Transmission and Morphological Studies of Diseased Helminthosporium Victoriae.

Basil Anastasiadis

Louisiana State University and Agricultural & Mechanical College

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The Department of Botany and Plant Pathology

by

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M.S., Louisiana State University, 1965
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ABSTRACT

Light microscopy of nonstained, diseased mycelium of Helminthosporium victoriae revealed: (1) cytoplasmic masses of different sizes and shapes in partially lysed cells; (2) spherical, compact cytoplasmic masses with an echinulated surface in cells with intact cytoplasm; and (3) multiangular granules of different sizes and shapes in nonlysed or partially lysed cells. In preparations of diseased mycelium stained with trypan blue, dark blue structures appeared in the apical, bulbous cells and blue-stained, cytoplasmic masses appeared in young portions of mycelium. Methyl green pyronine B stained certain materials red in some cells from older portions of the diseased colony. The red stained material appeared to be made up of small units, which oftentimes aggregated into larger particles. Giemsa revealed a deformity in the nuclei of diseased mycelium, as well as, two other types of blue-stained particles, one of which seemed to be present in the healthy mycelium. Feulgen and Feulgen-carmine were unsuccessful while azure B stained some particles blue.

Of over 3000 transfers from cultures inoculated with nematodes fed on diseased isolate D-1A, 173 were diseased. Of a similar number of transfers from cultures inoculated with nematodes fed on healthy H. victoriae, 31 were diseased and in the noninoculated control, 31 of over 3000 transfers were diseased. There were no statistically significant differences between the number of diseased colonies in
transfers from colonies inoculated with nematodes from diseased fungus and colonies inoculated with nematodes from healthy fungus.
INTRODUCTION

Lindberg (28) observed in studies on *Helminthosporium victoriae* Meehan and Murphy the causal agent of victoria blight of oats (*Avena sativa* L.), that some isolates were stunted and showed other abnormal characteristics of growth. The abnormality, which appeared to have characteristics of an infectious disease, was transmitted through hyphal contact with normal fungus. Bacteria or a toxin was excluded as a possible causal agent of the abnormal growth, but the precise nature of the causal agent was not determined. In 1962, Psarros and Lindberg (43) reported on the comparative morphology of diseased and healthy *H. victoriae*. They found that young cells of diseased mycelium were lysed, and were partially or totally devoid of protoplasm. Nuclei were not detected in the lysed cells. Branching was abnormal and projected in different directions. Branches were twisted and short, Cells in the mycelium were short and swollen with pronounced constrictions at the septa. It was shown further in these studies that in cells that were not lysed, cytoplasmic granules of different sizes and shapes were present.

In 1959, Jinks (23) described a disease in the fungus *Aspergillus glaucus* (L.) Lk. The disease designated "vegetative death" occurred in aged clones of the fungus, as if spontaneously. The disease was transmitted in crosses between mycelium of a normal homokaryon and the diseased or vegetative death mutant, and moved far beyond the maximum point of penetration of known chromosomally determined
characters. The vegetative death type gradually replaced the normal homokaryon. Jinks (24) considered this as unambiguous evidence of extrachromosomal inheritance.

In 1960, Lindberg (29) reported the isolation of a mutant from colonies of a diseased isolate (D-1A) of \textit{H. victoriae}. This mutant contained the causal agent of disease but showed almost no symptoms. Later work (32) demonstrated that when healthy \textit{H. victoriae} was inoculated with the symptomless diseased mutant, symptoms identical to those of the diseased isolate D-1A developed. The character in the mutant colony, which suppressed severe disease symptoms, however, was not transmitted to healthy \textit{H. victoriae}. It was concluded that if the mutant character that suppressed severe symptoms of disease was controlled in the nucleus, it would appear likely that the agent of the disease was cytoplasmic.

Cell free transmission is perhaps the best way to demonstrate whether or not a disease is caused by a virus. Extensive studies with cell free preparations of the diseased fungus failed to demonstrate with certainty that the agent of disease in \textit{H. victoriae} is a virus (3, 28, 29).

Production of disease in \textit{H. victoriae} with phenol extracts of two diseased isolates was reported (31). With respect to the number and type of diseased sectors produced, the phenol extracts of the diseased isolates showed marked specificity. It was concluded that either phenol extracts were infectious or that they induced disease development in inoculated test colonies. Although all previous studies of cell free transmission were inconclusive, cell free
transmission was still considered the best way to demonstrate an infectious causal agent of disease in *H. victoriae*.

Vector transmission was considered another method that might provide evidence for an infectious causal agent, and a mycophagous nematode was used for this purpose. *Aphelenchus avenae* Bastian (38), vector of some plant viruses (39), was used in a vector transmission study (3). Diseased cultures were observed among those inoculated with nematodes that fed on diseased *H. victoriae*. Such studies did not prove vector transmission of the disease in *H. victoriae* because viable fragments of the diseased fungus may have been transferred with the nematodes. Control nematodes from colonies of diseased *H. victoriae* were washed and transferred to blank culture plates without any sign of diseased fungus growth. The controls were not considered adequate because viable fragments of mycelium, not capable of producing visible diseased colonies, may have been plated.

Another serious criticism of the earlier vector transmission work was that the nematodes were not completely free of contaminant bacteria.

In order to continue the vector transmission studies it was essential that the above problems of contamination be eliminated so that adequate controls could be established.

In 1967, Lindberg (32) reported the separation of a mild disease from two severely diseased isolates of the fungus. Where both the mild and severe diseases were present in an isolate, symptoms of the mild were completely masked and only the severe disease could be detected. From earlier studies it was recalled that symptoms that developed in
colonies inoculated with nematodes were usually not those of the severe disease but may have been symptoms of the mild disease. If this was the case, further studies on vector transmission of the disease in *H. victoriae* were suggested. A continuation of vector transmission was further encouraged by obtaining a nematode culture free of bacteria. The diseased colonies that developed among colonies inoculated with nematodes were invariably those of the mild disease and occasionally those of the severe disease.

Because of the use of an effective method to check whether viable fragments of D-1A were carried with nematodes and because a nematode culture free of bacteria was obtained, in the present work further studies on the transmission of the disease agent from the diseased to the healthy *H. victoriae* with the nematode vector were carried out.

The present investigation also included a more detailed study of the cytoplasmic granules reported by Psarros and Lindberg (43) in the diseased mycelium. For this study living nonstained and stained preparations of diseased and healthy *H. victoriae* were examined by bright light microscopy. The stains trypan blue, methyl green, pyronine B, Giemsa, Feulgen and azure B were used to either enhance the visibility of cytoplasmic structures; reveal proteinaceous, ribonucleic acid (RNA); or desoxyribonucleic acid (DNA) containing materials.
Abnormal growth often resulting in death, was reported in fungi (10, 14, 20, 28, 37) and amoebae (46, 53). Some abnormalities were transmissible by contact with normal organisms, whereas, others were not.

**Characteristics of Diseases**

Some of the amoebae *Paramecium aurelia* Müller, called "killers" produced an agent capable of killing other nonkillers or "sensitive" animals. Some "sensitives" when placed with killers, with breiss of killers, or in an animal-free medium where "killers" lived, became abnormals and died in a few hours (50, 53). Spinning motion, aboral humps, paralysis, vacuolization and rapid lysis followed by death were the general symptoms of sensitives exposed to action of the killers.

In the yeast, *Saccharomyces cerevisiae* Meyen ex Hansen, a constant proportion of cells (about 1 percent) gave rise to dwarf colonies (11, 48). This dwarffing was caused by the absence of the respiratory enzymes cytochrome oxidase and succinic dehydrogenase (11, 48). Dwarfing, once started, was irreversible and was retained permanently and indefinitely during vegetative reproduction. In crosses between dwarf, adenine-dependent and normal, adenine-independent clones, followed by four successive backcrosses to the mutant clone, the mutant character of slow growth did not reappear in ascí (10, 12).
A slow type of growth called "poky" was observed in *Neurospora crassa* Shean and Dodge when the wild type 7A was crossed with four different strains of the fungus (37). Poky, characterized by slow growth, required 10 to 12 days to reach full growth whereas 3 to 4 days were required for the wild strain to reach full growth. The mycelium of poky was less dense than that of the wild type and had a characteristic red color. Any supplement added to the medium did not restore the growth of poky to the normal rate.

Abnormal type of growth in *N. crassa* also was reported by Garnjobst et al. (14). This abnormality was characterized by slow growth which only occasionally was stopped permanently. Hyphae in this abnormal growth were finer than those of the normal mycelium and laid flat on the surface of the medium without aerial growth. Occasionally short twisted tufts of hyphae developed. Conidia were produced in small numbers and their size was small compared to conidia from isolates of normal growth. Protoperithecia and perithecia were not produced in crosses between different abnormal isolates. Different temperatures, increased air and oxygen flow, different growth substances, and antibiotics were used to try to alter the pattern of growth of abnormal cultures but the slow growth rate remained practically unchanged.

Lindberg (28) reported the appearance of areas of stunted growth in normal colonies of *H. victoriae*. The abnormality spread rapidly in culture causing stunted growth and collapsed aerial mycelium. Infected hyphae branched profusely and abnormally and cells were of various sizes and irregular shapes. Cells of diseased hyphae showed different
degrees of lysis of the cell wall accompanied by partial or total leakage of protoplasm (43).

In 1959, Jinks (23) described an abnormality that developed, as if spontaneously, in certain aged clones of *Aspergillus glaucus*. The mutant cultures, designated as "vegetative death," were characterized by an irreversible cessation of growth followed by death of mycelial types. Vegetative death mycelium had swollen tips, and the cytoplasmic content disappeared or leaked into the medium. The protoplasm disintegrated in cells behind the growing tips and such dying cells excreted a brown pigment into the surrounding medium.

A deterioration of mycelium and sporophores in the cultivated mushroom, *Agaricus bisporus* (Lange) Sing, was described. (20, 47). Growth of the mycelium weakened and then disintegrated and disappeared from compose inoculated for mushroom production. On agar, diseased mycelium was sparse, of slow growth, and appressed to the surface of the medium. Colonies appeared irregular with no rhizomorphs. Production of a brown color also was noticed. Fruiting was either suppressed in the entire bed or in definite patches. Yield was affected adversely depending upon the time of onset of the disease and the variety of mushroom. Diseased sporophores showed rapid necrosis, wet soft-rotting decay, followed by complete decomposition within a few days. Other symptoms were, dwarfing, early maturity, various deformities in the pileus and stipe, and changes in color from white to offwhite, ashen or tan.

**Transmission**

The "poky" character in *N. crassa* was found not to be readily
transmissible by contact when poky and wild isolates were placed side by side. The poky type of slow growth in *N. crassa* reported by Mitchell and Mitchell (37) and the abnormal growth of the same fungus described by Garnjobst et al. (14) were transmitted only in crosses where the maternal or protoperithecial parent was the slow or abnormal part. The slow growth type in yeast was not transmitted when isolates of slow growth were used as the fertilizing parent (10, 37). This observation was used to point out that the causal agent of slow growth was cytoplasmic.

Transfers of mycelial tips from areas showing dwarfed or diseased symptoms or from the vicinity of such growth always gave rise to diseased cultures in *H. victoriae* (30, 43), in *A. glaucus* (23), in *N. crassa* (14) and in the cultivated mushroom *A. bisporus* (20, 47). Spores obtained from areas of cultures showing the abnormal, stunted growth germinated poorly or produced diseased cultures (14, 23, 47). This was not the case with *H. victoriae*, however, where conidia from diseased mycelium produced, in many of the cases, normal growth (30). Not only did the stunted abnormal growth spread from the point of its appearance to surrounding areas of normal growth but it also moved into normal isolates following hyphal contact with diseased mycelium. Mycelium of a once normal colony stopped growing, collapsed and was eventually replaced by the diseased type (14, 23, 28, 30, 47).

Viable mycelial cells from the diseased fungus were necessary to transmit the disease in *H. victoriae* (30) and in *A. glaucus* (23). Several attempts to transmit the abnormality in *H. victoriae* (28) and in *N. crassa* (14) with cell-free extracts were not successful. Lindberg (31), however, reported production of diseased sectors in normal
isolates inoculated with phenol extracts of diseased isolates.

Lindberg reported (29, 31, 32) a type of resistance of certain isolates of *H. victoriae* to the abnormality. Schisler et al. (47) found that the disintegration of mycelium was less severe in the "Golden white" variety of the cultivated mushroom than in the "Snow white" variety. Differences in killing activity and susceptibility also were observed in the Paramecium (4, 53).

**Agents of Disease**

The agents responsible for the abnormalities in most of the cases reported have not been identified. In some cases the causal agent was considered a virus (20, 28, 31, 37, 47, 53) and in other cases a cytoplasmic factor confined to mitochondria (10, 14, 23). Preer (40), using Feulgen and Giemsa staining techniques, found a large number of particles which he considered to be "kappa" in the cytoplasm of "killer" Paramecia. Such particles were not detected in sensitive Paramecia. Particles were distinguished as "brights" and "not brights" by the presence or absence of one or more refractile bodies (42). Particles not containing the refractile body were small and divided actively by transfer division, whereas particles that contained the refractile body "brights," had limited dividing power. The smaller particles probably developed into the "brights." Killing activity was associated with the particles that contained the refractile bodies. The particles were of a size between 2.25 u x 0.6 to 1.0 u. Electron micrographs showed that both types of particles were surrounded by a double membrane. Internally, the particles contained
filamentous materials with minute dense particles (granules) of irregular shapes and an extensive clear region. The refractile body (0.4 to 0.6 μ in diameter) appeared to contain a granular material as dense as the material found outside the body (9). Particles stained with Feulgen appeared to contain DNA uniformly distributed in all parts of the particle except in the refractile body. Sudan stains for lipids, the MacManus technique for polysaccharides, the Nadi reagent to identify cytochrome oxidase and the neotetrazolium test for reducing enzymes were negative. Other tests indicated the existence of an alkaline phosphatase and the presence of basic proteins (42).

Desoxyribonucleic acid was found to have an essential role in the development of killing activity although it was not necessary for activity of the mature particles. A toxin (paramecin) produced by "killer" Paramecia was compared to the colicin produced by certain colicinogenic bacteria (53). The size, shape, transverse division and specific gravity of the particles were considered as characters of bacteria and the refractile body was compared to bacterial spores (40, 42). Chemical composition of kappa particles was found to be very close to that of the bacteria (49). The negative reaction of the refractile body to carbolfuchsin, the failure of different techniques to reveal a chromatinic body and the negative test for enzymes, however, made kappa particles appear to be distinct from bacteria (42).

The existence of a virus was suspected (52) before the kappa particles were seen. The distribution of DNA as revealed by the Giemsa and Feulgen techniques, and the low content or absence of
enzymes were arguments that kappa particles were close to the viruses. The refractile body resembled that of a viral inclusion but other than that there was no evidence that it contained a virus (53). Preer (41) found a close resemblance of kappa to rickettsiae because of similarities in size, restricted intracellular habitat and appearance. One important difference, however, was that the cytoplasm surrounds the nucleus in rickettsiae whereas cytoplasm was not detected in kappa particles. Preer (41) observed also a closer resemblance of kappa particles to the nuclear part of the rickettsiae or bacteria than to the entire body of these organisms. Kappa also was considered as being derived from green algae (2) but this was not strongly supported. Hamilton and Gettner (16) and Preer and Stark (42) advanced the possibility that kappa were mitochondria because of the size, shape and the existence of membranes. In addition mitochondria might be expected to reduce cytochrome oxidase activity, as was found in kappa (53). Although kappa particles do not fit into any of the present taxonomic groups it was concluded that they are cytoplasmic genetic factors of Paramecia. By virtue of kappa being hereditary symbionts they are as well integrated into the genetic system of paramecia as bacteriophage are in the genetic system of bacteria (53).

Several different sizes and types of vesicles containing one or more inclusions were found in the cytoplasm of Amoeba proteus Pallas. These inclusions, of a diameter between 0.3-0.7 u, had an exterior membrane and a homogeneous interior. The structures were interpreted by Roht and Daniels (46) as infective organisms. Rod-shaped vibrio or
bacterium-like organisms, resembling the structures seen in the cytoplasm of amoeba, were found in the growth medium of the stock cultures. The authors, however, presented no evidence to prove that the inclusions were infectious. In a later paper Wolstenholme and Plant (56) observed high concentrations of DNA-containing particles in centrifuged *A. proteus*. Each particle consisted of a large number of bodies of a mean diameter of 0.5 μ. These DNA particulate bodies appeared to increase in number by division inside the amoeba cytoplasm. The authors speculated that the particles may be symbionts and of nuclear origin.

Early observations on poky in *N. crassa* led Mitchell and Mitchell (37) to suggest that the slow growth might be due to a virus. It was found later, however, that the poky type of growth was due to reduced respiratory activity caused by deficiencies of certain respiratory enzymes (18). Dwarf colonies (petites) in the yeast, *S. cerevisiae*, also were caused by deficiencies in certain respiratory enzymes (10, 12). Genetic studies showed the existence and operation of a nonchromosomal mechanism, a system of cytoplasmic units or components that control the characters of abnormal growth in *S. cerevisiae* (10), *N. crassa* (14, 37) and in *A. glaucus* (23). Genetic and cytochemical studies showed that mitochondria may be the cytoplasmic factors that cause abnormal growth (8, 18). Diacumakos et al. (8) microinjected mitochondria from the abnormal mycelium of *N. crassa* into cells of normal fungus and observed the development of abnormal growth. Ephrussi (10) drew similar conclusions from cytochemical and genetic studies.
When "vegetative death" mycelium and a normal homokaryon of A. glaucus were grown together in culture plates, vegetative death characters moved into the latter far beyond the maximum point of penetration on known chromosomally determined characters. Jinks (23) considered this an unambiguous criterion of extrachromosomal inheritance and called the phenomenon "infective heredity" (24).

In the disease of H. victoriae, bacteria and toxins were excluded as agents of the abnormality (28). It was shown in the same fungus that the respiratory rate of 2- to 6-day-old shake cultures of diseased fungus was greater than that of healthy fungus. When low concentrations of DNP (2,4-dinitrophenol) were added to the culture the healthy fungus responded with an increased respiratory rate, but the diseased fungus did not. Such respiratory responses are typical of organisms with infectious disease (1, 26, 55). Lindberg (31), reported production of diseased sectors in H. victoriae inoculated with phenol extracts of diseased mycelium and concluded that either the nucleic acid extracts were infectious and caused the disease, or that the extracts induced the disease.

Among the discussed abnormalities in amoebae and fungi only the "La France" or dieback disease of cultivated mushroom, A. bisporus, was proved to be caused by a virus (20, 47). Hollings et al. (20) demonstrated the presence of three types of virus particles in the infected tissue of mushroom. Infectivity studies using purified virus, however, were only partially successful.

Besides the direct damage caused in plants by the feeding of nematodes, some are carriers of viruses from diseased to healthy
plants. Virus transmission by the nematode *Xiphinema index* (19) was reported in 1958, and a review of nematode transmission of plant viruses was published by Raski and Hewitt (44) in 1963.
MATERIALS AND METHODS

In this investigation the healthy and diseased isolates designated N-1 and D-1A, respectively, were, unless otherwise mentioned, cultures on potato dextrose agar (PDA) and incubated at 22°C or at room temperature. The healthy isolate was propagated by transferring small bits of aerial mycelium plus conidia to fresh culture plates of PDA. All diseased fungus was propagated by inoculation of healthy H. victoriae according to the method described by Lindberg (28). A 3- to 4-day-old colony of D-1A ground in a sterile mortar and diluted with about 10 ml of sterile distilled water constituted a suspension of diseased fungus. Small cubes of mycelial mat plus agar of 2- to 3-day-old N-1 were dipped into the diseased fungus preparation and placed on PDA.

In the comparative morphology study, diseased and healthy H. victoriae were prepared using the cellophane strip method of Bakerspigel (5). Cellophane squares 1 x 1 cm were sterilized by autoclaving and dried between layers of sterile filter paper. Pieces of cellophane were placed on the medium surface of newly prepared cultures of both diseased and healthy fungus.

The cellophane pieces on which diseased and healthy fungus had grown, were stripped off the surface of the medium and mounted for observations. Observations were made on nonstained or stained mycelium at 72 hours for diseased and at 24 hours for healthy mycelium using a Bausch and Lomb microscope. Phase contrast microscopy was used originally for studies of the nonstained mycelium but it was
found of little value and the observations, therefore, were com-
pleted using bright-light microscopy.

The cellophane strip preparations were stained in 0.05 percent
trypan blue (36) for 1, 5, 10, 15, 20, and 30 minutes, rinsed and
mounted in physiological saline solution.

The methods of Hrushovetz (21) and Rawlins (45) for staining
with Giemsa were used. The Feulgen stain was used according to the
technique recommended by Hartman (17) and Gurr (15). The Feulgen-
carmine method of McIntoch (35) also was used. The methyl green
pyronine B method for staining DNA and RNA was used according to
Brachet (7) and Taft (54). Cellophane strips were left in the fixing
and killing solution (3 parts of 95 percent ethyl alcohol and 1 part
of glacial acetic acid) for 10-15 minutes. Strips were passed rapidly
(about 10 seconds) through 95, 85, 70, 50, 35, and 20 percent ethyl
alcohol and washed briefly (about 30 seconds) in distilled water.
This technique was later modified by passing strips slowly through
the alcohol series and distilled water was substituted for physio-
logical saline solution (PSS). Preparations were stained with methyl
green pyronine for about 30 seconds when the Taft method (54) was
used, but for 10-15 minutes when the Brachet method (7) was used.
Strips then were washed two times for 1 minute in distilled water,
transferred to 95 percent alcohol for 1 minute, and mounted in
Permout brand mounting medium.

Azure B was used according to the method of Flax and Himes (13)
as outlined by Jensen (22). Sudan III and IV were used to stain oil
globules. Preparations were stained for 30-90 minutes, washed in 50
percent ethyl alcohol, and mounted in glycerin (25).
For vector transmission studies the mycophasus nematode *Aphelenchus avenae* Bastian (34), vector of grape vine viruses (39), was used. A population of *A. avenae* was obtained by the sift and gravity method from soil of a sugarcane field at Louisiana State University. Since pure culture methods were used in all studies of the disease in *H. victoriae*, it was necessary that the nematodes be decontaminated of all bacteria. In a preliminary study (3) nematodes were treated for 1 minute with 0.1 percent HgCl$_2$, washed three times in sterile distilled water, and transferred to colonies of *H. victoriae*. The chemical was partly effective in killing contaminating bacteria but it was found to be toxic to the nematode. During the same study better results were obtained by treating nematodes with streptomycin sulfate (3, 38). In the present investigation nematodes in groups of 15 to 20 were treated with 0.1 percent streptomycin sulfate for: 10, 20, or 30 minutes; 1 or 2 hrs.; and overnight. Even the longest time of treatment did not affect the vitality of the nematodes. The nematodes treated overnight in the antibiotic were rinsed three times in sterile distilled water and transferred to cultures of *H. victoriae*. A nematode population free of bacterial contaminants was obtained by this method. Nematodes used in this study were propagated on cultures of healthy *H. victoriae* incubated at 26°C. Small pieces of mycelial mat with nematodes were transferred from these colonies to diseased and healthy cultures of *H. victoriae*. Diseased cultures were prepared by adding 0.5 to 1 ml of the diseased fungus suspension to PDA plates. Normal cultures were prepared by transferring small pieces of mycelial mat to fresh culture plates of PDA. The pieces of mycelial
mat with nematodes were transferred to 2- to 3-day-old cultures of
diseased and healthy *H. victoriae*, and incubated for 5 to 7 days at
26°C. Substantial increases in the nematode population were achieved
by this time and nematodes from such cultures were harvested for 2 to
3 days and then discarded.

Three transfers were made from the incubated cultures:
(a) Sixty nematodes mature, or at the 4th larval stage were picked
individually from diseased cultures with a loop and placed in a
Syracuse watchglass containing 0.5 ml of sterile distilled water.
Nematodes were passed individually through three sterile water baths
to eliminate any adhering fungal spores or cells. After the third
bath, nematodes were divided into three groups of 20 nematodes each.
Two of the groups of nematodes were transferred with a sterile micro­
pipette to two, 24-hour-old colonies of healthy fungus and the third
group was transferred to a blank culture plate of PDA to check for
diseased fungus growth or other fungal and bacterial contaminants.
The young healthy cultures were grown on oatmeal agar (OMA) (100 g
of oatmeal and 3 g of yeast extract per 1000 ml of distilled water).
A depression (well) was made at the center of the medium in which the
young colony was growing. The nematodes were pipetted into the well
to avoid the possibility of the suspension rolling off the fungus
colony. (b) From the nematodes feeding on healthy fungus cultures
40 were picked individually, washed three times, divided in two groups
of 20 nematodes each and transferred to two healthy cultures as
described above. (c) Two other young healthy control cultures received
no nematodes.
The inoculated and control culture plates were incubated at 26°C for 3 days. After this period the check plate was examined micro- and macroscopically for bacterial contaminants and growth of diseased *H. victoriae*. The test was continued when the control plate showed no growth and other cultures also were growing free of bacteria.

To determine whether or not nematodes were vectors of the agent of disease in *H. victoriae* 10 transfers were made from each of the inoculated colonies. Ten transfers also were made from control cultures which had received no nematodes. The transfers consisted of 10 small bits of aerial mycelium from each plate taken from the central areas of the colony where the nematodes were deposited. Transfers were placed on culture plates of PDA, in some tests one per plate, and in other tests two per plate. The inoculated plates were sampled three times at 3-day intervals, at the third, sixth, and ninth days after inoculation. The numbers of diseased and healthy colonies that developed were recorded after an additional 4 or 5 days.

A second method only slightly modified from the method discussed above also was used in the vector transmission studies. The nematodes were picked and washed as described above but the young N-I colonies to be inoculated were prepared differently, larger numbers of nematodes per inoculated colony were used, and inoculated colonies were assayed slightly differently.

The healthy N-I colonies to be inoculated with nematodes were prepared by grinding a small piece of mycelial mat (about 1 x 1 cm) from a young N-I colony in a sterile mortar with 10 ml of sterile,
distilled water. One-half ml of the suspension was pipetted onto three small Petri plates (6 cm diameter) containing OMA. The fungus covered the plate uniformly after 24 hours. Of 120 nematodes from diseased cultures of *H. victoriae* (D-1A), 100 were pipetted over the surface of the N-1 fungus of one plate and 20 were pipetted to a blank culture plate of PDA. One hundred nematodes from healthy N-1 cultures were pipetted to the second plate of N-1 and the third plate of N-1 received no nematodes. The plates were incubated at 26°C and after 3 days, the inoculated colonies and the one which received no nematodes were cut into 50 small squares. The squares were transferred, 1 per plate, to culture plates of PDA. The number of diseased and healthy colonies for each treatment was recorded after an additional 4 or 5 days.

Cultures of diseased and normal fungus were checked periodically for bacterial contaminants. A sterile needle was inserted into the culture and then streaked on PDA or a mycelial piece from the inoculated colonies was transferred to nutrient broth and later examined microscopically and macroscopically for the presence of bacteria.
RESULTS

A. Results of Microscopic Studies

In a light microscopy, morphological study of diseased and healthy *H. victoriae*, Psarros and Lindberg (43) found that cells of newly infected mycelium were distorted and lysed, resulting in partial or total leakage of their protoplasm. The authors also reported the presence of granules of different sizes and shapes in the cytoplasm of diseased cells (43). Since the cytoplasmic granules in cells of the diseased fungus were treated in only a limited way by Psarros and Lindberg (43) it was decided to study these anomalous structures more thoroughly.

The objective of the present investigation as outlined in the introduction was to compare the morphology of diseased and healthy *H. victoriae* by phase contrast microscopy. It soon became evident that the phase contrast observations were contributing little to the previous work, due in part to the apparatus available for the study. Therefore the morphological studies were carried out with bright light microscopy. Healthy mycelium grew relatively fast and covered the cellophane strips in about 24 hrs., whereas diseased mycelium grew very slowly and reached a length of 3-5 mm in about 72 hrs.

Unstained, healthy mycelium appeared as described earlier (43) with long straight hyphae arranged in bundles and with cells that contained uniform finely granular cytoplasm (Fig. 1 and 2). Cells in older mycelium contained many oil globules.

21
Before describing the different anomalous structures observed in nonstained and stained diseased mycelium, some abnormal characteristics of growth, in addition to those reported by Psarros and Lindberg (43), will be briefly described.

A type of finger-like branching was commonly observed at the edges of diseased colonies (Figs. 3, 4, and 6). The cells produced in the branching areas usually were bulbous or cylindrical to sausage-shaped. Although extensive lysis was observed in some elongated cells associated with the branching, bulbous cells commonly had intact cytoplasm.

Another characteristic feature of diseased colonies was the presence of hyphae at the edge of the colony with cells which were not showing any deformity or lysis. These hyphae were distinctive because of their limited or stunted growth and extensive curvature (Figs. 5 and 6). The cytoplasm of these cells was intact and occasionally only a few oil globules were present. At 72 hrs. after inoculation, the cytoplasm of some of these cells started to degenerate and this phenomenon gradually spread to almost all the cells.

**Anomalous structures observed in unstained mycelium.**

Three groups of anomalous structures were observed in the cells of diseased mycelium:

1. The cytoplasm of many severely diseased cells separated into masses of indefinite to spherical shape that varied considerably in size. Portions of cytoplasm of indefinite shape (Fig. 7) were seen in cells of mycelium that had already lysed before the observations were started (72 hrs.). In portions of mycelium that started
degenerating at the time observations were started, cytoplasmic masses appeared compact and had a shape close to spherical (Figs. 8 and 9). The surface of the masses was coarse to rather smooth as their shape changed from indefinite to compact spherical. Often there was considerable leakage of the granular cytoplasm. The compact masses were scattered in the cell lumen without any surrounding materials. This separation and the light-yellowish color of the masses made them prominent and easily distinguishable. Cytoplasmic masses of different sizes were found in the same cell. The size of the masses appeared to change and large masses were observed breaking down to smaller ones. The cytoplasmic structures in fixed and stained preparations either were not visible or stained as the rest of the cytoplasm.

(2) In cells with their cytoplasmic content intact, compact spherical structures of granular appearance were observed which had a slightly darker color than the rest of the cell contents (Figs. 10 and 11). The size of these masses varied from about 1 u to large spherical masses with the same diameter as that of the cell. The average diameter measured between 2.5 and 3 u. One to three spherical structures, of the same or different size, were seen in an individual cell. Large or small, all were granular with echinulated edges.

(3) Some cells in the older portions of diseased colonies contained very prominent and conspicuous structures. Unlike the two types of structures described above, they were brown to greenish-yellow, multiangular particles that varied considerably in size (Fig. 12). In some cells the particles varied in size from a fraction
of a micron to 2 or 3 u long, whereas other cells contained small or large particles only. The structures were seen in individual cells or in two to three contiguous cells. Cells that contained the structures were not uniformly distributed in the colony. In some areas there were particles in several cells while in other areas particles were not detected in any cells. The amount of particles that a cell contained was variable from a few to a number large enough to fill the whole cell lumen. The number of particles per cell varied according to their size. It was possible to distinguish the particles in a cell due to the difference between their color and the hyaline color of the rest of the cell. When the particles were few in number they were interspersed in the cytoplasm, but when many particles occupied most of the cell the cytoplasm was not detected.

Older portions of diseased mycelium also had cells with cytoplasm that showed rough granulosity and contained a large number of oil globules.

Normal, nonstained mycelium contained none of the anomalous structures described in diseased mycelium. Healthy *H. victoriae* contained oil globules in older portions of mycelium but they were neither as numerous nor as large as those found in diseased mycelium.

Different stains were used in this study to test whether some of the structures observed in the diseased, nonstained mycelium could be stained. It was also considered possible that stains might enhance visibility of some other structures not well distinguished in nonstained mycelium. A number of specific stains employed to detect the presence of virus inclusions or DNA and RNA containing material (7, 13, 15, 36, 45) also were used.
Trypan blue stain: 24- and 72-hr.-old healthy and diseased mycelium, respectively, were immersed for 15-20 minutes in 0.05 percent solution of trypan blue (36), rinsed in PSS and mounted. Some apical portions of healthy mycelium suffered slightly from the treatment. In these cases, the protoplasm appeared slightly altered in its form, large vacuoles were produced even at the very tip of hyphae and staining was poor. Other portions of the colony were not similarly affected by the treatment and apical or older cells had a finely granular cytoplasm and stained uniformly light blue (Fig. 13). Diseased, nonlysed cells stained blue and some showed slight vacuolization. In the portion of diseased colonies with the finger-like branching, bulbous cells contained structures that stained dark blue (Fig. 14). There were about 4 or 5 blue stained structures per cell. The structures were either scattered or located in a circle with one at the center. The average diameter of the structures was 3 to 3.5 μ, but some larger ones measured as much as 5 μ. The shape of the structures was close to spherical but the exact shape was difficult to determine because the edges were hazy. Increased staining time did not improve the visibility of the edges. Only 5 to 10 percent of the bulbous cells contained the structures. Occasionally the structures were observed in nonstained material (Fig. 15) but were not as well distinguished as in cells stained with trypan blue.

Other cells of young portions of diseased mycelium contained blue-stained structures somewhat different from those found in bulbous cells (Fig. 16). The structures were smaller in size (1-2 μ), lighter in color, granular in appearance, and had a sharp outline. Neither of these types of structures were seen in healthy mycelium.
Methyl green pyronine B stain: This stain was used to detect DNA and RNA containing material. Both the Taft (54) and Brachet (7) methods caused extensive rupture of terminal hyphal cells in healthy *H. victoriae*. The cytoplasm leaked out of the cells and remained attached to the outer surface of cell walls as a spherical compact mass. Older cells did not burst, but had a granular cytoplasm which stained poorly. Injury to the terminal and older cells of the healthy fungus was reduced and the cytoplasm was uniformly stained with methyl green pyronine when PSS was substituted for distilled water and the tissues were transferred slowly through the alcohol series (Figs. 17 and 18). In contrast to terminal or apical cells of the healthy fungus, diseased cells showed little or no bursting that could be attributed to staining.

The most characteristic structures observed in older sections of diseased mycelium stained with methyl green pyronine B, especially with the Brachet method, were of a deep red color (Figs. 19, 20 and 21). The material was composed of particles from a fraction of 1 μ to 2-3 μ or larger. There were cells with only small particles, others with larger particles, while some cells contained a mixture of both and often completely filled the cell. The shape of particles was irregular to roughly spherical and multiangular. Stained material was neither found in all cells nor was it found in all portions of the colony.

Nonlysed cells of the terminal portions of diseased hyphae contained another type of structure. These structures were dark blue, spherical granules of a diameter of 1-1.5 μ (Fig. 22). Some of the granules
were found in groups of 3 to 4 or more and had a very well defined outline. These granules were seen particularly well when stained by the Taft method.

Giemsa stain: Giemsa stain was used in cytology for nuclear staining (21, 43) and to detect inclusions in virus infected plants (45). Both the Hrushovetz (21) and Rawlins (45) methods for Giemsa staining were used in this study. Giemsa, according to the Rawlins method (45), seemed to best stain particles in diseased mycelium. With both methods nuclei of healthy mycelial cells appeared as illustrated and described by Psarros and Lindberg (43). In addition to the stained nuclei, blue spherical particles were observed in the healthy mycelium. From 3-5 of the particles (1 μ in diameter) were scattered in both young and old mycelial cells. All cells, however, did not contain the particles.

Diseased mycelium contained nuclei which often resembled those of the healthy fungus. In cases where the disease was well developed nuclei, unlike those of the normal mycelium, appeared deformed and variable in size (Fig. 23).

Various other structures were observed in the diseased mycelium stained with Giemsa. These can be grouped into two categories. In the first group blue particles of different sizes were commonly found in older portions of mycelium. These particles ranged in size from a fraction of a micron to 2-3 μ long (Figs. 24 and 25) and were highly variable in shape.

The second group of particles that stained blue with Giemsa had a spherical shape and a uniform size of around 1 μ (Fig. 26). The
uniform particles ranged in number per cell from 10-15 or more and sometimes almost filled the cell lumen. The particles were either uniformly distributed in the cell or clustered in small groups depending upon their number. The particles described in the normal mycelium stained with Giemsa resembled this type.

Feulgen staining method: The Feulgen staining method of Hartmann (17) was used for detection of DNA-containing material. The fungus preparations were not stained successfully although several attempts were made. McIntosh's (35) Feulgen-carmine staining technique gave somewhat better results. Nuclei stained light pink in some areas of the diseased mycelium. In the healthy mycelium nuclei stained very poorly and were seen only with great difficulty. No other cellular material was stained in either healthy or diseased mycelium.

Azure B stain: Azure B was used to detect DNA and RNA containing materials. The staining method of Flax and Himes (13) was used and gave almost the same results as the methyl green pyronine stain. The cytoplasm of healthy mycelial cells treated with azure B appeared to coagulate or shrink in response to the stain and in addition, small blue granules were seen in the injured cytoplasm. A small number of blue granular particles appeared in a few cells of the healthy mycelium. In the diseased mycelium, however, a large number of cells contained blue particles and the number per cell was far greater than in the normal fungus. The material which appeared as distinct particles in fresh mycelium and stained red with methyl green pyronine remained either nonstained or stained a light blue color in response to azure B.
Sudan III and IV were used to detect oil globules (25) which stained an orange yellow color in both healthy and diseased mycelium. Older portions of both diseased and healthy mycelium were rich in oil globules. Young diseased cells contained few oil globules even at the very tips of the hyphae. Oil globules were not detected with any of the other staining techniques described. In fresh material stained with Sudan it has been possible to distinguish oil globules from other intracellular structures in the diseased mycelium.

B. Results of Vector Transmission Studies

Preliminary vector transmission studies (3) were inconclusive because of the lack of adequate controls and the contaminant bacteria in nematode cultures. As described in the methods above the problem of contaminant bacteria was eliminated by treatment of nematodes with 0.1 percent streptomycin sulfate.

In the preliminary study, controls were not adequate to demonstrate that fragments of diseased fungus were not transferred with the nematodes. It was correctly pointed out that even with careful washing of the nematodes small, viable fragments of the diseased fungus that could not produce visible colonies might have been transferred.

As stated earlier Lindberg (32) in 1967 reported the isolation of a mild disease from the severe D-1A isolate. The mild disease was masked and was undetectable in the presence of the severe disease but it was expressed in the absence of the severe disease.

Where transmission was observed in the earlier vector studies it was invariably not the severe type of disease that was encountered. It
was known that if viable fragments of the severe diseased fungus were transferred with the nematode, symptoms of the severe disease would have developed in the test colonies in every instance. If, on the other hand, symptoms of the mild disease were the ones most frequently detected in the inoculated test colonies, vector transmission might have occurred and the problem of transferring fragments of the diseased fungus with the nematode would have been eliminated.

To provide adequate controls, the present vector transmission studies included three treatments. Treatment one consisted of nematodes feeding on mycelium of diseased *H. victoriae* which were transferred to colonies of healthy *H. victoriae*. For each two groups of nematodes transferred to young healthy colonies an additional group of 20 nematodes, drawn from the same pool as the previous two groups, was transferred to a blank culture plate to check whether nematodes were free of bacterial contaminants and fragments of diseased fungus mycelium. Treatment two served as a control of the first treatment with nematodes feeding on mycelium of healthy *H. victoriae* transferred to healthy cultures of the fungus. Treatment three was a second control and consisted of young colonies of healthy fungus which received no nematodes. There were 112 cultures of healthy fungus used for each treatment. All test cultures, whether inoculated or not with nematodes, with one exception, grew normally without showing any symptoms of disease (Fig. 27a). To test whether there was development of disease in the inoculated test colonies, transfers of small bits of aerial mycelium were made. Ten transfers were taken from the center of the cultures at each of the third, sixth, and ninth day after transfer of nematodes.
When all transfers from a culture were healthy, that culture was considered healthy. The data for such cultures are not presented. Cultures were considered diseased when one or more of the colonies that developed from the 30 transfers was diseased. Disease symptoms that developed in the colonies were almost always those typical of the mild disease as described by Lindberg (32) (Fig. 27b). Of 3360 transfers in treatment one, 173 produced disease colonies; of these 3 or 4 were typical of the severe diseased isolates D-1A (Fig. 27c₁). The remainder were all of the mild disease. The severe diseased colonies, due to their small number, were not recorded separately. In treatment two and three a similar low frequency of severe diseased colonies developed.

The number of cultures from each treatment that produced diseased colonies showed that of the 112 colonies inoculated with nematodes fed on diseased fungus in treatment one, 27 or 24.1 percent were diseased (Table 1). In the inoculated controls of treatment two, 11 of 112 colonies were diseased (9.6 percent), and in the noninoculated controls of treatment three, 7 of 112 colonies were diseased or 6.2 percent. The number of diseased colonies from 10 transfers at each of the third, sixth, and ninth day after inoculation, as well as, the total number of diseased colonies that developed from the transfers in each treatment are presented (Table 1). The number of diseased colonies that developed in all transfers from each of the inoculated cultures and noninoculated controls were analyzed statistically. Two statistical methods of analysis of data were used.
Table 1. The data show the number of diseased colonies among transfers, at 3-day intervals, from cultures of 3 treatments: 1) inoculated with nematodes from diseased *H. victoriae*, 2) inoculated with nematodes from healthy fungus, and 3) noninoculated *H. victoriae*.

<table>
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<th>3 - Noninoculated</th>
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Table 1. Continued

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<th>3 Noninoculated Transfers</th>
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(1) The t test, between each of the 3 pairs of treatments, and
(2) the analysis of variance (Tables 2 and 3). The mean differences obtained between the 3 pairs of treatments were 3.59 for treatments one and two, 1.98 for treatments one and three, and 1.61 for treatments two and three. These differences were not statistically significant by t test. In the analysis of variance the treatment component was not statistically significant.

In all treatments an increase in the number of diseased colonies in transfers from the third to the ninth day was observed. The first treatment, however, had a high number of diseased colonies in the first transfer with a similarly high number of diseased colonies in the following two transfers. Only one diseased culture from the third treatment gave high numbers of diseased colonies in the first set of transfers and continued high during the second and third transfers. This culture, in contrast to all other inoculated or non-inoculated cultures, developed disease sectors in parts of its periphery.
Table 2. *t* values obtained and *t* values required for each of the 3 pair of treatments.

<table>
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<th>Comparisons</th>
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<th><em>t</em> required</th>
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Table 3. Analysis of variance.

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<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th><em>F</em> value</th>
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<td>105.90</td>
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<td>Within treatments</td>
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<td>1105.88</td>
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<td>Total</td>
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*F* value obtained = 2.01

*F* value required for 5% level of probability for 2 and 42 degrees of freedom = 3.23
Figure 1. Unstained hyphal tips of healthy *H. victoriae*. (Approx. X1100)

Figure 2. Unstained hyphae of *H. victoriae*. (Approx. X1800)
Figure 3. Unstained finger-like branching with few oil globules. (Approx. X1100)

Figure 4. Unstained diseased mycelium tip with finger-like branching and bulbous cells. (Approx. X1100)
Figure 5. Unstained diseased mycelium without any deformity and lysis (about 72 hrs. old). (Approx. X1100)

Figure 6. Unstained diseased mycelium without any deformity and hyphal tip branching showing bulbous cells. (Approx. X1100)
Figure 7. Unstained diseased mycelium containing degenerating protoplasm in masses of irregular shape and size. (Approx. X2200)

Figure 8. Unstained diseased mycelium with spherical-like masses of degenerating protoplasm. (Approx. X1100)
Figure 9. Unstained diseased mycelium with spherical-like masses of degenerating protoplasm. (Approx. X1100)

Figure 10. Unstained diseased mycelium with intact protoplasm and containing spherical cytoplasmic structures. (Approx. X2200)
Figure 11. Unstained diseased hypha showing cells with intact cytoplasm and containing spherical cytoplasmic structures. (Approx. X2000)

Figure 12. Unstained diseased mycelium with cells containing numerous angular particles. (Approx. X2200)
Figure 13. Healthy tips of *H. victoriae* stained uniformly blue with trypan blue. (Approx. X1800)

Figure 14. Diseased *H. victoriae* stained with trypan blue. Structures in bulbous cells were stained blue. (Approx. X1800)
Figure 15. Unstained diseased mycelium showing bulbous cells containing structures which appeared dark blue when stained with trypan blue. (Approx. X2200)

Figure 16. Diseased mycelium stained with trypan blue. Cells contained blue round structures. (Approx. X900)
Figure 17. Healthy mycelium stained with methyl green pyronine. (Approx. X900)

Figure 18. Healthy mycelium stained with methyl green pyronine. Blue filter was used. (Approx. X1800)
Figure 19. Diseased mycelium stained with methyl green pyronine B. Cells contain red material. (Approx. X1800)

Figure 20. Diseased mycelium stained with methyl green pyronine B. Cells contain red material. Blue filter was used. (Approx. X1800)
Figure 21. Diseased mycelium stained with methyl green pyronine B. Cells contain red material. Blue filter was used. (Approx. X1800).

Figure 22. Diseased mycelium stained with methyl green pyronine B. Cells contain small dark blue granules. Blue filter was used. (Approx. X900)
Figure 23. Diseased mycelium stained with Giemsa showing deformed nuclei. (Approx. X900)

Figure 24. Diseased mycelium stained with Giemsa showing dark blue stained particles. Blue filter was used. (Approx. X1800)
Figure 25. Diseased mycelium stained with Giemsa. Cells contain dark blue particles. Blue filter was used. (Approx. X900).

Figure 26. Diseased mycelium stained with Giemsa. Cells contain blue spherical particles. (Approx. X900)
Figure 27. (a) Ten-day-old culture of *H. victoriae* inoculated with 20 nematodes fed on diseased mycelium. Transfers from the center of such cultures resulted in (b) colonies with mild symptoms of disease and (c1) healthy growth. (c2) colonies of severe disease developed from an occasional transfer.
DISCUSSION

The purpose of the microscopic study was to make thorough observations of anomalous structures in cells of diseased mycelium. In nonstained preparations of diseased mycelium, granules of different types were found. Granules were first observed with early changes in the cytoplasm. Different stages in the breakdown of the cytoplasm could be seen in the mycelium. Among the first was detachment and removal of a few granules from one or more points along the periphery of the cytoplasm. This caused a depression which deepened by further removal of cytoplasmic material. The separation advanced until the two adjacent parts of the cytoplasm were completely separated. Masses of cytoplasm often developed into round masses which later broke down into smaller pieces and leaked out of the cell. Other granular material had a compact granular appearance resembling the X bodies of virus inclusions as described by Bawden (6). These structures, however, did not resist fixing and killing treatments. Two other types of granules resembled more closely the inclusion bodies discussed by Bawden (6). The first type was a granular material made up of both small and large multiangular particles. In nonstained mycelium the granules were yellowish-green to brown and had a sandy or gravel-like appearance. Stained with methyl green pyronine B, the granules appeared red. This color indicated that the granular material contained RNA. Another type of anomalous structure appeared exclusively in the bulbous cells at the edges of severely diseased colonies.

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These structures appeared as gray, compact masses in nonstained mycelium but stained dark blue with trypan blue, a material used to stain proteinaceous inclusions in virus-infected plants. Some particles stained with Giemsa in both diseased and healthy mycelium. Particles reported by Knox-Davies and Dickson (27) in *H. turcicum* stained blue with Giemsa.

Although most of the stains used revealed some structures, especially in the diseased mycelium, Feulgen was of no value. This may be due to the fungus rather than to the technique used because it is admitted that the Feulgen technique has rarely been found in demonstrating nuclei in fungi (35).

Healthy mycelium suffered from severe to light injuries with certain treatments whereas diseased mycelium was not seriously affected. It is possible that the cellular changes caused by the disease preclude expression of injury by the stain.

Previous studies on transmission of the agent of disease from diseased to the healthy *H. victoriae* by using a nematode vector were inconclusive (3). There was the possibility that viable fragments of diseased mycelium capable of transmitting the disease to the test colonies were transferred with the nematodes. Lindberg (32) isolated a mild type of disease whose expression was inhibited and masked in the presence of the severe diseases but which could be detected in the absence of the severe disease. In the present experiments it was the mild type of disease that developed in almost all of the inoculated and control cultures. The results indicated that fragments of mycelium of the severe disease were not transferred with the nematodes. If
such fragments were transferred, the severe type of disease would have been transmitted to the test colonies and prevented detection of the mild disease. The occasional appearance of the severe type of disease in transfers from test colonies of treatment one, where nematodes fed on diseased fungus were transferred to healthy fungus, might be attributed to "spontaneous" diseased sectoring. "Spontaneous" development of diseased sector in apparently healthy isolates of *H. victoriae* was reported (28). The occurrence of the severe type of disease also was observed occasionally in treatment two and three where there was no chance to transfer mycelium of the severe disease. In treatment two nematodes fed on healthy mycelium were transferred on healthy colonies. Similar results were observed in treatment three in which no nematodes were added to the healthy colonies.

There was no statistically significant difference between the number of diseased colonies in the transfers from colonies either inoculated with nematodes from diseased fungus or with nematodes from healthy fungus. The development of colonies with symptoms of the mild disease in the transfers of treatments two and three was expected. It was reported (32) that the mild disease is endemic in the apparently healthy isolates N-1 and 7002, as well as, in the severe diseased isolates of *H. victoriae*.

It is concluded that the results of the vector transmission studies could be explained by one or more of the following hypotheses. Either the nematodes were vectors of the agent of the disease designated as "mild," that the mild disease was induced in apparently
healthy fungus by the feeding of the nematodes, or that the agent of
the mild disease was a natural course of development in apparently
healthy fungus, especially as the so-called healthy cultures aged.

In many experiments diseased colonies failed to develop in any
of the transfers from inoculated cultures. Development of the mild
disease seems to occur only under certain environmental conditions.
Mild diseased colonies were detected in the transfers for only a short
period during colder weather whereas the disease was never detected at
room temperature above 26°C. In addition, the mild disease seems not
to develop readily when cultures grown for extended periods at
temperatures above 26°C are exposed to temperatures below 26°C. It
seems that something must first trigger development of the mild
disease which will then continue to develop if the cultures are
exposed to cooler temperatures.

The second method used in the vector transmission studies was
found to be unsatisfactory. Transfers from both inoculated and con­
trol cultures consisted of cubes of mycelial mat plus agar. The
colonies that developed often showed a restricted type of growth
which made symptoms of disease difficult to detect. Little or no
differences were detected between the treatments. Therefore, the
results were considered unreliable; and were not presented.
SUMMARY

Studies of unstained, diseased mycelium of *Helminthosporium victoriae* with the light microscope showed: (1) cytoplasmic masses of different sizes and of irregular or spherical shapes in partially lysed cells, (2) round masses of compact, granular cytoplasm and with an echinulated surface in cells with intact cytoplasm, and (3) yellowish green to brown, granular particles with multiangular shapes, and of different sizes and number in nonlysed or partially lysed cells.

Bulbous cells of diseased mycelium stained with trypan blue were found to contain structures stained dark blue; other cells contained cytoplasmic masses stained blue. When methyl green pyronine was used as a stain, a material in the older portions of the colony made of particles of different sizes and multiangular shapes, stained red. Giemsa stained nuclei a reddish color and some nuclei were deformed and of different sizes. Two other cellular materials stained blue with Giemsa in diseased mycelium. One of these materials also stained blue in healthy mycelium. Feulgen and Feulgen-carmine techniques were unsuccessful and azure B stained some particles blue.

In vector transmission studies, from cultures which received nematodes fed on diseased isolate D-Al, 173 transfers from over 3000 were diseased. In a similar number of transfers from cultures which received nematodes fed on healthy fungus there were only 31 which showed disease. The same number of transfers from cultures which
received no nematodes gave 31 diseased cultures. There were no statistically significant differences between the number of diseased colonies that developed from cultures that received nematodes fed on diseased and healthy mycelium.
BIBLIOGRAPHY


40. Preer, J. R., Jr. 1948. Microscopic bodies in the cytoplasm of "killers" of Paramecium aurelia and evidence for the identification of these bodies with the cytoplasmic factor, kappa. Genetics 33: 625. (Abstr.)


VITA

Basil Anastasiadis was born in Athens, Greece on April 5, 1934. He received his elementary education in Pireaus and his high school education at the Greek French College at the same town and was graduated in 1951. The same year he was enrolled at the University of Naples (Portici), Italy, Faculty of Agriculture and was graduated in 1956 with a degree in Agricultural Sciences. He served in the Greek Army for two years and then worked at the Benaki Phytopathological Institute in Athens as an Employee of the Greek Department of Agriculture for four years. He began graduate work in the Department of Botany and Plant Pathology at Louisiana State University in February 1964 and received his Master of Science degree in July 1965. He is now a candidate for the degree of Doctor of Philosophy.
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Major Field: Plant Pathology

Title of Thesis: Transmission and Morphological Studies of Diseased H. victoriae

Approved:

Major Professor and Chairman

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