Spore wall development and septal pore ultrastructure in four species of Pachyphloeus

Rosaria Ann Healy
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Recommended Citation
Healy, Rosaria Ann, "Spore wall development and septal pore ultrastructure in four species of Pachyphloeus" (2002). Retrospective Theses and Dissertations. 19870.
https://lib.dr.iastate.edu/rtd/19870

This Thesis is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Spore wall development and septal pore ultrastructure in four species of *Pachyphloeus*

by

Rosaria Ann Healy

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Botany (Mycology)

Program of Study Committee:
Lois H. Tiffany (Major Professor)
Harry T. Horner
Edward Braun

Iowa State University

Ames, Iowa

2002
Graduate College
Iowa State University

This is to certify that the master’s thesis of

Rosaria Ann Healy

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
Dedication

I dedicate my thesis work with all my love to my husband who has made it possible for me to see this project through, and patiently waited for my return to a normal life. I am deeply grateful for his steadfast encouragement, support and love.
TABLE OF CONTENTS

INTRODUCTION 1
   Background information on hypogeous Ascomycetes 1
   Research question 3
   Organization of thesis 3

LITERATURE REVIEW 4
   Taxonomy 4
   Ecology 5
   Hypothesized relationship of *Pachyphloeus* to the Pezizaceae 7
   Molecular evidence for relationship of *Pachyphloeus* to the Pezizaceae 8
   Septal pore ultrastructure as a phylogenetic indicator 9
   Spore wall ontogeny and ornamentation as a phylogenetic indicator 11
   Spore ontogeny in the Pezizales 12
   Spore wall terminology 14

MATERIALS AND METHODS 18
   Field survey methods 18
   Herbarium loans of *Pachyphloeus* specimens 20
   Collections of *Pachyphloeus* and *Scabropezia* examined 20
   Fixation for light microscopy (LM) 22
   Fixation for scanning electron microscopy (SEM) 22
   Fixation for transmission electron microscopy (TEM) 23
   Material fixed for TEM and SEM 24
   Image capturing and processing 24

RESULTS 26
   Field data on *Pachyphloeus* species 26
   Species descriptions 29
   Septal pore ultrastructure 46
   Spore wall ontogeny 69

DISCUSSION 88
   Identity of *Pachyphloeus citrinus* 88
   Dispersal of *Pachyphloeus* propagules 88
   Phylogeny of *Pachyphloeus* 89
   Septal pore ultrastructure 91
   Secondary spore wall ontogeny 96

SUMMARY 100
INTRODUCTION

Background information on hypogeous Ascomycetes

The Ascomycota are a class of fungi characterized by the ascus, a sac-like cell in which ascospores, the sexual spores are produced. In the Euascomycetes, the filamentous ascomycota, asci are produced from ascogenous hyphae, the sexual hyphae formed through dikaryotization. The dikaryon typically fuses in the ascus, followed by meiosis and mitosis, which usually results in eight nuclei. Subsequent free cell formation usually results in eight ascospores. Classification of the Euascomycetes at the ordinal level is considered on the following characteristics: extent of exposure of the hymenium at maturity; whether an ascocarp is formed within or on the surface of its substrate, whether it is solitary or clustered, whether it develops an opening for release of spores, and if so, the characteristics of the opening; and morphology and discharge apparatus of the ascus.

An ecological subgroup of Euascomycetes, informally referred to as truffles, form fruiting bodies that are usually larger than 2 mm in diameter when mature and develop under the soil surface, a hypogeous situation. Species of these hypogeous Ascomycetes have been traditionally classified in the order Tuberales. The hymenium of a truffle is retained within a globose to laterally flattened or lobed, closed ascocarp. The relatively thick-walled spores are potentially dispersed long distances through animal consumption of the truffle and spore passage through the digestive tract; or dispersed short distances with soil disturbance or movement of soil water after ascocarp decomposition. The morphology of truffles is well suited to their peculiar growth habit and ecology, but difficult to interpret from a phylogenetic standpoint. Mycologists who study these fungi believe that the majority of species are related to the Pezizales, an order characterized by cup- or disc-shaped ascocarps usually developed above ground, an epigeous situation. Apothecia of the Pezizales open to varying extents exposing their hymenia of cylindrical operculate asci. Operculate asci form a lid-like structure at the tip of the ascus which opens at maturity with forcible spore discharge.

Ascocarp and ascospore features in the Pezizales and Tuberales are similar, but unlike taxa in the Pezizales, truffles lack forcible spore discharge. Therefore, the truffles have presented a systematics challenge to ascertain which characters are stable enough to be
phylogenetically informative at various levels of classification in both epigeous and hypogeous Ascomycetes such that the evolutionary history of truffles may be ascertained. Molecular data combined with ultrastructural data has provided evidence that hypogeous fungi have evolved independently numerous times from species in different families within the Pezizales. Currently, most genera of hypogeous Ascomycetes are retained in “truffle families” within the Pezizales or Tuberales until their relationships to each other and to genera in the Pezizales are more certain.

Ultrastructural features that have proved to be phylogenetically informative in the Ascomycetes at the ordinal and familial levels of classification are septal pore structures and, to a lesser extent, spore wall ontogeny. A lamellate structure, with alternating electron-dense and electron-translucent bands, associated with septal pores in somatic hyphae has been a definitive feature of all species of Pezizales examined, and therefore important at the ordinal level. The ultrastructure of ascal septae and of spore wall development has been used as evidence for hypothesized relationships between a few truffle species and epigeous families within the Pezizales. Characters believed to be phylogenetically informative at the family level are: the size and shape of Woronin bodies associated with septal pores of vegetative hyphae and the ultrastructure of the septal pore at the base of the younger ascus. A feature that may be conserved at the generic, and possibly family levels, is the manner and order in which secondary wall material is deposited on the primary wall of ascospores.
Research question

Septal pore ultrastructure and spore wall development were examined in four species of *Pachyphloeus* with the goal of clarifying the current ambiguous taxonomy surrounding this genus. Following methods delineated in Curry and Kimbrough (1983), a hypothesized phylogenetic relationship of *Pachyphloeus* to the Pezizaceae was assessed by examining the septal pore ultrastructure of ascal and vegetative hyphae and ascospore wall development in four species of *Pachyphloeus*. The final stages of spore wall development were used to provide additional characters for distinguishing an undescribed species of *Pachyphloeus* from the three described species used in this study.

Organization of thesis

The field methods and electron microscopy methods used in this study generated information that was different enough to warrant reporting and discussing in separate sections for each. Since there are only six species of *Pachyphloeus*, three of which occur in Iowa, this study provided an opportunity to integrate the history, generic and species concepts, and new information on *Pachyphloeus*. The Literature Review, Materials and Methods, and Results sections begin with field observations, including macro and micro descriptions of species of *Pachyphloeus*, followed by information on septal pore ultrastructure, and conclude with information on spore wall development. Discussion follows roughly the same order, with synthesis and interpretation of all pertinent available information. The most significant information from this study is listed in the Summary.
LITERATURE REVIEW

Taxonomy

The Genus *Pachyphloeus* was described by L.R. and C. Tulasne in 1844, based on *P. melanoxanthus* (Tul.) Tul. & C. Tul with the following characteristics: a thick peridium with an outer layer of pigmented cells; a thick margined orifice stuffed with hyphae, where the interstitial veins come to the surface in a depression on the ascoma; a prominent basal mycelial tuft; sterile veins that are initially a different color from the fertile tissue, but later more uniform in color; and asci with eight globose spores ornamented with spines, irregularly arranged in the ascus. The name *Pachyphloeus* was derived from a combination of Greek words for “thick” and “cortex” (Pegler et al., 1993).

To date there are six generally accepted species and one variety of *Pachyphloeus*: *P. melanoxanthus*, *P. citrinus* Berk. & Br., *P. conglomeratus* Berk. & Br., *P. ligericus* Tul. & Tul., *P. saccardoi* Matt., *P. virescens* Gilkey, and *P. melanoxanthus* Berk. & Br. variety *xanthocarnosus* Soehner. *Pachyphloeus melanoxanthus* was described as a black, warted ascoma, with a dull brown gleba, clavate asci arranged more or less regularly in the hymenium, and spiney, globose brown spores (Tulasne and Tulasne, 1844). *Pachyphloeus citrinus* differed from *P. melanoxanthus* in the dark brown peridium powdered with lemon yellow particles, and the comparatively large, lemon yellow orifice (Berkeley and Broome, 1846). *Pachyphloeus conglomeratus* differed from the others in that its peridium was smooth or rough, but lacked warted or verrucose sculpturing (Berkeley and Broome, 1846). *Pachyphloeus ligericus*, described by the Tulasnes in 1851, had a warted, sooty-green surface, and oval to spherical asci (Fischer, 1938). *Pachyphloeus saccardoi* was described from a specimen found in Italy with a warted, dark brown ascoma, very long, clavate asci, and minutely spiney, globose, dark brown spores which were large for the genus, being 18 – 24 µm diameter excluding sculpturing (Mattirolo, 1903). There are no reliable reports that either *P. saccardoi* or *P. ligericus* were ever found again (Soehner, 1936).

*Pachyphloeus virescens* was described from the United States (Gilkey, 1939) with a verrucose, dull greenish ascoma; “livid yellow” gleba; spherical to rarely calvate asci; and
globose spores ornamented by spines with thickened tips. From Germany, Soehner (1936) described *P. melanoxanthus* var. *xanthocarnosus*, though it did not resemble *P. melanoxanthus* very closely. He had only a single specimen, and hesitated to describe it as a new species. The description is similar to that of *P. virescens*, which has not been reported from Europe.

*Pachyphloeus carneus* Hark. was described from North America in 1899 (Harkness, 1899). Gilkey sent a specimen of *P. carneus* to Fischer who compared it with the type of *P. citrinus* and determined them to be synonymous (Gilkey, 1916). Gilkey tentatively accepted his opinion, but with reservations, because the ascocarps of North American specimens were consistently bright orange in color compared to the dark brown ascocarps with yellow papillae described for European specimens. She also noted a size difference, with North American specimens being larger.

Currently, *Pachyphloeus* is classified in the following families of the Pezizales: Pezizaceae (Castellano et al., 1989; Dissing and Korf, 1980), or Terfeziaceae (Trappe, 1979; Eriksson and Hawksworth 1993; Eriksson, 2000); or in the following families of the Tuberales: Eutuberaceae (Fischer, 1938; Gilkey, 1954; Hawker, 1954; Dennis, 1978), or Tuberaceae (Lange, 1956; Korf, 1973a).

Ecology

*Pachyphloeus* species have been collected in temperate forests and woodlands and are apparently absent from arid regions (Lawrynowicz, 1990). Collections of *P. citrinus* are recorded from Europe (Lawrynowicz, 1990), China (Zhang, 1992), Japan (Trappe, 1976), Mexico (Cázares et al., 1992) and the United States; *P. melanoxanthus* from Europe (Lawrynowicz, 1990), and Eastern North America (Gilkey, 1954); and *P. virescens* from North America (Gilkey, 1954; Cázares et al., 1992) and China (Zhang, 1992). *Pachyphloeus conglomeratus*, *P. ligericus* and *P. saccardoi* are recorded only from Europe (Soehner, 1936; Lawrynowicz, 1990). The first reported collection of *Pachyphloeus* in Iowa (Shearer and Tiffany, 1982) was from under *Quercus alba* L. in a pastured field (Tiffany, personal communication).
*Pachyphloeus* species are associated with a broad range of woody plant species. They are most often collected under trees in the Fagaceae. Woody associates recorded with collections of *Pachyphloeous* include *Fagus, Quercus, Carpinus, Corylus, Salix* and *Crategus* in Europe (Lawrynowicz, 1990); *Quercus* in China (Zhang, 1992); *Quercus, Arbutus* (Arora, 1986) and *Sequoia* in the United States (Harkness, 1899); *Quercus* in Mexico (Cázares et al., 1992); and *Abies* in Japan (Trappe, 1976).

Ascomata are usually collected close to the surface of the soil, emergent or on the soil surface under down wood or leaf litter. One of the peculiar growth habits noted in descriptions of collections is the association of the ascoma with twigs or other woody debris in the soil (Berkeley and Broome, 1846). However, there is not a consistent association with wood. While there are reports that many genera of hypogeous fungi form ectomycorrhizae with woody plant associates (Maia et al., 1996), there is no information on the nutritional sources of *Pachyphloeus*.

There is no information about mode of spore dispersal of *Pachyphloeus* species. The ascomata, like those of most other species of hypogeous fungi, do not open to release their spores, and their asci do not appear to have an active mechanism for forcible spore discharge. There are reports that some hypogeous fungi release their spores as a result of ascus and ascocarp break down in the soil through the action of microbes or invertebrates (Fogel and Peck, 1975). There are numerous reports of truffles and false truffles being eaten by small mammals and their spores dispersed in the scat (Trappe and Maser, 1977; Fogel and Trappe, 1978; Maser et al., 1978; Johnson, 1996). Unfortunately, because of the difficulty in germinating spores of these fungi, none of the studies involving mycophagy of ectomycorrhizal hypogeous fungi was followed by successful germination experiments. However, successful mycorrhizal synthesis was achieved using tassel-eared squirrel scat that was laden with spores from the Basidiomycetous truffles *Rhizopogon* Fr. Emend. Tul. & C. Tul. ssp., *Sclerogaster xerophilum* Fogel, *Hysterangium separabile* Zeller, *Sedecula pulvinata* Zeller, and the Ascomycetous truffles *Elaphomyces* Nees v. Esenbeck: Fr. spp., and *Tuber* Micheli ex Wiggers: Fr spp. (Kotter and Farentinos, 1984). Whether *Pachyphloeus* ascocarps are consumed by mammals is unknown. *Pachyphloeus* was not identified in any published studies on mycophagy. The odor of mature ascocarps is reported
by some workers to be strong (Berkeley and Broome, 1967), as also reported for hypogeous fungi that are consumed by animals (Castellano et al., 1989).

_Pachyphloeus_ has a definite season during which it fruits, depending on the climatic region. All species fruit around the same time of year. In northern Mexico, _P. citrinus_ and _P. virescens_ were collected from July through October (Cazares et al., 1992). In California, _P. citrinus_ was collected in January, and March through May, and July (Gilkey, 1916). _Pachyphloeus citrinus_ was collected in July in Japan (Trappe, 1976). In Denmark, _P. melanoxanthus, P. conglomeratus_ and _P. citrinus_ were collected in August and September (Lange, 1956); in Germany, _P. citrinus_ was collected from July to November, _P. conglomeratus_ in August, and _P. melanoxanthus_ from September to November (Soehner, 1936); and in Great Britain species were collected in autumn (Hawker, 1954).

**Hypothesized relationship of _Pachyphloeus_ to the Pezizaceae**

For many years the Tuberales were considered related to the Pezizales because of similarities between the peridial structure of truffles and the peridial (ectal excipular) structure of the apothecium of Pezizales, and because of similarities between spores (Kimbrough et al., 1991). They were retained in separate orders because one character that distinguishes the Pezizales as a monophyletic order is the operculate ascus, a character lacking in truffles. When spores of a fungus of the Pezizales are mature, the ascus opens by an operculum, and the spores are forcibly discharged to be dispersed by air currents. Most truffle asci have no specialized discharge mechanism. However, one of the hypogeous Ascomycetes, _Geopora cooperi_ Hark., was observed to forcibly discharge its spores some time after the ascocarp had been accidently broken. Closer examination revealed that the asci were operculate (Burdsall, 1965). This discovery fueled opinion that Tuberales should be classified as Pezizales (Rifai, 1968; Eckblad, 1968; Korf, 1973a; 1973b; van Brummelen, 1978; Dennis, 1978). In fact, Korf (1973a) commented that taxa in the Tuberales differed from those in the Pezizales only in their lack of an operculum.

Trappe agreed with this assessment, and based on macro and micro characters, transferred all Tuberales, except for _Elaphomyces_ Nees v. Esenbeck: Fr., to the Pezizales. However, he retained most of the genera in their truffle families (Trappe, 1979) and this disposition has in
general been accepted. However, data demonstrating a different type of ascospore
delimitation for several species of *Tuber* and for a species of *Terfezia* (Tul. & C. Tul.) Tul. &
C. Tul. (Berta and Fusconi, 1983; Janex-Favre et al., 1988) suggested that the Tuberales
should be retained (Parguey-LeDuc et al., 1987; Bellemère, 1994; van Brummelen, 1994).

Dissing and Pfister (1981) hypothesized a relationship between *Pachyphloeus* and a taxon
of the Pezizaceae they described as *Scabropezia* Dissing & Pfister. They were impressed by
similarities between structure of the ectal excipula, shape of the spores, and sculpturing of the
spores in the two genera. They observed that the main difference between the ectal
excipulum in the two genera is thicker cell walls in *Pachyphloeus*.

**Molecular evidence for relationship of *Pachyphloeus* to the Pezizaceae**

A salient feature of fungi in the Pezizaceae is that their asci turn blue in Melzer’s solution,
whose reaction with fungal tissue, though not understood (Read and Beckett, 1996), is
considered to be significant. Molecular evidence that this is a phylogenetically informative
character in the Pezizales was demonstrated by Landvik et al. (1997) who compared
sequences from the nuclear gene that codes for the small subunit of ribosomes (18S rDNA)
from Pezizalean taxa. Those with amyloid asci separated into a monophyletic group from the
rest of the Pezizales. Within the amyloid Pezizales, there are different extents and patterns of
bluing (Baral, 1987). Comparison of sequences from part of a nuclear gene that codes for the
large subunit of the ribosome (LSU rDNA) supports groupings by the type of amyloid
reaction (Hansen et al., 2001). An amyloid reaction to Melzer’s solution has been detected in
a few truffles. Lange (1956) reported a diffuse, but apparent amyloid reaction of asci of *P.
melanoxanthus*, and Kimbrough et al. (1991) reported an amyloid reaction in asci of
*Hydnobolites cerebriformis* when its ascocarp tissue was revived in KOH prior to treatment
with Melzer’s solution.

Recent molecular evidence supports the interpretation of an affinity of *Pachyphloeus* with
the Pezizaceae, and specifically with *Scabropezia*. Percudani et al. (1999) included
representative taxa from the truffle families Terfeziaceae, Balsamiaceae and Tuberaceae
along with epigeous taxa from nine Pezizalean families in a phylogenetic analysis of
nucleotide sequences from the 18S rDNA. They included *Pachyphloeus melanoxanthus* as a representative of the Terfeziaceae. Results of the analysis indicated that *Pachyphloeus*, along with other taxa of Terfeziaceae, nested within the Pezizaceae. In a different 18S rDNA analysis in which *Pachyphloeus melanoxanthus* was compared with taxa from the Pezizaceae, *Pachyphloeus* sequences were closest to those of *Scabropezia*. In addition, a close relationship was suggested between these two taxa and the anamorph *Glischroderma cinctum* Fuckel (Norman and Egger, 1999).

The rate of divergence between *Scabropezia* and *Pachyphloeus* at the molecular level was interpreted to be less rapid than at the morphological level. The reason for a more rapid morphological rate of change could be the selective pressures of adaptation to a hypogeous habit (Norman and Egger, 1999). A similarly rapid morphological evolution in the Basidiomycetes between *Suillus* (Micheli) Gray and the hypogeous genus *Rhizopogon* was ascertained using molecular clock analysis (Bruns et al., 1989). The similarities between the 18S rDNA sequences of *Pachyphloeus* and *Scabropezia* suggested to Norman and Egger (1999) that these two taxa may be synonymous.

**Septal pore ultrastructure as a phylogenetic indicator**

One of the most informative morphological characters for both operculate and inoperculate discomycetes is ascal pore ultrastructure (Gernandt et al., 2001; Kimbrough, 1994). Septal pores in the hyphae of Basidiomycetes and Ascomycetes provide cytoplasmic continuity between cells in a filament. Various structures associated with them are hypothesized to play a role in regulating and/or preventing cytoplasmic and organellar passage between cells, providing compartmentalization of sexual cells, and regulating senescence (Buller, 1958; Beckett et al., 1974; Gull, 1978). As the septal pores are vital to basic cellular function in both classes, their regulation is equally important (Markham, 1994). Therefore it is reasonable to surmise that pore associated structures are highly conserved. Evidence for this comes from phylogenetic analysis using 18S rDNA, which supports relationships inferred from septal pore ultrastructure (Norman and Egger, 1999).

There are several types of structures associated with septal pores in the Ascomycetes, some found mainly in somatic cells, some mainly in reproductive cells, and some in both.
Structures associated with septal pores of Ascomycetes include Woronin bodies, an amorphous electron-dense matrix and "secondary wall" (term of Kimbrough and Curry, 1985). Structures associated with septal pores of the Pezizales include those listed above as well as lamellate structures and a variety of structures conserved at the family level associated with the septal pore of the ascus (Kimbrough, 1994).

An organelle unique to the Ascomycetes is the Woronin body. Woronin bodies are membrane bound; electron-opaque; proteinaceous; (McKeen, 1971; Mason and Crosse, 1975; Turnau et al., 1993), spherical, or oblong to greatly elongated, or hexagonal in shape; and always located near septal pores of somatic cells (Markham, 1994). They have been observed to plug the pores of injured cells, old cells, or cells under physiological stress (Gull, 1978; Markham, 1994), effectively walling them off from healthy, metabolically active cells. They have been observed to plug the pores of healthy cells (McKeen, 1971), which has stimulated conjecture about other regulatory functions (Camp, 1977). They are formed in microbodies and therefore suspected of producing enzymes (Wergin, 1973; Markham, 1994). A role of differentiation through compartmentalization has been hypothesized (Trinci and Collinge, 1973; Gull, 1978), which is a notion strengthened by data indicating that hyphal branching is correlated with pore plugging (Trinci and Collinge, 1973).

There is less known about the other structures observed in septal pores. An amorphous electron-dense matrix has been observed, to varying extents, in pores of all types of hyphae in all classes of fungi (Markham, 1994). Its composition has not been determined. It is not membrane bound, and has not tested positive for protein, lipid or polysaccharides (Beckett, 1981; Kimbrough and Gibson, 1990). The origin of the material is unclear, but there is some evidence that it is synthesized on site (Littlefield and Bracker, 1971; Camp, 1977; Berndt and Oberwinkler, 1992). The function assumed for the amorphous matrix is to seal off one cell from another for purposes of differentiation, or to wall off old or degenerating cells (Markham, 1994).

In a wide variety of Pezizales, the septal walls in the pores of mature asci become enlarged around the pore, effectively reducing the diameter of the pore. This augmentation has been called "secondary wall" (Kimbrough and Curry 1985; Kimbrough and Curry, 1986b; Kimbrough, 1991; Li and Kimbrough, 1995a, 1995b; Kimbrough et al., 1996; Li,
1997), although there is no indication that the additional material is different from the original wall polymers (Markham, 1994). It has occasionally been observed in septal pores of other types of cells besides asci in the Pezizales (Li, 1994, Li and Kimbrough, 1995a).

There is little information on the composition or function of the septal pore associated lamellate structure unique to the Pezizales (Gull, 1978; Kimbrough, 1994), or of the various Pezizalean structures associated with ascal pores, but it is these structures that are the most phylogenetically informative (Kimbrough, 1994). Some somatic hyphae possess a lamellate structure of alternating electron-dense and electron-translucent bands associated with their septal pore rims. Because this lamellate structure is apparently unique to the Pezizales (Kimbrough, 1994), it is referred to as the “Peziza septal type” to distinguish it from the simpler types found in other orders, the “Neurospora type” (Curry and Kimbrough, 1983) and the “Sclerotinia-type” (Kimbrough, 1994). Extensive studies of taxa in various families of the Pezizales have revealed that the structure at the base of the ascus is a character stable at the family level, and variable at higher levels. Septal pores at the base of asci in the Pezizaceae have electron-dense uni- or bi-convex bands (Kimbrough, 1991), the simplest septal structures observed in the Pezizales, and referred to as the “pezizoid septal types” (Curry and Kimbrough, 1983). In the Pezizaceae, somatic cells may have uni- or bi-convex bands, but more often have one or more of the following structures: an amorphous electron-dense matrix, lamellate structures, and spherical Woronin bodies (Kimbrough, 1994).

By combining data of septal pore ultrastructure with morphological and cytological observations, Kimbrough and his associates were able to link the following truffle genera to Pezizalean families: Barssia to the Helvellaceae (Kimbrough et al., 1996); Genea to the Otideaceae (Li and Kimbrough, 1994); two species of Tuber to the Otideaceae (Li and Kimbrough, 1995b); and Hydnobolites to the Pezizaceae (Kimbrough et al., 1991).

**Spore wall ontogeny and ornamentation as a phylogenetic indicator**

Another character that has been used for assessing phylogenetic relationships within the Ascomycetes is spore wall ontogeny and ornamentation. Initial spore wall ontogeny is the same for all Ascomycetes and consists of the delimitation of spores by two unit membranes, the development of spore walls between these two membranes, and the initial deposition of
primary wall (Beckett, 1981). Secondary wall ontogeny varies, and is responsible for the color and sculpturing of spores (Beckett, 1981). In the classification of Pezizales, traditional spore characters used in taxonomy are color, shape, ornamentation, and number of nuclei. Spores of the Pezizales are one-celled, radially or bilaterally symmetrical, usually hyaline except in Ascobolaceae and Pezizaceae, and usually larger than 10 µm. Some investigators believe that spore ornamentation is of value at the generic and family levels (Kimbrough, 1970; Dyby and Kimbrough 1987), while others caution that spore ornamentation is valuable only at low taxonomic levels (Bellemère, 1994; van Brummelen, 1978). Opinions vary also about the value of secondary wall ontogeny as a phylogenetic indicator. Wu (1991), who studied spore ontogeny in the Humariaceae, commented that it is as informative as septal pore ultrastructure; but Li (1994), who studied spore wall development in Sarcosomataceae and Sarcoscyphaceae, concluded that spore wall ontogeny in the Pezizales is too inconsistent to be a good phylogenetic character. While there is disagreement about the phylogenetic usefulness of spore morphology and spore development, all investigators agree that these are useful characters for distinguishing among species (Li, 1994; Bellemère, 1994).

**Spore ontogeny in the Pezizales**

Spore ontogeny is similar in most Ascomycetes, and is unique among Eukaryotes (Bracker, 1967). Ascospores are produced through “free cell formation” (Harper, 1899), of which there are two types: one in the Hemiascomycetes and in some of the Tuberales; and the other in the Euascomycetes, which includes the Pezizales (Beckett, 1981). In the Euascomycetes, a double-membrane system (Bracker, 1967), originates in the ascus plasmalemma (Syrop and Beckett, 1972; Bracker, 1967; Mims et al., 1990, Bellemère, 1994), and forms an ascus vesicle that surrounds the usually eight haploid daughter nuclei, the products of meiosis and mitosis after karyogamy in the ascus cell. The ascus vesicle becomes constricted around each primordial spore (Mainwaring, 1967), breaks so that there is a double-membrane around each nucleus and its surrounding ascoplasm, and the free ends of the double-membrane fuse (Bracker, 1967).

Delimitation in some of the Tuberales is different from that of the other Euascomycetes. Ascospores in some species of *Tuber* and *Terfezia* are individually delimited by single unit
membranes arising from the ascus plasmalemma in different places along the apical region of the ascus. After delimitation, the delimiting membrane becomes a double-membrane between which the spore walls are formed (Parguey-Leduc and Janex-Favre, 1977; Janex-Favre and Parguey-Leduc, 1983; Janex-Favre et al., 1988). Individual delimitation was also reported in *Tuber magnatum*, but the delimiting membranes were double (Berta and Fusconi, 1983).

In all Ascomycetes, the spore wall develops between the two delimiting membranes (Conti and Naylor, 1960; Bracker and Williams, 1966; Bracker, 1967; Bandoni et al., 1967; Reeves, 1967; Lynn and Magee, 1970; Beckett et al., 1973; Read and Beckett, 1996). Development of the wall varies, resulting in one or more distinct layers, which may or may not be ornamented with a variety of different types of structures. The order of development varies also. The outer of the two delimiting membranes becomes the investing membrane, and may persist or disappear by ascospore maturity (Merkus, 1973). The inner membrane becomes the sporoplasmalemma. Both membranes may be involved in the synthesis of wall layers (Read and Beckett, 1996). The ascoplasm that is not included in spore delimitation becomes the epiplasm. By the time the spores are mature, most of the epiplasm has degenerated and disappeared (Dyby and Kimbrough, 1987). After spore delimitation, the enveloped ascoplasm, with nucleus or nuclei, becomes the sporoplasm.

The primary wall, composed of a homogeneous, electron-translucent material, develops centrifugally, and is thought to originate from the sporoplasm (Bellemère, 1994) or from both the sporoplasm and the epiplasm (Gibson and Kimbrough, 1988; Li, 1994; Read and Beckett, 1996). Up to this point, with the exception of *Tarzetta* (Cook) Lambotte (Humariaceae) that initially develops an electron-dense wall (Wu, 1991), there is little variation among Euascomycetes. In the Pezizales, the primary wall sometimes differentiates into two or three layers. The outermost layer, referred to as the epispore by some workers (Merkus, 1976), is often striated, and is reported by some to be developed from the secondary wall rather than the primary wall (Beckett, 1981; Gibson and Kimbrough, 1987). The innermost layer, referred to by some workers as the endospore, sometimes has an undulating base (Bellemère, 1994).
A secondary wall is deposited on the spore primary wall centripetally. Some investigators dislike the term “secondary wall” because it implies development of two distinct walls, when it is not clear that this is so. The secondary wall may simply be a modification of the primary wall (Bellemère, 1994). However, the majority of investigators refer to it as a secondary wall because it is different in appearance (eg. Merkus, 1973; Dyby and Kimbrough, 1987; Kimbrough et al., 1991; Wu and Kimbrough, 1991; Read and Beckett, 1996). The secondary wall is usually electron-dense. In some taxa, the secondary wall becomes smooth or ornamented and, in others, it forms but disappears by maturity. The secondary wall is not present in all Ascomycetes (Moore and McAlear, 1962; Merkus, 1973), but in the Pezizales it develops as a temporary if not a permanent layer (Merkus, 1976). In some cases, it is present merely as a flocculose tissue in the perisporal sac that disappears with maturity (Li and Kimbrough, 1995c). Major differences between species occur in the latter stages close to spore maturation and usually result in secondary wall variability (Gibson and Kimbrough, 1988; Read and Beckett, 1996).

There are conflicting opinions about whether the secondary wall has its origins in the sporoplasm (Read and Beckett, 1996), the epiplasm (Bellemère, 1994; Merkus, 1973; 1974), or both (Merkus, 1973; Read and Beckett, 1996). Read and Beckett (1996) reason that in species where epiplasmic degeneration occurs before spores are mature, further deposition must originate in the sporoplasm, but that in the Pezizales where epiplasm persists well into maturity, epiplasm may play a role in secondary wall development.

Ascus development within an ascoma is sequential (Read and Beckett, 1996), but spore development within an ascus is simultaneous in most species of Pezizales studied. Exceptions occur in the Sarcoscyphineae where basal spores in an ascus mature more quickly than apical spores (Merkus, 1976; Li, 1994); and in Leucangium carthusianum, one of the hypogeous Ascomycetes, which has nonsynchronous development of spores in an ascus (Li, 1997).

**Spore wall terminology**

The terminology employed by various researchers to describe spore wall ontogeny is confusing and there is no agreement on a standard terminology among researchers.
Ascoplasm: the cytoplasm, including organelles and other structures present in the ascus before spore delimitation.

Delimiting unit membrane system (Carroll, 1969): a double-membrane that delimits the spores. It is derived from the plasmalemma, and is sometimes referred to as an ascus vesicle. The spore wall forms between these two membranes. The ascus vesicle is a feature of all euascomycetes except some of the Tuberales (Read and Beckett, 1996).

Endospore (SW2): the region along the inner periphery of the primary wall that sometimes differentiates late in spore wall development of some species (Bellemère, 1994). It develops in all classes of fungi, but only in some species, so the taxonomic value is low (Bellemère, 1994). In *Pachyphloeus* it can be distinguished about the same time that the epispore (SW1) becomes striate.

Epiplasm: the ascoplasm that is not enveloped by the delimiting membranes of the spores. It usually degenerates during sporogenesis, but persists well into the maturation of spores in the Pezizales (Wells, 1972).

Epispore (SW1): the electron-dense striate layer over the primary wall (Merkus, 1973). The epispore may be derived from the primary wall (Read and Beckett, 1996) or the secondary wall (Gibson and Kimbrough 1988). In *Pachyphloeus*, this is the first material deposited after the primary wall is formed.

Investing membrane: the outer of the two delimiting membranes (Mainwaring, 1967).

Perisporal sac: the area bordered by primary wall and the investing membrane (Wells, 1972).

Primary wall: the first deposition between the spore delimiting membranes (Merkus, 1973). It is initially homogeneous and electron-translucent, but may become modified with an epispore and/or an endospore. Opinions vary about its origins, but it is a universal feature of ascospores (Read and Beckett, 1996).

Secondary wall (SW1, SW2, SW3, SW4, SW5): the electron-dense layer deposited on the primary wall of many species of Ascomycetes. It begins forming after the primary wall is completed, although the primary wall may become modified after secondary wall
deposition begins. In some species of the Pezizales, it forms and then later disappears (Merkus, 1973). Spore wall ornamentation is usually due to secondary wall material. In this thesis, secondary wall terminology follows suggestions of Read and Beckett (1996). Numerical designation is given to every modification or deposition in the order it occurs after the primary wall is formed in *Pachyphloeus*. SW1 refers to the epispore (see “epispore” definition above). SW2 refers to the endospore (see “endospore” definition above). SW3 designates the homogeneous material deposited on top of the epispore (SW1) around the same time as the epispore becomes striate. SW4 designates the fibrillous fibers deposited on top of SW3. SW5 designates the fasciated fibrils that are modified from the border of SW3 close to the time of maturation.

**Sporoplasm**: cytoplasm along with all organelles enveloped by the sporoplastomainema (Wells, 1972).

**Sporoplastomainema**: the inner of the two delimiting membranes of a spore (Bracker, 1967).

**Tonoplast**: membrane of a vacuole.
Figure 1 Spore terminology
MATERIALS AND METHODS

Field survey methods

Collection of *Pachyphloeus* species was part of a wider effort to survey woodlands in Iowa for hypogeous fungi. To optimize the chances of collecting a representation of the hypogeous fungi in Iowa, sites were selected from diverse geologic regions in the state. To gauge their seasonal development search was undertaken three times per year between April and November for three years. Twenty sites were chosen based on their representation of woodland type (upland deciduous or coniferous), location in diverse landform regions, and accessibility (Fig. 2). Some collections were made at additional sites opportunistically. A “site” in this study refers to the entire park or wildlife area. Within each of these sites, there were specific areas that were searched each time the site was visited. New areas within each site were searched as time permitted.

![Figure 2](image-url) Sites searched for truffles from 1998-2000. Map of Iowa landforms redrawn from page 31 of Prior (1991). See Table 2 for sites corresponding with numbered dots.
Table 1  Sites with their corresponding numbers as depicted in Figure 2

<table>
<thead>
<tr>
<th>Site Number</th>
<th>Site</th>
<th>Landform</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pilot Knob State Preserve</td>
<td>Des Moines Lobe</td>
</tr>
<tr>
<td>2</td>
<td>Fort Defiance State Park</td>
<td>Des Moines Lobe</td>
</tr>
<tr>
<td>3</td>
<td>Woodman Hollow State Preserve</td>
<td>Des Moines Lobe</td>
</tr>
<tr>
<td>4</td>
<td>Ledges State Park</td>
<td>Des Moines Lobe</td>
</tr>
<tr>
<td>5</td>
<td>Reactor Woods Park</td>
<td>Des Moines Lobe</td>
</tr>
<tr>
<td>6</td>
<td>McFarland Park</td>
<td>Des Moines Lobe</td>
</tr>
<tr>
<td>7</td>
<td>Hickory Grove Park</td>
<td>Des Moines Lobe</td>
</tr>
<tr>
<td>8</td>
<td>Grammer Grove Park</td>
<td>Southern Iowa Drift Plains</td>
</tr>
<tr>
<td>9</td>
<td>Cardinal Marsh Wildlife Area</td>
<td>Iowan Surface</td>
</tr>
<tr>
<td>10</td>
<td>White Pine Hollow State Preserve</td>
<td>Paleozoic Plateau</td>
</tr>
<tr>
<td>11</td>
<td>Mines of Spain State Park</td>
<td>Paleozoic Plateau</td>
</tr>
<tr>
<td>12</td>
<td>Palisades Kepler State Park</td>
<td>Southern Iowa Drift Plains</td>
</tr>
<tr>
<td>13</td>
<td>Woodthrush Woods State Preserve</td>
<td>Southern Iowa Drift Plains</td>
</tr>
<tr>
<td>14</td>
<td>Lacey-Keosaqua State Park</td>
<td>Southern Iowa Drift Plains</td>
</tr>
<tr>
<td>15</td>
<td>Shimek State Forest (Donnelson Unit)</td>
<td>Southern Iowa Drift Plains</td>
</tr>
<tr>
<td>16</td>
<td>Stephens State Forest (White Breast Unit)</td>
<td>Southern Iowa Drift Plains</td>
</tr>
<tr>
<td>17</td>
<td>Waubonsie State Park</td>
<td>Loess Hills</td>
</tr>
<tr>
<td>18</td>
<td>Preparation Canyon State Park</td>
<td>Loess Hills</td>
</tr>
<tr>
<td>19</td>
<td>Stone State Park</td>
<td>Loess Hills</td>
</tr>
<tr>
<td>20</td>
<td>Oak Grove State Park</td>
<td>Northwest Iowa Plains</td>
</tr>
</tbody>
</table>

White Pine Hollow was the only site to contain native white pine. Sites that included planted conifers that were searched under were McFarland Park, Cardinal Marsh, Oak Grove Park, Shimek Forest, Stephens Forest and Grammer Grove Park.
Data for each collection included location, date, canopy species, position of the sporocarp relative to the soil surface, fresh color of the sporocarp, and presence of leaf or twig litter. Search was carried out with a three-pronged hand cultivator, and vertical exploration was limited to five centimeters. Sporocarps were kept in a cooler or refrigerator until microscopic data could be obtained. Each species was visually recorded using a 50 mm macro lens mounted on a Pentax 35 mm camera using Ektachrome Elite Daylight slide film, ASA 64 or 100.

Measurements were taken of fresh ascocarps. Fresh free hand sections were mounted in water for observing peridial and spore color with light microscopy, and for measuring spores, asci, and excipular cells. Dried sections of selected ascocarps with mature spores from each species were rehydrated for 15 minutes in 2% KOH or 10% KOH, rinsed in deionized water, and mounted in Melzer's solution to test for a bluing reaction following the method of Kohn and Korf (1975). Spores were assumed to be mature if they were pigmented and ornamented. Colors of mature and immature ascocarps follow Ridgeway (1912). Species descriptions follow the suggestions by Weber et al. (1997). After recording details of macro and micro morphology of the ascocarps, specimens were cut in half, air dried and deposited in the Ada Hayden Herbarium at Iowa State University.

**Herbarium loans of *Pachyphloeus* specimens**

Collections of *Pachyphloeus* were examined from the following herbaria: National Fungus Collections, Crops Research Division, Plant Industry Station, Beltsville, Maryland, USA (BPI); Muséum National d'Histoire Naturelle, Laboratoire de Cryptogamie, Paris, France (PC); and the University of Nebraska State Museum, Lincoln, Nebraska, USA (NEB).

**Collections of *Pachyphloeus* and *Scabropezia* examined**

9/15/00; Emmet Co., Fort Defiance State Park 8/7/98, 8/8/98, 7/15/99, 9/11/99; Fremont Co.,
Waubonsie State Park 6/20/00; Hancock Co., Pilot Knob State Preserve 8/28/98; Jasper Co.
Rock Creek State Park 8/12/00; Jefferson Co., Lamson Woods State Preserve 9/2/00;
Forest, Farmington Unit 7/7/00, 9/1/00; Linn Co., Palisades-Kepler State Park 9/10/98,
7/31/99; Lucas Co., Stephens State Forest, Whitebreast Unit 7/11/98; Marshall Co.,
Gramer Grove Park 7/30/98, 7/29/00; Monona Co., Preparation Canyon State Park 6/21/00;
Sac Co., Whitehorse Access 7/20/98; Sioux Co., Oak Grove State Park 8/9/98, 8/7/99,
8/18/00; Story Co. Christianson Preserve 8/24/96; Story Co., Hickory Grove Park 8/6/99,
9/9/99, 7/28/00, 8/26/00; Story Co., McFarland Park 8/24/96, 8/31/96, 7/21/99, 8/4/00; Story
County private woods 7/26/95, 8/3/95, 6/29/97, 7/21/97, 7/27/97, 7/4/99, 7/24/00; Story Co.,
Reactor Woods Park, Ames 7/28/96, 7/22/99, 8/28/99, 8/9/00, 9/10/00; Story Co., Robison
Wildlife Acres 9/2/96; Van Buren Co., Lacey-Keosaqua State Park 7/10/99, 7/8/00; Webster
Co., Dolliver State Park 7/21/98, 9/8/00; Webster Co., Woodman Hollow State Preserve
7/22/00, 9/8/00; Winneshiek Co., Will Baker Park, Decorah 10/2/98; Winneshiek Co,
Cardinal Marsh Wildlife Area 10/3/98; Woodbury Co., Stone State Park 7/19/98, 8/10/98,
8/7/99, 7/27/00. FRANCE: Leg. L. Quelet, 7/21/1879 (PC); Leg. LR Tulasne, 1873 (PC).
ENGLAND: Bristol, Berkeley and Broome, July 1841 (PC).

_Pachyphloeus conglomeratus_ Berk. & Br. FRANCE: Bains de Landes, September 1846
(PC).

_Pachyphloeus melanoxanthus_ Tul:: FRANCE: Meudon pres Paris, Bois de la Dane-Rose,
September 1823 (PC); Port pres Gien, leg. Tulasne, October 1843 (PC). IOWA: Boone Co.
private woods 8/4/98; Buchanan Co., Jakway Forest Area 7/23/98; Delaware Co., Backbone
State Park 7/24/98; Story Co., Hickory Grove Park 7/28/00.

Dubuque Co., White Pine Hollow State Preserve 8/20/00; Emmet Co., Fort Defiance State
Park 8/8/98; Lee Co., Shimek State Forest, Farmington Unit 7/7/00; Lucas Co., Stephens
State Forest, White Breast Unit 6/29/00; Marshall Co., Grammer Grove Park 7/29/00;
Monona Co., Preparation Canyon State Park 7/24/99; Story Co. private woods 7/24/00; Story
Co., Reactor Woods Park, Ames 8/9/00; Van Buren Co., Lacey-Keosaqua State Park 7/21/99, 7/8/00; Webster Co., Woodman Hollow State Preserve 7/22/00; Woodbury Co., Stone State Park 7/27/00;


Scabropezia flavoviresens (Fuckel) Dissing and Pfister: IOWA, Emmet Co., Fort Defiance State Park 8/17/00. NEBRASKA: Barney Creek, Niobrara River, coll. ME Moore, 8/13/1897 (as *Sphaerospora flavoviresens* (Fuckel) Saccardo, BPI 573895).

Scabropezia scabrosa (Cooke) Dissing and Pfister: WEST VIRGINIA, Cheat Bridge, 8/18/1906 (as *Phaeopezia scabrosa* (Cooke) Saccardo, BPI 662493).

**Fixation for light microscopy (LM)**

For paraffin sectioning, ascocarps were fixed in formalin/acetic acid/alcohol (FAA), dehydrated in an ethanol (ETOH) series (50%, 70%, 95%, several changes in 100%), followed by several changes in tertiary butyl alcohol (TBA), infiltrated with paraffin in a 60°C oven, and embedded in paraffin with a melting point of 56°C. Sections 8 µm thick were cut on a rotary microtome, mounted on glass slides with Haupts adhesive and 2% formalin in water, dried down on a warming tray, deparaffinized to water, and stained for viewing of general morphology using iron hematoxylin in water. Stained sections were dehydrated in an ethanol series to xylene, mounted in Permount on a drop of xylene and coverslipped. Ascocarps that were fixed for transmission electron microscopy (TEM) were also studied at the LM level. Sections 1 to 2 µm thick were cut from resin-embedded blocks into a drop of water on a glass knife mounted on an ultramicrotome, dried down on a glass slide, stained with toluidine blue, water rinsed, dried down, and a coverslip attached with Permount on a drop of xylene.

**Fixation for scanning electron microscopy (SEM)**

*Pachyphloeus citrinus, P. virescens* and *Pachyphloeus* sp. nov. were fixed as described for LM, and 50 µm sections were cut on a rotary microtome. Sections were deparaffinized in xylene and transferred to 100% ETOH. *Pachyphloeus melanoxanthus* and additional
specimens of *P. citrinus* were fixed in 2.5% glutaraldehyde (GA) + 2% paraformaldehyde (PF) + 0.2 mM calcium chloride (CaCl$_2$) in 0.1 M sodium cacodylate buffer (SCB), pH 7.2, postfixed in 1% osmium tetroxide (OsO$_4$) for 1.5 hrs, dehydrated to 100% ETOH, and freeze fractured in liquid nitrogen. All specimens were subsequently critical point dried in a DCP-1 Denton Critical Point Drying Apparatus (Denton Vacuum Inc., Cherry Hill, NJ) mounted on aluminum stubs, with double-sided sticky tape, silver painted around the specimen edges, and the paint allowed to air dry in a sealed dessicator with Dry Rite, where the specimens were stored. Specimens were sputter coated with a Denton Vacuum LLC Desk II Cold Sputter Unit (Denton Vacuum Inc., Moorestown, NJ) for 120 seconds with Au/Pd and viewed with 10-15 kV in a JEOL 5800LV SEM (JEOL USA, Inc., Peabody, MA). Images were digitally captured.

**Fixation for transmission electron microscopy (TEM)**

Most of the ultrastuctural data for this study was gathered from *P. citrinus*, which was represented by the greatest number of samples at various stages of maturity. The other three *Pachyphloeus* species were studied less extensively.

Fixation for TEM followed that of Curry and Kimbrough (1983). Specimens were fixed in 2.5% GA + 2% PF + 0.2mM CaCl$_2$ in 0.1 M SCB, pH 7.2, overnight at 4°C. Fixed samples were washed 3 times in SCB, post fixed in 1% OsO$_4$ for 1.5 hrs, washed 2 times in SCB followed by a deionized water (diH$_2$O) wash and dehydrated in an ETOH series followed by several changes in acetone, infiltrated with a Spurr’s resin (Spurr, 1969): acetone series (1:3, 1:1, 3:1, pure resin) and embedded in pure resin, polymerized at 70°C for 2 days, cut with a Diatome diamond knife (Diatome-U.S., Fort Washington, PA) on a Reichert Ultracut S ultramicrotome (Leica, Wien, Austria) mounted on copper slot grids and deposited on Formvar-coated racks according to the method of Rowley and Moran (1975). Sections were post stained with 0.4% uranyl acetate (UA) in 100% methanol for 40 minutes and in lead citrate (PbC) for 20 minutes.

In addition, two ascocarps of *P. citrinus* were fixed in ruthenium red (RR) following the schedule of Handley et al. (1988). Material was fixed in equal parts of 2% GA, 0.2M SCB, pH 7.2 and 1% RR in diH$_2$O overnight at 4°C, washed 3 times in 0.066M SCB, pH 7.2;
postfixed in equal parts 4% OsO₄, 0.2 M SCB, pH 7.2 and 1% RR in diH₂O overnight at room temperature, in a container wrapped in aluminum foil to exclude light; washed in 0.066M SCB, and processed for embedding in Spurr’s resin (see previous section). The sections were not post stained except where noted in text and plates. Sections were viewed with 80 kV on a JEOL 1200EX (JEOL USA, Inc., Peabody, MA) scanning transmission electron microscope (STEM).

Ultrastructure was examined in median sections through the septal pores of the following types of hyphae: ascal, ascogenous, paraphyses and excipular. *P. citrinus* was the primary species examined because all four types of hyphae were clearly differentiated in this species. The other three species were included to compare with *P. citrinus* where possible. Ascogenous hyphae were identified as such only if their connection to an ascus was observed. Paraphyses were identified by their filiform shape and by their position with respect to asci. Outermost excipular cells were identified in all species based on their location in the peridium and their pseudo-parenchymatous form. Light micrographs of *P. citrinus* depict paraphyses in Figs. 6, and 8-11; ascogenous hyphae in Figs. 10 and 11; and excipular hyphae in Figs. 6 and 7.

**Material fixed for TEM and SEM**


**Image capturing and processing**

Light micrographs were obtained on a Leitz Wetzlar (West Germany) Orthoplan compound microscope with a Leica WILD MPS 52 (Leica, Wien, Austria) camera system and Kodak Technical Pan film (Eastman Kodak, Rochester, NY). Film was developed in the dark in 1:1 D-76 developer: water for 9 min., development stopped in a water bath for 30
sec., fixed for 4 min. in Kodak Fixer, washed in a water bath for 15 min., dipped in water with a small amount of Kodak Photo-Flo, and air dried.

TEM micrographs were obtained using Kodak Electron Image film SO-163. Film was processed under a No. 2 red safety light in 1:1 Kodak D-19 developer: water for 2 min., development stopped in an acidified water bath for 30 sec., fixed for 10 min., washed in a running water bath for 30 min., dipped in water with a drop of Kodak Photoflo and air dried.

TEM and LM negatives were scanned on a UMAX Powerlook 1100 scanner, using UMAX Magiscan 4.5 software in the Design Center of ISU. The 35 mm slides were scanned on a UMAX Powerlook 3000 scanner, using UMAX Magiscan 32V4.3 software in the Bessey Microscopy Facility. In the Ada Hayden Herbarium, scanned images were processed in Adobe Photoshop 5.5 and put into plates, with text added using Adobe Illustrator 8.
RESULTS

FIELD DATA ON PACHYPHOEUS SPECIES

Species collected in Iowa and their season

Four species of *Pachyphloeus* were collected: *P. citrinus*, *P. virescens*, *P. melanoxanthus*, and an undescribed species. In addition, one ascocarp of *Scabropezia* was collected, providing a chance to compare fresh characters of *Pachyphloeus* with its putative closest epigeous relative. Most collections were made in July and August of all years (Fig. 3).

![Graph showing percentage of sites in Iowa from which *Pachyphloeus* species were observed during months of 1997 to 2000](image)

First collections were made in mid-June, and final collections in mid-October. In maturing sporocarps, spores became ornamented in mid-July. Sporocarps with mature spores were collected late July through mid-October. Most collections included immature ascocarps or ascocarps with immature spores.

*Pachyphloeus citrinus* was collected from all sites searched (Fig. 3), and *P. virescens* was collected more frequently than *P. melanoxanthus* or *Pachyphloeus* sp. nov. (Fig. 4).
Fig. 4 Numbers of Iowa collections of *Pachyphloeus* species between 1997 and 2000 as an indicator of relative abundance

**Habitat and distribution of *Pachyphloeus* species**

Ascocarps were collected on the soil surface, often under litter or by down logs, or they were shallowly hypogeous, usually less than 2 cm deep. Fruiting bodies were often found under *Quercus macrocarpa*, *Q. alba*, *Q. rubra*, and *Tilia americana*. *Pachyphloeus citrinus* and *P. virescens* were collected from disturbed woods, such as in wooded housing developments and pastures, as well as from rich woods. There is not enough data to draw any correlations between landform and *Pachyphloeus* species occurrence, or between precipitation patterns and occurrence or abundance of species.

**Characteristics of *Pachyphloeus***

*Pachyphloeus* ascocarps can be recognized by the following combination of characteristics: 1.) Ascocarp a “stereothecium” (term coined by Weber et al., 1997), a solid sporocarp with a peridium of somatic tissue completely and continuously enclosing sporogenous tissue in which the asci are persistent; 2.) Ascocarp with round to oblong orifice stuffed with interwoven hyphae, usually pallid and of a different texture than the rest of the peridium (Fig. 5); 3.) Tuft of hyphoid, undulate, septate mycelia that holds dirt and organic debris (Fig. 5), often a different color than the surface of the ascocarp; 4.) Thick excipulum usually sculptured with small cone-shaped verrucae, polygonal warts, or low-beveled pyramidal warts (Fig. 5), of textura angularis (Fig. 7); 5.) Gleba with veins of textura intricata (Fig. 14); 6.) Convoluted hymenium resulting in a marbled appearance (Fig. 12);
and, 6.) Globose spores with coarse to slender spines. The hypha-stuffed orifice and mycelial tuft are usually, but not always, on opposite poles of the ascocarp.

Asci of sporocarps from mature and immature specimens of each species rehydrated in KOH and tested with Melzer's reagent could not be interpreted as producing an amyloid reaction, even when viewed under oil immersion. In comparison, asci of Scabropezia were diffusely and obviously blue with no pretreatment.

Young ascocarps of P. citrinus resemble young unopened apothecia of some cup fungi (Fig. 6), with a thick excipulum surrounding the somatic hyphae that form paraphyses and fertile ascogenous hyphae. The paraphyses are aligned in a palisade along the hypothesized homologous surface of the apothecium, over which the hypha-stuffed orifice develops. Sections of ascocarps that were fixed in RR demonstrate the existence of an extracellular matrix (ECM) surrounding hyphae throughout the excipular and hymenial areas (Figs. 9, 31, 74). The orifice is stuffed with loosely interwoven filiform hyphae that grow over the paraphyses (Figs. 5, 8, 10, 17, 27). These hyphae are different in color and texture from the excipulum, which is composed of large-celled, somewhat isodiametric pseudo-parenchyma (Figs. 5, 7, 13, 17, 19, 23, 27, 28).

Dikaryotic fertile hyphae, ascogenous hyphae, give rise to crosiers (Fig. 31) from which the asci develop. Asci elongate between the paraphyses, as in the other cup fungi, although some asci continue their development in the subhymenial hyphae (Fig. 14), or grow over the top of the paraphyses (Figs. 10, 14) resulting in an irregular hymenium. The hymenium and subhymenium continue to expand within the closed ascocarp, resulting in a highly convoluted arrangement (Figs. 12, 14). This arrangement results in the marbled appearance of the gleba. The pigmented veins consist of asci and paraphyses, and are referred to as fertile veins. The pallid veins consist mostly of subhymenial and somatic hyphae, and are referred to as sterile veins.

Sometimes ascocarps were infested with larval mycophagists. The larvae were observed to ingest material around the spores, effectively releasing them to the outer environment. On a few occasions, spore masses were encountered during collecting, where the soft ascocarp tissue had been mostly consumed.
SPECIES DESCRIPTIONS

*Pachyphloeus citrinus* Berkeley & Broome

**Habitat, season, and distribution**

Fruiting bodies shallowly hypogeous, usually less than 2 cm deep, and often on the soil surface beneath leaf and twig litter under *Quercus macrocarpa*, *Q. alba*, *Q. rubra*, *Q. bicolor*, *Tilia americana*, *Carya ovata* and *C. cordiformis*.

**Season:** Ascocarps collected from mid-June through early October, but most often in July and August. Spores became ornamented beginning mid-July. Sporocarps with mature (ornamented, pigmented) spores collected late July through early October. Immature sporocarps collected late June through August.

**Known distribution:** Japan (Trappe, 1976), China (Zhang, 1992), Europe (Lawrynowicz, 1990), and North America (Gilkey, 1954; Cázares et al., 1992).

**Macro characters**

**Shape and texture:** Stereothecium, radially symmetrical to compressed; globose to laterally flattened and subglobose, with a basal tuft of mycelia. Ascocarp surface sculptured with beveled or polygonal, pyramidal warts 1.5 to 3 mm in diam.

**Size:** Mature ascocarps 3 mm diam. to 15 x 13 x 12 mm.

**Color:** Xanthene orange to amber brown (Fig. 12), occasionally greenish-orange, yellow, or cream-colored. Hypha-stuffed orifice, often in a depression, light orange to pale yellowish-orange to tan in color. Mycelial tuft white to cream in color.

**Odor:** Of burned potatoes, odor more pronounced after storage. When cut open immature specimens emit odor after storage.

**Gleba:** Immature ascocarps have translucent white fertile veins and solid white sterile veins, darkening after being cut, and drying to dark gray with white veins. Mature ascocarps marbled with deep neutral gray to brown fertile veins, and white sterile veins (Fig. 12).

**Micro characters**

**Peridium:** Verrucose, with warts up to 420 µm high, the pigmented portion 30 to 50 µm thick, and outermost tissue light brown or golden-brown under LM; total thickness of
peridium 150 to 850 µm, of textura angularis, with cells more or less isodiametric, and of variable size.

**Sterile veins:** Textura intricata.

**Paraphyses:** Paraphyses arranged in a palisade with asci (Figs. 10, 11), 130 to 278 µm x 5 to 7.5 µm. Paraphysis tip sometimes swollen to 11.7 µm.

**Asci:** Asci typically cylindrical, sometimes clavate, 183.2 to 262 µm x 24 to 37.5 µm with 8 spores in a uniseriate arrangement by maturity (Fig. 15).

**Spores:** Globose, ornamented with fused-tipped coarse spines more or less evenly distributed across the spore. Fusion of spine tips may obscure spines (Figs. 16, 107). Size including sculpturing 15.3 to 25 µm diam., with average range of 17.5 to 22.5 µm diam.; excluding sculpturing 13.9 to 20 µm diam. Spines 1.5 to 5 µm long. Mature spores medium brown in color.

**Comments**

*Pachyphloeus citrinus* can be distinguished from other species of *Pachyphloeus* by the arrangement of paraphyses in a distinct palisade, and by their cylindrical asci with mostly uniseriate spores that have spines with fused tips. No other species have orange colored ascocarps, but the color of the ascocarp is variable such that some may resemble *P. virescens*. The odor of *P. citrinus* collected during this study was the most pungent of the four species collected. Berkeley and Broome (1846, p. 79) likened the odor to “rotting sea-weed”. It was even detectable in the packaging of dried specimens two years old. Although reported to be a widespread species in the U.S. (Arora, 1986), there is little published information on American collections.

**Pachyphloeus virescens** Gilkey

**Habitat, season, and distribution**

Fruiting bodies hypogeous, usually less than 2 cm deep, or on the soil surface beneath leaf or twig litter. The canopy consisted of two or more of the following species: *Quercus macrocarpa*, *Q. alba*, *Q. rubra*, *Q. palustris*, *Ostrya virginiana*, *Tilia americana*, *Carya ovata*, *C. cordiformis*, and *Pinus strobus*
Season: Ascocarps collected from late June through mid-August. Fruiting bodies with mature (ornamented, pigmented) spores collected from late July and immature specimens collected throughout the season.

Known distribution: California, Nebraska (Gilkey, 1939), northern Mexico (Cázares et al., 1992), and China (Zhang, 1992).

Macro characters

Shape and texture: Stereothecium, radially symmetrical to compressed; globose to laterally flattened and subglobose, with a basal tuft of mycelia. Peridium warty with cone shaped verrucae.

Size: Mature ascocarps 4 x 3 x 3 mm to 13 x 9 x 11 mm.

Color: Ascocarps greenish-yellow to raw umber with mummy brown tips of verrucae and cadmium yellow to mustard yellow hypha-stuffed orifices (Fig. 17). The basal tuft of mycelia white to yellow cream to cinnamon brown in color.

Odor: Of burned potatoes, like P. citrinus.

Gleba: Immature ascocarps have fertile veins of olive brown to yellowish-gray, and sterile veins of cream to bright yellow. Mature ascocarps have fertile veins of Saccardo’s umber to medal bronze and sterile veins of cream to bright yellow (Fig. 17).

Micro characters

Peridium: Verrucose, with warts up to 245 µm high, the pigmented portion up to 588 µm thick, and of a bright yellow to golden brown color under LM. Total peridium thickness 196 to 980 µm, and composed of textura angularis, with cells more or less isodiametric, 20 to 50 µm diam.

Sterile veins: Textura intricata.

Paraphyses: 5 to 7.5 µm diam. Distributed among asci, but not in distinguishable palisade.

Asci: Globose or subglobose to clavate (Fig. 18), each with 8 spores irregularly arranged. Size 49.5 µm diam. to 150 x 55 µm.

Spores: Globose, with coarse spines evenly distributed across surface (Figs. 18, 20). Size including sculpturing 16 to 21.3 µm diam., excluding sculpturing 14.6 to 18.8 µm diam. Spines capitate, 1.4 to 2.5 µm long. Mature spores hyaline, occasionally bright yellow.
Comments

*P. virescens* can be distinguished from other species of *Pachyphloeus* by their verrucose ascocarps, composed of cone-shaped rather than hexagonal warts, their spore ornamentation of relatively slender capitate spines, and their subglobose asci arranged irregularly in the gleba. Asci tend to be suglobose although some are clavate. This is the only species collected in Iowa with bright yellow hyphae in the orifice and in the sterile veins, although not all specimens are so brightly colored.

*Pachyphloeus melanoxanthus* Tul and C. Tul

Habitat, season, and distribution

Fruiting bodies hypogeous, usually less than 2 cm deep, or on soil surface beneath litter. *Quercus alba* or *Q. macrocarpa* and *Ostrya virginiana* were present in the canopies of all four collections. Other species present in the canopy of one or more collections included *Prunus* sp., *Tilia americana* and *Juglans nigra*

Season: From late July through early August. There were only four collections, and all appeared to be mature.

Known distribution: Europe (Lawrynowicz, 1990), Maine, and New Hampshire (Gilkey, 1939).

Macro characters

Shape and texture: Stereothecium radially symmetrical to slightly compressed, and globose to subglobose, with a localized tuft of mycelia. Peridium sculptured with hexagonal, pyramidal warts.

Size: Ascocarps 9 x 8 x 8 mm to 15 x 13 x 12 mm.

Color: Purplish black to black (Figs. 21, 22), with reddish brown mycelial tuft. Hypha-stuffed orifice concolorous with rest of ascocarp.

Odor: Little to none.

Gleba: Fertile veins drab to raw umber to light or deep olive gray, sterile veins cream to maize-yellow.

Micro characters
Peridium: Peridium with polygonal warts up to 245 µm high, bright to deep reddish brown under LM; total peridial thickness up to 686 µm, of textura angularis. Cells more or less isodiametric, 60 to 88 µm in diam.

Sterile veins: Textura intricata, hyphae often swollen.

Glebal hyphae: 7.3 µm diam. At septum, some cells swollen to 11.7 µm diam.

Asci: Mostly clavate to pyriform (Fig. 24), with 8 spores biseriately arranged in the ascus. Size 87.5 x 69.5 µm to 107.5 x 48.8 µm.

Spores: Globose, and densely ornamented with slender spines (Figs. 24, 25, 121). Size including sculpturing 16 to 24 µm diam., with average range of 20 to 24 µm diam. Spore size excluding sculpturing 13 to 17.5 µm diam. Spines 1.3 to 6.5 µm long. Mature spores hyaline to orange-brown.

Comments

Iowa collections of *P. melanoxanthus* fit the description of Berkeley and Broome (as *Choireomyces melanoxanthus* Berkeley & Broome, 1967). This species can easily be distinguished from all other species by the sporocarp color, black at all stages (Berkeley and Broome, 1846), and by the spore sculpturing of slender spines, which are never with swollen tips. The paraphyses are not identifiable as the glebal hyphae are swollen.

*Pachyphloeus* sp. nov.:

Habitat, season, and distribution

Fruiting bodies hypogeous, usually less than 2 cm deep, or on the soil surface beneath litter. *Quercus* sp. alone in the canopy of half the collections, and present with other species in 100% of the collections. Canopy species included *Quercus macrocarpa*, *Q. alba*, or *Q. rubra*, *Prunus serotina* and *Ostrya virginiana*.

Season: collected July and October.

Macro characters

Shape and texture: Stereothecium radially symmetrical to slightly compressed, with basal tuft of mycelia. Ascocarp surface sculptured with low, flat, polygonal verrucae evenly dispersed over the surface except for the margin of the hypha-stuffed orifice which
is rough; verrucae 0.3 to 1 mm in diam., 4- to 5-sided, tips darker in color than rest of the surface.

**Size**: Mature ascocarps 8 x 8 x 6 mm to 17 x 15 x 13 mm.

**Color**: Morocco Red, hypha-stuffed orifice orange rufous (Figs. 26, 27). Mycelial tuft white to cream-colored.

**Odor**: No odor at first, but strongly of burned potatoes after confinement.

**Gleba**: Pallid at all stages (Fig. 26). Sterile veins white becoming grayish on drying, fertile veins white becoming light drab to cream-colored.

**Micro characters**

- **Peridium**: Orangish-brown under LM, 368 to 630 µm thick including verrucae.
- Verrucae 158 to 263 µm high. Composed of textura angularis changing to textura intricata next to the gleba. Cells more or less isodiametric, 26 to 35 µm in diam. Walls 2.5 to 3.8 µm thick. Basal mycelial tuft light brown, the hyphae 5 to 10 µm in diam., some with granular incrustations along the exterior.
- **Sterile veins**: Textura intricata, 8.7 µm in diam.
- **Glebal hyphae**: 7.3 to 10 µm diam. at septum, some cells swollen to 11.7 µm diam.
- **Asci**: Asci formed more or less in a palisade, clavate to clavate-cylindrical, usually wider in the center than at either end and pedicellate (Fig. 29). Asci 162.5 x 40 to 200 x 37.5 µm, 8-spored, spores in a uniseriate to biseriate arrangement.
- **Spores**: Spores globose, ornamented with coarse spines dispersed evenly over the spore surface. Spines with acute or truncate tips, occasionally capitate (Figs. 30, 129). Mature spore color usually chlorinous, occasionally greenish-yellow or light brown. Size including spines 20 to 21.3 µm diam; excluding spines 15 to 17.5 µm diam. Spines 2.5 to 5 µm in length.

**Comments**

This species differs from the others in the reddish-brown color of the peridium and the pallid color of the gleba at maturity. The spines on the spores are often truncate and coarse rather than acute and slender as in *P. melanoxanthus* or capitate and slender as in *P. virescens*, or with fused spine tips as in *P. citrinus*. The asci are clavate, not pyriform as in *P. melanoxanthus*, cylindrical as in *P. citrinus* or subglobose as in *P. virescens*. 
Scabropezia flavovirens (Fuckel) Dissing and Pfister,

Habitat, season, and distribution: On soil under down log.

Canopy species included Quercus rubra, Tilia americana and Ostrya virginiana.

Season: Collected only once, in August.


Macro characters

Shape and texture: Excipulum exterior with pronounced warts, and basal mycelial tuft; medullary excipulum gelatinized with drying,

Size: Ascocarp 7.5 mm diam.

sculpturing, and 11 to 14 μm diam., excluding sculpturing. Spines 1 to 2 μm high.

Comments

The excipulum of S. flavovirens is similar to that of all Pachyphloeus, and the spore morphology like that of P. virescens. Scabropezia fruiting bodies are apothecia, while Pachyphloeus are stereothecia. Scabropezia fruits on the soil, while Pachyphloeus usually fruits under the soil surface. The asci of S. flavovirens are much narrower and the spores appreciably smaller than those of any Pachyphloeus. The paraphyses are considerably longer than those of P. citrinus. The number of differences between Pachyphloeus and Scabropezia suggests that these two taxa should be retained as separate entities. There are good photographs of Scabropezia in Dissing and Pfister (1981, pp. 103-107, Figs.1-7); and in Hansen et al. (2001, p. 959, Fig. 7).
Fig. 5  Ascocarps of *Pachyphloeus virescens* (left), with verrucose surface; *P. citrinus* (middle) with polygonal warts; and *P. citrinus* (right) with low-beveled pyramidal warts. Ascocarps in top row show hypha-stuffed orifice (O), those in bottom row show basal mycelial tuft (BM). Bar = 2.5 mm

Figs. 6-8  LM of resin embedded *Pachyphloeus citrinus*

6. Cross-section of young ascocarp showing thick excipulum (E), and palisade of paraphyses (P) below the hypha-stuffed orifice (O). Bar = 250 µm
7. Excipulum (E). Fixed in RR. Bar = 100 µm
8. Paraphyses (P) lining hypha-stuffed vein (SV). Fixed in RR. Bar = 50 µm

Fig. 9  TEM of *Pachyphloeus citrinus* fixed in RR showing extracellular matrix (ECM) around paraphyses (P) to the right and under hypha-stuffed vein (SV). Bar = 4 µm

Figs. 10-11  LM of resin embedded *Pachyphloeus citrinus*

10. Hymenium of asci (As) and paraphyses (P). Note hyphae and asci over the paraphyses tips (SV), resulting in irregular hymenium in slightly older ascocarp. Bar = 50 µm
11. Development of asci (As) from ascogenous hyphae (AH) in subhymenium. Asci elongate between the paraphyses (P). Bar = 50 µm
Figs. 12-16 *Pachyphloeus citrinus*

12. Mature ascocarp half on left showing excipulum (E), gleba (G) with white veins mostly of sterile hyphae (SV), brown veins mostly of asci (FV); and ascocarp half on right showing peridial surface with low polygonal warts (P). Bar = 2 mm

13. LM paraffin section showing excipulum (E) and gleba (G). Bar = 100 µm

14. LM paraffin section showing hymenium in fertile vein (FV) with irregular palisade of asci. Note asci (As) developing in the ‘sterile’ vein (SV). Bar = 100 µm

15. LM of fresh ascus with ascospores, mounted in water. Bar = 25 µm

16. SEM of spore in ascus. Bar = 1 µm
Figs. 17-20  *Pachyphloeus virescens*

17. Top half of mature ascocarp (left) showing peridial surface with dark brown-tipped verrucae (arrows) and hypha-stuffed orifice (O), bottom half of mature ascocarp (right) showing marbled gleba (G) composed of cream-colored veins of mostly sterile hyphae (SV) and brownish gray veins of asci and spores (FV). Bar = 4 mm

18. LM of fresh asci with ascospores, mounted in water. Bar = 10 μm

19. LM paraffin section showing excipulum (E) and gleba (G). Bar = 100 μm

20. SEM of spore. Bar = 1 μm
Figs. 21-25 *Pachyphloeus melanoxanthus*

21. Mature ascocarp with marbled gleba (G) composed of cream-colored veins of mostly sterile hyphae (SV) and raw umber veins of asci and ascospores (FV). Bar = 3 mm

22. Mature ascocarp halves showing marbled gleba (left) and black peridial surface with polygonal warts (right). Bar = 3 mm

23. LM of paraffin section showing excipulum (E) and gleba (G). Bar = 50 µm

24. LM of fresh ascus with ascospores, mounted in water. Bar = 25 µm

25. SEM of spore. Bar = 10 µm
Figs. 26-30 *Pacyphloeus* sp. nov.

26. Mature ascocarp halves. Left half showing pallid gleba (G) composed of opaque white veins of mostly sterile hyphae (SV) and translucent white veins of asci and spores (FV), right half showing peridial surface. Bar = 4 mm

27. Top of mature ascocarp showing hypha-stuffed orifice (O). Bar = 4 mm

28. LM paraffin section showing excipulum (E) and gleba (G). Bar = 50 µm

29. LM of fresh ascus with ascospores, mounted in water. Bar = 20 µm

30. SEM of spore. Bar = 2 µm
SEPTAL PORE ULTRASTRUCTURE

Septal pore apparatus

Five consistent features were observed in septal pores of *P. citrinus*: uni- or bi-convex bands; lamellate structures; Woronin bodies; septal wall augmentation; and an amorphous electron-opaque matrix (Table 2, p. 52). The uni- and bi-convex bands both consist of a homogeneous, moderately electron-dense material and were present at the bases of asci. The lamellate structures are a series of electron-dense and electron-translucent bands arranged transverse to the septum. The electron-dense bands are similar in appearance to unit membranes, with an electron-translucent lumen sandwiched between two electron-dense sheets. Up to four lamellate structures were observed in a single section through a pore. They are present in reproductive and vegetative hyphae. No differences were observed between lamellate structures in different types of hyphae. The Woronin bodies are present in all but ascal cells. They are globose to subglobose and larger than the associated pores, except for a few observed within the pores that were the same diameter as the pore, and ovoid. Septal wall augmentation was viewed only in ascal septae. Amorphous electron-opaque material was observed in pores of hyphae of all types (Table 2, p. 52).

Septal pores of asci

Septal pores at the base of asci were viewed only in *P. citrinus* and *P. virescens*. In *P. citrinus* the structure associated with the septal pore at the base of a young ascus was a bi-convex band. Structures observed in the pores of older asci included uni-convex bands, a lamellate structure, and septal wall augmentation which frequently encompassed an amorphous electron-opaque matrix.

Bi-convex bands occurred in the septal pores of a young ascus (Figs. 31-33). One of these bands was clearly membrane bound (Fig. 32). The fusion region of the ultimate and antipenultimate cells of the crozier was open (Fig. 31). A uni-convex band on the ascus side of the septum was observed in other asci (Figs. 34, 35). In one of these, a lamellate structure
projected into the pore from the septal pore plate rim on the side of the cell subtending the ascus (Fig. 34). On the ascus side, electron-dense condensed ascoplasm with irregularly arranged endoplasmic reticulum (ER) formed a hemispherical mound over the septum at the base of the ascus (Fig. 35). This differentiation of ascoplasm in the ascus could be viewed also at the LM level in fixed, stained material (Fig. 38).

In older asci of *P. citrinus* and *P. virescens*, septal wall augmentation was observed to form thick hemispherical evaginations into cells on either side of the septum (Figs. 36, 37). The augmented septal wall had a core of electron-opaque amorphous material that was presumably completely enveloped by the thickened wall.

**Septal pores of ascogenous hyphae**

Septal pores of ascogenous hyphae were observed only in *P. citrinus*. Observed septal pore features included a bi-convex band, lamellate structures and Woronin bodies. Some pores had no occluding apparatus visible.

A bi-convex band was observed in the septal pore of ascogenous hyphae leading to a young ascus (not shown). Lamellate structures were often directed away from the pore, or partially obstructed the pore (Figs. 39-42). Woronin bodies were occasionally observed to plug the pore in ascogenous filaments where a healthy cell adjoined a cell with deteriorating cytoplasm (Fig. 39). Pores with no occluding apparatus often had structures within them, but it could not be determined if these had been stationary or migrating (Figs. 40, 43, 44). There was a mitochondrion situated in the middle of one pore (Fig. 40), and vacuoles straddled two others (Fig. 43, 44). A biperforate septum was observed in one filament (Fig. 44).

**Septal pores of paraphyses**

Paraphyses were identified only in *P. citrinus*, but glebal septal pore features in other species were similar to those of paraphyses in *P. citrinus*. Structures associated with the septal pores of paraphyses commonly included Woronin bodies, lamellate structures, uni-convex bands, and amorphous electron-opaque material.

Woronin bodies were observed in the vicinity of most pores (Figs. 45-50). In Fig. 45 the pore does not appear to be completely sealed off by the Woronin body, and there is an
electron-translucent zone, devoid of organelles or ribosomes, around the Woronin body. A uni-convex band was observed in one of the upper cells of a branched paraphysis (Fig. 77), and another was apparently displaced from a pore plugged with a Woronin body (Fig. 78).

Lamellate structures spanned the septal pores, or projected away from them (Figs. 46, 48, 78). Amorphous electron-opaque material completely occluded the pore, and wrapped around the septal pore rim (Fig. 47). In some sections, two projections on the septal pore rim were observed, which were obscured for the most part by the lamellate structure (Figs. 46, 48).

Uni-convex bands were observed in unidentified glebal hyphae (Figs 49, 50). In these, the convex bands were accompanied by either the projections from the pore rim or another band spanning the pore. In Fig. 50, the other band was clearly a lamellate structure. The accompanying structures in Fig. 49 are similar to the projections described previously. The projections appear to span the pore in the off-median section in Fig. 49.

Ultrastructural features in septal pores of glebal hyphae of the other species were similar to those of the paraphyses of *P. citrinus*. Features commonly included lamellate structures spanning the septal pores (Figs 51, 53, 54) or projecting away from the pores (Figs. 52, 57), Woronin bodies (Figs. 53-55, 57-59), amorphous electron-opaque pore plug material (Figs. 52, 59, 60), and uni-convex bands (Figs. 54, 56).

**Septal pores of excipular cells**

Structures associated with the septal pores of excipular cells were similar in all species. These structures commonly included amorphous electron-opaque material, Woronin bodies, and lamellate structures.

In a young ascocarp of *P. citrinus*, a Golgi-like structure (Figs. 61, 62) occluded the pore of two adjacent excipular cells proximal to the glebal area. Some pores were unoccluded, or only partially occluded, especially in excipular cells relatively close to the gleba (Fig. 63). Amorphous electron-opaque material (Fig. 64 in *P. citrinus*, Fig. 67 in *P. melanoxanthus*) and Woronin bodies were commonly found within the pores (Fig. 65 in *P. citrinus*, Fig. 66 in *P. virescens*).
*Pachyphloeus melanoxanthus* differed from the other species in the presence of small hexagonal structures in the cytoplasm (Fig. 51). They were approximately 30 nm in diameter, hexagonal in shape, membrane bound, sometimes with an electron-dense core, and with electron-dense spines radiating from the angles of the hexagon. These are interpreted as virus-like particles because they were found in only one species, represented by a single specimen, and appear similar in shape and size to viruses depicted in the cytoplasm of other fungi (Weber, 1979). *Pachyphloeus virescens* also possessed structures not seen in the other species (Figs. 55, 66). They were globose, membrane-bound, electron-dense and approximately 100 nm in diameter. These also fit the criteria for virus-like particles in their uniform shape and size, membrane, cytoplasmic location, and the fact that they were not observed in the other species (Weber, 1979).

Septal pore ultrastructures observed in *Pachyphloeus* species were very similar to septal pore ultrastructures observed by Kimbrough and his associates for species in the Pezizaceae (see Table 2 for references).
Table 2  Comparison between septal pore apparatus reported for somatic and reproductive hyphae in the Pezizaceae\(^1\) and septal pore apparatus observed in *Pachyphloeus citrinus*\(^2\)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Ascal septal pores</th>
<th>Ascogenous septal pores</th>
<th>Paraphysis septal pores</th>
<th>Excipular septal pores</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Woronin bodies</strong></td>
<td>Rare(^1)</td>
<td>Occasional(^1)</td>
<td>Common(^1)</td>
<td>Common(^1)</td>
</tr>
<tr>
<td></td>
<td>Not observed(^2)</td>
<td>Occasional(^2)</td>
<td>Common(^2)</td>
<td>Common(^2)</td>
</tr>
<tr>
<td><strong>Uni-convex or bi-convex bands</strong></td>
<td>At base of young asci(^1)</td>
<td>Common(^1)</td>
<td>In basal cell(^1)</td>
<td>Occasional(^1)</td>
</tr>
<tr>
<td></td>
<td>At base of young asci(^2)</td>
<td>Occasional(^2)</td>
<td>Not uncommon(^2)</td>
<td>Not observed(^2)</td>
</tr>
<tr>
<td><strong>Lamellate structure</strong></td>
<td>Occasional, associated with convex band(^1)</td>
<td>Occasional, associated with convex band(^1)</td>
<td>Common(^1)</td>
<td>Common(^1)</td>
</tr>
<tr>
<td></td>
<td>Occasional, associated with convex band(^2)</td>
<td>Not uncommon(^2)</td>
<td>Common(^2)</td>
<td>Common(^2)</td>
</tr>
<tr>
<td><strong>Amorphous electron-opaque material</strong></td>
<td>In pore, surrounded by secondary wall(^1)</td>
<td>In pores of older cells(^1)</td>
<td>In pores of older cells(^1)</td>
<td>Occasional(^1)</td>
</tr>
<tr>
<td></td>
<td>In pores of older asci, surrounded by septal wall thickenings(^2)</td>
<td>Occasional(^2)</td>
<td>Not uncommon(^2)</td>
<td>Common(^2)</td>
</tr>
<tr>
<td><strong>No septal pore apparatus</strong></td>
<td>Occasional(^1)</td>
<td>In upper cells(^1)</td>
<td>Occasional(^1)</td>
<td>Occasional(^1)</td>
</tr>
<tr>
<td></td>
<td>Not observed(^2)</td>
<td>Common(^2)</td>
<td>Not observed(^2)</td>
<td>Common proximal to gleba(^2)</td>
</tr>
<tr>
<td><strong>Secondary wall material or septal wall augmentation</strong></td>
<td>In pores of mature asci(^1)</td>
<td>Occasional(^1)</td>
<td>Not reported(^1)</td>
<td>Not reported(^1)</td>
</tr>
<tr>
<td></td>
<td>In pores of older asci(^2)</td>
<td>Occasional(^2)</td>
<td>Not observed(^2)</td>
<td>Not observed(^2)</td>
</tr>
</tbody>
</table>

\(^1\) Pezizaceae data in top row cells, from Curry and Kimbrough (1983); Kimbrough et al. (1991); Kimbrough (1994)

\(^2\) *Pachyphloeus citrinus* data in bottom row cells
Figs. 31-36 TEM of septal pores at the base of asci of *Pachyphloeus citrinus*. Figs. 31-33 of material fixed in RR.

31. Young ascus (As) formed from crozier. Ultimate cell (U) and antipenultimate cells (APU) of crozier are fused. Note extracellular matrix (ECM). Bar = 2 μm

32. Bi-convex band (arrows) in septal pore between ascus (AS) and ultimate cell (U). Note electron-opaque inclusion (EOI) in ultimate cell. Bar = 100 nm

33. Bi-convex band in septal pore between ascus (As) and antipenultimate cell (APU). Bar = 100 nm

34. Uni-convex band (CB) on ascus (As) side of pore, and lamellate structure (arrow). Bar = 100 nm

35. Uni-convex band (CB) with superimposed condensed electron-dense material on ascus (As) side of pore. Bar = 300 nm

36. Thickened septal wall (Spl) around electron-opaque matrix (MX) in septal pore. Unstained section. Bar = 200 nm

Fig. 37 TEM of ascal septum of *Pachyphloeus virescens* with electron-opaque material (MX) in septal pore surrounded by augmented septal wall (Spl). Note sporoplasmalemma (arrows) bordering septal wall on ascus side (As) of pore. Bar = 200 nm

Fig. 38 LM of *Pachyphloeus citrinus* ascus (As) with hemispherical mounds of differently staining material over septae (arrows). Bar = 10 μm
Figs. 39-44 TEM of ascogenous hyphal septae of *Pachyphloeus citrinus*

39. Woronin body (W) in pore. Material fixed in RR. Bar = 200 nm
40. Pore with mitochondrion (arrow) and lamellate structure. Bar = 100 nm
41. Branching ascogenous filament (AH). Ascus (As). Bar = 2 µm
42. Lamellate structures (arrows). Bar = 100 nm
43. Unobstructed pore straddled by vacuole (V). Bar = 200 nm
44. Biperforate septum, one pore with vacuole (V), the other unobstructed. Bar = 200 nm
Figs. 45-48  TEM of septal pores in paraphyses of *Pachyphloeus citrinus*

45. Woronin body (W) with electron-translucent zone (arrow) in pore. Material fixed in RR and post stained with UA and PbC. Bar = 200 nm

46. Lamellate structure spanning pore, with associated Woronin body (W). Septal plate rims with projections (arrows). Bar = 200 nm

47. Electron-opaque pore plug matrix (MX) with Woronin body (W). Bar = 200 nm

48. Lamellate structure spanning pore, septal pore rim with projections (arrows). Bar = 200 nm

Figs. 49, 50  TEM of septae in unidentified glebal hypae of *Pachyphloeus citrinus*

49. Uni-convex band and pore rim projections in off median section (arrow). Bar = 200 nm

50. Uni-convex band with lamellate band enlarged to show two narrow membrane-bound electron-translucent structures (arrows) abutting each other and projecting from rim of septal pore plate into pore, beneath the electron-dense lamellate band (arrows). Bar = 200 nm
Figs. 51-54  TEM of septal pores in glebal hyphae of *Pachyphloeus melanoxanthus*

51. Lamellate structure spanning pore. Virus-like particles (arrows and insert). These same structures are observed in Figs. 52-54. Bar = 200 nm

52. Lamellate structures, electron-opaque amorphous material (MX). Bar = 200 nm

53. Woronin body plugging pore, note degenerated membrane; lamellate structures spanning pore; scant accumulation of electron-opaque material (arrow). Bar = 200 nm

54. Uni-convex band (CB) and lamellate structures (arrows). Bar = 200 nm
Figs. 55-57 TEM of septal pores in glebal hyphae of *Pachyphloeus virescens*

55. Woronin body in pore, lamellate structures. Virus-like particles (arrows). Bar = 200 nm

56. Uni-convex band and lamellate structure. Bar = 100 nm

57. Woronin body in pore, lamellate structures, electron-opaque material (arrows). Bar = 200 nm

Figs. 58-60 TEM of septal pores in glebal hyphae of *Pachyphloeus* sp. nov.

58. Woronin body in pore spanned by lamellate structure. Bar = 100 nm

59. Electron-opaque matrix (MX), lamellate structure (see Fig. 60), and Woronin body. Bar = 200 nm

60. Enlarged portion of Fig. 59 showing lamellate structure (arrow). Bar = 50 nm
Figs. 61-65  TEM of septal pores of excipular hyphae of *Pachyphloeus citrinus*

61. Short lamellate structure, pore occluded by membranous structure. Bar = 300 nm
62. Enlargement of lamellate structures (arrows) in Fig. 61. Bar = 100 nm
63. Electron-opaque amorphous material (MX), lamellate structure (arrow). Bar = 100 nm
64. Electron-opaque amorphous material (MX), Woronin body (W). Bar = 100 nm
65. Woronin body (W) in pore of two vacuolate cells. Vacuoles (V). Bar = 200 nm

Figs. 66-67  Septal pores of excipular hyphae in species of *Pachyphloeus virescens*

66. Woronin body (W) in pore between vacuolate and cytoplasmically rich cells. Virus-like particles in right cell (arrows) Bar = 200 nm
67. Woronin-like body (*) in pore, with electron-opaque plug material (MX) and lamellate structures (arrows). Bar = 100 nm
Septal pore occlusion

In addition to morphological data for phylogenetic information, there were general observations regarding septal pore occlusion and the amorphous electron-opaque matrix. Electron-opaque matrix material and septal pore occlusion are universal features in fungi (Markham, 1994), and since no differences were visualized among species of *Pachyphloeus*, observations from all species are combined.

Septal pore occlusion is interpreted here to be progressive. Lamellate structures rarely appeared to play a role in total occlusion except where they were seen to span the pore. However, the extent of occlusion by lamellate structures and uni- or bi-convex bands could not be assessed without serial sections (which were not made in this study). In an occluded pore, the occluding structure was often a Woronin body, but it did not always appear to provide a complete seal (Fig. 45). A septal pore between a cell with degenerating cytoplasm and a cell that was cytoplasmically rich, was occluded by a Woronin body from the cytoplasmically rich cell (Figs. 39, 45, 55, 66, 78). A Woronin body was always larger in diameter than the pore with which it was associated, except where an ellipsoid Woronin body was completely lodged in the middle of a pore (Fig. 69). No ellipsoid Woronin bodies were observed outside of the pores, thus it is assumed that in these cases the shape was modified during the plugging process.

Various amounts of amorphous electron-opaque material accumulated in and around septal pores. Accumulation appeared to begin around the Woronin body in the pore (Figs. 68, 69, 71) or along the septal pore wall in the vicinity of the pore (Figs. 57, 63, 78). Complete occlusion appeared to be achieved with the electron-opaque matrix material alone (Fig. 64) or typically in conjunction with other pore structures (Figs. 71-73, 75, 77-80). With accumulation of amorphous electron-opaque material, the membrane of the Woronin body became less distinct, as though ‘nibbled’ (Figs. 53, 64, 69), and finally disappeared (Fig. 71). By definition, a Woronin body is membrane-bound (Curry and Kimbrough, 1983; Markham, 1994), therefore the structure in a pore that resembles a Woronin body in shape, location, and electron-density; but lacks a membrane, is referred to as a Woronin-like body. The Woronin-like bodies in the center of some occluded pores, appeared to be integrated along their
margins with the matrix material (Figs. 72, 75). These were not as electron-dense as membrane-bound Woronin bodies.

It is of interest to note the effect of RR fixation on septal pore ultrastructure of *P. citrinus*, as it may shed light on the character of the amorphous electron-dense matrix material. In RR fixed material that had not been post stained (Figs. 31-33, 68, 69, 74-78), some of the amorphous electron-opaque material stained heavily (Figs. 68, 69, 74-78), and in some places appeared to be fibrillar (Figs. 77, 78). Near the septal pore, it appeared to originate between the plasmalemma and the septal pore wall (Figs. 76, 79). The side of the plasmalemma that faces the cell wall stains particularly heavily. For the most part, the structures that stained as heavily as the amorphous electron-opaque matrix were the plasmalemma, certain vesicles along with their contents (Fig. 32), material in a few vacuoles (Fig. 78), and the ECM (Figs. 9, 31, 33). Some of the vesicles with densely staining membranes were observed to originate from or fuse with the plasmalemma. It is possible that the fibrillar appearance of plasmalemma-associated electron-opaque material is an artifact of the RR fixation, but it appears to be specific to the plasmalemma, especially near the septal pore and occasionally in vesicles in the cytoplasm.

Figure 80 shows the comparative opacity of matrix stained with OsO$_4$ alone. In this case, material was kept in OsO$_4$ for 1.5 hrs rather than overnight. One of the most notable differences between this fixation and fixation that included RR is the relative lack of staining of the plasmalemma in the former.
Figs. 68–73 TEM of electron-opaque material in septal pores

68. Excipular pore of *P. citrinus* fixed in RR. Woronin body in pore with a little pore plug material (arrow), vesicles (Vs) developing from plasmalemma. Bar = 200 nm

69. Paraphysis cell of *P. citrinus* fixed in RR. Pore plugged by ellipsoid Woronin body (W) associated with vacuole, and amorphous electron-opaque material apparently continuous with vesicles from plasmalemma (arrow). See Fig. 76 for enlargement. Bar = 200 nm

70. Glebal hypha of *P. melanoxanthus*. Electron-opaque pore plug (MX) and Woronin body (W) in pore with lamellate structures possibly displaced from pore (arrows). Bar = 200 nm

71. Paraphysis hypha of *P. citrinus*. Woronin body (W) with indistinct boundaries in pore, with associated electron-opaque material (MX). Bar = 200 nm

72. Glebal hypha of *P. melanoxanthus*. Electron-opaque pore plug (MX), lamellate septal structure (arrow), and Woronin-like body in pore (WL). Bar = 200 nm

73. Glebal hypha of *Pachyphloeus* sp. nov. Electron-opaque material (MX) and convex band (arrow) in pore. Bar = 300 nm
Figs. 74-80 TEM of *Pachyphloeus citrinus*

74. Global hypha: paraphysis (P), extracellular matrix (ECM), ascus (As). Fixed in RR, not post stained. Bar = 3 µm

75. Subhymenial hypha septal pore occupied by Woronin-like body (WL) surrounded by electron-opaque material, and vesicles developing from plasmalemma. Fixed in RR, not post stained. Bar = 200 nm

76. Enlargement of Fig. 69 showing connection of electron-opaque material with plasma membrane (PM) proliferation along septal wall. Fixed in RR, not post stained. Bar = 50 nm

77. Paraphysis septal pore with convex (possibly bi-convex) band (CB) and electron-opaque fibrillar substance (arrow) adhering to band in cell closer to the tip of the paraphysis. Fixed in RR, not post stained. Bar = 100 nm

78. Paraphysis septal pore with Woronin body (W) in pore, lamellate structures, displaced convex band (CB), and electron-opaque amorphous material (arrow). Fixed in RR, not post stained. Bar = 200 nm

79. Paraphysis septal pore with Woronin body (W), electron-opaque material, proliferation of plasmalemma and vesicles along the septal wall. Fixed in RR and post stained with UA and PbC. Bar = 200 nm

80. Paraphysis septal pore in material fixed in GA and PF and for 1.5 hrs in OsO₄, but with no RR. Not post stained. Note lighter staining of the electron-opaque matrix (MX). Woronin body (W). Bar = 200 nm
SPORE WALL ONTOGENY

Ascospore wall ontogeny was followed in *P. citrinus* to a greater degree than in the other three species.

**Primary wall development**

The earliest stage of ascosporogenesis detected was after spore delimitation by a double-membrane (Figs. 81, 82). The delimiting membranes were each approximately 4.5 nm in diameter, and had approximately 21 nm of primary wall between them. The sporoplasm was identical in appearance to the epiplasm except for a lack of nuclei in the epiplasm. Each spore had one nucleus, more or less centrally located with a zone of organellar exclusion around it. The nucleus on the right in Fig. 81 has the remains of a spindle apparatus at the two poles, indicating recent division.

In the next stage observed, the primary wall of the spore was approximately 490 nm thick, composed of a homogeneous electron-translucent material, and superimposed by the investing membrane (Fig. 83). Tubular structures approximately 12-14 nm in diameter (Fig. 84) were observed along the perimeter of the primary wall, close to the investing membrane. The sporoplasm was filled with mitochondria, small vacuoles with a few lipid bodies, and a single nucleus. The investing membrane evaginated slightly into the epiplasm irregularly along the primary wall, and this evaginated area contained condensed relatively electron-dense material (Fig. 85).

**Expansion of the investing membrane**

During the next stage, the investing membrane expanded into the epiplasm. The space between the investing membrane and the primary wall was relatively homogeneous except for the presence of the tubular structures described in the previous stage (Fig. 86). Fibrils were visualized throughout the perisporal sac, and connections with the primary wall were numerous (Fig. 87). The numbers of lipid bodies and ER increased in the epiplasm. In the
sporoplasm, ER increased, the nucleus was still centrally located, and there were many mitochondria and a few small lipid bodies.

The epiplasm became increasingly vacuolated as it degenerated (Figs. 88, 89) until vacuoles surrounded the spores, and tonoplast membranes aligned in many places with the investing membrane (Figs. 89-92). At the same time, the investing membrane continued to expand, and the perisporal sac accumulated a greater density of flocculose material (Figs. 88-92) that had numerous contacts with the primary wall (Fig. 92). Vesicles, remnants of epiplasm containing lipid bodies, among other substances, were in contact with the tonoplast and investing membranes, and often constricted between them (Figs. 88-92). Bridges of epiplasm containing heterogeneous materials between vacuoles were in contact with the investing membrane (Figs. 89, 91). Flocculose material in the perisporal sac increased as the epiplasm decreased. Tubular structures were present in the perisporal sac throughout this stage of spore development, and the nucleus was located in the center or off to one side. Mitochondria were enlarged, and along with ER, fewer in number, indicating a slowdown in metabolic processes within the sporoplasm.

**Epispore (SW1) development**

The first observed deposition on the primary spore wall was of material destined to become epispore (SW1). This electron-dense, fibrillar material accumulated in a thin layer, unevenly distributed over the primary wall (Fig. 93). This stage is interpreted here as the initiation of secondary wall development, and is labeled SW1 to indicate that it is the first layer of secondary wall. SW1 uniformly covered the primary wall. In the sporoplasm, the nucleus maintained its central position, and lipid bodies increased in size (Fig. 94). In the perisporal sac, tubular structures were absent, the flocculose material increased appreciably in density, and fibrils were in contact with the secondary wall in numerous places (Figs. 95, 96). The investing membrane and tonoplast together formed a double-membrane around the spore wall (Figs. 92, 95, 96). Flocculose material in the perisporal sac continued to increase in density. The epispore became evident as a laminate and relatively electron-opaque region with secondary wall material deposited over it (Figs. 100, 103, 104, 106). The mature
epispore was approximately 42 nm thick and consisted of up to five alternating electron-translucent and electron-dense bands of various diameters.

**Endospore (SW2) development**

The sporoplastalemma became undulate, and a thin electron-dense band was discernable at the base of the primary wall (Figs. 96, 97). This is interpreted as the beginning of endospore (SW2) formation. The endospore developed between the sporoplastalemma and the inner face of the primary wall (Fig. 97), and is interpreted here as an addition to the primary wall rather than a modification of it. Therefore, the endospore is labeled SW2 for the second deposition of wall material on the primary wall. Endospore was not always discernable. The mature endospore is visible in Figs. 101, 103, and 106.

**Secondary wall (SW3) elaboration**

An uneven accumulation of electron-dense secondary wall material (SW3) over the epispore (SW1) resulted in mounds or ornaments of a homogeneous composition (Figs 97, 98). The ornaments had lacunae that were continuous, in some cases, with the perisporal sac (Figs. 100, 103). Lipid bodies within the sporoplasm increased markedly in size (Figs. 99, 102). There was some variation among spores in the timing of development of a single, large central lipid body. In some spores, it occurred at this stage, and in others, it occurred later.

**Production of spines coated with filiform fibers (SW4)**

Mounds of SW3 continued to accumulate perisporal fibers until spines formed. At the same time, there was an accumulation of SW3 along the inner perimeter of the investing membrane. The sporoplasm was occupied for the most part by a large central lipid body.

Subsequently, a loose weft of long filiform fibers (SW4), accumulated along the spine columns and bases, but not along the spine tips nor on SW3 along the investing membrane (Figs. 101, 102). These fibers were approximately 6 nm in diameter and were arranged in pairs with an electron-translucent lumen between two electron-dense strands.

The final development was of short fasciated fibrils (SW5) approximately 50 to 105 nm long radiating from SW3 of the spine column, base, and along the investing membrane (Figs.
The filiform fibers (SW4) overlaid these radiations. At last, the flocculose material in the perisporal sac became disorganized, less dense (Fig. 105), and largely absent in older spores (Fig. 104), and an electron-dense band underlaid the fibrils (Fig. 104). At this point, the investing membrane was separated from the tonoplast (Fig. 104). The fasciated fibrils (SW5) could be viewed in grazing sections through the spore as well (Fig. 105). In SEM view, the spore is obscured by the SW3 connections between spines (Fig. 107).

**Secondary wall development in P. virescens**

Early epispore (SW1) deposition in *P. virescens* differed from *P. citrinus* in the formation of condensed electron-opaque globules in the perisporal sac, and deposition of these globules on the primary wall (Fig. 108). Globules flattened where they contacted the primary wall. Flocculose material in the perisporal sac had numerous connections with the globules and the primary wall (Fig. 109).

The next stage appeared similar to *P. citrinus*. The epispore (SW1) was evenly distributed along the primary wall, and mounds of secondary wall material (SW3) with lacunae formed on top of the epispore (Fig. 110). Secondary wall (SW3) material accumulated on the tops of the mounds until spines were formed with electron-dense caps. Deposition was more concentrated at the spine tips (Fig. 111). Electron-dense caps were not observed on spore spines of *P. citrinus*. One feature observed only in *P. virescens* was the formation of electron-dense globules in the perisporal sac during spine formation (Fig. 111). *P. virescens* had a well formed endospore (SW2), approximately 166 nm thick, an epispore (SW1) approximately 44 nm thick, and the primary wall was 440 nm thick. There was an accumulation of secondary wall (SW3) material along the inner perimeter of the investing membrane, but only in some spores and not to the same extent as in *P. citrinus* (Fig. 112). Filiform fibers (SW4) similar to those in *P. citrinus* overlay the SW3 material along the columns, base of spines, and tips as well (Fig. 113). The perisporal sac became highly vacuolated (Fig. 114), and collapsed around the spines (Fig. 113). The SW3 did not become appreciably fimbrillate as in *P. citrinus*. Views with SEM revealed spines thickened at the tips on some spores, while other spores had spines joined one to another by SW3 material (Fig. 116).
Secondary wall development in *P. melanoxanthus*

Early secondary wall deposition in *P. melanoxanthus* was not observed, but mounds of material with lacunae (SW3) accumulated over the epispore as in the other species (Fig. 117). Spine tips had electron-dense caps, where deposition was concentrated (Fig. 118). The epispore (SW1) was approximately 42 nm, the endospore (SW2) 37 nm, and the primary wall 295 nm in diameter. Filiform fibers (SW4) covered all SW3 deposition. These fibers were paired, electron-dense with an electron-translucent center, approximately 3-4 nm in diameter, and in a tight, parallel arrangement. There were no fasciated fibrils (SW5) in this species. The final structure of the ornamentation was of long slender spines composed of a core of homogenous material (SW3) wrapped in fibers (SW4), with the investing perisporium collapsed around them (Fig. 118). Figure 118 shows the view in SEM, with a small amount of investing membrane adhering to some of the spore spine tips.

Secondary wall development in *Pachyphloeus sp. nov.*

As in *P. virescens*, epispore (SW1) deposition resulted from condensation of electron-dense globules in the perisporal sac (Figs. 122, 124). Granular-flocculose material in the perisporal sac had numerous connections with the electron-dense globules and the primary wall. Globules in contact with the primary wall flattened (Figs. 122, 123). Ornamentation (SW3) occurred as in the other species, with deposition concentrated at the electron-dense caps of the spine tips (Fig. 125). The epispore (SW1) was 34 nm, the endospore (SW2) was 50 nm and the primary wall was 442 nm thick. Long filiform fibers (SW4) similar to those seen in the other species were observed when the spines came in contact with the investing membrane. The fibers were approximately 10 nm in diameter, and developed from flocculose material in the perisporal sac. Flocculose material became organized as short parallel fibers, most clearly viewed near the IM in the two spores in Fig. 127.

Final development was of fasciated fibrils (SW5) approximately 123 nm long radiating from the SW3 material, and under the long, loose weft of fibers of SW4 (Fig. 128). The tonoplast and investing membranes collapsed over the spines. In SEM view, the mature spore spines are plainly visible, and connections between the spine tips rare (Fig. 129).
Figs. 81-85 TEM of young ascospores of *Pachyphloeus citrinus*

81. Two ascospores with delimiting membranes (arrows), nuclei (N), and spindle pole bodies (arrowheads, insert of top spindle pole body). Bar = 1 µm

82. Delimiting membranes: sporoplasmalemma (SM) and investing membrane (IM) with incipient primary wall between them. Bar = 200 nm

83. Older spore with well-developed primary wall (PW), note also epiplasm (Epl); investing membrane (IM); lipid body (L); nucleus (N); sporoplasmalemma (SM); sporoplasm (Spl); vacuole (V). Bar = 1 µm

84. Higher magnification of primary wall in Fig. 83, showing tubular structures between primary wall and investing membrane (arrows, insert of structures indicated by bottom arrow). Bar = 200 nm

85. Primary walls with investing membrane evaginating into epiplasm (arrow). Bar = 200 nm

Figs. 86-88 TEM of expansion of perisporal sac in *Pachyphloeus citrinus*

86. Perisporal sac (PS) containing fibrillar material. Note also Epiplasm (Epl) with small lipid bodies (L); and sporoplasm (Spl) with central nucleus and numerous ER and mitochondria. Bar = 1 µm

87. Higher magnification of spore in Fig. 86 showing primary wall (PW) and perisporal sac between investing membrane (IM) and sporoplasmalemma (SM). Note numerous ER (arrows) in sporoplasm. Bar = 200 nm

88. Perisporal sac with condensed fibrillar material and tubular structures (arrowheads) in evaginations of investing membrane (IM), which is aligned in places with tonoplast (T) of vacuoles (V) in epiplasm. Note lipid body (L) in epiplasm. Bar = 200 nm
Figs. 89-92 TEM of continued expansion of perisporal sac in spores of *Pachyphloeus citrinus*

89. Expanded perisporal sac (PS), vacuolated (V) epiplasm. Note small lipid bodies (L) in sporoplasm. Bar = 1 μm

90. Expanded perisporal sac (PS) with condensed flocculose material, lipid bodies (L) in epiplasm, between vacuoles (V). Bar = 1 μm

91. Expanded perisporal sac (PS), cytoplasmic bridges containing lipid bodies (L) between vacuoles (V) in epiplasm. Bar = 1 μm

92. Higher magnification of spore in Fig. 91 showing alignment of the investing membrane (IM) with the tonoplasts (T) of vacuoles (V), and tubular structures (arrowhead) in the perisporal sac (PS). Sporoplasm (Spl) Bar = 200 nm

Figs. 93-96 TEM of initiation of secondary wall with epispore deposition (SW1) in *Pachyphloeus citrinus*

93. Beginning of epispore deposition (SW1) on the primary wall. Note numerous connections of epispore material with flocculose material in perisporal sac (PS). Bar = 100 nm

94. Continued expansion of perisporal sac, increased density of flocculose material, and uniform distribution of epispore (SW1) over primary wall. Note increased size of lipid bodies (L) in sporoplasm. Bar = 2 μm

95. Higher magnification of spore in Fig. 94 showing epispore (SW1) on primary wall (PW). Perisporal sac (PS). Bar = 200 nm

Fig. 96 TEM of initiation of endospore formation with invagination of sporoplasmalemma (SM) into sporoplasm (Spl). Bar = 200 nm
Figs. 97-103 TEM of secondary wall ornamentation on spores of *Pachyphloeus citrinus*


98. Older spore with endospore (arrowhead) and ornamentation mounds. Bar = 500 nm

99. Older spore with lacunae in ornamentation mounds. Note enlarged lipid bodies (L) in sporoplasm. Bar = 1 µm

100. Ornamentation (SW3) of secondary wall showing lacunae (arrowhead) continuous with the perisporal sac (PS). Note epispore (SW1) and large lipid body (L). Bar = 200 nm

101. Older spore with well developed ornamentation of the secondary wall (SW3) and with fibrilous fibers (SW4) overlaying the ornaments. Note well developed epispore (SW1) and endospore (SW2). Primary wall (PW). Bar = 200 nm

102. Nearly mature spore. Note large lipid body (L), and fusion of spine tips through deposition of SW3 along investing membrane (arrows). Bar = 1 µm

103. Higher magnification of spine from spore in Fig. 102, showing lacuna (arrowhead) in continuity with perisporal sac (PS), and SW3 overlayed with fibrilous fibers (SW4). Note secondary wall material (SW3) deposited along investing membrane (IM) and epiplasmic material deposited along tonoplast (T). Primary wall (PW) between striated epispore (SW1) and endospore (SW2). Bar = 200 nm
Figs. 104–106 TEM of final stage of spore wall development in *Pachyphloeus citrinus*

104. Thickened spine tips of SW3 ornamentation is continuous with SW3 material deposited along investing membrane, except for small gaps (arrowhead). Short fibrils (SW5) perpendicular to secondary wall radiating uniformly from SW3, overlayed by fibrilous fibers (SW4) along spine column and along SW3 over primary wall. Fixed in RR. Bar = 200 nm

105. Grazing section through spore showing SW3 deposition along the investing membrane, disorganization of the IM and tonoplast (arrowhead), the layering of loosely arranged fibrilous fibers (SW4) along the spine columns and thickened tips (SW3), and the short fibrils (SW5) radiating at right angles from SW3. Bar = 200 nm

106. Arrangement of loose fibrilous fibers (SW4) over short fibrils (SW5) radiating from SW3. Note striated epispore, well developed endospore, vacuole in epiplasm (V) and tonoplast (T). Fixed in RR. Bar = 200 nm

Fig. 107. SEM of *P. citrinus*. Note holes in SW3. These correlate with gaps observed in deposition of SW3 along the investing membrane. Mounds indicate spine tips. Bar = 1 µm
Figs. 108-115 TEM of secondary wall development in *Pachyphloeus virescens*

108. Beginning of epispore development with deposition of electron-dense condensed globules. Bar = 1 µm

109. Higher magnification of condensed globule (SW1) flattened where it contacts primary wall. Note intimacy of tonoplast with investing membrane, most evident when compared with places where they split away as separate unit membranes (arrow). Bar = 200 nm

110. Lacunae in SW3 ornamentation superimposed over SW1 in *P. virescens*. Bar = 200 nm

111. Spine development (SW3) with electron-dense caps on spine tips. Note relatively thick endospore (SW2), striated epispore (SW1), primary wall (PW) and condensed granules (arrows) in the perisporal sac (PS). Bar = 200 nm

112. Deposition of secondary wall (SW3) along investing membrane (IM). Bar = 300 nm

113. Nearly mature spore with capitate spines (SW3), and fibrilous fibers (SW4) along spine columns and bases. Investing membrane and tonoplast are collapsed around spines, and vacuoles occur between spines. Bar = 200 nm

114. Nearly mature spore with perisporal sac becoming vacuolated. Note large lipid body (L) and relative lack of SW3 along investing membrane. Bar = 1 µm

115. Oblique section through nearly mature spore showing fibrilous fibers (SW4) wrapped around SW3. Bar = 200 nm

Fig. 116 SEM of *Pachyphloeus virescens* spore halves in ascus demonstrating capitate spines on one spore, and spines connected by SW3 material on another spore. Bar = 5 µm
Figs. 117-120  TEM of secondary wall development in *Pachyphloeus melanoxanthus*

117. Secondary wall development with lacunae in the ornamentation. Note epispore. Bar = 200 nm

118. Spines with electron-dense caps and lacunae. Note epispore, and thin endospore. Bar = 200 nm

119. Cross section through spines of nearly mature spore demonstrating SW3 core wrapped in fibrilous fibers (SW4). Note disorganization of IM and T (arrows). Bar = 200 nm

120. SW4 encased spines on mature spore. Bar = 200 nm

Fig. 121  SEM of mature spores of *Pachyphloeus melanoxanthus* in ascus. Note perispornium, present as thin membrane adhering to spine tips of spore at top. Bar = 5 µm
Figs. 122–128 TEM of secondary wall development in *Pachyphloeus* sp. nov.

122. Beginning of epispore development with condensation of electron-dense globules in perisporal sac, flattened where they contact primary wall. Bar = 1 µm

123. Higher magnification of spore in Fig. 122 showing flattened, condensed material along primary wall. Bar = 200 nm

124. Higher magnification of Fig. 122 showing condensed globules in contact with flocculose fibers in the perisporal sac. Bar = 200 nm

125. Development of spine (SW3) with electron-dense cap surrounded by relatively electron-dense material in the perisporal sac (PS). Bar = 200 nm

126. Developing spines with electron-dense caps and lacunae, but no condensed globules in the perisporal sac (PS). Note large lipid bodies (L) in the sporoplasm. Bar = 2 µm

127. Beginning of SW4 with layering of long fibrilous fibers along SW3. Note synthesis of fibers from flocculose material in the perisporal sac (arrowheads and insert). Bar = 200 nm

128. Mature spore with radially arranged fibrils (SW5) perpendicular to primary wall, and fibrilous fibers (SW4) over SW5 along spine column and base. Note disorganization of IM and T (arrowhead). Bar = 200 nm

Fig. 129 SEM of mature spores of *Pachyphloeus* sp. nov in ascus. Bar = 5 µm
Identity of *Pachyphloeus citrinus*

When Fischer (Gilkey, 1916) declared *P. carneus* to be conspecific with *P. citrinus*, Gilkey accepted his opinion, but with reservations. During this study, the misgivings that Gilkey communicated resurfaced because the characters that concerned her are the same as characters in the Iowa specimens that consistently differ from the type specimen description. As Gilkey noted, the majority of North American ascocarps are orange, a prominent characteristic that is not mentioned for the type specimen, or for any other British specimens (Hawker, 1954; Dennis, 1978). Ascocarps of the North American taxon lack powdery lemon yellow particles and do not blacken with age as described for the type specimen (Berkeley and Broome, 1846). These discrepancies cannot be resolved by examining the type specimen because the type description was based entirely on fresh macro features of the ascocarp (Berkeley and Broome, 1846) that are not preserved when ascocarps are dried. However, mature spores are well preserved in herbarium specimens. Spores studied from herbarium specimens collected in France differed from the North American taxon in their relatively slender spines, some of which are capitate like the North American taxon, but connections between spine tips were not visualized. It is possible that mature spores were not present in the slides examined. By comparison, a specimen from California, initially identified as *P. carneus* and annotated as *P. citrinus* by Fischer, has spores resembling those in Iowa collections in their relatively coarse spines that tend to be capitate and connected at the tips. Until study of the type specimen can be made, the North American taxon should be retained as *P. citrinus*.

Dispersal of *Pachyphloeus*

The mode of *Pachyphloeus* spore dispersal is incompletely known. Many truffle species are apparently distributed through mycophagy (Castellano et al., 1989). Observations during this study indicate that invertebrate (larval) mycophagy is one way that spores are released from the sporocarp. Presumably, the spores are subsequently carried by soil water for short distances in the soil. It is difficult to explain the widespread occurrence of *Pachyphloeus*
species solely through passive spore dispersal. No mycophagy studies have identified *Pachyphloeus* in their analyses. Given the wide distribution of *P. citrinus*, and the fact that mycophagy studies were undertaken in woodlands where this species occurs (Maser et al., 1978; Maser and Maser, 1987; Pastor et al., 1996) the absence of *Pachyphloeus* spores from animal stomachs or scat indicates that if mammal mycophagy occurs at all, it may not be the most common way that *Pachyphloeus* is dispersed. This is surprising because the sporocarps have traits that suggest mammalian mycophagy as a major mechanism in dissemination. They exude a strong aroma, and the spores are large and thick walled compared to those of their putative closest epigeous relative *Scabropezia*. It would be of interest to undertake a mycophagy study in Iowa woodlands during July and August, the peak fruiting months of *Pachyphloeus*.

There may be other ways that *Pachyphloeus* is dispersed. The linking of *Pachyphloeus* to the anamorph *Glischroderma* (Norman and Egger, 1999) is intriguing. *Glischroderma* produces conidia in an ostiolate peridiate structure on the soil surface (Malençon, 1964). If *Pachyphloeus* has a conidial stage, and the asexual propagules are produced on the soil surface, disturbance of the conidia followed by wind dispersal could help to explain the widespread distribution of species of *Pachyphloeus*. Norman and Egger did not find a match between molecular sequences from the 18S rRNA gene of *P. melanoxanthus* and *Glischroderma*, but it would be logical to look for the teliomorph in North American samples of *P. citrinus* since this is reported to be the most common species in the United States (Arora, 1986), and the analyzed sample of *Glischroderma* originated in New York (Norman and Egger, 1999). The identification of an anamorph in a hypogeous genus of the Pezizaceae would not be surprising given the occurrence of anamorphs among other taxa in this family (Eckblad, 1968), and has implications of potential anamorphs for other Pezizaceous truffles such as *Hydnobolites*.

**Phylogeny of Pachyphloeus**

Septal pore data from this study provides morphological evidence for affiliation of the hypogeous truffle genus *Pachyphloeus* with the predominantly epigeous Pezizaceae in the Pezizales, and complements molecular data (Percudani et al., 1999; Norman and Egger,
that lead to the same phylogenetic conclusion. Genetic distances in molecular data analyzed by Percudani et al. (1999) were interpreted to indicate derivation of hypogeous Pezizales from epigeous Pezizales rather than the reverse. Molecular evidence suggests that *Pachyphloeus* shares a common ancestor with the epigeous cup fungus *Scabropezia*, and that species divergence took place relatively rapidly (Norman and Egger, 1999). Van Brummelen (1978) interpreted lack of operculae in truffles as a reduction that took place in response to change in mode of spore dispersal. Based on these interpretations, the common ancestor of *Pachyphloeus* and *Scabropezia* had operculate asci and forcibly discharged its spores. While *Scabropezia* retained the ancestral traits of operculate asci and forcible spore discharge, *Pachyphloeus* lost both of these traits after a change in mode of spore dispersal during its adoption of a hypogeous lifestyle. Since ascospores of *Pachyphloeus* are not dispersed by wind, there is no advantage to forcibly discharge them, and therefore no advantage to form an operculum. Consequently, mutations affecting these two features would not be selected against.

Morphological differences in the asci of *Pachyphloeus* species show a progression from the pleisiomorphic cylindrical shape shared by *Scabropezia* and *P. citrinus* to the highly derived subglobose to globose shape in *P. virescens*. *Pachyphyloeus melanoxanthus* and *Pachyphloeus* sp. nov., with their clavate to pyriform asci, fall between these two extremes. The salient ascus features of the Pezizaceae that are lacking in *Pachyphloeus* are apparently not too genetically complex to change relatively rapidly. An ascus with a cylindrical shape is necessary for the build up of pressure needed to forcibly discharge spores (Raju, 1988), and it takes a single gene mutation to change the shape of an ascus (Berbee and Taylor, 1995). Therefore, if there is no selective advantage for forcible spore discharge, there is no selective advantage in the production of cylindrical asci over asci of other shapes.

Thiers (1984) speculated that derivation of hypogeous from epigeous taxa in both Basidiomycetes and Ascomycetes resulted from neotony. Study of young specimens of *Pachyphloeus* gave credence to this view. Ascocarp development in some Pezizalean taxa passes through a cleistohymenial stage before opening into an apothecium (Kimbrough, 1970). The young ascocarp of *Pachyphloeus* is similar to that of a young cup fungus in which the margin of the excipulum exceeds the apices of the paraphyses, nearly enclosing
them. Hyphae that overgrow the surface of the hymenium in a loose interwoven weft complete the enclosure, resulting in the hyphal-stuffed orifice. The continuous development of a cleistohymenium that fails to open could result in the convoluted gleba of mature *Pachyphloeus*. Dennis (1978) envisioned an evolutionary progression in the Tuberales from an organized hymenium with a palisade of asci, the situation in the Pezizales, to a disorganized hymenium of asci scattered in the gleba, the situation in many of the Tuberales, and interpreted the latter situation as advanced. His interpretation is followed here. The hymenial organization in species of *Pachyphloeus* exhibit the same pattern of relative species divergence as their asci, with a highly organized and recognizable hymenium in *P. citrinus*, a less organized gleba with swollen hyphae in *P. melanoxanthus* and *Pachyphloeus* sp. nov., and an irregular distribution of asci in the gleba of *P. virescens*. The evolutionary progression of an organized to a disorganized hymenium could result from the loss of selective pressure to produce cylindrical asci in a palisade designed to forcibly discharge spores simultaneously for wind dispersal. Therefore, it is reasonable to think that there would be no selection against a disorganized hymenium in a hypogeous Ascomycete.

The presence of this range of ascus morphology and hymenial organization within a single genus implies a low taxonomic value for these characters in hypogeous fungi. However, perhaps sequence of divergence may be hypothesized based on these characters. If an evolutionary progression among species that occur in Iowa can be inferred from ascus and hymenial structure, then *P. citrinus* is the least derived from a common ancestor, *P. virescens* the most derived, and the other species fall between these two.

**Septal pore ultrastructure**

*Pachyphloeus* species have the following ultrastructural similarities with septal pores of taxa in the Pezizaceae: a uni- or bi-convex band at the base of asci (the “pezizoid septal type” of Curry and Kimbrough, 1983), and large, spherical Woronin bodies in all cells except asci. Woronin bodies have been observed in ascogenous hyphae of the Pezizaceae (Curry and Kimbrough, 1983), Pyronemataceae (Kimbrough and Curry, 1986b), Ascobolaceae (Kimbrough and Curry, 1985) and in asci of *Leucangium* and *Thelebolus* (Li, 1997). Woronin bodies of *Pachyphloeus* were of average size for Pezizaceous species, although
some surpassed 750 nm diameters, the top diameter in the range cited by Markham (1994). In keeping with their presumed function of rapid occlusion of septal pores of old or injured cells, they were usually larger in diameter than the septal pores with which they were associated. Ovoid forms found within a couple of septal pores may have become misshapen during the process of plugging pores.

The lamellate structure (the "peziza septal type" of Curry and Kimbrough, 1983) common in pores of vegetative hyphae, and present in pores of ascogenous hyphae of *Pachyphloeus* are common in all other Pezizalean taxa studied. The composition, origin and function of lamellate structures and convex bands are not known (Kimbrough, 1994; Markham, 1994). They have been postulated to function as "subcellular sieves" (pg. 1102 of Markham, 1994).

The two septal pore rim projections observed in association with some of the lamellate structures are strongly reminiscent of the projections associated with the amorphous electron-dense pore plug in *Neurospora*-type septae found in Pyrenomycetes (see Figs. 2, and 9b; in Trinci and Collinge, 1973; and Fig. 16A in Curry and Kimbrough, 1983). This similarity in structure is intriguing, and is perhaps visual evidence for homology. Perhaps the *Peziza*-type septum is an elaboration of the *Neurospora*-type septum, through derivation of the first or reduction of the latter.

The augmented septal pore walls in older ascogenous and ascal hyphae of *Pachyphloeus* have also been reported in the Ascobolaceae (Kimbrough and Curry, 1985), the Pyronemataceae (Kimbrough and Curry, 1986a), the Sarcoscyphaceae, the Sarcosomataceae (Li and Kimbrough, 1995a), and the Helvellaceae (Kimbrough, 1991; Kimbrough et al., 1996) of the Pezizales. Septal wall augmentation around the pore may serve a dual function of maintaining turgor in the ascus, and preventing anything from entering or leaving the ascus (Wells, 1972). A similar phenomenon, termed consolidation, has been reported in old or injured cells of Ascomycetes, Basidiomycetes and deuteromycetes, and is thought to function as a permanent walling off of one cell from another (Markham, 1994).

Amorphous electron-opaque pore plug material similar in appearance to that observed in *Pachyphloeus* has been reported for a wide variety of Ascomycetes (Markham, 1994). Despite the prevalence of this material in septal pore plugs throughout the Ascomycota, surprisingly little is known about its composition or origin. Cytochemical tests for non-
water-soluble polysaccharides in this material have been negative (Markham, 1994). It does not appear to consist of protein as it withstood pronase digestion (Pellegrini et al., 1989). It is involved in pore plugging in old or injured cells, healthy somatic cells (McKeen, 1971, Markham, 1994), and ascal cells. There is evidence that deposition of the matrix in the pore plug is in some cases, in response to ageing (Markham, 1994). In this study, the electron-opaque matrix appeared to envelope all other septal pore structures during pore plugging, and the Woronin body appeared to degenerate and become integrated with it.

The function of lamellate structures is unknown. The lamellate structures have substructural units that are membrane-like in appearance. If the lamellate structures are membranous, these membranes would greatly expand the area over which plasmalemma function occurs, increasing the efficiency of cellular exchange in a filament. Microscopy alone cannot resolve the extent to which a particular type of occlusion serves as either a selective barrier allowing cytoplasmic continuity and ion exchange while preventing organelle migration; or as an impenetrable barrier separating old or injured cells from healthy ones; or as suggested by Gull (1978) walling off cells to allow differentiation to occur within the ascocarp.

Previous researchers have illustrated their interpretations of pore plugging as a progressive deposition of electron-dense pore plug material (Trinci and Collinge, 1973; Markham, 1994). While there is no proof that pore plugging follows such a sequence, that interpretation has not been disproved and is a logical scenario. A similar sequence is proposed here for *Pachyphloeous*. This sequence of plugging was deduced with the assumption that pores connecting cytoplasmically-rich cells with Woronin bodies enveloped by a complete membrane represent the youngest pores, and pores connecting empty or nearly empty cells represent the oldest pores. It was assumed that pores with recognizable Woronin bodies in various stages of disorganization are intermediate between these two extremes. Occlusion of a septal pore begins with a Woronin body that possesses a complete membrane, and no electron-opaque matrix material (Fig. 55). A minute amount of electron-opaque material forms between the septal pore rim and the Woronin body (Fig. 68). The margin of the Woronin body becomes irregular (Fig. 64). Electron-opaque material appears to be integrated with the Woronin body in some places (Figs. 69-71), and the membrane continues
to degenerate. Finally, the membrane disappears, the margin of the Woronin body is no longer discrete, and electron-opaque material appears to be integrated with it (Fig. 71). Figures 72 and 75 show a Woronin-like body in the center of a pore surrounded by electron-opaque matrix. These structures have no discernable membranes, and fibrils of electron-opaque matrix are integrated with them. The structure in Fig. 75 is less electron-dense than other Woronin bodies. If it represents the remains of a Woronin body, it appears to be much altered. Pore plug occlusion appears to occur in more than one way, and complete occlusion appears to be accomplished by more than one type of material.

The heavy staining of electron-opaque pore plug material by RR/OsO₄ fixation has not been previously reported. Ruthenium red in conjunction with OsO₄ is thought to bind to free carboxyl or hydroxyl groups and has been used to fix and/or stain extracellular matrices of bacteria and fungi (Erdos, 1986; Handley et al., 1988). The other two fixatives used on this material, OsO₄ and GA, fix lipids and proteins, respectively (Handley et al., 1988). It is not known whether RR is converted to ruthenium tetroxide during fixation. Ruthenium tetroxide is capable of binding to lipids and proteins as well as to polysaccharides. Unfortunately, a control for OsO₄ was not included during fixation of this material. The fixation protocol called for overnight exposure to a combination of OsO₄ and RR. Such a long exposure time in osmium could increase the amount of lipid fixation and intensity of stain. However, the fact that staining of the plasmalemma in osmium alone was light compared to the intensity of staining by the layer of the membrane that faces the cell wall (the exterior face) in material fixed in RR/OsO₄, could implicate a glycoprotein component asymmetrically associated with one polar end of the lipid bilayer.

Origin of the amorphous pore plug material was not resolved in this study, but some of the material appears to originate in the plasmalemma, or in vesicular membranes that originate from the plasmalemma. Previous investigators suggested that degradation products from Woronin bodies contribute to the pore plug matrix (Markham, 1994), an idea supported by data from this study. Since RR did not stain all of the pore plug matrix material equally, and since a variety of structures are enveloped and possibly degraded in and incorporated by this matrix, it is probable that the complete pore plug is composed of more than one type of material.
Evidence in this study indicates that a variety of structures may contribute to the initial plugging of septal pores. If septal pore structures serve other functions, those functions are not known. It appears that the convex bands tend to remain in place, but the lamellate structures do not permanently occlude the pore. The lamellate structures have substructural units that are membrane-like in appearance. If the lamellate structures are membranous, these membranes would greatly expand the area over which plasmalemma function occurs, increasing the efficiency of cellular exchange in a filament. Studies utilizing microscopy alone cannot resolve the extent to which a particular type of occlusion serves as either a selective barrier allowing cytoplasmic continuity and ion exchange, while preventing organelle migration; or as an impenetrable barrier separating old or injured cells from healthy ones; or as suggested by Gull (1978) walling off cells to allow differentiation to occur within the ascocarp.

There are no previous reports of extensive ECM in the ascocarps of truffles. There are reports of a mucilaginous sheath detected at the EM level on the outer ascus walls of a number of Pezizalean species, especially in those that were freeze substituted (Kimbrough and Gibson, 1990; Mims et al., 1990; van Brummelen, 1993). Samuelson (1978), who fixed material conventionally, but without RR reported a correlation between asci of the Pezizales with mucilaginous sheaths and asci with amyloid reactions to Melzer’s solution. Those that were amyloid (taxa of the Pezizaceae and Ascobolaceae sensu Rifai, 1968) had mucilaginous sheaths. Since the ECM in those species was retained without special fixation methods, they apparently possess an ECM with a different composition and/or organization than those with ECM that is only retained and/or stained with RR/OsO₄ fixation, such as found in Pachyphloeus asci, which are not amyloid. The function of ECM in sporocarps of nonpathogenic fungi is unknown. Roles could include protection of asci and developing ascospores from dessication, a medium in which aromatic compounds could be stored or released into the environment under the right conditions, and/or a medium in which protective compounds such as antibacterial agents could be located.
Secondary spore wall ontogeny

Evidence from this study suggests that both the sporoplasm and the epiplasm contribute to secondary spore wall development in *Pachyphloeus*. Vesicles with epiplasmic materials were frequently seen in close association with the investing membrane, and amorphous electron-dense material was observed between the investing membrane and associated tonoplast. Concurrent with expansion of the perisporal sac and vacuolization of the epiplasm, lipid bodies increased in the epiplasm, and are interpreted as storage bodies for the break-down products of epiplasmic organelles. Lipids and other epiplasmic products are presumably modified for crossing the investing membrane, to enter the perisporal sac as the homogeneous fibrillar material deposited on the primary wall and subsequent secondary walls SW1 and SW3. The SW4 appears to be modified and assembled from flocculose material in the perisporal sac. The SW5 appears to be a modification of SW3.

The tubular bodies observed in the perisporal sac could be a result of sectioning through convolutions of the investing membrane. During spore ontogeny, the investing membrane extends a great deal from the original delimiting length. It appears to enlarge and expand prior to secondary wall development, and stabilize about the time secondary wall development begins. If the investing membrane is fully expanded when secondary wall development begins, this membrane would be taut, and sectioning through folds would not be possible. This situation would explain the absence of “tubular bodies” at the time of epispore formation.

The numerous mitochondria and ER in the sporoplasm of the youngest spores indicates high metabolic activity, which might implicate the sporoplasm as a source for primary wall material. During secondary wall construction, the sporoplasm has relatively few mitochondria, and a marked increase in size of lipid bodies, which would imply a conversion of carbohydrates and proteins in the sporoplasm to lipid for storage. The lipid bodies are centrally located in the sporoplasm. The sporoplasm is interpreted in this study to contribute the endospore (SW2) because the sporoplast membrane becomes undulated during endospore development, and the primary wall does not change in diameter. Other secondary wall
developments did not appear to originate in the sporoplasm as no electron-dense materials passed through the primary wall.

In contrast, epiplasmic material was constantly in contact with the investing membrane, vesicles were abundant and the epiplasm steadily became vacuolate. The epiplasm and investing membrane/tonoplast membrane complex persisted almost to maturity in *Pachyphloeus* species. Persistent epiplasm and persistent involvement of epiplasmic tonoplasts and investing membranes in spore wall development are features common in the Pezizales, but not in other Ascomycetes (Read and Beckett, 1996). If the epiplasm contributes to secondary spore wall development, and if epiplasmic persistence results from a high proportion of this material relative to the amount present in other Ascomycetes, one may infer that the epiplasm contributes to the relatively elaborate ornamentation of spores in some of the Pezizales.

While asynchronous spore development was observed in all species of *Pachyphloeus*, the difference between stages of spore development within an ascus was not great. A more dramatic asynchronous development of spores within an ascus has been reported in another truffle *Leucangium* (Li, 1997). It would be interesting to know if asynchronous spore development is associated with individual spore delimitation as found in *Tuber* and *Terfezia* versus delimitation by an ascus vesicle as found in epigeous Pezizales. *Tuber* species also have asynchronous spore development (personal observation). Unfortunately, the type of delimitation in *Pachyphloeus* was not determined.

Species of *Pachyphloeus* differed in the way initial deposition of the epispore occurred. Material destined for epispore development was deposited directly on the primary wall in *P. citrinus*, but condensed first into electron-opaque globules in the perispore prior to deposition in *P. virescens* and *Pachyphloeus* sp. nov. This variation is probably not significant. Two species of *Sarcoscypha* (Fr.) Boud. in the Sarcoscyphaceae, *S. coccinea* (Jacq.: Fr.) Lambotte and *S. occidentalis* (Schw.) Sacc., vary in the same way (Li, 1994). Lacunae in the secondary wall ornamentation of *Pachyphloeus* is reported to be uncommon, but has also been documented in the non-Pezizaceous genera *Rhizina* Fr.: Fr., *Geopyxis* (Pers.: Fr.) Sacc., and *Gyromitra* Fr. (Kimbrough and Gibson, 1990). For this reason, lacunae are not interpreted here as phylogenetically important at the family level.
Merkus found seven different types of spore wall development in her extensive ultrastructural study of taxa in the Pezizales (Merkus, 1976). Taxa in the Pezizaceae followed one of two patterns: secondary wall material accumulated on the epispore to became either a permanent ornamentation or a permanent smooth layer uniform in thickness. Secondary spore wall development in *Pachyphloeus* followed the first of these developmental patterns, consistent with its hypothesized placement in the Pezizaceae. The general pattern of primary spore wall development, epispore formation and endospore formation were similar to those seen in species of *Peziza* Dill.: Fr. (Merkus, 1975; Dyby and Kimbrough, 1987), *Hydnobolites* (Kimbrough, 1991) and *Terfezia* (Janex-Favre and Parguey Leduc, 1985; Janex-Favre et al., 1988). The spore wall development of *Plicaria trachycarpa* (Curr.) Boud. (cited as *Peziza trachycarpa* Curr.) was remarkably similar to that of *Pachyphloeus* species in its capitate spines that were sometimes connected with each other, and the fibrillar fringe along the ornaments (Merkus, 1975). This species was included in a molecular phylogenetic analysis of the Pezizaceae, but came out in a different clade than *Pachyphloeus* (Hansen et al., 2001). Differences occurred among species of *Pachyphloeus* and species of the Pezizaceae during secondary wall development, especially in the quantity and location of deposition, and in the resulting architecture.

In comparison with other truffles, secondary wall development in *Pachyphloeus* was similar to *Terfezia* and *Hydnobolites* until final deposition. Both genera differed from *Pachyphloeus* during final wall deposition in the lack of filiform fibers (SW4) or fasciated fibrils (SW5) (Janex-Favre et al., 1988; Kimbrough, 1991). In contrast, secondary wall development in species of *Tuber* is very different from *Pachyphloeus*. In spore walls of *Tuber*, there does not appear to be an endospore (SW2) or material comparable to SW3. Instead, the ornamentation appears to consist solely of filiform fibers similar to those of SW4 (Janex-Favre and Parguey-Leduc, 1980; Janex-Favre and Parguey-Leduc, 1983; Parguey-Leduc and Janex-Favre, 1987).

Final ornamentation of spores in *Pachyphloeus* is considerably more elaborate than in *Peziza* or *Plectania* (Li and Kimbrough, 1995c). It is also more elaborate than in *Terfezia* or *Hydnobolites*. An elaborate secondary wall is thought to be important in spores that are dispersed through mycophagy, a mode of dispersal not yet demonstrated for *Pachyphloeus*. 
Major differences among species of *Pachyphloeus* were not seen until the final stages of spore ornamentation. These differences were in the amount and location of SW3 deposition, in the amount of filiform fibers (SW4) that accumulated over the ornaments, and in the modification of SW3 in the development of SW5. The coarseness of the spines on spores of *Pachyphloeus* sp. nov. appears to correlate with the amount of, or possibly the spaces between, filiform fibers (SW4) covering the SW3 deposition.
SUMMARY

1.) *Pachyphloeus citrinus* was the most commonly collected species of *Pachyphloeus* in Iowa during four consecutive field study years.

2.) Four species of *Pachyphloeus* were collected in Iowa: *P. citrinus*, *P. melanoxanthus*, *P. virescens* and *Pachyphloeus* sp. nov.

3.) Species of *Pachyphloeus* were collected less than two cm deep in soil under *Quercus* sp. and *Tilia americana*.

4.) *Pachyphloeus* species fruit from mid-June through mid-October, and species richness and abundance are highest in July and August.

5.) While similarities between *Scabropezia* and *Pachyphloeus* are acknowledged, especially regarding spore morphology and excipular cells, the differences between their spore sizes, ascus tip morphologies, ascus amyloidity, growth habits, and ascocarp structures suggest keeping these two taxa as separate genera rather than synonymizing them as suggested by Norman and Egger (1999).

6.) In some cases, ascospores were released passively through disintegration of the ascocarp, which was sometimes facilitated by insect mycophagy.

7.) Observations of uni- and bi-convex bands at the base of asci in *Pachyphloeus citrinus*, are new ultrastructural evidence to add to molecular evidence (Percudani et al., 1999; Norman and Egger, 1999), and macromorphological evidence (Dissing and Pfister, 1981) that *Pachyphloeus* is a member of the Pezizaceae (Pezizales).

8.) All internal hyphae of ascocarps of *Pachyphloeus citrinus* are enveloped by extracellular matrix.

9.) Spore wall development in *Pachyphloeus* is consistent with that of taxa in the Pezizaceae that form permanent ornamentation on top of the epispore.

9.) *Pachyphloeus* spores develop asynchronously in an ascus and asci develop asynchronously in an ascocarp, thus ascocarps are often found with a combination of young asci and asci with mature spores.
10.) Secondary spore wall ornamentation in *Pachyphloeus* is among the most elaborate described for taxa in the Pezizales and more elaborate than secondary spore wall development of genera in the Terfeziaceae.

11.) *Pachyphloeus citrinus* spores are ornamented with spines whose tips are fused.

12.) A new species of *Pachyphloeus* is distinguished by macro, micro and ultrastructural features.

13.) Based on old and new observations of *Pachyphloeus* species, the following definition for this genus is offered: ascocarp a steriothecium with solid, marbled gleba; hypha-stuffed orifice of textura intricata, continuous with sterile veins of the gleba, basal tuft of filiform mycelia often on opposite pole of ascocarp from orifice, thick excipulum of textura angularis, with pigmentation in the outermost cells; asci with eight globose spores; and spores ornamented with spines whose tips may be fused, capitate or acute.
LITERATURE CITED


Dennis, RWG 1978 *British Ascomycetes* Cramer, Vaduz.


Handley, PS, J Hargreaves, and DWS Harty 1988 Ruthenium red staining reveals surface fibrils and a layer external to the cell wall in *Streptococcus salivarius* HB and adhesion deficient mutants. *Journal of General Microbiology* 134: 3165-3172.


Lange, M 1956 Danish hypogeous macromycetes. Dansk Botanisk Arkiv 16: 1-84.


McKeen, WE 1971 Woronin bodies in *Erysiphe graminis* DC. *Canadian Journal of Microbiology* 17: 1557-1560.

Merkus, E 1973 Ultrastructure of the ascospore wall in Pezizales (Ascomycetes)-I


Merkus, E 1974 Ultrastructure of the ascospore wall in Pezizales (Ascomycetes)-II.


Merkus, E 1975 Ultrastructure of the ascospore wall in Pezizales (Ascomycetes)-III


Ridgeway, R 1912 *Color Standards and Color Nomenclature.* Published by author. Washington, D.C.


Shearer, JF and LH Tiffany 1982 A preliminary report of some Tuberales in Iowa.  


Spurr, AR 1969 A low-viscosity epoxy resin embedding medium for electron microscopy.  
*Journal of Ultrastructural Research* 26: 31-42.


ACKNOWLEDGEMENTS

It is my pleasure to acknowledge my advisors Dr. Lois H. Tiffany and Dr. Harry T. Horner for their many provocative questions, good suggestions, and timely words of encouragement. It is with gratitude that I acknowledge the helpful suggestions of Dr. Ed Braun. Anna Gardner is gratefully acknowledged for her computer art wizardry in generating the spore model, the map of Iowa with landforms, and for her patient assistance with scanning and computer software programs. Many thanks are due to Mary Jane Hatfield and Sibylla Brown for contributing ascocarps for this study. I am indebted to the University of Nebraska Herbarium and the National Fungus Collection for their loan of specimens, and to Dr. B. Buyck at the Cryptogamie Herbarium in Paris for accommodating my examination of specimens of *Pachyphloeus*. Deborah Lewis, curator of the Ada Hayden Herbarium at ISU, is especially thanked for her assistance in arranging herbarium visits and borrowing specimens. Marianne B. Smith is gratefully acknowledged for translating a portion of Soehner (1936) from German to English. It is with deep gratitude that I acknowledge the financial support of this project by the Iowa Science Foundation and the Gilman Fund of the Department of Botany at Iowa State University. All microscopy was accomplished in the Bessey Microscopy Facility, and much appreciation is extended to Dr. Horner and Tracey Pepper who keep the facility running smoothly.