie close to each other or they may separate, forming cisternae of varying sizes (Fig. 19). Some vacuoles start to develop and spherosome-like bodies are distributed throughout the cytoplasm. As the germination progressed, a large vacuole appeared in the spore. The contents of the spore migrated into the growing germ tube (Fig. 20). Mitochondria seem to increase in number. A nucleus is observed with no nucleolus (Fig. 20).

3. Germ tube

The germ tube cytoplasm is filled with ribosomes, mitochondria, cisternae of endoplasmic reticulum and a few small vacuoles (Fig. 21). The cytoplasm, delimited by plasmalemma, is enclosed in a thin layer of cell wall. The nucleus, with characteristic double membrane interrupted by pores, has homogeneous nucleoplasm. The endoplasmic reticulum
appears as elongated strands with many ribosomes (Fig. 21).

4. Hypha

In a young hypha multivesicular structures are frequently encountered and a few small spherosome-like bodies also appear (Fig. 22). The endoplasmic reticulum appear as smooth-surface, tubular structures aggregated together. The nucleoplasm is no longer homogeneous and it contains dense nucleoprotein particles.

A mature hypha possesses numerous spherosome-like bodies with a varying degree of stainability (Fig. 23). Staining with Sudan IV reveals numerous small dark lipid bodies in the hypha under the light microscope (Fig. 24). The lipid bodies are so numerous that they obscure the other organelles in the cytoplasm. The nucleus appears to contain a nucleolus but it is not as well defined as in the host cell (Figs. 7, 8, 12). The cell wall remains smooth and is not differentiated into layers.
II Difference between the parasitic and axenic culture spores

A. Germination in the presence and absence of a host

When the mixed suspension of the spores of *C. cucurbitarum* and *P. virginiana* is spread on the same plate containing the rich medium, the spores of the host germinate within 5 hours. Whereas the first germ tube of the parasitic fungus is not observed till after 16-18 hours. It was also observed that the spores of *P. virginiana* near the host hypha germinate faster than the spores far away. Each spore produces 2-6 germ tubes (Fig. 25) which may advance or branch at random.

After the infection peg penetrates the host cell, the external parasitic hypha turns aside and grows parallel to the host hypha, sending out lateral branches in all directions (Fig. 26). These branches form a network around the host and attack the host at numerous points.

Although water, minimal medium and other materials do not support the germination of *P. virginiana* spores in the absence of a host (Berry and Barnett 1957), they do germinate in the rich medium, and germ tubes normally develop into hyphae. Some of them even give rise to sporophores after 48 hours (Fig. 27) and produce spores (Fig. 16). These spores are, hereafter, referred to as axenic culture spores. They
are no smaller than the parasitic culture spores. When these spores are collected and placed on the other plates containing a rich medium, about two-third of them fail to germinate (compare it with 92% germination of parasitic spores, Appendix I). The germinating spore from the axenic culture remains short and never gives rise to a conidiophore again. However, the germ tube is still viable to parasitize if the host is available. This indicates that the parasitic culture spore can produce one generation of spore, but the axenic culture spores cannot grow to produce another generation in the absence of the host.

B. Morphological and biochemical differences between the parasitic and axenic culture spores

The observations from the germination of P. virginiana spore in the presence or absence of a host suggest that the parasitic culture spores are much more viable than axenic culture spores. This may be explained on the basis that the spores produced by the parasite growing on the host contain certain stored material which enables them to grow in the absence of a host, while this stored material may not be synthesized and stored in the axenic culture spores.

The light microscopy fails to reveal any significant difference in size or general morphology of the parasitic
and axenic culture spores. However, there are two clear spots at the terminal ends of the parasitic culture spores and no such spots are found in the axenic culture spores when stained with cotton blue solutions (Figs. 14, 16). When the Sudan IV stain is applied, the parasitic culture spores show two dark spots at the terminal ends (Fig. 15).

The electron microscopy also shows that there is no difference in cell wall, mitochondria, nucleus, endoplasmic reticulum or other organelles (Figs. 17, 18), but in the parasitic culture spore two large terminal electron transparent areas become apparent when the spore is fixed in KMnO₄ (Fig. 17). These areas do not occur in the axenic culture spore (Fig. 18).

From the light microscopy and ultrastructure findings, it is obvious that the basic difference between the parasitic culture and axenic culture spore is in their lipid contents. Further evidence to demonstrate the difference in lipid content, was obtained by extracting lipid from the spores. The extraction of the amount of lipid contents from the parasitic culture spore is far more than axenic culture spore as shown in the thin layer chromatography (Fig. 28).
III Host-parasite interaction

A. Penetration of the host cell

As the germ tube approaches the host cell, the tip of the germ tube swells over the surface of the host cell wall to form an appressorium (Fig. 29). There is a layer of amorphous electron dense material which occurs between the appressorium and the host cell wall (Fig. 29). When the appressorium sends an infection peg to start the penetration, the host cell wall is invaginated and looks like a dome-shape protuberance. The invading infection peg is adpressed tightly to the host cell wall and it is quite difficult to distinguish the wall of the infection peg from the cell wall of the host. No papilla formation is detected (Fig. 29).

The host cell wall is eventually penetrated (Fig. 30). The fine structure of the host cell at the point of penetration does not show any change (Fig. 30). The passage made through the host cell wall is of the same diameter as the infection peg. The penetrated host cell wall fits tightly around the neck of the haustorium and becomes a collar. The collar is merely an extension of the host cell wall (Fig. 30). The fine structure and electron density of the collar is apparently the same as the host cell wall.
The present finding does not agree with Bracker (1968), who stated that the collar is the result of deposition of host cellular material.

To check the possibility of enzymatic action on the penetration of the host cell wall, a histochemical test described by Mckeen et al. (1969) was followed. No morphological or chemical change was observed under the light microscope.

B. Fine structure of the haustorium

Following the penetration of the host cell wall, the infection peg elongates and becomes a short haustorial neck (Fig. 30). The latter is surrounded by a collar. The cellular organelles seem to migrate from the haustorial mother cell into the haustorium (Fig. 30).

In a young haustorium, 22-24 hours after inoculation, many ovoid osmiophilic bodies are present in the cytoplasm (Fig. 31). These bodies appear only during the early expanding stage of the haustorium and are seldom found in the older haustorium (Figs. 37-39). The cytoplasm is electron dense with a high concentration of ribosomes, and relatively few vacuoles (Figs. 31, 32, 33). Sometimes a mylin-like body is observed in the haustorium (Fig. 32). The mitochondria are elongated in shape with well developed cristae (Fig. 33). The haustorium cytoplasm is enclosed in a thin cell wall.
(Figs. 31, 32, 33). No nucleus is observed in a young haustorium.

The fine structure and organelle composition of the haustorium changes with age. In older haustorium, 36 hours after inoculation, membranous structures become loose and are always associated with the vacuoles (Figs. 37, 38, 39). Sometimes a nucleus with a characteristic double membrane is observed (Fig. 37). There is a general decrease in the number of organelles of the haustorium and the cytoplasm is gradually occupied by vacuoles which indicates a decline in its metabolic activity.

C. Fine structure of an interface

As shown in other obligate parasites, there is a zone surrounding the haustorium of *P. virginiana* in *C. cucurbitarum*. During the earlier stages of haustorium development, no sheath is formed around the haustorial neck and the haustorium. There is only a sheath membrane around the haustorium to separate it from the host cytoplasm. The sheath membrane, in fact, is an extension of the host plasmalemma (Fig. 30) but appears thicker than the plasmalemma at a later stage (Figs. 31, 32, 33, 34,) which indicates that the sheath membrane is differentiated from plasmalemma and may have a special function. This finding agrees with
the previous reports by Ehrlich and Ehrlich (1963) in *Puccinia graminis*-wheat system and Bracker (1968) in *Erysiphe graminis*-barley system.

At 22-24 hours after inoculation, a thin layer of clear zone becomes visible along the side of the haustorium (Figs. 31, 32, 33, 34). This zone is bounded by a sheath membrane which is never observed to be penetrated by the haustorium. However, the sheath membrane is frequently observed to be invaginated in the host cytoplasm. (Fig. 33). Haustoria are often observed in the vicinity of the host nucleus (Figs. 30, 34, 36).

Behind the sheath membrane, there is an extensive network of smooth surface endoplasmic reticulum (Figs. 31, 32, 33, 34, 35) and sometimes the cisternae of endoplasmic reticulum completely enclosed the haustorium along with its sheath membrane. While the healthy host mycelium is characterized by relatively few cisternae of endoplasmic reticulum (Figs. 7, 8, 12), the extensive development of endoplasmic reticulum is considered a noticeable change. The vesicular activity of host cytoplasm also increases in the vicinity of the haustorium (Fig. 35) and some dense vesicles, probably polysaccharides in nature, are observed at the haustorium surface.

Around an older haustorium, 36 hours after inoculation,
a definite region of electron dense sheath is developed (Figs. 36, 37, 38, 39). There is an indication of sheath zone differentiating into two layers (Figs. 37 & 38). This coincides with the host cell wall which differentiates from one layer at germ tube (Fig. 7) to 2 layers at old hypha stage (Fig. 12). As the haustorium becomes completely ensheathed the material in the sheath zone shows the same electron density as that of the host cell wall (Fig. 39). The endoplasmic reticulum which has developed during the early stage of haustorium development is completely disappeared now. The ensheathed haustorium begins to show signs of starvation and vacuolation (Figs. 38, 39).
IV Autoradiography

A. Incorporation of the label in germinating spore and hyphae

During the germination of the spore of _C. cucurbitarum_ a new layer develops inner to the three original layers of the cell wall (Fig. 6). When the spore wall ruptures, the new layer extends and becomes the wall of the young germ tube (Fig. 6). In case of the spores germinating on the rich medium, supplemented with $^3$H N-acetyl-glucosamine the newly developed layer is heavily labelled as indicated by the number of grains (Figs. 40 & 41). Most of the grains are located on the new inner layer and many of them are distributed along the wall of the young germ tube (Fig. 41). These results clearly show that the germinating spores incorporate the free chitin precursor from the medium and utilize it in the synthesis of a new inner wall.

Under identical treatment, the tip of the hypha shows more grains (Fig. 42) as compared to the mature hypha (Fig. 43). It indicates a higher metabolic activity of cell wall synthesis in the region of the tip than in the other part of a hypha. A careful examination of Figs. 40-43 would suggest that N-acetyl-glucosamine has been specifically incorporated into the host cell wall region.
On the other hand, no grains were observed on the parasitic hypha or germ tube treated in the same fashion as the host fungus. In fact, Figures 44 & 45 show the host and the parasitic fungi, treated together with the same amount of tritiated N-acetyl-glucosamine for 2 hours, with grains on the former and no grain on the latter.

B. Incorporation of the label in the sheath

When the young infected host (18 hours after inoculation) is fed with $^3$H N-acetyl-glucosamine, the grains are detected only along the host cell wall and no grain is observed around the haustorium (Fig. 46). No sheath is detected at this stage of infection. Since the thin cell wall of the young haustorium shows no label, it strengthens our finding that the cell wall of the parasitic fungus does not incorporate N-acetyl-glucosamine.

An infected host cell (24 hours after inoculation) shows the beginning of sheath development around the haustorium and also the presence of numerous grains in its vicinity (Fig. 47). This is an indication of the metabolic activity of cell wall-synthesis in this region. As the haustorium ages (30-36 hours after inoculation), a well developed sheath can be observed around the haustorium (Figs. 48, 49).
The grains are localized on the sheath zone. Figure 49 shows a greater number of grains on the sheath than on the host cell wall. This is not surprising because there is more wall synthesis in the sheath zone than in the cell wall.

The distribution of grains counted from 15 micrographs was as follows: host cell wall 81% (222); host cytoplasm 9-4% (26); parasitic fungus 2-8% (7); Background 6-8% (16); total number of grains 271 (Appendix II).
DISCUSSIONS AND CONCLUSIONS

I Spore germination

Piptocephalis virginiana is considered an obligate mycoparasite, but its spores behave differently than other obligate parasites. The uredospores of Puccinia graminis and conidia of Erysiphe graminis (Burnett 1968), can germinate in water without the supply of external nutrients, but the spores of P. virginiana do not germinate in water. They germinate only in a rich medium with yeast extract, or in the filtrate of an host fungus (Berry and Barnett, 1957). This indicates that the spores of P. virginiana needs some special substance which is present in yeast extract or host mycelium. Whether this substance serves as an external nutrient or a stimulator for germination is not known because the complex nature of yeast extract and host filtrate makes it difficult to determine, with any degree of accuracy, the requirements of germination. It is very likely that this substance, probably functions as an activator which removes the block of constitutional dormancy factor. The presence of large amounts of lipids in P. virginiana spore, utilized as endogenous source of energy, supports this suggestion.

During the spore germination, there are two basically
different mechanisms of vegetative wall formation, each
type being characteristic of certain groups of fungi.
In the first type there is development of a new layer
of cell wall as observed in *Rhizopus* (Hawker and Abbott,
1963). In the second type there is no development of the
new layer and the innermost layer breaks through the outer
wall and becomes the vegetative wall of germ tube. This
kind of germination appears throughout the higher fungi,
such as ascospores of *Neurospora* (Sussman, 1966), conidia
of *Botrytis* (Hawker and Hendy, 1963) and Uredospores of
*Melampsora* (Manocha and Shaw, 1967). In the present study,
the host spores form a new cell wall during spore germination
which belongs to the first type of spore germination.
Further evidence is provided by feeding the germinating
spores with $^3H$ N-acetyl-glucosamine and by locating the
label in the new cell wall.

II The axenic and parasitic culture spores

The study on the parasitic and axenic culture spores
of *P. virginiana*, show that the former has more lipid content
than the latter. De Robertis, *et. al.* (1965) have described
that Sudan IV has a greater histochemical value and is
accumulated in the interior of the lipid droplets by a simple
process of diffusion and solubility. Jensen (1962) also
pointed out that Sudan IV will stain fats. In light microscopy, when parasitic culture spore was stained with Sudan IV, 2 terminal dark bodies appeared but these bodies were not seen in the axenic culture spore.

In fungi, lipids are found abundantly in spores (Bracker, 1967). Especially, the obligate parasites spores contain a large amount of lipid as their endogenous source of energy. Since lipids serve as reservoir of energy rich material, it is apparently used to support the spore germination. Shu et. al. (1954) reported the lipid contents of rust uredospores dropped appreciably from 21% to 7.8% of total dry weight of spores during germination. This indicates that the lipids are consumed during spore germination. Since the parasitic culture spore has large amounts of lipid, it germinates normally and the failure of the axenic culture spore to germinate may be due to the deficiency of endogenous source of energy to support its germination.

In the host spore, the lipid content is not as prominent as parasitic fungus spore, instead, it contains a fairly large number of glycogen particles. Glycogen is also generally considered as a primary storage polysaccharide in fungi (Bracker, 1967). Glycogen is believed to break down to glucose and the enzymatic degradation of glucose, releases energy (Zaloker, 1959).
III The Ultrastructure of host and parasite fungi

The present study has revealed interesting differences in fine structures between the host and the parasitic fungi. There are three distinct layers of different electron density in the cell wall of the host spore whereas the spore of the parasite has only two. In fungi the spore cell wall composed of 2-3 layers is quite common (Sussaman, 1966). The cell wall of the host vegetative hypha differentiates into two layers. Agar and Douglas (1955) have shown two layers in a yeast, an outer electron-dense layer and an inner less dense layer. On the other hand, the cell wall of the parasitic fungus has one layer in all stages. Both the host and parasitic fungi lack the recognizable Golgi dictyosome. In the healthy host cell of all stages, the endoplasmic reticulum is sparse and rarely visible. However, it develops extensively in number as the host is infected by the parasitic fungus. Endoplasmic reticulum of the parasitic fungus, forms parallel strands in the swollen spore, elongated cisternae in the germ tube and an irregular network in hypha and haustorium. The mitochondria of the host cell are regular and the cristae are well developed. In the parasitic fungus the mitochondria are much smaller than the host and are more or less irregular in shape. During infection the host mitochondria swell and
their shape is slightly changed. Since mitochondria are concerned with respiration and convert the energy released by oxidation to the bond energy of ATP, the change in shape and size may correlate with the metabolic state of the host cell. The ribosome concentration seems to increase after infection.

The myelinoid body which is rarely observed in the host generally prevails in the haustorium. Some of them are associated with vacuoles and vesicles. Linnane et al. (1962) reported that this body functions like a mitochondrion and contains some enzymes. The large number of folds that make up the system of membranes provides a greatly increased surface area for enzyme activity. Most of these bodies are associated with the vacuoles and this agrees with Mitchell and Mckeen (1970) who suggested myelinoid bodies in the vacuoles of powdery mildew to be synthetic in function. Since it is always found in the haustorium, it may play a role in the process of parasitism.

Careful examination of micrographs of host and parasitic fungi, fails to show an organelle in the germ tube of the latter equivalent to the nucleolus of the host. Birnstiel (1967) has reported that the major task of a nucleolus is to synthesize ribosomal RNA and its absence in the organism would result in the loss of capacity to
convert amino acids into proteins. The young hypha of the parasitic fungus has some small groups of ribonucleo-protein particles and the mature hypha has a definite nucleolus. It is quite likely that the hypha starts its protein synthesis after the germ tube has penetrated the host and has obtained nutrients from the host. It may have stimulated the formation of a nucleolus. The frequent occurrence of a haustorium close to the host nucleus may enhance direct control of certain metabolic activities which benefit the parasitic fungus.

IV Mode of penetration

The study of the nature of penetration of the host cell wall by the parasite is important since it is the first step towards the establishment of the host-parasite relationship. Two major mechanisms have been proposed to explain the process of fungal penetration, mechanical or enzymatic. Recently, Edward and Allen (1970) have suggested that the penetration in *Erysiphe graminis* consists in the dissolution and digestion of the cellulose portion of the epidermal wall by an enzyme apparently secreted by the infection peg followed by a mechanical push of the infection peg through the papilla. The present observations indicate that penetration of *P. virginiana* in *C. cucurbitarum* probably involves a mechanical process. The appressorium is observed
to bind tightly to the surface of the host cell wall probably by the mucilage secreted by the former (Dickinson, 1960). The cell walls of the host and parasitic fungus are hardly distinguishable in the zone of penetration probably due to the pressure from the infection peg. Changes in the structure of cell wall of the host generally accompanied by enzymatic penetration (McKeen et. al., 1969, Kunoh & Akai, 1969), were not observed. The presence of crushed cell wall surrounding the developing haustorium also gives an evidence for the mechanical pressure as the mode of penetration. It is further supported by the work of England (1969), who showed that the cell wall of C. cucurbitarum, susceptible to P. virginiana, is more easily broken down by mechanical method (sonication) than the cell wall of Phycomyces which is a resistant fungus.
V Collar formation

Armentrout and Wilson (1969) observed no collar around the haustorial neck of *P. virginiana* penetrating *Mycotypha microspora*, whereas a definite collar was observed around the haustorial neck of *P. virginiana* invading *C. cucurbitarum*. It appears that formation of a collar region is a feature of host response to penetration. Similar structures have been reported in different host-parasite interactions and have been given various names such as callosities (Young, 1926), sheath (Smith, 1900; and Fraymouth, 1956) and collar (Bracker, 1968). Bracker (1968) believes that the collar is not an ingrowth of the host cell wall but a deposit of the cellular materials. In the present study, the collar is observed as an extension of the host cell wall and both possess the same electron density and fibrillar orientation. It is quite likely that the collars of fungi and vascular plants are different in origin. The vascular plants generally form papilla (Hess, 1969; McKeen et al., 1969; Stavely et al., 1969; Temmink and Campbell, 1969) by depositing electron-dense amorphous material on the inner surface of the cell wall as a response to infection. The papilla probably serves as a physical barrier but after the penetration of the infection peg it becomes a collar. In the present system, no papilla develops and the
host cell wall is the only barrier to resist the penetration of the parasite and later becomes a collar.

VI Spherosome

Spherosome-like bodies of variable sizes are frequently seen in the germ tubes and hyphae of both the fungi. They are believed to be composed of lipids as revealed by simple staining with Sudan IV under the light microscope (Jensen 1962) and by sodium methoxide, dissolved in a methanol-benzene mixture, treatment of thin sections under the electron microscope (Eurenius & Jarskär, 1970). Similar organelles have been described by Frey-Wyssling et. al., (1963) as sites of lipid synthesis and by Jacks et. al., (1967) as structures of lipid storage. Mishra and Colvin (1970) have suggested that spherosomes are storage or transport organelles which are consumed during growth of the organism. This is in confirmation with our results.

The young haustorium contains many spherosome-like bodies and only a few are observed in the mature haustorium. These bodies are either consumed during growth of the parasite or have migrated to the developing sporophores where they become the main constituent of the spore. In another mycoparasite system, P. virginiana on Mycotypha microspora, Armentrout and Wilson (1969) have observed spherosomes moving down the hypha of the parasitic fungus
towards the haustorium under the light microscope. On this basis they concluded that spherosomes of the parasite could contribute enzymes to the sheath, which gave a positive acid phosphatase test. Armentrout and Wilson (1969) proposed that spherosomes of fungi are comparable to animal lysosomes and they seem to be actively involved in the process of parasitism. We did not observe acid phosphate activity in the parasite at an electron microscope level after following the procedures of Armentrout and Wilson (1969). Further, the identification of spherosomes on their acid phosphatase activity alone does not seem to be valid since this enzyme is also present in other organelles such as cell wall and golgi bodies. More recently Rosenthal et. al., (1970) have warned about the interpretation of phosphatase cytochemical data and have suggested that complex chemical events which influence localization of the reaction product, be it of enzymatic or nonenzymatic origin, should be taken into account.
Many specialized host-parasite interactions have drawn attention to the origin and organization of sheath around the haustorium. Ehrlich and Ehrlich (1963) have observed a continuity of the haustorial cytoplasm through small channel-like areas in the haustorium wall to the zone of encapsulation (sheath). The substance composing the zone of encapsulation appeared similar to the cytoplasm of the haustorium. Peyton & Bowen (1963) observed numerous vesicles around the haustorium of *Peronospora manshurica* and have interpreted these as secretory bodies derived from the host cytoplasm and fusing with the plasma membrane to discharge material into the zone of apposition (=sheath). The observations of Shaw & Manocha (1965) on wheat rust agreed with the formation of vesicles and extensive development of smooth surfaced ER in the host cytoplasm around the haustorium of *Puccinia graminis*, which may be associated with the origin of the sheath. Armentrout and Wilson (1969) have described the formation of "blebs" from the sheath of *P. virginiana* which appear to be releasing material from the parasite via the sheath matrix into the cytoplasm of the host cell. We did not observe any "blebs" or similar bodies in the haustorium sheath or host cytoplasm. A few vesicles in the host cytoplasm and some fusing with the
host plasmalemma were observed. Young haustoria with no electron-dense material in the sheath were completely surrounded by long cisternae of endoplasmic reticulum. Mature haustoria appeared to contain electron-dense material in the sheath zone and the ER system of the surrounding host cytoplasm disappeared at this stage. Electron density of sheath material was the same as that of the host cell wall. Numerous references are available in literature to link the role of ER in cell wall synthesis. Recently Lynn & Magee (1970) have described the cell wall synthesis through the mediation of endoplasmic reticulum in the ascospores of a yeast. The disappearance of ER after the completion of the sheath and electron density of the latter suggested that sheath was composed of cell wall material. The exact nature of the sheath material and details of the role of ER remains to be confirmed by high resolution autoradiography. It is likely that formation of new cell wall restrict the further branching and growth of the haustorium and reduce the metabolic interchange between the host and the parasite.
VIII Autoradiography

This technique is based on the incorporation of the radioactive isotopes into various cellular constituents by biological material. Many workers have applied this technique to the non-pathogenic and pathogenic fungi (Garraway and Pelletier, 1966, Mount and Ellingboe, 1969, and Sivak and Shaw, 1970). However, the use of high resolution autoradiography to study the host-parasite relations at the cellular level has not been reported before.

The present observations indicate that the $^3$H N-acetyl-glucosamine incorporated specifically into the host cell wall area and was rarely incorporated by the parasitic fungus. Although both host and parasitic fungi are members of Mucorales, the chemical composition of the cell wall of the parasitic fungus is unknown. According to the results, the cell wall material of the parasitic fungus may be something other than chitin or, alternatively, the parasitic fungus is not capable of utilizing the free precursor, N-acetyl-glucosamine, for the synthesis of its own cell wall.

The young haustorium does not incorporate label and this agrees with the present findings that the cell wall of the parasitic fungus does not incorporate N-acetyl-glucosamine. As the haustorium ages, it is enclosed in the sheath, when the label was fed to such haustoria grains were
observed on the sheath. The number of grains on the sheath were more than in the host cell wall. This may be due to more wall synthesis in the sheath than the hypha wall. This finding permits to conclude, within reasonable limits, that the sheath is basically composed of the same material as the host cell wall and support the ultrastructural findings.

IX. Comparison with other parasites

The information obtained from the present study can allow an ultrastructural comparison with other obligate parasites of higher plants and mycoparasite. This may serve a useful purpose of assembling the data and clarifying the similarities and differences among different host-parasite systems.

Comparing the mycoparasite to an obligate parasite of a higher plant, a conclusion can be drawn that the basic ultrastructure of the haustorium apparatus of all the organism is essentially the same although some phenomena have been noticed and interpreted differently by different investigators.
<table>
<thead>
<tr>
<th>Parasite</th>
<th>Puccinia graminis</th>
<th>Erysiphe cichoracearum</th>
<th>Piptocephalis virginiana</th>
<th>P. virginiana</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host</td>
<td>Triticum aestivium</td>
<td>Helianthus annuus and other host</td>
<td>Mycotypha microspora</td>
<td>C. cucurbitarum</td>
</tr>
<tr>
<td>1. Mode of penetration</td>
<td>Not reported</td>
<td>Enzymatic degradation</td>
<td>Not reported</td>
<td>Primarily mechanical</td>
</tr>
<tr>
<td>2. Collar</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>3. Papilla</td>
<td>Not reported</td>
<td>Yes</td>
<td>Not reported</td>
<td>No</td>
</tr>
<tr>
<td>4. Lomasomes at sheath boundary</td>
<td>Yes</td>
<td>Not reported</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5. Endoplasmic reticulum associate with sheath</td>
<td>Yes</td>
<td>Not reported</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>6. Vesicles</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>7. Spherosomes</td>
<td>Not reported</td>
<td>Lipid bodies</td>
<td>Contain acid phosphatase</td>
<td>Contain lipid</td>
</tr>
<tr>
<td>8. Sheath haustorium</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>9. Nucleate haustorium</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

A - Appressorium
C - Collar
Ci - Cisternae
ER - Endoplasmic reticulum
H - Host fungus
Ha - Haustorium
HW - Host fungus cell wall
IP - Infection Peg
LB - Lipid Body
M - Mitochondrion
My - Myelin-like body
N - Nucleus
NM - Nuclear membrane
NP - Nuclear pore
Nu - Nucleolus
P - Parasitic fungus
R - Ribosomes
S - spherosome-like body
Sh - sheath
SM - sheath membrane
V - Vacuole
Ve - Vesicle
Fig. 1. The conidiophore of *Choanephora cururbitarum* X 400.
Fig. 2. The sporangium of *C. cucurbitarum*. X 800.

Fig. 3. A single conidium of *C. cucurbitarum*. X 4000.

Fig. 4. A single spore of *C. cucurbitarum*. X 3500.
Fig. 5. Thin section of a spore of *C. cucurbitarum*. Note the three layered cell wall. X 14,200.

Fig. 6. Thin section of a germinating spore of *C. cucurbitarum*. Note the new inner layer wall. X 18,000.
Fig. 7. Thin section of a young hypha of *C. cucurbitarum* showing numerous ribosomes, mitochondria and two nuclei with nucleoli. Note a single thin layer cell wall. X 30,000.

Fig. 8. Thin section of a mature hypha of *C. cucurbitarum*. Note the double layer cell wall. X 15,000.

Fig. 9. A hypha of *C. cucurbitarum* stained with Sudan IV showing numerous lipid bodies under the light microscope. X 3500.
Fig. 10. Thin section of C. cucurbitarum before the treatment of lipid solvent. Note the lipid bodies. X 36,000.

Fig. 11. Thin section of C. cucurbitarum after the treatment of lipid solvent. Note the absence of lipid bodies. X 36,000.
Fig. 12. Thin section of an old hypha of *C. cucurbitarum*. Note the vacuoles and multivesicular structures and double layer cell wall. X 30,000.
Fig. 13. The sporophore of *Piptocephalis virginiana*. X 800.
Fig. 14. Parasitic culture spore of *P. virginiana*. X 3600.

Fig. 15. Parasitic culture spore of *P. virginiana*. Stained with Sudan IV. X 3600.

Fig. 16. Axenic culture spore of *P. virginiana*. X 3600.
Fig. 17. Thin section of a parasitic culture spore of *P. virginiana*. X 30,000.

Fig. 18. Thin section of a axenic culture spore of *P. virginiana*. X 30,000.
Fig. 19. Thin section of a swelling spore of P. virginiana. X 22,000.

Fig. 20. Thin section of a germinating spore of P. virginiana. Note the big vacuole. X 14,000.
Fig. 21. Cross section of a germ tube of *P. virginiana*. X 60,000.

Fig. 22. Thin section of a young hypha of the *P. virginiana*. Note multivesicular structure, lipid bodies, and dense particles in the nucleus. X 45,000.
Fig. 23. Thin section of a mature hypha of the *P. virginiana* showing numerous lipid bodies and a nucleus with nucleolus. X 54,000.
Fig. 24. A hypha of *P. virginiana* stained with Sudan IV showing numerous lipid bodies under light microscope. X 4,000.

Fig. 25. The germinating spores of *P. virginiana* under light microscope. X 2,000.
Fig. 26. Parasitic hyphae encircling around the host fungus. X 950.
Fig. 27. The sporophore of axenic culture of *P. virginiana*. X 400.
Fig. 28. Lipid extraction from (A). Parasitic culture spore and (B). Axenic culture spore on the thin layer chromatography.
Fig. 29.  Thin section of an infection peg. Note electron-dense material between appressorium and host cell (arrow). X 46,000.

Fig. 30.  Thin section of a penetrated host cell. Note the formation of a collar and its resemblance to cell wall. X 54,000.
Figs. 31-32. Thin section of young haustoria (22-24 hours after inoculation).

Fig. 31 shows spherosome-like bodies. X 75,000.

Fig. 32 shows mylin-like body. X 72,000.
Figs. 33-34. Thin section of young haustoria (22-24 hours after inoculation)

Fig. 33, shows the electron-transparent zone between the haustorium and the host. X 54,000.

Fig. 34 shows the elongated cisternae of endoplasmic reticulum completely encircling the haustorium. Fixed in KMnO₄. X 72,000.

Fig. 35. Thin section of a haustorium (28 hours after inoculation). Note the presence of numerous dense vesicles. Fixed in KMnO₄. X 54,000.
Figs. 36-37. Haustorium (36 hours after inoculation).

Fig. 36 shows the disappearance of the endoplasmic reticulum. X 36,000.

Fig. 37 shows the development of the electron-dense sheath. X 22,000.
Fig. 38. Haustorium (36 hours after inoculation) shows the sheath differentiated into two layers. X 92,000.
Fig. 39. Haustorium (36 hours after inoculation) shows the electron density of the sheath is the same as the host cell wall. X 60,000.
Fig. 40. Portion of a germinated spore of *C. cucurbitarum*. Note the grains located at the inner layer of the wall. X 15,000.

Fig. 41. Germinating spore of *C. cucurbitarum*. Note the grains distributed along with the outgrowing germ tube. X 20,000.

Fig. 42. Tip of the germ tube of *C. cucurbitarum*. Note the numerous grains on the tip. X 15,000.
Fig. 43. Hypha of the C. cucurbitarum. Note the grains around the cell wall area. X 12,000.

Figs. 44 & 45. Hypha of C. cucurbitarum, and P. virginiana. Note the grains at the host cell wall but not in the parasitic fungus. X 24,000, X 14,000.
Fig. 46. Young Haustorium (18 hours after inoculation) without a sheath and also without any grains. Note the presence of grains on the host cell wall. X 35,000.

Fig. 47. Haustorium (24 hours after inoculation). Note with sheath just starting. Note numerous grains in vicinity of the haustorium. X 40,000.

Fig. 48. Haustorium (30 hours after inoculation). Note the grains at the developed sheath. X 30,000.
Fig. 49. Haustorium (30 hours after inoculation). Note the grains at the sheath as well as at the host cell wall region. X 38,000.
LITERATURE CITED


*Original not seen.
Literature Cited Cont'd


Literature Cited Cont'd


Literature Cited Cont'd


Literature Cited Cont'd


Literature Cited Cont'd


Literature Cited Cont'd


APPENDIX I

A) Percentage of spore germination in parasitic culture (counting at 100 x)

<table>
<thead>
<tr>
<th>Spore Counted</th>
<th>Spore Germinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>70</td>
<td>69</td>
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<tr>
<td>35</td>
<td>30</td>
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<td>43</td>
<td>38</td>
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<td>23</td>
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<td>32</td>
<td>29</td>
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<tr>
<td>45</td>
<td>42</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td><strong>27</strong></td>
<td><strong>26</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>437</strong></td>
</tr>
</tbody>
</table>

Percentage of germination \( \frac{403}{437} \times 100 = 92\% \)

B) Percentage of spore germination in axenic culture (counting at 100 x)

<table>
<thead>
<tr>
<th>Spore Counted</th>
<th>Spore Germinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>13</td>
</tr>
<tr>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>39</td>
<td>12</td>
</tr>
<tr>
<td>26</td>
<td>9</td>
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<td>25</td>
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<td>10</td>
</tr>
<tr>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td><strong>30</strong></td>
<td><strong>11</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>300</strong></td>
</tr>
</tbody>
</table>

Percentage of germination \( \frac{99}{300} \times 100 = 33\% \)
APPENDIX II

Distribution of silver grains over the sections incorporated with $^3$H-N-acetyl-glucosamine

A. Cell wall region
   1. Host fungus cell wall area.
      6, 21, 7, 13, 15, 19, 8, 18, 3, 20, 5, 26, 9, 7, 25.
   2. Host fungus cytoplasm region.
      1, 3, 2, 1, 3, 1, 4, 2, 5, 1, 1.
      1, 1, 2, 1, 2, 1.
   4. Background.
      3, 2, 3, 1, 1, 2, 2, 1, 1.

Total grains counted: 271.
Total grains on host fungus cell wall: 222. 81%
Total grains on host fungus cytoplasm: 26. 9.4%
Total grains on parasitic fungus: 7. 2.8%
Total grains on background: 16. 6.8%