Aspects of Ceratocystis montia pathology and ontogeny

Russell Albert Blauel
The University of Montana

Follow this and additional works at: https://scholarworks.umt.edu/etd
Let us know how access to this document benefits you.

Recommended Citation
https://scholarworks.umt.edu/etd/1609

This Thesis is brought to you for free and open access by the Graduate School at ScholarWorks at University of Montana. It has been accepted for inclusion in Graduate Student Theses, Dissertations, & Professional Papers by an authorized administrator of ScholarWorks at University of Montana. For more information, please contact scholarworks@mso.umt.edu.
ASPECTS OF CERATOCYSTIS MONTIA PATHOLOGY AND ONTOGENY

By

Russell A. Blauel

B.A. University of Montana, 1964

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF ARTS IN

BOTANY

UNIVERSITY OF MONTANA

1967

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

FEB 22 1968
Date
UMI Number: EP34285

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent on the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI EP34285
Copyright 2012 by ProQuest LLC.
All rights reserved. This edition of the work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346
ACKNOWLEDGMENTS

Special thanks go to Dr. C. C. Gordon for his support during the entire study.

Appreciation is also extended to Dr. H. S. Whitney, Dr. M. Chessin, Dr. J. Taylor, G. Blauel, A. Dale, M. Thornton and Dr. F. W. Cobb, Jr. for their generous assistance.
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td></td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td></td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>Pathology</td>
<td>1</td>
</tr>
<tr>
<td>Ontogeny</td>
<td>3</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>6</td>
</tr>
<tr>
<td>Pathology</td>
<td>6</td>
</tr>
<tr>
<td>Ascospore dispersion</td>
<td>7</td>
</tr>
<tr>
<td>Ontogeny</td>
<td>8</td>
</tr>
<tr>
<td>OBSERVATIONS AND RESULTS</td>
<td>9</td>
</tr>
<tr>
<td>Pathology</td>
<td>9</td>
</tr>
<tr>
<td>Ascospore dispersion and germination</td>
<td>12 &amp; 13</td>
</tr>
<tr>
<td>Ontogeny</td>
<td>14</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>17</td>
</tr>
<tr>
<td>Pathology</td>
<td>17</td>
</tr>
<tr>
<td>Ascospore dispersion</td>
<td>18</td>
</tr>
<tr>
<td>Ontogeny</td>
<td>20</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>23</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>36</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1. Ascospore separation tests</td>
<td>12</td>
</tr>
<tr>
<td>2. Ascospore germination tests</td>
<td>13</td>
</tr>
</tbody>
</table>
# LIST OF PLATES

<table>
<thead>
<tr>
<th>Plate</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
</tr>
</tbody>
</table>
**ASPECTS OF CERATOCYSTIS MONTIA PATHOLOGY AND ONTOGENY**

**Introduction**

The blue stain disease of pines, apparently endemic in northwestern Montana, from time to time becomes epidemic and causes the death of large numbers of merchantable trees (Tunnock, 1965). It is a complex disease involving several microorganisms vectored to several species of pines by the mountain pine beetle *Dendroctonus ponderosae* Hopkins. The microorganisms consistently associated with this disease are species of *Ceratocystis, Europhium*, yeast, bacteria, mites and often nematodes. The bark beetle (*Dendroctonus ponderosae*) attacks ponderosa pine (*Pinus ponderosa* Laws.), lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.), western white pine (*Pinus monticola* Dougl.), and limber pine (*Pinus flexilis* James). Several authors have described the blue stain disease on pines in detail (Boyce, 1948; Francke-Grosmann, 1960).

*Ceratocystis montia* (Rumb.) Hunt, an ascomycetous fungus, is consistently found in blue stained sapwood. Although not a proven primary pathogen, *C. montia* is strongly implicated in tree death because it rapidly colonized the living sapwood rendering it nonfunctional (Rumbold, 1941; Mathre, 1964; Reid, et al., 1967).

The main purpose of this thesis is to present aspects of pathology and ascocarpic ontogeny of *C. montia*. In addition, a new method of isolating single ascospores is described.

**Literature Review**

Pathology — *C. montia* and its association with bark beetles and the blue stain disease were first described by C. T. Rumbold (1941). Her
report stated that the bark beetle and its associated microorganisms were responsible for the death of many pines in northwestern United States and Canada. A description of *C. montia* was given that included the mycelium, conidial stages, perithecia, asci and ascospores. *C. montia*'s sexual fruiting body is a well-developed, darkly pigmented perithecium possessing a long slender neck with an apical ostiole. The perithecia are produced either singly or in clusters, superficially or with bases immersed in the substrate, but they are not formed in or on a stroma. Deliquescent, irregularly arranged asci are borne inside the perithecium, and there are no true paraphyses or periphyses present. Ascospores are enveloped in a gelatinous sheath which imparts a rectangular shape with flanged corners at the end.

R. C. Robinson (Jeffrey, 1962) studied the blue stain disease in lodgepole pine infested with the mountain pin beetle and concluded that *C. montia* was a prevalent blue stain fungus among the microorganisms present. Robinson's studies indicated that *C. montia* is the first blue stain fungus to develop during invasion of host tissues.

More detailed work concerning the pathological relationships of *C. montia* and *D. ponderosae* has been carried out by R. W. Reid, et al., (1967) working with lodgepole pine. They confirmed the pathogenicity of this blue stain fungus and described a sequence of changes that occur in and adjacent to infected pine tissue. Differences in secondary resinosis abilities of individual lodgepole pines accounted for successful and non-successful attack by the blue stain fungi. Additional evidence has been obtained which shows that *C. montia* is less affected by resinous substances than is the blue stain fungus *Europhium* sp. (Shrimpton & Whitney, personal communication).
Ontogeny — Four different concepts concerning the ascocarp development of the genus *Ceratocystis* have been presented by Elliott (1923), Andrus & Herter (1933), Gwynne-Vaughan & Broadhead (1936) and Moreau & Moreau (1952). Elliott (1923, 1925) working with *C. fimbriata* Ell. & Halst. stated the perithecial body developed from a hyphal knot originating from two separate hyphae which had differentiated into an antheridium and oogonium. Nuclear transfer through a trichogyne with karyogamy following soon after was reported. Nuclear divisions producing eight nuclei then occurred, followed by migration of nuclear pairs into coenocytic hyphae which had arisen from the carpogonium. These ascogenous hyphae continued to grow inside the perithecial cavity between large thin-walled sterile cells which acted as nutritive tissue. Wherever an ascogenous hypha contacted a nutritive cell or the perithecial wall, a permanent attachment formed, giving the impression that ascus mother cells arose from many different points inside the perithecium. Ascus mother cells originated as terminal swellings of the coenocytic ascogenous hyphae. Nothing resembling crozier hooks was produced, with asci first being developed at the perithecial apex where a beak was concurrently being formed. Ascus development continued downwards toward the base and outer walls of the perithecium. Just before full ascospore maturity the ascus wall disintegrated.

Andrus and Harter (1933) interpreted *C. fimbriata* as being homothallic and parthenogenic. All cells involved in the perithecial initial were uninucleate until a nuclear migration occurred from the terminal cell into the subterminal cell (oogonium). The binucleate condition in the oogonium prevailed until ascus formation. Then the oogonial wall dissolved, leaving its contents lying free in the perithecial cavity to act as the primary
ascus. Nuclear division then occurred in the primary ascus resulting in a 4 to 8 nucleated plasmodium. This fragmented, and the daughter plasmodia and their progeny provided many unattached, naked n + n plasmodia. The n + n plasmodia eventually stopped dividing and the nuclei within them fused. During the first nuclear division following karyogomy an ascus wall formed within the plasmodium from the fusion nuclei membrane. All three divisions and final ascospore delimitations took place within this ascus wall.

Gwynne-Vaughan and Broadhead (1936) published observations on the development of *C. fimbriata* which stated that the perithecial initial had one cell which became binucleate by mitosis with no wall formation. This cell later became multinucleate and divided to form several smaller multinucleate cells which branched to form ascogenous hyphae and crozier hooks. The asci reportedly grew inward, with the ascogenous hyphae and asci still attached to the cavity walls. Beak development originated from a meristematic region of an inner sheath which pushed through an outer sheath and elongated.

Moreau and Moreau (1952) claimed a much different perithecial development occurring in the ascogenous system of *C. moniliformis* (Hedge.) Moreau stating that a lysigenous process was responsible for creating the central cavity. This process involved central cells becoming vacuolate with cell membranes breaking down, leaving a multitude of nuclei enclosed within a common protoplasm. Degeneration apparently occurred throughout the central perithecial region, with only the cells lining the cushion region escaping. Radial files of cells were illustrated, both uni- and binucleate, within the perithecial cavity. Moreau and Moreau stated that
an ascus could be produced by simple partitioning, or karyogamy could occur within any cell of a file. This cell could then act as a mother cell producing one or more asci near its apex. It is important to note that Moreau and Moreau claimed that because the files of ascogenous cells developed basipetally, they were not ascogenous hyphae.

More recent studies of the genus *Ceratocystis* have been carried out by Tiffany (1956), Wilson (1956), Rosinski (1961), and Bannville (1966). Their conclusions paralleled those of Gwynne-Vaughan and Broadhead. Taylor-Vinge (1940) studied *C. montia* and published the only report of perithecial development for the species. She concluded that perithecial initials appeared as recurved branches with no antheridia present. The hyphal branch coiled and became the fertile coil of the perithecium, the tip cell became binucleate by undetermined means, and the adjacent sterile cells gave rise to a sterile sheath. The rest of the coil cells became multinucleate through karyogamy without cytokinesis while some cells gave rise to ascogenous hyphae, at whose tips asci were formed by typical crozier development. Perithecial beak formation occurred simultaneously with this process. Inside the ascus three nuclear divisions produced eight nuclei which developed into ascospores through free cell formation. The spores were initially round but later became polyhedral in shape, bearing flanges. The ascus wall deliquesced shortly before spore maturity, freeing the spores into the central perithecial cavity. The perithecial sheath was composed of a thick-walled outer layer of dark brown cells and a thin-walled inner layer of pseudoparenchymatous cells.
Materials and Methods

Pathology — Sample materials were collected from various ponderosa pine stands within a twenty-five mile radius of Missoula, Montana. All trees sampled were infested with D. ponderosae. Samples were gathered by chiseling out portions of standing or felled trees. Outdoor pictures were taken with a Nikromat F.T. 35mm camera equipped with 3.5f 43-86mm lens. Kodak pan X black and white film was used and developed with Ethol TEC. Prints were made on F2, F3, and F4 Kodabromide single weight paper.

Cultures were started from ascospores found on the tips of C. montia perithecia growing in D. ponderosae galleries. Positive identifications were made from culture-grown material by following the criteria in Hunt's (1956) key to Ceratocystis spp., by comparing material with known cultures of C. montia, and by verifying identifications through H. S. Whitney. Culturing involved transferring cirri of ascospores to either 2% water agar plates or 2% malt extract agar plates. Transfer tasks were performed with a flame sterilized micro-needle knife tipped with a tiny piece of firm sterile water agar. Subculture transfers to tubes and plates of malt extract agar were made using single ascospores, ascospore cirri, perithecia and hyphal tips.

Examinations of insects, fungus, colonized pine tissue and fungus cultures were made with a ten-power hand lens, a Bausch and Lomb binocular dissecting microscope and a Reichert Zetopan phase-equipped microscope. Photomicrographs were taken with a Leica 35mm camera, metered by a Gossen micro Six "L," on Kodak pan X black and white film, developed with Ethol TEC and printed on F2, F3, and F4 Kodabromide single weight paper.
Temporary slides were made of perithecial squashes, sporodochial squashes, ascospore cirri squashes, hand sections of wood tissue and perithecia grown on wood tissue. Sterile water, lactophenol, lactophenol with cotton blue and lactophenol with carmine red were all used as mounting media.

Ascospore dispersion -- Materials tested as ascospore dispersers were bio-degradable surfactants, detergents, beetle enzymes, chemical solvents, pine resin, and ponderosa pine resin terpenes as specifically listed in Table 1.

Attempts were made to prepare enzyme extracts from adult beetles in order to test them as ascospore dispersing agents. One-hundred mature D. ponderosae beetles were placed into a thermal jug of acetone that had been previously cooled with dry ice. The beetles were left until frozen (approximately two minutes) and then ground up in a cold mortar containing crushed dry ice. The crushed mixture was then washed into an acid-washed evaporator flask with acetone. The acetone was allowed to evaporate at room temperature. The resulting powder was divided into four parts and taken up in a minimum of one of four different solvents. Buffers were made up from K₂HPO₄ set with one normal HCL at 0.1M having pH's of 5.5, 6.5, and 7.3. Distilled water was used as the fourth solvent. A second extract was prepared using glycerine. Fifty mature beetles were ground up in mixtures of 60% glycerine and 40% phosphate buffers of pH's 5.5, 6.5, 7.3 and in pure glycerine.

Potential ascospore dispersers were tested as follows: Four milliliters of sterile 2½% water agar were poured into 6 cm. sterile Petri
plates. Immediately before testing, a drop of disperser was placed on a water agar surface. Ascospores were collected from ostiolar tips of *C. montia* perithecia with the aid of a binocular dissecting microscope. A sterile micro-needle knife was used to wipe spores from the beaks and transfer them on the tip of a tiny piece of water agar into the drop of prospective disperser. A quick stir with the micro-needle knife was then given to the drop. Plates were examined under the dissecting microscope for two minutes and then again at intervals of 5 and 20 min., and 2, 3, 4, 15, 30, and 48 hours in order that the degree of ascospore separation could be judged.

Germination tests of separated ascospores were performed using only the best dispersers. Sterile distilled water was used as a control. Once separation had been completed, the drop containing the ascospores was streaked over the surface of the water agar plate with a sterile wire loop. The plates were then placed in an incubator (18°C) for 48 hours at which time germination counts were made using a microscope (400x).

Ontogeny — Perithecia grown in vitro and found in vivo were used in ontogenetic studies. Small pieces of wood tissue (approximately 1mm x 5mm) bearing perithecia were cut from *D. ponderosae* galleries and fixed immediately in vials of FAA (formalin, glacial acetic acid, ethyl alcohol). Pure cultures were obtained using ascospore cirri* from *D. ponderosae* galleries. After ten days growth in culture, specimens were taken every 25 hours until the 20th day. Specimens consisted of small pieces of agar bearing perithecia cut from the cultures and fixed immediately in vials

*cirri: plural of cirrus; a curl-like tuft of forced-out spores.*
of FAA. The fixed materials were thoroughly aspirated overnight and then run through the tertiary butyl alcohol-paraffin embedding series (Johansen, 1940). Once embedded in hard paraffin (Paraplast)\(^1\) the material was cut on a rotary microtome to thicknesses of 7, 10, 12, and 15 micra. The wood tissue required some aerosol\(^2\) soaking. The ribbon was then mounted on glass slides using Haupts' adhesive. Approximately 700 slides were stained using Feulgen's-fast green stains and Giemsa stain. Cover slips were mounted using Harleco's Synthetic Resin.\(^3\) The slides were examined and photos taken using the Reichert phase microscope and photo equipment previously mentioned.

**Observations and Results**

Pathology — Ponderosa pine attacked by *D. ponderosae* and the blue stain complex stood out from healthy trees by a display of the following symptoms: (1) all tree trunks had many pitch tubes (Fig. 3), (2) newly attacked trees showed fresh frass accumulating at the tree base, (3) brood trees showed a marked sparseness of foliage, slight chlorosis, and much reduced vigor, and (4) older attacked trees were either dead or nearly so and displayed browned needles or branches stripped of their foliage (Fig. 1, 2). When bark was removed from current year brood trees, a well-developed *D. ponderosae* brood accompanied by the blue stain fungi was found (Fig. 4). Cross sections of attacked trees showed varying

---

\(^1\)Scientific Supplies Company, Seattle, Washington.  
\(^2\)QT 100, Scientific Supplies Company, Seattle, Washington.  
amounts of blue stain. The degree of blue stain girdle also varied with height within a given tree. However, most brood trees showed complete or almost complete girdling within ten feet of the tree base (Fig. 5). Upon close examination of the sapwood, rays appeared distinctly pigmented (Fig. 6).

Adult female *D. ponderosae* constructed egg galleries and laid eggs in tiny niches in the side walls of the galleries (Fig. 4, 7-10). These eggs hatched, and the larvae mined in the inner bark more or less at right angles to the main gallery. *Dendroctonus ponderosae* pupal cells were lined with sporodochia of the blue stain fungi, *C. montia*, and *Europhium sp.* (Fig. 8, 9). Sexual fruiting bodies of *C. montia* were common in *D. ponderosae* galleries (Fig. 11-14). Bases of the perithecia were partially buried in wood tissue with the necks sticking out into the open tunnels (Fig. 11, 12, 14). A cirrus of sticky ascospores was present at the tips of most beaks (Fig. 11, 14). Microscopic examination of separated ascospores revealed a rectangular gelatinous sheath with flanges surrounding a somewhat round-ended, elongate ascospore (Fig. 33-35). Ascospores are believed to be binucleate (Fig. 35). *Dendroctonus ponderosae* adult beetles were found carrying *C. montia* and *Europhium sp.* through culture isolation techniques. Possible inocula were ascospores (exuded in sticky cirri from perithecial tips), conidia (produced almost any place vegetative hyphae were present, with large clusters lining the pupal cells), micro-endospores (possibly produced within the major

---

4 *Europhium sp.*: a recently discovered sexual stage of one *Leptographium* species.
mycelia with potential for spreading systemically throughout the tree),
and hyphal fragments. *Ceratocystis montia* was vectored in one or more
of these forms by the bark beetle *D. ponderosae* (Fig. 7-10). Evidence
that supported this was that the fungus was consistently isolated from
newly emerged tenerals\(^5\) and older adult beetles. Also no attacks of *D.*
ponderosae were found in ponderosa pine that did not contain *C. montia.*

\(^5\)Tenerals are juvenile forms of beetles that have emerged from
the pupal stage but are not yet fully matured adults.
### Table 1

**Results of Ascospore Dispersal**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2, 3, t</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>under 1 min.</td>
</tr>
<tr>
<td>Distilled sterile water (control)</td>
<td>nil</td>
</tr>
<tr>
<td>Acetone enzyme extract</td>
<td>nil</td>
</tr>
<tr>
<td>in 5.5 pH</td>
<td>nil</td>
</tr>
<tr>
<td>in 6.5 pH</td>
<td>nil</td>
</tr>
<tr>
<td>in 7.3 pH</td>
<td>nil</td>
</tr>
<tr>
<td>in distilled water</td>
<td>nil</td>
</tr>
<tr>
<td>Glycerol enzyme extract</td>
<td>nil</td>
</tr>
<tr>
<td>in pH 5.5</td>
<td>nil</td>
</tr>
<tr>
<td>in pH 6.5</td>
<td>nil</td>
</tr>
<tr>
<td>in pH 7.3</td>
<td>nil</td>
</tr>
<tr>
<td>Tween 20</td>
<td>nil</td>
</tr>
<tr>
<td>Aniline</td>
<td>nil</td>
</tr>
<tr>
<td>Acetone</td>
<td>nil</td>
</tr>
<tr>
<td>Methyl Benzoate</td>
<td>nil</td>
</tr>
<tr>
<td>Liquinox surfactant</td>
<td>nil</td>
</tr>
<tr>
<td>Sparkleen</td>
<td>nil</td>
</tr>
<tr>
<td>Tide detergent</td>
<td>nil</td>
</tr>
<tr>
<td>ETOH</td>
<td>nil</td>
</tr>
<tr>
<td>Tertiary Butinol</td>
<td>nil</td>
</tr>
<tr>
<td>Methyl Cellosolve</td>
<td>nil</td>
</tr>
<tr>
<td>Aerosol</td>
<td>nil</td>
</tr>
<tr>
<td>Toluene</td>
<td>nil</td>
</tr>
<tr>
<td>Benzene</td>
<td>nil</td>
</tr>
<tr>
<td>Ligroine</td>
<td>nil</td>
</tr>
<tr>
<td>Xylene</td>
<td>(40sec.) s.a.</td>
</tr>
<tr>
<td>Pinene</td>
<td>(30sec.) s.s.</td>
</tr>
<tr>
<td>Liquid pine resin</td>
<td>(50sec.) s.s.</td>
</tr>
<tr>
<td>α pinene*</td>
<td>(30sec.) s.s.</td>
</tr>
<tr>
<td>β pinene*</td>
<td>(30sec.) s.s.</td>
</tr>
<tr>
<td>Δ³ carene*</td>
<td>(40sec.) s.s.</td>
</tr>
<tr>
<td>undecane*</td>
<td>(50sec.) s.s.</td>
</tr>
</tbody>
</table>

*purified fractions of pine resin supplied by F. W. Cobb, Jr., and E. Zavarin, Department of Plant Pathology, University of California, Berkeley.

**small aggregates

***single spores
Table 2

<table>
<thead>
<tr>
<th>Dispersal agents</th>
<th>Avg.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>0%</td>
<td>0-0</td>
</tr>
<tr>
<td>Benzene</td>
<td>0%</td>
<td>0-0</td>
</tr>
<tr>
<td>Ligroine</td>
<td>0%</td>
<td>0-0</td>
</tr>
<tr>
<td>Xylene</td>
<td>0%</td>
<td>0-0</td>
</tr>
<tr>
<td>Pinene</td>
<td>0%</td>
<td>0-0</td>
</tr>
<tr>
<td>α pinene</td>
<td>0%</td>
<td>0-0</td>
</tr>
<tr>
<td>β pinene</td>
<td>0%</td>
<td>0-0</td>
</tr>
<tr>
<td>Δ³ carene</td>
<td>8.9%</td>
<td>7-12</td>
</tr>
<tr>
<td>Undecane</td>
<td>0%</td>
<td>0-0</td>
</tr>
<tr>
<td>Ponderosa pine resin</td>
<td>51.2%</td>
<td>38-69</td>
</tr>
<tr>
<td>Distilled sterile H₂O</td>
<td>58.4%</td>
<td>34-78</td>
</tr>
</tbody>
</table>

The chart above represents compilation of 10 different tests, each test consisting of at least 100 ascospores placed in each liquid. Although Δ³ carene showed 8.9% germination, the fungus showed very poor hyphal growth with budding predominating the germinations (Fig. 20). The growth of hyphae under natural pine resin was good although not equal to hyphal growth under sterile distilled water (Fig. 19). Ascospores failed to germinate in the other terpenes tested (Fig. 21).
Ontogeny - Vegetative hyphae of *C. montia* grown in malt agar displayed a sequence of changes during growth. Hyphae first grew out in a pattern radiating distally from the inoculum. The hyphae became darkly pigmented and reached diameters ranging from 2 to 7 micra. These mature hyphae appeared rough-walled, had many tiny protrusions, branched frequently, and displayed uni- to multi-nucleate conditions (Fig. 17, 18, 22-26, 28, 29). A system of young hyaline hyphae developed, originating as lateral branches from the mature hyphae. These hyphae had diameters ranging from 0.75 to 2.0 micra, possessed relatively smooth walls, and had nuclear conditions which were not discernable. The hyaline hyphae increased the intricacy of the mycelial system, criss-crossing between matured hyphae and anastomosing with one another (Fig. 36).

Sexual fruiting bodies were not observed in cultures started from single ascospores or single hyphal tips, but were produced profusely in cultures started from several ascospores. Few were produced in cultures started from vegetative or asexual inoculum. Perithecial initials arose from anastomosis between filaments of the thin hyaline hyphal network (Fig. 31, 32). Initials originated as a small coil of intertwisting cells, primordial mass increased (Fig. 37, 38, 43). Nuclear conditions inside the primordial cells (as in later ascocarpic stages) were difficult to discern throughout this study due to the granular nature of the cell materials. Hyaline hyphae originating from peridial cells of the primordium were apparent soon after primordial initiation (Fig. 40). Hyaline hyphae originating from the vegetative hyphae in the agar near the developing ascocarp often grew toward it. Anastomosing between these two hyphal systems (primordial and vegetative) was observed (Fig. 41, 47).
Undifferentiated cells (pseudoparenchymatous) continued to be produced centrifugally by each layer of peridial cells until the ascocarp was 3/4 its mature size. When an ascocarp reached approximately 3/4 its full size, the newly-formed peridial cells became thick-walled and darkly pigmented. The central core of the ascocarp at this time was composed of thin-walled pseudoparenchymatous cells (Fig. 37, 38).

The following sequence of events led to ascospore formation. A zone consisting of from 2-3 layers of radially arranged centrum cells in the perithecial apex became less dense in cytoplasmic consistency (Fig. 44, 46). These apical cells then lysed leaving a small cavity (Fig. 49, 51). The cells below this cavity then commenced to differentiate with differentiation continuing basipetally throughout the central core region. Lysing followed the differentiation but proceeded in a selective manner. Single rows of "fertile" cells were separated out by the lysing of the "infertile" cells between the rows. These lysed cells formed granular "sticky" matrix material. The remaining cell tiers (each tier composed of from 2-4 cells) lost their cross walls and formed elongate cocnocyteic cells which remain attached to the pseudoparenchymatous region below. The elongate structures then differentiated through indiscernable means into asci, each containing binucleate ascospores (Fig. 16, 33-35). Soon after ascospore formation, the asci deliquesced releasing immature ascospores into the cavity above (Fig. 52, 53). The lysigenous-asci differentiation zone moved basipetally through the pseudoparenchymatous cells transforming the central core into ascospores and sticky matrix material (Fig. 49-53).
Primordia of sterile perithecia originated in the same manner as the fertile ones. Growth of the primordium resulted from cell multiplication in the external layer. The number of hyaline hyphae growing from the primordia increased (Fig. 39, 42). There was a noticeable lack of hyaline vegetative hyphae growing toward the primordial body. Peridium differentiation proceeded as usual. Internal lysing occurred in the pseudoparenchymatous cells, but all the cells completely disintegrated leaving no elongate structures as found in fertile perithecia (Fig. 42, 52). The sterile perithecium was left as a peridial shell encasing some lysed cell remnants (Fig. 42). Sometimes vegetative hyphae penetrated the sterile perithecium and occupied the hollow interior (Fig. 45, 48).
Discussion

Pathology -- The predominant fungi involved in the blue stain disease of ponderosa pine in Montana, *C. montia* and *Europhium* sp., are the same as those reported by Robinson (1962) on lodgepole pine in British Columbia, Canada. *Ceratocystis montia* produced four forms of inocula: ascospores, conidia, micro-endospores and fungal fragments. Perithecia, while not prevalent in pupal chambers, were produced in most other open spaces under the bark, especially in egg and larval galleries. Tips of the beaks of *C. montia* perithecia were generally laden with sticky ascospore cirri at teneral emergence time (Fig. 14). The fact that tenerals usually do not take flight immediately, but rather move around feeding under the bark through the galleries, gives ample opportunity for them to pick up or ingest inoculum. *D. ponderosae* beetles involved acted in behavior patterns similar to those described by Reid (1959, 1962, 1963) for lodgepole pine. Species of *Ips* had also attacked the brood trees studied, but their distribution was such that *D. ponderosae* could easily be studied separately.

*Dendroctonus ponderosae* beetles have three possible ways of carrying inoculum. First, their bodies are covered with short bristles among which inocula can easily lodge (Fig. 10). Second, *D. ponderosae* possess structures termed mycangia which are especially adapted for transporting fungal spores (Farris, 1965). Third, viable inocula such as ascospores and conidia have been found in beetle guts (H. S. Whitney, 1962).  

6*Ips* spp. are beetles that often attack trees first weakened by *D. monticola*-*G. montia* attack (Robinson, 1962).
personal correspondence). The mountain pine beetle's pattern of behavior contributes to the spreading of the inoculum. The adult beetle has a short flight and attacks individual trees in large numbers. Host penetration through the outer bark down to the cambium and sapwood surface is accomplished by the insect, permitting infection by *C. montia* (Fig. 3, 4).

The actual pigmentation within the wood (from light brown to darker brown and finally to steel blue) follows some distance behind fungal invasion because the color is that of the mature fungal hyphae (Fig. 17, 18). Growth and spread of the fungus inside the host, host tissue reactions (such as resinosis) and the pattern of fungus colonization appeared similar to that which occurs in lodgepole pine (Reid, et al., 1967). *Ceratocystis montia* appears to grow very quickly parallel to the longitudinal axis of the tree through the cambial tissues but progresses at a somewhat slower rate in the sapwood, both longitudinally and centripetally. Circumferential growth in the sapwood is very slow, and complete fungal girdling of the tree results from overlapping of large numbers of insect hits. Hyphae grow rapidly through rays of the sapwood, and no extended incubation period is required prior to asexual inocula formation. When the blue stain attack has been successful and encirclement of the bole accomplished by the hyphae, tree mortality follows the next growing season (Fig. 1, 5).

Ascospore Dispersion -- Results from the ascospore dispersion tests showed that enzymes extracted from *D. ponderosae* beetles by the
acetone freezing method and glycerine emulsion method were not success-
ful in separating C. montia ascospore cirri into single ascospores.
Reasons for this could be either that enzyme inactivation occurred or
that enzymes are not a factor in dispersion. Detergent solutions and
surfactants also had little effect on cirri. Chemicals showing strong
lipid solvancies, natural pine resin and pure fractions of pine resin,
proved to be the most effective ascospore dispersers.

The relationship between in vitro and in vivo ascospore disper-
sions were as follows. (1) Inside the "old" brood tree initial
copious resin production had slowed considerably by the time perithecia
bearing cirri of ascospores were produced; thus no washing away of the
cirri by resin occurred. (2) Accumulated evidence (Rumbold, 1941;
Robinson, 1962; & H. S. Whitney, personal communication) verifies the
beetle D. ponderosae as the important vector of C. montia. (3) Pure
mechanical ascospore separation by the beetles must not be dismissed.
(4) Ascospore dispersion, while not necessary for infection, facili-
tates spread of the fungus in freshly attacked trees.

Tests concerning effects of ascospore dispersing agents on spore
viability showed that chemically pure lipid solvents were toxic to
C. montia ascospores and no germination ensued. Fresh ponderosa pine
resin was found to be an excellent ascospore disperser and proved rela-
tively innocuous to ascospore germination (Table 1 and 2). In efforts
to locate a chemical capable of harmless separation, four pure frac-
tions of ponderosa pine resin showing lipophyllic tendencies were
tested and found to be toxic to ascospores (Fig. 19, 20, 21). Reasons
for phytotoxicity are not known. Results of the dispersion study led to the conclusion that natural pine resins are probably involved in natural ascospore dispersion.

Ontogeny -- Three possible explanations for the presence of protrusions appearing on mature vegetative hyphae exist (Fig. 18, 19). They may be: (1) sites of production and emergence of germinated micro-endoconidia; (2) areas in the hyphal wall where enzymes have been released; or (3) wall protrusions involved in food absorption or some other nonapparent function.

Nuclear conditions of C. montia vegetative hyphae were found to vary from uni- to multinucleate (Fig. 22-29). With clear evidence for nuclear migration in ascomycetes through septal pores (R. T. Moore & J. H. McAlear, 1962), it follows that nuclear migration possibly occurs in this fungus. Nuclear division rate differing from cytokinesis rate could also account for these conditions. The results obtained from single spore and hyphal tip cultures prove conclusively that the strains of C. montia used in this study were all heterothallic. Parthenogenesis of the fungus studied can be dismissed on the grounds that anastomosing between vegetative hyphae is required for primordial initiation and between the perithecium and the vegetative system for production of asci and ascospores.

The sequence of ascocarp development was based on observations of prepared slide material. The basic physical problem encountered was the accurate interpretation of a complex 3-dimensional object (the perithecium) through series of 2-dimensioned slides. Due to the
granular nature of materials inside the perithecium, clear sharp photography was made difficult (Fig. 54-56). Further work utilizing culture techniques, radiation labeling, histochemical analysis, the vital stain techniques and time lapse photography is desirable. The paraffin slide technique is the important first stage in ontogenetic studies and often indicates what other methods need to be utilized.

The conclusion that fertilization probably occurs during enlargement of the ascocarp and not during ascocarp initiation was based on the following two observations. Although perithecia are always initiated by anastomosis between two hyphae, they sometimes developed into a sterile perithecium. Also, sterile perithecia noticeably increased the number of lateral hyphae produced (Fig. 29, 42).

Internal perithecial development through a lysigenous—differentiation process is supported by the following: (1) no ascogonial system was observed in the primordium; (2) no disruption of the pseudoparenchymatous tissue by a developing ascogonial system was observed; (3) since peridium differentiation and beak formation in *Ceratocystis* sp. are classically recognized, differentiation is not a new concept in the genus; (4) lysing of pseudoparenchymatous tissue (nutritive cells) and of the ascus wall has been described in detail in *Ceratocystis* sp. literature; (5) the cytoplasmic contents of all the pseudoparenchymatous cells become more dense (proceeding in sequence just ahead of the lysing (Fig. 46, 49); (6) a sequence of lysing precedes the formation of asci (Fig. 52, 56); (7) the development of asci in relatively uniform layers indicates a spatial phenomenon (Fig. 52, 53); and (8) there is a sequential development of asci starting at the ascocarp apex and progressing basipetally.
Under culture conditions (i.e., malt agar) one obvious deviation noted was that a few parithecinal beaks pointed downward into the agar in both fertile and sterile perithecia (Fig. 45). The phenomenon has never been observed in wood tissue. Less obvious deviations also occur.
Summary

A blue stain complex attacking ponderosa pine, having *C. montia* as one of the major pathogens, was described. Relationships between the host, disease and vector were examined with similarities to the blue stain complex on lodgepole pine noted (Robinson, 1962).

Natural ascospore dispersion through pine resins was shown to be probable and a method of single ascospore isolation in the lab explained. Aspects of *C. montia* grown in malt agar culture were noted. The strains studied proved to be heterothallic and non-parthenogenetic. Ontogenetic investigations revealed primordia originating as intertwined coils of cells that increased in mass by external circumferential cell growth. Hyaline appendages were produced from the primordia and anastomosed with the surrounding vegetative hyphae. When fertile perithecia reached approximately 3/4 full size the outer cell layers differentiated into the dark, thick-walled peridium. Internally, a sequence of pseudoparenchymatous cell differentiation and lysing resulted in a final product of binucleate ascospores embedded in a gelatinous matrix.
PLATE #1

Figure

1  Ponderosa pine stand attacked by D. ponderosa and Blue stain complex.

2  Comparison between unattacked (left) and current attacked brood tree (right).

3  Beetle "pitch holes" on outside of bark.

4  Portion of brood tree with bark removed exposing D. ponderosae galleries and discolored sapwood.

5  Cross section of attacked tree showing complete blue stain girdling.

6  Closeup of blue pigmented rays.

7  Stages of insect development showing 2 larval instars (left) pupa (bottom right) teneral (mid right) and adult (top right).

8  Late stage pupa in pupal cell surrounded by sporodochia of the blue stain complex. Inner bark also stained.

9  Same (cf. 8) pupal cell with pupa removed showing complete sporodochial mass.

10 D. ponderosae adult.
Figure

11 *D. ponderosae* gallery lined with perithecia of *C. montia*. Note beaks protruding into gallery.

12 Numerous *C. montia* perithecia.

13 Single *C. montia* perithecium

14 *C. montia* beaks loaded with cirri of ascospores.

15 Squash of *C. montia* perithecium.

16 Enlargement of *C. montia* perithecial squash showing ascospores within asci.

17 *C. montia* vegetative hypha grown in malt extract agar. Note rough walls and protrusions.

18 Another *C. montia* vegetative hypha (cf. 17).

19 Germinated *C. montia* ascospore under Ponderosa pine resin after 48 hrs.

20 Budding *C. montia* ascospores under Ponderosa pine resin after 48 hours.

21 Ungerminated *C. montia* ascospores under β pinene.
Figure

22  *C. montia* vegetative hypha showing uninuclear condition.

23  *C. montia* vegetative hypha showing trinuclear condition.

24  *C. montia* vegetative hypha showing multinuclear condition.

25  *C. montia* vegetative hypha showing close up of binuclear condition.

26  *C. montia* vegetative hypha showing trinuclear condition.

27  *C. montia* primordium.

28  *C. montia* vegetative hypha showing multinuclear condition.

29  *C. montia* vegetative hypha showing binuclear condition.

30  *C. montia* primordium.

31  *C. montia* primordium.
    Note hyaline hyphae (anoptrol contrast).

32  *C. montia* primordium.

33  Side view of mature *C. montia* ascospore.
    Note gelatinous sheath.

34  End view of mature *C. montia* ascospore.
    Note flanges.

35  Side view of mature *C. montia* ascospore.
    Note binucleate condition.
PLATE #4

Figure

36  C. montia hyphal system growing in malt extract agar.  
     Note dark and hyaline hyphae.

37  Young developing perithecium of C. montia.

38  Young developing perithecium of C. montia.

39  Serial section (cf. Fig. 42) of sterile perithecium.  
     Note numerous appendages.

40  Young developing coils of C. montia.

41  Anastomosis between hyphae communicating with C. montia  
     primordium.

42  Serial section (cf. Fig. 39) of sterile perithecium.  
     Note lysing pseudoparenchymatous tissue.

43  Developing C. montia perithecium and appendages.

44  Beginning of differentiation in C. montia perithecium.

45  Sterile perithecium showing vegetative hyphal penetration  
     and growth inside hollow shell.  
     Note beak down into agar.

46  Off median section of C. montia perithecium showing cyto-
     plasmic differentiation process.

47  Developing primordium of C. montia showing many hyphal  
     connections.
PLATE #5

Figure

48  Sterile *C. montia* perithecium on agar surface with vegetative hyphae growing inside.

49  Developing *C. montia* perithecium showing lysed apical zone and cytoplasmic differentiation below.

50  *C. montia* perithecium showing lysing (serial section).

51  *C. montia* perithecium showing lysing (serial section).

52  Maturing *C. montia* perithecium showing asci developing in layers with free ascospores above and lysigenous process below.

53  Another maturing perithecium (cf. 52).
Figure

54 Serial sections (cf. 55, 56) of maturing *C. montia* perithecium showing internal development.

55 Maturing perithecium—median section

56 Maturing perithecium.
REFERENCES


Elliott, J. A. The ascigerous stage of the sweet potato black-rot fungus. *Phytopath.*, 1923, 13: 56. (Abst.)


