MORPHOLOGICAL AND CYTOLOGICAL STUDIES AND CHARACTERIZATION OF A NEW ASCOMYCETE: ATTENUOSPORA SILVESTERRA

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ABSTRACT

Information was obtained through morphological and cytological studies in order to describe and classify a recently isolated Pyrenomycetous fungus. The fungus was isolated with Gliocladium roseum from soil samples of maple-elm-ash forest. Serial sections obtained from paraffin embedded materials stained with Hematoxylin Iron Alum and Lactophenol cotton blue revealed that this organism's perithecial initials arise as a single-celled archicarp, which grows out as a side branch from a vegetative hypha. The single-celled archicarp becomes transversely septate and elongated. The cells from the basal regions of the archicarp send out hyphae growing around the archicarp forming a primary ascocarp wall which encloses a pseudoparenchymatous centrum. The perithecium is globose to flask-shaped, dark and ostiolate. The asci are fusiform, curved or straight which arise without crozier formation from the ascogenous hyphae. No paraphyses have been found. The ascospores are hyaline and spindle-shaped with a spine-like appendage at each end. The fungus also bears hyaline and cylindrical conidia on phialides. The phialides are subhyaline to brownish, smooth and simple with a single septum located near the base of each phialid. In the light of these morphological and cytological evidences, the fungus is placed in the order Sphaeriales, and is designated as Attenuospora silvesterra Totaram & Nelson gen. nov. sp. nov.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

A Pyrenomycetous fungus was isolated with Gliocladium roseum from soil samples of maple-elm-ash forest near the town of Avon Wisconsin. Preliminary identifications of the fungus placed it in the order Sphaeriales mainly because of its perithecium, type of asci and ascospores, and in the family Sphaeriaceae because of its perithecium. It is currently designated as Attenuospora silvesterra gen. nov. sp. nov. (Latin adj. Attenuo, attenuated, Latin adj. spora, spore, Latin adj. silves, forest, Latin adj. terra, soil).

The purpose of this study was to identify the Pyrenomycetous fungus to genus and species; to determine its relationship with Gliocladium roseum, to know whether it is parasitic and its mode of parasitism; and to study the morphology and the morphological development of its perithecium.

LITERATURE REVIEW

The Classification of the Ascomycetes is perhaps the most difficult taxonomic problem in mycology (Luttrell, 1951). Identifications in this, the largest class of the Fungi, have always been very difficult.

Miller (1949) tended to shift the emphasis in classification from such apparent superficial criteria as habitat, insertion of the ascocarp, presence or absence of a stroma, and color and consistancy of perithecial walls and stroma, to what seem to be more fundamental criteria derived from ascus structure and centrum structure. Consequently, numerous changes in the classification of the Pyrenomycetes have been proposed, and the classification of this group of fungi is in an unstable state of transition. Thus, Luttrell (1951) examined critically the data on the structure of the ascus and the ascocarp centrum in this group. He mentioned that the Pyrenomycetous fungi have unitunicate asci from which the ascospores are discharged through a pore. The unitunicate asci are surrounded by a single wall. The ascus wall gives a blue reaction with iodine, at least in certain parts at some stage of development in the majority of the species. Upon the basis of the numerous variations in wall structure and manner of ascospores discharge, the asci in this group are separated into a number of types.

Luttrell described the Claviceps Type of asci as being long and cylindrical with thin lateral walls, the apex consists of a thickened hemispherical cap penetrated by a fine pore. The Xylaria Type asci are cylindrical with thin lateral walls, of which the

apex is thickened with a pore; and an annular ridge projects from the inner surface of the thickened apical wall surrounding the pore and forming a pecular crown in the apex of the ascus. In the Diatrype Type, the asci are clavate with long stalks. The ascus wall is thin except at the apex where it is thickened and penetrated by a pore. The Endothia Type asci are also clavate, but short-stalked and thin-walled. The ascus wall is thickened at the apex and is provided with a pore. The Erysiphe Type asci are globose, short stalked, uniformly thin walled and lack a pore. The Corynelia Type asci are ovoid to clavate with very long, delicate stalks, uniformly thin-walled, and lack a pore. The Ophiostoma Type asci are globoid, nonstipitate, uniformly thin-walled and lack a pore. The Nectria Type asci are cylindrical with stalks and are uniformly thin-walled. Often the ascus is hardly more than a membranous sac surrounding the ascospores. Usually there is no distinct pore.

Luttrell also emphasized on the increasing knowledge of the internal structure of the ascocarp - the centrum which includes the ascogenous hyphae and asci, and the sterile tissues which function as nourishment for the asci. He described many types of ascocarp centrum as in the case of the asci. In the Dothidea Type, Pleospora Type and the Elsinoe Type the ascogonia are formed within a plectenchymatous or pseudoparenchymatous stroma. However, the Dothidea Type centrum varied, in that, the expansion and ultimate disintegration of the sterile pseudoparenchymatous tissues creates the locule within which the asci develop. In the

Pleospora Type the region of the stroma occupied by the ascogonia, a group of vertically arranged, separate hyphae appears. These hyphae are pseudoparaphyses among which the asci originate and form a concave layer across the base of the centrum. In the Elsinoe Type the ascogenous hyphae are long and penetrate the stroma, producing asci at intervals. Each ascus develops independently. Consequently, the asci are scattered in monascous cavities in the stroma.

In the next three types of centrum - Diaporthe Type, Ophoistoma Type and Xylaria Type, the ascogonia are produced free upon the mycelium or formed within a stroma. The major differences are, in the Diaporthe Type the ascogonium forms a spherical mass of pseudoparenchymatous tissue - the perithecial initial; and the asci expand as a group into the disintegrating centrum pseudoparenchyma and ultimately form a layer lining the base of the perithecial cavity. In the Ophiostoma Type the asci matured progressively from the apex to the base of the perithecium and never form a definite wall layer. As the asci mature the sterile cells of the centrum collapse and disintegrate to form the perithecial cavity. The walls of the asci deliquesce and free the ascospores within the perithecial cavity. In the Xylaria Type hyphal branches with free tips - paraphyses grow upward and inward from the inner surface of the wall over the base and sides of the perithecium. Pressure exerted by the growth of opposed paraphyses expands the perithecium and creates a central cavity. The perithecium becomes pyriform as a result of growth of hyphae in the apical region of the wall to form the neck.

Another type, the Nectria Type, still differs from the others. The ascogonia, which are formed within a stroma, become surrounded by concentric layers of vegetative hyphae which form a true perithecial wall. The cells of the inner layer of the wall in the apical region of the young perithecium produce a palisade of inward-growing hyphal branches-pseudoparaphyses which exert pressure by expansion of the wall and create a central cavity within the perithecium. The last type of centrum described is the Phyliactina Type, in which the ascogonia are produced free upon the superficial mycelium. They are unicellular and are formed in association with unicellular antheridia. Hyphae arise from the stalk cells of the ascogonium and the antheridium and sometimes from the hyphae to form a sheath about the ascogonium which initiates the perithecium.

In 1973 Ainsworth, Sparrow and Sussman also studied the Pyrenomycetous fungi in order to classify them. According to them some of these fungi are easily recognized by the size or arrangement of the asci in the hymenial layer as well as by the more complicated form of the ascospores. However, they emphasized the morphology of the ascus especially of the ascus apex.

Presently, it seems that the ascocarp centrum, the ascus apex and the different forms of the ascospores are the major emphasis in classifying a fungal organism which is found in the class Ascomycetes. Thus studying the morphological development of the ascocarp will reveal the centrum and ascus structures.

The convincing work of Harper (1900) led to greater emphasis being directed to that course of development, represented by

Pyronema and Discomycetous species in general, wherein asci are produced following a characteristic crozier device either terminally or laterally on hyphae that are direct outgrowths from an ascogonium or group of ascogonial cells. A few investigators in this field who ventured outside the group of Discomycetous species have been inclined to interpret their findings in accordance with the above process, even though it was not possible in some cases to follow the various stages intermediate between ascogonium and ascus. This applies in part to the work of Elliott (1925) on Ceratostomella, of Cookson (1928) on Melanospora, and in fact to most of the species of the Pyrenomycetes. Other investigators, unable to follow the stages intermediate between ascogonium and ascus, have concluded that the ascogonium is abortive and that the ascogenous hyphae later grow out from purely vegetative cells within the perithecium. A review of early literature in this field has been made by Overton, 1906.

Blackman and Welsford (1921) described a much-coiled and septate ascogonium in Polystigma rubrum D.C. but they considered it to be abortive - ascogenous hyphae developing later from purely vegetative cells within the perithecium. McIntosh (1927) considered the hyphal outgrowth from ascogonia of Nectria mammoidea to be abortive structures. He says, "The true ascogenous hyphae arise directly from the vegetative cells at the foot of the cavity. These hyphae consist of a single cell which is generally binucleate."

A few investigators have recognized that individual cells of the ascogonium may become separated and proliferate independently.

Brown (1913) observed such to be the case in Xylaria tentacularia B. & Br., and Lupo (1922) in Hypoxylon coccineum.

Rogers (1965) did a detail study of the cytology of the ascus of Hypoxylon fuscum. The general sequence of events leading to the mature ascocarp, ascus and ascospore is similar to that described for many other Pyrenomycetes. The ascogenous hyphae develop as short outgrowths from two to eight nucleate ascogenous cells located along the lower portion of the perithecial wall. The short ascogenous hyphae, terminate in croziers. Cells of another kind, of unknown function, often are found among the ascogenous cells. These cells are uninucleate, the nucleus often being larger than the late prophase ascal nucleus. Croziers are formed in the well-known manner. Proliferation of croziers usually occurs through the fusion of the ultimate and antepenultimate cells. The first proliferation may be initiated prior to karyogamy in the penultimate cell.

Mhaskar and Rao (1976) studied the development of the ascocarp of Epichloe cinerea. At an early stage of development, deeply stained meristematic cells appear at the subperipheral region of of the ascocarpic stroma. These cells can be differentiated from the rest of the tissue by their dense cytoplasm and prominent nuclei and constitute the archicarp. During the later stages of development, some of the sterile cells from the basal part of the archicarp elongate and grow parallel to the long axis of the developing ascocarp. These cells ramify towards the apex, turn inward and completely surround the fertile mass of the archicarp. In the later stages, the fertile mass of the archicarp does not

increase much in size but the sterile hyphae surrounding it elongate and reach the periphery of the developing ascocarp. The ascogenous hyphae form a fascicle of asci in the central perithecial cavity by the production of croziers.

Cookson (1928) published a well illustrated account of development of the perithecium in Melanospora zamiae, Corda. She believed the central core of cells to be hyphal in origin, rather than parenchymatic. The core was thought to become differentiated into fertile and sterile elements, yet she was uncertain whether the two elements had a common or an independent origin. Although unable to trace the origin of the binucleate ascogenous cells, Cookson presumed that they developed from hyphal outgrowths of an enlarged cell of the archicarp.

Hutting (1935) dealt with the initation and development of the perithecium of Glomerella. He found two branches arising from one cell of the haploid mycelium of Glomerella which he desiginated as male and female sex organs. The ascogonium branch, consisting of uninucleate cells rich in cytoplasmic contents, wound spirally, with its terminal cell somewhat swollen. In contrast the antheridal branch was thinner and not as rich in cytoplasm. From these two branches a sheath of hyphae arose which formed the peridium of the perithecium. When fertilization occured the nucleus of one of the cells of the antheridium passed over to the tip cell of the ascogonium. According to Hutting, the two-nucleate phase has its beginning here and is ended with the reduction division. Lucas (1946) working with sectioned material of the ipomoea Glomerella, reported fusion of two haploid nuclei in the young

ascus and three subsequent nuclear divisions resulting in the formation of eight haploid nuclei, each of which becomes included within an ascospore. Wheeler et al (1948) presented a detailed account of the cytological aspects of crozier and ascus development as determined from squash preparation stained with propionocarmine. They reported that the ascogenous system developed by crozier proliferation from a binucleate cell of a deeply staining coil of cells which was centrally disposed in the young perithecium. Ernest (1949) studied the initiation and early development of the perithecium in self-fertile, scattered-perithecial cultures of Glomerella. She found that the perithecia were produced on definite strands of the mycelium rather than at random; these strands usually were larger and richer in cytoplasm than the surrounding vegetative hyphae. McGahen and Wheeler (1951) studying the Genetics of Glomerella showed that the conidia and the young hyphal cells were uniformly uninucleate. The initials arise from adjacent cells either from within a coarse hyphal branch or at the end of a lateral branch of this hyphal strand. However, in all cases, the initials are uninucleate at origin, and the resultant coils contain uninucleate cells.

A number of cytological studies have been made with members of the genus Ceratostomella and in some respects the development reported appears to be similar to that found in Glomerella. Andrus and Harter (1933)reported on the morphology of reproduction in Ceratostomella fimbriata. These authors studied the homothallic strain in which they found the antheridal and ascogonial branch to be produced on the same hypha. The antheridium was functionless.

The subterminal cell of the ascogonial branch, which was generally binucleate, became the ascogonium which eventually give rise to asci. In the case of the heterothallic Ceratostomella multiannulata, Andrus (1936) observed a binucleate subterminal cell, termed the ascogonium, in the ascogonial coil. There was present, at times, an antheridium. These structures became enveloped with sterile hyphae. After elongation of the ascogonium, the nuclei repeatedly divided conjugately. The ascogonium then broke up into two to four nucleate cells which proliferated by typical crozier formation.

There have been numerous reports on the cytology of Neurospora as far as the ascogenous hyphae, crozier, and asci are concerned. Colson (1934) working with N. tetrasperma, reported that the archicarp was a small coiled multinucleate hyphae with densely granular cytoplasm. It consisted of more than one cell, but the cells were very long like those of the vegetative hyphae in the young stages. No antheridia were observed. The archicarp became enveloped in a sheath of sterile hyphae. The hyphae of the fertile region at the base of the perithecium branched and rebranched becoming so complicated that it was impossible to follow the course of any one hypha. The development continued, finally forming ascogenous hyphae which contained what were thought to be binucleate cells. This was followed by the formation of croziers and then asci. Dodge (1935) studied the mechanics of sexual reproduction in Neurospora tetrasperma, a heterothallic species. He described three distinct stages of development of the perithecium. Firstly there appears the perithecial fundament, which quickly becomes surrounded

by several layers of compact hyphal growth to form the incipient ascocarp. Secondly, nuclei of opposite sex reaction then come together in the ascogenous cells if these cells do not already contain both kinds as is normally the case in N. tetrasperma. This stimulates further growth of the fruiting body, the differentiation of wall tissue, the formation of the ostiolar papilla, and finally the development of the ascogenous hyphae. Thirdly, fertilization is consummated with nuclear fusion in the ascus, which is followed by the reduction divisions and the delimitation of ascospores. Nelson and Backus (1968) also described the ascocarp development in two homothallic Neurosporas. The incipient perithecial development of N. terricola and N. dodgei are initiated by the formation of a coiled archicarp, the terminal portion of which is soon differentiated into an ascogonium consisting of several cells. No trichogynes were identified.

Many studies have been done with the genus Gelasinospora. Ellis (1960) studied the plasmogamy and ascocarp development in G. calospora. According to Ellis, G. calospora forms no extensive ascogenous hyphae, and the croziers are formed from a compact tissue at the base of the immature perithecium. A protoperithecial coil arises usually as a short side branch from an intercalary cell of a hypha densely filled with cytoplasm, and plasmogamy occurs shortly before or, more frequently, shortly after the formation of the first branch. Lu (1967) studied the meiosis and centriole behaviour during the ascus development of G. calospora. The development of the asci is basically the same as that of Neurospora and that of Sordaria fimicola.

During meiotic prophase, the ascus nucleus and nucleolus increased in size. The haploid number of chromosomes in this species was demonstrated to be seven. The centrioles are found to increase in size from division 1 to division 111, within the ascus, but decrease in size again by division 1V.

A complete description of the development of the perithecium of Sordaria fimicola was presented by Ritchie (1937). At the time of the formation of the perithecial fundaments, certain of the ends of otherwise undifferentiated hyphae become coiled. This coil or archicarp, twists about itself for several turns, one or more hyphae may contribute to the formation of the archicarp, which soon becomes invested with smaller, numerous vegetative hyphae. Usually there is no structure that can be interpreted as a trichogyne, though there appear occasionally unaccountable outgrowths from the archicarp. As development proceeds the paired nuclei fuse in the primary ascus cell and the ascus grows upwards without forming the typical crozier, although occasionally regular croziers are formed. Mirza and Khatoon (1937) reported that in S. humana, ascogonia arise as coiled mulitnucleate side branches and have neither trichogynes nor antheridia, though anastomoses were noted between somatic hyphae. The peridium and sterile centrum elements arise from hyphae developing from the subtending hyphae of the ascogonium; and that inside the two-layered peridium a perilocular layer gives rise to the schizogenously developed neck and ostiole. The ascogenous hyphae develop from the ascogonium and the asci arise in the usual way. Uecker (1976) also described the development and cytology of S. humana

and the developmental stages of the perithecium and ascus are basically the same as described by Mirza and Khatoon. However, Uecher gave more detail description of the developmental stages of the coiled, septate, multicleate ascogonium and the ascogenous hyphae, and also stated that the haploid chromosome number was seven.

Another minute member of the Pyrenomycetes - Ophiobolus graminis was studied by Jones (1926) who described the development of the perithecium. O. graminis causes the 'Take-all' or 'White-head' disease of cereals. The early stages in the formation of the perithecia are found at about the same time as the appearance of the spermogonia, but these stages are strikingly different, so that there is no confusion between them. The ascogonial coils can be seen in the epidermal cells and mesophyll of the leaf-sheath of cereals. The coils are deeper stained than the vegetative mycelium and are divided into a number of uninucleate cells. Further stages show that any number of these cells may branch towards the interior of the coil, each to give rise to another cell but further stages in the development of the perithecium of Ophiobolus will show that the archicarps are abortive structures.

Jenkins (1934) studying the development of Cordyceps agariciformia, described the ascogonia to be short, usually slightly coiled three to five septate hyphae, of uninucleate or binucleate cells. As the ascogonial cells increase in size they likewise tend to become more coiled and their cells become multinucleate. The ascogonia originate as branches from various hyphae of the

interzonal region. Some ascogonia originate from hyphae of the peripheral layer and a few, from hyphae within the periphery of the core. The ascogonia are distinguishable from the vegetative hyphae in their stage only by the fact that they are shorter, somewhat thicker, their nuclei stain more densely and they retain the stain more tenaciously than do the vegetative hyphae. In some instances, these structures arise in the larger interhyphal spaces of the interzonal region, and usually occur in groups of three to four. Asci grow out from ascogenous hyphae which have in turn arisen from the plectenchymatous floor of the perithecial cavity. In some cases asci arise from typical croziers.

The taxonomic position of Acrospermum is clearly in doubt. This organism was placed in the order Hysteriales by Rehm (1887), in the order Hypocerales by Ellis and Everhart (1892), in the order Sphaeriales by Von Hohnel (1917) and in the order Coryneliales by Arnand (1930), (see Brandriff, 1936). Basically there is no archicarp observed according to Brandriff, and the earliest stage is merely a protruding lobe of homogenous, pseudoparenchymatous, stromatic tissue. As the ascocarp undergoes further development and pushes upward to assume the typical columnar form, the hyphae composing its interior become much elongated in the vertical direction and are narrow and only slightly branching. These hyphae soon undergo disintegration, and the ascigerous cavity of locule begins to make its appearance. As the stromatal hyphae disappear the interior of the young ascocarp becomes filled with ascogenous hyphae and later with paraphyses and asci. Brandriff concluded that Acrospermum is in the order Hypocereales.

Wehmeyer (1955) described the ascocarp development in Pseudoplea gaeumannii. He observed that the ascocarp primordia begin to appear about thirty-six hours after inoculation. At this time many hyphae increase in diameter, become septate into shorter cells with a smaller number of nuclei, show a thickening and pigmentation of their walls and tend to become swollen just below the septa. Such hyphae may form short side branches which coil irregularly about the main branch. The cell or cells of such a clump of hyphae give rise to the ascocarp stroma, and certain favoured cells attain some physiologic advantage by undergoing a "progressive" and "retrogressive" development. The cells undergoing progressive development finally become the ascogenous tissue whereas the cells undergoing retrogressive development ultimately become dead structural elements. The ascogonial cells are larger, irregularly shaped, multinucleate and usually granular, but the asci arise by fusion of two nuclei of binucleate cells.

Kowalski (1965) studied the development of the ascocarp of Didymocrea sadasavanii in which he mentioned that the perithecial primordia begin to appear three or four days after inoculation. The earliest stage consist of two enlarged intercalary cells. These cells then divide to form a chain of uninucleate, unbranched cells. The primordial cells are slightly wider but approximately one-third the length of the vegetative ones. The primordial chain attains a length of approximately ten to fifteen cells and then it branches in random fashion. After a small amount of branching occurs, narrow, elongated hyphae grew out from the primordium. These strongly resemble trichogynes but it is difficult to

attribute a fertilization-related function to the trichogyne-like hyphae since this species is homothallic and lack spermatia. The development of the ascus is initiated by crozier formation, and pseudoparaphyses grow to the bottom of the locule, formed by the dissolution of the centrum and are attached to the bottom of the perithecium. Didymocrea is described to have an ascohymenial type of development.

The organism Melanospora tiffanii was also studied by Kowalski (1965). The ascocarp primordium originates as a single-celled, multinucleate gametic hypha which grows out as a side branch from the vegetative hypha. It is wider and more darkly stained. The gametic cell becomes transversely septate and slightly elongated, forming the archicarp. This structure typically contains eight to twelve cells with the apical and basal cells elongated. Cells from the apical and basal regions of the archicarp sent out hyphae which grow around the intercalary cells of the archicarp. One or two intercalary cells enlarge greatly in size and become the multinucleate ascogonium which later gives rise to the ascogenous hyphae. The cells of the archicarp just basipetal to the ascogonium from the centeum which is pseudoparenchymatous, whereas those furthest from both ends of the ascogonium give rise to the perithecial wall. In time the primordium becomes spherical in shape. The ascus in this organism develops without crozier formation. The nuclei of the binucleate ascogenous cells fuse and the cells then elongate to form asci.

Rai and Wadhwani (1970) reported on the development of the ascocarp and ascus cytology in Anixiella indica. The ascocarp

arises by the coiling of a hyphal branch. The septate ascogonial coil soon becomes invested with vegetative hyphae forming a spherical ball of pseudoparenchymatous tissue. As the development proceeds, the pseudoparenchymatous tissue becomes distinguishable into two layers - the peridium and the central sporogenous tissue. The asci develop from croziers, which were produced by binucleate ascogenous cells, that develop from the basal compressed sporogenous tissue. The haploid number of chromosomes is four.

The development of the ascocarp of Lophodermella morbida is typically the same as many other Ascomycetes. L. morbida causes severe disease of new leaves of Pinus ponderosa. The uniquness of this organism according to Uecker and Staley (1973), causes the leaf of P. ponderosa to brown in June. Acervuli (probably the imperfect stage of L. morbida) appear in September, paraphyses and ascogenous hyphal initial cells develop in November from the basal pseudoparenchyma, croziers form in May, and spores are released from June to August. The ascostroma is subhypodermal and the haploid chromosome number is eight or nine.

Although many cytological studies with various Ascomycetes have been made, very few of these include a detailed description of the initiation and early development of the ascocarp. This seems to be especially true of the Pyrenomycetes. Even in the cases in which the early stages are described there is much disagreement in reports by different investigators and many different types of development have been described. Some reports are given for the early development of the perithecia and in the cases in which more than one investigator has reported on one fungus, their

result do not always agree. In the lower forms the fruiting structure consists of hyphal tips that give rise apogamously to multi-spored asci. The opposite extreme consists of the production of well-differentiated antheridia and oogonia. In some cases only one of the sexual structures is well differentiated while in others, in which the two are present, one may be functionless.

MATERIALS AND METHODS

The fungus used in this study is in the class Ascomycetes within the series Pyrenomycetes. The Pyrenomycetous organism was isolated with Gliocladium roseum from soil samples of a maple-elm-ash forest near the town of Avon, Wisconsin by Dr. Robert O.Novak. G. roseum is another fungus in the class Deuteromycetes. These organisms were maintained on corn meal agar for experimental use throughout the course of this study.

SLIDE CULTURES

Temporary mount preparations were made using the standard microslide technique. A slide on a bent-glass rod in the bottom of a petri dish, a cover slip and a piece of brown paper or filter paper, were covered and sterilized. A corn meal agar plate about 10 ml. of agar was prepared, allowed to solidify and dry. An agar block was cut about 1 cm. square or punctured with a sterilized glass test tube. The block of agar was then carefully transfered with a flamed loop or forceps to the center of the slide in the petri dish. The fungi were inoculated at four places around the agar block and a sterilized cover slip, 22 mm. square was placed on the inoculated block. The plate was then incubated at room temperature (approximately 20-22^{o}C) for two to three weeks. The slide preparation was checked periodically under the low power of a microscope for sporulation. Aseptic technique was used and all sterilization was done by either autoclaving or heat flaming.

CULTURES GROWN ACROSS STANDARD MICRO SLIDES

Standard micro slides were sterilized by autoclaving, and with the use of a forceps, the slides were dipped into melted corn meal agar. The agar coated slides were then placed in petri dishes containing corn meal agar. The fungi were inoculated with an inoculating needle at the center of the slides and also at one edge of the slides to ensure growth. The plates were incubated at room temperature (approximately 20-22°C) for two to three weeks. The cultures were used mostly for permanent mounts.

CELLOPHANE MOUNTS

Strips of cellophane were cut approximately 1 cm square and were sterilized by autoclaving. The sterilized strips were aseptically placed with a forceps into petri dishes containing corn meal agar. The fungi were inoculated with an inoculating needle on the center of the cellophane strips. They were then incubated at room temperature (approximately 20-22°C) for one to two weeks. When the resulting cultures reached the desired stages, they were removed, fixed and stained as described below.

Basically, the three culture techniques used were serving a similar purpose, that is to provide a microscopic preparation showing various developmental stages. To maintain the two fungi in culture, they were grown in corn meal agar. The corn meal agar was used because it supresses vegetative growth while stimulating sporulation of the fungi. The two fungi were also grown in petri dishes on a variety of common laboratory media which included potato dextrose agar, Sabouraud agar, malt agar, malt yeast extract

agar, lactose agar, corn meal agar and a modified dilute corn meal agar (Difco Laboratories).

METHODS FOR FIXING AND STAINING

Paraffin-imbedded material for sectioning was prepared, using various modifications of the technique described by Sass (1968). The organisms were cultured on corn meal agar plates, from which, at intervals of 3-16 days after inoculation, small blocks of agar were removed and fixed in Craft's 111 killing solution and formalin-aceto-alcohol (FAA) solution as described by Johansen, 1940. The specimens were dehydrated with a series of teritary butyl alcohols (TBA) and embedded in paraffin. Embedded material was sectioned on a rotory microtome at approximately 6-8 um and stained with the hematoxylin iron alum stain (Sass, 1968). A saturated solution of violet green in clove oil was used for destaining the slides in an attempt to bring out the nuclei of the initial stages of the perithecium. The stained slides were sealed with Pro-Texx mounting medium.

Permanent mounts were also made by using the HCL-Giemsa stain method as described by Ward and Ciurysek (1962). Cultures grown across standard micro slides and cellophane strips were used. The slides and cellophane strips were hydrolized at 63°C with 1N HCL and stained with the Giemsa stain. The stained cellophane strips were placed on standard micro slides, flooded with Pro-Texx mounting medium and covered with cover slips. The stained slides with cultures grown across them were also flooded with Pro-Texx mounting medium and covered with cover slips.

TEMPORARY MOUNTS

Temporary mounts using the New Fixation method by Lu (1962) were used. The fixative used in this technique was composed of 9 parts of n-butyl alcohol, 6 parts of glacial acetic acid and 2-3 parts of 10% aqueous chromic acid. This method reveals, within the ascus, the nuclei, stains cytoplasm only slightly and reveals all stages of dividing nuclei. The staining process was done by using the propionocarmin stain, as described by Lu.

Temporary mounts were also made with stain-fixative such as 60% aceto-carmin and aceto-orecin (Sass, 1968). These stain-fixative methods were also used to trace the development of the perithecial initials and nuclear conditions.

Young perithecia were placed on a slide with a drop of either 60% aceto-carmin or 60% aceto-orecin, and were pressed with a needle so that the asci were extruded through the ostiole or broken perithecial wall. Fragments of the perithecial walls were removed, a cover slip was applied and the slide was gently heated on a low flame and left for 24 hours, then observed under the microscope.

Finally, temporary mounts, especially of young cultures, 3-7 days old, were frequently made and stained with lactophenol cotton blue (LCB). The LCB acts as a killing and mounting media, while at the same time, it makes the hyaline fungal structures more distinct and it is a fast method used to study the gross morphology of the organisms.

The photomicrographs were made with a Leitz microscope and camera using Kodak 35 mm Panatomic-X film.

RESULTS

Gliocladium roseum showed similar growth characteristic on potato dextrose agar, malt agar, malt yeast extract agar and Sabouraud agar (Fig. 1). The colonies on these agar slants are loose floccose, with an abundance of hyphae and conidiophores with conidia. The surface is white to pink in color. However, there is a minimal amount of growth on lactose agar and corn meal agar, and the surface is mostly white in color. Hyphal growth could be seen within 24 hours after inoculation and maximum fruiting bodies appeared within 3-4 days. The conidiophores are usually in relatively dense clusters, enclosed in slime, but occasionally (particularly in young growth) the conidiophores are isolated in whorls - The Verticillium Type (Fig. 2). The conidia are colorless, elliptical, smooth, and are approximately 5-7 X 3-5 um.

When both fungi - Gliocladium roseum and Attenuospora silvesterra gen. nov. sp. nov. are cultured together, they also showed similar growth characteristic on the same type of agar slants - potato dextrose agar, malt agar, malt yeast extract agar and Sabouraud agar (Fig. 3), as in the case when G. roseum is growing alone. The only difference is that the sexual fruiting bodies - dark perithecia can be seen on the lactose agar and more so on the corn meal agar. Attenuospora silvesterra gen. nov. sp. nov. also produces conidiophores bearing conidia after five days of growth, and young immature perithecia appear on the tenth or eleventh day with mature ascospores by the fifteenth day.

The immature perithecia are light brown in color and become black when mature. The mature perithecia are globose to flask-shaped, 57-100um in diameter, with a blunt or conical ostiole (Figs. 4B, 5, 17). The asci are fusiform, slightly curved with a long stalk, 3.5-5 X 15-25 um (Figs. 7, 8, 18). Each ascus contains eight hyaline, spindle-shaped ascospores with spine-like projection at each end (Figs. 9, 19). The overall length of the ascospores is about 9-17 um, mostly 10-12um, but the main body of the spore is 4.5-8 X 1.5-2 um. Conidia are produced on upright phialides, 60 um in length, 3-5 um in diameter at the base and then tapering upwards. The phialides are sub-hyaline to brownish, smooth and simple with a single septum located near the base of each phialid (Fig. 10). The conidia are produced endogenously and are smooth, hyaline and cylindric, 1.5-2 X 4-6 um.

Attenuospora Totaram & Nelson, gen. nov.

Perithecia, 57-100 um in diameter, scattered, nonstromatic, ostiole blunt or conical, globose to subglobose, black; peridium pseudoparenchymatous in longitudinal section, 3-4 layered in section. Asci, 3.5-5 X 15-25 um, unitunicate, nonamyloid, fusiform, straight or curved. No paraphyses and no periphyses. Ascospores, 9-17 um in length from tip to tip, mostly 10-12 um, one celled, hyaline, smooth, spindle-shaped with spine-like tips.

Conidial stage: Phialides, 60 um in length and 3-5 um in diameter at the base, subhyaline to brownish, smooth, simple with a single septum located near the base of the phialides which is wider and tapering upward. Conidia, 1.5-2 X 4-6 um, produced

endogenously, hyaline, smooth, cylindrical.

Attenuospora silvesterra Totaram & Nelson, sp. nov.

Habitat: Soil of maple-elm-ash forest within the vicinity of Avon, Wisconsin.

DEVELOPMENT OF THE ASCOCARP

The perithecia are produced at random on the surface of the medium (Fig. 11) and scattered around the point of inoculation. The perithecial initials appear after five to six days of incubation at room temperature.

The ascocarp primordium originates as a single-celled ascogonium which grows out as a side branch from a vegetative hypha. The primordial hyphae are wider than the vegetative cells and stain more darkly with cotton blue. The single-celled primordium becomes transversely septate and elongates forming the archicarp (Figs. 12, 20). The young archicarp typically contains 5-7 cells, reaching approximately 25 um in length (Figs. 13, 21). The archicarp tapers upward from a large basal cell and becomes extended at the tip to appear as a trichogyne-like structure (Figs. 14, 22). This trichogyne-like structure can also be seen during the protoperithecial stage of development (Figs. 16, 23). However, no trichogyne-spermatium interaction was actually observed.

The perithecial wall is initiated by a branch from the parent hypahe (Figs. 13, 21) or from the lower part of the archicarp (Fig. 14) which wraps around the archicarp forming a spherical ball of pseudoparenchymatous cells (Fig. 15). These pseudoparenchymatous

cells continued developing into a protoperithecium about 20-30 um in diameter (Figs. 16, 23). Through growth and proliferation of the pseudoparenchymatous cells a prosenchymatous periderm many cell layers thick soon develops. The outer periderm layer consisted of globular or spherical thick walled cells, 3-4 cells thick. While the periderm layer develops, the ascogonium remains centrally located (Fig. 16).

As the perithecium increased in size, the nucleated ascogenous cells formed a layer near the base of the centrum. The centrum of the protoperithecium (Figs. 4A, 6) or also the mature perithecium (Figs. 4B, 5, 17) is completely filled with loosely arranged structures resembling cells. No paraphyses have been observed and the young asci arise from the base of the centrum, (Figs. 4A, 6) then become elongated, forcing themselves upwards and tearing away some of the supra-pseudoparenchymatous tissue. The ostiolar canal was formed at the apex of the perithecium while the latter was still immature. As the perithecium matures the ostiole becomes blunt or conical in shape but no distinct periphyses have actually been observed (Figs. 4B, 5, 17). The wall structure of a mature perithecium was 2-4 cell layers thick. Some of the cells were small and spherical while others were large and globose, elongated or polygonal in shape.

Fig. 1 Growth of *Gliocladium roseum* on potato dextrose agar, malt agar, malt yeast extract agar, Sabouraud agar, lactose agar and corn meal agar (from left to right).

Fig. 2 *Gliocladium roseum* showing the *Verticillium* Type of conidiophores with conidia (from LCB preparation).

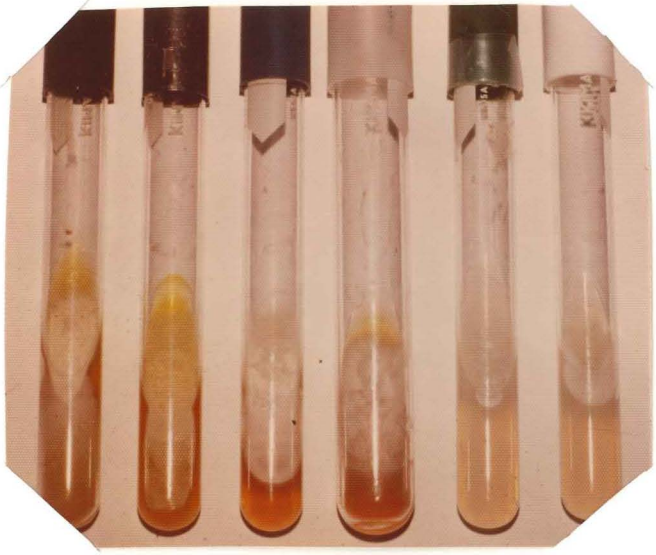

Fig. 1

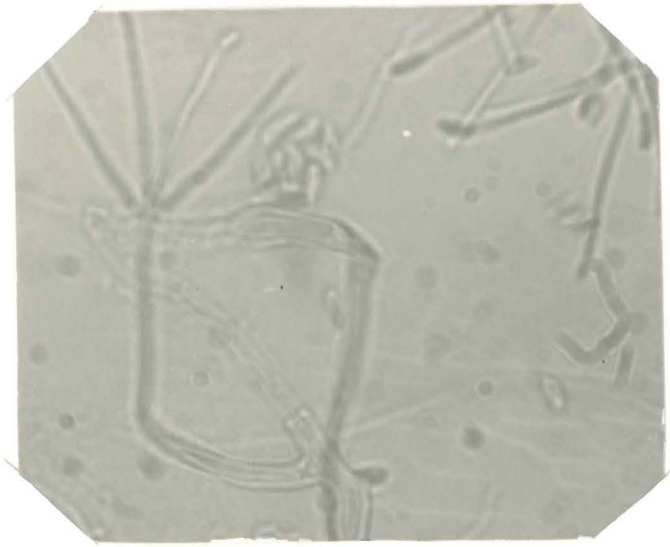

Fig. 2

Fig. 3 Growth of Gliocladium roseum and Attenuospora silvesterra gen. nov. sp. nov. on potato dextrose agar, malt agar, malt yeast extract agar, Sabouraud agar, lactose agar and corn meal agar (from left to right).

Fig. 4 Longitudinal section of two perithecia.

4A. Young perithecium showing asci arising from the base of the centrum.

4B. Matured perithecium showing the centrum, ostiole and perithecial wall (from hematoxylin iron alum stain). 600 X.

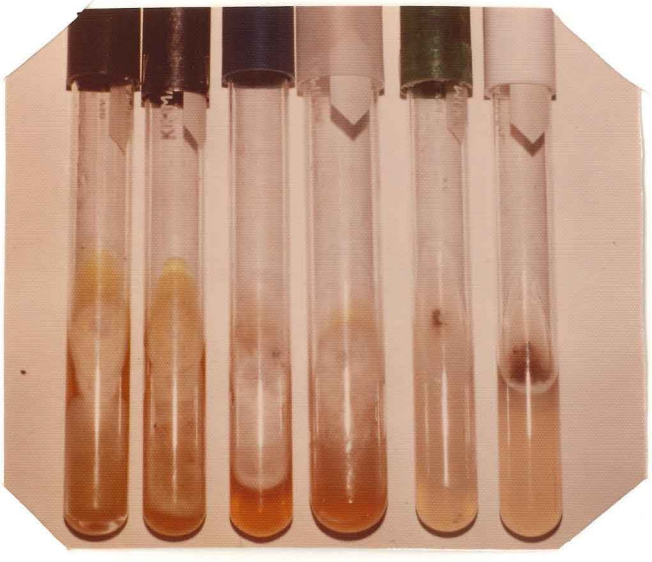

Fig. 3

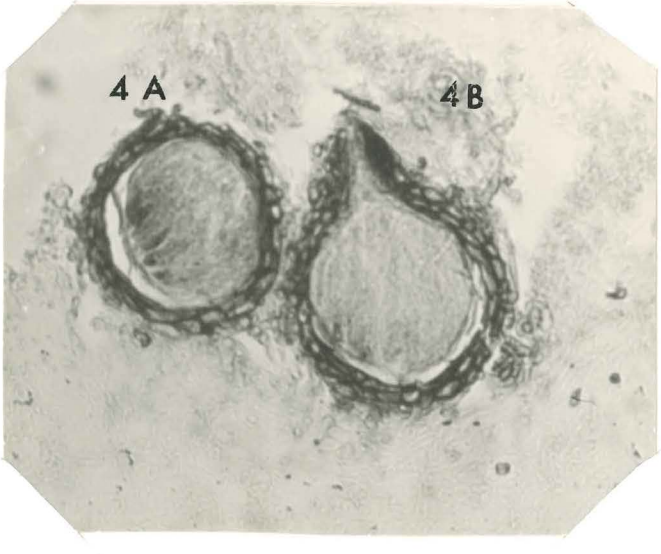

Fig. 4

Fig. 5 Longitudinal section of a perithecium showing the centrum, ostiole and perithecial wall (from hematoxylin iron alum stain). 1250 X.

Fig. 6 Longitudinal section of a young perithecium showing asci arising from the base of the centrum (from hematoxylin iron alum stain). 1250 X.

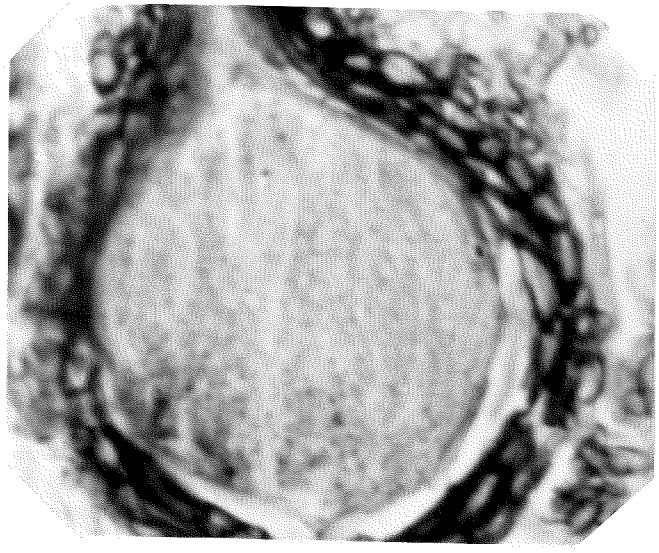

Fig. 5

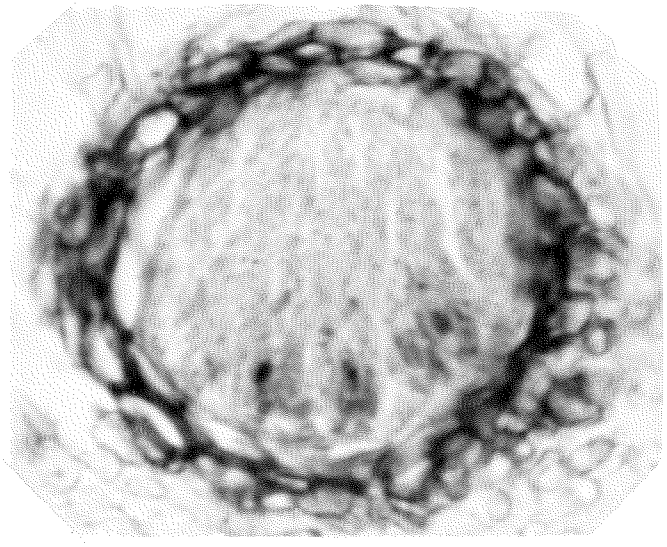

Fig. 6

Figs. 7, 8 Mature asci (Iodine preparation). 1750 X.

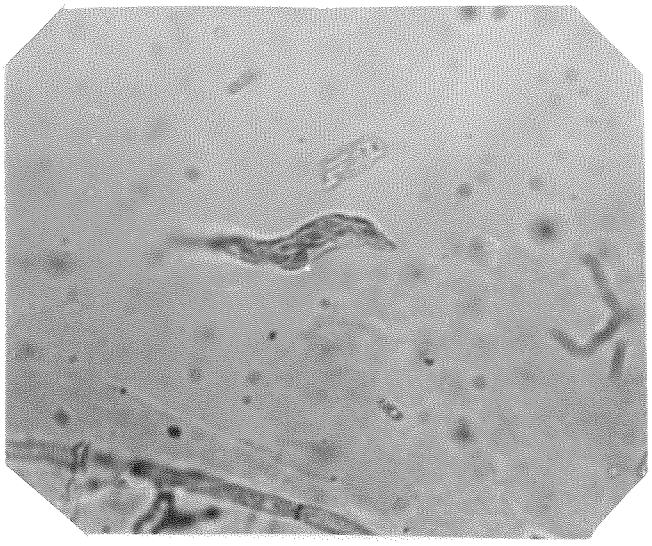

Fig. 7

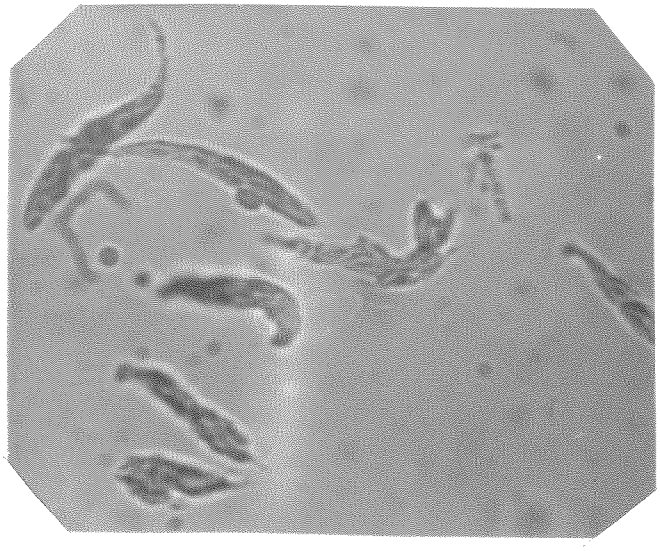

Fig. 8

Fig. 9 Mature ascospores (acetocarmin preparation). 1400 X.

Fig. 10 Conidiophore with conidia of *Attenuospora silvesterra* gen. nov. sp. nov. (from HCL-Giemsa preparation).

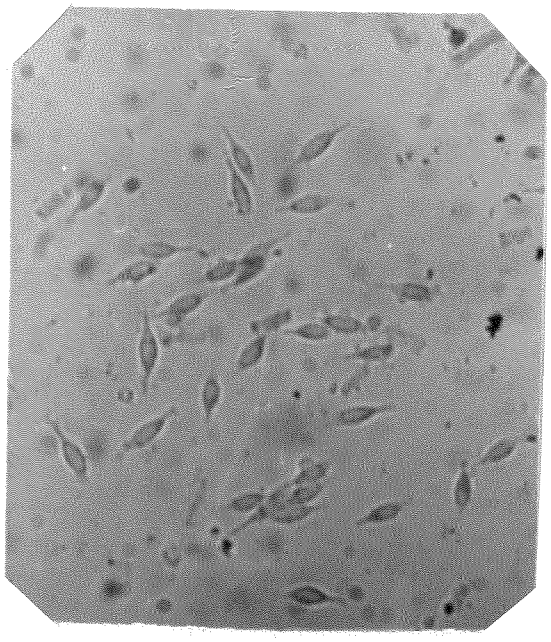

Fig. 9

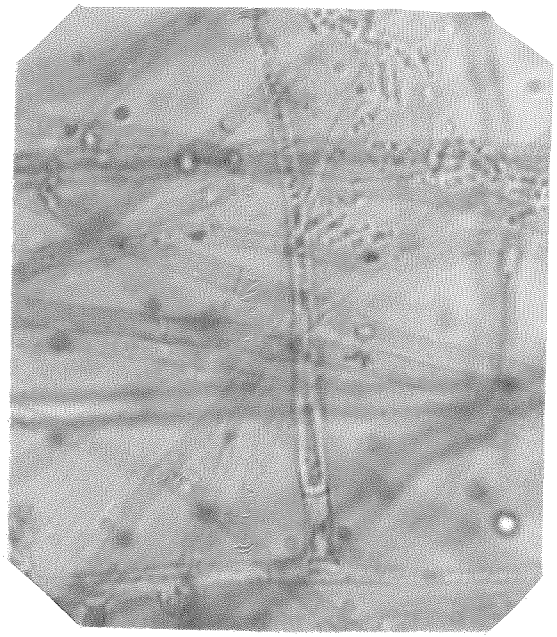

Fig. 10

Fig. 11 A mature perithecium (from slide culture preparation stained with LCB). 400 X.

Fig. 12 Two celled archicarp (from LCB preparation). 1200 X.

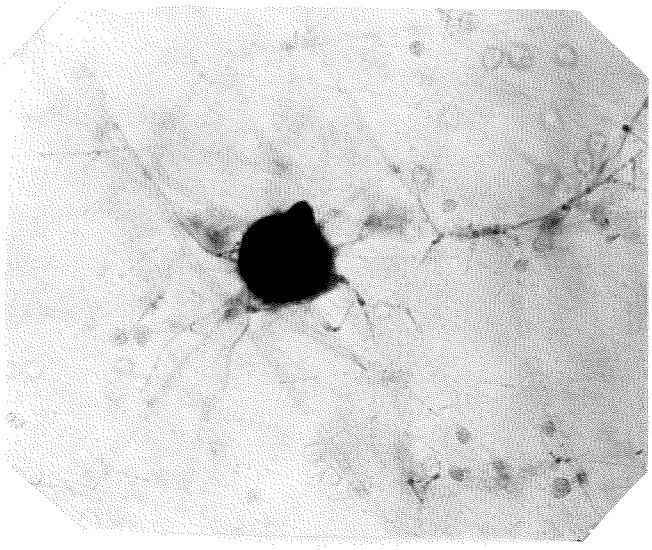

Fig. 11

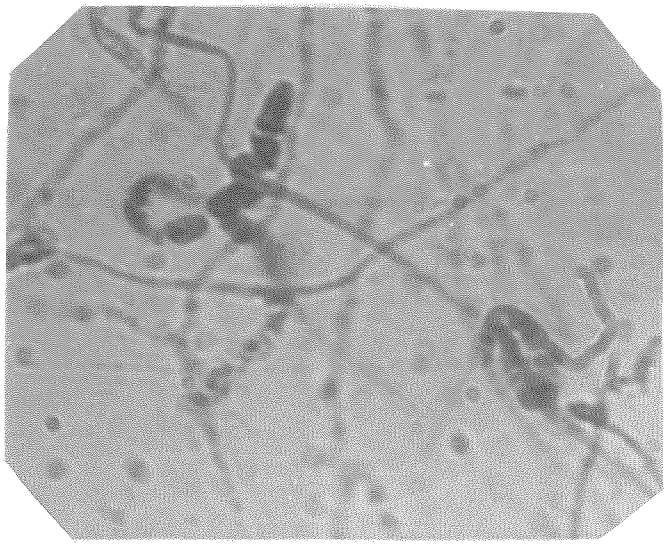

Fig. 12

Fig. 13 Five celled archicarp showing a branch from parent hypha (from LCB preparation). 1520 X.

Fig. 14 An early stage of perithecial development, showing a trichogyne-like structure and early stages in wall formation (from LCB preparation). 2500 X.

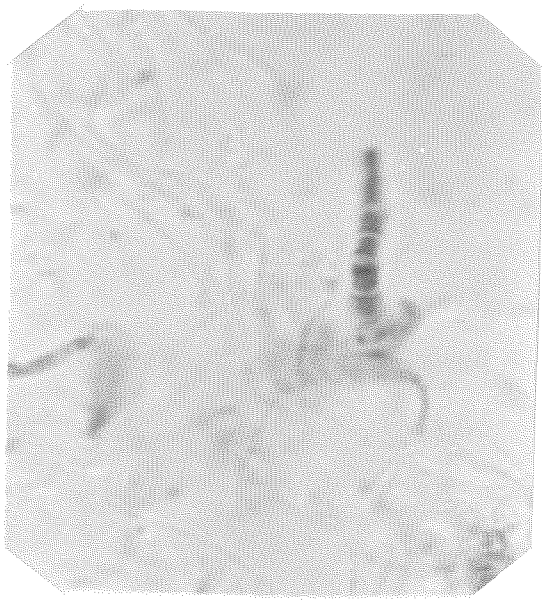

Fig. 13

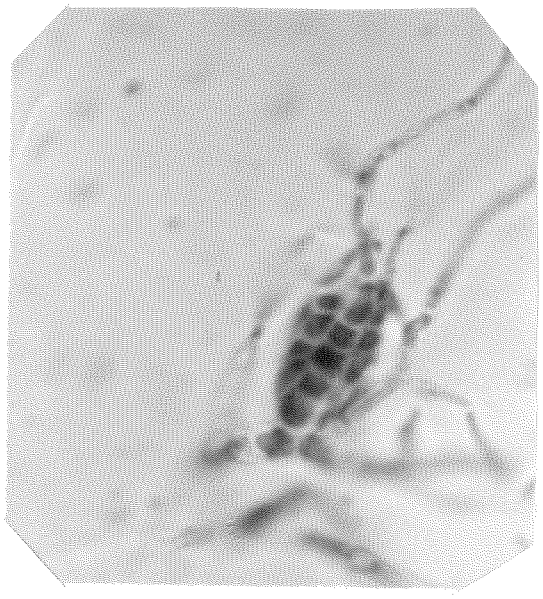

Fig. 14

Figs. 15, 16 Early stages of perithecial development (from LCB preparation). Fig. 15 shows a few layers of cells around the archicarp. Fig. 16 shows cells around the archicarp. 1500 X.

Figs. 15, 16 Early stages of perithecial development (from LCB preparation). Fig. 15 shows a few layers of cells around the archicarp. Fig. 16 shows cells around the archicarp. 1500 X.

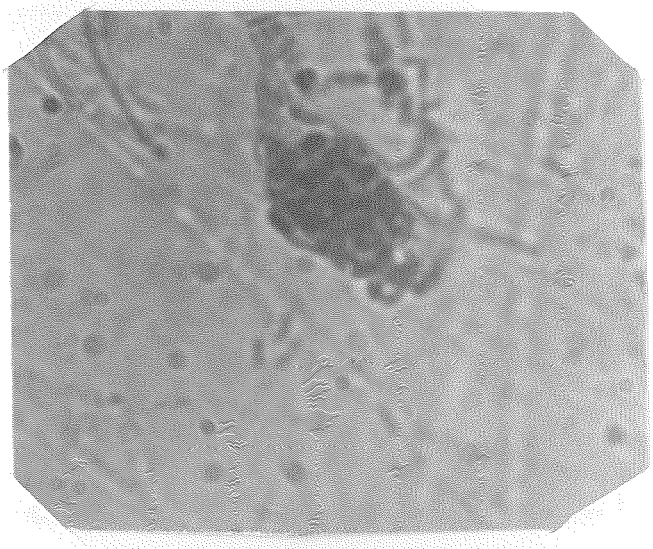

Fig. 15

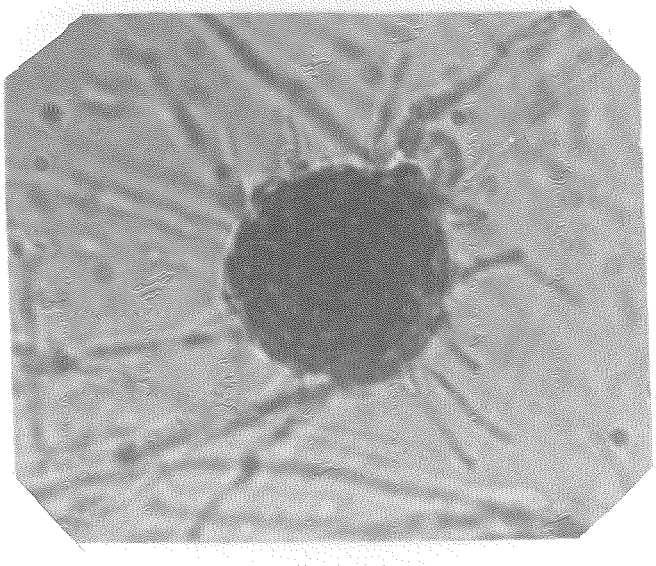

Fig. 16

Figs. 17-19 Fig. 17 Diagram of centrum. Fig. 18 Diagram of asci. Fig. 19 Diagram of ascospores.

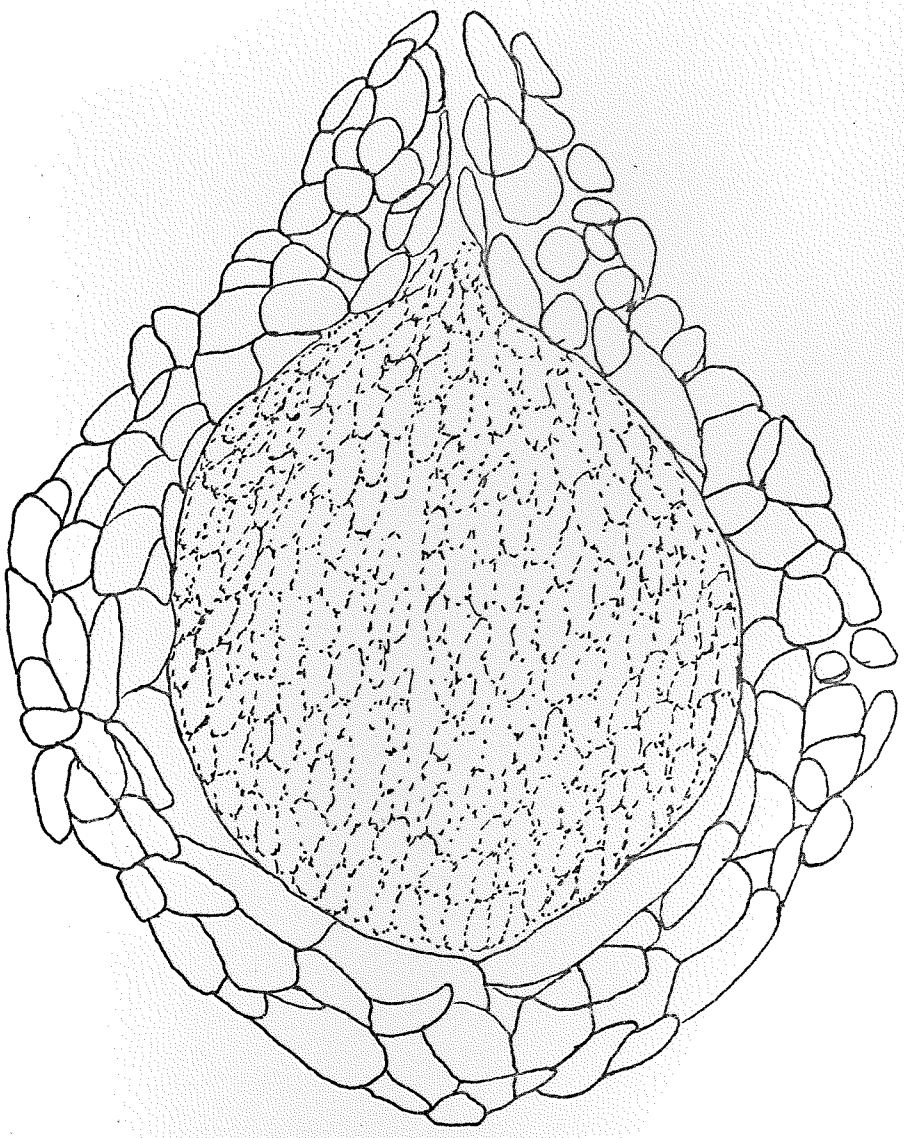

Fig. 17

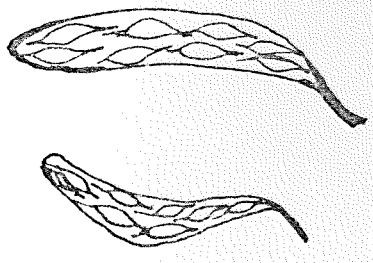

Fig. 18

Fig. 19

Figs. 20-23 The early stages of perithecial development. Fig. 20 Two celled archicarp. Fig. 21 Five celled archicarp, showing a branch from the parent hypha. Fig. 22 Shows a trichogyne-like structure. Fig. 23 Shows a spherical ball of pseudoparenchymatous cells around the archicarp.

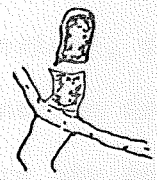

Fig. 20

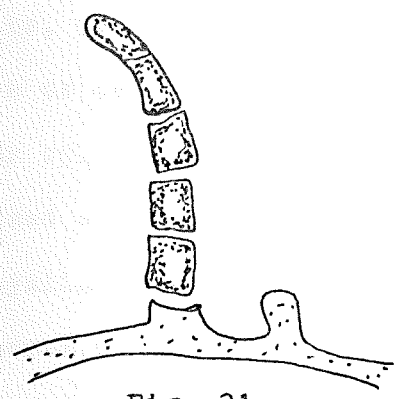

Fig. 21

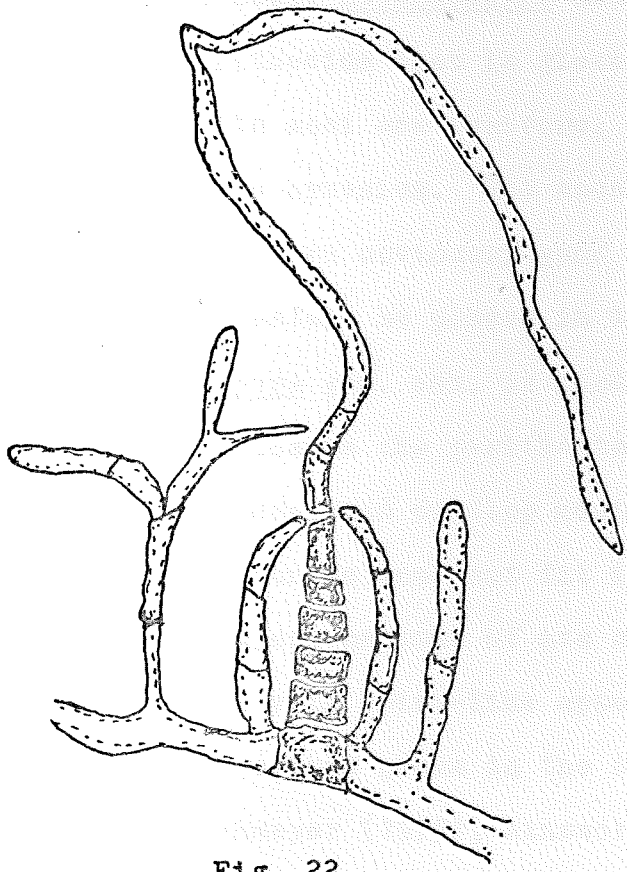

Fig. 22

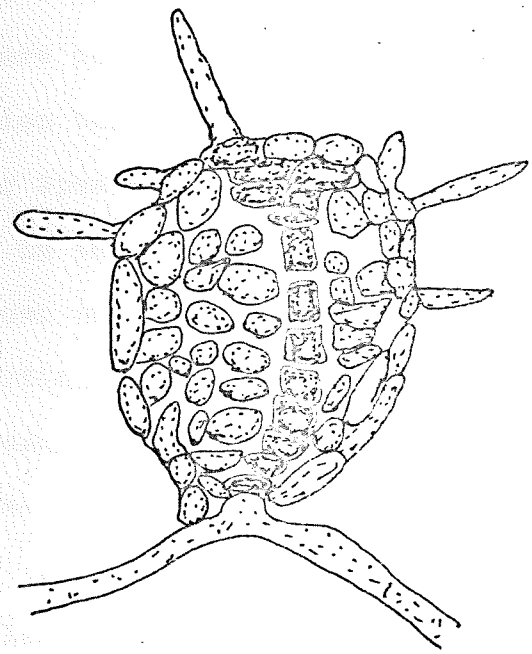

Fig. 23

DISCUSSION

The Sphaeriales are defined in a very broad sense. In this order, the ascomata are spherical, hemispherical or flask-shaped. They are mostly ostiolate, which is papillate, or elongated cylindrical and are provided with periphyses or ostiolar hairs. The asci are spherical, clavate, fusiform or cylindrical and arranged in a hymenium or irregularly disposed at different levels. The ascospores are one celled or septate, hyaline or colored, paraphyses are mostly present, often evanescent in an early stage, or they may be absent, Ainsworth et al (1973).

In this study Attenuospora gen. nov. has been shown to have a perithecium that is globose to flask-shaped, dark and ostiolate. Its asci are fusiform, curved or straight and are arranged in the hymenium. The ascospores are one celled and are hyaline. These are obviously good indication that Attenuospora gen. nov. can safely be placed in the order Sphaeriales.

Attenuospora gen. nov. is also placed in the family Sphaeriaceae because the perithecia are brown when immature and black when mature, and develop mostly superficially. However, the described fungus does not fit in any described genus in the order Sphaeriales, because of its ascospores which are one celled spindle shaped with spine-like appendages. There is not any previously described genus in the Sphaeriales with this type of ascospore, Wehmeyer (1975), Ainsworth et al (1973), Luttrell (1951), Miller (1949), and Hansford (1946).

The fungus is now placed in a new genus Attenuospora gen. nov.

It is closely related to the genus Hyaloderma within the family Sphaeriaceae according to Ainsworth et al (1973), but Hyaloderma is a parasite of a fungus that is a leaf parasite. The asci are obovate, aparaphysate, the spores are acircular and multiseptate, Hansford (1946).

The species - A. silvesterra sp. nov. which is also given to this fungus is chiefly derived from the habitat where the fungus was isolated. Attenuospora silvesterra gen. nov. sp. nov. was isolated with another fungus - Gliocladium roseum from soil samples of maple-elm-ash forest near the town of Avon Wisconsin.

Gliocladium roseum is a soil saprophyte, it can show antagonism only on direct contact with some fungi, and is found frequently in nature growing on other fungi, Barnett and Lilly (1962). Gliocladium roseum is also a destructive mycoparasite where it parasitizes Trichothecium roseum, Rhinotrichum macrosporum, Helminthosporium sativum and the conidia of Ceratocystis fimbriata, Barnett and Lilly (1962). However, it seems as if Attenuospora silvesterra gen. nov. sp. nov. is parasitizing Gliocladium roseum. Probably it is a 'balance' or a biotrophic type of parasitism. Biotrophic parasitism is where the parasite causes little or no apparent damage to its host, Barnett (1963) and Barnett (1964). The mode of parasitism of Attenuospora silvesterra is uncertain, but it may probably be unique as in the case of Gonatobotryum fuscum and Gonatobotrys simplex which have contact with their hosts by means of small specialized absorptive hyphae, Shigo (1960).

A definite pattern of growth has always formed on the corn meal agar when both fungi are inoculated. Gliocladium roseum begins to grow quickly at 25°C, producing an abundance of Verticillium type conidia within 3-4 days after inoculation and the Gliocladium type conidia are abundant within 4-6 days. By 5-7 days the cylindrical conidia of Attenuospora silvesterra gen. nov. sp. nov. appear while the perithecia appear approximately 7 days later.

Through observation, the development of the perithecium and the cytology of the ascus of Attenuospora silvesterra gen.nov. sp. nov. is quite unlike most of the other Pyrenomycetous fungi. No ascogonial coils have been encountered as in the case of the Neurosporas, Colson (1934), Backus (1939), Nelson and Backus (1968); Gelasinosporas, Cain (1950), Ellis (1960); Sordaria fimicola, Carr and Olive (1958). In these forms the young ascogonial coils generally arise as a result of coiling of a hyphal branch, containing an irregular number of nuclei. The septate coils soon become invested with vegetative hyphae forming a several-layered pseudoparenchymatous peridium.

However, the development of the perithecium of Attenuospora silvesterra gen. nov. sp. nov. is similar to that of Melanospora tiffanii, Kowalski (1965). The primordium originates as a single-celled archicarp which grows out as a side branch from a vegetative hypha. The single-celled archicarp becomes transversely septate and elongated. The cells from the basal regions of the archicarp send out hyphae growing around the archicarp forming a primary ascocarp wall which encloses a pseudoparenchymatous centrum. The

archicarp then gives rise to the ascogenous system, and the initial ascocarp wall becomes thickened to develop the mature perithecial wall. As the perithecium increases in size the asci develop without crozier formation from the ascogenous hyphae as in the case of Melanospora tiffanii.

Although the development of the perithecium of Attenuospora silvesterra gen. nov. sp. nov. is similar to that of Melanospora tiffanii, it is still placed in a new genus. This is because of the size and shape of its asci, the size and shape of its ascospores, the absence of periphyses and paraphyses, and its habitat.

SUMMARY AND CONCLUSION

1 The Pyrenomycetous fungus was isolated with Gliocladium roseum from soil samples of maple-elm-ash forest. It is currently designated as Attenuospora silvesterra gen.nov. sp. nov. because of its ascospores which are spindle-shaped with spine-like appendages at each end, and because of its ascus and centrum characteristic.

2 Morphological studies have shown that the perithecial initial of Attenuospora silvesterra gen. nov. sp. nov. originates as a single-celled archicarp which grows out as a side branch from a vegetative hypha. The asci develop without crozier formation from the ascogenous hyphae, and no paraphyses have been seen.

3 The perithecium is globose to flask-shaped, dark and osticlate. The asci are fusiform, curved or straight, and the ascospores are hyaline, one celled, spindle-shaped with a spine-like appendage at each end. The fungus also bears hyaline and cylindrical conidia on simple phialides.

4 These studies indicate that Attenuospora silvesterra gen. nov. sp. nov. is best classified in the order Sphaeriales and in the family Sphaeriaceae.

5 Attenuospora silvesterra gen. nov. sp. nov. is evidently parasitizing Gliocladium roseum, but the mode of parasitism is not clearly understood.

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