The objectives of this study were to examine and describe the mycorhizae associated with Botrychium dissectum and Botrychium virginianum. The objectives also included trying to isolate the fungi associated with them and to analyze the association.

Root sections were cleared and stained by placing them in a saturated solution of chloral hydrate and .01% acid fuchsin. These sections were either boiled for fifteen minutes or allowed to stand in the solution for at least two days. Hand sections were mounted in glycerine.

Examination of these sections showed fungi to be present in the younger roots of B. dissectum and in smaller amounts in all of the roots of B. virginianum. Infection occurred through the epidermis by aseptate, multinucleate hyphae which penetrated to the fourth cell layer of the cortex. Hyphenknäuel (hyphal coils) were found in this area. Within the fifth and sixth cortical layers of both species were Pilzverdauungszellen (fungal digesting cells) and sporangioles. Vesicles were observed only in B. virginianum. Following the initial infecting, no hyphal connections to the epidermis were present.

The infecting hyphae were irregular varying in diameter from 4 to 12 microns in B. dissectum and from 2.5 microns in B. virginianum. Swellings often occurred in hyphae where they passed through cell walls of the roots. In B. virginianum these
swellings were often dark colored. Septations were seen in hyphae on the outside of the roots of *P. virginianum*. None were observed in the hyphae within the roots. Because of the size differences and habit of growth of fungi within the two ferns, it is proposed that different fungi infect them.

Thin sections of roots were surface sterilized and placed upon Czapek’s and malt extract media. Fungal isolates from these were compared to fungi seen within the roots. Since no reproductive structures were observed in the hyphae within the roots and all comparisons had to be made with somatic hyphae, identification of the fungi was not possible.

Several methods of spore germination were tried. Since these were not successful and since no gametophytes were found, no studies of the reported endophytic fungi could be made.
A STUDY OF MYCORHIZAL ASSOCIATIONS
IN TWO SPECIES OF BOTRYCHIUM

A Master's Thesis
Presented to
the Faculty of the School of Graduate Studies
Indiana State University
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In Partial Fulfillment
of the Requirements for the
Master of Arts Degree

by
Rebecca A. Carr
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THESIS APPROVAL SHEET

The thesis of Rebecca A. Carr, contribution of the School of Graduate Studies, Indiana State University, Series I, Number 865, under the title, "A Study of Mycorhizal Associations in Two Species of Botrychium," is approved as counting toward the completion of the Master of Arts Degree in the amount of six semester hours of graduate credit.

APPROVAL OF THESIS COMMITTEE:

William J. Brett
(Signature of Committee Member)

Fred Rathwell
(Signature of Committee Member)

Joe F. Henner
(Signature of Committee Chairman)  [Date: 7-20-68]

APPROVAL FOR SCHOOL OF GRADUATE STUDIES:

[Signature]
(Dean of Graduate Studies)  [Date: 7-20-68]
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A mycorhiza is the association of a fungus with the roots or rhizomes of another plant. The relationship is a symbiotic one in which the two live together doing no harm to each other, and perhaps one or both benefiting from the association. Most of the associations seem to be obligate.

The first recognized mycorhizal association was in some of the conifers with the Homobasidiomycetes. Since then it has been found that many of the vascular plants have such an association with fungi. The mycorhiza within these plants may be one of the lower fungi instead of a higher fungus.

The objectives of this research were to examine and describe the fungi associated with two species of the fern family Ophioglossaceae, Botrychium dissectum Clute and Botrychium virginianum S.W. Other objectives included trying to isolate the fungus involved in the association, and if this were accomplished to synthesize the association. This is the actual proof that a particular isolate is the fungus involved in a mycorhizal association. It would be desirable to investigate the nutritional relationship of the association.
LITERATURE REVIEW

Much of the significant work pertaining to mycorhizae in ferns has been collected by Burgeff (1938) in the chapter Mycorhiza in the Manual of Pteridology. Kelly (1950) has also included fern mycorhizae in his book Mycotrophy. The following is a summary from these and other sources.

The study of fern mycorhizal associations still remains in its early stages since the fungi have not been successfully cultured separately, nor have the associations been synthesized. Most of the research done with ferns has been limited to the Ophioglossaceae. The fungi associated with the species of this family have been the phycomycetous type. Burgeff calls the fungi found in this family the thamniscophage mycorhiza. Kelly refers to the fungi as the vesicular-arbuscular type.

The thamniscophage mycorhiza is characterized as having multinucleate, aseptate mycelia which form vesicles and arbuscles. Vesicles are terminal swellings which store reserve materials. The arbuscles are formed from finely branched hyphae which penetrate the cells like haustoria. These are the source of the sporangioles described by Jansa (in Burgeff). Gallaud (in Burgeff) interpreted the sporangioles as the remains of digested hyphae which stain according to their organic contents.

Mycorrhizae have been observed and noted to some degree in all of the species of Botrychium and Ophioglossum examined. Grevillius (in Burgeff) described the Hyphenknäuel (hyphal coils) within the cells which are connected by intercellular, longitudinal strands of hyphae. He found these in all of the European species he studied. Burgeff described the method of infection of the sporophyte of Ophioglossum pendulum and the development of the
fungus. Only the thin roots are usually infected. This occurs through the epidermal cells of the root by the coenocytic hyphae. These penetrate to about the fourth layer of parenchyma cells of the root cortex. This layer of cells becomes the fungal host cells. The outer layers of root cells following the initial infection are free from the fungus. The fifth and sixth cortical layers contain fungus cells called Pilzverdauungszellen (fungal digesting cells). The Sternarbuskeln (star arbuscles) are formed also in these cortical layers as a result of outpocketing of the finely branched, thin walled haustoria like hyphae. The starch of the infected cells is slowly digested by the fungus. Cells which have been infected for a period of time will contain excretory bodies as well as the Sternarbuskeln.

The gametophyte of O. pendulum was described by Lang (in Kelley). Infection occurs through a rhizoid with the superficial cells containing only the infecting hyphae. The fungus is limited to the central areas. The basic difference seen by Burgeff between the fungus found in the sporophyte and the gametophyte was the lack of clear separation between the fungal host cells and the digesting cells in the latter.

In Ophioglossum vulgatum as observed by Bruchmann (in Kelley), the infection occurs through the epidermal wall. The hyphae spread throughout the mid-portion of the prothallus with the inner and outer layers free of the fungus. These cells free of fungus are filled with starch granules. The amount of starch present is in an inverse proportion to the fungus. The hyphae are filled with oil and protein. The advantage of the association for the prothallus and the fungus seems to be the holding and storing of reserve materials, especially the oil. It is of value during the summer heat and the winter cold in protecting the prothallus from drying.

Gewirtz and Fahn (1960) briefly described the endophytic
fungus found in the sporophyte of Ophioglossum lusitanicum L. from Israel. The fungus forms the usual ring-shaped zone in the middle of the root cortex. They were unable to culture any of the spores. Further observation based upon on gametophyte showed infection in the upper regions rather than the lower portion of the gametophyte.

Nishida (1956) described the gametophyte of Botrychium virginianum found in Japan and its endogenous fungus. Jeffery made the original morphological description in 1898 of this gametophyte which included observations about the fungus. He suggested that it was an intermediate between Pythium and Completoria.

Nishida was unable to culture the fungus separately so his description is based upon his observation as it is found in the gametophyte. The young hyphae are 1 to 2 microns in diameter becoming 3 to 4 microns in diameter as they grow older. They usually are aseptate, but septations may occur as an older hypha loses its cytoplasm separating it from the younger portion of the same hypha.

Infection takes place through the rhizoids which he did not find to be septate as reported by Jeffery. Infection may also occur through the cell walls of the epidermis of the gametophyte. The cells of the second or third layer become the fungus host cells. The hyphae then branch profusely and form Pilzknäuel (fungal coil) described by Burgeff for Lycopodium or Hyphenknäuel described by Grevillius (in Burgeff). When the fungus reaches the fifth or sixth layer, it forms Sternarbuskeln. These appear to be homologous to haustoria formed by members of the Peronosporales. The sporangioles described by Janse are formed as a result of the loss of individuality of hyphae of the arbuscles. These compose the "structureless, granular conglomerate" of the sporangiole. The process of change is called digestion of the fungus and occurs in the Pilzverdauungszellen. The vesicles are usually
terminal, but Nishida reports a few intercalary ones. He agrees with Gallaud that they are probably storage structures.

Nishida also reports the presence of structures suggesting oogamy. He found a small sac structure similar to a vesicle with a thin wall surrounded by a hypha that might be interpreted as an oogonium with an antheridium. On the basis of these structures, he agrees with the earlier writers that the fungus is probably one of the Peronosporales.
MATERIALS AND METHODS

Specimens of _B. dissectum_ were collected from Owen and Vigo counties, Indiana, and specimens of _B. virginianum_ were collected from Clark county, Illinois. These plants were collected at various times during the year and kept in flowerpots in a shaded east window until needed. _B. dissectum_ is easier to maintain this way than _B. virginianum_. The plants of _B. virginianum_ collected in the fall did not send up new leaves although the roots remained alive throughout the year. The spores used from _B. dissectum_ were collected in October; the ones used from _B. virginianum_ were collected in May.

The roots of both species were cleared with a saturated solution of chloral hydrate and stained with 0.01% acid fuchsin (Gerdemann, 1955). The clearing was necessary to remove the starch granules which tend to obscure the field of study. The roots were cut into 1 to 2 cm sections before being placed into the solution. They were either boiled for about fifteen minutes, or allowed to remain in the solution for at least two days. The second method did not cause as much distortion to the outer cell layers. Root sections were kept in this solution over two months to use for later comparisons. Hand sections were made using a razor blade and pith sections as a holder. The sections were mounted in glycerine.

Attempts to germinate the spores were unsuccessful. Spores were washed with sterile water, centrifuged, and placed in a medium of Moore's solution and 1% sucrose as described by Freeburg and Wetmore (1957). Spores directly from the sporangia were also placed on soil taken from an area where the ferns grew. Some of the spores were placed on sterilized soil; others were placed on unsterilized soil.

Attempts to isolate fungi found within the roots have been
inconclusive. The following methods were tried. Roots were washed and sterilized with clorox, and thin sections of these roots were placed upon malt extract agar (Johansen, 1940) and upon Czapek's agar. With the aid of a dissecting microscope, hyphae on the outside of the roots were removed and placed upon the same media.

The soil from around the roots of the plants was screened through fine wire mesh or cheese cloth. The particles which were too large to pass through were examined under a dissecting microscope for gametophytes. None were found.
RESULTS

Fungi were found in some stage in nearly all of the root sections examined except the older and thicker roots of B. dissectum. All of the roots of B. virginianum are nearly the same size with little indication of their age by size or color of the epidermis. Relative age of the roots may be determined by their position on the rhizome with the youngest root nearest the apical region of the rhizome.

The amount of fungus was greatest in the young roots of B. dissectum. In many of these roots, the fungus formed a complete ring in the middle of the cortex. The ring was five to six cell layers from the epidermis and two to three cell layers thick. When this condition existed usually there were no hyphal connections to the epidermis. The cells were filled with either hyphal coils or the granular masses shown in Fig. 1. The granular masses are usually yellow which persists even after staining.

Infection occurs in B. dissectum through the epidermal cells. The hyphae are coenycytic and very irregular in diameter. The size may vary from 4 to 12 microns. The average diameter is 6 to 8 microns. The hyphae proceeds toward the center of the root, but it does not go past the seventh layer of cells. The hyphae while growing toward this layer branches several times. As the hypha passes through the cells there is often a swelling. This is shown in Fig. 2 and 3. The infecting mycelium may pass along the outside of the root, and more than one hypha may enter into the root as is seen in Fig. 2.

The amount of fungus found within a root section of B. virginianum is less, but the incidence is greater than in B. dissectum. Some of the hyphae seen along the epidermis of B.
virginianum were obviously septate. This is illustrated in Fig. 6 and 7. These hyphae were brown while most of the hyphae within the roots were hyaline. Septations were not evident in the hyphae within the roots. The diameter of the hyphae was 2.5 to 10 microns with an average diameter of 4 to 6 microns.

Infection occurs by the penetration of hyphae through the epidermis. The hyphae are often branched, and they are usually swollen where they pass from one cell to another. The hyphae seem to be darker in this area as well as thicker. This is illustrated in Fig. 4. While no arbuscles were seen the masses at the ends of the hypha as shown in Fig. 4, appear to fit Janse's description of sporangioles. Fig. 5 shows a hypha with a swelling at the end which is presumed to be a vesicle.

Several kinds of fungi grew as a result of inoculation of root sections on the different media. However, there was no direct evidence that any of these fungi were the same as the mycorhiza. No reproductive structures were seen on the hyphae within the root so all comparisons with the isolates had to be based upon the vegetative mycelia.
DISCUSSION

The appearance of mycorhiza as seen in both species agrees in many ways with the description of mycorhiza of other species of Botrychium and Ophioglossum. The location of fungi within the roots of *B. dissectum* corresponds very closely with the location described by Burgeff for mycorhiza of *O. pendulum*. The Hyphenknüel were observed, but no Sternarbuskeln were seen. However, if the sporangioles are formed as result of the break down of the arbuscles as reported by Janse, it is possible that they may be present at some time since structures similar to the sporangioles were seen in several sections of roots.

Many root sections of *B. dissectum* as well as some of the sections of *B. virginianum* had the granular masses within the cells of the sixth layer. These appear to be the Pilzverdauungszellen described by Burgeff. The other cells near these which have the Hyphenknüel within them would correspond to the fungal host cells.

The septations observed in some of the hyphae along the outside of the roots of the sporophyte *B. virginianum* have not been reported before. Nishida observed some septations between the older and younger portions of a hypha within the gametophyte of the same species. Since septations were not observed in the fungus within the root, it is possible that the septate hyphae were different fungi than the mycorhizal hyphae.

The method of infection appears to be the same in both species; it is similar to the method described by others for the sporophytes of the Ophioglossaceae. The amount of fungus within the root sections does vary between the two species.

The roots of *B. dissectum* change in size and appearance as they grow older. The young roots are thinner, lighter colored and the epidermal cells are thin walled. The older roots are thicker,
darker colored, and the epidermal cells are thicker. The roots of B. virginianum are nearly the same in appearance regardless of age. They look like the younger roots of B. dissectum except that the total number of roots is much greater.

Since the fungus is found in the younger roots of B. dissectum and it is found in all of the roots of B. virginianum, it could be assumed that there is some relationship between the thickness of the epidermal cells and the presence of the fungus. In the young roots of B. dissectum, there are generally more cells infected by fungus in a section than in a similar section from B. virginianum, but the number of cells infected per plant may be nearly the same. No quantitative analysis was made as this would require a total count of all the infected cells in all of the roots of sample plants of both species.

A question is posed concerning the fate of the fungus which may have been present in the now older roots. It would be assumed that they had the same type of infection within the cells at one time. Why is the fungus no longer there, and what causes it to disappear? The reason that it is not reinfected could be due to the thickness of the epidermal cell walls. This does not explain why the fungus was not retained within the cortex since after the initial infection there is no direct connection to the outside of the root.

Because of the size variation and the habit of growth of the fungi within the two fern species, it is proposed that different species infect them. Since the ferns grow under different environmental conditions, it is likely that the microflora would vary. B. virginianum is found in lightly shaded areas within a woods which have acid soil rich in humus. They will not survive in open sunlight. B. dissectum is more adaptable as to light requirements and soil types. It has been assumed that the source of infection is from fungi within the soil in the immediate area in which the plants grow.
As no spores of any of the Ophioglossaceae have been cultured in vitro, and all gametophytes found in the field have endogenous fungi associated with them, it may be concluded that the fungus is necessary for the growth of the spores into mature gametophytes. The exact mycorhizal relationship has not been determined although it has been suggested by Bruchmann that the oil contained within the fungal hyphae helps to prevent drying of the gametophyte. It is possible that the same role is played by a fungus in the roots of the sporophyte as it is found only in roots which have not developed thicker walls in the epidermis.

Another possible role of the fungus is as an aid in water and mineral uptake into the roots since there are no root hairs on any of the roots of the Ophioglossaceae. At this time there is no evidence to support this suggestion.

The roots evidently provide the food source for the fungus as there is an inverse proportion of starch to the fungus within the root cells. However, in the gametophyte which has no chlorophyll, this nutritional relationship is not as clear. Again there is the same lack of starch in the cells infected by the fungus, but there is starch in the other cells. What is the source of starch in these cells? It may be possible that the fungus helps to supply the nutrients necessary for the growth of the gametophyte. Since it has not been possible to culture the spores in vitro, it may be postulated that the fungus does supply some of the essential nutrients. Freeburg and Wetmore (1957) have succeeded in culturing the spores of Lycopodium in vitro so it may eventually be possible to do the same with the spores of the Ophioglossaceae.

Until it is possible to culture the fungus and the ferns separately and to synthesize the association, it is not possible to give more than a morphological description of the association.
A microscopic study was made of root sections from Botrychium dissectum and Botrychium virginianum to determine the presence of a mycorhizal association. Fungi were found in both species. B. dissectum had the larger amounts of fungus in the young roots with none present in the older roots. B. virginianum had hyphae present in all of its roots, but it was found in relatively smaller amounts than in the young roots of B. dissectum.

Attempts to isolate the fungi involved in the mycorhizal association were inconclusive. Several methods were tried for germinating the spores. None were successful.

The fungi had multinucleate, aseptate hyphae which formed Hyphenknüel and sporangioles within the cortical cells of the root of both ferns. Vesicles were found in B. virginianum only. The fungus host cells and Pilzverdauungszellen were located in the fifth and sixth cell layers from the outer epidermis of the root of both species. After the initial infection there were no hyphal connections to the outside of the root in both. Because of size and growth variations of the hyphae in the two species of ferns, it is proposed that different species of fungi are involved in the mycorhizal association.
LITERATURE CITED


Fig. 1

Cross-section of root of *B. dissectum* with Pilzverdauungszellen (a) and Hyphenknäuel (b). X ca. 400.
Fig. 2.

Cross-section of root of *B. dissectum* with infecting hyphae. *X ca. 400.*
Fig. 3.

Longitudinal-section of root of *B. dissectum* with infecting hyphae. X ca. 400.
Fig. 4.

Cross-section of root of *B. virginianum* with infecting hyphae and sporangioles. X ca. 400.

Fig. 5.

Cross-section of root of *B. virginianum* with vesicle at end of hyphae. X ca. 800.

Figs. 6 and 7.

Cross-sections of roots of *B. virginianum* with septate hyphae along the outer root epidermis. X ca. 400.