Microspore Wall Morphogenesis and Orbicule Ultrastructure of *Isoetes*

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MICROSPORE WALL MORPHOGENESIS AND ORBICULE

ULTRASTRUCTURE OF *ISOETES*

by

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B.Sc. May 1994, University of Jordan
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Old Dominion University in Partial Fulfillment of the
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The microspore wall morphogenesis and orbicule ultrastructure of Isoetes were studied using electron microscopy. Microspore wall morphogenesis in five species of Isoetes (I. appalachiana, I. engelmannii, I. hyemalis, I. piedmontana, and an undescribed species from York Co., VA, USA) was studied using scanning and transmission electron microscopy. Results show that they generally have the same developmental stages in terms of microspore wall morphogenesis. The mature microspore wall consisted of four layers: perispore, paraexospore, exospore and endospore. Paraexospore formation began during the tetrad stage. The exospore was then formed between the paraexospore and the plasma membrane. During the free spore stage, two walls were formed, the perispore and the endospore. The perispore develops on the surface of the paraexospore while the endospore was formed interior to the exospore and plasma membrane. Orbicule ultrastructure of nine species of Isoetes from the USA, Canada and Syria was studied, for the first time in the genus, using scanning electron microscopy. Orbicules are minute granules of sporopollenin observed on the inner wall of the secretory tapetum and their function remains unknown. Ultrastructural variation in orbicules of the nine studied species was documented. Orbicules were very variable in both shape and size.
This dissertation is lovingly dedicated to my mother, my wife Raeda and my daughter Maya for their support, encouragement and love.
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I would first like to thank my advisor, Dr. Lytton Musselman, for his continuous encouragement and support, for his valuable feedback on my work since its onset and for the countless invitations to dinners and coffee.

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# TABLES OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>GENUS DESCRIPTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>2</td>
</tr>
<tr>
<td>II. MICROSPORE WALL MORPHOGENESIS OF <em>ISOETES PIEDMONTANA</em></td>
<td>5</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>5</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>5</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>9</td>
</tr>
<tr>
<td>RESULTS</td>
<td>10</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>17</td>
</tr>
<tr>
<td>III. A COMPARATIVE STUDY OF MICROSPORE WALL MORPHOGENESIS OF <em>ISOETES</em></td>
<td>19</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>19</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>19</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>21</td>
</tr>
<tr>
<td>RESULTS</td>
<td>34</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>37</td>
</tr>
<tr>
<td>IV. MORPHOLOGY OF ORBICULES IN <em>ISOETES</em></td>
<td>40</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>40</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>40</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>42</td>
</tr>
<tr>
<td>RESULTS</td>
<td>44</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>49</td>
</tr>
<tr>
<td>V. SUMMARY</td>
<td>52</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>58</td>
</tr>
<tr>
<td>VITA</td>
<td>67</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Orbicules and microspore morphology, microspore surface ornamentation, orbicule shape, size and surface</td>
<td>51</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 3. Tetrad stage</td>
<td>12</td>
</tr>
<tr>
<td>4 – 6. Free spore stage</td>
<td>13</td>
</tr>
<tr>
<td>7 – 8. Mature stage</td>
<td>15</td>
</tr>
<tr>
<td>9 – 11. <em>Isoetes piedmontana</em> SEM micrographs</td>
<td>16</td>
</tr>
<tr>
<td>12 – 15. Tetrad stage</td>
<td>24</td>
</tr>
<tr>
<td>16 – 18. <em>Isoetes appalchiana</em></td>
<td>26</td>
</tr>
<tr>
<td>19 – 21. <em>Isoetes engelmannii</em></td>
<td>27</td>
</tr>
<tr>
<td>22 – 23. <em>Isoetes hyemalis</em></td>
<td>28</td>
</tr>
<tr>
<td>24 – 25. York County tetraploid</td>
<td>29</td>
</tr>
<tr>
<td>26 – 27. York County tetraploid</td>
<td>30</td>
</tr>
<tr>
<td>28 – 30. <em>Isoetes appalchiana</em></td>
<td>31</td>
</tr>
<tr>
<td>31 – 33. <em>Isoetes hyemalis</em></td>
<td>32</td>
</tr>
<tr>
<td>34 – 36. <em>Isoetes engelmannii</em></td>
<td>33</td>
</tr>
<tr>
<td>37 – 42. Microspore SEMs of orbicules ( <em>Isoetes hyemalis</em>, <em>I. ×bruntonii</em>, <em>I. engelmannii</em>)</td>
<td>46</td>
</tr>
<tr>
<td>43 – 48. Microspore SEMs of orbicules ( <em>Isoetes appalachiana</em>, <em>I. mattaponica</em>, <em>I. ×altonharvillii</em>)</td>
<td>47</td>
</tr>
<tr>
<td>49 – 54. Microspore SEMs of orbicules ( <em>Isoetes tuckermannii</em>, <em>I. valida</em>, <em>I. olympica</em>)</td>
<td>48</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

GENUS DESCRIPTION

*Isoetes*, known as quillworts, is a genus in the monotypic family Isoetaceae (Lycophyta). These plants are small to medium in size and resemble rushes, both in their aquatic or amphibious habitat preference and appearance. They grow in lakes, ponds, rivers, and ephemeral pools. The axis of the plant is a short, erect structure commonly referred to as a corm. The basal portion of the young plant is usually two lobed in North American species. The upper part of the axis is covered with a dense cluster of subulate leaves, which are usually pale at the base. Leaves can range from few centimeters to 50 centimeters or more in length. A single, unbranched, central vein runs from the base to the leaf apex. Unlike most plants, *Isoetes* leaves contain four air chambers with cross walls at irregular intervals along their length.

*Isoetes* roots branch dichotomously after they emerge from the corm ground tissue. A single, oval to orbicular, sporangium forms on the adaxial side of the leaf. The sporangia are covered partially or entirely by a pale membrane called the velum. The sporangial wall may be dark spotted or striped. A layer of tapetum lines the inner sporangial wall. The main function of the tapetum is believed to be nutrient supply for the newly formed spores.

*Isoetes* is heterosporous, producing megaspores within the megasporangium and microspores within the microsporangium. Microsporangia and megasporangia are indistinguishable during early stages of development.

This dissertation follows the format of *American Journal of Botany*.
In microsporangia, an irregular group of deeply staining cells ultimately become the microsporocytes, which separate from each other prior to meiosis. Estimates of the number of bifacial (monolete) microspores produced by a single sporangium range from 300,000 – 1,000,000. The spore number of each sporangium in *Isoetes* is probably greater than in any other vascular plant. Monolete microspores are small, ranging from 20 – 40 \( \mu m \) in diameter. Megasporangia, may contain from 8 to 300 tetrahedral (trilete) megaspores, which range from 200- 900 \( \mu m \) in diameter. Megaspores may be white, green, gray, or black at maturity. The surface may be smooth or have distinctive ornamentation (Lellinger and Taylor, 1997).

The chromosomes of *Isoetes* range in length from 1-2 \( \mu m \) to 7-8 \( \mu m \). The basic chromosome number for the genus is \( x=11 \) and allopolyploidy is common. A number of species are diploid as in *Isoetes engelmannii* (2n=22), tetraploid as in *Isoetes hyemalis* (2n=44) or hexaploid as in *Isoetes japonica* (2n= 66) from Japan. (Masayuki, 1996).

LITERATURE REVIEW

The genus *Isoetes* is considered one of the most poorly studied genera of fern allies; the number and status of the species will certainly be revised as studies progress. Megaspores have been the most frequently used character in identifying species, mainly due to their size and surface patterns. Megaspore surface features differ markedly among species and have therefore been used to distinguish taxa. Pfeiffer (1922) studied the characteristics of megaspores and used them in species delineation within the genus. She also suggested that evolutionary relationships were indicated by the ornamentation of the megaspores; however, recent studies show that megaspore ornamentation was not
sufficient to establish a relationship within the genus due to convergence of spore features (Hickey 1986).

Microspores have also been used in delimiting species of *Isoetes*; however, their small size as well as the limitations of the light microscope (which cannot be used to observe the surface details of the spores) did not prevent taxonomists from including limited information about the diversity of microspore ornamentation (Pfeiffer 1922). More than one scientist has recently studied microspore ornamentation using scanning electron microscopy. Musselman (2002) studied the ornamentation of *Isoetes* microspores of 52 taxa from Africa, Asia, Australia, Europe, North America and South America. He found species with higher ploidy levels have larger microspores, but no clear relationship between microspore ornamentation and ploidy levels was established. Since neither megaspore characters nor microspore characters could help in solving taxonomic problems of *Isoetes*, scientists looked at features that might help in elucidating relationships within the genus. Lugardon (1973) studied microspores of five species of *Isoetes* and divided the microspore wall into four sublayers: perispore (exterior), paraexospore, exospore and endospore (interior).

Lugardon (1991) also investigated microsporogenesis in *Isoetes duriei* Bory. He found that the mature wall also consists of 4 layers, which he termed perispore, paraexospore, exospore and endospore. At early stages of sporoderm formation, the tetraspore, one of the four spores in a spore mother cell, enveloped by a special wall that is usually thin and nearly without callose. The exospore and paraexospore formed directly after release of the tetraspores from the sporocyte wall, which surrounds them at the tetrad stage. After the two walls were formed, fibrillar-granular material accumulated
on the outer surface, forming the perispore. Uehara et al. (1991) studied microspore wall morphogenesis in *Isoetes japonica* using transmission electron microscopy. Four layers were found to form the microspore wall, which they named the perispore, outer exospore, inner exospore, and endospore. The perispore consisted of electron dense material derived from the tapetum, while the exospore was divided into an outer and an inner layer with a large gap between the two. The outer exospore appeared as an undulating plate, consisting of tripartite lamellae on the microspore cell membrane. Uehara et al. (1991) concluded that lamellae of the outer exospore, inner exospore and endospore are formed and derived in that order from the cytoplasm of the microspore and the homogeneous sporopollenin material of the perispore may be derived from the sporophytic tapetal cytoplasm.

Macluf et al. (2003) examined the morphology and ultrastructure of microspores of *Isoetes savatieri* Franchet. The microspores were elliptic and microechinate; however, only three layers were recognized: perispore, exospore and endospore. The perispore was composed of thin joined threads that formed a lacunose layer. Two structurally different parts of the underlying exospore are described them. The endospore is fibrillar.

None of the previous studies have emphasized the relationship between developmental patterns and the final surface structure of the spore, or include the ploidy levels, although polyploidy is very common in this genus.
CHAPTER II
MICROSPORE WALL MORPHOGENESIS OF *ISOETES*
*PIEDMONTANA*

ABSTRACT

Microspore wall morphogenesis of *Isoetes piedmontana* was studied using scanning and transmission electron microscopy. The microspore wall consisted of four layers: perispore, paraexospore, exospore and endospore. Immediately after meiosis, the paraexospore was formed around the microspore. The exospore was formed next between the cell membrane and the paraexospore. Finally, the perispore was deposited on the paraexospore and the endospore is formed within the exospore. Paraexospore, exospore and endospore were largely derived from the cytoplasm of the microspore. The sporopollenin materials of the perispore were derived from the secretory tapetal cells along the inner sporangial wall.

INTRODUCTION

The genus *Isoetes* includes 150 species distributed worldwide (Taylor et al. 1985). Plants are small to medium in size and resemble rushes, both in their aquatic or amphibious habitat preference and appearance. They grow in lakes, ponds, rivers, and ephemeral pools. The axis of the plant is a short, erect structure commonly referred to as a corm. Leaves range from a few centimeters to 1.0 meter or more in length. *Isoetes* roots branch dichotomously after they emerge from the corm ground tissue. A single, oval to orbicular, sporangium formed on the adaxial side of the leaf. A layer of the
tapetum forms the inner sporangium wall. The main function of the secretory tapetum is believed to be nutrient supply for the newly formed spores (Pacini et al. 1985).

*Isoetes* is heterosporous, producing megaspores formed within the megasporangium and microspores formed within the microsporangium.

In microsporangia, an irregular group of deeply staining cells ultimately becomes the microsporocytes, which separate from each other prior to meiosis. Estimates of the number of bifacial (monolete) microspores produced by a single sporangium range from 300,000 – 1,000,000. Microspores are small, ranging from 20 – 40 μm in length. The chromosomes of *Isoetes* range from 1-2 μm to 7-8 μm. The basic chromosome number for the genus is x=11 and allopolyploidy is common. (Taylor et al. 1985)

The genus *Isoetes* is one of the most poorly studied genera of fern allies, and the number and status of the species will certainly be revised with further work. Megaspores are the most frequently character used in identifying *Isoetes* species, mainly due to their size and surface patterns. Megaspore ornamentation differs markedly among species and has been used to distinguish taxa. Pfeiffer (1922) studied the characteristics of megaspores and used them in species delineation within genus. She also suggested an evolutionary relationship indicated by the ornamentation of the megaspores; however, recent studies showed megaspore ornamentation was not sufficient to establish a relationship within the genus due to convergence of spore features (Hickey 1986).

Microspores have also been used in delimiting species of *Isoetes* (Musselman, 2002); however, their small size as well as the limitations of the light microscope (which can't be used to observe the surface details of the spores) did not prevent taxonomists from including limited information about the diversity of the microspore ornamentation.
Several investigators have recently studied microspore ornamentation using scanning electron microscopy. Musselman (2002) studied microspore ornamentation of 52 taxa from Africa, Asia, Australia, Europe, North America and South America. He found species with higher ploidy levels have larger microspores, but no clear relationship between microspore ornamentation and ploidy levels were established. Since neither megaspore characters nor microspore characters help in solving taxonomic problems of *Isoetes*, researchers looked at features that might help in elucidating relationships within the genus.

Using Transmission electron Microscope, Lugardon (1973) studied microspores of five species of *Isoetes* and divided the microspore wall into 4 sublayers: perispore (exterior), paraexospore, exospore and endospore (interior). Lugardon (1991) investigated microsporogenesis in *Isoetes duriei* Bory. He found, at early stages of sporoderm formation, the tetraspore, one of the four spores in sporocyte, was found to be enveloped by a special wall that is usually thin and nearly without callose. The exospore and paraexospore formed directly after release of the tetraspore from the envelopes that surround them at the tetrad stage. When these two walls were formed, fibrillar-granular material accumulated on the outer surface, forming the perispore. Uehara et al. (1991) studied the microspore wall morphogenesis in *Isoetes japonica* A. Br. using transmission electron microscopy. Uehara et al. (1991) referred to microspore wall layers as perispore, outer exospore, inner exospore, and endospore. The perispore consisted of electron dense material derived from the tapetum, while the exospore was divided into an outer and an inner layer with a large gap between them. The outer exospore appears as an undulating plate, consisting of tripartite lamellae on the microspore cell membrane. Uehara et al.
(1991) concluded that lamellae of the outer exospore, inner exospore and endospore are formed and derived in that order from the cytoplasm of the microspore; the homogeneous sporopollenin material of the perispore may be derived from the sporophytic tapetum cytoplasm. Macluf et al. (2003) examined the morphology and ultrastructure of microspores of *Isoetes savatieri* Franchet. The microspores were elliptic and microechinately sculptured; however, only three layers were recognized: perispore, exospore and endospore. The perispore was composed of thin joined threads that formed a lacunose structure, which could be distinguished in the microspore wall. Two structurally different parts of the underlying exospore were described. The endospore is fibrillar.

This paper describes the wall morphogenesis in *Isoetes* microspores, focusing on the relationship between wall morphogenesis and mature microspore ornamentation.
MATERIALS AND METHODS

Specimens of *Isoetes piedmontana* (Pfeiffer) Reed were collected from Franklin County, E of Louisburg on Walter Collins Rd. at Stallings crossroad area, North Carolina. 3 May 2004, K. Alarid & R. Bray, 7/2004, a voucher specimen is deposited in the herbarium. Old Dominion University, Norfolk, Virginia, USA.

Microsporangia at various developmental stages were carefully excised from the specimens manually.

For scanning electron microscopy, microspores were collected from the sporangia and placed on double stick tape on stubs. The samples were coated with gold-palladium and examined with LEO 435VP. For transmission electron microscopy, microsporangia were cut into about 1-2 mm slices, and fixed (primary fixative) for 24 hours in 2.5% gluteraldehyde- 2% formyaldehyde in 0.1 M sodium calcodylate buffer (pH 7.4) at 4°C then washed three times (ten minutes per wash) in cacodylate buffer (pH 7.4) at room temperature. Microsporangia were post fixed (secondary fixative) for 2 hours with 2% osmium tetra-oxide in 1M CaCo (pH 7.4) at 4°C. Microsporangia were finally washed in calcodylate buffer (pH 7.4) three times (each time for 10 minutes), at room temperature. At this stage, microsporangia were moved through a series of ethanol concentrations (30%, 50%, 70%, 80%, 90%, 95%, and 100%). They were left for 40 minutes in each concentration.

By the end of the dehydration process, microsporangia were transferred to 100% propenyl oxide solution and left for 60 minutes at room temperature. Microspores were placed in Spurr resin, prepared as follows: 1: 2 Spurr: propenyl oxide, 1: 1 Spurr:
propenyl oxide, 2: 1 Spurr: propenyl oxide. Microsporangia were left in the first two dilutions for 48 hours and 72 hours in the last dilution.

After infiltration, microsporangia were embedded in the Spurr resin for 24 hrs at 65°C. Silver thin sections of microspores were cut with a diamond knife on a RMC MT2C ultramicrotome and placed on naked copper grids. The sections were then stained with uranyl acetate for 20 mins and lead citrate for 3 mins. The tissues were viewed and photographed using a JEOL 100 CXII transmission electron microscope (TEM).

RESULTS

Tetrad Stage

The developmental stages began immediately after meiosis. Large numbers of tetrads (sporocytes) were observed in the microsporangium (Fig. 1). The tetraspores represent the products of meiosis in microspore mother cells. Tetrads were surrounded by two to three layers of tapetal cells, which were enclosed by the sporocyte wall. The tapetum forming the inner surface of the microsporangial wall was of the secretory type (Uehara et al. 1991). Tapetal cells had thin cellulosic walls with plasmodesmata. Some vacuoles, endoplasmic reticulum, dictyosomes and a few plastids occurred in the microspore cytoplasm. The four immature microspores were attached to each other along their proximal face where apertures formed later, with a space between the sporocyte wall and the microspores. At this stage, the initial paraexospore layer consisted of electron dense lamellae that formed on the spore plasma membrane. The paraexospore at this stage consisted of three to six sheets of lamellae (Fig. 2). The thickness of the paraexospore varied along the microspore surface. The greatest thickness was observed at the distal face of the microspore while the least thickness was observed at the area of the
aperture. A large protrusion occurred at the proximal face of the microspore. At the top of this protrusion, the paraexospore plates were discontinuous (Fig. 1). There was a large space between the plasma membrane and the paraexospore at the proximal angle of the microspore. Another layer, the exospore, was formed between the paraexospore and the plasma membrane of the microspore. The exospore layer in the aperture area was thicker than along the distal surface.
Figs. 1-3. Tetrad stage. 1- The spore mother cell wall surrounds each tetrad. Many plastids are seen in the cytoplasm of each tetraspore. The figure shows three of the four tetraspores. The paraexospore wall is discontinuous at the aperture region. 2- The paraexospore wall: undulating plates surround each microspore. 3- A tetrad showing one large tetraspore and a portion of two other tetraspores. PE, paraexospore; AP, aperture; NU, nucleus; SPC, spore mother cell wall.
Figs. 4-6. Free spore stage. 4- After release from the tetrad wall of the spore mother cell, the shape of the microspore undergoes dramatic changes. 5- Two more walls are formed, the exospore and the endospore. 6- The paraexospore increases in thickness to about 0.4μm. The endospore is thicker at the aperture area than on the distal side. PE, paraexospore; AP, aperture; NU, nucleus; SPC, spore mother cell wall; EX, exospore; EN, endospore; PEN, pseudoendospore.
Free Spore Stage

At the free spore stage, the sporocyte wall breaks down, releasing the microspores, which were larger in size than at the tetrad stage. Rough endoplasmic reticula were also abundant, indicating protein synthesis and suggesting that the materials constituting the walls were formed internally rather than externally. Large vacuoles were observed in the cytoplasm of some microspores. As the microspore increased in size, two more layers were added to the microspore wall: endospore and perispore (Fig. 4).

Prior to the formation of the endospore, a pseudoendospore was formed inside the exospore (Fig. 5). The pseudoendospore consisted of an irregularly granular layer on the inner surface of the exospore. This layer will be disappeared later and fused with the endospore layer. The endospore was formed interior to the pseudoendospore between the exospore and plasma membrane and was the thickest layer of the mature microspore wall (0.6μm). As the three inner layers of the microspore matured, the perispore began to form on the surface of the paraexospore. The plates of the paraexospore increased in thickness and appeared to undulate, forming a multi-laminar substructure with irregular spaces. The thickness of the exospore increased to 0.3 μm. The mature microspores of *Isoetes piedomontana* were 20-30 μm in diameter and have an echinate surface (Fig. 10). They had a thick and fully developed perispore which is 2μm thick distally and 5μm thick proximally (Fig. 5). This outermost layer consisted of branching threads of sporopollenin which were closely and irregularly spaced. At this stage, the cytoplasm of the mature microspore was filled with many fat bodies (oil droplets).
Figs. 7-8. Mature stage. 7-Mature microspore showing fully developed perispore.
8-Magnified image of the four wall layers; endospore, exospore, paraexospore and perispore. The perispore consists of thin interconnected threads. The cytoplasm is filled with vacuolar fat bodies (FB). PE, paraexospore; EX, exospore; EN, endospore; FB, fatbodies.
Figs. 9-11. *Isoetes piedmontana* SEM micrographs. 9. A microspore showing the echinate ornamentation, the distal echinae are longer than the proximal ones. 10. Microspore showing the aperture area. 11. Echinate surface.
DISCUSSION

This study is one of the few on Isoetes microspore wall ultrastructure (Macluf et al. 2003). The wall morphogenesis of I. piedmontana is summarized as follows: The process of maturation started directly after meiosis II. Four tetraspores, products of cytokinesis following meiosis of the spore mother cells, were formed, each with one wall, the paraexospore. Before the tetraspores leave the tetrad, the exospore formed inside the paraexospore. At the end of the tetrad stage, the wall of spore mother cell is ruptured and the microspores were released. Subsequently, the endospore is formed inside the exospore and the perispore formed on the surface of the paraexospore. In I. piedmontana, the shape of the microspore at the tetrad stage is determined by the outer line of the paraexopore wall, consisting of anatomsing laminar plates that loosely covered the exospore with a narrow discontinuity in the aperture region (Lugardon 1973). The present work, in addition to Tyron and Lugardon (1991), and Uehara et al. (1991) confirms earlier descriptions. It is, however, difficult to distinguish between the paraexospore and the exospore when the microspore is fully mature, as noted by Mcluff et al. (2006). Uehara et al. (1991) used the term tripartite lamella to describe the structure of the paraexospore and exospore, a common feature of the sporoderm in higher plants. Steinkamp and Doyle (1981) and others refer to lamellae in pollen of higher plants. Brown and Lemmon (1982) considered the lamellae of the bryophytes, Sphagnum lescurii (Sphagnaceae) to be grown centripetally from the cytoplasm of the microspore. Uehara and Kurita (1991) reported the same lamellar nature in the exospore of Lycopodium sp. (Lycopodiaceae) The paraexospore, which resembles the exospore, is made of the same materials as the exospore, which apparently originated from the
cytoplasm of the microspore. The aperture region, where the four tetraspores are joined inside the spore mother cell, is a unique feature of this genus and differs from other Pteridophyte genera. The same structure ( aperture) was described by Lugardon (1973) for *I. echinospora, I. setaceum, I. duriei* and *I. andicola* except for the lack of multi-bedded areas observed in *I. japonica* (Uehara 1991) and *Selaginella* (Selaginellaceae) species (Lugardon, 1972, 1978). The thickening of the undulating plates of paraexospore occurred directly after the formation of the exospore. I believe part of the thickening occurred as a result of external deposition of sporopollenin coming from outside the cytoplasm of the microspore. The tapetum is believed to play a major role in the thickening of the paraexospore and subsequently the formation of the perispore (Pettit, 1979). Uehara and Kurita (1991) observed the same pattern in *Lycopodium clavatum*.

The endospore, formed internal to the exospore, originated from the cytoplasm of the microspore. Macluf et al. (2006) described a complex network of two types of endospore in *I. savatieri*; fibrillar and reticulate. In *I. piedmontana*, only the fibrillar type was observed. The third type which was observed in my study is the pseudoendospore, described by Tyron and Lugardon (1991). The difference between the endospore and pseudoendospore is that the endospore forms at the time of germination while the pseudoendospore forms as a thick layer below the exospore as the spores expand and mature. The shape of the perispore layer determines the ornamentation of the microspore; the spiny projections on the echinate surface of the microspore of *I. piedmontana* as revealed by the scanning electron micrographs (Fig. 11) mirror the protrusions of the perispore in the transmission electron micrographs (Fig. 8). This indicates that the structure of the perispore is responsible for the sculpturing of the mature microspore wall.
CHAPTER III

A COMPARATIVE STUDY OF MICROSPORE WALL

MORPHOGENESIS OF *ISOETES*

ABSTRACT

Microspore wall morphogenesis of four species of *Isoetes* was studied using scanning and transmission electron microscopy. The microspore wall consisted of four layers: perispore, paraexospore, exospore and endospore. Immediately after meiosis, the paraexospore was formed around the microspore. The exospore was formed next between the cell membrane and the paraexospore. Finally, the perispore was deposited on the paraexospore and the endospore is formed within the exospore. Paraexospore, exospore and endospore were largely derived from the cytoplasm of the microspore. The sporopollenin materials of the perispore were derived from the secretory tapetal cells along the inner sporangial wall.

INTRODUCTION

*Isoetaceas* constitutes a family with worldwide distribution. They live in temperate regions of all continents in lakes, ponds, rivers, swampy areas, and ephemeral pools. *Isoetes* is heterosporous, producing megaspores in megasporangia and microspores in microsporangia. Microspores and megaspores are strikingly different in size and shape. Megaspores are trilete, globular in shape and range from 200-750 μ in diameter. Microspores of *Isoetes* are monolette, bilaterally symmetrical with a single aperture. They are usually between 10 and 30 μ in length. (Lellinger and Taylor, 1997).
A layer of secretory tapetum lines the inner wall of the sporangium. The main function of the tapetum is to provide nutrition to the newly formed spores (Pacini et al. 1985). Despite numerous studies on spore morphology in Isoetes, the development of the microspore wall remains poorly understood. Microspores have been used to delimit species of Isoetes (Musselman 2002); however their small size as well as the limitation of light microscopy did not prevent taxonomists from including limited information about the diversity of microspore ornamentation (Pfeiffer 1922). Recently, microspore ornamentation has been widely investigated using the scanning electron microscope. Lugardon (1973) studied the microspore walls in five species of Isoetes and divided the microspore wall into 4 sublayers: perispore, paraexospore, exospore and endospore. Uehara et al. (1991) studied the microspore wall morphogenesis in Isoetes japonica A. Br from Japan. Four layers formed the microspore wall (perispore, outer exospore, inner exospore and endospore).

Musselman (2002) studied microspore ornamentation of 52 taxa from Africa, Asia, Australia, Europe, North America and South America. He found species with higher ploidy levels have larger microspores.

Macluf et al. (2003) examined the morphology and ultrastructure of megaspores and microspores of Isoetes savatieri Franchet. Three wall layers were recognized: perispore, exospore and endospore.

For this study, the following four species were studied: Isoetes engelmannii is widely distributed in the eastern United States. It is considered to be a basic diploid. Plants grow in shallow lake and river shores in subacid to calcareous substrate (Musselman and Knepper 1994).
*Isoetes appalachiana* occurs in submerged to emergent along creek banks, woodland pools and lakes in acidic clay or gravel substrate. The plant is distributed the Eastern of North America, from Pennsylvania to Florida. (Brunton et al. 1997).

*Isoetes hyemalis* is typically a species of shallow, running water in creeks, sloughs, and along river shores under dense shade of deciduous or mixed swamp forest. *Isoetes hyemalis* is known from sites in the lower Piedmont of Virginia and North Carolina and across the adjacent Coastal Plain of the Carolina, Georgia and Florida according to Brunton and Britton (1994).

York County tetraploid occurs in shallow depressions and streams in deciduous coastal plain forests. This species is collected from York County, Virginia.

MATERIALS AND METHODS

Specimens of *I. engelmannii* A. Braun, *I. appalachiana* Brunton and Britton, an undescibed species (York Count, VA), are *I. hyemalis* Brunton were studied. Voucher specimens have been deposited in the Old Dominion University Herbarium (ODU).


- *Isoetes* sp. SR 173, Godwin Neck Road, on the east side of the road. York County, VA.

Microsporangia at various developmental stages were carefully excised from the
dried pressed specimens manually.

For TEM studies, microsporangia were cut into about 1-2 mm slices, and fixed
(primary fixative) for 24 hours in 2.5% gluteraldehyde- 2% formyraldehyde in 0.1 M
sodium calcodylate buffer (pH 7.4) at 4°C then washed three times (ten minutes per
wash) in cacodylate buffer (pH 7.4) at room temperature. Microsporangia were post fixed
(secondary fixative) for 2 hours with 2% osmium tetra-oxide in 1M CaCo (pH 7.4) at
4°C. Microsporangia were finally washed in calcodylate buffer (pH 7.4) for three times
(each time for 10 minutes), at room temperature. At this stage, microsporangia were
moved through a series of ethanol concentrations (30%, 50%, 70%, 80%, 90%, 95%, and
100%). They were left for 40 minutes in each concentration.

By the end of the dehydration process, microsporangia were transferred to 100%
propenyl oxide solution and left for 60 minutes at room temperature. Microspores were
placed in Spurr resin, prepared as follows: 1: 2 Spurr: propenyl oxide, 1: 1 Spurr:
propenyl oxide, 2: 1 Spurr: propenyl oxide. Microsporangia were left in the first two
dilutions for 48 hours and 72 hours in the last dilution.

After infiltration, microsporangia were embedded in the Spurr resin for 24 hrs at
65°C. Silver thin sections of microspores were cut with a diamond knife on a RMC
MT2C ultramicrotome and placed on naked copper grids. The sections were then stained
with uranyl acetate for 20 mins and lead citrate for 3 mins. The tissues were viewed and
photographed using a JEOL 100 CXII transmission electron microscope (TEM). For
scanning electron microscope studies, microspores were collected from the sporangia and placed on double stick tape on stubs. The samples were coated with gold-palladium and examined with LEO 435VP.

12. One tetraspore surrounded by plasma membrane. Well defined nucleus, high number of ER and elongated plastid are observed in the cytoplasm. 13- Two tetraspores, one showing a clear nucleus and thin plasma membrane wall. 14- Tetraspores with 4 immature microspores surrounded by sporocyte wall. 15- Enlarged micrograph showing the early microspore of *Isoetes appalachiana* with 2 walls; paraexospore and exospore. The paraexospore wall is discontinuous at the aperture area. Figure abbreviations: PE,
paraexospore; EX, exospore; AP, aperture; NU, nucleus; PM, plasma membrane; SPC, spore mother cell wall; ER, endoplasmic reticulum
Figs. 16-18. *Isoetes appalachiana*. 16- After the microspore departs the spore mother cell, more walls are formed; endospore and perispore. The endospore is thicker along the distal side of the spore. 17- A highly magnified micrograph shows the wall layers of the microspore. The perispore is accreted from compounds of the tapetal cells of the sporangial wall. 18- A magnified TEM image of the aperture area showing the four walls, the endospore is very thick compared to other walls. Figure abbreviations: PE, paraexospore; EX, exospore; AP, aperture; Nu, nucleus; P, perispore; EN, endospore
Figs. 19-21. *Isoetes engelmannii*. 19- A microspore showing the four wall layers. Large numbers of endoplasmic reticula with well defined nucleus are observed in the cytoplasm.

20- A high resolution image showing the pseudoendospore wall that will eventually disappear and fuse with the endospore layer. 21- A magnified image of the wall shows the 4 layers; outer perispore, paraexospore, exospore, pseudoendospore and inner endospore. Figure abbreviations: PE, paraexospore; EX, exospore; AP, aperture; NU, nucleus; P, perispore; EN, endospore; PEN, pseudoendospore.
Figs. 22-23. *Isoetes hyemalis*. 22- A microspore section showing the 4 wall layers with many fat bodies which at maturity will fill the cytoplasm. Four walls are formed; endospore and exospore completely surround the cytoplasm while the paraexospore and perispore are discontinuous at the aperture area. 23- A highly magnified image, showing the four walls with large space between the paraexospore and the exospore. Figures abbreviations: PE, paraexospore; EX, exospore; NU, nucleus; P, perispore; EN, endospore
Figs. 24-25. York county tetraploid. 24- Mature microspore showing the four wall layers. The paraexospore is attached to the exospore at the proximal aperture side. The perispore layer is very thin at this stage. 25- A magnified image of the wall of the microspore.

Figure abbreviations: PE, paraexospore; EX, exospore; AP, aperture; P, perispore; EN, endospore
Figs. 26-27. York County tetraploid. 26- Mature microspore, showing the four wall layers. The cytoplasm is filled with vacuolar fat bodies (FB). The perispore (P) consists of thin interconnected threads, it is wider on the distal side. 27- SEM micrograph showing the echinate surface. Figure abbreviations: PE, paraexospore; EX, exospore; P, perispore; EN, endospore
Figs. 28-30. *Isoetes appalachiana*. 28- Mature microspore showing fully developed perispore (P). Large vacuole is observed in the cytoplasm with many fat bodies. 29- An enlarged image of the microspore wall. 30- SEM showing sparsely low tuberculate ornamentation. Figure abbreviations: PE, paraexospore; EX, exospore; P, perispore; EN, endospore
Figs. 31-33. *Isoetes hyemalis*. 31- Mature microspore with a fully developed endospore and perispore, the perispore and paraexospore wall layers are discontinuous at the aperture area. 32- A magnified micrograph showing the four wall layers; perispore, paraexospore, exospore and endospore. 33- SEM micrograph showing moderately dense echinate ornamentation. Figure abbreviations: PE, paraexospore; EX, exospore; P, perispore; EN, endospore.
Figs. 34-36. *Isoetes engelmannii*. 34- Mature microspore showing the cytoplasm filled with fat bodies. The wall is thinner compared to other species. 35- An enlarged image of the wall showing the four wall layers. 36- SEM micrograph showing psilate surface of the microspore- equatorial view. Figure abbreviations: PE, paraexospore; EX, exospore; P, perispore; EN, endospore; FB, fat bodies.
RESULTS

Tetrad Stage

Two main stages were observed in development: the tetrad stage and the free spore stage. The tetrad stage is observed directly after meiosis. Tetrads were surrounded by a 3-4 cell layer of tapetum. The tapetum covering the inner wall of the microsporangium was secretory. Tapetal cells were characterized by their thin cellulosic walls with plasmodesmata. Each group of four tetraspore was enclosed by a thick wall (sporocyte wall) approximately 1-2 μ in diameter. At this stage, tetraspores enclosed by, the only layer observed is the cell membrane. The TEM micrographs of the York County tetraploid (Figs. 12, 13) show a thin cell membrane enclosing each single tetraspore. Endoplasmic reticula, golgi apparati, dictyosomes and a nucleus were observed in the microspore cytoplasm. Prior to the formation of any other wall layer, a large number of rough endoplasmic reticula were observed; they will eventually form the paraexospore and exospore and endospore walls. The four immature microspores were aligned adjacent to each other along their proximal faces where the aperture will later form (Fig. 14). The next stage showed the formation of the first layer named paraexospore, formed inside the plasma membrane (Figs. 14, 15). This wall was formed while the tetraspores were still enclosed by the sporocyte wall. The organelles inside the cytoplasm of the tetraspore were responsible for the formation of the paraexospore layer. In I. appalachiana (Fig. 15), the paraexospore appeared as electron dense lamellae. This layer was similar in the other species in the study. The thickness of the paraexospore increased significantly as the tetraspore size increases before release from the sporocyte wall. The thickness of the paraexospore was not uniform across the whole microspore.
In *I. appalachiana* (Fig. 16), the greatest thickness was observed along the distal face of the microspore while the least thickness was at the area of the aperture. A large protrusion (aperture area) occurred at the proximal face of the microspore. At the distal end of the protrusion, the paraexospore plates were discontinuous (Figs. 16, 22, 31). There was a large space between the plasma membrane and the paraexospore at the proximal side of the microspore was observed. The exospore formed inside the paraexospore, developing between the paraexospore and the plasma membrane. The thickness of the exospore was uniform except in the area of the aperture which had the thinnest wall and clearly is seen in *I. appalachiana* (Fig. 16), *I. hyemalis* (Fig. 22), York county tetraploid (Fig. 24).

In *I. engelmannii*, the paraexospore and exospore were uniformly thick over the microspore (Fig. 19). A clear space occurred between the exospore and the paraexospore. Immediately, before microspores leave the sporocyte wall, the size of the paraexospore was 0.2 μ in diameter while the thickness of the exospore was 0.4 μ.

*After Tetrad Release from Sporocyte*

At this stage, the wall of the sporocyte broke down and the microspores were free in the microsporangium. This stage was characterized by an increase in size of both exospore and paraexospore layers. The size of the microspore increased to 20 –25 μ. A large vacuole was observed in the cytoplasm of the microspore (Fig. 28). A large number of endoplasmic reticula were present, indicating an active protein synthesis taking place.

Two more layers were added. The perispore was formed on the outer surface of the paraexospore and the endospore was deposited inside the exospore layer. In *I.*
engelmannii (Fig. 20), prior to the formation of the endospore, the granular pseudo-endospore layer was formed inside the exospore. This disappeared later and fused with the endospore layer.

The endospore is formed interior to the pseudo-endospore between the exospore and the plasma membrane. The thickness of the endospore will increase to 0.6 μ thickness. The endospore thickness varies across the lumen of the microspore. In I. appalachi ana, I. hyemalis and the York County tetraploid (Figs. 16, 22 and 26) the endospore is thicker near the aperture area, while the thickness of the paraexospore and exospore is thinner near the aperture area.

In I. engelmannii the thickness of the endospore is uniform around the microspore (Fig. 34). The perispore layer consists of threads of sporopollenin more or less regularly juxtaposed and closely fused showing small discontinuity. The appearance of the perispore as the outermost layer is responsible for the outer wall sculpturing seen in the mature microspore. In I. hyemalis and the York County tetraploid, the protrusions of the perispore (Figs. 26, 31) mirror the projections of the echinate surface shown in the SEM micrograph (Figs. 33, 27).

In I. appalachi ana, the SEM micrographs reveal a sparse to dense low tuberculate surface (Fig. 30). The TEM micrographs of I. appalachi ana show the perispore with low projections like in the SEM micrograph (Fig. 30).

In I. engelmannii, the surface is psilate (Fig. 34) and the perispore is smooth as well. The deposition of sporopollenin which contributes to the formation of the surface sculpturing comes from outside the spore. The secretory tapetum is believed to be responsible for the formation of the perispore.
DISCUSSION

Spore mother cells were surrounded by a thick coat and was considered to be derived from the dictyosomes of the cytoplasm. Similar results were observed in spore mother cells of some other Pteridophyta. (Lugardon 1972; Pettitt 1978).

The shape of the tetraspores at the tetrad stage was determined by the outer line of the paraexospore wall formed inside the plasma membrane of the microspore. Large numbers of endoplasmic reticula and dictyosomes are observed in the cytoplasm of each tetraspore, directly before formation any wall layer, and these organelles were thought to be responsible for the formation of the paraexospore.

Brown et al. (1982) considered the lamellae of the bryophyte, *Sphagnum lescurrii* (Sphagnaceae) formed centripetally from the cytoplasm of the microspore. Uehara and Kurita (1991) reported the same lamellar structure in the exospore of *Lycopodium* sp. The paraexospore, which resembles the exospore, was made of the same materials as the exospore and was produced by organelles in the cytoplasm of the microspore.

The formation of the undulating plates of paraexospore, as mentioned before, resulted chiefly from the cytoplasm of the microspore, but not completely. After the microspore was released from the spore mother cell, the paraexospore thickens as it is deposited externally. The tapetum is considered the source of the outer paraexospore (Pettit 1979).

Uehara and Kurita (1991) observed the same pattern in *Lycopodium clavatum*. The aperture region, in which the four tetraspores were closely aligned inside the spore mother cell, is a unique feature of this genus and differs from other pteridophyte genera. The paraexospore and exospore of each tetraspore showed some discontinuity near the
aperture area. This area is believed to be the area of sperm release after the microgametophyte becomes fully mature. In the microspore wall, the thickness of the distal side of the spore wall is too thick to allow the sperm to escape. The wall discontinuity is seen in all species except in *I. engelmannii*. *Isoetes engelmannii* has the thinnest wall of all species studied. The endospore formed internal to the exospore, originated from the cytoplasm of the microspore in *I. engelmannii*. In *I. savatieri* Franchet. Macluf et al. (2006) described a complex network of two types of endospore: fibrillar and reticulate. In my study, only the fibrillar type was observed.

The third wall observed was the pseudo-endospore, described by Tyron and Lugardon (1991). The pseudo-endospore was observed only in *I. engelmannii*. The difference between the endospore and pseudo-endospore is the endospore forms at the time of germination while the pseudo-endospore forms a thick granular layer below the exospore as the spore expands and matures. The pseudo-endospore will later disappear and fuse with the endospore. The thickness of the endospore layer was not uniform. The area adjacent to the aperture had the thickest endospore layer while the thickness of the exospore, paraexospore and perispore was thinner in that region. That observation held true for all species in this study except *I. engelmannii*.

The perispore layer determined the ornamentation of the microspore. The projections on echinate surface of the microspores in *I. hyemalis* and the York County tetraploid as revealed by scanning electron micrographs resembled the protrusions of the perispore in the transmission electron micrographs. Also the smooth surface of the microspore of *I. engelmannii* and the projections on the microspore surface of *I.*
appalachiana revealed by the scanning electron micrographs that they resemble the perispore morphology on the transmission micrographs.

In summary, wall morphogenesis of Isoetes microspores starts directly after meiosis II. In the spore mother cell, four tetraspores, the products of cytokinesis following meiosis of the spore mother cells formed, each with one wall, the paraexospore. Before the tetraspores were released from the spore mother cell, the exospore formed inside the paraexospore. At the end of the tetrad stage the wall of the spore mother cell ruptured and the microspores were released. Subsequently, the endospore formed inside the exospore and the perispore is formed on the outer surface of the paraexospore. The perispore was responsible for the sculpturing of the mature microspores and the tapetum is thought to be the origin of sporopollenin making up the perispore.
CHAPTER IV

MORPHOLOGY OF ORBICULES IN Isoetes

ABSTRACT

Orbicules or Ubisch bodies were observed as minute granules of sporopollenin on the innermost tangential and/or radial walls of secretory tapetum cells. Although studied in angiosperms, orbicules have not been reported from pteridophytes. Orbicules were studied with scanning electron microscope in nine species of Isoetes. The orbicule types are described based on morphological and ultrastructural variation. The size, shape, surface, location, distribution and density of orbicules were investigated. Orbicule characteristics may prove to be of systematic value for the genus Isoetes.

INTRODUCTION

Orbicules are tiny granules located on the inner surface of the microsporangial wall. They were discovered by Rosanoff (1865) who studied the anatomy and morphology of anthers and observed small tiny granules covering the inner wall of the tapetum. He treated these structures with sulfuric acid and discovered they were resistant to the acid. Von Ubisch (1927) and Kosmath (1927) studied a group of taxa with and without orbicules, focusing on those with orbicules. The orbicules were treated with different chemicals and stains to better understand their nature. The study showed orbicules share characteristics with pollen. The term orbicules was coined by Erdtman (1961) and was defined as “small granules spread over the exine surface in certain gymnosperms’ pollen”. Rowley (1962) referred to these granules as Ubisch bodies. The function of the orbicules remains unknown. Echlin (1971) and Bhandari (1984) reviewed
the literature on the different hypotheses that have been proposed concerning orbicule function, one of which is a transport mechanism for sporopollenin between tapetum and the developing microspore.

Orbicules are well studied in the flowering plants and are variable in both shape and size. Size ranges from 0.14 \( \mu \) in *Rondeletia ordata* (Rubiaceae) (Huysmans et al., 1997) to 15\( \mu \) in *Saxifraga cymbalaria* var. luetiana, (Saxifragaceae) (Abadie & Hideux, 1979); commonly they are less than 5 \( \mu \) in diameter. There are always similarities between the surface of the orbicules and the exine of the pollen grain or spore. Orbicules are resistant to acetolysis and show the same reaction as exine to all histochemical stains indicating they are made of sporopollenin.

In higher plants, two main types of tapetum are known; the secretory (parietal, grandular, or cellular) tapetum and the amoeboid (syncytial invasive or plasmodial) tapetum. Secretory tapetum is believed to be the most primitive. The tapetal cells' main function is to provide nutrients for the developing microspores and contribute to the formation of the outer spore surface. Orbicules are only known from species with secretory tapetum; however, some species with secretory tapetum do not show development of any orbicules.

The literature is lacking studies investigating microsporogenesis in the genus *Isoetes*. Lugardon (1981) investigated the orbicules in a group of spermatophytes using transmission electron microscopy. He observed small granules on the surface of the tapetal layer and found that the orbicules surface is similar to the surface (exine) of the spore.
MATERIAL AND METHODS

Specimens Examined

This study was based on samples from 9 species of Isoetes collected from different locations in Virginia, North Carolina, Canada and Syria: I. hyemalis Brunton, I. mattraponica L.J. Musselman and W.C. Taylor, I. engelmannii A. Braun, I. tuckermannii Britton and Brunton, I. appalachiana Brunton and Britton, I. ×bruntonii Knepper and Musselman, I. ×altonharvillii Musselman and Bray, I. olympica A. Braun. I. valida, (Engelm) Clute.

Voucher specimens have been deposited in the Old Dominion University Herbarium (ODU).


- *Isoetes tuckermannii* Britton and Brunton. 50 to 100 m North from outlet along E shore Ainslie Lake opposite S end of Ainslie, Village Campground, Cape Breton Island, Inverness CO, Canada. 1 September 1995. D. Brunton & K. McIntosh. 12,318.


**Scanning Electron Microscopy**

Fresh and dried herbarium specimens were used for this study. Herbarium material was collected by the author and vouchers were deposited in the herbarium of Old Dominion University, Norfolk, VA, USA (ODU).

The microsporangia of each species were cut into halves and cleared of microspores to expose the inner microsporangial wall which is believed to be the tapetal layer. Small pieces of the inner wall were placed on a stub. Stubs were sputter coated with gold palladium. Samples were then examined on a Leo 345 VP Scanning Electron Microscope.
RESULTS

The size of orbicules in *Isoetes* ranges from 1 µ in *I. × altonharvillii* (Figs. 48) to 6 µ in *I. tuckermannii* (Fig. 50). The shape of orbicules also varied among species in the genus *Isoetes*. Each species studied had a unique shape. Two species were found to have irregular shapes, *I. × altonharvillii* and *I. appalachiana*. The irregular orbicules were asymmetrical and possessed irregularly lobed margins. *Isoetes × altonharvillii* exhibited hybrid vigor in which many aborted and broken spores were observed. The orbicule surface had a range of ornamentation patterns intermediate between its parents (*I. engelmannii* and *I. valida*), scabrate to tuberculate. In *I. hyemalis*, doughnut shaped orbicule were observed. The surface of orbicules had small perforations. The size of *I. hyemalis* orbicules was 3.5-4.5 µ while the surface of the microspore was echinate (Figs. 37, 38). In *I. appalachiana*, orbicules were irregularly shaped with a surface of tiny granules. The size of the orbicules ranged from 2-3.5 µ. Microspore ornamentation of *I. appalachiana* was sparsely to densely low tuberculated (Figs. 43, 44).

*Isoetes × bruntonii* had a large square shaped orbicules embedded in the wall of the tapetum. The average size of the orbicules was 5 µ with a rough surface (scabrate). The microspores of *I. × bruntonii* were echinate, resembling *I. hyemalis*, one of its parents (Figs. 39, 40).

The orbicules of *I. engelmannii* were regular and mostly spherical. They were characterized by a scabrate surface and range from 1-2 µ, while the microspore surface was psilate (Figs. 41, 42).

Smooth cuplike orbicules were found in *I. valida*; the microspore surface was echinate.
In *I. olympica*, thin threads of sporopollenin connected the orbicules to each other and with the tapetal membrane. An aggregate of spherical orbicules distinguished this species. The size ranged from 4-6 μ. The microspores of *I. olympica* had a strongly echinate surface (Figs. 53, 54).

*Isoetes tuckermanii* had two different types of orbicules, the first was cuboidal while the other resembled a volcano with a large central opening (Fig. 50). The size of the two kinds ranges from 3 to 6 μ. The orbicule surface was smooth. The microspores were scabrate to echinate. *I. mattaponica* had cup like orbicules with clear core and rough surface. Orbicules ranged from 2.5-4 μ. The microspore surface was psilate (Figs. 45, 46).
Figs. 37 – 42. Microspore SEMs of orbicules (Isoetes hyemalis, I. ×bruntonii, I. engelmannii). Isoetes hyemalis (37, 38), 37- Microspore echinate. 38- Orbicules doughnut shaped with perforated surface. I. ×bruntonii (39, 40), 39- Microspore is scabrate to echinate. 40- Orbicules cuboidal with smooth surface. I. engelmannii (41, 42), 41- Microspore psilate. 42- Orbicules spherical with scabrate surface.
Figs. 49-54. Microspore SEMs of orbicules (*Isoetes tuckermannii, I. valida, I. olympica*).

*Isoetes tuckermannii* (49, 50) 49- microspore with scabrate to echinate surface 50- Orbicules are cuboidal in shape with smooth surface. *Isoetes valida* (51, 52) 51- Microspore sparsely echinate. 52- Orbicules cup-like with smooth surface. *Isoetes olympica* (53, 54) 53- Microspore strongly echinate 54- Orbicules spherical with sporopollenin threads on the surface.
DISCUSSION

A remarkable correlation occurs in higher plants between the ornamentation of the pollen exine and that of the orbicular wall (Hesse 1986, Al-Ghazaly and Jensen, 1986). Other workers, (Vinckier et al 2000) found a different pattern in their study of orbicules of Ixoroideae. The orbicules of Ixoroideae did not show the same patterns between the pollen exine and the orbicular wall ornamentation. My results agree with Vinckier et al. (2000).

None of the species examined in this study have similar patterns of both orbicule and microspore surface. Based on the results from this study, each species of Isoetes is believed to have a unique orbicule type, though further studies are needed. My study suggests that orbicules may be used as a diagnostic character to differentiate among species. The microspore morphology also varied among species examined as well (Figs. 37-54). Different ornamentation types were observed ranging from a psilate surface in I. engelmannii to a strongly echinate surface in I. olympica. Orbicules were mostly regular in shape except in I. ×altinharvillii, with irregularly distributed aggregates embedded in the tapetal wall. Circular orbicules with a flattened core were observed in I. tuckermannii; they are similar to the orbicules of Caffea and Psilanthus (Ixoroideae: Rubiaceae) (Vinckier et al. 2000). Hesse (1988) pointed out that some individuals of one species may produce orbicules while others do not. He also noted that different kinds of orbicules occur in a single species; in the current study, I. tuckermannii (Fig. 50) had two kinds of orbicules.

I found the tapetum was responsible for the formation of the spore surface (perispore) layer that forms the surface of the microspore wall, suggesting that the spore
surface should have the same ornamentation as the orbicular wall, as observed in flowering plants (Al-Ghazaly and Jensen, 1986). Contrary to that expectation, orbicules of *Isoetes* had a different pattern in the species examined. Table 1 summarizes these microspore and orbicule features. Also the appearance of orbicules may go through different developmental stages depending on the time of observation. In *I. tuckermannii*, two kinds of orbicules were seen, and each could be a different developmental stage. Cerceau- Larrivée et al (1981), found in *Saxifraga cymbalara* (Saxifragaceae) both disc shaped and spherical shaped orbicules, with the spherical orbicules develop later in the ontogeny than disc shaped ones.

All species had a narrow range of orbicular diameters. The parental relationships among different tetraploid species were not indicated by the morphology of orbicules.

In conclusion, *Isoetes* is frequently mentioned as a genus with a reduced number of stable diagnostic characters and some vegetative traits such as the extension of velum and lobing of the corm. I found that the presence and type of orbicules are stable characters and may prove to be as a taxonomic tool.
<table>
<thead>
<tr>
<th>Species</th>
<th>Microspore Surface</th>
<th>Orbicule shape</th>
<th>Orbicule Size</th>
<th>Orbicule surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>• <em>I. engelmannii</em></td>
<td>Psilate</td>
<td>Spherical</td>
<td>1-2 μm</td>
<td>Scabrate</td>
</tr>
<tr>
<td>• <em>I. valida</em></td>
<td>Sparsely echinate</td>
<td>Cup like</td>
<td>2-3 μm</td>
<td>Smooth</td>
</tr>
<tr>
<td>• <em>I. × altonharvillii</em></td>
<td>Scabrate-tuberculate</td>
<td>Irregular</td>
<td>1-1.9 μm</td>
<td>Pitted</td>
</tr>
<tr>
<td>• <em>I. appalachiana</em></td>
<td>Echinate</td>
<td>Irregular</td>
<td>2-3.5 μm</td>
<td>Perforated</td>
</tr>
<tr>
<td>• <em>I. tuckermannii</em></td>
<td>Scabrate-echinate</td>
<td>Cuboidal</td>
<td>3-6 μm</td>
<td>Smooth</td>
</tr>
<tr>
<td>• <em>I. mattaponica</em></td>
<td>Psilate</td>
<td>Cup like</td>
<td>2.5-4 μm</td>
<td>Rough</td>
</tr>
<tr>
<td>• <em>I. olympica</em></td>
<td>Echinate</td>
<td>Spherical</td>
<td>4-5.5 μm</td>
<td>Striated</td>
</tr>
<tr>
<td>• <em>I. hyemalis</em></td>
<td>Echinate</td>
<td>Doughnut</td>
<td>3.5 – 4.5 μm</td>
<td>Perforated</td>
</tr>
<tr>
<td>• <em>I. × bruntonii</em></td>
<td>Echinate</td>
<td>Scabrate</td>
<td>4-5.5 μm</td>
<td>Scabrate</td>
</tr>
</tbody>
</table>

Table 1: Orbicules and microspore morphology, microspore surface ornamentation, orbicule shape, size and surface.
CHAPTER V

SUMMARY

The microspore wall morphogenesis of five species of *Isoetes* was investigated using the transmission and scanning electron microscopes. The process of microspore wall formation in *Isoetes* includes two main stages: the tetrad and the free spore stage. Immediately after meiosis, large numbers of tetrads enclosed by the sporocyte wall were observed in the cytoplasm. At this stage, the initial paraexospore layer began to form on the spore plasma membrane. Another layer, the exopsore, was formed between the paraexospore and the plasma membrane of the microspore. During the free spore stage, the sporocyte wall breaks down, releasing the microspores. As the microspore increases in size, two more layers were added to the microspore wall, endospore and perispore. Pseudoendospore layer was formed only in *I. engelmannii* and *I. pietmontana*. The pseudoendospore will later fused with the endospore layer. The endospore was formed interior to the pseudoendospore between the exospore and plasma membrane. The perispore formed on the surface of the paraexospore. The perispore was responsible for the appearance of the microspore. The perispore layer for *I. hyemalis* had an echinate surface, *I. engelmannii* a psilate surface, the York County tetraploid an echinate surface, and *I. appalachiana* a sparsely to densely tuberculate.

Orbicule morphology of nine *Isoetes* species were investigated using scanning electron microscope. Orbicules are tiny granules located on the inner surface of the microsporangia wall. The size of orbicules ranged from 1 μ in *I. ×altonharvillii* to 6 μ in *I. tuckermannii*. The shape of orbicules varied among species in the genus *Isoetes*. Two species were found to have irregular shapes, *I. ×altonharvillii* and *I. appalachiana*. The
orbicules of *I. engelmannii* were spherical while *I. hyemalis* had doughnut shaped orbicules. *Isoetes valida* exhibited cup like orbicules and *I. ×bruntonii* had square shaped orbicules. Threads of sporopollenin connecting the spherical orbicules were observed in *I. olympica*. 
LITERATURE CITED


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