

TAXONOMIC RE-EVALUATION AND SPECIES RECOGNITION  
OF **AZOLLA** LAM., WITH PARTICULAR REFERENCE TO  
SECTION **AZOLLA**.

VOL I

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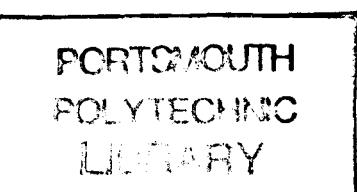
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**FOR KATHY... WITH ALL MY LOVE.**

THANK YOU FOR YOUR  
PRESENCE IN MY LIFE...  
YOU ENCOURAGE ME  
TO GO BEYOND MYSELF,  
YOU HAVE THAT INVISIBLE TOUCH...

## ABSTRACT.

DAVID G. DUNHAM - TAXONOMIC RE-EVALUATION AND SPECIES RECOGNITION OF *AZOLLA* LAM., WITH PARTICULAR REFERENCE TO SECTION *AZOLLA*.

PART A: Lack of a stable taxonomic framework for *Azolla* Lam. hampers research on the *Azolla-Anabaena* symbiosis which has special economic importance in flooded agricultural systems.

Using material from twenty-one major world herbaria and some fresh material the present investigation aims to critically re-evaluate the taxonomy of Sect. *Azolla*. Scanning electron and light microscopy are employed and some characters quantified. Vegetative and reproductive characters, often poorly defined and undelimited, are evaluated to assess the extent of phenotypic variation; those specimens examined include nomenclatural Type material.

Some terminological confusion concerning megasporangium apparatus structure is revealed and recommendations are made. Extensive sampling and description of phenotypic variation provides a more rational approach to species recognition. The megasporangium apparatus, particularly sporoderm structure, is confirmed as the best taxonomic indicator. In contradiction to previous reports, massula characters are of some taxonomic use, particularly when quantified (eg. glochidial septation). Phenotypic polymorphisms render vegetative characters of little or no diagnostic value, except for the dorsal leaf lobe trichomes. The evolutionary significance of some features is reviewed in the context of fossil and extant *Azolla*.

The following taxonomic proposals are made:- *A.filiculoides* Lam. is a valid taxon, with two subspecies to account for phenotypic variation; *A.caroliniana* Willd. and *A.microphylla* Kaulf. are synonymous with *A.filiculoides*. However, the name *A.microphylla* has been used for a valid taxon which now requires typification and a new name; *A.mexicana* Presl is a valid taxon and intraspecific taxa are not fully substantiated; an un-named taxon requires typification, it has previously been called *A.caroliniana* by some workers, but is not *A.caroliniana* of Willdenow. A preliminary study of Sect. *Rhizosperma* (Meyen) Mett. is also made, with *A.pinnata* R.Br. and *A.nilotica* Descne. ex Mett. being recognised. Future taxonomic research is suggested, and should be more applied.

PART B: Aspects of the reproductive biology of *A.filiculoides* are investigated. Inoculation of the embryo and leaf cavity with *Anabaena azollae* Stras. are described, illustrating the subtlety of morphogenesis in maintaining the symbiosis.

DECLARATION

I hereby certify that the work submitted in this thesis  
is my own and has not been presented previously, or  
separately, for any other degree.

Signed. \_\_\_\_\_

D.G. Dunham.

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Thanks extend to all those who have given technical advice, especially Colin Derek and the late Eddie Hawton for their willing assistance with photography.

Many thanks to Jane Forbes for typing this thesis from an often illegible handwritten manuscript; errors and omissions are therefore of my own making.

Finally, very special thanks are given to Kathy for her patience and encouragement through the frustrations of preparing this thesis.

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**VOLUME I**

**PART A**

1. INTRODUCTION

## 1. INTRODUCTION

### 1.1 THE SIGNIFICANCE OF THE AZOLLA-ANABAENA ASSOCIATION

**Azolla** Lam. is a genus of heterosporous water ferns with a worldwide distribution in tropical to temperate regions. Considerable attention has been focused on this small, but elegant free-floating fern because of its symbiotic association with the nitrogen-fixing cyanobacterium **Anabaena azollae** Stras. This unique association within the pteridophytes has been used for centuries in China and Vietnam (Lumpkin & Plucknett, 1982). According to these authors it was used as a fodder for animals such as pigs and ducks long before it was cultivated as a green manure. However, its principal use is now as a green manure, and interest in this aspect has extended throughout Asia, and even to Africa and South America. Since the beginning of the 'Green Revolution' in the early 1960's more attention has been paid to **Azolla** and its potential as a green manure for flooded crops, in particular rice. New high yielding rice varieties require high nutrient inputs, and **Azolla**, by virtue of being a solar powered nitrogen source, aquatic and fast growing, has a potential use in lowland rice production. Although most cultivated soils are deficient in assimilatable nitrogen, the paddy system is particularly prone to nitrogen depletion through leaching. Therefore, particularly in under-developed countries, a cheap source of nitrogen is required instead of the expensive inorganic nitrogen fertilisers derived from the Haber-Bosch Process. Much of the nitrogen fixed by the **Azolla-Anabaena** association becomes available to rice plants through decomposition

after the *Azolla* has been incorporated into the paddy soil. It was estimated that 25 - 35 days growth of *Azolla* could supply enough nitrogen for a 3 - 8 tonne per hectare crop of rice (Lumpkin & Plucknett, 1982). In short field trials, Talley & Rains (1980) reported that 2.7 kg nitrogen per hectare per day was fixed by the association, although Watanabe et al. (1980) suggested an annual average of 1.4 kg nitrogen per hectare per day.

Peters et al. (1985) showed that nitrogen fixed by the endosymbiont was released and taken up by *Azolla*, probably through unusual hair-cells in the leaf cavity; the nitrogen was transferred as ammonium, and its release and uptake appeared to be under the control of *Azolla*. The physiological complexity and intimacy of the *Azolla-Anabaena* association illustrated by these authors was also reflected by morphological and structural studies. A colony of the endosymbiont immediately behind a stem apex gave rise, via an effective inoculation process, to the colony found in the cavity of each dorsal leaf lobe (Peters & Calvert, 1983; Dunham & Fowler, 1985, and in prep.). Subsequent growth of these cavity colonies was both co-ordinated and synchronised, probably by the fern symbiont (Peters et al., 1985). Continuation of the association through the sexual phase of the life cycle was recently illustrated in detail by Dunham & Fowler (1985, and in prep.); as expected it appeared both complex and intimate. Cells of *Anabaena* became incorporated into a developing sporocarp, probably by a similar method to leaf cavity inoculation, but only in the mega-sporocarp did these cells persist as 'resting cells'. After fertilisation of an oosphere these 'resting cells' divided to

form an active colony which was collected on the stem apex of the emerging embryo (Dunham & Fowler, and in prep., see Part B).

Intimacy is further illustrated by the fact that free-living *Anabaena azollae* has not been found, and that the culturing of isolated *A.azollae* was extremely difficult (Lumpkin & Plucknett, 1980). A study by Ladha & Watanabe (1982) indicated that cultured isolates of the endosymbiont were contaminants or mutants selected by the isolation and culture methods. Although these authors (pers. comm.) believed that *Anabaena*-free *Azolla* cannot exist in nature, there are some reports of it having been found (Marsh, 1914; Fremy, 1930; Hill, 1977). Huneke (1933) and Bortels (1940) reported unsuccessful attempts to recombine isolated *Anabaena azollae* and *Anabaena*-free *Azolla*; at present this is being attempted by workers in China and the Philippines (Ladha, pers.comm.)

The International Rice Research Institute (IRRI) at Los Baños, Philippines recognises the contribution that *Azolla* can make to rice production in under-developed countries. To this end IRRI encourages all research on *Azolla*, although it concentrates on screening 'strains' (populations) for use under a variety of environmental and cropping systems. The results have suggested that suitability of *Azolla* to a particular set of conditions may be at the level of the 'strains'. This level of variation in the growth, nitrogen fixation and requirements of *Azolla* 'strains' was also suggested by Peters et al. (1980). Although this and other studies were aimed at the primary use of *Azolla*, namely in rice production, a definite move to exploiting it as a fodder crop for cattle, pigs, fowl and fish was proposed by the First International

Workshop on *Azolla* use, held at Fuzhou, China in April 1985. *Azolla* has also been shown to have a potential use in water purification, potassium accumulation and weed control in paddy fields. In addition to management problems of *Azolla*, the Workshop highlighted the lack of basic knowledge of this interesting fern, particularly in respect to taxonomic and sporulation studies, without which the exploitation of *Azolla* cannot progress.

Prior to the Workshop, palaeobotanical studies on *Azolla* had already revealed that a comprehensive taxonomic treatment of the genus had been neglected and was long overdue. Fowler (1975, 1981) and Fowler & Stennett-Willson (1978) were perhaps instrumental in this, and this research at Portsmouth Polytechnic formed the basis upon which the present investigation was initiated; this was to re-evaluate the taxonomy of extant *Azolla*, with particular reference to Section *Azolla*. Furthermore, the approach for this was provided by Fowler's investigations. Justification for the present research programme was forwarded by the increasing awareness at IRRI and other establishments for a clear and workable taxonomic framework for *Azolla*. Such a framework is essential for the correct identification and development of useful strains in their 'mainstream' research programmes. There appears to be little continuity in the species names used, and this must result in any conclusions being of little or short-lived value.

In addition to taxonomic confusion, a vagary of structural and morphological terminology has been used to describe reproductive

structures of *Azolla* (Fowler, 1981); to remedy this <sup>the</sup> unified scheme proposed by Fowler & Stennett-Willson (1978) appears to require re-evaluation and consolidation. Current taxonomic information, including species recognition, is open to various interpretations because previous authors have examined too few specimens, provided poor descriptions and illustrations, overlooked some characters and excluded nomenclatural Type material. These factors, in the light of undelimited phenotypic variation and disparate terminology, compound to result in synonymy of different taxa and incorrect designation of specimens.

## 1.2 TAXONOMIC HISTORY

### 1.2.1 Suprageneric taxa

The genus *Azolla* was established by Lamarck in 1783 when he described, as *A.filiculoides*, specimens supposedly collected from the Magellan area of Chile. The generic name is of latin derivation meaning that it is killed by drying (*azo* = to dry and *allyo* = to kill). There are various interpretations and names used in the classification of ferns (see Reed, 1954 and Sporne, 1975). It was beyond the scope of this thesis to discuss the merits of using names above the Family level. The scheme adopted here expressed the position of *Azolla* in the Leptosporangiateae, which contains the Salviniales (Sporne, 1975). According to him, the Salviniales possessed an obscure relationship with other fern groups. Based on soral features and aquatic habit, Bower (1935) and Wagner (1969) suggested a phylogenetic relationship with the Hymenophyllaceae.

Although Sporne (1975) stated that ". . . this hardly seems acceptable in view of the many extraordinary features that mark them (the Salviniaceae) off from all other ferns", he did not propose an alternative relationship.

At the Family level *Azolla* has been placed in the Marsileaceae R.Br., Salviniaceae Reichenb. and the monogeneric Azollaceae C.Chr. Griffith (1845) reviews early proposals, which were based primarily on vegetative morphology, and using a few reproductive features placed *Azolla* in the Salviniidae Bartl. (= Salviniaceae). The literature indicates periods when *Azolla* was placed in the Salviniaceae or the Azollaceae. This was despite the latter being characterised by bilobed leaves, presence of roots and unisporangiate megasporocarps, while the former had entire leaves, absence of roots and multisporangiate megasporocarps. These differences were further reinforced by other characters illustrated by Bonnet (1956), which may explain why the familial name, Azollaceae, has recently received wide acceptance. However, in palaeobotanical studies *Azolla* was placed in the Salviniaceae (Hills & Gopal, 1967; Sweet & Hills, 1974; Hall, 1975; Zhou, 1983).

#### 1.2.2 Generic and subgeneric taxa

Although *Azolla* has been considered distinct from other genera, there has been considerable controversy and confusion surrounding the subgeneric taxa. Meyen (1834, 1836) proposed two genera, *Azolla* and *Rhizosperma*, based on the number of floats on the megasporangium and the type of massula process. However,

these criteria and names were later used to define Sections (Mettenius, 1847). Since then sub-generic (Strasburger, 1873) and sectional status have been given to these taxa with equal frequency and without apparent justification. The name '*Euazolla*' instead of *Azolla* was used by Baker (1887) for Section, presumably because it was fashionable at that time to use the prefix 'Eu' to indicate that a supraspecific taxon included the generic Type. Although use of this prefix is outlawed in the International Code of Botanical Nomenclature (Article 21.3) (Stafleu, 1978), the epithet '*Euazolla*' was cited up until recently; for example, Peters et al. (1980), Calvert et al. (1983) and others. Some authors have added to Meyen's (1836) original criteria; Baker (1887) added "solitary" and "fascicled" roots, whereas Lumpkin & Plucknett (1982) added distribution of trichomes. With the discovery of fossil *Azolla* palaeobotanists erected new infrageneric taxa, but the features used remained essentially the same as Meyen's (1836). Sweet & Hills (1974, 1976) were more precise when they argued that Subgenus should be defined on the morphology of the massula and Sections on the morphology of the megaspore apparatus. This was to encompass fossil species with circinate massula processes. Although fossil studies have contributed much to the taxonomy of *Azolla*, no attempt has been made to apply this dual hierarchy proposed by Sweet & Hills. This suggests that there is little exchange of proposals between researchers of fossil and extant *Azolla*. However, in respect to the latter, the emphasis has been on the taxonomy of species rather than higher taxa.

With the brief description of habit, leaf shape and roots Lamarck

(1783) suggested, in his description of *A.filiculoides*, that *Azolla* was a new genus of the "Naiades". Subsequently other species were described by Brown (1810), Molina (1810), Willdenow (1810), Kaulfuss (1824), Desvaux (1827), Schumacher (1827), Sprengel (1827), Presl (1845), Zollinger (1854), Bertoloni (1864), Decaisne in Mettenius (1867) and Franchet & Savatier (1876) (see Table 1.1). The protalogues for all the species, with the exception of *A.nilotica* Decsne ex Mett., only provide very brief descriptions of habit, frond and leaf shape, nature of the leaves and locality, and only the protalogues of *A.microphylla* Kaulf. and *A.nilotica* recognise the presence of reproductive structures. Clearly, most protalogues offer no assistance in species differentiation. In contrast, the detailed protologue of *A.nilotica* by Mettenius (1867) described many morphological and anatomical features of vegetative and reproductive structures. Furthermore, although Type material of *A.filiculoides* and *A.pinnata* R.Br. are excluded, Mettenius compares both these species with *A.nilotica*. This was based on a manuscript by Decaisne in the Museum d'Histoire Naturelle, Paris, in which he described and named *A.nilotica*. Similarly, the name *A.mexicana* was first published, without description, by Schlechten-dal & Chamisso (1830), but later described by Presl (1845) and validly published.

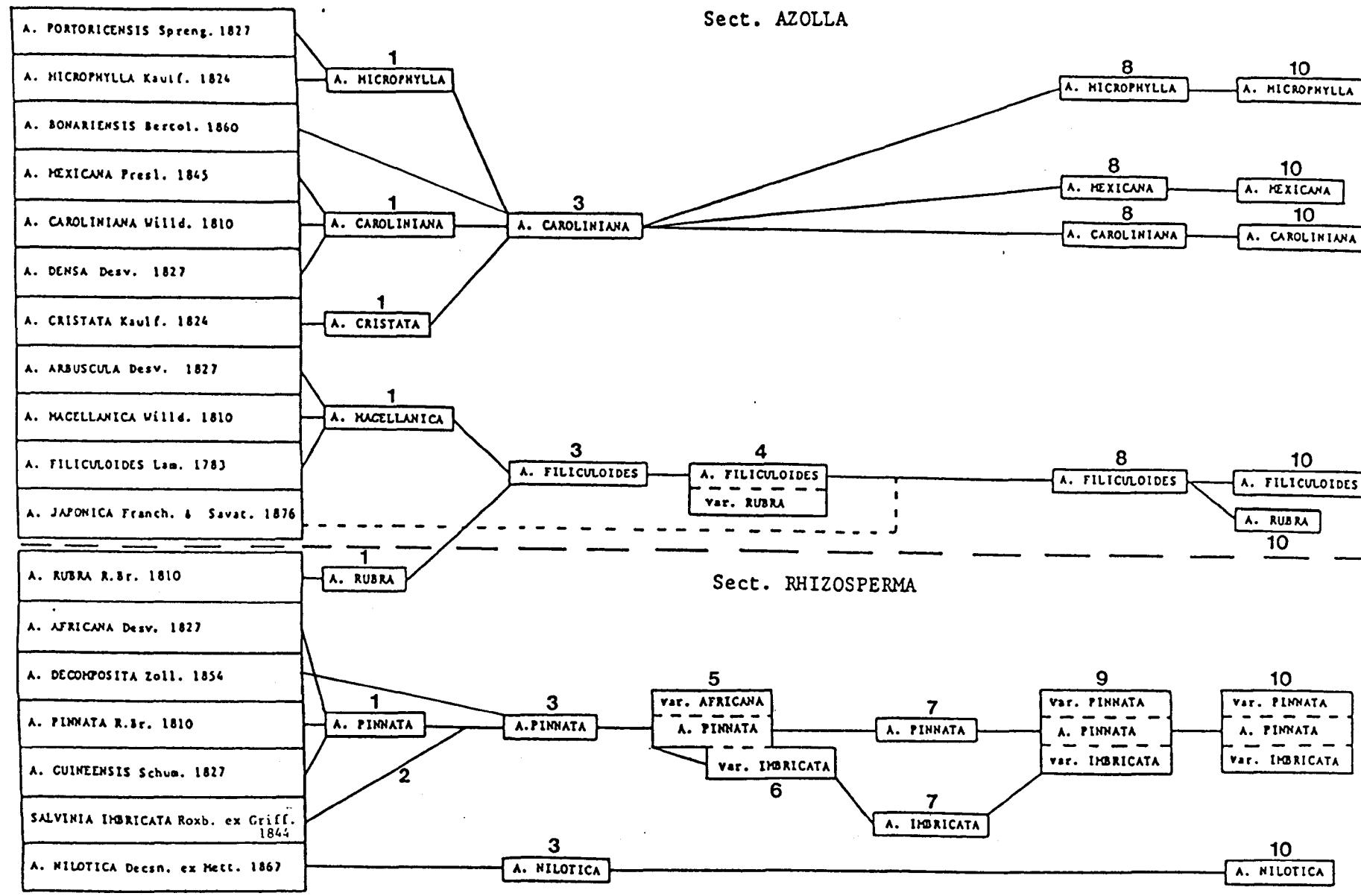
Brown (1810) was first to recognise reproductive structures, although these were not noted in the protalogues; however, like Martius (1834) and Meyen (1834, 1836), he confused male and female sporocarps. Griffith (1845) rectified this error in his study of *A.pinnata* in which he also furthered the understanding of structure and morphology of the reproductive organs.

TABLE 1.1

Table illustrating the taxonomic history of *Azolla*,  
the lines representing how the present worker believes  
the concepts of previous authors' taxa are related.  
(only significant classification changes are included)

- 1= Mettenius (1847).
- 2= Griffith (1845).
- 3= Mettenius (1867).
- 4= Strasburger (1873).
- 5= Baker (1897).
- 6= Bonaparte (1918)
- 7= Nakai (1925).
- 8= Svenson (1944).
- 9= Sweet & Hills (1971).
- 10= Lumpkin & Plucknett (1982).

TABLE I.1



Three years later, in 1847, Mettenius published the first comparative study of seven proposed species; these were - *A.magellanica* Willd. (=*A.filiculoides* and *A.arbuscula* Desv.), *A.caroliniana* Willd. (=*A.densa* Desv. and *A.mexicana* Schlecht. & Cham.ex Presl), *A.microphylla* (=*Salvinia Azolla* Raddi and *A.portoricensis* Spreng.)- and *A.cristata* Kaulf. in Section *Azolla* Meyen (Mett.) and *A.rubra* R.Br., *A.africana* Desv. and *A.pinnata* (=*Salvinia imbricata* Roxb.ex Griff.) in Section *Rhizosperma* Meyen (Mett.) (Table 1.1). According to Svenson (1944), Mettenius (1847) based his description of *A.microphylla* on the one by Martius (1834), but there was little improvement. Unlike his predecessors Mettenius (1847) placed more emphasis on reproductive characters than vegetative ones, and his descriptions therefore included megasporangium, massula and leaf morphology.

A year after his death in 1866, saw the publication of Mettenius's final work on *Azolla*. He had completely revised his earlier proposals after apparently placing more emphasis on vegetative characters (Mettenius, 1867). He recognised the priority of the name *A.filiculoides* over *A.magellanica*, in which he placed *A.rubra* (transferred from Section *Rhizosperma* to Section *Azolla*) and redefined *A.caroliniana* in which he included *A.microphylla*, *A.cristata*, *A.bonariensis* Bertol. and *Salvinia Azolla*. In Section *Rhizosperma* he included *A.pinnata*, comprising *A.africana*, *A.guineensis* Schum. and *A.decomposita* Zoll., and *A.nilotica*. Therefore, at this time, Mettenius (1867) recognised two Sections, each containing two species (Table 1.1).

Following Mettenius' (1867) prospectus, Strasburger (1873) was the first to describe, illustrate and compare sporoderm structure in *Azolla*. Furthermore, he erected *A.filiculoides* Lam. var. *rubra* R.Br. (Stras.) based on glochidial septation. However, as in previous and subsequent studies this character was poorly defined. Strasburger's main contribution was to the understanding of morphology and anatomy of *A.filiculoides*, with the noteworthy appreciation of interspecific differences in the number of cells comprising the leaf trichomes. Although not popularised until 1982 by Lumpkin & Plucknett, this character was also used by Martius (1884), van Ooststroom (1948), di Fulvio (1957) and Peiterse et al. (1977). Through his detailed anatomical investigation Strasburger (1873) was the first to describe and name the endosymbiont *Anabaena azollae*, but at this time the concept of a symbiotic association was not appreciated.

Two species described by Mettenius (1867) were redescribed by Martius (1884) in the revised "Flora Brasiliensis". Apart from size and geographical information for South American specimens, the descriptions of *A.filiculoides* and *A.caroliniana* were unaltered. Then followed a period when inferior descriptions of taxa were published and new names and ranks introduced. Baker (1887) proposed *A.pinnata* R.Br. var. *africana* Desv. (Baker) based on frond morphology, and Nakai (1925) proposed the new combination, *A.imbricata* Roxb. ex Griff. (Nakai) based on leaf morphology. Between these two publications Sadebeck (1902), following Mettenius' (1867) classification, failed to enhance any descriptions because he used undelimited vegetative and

reproductive characters. Therefore, few worthwhile changes were made to the circumscriptions of taxa between 1867 and 1925. The only real improvement was made by Strasburger (1873) who included sporoderm structure in his descriptions which were otherwise based on those of Mettenius (1867). In many respects the literature and herbarium determinations suggest that the taxonomy of *Azolla*, particularly Section *Azolla*, was relatively stable. The two Sections were easily distinguished, and the two species in each had such wide circumscriptions that little controversy surrounded them. However, this changed when Svenson (1944) proposed a new scheme for what he called Section 'Euazolla'.

Four species were recognised in Section 'Euazolla', namely *A.filiculoides*, *A.caroliniana*, *A.microphylla* and *A.mexicana*. From 1847 until Svenson's investigation the latter species had been considered synonymous with *A.caroliniana*, as was *A.microphylla* according to Mettenius (1867) (Table 1.1). However, Svenson (1944) suggested that a change in the application of the names *A.mexicana* and *A.microphylla* may be necessary when the Type material of each was examined. *A.rubra* was not considered as a separate taxon, but merely as a variant of *A.filiculoides* in respect to glochidial septation (Svenson, 1944). Interestingly, Svenson did not consider that geographical differences were important in the distinction of *A.rubra*, although he did place some significance on geographical distribution when describing other taxa. Of the two publications by Mettenius (1847, 1867), Svenson (1944) only cited the earlier one. Furthermore, he dismissed Strasburger's (1873) study as "... contributing nothing to taxonomy". This dismissal was unfo-

-rtunate because Strasburger's study not only indicated that sporoderm structure was potentially useful, but provided the only link to Mettenius's (1867) publication. Therefore, Svenson (1944) appears to have broken the continuity in the taxonomic history of *Azolla*. Clearly Mettenius' (1867) and Svenson's (1944) proposals cannot be reconciled because their species circumscriptions, except *A.filiculoides*, were different (see Table 1.1). This has undoubtedly fostered taxonomic confusion because subsequent authors have followed either Mettenius (1867) or Svenson (1944). Further confusion resulted from Svenson's study because he placed too much significance on undelimited characters such as branching, size and glochidial septation, the vegetative features being used in the absence of reproductive structures. However, although Svenson (1944) distinguished three of his four species by their sporoderm sculpturing, his descriptions of it are inadequate. Svenson (1944) was unable to find megasporocarps in specimens which he attributed to *A.caroliniana*, and this undoubtedly initiated the search for megaspores of this species; as a result any megaspore apparatuses not readily attributable to the other species described by Svenson were identified as *A.caroliniana*. However, Svenson's use of glochidial septation, branching, leaf shape and size as distinguishing characters were questioned (Godfrey et al., 1961; Correll & Correll, 1972; Ott & Petrik-Ott, 1973). Furthermore, Godfrey et al., (1961) reported that Svenson himself was not confident about separating species, particularly using vegetative features. Despite these doubts over Svenson's (1944) diagnosis, his prospectus received wide acceptance.

In her little cited study on *Azolla* from Central Argentina, di Fulvio (1957) described *A.filiculoides* and *A.caroliniana*. She indicated that *A.microphylla* sensu Svenson (1944) was not equivalent to *A.microphylla apud* Kaulfuss (1824). Di Fulvio (1957) re-evaluated reproductive and vegetative characters which included sporoderm structure and sculpturing, glochidial septation and leaf trichomes. *A.caroliniana* was reported as having one or two celled leaf trichomes and the glochidia were one to many septate. Di Fulvio (1961) described the sporoderm structure of *A.mexicana* and compared it with that of *A.filiculoides* and *A.caroliniana apud* di Fulvio (1957). These studies by di Fulvio (1957, 1961) represented the realisation of the importance of sporoderm structure, and the later publication was the first detailed study of *A.mexicana*. These observations were made using a light microscope, as were the observations by Demalsey (1953), on *A.nilotica*, and by Bonnet (1957), on *A.filiculoides*. However, these latter two investigations were morpho-anatomical, and contributed little to the taxonomy.

More recently, the scanning and transmission electron microscopes enabled more critical investigations. In a transmission electron microscope study of *Azolla*, Kempf (1969a) described the megaspore apparatus of *A.pinnata*, and indicated that sporoderm structure was a useful taxonomic tool in both fossil and extant *Azolla*. In a wider treatment of *A.pinnata*, Sweet & Hills (1971) proposed two varieties, *A.pinnata* R.Br. var. *pinnata* and *A.pinnata* R.Br. var. *imbricata* (Roxb. ex Griff.) Bonap. distinguished on frond and leaf morphology. However, these authors found no discernible differences between the varieties from the megaspore apparatus.

This cautious investigation was in contrast to that by Martin (1976a), in which *A.filiculoides*, *A.caroliniana*, *A.pinnata*, *A.imbricata* and *A.nilotica* were described from only the megaspore apparatus. Although Martin (1976a) used information from fossil studies, he appears to have introduced more confusion to *Azolla* taxonomy and megaspore apparatus terminology. Two species found in the Netherlands, namely *A.filiculoides* and *A.caroliniana*, were compared by Pieterse et al. (1977), although the latter combination was only tentatively used. This possibly resulted from an appreciation of both the circumscriptions of *A.caroliniana* by Mettenius (1867) and Svenson (1944), and was the first time that such a comparison had been made. Although Pieterse et al. (1977) studied both vegetative and reproductive characters, they were unable to provide useful taxonomic proposals. This study illustrated that not only were the descriptions and illustrations by Mettenius (1867) and Svenson (1944) inadequate, but their respective definitions of *A.caroliniana* were not equivalent.

In their study of *A.filiculoides*, *A.microphylla*, *A.pinnata* and *A.nilotica*, Fowler & Stennett-Willson (1978) have provided the most critical descriptions and illustrations of extant *Azolla* to date. They employed scanning electron microscopy and light-microscopy to examine sporoderm sculpturing and structure respectively. Apart from proposing a standardised terminology for the megaspore apparatus, Fowler & Stennett-Willson (1978) suggested that the megaspore apparatus provided the best means of separating taxa. This was, in part, illustrated through the tentative suggestion that *A.filiculoides* var. *rubra* might represent a separate species

on the basis of sporoderm structure. Unfortunately, Bates (1980) did not examine sporoderm structure in his study, instead he concentrated on megaspore apparatus morphology and sporoderm sculpturing. Although he employed scanning electron microscopy and examined many different populations, his descriptions were inadequate. Consequently, only *A.filiculoides* and probably *A.caroliniana* were recognised in North America (Bates 1980). It appeared that, like Mettenius (1847, 1867), Bates (1980) considered *A.mexicana* synonymous with *A.caroliniana*, which is surprising because Bates found considerable variation in sporoderm sculpturing. Although he made no attempt to explain the variation, it was suggested later that *A.caroliniana* might have been a hybrid (Bates & Brown, 1981). In the same year that Bates released his MSc thesis, Lumpkin & Plucknett (1980) published a review of *Azolla* in which the section on taxonomy essentially followed Svenson (1944) for Section 'Euazolla' and in Section Rhizosperma only *A.pinnata* and *A.nilotica* were recognised. Two years after their review the first comprehensive book on the biology and use of *Azolla* was published by Lumpkin & Plucknett; this expressed the increasing significance and importance of *Azolla*. In the book, Lumpkin & Plucknett (1982) had completely revised their taxonomy since 1980. Section Rhizosperma contained *A.pinnata* with varieties *pinnata* and *imbricata*, and Section Azolla contained *A.filiculoides*, *A.mexicana*, *A.microphylla*, *A.rubra* and *A.caroliniana*; this it was claimed was consistent with proposals by Svenson (1944), Moore (1969) and Fowler & Stennett-Willson (1978). It appeared that Lumpkin & Plucknett (1982) followed the suggestion made by Fowler & Stennett-Willson (1978), that *A.filiculoides* var. *rubra* might warrant

specific status, and, furthermore, the megaspore apparatus of *A.caroliniana* was described. Most of Lumpkin & Plucknett's taxonomic treatment was in the form of a 'tentative' key which relied on vegetative characters such as frond morphology, colour and frequency of sporulation, whereas sporoderm sculpturing was only superficially described, and sporoderm structure and glochidial septation were excluded. It appeared that Lumpkin & Plucknett (1982) failed to provide original proposals, instead they highlighted dispersed suggestions and proposals made by previous authors.

The transmission and scanning electron microscopical studies of Quaternary megaspore apparatuses of *A.pinnata* by Zhou (1983) found geographically correlated differences in sporoderm ultrastructure. His tentative approach to taxonomically explaining these differences was justified because relatively few populations were examined. Even fewer populations were examined by Calvert et al. (1983). In this scanning electron microscope study of *A.mexicana*, only one population was studied, but it provided the most detailed description to date. However, the description pertains mainly to the megaspore apparatus, with glochidial septation and vegetative features being mostly ignored.

In Ashton & Walmsley (1984) *A.filiculoides*, *A.pinnata* var. *pinnata* and *A.nilotica* were described from southern Africa, but their treatment followed Sadebeck (1902), Svenson (1944) and Sweet & Hills (1971). It appears, therefore, only to contribute to the current taxonomic confusion. Calvert et al. (1985) suggested that sporoderm structure could be used to distinguish six "currently

"recognised" species, namely, *A.filiculoides*, *A.mexicana*, *A.microphylla*, *A.caroliniana*, *A.pinnata* and *A.nilotica*. Furthermore, they reported one population with features intermediate between *A.filiculoides* var.*rubra* and *A.microphylla*.

The investigations outlined in Section 1.2 usually had two common failings. Firstly, only a few different populations of each taxon were examined in any one study. Svenson (1944), Sweet & Hills (1971) and Bates (1980) were perhaps exceptions because more than twelve populations were examined. However, Svenson (1944) does not indicate how many populations of *A.caroliniana* he examined.

Secondly, nomenclatural Type material, which is the reference base for naming taxa, has consistently been neglected, thus rendering any taxonomic conclusions highly suspect. However, some studies made during the 1800's may not have excluded Types. For example, Mettenius (1847) cited herbaria from which he obtained specimens, but not Type material, and some of these herbaria may have contained Types. Indeed, he cited and illustrated specimens collected by Schiede from Mexico which were possible syntypes of *A.mexicana*. The only other indication that Mettenius (1847) examined Types was that he possessed material from Sprengel's herbarium, and it was Sprengel who typified *A.portoricensis*. Although Nakai (1925) examined a few Type specimens, he provided poor descriptions of them. More recently, di Fulvio (1961) claimed that her specimens were probable isotypes of *A.mexicana*, and Sweet and Hills (1971) similarly examined "probable isosyntypes" of *A.pinnata*.

### 1.3 STRUCTURAL TERMINOLOGY

Although much controversy surrounds the terminology applied to the structure of the megaspore apparatus of *Azolla*, the structure of the sporocarp is quite well understood, mainly through the accounts of Strasburger (1873), Berggren (1879-80, 1882), Rozé (1888), Campbell (1893), Pfieffer (1907), Rao (1935) and Bonnet (1957), Konar & Kapoor (1974), Becking (1978) and Calvert et al. (1983).

In respect to the megaspore apparatus, much of the confusion was created by the use of pollen wall terminology. Indeed, it is suggested that this can be levelled as a source of confusion to pteridophyte spore wall terminology in general (Lugardon, 1978b).

In the early years of *Azolla* descriptions there was no established pollen terminology, and authors appear to have used appropriately descriptive terms (e.g. Griffith, 1845; Mettenius, 1847, 1867; Strasburger, 1873; and Campbell, 1893). During this time, the megaspore apparatus was termed a "spore", or prefixed with "macro". However, the use of the prefix "mega" is more common today. Although Hall (1975) refers to fossil taxa with "megaspores", most authors use the term megaspore apparatus or megaspore complex to indicate that the structure consists of a megaspore proper surrounded by perinous accessory material (Jain, 1971; Sweet & Hills 1971, 1974, 1976; Fowler & Stennett-Willson, 1978; Calvert et al. 1983 and others). As early as 1834 Martius illustrated the basic structure of a megaspore apparatus, but it was not until Strasburger's investigation in 1873 that the float region ("Schwimmapparat") and the basal region ("macrosporenhalfte-") were adequately described. More recently the float and basal

regions were termed supraspore and infraspore respectively (Sweet & Hills, 1971).

The collar region separates the supra- and infraspore in surface view, and although quite distinct in most species it is rarely acknowledged in early publications. However, more recently it is noted and termed the collar or girdle (Sweet & Hills, 1971; Martin, 1976a, Fowler & Stennett-Willson, 1978; Calvert et al. 1983 and others). Interestingly, Fowler (1981) defined collar morphology and proposed a phylogeny for extant *Azolla* based on sectional morphology of the collar. A conical mass (Campbell, 1893) between the floats has since been termed the gula, columella, column, labrum or acrolamella (Fowler & Stennett-Willson, 1978). These latter authors suggested that confusion over the term columella was related to its use for the proximal triseptate structure or the superstructure of hairs on the proximal surface or both. Consequently, Fowler & Stennett-Willson (1978) clarified the definition by reserving the term columella for the triseptate structure and introduced a new term, suprafilosum, for the suprastructure of hairs. However, Hall (1975) argues that the columella should correctly be called acrolamella (following Tschudy, 1966) and that it is unique to the Salviniales, including ancestral members. Hall's argument relies on the wall stratum from which the acrolamella is composed. It is this subject, wall stratification, that causes the greatest terminological confusion.

It is not within the scope of the present work to investigate the development of wall strata, which, in *Azolla* is complex and

potentially taxonomically important (Kempf, 1969a; Fowler & Stennett-Willson, 1978 and others). However, it is appropriate to outline some of the terms adopted by previous authors. (Fig.1.1). By the late 1800's two wall layers were recognised - the inner exospore (= megaspore wall proper) and an outer epispose which was sub-divided into two layers (Campbell, 1893). Later, researchers such as Demalsey (1953) and Bonnet (1957) distinguished an innermost endospore from the exospore. As indicated by Bonnet (1957) perispore was being used as an equivalent term for epispose. Hall & Swanson (1968) and Jain & Hall (1969) drastically changed the definitions of these terms. In their terminology the exospore became the endospore and most of the epispose/perispore became the exospore, and to add to the confusion their perispore was only the outer hairy zone of the epispose/perispore. Yet another confusing scheme was used by Jain (1971). Using angiosperm terminology he termed the exospore the endexine and the perispore, as defined by Bonnet (1957), was termed the ectexine. The perine *sensu* Jain (1971) was reserved for only the outer hairy zone of the perispore (= perispore *sensu* Hall & Swanson (1968) and Jain & Hall (1969)). Use of the terms exine and perine by Jain (1971) were not only inconsistent with the definitions in Kremp (1965), but also appeared to be a confusion of these terms as applied by Kempf (1969a, 1969b). This latter author considered exine equivalent to both endospore and exospore, and the perine equivalent to epispose and perispore *sensu* Bonnet (1957) and others. During the 1970's the term perine became more acceptable to workers of both fossil and extant species (Fowler, 1975, 1981). This was illustrated by the indirect use of the term perine in the terms inperine and

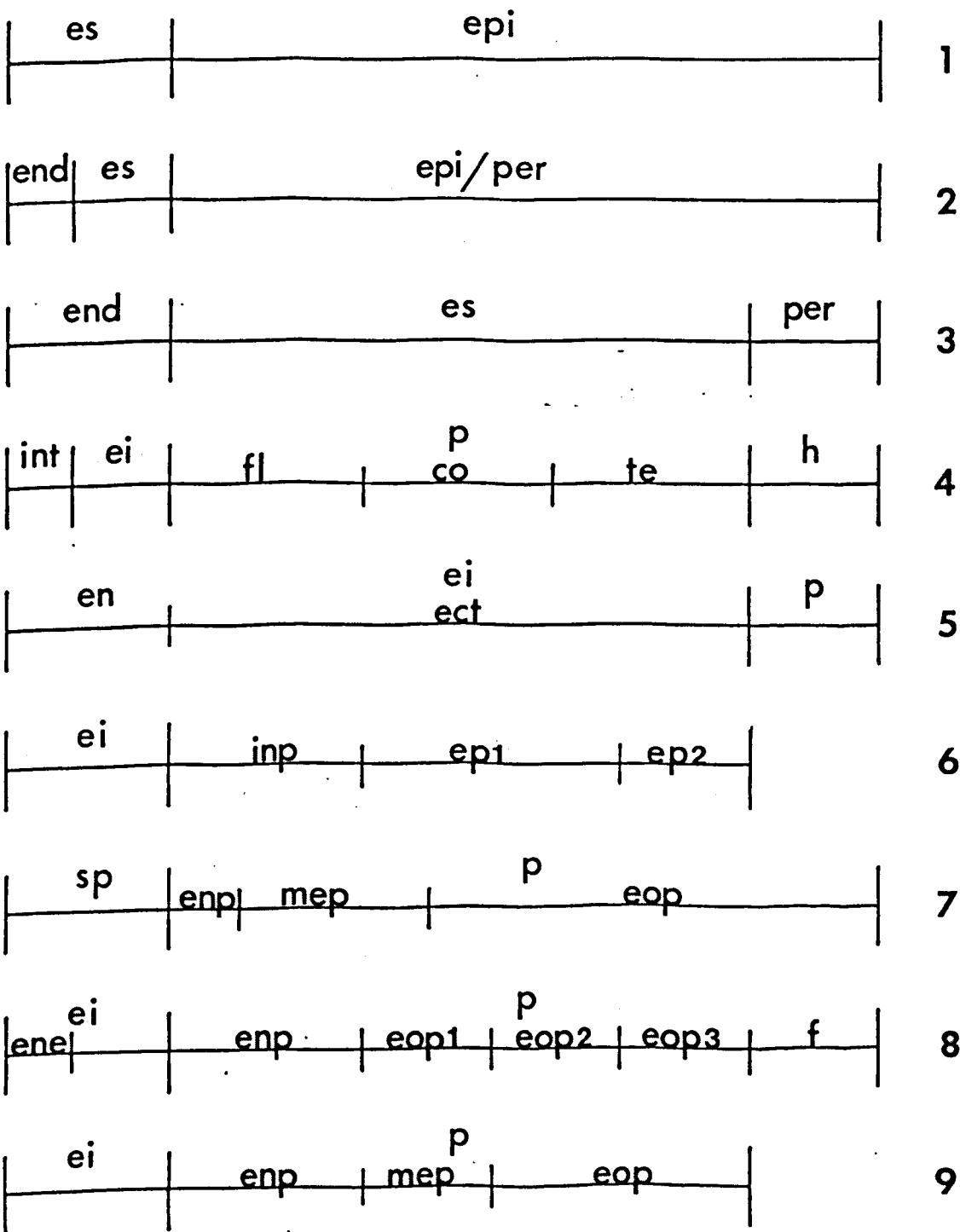
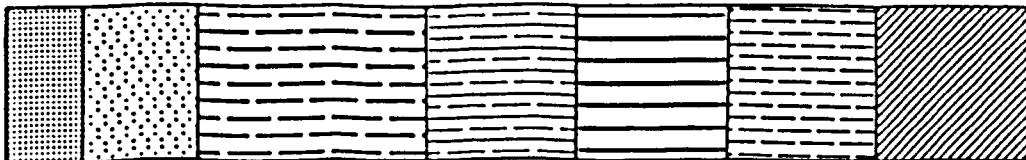
FIGURE 1.1

History of terminology used to describe wall strata of *Azolla*.

Key: co = columella; ect = ectexine; en = endexine; end = endospore; ene = endoexine; enp = endoperine; eop = exoperine; eopl = exoperine 1; eop2 = exoperine 2; eop3 = exoperine 3; epi = epispose; es = exospore; ei = exine; epl = experine 1; ep2 = experine 2; f = filosum; fl=foot layer; h = hair; inp = inperine; int = intine; mep = mesoperine; p = perine; per=perispore; sp = sporoderm; te = tectum.

1 = Campbell (1893); 2 = Demalsey (1953), Bonnet (1957);  
3 = Hall & Swanson (1968), Jain & Hall (1969); 4 = Kempf  
(1969a, 1969b); 5 = Jain (1971); 6 = Sweet & Hills  
(1971); 7 = Martin (1976a); 8 = Fowler & Stennett-Wills-  
on (1978); 9 = Calvert et al. (1983).

# SPORODERM STRATA



exine; the exine being *sensu* Kempf (1969a, 1969b). Martin (1976a) continued this acceptance by describing the perine as divided into three layers - the endoperine, mesoperine and exoperine, but termed the exine the sporoderm which, in most palynological definitions is considered to be composed of all wall strata (exine and perine) (Kremp, 1965). Martin's (1976a) terms for perine strata were perpetuated by Calvert et al. (1983). Introducing a standardised terminology Fowler & Stennett-Willson (1978) used essentially the same terms as Sweet & Hills (1971), but provided new definitions which appeared homologous throughout fossil and extant *Azolla*. It appeared that Calvert et al. (1983) had misinterpreted Martin's (1976a) scheme, illustrating that it could not be widely used. To conclude this section on wall strata it is worth noting that publications by Lugardon (1978a, 1978b), although not including *Azolla*, are pertinent to the general discussion of wall strata in relating to their ultrastructure terminology and development.

It appeared that Mettenius (1847, 1867) and Svenson (1944) had the most profound influence on the taxonomy of extant *Azolla*. Not only had they caused controversy and confusion by their very different proposals, but it was subsequently found that their distinguishing characters were of little use. This undoubtedly prompted the recent proposals by Lumpkin & Plucknett (1982). However, this study (and indeed all other taxonomic studies) has made the same mistakes as Mettenius (1847, 1867) and Svenson (1944), namely inadequately described undelimited characters. Furthermore, all these and other investigations have neglected nomenclatural Type

material. It is clear, therefore, that a comparative phenetic study critically appraising old and new characters, thereby establishing useful ones, from many geographically diverse populations, and including Type specimens, has not been attempted by previous authors. The current concensus amongst researchers of extant *Azolla* is that existing taxonomic proposals are inadequate; a fact that has long been realised through fossil investigations. Furthermore, more information is required to endorse the unified terminology proposed by Fowler & Stennett-Willson (1978).

#### 1.4 OBJECTIVES

Recent investigations by Fowler (1975, 1981) and Fowler & Stennett-Willson (1978) have clearly shown that in a limited sample, the megaspore apparatus provides the best means of separating species of *Azolla*. These three studies formed the basis for establishing a research programme at Portsmouth Polytechnic which aimed to critically re-evaluate the taxonomy of *Azolla*, with particular reference to species of Section *Azolla*. To achieve this, it is necessary to evaluate sculptural and structural megaspore apparatus characters and establish megaspore apparatus types; this will also include a study of megaspore apparatus structure. Such an approach would be a continuation of the work initiated by Fowler & Stennett-Willson (1978). Having established megaspore apparatus types, other reproductive and vegetative characters can be evaluated and correlated with the megaspore apparatus types to establish a suite of taxonomically useful characters. Finally, these types will be compared with

nomenclatural Type specimens. This should lead to the correction of nomenclatural confusion and provision of a unified taxonomic prospectus. Light microscopy and scanning electron microscopy will be employed and, where appropriate, characters quantified. Herbarium specimens and limited fresh/fixed material will be used in this investigation. Aspects of the reproductive biology in *Azolla*, using cultured material, will also be investigated and described in Part B.

**2. MATERIALS AND METHODS**

## 2. MATERIALS AND METHODS

### 2.1 SELECTION AND ACQUISITION OF MATERIAL

#### 2.1.1 Fresh Material

The International Rice Research Institute (IRRI), Los Baños, Philippines maintains a phytotron culture collection and duplicate greenhouse collection of 131 'strains' of *Azolla* which were collected from worldwide locations. A 'strain' represents one population collected from one locality. Twenty representative 'strains' were fixed in buffered glutaraldehyde and brought from IRRI to Portsmouth in 1982 by Dr. K. Fowler. A further eighty 'strains' were similarly brought to Portsmouth in 1985 by Dr. K. Fowler and the present worker. These 'strains' were added to the collection at Portsmouth. In addition to this, fresh specimens from the IRRI collection, but grown in the Department of Botany, University of Manchester, were received for study. Other sources of fresh material included Portsmouth Polytechnic culture collection, a canal at Greywell, U.K. and a stream at Fordingbridge, U.K.

'Strains' maintained at Portsmouth were kept either in a greenhouse or in a crude growth room. In the former, the 'strains' were grown in plastic trays containing approximately 2 cm of sterilised potting compost and three quarters filled with nitrogen-free culture solution (see Appendix II). These cultures received little attention except for thinning every fourteen days, replenishment of culture solution when required

and change of compost every twenty-eight days. One 'strain' was maintained in outdoor tubs, which were replenished with tap water when required. 'Strains' maintained in the growth room were in 250 cm<sup>3</sup> beakers containing a nitrogen-free culture medium (see Appendix II). A bank of three 'Grolux' (Thorn U.K.) lamps were placed 30 cm above the beakers. The growth room facilitated control of light and dark, temperature and humidity. The culture medium was changed every seven days, and the cultures thinned when required.

### 2.1.2 Herbarium Material

"Index Herbariorum" by Holmgren et al. (1981-82) was used to identify major world herbaria. Replies from 39 herbaria to questionnaires on their holdings of *Azolla* were received and used to select twenty-two collections, in whole or part, for loan. Collections are referred to throughout this work by the Index Herbariorum codes.

Table 2.1 lists the herbaria which supplied specimens, together with information on the number and potential use of the herbarium sheets received. Some 2,550 sheets were received either at Portsmouth Polytechnic or at the British Museum (Nat. Hist.). A total of 1,331 sheets were catalogued, providing information on collector, date, location, etc. Individual sheets could then be located when required and duplicate sheets recognised. Important collections from the National d'Histoire Naturelle, Paris (P; those of Lamarck and

Jussieu) and the Botanischer Garten and Botanisches Museum, Berlin (B; that of Willdenow), were not available on loan; visits were made to examine that material.

The entire IRRI culture collection was collected as herbarium specimens in 1982 and again in 1985. Dried specimens of some IRRI strains were collected from a field station at Bagiuo, Philippines in 1985. In addition to this, as 'strains' sporulated at IRRI, dried samples were forwarded to Portsmouth Polytechnic for examination.

Specimens in each collection were identified as to their potential use by the following criteria:-

- (i) presence of sporocarps
- (ii) presence of three floated megaspore apparatuses
- (iii) nomenclatural/historic importance (i.e. Type material).

Further selection of specimens, if required, was based upon the nature of the leaf surface (as seen under LM) and collection site. Specimens selected in this way were catalogued on index cards and assigned the herbarium code letter and a sequential number from 1 to n, where n was the number of selected specimens from that herbarium collection. This coding was unique to this study and enabled specimen samples and information on specimens to be retrieved quickly. Appendix I gives a complete listing of the specimens examined in this study.

TABLE 2.1

Table to show the sources of herbarium and fresh material used in this investigation. Also indicated are the number of sheets, the number of useful sheets and the presence of Type specimens. See also Appendix I

TABLE 2.1

<u>HERBARIUM/SOURCE CODE</u>	<u>TOTAL NO. OF SHEETS</u>	<u>NO. OF USEFUL SHEETS</u>	<u>NO. OF TYPE SPECIMENS</u>
ADELAIDE (AD)	46	1	0
ALABAMA (UNA)	40	1	0
BERLIN (B)	271	76	9
BRITISH MUSEUM (NAT.HIST.) (BM)	80+	11	4
BROOKLYN (BKL)	32	6	0
EDINBURGH (E)	2	1	1
GRAY (GH)	86	18	0
HALLE (HAL)	10	5	2
LEIDEN (L)	339	31	0
MEISE (BR)	318	10	1
MEXICO (ENCB)	23	4	0
MILWAUKEE (MIL)	10	7	0
MISSOURI (MO)	211	51	0
MUNCHEN (M)	113	15	0
NANJING (NAJ)	7	0	0
NEW YORK (NY)	56	17	0
PARIS (P.incl.P-LAM, P-J)	633	31	9
PEKING (PE)	61	2	2
SYDNEY (NSW)	20	15	0
UTRECHT (U)	84	5	0
WASHINGTON (US)	122	4	0
IRRI (incl. fresh)	131	20+	0
MISCELLANEOUS (fresh)	5	5	0
MISCELLANEOUS (Herbarium)	6	6	0

2.2 TECHNIQUES FOR EXAMINING MATERIAL USING THE SCANNING ELECTRON MICROSCOPE

2.2.1 Fresh Material

Standard methods were employed to prepare fresh vegetative material for examination by scanning electron microscopy. This involved fixation in buffered 2.5% glutaraldehyde solution, dehydration in ethanol, critical point drying in CO<sub>2</sub> and sputter-coating with gold (See Appendix III Methods A). Megaspores were prepared using the method described in section 2.2.2.1. This methodology was also used for scanning electron microscopical examination in the study of the reproductive biology of *A.filiculoides*.

2.2.2. Herbarium material

2.2.2.1. MEGASPORE APPARATUS

At least three megasporocarps judged to be mature by their size and position on the fronds were removed and placed in distilled water for 10 minutes. The sporocarp and sporangial walls were removed to release the megaspore apparatuses. These were then placed in 50% acetone for at least 4 hours, and then transferred to 100% acetone for 24 hours. Subsequently, the dehydrated and lipid free megaspore apparatuses were cut longitudinally in half with a grease free razor blade to show sporoderm structure, or were left whole to show morphology and sculpturing. The halved and whole megaspore apparatuses were mounted on stubs with double sided sellotape, air dried and stored as necessary.

in a vacuum desiccator and then sputter coated with gold (see Appendix III Methods B (i)).

Treatment with acetone not only dehydrated the specimens but also removed any lipids which might foul the cut surfaces. Calvert et al. (1983) first used the above cutting method in extant *Azolla*, however, this followed dehydration and critical point drying similar to the methodology in section 2.2.1. In the present study the lengthy procedure used by Calvert et al. (1983) was found to be unnecessary. The method outlined above was compared with the method outlined in section 2.2.1., and a considerable saving in time and cost was achieved without inducing any artefacts.

#### 2.2.2.2. MASSULA

Massulae were prepared by teasing them from a few microsporangia mounted on a stub using fine tungsten needles. The massulae were then air dried in a vacuum desiccator and sputter coated with gold (see Appendix III Methods B (ii)).

#### 2.2.2.3. VEGETATIVE MATERIAL

A small sample of vegetative material was mounted on a stub with carbon adhesive, air dried in a vacuum desiccator and then sputter coated with gold for examination of the leaf surfaces. If the nature of the trichomes on the abaxial surface of the dorsal leaf lobe could not be discerned, another sample of the material was 'reflated'. This involved the impregnation of the sample with 10% hydrogen peroxide solution and the subseque

-nt release of oxygen from the latter which 'reflated' much of the tissue. Subsequent treatment was the standard method of dehydration, critical point drying and sputter coating with gold (see Appendix III Methods B (iii)). This method for 'reflation' of tissues was developed during this study as a result of attempts made using a number of methods.

It was found that ringing the stub with carbon adhesive considerably reduced 'charging' when examining the specimens. The megasporo apparatuses, massulae and vegetative sample were, by careful arrangement, mounted on the same stub.

All observations and photography for section 2.2 employed a JEOL T20 scanning electron microscope fitted with a medium format (6x7) camera.

## 2.3 TECHNIQUES FOR EXAMINING SPECIMENS USING THE LIGHT MICROSCOPE

### 2.3.1. Reproductive Structures

General and qualitative observations were made in relation to the abundance and shape of mega- and microsporocarps. For the latter structures, the relative quantity of contained microsporangia was noted.

#### 2.3.1.1. MASSULAE, GLOCHIDIA AND MICROSPORES

Microsporangia from at least four microsporocarps were collected from four different fronds on a herbarium sheet and placed in a drop of water on a glass slide and then stirred to mix the

sample. Massulae were teased from twenty microsporangia and the number of massulae in each was recorded on a data sheet.

A permanent preparation was then made using 'Hydromount' (National Diagnostic, U.S.A.) and sealed with 'DPX' mountant.

The following characters were recorded on a data sheet:-

- (i) number of septa per glochidium from 100 glochidia
- (ii) distribution of septa in glochidia (qualitatively)
- (iii) presence/absence of intrusions in glochidia (qualitatively)
- (iv) length of glochidia from 50 glochidia
- (v) taper (shape) of the glochidial shaft region (qualitatively)
- (vi) shape of glochidial apex (qualitatively)
- (vii) number of microspores per massula from at least 30 massulae

A strict sampling method was used (see Appendix III Methods C) to make a more objective appraisal of characters. This study makes the first attempt to quantify these characters in extant *Azolla*. The number of observations for each character was determined from a knowledge of the characters concerned and the time limits imposed on collecting data from the material available.

'Hydromount' is a water soluble mountant developed for fluorescent microscopy. However, it was used in this study because glochidia only assume their true shape and orientation in water. The use of 'Hydromount' removed the need for lengthy

dehydration to obtain permanent preparations. In addition to this, the massulae can be retrieved from the preparations and re-mounted if necessary.

It was necessary to define certain characters such as septum, intrusion, shapes and lengths. This was to reduce ambiguities and confusion that may arise; these definitions can be found in section 3.

### 2.3.2. Vegetative Structures

#### 2.3.2.1. HERBARIUM MATERIAL

Fronds were soaked in 10%  $\text{Na}_3\text{PO}_4$  solution for at least 30 minutes, or until saturated, to restore the tissue to a more life-like condition. The following observations were then made, employing the procedure in Appendix III Methods D, and recorded on a data sheet:-

- (i) maximum length and width of dorsal and ventral leaf lobes
- (ii) maximum width of hyaline margin
- (iii) number of leaves between branches
- (iv) shape of apex of the dorsal and ventral leaf lobes
- (v) nature of the hyaline margin
- (vi) nature of the trichomes on the abaxial surface of the dorsal leaf lobe (see section 2.2.2.3.)
- (vii) general habit of the fronds.

The sampling procedure for (i), (ii) and (iii) was an attempt to

make a more objective appraisal of these characters by making ten measurements per sample (see Appendix III Methods D (ii)). (In practice, it proved impossible to make accurate measurements of the ventral leaf lobes. This leaf lobe was membranous and became contorted during drying, therefore characters (i) for the ventral leaf lobe were not scored).

#### 2.3.2.2. FRESH MATERIAL

Only fresh/fixed material from the IRRI culture collection and Portsmouth were used. The methods outlined below can be used for any light microscope examination of fresh material where sectioning is required. For the study of the reproductive biology of *A.filiculoides*, the precise details of sporocarp collection and culturing are given in Appendix III Methods F.

**Wax embedded material:-** Specimens were fixed in buffered 2.5% glutaraldehyde solution, washed, dehydrated in 2 methylpropan 2-ol , transferred by dilution to liquid paraffin and then embedded in 'Fibrowax'. 8um thick sections were cut on a Cambridge or Jung Rotary microtome fitted with steel knives, stained with haematoxylin and eosin (Smith & Bruton, 1977) or safranin and fast green (Johansen, 1940), and permanently mounted on glass slides with 'DPX' mountant (see Appendix III Methods E (i)).

**Resin embedded material:-** Specimens were fixed in buffered 2.5% glutaraldehyde solution, washed, dehydrated in a graded series of ethanol and embedded in methacrylate (JB-4) resin (Polysci-

nces Inc., U.S.A.). 4 $\mu$ m thick sections were cut on a Pyramitome fitted with glass knives, floated on to glass slides, stained with 1% toluedine blue solution and permanently mounted in 'DPX' mountant (see Appendix III Methods E (ii)).

All observations and photography for section 2.3 employed Wild M5 and M20 light microscopes fitted with eye-piece graticules and photoautomat.

#### **2.4 DATA SIMPLIFICATION**

The numerical data of leaf dimensions and glochidial characters were simplified to mean, variance and standard error for each population using a TEXAS Ti 58C programmable calculator. The means (and variances of glochidial length) were then used to create datafiles stored on disc. The datafiles were further simplified and/or compared using the UNISTAT package in a BBC B Microcomputer. The F test, Student's t-test, regression analysis and Basic statistic programmes were used from the package. Several hypotheses were offered for the t-test, and the F test was used to determine which was the most appropriate for a particular data set.

Data from each population sampled for glochidial septation are presented in Appendix IV because the data are so important compared with other quantified characters.

3. DEFINITIONS

### **3. DEFINITIONS**

A proposed glossary.

It has already been shown that the terminology applied to *Azolla* is confused because many researchers have not clearly defined terms which they have used. Some have misinterpreted other workers' definitions, have used ambiguous terms (without re-defining them) and others have used terms with overlapping meanings or a combination of some or all these. In an attempt to provide a unified terminology, with unambiguous practical application, this section contains definitions of terms used in the present study. Although most of the terms have been used previously, observations from this investigation are used to modify and clarify many definitions. To prevent confusion many structures, or part thereof, that were measured have been defined; this will enable continuity and comparability of results, within this and future investigations. It is also hoped that many terms, particularly those for reproductive features, can be applied to fossil as well as extant *Azolla*. Discussion of certain terms will be dealt with in section 5.

#### **3.1 REPRODUCTIVE STRUCTURES AND FEATURES**

3.1.1 Sporocarp: Unlike the Marsileales the sporocarp in the Salviniales (including *Azolla*) is a modified indusium, forming a two-cell thick sporocarp wall. Unlike *Salvinia*, extant *Azolla* sporocarps are different sizes and occur in pairs or fours(Fig.-3.1).

Megasporocarp: Sporocarp containing a single megasporangium (in *Azolla*). The modified indusium completely encloses the megasporangium except for a small apical pore. The dehiscence zone is equatorial in extant *Azolla*, although no obvious line is demarcated. In extant *Azolla*, *Anabaena azollae* is usually located between the megasporangial and megasporocarp walls. This sporocarp is smaller than the microsporocarp (Fig.3.1a).

Microsporocarp: Sporocarp containing microsporangia. The modified indusium completely encloses the microsporangia except for a small apical pore. Development of microsporangia is gradate and there is no demarcated zone of dehiscence. Viable *Anabaena azollae* is absent from the mature microsporocarp of extant *Azolla*. This sporocarp is larger than the megasporocarp (Fig.3.1).

Indusial cap: Persistent, thickened apical region of the megasporocarp wall that remains capping the apical half of a released megaspore apparatus.

3.1.2 Megasporangium: The wall is one-cell thick, develops from the placenta (=columella, receptacle) and usually completely encloses one megaspore apparatus (Fig.3.1a). Like the megasporocarp wall the dehiscence zone is equatorial, and there is no obvious line demarcated.

Megaspore apparatus: A megaspore proper surrounded by mainly perinous material which is usually elaborated to form the

collar, acrolamella, suprafilosum and distal sculptured surface. Floats are usually accommodated in an apical position and attached to the suprafilosum (Fig.3.2).

Megaspore: A megaspore proper bounded by the exospore and surrounded by perine, which is elaborated to form a distal sculptured surface, a more proximal collar and a proximal acrolamella. In heterosporous tracheophytes the term 'megaspore' usually means only the megaspore proper (Fig.3.2).

Collar: Present in all extant taxa; delimits periphery of the proximal surface of the megaspore proper; appears raised and contoured due to increased thickness, by alveolation, of the endoperine. In extant Section *Azolla* the collar is tricuspid, the cusps extending between the floats. The external surface is seen in surface view and may be wholly or partly covered by a thin layer of exoperine (Fig.4.4a & b). The internal surface is adjacent to the internal surface of a float. Suprafilosum arises from the internal collar surface (Fig.4.1c & d). In extant taxa of Section *Azolla* the external collar surface possesses a downwardly directed flange with an associated groove (Fig.3.2).

Waist: A marked constriction of the megaspore at the base of the collar where it meets the distal megaspore region; most commonly seen in the *A.filiculoides* type (Fig.4.6a & c).

Acrolamella (syn. *gula*, *columella*, *column*, *labrum*, *trifolium*): Conical, triseptate, centrally positioned elaboration of the proximal perine composed of alveolate endoperine. The septa divide the proximal surface into three equal sectors, and partially support the floats. Suprafilosum originates from the surface and intertwines with filosum from the floats, thereby affording anchorage for them (Figs.3.4 & 4.2).

Filosum: Hair-like filaments arising from the perine. When the megasporangium has distinct polarity it is necessary to distinguish whether filosum arises from the distal perine or forms a proximal suprastructure.

Suprafilosum: Filosum arising from the proximal perine surface. Suprafilosum forms a suprastructure which extends up between the floats and forms a funnel at the float apices (Figs.3.2 & 3.4).

Infrafilosum: Filosum arising from the distal perine.

Float: Alveolate, pseudocellular structure which is attached to a megasporangium. Filaments of filosum arise from certain areas and surfaces; this filosum serves to attach a float within the megasporangium (Figs.3.2 & 4.1). In order to describe the origin of filosum on a float it is necessary to define float surfaces in relation to their orientation and/or position to other floats.

External surface: Seen in surface view of a megasporangium (Fig.3.3).

Internal surface: Adjacent to the acrolamella and internal surfaces of other floats (Fig.3.3).

Longitudinal surface/edge: In contact with the same of an adjacent float, in the longitudinal plane of a megasporangium (Fig.3.3).

Transverse surface/edge: In contact with the same of the upper or lower float tier, more or less in the transverse plane of a megasporangium; only found in taxa with more than three floats (Fig.3.3).

Funnel (of suprafilosum): Mesh-work of irregularly arranged suprafilosum at the apex of a megasporangium. Float filosum intertwines with the funnel thereby holding the float apices together. When the indusial cap is in position the funnel closely adheres to the megasporangial wall, is umbrella-shaped and caps the apical region of the megasporangium (Fig.3.1a). Reversion to the funnel-shape usually occurs when the indusial cap is removed. Part of the megasporangial wall remains attached to the inside of the funnel when the indusial cap is removed (Fig.3.2).

Apical membrane: Remnant of the megasporangial wall which is attached to the funnel after the indusial cap is removed (Fig.3.2).

Sporoderm: Wall of the megaspore comprising the innermost exospore and a two-layered perine (Fig.3.5).

Perine: Mostly two-layered wall radially external to the exospore and gives rise to filosum. The outer layer is often distinctly sculptured in the distal region of the megaspore (Fig.3.5).

Exospore (syn. exine): Wall surrounding the megaspore proper. Under the light microscope a thin basement layer, the endospore, may be distinguished from a thicker striated layer. Under the scanning electron microscope the thin basement layer is not readily discernible, however, the striations are probably caused by radially arranged sinuous alveolae, which are particularly obvious in the outer three quarters of the exospore (Figs.3.2 & 3.5).

Endoperine: Innermost homogeneous to alveolate layer of the perine located radially external, and closely attached, to the exospore. Ultrastructurally (under SEM) the endoperine may be seen to be composed of irregularly arranged, anastamosing, vermiciform elements which are simple or branched. However, these elements may not be distinct. Extensive development of the endoperine is significant in the formation of the collar, acrolamella and sculpturing of the megaspore (Fig.3.5).

Exoperine: Outermost layer of the perine with two or three zones usually recognisable, but excluding the infrafilosum (Fig.3.5).

Exoperine 1: Thin foot zone of exoperine, found in all extant taxa; composed of irregular and often anastamosing bacula which are predominantly radially orientated (Fig.3.5).

Exoperine 2: Relatively thick zone, radially external to exoperine 1; highly variable structure. Usually contributes significantly to sculpturing on the distal megaspore (Fig.3.5).

Exoperine 3: Reserved for the thin outer zone of the exoperine 2 composed of small elements which are mainly tangentially orientated or of solid material. In some extant taxa this zone is usually localised in specific surface areas (usually sculpturally raised areas) rather than, as in other taxa, forming a continuous zone (Fig.3.5).

Excrescence: Large protuberance from the perine surface composed of both exoperine and endoperine (Fig.3.5).

Endoperine intrusion: Localised, conical thickening or small eruption of endoperine covered by exoperine.

Such a structure usually contributes to the sculpturing of the distal megaspore. However, endoperine intrusions can only be identified by sectioning the distal sporoderm (Fig.3.5).

- 3.1.3    Microsporangium: A sporangium that gives rise to microspores: the wall is one-cell thick and completely encloses one or more massulae. A microsporangium is attached to the placenta (=columella, receptacle) by a stalk. There is no annulus in extant **Azolla** (Fig.3.1b).

Massula: Highly alveolate structure with embedded microspores; chemically similar to perine of the megaspore apparatus. Adjacent to a microspore containing cavity is a flask-shaped cavity which provides access to the exterior. Massulae may have long appendages (trichomes and glochidia) which are of taxonomic significance. These appendages may be located in specific areas of a massula surface which necessitates their definition (Figs.3.6 & 3.7).

External surface: The surface in contact with the microsporangial wall when the massula is enclosed by it; usually convex (Fig.3.6).

Internal surface: Any surface in contact with other massulae within the microsporangium; often concave (Fig.3.6).

Massula trichome: Spiny or hair-like, simple or branched appendage arising from a massula surface; probably serving to attach a massula to the megaspore apparatus. These trichomes may be confined to certain massula surfaces (Fig.3.7a). In extant and fossil taxa (including ancestral Salviniaceae) massula appendages have been termed glochidia, but are without barbed or fluked heads (Sweet & Hills, 1974; Hall, 1975; Lumpkin & Plucknett, 1982). However, the term 'glochidium' implies a fluked head and should not be used for non-fluked appendages. Such forms have been found with and without barbs along their length, and with circinate tips in fossil taxa.

Glochidium: Massula appendage with a fluked head (i.e. glochidi-ate) confined to certain areas of massula surfaces (Figs.3.6b,3-7b,4.32); found in extant and fossil taxa. To adequately describe glochidial characters it is necessary to define these characters.

Glochidial length: apex to insertion on massula surface (Fig.3.8).

Glochidial shaft: whole glochidium except for the distended base and fluked head; often corresponds to much of the glochidial vacuole (Fig.3.8).

Glochidial vacuole: apparent vacuole in glochidial shaft; may be septate or non-septate, but clearly delimited at apex and base (Fig.3.8).

Glochidial stalk: non-vacuolate, probably solid region between the basal end of the vacuole and the insertion on to the massula. The stalk may be relatively long or short (Fig.3.8).

Glochidial shape: defined by tapering (basal and/or apical) of the region delimited by the glochidial vacuole (Fig.3.9).

Septum: a complete, more or less transverse wall in the glochidial vacuole; may be distinct or faint (Fig.3.10). For positions of septa see Figure 3.8b.

Glochidial intrusion: outgrowth of the glochidial wall into the vacuole; may appear to be an incomplete septum (Fig.3.10).

N.B. Twists and contortions of a glochidium may appear as septa, however, with careful observation of the glochidial shaft it is possible to distinguish septa from twists.

Microspore: microspore proper, bounded by the exospore with laesurae, and embedded within a massula; not surrounded by elaborated perine as is the megaspore proper in a megaspore apparatus. (Figs.3.6a,4.4d).

### 3.2 VEGETATIVE FEATURES

#### 3.2.1 Branching

Flabellate: successive bifurcations in one plane forming a dorsiventral system.

Isotomous dichotomy: successive pairs of branches attaining approximately equal size and development. (Troll, 1937); dominance of one branch not apparent (Fig.3.11a).

Anisotomous dichotomy: one member of each bifurcation developing more strongly than the other (Troll, 1937); dominance of one axis apparent (Fig.3.11b).

Pinnate: main axis with pairs of approximately opposite branches; each pair being more or less equal in size.

Sub-pinnate: main axis with pairs of branches, each member of a pair being alternate and more or less equal in size.

### 3.2.2 Trichomes

Leaf trichome: as applied to taxonomic characters, confined to the abaxial surface of the dorsal leaf lobe. Composed of one or more cells which protrude above the other epidermal cells, and usually associated with a stoma (Fig.3.12a-c).

Stem trichome: only found in extant taxa of Section **Rhizosperma** on the rhizome.

Cavity trichome: confined to the cavity containing, and closely associated with, *Anabaena azollae* in the dorsal leaf lobe. Usually composed of a stalk cell attached to the cells lining the cavity, a bulbous body cell and one to several terminal cells (Fig.3.12d).

Apical trichome: similar to some cavity trichomes, but confined to the apex of the rhizome (=primary branch hair, Calvert et al., (1981) ). Closely associated with the colony of *Anabaena azollae* around the rhizome apex and each developing leaf; innoculates each developing leaf cavity with **A.azollae** and becomes incorporated in to the leaf cavity.

### 3.2.3 Leaves

Bilobed (dorsal and ventral) and alternately inserted.

Width: for the dorsal and ventral lobes this was measured at the point of maximum width, and included width of the hyaline margin.

Length: for the dorsal and ventral lobes this was measured at the point of maximum length.

Hyaline margin: found only on the dorsal leaf lobe, is a translucent, achlorophyllous margin of several cells wide but only one cell thick in section. Measurements were made at the point of maximum width of the dorsal leaf lobe.

FIGURE 3.1

Sporocarp structure

(a) Diagrammatic median L.S. of a megasporocarp illustrating its structure. (Mag. ca X60). (Typical of Sect. *Azolla*).

(b) Diagrammatic median L.S. of a microsporocarp illustrating its structure. (Mag. ca X25).

(ap = apical pore; Aa = *Anabaena azollae* resting cells; fs = funnel of suprafilosum; FL = float; C = collar; ex = exospore; M = megasporangial wall; Mw = megasporocarp wall; m = microsporangium; ms = microsporangial stalk; mw = microsporocarp wall; P = placenta; p = perine).

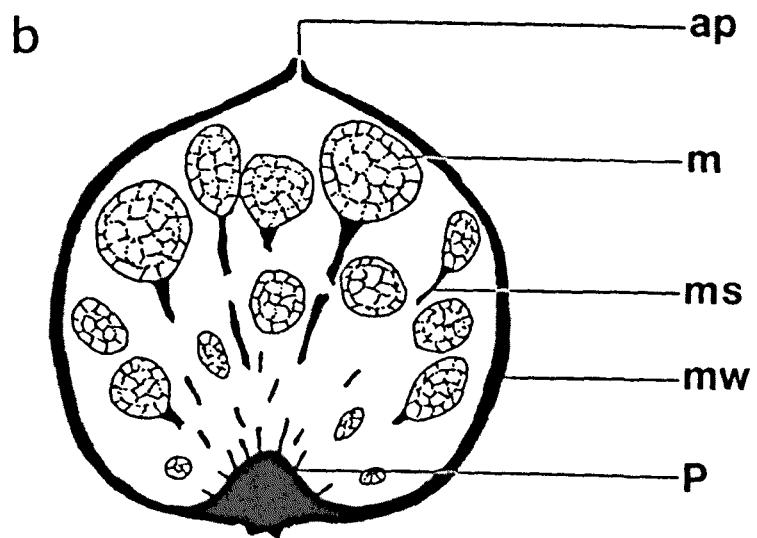
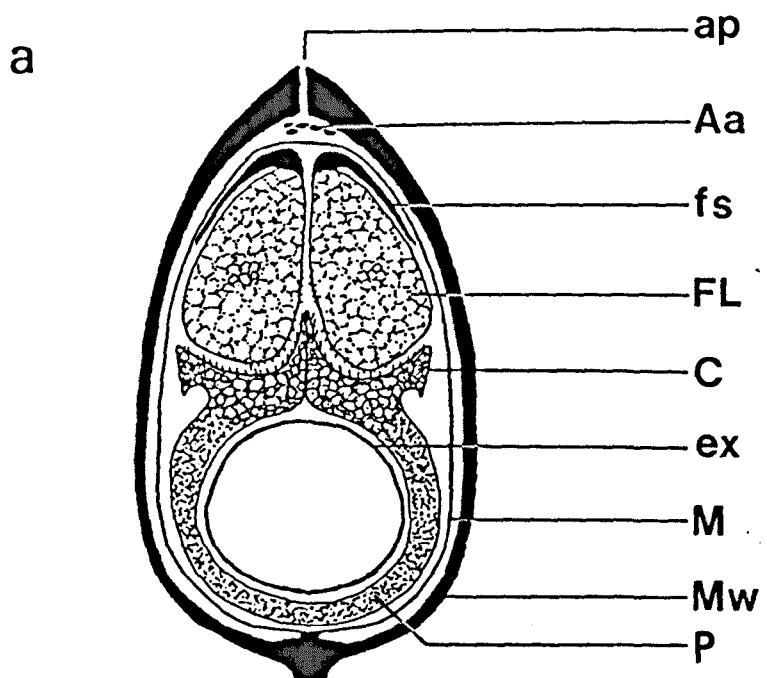


FIGURE 3.2

Megaspore apparatus structure

Diagrammatic median L.S. of a megaspore apparatus illustrating its structure. (Mag. ca X100).

(fs = funnel of suprafilosum; sf = septum of suprafilosum; AM = apical membrane; as = acrolamella septum; C = collar; SP = sporoderm; ex = exospore; en = endoperine; ep = exoperine)

FIGURE 3.3

Structure of the float region in respect to float surfaces

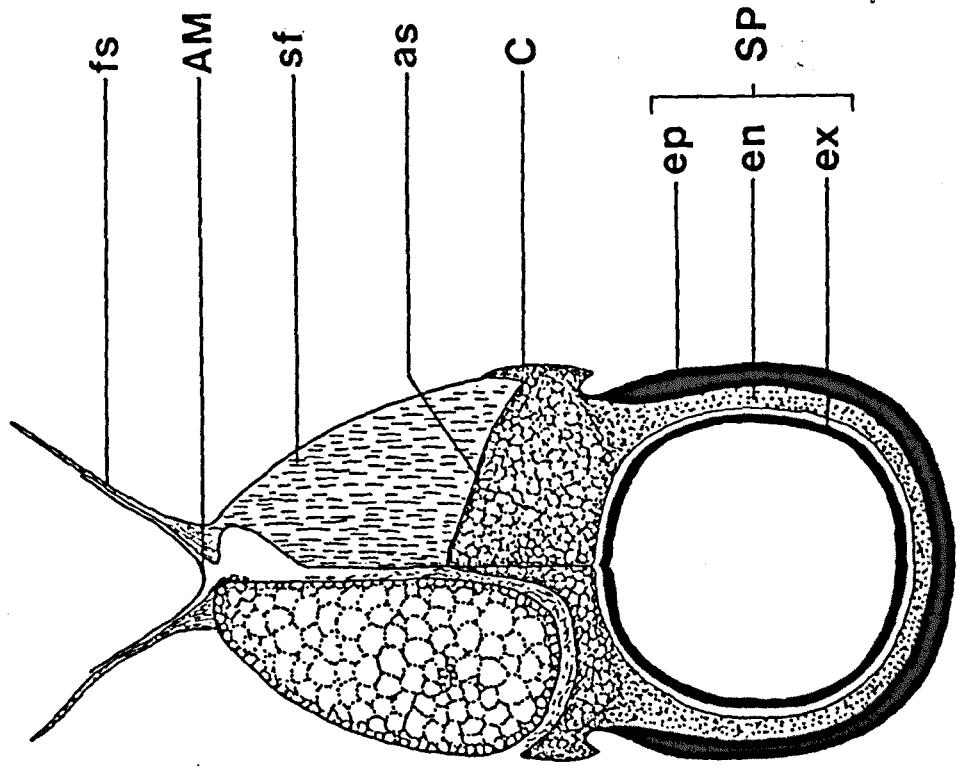
(a) Diagrammatic median L.S. of a 3-floated taxon (filosum omitted).  
(Mag. ca X80). (Typical of Sect. *Azolla*).

(b) Diagrammatic median L.S. of a 9-floated taxon (filosum omitted).  
(Mag. ca X75). (Typical of Sect. *Rhizosperma*).

(c) Diagrammatic T.S. above the acrolamella of a 3-floated taxon.  
(Mag. ca X100).

(L = longitudinal float surface; T = transverse float surface; 1 = external surface; 2 = internal surface; A = upper float tier; B = lower float tier; a = adjacent float surface)

3.2



3.3

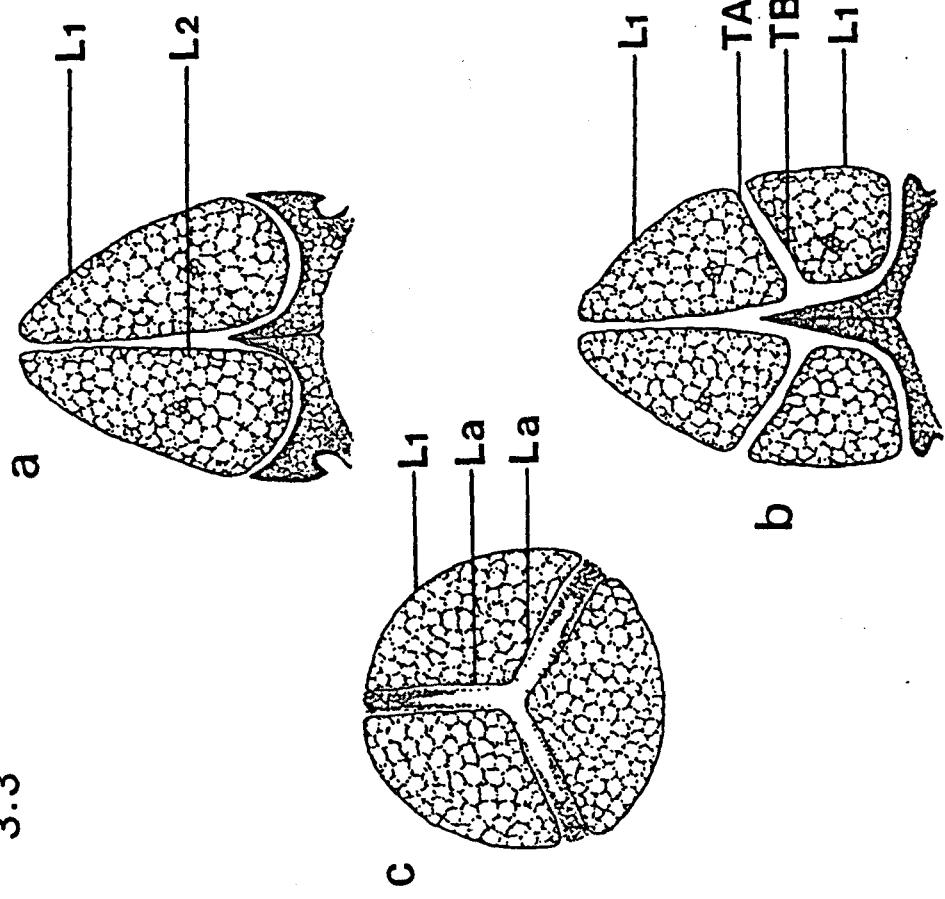


FIGURE 3.4

Elaboration of the proximal perine and the superstructure of suprafilosum

(a) Diagrammatic representation of extant Sect. *Azolla*. Note the break in the suprafilosum septum (arrow), collar cusps and less angular acrolamella septa compared to extant Sect. *Rhizosperma*.  
(Mag. ca X150).

(b) Diagrammatic representation of *A.pinnata* (Sect. *Rhizosperma*). Note the complete supra- filosum septum, lack of collar cusps and angular acrolamella compared to extant Sect. *Azolla*. (Mag. ca X150).

(fs = funnel of suprafilosum with attached apical membrane; sf = suprafilosum septum; as = acrolamella septum; C = collar; cc = collar cusp)

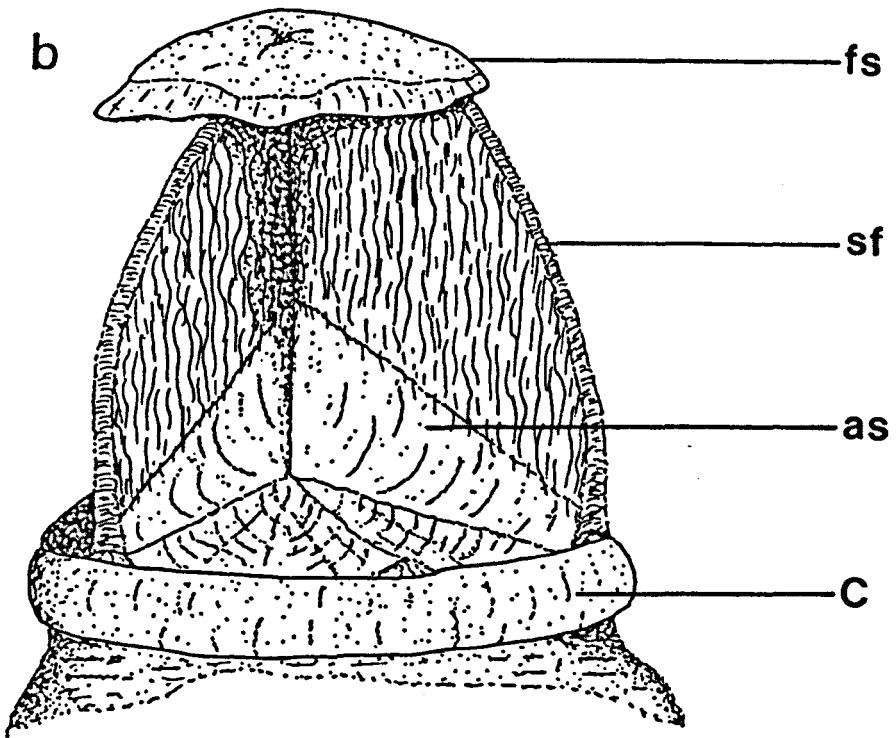
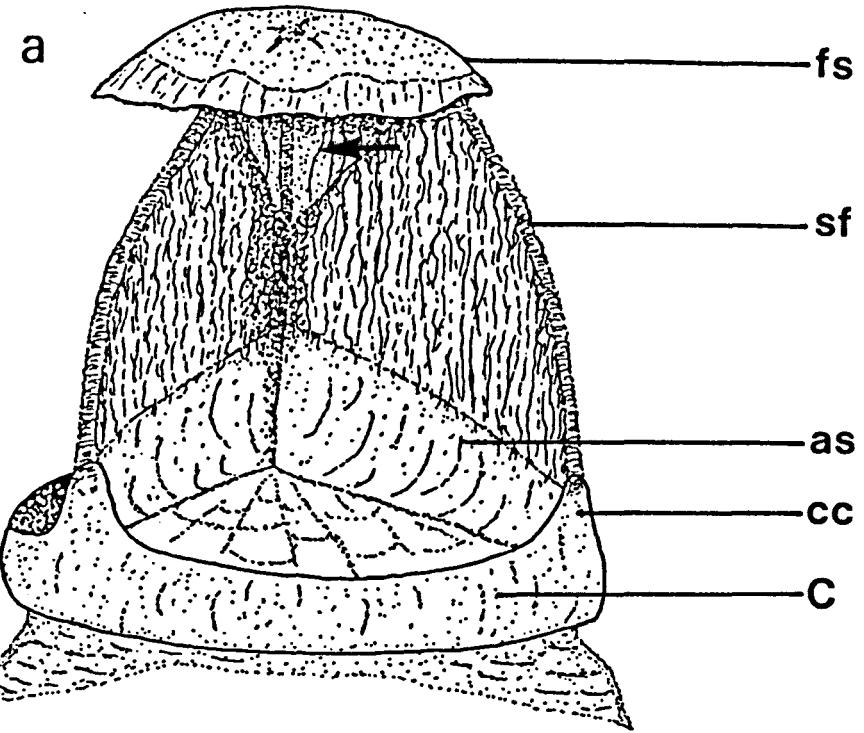


FIGURE 3.5

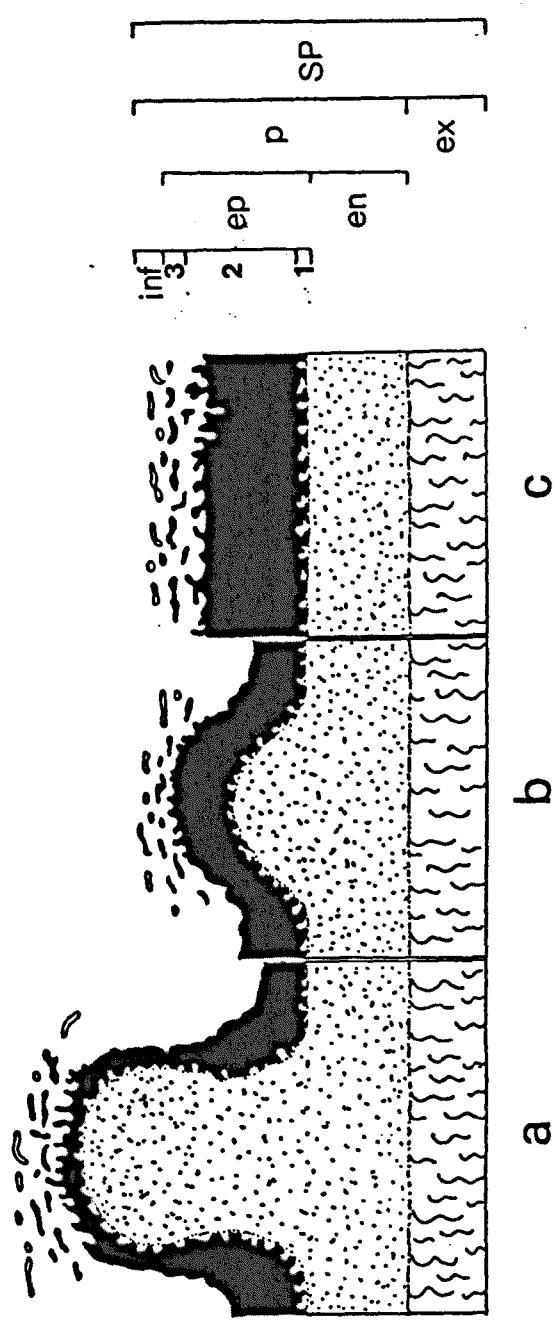
Diagrammatic representation of sporoderm stratification

(a) Stratification in an excrescence.

(b) Stratification with an endoperine intrusion.

(c) Stratification with uniform thickness of endoperine.

(SP = sporoderm; ex = exospore; p = perine; en = endoperine; ep = exoperine; 1 = exoperine 1; 2 = exoperine 2; 3 = exoperine 3; inf = infrafilosum)



c

b

a

FIGURE 3.6

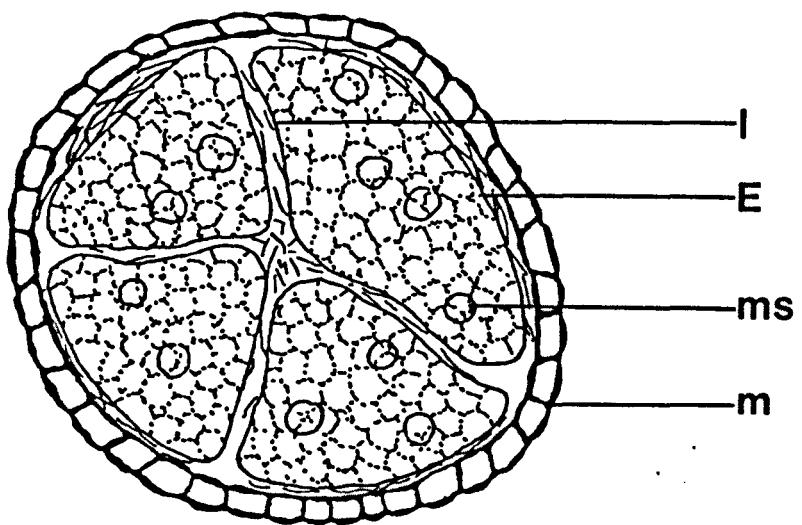
Massula surfaces

(a) Diagrammatic median section of a microsporangium illustrating massula surfaces.

(b) Diagrammatic representation of a massula illustrating the distribution of glochidia in extant Sect. *Azolla*.

(I =internal surface; E = external surface; m = microsporangial wall;  
ms = microspore; g = glochidium)

a



b

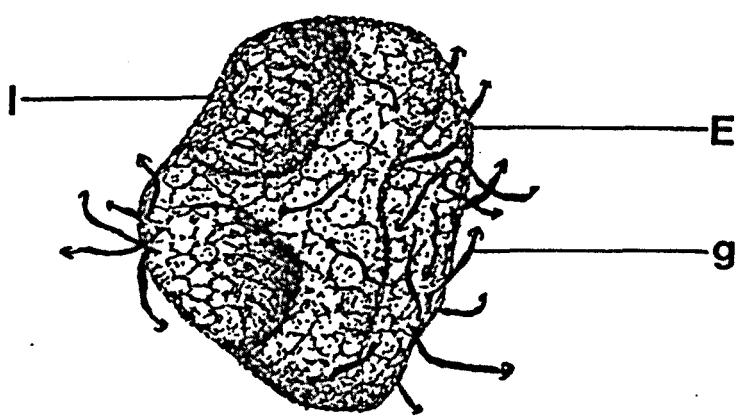


FIGURE 3.7

Massula appendages

- (a) SEM of massula trichome on the internal surface of a massula.  
(Sect. *Rhizosperma*, *A. pinnata*).
- (b) SEM of glochidia on the external surface of a massula.  
(Sect. *Azolla*).

(MT = massula trichome; g = glochidium; Scale bar = 50 $\mu$ m)

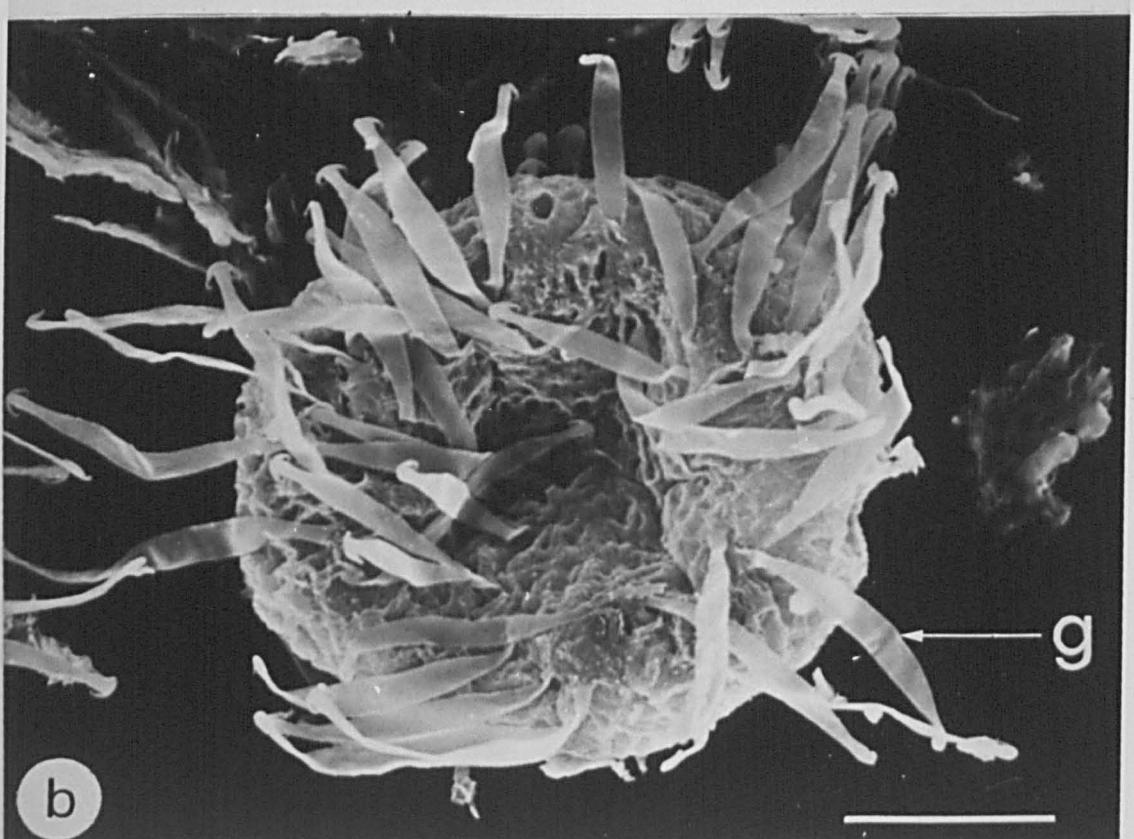
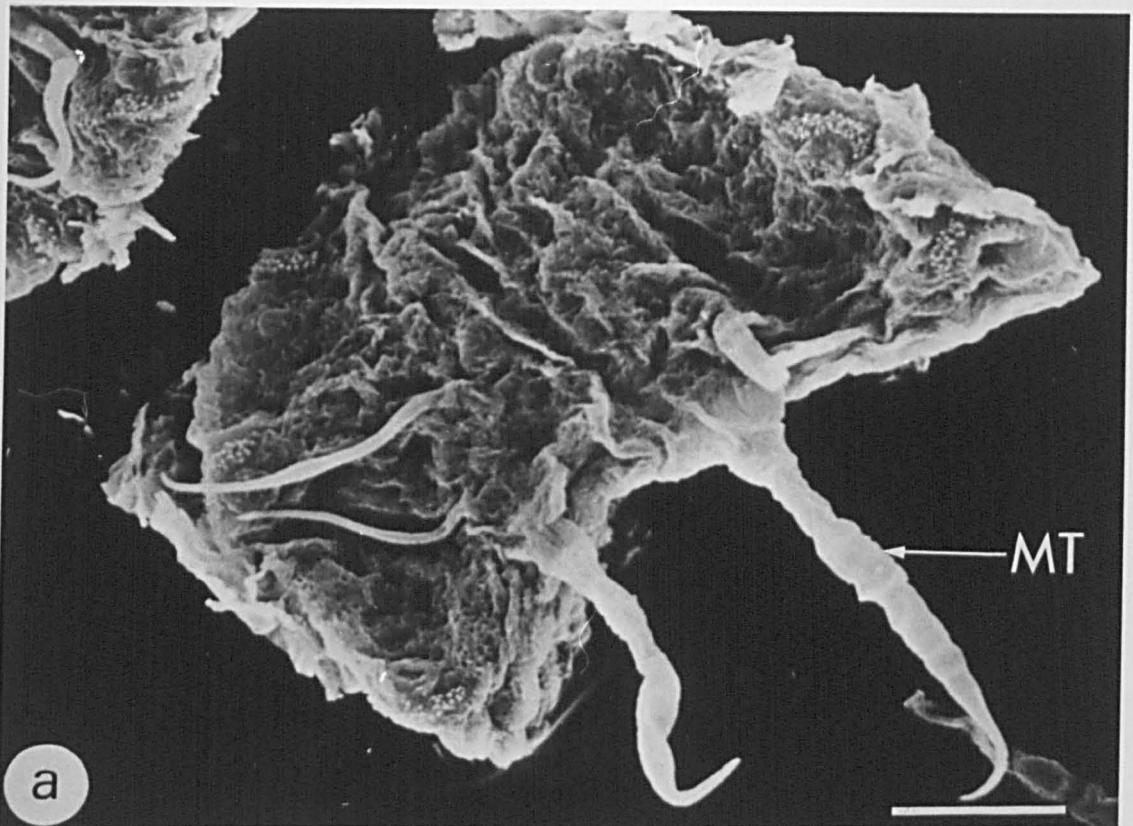


FIGURE 3.8

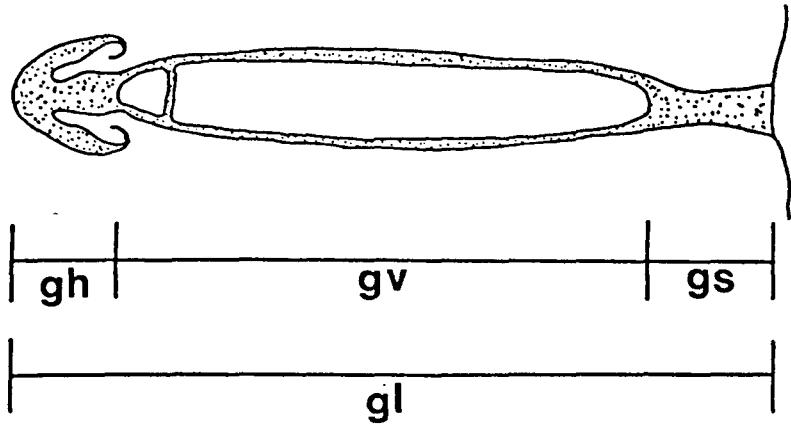
Glochidial characters

(a) Diagrammatic representation of a glochidium defining characters investigated (gv + gs = glochidial shaft).

(b) Diagrammatic representation of a glochidium defining positions in a glochidial vacuole.

(gl = glochidial length; gh = glochidial head; gv = glochidial vacuole; gs = glochidial stalk; A = apical; M = median; B = basal)

**a**



**b**

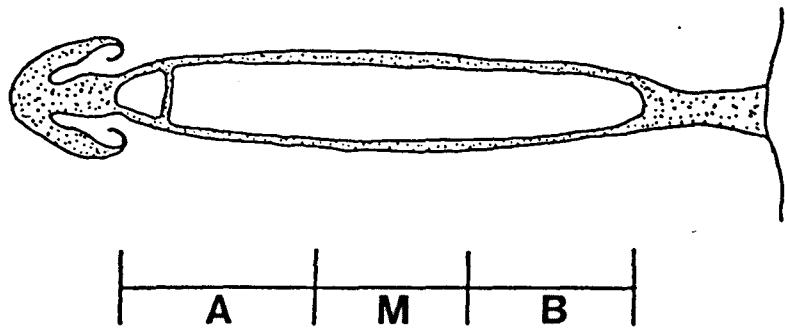


FIGURE 3.9

Glochidial shape (defined by shape of the glochidial vacuole)

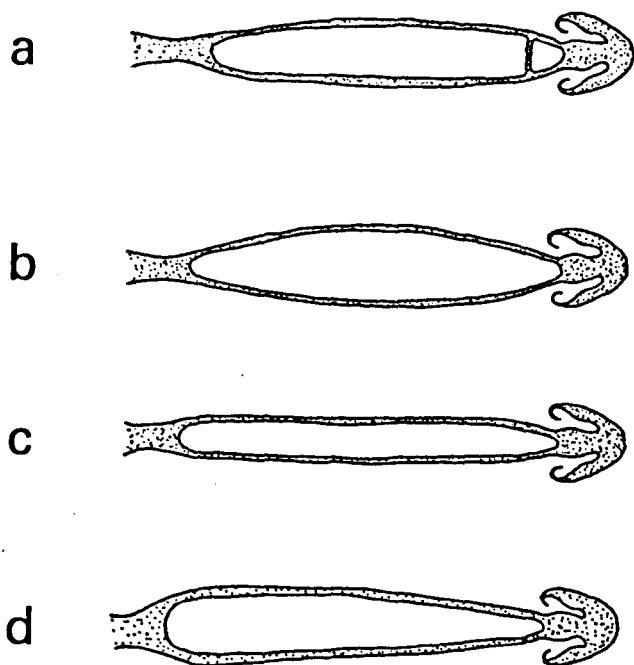
- (a) More less basal and apical tapering.
- (b) Basal and apical tapering.
- (c) Parallel sided glochidium.
- (d) Apical tapering.

FIGURE 3.10

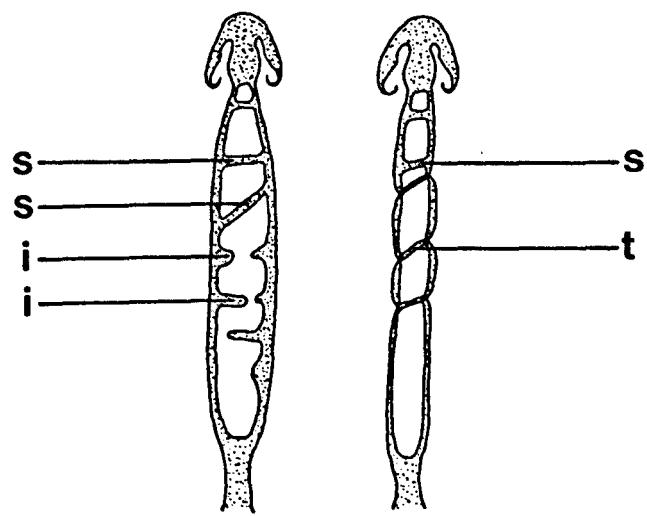
Diagrammatic representation of septa, intrusions and twists in the glochidial vacuole.

(s = septum; i = intrusion; t = twist)

3.9



3.10

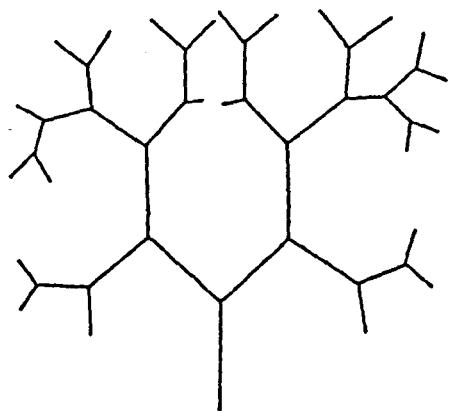


**FIGURE 3.11**

**Branching pattern**

- (a) Diagrammatic representation of isotomous branching giving rise to a rounded frond shape.
- (b) Diagrammatic representation of anisotomous branching giving rise to an elongated frond shape.

a



b

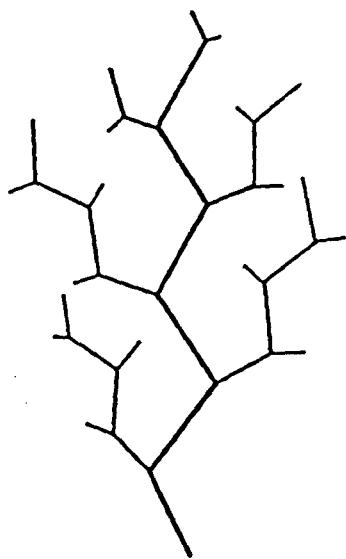
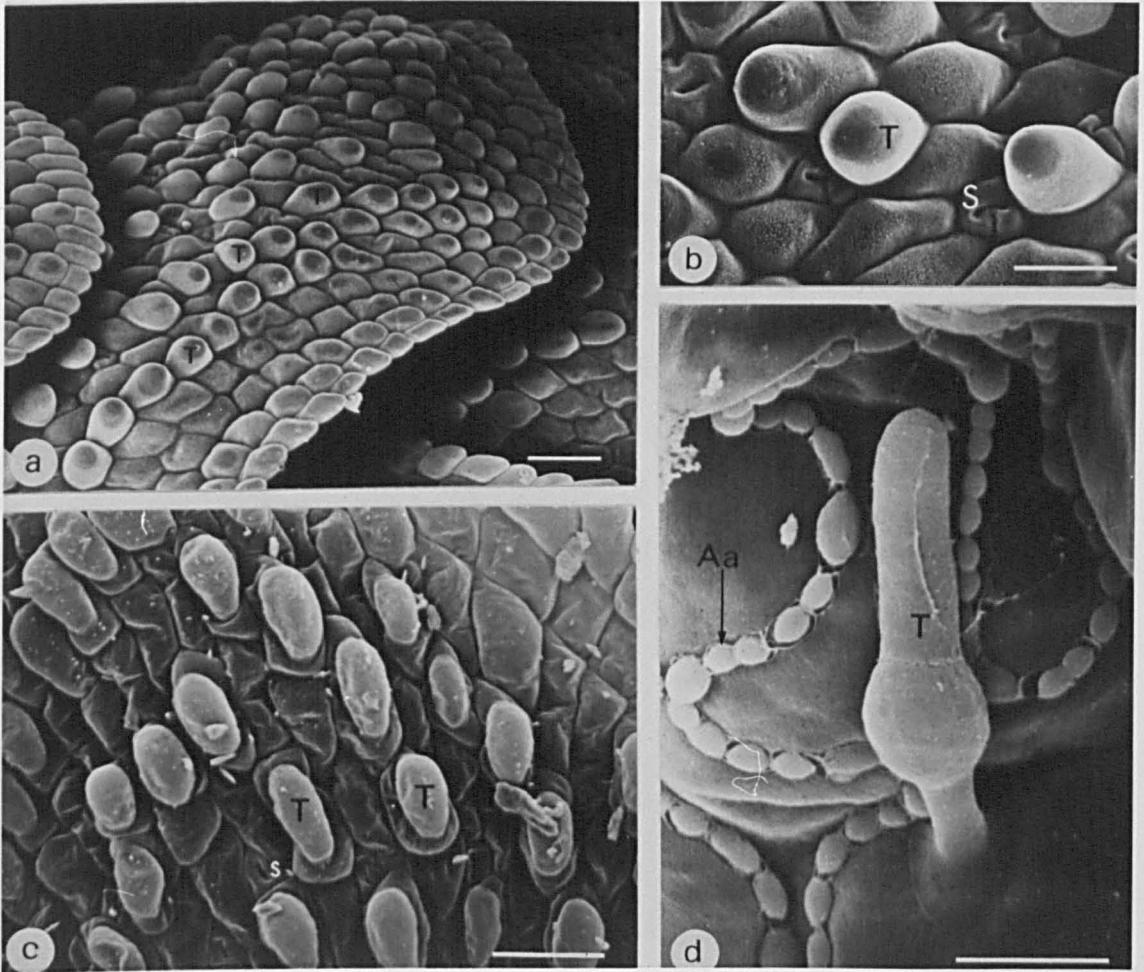


FIGURE 3.12

Trichomes

- (a) SEM of the abaxial surface of a dorsal leaf lobe with one-celled trichomes. (Scale bar = 1000 $\mu$ m).
- (b) SEM of one-celled trichomes protruding above the other epidermal cells. These trichomes are usually associated with stomates. (Scale bar = 50 $\mu$ m).
- (c) SEM of two-celled trichomes protruding above the other epidermal cells. (Scale bar = 50 $\mu$ m).
- (d) SEM of a simple trichome found in the dorsal leaf lobe cavity which contains *Anabaena azollae*. (Scale bar = 25 $\mu$ m).

(T = trichome; S = stomate; Aa = *Anabaena azollae*)



#### **4. RESULTS**

## 4.1.1

The Megasporocarp

Each megasporocarp accommodated one megasporangium in which was found a single functional megaspore proper and accessory material that together constitute a megaspore apparatus. A two-cell thick megasporocarp wall enclosed the megasporangium except for an apical pore. The basal megasporocarp wall was usually more or less translucent and fragile, but approximately half-way towards the apex, it became highly thickened, forming a persistent indusial cap. Inside the sporocarp wall and completely surrounding the megaspore apparatus was the one-cell thick megasporangial wall. At maturity, this wall became compressed which rendered the radial cell walls indistinguishable. In the apical region of the sporocarp the megasporangial wall was consistently found closely adhered to both the sporocarp wall and funnel of suprafilosum. This latter structure cascaded down over the external faces of the floats. At the apex of a sporocarp, between the megasporangial wall and the sporocarp wall, the endosymbiont, *Anabaena azollae* was almost invariably located. Features described above are illustrated in Figures 3.1, 4.1a & b, 4.2a. Although there was usually a single megaspore apparatus per megasporangium, it was observed that two megaspore apparatuses were very occasionally found. However, as shown in **Figure 4.47b** the float region was always highly reduced, or even absent.

#### 4.1.2 Structure of the megaspore apparatus

The objectives of this investigation are, in part, to sample the phenotypic variation of the megaspore apparatus. This variation can then be used to group together populations with overall similarity to each other. Each of these groups can then be used to establish megaspore apparatus types. Using these types it is possible to evaluate the taxonomic value of characters from other organs of the plant. Ultimately, it may be possible to provide descriptions of taxa using useful taxonomic characters with known phenotypic variation. The correct names for the types can then be assigned after examination of nomenclatural Type material. However, prior to establishing the megaspore apparatus types it is necessary to understand the complex structure of a megaspore apparatus in extant *Azolla*.

In surface view the apical region, which accommodates the floats, was separated from the truncated spherical basal region by the collar (Figs.3.2,4.5,4.6). In extant *Azolla* float number separated Section *Azolla* (three floats) from Section *Rhizosperma* (nine floats). However, float structure was common to all extant taxa, composed of many isodiametric pseudocellular cavities (=alveolae). These alveolae were not visible in surface view because of a thin covering of material which was variously perforated(Figs.4.1,4.7b&c). In members of Section *Azolla* each float possessed two surfaces (for definition see section 3). The external surface was often subtriangular in shape with rounded corners. In longitudinal median section each

float was almost biconvex, with the external surface being more flattened (Figs.4.2b). Filosum was consistently found attached to the apical half of the longitudinal edges, and the apical eighth of the internal and external float surfaces. Filosum from the latter two surfaces intertwined with the funnel of suprafilosum. This not only anchored the funnel to the float apices, but also held the float apices together. Other float filosum from the internal surface intertwined with suprafilosum (Fig.4.1b). The filosum elements were ca. 0.5μm to 1μm in diameter.

The funnel of suprafilosum, in an intact megasporocarp, was always folded back and capped the apical third of the floats. However, the characteristic funnel shape was only assumed when the indusial cap was removed during peeling or embryo development (Figs.3.2,4.5c). During peeling, part of the megasporangial wall remained fused to the inside of the funnel, providing a seal across the float apices; this seal has been termed the apical membrane (Fig.3.2).

Extending up between the floats from the collar level to approximately the apical third of the floats was the angled, but conically shaped acrolamella. It was composed of alveolate endoperine and was continuous with the more dense endoperine of the distal megaspore region, suggesting that the acrolamella was an elaboration of the proximal perine. Although the acrolamella was triseptate and divided the proximal megaspore surface into three equal sectors with concave bases in extant *Azolla*, the

*suprafilosum* was found to significantly contribute to forming three sectors in which the floats were accommodated (Figs.3.4,-4.1c & d,4.2,4.3a&b).

Depending upon the taxonomic Section, three or a single float nestled in each concave sector of the apical region of a megaspore apparatus. In Section *Azolla*, with a single float per sector, the acrolamella septa extended radially and joined on to the collar at the cusps; these cusps were not seen in Section *Rhizosperma* (Fig.3.4). In the longitudinal plane of the acrolamella septa the *suprafilosum* was found to be in continuation with the funnel. In this same plane float filosum from the longitudinal edges of the floats was observed, and presumably helped anchor the floats by intertwining with the filaments of *suprafilosum*. During the present investigation no evidence was found for a complete tube of *suprafilosum* from the acrolamella to the funnel in members of Section *Azolla*. Sections and dissections of the megaspore apparatus revealed that *suprafilosum* was consistently absent from the more apical float region of the longitudinal median plane of each float. However, at the float apex in this region float filosum originated and intertwined with the funnel. The distribution of float filosum and the superstructure of *suprafilosum* are illustrated in Figures 4.1,4.2.

In the *A.pinnata* type (Section *Rhizosperma*) a similar triseptate acrolamella and superstructure of *suprafilosum* was observed (Fig.4.3). However, each of the three sectors accommodated

three floats; two in a lower tier and one in an upper tier. With this arrangement alternate longitudinal float surfaces in the lower tier lacked filosum. The distribution of filosum in the upper tier of floats was similar to that of members of Section *Azolla*. However, *A.nilotica* (Section *Rhizosperma*) lacked suprafilosum except rudimentary filaments on the internal collar surface. All other filosum appeared to emanate from the floats. Where filosum was absent between adjacent float surfaces, small hooks were commonly observed on the edges of the surface in taxa of Section *Rhizosperma*. These hooks appeared capable of linking on to adjacent floats. The arrangement of the acrolamella suprafilosum and floats are illustrated in Figure 4.3.

Like the acrolamella, the collar was consistently found to be an elaboration of the megasporangium perine. In sectional view the collar delimited the periphery of the proximal surface megasporangium proper. As previously stated a tricuspid collar was only found in taxa attributed to Section *Azolla*, furthermore, the collar in these taxa was consistently larger than members of Section *Rhizosperma*. A further difference between the two Sections was the presence of a flange in taxa attributed to Section *Azolla*; this flange was best observed in sectional view (Figs. 4.4a, 4.9, 4.8). However, there were similarities between the Sections in respect to the collar. Composed mainly of alveolate endoperine, the collar was often covered by a thin layer of exoperine extending from the distal perine surface. In the *A.nilotica* type this exoperine extended on to the external surface of the lower float tier rendering the collar inconspicuous.

-ous in surface view. However, in other types the exoperine was often observed to die-out completely within the length of the collar (Fig.4.4a & b). Originating from the internal surface of the collar were numerous filaments of suprafilosum. In the *A.pinnata* and *Azolla* sp. types some of these filaments appeared to cascade over the external surface of the collar on to the distal perine surface. In these two types, like the *A.nilotica* type, the collar<sup>surface</sup> was consistently obscured (Figs.4.25,4.59a & b).

Below the collar, in surface view, the spherical basal region of the megasporangium contains the megasporangium proper which was bounded by the exospore. Laesurae were located immediately below the acrolamella; however, it was not possible to demonstrate convincingly that the laesurae were in the same longitudinal plane as the septa union lines of the acrolamella. Surrounding most of the dense exospore was the perine, and together these are termed the sporoderm. The perine was consistently found to be divided into two layers, but in the proximal region of the exospore only the inner perine layer, the endoperine, was present (Figs.3.2,4.4a & b). The outer layer, the exoperine, may be divided into at least two zones. (Fig.3.5). The innermost of these the exoperine 1, was common to all taxa, and probably gave rise, at least in part to the infrafilosum (Fig.4.4c). However, infrafilosum was rarely observed originating from the exoperine 1, even in specimens with much infrafilosum. Therefore, it is probable that it could originate from a more superficial exoperine layer. Exoperine 2 and/or 3

gave rise to the highly sculptured perine surface (Fig.3.5). Although often relatively dense, the complex architecture and sculpturing of the perine formed the basis for defining the megaspore apparatus types (=types) in this investigation. Detailed descriptions of sporoderm structure and sculpturing are given for each megaspore apparatus type in Section 4.2.

## 4.2 MEGASPORE APPARATUS TYPES

### 4.2.1 A.filiculoides type

#### Megaspore apparatus

##### 4.2.1.1 GENERAL APPEARANCE

The megaspore apparatus was ovoid with a mean length of ca 546 $\mu$ m (range 490 $\mu$ m - 607 $\mu$ m) and an equatorial diameter of ca 364 $\mu$ m (range 304 $\mu$ m - 412 $\mu$ m). However, the floats often collapsed which distorted this shape. The collar region usually had a distinct waist (Figs.4.5,4.6). The distal megaspore region was spheroidal in shape with more elongated forms resulting from excess sporoderm distally compared with other regions (Figs.4.5, 4.6).

##### 4.2.1.2 FLOATS (Surface view)

These appeared more or less triangular in surface view, although the basal end was usually somewhat rounded. Surface puncturing of floats was characteristically of small, irregularly shaped holes (size ca 3 $\mu$ m) (Figs.4.5,4.6,4.7b & c). Float filosum was clearly seen attached to edges of the apical half and apical external surface of each float (Figs.4.5,4.6,4.8a) and this filosum intertwined with the suprafilosum funnel. Filosum filaments were ca 0.6 $\mu$ m in diameter. Supernumerary floats were rarely observed in this type.

#### 4.2.1.3 COLLAR

In surface view the narrowest region was at the longitudinal mid-point of each float, the width gradually increasing towards the cusp region where the collar often extended between the floats. The lower edge of the collar always had inwardly curved spines extending towards the base of the megasporangium. These spines were often elongated and formed short filosum elements which extended on to the sporoderm surface (Fig.4.8b-d). The lower edge of the collar, in surface view, was the lowest point of the flange, however, this was not always distinct when the flange 'met' the sporoderm (Fig.4.8). In sectional view the collar varied in angle to the equatorial axis from ca 60° to ca 22°. The greater the angle the more likely a waist was observed. The flange consistently originated at a level from half to two-thirds the way up the collar (Fig.4.9); this was characteristic of the *A.filiculoides* type, with Australian populations tending to have the lower flange insertion.

#### 4.2.1.4 SPORODERM

Sculpturing - Raised angular areas interconnected by narrow ridges or bridges of exoperine material. The rounded nature of the depression areas being caused by the ridges/bridges (Figs.4.10c,4.7a). The entire sporoderm surface was covered by a weft of infrafilosum, of varying amount, which often obscured the surface sculpturing to a variable degree. Infrafilosum therefore imparted variation to the distinctness of the raised areas (Fig.4.10).

This study showed that in a few specimens the raised areas, or excrescences, were taller towards the distal region of the megaspore apparatus (Fig.4.6b). Closeness of excrescences also influenced the obviousness of the depressions. In some specimens there appeared to be little or no depression area, particularly in <sup>the</sup> proximal half of the sporoderm surface. In other specimens with many excrescences and much infrafilosum the depressions appeared to be like foveae (Fig.4.10). Extent of the exoperine 3 also influenced the sculpturing of the *A.filiculoides* type. Indeed, this type was occasionally seen to resemble the *A.mexicana* type at high magnifications. At such magnifications sculpturing was also found to exhibit variation in the *A.filiculoides* type. The depression areas, when visible, had an undulating surface which was often minutely reticulate to verrucose. This latter extreme was commonly observed in Australian populations where the verrucose bodies sometimes almost filled the depressions (Figs.4.10d,4.7a,4.12c). Sculpturing, under high magnification, towards the apex of an excrescence was usually not visible, particularly towards the proximal sporoderm region. However, when visible it was consistently observed to be relatively smooth, although towards the excrescence apex the perine surface developed anastomosing vermiform ridges. These ridges became more distinct to form the baculate exoperine (see section 4.1.2). However, at high magnifications only the verrucate to rugulate surface of this layer was usually visible (Figs.4.7a,4.-10,4.14a-c). Filaments of exoperine 3 were usually observed arising from the exoperine 2 at the often flattened excrescence apex. These filaments interconnected adjacent excrescences and were

almost indistinguishable from the infrafilosum elements which emerge from small foveae in the verrucate-rugulate sculpturing (Fig.4.10).

Considerable variation in the sculpturing was observed at high magnification, and was attributed to the degree of fusion and quantity of perine elements (Fig.4.10). This variation was further compounded by the previously described variation seen at low magnification. This study revealed that the interconnecting ridges and bridges between the excrescences were either ridges of solid exoperine 2 which, in surface view, were hidden by filaments of exoperine 3 and infrafilosum, or, the exoperine 2 ridges were absent and only bridges of exoperine 3 and infrafilosum connected adjacent excrescences (Figs.4.7a,4.10c). Where excrescences were tall and relatively isolated, the difference between a ridge and a bridge could be observed. However, the difference was best observed while studying sporoderm structure. Furthermore, observation of structure gave a better insight into the derivation of the sculpturing.

Structure - Sporoderm thickness in the 43 populations measured, varied from ca 17 $\mu$ m (range ca 13 $\mu$ m - 22 $\mu$ m) in the depressions to 45 $\mu$ m (range ca 26 $\mu$ m - 50 $\mu$ m) in the raised areas which were large interconnected excrescences. The sporoderm was divided into three basic layers which are defined in section 3.1.1. The inner most layer, the exospore, had a mean thickness of 3.7 $\mu$ m (range 2.5 $\mu$ m - 4.0 $\mu$ m). It appeared to be composed of dense material with variable numbers of generally radially elongated 'cavities'

(Figs. 4.12e, 4.14e) which often appeared to increase in frequency towards the external edge of the exospore. The external surface of the exospore (i.e. surface adjacent to the endoperine) was a meshwork of fine elements which appeared to become fused; with Australian populations usually having <sup>the</sup> latter extreme (Fig. 4.14f & g).

The endoperine under high magnification was composed of closely packed tiny rods. These rods were simple and branched and irregularly vermiform, and were particularly obvious in Australian populations, but in North American and European populations, were not. In these populations the endoperine often appeared more or less solid (Fig. 4.14d). South American populations usually appeared, under high magnification, intermediate between the two previously described types. Under low magnification the endoperine varied considerably and is best described in relation to the depression and excrescence regions of the perine. Beneath depressions the endoperine was 3.3 $\mu$ m (range 2.5 $\mu$ m - 5.3 $\mu$ m) thick often with small rounded alveolae. Usually, and particularly in Australian populations, these alveolae were irregularly arranged and confined to the radially external half of the endoperine. In populations from other regions the alveolae often formed one or two rows in the depression region, often interdispersed with small alveolae. In excrescences one, two or a few large alveolae were commonly observed. However, the Australian populations only rarely had large alveolae; in these populations the excrescence endoperine often had similar sized alveolae to the depression region.

However, large alveolae were occasionally observed. A dense 'plug' of endoperine may be found centrally at the base of an excrescence in some Australian populations. Variation in endoperine vacuolation can be seen in Figures 4.11 - 4.14. In Australian populations the endoperine (both depression and excrescence) consistently appeared to be denser than in populations from other regions; Figures 4.11 & 4.12 illustrate this variation.

The exoperine 1 was ca 1.3 $\mu$ m thick and irregularly baculate. However, towards the apex of an excrescence the exoperine 1 became indistinguishable (Figs.4.12a & c,4.13b). The exoperine 2 in the depressions was solid and ca 7.2 $\mu$ m thick, but exhibited considerable variation in respect to its thickness, this was often observed within a single population. Towards an excrescence the exoperine 2 usually thinned until at the apex of an excrescence it was ca 3 $\mu$ m thick. Here the exoperine 2 was usually baculate or shortly columellate. The bacula tended to anastomose and form the verrucate-rugulate sculpturing (Figs.4.1-2a & c,4.13). Arising from the apices of the radial bacula of the exoperine 2 were the tangential exoperine 3 filaments. This zone, which only originated from an excrescence apex in the *A.filiculoides* type, comprised of filaments which were ca 2 $\mu$ m in diameter, giving this layer a thickness of ca 2.5 $\mu$ m. The filaments tended to anastomose and extend to, and thereby link, adjacent excrescences (Figs.4.11d,4.13a & b). Variation was observed at the intra- and inter-population levels in respect to the exoperine 2 and 3 zones and the degree of fusion of their

respective elements. Some specimens possessed solid exoperine 2 at excrescence apices and the exoperine 3 elements were sometimes fused, although still discernible (Figs. 4.12a-c, 4.14a-c). The variation in the exoperine 2 and 3 zones was, in this study, found to greatly influence the sculpturing. This influence was seen at both high and low magnifications. The thickness of exoperine 2 in the depressions and the thickness of endoperine in the adjacent excrescences both influenced the depth of a depression and hence the prominence of excrescences; this is illustrated in Figures 4.12a-c, 4.14a-c. In some specimens adjacent excrescences had little or no depression area which influenced their prominence.

#### 4.2.2 A.mexicana type

##### Megaspore apparatus

###### 4.2.2.1 GENERAL APPEARANCE

Megaspore apparatus usually ovoid with a mean length of ca 518 $\mu$ m (range 475 $\mu$ m - 560 $\mu$ m) and an equatorial diameter of ca 343 $\mu$ m (range 312 $\mu$ m - 387 $\mu$ m). The collar region always had a shallow waist. Although not a consistent feature, this megaspore type often had a more hemispherical apical region than other megaspore types (Fig.4.15).

###### 4.2.2.2 FLOATS (Surface view)

These were subtriangular, but truncated at their apices which contributed to the hemispherical shape of the apical region of the megaspore apparatus (Figs.4.16a,4.15). Float surface

punctured by characteristically minute holes which were often with relatively smooth sides (Fig.4.16a - c). In some specimens the holes were so minute that they appeared to be absent. Float filosum was usually seen attached to the edges of the apical half and apical external surface of each float where the filaments intertwined with the suprafilosum funnel. Filosum filaments ca 0.5 $\mu$ m - 1.0 $\mu$ m diameter. Supernumerary floats were observed in several populations of this type; either an equal division of each float or one float of a specimen was unequally divided (Fig.4.15b). All specimens sampled from one population (NY1) were found to possess a small supernumerary float in each float sector.

#### 4.2.2.3 COLLAR

In surface view the narrowest region was at the longitudinal mid-point of each float. The width gradually increased towards the cusp region where the collar clearly extended between the floats (Fig.4.15). The collar surface was usually relatively smooth, with only small undulations. Although the Type material possessed a few filaments of filosum on the collar, this was rarely seen in other populations. The lower collar edge was the lowest point of the flange, and it always 'met' the perine surface. Extending basally from this lower edge were inwardly curved spines which often elongated to form short filosum elements. In longitudinal sectional view the collar was at an angle of between 30° and 60° to the equatorial axis but in some specimens this variation was observed between the cusp and longitudinal mid-point of the floats. In this latter region the

collar was often considerably broader, in sectional view, compared with the cusp region. The flange always originated in the lower third of the collar; the groove was therefore in a relatively low position (Fig.4.16d & e).

#### 4.2.2.4 SPORODERM

Sculpturing - General appearance at low magnifications, under the scanning electron microscope, usually of a finely sculptured surface with crater-like foveae (ca 20 $\mu$ m diameter) (Fig.4.17).

These were often obscured by a variable quantity of infrafilosum which reduced their apparent size (Fig.4.17). A size reduction in foveae also occurred when the perine surface elements were fused; this fusion was only seen at higher magnifications.

At such magnifications the sculpturing was verrucate to rugulate with many small foveae (ca 3 $\mu$ m). Variation consistently observed during this study was in respect to the degree of fusion of the elements comprising the surface (Fig.4.17). In addition, the smaller foveae were found in the more fused (more rugulate) condition. Occasionally <sup>a</sup>infrafilosum filaments (ca 0.8 $\mu$ m diameter) were observed emerging from the small foveae. The previously described sculpturing extended down the sides of the large crater-like foveae and occasionally completely lined them (Fig.4.17b & c). More commonly, however, the bottom of these large foveae could be seen to have relatively smooth sculpturing. The considerable variation in the sculpturing seen at high magnification was occasionally found within one specimen; the more fused and smooth type being observed in the proximal region of the distal sporoderm surface. The sculptural variation

described here was influenced by the quantity of infrafilosum, furthermore variation was at both intra- and inter-population level. The sculpturing at the bottom of the large foveae was better observed from sporoderm sections.

Structure - The mean maximum sporoderm thickness in 27 populations measured, was ca 32 $\mu$ m (range ca 21 $\mu$ m - 37 $\mu$ m) and to the base of a large fovea the thickness was ca 19 $\mu$ m (range ca 13 $\mu$ m - 21 $\mu$ m). The sporoderm was divided into three basic layers which are defined in section 3.1.1. The innermost layer, the exospore, had a mean thickness of 4.2 $\mu$ m (range 3.1 $\mu$ m - 5.0 $\mu$ m). It appeared to be composed of more or less dense material. Within this matrix were variable numbers of generally radially elongated 'cavities' (Fig.4.18b & c). These 'cavities' often appeared to increase in size towards the radially external surface of the exospore. This surface of the exospore appeared to be meshwork of fine filaments tending toward fusion (Fig.4.20-a). Compared with other megaspor types, the fused state was perhaps less common in the *A.mexicana* type.

The endoperine structure, under high magnification, was of closely packed tiny rods. These rods were simple and branched, and irregularly vermiform (Fig.4.20b). This study found variation in respect to the degree of fusion of these endoperine rods which was commonly at the inter-population level and more rarely at the intra-population level. Endoperine structure, at low magnification, exhibited similar levels of variation which were manifested in density of alveolae. In most specimens there

appeared to be a basal zone of endoperine with few or no alveolae (with the exception of small 'cavities') (Figs.4.18,4.19). Although the endoperine alveolae varied in density, in any one specimen of the *A.mexicana* type, they were usually of relatively uniform dimensions. However, some specimens lacked alveolae and large isolated alveolae were very rare (Figs.4.18c,4.20b). Thickness of the endoperine was not always uniform (range ca 3 $\mu$ m - 30 $\mu$ m) except in some populations and/or specimens of a population. In such instances endoperine thickness was ca 6 $\mu$ m while in other specimens the endoperine intruded into the exoperine layer in localised areas. The endoperine thickness of these intrusions was ca 18 $\mu$ m (range 11 $\mu$ m - 30 $\mu$ m). The *A.mexicana* type intrusions very rarely attained prominence as in the *A.filiculoides* type (Fig.4.19). A specimen from one population (M041) had two massive excrescences at the distal end of the megasporangium; such features were considered to be abnormalities because other specimens of the population were typical of the *A.mexicana* type. The intrusion endoperine of this type was similar to the endoperine below the foveae, however, there were often few or no alveolae in the central region of the excrescence. Although some populations completely lacked intrusions, others possessed large, small or few intrusions, or combinations of these, with variation being consistently observed at the intra population level (Fig.4.19). Furthermore, structural variation, so far described, could not be correlated with any other characters or geographical distribution. The presence or absence of intrusions could not be detected from the surface sculpturing because of the complex and variable configuration of the perine layers; this was elucidated during this investigation.

The exoperine usually consisted of three zones. The innermost was the exoperine 1 which, like all the other megaspore types, was baculate in structure. The bacula fused at the tips and formed the basement zone for the exoperine 2 layer (Fig.4.18b). The exoperine 1 was ca 1.6um thick. Structure of the exoperine 2 varied from columellate to solid (Figs.4.18,4.19); this variation was occasionally observed within one population. The range in thickness was ca 3um - 17um. This study revealed that the pattern of variation in exoperine thickness created the distribution of large foveae in the absence of endoperine intrusions. Large foveae were formed where the columellae became short and baculate or were fused to form solid exoperine 2 (Fig.4.18). Sections through these foveae revealed the true nature of the perine sculpturing which may not be seen from the surface. Where there was no or little fusion of the bacula, the sculpturing at the foveae base was verrucate (Fig.4.17a-b). With increased fusion of the bacula, the sculpturing became rugulate and eventually gently undulating, with small verrucae (Fig.4.17c-g). The 'raised' areas were formed from tall, irregularly divided and arranged radial columellae. Often these columellae were arranged so as to leave a space within the raised area (Fig.4.18a). Where an endoperine intrusion occurred, irregularly arranged radial bacula of exoperine 2 were found, particularly towards the summit of the intrusion. In foveae the exoperine 2 ~~intergrad~~<sup>d</sup> between baculate and solid. The above variation contributed to the sculpturing described previously and is illustrated in Figure 4.17. Much of this considerable variation, was related to the degree of fusion of the exoperine 2 and the occurrence of

endoperine intrusions. In respect to intrusions, it was found that in some populations, where intrusions occurred, not every 'raised' area was associated with an intrusion. To account for all this variation, unsuccessful attempts were made to correlate it with other characters and geographical distribution. The exoperine 2 columellae consistently divided at their apices, which then anastomosed and formed the verrucate-regulate sculptured exoperine 3 which was ca 2um thick. This zone comprised of tangentially orientated, short vermiform elements which, with the exception of the bottom of most foveae, completely covered the underlying perine (Figs.4.18a & b, 4.19a-c). Therefore, the considerable structural variation was not manifested in the sculpturing. As described in the section on sculpturing, the exoperine 3 elements exhibited varying degrees of fusion.

#### 4.2.3 A.microphylla type

##### Megaspore apparatus

###### 4.2.3.1 GENERAL APPEARANCE

Megaspore apparatus usually ovoid with a mean length of ca 511um (range 462um - 600um) and mean equatorial diameter of ca 338um (range 269um - 572um). In the collar region there was a shallow waist. The basal region of the megaspore apparatus was spheroidal in shape (Fig.4.21).

###### 4.2.3.2 FLOATS (Surface view)

These appeared more or less triangular with the basal end somewhat rounded. However, some specimens bore floats that were

less tapered towards the apex (Fig.4.21). The surface puncturing was not unlike that of the *A.filiculoides* type, with irregularly shaped holes (ca 4um). However, these holes were often large (ca 7um) or almost indiscernible (Figs.4.21,4.22a). Float filosum was usually observed arising from the apical half and apical external surface of each float (Fig.4.21b). At the latter site the filaments intertwined with the filaments of the filosum funnel. The suprafilosum filaments were ca 0.6um in diameter. Supernumerary floats were very rarely observed in the *A.microphylla* type.

#### 4.2.3.3 COLLAR

In surface view the narrowest region was usually at the longitudinal mid-point of each float. The width gradually increased towards the cusp region. Very occasionally the collar was found to have distinctive narrow regions either side of the cusp (Fig.4.21d). The collar surface was relatively smooth with only small undulations and the occurrence of even a few filaments of filosum on the collar surface was rare (Fig.4.21). The lower edge of the collar was the lowest point of the flange, and it always 'met' the perine surface. Extending basally from this lower edge were inwardly curved spines which often elongated to form short filosum elements (Fig.4.22b & c). In sectional view the collar was at an angle of between 20° and 85° to the equatorial axis. More commonly, however, the angle was between 30° and 60° with the greater angles being found in the cusp region; here the sectional width of the collar was the smallest (Fig.4.22b-d). The origin of the flange was probably at its

lowest position on the collar in the *A.micropylla* type, being consistently found in the lower third. However, it was commonly in the proximal quarter of the collar in sectional view (Fig.4.2-2b-d). Consequently the groove was in its lowest position in this type when compared with all the other megaspore types.

#### 4.2.3.4 SPORODERM

Sculpturing - General appearance at low magnification under the scanning electron microscope was usually of a relatively smooth surface, albeit finely sculptured. However, shallow foveae, ca 30 $\mu$ m diameter, were often observed towards the basal megaspore region, and rarely just below the collar (Fig.4.2la). By re-orientating the megaspore apparatus the foveae were occasionally seen over the entire perine surface with the deepest foveae at the basal end of the megaspore apparatus. At high magnifications the sculpturing was rugulate-verrucate to reticulate with numerous irregularly arranged small foveae of varying size (Fig.4.23). Filaments of infrafilosum were occasionally seen emerging from these foveae (Fig.4.23f). The amount of infrafilosum varied considerably and, as in other megaspore types, obscured the sculpturing to variable degrees. Variation of the sculpturing at high magnification was evident even in a single specimen. In general the sculptural elements were more fused (and hence rugulate) just below the collar. Whereas towards the basal region of the megaspore the sculptural elements were usually less fused (and hence reticulate). This was more obvious in some specimens than others. At high magnification there appeared to be no differences in the sculpturing within the large foveae.

Structure - Sporoderm thickness in 26 populations measured was ca 21 $\mu$ m (range ca 17 $\mu$ m - 25 $\mu$ m) and was divided into three basic layers which are defined in section 3.1.1. The innermost layer, the exospore, was ca 4 $\mu$ m thick (range 3.4 $\mu$ m - 5.3 $\mu$ m). It appeared to be composed of dense material with radially elongated 'cavities'. On closer examination the exospore was structurally similar to other megaspore types, some specimens possessing apparently fused elements (foliations) while others possessed a dense matrix. There was no obvious increase in size or frequency of the 'cavities' towards the external surface of the exospore. The exospore surface was a meshwork of filaments which were in various states of fusion (Fig.4.24b).

The endoperine, which was ca 4 $\mu$ m thick (range ca 2.4 $\mu$ m - 5.3 $\mu$ m) and mostly uniformly thick. However, it was often seen to have localised thicker areas. This gave an undulating sectional outline to this layer. Occasionally towards the distal region intrusions of endoperine were observed (Fig.4.24a). In respect to its structure at high magnification, the endoperine was composed of closely packed minute rods, probably like those of the other megaspore types, by being simple and branched, and irregularly vermiciform. However, in the *A.microphylla* type the rods were almost indiscernible in most specimens. Endoperine alveolation of this type was the least of all the megaspore types, and the alveolae were the least rounded (Fig.4.24).

The exoperine was usually divided into three zones. The innermost zone, the exoperine 1, was similar to the exoperine 1 in

other megaspore types, being baculate and ca 1.5 $\mu$ m thick (range 0.8 $\mu$ m - 2.3 $\mu$ m). The bacula were often observed to fuse and form a basement layer for the exoperine 2 layer (Fig.4.24b). Variation was observed in this layer in respect to the degree of fusion of the elements. In some specimens the exoperine 2 comprised of irregularly arranged radial columellae, while in other specimens the columellae tended to fuse or ultimately were completely fused forming an almost solid exoperine 2; the only 'breaks' being occasional cavities (Fig.4.24c). It was 1 $\mu$ m thick. This variation was commonly observed in this study at the intra- and inter-population level, although the latter level was more common. Within a single specimen the exoperine 2 was often observed to thin considerably just below the collar (Fig.4.24d). Here the exoperine 2 was usually more or less solid and in order to describe the true sporoderm structure observations were made of the distal sporoderm area. Although thinning of the exoperine 2 was occasionally seen in other types it was most noticeable in the *A.microphylla* type. Variation in the thickness of the exoperine 2 gave rise to the shallow foveae as did variation in the thickness of the endoperine when the exoperine 2 was uniformly thick. Obvious intrusions of the endoperine were occasionally observed, and these gave rise to deep foveae on their flanks; these were only found in the distal sporoderm region (Fig.4.24a). Therefore, several structural causes of large foveae were found, but these could not be discerned from the sculpturing. The exoperine 3, the zone that gives rise to the sculpturing seen at high magnifications, was not always distinguishable in sectional view because it was

probably fused to a more or less solid exoperine 2. However, when the exoperine 2 was columellate or nearly so, an exoperine 3, with tangential elements, was distinguishable and ca 2 $\mu$ m thick (Fig.4.24a). When an exoperine 3 was distinct, its elements formed reticulate sculpturing, whereas a rugulate-verrucate sculpturing indicated that the exoperine 3 was not always distinct (in sectional view) from the exoperine 2.

#### 4.2.4 Azolla sp. type

Megaspore apparatus.

##### 4.2.4.1 GENERAL APPEARANCE

Megaspore apparatus was more or less ovoid with a mean length of ca 485 $\mu$ m (range 437 $\mu$ m - 543 $\mu$ m) and a mean equatorial diameter of ca 321 $\mu$ m (range 282 $\mu$ m - 341 $\mu$ m). The collar region was not distinct because it was almost invariably covered by filosum, a waist was therefore rarely observed. The basal region of a megaspore apparatus was usually spheroid in shape. These features are illustrated in Figure 4.25.

##### 4.2.4.2 FLOATS (Surface view)

These were usually sub-triangular, the angles of which were decidedly rounded. Float puncturing was rare in most populations. When it was present it was not unlike the puncturing in the *A.filiculoides* type with irregularly shaped holes (Figs.4.25, 4.27e). The surface ornamentation was unlike other megaspore types, being characteristically rugulate

verrucate or there were narrow vermiculate ridges on the float surface (Fig.4.27e & f). Filosum was always attached to the edges of the apical half and apical external surface of each float. The filaments of filosum were ca 1 mm in diameter and intertwined with the suprafilosum from the cusps and suprafilosum funnel (Fig.4.25). Supernumerary floats were occasionally observed in the *Azolla* sp.type.

#### 4.2.4.3 COLLAR

In surface view the collar was rarely discernible (Fig.4.25.). In a few specimens it was possible to see that the narrowest point of the collar was at the longitudinal mid-point of each float. The collar width gradually increased towards the cusp region, which was also covered by filosum. It was consistently found in this study that the filosum covering the collar came from beneath the floats and the collar surface itself. This filosum cascaded down on to the perine surface, and therefore usually obscured any waist that may have been present in the collar region (Fig.4.25.). In sectional view the collar was at an angle of ca 20° to 60° to the equatorial axis; this variation was occasionally seen between adjacent cusp regions. Associated with this was variation in the sectional width of the collar. In the cusp region the collar was relatively narrow, whereas in other regions it was relatively wide (Fig.4.26a-c). When the flange could be seen in sectional view it usually originated just below half way up the collar. However, it was very occasionally observed to originate in the lower quarter of the collar. In two populations from Holland the collar, in sectional view, was found

to be dissected and lacking a flange. However, only one specimen from each population was available for sectioning.

#### 4.2.4.4 SPORODERM

Sculpturing - General appearance, at low magnification under a scanning electron microscope, was usually of a 'hairy' surface with few to many steep sided foveae (Fig.4.27a-d). One population (NY11) appeared to have 'excrescences' which were almost completely obscured by infrafilosum. However, where there was less infrafilosum foveae were occasionally visible in a finely sculptured surface (Fig.4.27a-c). When a whole specimen had little infrafilosum it resembled the *A.mexicana* type (Fig.4.-25a). One population (B17) was found to resemble the *A.microphylla* type, with large foveae toward the base of the megasporangium in an otherwise relatively smooth perine surface. Under higher magnifications the sculpturing was also not unlike that of the *A.microphylla* type (Fig.4.27b). The surface of the perine in the *Azolla* sp.type varied from rugulate-verrucate to reticulate depending upon the degree of fusion of the surface perine elements. Occasionally these surface elements appeared to be vermiculate. Numerous irregularly arranged and sized small foveae were also present, often with filaments of infrafilosum emerging from them. The sculpturing was usually difficult to discern because of the large amounts of infrafilosum on the perine surface. Amongst the filaments of infrafilosum, which were ca 1  $\mu$ m in diameter, large steep sided foveae were seen (Fig.4.27a-d). Where there was less infrafilosum, some of these foveae were found to be lined with sculptural elements

described above; such specimens resembled the *A.mexicana* type. The considerable variation found during this investigation, in the sculpturing of the *Azolla* sp.type was usually at the inter-population level.

Structure - Mean sporoderm thickness in 12 populations measured was ca 30 $\mu$ m (range ca 16 $\mu$ m - 52 $\mu$ m). This range included those specimens with excrescences which were up to ca 52 $\mu$ m). The sporoderm was divided into three basic layers which are defined in section 3.1.1. The innermost layer, the exospore, was ca 4 $\mu$ m thick. Under the scanning electron microscope it appeared to be composed of a dense material with a few to many radially elongated cavities. Therefore, the exospore structure was essentially similar to that of other megaspore apparatus types (Fig.4.28c). The external surface of the exospore was found to be a meshwork of fine filaments, however, some fusion of these filaments was observed in the specimens examined; fusion was more common than in the *A.mexicana* type (Fig.4.28d).

The endoperine was composed of usually closely packed minute rods, which were shortly vermiform (Fig.4.28c). The *Azolla* sp.type exhibited considerable variation in the density of these rods. In one population (B17), considerable variation was observed within one specimen (Fig.4.28d) giving rise to an apparently two zoned endoperine. The minute endoperine rods were found to be simple and branched and irregularly vermiform in the outer zone. In the inner zone, the rods were somewhat columellate, but were also branched and irregular (Fig.4.28d).

Close examination revealed that this zone was firmly attached to the exospore and the 'outer' endoperine zone, and in the proximal sporoderm the inner endoperine zone was less distinct. This endoperine structure was unique to population B17.

Compared with high magnifications, endoperine structure at low magnifications was even more variable. In some populations variation ranged from no alveolae to a few large isolated alveolae and a few isolated groups of large alveolae; the large alveolae causing the endoperine to intrude into the exoperine. Other populations possessed a few small endo~~perine~~ alveolae while specimens of the same population possessed highly alveolate endoperine (Figs.4.28,4.29). Very occasionally this range of variation was approached in a single specimen. The highly alveolate condition (Fig.4.28a-c) appeared to be more common, and one population sampled was found to have only this type of endoperine. In addition to this considerable variation, the endoperine intruded into the exoperine. These intrusions often had larger vacuoles than the surrounding endoperine (Figs.4.28b & e,4.29d). Several populations were found to have massive excrescences which resembled those of some Australian populations of the *A.filiculoides* type (i.e. without large vacuoles) (Fig.4.-28e). The endoperine in the *Azolla* sp.type exhibited the greatest variation of all the megasporangium types, and some of this variation was at the intra-population level. Endoperine thickness ranged from ca 4 $\mu$ m upto ca 38 $\mu$ m in an excrescence.

The exoperine was ca 12 $\mu$ m thick (range ca 8 $\mu$ m - 16 $\mu$ m) and could

often be divided into three zones. The exoperine 1 was always distinguishable except at the apex of an excrescence (cf. the *A.filiculoides* type). This zone was, as in the other megaspore types, composed of baculate radially arranged elements and was ca 2 $\mu$ m thick. The tips of the bacula tended to fuse thereby forming the basement 'layer' of the exoperine 2 (Figs.4.28,4.29). This latter zone was ca 8 $\mu$ m thick (range 3.8 $\mu$ m - 13.3 $\mu$ m). The exoperine 2 was commonly columellate or nearly so. However, the columellae were not always clearly radial in their orientation. Furthermore, they tended to fuse, forming a more solid exoperine 2 (Figs.4.28,4.29); this was not unlike the same zone in the *A.microphylla* type. In one population (B48), which had excrescences, all the exoperine 2 was solid except where it became baculate at the apex of an excrescence (cf. the *A.filiculoides* type). Further variation in the exoperine 2 was found where the endoperine was highly alveolate. In such specimens the exoperine (s.l.) was consistently very thin compared with other specimens. This study revealed that variation in the thickness of the exoperine 2 zone often reduced or cancelled out the sculptural expression of the excrescences; the exoperine 2 often filled the depression regions (Figs.4.28a & b,4.29d). Alternatively, variation of exoperine thickness created the foveae observed in the sculpturing in the absence of an endoperine intrusion (Fig.4.29a). In addition to this, a raised area may be formed by a combination of endoperine intrusion and variation in the exoperine thickness. Steep sided foveae observed in the sculpturing were the thin areas of the perine and may be formed between two closely located endoperine intrusions or by small areas of

short exoperine 2 columellae (i.e. small areas of thin exoperine 2) (Figs.4.28,4.29). Shallow sided foveae were formed where two intrusions were relatively far apart, as in the *A.mexicana* type (Fig.4.29a). Therefore, there were several structural causes of the sculptural features observed. By studying sporoderm structure it was possible to show that infrafilosum reduced or covered some foveae (Figs.4.28e,4.29a). Towards the external surface of the exoperine 2, its elements divided and formed tangentially orientated elements of the exoperine 3, which were ca 2 $\mu$ m thick. This layer formed the rugulate-reticulate to vermiciform sculpturing that was described previously. Where the exoperine 2 columellae were more or less fused (i.e. + solid) and/or was thin, an exoperine 3 was not discernible (Figs.4.28d,4.29a). In such specimens the exoperine surface may have been rugulate to verrucate. Variation in the degree of fusion of the exoperine 2 columellae was commonly observed within one specimen of a population. Furthermore, the exoperine often, and occasionally the endoperine, became thinner towards the collar region, and like the *A.microphylla* type, the exoperine 2 was usually solid in this region. This study also revealed that excrescences were more likely to be found and were larger in the basal region of the megaspore apparatus. Attempts were made to correlate the variation in the sporoderm structure with other characters and geographical distribution. However, they met with little success, although, specimens with more alveolate endoperine and associated structural features were commonly found in central and eastern regions of the USA where the distribution of the *A.filiculoides* and *A.mexicana* types overlapped. Other such populations

were found in Holland. All other populations were collected from Central and South America.

#### 4.3

##### CHARACTERS ASSOCIATED WITH SPOROCARPS

Having described and established the megaspore apparatus types, it was possible to delimit the variation of other characters for each of the types. The usefulness of these characters may then be evaluated by assessing their degree of overlap with those of other types. This next section describes the variation in characters other than megaspore apparatus characters and the evaluation of their usefulness.

#### 4.3.1

##### Megasporocarps and microsporocarps

These occurred in pairs on the ventral surface of a frond at a branch point. The pair appeared to replace the ventral leaf lobe of the first branch leaf with the often shrouding 'involucr' appearing to be a downward extension of the dorsal leaf lobe. A pair of sporocarps comprised two megasporocarps, two microsporocarps or one of each. Although not quantified the ratio of mega- to microsporocarps appeared variable in all the megaspore types. Some populations produced mainly one type of sporocarp, while others had produced apparently equal numbers of each type. It was interesting to note that the majority of Californian populations attributed to the *A.filiculoides* type produced mainly microsporocarps in great abundance. There appeared to be no common factor, except geographical location, that linked these populations.

Megasporocarps were distinguishable from microsporocarps by their relatively small size (ca 0.8mm long and ca 0.4mm diameter) and ovoid shape, whereas the microsporocarps were spherical and ca 2.5mm diameter. The cells comprising the megasporocarp wall were probably tabular when fresh. Towards the base they were fragile, but towards the apex they were brown and highly thickened forming the indusial cap. Although no formal dehiscence line was apparent, the wall always split equatorially in the collar region of the megaspore apparatus. Megasporocarps appeared similar in all megaspore types.

#### 4.3.2 Microsporocarps

Microsporocarp size was difficult to measure in herbarium material because deformation had occurred during drying. However, although no data are available, there appeared to be no difference between the types. The shape of microsporocarps was usually spherical but very occasionally ovoid shapes were observed in the *A.filiculoides*, *A.microphylla* and *Azolla* sp.type-s. There were no differences between the types in respect to shape of the cells comprising the microsporocarp wall which was two celled thick; cells of the outer layer being tabular.

#### 4.3.3 Microsporangia

The sorus was clearly seen to be gradate in fresh and herbarium specimens. Number of microsporangia per microsporocarp varied from few to many in all types. The gradate habit and deformation during drying made recording measurements of microsporangium size unreliable. However, this character appeared equally

variable in all the megaspore types. No annulus was observed in any type and the microsporangial wall cells appeared similar in all types; i.e. tabular and more or less isodiametric in surface view.

#### 4.3.4 Massulae

The number of massulae per microsporangium varied from 2 to 11 across all the types. When the mean number for each population was considered for each type the range showed considerable overlap (Table 4.1, Fig. 4.31). It would appear that the mean of the population means for the American and European *A.filiculoides* type was significantly different from the *A.mexicana*, *A.microphylla* and Australian *A.filiculoides* types (Table 4.2). When rounded to the nearest whole number, the mean number of massulae per microsporangium was 5 for each megaspore type. The shape and size of massulae appeared to vary with the number of massulae per microsporangium. It was consistently observed that when four massulae occupied a microsporangium the massulae were usually equal in size. However, when there were more than four massulae per microsporangium at least one massula was smaller than the others.

TABLE 4.1

MEGASPORE APPARATUS TYPE	MEAN	MIN - MAX	S <sup>2</sup>	n
<u>A.filiculoides</u> (f)	5.30	3.80 - 6.55	0.51	19
<u>A.filiculoides</u> (r)	4.60	3.20 - 5.80	0.32	24
<u>A.mexicana</u>	4.60	3.25 - 6.15	0.74	13
<u>A.microphylla</u>	4.70	3.15 - 7.40	1.51	18
<u>Azolla</u> sp.	4.80	3.35 - 6.63	0.83	11

Table to show the mean ( $\bar{x}$ ), minimum, maximum, variance ( $S^2$ ) of the number of massulae per microsporangium for each megaspor type. n = number of populations sampled; (r) = Australian populations; (f) = non-Australian populations.

TABLE 4.2

COMPARISON IN t-TEST		COMPARISON IN t-TEST	
<u>A.filiculoides</u> (f) v <u>A.filiculoides</u> (r)	X	<u>A.filiculoides</u> (r) v <u>A.microphylla</u>	✓
<u>A.filiculoides</u> (f) v <u>A.mexicana</u>	X	<u>A.filiculoides</u> (r) v <u>Azolla</u> sp.	✓
<u>A.filiculoides</u> (f) v <u>A.microphylla</u>	X	<u>A.mexicana</u> v <u>A.microphylla</u>	✓
<u>A.filiculoides</u> (f) v <u>Azolla</u> sp.	✓	<u>A.mexicana</u> v <u>Azolla</u> sp.	✓
<u>A.filiculoides</u> (r) v <u>A.mexicana</u>	✓	<u>A.microphylla</u> v <u>Azolla</u> sp.	✓

Table to show comparisons made using the t-Test and whether there was a significant difference between the megaspor types in respect to the mean number of massulae per microsporangium.

X = significant difference ( $p > 95\%$ ); ✓ = no significant difference ( $p > 95\%$ ); (r) and (f) = see Table 4.1

A character found to be correlated with the number of massulae per microsporangium was the number of microspores per massula.

Figure 4.31b illustrates that the more massulae there were per microsporangium the fewer microspores were present in each

massula; the corrected r value of regression = 0.682. Furthermore, the mean number of massulae per microsporangium was 4.8 and the mean number of microspores per massula was 13.4. By multiplication, the mean number of microspores per microsporangium was therefore 64.3 (= 64).

Regardless of number, each massula in a microsporangium had one surface in contact with the microsporangial wall. This surface was termed the 'external surface'; and other surface(s) in contact with adjacent massulae were termed the 'internal surface s'. In the *A.mexicana* type the sculpturing of both surfaces was usually of obvious ovoid depressions, which were often disfigured by drying (Fig.4.30b). There was no apparent difference between the other types which possessed smooth surfaces or they had very shallow depressions. Disfiguration through drying prevents further description here; any descriptions being of desiccation artefacts. However, within the *A.filiculoides* type, Australian populations only commonly possessed short blunt processes which were always present on the external surface, and occasionally on the internal surface (Fig.4.30d & e). Long processes emanating from the massula surfaces were called glochidia. On the external surface these were often observed in distinct groups towards the periphery of the surface and in the central region (Fig.4.30b). On the internal surface glochidia were usually located in a more or less central position where all the massulae would be in contact with each other within the microsporangium (Fig.4.30a & c).

## 4.3.5

Glochidia

The glochidium presents a potential wealth of characters, and these were studied in this investigation. Shape of the glochidial shaft was scored for each population in respect to dominant and subordinate shape; these being described from basal and apical tapering of the shaft as seen under the light microscope (see Definitions section 3.1.3). Table 4.3 shows the results for this character which appeared to overlap between the types. In the *A.filiculoides* type the dominant tapering was clearly basal and apical (B-A). Australian populations of this type generally had less pronounced B-A tapering compared with other populations of this type and only two populations had parallel sided glochidial shafts (PARA); again only found in Australian populations. The *A.microphylla* type also had predominantly B-A tapering which may be very pronounced. However, in a few populations this tended to be PARA. Apical tapering was observed as a subordinate state in only two populations of the *A.microphylla* type. Although tapering in the *Azolla* sp. type was usually B-A, it was not at all pronounced and tended towards the parallel condition. Only two populations of this type exhibited apical tapering. The *A.mexicana* type exhibited the greatest variation in respect to glochidial shape, with B-A, PARA and apical tapering being observed. However, the former state was most common (Table 4.3). Figure 4.32 illustrates some of the features described above.

Table 4.4 to 4.8 and Figure 4.33 illustrate the results for glochidial length (the minima and maxima are population means).

When all the populations of each type were considered the range of glochidial length was 51um to 171um (*A.filiculoides* type), 51um to 190um (*A.mexicana* type), 47um to 122um (*A.microphylla* type) and 29um to 87um (*Azolla* sp. type). The range of all the population means was 112um while the range of the means between the types was only 26.3um. Despite an overlap between the types in the ranges of mean glochidial length, the *Azolla* sp. type generally possessed the shortest glochidia, whereas the *A.mexicana* type often possessed the longest glochidia. This latter type also had the largest range of lengths and the *A.microphylla* type had the smallest range. However, when Australian populations of the *A.filiculoides* type were considered separately, the variation in the Australian populations was over twice that of other populations of this type. These 'other populations' having the least variation of all the types (Table 4.4., Fig.4.33). The variance and skewness of the data suggested that little weight should be attached to significances obtained from the t-Test. However, it is interesting to note that at the 5% level, the mean glochidial lengths of the types were different except the *A.filiculoides* and *A.mexicana* types, and the *A.microphylla* and *Azolla* sp. types (Table 4.5). The similarity between the former pair appeared to be caused by the Australian populations of the *A.filiculoides* type. When considered separately, these Australian populations were similar to the *A.mexicana* type; the other populations of the *A.filiculoides* type being significantly different (Table 4.6). A visual comparison of the mean variances of glochidial length for each type indicated that the *A.mexicana* type was very different from the other types (Table 4.7).

However, the t-Test contradicted this, indicating that there was no significant difference between the *A.filiculoides* and *A.mexicana* types (Table 4.8). This further suggested that very little weight can be placed on differences and similarities indicated by the t-Test on population variances of glochidial length.

However, the mean variance was interesting when compared with the mean glochidial length of each type. In all types, except the *A.mexicana* type, the ratio of mean length to mean variance approximated to one. In the *A.mexicana* type this ratio was ca 0.5. The ratio of variances about the two aforementioned means gave an indication of intra-type variability of glochidial length; this increased from the *Azolla* sp. to *A.microphylla* to *A.filiculoides* to *A.mexicana* types. However, the increasing order of variance, which was an indication of inter-population variability, was *A.microphylla*, *A.filiculoides*, *Azolla* sp. and *A.mexicana*. Therefore, intra-type and inter-population variability showed some independance indicating that the sampling method was sufficient to detect differences.

Another feature of glochidia investigated was presence or absence of septa within the glochidial vacuole. Results of the number and predominant position of these septa are summarised in Tables 4.9 - 12 and Figures 4.34 - 4.39 (for definitions see section 3.1.3; population data are presented in Appendix IV). For each population the mean number of septa was determined and used to determine the mean range of septation. The range (not mean range) was the minimum and maximum number of septa per glochidium from populations of the types. The grand mean number of septa

was determined from the population means. The histograms were compiled by determining the mean number of glochidia with a particular number (class) of septa for each type. Therefore, Figures 4.34a, 4.35a, 4.38a & 4.39a are histograms of mean frequency of the number of septa per glochidium for each type. A comparison of these Figures indicated that only the *A.filiculoides* type may be different from the other three types. The ranges, mean ranges and means for each of the four types are given in Table 4.9.

The *A.filiculoides* type had a range of septation (i.e. range of the number septa per glochidium) of 0 to 12 (mean range of 0.02 to 7.19). The grand mean was 1.44 (1 rounded to the nearest whole number). However, the mode class of septa was 0. Within the distribution represented by Figure 4.34b there appeared to be two groups of populations (called here sub-types). These two sub-types can be correlated with their biogeography. One sub-type had a range of 0 to 6 (mean range of 0.02 to 2.17) septa and a grand mean of 0.47 (Table 4.9). In addition, the septa of this sub-type were consistently observed in the apical region of the glochidial shaft (Table 4.12, Fig. 4.32a). This sub-type was always found in Europe and in North and South America. The other sub-type was only found in Australia and New Zealand. This sub-type had a range of 0 to 12 septa (mean range 0.65 to 7.90) and a grand mean of 2.42, (or 2 rounded to the nearest whole number) (Table 4.9). Septa in the Australian and New Zealand sub-type were located in all positions in the glochidial vacuole. However, it was found that when there were few septa in a

glochidium, the septa were usually apical. In general, the more septa found in a glochidium the more likely they were to be uniformly distributed (Table 4.12). Although the two sub-types within the *A.filiculoides* type had an overlapping distribution in respect to the number of septa, the t-Test (using the population mean number of septa) indicated that they were significantly different (Fig.4.34b, Table 4.11). It was possible to correlate differences in the sub-types with biogeography and, as already shown, sporoderm structure.

The *A.mexicana* type had a range of 0 to 12 septa (mean range 0.26 to 8.7) with a grand mean of 3.78 (or 4 rounded to the nearest whole number) (Table 4.9). However, Figure 4.35b shows that the mode class of septa was 3. Within the *A.mexicana* type there appeared to be two sub-types which can only be loosely correlated with biogeography. It was found that, with the exception of four, all populations studied from the western seaboard of the U.S.A., Canada and South America had a mean number of septa less than 3.00. Those populations with a mean greater than 3.00 were from central eastern U.S.A., with the exception of three populations (Fig.4.35b). Histograms illustrating the septation of these two sub-types within the *A.mexicana* type are shown in Figure 4.36b. The range of one sub-type was 0 to 6 septa (mean range 0.26 to 2.77) with a grand mean of 1.91 (or 2 rounded to the nearest whole number). The other sub-type had a range of 1 to 12 septa (mean range 3.23 to 8.67) and a grand mean of 5.80 (or 6 rounded to the nearest whole number) (Table 4.9). As in the two sub-types of the *A.filiculoides* type, the mode class of

septa was also the mean class when two sub-types were considered. The t-Test suggested there was a significant difference between these two sub-types despite there being an overlap between them in respect to septation (Fig. 4.36a Table 4.11). (The t-Test was performed on the mean number of septa in each population). Attempts were made to further sub-divide the *A.mexicana* type into three or four sub-types based on septation (Figs. 4.36b, 4.37). However, the 'correlation' with geographical distribution was not improved. This indicated variation in septation of the *A.mexicana* type was best accounted for by two geographical subtypes. It was not possible to correlate septation differences with any other character except geographical distribution.

The predominant position of septa in the glochidial vacuole of the *A.mexicana* type was uniformly distributed. However, if there were only a few septa in any one glochidium the septa were usually apical, with one or two basal septa (Table 4.12).

The *A.microphylla* type had a range of 0 to 10 septa (mean range 0.04 to 6.90) with a grand mean of 3.39 (or 3 rounded to the nearest whole number). Unlike the *A.filiculoides* and *A.mexicana* types the mode class of septa per glochidium was also the mean (Fig. 4.38a). Although Figure 4.38b indicated two sub-types based upon those populations with at least some aseptate glochidia and those with all septate glochidia, their distributions were highly overlapping. Attempts to correlate these two 'sub-types' with other characters (including geographical distribution) were unsuccessful. The predominant position of septa in the *A.microphylla* type was apical and basal

(i.e. one or more apical with one or two basal septa). However, where there were many septa they were often uniformly distributed in the glochidial vacuole (Table 4.12).

The *Azolla* sp. type had a range of 0 to 15 septa (mean range 0.59 to 5.21) and a grand mean of 2.73 (or 3 rounded to the nearest whole number). As indicated by the standard error of the mean ( $SE\bar{x}$ ), this type exhibited the greatest variation in respect to septation (Table 4.9); this was also illustrated in Figure 4.39a. Two populations of the *Azolla* sp. type from Holland had highly irregular septa which were difficult to define and count (Fig.4.32h). Despite this, these populations did have more septa per glochidium than their counterparts from North and South America. Figure 4.39b illustrates the septation differences between the populations from Holland and those from other regions. There appeared to be no such differences between the populations from North America and South America; this was also indicated by the t-Test. The predominant position of septa in the *Azolla* sp. type was apical where there were few septa, and apical and basal as well as uniform, where there were many septa per glochidium (Table 4.12). The *Azolla* sp. type often possessed a typical septa in that they were wide, giving the appearance that the glochidial vacuole was compartmented (Fig.4.32h). With the indication that there were differences in septation of the *A.filiculoides* and *A.mexicana* types the t-Test was used to compare the types and sub-types; the mean number of septa for each population was used. The *A.filiculoides* type was different from all the other three types. However, the *A.mexicana*,

*A.microphylla* and *Azolla* sp. type were all similar (Table 4.10). This supports the indication from the histograms (Fig.4.34a,4.35-a,4.37a,4.38a,4.39a). When the sub-types were included in the t-Test the following differences and similarities were indicated (see Table 4.9 for sub-type symbols).

The sub-types *A.filiculoides* (f) and *A.mexicana* (+3) were different from all other types and sub-types (Table 4.11). However, the sub-type *A.filiculoides* (r) was only different from aforementioned sub-types and the *A.microphylla* type. The sub-type *A.mexicana* (-3) was different from the *A.filiculoides* (f) and *A.mexicana* (+3) sub-types and the *A.microphylla* type. The *A.microphylla* type was different to all the sub-types and was only similar to the *Azolla* sp. type (Table 4.11). However, this latter type was different from all sub-types except the *A.filiculoides* (r) and *A.mexicana* (-3) sub-types.

The last glochidial feature to be investigated was the predominant shape of the apex of the glochidia. In all types the glochidial apex was usually rounded. All the populations of the *A.filiculoides* type tended towards having obtuse glochidial apices. In the *A.mexicana* and *Azolla* sp. types the glochidial apices were usually rounded, with a few populations of each possessing acute apices.

TABLE 4.3

MEGASPORE APPARATUS TYPE	n	B-A	PARA	A
<u>A.filiculoides(f)</u>	28	28	-	-
<u>A.filiculoides(r)</u>	26	24	2	-
<u>A.mexicana</u>	28	15*	10*	5*
<u>A.microphylla</u>	23	19*	4*	2*
<u>Azolla</u> sp.	10	7*	3*	2*

Table to show the shape of the glochidial shaft in each megasporous apparatus type.

n = number of populations sampled; B-A = basal and apical tapering;

PARA = parallel sided glochidial shaft; A = apical tapering;

\* = tied scores; (f) and (r) see Table 4.1

TABLE 4.4

MEGASPORE APPARATUS TYPE	$\bar{x}$	min - max	SE $\bar{x}$	$S^2$	n	Populations min - max
<u>A.filiculoides(f)</u>	81.56	69.9- 94.5	1.06	35.1	32	51 - 120
<u>A.filiculoides(r)</u>	93.46	76.7-119.7	2.11	93.5	22	57 - 171
<u>A.mexicana</u>	90.95	61.9-147.0	4.13	340.3	21	51 - 190
<u>A.microphylla</u>	72.76	56.6-90.3	1.62	52.3	21	47 - 122
<u>Azolla</u> sp.	64.56	35.0-74.6	3.77	127.6	16	29 - 87

Table to show mean ( $\bar{x}$ ) population mean minima and maxima, standard error (SE $\bar{x}$ ) and variance ( $S^2$ ) of glochidial length (measured in  $\mu\text{m}$ ) for each megasporous apparatus type. The minima and maxima from populations also shown.

n = number of populations sampled; (r) and (f) see Table 4.1.

TABLE 4.5

COMPARISON IN t-TEST		COMPARISON IN t-TEST	
<u>A.filiculoides</u> v <u>A.mexicana</u>	✓	<u>A.mexicana</u> v <u>A.microphylla</u>	X
<u>A.filiculoides</u> v <u>A.microphylla</u>	X	<u>A.mexicana</u> v <u>Azolla</u> sp.	X
<u>A.filiculoides</u> v <u>Azolla</u> sp.	X	<u>A.microphylla</u> v <u>Azolla</u> sp.	✓

As Table 4.6 with A.filiculoides(f) and A.filiculoides(r) combined.

TABLE 4.6

COMPARISON IN t-TEST		COMPARISON IN t-TEST	
<u>A.filiculoides</u> (f) v <u>A.filiculoides</u> (r)	X	<u>A.filiculoides</u> (r) v <u>A.microphylla</u>	X
<u>A.filiculoides</u> (f) v <u>A.mexicana</u>	X	<u>A.filiculoides</u> (r) v <u>Azolla</u> sp.	X
<u>A.filiculoides</u> (f) v <u>A.microphylla</u>	X	<u>A.mexicana</u> v <u>A.microphylla</u>	X
<u>A.filiculoides</u> (f) v <u>Azolla</u> sp.	X	<u>A.mexicana</u> v <u>Azolla</u> sp.	X
<u>A.filiculoides</u> (r) v <u>A.mexicana</u>	✓	<u>A.microphylla</u> v <u>Azolla</u> sp.	✓

Table to show comparisons made using the t-Test and whether there was a significant difference between the megasporule apparatus types in respect to mean length of glochidia.

X = significant difference ( $p > 5\%$ )

✓ = no significant difference ( $p > 95\%$ )

TABLE 4.7

MEGASPORE APPARATUS TYPES	$\bar{x}$	min-max	SE $\bar{x}$	$s^2$	n
<u>A.filiculoides(f)</u>	69.36	38.9-176.3	9.3	1216.2	15
<u>A.filiculoides(r)</u>	91.58	35.3-331.0	13.1	3615.7	22
<u>A.mexicana</u>	201.93	70.1-445.2	57.4	19000.0	7
<u>A.microphylla</u>	76.97	56.4-106.4	15.1	454.6	3
<u>Azolla</u> sp.	50.28	23.6-61.7	5.7	162.7	6

Table to show the mean ( $\bar{x}$ ), range (min-max), standard error (SE $\bar{x}$ ) and variance ( $s^2$ ) of the variances of glochidial length for each megaspore apparatus type.

n = number of populations sampled; (r) and (f) see Table 4.1

TABLE 4.8

COMPARISON IN t-Test	COMPARISON IN t-Test		
<u>A.filiculoides</u> v <u>A.mexicana</u>	✓	<u>A.mexicana</u> v <u>A.microphylla</u>	X
<u>A.filiculoides</u> v <u>A.microphylla</u>	✓	<u>A.mexicana</u> v <u>Azolla</u> sp.	X
<u>A.filiculoides</u> v <u>Azolla</u> sp.	✓	<u>A.microphylla</u> v <u>Azolla</u> sp.	✓

Table to show comparisons made using the t-Test, and whether there was a significant difference between the megaspore apparatus types in respect to the variances of glochidial length.

X = significant difference ( $p < 5\%$ ); ✓ = no significant difference ( $p > 95\%$ ).

TABLE 4.9

MEGASPORE APPARATUS TYPES	$\bar{x}$	range	SE $\bar{x}$	$S^2$	n	POPULATIONS min-max	Grand	ROUNDED Grand
<u>A.filiculoides(f)</u>	0.47	0.02-2.17	0.076	0.21	37	0 - 6		
<u>A.filiculoides(r)</u>	2.42	0.65-7.90	0.264	1.74	26	0 - 12	1.44	1
<u>A.mexicana(+3)</u>	5.80	3.23-8.67	0.436	2.09	12	1 - 12		
<u>A.mexicana(-3)</u>	1.91	0.26-2.77	0.242	0.71	13	0 - 6	3.78	4
<u>A.microphylla</u>	3.39	0.04-6.90	0.337	2.38	22	0 - 10	3.39	3
<u>Azolla</u> sp.	2.73	0.59-5.21	0.475	2.03	10	0 - 15	2.73	3

Table to show mean ( $\bar{x}$ ) mean range, standard error (SE $\bar{x}$ ) and variance ( $S^2$ ) for each megaspore apparatus type in respect to glochidial septation. Population ranges also shown. The grand $\bar{x}$  represents combination of the groups within a megaspore apparatus type. This grand $\bar{x}$  is rounded to the nearest whole number.

The A.filiculoides and A.mexicana types are each divided into two groups (f) and (r) see Table 4.1; (+3) and (-3) populations with mean number of septa greater and less than 3 respectively.

TABLE 4.10

COMPARISON IN t-Test		COMPARISON IN t-Test	
<u>A.filiculoides</u> v <u>A.mexicana</u>	X	<u>A.mexicana</u> v <u>A.microphylla</u>	✓
<u>A.filiculoides</u> v <u>A.microphylla</u>	X	<u>A.mexicana</u> v <u>Azolla</u> sp.	✓
<u>A.filiculoides</u> v <u>Azolla</u> sp.	X	<u>A.microphylla</u> v <u>Azolla</u> sp.	✓

Table to show comparisons made using the t-Test and whether there was a significant difference between the megasporule apparatus types in respect to glochidial septation.

X = significant difference ( $p > 5\%$ ); ✓ = no significant difference ( $p > 95\%$ ).

TABLE 4.11

COMPARISON IN t-Test		COMPARISON IN t-Test	
<u>A.filiculoides(f)</u> v <u>A.filiculoides(r)</u>	X	<u>A.filiculoides(r)</u> v <u>A.microphylla</u>	X
<u>A.filiculoides(f)</u> v <u>A.mexicana</u> (+3)	X	<u>A.filiculoides(r)</u> v <u>Azolla</u> sp.	✓
<u>A.filiculoides(f)</u> v <u>A.mexicana</u> (-3)	X	<u>A.mexicana</u> (+3) v <u>A.mexicana</u> (-3)	X
<u>A.filiculoides(f)</u> v <u>A.microphylla</u>	X	<u>A.mexicana</u> (+3) v <u>A.microphylla</u>	X
<u>A.filiculoides(f)</u> v <u>Azolla</u> sp.	X	<u>A.mexicana</u> (+3) v <u>Azolla</u> sp.	X
<u>A.filiculoides(r)</u> v <u>A.mexicana</u> (+3)	X	<u>A.mexicana</u> (-3) v <u>A.microphylla</u>	X
<u>A.filiculoides(r)</u> v <u>A.mexicana</u> (-3)	✓	<u>A.mexicana</u> (-3) v <u>Azolla</u> sp.	✓
		<u>A.microphylla</u> v <u>Azolla</u> sp.	✓

Table to show comparisons made using the t-Test and whether there was a significant difference between the megasporule apparatus types in respect to glochidial septation.

The A.filiculoides and A.mexicana types are each divided into two groups.

(f) and (r) see Table 4.1; (+3) and (-3) see Table 4.9; X = significant difference ( $p > 5\%$ ); ✓ = no significant difference ( $p > 95\%$ ).

TABLE 4.12

MEGASPORE APPARATUS TYPES	POSITION OF SEPTA IN GLOCHIDIAL SHAFT						
	A	A-M	M-B	M	B	u	n
<u>A.filiculoides</u> (f)	28	-	-	-	-	-	28
<u>A.filiculoides</u> (r)	14	11	1	5	1	3	26
<u>A.mexicana</u>	9	4	1	2	8	22	28
<u>A.microphylla</u>	15	2	1	-	15	9	23
<u>Azolla</u> sp.	9	1	-	3	6	7	10

Table to show the position of septa within the glochidial shaft for each megasporae apparatus type. Scores are based on the number of populations having a particular character state. It is possible for a population to possess more than one character state.

(f) and (r) see Table 4.1; A = apical; M = median; B = basal; u = uniformly distributed; A-M = apical to median; M-B = median to basal; n = number of populations sampled.

For definition of positions see Section 3

TABLE 4.13

MEGASPORE APPARATUS TYPES	HABIT	SHAPE	BRANCHING	n
<u>A.filiculoides</u> (f)	H - V	R-D-ED	ID-AD-SP	+80
<u>A.filiculoides</u> (r)	H - V	R-D-ED	ID-AD	
<u>A.mexicana</u>	H	R	ID	+30
<u>A.microphylla</u>	H (V)	R-D	ID-AD-SP	+30
<u>Azolla</u> sp.	H	R (D)	ID-AD	+12

Table to show frond habit, shape and branching in the megasporangium apparatus types.

H = horizontal; V = vertical; R = rounded; D = deltoid;

ED = elongated deltoid; ID = isotomous dichotomy

AD = anisotomous dichotomy; SP = sub-pinnate; n = number of populations sampled; ( ) = uncommon character state;

(r) = Australian populations; (f) = non-Australian populations.

TABLE 4.14

MEGASPORE APPARATUS TYPES	TIP SHAPE	LOBE LENGTH (um)			LOBE WIDTH (um)			L:W	n
		$\bar{x}$	min-max	SE $\bar{x}$	$\bar{x}$	min-max	SE $\bar{x}$		
<i>A. filiculoides</i> (F)	R-SA	963.0	698-1260	94.8	747.7	522-1120	90.8	1.31	6
<i>A. filiculoides</i> (R)	R-SA	1424.7	864-2065	113.7	1237.0	906-1655	75.3	1.14	13
<i>A. filiculoides</i> (S)	R-SA	935.0	650-1182	58.85	848.2	513-1107	58.9	1.11	10
<i>A. mexicana</i>	R-SA-A	982.9	806-1192	42.4	810.9	622-1128	47.8	1.23	10
<i>A. microphylla</i>	R-SA	1018.8	864-1184	26.8	826.8	656-944	27.4	1.24	10
<i>Azolla</i> sp.	R-A	940.1	602-1268	67.5	734.9	308-997	61.8	1.32	10
<i>A. filiculoides</i> (F)(R)(S)*	R-SA	1160.3	650-2065	72.3	1001.7	513-1655	58.6	1.17	29

Table of simplified data of dorsal leaf lobe characters for each megasporangium apparatus type.

$\bar{x}$  = mean; SE $\bar{x}$  = standard error; L:W = length to width ratio; n = number of populations sampled; (F) = fertile non-Australian populations; (S) = sterile non-Australian populations; (R) = Australian populations; \* = (F)(R) and (S) combined; R = rounded; SA = sub-acute; A = acute.

TABLE 4.15

COMPARISON	t-Test		COMPARISON	t-Test	
	L	W		L	W
<u>A.filiculoides</u> v <u>A.mexicana</u>	X	X	<u>A.mexicana</u> v <u>A.microphylla</u>	✓	✓
<u>A.filiculoides</u> v <u>A.microphylla</u>	X	X	<u>A.mexicana</u> v <u>Azolla</u> sp.	✓	✓
<u>A.filiculoides</u> v <u>Azolla</u> sp.	X	X	<u>A.microphylla</u> v <u>Azolla</u> sp.	✓	✓

Table to show comparisons made using the t-Test and whether there was a significant difference between the megasporangium apparatus types in respect to dorsal leaf lobe mean length and mean width.

The A.filiculoides(F)(R) and (S) groups as in Table 4.14 are combined.

X = significant difference ( $p > 5\%$ ); ✓ = no significant difference ( $p > 95\%$ ).

TABLE 4.16

COMPARISON	t-Test		COMPARISON	t-Test	
	L	W		L	W
<u>A.filiculoides</u> (F) v <u>A.filiculoides</u> (R)	X	X	<u>A.filiculoides</u> (R) v <u>A.microphylla</u>	X	X
<u>A.filiculoides</u> (F) v <u>A.filiculoides</u> (S)	✓	✓	<u>A.filiculoides</u> (R) v <u>Azolla</u> sp.	X	X
<u>A.filiculoides</u> (F) v <u>A.mexicana</u>	✓	✓	<u>A.filiculoides</u> (S) v <u>A.mexicana</u>	✓	✓
<u>A.filiculoides</u> (F) v <u>A.microphylla</u>	✓	✓	<u>A.filiculoides</u> (S) v <u>A.microphylla</u>	✓	✓
<u>A.filiculoides</u> (F) v <u>Azolla</u> sp.	✓	✓	<u>A.filiculoides</u> (S) v <u>Azolla</u> sp.	✓	✓
<u>A.filiculoides</u> (R) v <u>A.filiculoides</u> (S)	X	X	<u>A.mexicana</u> v <u>A.microphylla</u>	✓	✓
<u>A.filiculoides</u> (R) v <u>A.mexicana</u>	X	X	<u>A.mexicana</u> v <u>Azolla</u> sp.	✓	✓
			<u>A.microphylla</u> v <u>Azolla</u> sp.	✓	✓

Table to show comparisons made using the t-Test and whether there was a significant difference between the megasporangium apparatus types in respect to dorsal leaf lobe mean length and mean width.

X = significant difference ( $p > 5\%$ ); ✓ = no significant difference ( $p > 95\%$ ); (F), (R) and (S) see Table 4.14; L = dorsal lobe length; W = dorsal lobe width.

TABLE 4.17

COMPARISON IN t-Test		COMPARISON IN t-Test	
<u>A.filiculoides</u> v <u>A.mexicana</u>	✓	<u>A.mexicana</u> v <u>A.microphylla</u>	✓
<u>A.filiculoides</u> v <u>A.microphylla</u>	✓	<u>A.mexicana</u> v <u>Azolla</u> sp.	✓
<u>A.filiculoides</u> v <u>Azolla</u> sp.	X	<u>A.microphylla</u> v <u>Azolla</u> sp.	✓

Table to show the comparisons made using the t-Test and whether there was a significant difference in the megasporangium apparatus types in respect to the dorsal leaf lobe length-width ratio. The A.filiculoides (F)(R) and (S) groups are combined.

X = significant difference ( $p > 5\%$ ); ✓ = no significant difference ( $p > 95\%$ ).

TABLE 4.18

COMPARISON IN t-Test		COMPARISON IN t-Test	
<u>A.filiculoides</u> (F) v <u>A.filiculoides</u> (R)	X	<u>A.filiculoides</u> (R) v <u>A.microphylla</u>	X
<u>A.filiculoides</u> (F) v <u>A.filiculoides</u> (S)	X	<u>A.filiculoides</u> (R) v <u>Azolla</u> sp.	X
<u>A.filiculoides</u> (F) v <u>A.mexicana</u>	✓	<u>A.filiculoides</u> (S) v <u>A.mexicana</u>	X
<u>A.filiculoides</u> (F) v <u>A.microphylla</u>	✓	<u>A.filiculoides</u> (S) v <u>A.microphylla</u>	X
<u>A.filiculoides</u> (F) v <u>Azolla</u> sp.	✓	<u>A.filiculoides</u> (S) v <u>Azolla</u> sp.	X
<u>A.filiculoides</u> (R) v <u>A.filiculoides</u> (S)	✓	<u>A.mexicana</u> v <u>A.microphylla</u>	✓
<u>A.filiculoides</u> (R) v <u>A.mexicana</u>	✓	<u>A.mexicana</u> v <u>Azolla</u> sp.	✓
		<u>A.microphylla</u> v <u>Azolla</u> sp.	✓

Table to show the comparisons made using the t-Test and whether there was a significant difference in the megasporangium apparatus types in respect to the dorsal leaf lobe length-width ratio.

X = significant difference ( $p > 5\%$ ); ✓ = no significant difference ( $p > 95\%$ ); (F)(R) and (S) see Table 4.14.

FIGURE 4.1

Megaspore apparatus structure

SEM's illustrating the distribution of filosum in the apical region.

(a) Float filosum entwining with the funnel of suprafilosum. Distorted resting cells of *Anabaena azollae*, indusial cap and apical membrane can also be seen.

(b) Illustration of the funnel of suprafilosum cascading down over the float region. The close adherence of the indusial cap, megasporangial wall and funnel of suprafilosum can be seen, together with the typical alveolate structure of a float.

(c) L.S. disrupted float region of a 3 floated taxon showing the attachment of a float to the suprafilosum.

(d) L.S. distorted float region of a 9 floated taxon showing the distribution of suprafilosum.

(Ff = float filosum; FS = funnel of suprafilosum; Sf = suprafilosum; IC = indusial cap; am = apical membrane; Aa = *Anabaena azollae* resting cells; m = megasporangial wall; FL = float; Scale bar = 50 $\mu$ m).

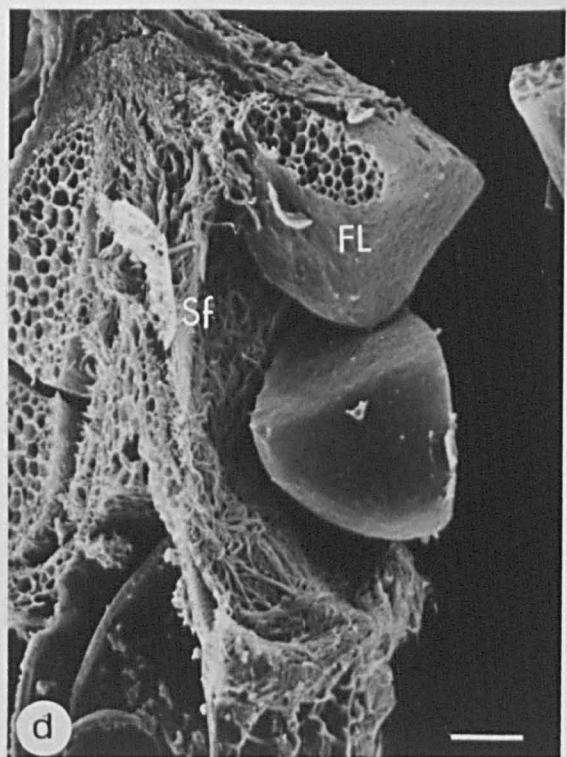
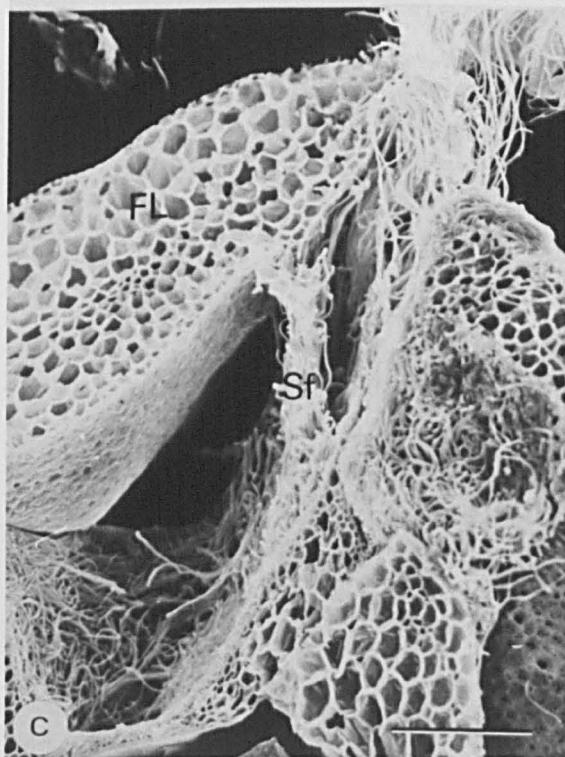
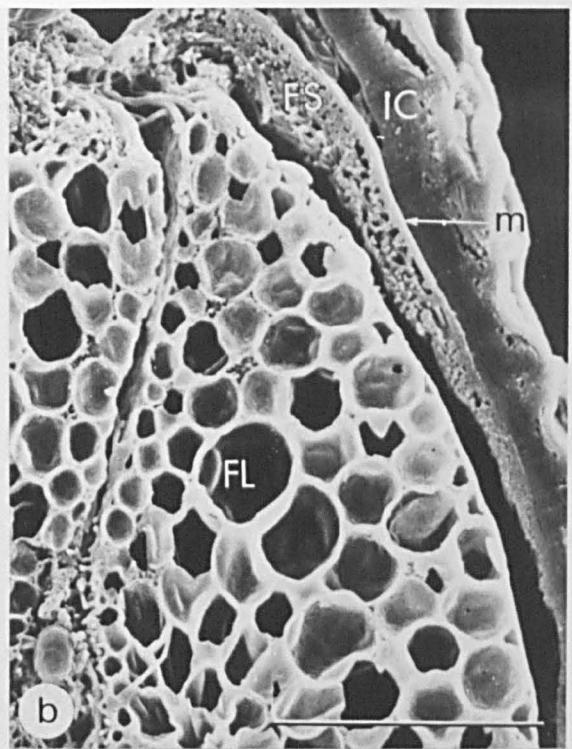
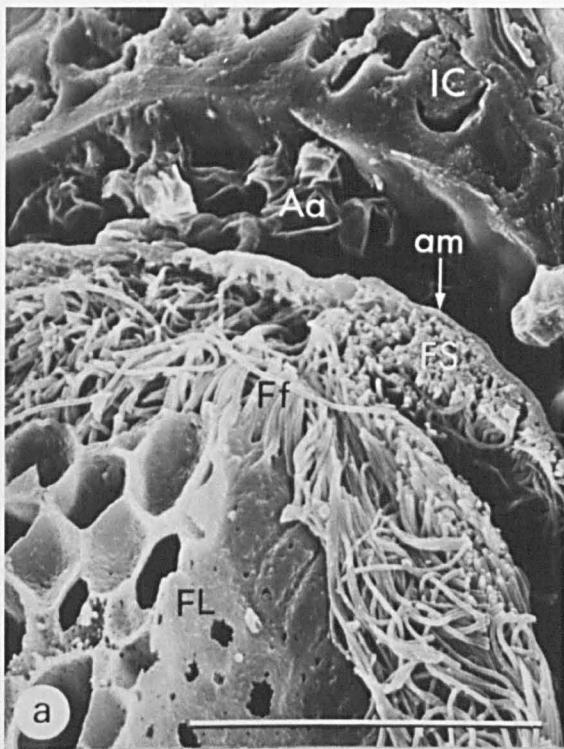


FIGURE 4.2

Megaspore apparatus structure

SEM's illustrating acrolamella morphology and structure and suprafilosum distribution.

(a) L.S. of apical region showing the acrolamella morphology in the mid-float sectional view, together with float structure and localised float filosum.

(b) L.S. showing the acrolamella morphology in the mid-float and cusp region. In the latter region the relationship of the collar to the acrolamella septum can be seen. The variation in morphology and supportive role of collar is also apparent.

Note the absence of suprafilosum in apical region of the floats (arrow) and the float filosum in the cusp region.

(c) L.S. showing the distribution of suprafilosum near the region of the acrolamella septum in 9 floated taxa.

(Fl = float; ac = acrolamella; as = acrolamella septum; Ff = float filosum; Sf = suprafilosum; sf = suprafilosum septum; C = collar; Scale bar = 50 $\mu$ m).

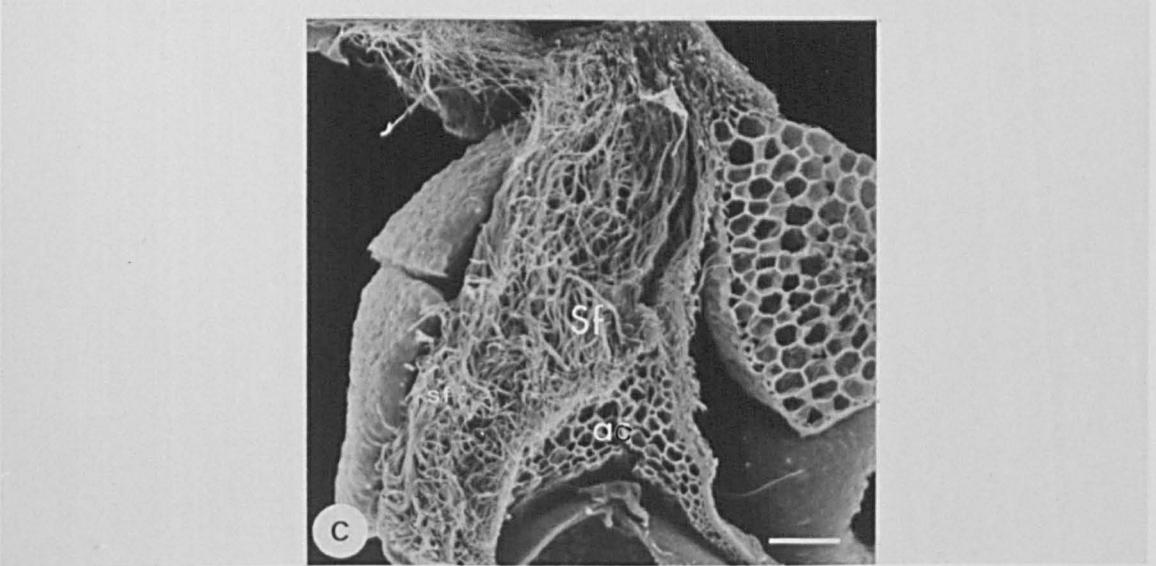
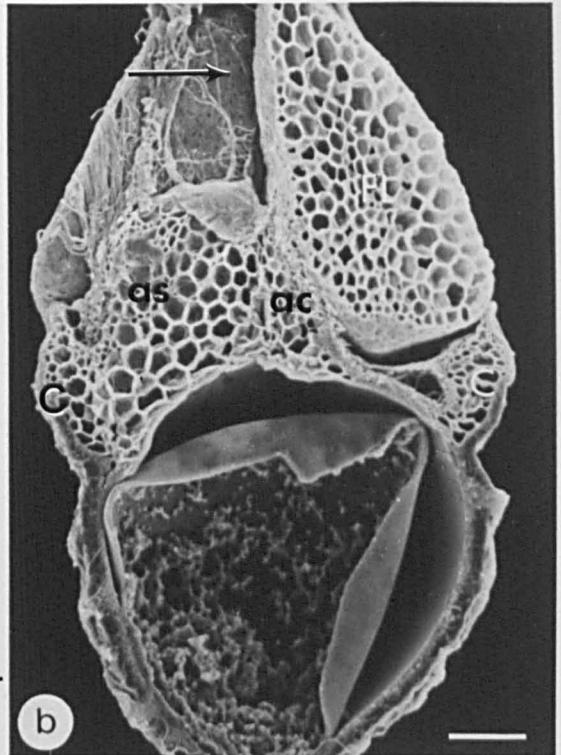
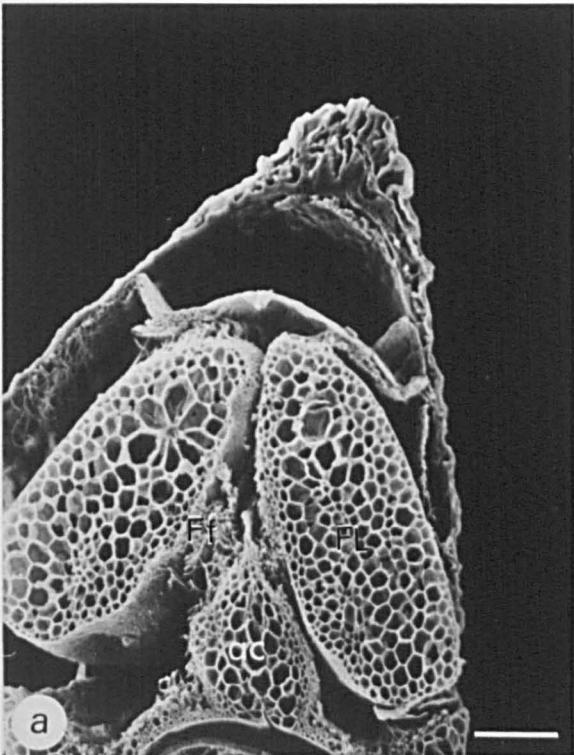


FIGURE 4.3

Megaspore apparatus structure in Sect. *Rhizosperma*

- (a) SEM illustrating a L.S. of the apical region. Note the distribution of suprafilosum, shape of floats and shape of the acrolamella.
- (b) SEM illustrating the distribution of float filosum between two acrolamella sectors.
- (c) SEM showing hooks on external float edges.
- (d) SEM showing hooks on internal float edges.

(Ff = float filosum; Fl = float; ac = acrolamella; h = hook;  
Sf = suprafilosum; Scale bar = 50 $\mu$ m).

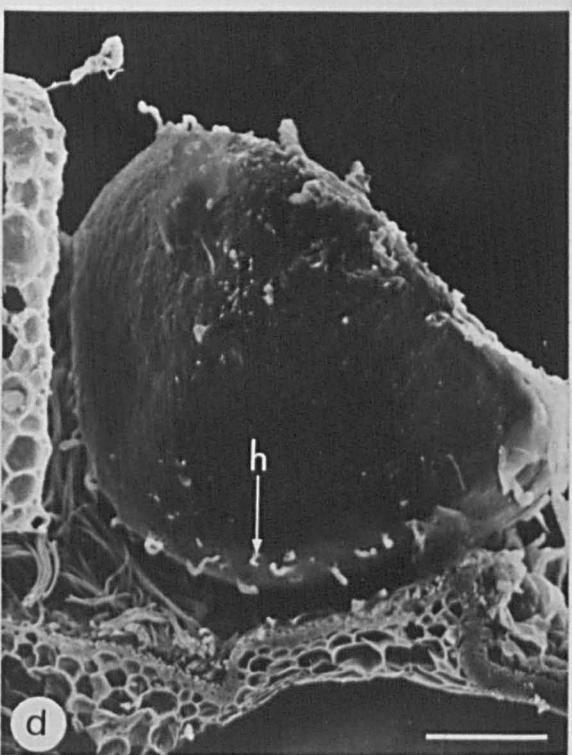
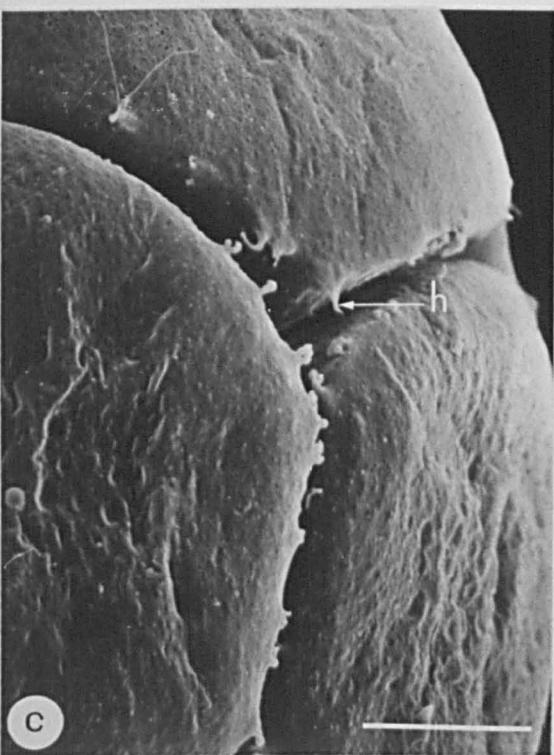
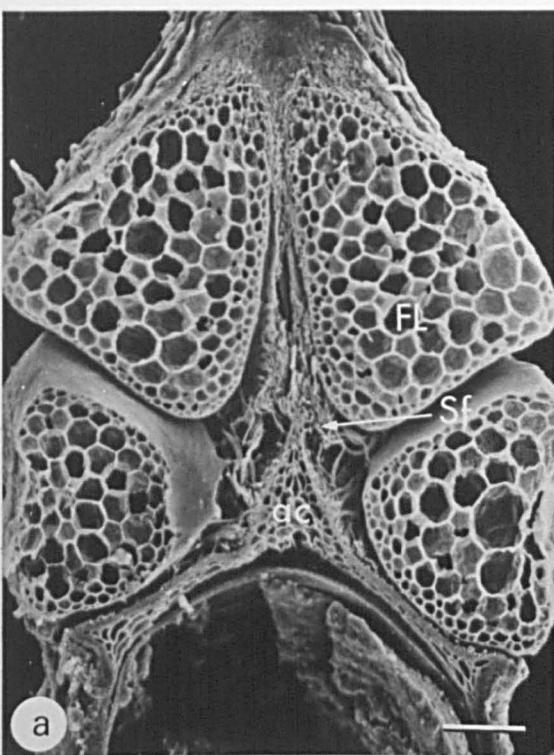


FIGURE 4.4

Megaspore apparatus structure

- (a) SEM of L.S. of a collar from 3 floated taxa illustrating the distribution of exoperine in the collar region. Note how the exoperine thins on the collar surface (arrow). (Scale bar = 50 $\mu$ m).
- (b) SEM of L.S. of a collar from the *A.nilotica* type illustrating the extension of exoperine over the collar on to the float (arrow). (Scale bar = 50 $\mu$ m).
- (c) SEM of sporoderm structure illustrating the origin of infrafilosum from the exoperine 1 zone. Note also endoperine and exospore structure at high magnification. (Scale bar = 5 $\mu$ m).
- (d) SEM of an isolated microspore with a trilete mark.  
(Scale bar = 10 $\mu$ m).

(C = collar; Fl = float; ep = exoperine; epl = exoperine 1; inf = infrafilosum element; en = endoperine; ex = exospore; f = flange).

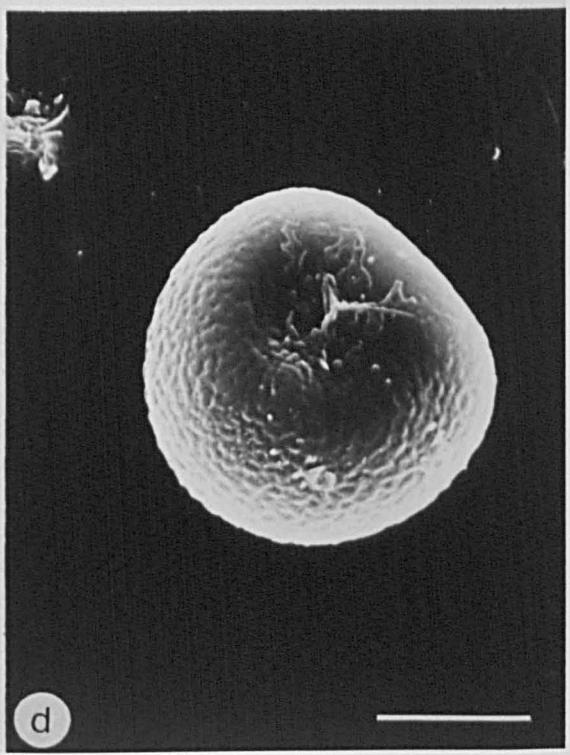
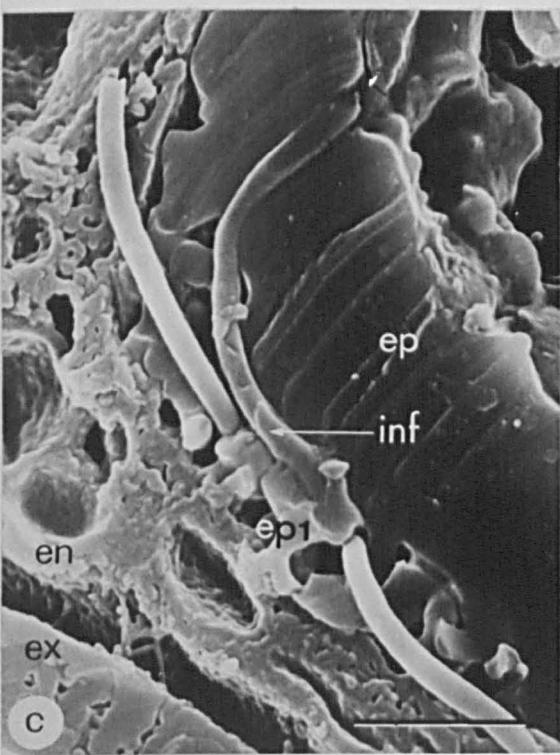
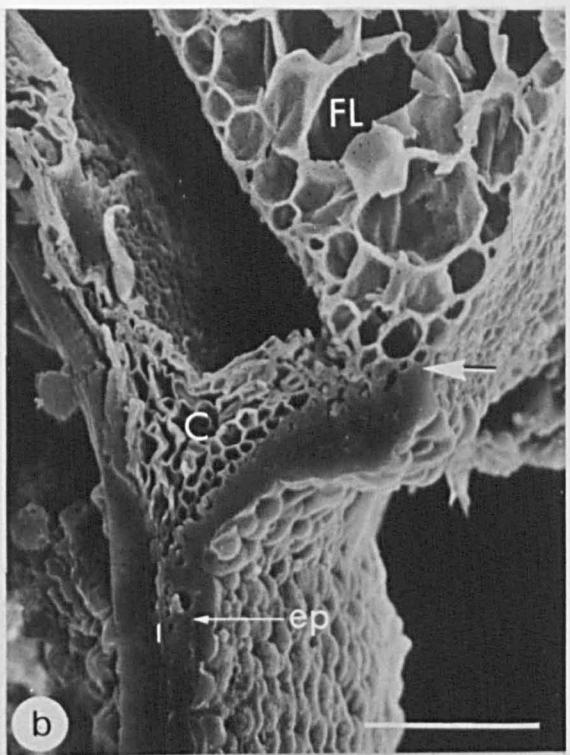
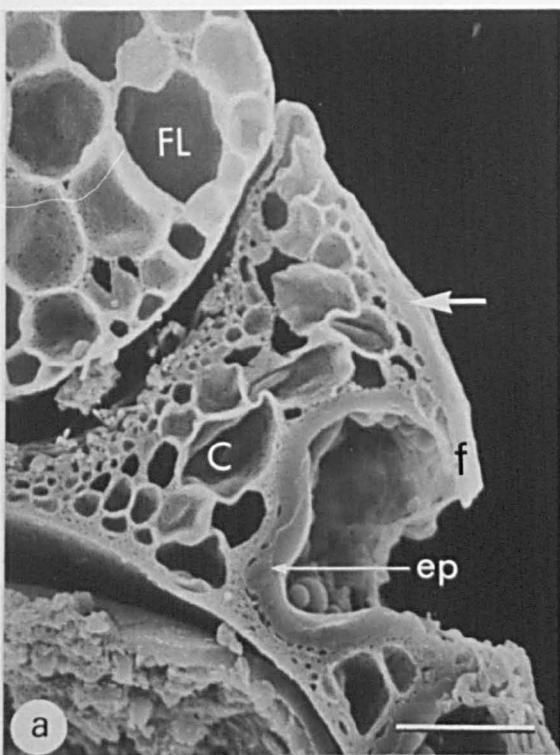


FIGURE 4.5

The *A.filiculoides* type

SEM's illustrating general morphology of megaspore apparatuses and sporoderm sculpturing at low magnification. Note variation in prominence of excrescences and amount of infrafilosum.

- (a) Australian population
- (b) North American population
- (c) South American population
- (d) Australian population

(Scale bar = 100 $\mu$ m).

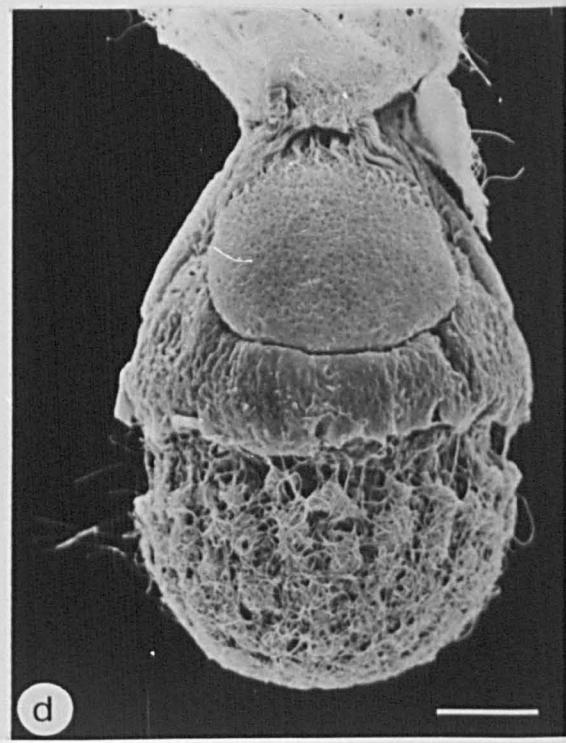
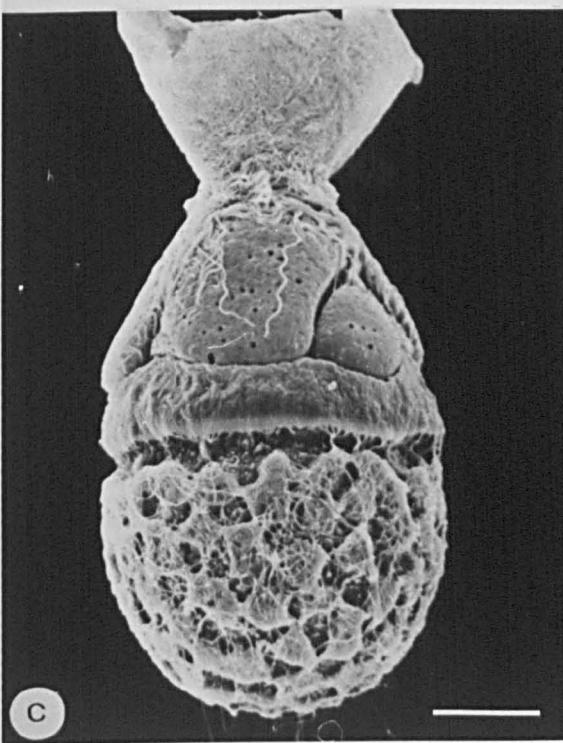
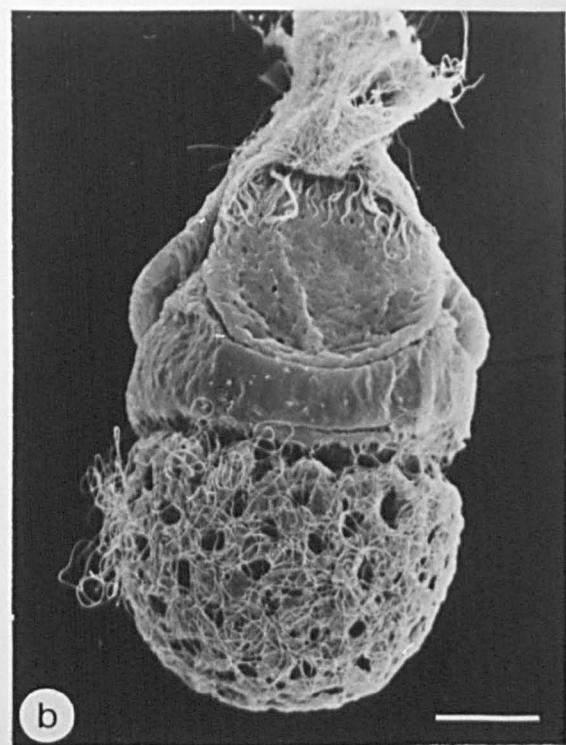
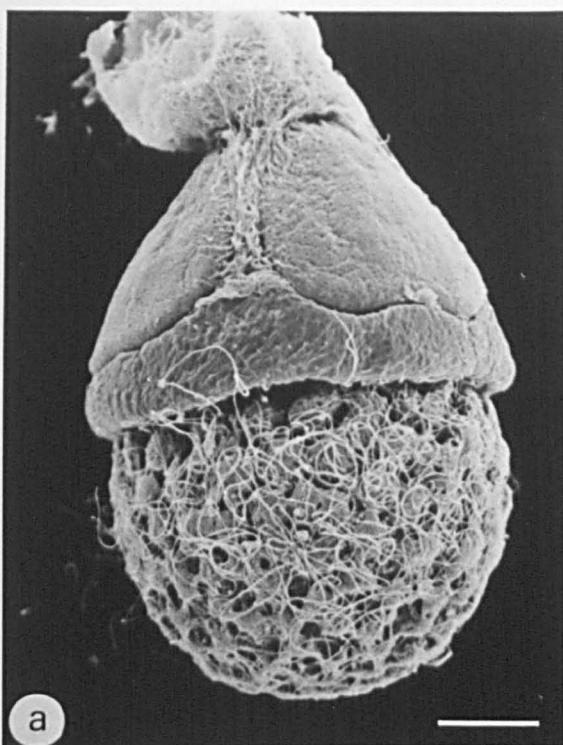


FIGURE 4.6

The A.filiculoides type

SEM's of Variation in general morphology and low magnification sporoderm sculpturing of megasporangia. Note variation in prominence of excrescences, amount of infrafilosum and the waist (arrow).

- (a) Australian population
- (b) South American population (note elongated distal megasporangium).
- (c) South American population
- (d) Hawaian population

(Scale bar = 100 um).

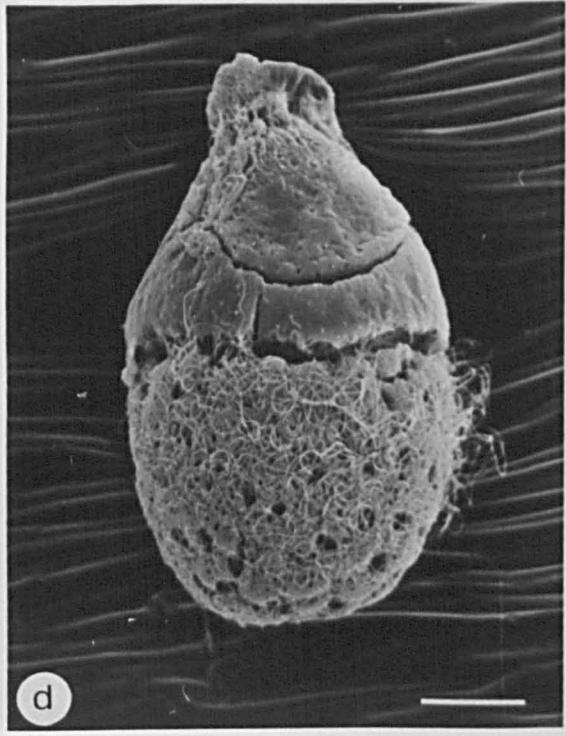
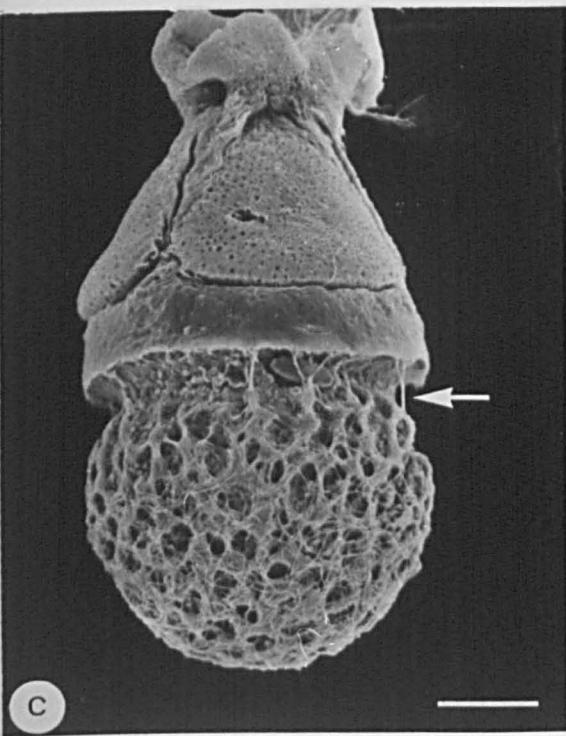
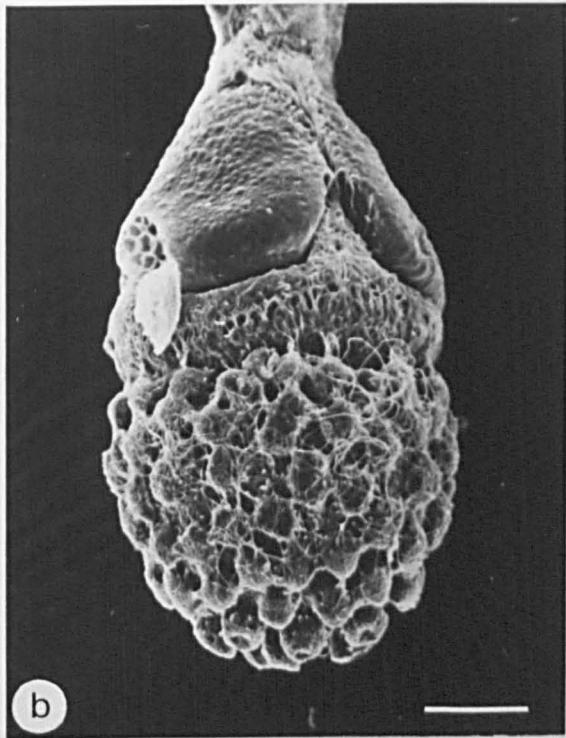
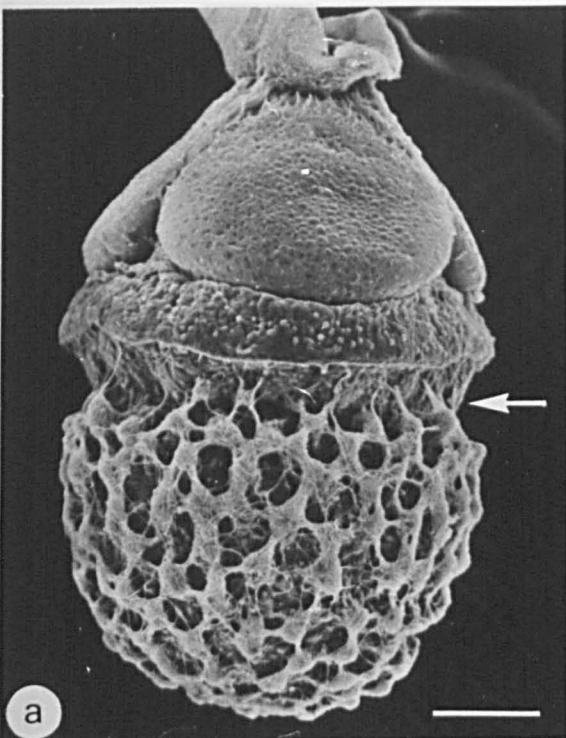


FIGURE 4.7

The A.filiculoides type

(a) SEM of sculpturing viewed at high magnification. Bridges and ridges can be seen connecting excrescences. This micrograph illustrates variation of sculpturing within a single specimen.

(b & c) SEM's of variation in float puncturing.

(For key see Figures 4.10; Scale bar = 50um).

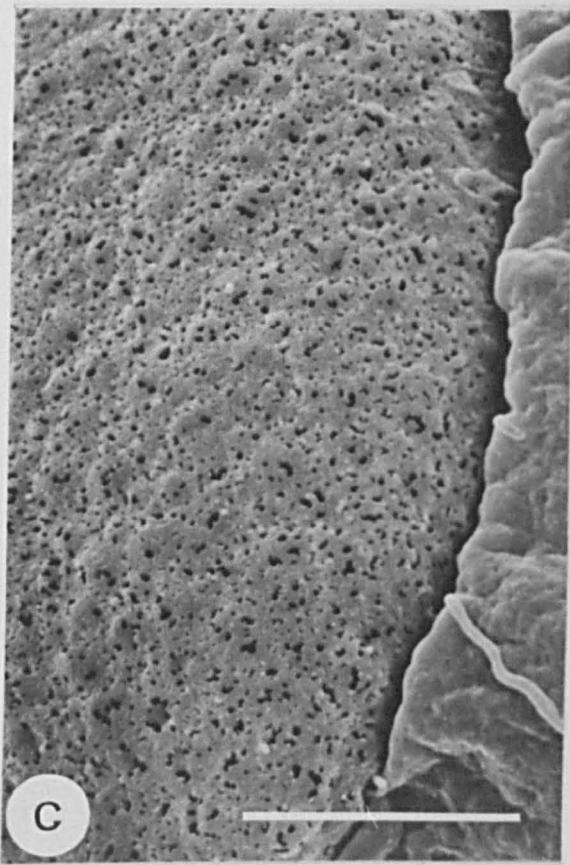
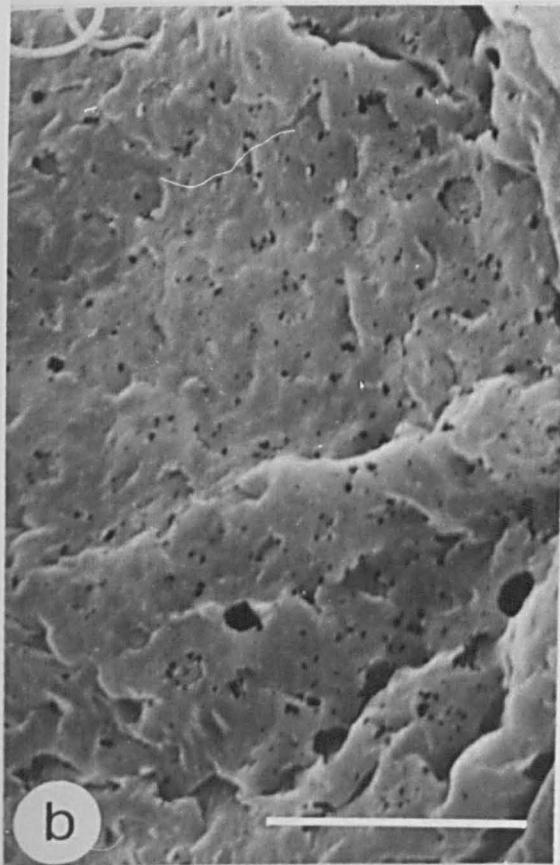
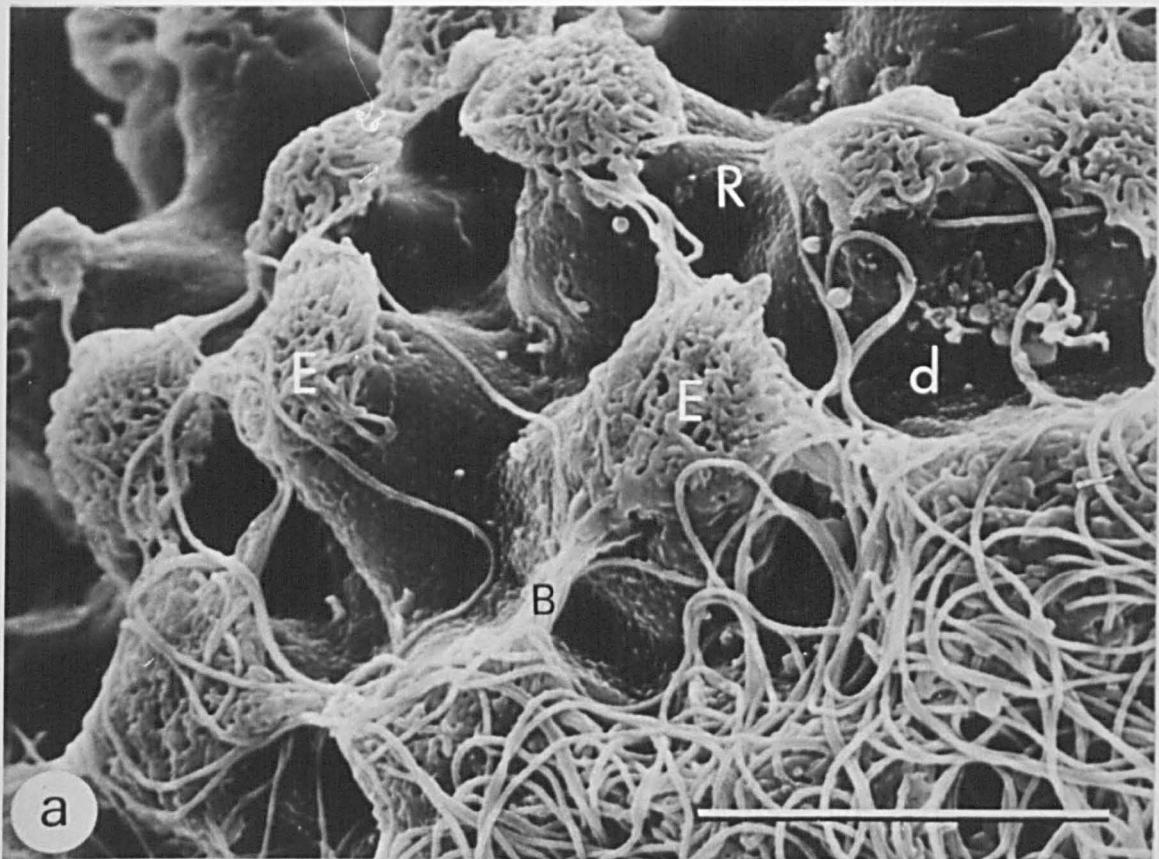


FIGURE 4.8

The *A.filiculoides* type

SEM's to show variation in surface view of the collar (a & c) and their respective morphology in sectional view (b & d).

(C = collar; Scale bar = 50um)

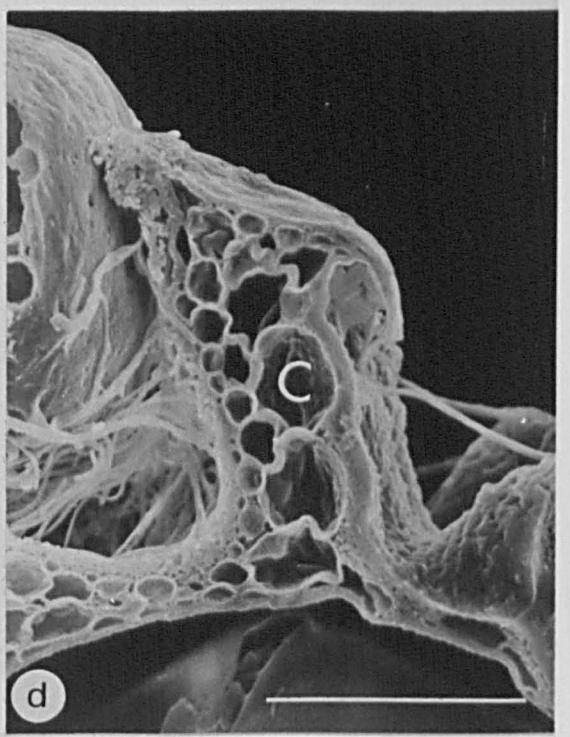
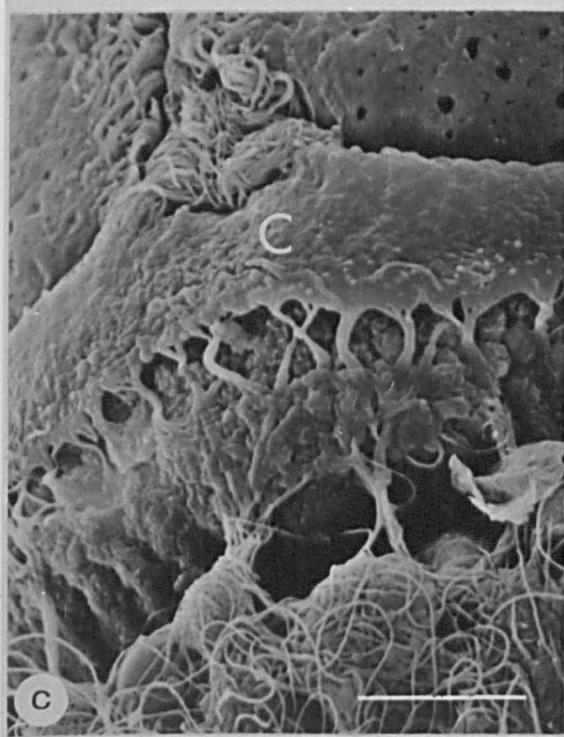
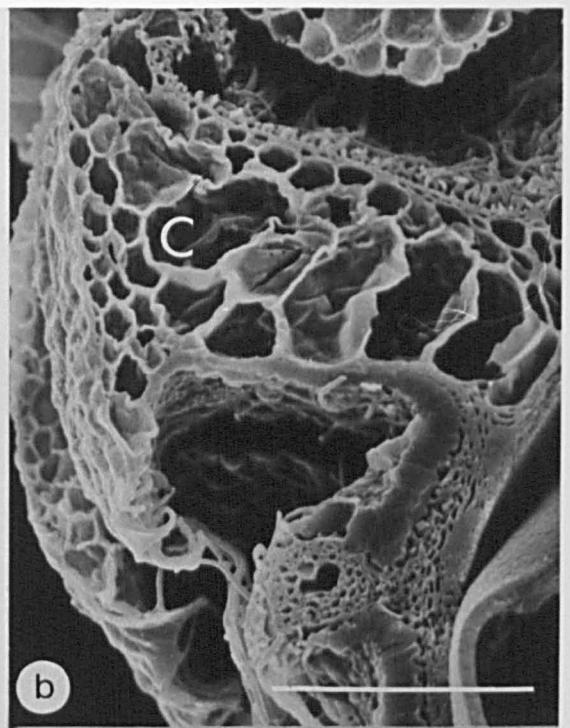
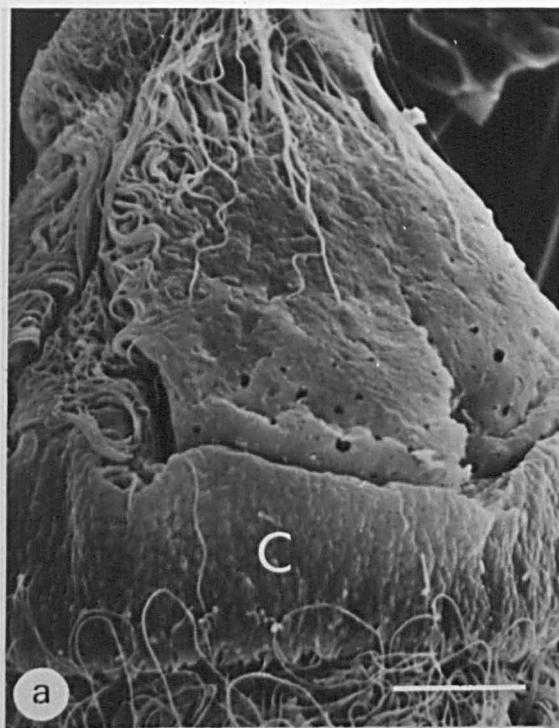


FIGURE 4.9

The A.filiculoides type

(a-f) SEM's illustrating variation in longitudinal section collar morphology; all sections made in the mid-float region. Note the position of the flange on the collar and the angle of the collar to the equatorial axis of the megaspore apparatus.

(f = flange; bar = equatorial axis; Scale bar = 25 $\mu$ m).

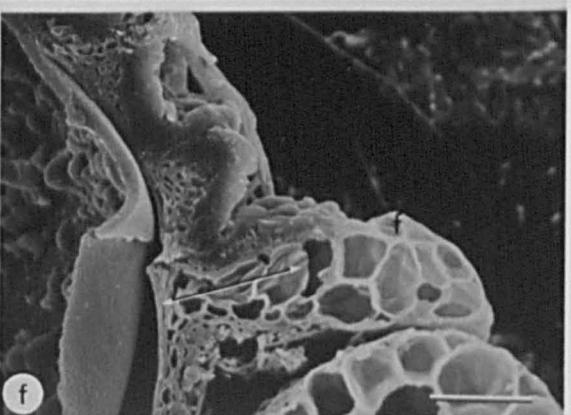
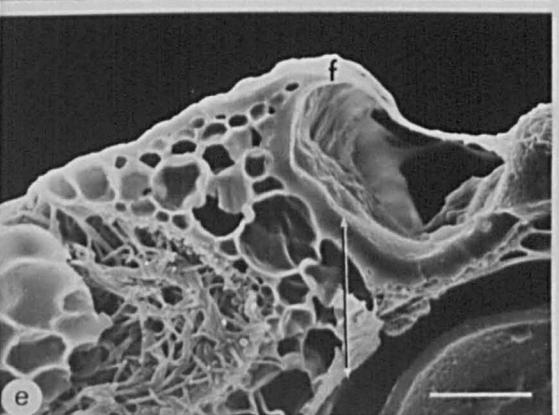
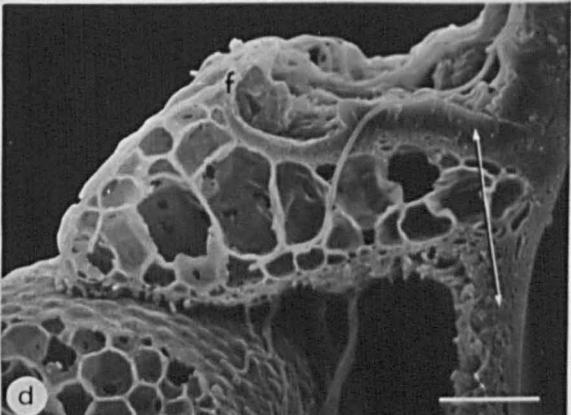
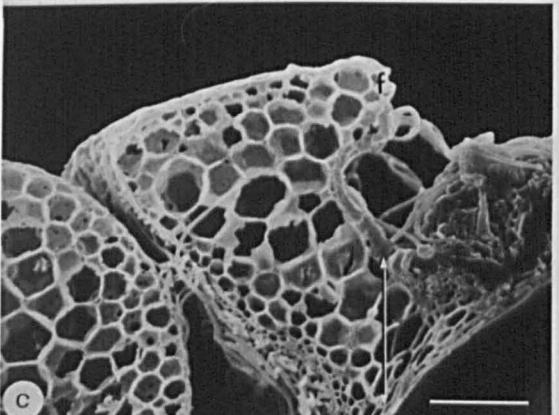
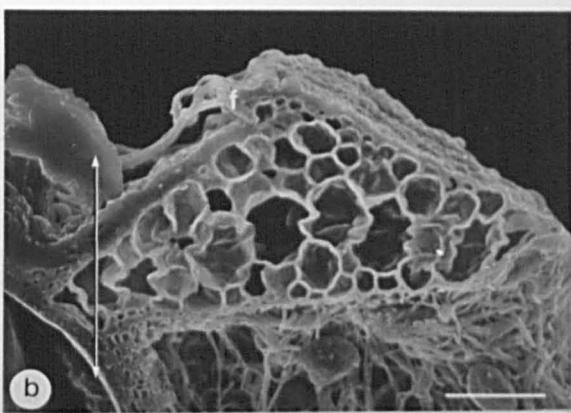
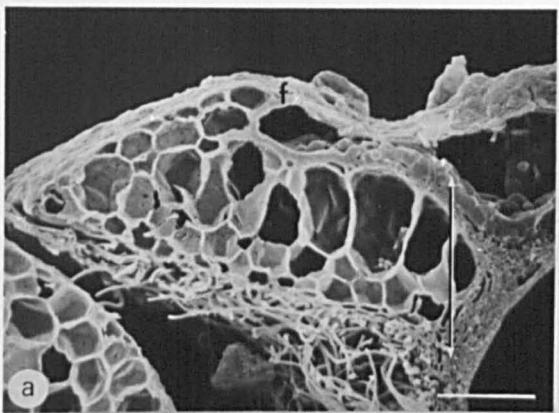


FIGURE 4.10

The A.filiculoides type

SEM's of Variation in sporoderm sculpturing seen at high magnification-

. Variation attributed to prominence of excrescences, degree of fusion of exoperine 3 elements and amount of infrafilosum obscuring the excrescences.

- (a) Prominent excrescences capped with exoperine 3.
- (b) Exoperine 3 causing excrescences to be indistinct.
- (c) Anastomosing exoperine 3 elements capping the excrescences. Perine surface in depression relatively smooth. Bridges of exoperine connecting excrescences.
- (d) As in (c), but perine in depressions undulating and with verrucose bodies.
- (e) Infrafilosum obscuring the underlying perine surface, however, excrescences capped with anastomosing reticulum of exoperine 3 can be seen.
- (f) Excrescences indistinct, the exoperine elements appearing fused to each other.

(E = excrescence; B = bridge connecting two excrescences; R = ridge connecting two excrescences; d = depression; inf = infrafilosum; vb = verrucose body; Scale bar = 50 $\mu$ m).

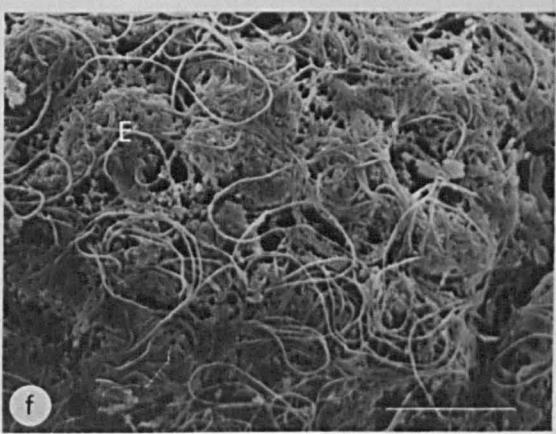
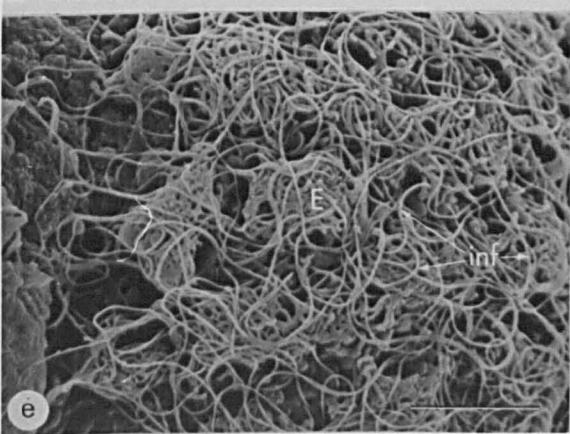
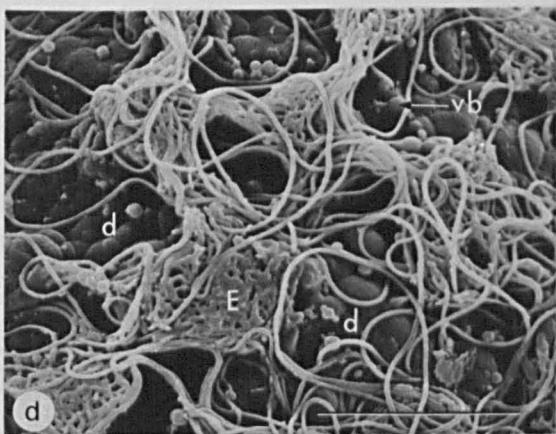
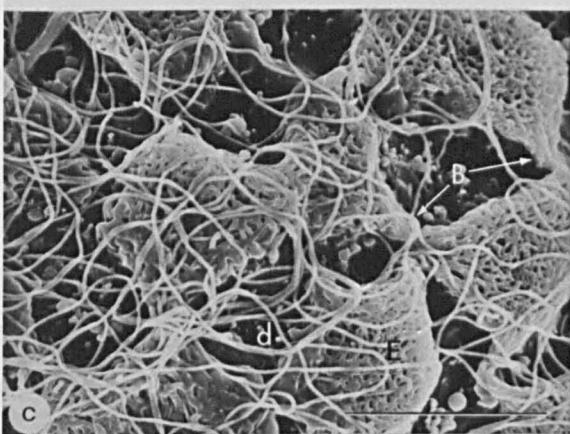
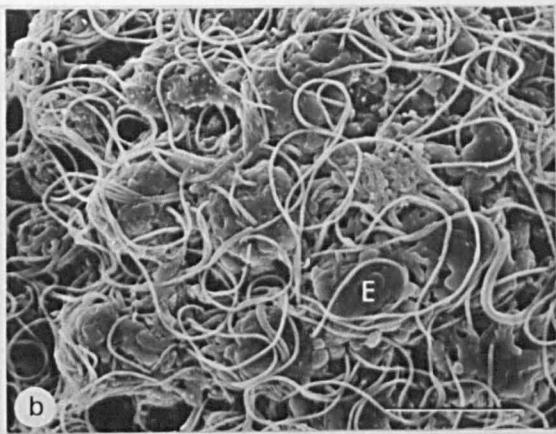
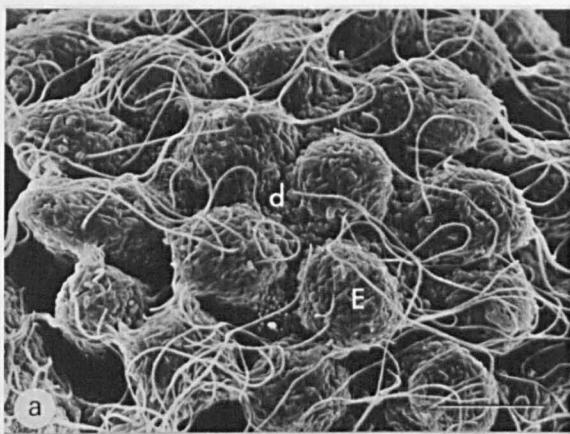


FIGURE 4.11

A.filiculoides subtype rubra

(a-d) SEM's illustrating variation in sporoderm structure, particularly the increasing endoperine alveolation from (a) to (d). Note the dense endoperine 'plug' in (b). (see also Figure 4.12 a & b).

(en = endoperine; enp = endoperine plug; ex = exospore; ep = exoperine;  
Scale bar = 10 $\mu$ m). (Exospore absent in (b)).

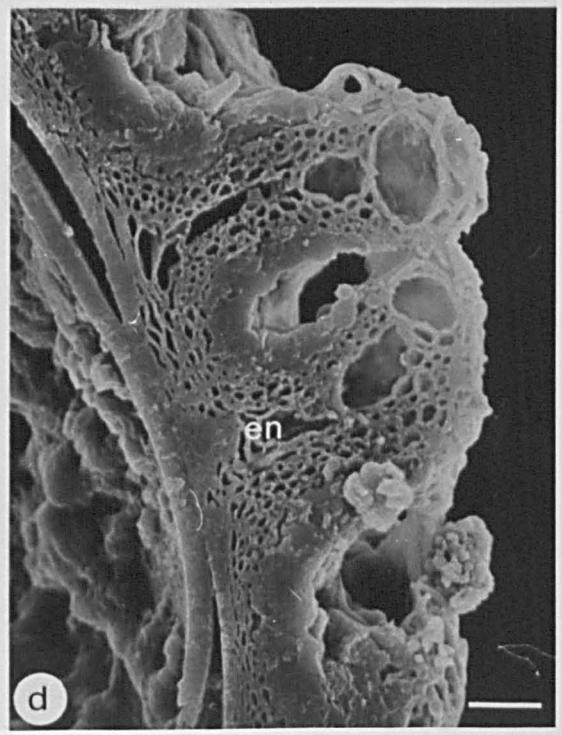
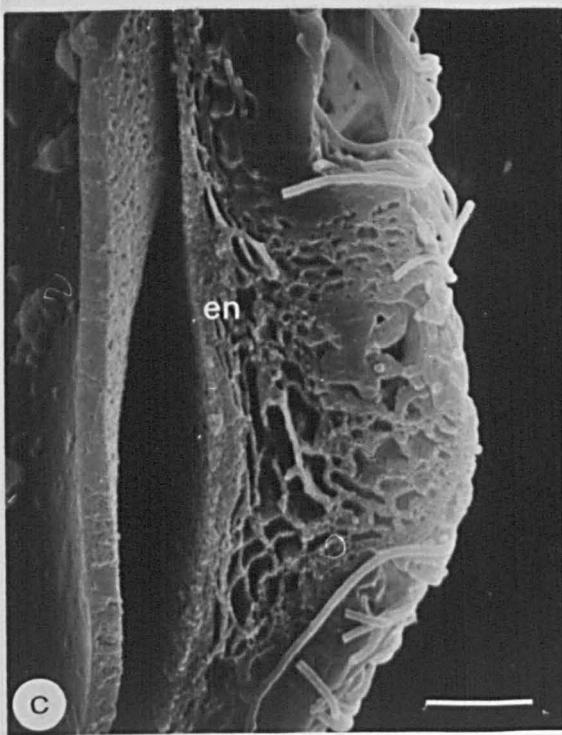
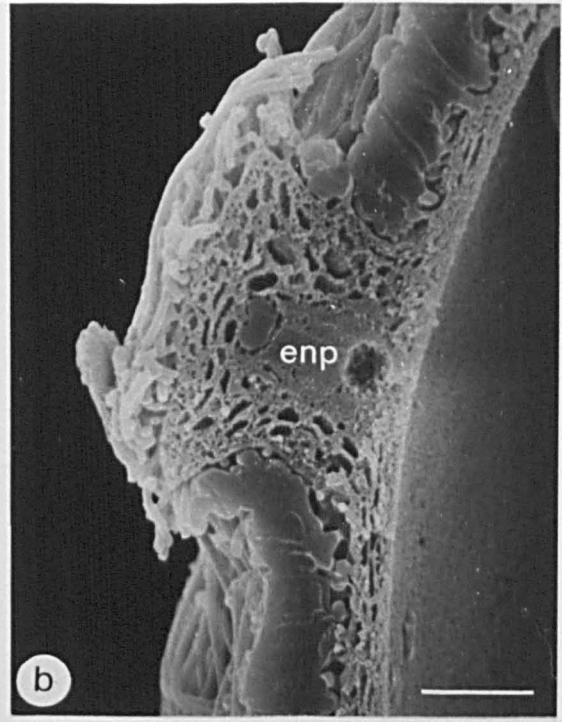
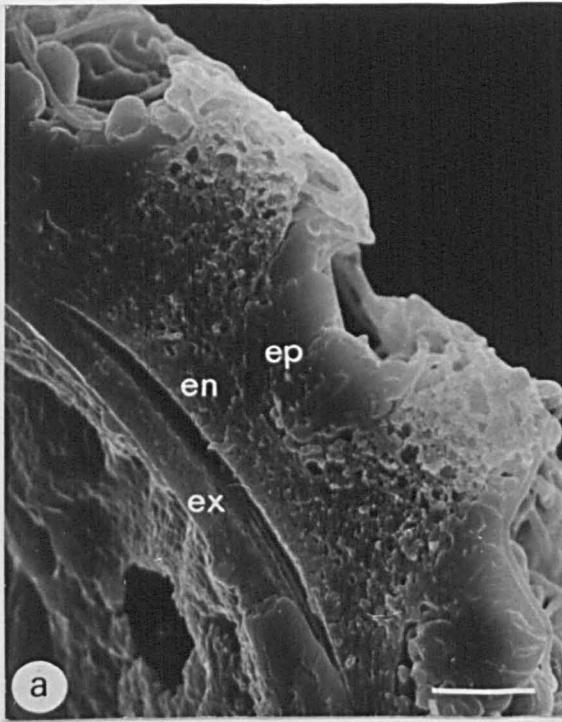


FIGURE 4.12

A.filiculoides subtype rubra

(a & b) SEM's illustrating variation in sporoderm structure, continued from Fig. 4.11. (exospore absent) (Scale bar = 10 $\mu$ m).

(c) SEM illustrating granular nature of endoperine matrix as seen at low magnification. Also note the verrucose exoperine bodies. (Scale bar = 10 $\mu$ m).

(d) SEM illustrating endoperine structure at high magnification. Note the small endoperine rods. (Scale bar = 5 $\mu$ m).

(e) SEM illustrating exospore structure which is common to both subtypes within the **A.filiculoides** type. (Scale bar = 5 $\mu$ m).

(ex = exospore; en = endoperine; ep = exoperine; vb = verrucose exoperine body; r = endoperine rods; ep3 = exoperine 3)

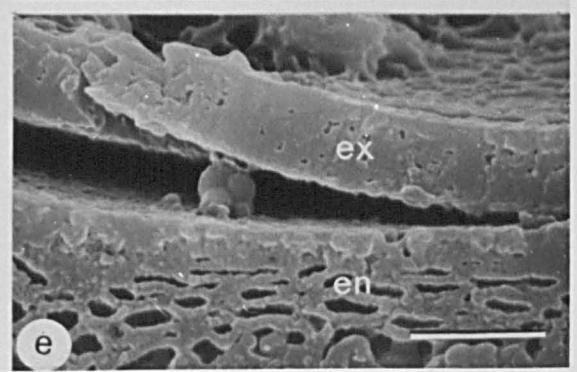
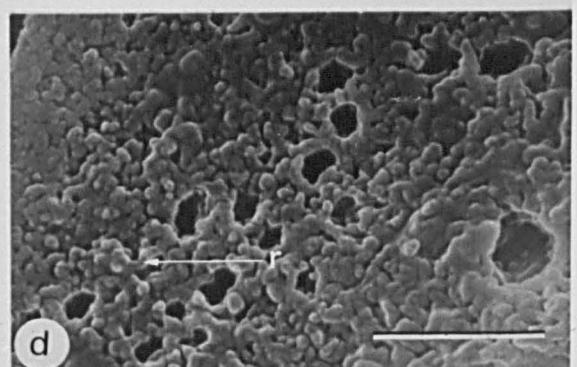
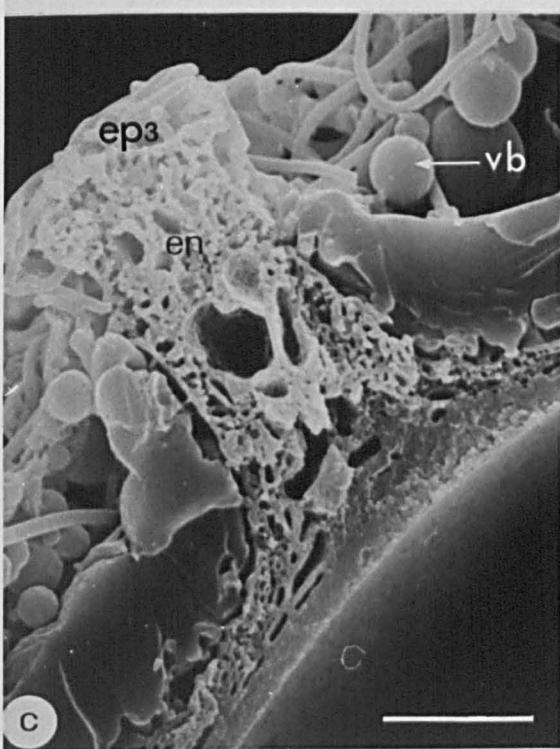
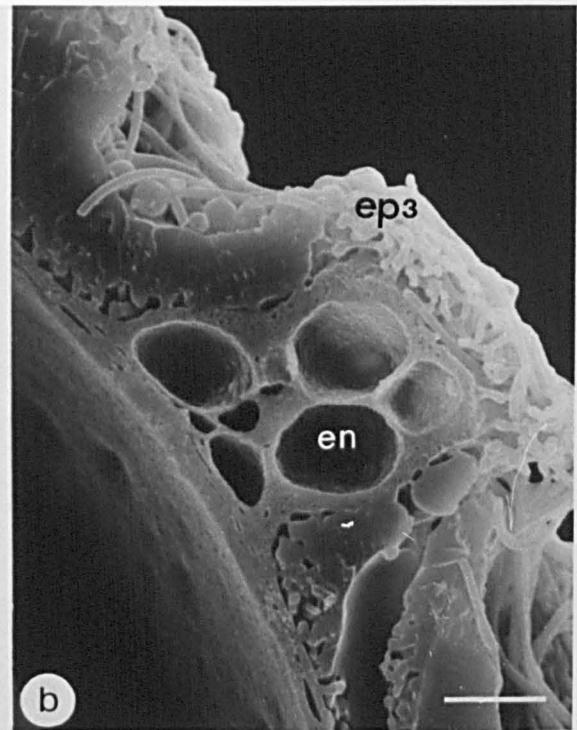
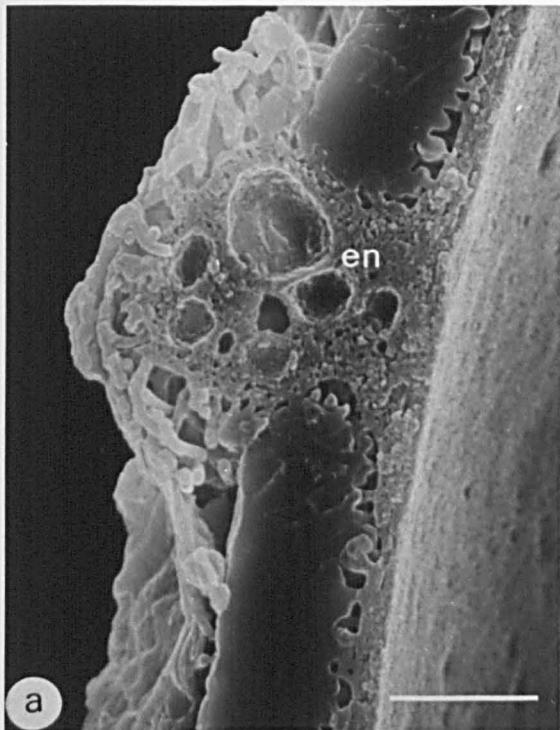


FIGURE 4.13

A.filiculoides subtype filiculoides

(a-d) SEM's illustrating variation in sporoderm structure, particularly in respect to endoperine alveolation. (See also Figure 4.14. a & b)

(b) Note the bridges of exoperine between excrescences.

(en = endoperine; B = exoperine bridge; Scale bar = 10 $\mu$ m).

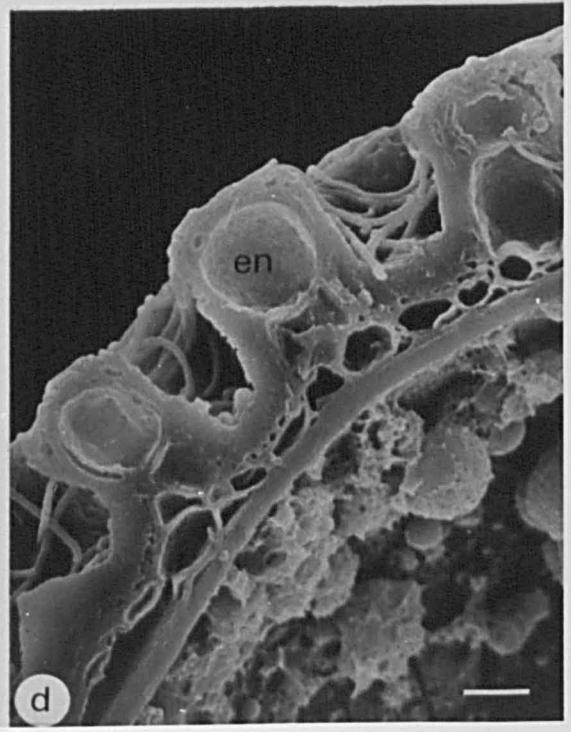
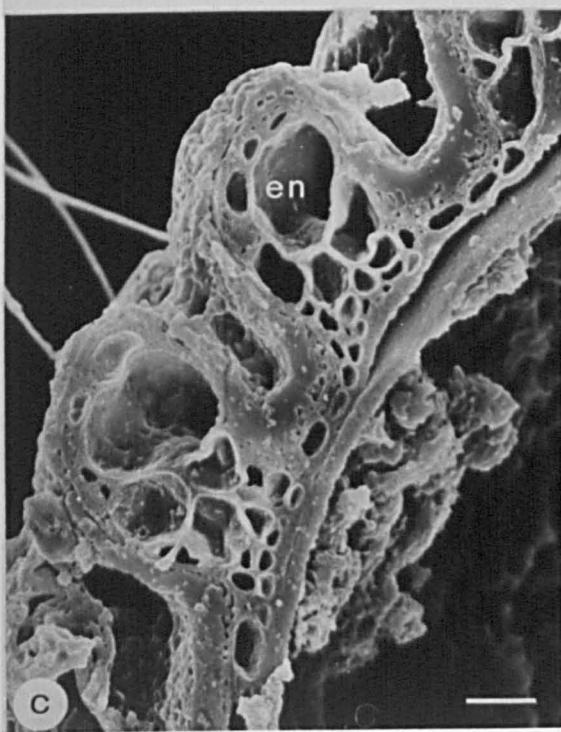
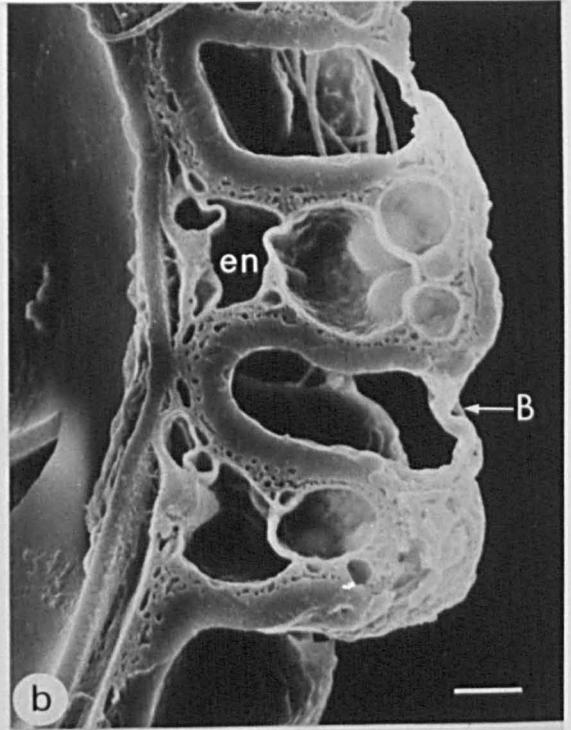
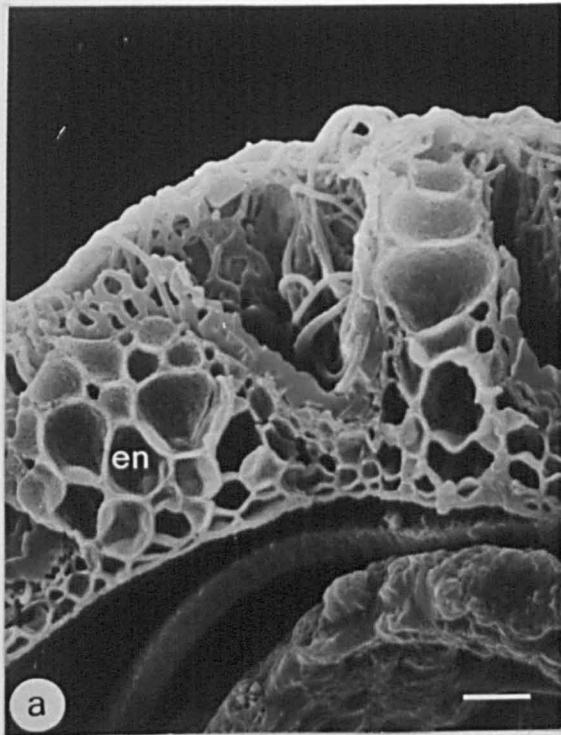


FIGURE 4.14

A.filiculoides subtype filiculoides

(a & b) SEM's illustrating variation in sporoderm structure, continued from 4.13 a - d. Note the net-like exoperine 3 zone covering excrescences in (a).

(c) SEM illustrating sporoderm structure, particularly the transition of exoperine 2 to exoperine 3 at the apex of an excrescence. (Scale bar = 10 $\mu$ m).

(d) SEM illustrating endoperine nature. (Scale bar = 5 $\mu$ m).

(e) SEM illustrating exospore nature in L.S. (Scale bar = 5 $\mu$ m).

(f & g) SEM's illustrating variation in sculpturing of the radially external exospore surface. (Scale bar = 2.5 $\mu$ m).

(ep.2 = exoperine 2; ep.3 = exoperine 3; en = endoperine; ex = exospore)

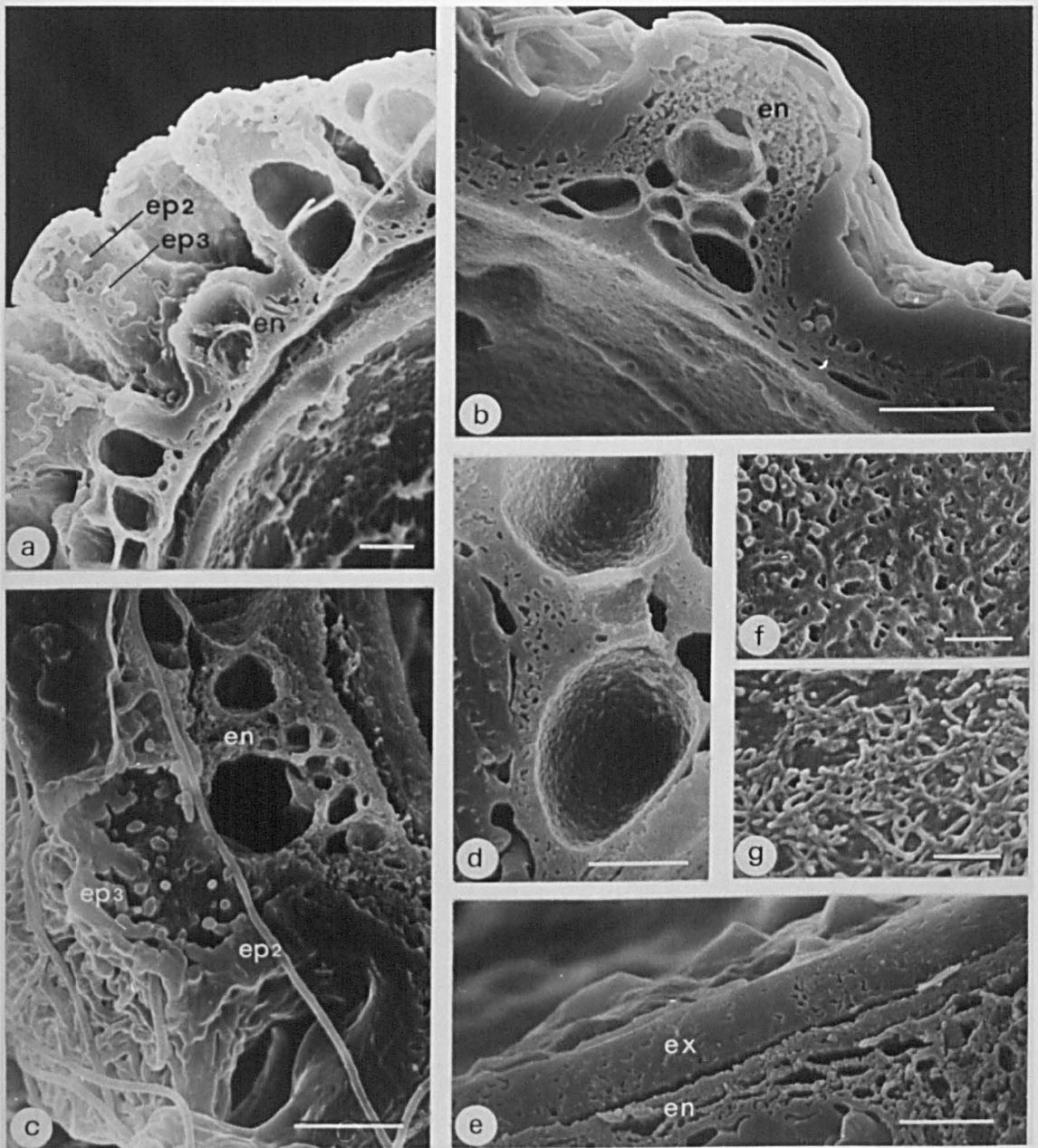


FIGURE 4.15

The A.mexicana type

(a-d) SEM's illustrating general morphology of megaspore apparatuses and sporoderm sculpturing at low magnification. The effect of *infrafilosum* quantity can be seen. A <sup>ar</sup><sub>x</sub> supernumerary float can be seen in (b).

(SF = supernumerary float; Scale bar = 100 $\mu$ m).

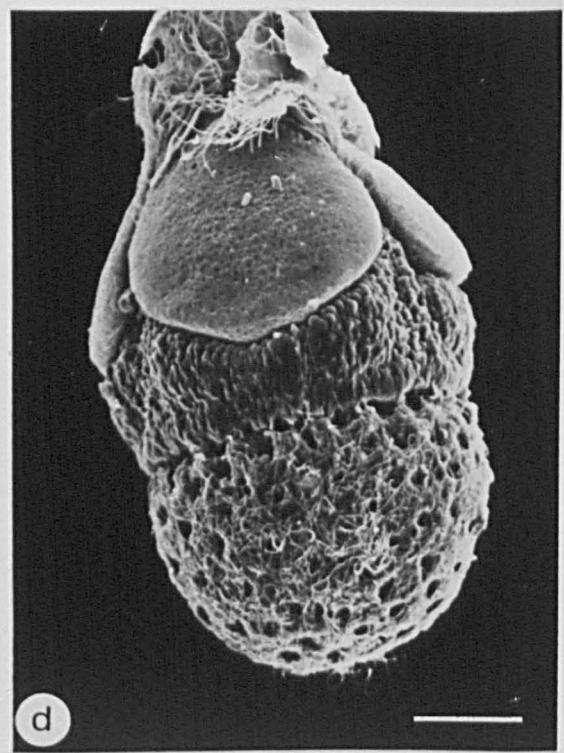
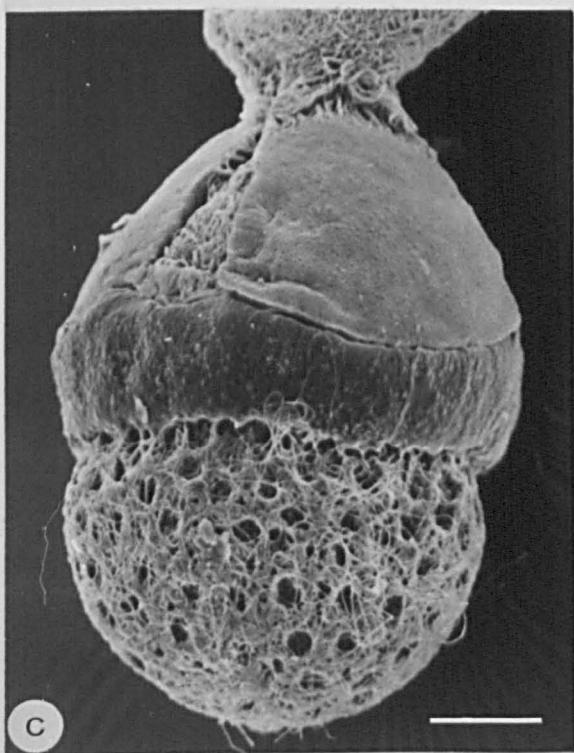
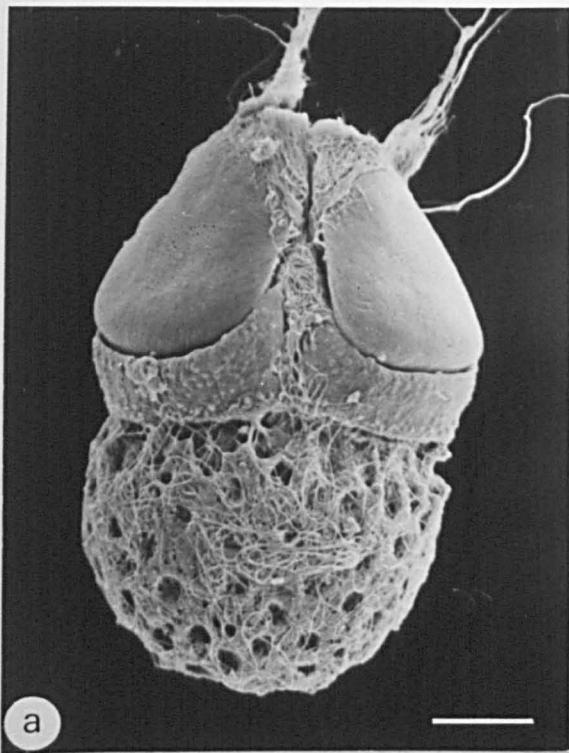


FIGURE 4.16

The A.*mexicana* type

- (a) SEM illustrating typical float shape. (Scale bar = 100um).
  - (b) SEM illustrating float surface and puncturing. (Scale bar = 10 um).
  - (c) SEM illustrating float surface and puncturing. (Scale bar = 10 um).
  - (d) SEM illustrating L.S. collar morphology in the cusp region. Note collar angle relative to the megaspore apparatus equatorial axis. (Scale bar = 25um).
  - (e) SEM illustrating L.S. collar morphology in the mid-float region. Note collar angle relative to the megaspore apparatus equatorial axis. (Scale bar = 25um).
- (Fl = float; C = collar; f = flange; bar = equatorial axis)

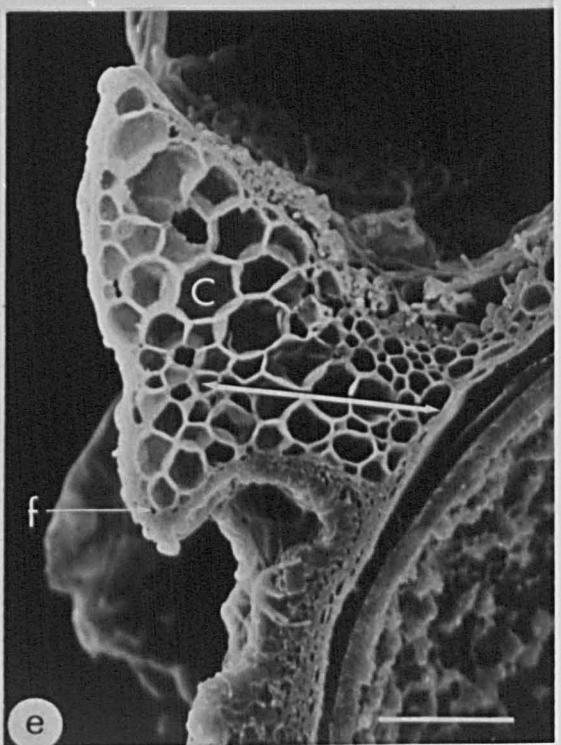
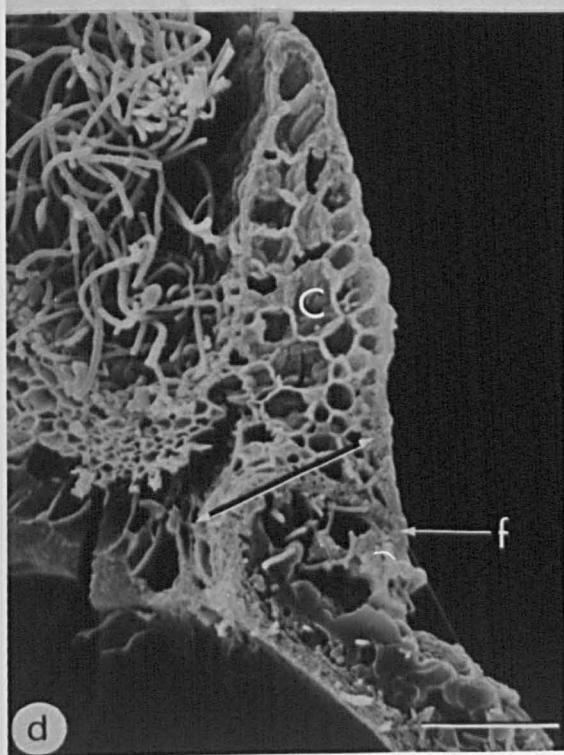
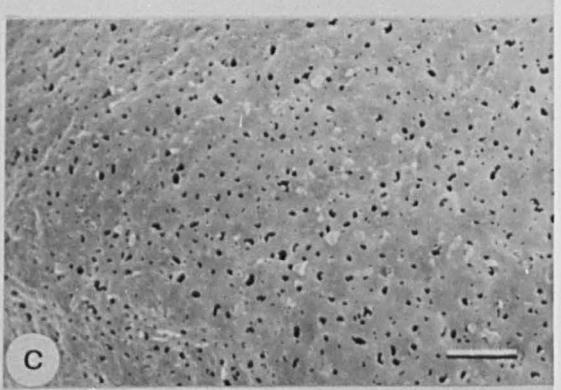
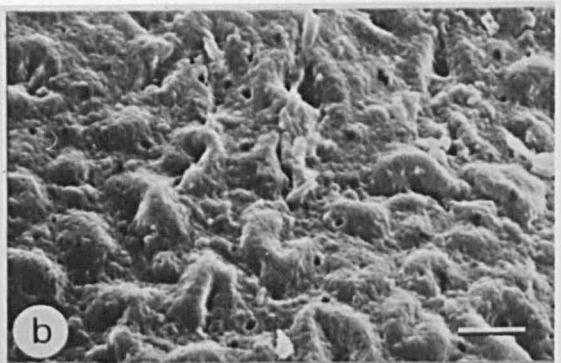
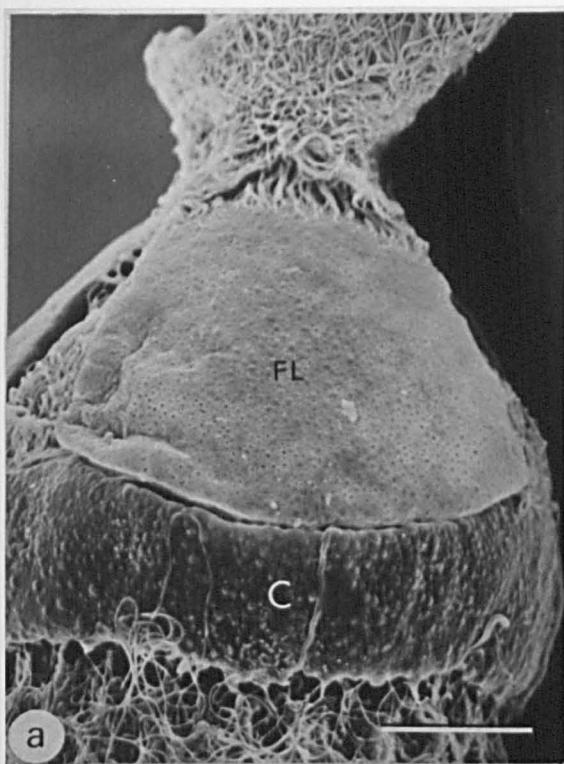


FIGURE 4.17

The *A. mexicana* type

(a-f) SEM's illustrating variation in sporoderm sculpturing at high magnification. Note the large and small foveae, the variation in fusion of the surface elements and the effect of variable quantities of infrafilosum.

(LF = large fovea; SF = small fovea; Scale bar = 50 $\mu$ m)

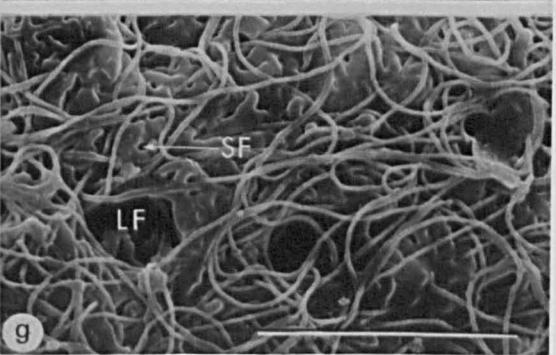
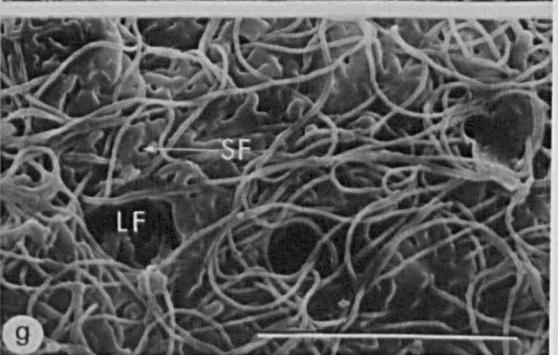
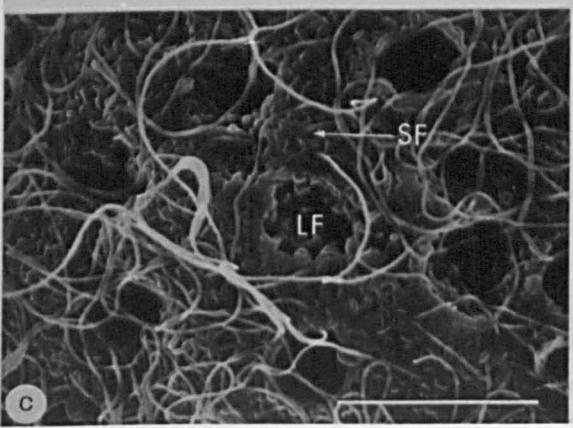
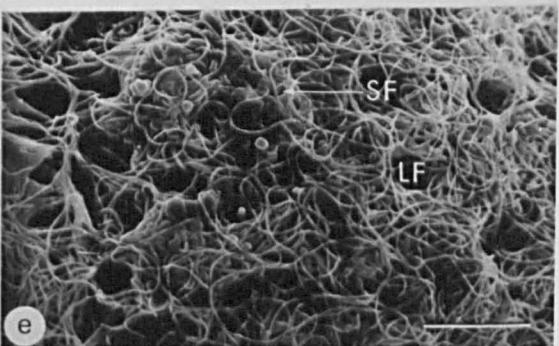
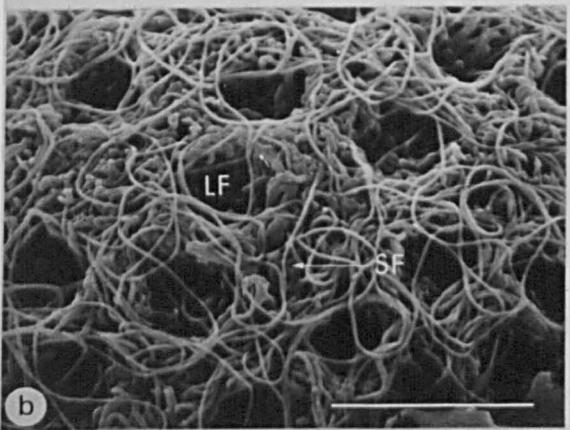
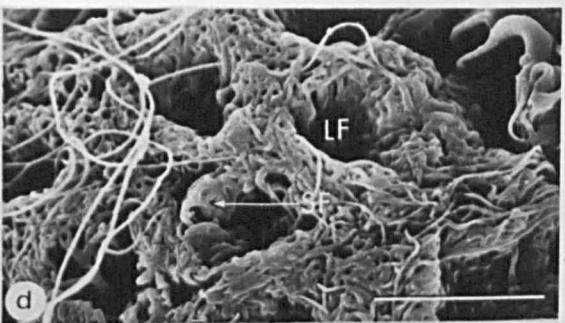
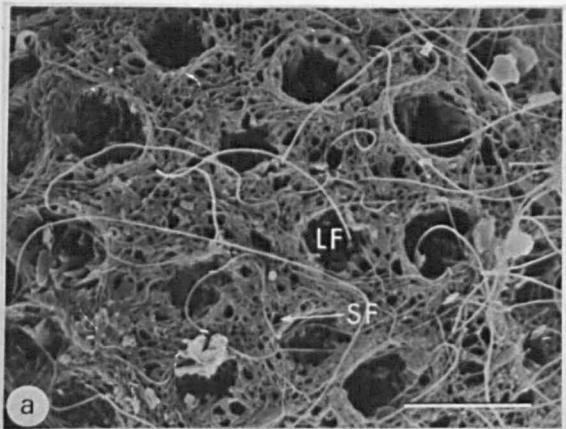


FIGURE 4.18

The A.mexicana type

(a-d) SEM's illustrating variation in sporoderm structure.

Note variation in respect to nature of the exoperine 2, also the almost uniform thickness of the endoperine; variation in exoperine 2 thickness creating the foveae and raised areas. (also see Figure 4.19).

(a) The relationship between structure and sculpture can be seen, also the exoperine 2 is columellate.

(d) Note origin of infrafilosum in the exoperine 1 zone.

(LF - large fovea; ex = exospore; en = endoperine; ep.1 = exoperine 1; ep.2 = exoperine 2; inf = infrafilosum filament; Scale bar = 10 $\mu$ m; ep3 = exoperine 3)

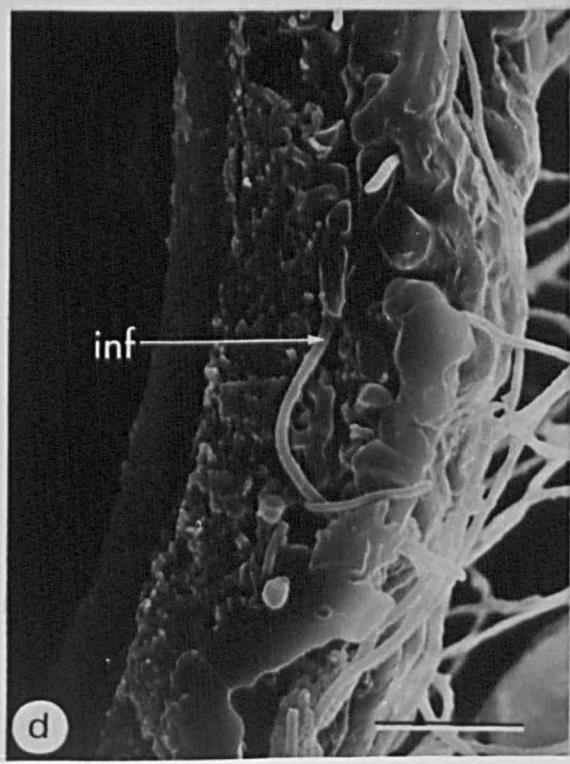
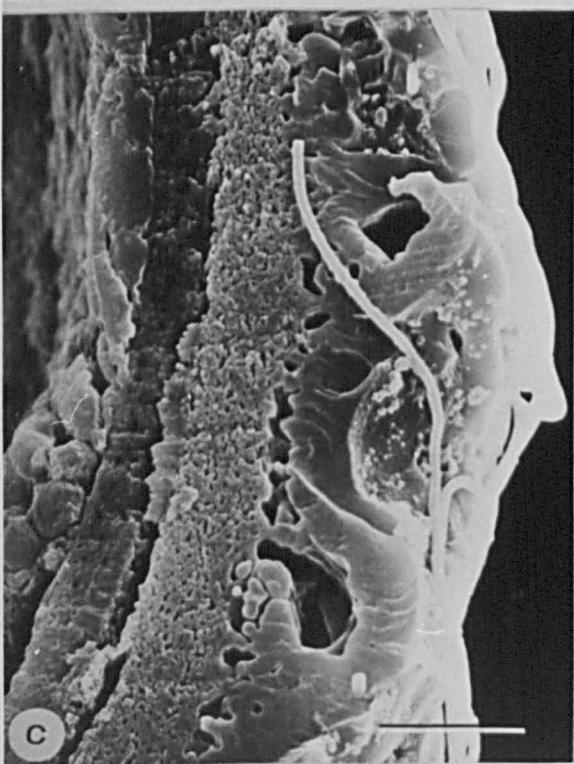
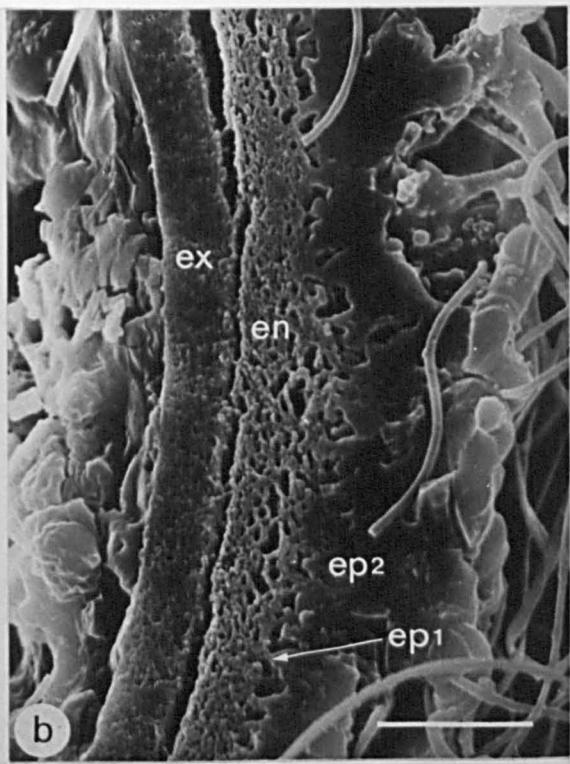
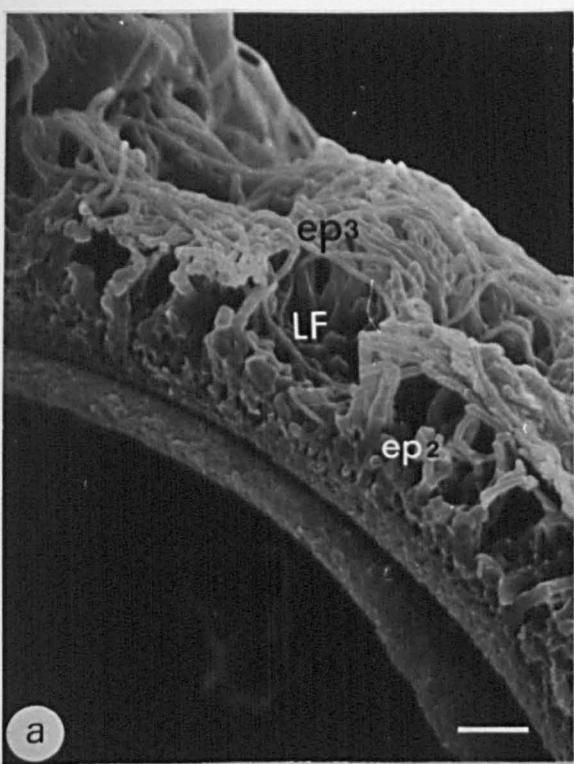


FIGURE 4.19

The A.mexicana type

(a-d) SEM's illustrating variation in sporoderm structure.

Note variation in respect to nature of the exoperine 2, also the endoperine intrusions which significantly contribute to the pattern of foveae and raised areas. (Also see Figure 4.18).

(d) The endoperine intrusion is unusually large.

(LF = large fovea; ex = exospore; eni = endoperine intrusion; ep.2 = exoperine 2; Scale bar = 10 $\mu$ m; ep3 = exoperine 3)

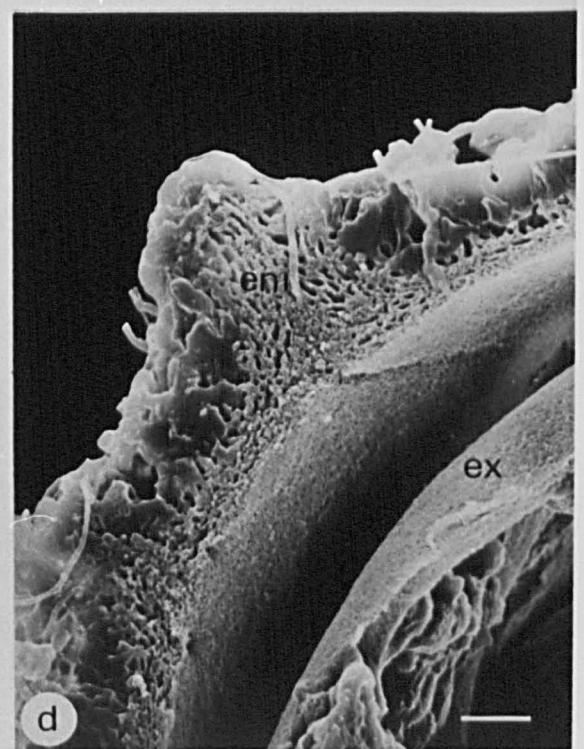
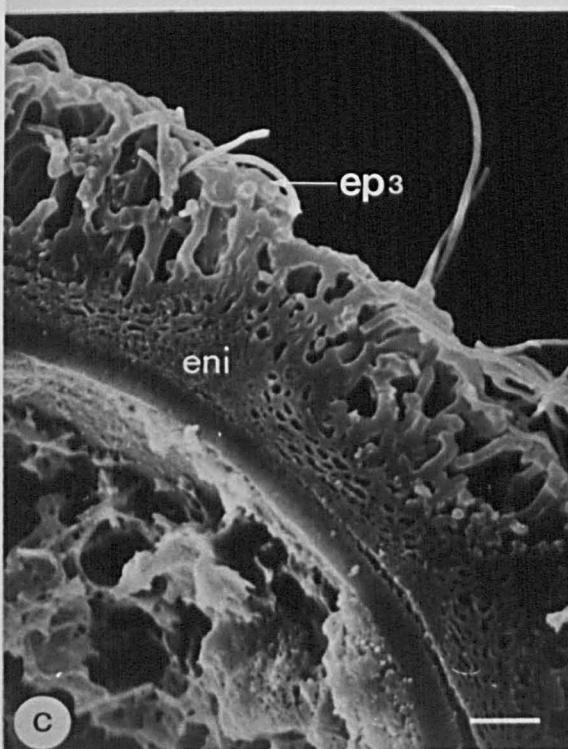
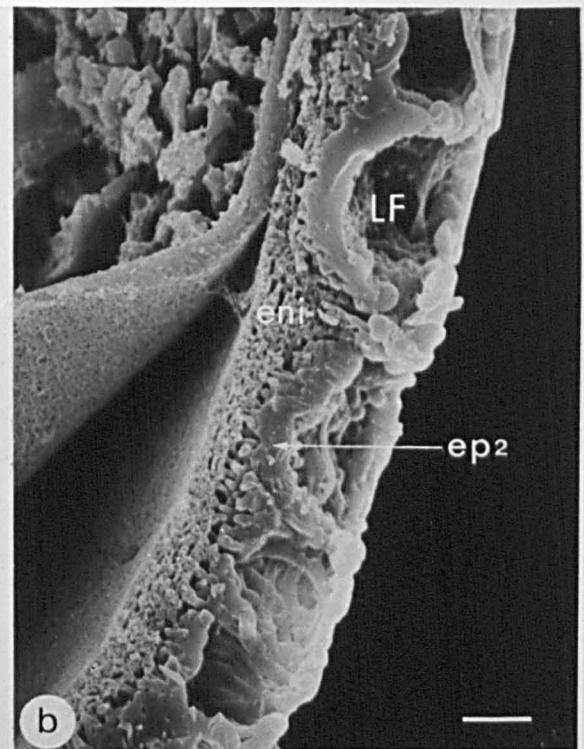
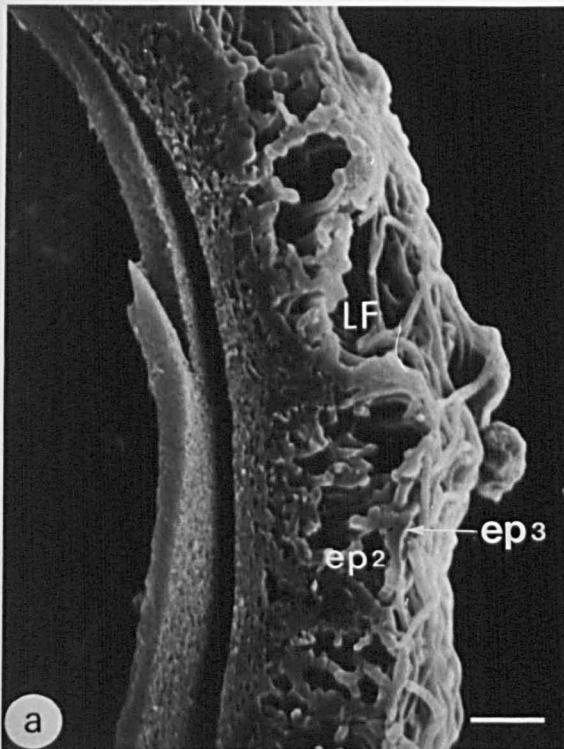


FIGURE 4.20

The A.mexicana type

(a) SEM illustrating the sculpturing of the external surface of the exospore.

(b) SEM illustrating the granular nature of the endoperine.

(ex = exospore; en = endoperine; ep = exoperine; Scale bar = 5 $\mu$ m).

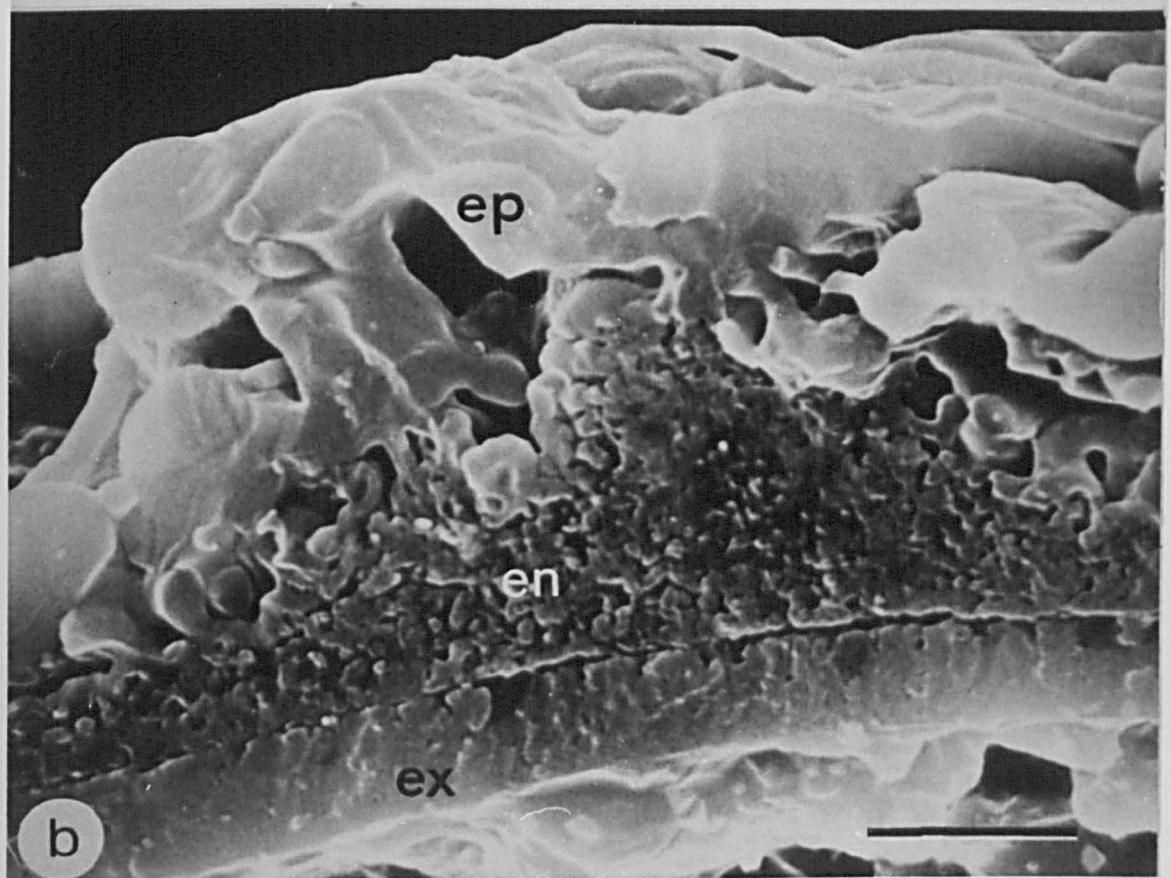
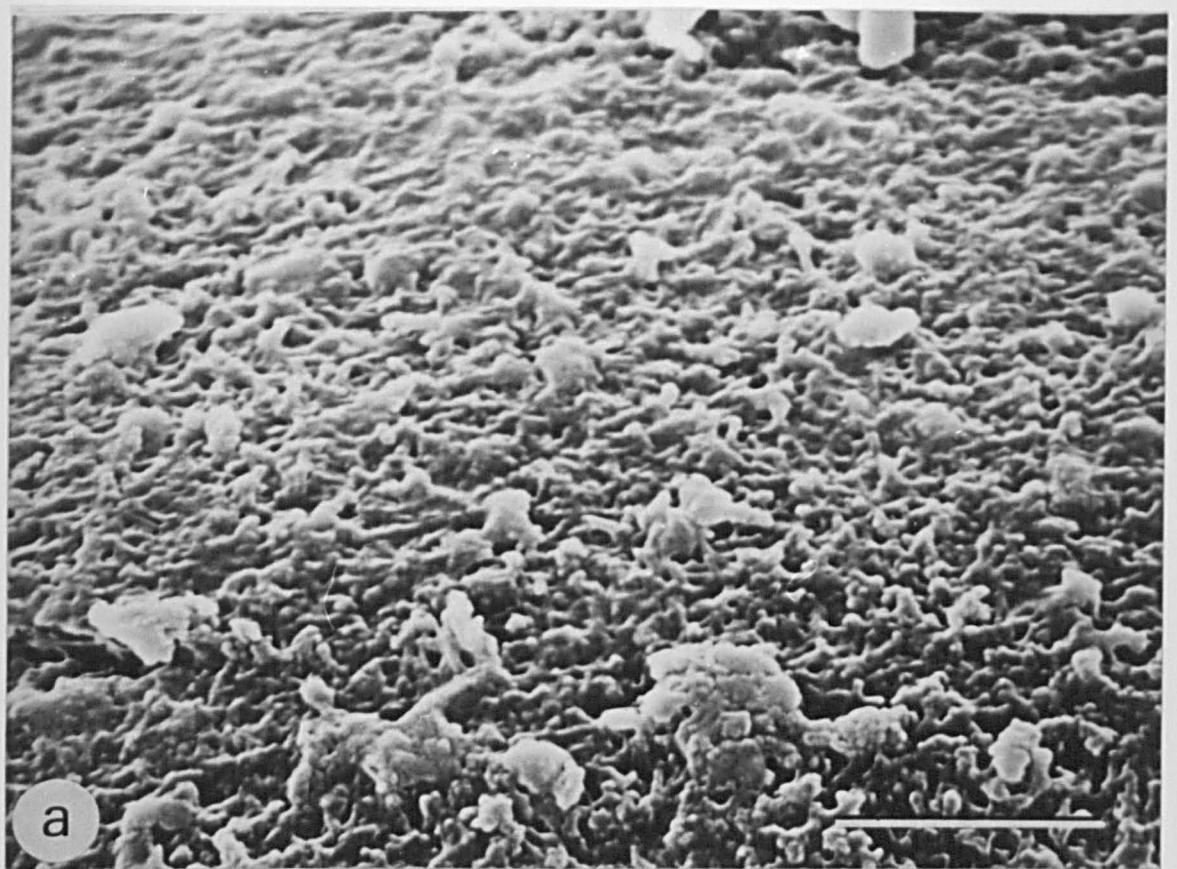


FIGURE 4.21

The A.microphylla type

(a-d) SEM's illustrating general morphology of megaspore apparatuses and sporoderm sculpturing at low magnification. Note the influence of variable quantities of infrafilosum, also the obvious extension of the collar between the floats in the cusp region.

(a) Note the presence of large foveae in the distal sporoderm surface.

(b) Infrafilosum almost completely absent.

(LF = large fovea; cc = cusp of collar; Scale bar = 100 $\mu$ m).

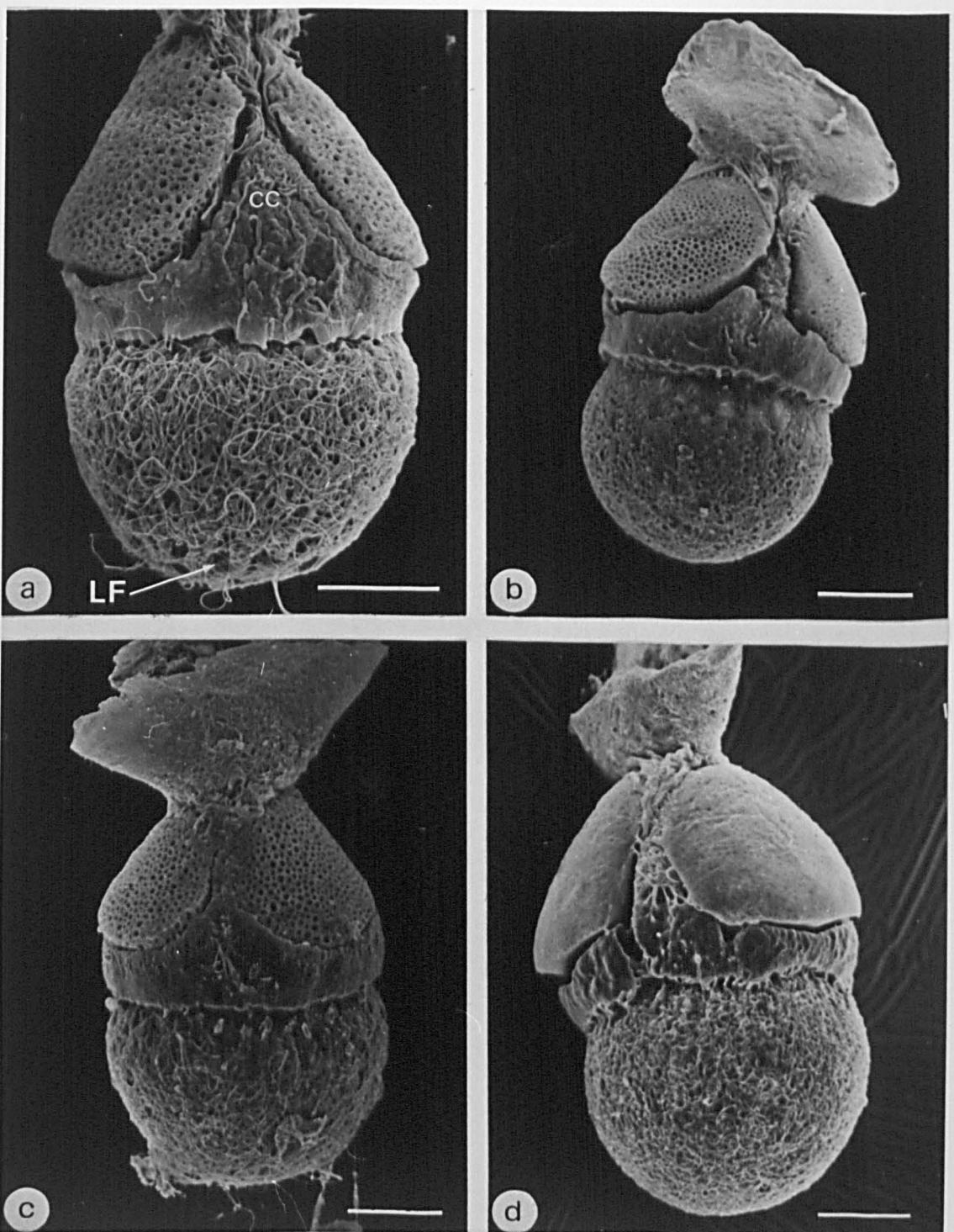


FIGURE 4.22

The A.microphylla type

- (a) SEM illustrating puncturing of the float surface.
- (b-d) SEM's illustrating variation in L.S. collar morphology. Note the low origin of the flange and the collar angle relative to the equatorial axis of the megaspore apparatus.
- (b) L.S. of collar in mid-float region.
- (c) L.S. of collar in mid-float region.
- (d) L.S. collar near the cusp region.
- (f = flange; bar = equatorial axis; Scale bar = 25 $\mu$ m).

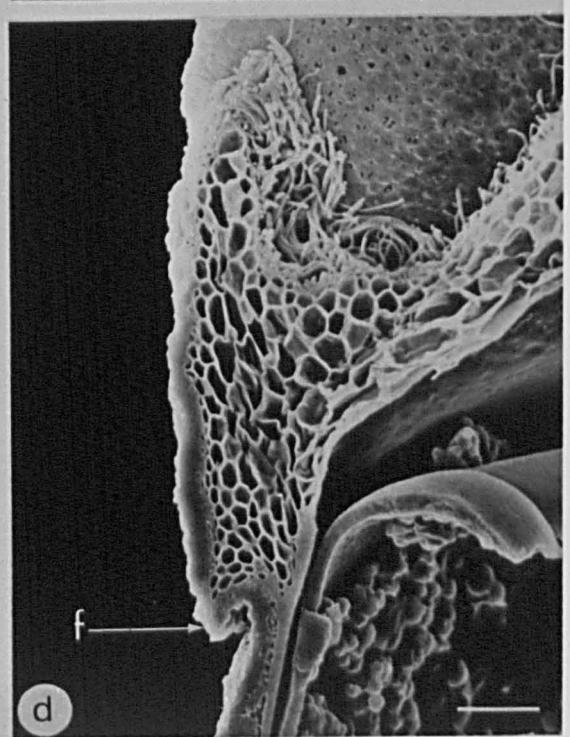
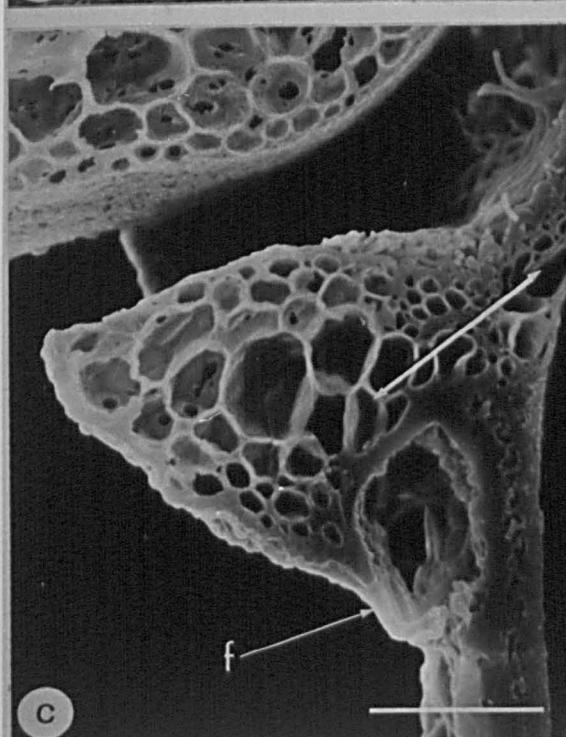
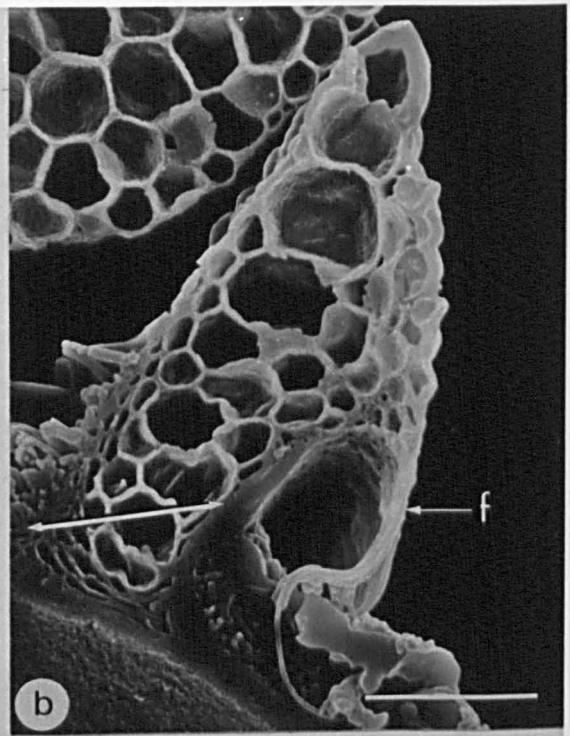
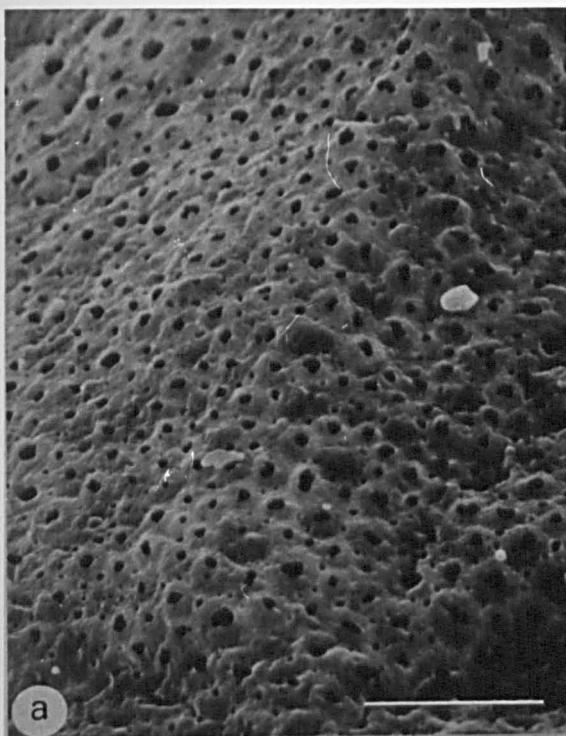


FIGURE 4.23

The A.microphylla type

SEM's illustrating variation in sporoderm sculpturing viewed at high magnifications. Note the effect of variable quantities of infrafilosum and the degree of fusion of the surface elements.

- (a) Little infrafilosum obscuring sculpturing. (Scale bar = 50 $\mu$ m).
- (b) Infrafilosum obscuring relatively unfused surface elements. (Scale bar = 50 $\mu$ m).
- (c) Large foveae in distal sporoderm surface, some small foveae obscured by infrafilosum. (Scale bar = 50 $\mu$ m).
- (d) Surface elements showing fusion. (Scale bar = 50 $\mu$ m).
- (e) Considerable fusion of surface elements resulting in few small foveae. (scale bar = 50 $\mu$ m).
- (f) Considerable fusion of surface elements. Filaments of infrafilosum can be seen emerging from small foveae (arrows). (Scale bar = 10 $\mu$ m).

(LF = large fovea; SF = small fovea)

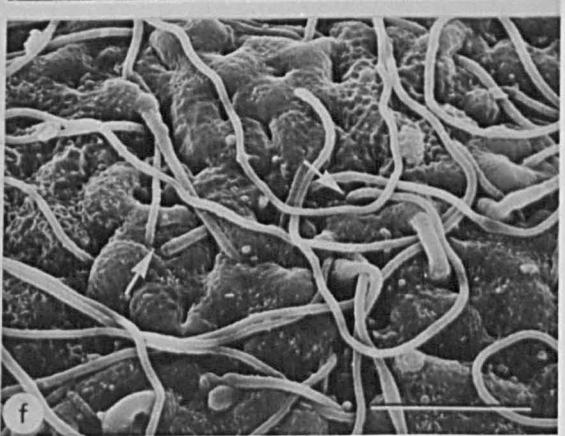
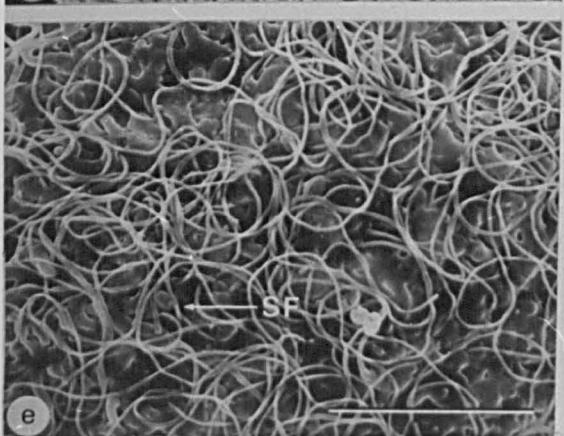
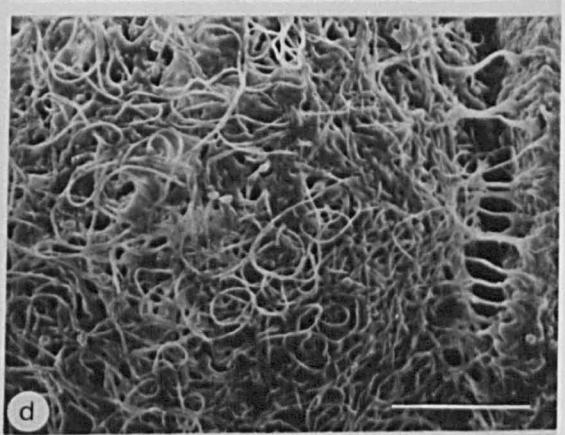
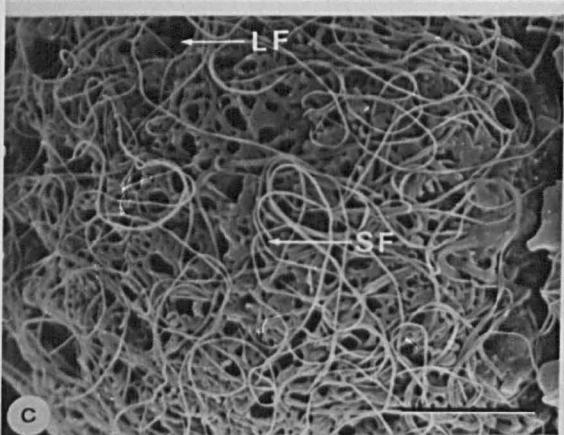
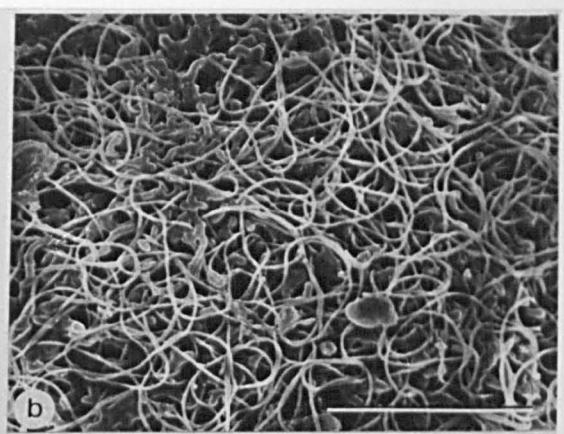
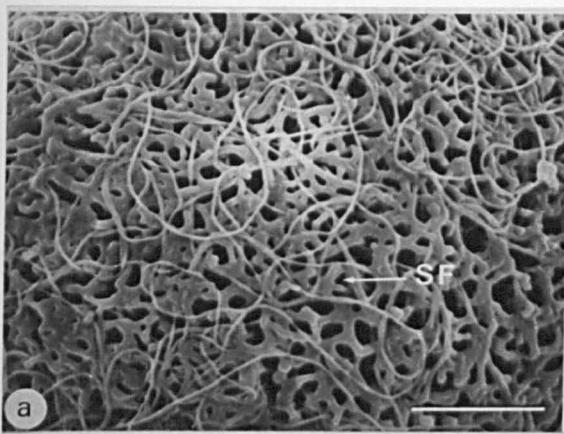


FIGURE 4.24

The A.microphylla type

SEM's illustrating variation in sporoderm structure.

Note the variation in respect to the fusion of exoperine 2 elements.

- (a) Illustration of the relationship between sporoderm structure and sculpturing. The endoperine intrusion forming a raised area is uncommon. Exoperine 2 columellae relatively unfused. (Scale bar = 10  $\mu\text{m}$ ).
- (b) Some fusion of exoperine 2 columellae. Note also exospore surface. (Scale bar = 5 $\mu\text{m}$ ).
- (c) Much fusion of exoperine 2 columellae. Note the dense nature of the endoperine and the nature of the exospore. (Scale bar = 5 $\mu\text{m}$ ).
- (d) Sporoderm structure towards the collar. Note how the exoperine thins and becomes solid in this region. (Scale bar = 25 $\mu\text{m}$ ).

(ex = exospore; en = endoperine; eni = endoperine intrusion; ep.1 = exoperine 1; ep.2 = exoperine 2; LF = large foveae; C = collar; ep3 = exoperine 3)

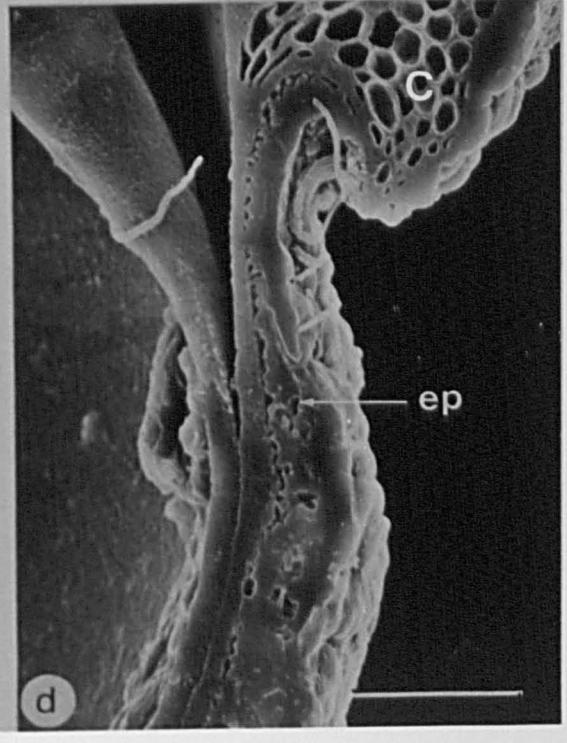
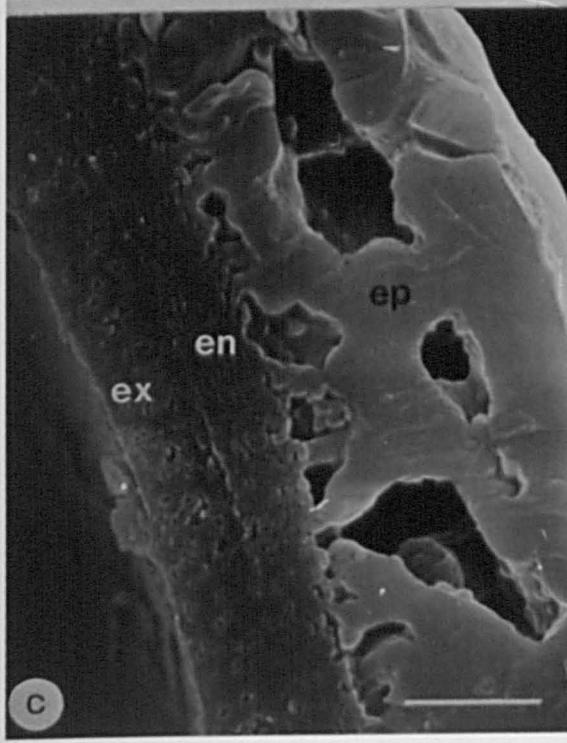
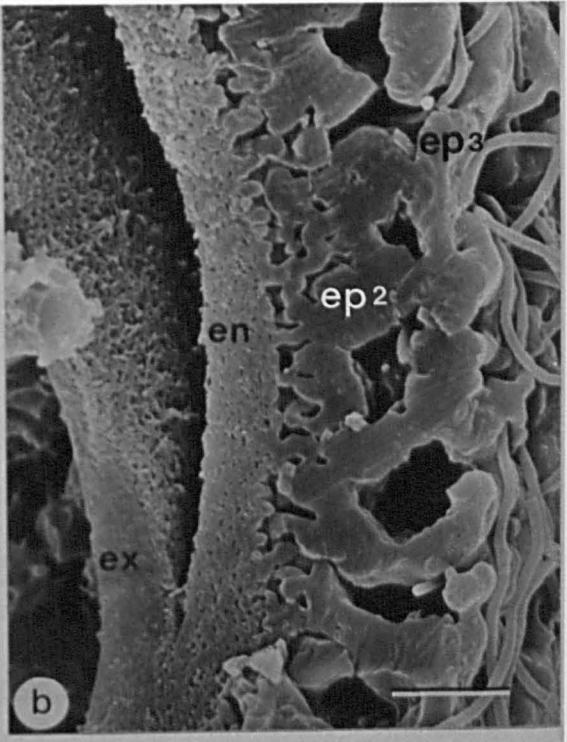
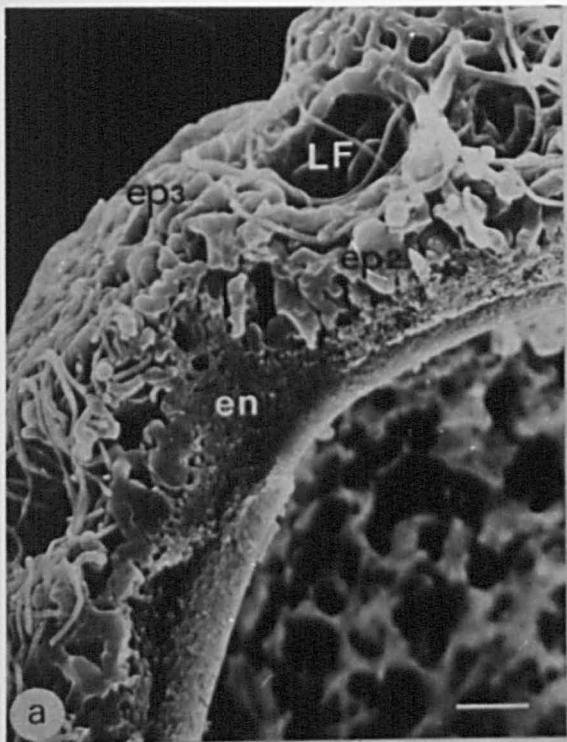


FIGURE 4.25

The Azolla sp. type

(a-d) SEM's illustrating general morphology of megaspore apparatuses and sporoderm sculpturing at low magnification. Note the amount of filosum covering the sporoderm and collar surfaces. (Scale bar = 100  $\mu\text{m}$ ).

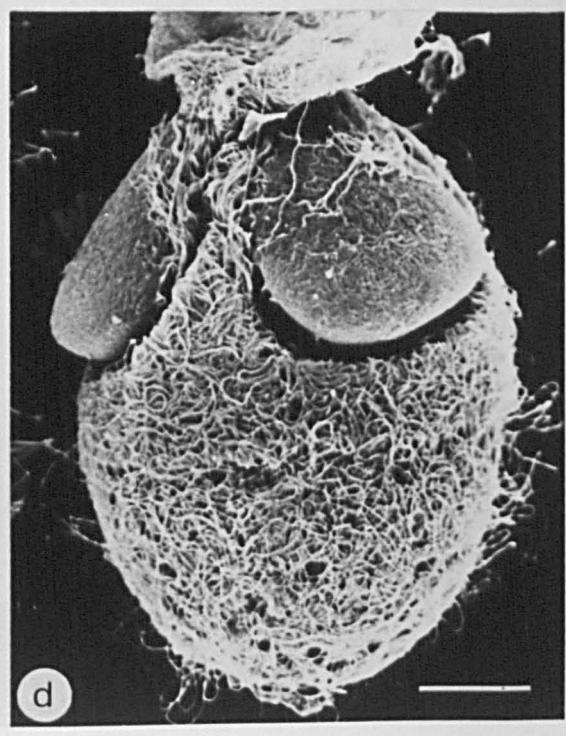
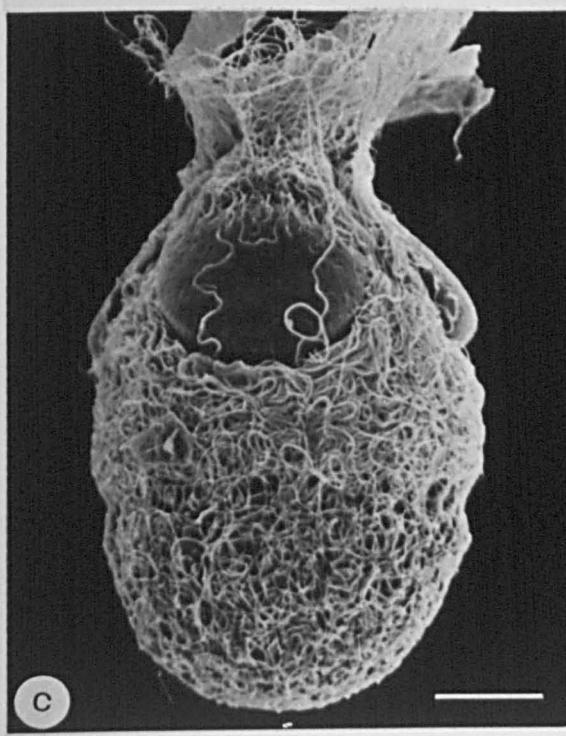
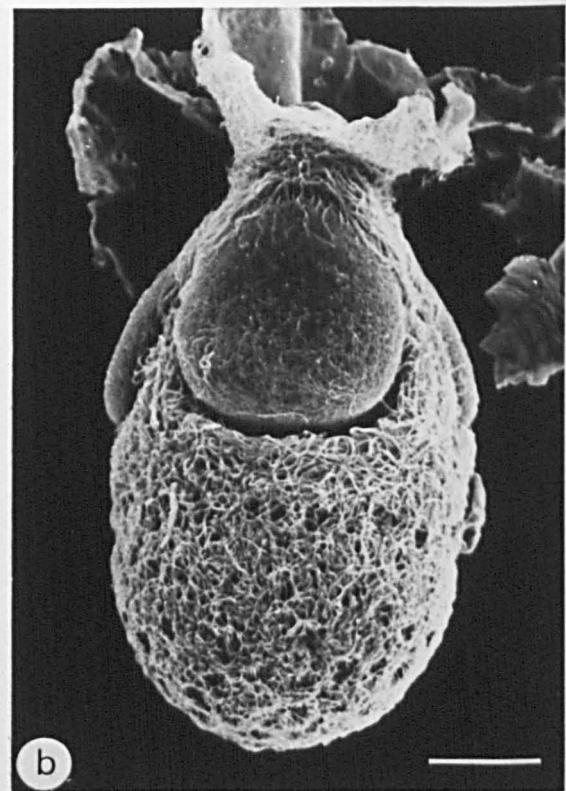
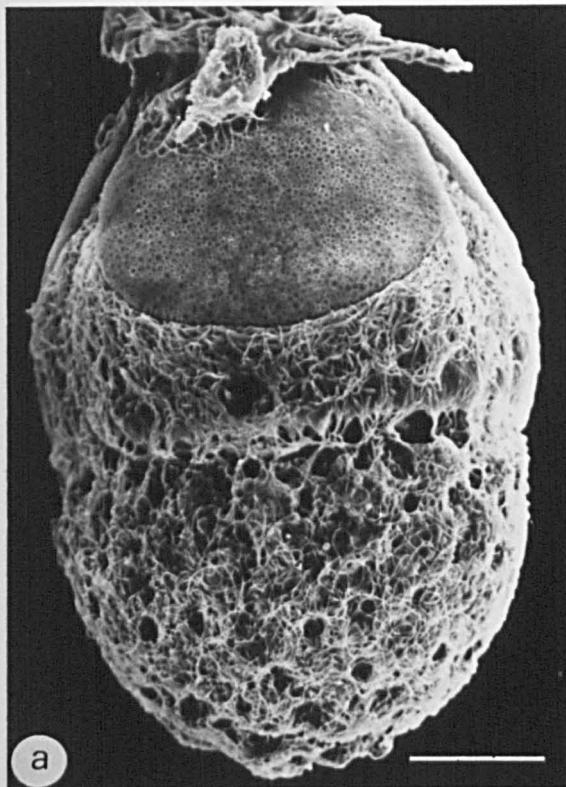


FIGURE 4.26

The Azolla sp. type

(a-c) SEM's illustrating L.S. of the collar and differences in its robustness.

(a) L.S. collar towards the cusp. Note the position of the flange.  
(Scale bar = 25 $\mu$ m).

(b) L.S. collar in the mid-float region. Note the position of the flange. (Scale bar = 25 $\mu$ m).

(c) Unusually robust collar apparently lacking a flange. This was common in specimens from Holland. (Scale bar = 25 $\mu$ m).

(d) Exospore sculpturing. (Scale bar = 5 $\mu$ m).

(f = flange)

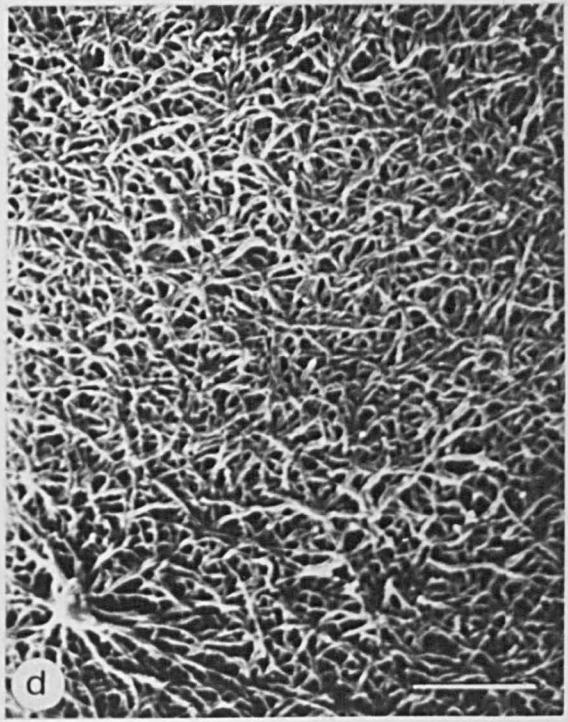
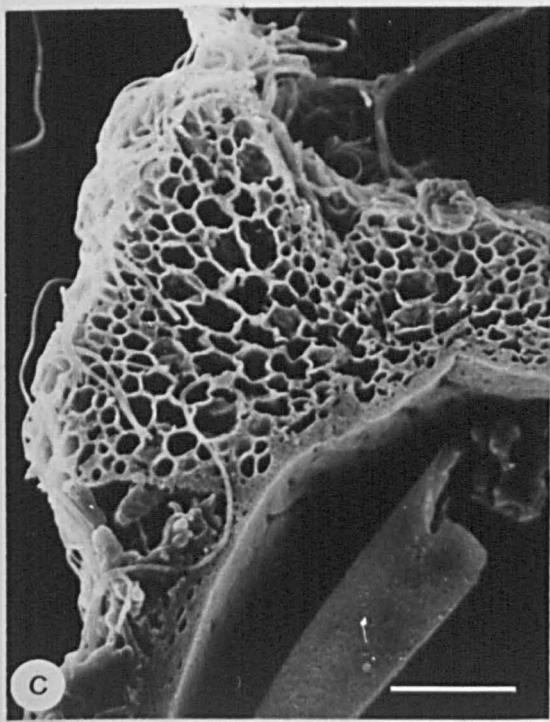
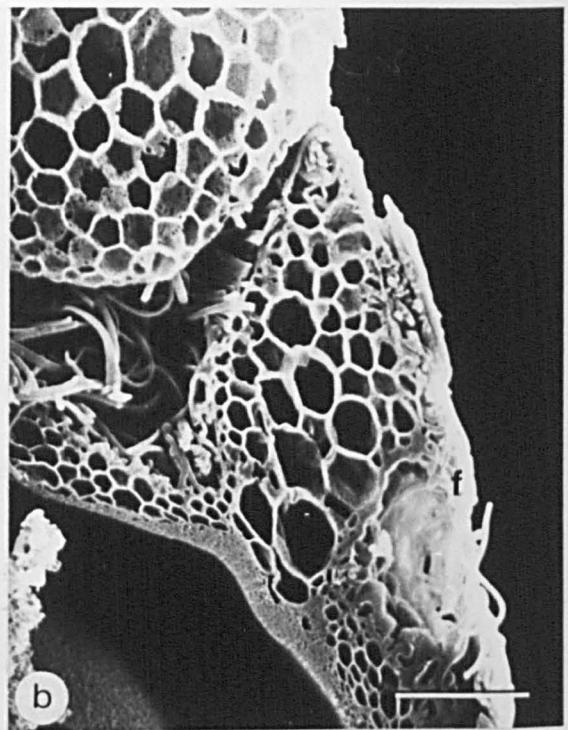


FIGURE 4.27

The Azolla sp. type

SEM's illustrating sporoderm and float sculpturing at high magnification. Note the effect of infrafilosum quantity in respect to obscuring the sculpturing.

- (a) Little infrafilosum present and the sculptural elements can be seen.
- (b) Sporoderm surface.
- (c) Sporoderm surface.
- (d) Much infrafilosum obscuring the sculptural elements and small fovea, however large foveae can be seen.
- (e) Float surface.
- (f) Float surface.

(LF = large fovea; SF = small fovea; Scale bar = 25 $\mu$ m)

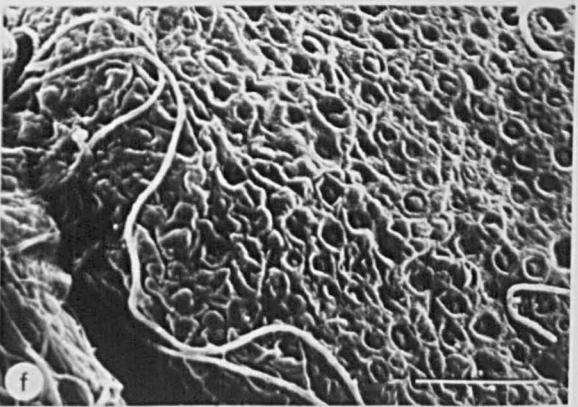
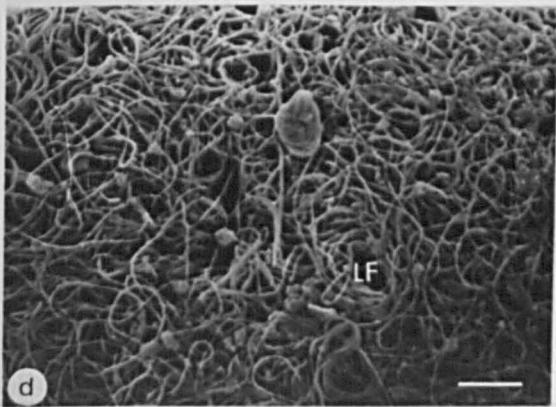
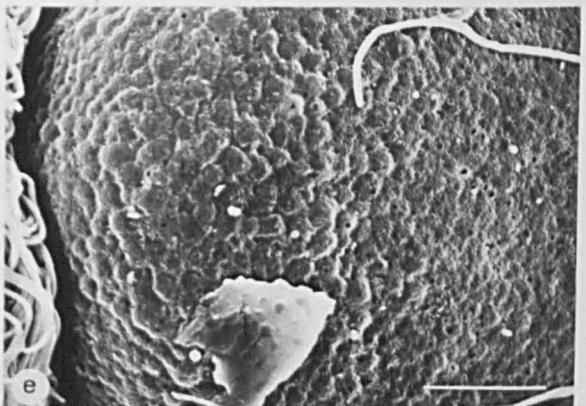
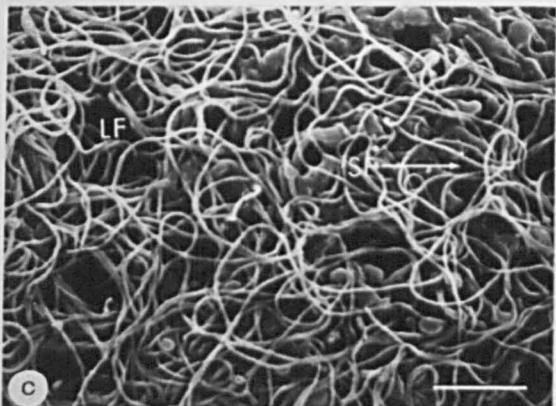
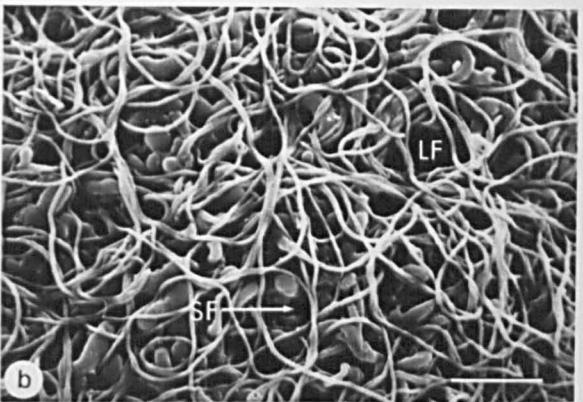
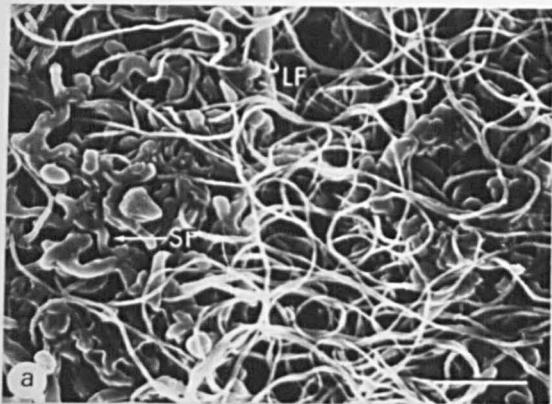


FIGURE 4.28

The Azolla sp. type

SEM's illustrating sporoderm structure. (a-c) note the alveolate endoperine. ( d & e) are ~~a~~ typical of this type and are from a single population (B48).

(a) Alveolate endoperine and columellate exoperine 2. (Scale bar = 25 um).

(b) Alveolate endoperine intruding into the exoperine 2 and forming a fovea. (scale bar = 10um).

(c) Alveolate endoperine, nature of the exoperine 1 and structure of the exospore. (Scale bar = 5um).

(d) Note the two endoperine zones (arrows). (Scale bar = 10um).

(e) Note the endoperine forming an excrescence. (Scale bar = 10um).

(ex = exospore; en = endoperine; ep.1 = exoperine 1; ep.2 = exoperine 2; ep = exoperine; E = excrescence).

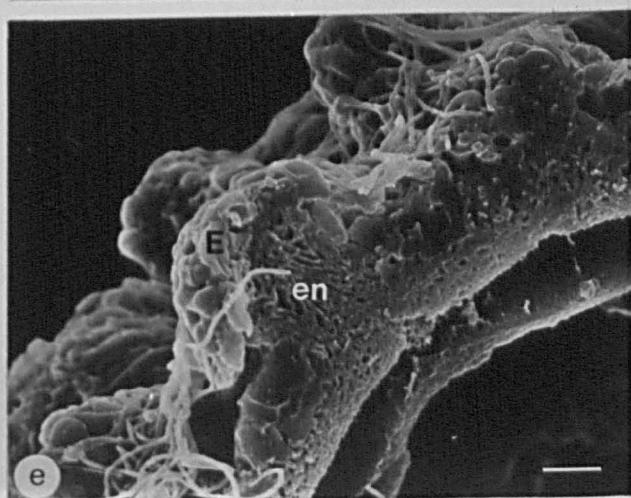
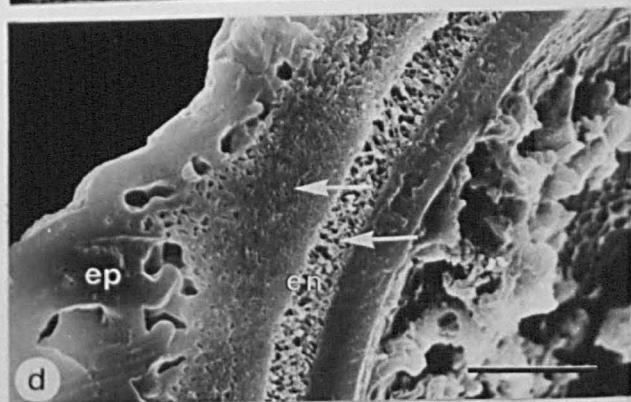
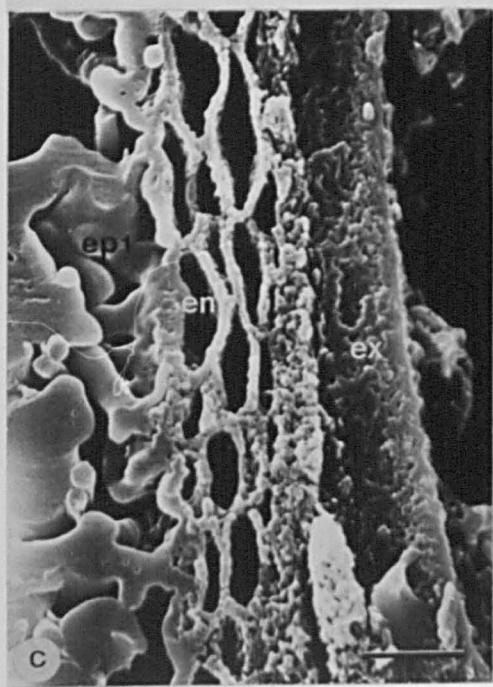
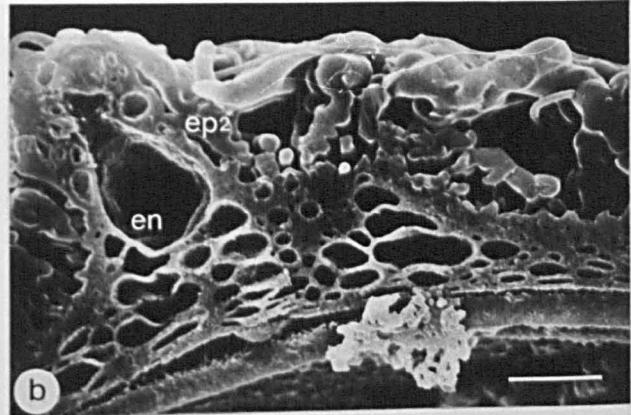
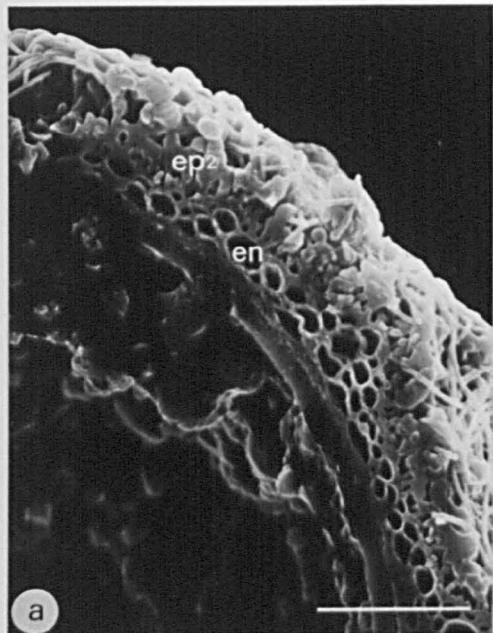


FIGURE 4.29

The Azolla sp. type

SEM's illustrating variation in sporoderm structure.

Note variation in the exoperine 2 and alveolation of the endoperine.

(a) Exoperine 2 solid and variation in its thickness forming a large fovea.

(b) Columellae of exoperine 2 discernible and variation in thickness of this zone forming a large fovea.

(c) A single alveolus in the endoperine forming an intrusion.

Exoperine 2 of short columellae (exospore not included).

(d) Several alveolae forming an endoperine intrusion. Exoperine 2 of irregularly arranged columellae.

(ep.2 = exoperine 2; LF = large fovea; a = endoperine alveolus; Scale bar = 10 $\mu$ m).



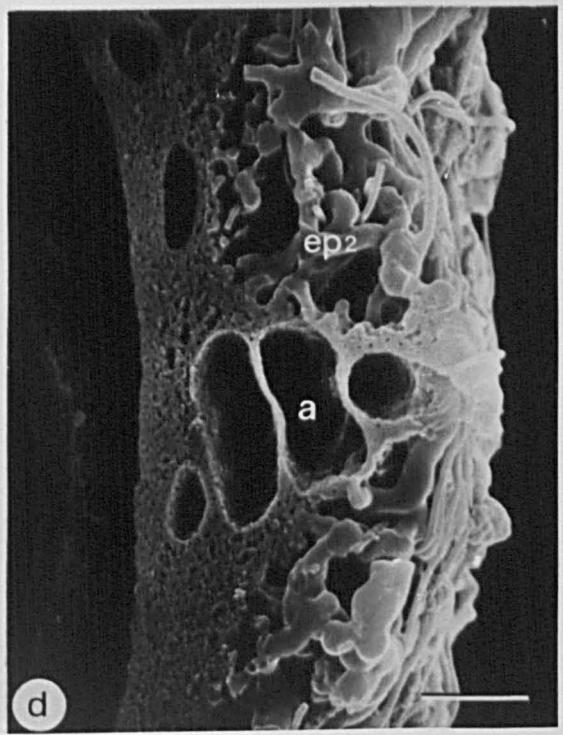
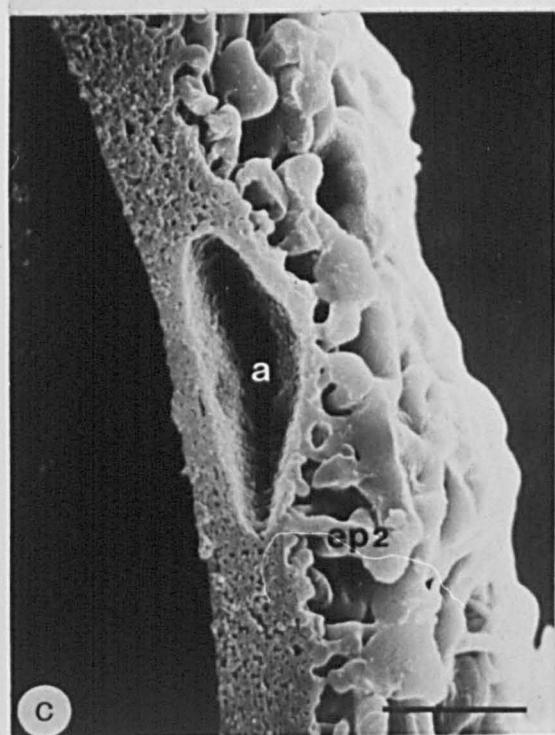
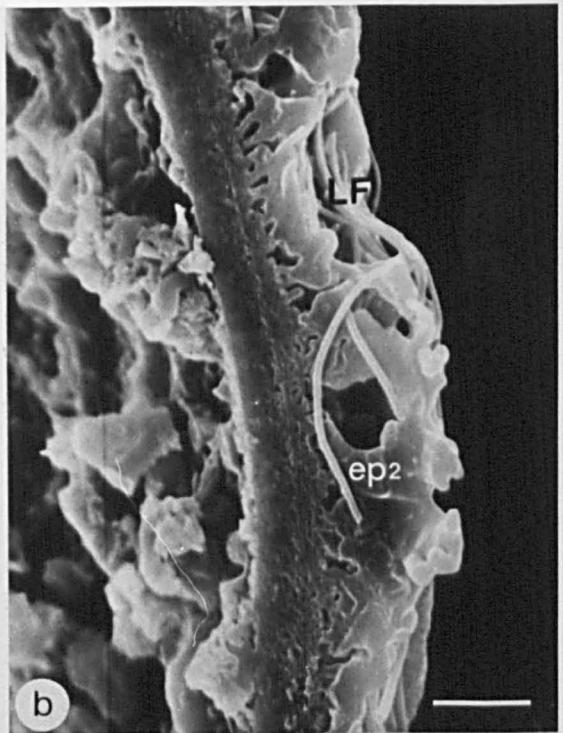
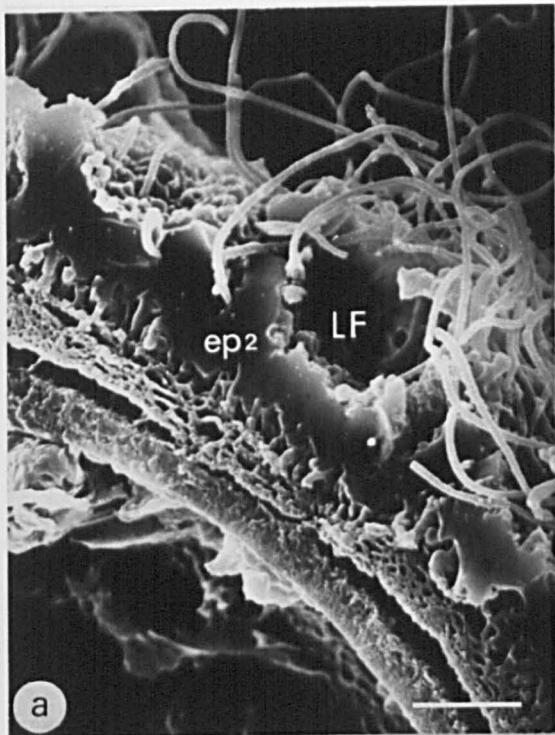


FIGURE 4.30

Massulae

(a) SEM of massulae from a microsporangium. The localisation of glochidia can be seen on the internal massula surfaces. (Scale bar = 25um).

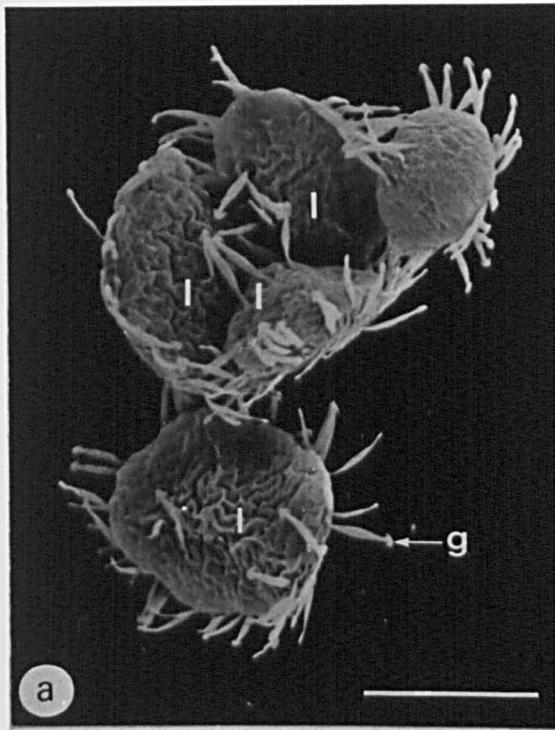
(b) SEM of the external surface of a massula of the *A.mexicana* type. Note the hollows in the massula surface which are characteristic of this megaspore apparatus type. (Scale bar = 25um).

(c) SEM of the internal surface of a massula of the *A.filiculoides* type. The relatively smooth surface is often contorted during drying. Note the more or less central group of glochidia (arrow). (scale bar = 25um).

(d) SEM of the external massula surface of *A.filiculoides* subtype *rubra* showing blunt protuberances (arrow) which give the massula surface a granular appearance. (Scale bar = 100um).

(e) High magnification of (d) illustrating the protuberances. (scale bar = 5um).

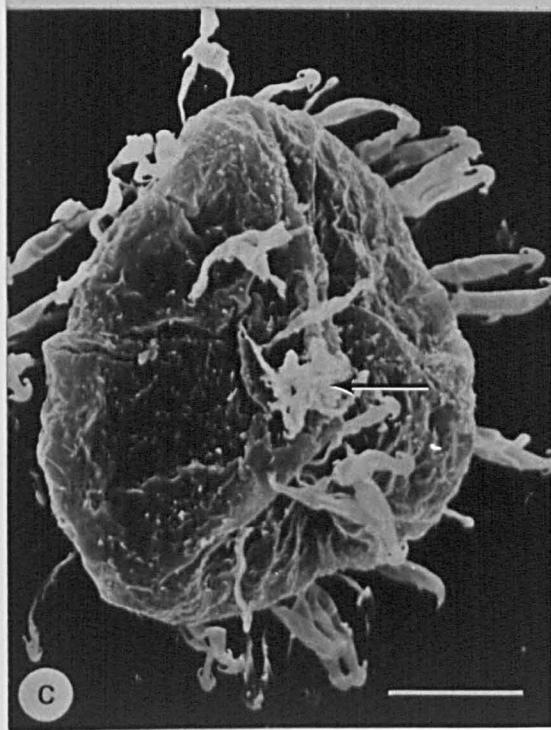
(g = glochidium; h = hollow; I = internal massula surface)



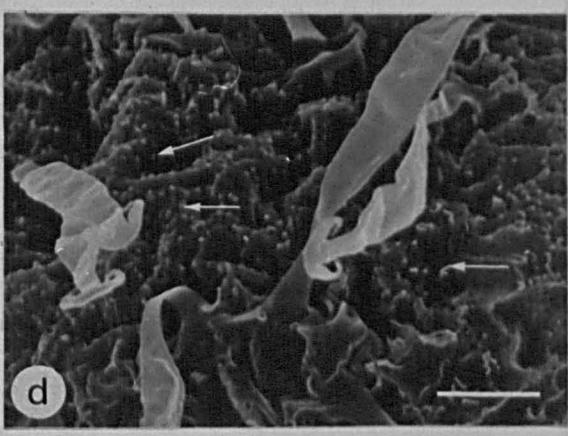
a



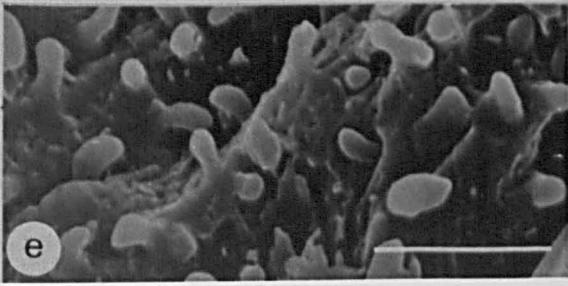
b



c



d



e

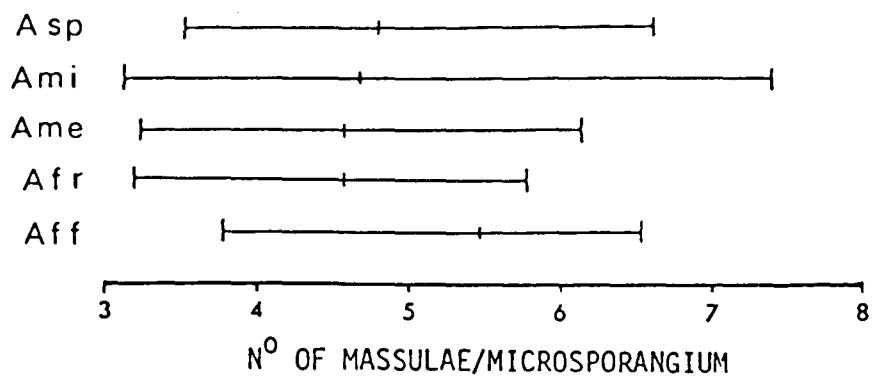
FIGURE 4.31

(a) Showing the population mean range and mean number of massulae per microsporangium in the megasporangium apparatus types.

(A.sp = *Azolla* sp. type; A.mi = *A.microphylla* type; A.me = *A.mexicana* type; A.fr = Australian populations of the *A.filiculoides* type; A.ff = American and European populations of the *A.filiculoides* type)

(b) Scatter plot of number of massulae per microsporangium against number of microspores per massulae together with the 'best fit' regression line.

a



b

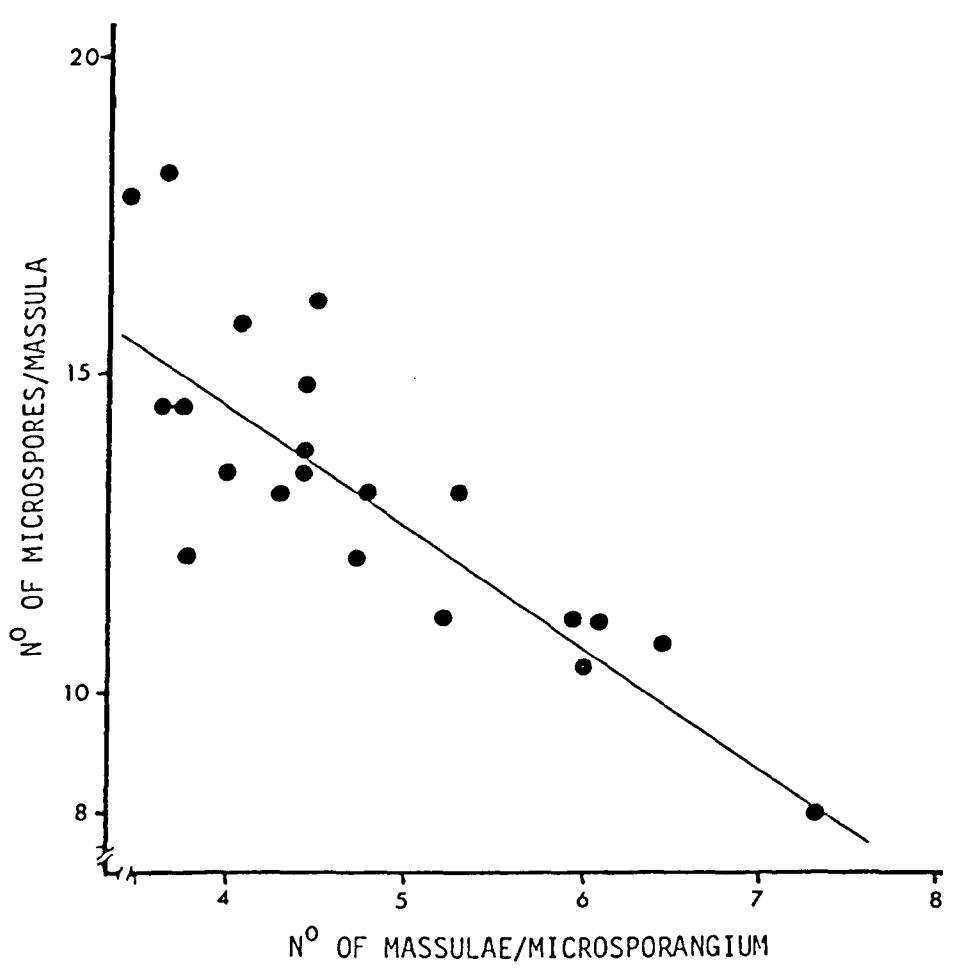
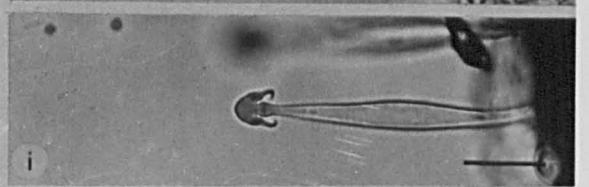
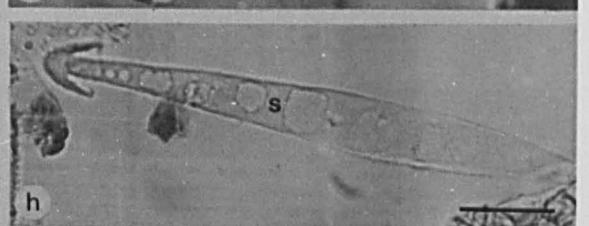
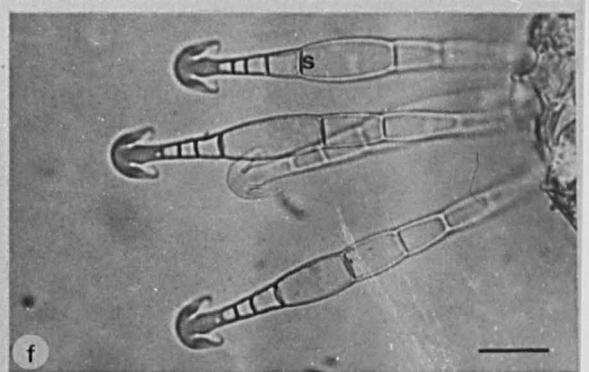
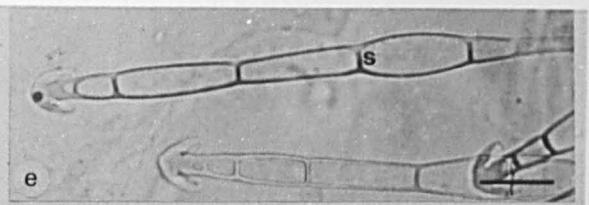
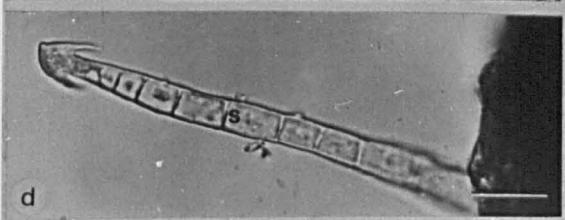
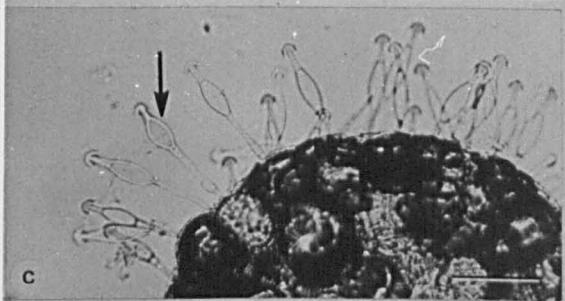
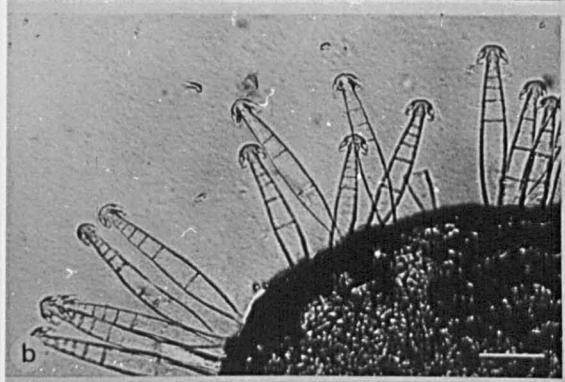
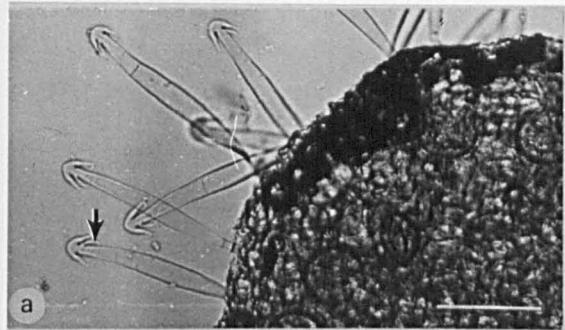


FIGURE 4.32

Glochidia

- (a) L.M. of non-septate and one and two septate glochidia found in the *A.filiculoides* subtype *filiculoides*. Note the apical and basal tapering of the glochidial shaft. The extra apical fluke is rare and occurs in all types (arrow). (Scale bar = 25 $\mu$ m).
- (b) L.M. of septate glochidia found in *A.filiculoides* subtype *rubra*. Note the apical and basal tapering of the glochidial shaft. (Scale bar = 25 $\mu$ m).
- (c) L.M. of glochidia of the *A.mexicana* type. They are atypical because of the apical dilation of the glochidial vacuole (arrow). (Scale bar = 25 $\mu$ m).
- (d) L.M. of a septate glochidium of the *A.mexicana* type with parallel sided walls of the glochidial shaft. (Scale bar = 10 $\mu$ m).
- (e) L.M. of septate glochidia of the *A.mexicana* type. (Scale bar = 10 $\mu$ m).
- (f) L.M. of septate glochidia of the *A.microphylla* type with apical and basal tapering of the glochidial shaft. (Scale bar = 10 $\mu$ m).
- (g) L.M. of septate glochidia of the *A.microphylla* type. The septa are basal and easily overlooked unless the whole glochidium can be viewed. (scale bar = 10 $\mu$ m).
- (h) L.M. of a glochidium of the *Azolla* sp. type from Holland. The septa are very wide, the glochidial shaft appearing to be composed of many small vacuoles. (Scale bar = 10 $\mu$ m).
- (i) L.M. of a glochidium of the *Azolla* sp. type, illustrating how diminutive the glochidia can be. (Scale bar = 10 $\mu$ m).

(S = septum)



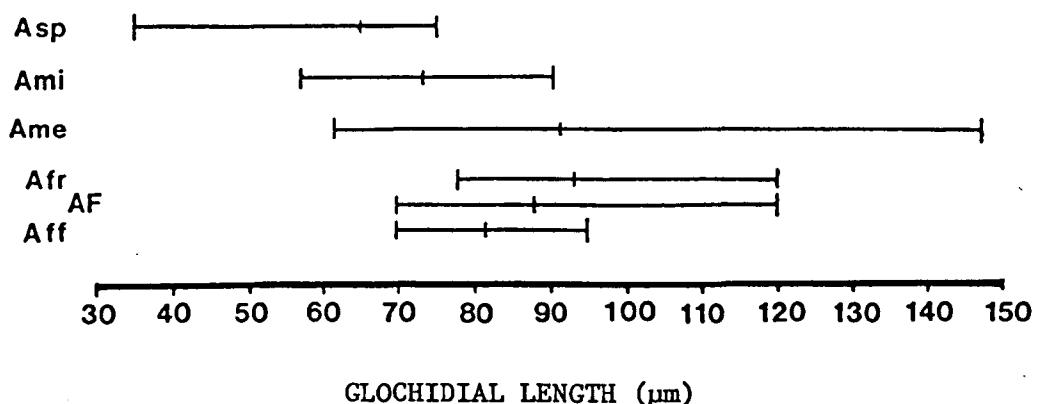


FIGURE 4.33

Showing the range and mean of glochidial length ( $\mu\text{m}$ ) in the megaspore types. (Ranges from population means, and the means are grand means for each type)

(Asp = the *Azolla* sp. type; Ami = the *A.microphylla* type; Ame = the *A.mexicana* type; AF = the *A.filiculoides* type; Afr = Australian populations of the *A.filiculoides* type; Aff = American and European populations of the *A.filiculoides* type)

FIGURE 4.34

(a) Histogram illustrating glochidial septation of the *A.filiculoides* type.

(b) Histogram illustrating glochidial septation of the *A.filiculoides* type with the populations divided into two geographical groups, namely American and European populations and Australian populations.



= American and European populations

(No. of populations = 35)

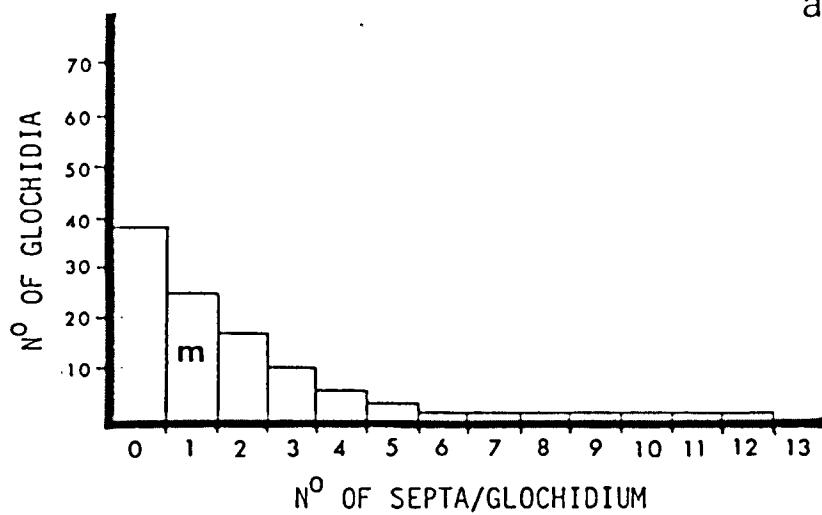


= Australian populations

(No. of populations = 22)

(m = mean class)

a



b

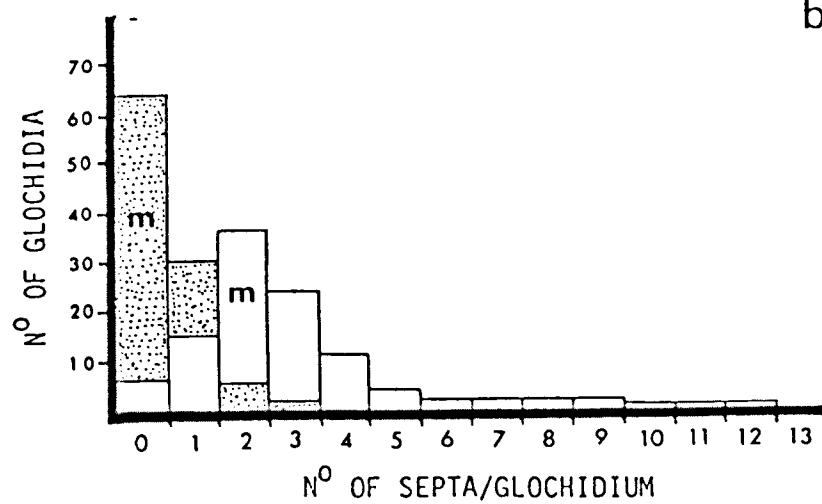


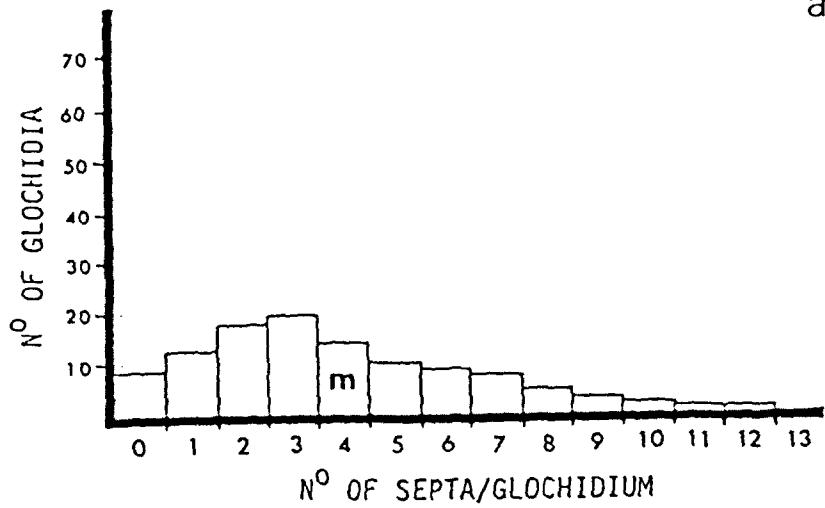
FIGURE 4.35

(a) Histogram illustrating glochidial septation in the *A.mexicana* type. ( $m$  = mean class).

(b) Map of Mexico and USA showing the distribution of possible subtypes of the *A.mexicana* type.

(- =  of Figure 4.36a; + =  of Figure 4.36a;  $\circ$  =  of Figure 4.36b;  $\Delta$  =  of Figure 4.36b;  $\square$  =  of Figure 4.36b;  $\bullet$  =  of Figure 4.37a;  $\blacktriangle$  =  of Figure 4.37a;  $\blacksquare$  =  of Figure 4.37b;  $x$  =  of Figure 4.37b)

a



b

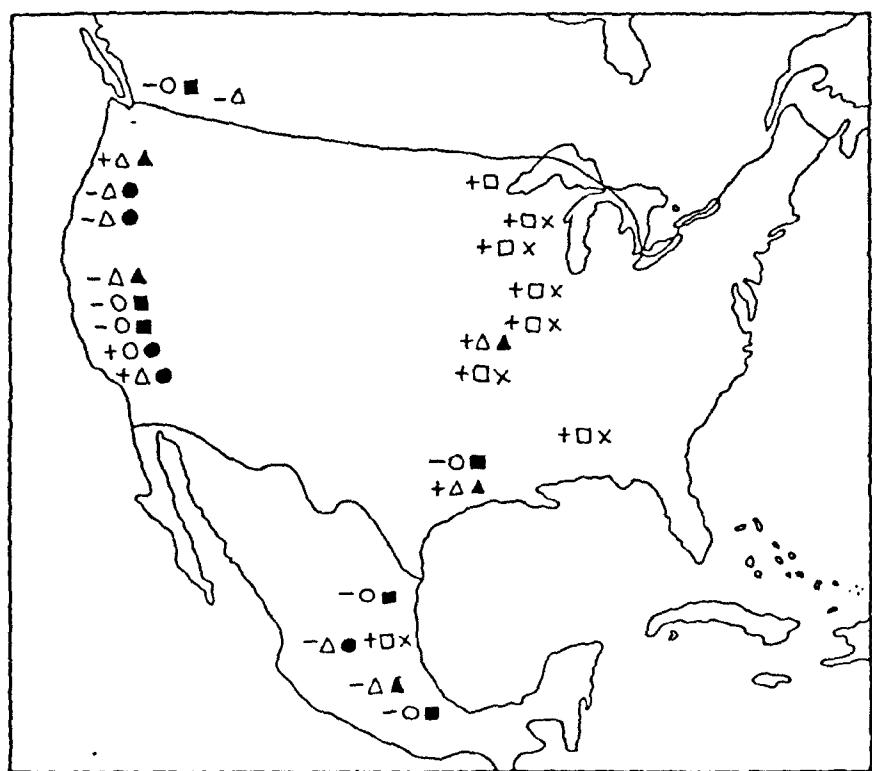


FIGURE 4.36

(a) Histogram illustrating glochidial septation of the *A.mexicana* type with the populations divided into two groups. The groups being defined by those populations with a mean of less than three (-) and those with a mean of more than three (+).



= - (number of populations = 13)



= + (number of populations = 11)

(m = mean class)

(b) Histogram illustrating glochidial septation of the *A.mexicana* type with the populations divided into three groups. The groups being defined by mean number of septa two or less (O), all glochidia with at least one septum (Δ) and mean number of septa more than 5 (□).



= O (number of populations = 10)

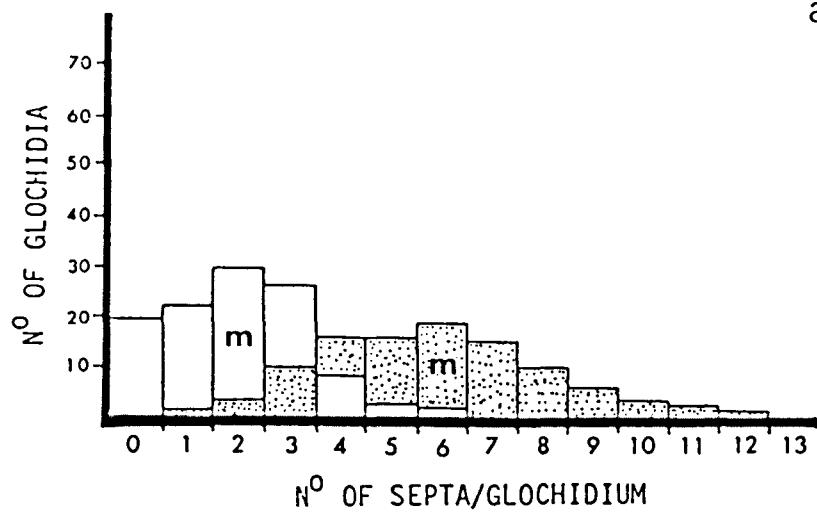


= Δ (number of populations = 10)



= □ (number of populations = 9)

a



b

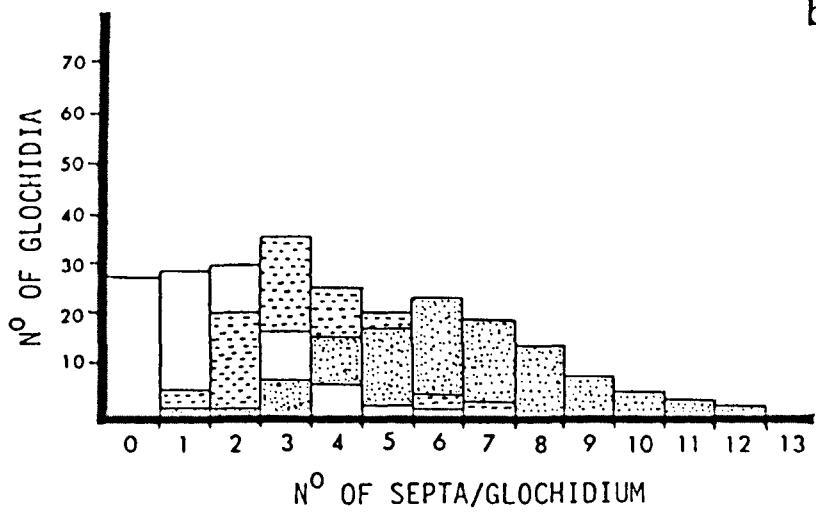


FIGURE 4.37

(a) Histogram illustrating two of four subtypes of the *A.mexicana* type in respect to glochidial septation.



= populations with at least one septum in some glochidia, but the mean number of septa being five. (No. of populations = 7).



= populations with at least two septa in some glochidia, but the mean number of septa being five. (No. of populations = 4).

(m = mean class, which is the same for each of the two subtypes)

(b) Histogram illustrating two of four subtypes of the *A.mexicana* type in respect to glochidial septation.



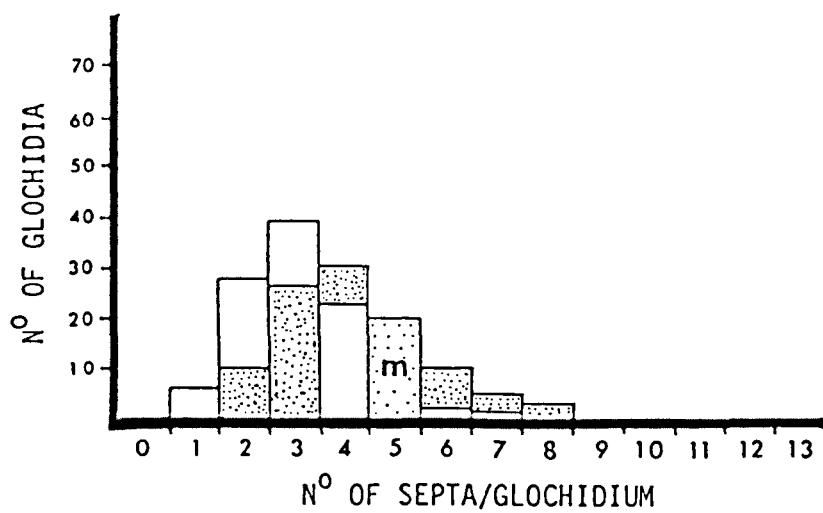
= populations with at least some glochidia without septa. (No. of populations = 10).



= populations with mean number of septa being six or more. (No. of populations = 9).

(m = mean class)

a



b

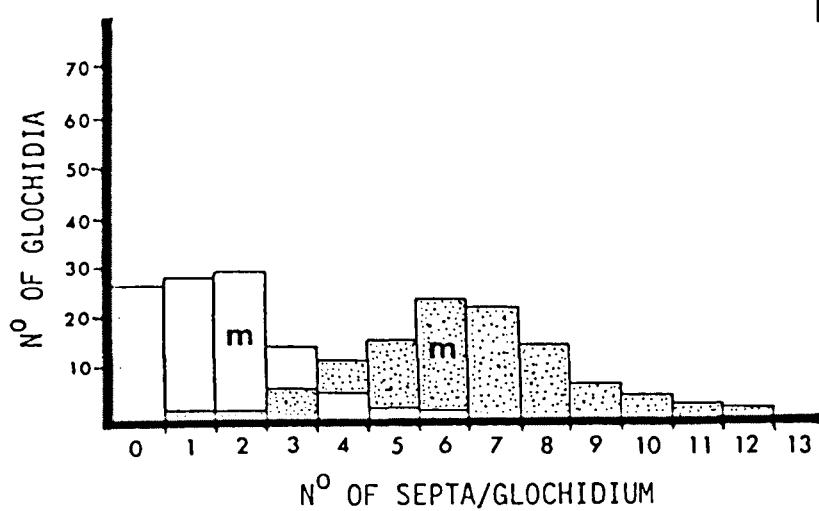


FIGURE 4.38

(a) Histogram illustrating glochidial septation of the *A.microphylla* type.

(b) Histogram illustrating glochidial septation of the *A.microphylla* type with the populations divided into two groups. The groups defined by at least some glochidia without septa, and all glochidia with at least one septum.



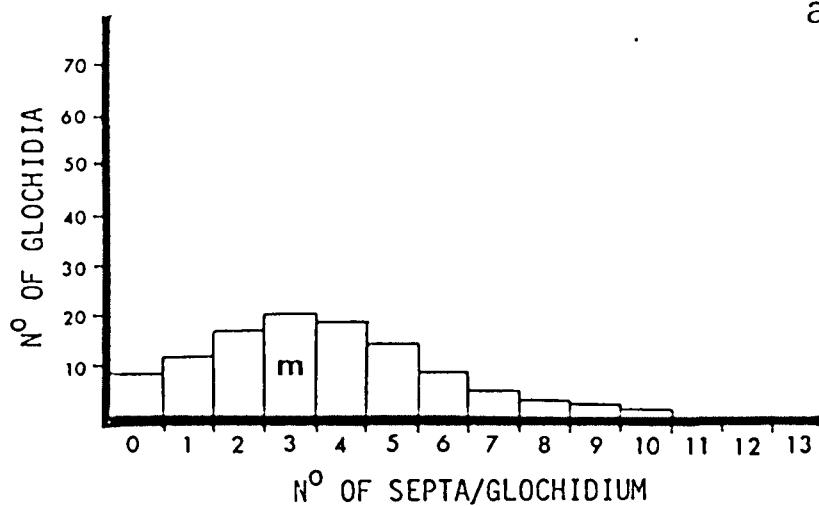
= populations with at least some glochidia without septa. (No. of populations = 9).



= populations with all glochidia with at least one septum. (No. of populations = 14).

(m = mean class)

a



b

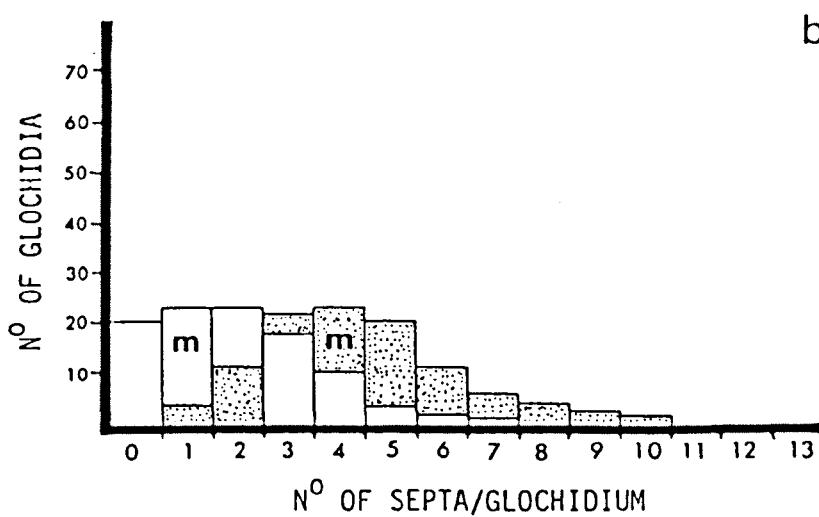


FIGURE 4.39

(a) Histogram illustrating glochidial septation of the **Azolla** sp. type. (No. of populations = 12).

(b) Histogram illustrating glochidial septation of the **Azolla** sp. type with the populations divided into two groups.



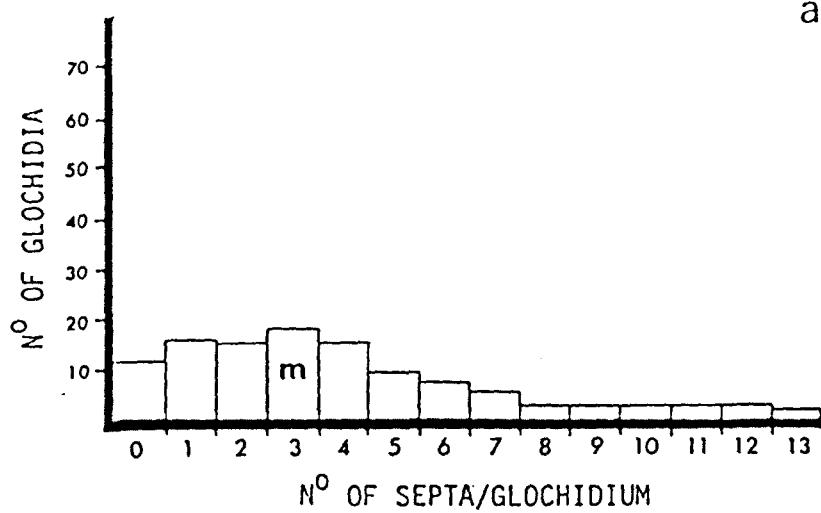
= American populations. (No. of populations = 10)



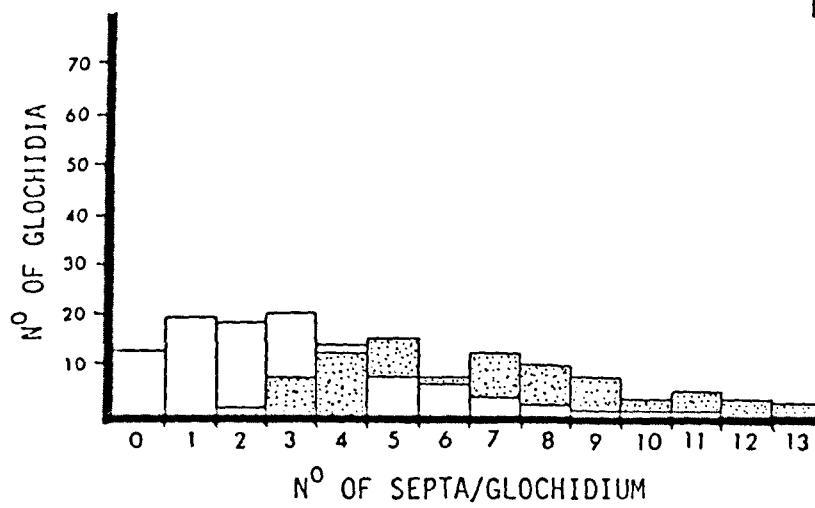
= Dutch populations. (No. of populations = 2)

(m = mean class)

a



b



4.4.1 Fronds

Frond Habit: This refers to the overall nature of the fronds in a population. In herbarium samples it was not possible to observe the orientation of fronds to the water surface. However, living populations attributed to the *A.filiculoides* type at IRRI, Fuzhou and Portsmouth became vertical, particularly when over-crowded. At Portsmouth, some populations of this type did not become vertical at all. At IRRI, several populations attributable to the *A.microphylla* type also exhibited vertical frond habit; this was particularly evident in IRRI 421 from the Galapagos Islands. Horizontal frond orientation was observed in uncrowded populations of the *A.filiculoides* and *A.microphylla* types and in all other types, even when over-crowded (Fig.4.40). Disturbance of the water surface often caused horizontal fronds to assume a near vertical orientation, however, frond shape and branching were not consistent with true vertical habit. In horizontal overcrowded populations fronds may grow in layers, whereas in the vertical form, fronds were clearly more or less perpendicular to the water surface and have a different branching pattern. These results are summarised in Table 4.13.

Frond Shape: In all the types in Section **Azolla** the branching pattern was based on a flabellate dichotomous system, and frond shape was related to the relative elongation of bifurcations. Isotomous dichotomies gave rise to rounded or elliptical fronds whereas, anisotomous dichotomies gave rise to deltoid shaped

fronds (Figs. 3.11, 4.41). The latter was often obscured by elongation of the interbranches and branches. Similarly, fragmentation also obscured frond shape in both isotomous and anisotomous forms. Although all types were found with elliptical fronds, only the *A.filiculoides* and *A.microphylla* types were observed with elongated-deltoid fronds, and hence anisotomous dichotomies, which very occasionally appeared sub-pinnate. This was usually, but not always, associated with sporulation. Compared with populations of the *A.filiculoides* type from other regions, Australian populations of this type commonly fruited when in the elliptical form. The deltoid shape was commonly obscured in the *A.filiculoides* type because elongation of interbranches and branches had occurred. This elongation was associated with vertical frond habit in living populations, however, in the deltoid form the tendency to become vertical could be recognised. These results are summarised in Table 4.13.

Frond size: In herbarium and living populations of *Azolla* frond size varied from ca 5mm to ca 80mm. Populations of the *A.filiculoides* and *A.microphylla* types often attained the larger sizes. Frond size was a difficult character to measure accurately because frond fragmentation rendered size impossible to define accurately. In living populations frond size varied considerably between environments and season. Evidence from this study suggested that frond habit, branching pattern and size were probably environmentally influenced. Furthermore, frond habit and branching were closely associated, i.e. vertical fronds were usually deltoid or elongated deltoid in shape and branching was

apparently lateral, or as in IRRI 421, sub-pinnate. Alternatively, horizontal fronds were usually rounded or elliptical and branching was isotomous. Some herbarium populations of the *A.filiculoides* type exhibited the whole range of morphologies, as did a living population of this type grown at Portsmouth over a four month period. Therefore, vegetatively *Azolla* taxa may be indistinguishable. However, the *A.filiculoides* and *A.microphylla* types may be distinct from the other types under certain environmental conditions, but not necessarily from each other. Figure 4.41 illustrates the above observations relating to the morphological plasticity of *Azolla*. Table 4.13 summarises frond habit, shape and branching in most populations studied, and illustrates the overlap in character states which were continuously variable from one state to another.

Fronds may be observed with highly overlapping leaves (imbricate) through to little or no overlap of leaves (lax). Both elliptical and elongated fronds may be observed with this range of imbrication, and was not correlated with any taxon. In general, the older regions of a frond were more lax than the younger regions, and in vertical fronds of the *A.filiculoides* type the leaves were perhaps the most lax. Associated with each branch was a subtending leaf which often appeared to be larger than other leaves. For this reason these leaves were not measured. Excluding these subtending leaves, the number of leaves between branches was two or three in each type of Section *Azolla*.

Although apparently more interbranch leaves were found in some populations where branches had been lost.

#### 4.4.2 Leaves

Leaves of *Azolla* were bilobed, with each lobe possibly providing potentially useful characters. The ventral leaf lobe, which was only a few cells thick, was usually in contact with the water surface. In herbarium specimens this ventral lobe was usually highly contorted and often damaged. Even after soaking in tri-basic sodium phosphate solution (see section 2.3.2) for at least 12 hours the ventral leaf lobe could not be measured accurately enough to obtain meaningful data. In fresh specimens the ventral leaf lobe was either more or less flat, broadly ovate and mostly in contact with the water surface, or it was contorted such that only a small percentage of its lower surface was in contact with the water. This contorted shape of the ventral leaf lobe was usually associated with the deltoid frond form in the *A.filiculoides* and *A.microphylla* types. However, some herbarium populations indicated that contorted ventral leaf lobes may also occur in other taxa which only exhibit the elliptical frond form. The dorsal leaf lobe was more robust and provided far more information than the ventral leaf lobe in herbarium specimens. Characters considered from the dorsal leaf lobe were maximum length, maximum width, length to width ratio, shape of apex, maximum width of the hyaline margin, nature and prominence of the hyaline margin and the number of cells comprising the trichomes on the abaxial surface of the dorsal leaf lobe. The results are summarised in Tables 4.14-18 and Figure 4.42. It was possible to

divide the results for the *A.filiculoides* type into three groups defined by geographical distribution and whether fertile or sterile. These groups were defined as *A.filiculoides* fertile, from the Americas and Europe (denoted by (F)); *A.filiculoides* sterile, from the Americas and Europe (denoted by (S)); *A.filiculoides* fertile or sterile, from Australia (denoted by (R)).

The ranges of dorsal leaf lobe length and width overlapped considerably between the types (Figs. 4.42a & b, Table 4.14). The *A.filiculoides* type exhibited the greatest size range, which, in general, was associated with frond habit and shape; in respect to the *A.filiculoides* (F) type the larger leaves were found on vertical and deltoid fronds. The *A.filiculoides* (R) type was different in that large leaves were found on elliptical and horizontal fronds. Using the t-Test, significant differences in lobe length and width were found between the *A.filiculoides* (R) type and all other types (including the *A.filiculoides* (F) and (S) type (Table 4.16)). There were no significant differences between the *A.filiculoides* (F) type and the *A.filiculoides* (S), *A.mexicana*, *A.microphylla* and *Azolla* sp. types; the *A.filiculoides* (S) type and the *A.mexicana*, *A.microphylla* and *Azolla* sp. types; the *A.mexicana* type and the *A.microphylla* and *Azolla* sp. types.; the *A.microphylla* type and the *Azolla* sp. type. When the three *A.filiculoides* type groups (F), (S) and (R) were combined the t-Test indicated a significant difference from all other types (Table 4.15). However, this difference was not reflected in the length to width ratio where it was found that:- the

*A.filiculoides* (F) type was different from the *A.filiculoides* (R) and (S) type; the *A.filiculoides* (R) type was different from the *A.microphylla* and *Azolla* sp.; *A.filiculoides* (S) was different from *A.mexicana*, *A.microphylla* and *Azolla* sp types. All other combinations of types were not significantly different (Table 4.18). The only significant difference between types, when the three *A.filiculoides* type groups were combined, was between the *A.filiculoides* and *Azolla* sp. (Table 4.17).

Regression of dorsal lobe length against width for all types indicated that longer dorsal lobes were associated with proportionally wider lobes ( $r = 0.64$ ). When each type was considered separately, only the *A.microphylla* type had a regression coefficient of less than 0.50 (i.e. 0.20). Similar results were obtained when dorsal lobe width was regressed against width of the hyaline margin (all taxa  $r = 0.60$ ). Only the *A.microphylla* type showed a negative correlation ( $r = -0.20$ ). Figure 4.42c illustrates that the ranges of width of the hyaline margin overlap considerably between the types. It was not surprising that Figures 4.42b and 4.42c are very similar because the width of the hyaline margin was a component of dorsal lobe width. Because of this, and the range of variation, width of the hyaline margin was not considered further. Nature and prominence of the hyaline margin appeared to be similar in all types. The hyaline margin varied from smooth to serrate in the basal half of the leaf. Only the *Azolla* sp. was consistently found not to have an obvious hyaline margin, although all other taxa may be found in this condition. Another character associated with the dorsal

leaf lobe was shape of the lobe apex. This was found to be variable in all taxa; from rounded to acute, although only the *A.mexicana* and *Azolla* sp. types exhibited this latter extreme, other taxa having sub-acute dorsal lobe apices (Table 4.14).

#### 4.4.3 Trichomes

The number of cells comprising the trichomes on the abaxial surface of the dorsal leaf lobe were initially studied in fresh specimens from Portsmouth and IRRI. Two types of trichome were recognised. One-celled trichomes were clearly seen to emerge from between and project above the surrounding epidermal cells; this was also seen on herbarium specimens. This type of trichome usually became more vertical towards the leaf base (Fig.3.12a & b). In some populations one-celled trichomes were not very distinct; this was particularly evident in populations from Australia and sterile populations from the New World. Two-celled trichomes comprised an enlarged epidermal cell with a smaller cell on top of it (Fig.3.12c). In fresh specimens the shape of the apical trichome cell varied considerably within types. In herbarium specimens this apical cell collapsed and assumed a distinctive boat-like shape.

The *A.mexicana* and *A.microphylla* types consistently possessed two-celled trichomes. In the latter taxon the apical cell often became more vertical towards the leaf base. Trichomes of the *Azolla* sp. type were only studied in herbarium specimens and appeared to be two-celled, usually with an apical prolongation or a third cell (Fig.4.43b & d). In some populations of this type

peculiar water soluble crystals were found encrusting the apical cell. This was not a consistent feature, but only occurred in this type (Fig 4.43c). X-ray analysis of the crystals revealed phosphorus to be the major elemental constituent. The prolongations and/or the crystals rendered trichomes of the *Azolla* sp. very distinctive under the scanning electron microscope.

The *A.filiculoides* type consistently had one-celled trichomes, but very rarely, several two-celled trichomes were found towards the leaf base. In types attributable to Section *Azolla* the trichomes were confined to the abaxial surface of the dorsal leaf lobe, whereas trichomes may also be found on the stem of types belonging to Section *Rhizosperma*.

#### 4.4.4 Colour

This character was extremely difficult to assess in herbarium specimens because of changes induced by drying and ageing. However, in the living populations observed at Portsmouth and IRRI, colour varied from various shades of green through various shades of red to brick red/brown in most taxa. These colours appeared to vary with environment, which included season and exposure to sun. A further complication of assessing colour by eye was that often this worker saw and described colours differently to other workers.

#### 4.4.5 Roots

These hung freely in the water and varied considerably in length within each taxon. Indeed the Type specimen of *A.filiculoides*

possessed roots upto 80mm long. More commonly **Azolla** roots were approximately 30 or 40mm long. When young, a root was encased in a root sheath which split during elongation, revealing an often translucent pale green root upon which whorls of root hairs may be observed. Number of root hairs per whorl could not be accurately recorded in herbarium specimens because of drying and collapse. In older roots, root hairs were apparently lost. The roots became brown with age which was caused by deposition of unidentified materials in thickened epidermal cells; this was observed in all taxa. All taxa developed roots with a coiled tip, although the **A.filiculoides** type did this more readily than others (Fig.4.44). It appeared that root tips coiled when exposed to light and this coiling may have stabilised fronds by holding them together or by hooking onto submerged objects. When **Azolla** formed a mat and light could not access the roots, coiling of the root tips did not occur or was very rare.

#### 4.5

#### BIOGEOGRAPHY

Although extant **Azolla** has a worldwide distribution from continental temperate to tropical climatic zones, some taxa are indigenous to certain regions. Section **Rhizosperma** was limited to the sub-tropical and tropical continents of Africa, Asia and Australia, whereas Section **Azolla** taxa were limited to temperate to tropical regions of Europe, North and South America and Australia. It was interesting to note that Australia was the only country where taxa from the two Sections naturally (i.e. without introduction) occurred. Indeed, during this

investigation one herbarium sheet of a collection made in south western Australia contained both the *A.pinnata* and *A.filiculoides* types and the arrangement of the fronds suggested that these two taxa were growing together in the same pond.

Before describing the distribution of taxa in Section *Azolla* in more detail, it should be noted that the distribution described here for each taxon (type) was dependant upon samples collected by botanists past and some present. It was very apparent that certain areas of the world were better represented than others in respect to the number of samples that had been collected. Therefore, the results presented here cannot be a definitive distribution for the taxa, as results are limited by the bias of the herbarium collections examined. However, every effort was made to obtain collections that would yield representative results.

The *A.filiculoides* type was found to have the widest geographical distribution of all the types. Apparently indigenous populations were found in south western Australia, New Zealand, central and south Japan, most of Europe (Britain, Holland, Belgium, France, West Germany, Italy, Portugal and Czechoslovakia), U.S.A. and most South American countries. As indicated previously (i.e. sections 4.2, 4.3 and 4.4), populations from Australia possessed certain features which separate them from populations in other countries, while populations from Japan and New Zealand appeared intermediate. Furthermore, this study indicated an apparent cline of variation in sporoderm structure from the U.S.A. to

South America to Australia. The Japanese and New Zealand populations appeared to be more similar to the South American populations. European populations of the *A.filiculoides* type had similar sporoderm structure to U.S.A. populations; this type was not the only one to be found in Europe. In Holland the *Azolla* sp. type was also found, but was limited to north and north Eastern regions and appeared less common than the *A.filiculoides* type. Although the latter type was found in most other regions of Holland, the only area of overlap in distribution of the two types was in the Leiden and Utrecht areas. In addition to these two types the *A.mexicana* type was also found in North America. Populations attributable to this type came from California, Oregon, Washington State and British Columbia on the west coast and Minnesota, Wisconsin, Nebraska, Kansas, Iowa, Illinois, Texas, Georgia and Arkansas in the central and south east. The *Azolla* sp. type was generally limited to an area within the central south east region - namely Nebraska, Kansas, Illinois, Missouri, Ohio, Indiana, Arkansas and Louisiana. Although the *Azolla* sp. and *A.mexicana* types may be considered sympatric, the former type appeared to be less abundant in herbarium collections than the latter type. The *A.filiculoides* type was the most abundant of all the types in herbaria, not only from the U.S.A., but worldwide. However, in North America it was consistently limited to the western and eastern states of California, New Jersey and New York, with only this type in the Hawaiian Islands. The *A.filiculoides* and *A.mexicana* types were sympatric in California, Oregon, Washington and Georgia, however, three types were found in Texas. It was interesting to find that in the

Californian Type locality of *A.microphylla* only the *A.filiculoides* and *A.mexicana* types were found. Only the *A.filiculoides* type was found in the Type locality (the most precise location is Carolina, USA) of *A.caroliniana*. In Mexico, which gives its name to *A.mexicana*, both the *A.filiculoides* and *A.mexicana* types were found, but the former type was apparently more common.

With both the *A.filiculoides* and *A.mexicana* types being indigenous to Mexico it was perhaps surprising to find that they did not extend into mesoamerica. Instead, the *A.microphylla* type was found in Honduras, Costa Rica, Cuba and the Dominican Republic with the *Azolla* sp. type only in Honduras and Cuba. However, it should be noted that there were certain differences between populations from the U.S.A. and South America (mesoamerica included) of the *Azolla* sp. type in respect to sporoderm structure. In South America (excluding mesoamerica) all three types were found. The *A.mexicana* type appeared to be confined to the northern countries of Columbia, Venezuela, Guyana and Ecuador. The *Azolla* sp. type was only found in Venezuela to the North and more centrally in north Argentina, Paraguay and east Brazil between Rio de Janeiro and Porto Alegre. The *A.microphylla* type was commonly found in this latter region of Brazil, northern Brazil, Paraguay, northern Argentina, Uruguay, Guyana, Surinam, Guyana and the Galapagos Islands. As in other regions of the world, the *A.filiculoides* type was the most widely distributed and common of all the types in South America. It was found in Colombia, Venezuela, Ecuador and Guyana in the north, Peru, Bolivia, Chile and Argentina to the south and

Uruguay and Brazil (between Rio de Janeiro and Porto Alegre) in the east. The most southern population was from the Rio Negro in Argentina. The lack of collecting sites from central Brazil was probably due to the inaccessability of that region. Figures 4.45 & 4.46 illustrate the distributions of all the types; interestingly the *Azolla* sp. type from South America was limited to regions where two other types were found.

A brief survey of the distribution of megasporic types in Section *Rhizosperma* revealed that the *A.pinnata* type had a wide distribution in the Australian and Asian continents, from Pakistan in the west to Japan in the east. The latter being the most northerly and Australia the most southerly extents. This type was also found from central to southern Africa. The *A.nilotica* type had, by comparison to the *A.pinnata* type, a limited distribution. It only occurred in the African continent from Sudan southward (in a central-eastern line) to Zimbabwe. See Figure 5.7 for the global distribution of extant *Azolla*.

**FIGURE 4.40**

L.M.'s illustrating frond morphology in populations grown under similar greenhouse conditions at IRRI.

(a & b) The **Azolla** sp. type.

(c) The **A.filiculoides** type.

(d) The **A.microphylla** type.

(e & f) The **A.mexicana** type.

(Scale bar = 2 cm)

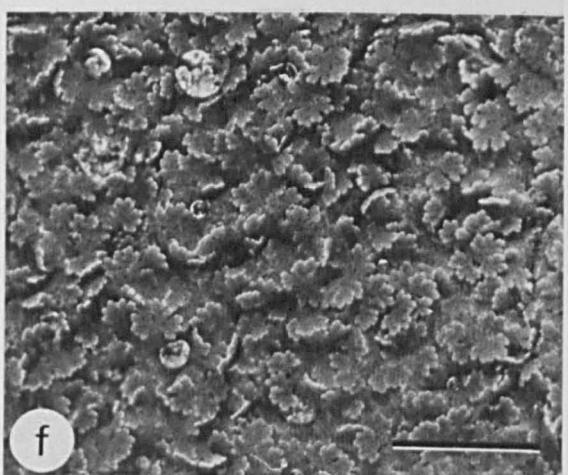
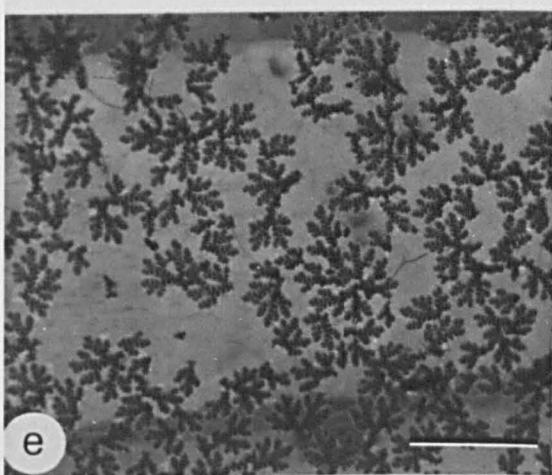
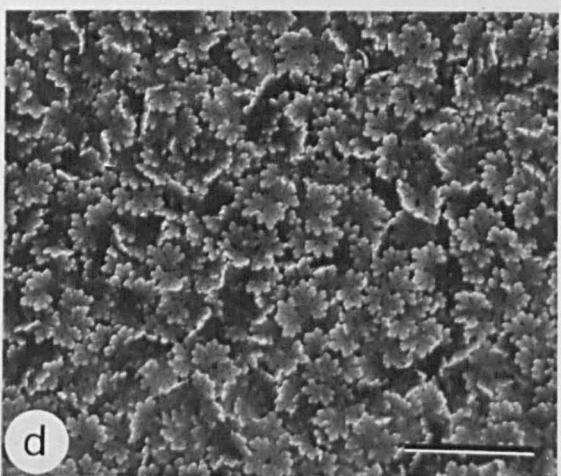
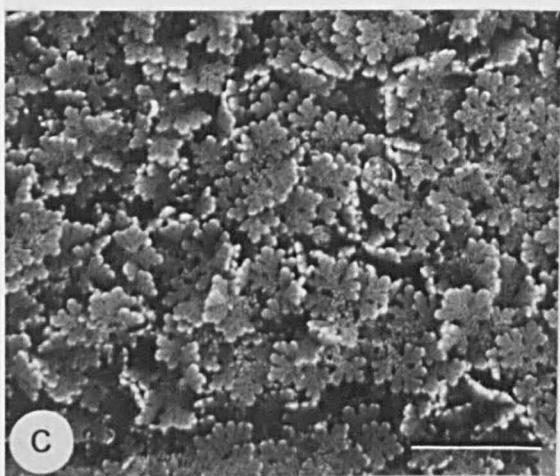
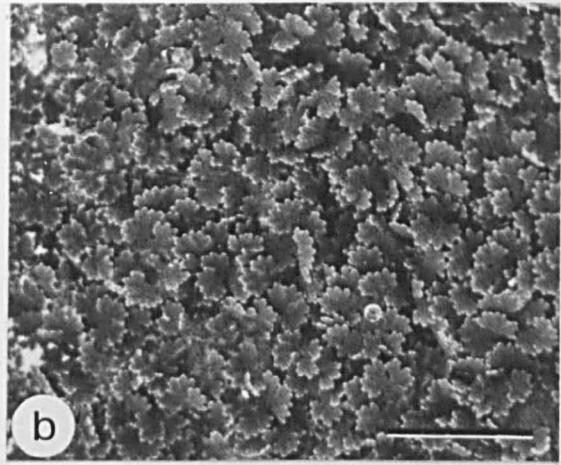
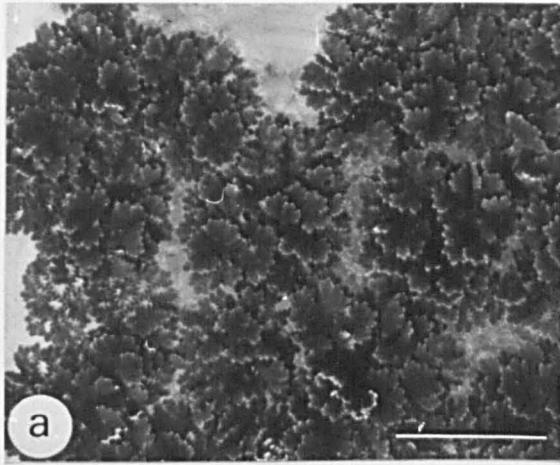
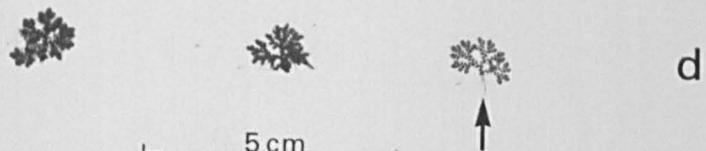
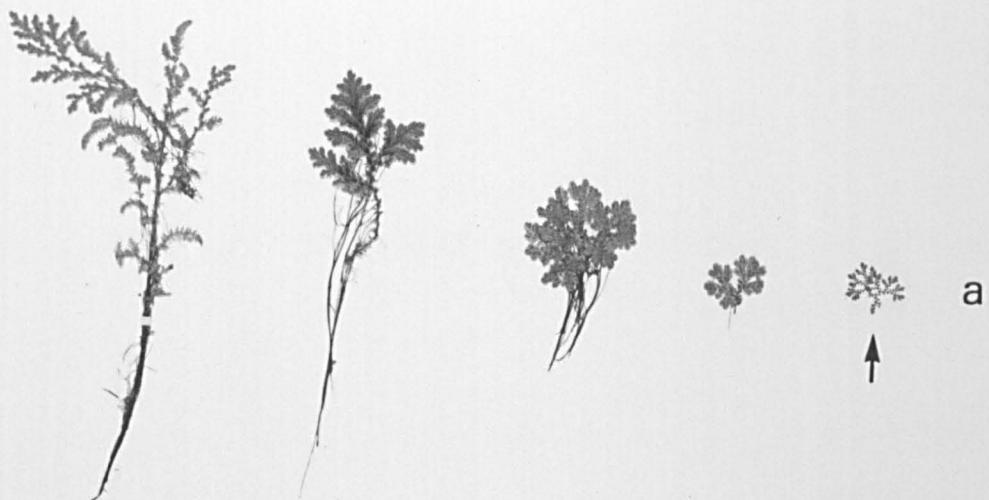


FIGURE 4.41

Vegetative Variation

L.M. illustrating variation of frond morphology within and between the megasporangium apparatus types. Note the polymorphisms in the *A.filiculoides* and *A.microphylla* types and the ranges of size in all the types. All specimens are herbarium specimens and are presumed to have grown under different environmental conditions except fronds indicated with an arrow; these are from the IRRI phytotron culture collection.

- (a) The *A.filiculoides* type.
- (b) The *A.microphylla* type.
- (c) The *A.mexicana* type.
- (d) The *Azolla* sp. type.



5 cm

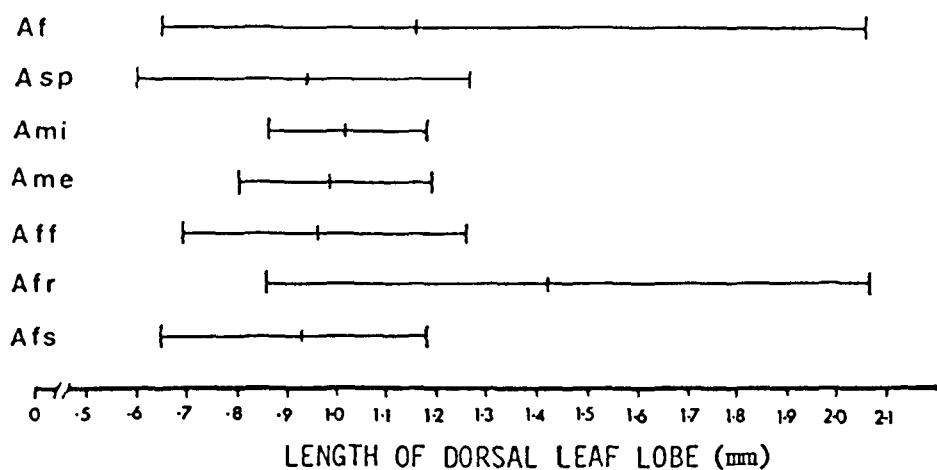
FIGURE 4.42

- (a) Showing the range and mean of dorsal leaf lobe length of the megaspore apparatus types.
- (b) Showing the range and mean of dorsal leaf lobe width of the megaspore apparatus types.
- (c) Showing the range and mean of hyaline margin width of the megaspore apparatus types.

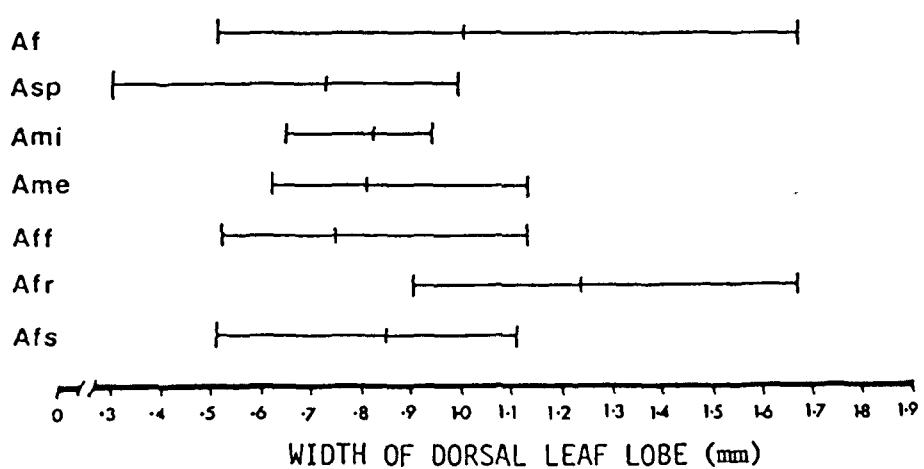
(Af = *A.filiculoides* type which includes Aff, Afr and Afs; Aff = fertile populations of the *A.filiculoides* type (excluding Australian populations); Afr = Australian populations of the *A.filiculoides* type; Afs = sterile populations of the *A.filiculoides* type (excluding Australian populations) Asp = *Azolla* sp. type; Ami = *A.microphylla* type; Ame = *A.mexicana* type)

(Similarity between (b) and (c) in respect to Asp, Ami, Ame, Aff, Afr).

a



b



c

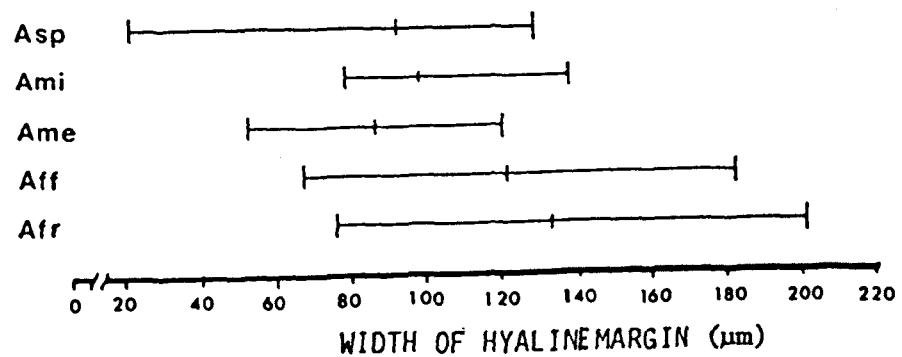
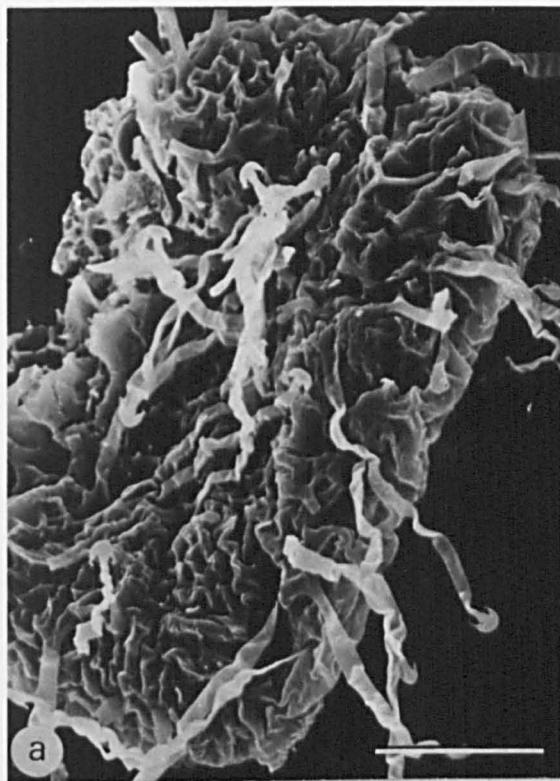


FIGURE 4.43

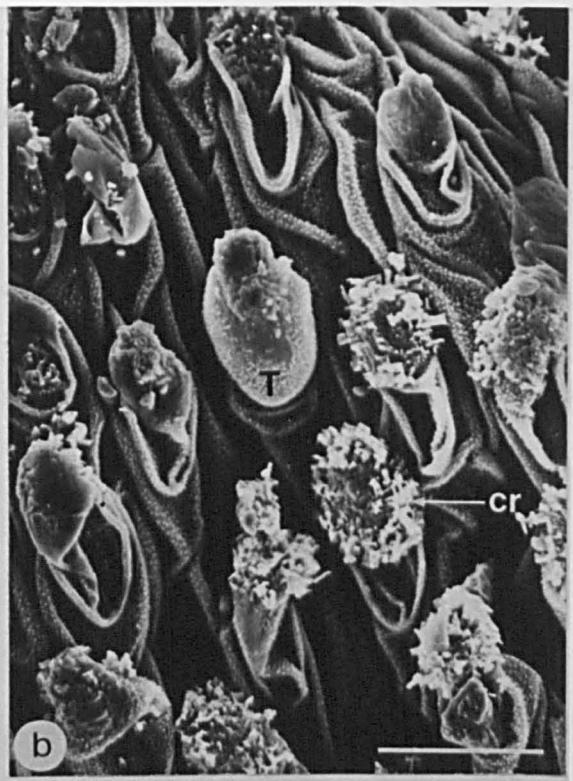
The Azolla sp. type

- (a) SEM illustrating the external massula surface. Distortion caused by drying masks the true nature of the surface. (Scale bar = 50 $\mu$ m).
- (b) SEM of trichomes on the abaxial surface of the dorsal leaf lobe with crystals on their apices. (Scale bar = 50 $\mu$ m).
- (c) SEM illustrating the crystals on the apex of a leaf trichome. (Scale bar = 10 $\mu$ m).
- (d) SEM illustrating trichomes on the abaxial surface of the dorsal leaf lobe without crystals. Note the prolongation or third cell of the trichomes. (scale bar = 50 $\mu$ m).

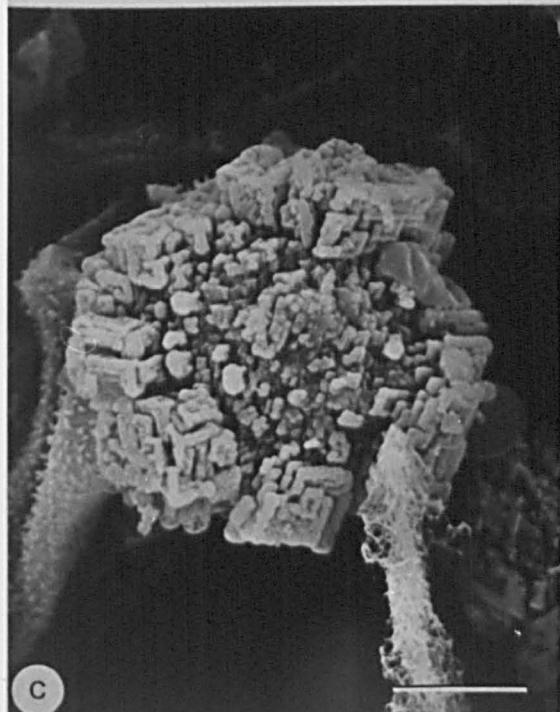
(T = trichome; cr = crystals; pr = prolongation or third cell of a trichome).



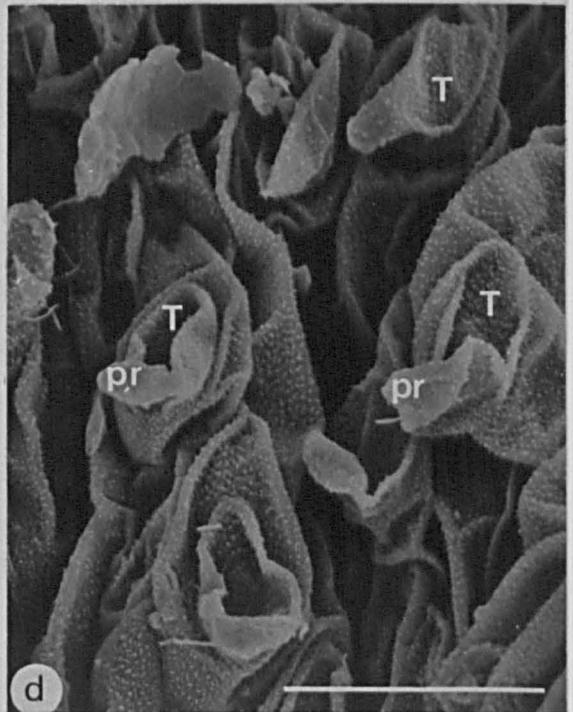
a



b



c



d

FIGURE 4.44

L.M.'s showing root-tip coiling occurring in all the types grown under similar greenhouse conditions at IRRI.

- (a) The *A.filiculoides* type.
- (b) The *A.mexicana* type.
- (c) The *Azolla* sp. type.
- (d) The *A.microphylla* type.

(Scale bar = 2 cm)

(Arrows illustrate coiled root-tip)

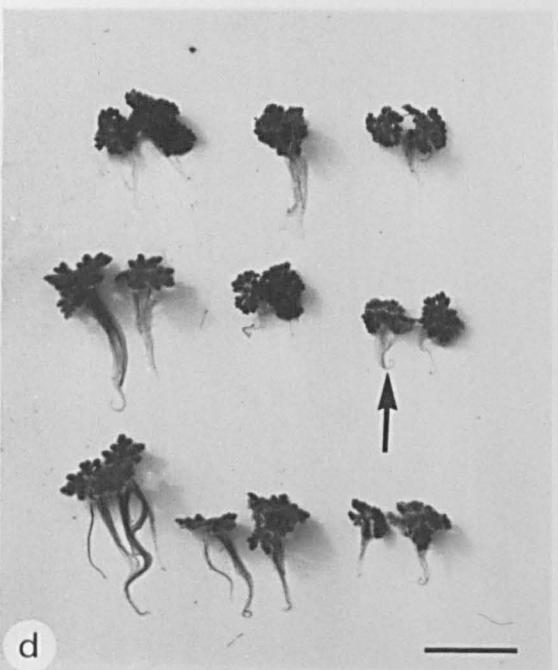
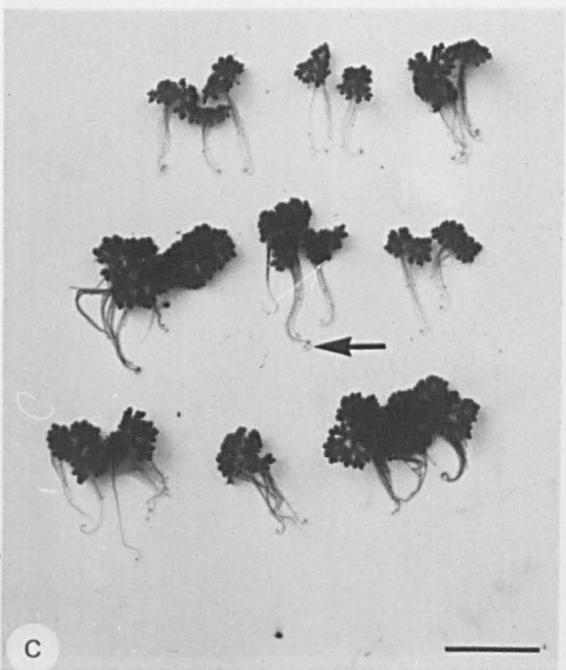
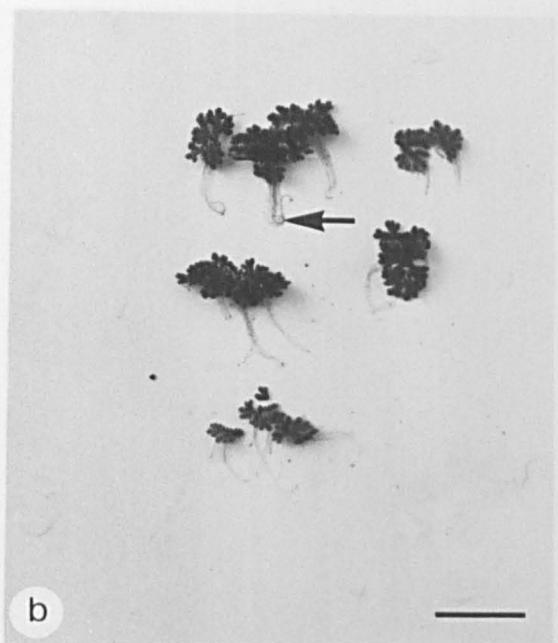
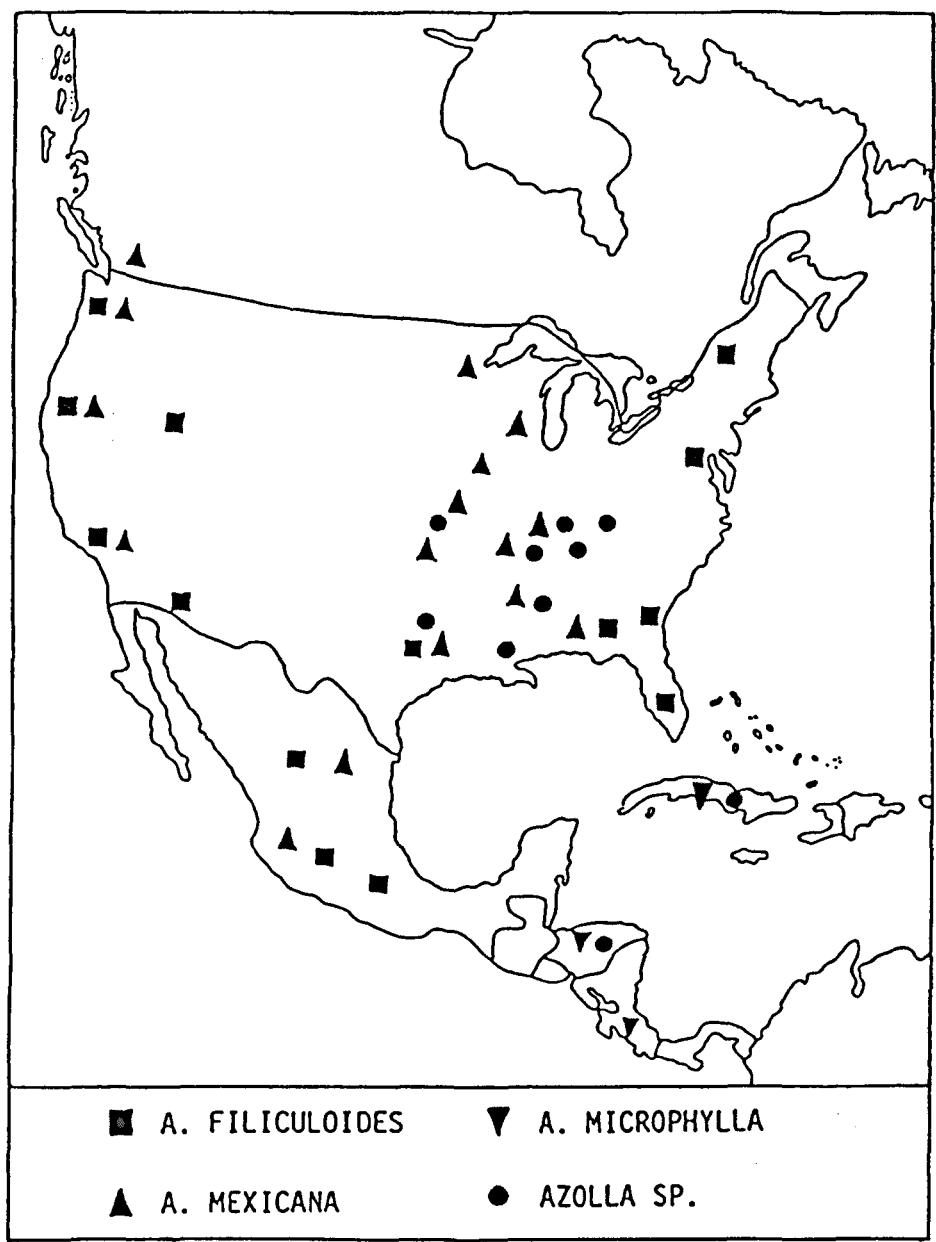


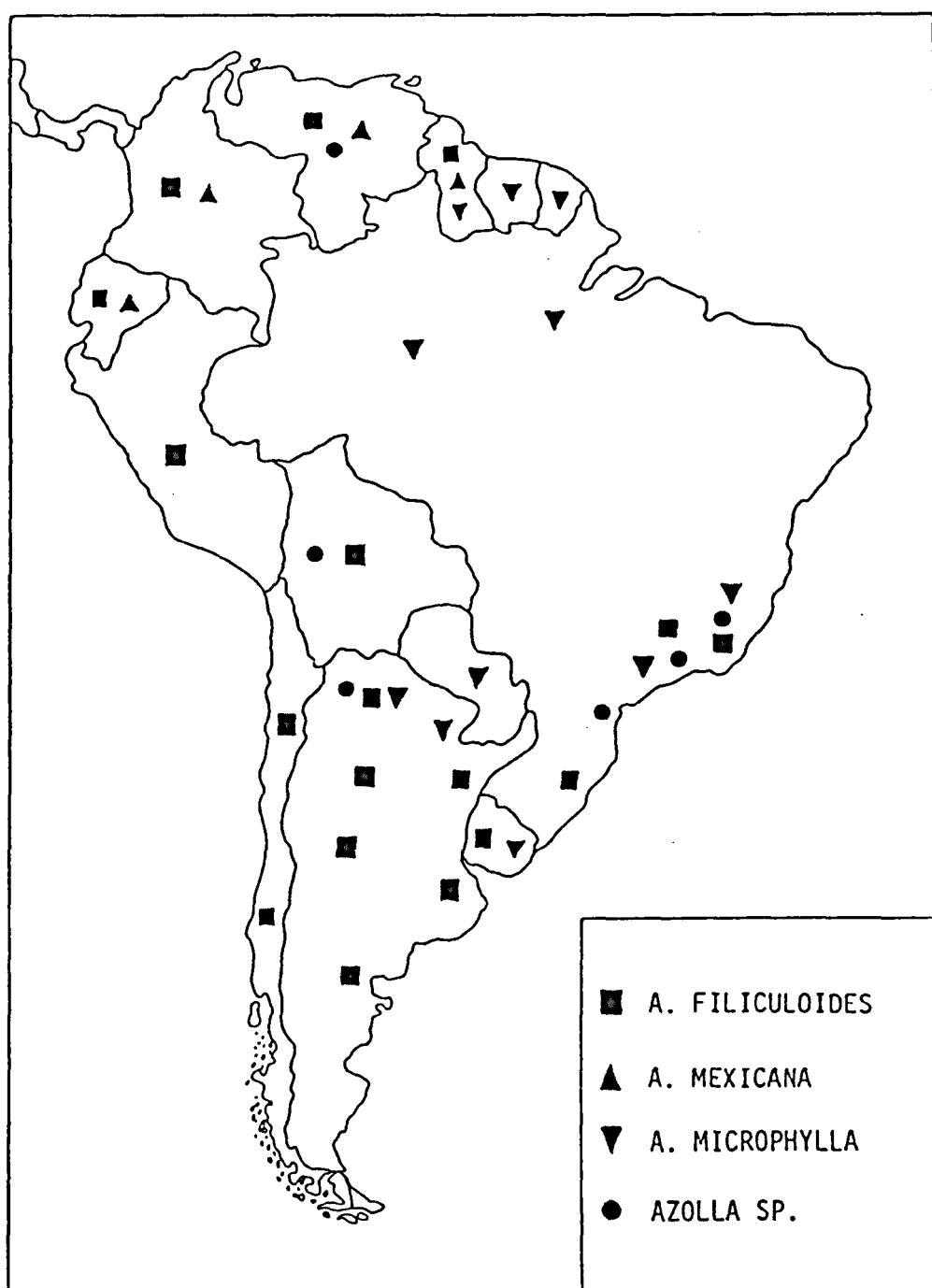
FIGURE 4.45

Map of North and Meso America showing the distribution of the four main megasporangium apparatus types.



**FIGURE 4.46**

Map of South America showing the distribution of the four main megaspore apparatus types.



## 4.6 MISCELLANEOUS OBSERVATIONS

### 4.6.1 Presence/Absence of *Anabaena azollae*

Sectioning the megasporangium apparatus for examination under the scanning electron microscope provided the opportunity to search for *Anabaena azollae* in the indusial cap. From some 80 populations examined for the presence of *Anabaena azollae* only five populations clearly lacked the endosymbiont, two from England and one from West Germany (the *A.filiculoides* type), one from Holland and one from Missouri (USA) (both the *Azolla* sp. type). Both populations from England (Greywell and Fordingbridge) were studied in culture at Portsmouth, and the leaf cavities also lacked the endosymbiont. However, the cavity trichomes were still present.

Under glasshouse culture conditions, the Greywell population was unable to tolerate high light intensities compared with other populations with *Anabaena azollae*. A further difference between these two types of population was that the *Anabaena*-free one senesced more quickly. Apart from these differences, the two 'types' exhibited similar morphological characteristics in vegetative and reproductive structures. The Greywell population was occasionally visited over a two year period. It thrived during the spring and summer months, and died back during the winter. Healthy fronds were readily found within the cover of emergent water vegetation even during severe frost periods. Morphological changes typical of the *A.filiculoides* and *A.microphylla* types were seen; however, the megasporangium apparatus was

clearly of the former type. It was possible that the Greywell and Fordingbridge populations were from the same original population because the two locations were once linked by canals. Furthermore, the Greywell population readily produced embryos under culture conditions, therefore, dispersal may have been by vegetative and/or reproductive means.

#### 4.6.2 Megaspore apparatus abnormalities

Apart from supernumerary floats, certain other abnormalities were very occasionally observed. The commonest of these was a megasporocarp containing two megaspores. In such specimens the collar region was disfigured and disrupted, but separated the two megaspores (Fig.4.47b). The floats were absent or diminutive, and occasionally a float-like structure was seen on the surface of the distal perine. Sections of one of these specimens revealed that each megaspore contained a megaspore proper. However, the perine was often abnormally structured. Similar abnormalities were found in IRRI 417 cultured at the Department of Botany, Manchester University. From a sample of fifty sporocarps, 50% were found to be abnormal in respect to the collar, floats and distal perine compared with IRRI 417 cultured at IRRI (Fig.4.47a). It was known that the IRRI specimens were grown in outdoor experimental plots, whereas the Manchester University specimens were cultured in a growth cabinet.

A further and apparently very rare abnormality was the inverted development of a normal megaspore apparatus within the sporocarp (i.e. the float region was basal and the basal megaspore region

was apical); only one sporocarp from one population was found in this condition.

#### 4.7

#### NOMENCLATURAL TYPE MATERIAL

In order to establish a more workable taxonomic framework for *Azolla* it was necessary to delimit the phenotypic variation of both reproductive and vegetative characters. Part of this involved establishing megaspore apparatus types which were assigned names. These names, in general, followed the current concensus. However, to provide a more correct taxonomy it was necessary to examine and describe nomenclatural Type material. The next section contains descriptions of the Type material that was located and obtained during this investigation. It should be noted that only the Types of Bertoloni (*A.bonariensis*), Molina (*A.squamosa*) and Schaffner (*A.mexicana*) were not definitely located.

Type material was defined as a specimen or specimens which were as far as could be ascertained, examined for description by the author who first validly published a name or epithet of taxon. Valid publication was defined in accordance with Articles 32 to 45 of the International Code of Botanical Nomenclature (Stafleu, 1978). Type material was identified by matching catalogue or specimen numbers and any other available information in the annotation and protologue. Identification of original author handwriting was made to determine the validity of any annotation.

4.7.1 A.filiculoides Lam. Encyclopedia Methodique: Botanique Vol. 1 P. 343, Paris (1783). The Type specimens of A.filiculoides (also the Type species of the genus) were in the Lamarck Herbarium (Herbier Historique) in the Museum d'Histoire Naturelle, Paris (P-LA). The specimens comprise many sterile fronds with the annotation "Pl. aquatique rapportee de Magellan par M. Commerçon. quid? (azolle, enc)", written in Lamarck's own hand (Fig.4.48a & b). There was no one frond designated as the holotype.

Description (see Figs.4.48a & 4.49)

Fronds elliptical to round, upto 23mm in diameter, although mostly ca 15mm; Branching isotomous, although tending to lateral; Roots prominent, upto 80mm long, dark brown and not coiled at their tips; Leaves more or less strongly overlapping, usually two per interbranch; Colour chocolate brown with some olive green; Ventral lobes probably horizontal and not contorted, ovate; Dorsal lobes more or less ovate 1.17mm long ( $\pm 0.023$ ) and 0.97mm wide ( $\pm 0.022$ ), apex rounded; Hyaline margin serrate, obvious, particularly towards the lobe base, mean maximum width 0.096mm ( $\pm 0.005$ ); Trichomes one celled and restricted to the abaxial surface of the dorsal leaf lobe. Reproductive bodies not seen.

Also at Paris, in the Jussieu Herbarium (Herbier Historique, (P-JU)), was one sample of specimens on a sheet containing three different samples which appeared to be syntypes of the Lamarck material. However, Jussieu probably considered that this sheet was representative of only two collections because only two

catalogue numbers were given (1601-A and 1601-B). 1601-B was clearly two separate samples under the same catalogue number. The top left and middle right samples on the herbarium sheet were similar to the Lamarck specimens. Annotation in Jussieu's handwriting suggested that 1601-B (two samples) were collected by Commerson from between Buenos Aires and Montevideo. Also in Jussieu's hand was a note indicating that Lamarck wrongly cites the location as Magellan. The other specimens under No. 1601-B were very similar to the other sheet of *A.filiculoides* in the Lamarck Herbarium; indeed they both contained the same flowering angiosperm (identity not determined) in the samples. Identification: both specimens are *A.filiculoides*. A third sample of *Azolla*, 1601-A, was determined as *A.pinnata* from Africa. Other specimens which were probably syntypes of *A.filiculoides* were in the General Herbarium at P and BM. These were from the Commerson collection and were cited as being collected from Montevideo and Buenos Aires.

#### 4.7.1.1 *A.rubra* R.Br. *Prodromus Florae Novae Hollandiae et Insulae van Dieman.* J. Johnson and Co. London., (1810).

Many fronds were attached to an herbarium sheet in the R. Brown collection at the British Museum (Natural History) (BM). The specimens were labelled as being collected from 'Port Jackson' (= Sydney) (Fig. 4.52a). Although fertile there were very few microsporocarps and many megasporocarps. A holotype was not designated but a duplicate (syntype) collection was located in the herbarium at the Royal Botanic Gardens, Edinburgh (E). A lectotype will be designated from the collection at BM.

Description (see Figs.4.50,4.51,4.52a).

Fronds elliptical to elongated (ca 10mm - 15mm 'diameter'); Branching isotomous; Roots broken, not obvious, dark brown; Leaves overlapping becoming less imbricate in older regions of fronds; Colour crimson to red-brown; Ventral lobes probably horizontal and uncontrolled when fresh, ovate (ca 0.9mm long by ca 0.8mm wide); Dorsal lobes ovate (ca 0.9mm long by ca 0.9mm wide; L : W = 0.98), apex rounded, occasionally becoming obtuse; Hyaline margin not obvious (ca 0.08mm wide) becoming serrate towards the lobe base; Trichomes one-celled and restricted to the abaxial surface of the dorsal leaf lobe, however, the trichomes were not obvious (Fig.4.51c).

Microsporocarps very few, usually damaged; Microsporangia mostly loose, but adhering to specimens; Massulae ca 4 per microsporangium; Glochidia 0, 1, 2 and 3 septate, rarely 4 septa (mean not determined because sample too small), glochidial taper was basal and apical (B-A).

Megaspore apparatus spheroidal with a distinct equatorial waist (ca 575μm by 362μm); FLOATS typical of the *A.filiculoides* type; Collar also typical of the *A.filiculoides* type with the flange originating approximately half way up the collar in sectional view; Sporoderm sculpturing at low magnification of raised angular excrescences connected by narrow bridges or ridges of exoperine; there was a thin weft of infrafilosum covering the perine surface; Sporoderm sculpturing at high magnification verrucose in the depressions, but the excrescence apices possess

-ed verrucate to vermiform sculpturing which extended as ridges and bridges between excrescences, it was typical of the *A.filiculoides* type; Sporoderm structure-the basic organisation of excrescence and depression areas was also typical of the *A.filiculoides* type; exospore ca 3.6 $\mu$ m thick, endoperine ca 2.7 $\mu$ m (depression region) to ca 26 $\mu$ m (excrescence region) thick, exoperine 1 ca 1.8 $\mu$ m thick, exoperine 2 ca 9.1 $\mu$ m thick and exoperine 3 ca 1.8 $\mu$ m thick. Although excrescences possessed small alveolae in the endoperine, some were large.

Remarks: alveolation was typical of Australian populations of the *A.filiculoides* type, as were the loosely packed rods comprising the endoperine. Figures 4.50 & 4.51 illustrate megasporangium features of this Type material.

#### 4.7.1.2 *A.caroliniana* Willd. in Linn. Sp. Plant.(ed. 4), 5: 541. (1810).

The herbarium sheet was in the Willdenow Herbarium (No. 20260) Botanischer Garten and Botanisches Museum, Berlin-Dahlem (B). The material was from the Richard collection and originated "in aquis carolina". A holotype was not designated (Fig.4.52b). The material was sterile, hence Willdenow's lack of mention of reproductive bodies.

Description (see Fig.4.52b).

Fronds elliptical to round (9.6mm and 8.3mm in 'diameter'); Branching isotomous; Roots broken, nature unknown, brown to black; Leaves overlapping becoming more lax in the older region of one frond, two leaves per interbranch; Ventral lobes probably horizontal and not contorted, ovate; Dorsal lobes ovate, apex

rounded, 1.07mm long and 0.88mm wide (means of five lobes); Colour-brick-red/brown and brown; Hyaline margin serrate, particularly towards the lobe base; not obvious (width ca 0.068mm); Trichomes one-celled, confined to the abaxial surface of the dorsal leaf lobe; Remarks-the two fronds were not identical in respect to the leaf imbrication and preservation and may have been from two different samples; Identification-these are the *A.filiculoides* type.

The Richard Herbarium is in the Museum d'Histoire Naturelle, Paris and it contained specimens similar in appearance and collection locality to the Willdenow Type specimens of *A.caroliniana* and can be taken as syntypes. It is doubtful that Willdenow saw these specimens however. Other sheets in the General Herbarium at P contained similar specimens, one of which was collected by Michaux "in aquis carolina". Furthermore, there were other specimens with a similar appearance in the Jussieu Herbarium (P-JU No. 1602). These were also collected by Michaux from South Carolina. Reproductive structures were not found on any of these 'duplicates', but they all possessed one-celled trichomes on the abaxial surface of the dorsal leaf lobe and are therefore the *A.filiculoides* type.

4.7.2 *A.mexicana* C.Presl. In Abh.Bohm.Ges.Wiss, Vol.3, p. 580 (1845).  
Schlecht.& Cham. Linnaea 5: 625 (1830).

This Type material was located in the Herbarium at the Martin Luther Universitat, Halle-Wittenberg (HAL). Subsequently, duplicates were found at B, BM and P. Specimens from the Presl

herbarium at PR were received after completing the practical work for this thesis. However, examination of the specimens revealed that they are very similar to those from HAL, B, BM, and P. According to the annotation, the material from HAL and other herbaria was collected by C.J.W. Schiede (1829) No. 839 from Mexico "inter Serpillo et Estero in aquis stagnantibus" (Fig. 4.53).

Similar information was found on the specimens at B (sheet No. 000936) and at P (Herb. Luerssen No. 5122). All samples of the material bore numerous reproductive structures, with the exception of the PR material which bore only a few sporocarps.

Description (see Fig. 4.54).

Fronds elliptical upto 20mm in diameter, however, they were mostly fragmented; Branching isotomous; Roots not obvious, diminutive, dark brown; Leaves strongly overlapping, usually with two per interbranch; Colour dark brown; Ventral lobes ovate, probably more or less horizontal and uncontorted when fresh; Dorsal lobes more or less ovate, (ca 0.8mm long by ca 0.7mm wide), apex obtuse; Hyaline margin serrate, becoming more so towards the lobe base (mean width ca 0.07mm), not obvious; Trichomes two-celled, restricted to the abaxial surface of the dorsal leaf lobe (Fig. 4.54e).

Microsporocarps-many damaged, but probably spheroid; Microsporangia many per microsporocarp, mean number of massula in each = 4 (range 3 - 5); Massulae typical of the *A. mexicana* type with ovoid depressions on the surfaces; Glochidia 0 to 6 septate (mean = 2.6), length ca 75 $\mu$ m (range 59 $\mu$ m to 95 $\mu$ m), taper of the

shaft was consistently basal and apical (B-A), apex was rounded (Fig. 4.54d).

Megaspore apparatus ovoid with only a slight equatorial waist (ca 525 $\mu$ m by 390 $\mu$ m); Floats with quite large, conspicuous and more or less rounded perforations; Collar typical of the *A.mexicana* type with the flange originating in the lower quarter of the collar in sectional view; Sporoderm sculpturing at low magnification also typical of the *A.mexicana* type with crater-like foveae (ca 20 $\mu$ m diameter), thin weft of infrafilosum covering the perine surface; Sporoderm sculpturing at high magnification was verrucate with small foveae, however, this was occasionally obscured by infrafilosum; Sporoderm structure-the basic organisation was typical of the *A.mexicana* type with endoperine intrusions, particularly towards the distal sporoderm region, the thickness of the sporoderm layers were:- exospore ca 4 $\mu$ m, endoperine 1 ca 2.5 $\mu$ m, exoperine 2 ca 7 $\mu$ m (in depression) to ca 3 $\mu$ m (on a raised area), exoperine 3 ca 2 $\mu$ m. Figures 4.54a-c illustrate megaspore apparatus features of this Type material.

#### 4.7.3 *A.microphylla* Kaulf. *Enumeratio Filicum*. 273 (1824).

The Type material was located in the herbarium in the Jardin Botanique National de Belgique, Meise (BR). Subsequently duplicate specimens were identified at B (sheet No. 000916) and P (No. 6484), with photographs of the BR sheet at BM. The specimens at BR were considered to be the Type by Alston when he annotated the specimens in 1948. It appeared that the specimens were once on a sheet with other *Azolla* material and have been separated then attached to another sheet. Unfortunately, this

resulted in an error and subsequent annotation for each new sheet was mixed up; comparison of the original and subsequent annotation reveals this error. The original annotation states that Chamisso collected the Type material from San Francisco, California in 1824. Similar information was found on the sheets from B and P. However, only the BR material bore one megasporocarp. This material also had an accompanying slide preparation of several massulae.

Description (see Fig.4.56).

Fronds elliptical ca 10mm 'diameter' and easily fragmented; Branching isotomous; Roots upto ca 18mm long, dark brown; Leaves overlapping becoming distant in older regions with two or three leaves per interbranch; Colour dull green to brown; Ventral lobes ovate, probably more or less horizontal when fresh, although they may have been slightly contorted; Dorsal lobes more or less ovate (ca 0.8mm long by ca 0.7mm wide), apex rounded; Hyaline margin serrate towards the lobe base (mean width ca 0.06mm), not obvious; Trichomes one-celled and confined to the abaxial surface of the dorsal leaf lobe (Fig.4.56e)

Microsporocarps and microsporangia not found. Massulae studied from the slide accompanying the specimens using only a light microscope. Glochidia 0 to 1 septate and very rarely 2 septate, glochidial shaft tapered strongly towards the base and apex (B-A), apex rounded (Fig.4.56d).

Megaspore apparatus ovoid with collapsed floats, size ca 550 $\mu$ m long by ca 350 $\mu$ m diameter indicating it was probably immature;

Collar this was disfigured by drying and may have obscured the waist typical of the *A.filiculoides* type; Sporoderm sculpturing there appeared to be foveae (ca 12 $\mu$ m diameter) in a surface that was greatly obscured by infrafilosum, close examination revealed excrescences with more or less verrucate sculpturing at their apices, bridges or ridges of exoperine 3 could be seen interconnecting the excrescences. There was only one megasporocarp, therefore, the megaspore apparatus was not sectioned, and sporoderm structure not examined. However, it was possible to identify the Type material of *A.microphylla* as belonging to the *A.filiculoides* type. This supports evidence from the nature of the leaf trichomes and glochidial characters. Furthermore, in his annotation Alston described the glochidia as non-septate and concluded that the specimens were *A.filiculoides*. Figure 4.56a-c illustrates megaspore apparatus features of this Type material.

#### 4.7.4 Other Type Material in Section Azolla

Having established the megaspore apparatus types, and evaluated potentially useful characters it was possible to make a more meaningful assessment of sterile Type specimens; particularly those with little material. Pre-empting later discussion, the most useful vegetative character was found to be the nature of the trichomes on the abaxial surface of the dorsal leaf lobe, other vegetative characters being of no consistent practical use. With this in mind the following Type specimens were examined.

***A.magellanica* Willd.non Bertol.** In Linn.Sp.Pl.(ed 4.)5: 541 (1810).

This was located in the Willdenow herbarium (No. 20258) in the herbarium at B. General appearance was of relatively large elongated specimens with anisotomous ('lateral') branching, and was similar to many specimens of the *A.filiculoides* type. The trichomes on the abaxial surface of the dorsal leaf lobe were clearly one-celled. Willdenow reported "*capsulam imperfectam*" (immature reproductive structures) in his description, but his herbarium material was sterile. Willdenow also cites the habitat as "in aquis regni" in Peru, Chile and Magellan. Identification-the *A.filiculoides* type.

***Salvinia azolla* Raddi Fl.Brazil. I.p.2,t.1,f.3. (1825).**

A possible candidate for the Type specimen was located in the general herbarium at B; it was part of the A.Braun Herbarium. It appeared to have been redetermined as *A.cristata*. The specimens were small with isotomous branching and one-celled trichomes on the abaxial surface of the dorsal leaf lobe. There was no indication of the collection locality. Identification-the *A.filiculoides* type.

***A.arbuscula* Desv., Ann.Soc.Linn. (Paris) 6: 178 (1827).**

This was located in the Desvaux herbarium at P. M. Tindale had identified it as a Type specimen in 1950 and this was confirmed by this study. The annotation, in Desvaux's handwriting, indicated that the specimen was collected from "*america calidior-e*" (Fig.4.57a). The specimen was somewhat elongated with anisotomous ('lateral') branching, and was sterile; it was similar to many specimens of the *A.filiculoides* type. Furthermore

-re the trichomes on the abaxial surface of the dorsal leaf lobe were one-celled. Identification-the *A.filiculoides* type.

***A.densa* Desv., Ann.Soc.Linn.(Paris)6: 178 (1827).**

This was also located in the Desvaux herbarium at P. In 1950 M. Tindale identified it as Type material; this was confirmed by this study. Annotation, in Desvaux's handwriting indicated that it was collected from Carolina in USA (Fig.4.57b). The specimens were small isotomously branched plants, with one-celled trichomes on the abaxial surface of the dorsal leaf lobe. Reproductive structures were not found. Identification-the *A.filiculoides* type.

***A.japonica* Franch. & Savat. In Enum.Pl.Jap.2: 195 (1876).**

The Type specimens were located in the General herbarium at P. The collector was A. Franchet (No. 1530 bis) (Fig.4.58b). It was apparently collected from the Yokoska area of Japan. In 1978 T. Nakaike designated the specimens as a holotype collection, which was confirmed by this study. In 1979 T.A. Lumpkin identified the specimens as *A.filiculoides* (Fig.4.58b). Although rather small leaved and lax, the specimens were anisotomously ('laterally') branched; not dissimilar to the *A.filiculoides* type. The trichomes on the abaxial surface of the dorsal leaf lobe were one-celled. The specimens were sterile. Identification-the *A.filiculoides* type.

***A.cristata* Kaulf. Enumeratio Filicum. 274 (1824).**

The Type specimens are probably at BR, the handwriting on the

specimen packet being the same as that on the Type specimens of *A.microphylla*; furthermore, 'Römer' is cited which also agrees with this Type (Fig. 4.58a). Doubtful syntypes were located at P with the number 6483 and were collected by Osenbaul. This information was not on the BR specimens.

The *A.cristata* specimens were small, the branching being isotomous. Leaves were small and the trichomes on the abaxial surface of the dorsal leaf lobe were two-celled. However, this was difficult to discern. Identification-either the *A.mexicana* or *A.microphylla* type.

*A.mexicana* sensu Schaffner. No known reference.

Type material and a protologue for this taxon could not be located. However, several populations found at B, MO and P were designated as *A.mexicana* Schaffner. The name appears not to have been published (presumably because *A.mexicana* C. Presl already existed) Examination of the megasporangium apparatus, glochidia and dorsal leaf lobe trichomes revealed that these populations were of the *A.filiculoides* type.

*A.portoricensis* Spreng. Syst. Veg. 4: 9 (1827).

Doubtful Type material was located in the General Herbarium at B. (sheet N°. 000916). It was unlikely to be the 'holotype' because Sprengel's herbarium was destroyed, and only some duplicates remain (Heine pers. com.). It appeared that the material was collected by Sprengel and then given to Mettenius. The Type locality of *A.portoricensis* was Puerto Rico and the Type

specimens represent the only population from that locality to be examined in this investigation.

The leaves were somewhat lax and possessed two-celled trichomes on the abaxial surface of the dorsal leaf lobes. The lack of reproductive structures prevented definite identification of the specimens. The only other populations from the same region were from the Dominican Republic and were of the *A.microphylla* type. Tentative identification-the *A.microphylla* type.

*A.squamosa* Molina. Saggio Storia Nat. Chile, ed.2, 125 (1810). The Type material was not located and no herbarium collections were found with that designation. However, on examination, all populations from Chile were identified as the *A.filiculoides* type.

*A.bonariensis* Bertol. Botan.Zeitung, 21: 342-343 (1861). The Type material was not located and may have been destroyed during World War II (Stafl u & Cowens, 1976). The specimens were collected from Buenos Aires, Argentina.

#### 4.8

#### TAXA OF SECTION RHIZOSPERMA

The major part of the present investigation aimed to re-evaluate the taxonomy of Section *Azolla*. However, the opportunity arose to make a preliminary study of Section *Rhizosperma*. This included examining a very limited number of populations of the two taxa currently recognised in this Section together with Type

material. Although many populations were superficially studied during the cataloguing process at the onset of the study, only three populations of the *A.nilotica* type and six of the *A.pinnata* type were examined in any detail; <sup>this study</sup> was mainly restricted to the megaspore apparatus. The Type material of *A.nilotica* was fertile and was included in the descriptions. However, the Type material of *A.pinnata* was sterile and was not included. The 'mainstream' investigation of Section **Azolla** and superficial observations of Section **Rhizosperma** suggested that vegetative features were highly variable and their description, from a very limited sample, would be fruitless. The aims of this preliminary study were to continue the study initiated by Fowler & Stennett-Willson (1978), relate Section **Azolla** to Section **Rhizosperma** and initiate future research. This was to be achieved by describing megaspore apparatus types.

#### 4.8.1 Megaspore Apparatus Types

##### 4.8.1.1 THE *A.pinnata* TYPE

###### Megaspore Apparatus

General appearance: Mature megaspore apparatus usually slightly elongated ovoid shape with a length of ca 610 $\mu$ m and an equatorial width of ca 390 $\mu$ m; the elongation being in the equatorial region. Only a slight waist was usually observed. The basal region was spheroidal but in immature specimens it was ovoid (Fig.4.59a & b).

FLOATS (in surface view): These were in two tiers, an upper tier of three floats and a lower tier of six floats. However, they

were arranged in three groups of three, each group occupying one of the three sectors created by the acrolamella and suprafilosum. Each group consisted of a sub-triangular upper float and two smaller more or less trapezoid lower floats (Fig.4.59a & b). Minute perforations were consistently observed in the float surfaces. The groups of floats were separated by longitudinal bands of filosum. The longitudinal radial surface of the upper float gave rise to filosum elements that intertwined with the band of suprafilosum. However, within a group of three floats filosum was not observed connecting adjacent floats (Fig.4.59b). Instead, small hooks and nodules were found on the outer transverse edge of adjacent floats (Fig.4.3c).

**Collar:** In surface view the collar region was distinct because large quantities of filosum originated from it and cascaded down onto the sporoderm surface. Furthermore, the collar filosum was consistently seen to be in continuity with the septum filosum (Fig.4.59a & b). In sectional view the collar was diminutive compared to the collar of the three-floated types. Furthermore, it was more or less horizontal to the equatorial plane of the megaspore apparatus, club-shaped and lacked a flange. Sections clearly revealed that both the external and internal surfaces of the collar were covered by filosum, and that on the former surface a thin layer of exoperinous material gave rise to the filosum (Fig.4.60c).

**Sporoderm sculpturing:** Apart from the filosum cascading down from the collar, the surface was consistently devoid of infrafilosum.

A conspicuous and distinctive feature of the surface at low magnification were large, prostrate, tuberculate and elongated excrescences (ca 20 $\mu$ m - 80 $\mu$ m long). They usually had constrictions and occasionally small blunt outgrowths along their length (Fig.4.59c & d). Just below the collar, the excrescences were ca 8 $\mu$ m in diameter, whereas toward the distal sporoderm surface their diameter was ca 16 $\mu$ m. At high magnifications the surface appeared to be composed of closely packed rods, some of which anastomosed giving almost rugulate elements within the granular surface (Fig.4.59c & d).

Sporoderm structure: Sporoderm thickness ranged from ca 13 $\mu$ m to ca 16 $\mu$ m, although in an excrescence it was ca 30 $\mu$ m. Three basic layers of the sporoderm were recognised. The exospore was similar to that of other extant *Azolla* types, being composed of dense material with elongated irregularly shaped cavities (Fig.4.60b). However, these were not clearly radially orientated. The exospore was ca 4 $\mu$ m thick. External to this layer was a ca 6 $\mu$ m thick endoperine composed of closely packed tiny rods which often appeared thread-like. These rods appeared to be simple or branched and irregularly vermiform (Fig.4.60a & b). Apart from spaces between the rods the endoperine was consistently without alveolae. Three zones were usually recognised in the exoperine. The exoperine 1 was ca 1 $\mu$ m thick and composed of bacula of variable size which graded into the exoperine 2 elements. These were branched anastomosing bacula forming a zone ca 7 $\mu$ m thick. Towards their apices the bacula tended to fuse forming a more solid 'zone' before branching and terminating in

rounded and mostly free apices (Fig.4.60a & b). These apices gave rise to the granular surface described previously. The bacula surrounding an excrescence were elongated and appeared to be somewhat fused to form a sheath around the endoperine in the centre. As an excrescence emerged above the granular perine surface the exoperine was solid and formed a covering of exoperine 3. Therefore, this zone was confined to the surface of excrescences.

#### 4.8.1.2 The *A.nilotica* TYPE

##### Megaspore Apparatus

General appearance: Ovoid, although the apical and basal regions were spheroidal with a distinct waist in the collar region (Fig.4.61a). The length was ca 480 $\mu$ m and the equatorial diameter was ca 300 $\mu$ m.

FLOATS (in surface view): These were in two tiers, an upper one of three floats and a lower tier of six floats. However, the floats were arranged in groups of three; each group occupying one of the three sectors created by the acrolamella. The upper float in a group of three was sub-triangular and the two smaller lower floats were more or less trapezoid (Fig.4.61a). A quite distinctive feature was that the three floats of each group appeared closely associated; more so than in the *A.pinnata* type. Filosum originated from the longitudinal radial outer edges of each upper float forming a band of filosum which separated each float group. However, within a group of floats, filosum was absent. Instead, minute hooks and nodules, which appeared to hold the

floats together, were found. The external float surfaces were relatively smooth and minutely perforated.

**Collar:** In surface view the collar was almost indistinguishable (Fig.4.6la). Filosum was always absent from the collar surface. In sectional view the collar was diminutive compared to the collar of three floated types. Also, it lacked a flange and was more or less horizontal to the equatorial plane, but with an upwardly curved end. Sections also revealed that exoperine extended from the sporoderm up over the collar and onto the external float surface in the *A.nilotica* type (Fig.4.6ld). This explains why the collar was almost indistinguishable in surface view. Where the internal collar surface was in contact with a float there were short filosum filaments (Fig.4.6ld). Dissections of the apical region of the megaspore apparatus revealed that suprafilosum septa were absent on the acrolamella. However, there was evidence of very short filaments on some specimens.

**Sporoderm sculpturing:** Infrafilosum was consistently absent from the perine surface. A conspicuous and distinctive feature of the surface, particularly the distal surface, were spine-like exoperine protuberance on a finely granular sculpturing. A protuberance possessed a blunt and often recurved apex and often appeared to comprise of several fused exoperine prolongations which branched and recurred at the apex (Fig.4.6lb). Although ca 16 $\mu$ m in length, they attained a length of ca 26 $\mu$ m. At high magnification sculpturing of the finely granular surface was

of closely packed (ca 5 $\mu$ m apical diameter) clavae, between which small foveae (ca 0.7 $\mu$ m diameter) were often observed (Fig.4.6lc). In the position of these foveae, globular, possibly perinous material was occasionally found. However, these globules may have been contaminants because they were often observed near the apices of clavae. Immediately below the collar, the surface of the clavae was usually granular.

**Sporoderm structure:** Sporoderm thickness ranged from ca 10 $\mu$ m to 18 $\mu$ m, although including a protuberance it was up to ca 30 $\mu$ m thick. Three basic layers were easily recognised in the sporoderm. The exospore was ca 6 $\mu$ m thick and composed of dense material with elongated irregularly shaped cavities, their elongation being predominantly radial thereby creating a striated appearance. However, a thin basal or innermost zone of the exospore appeared more dense and lacked the cavities (Fig.4.6lc). External to the exospore was the endoperine which was ca 1.5 $\mu$ m thick. Its appearance was coarsely granular with small cavities. However, the granularity was caused by closely packed tiny rods which were simple or branched and irregularly vermiciform. Although the exoperine was clearly recognisable, the exoperine 1 was thin (ca 0.8 $\mu$ m) and barely discernible. It was consistently found to be composed of short bacula which became fused at their apices to form the exoperine 2. This zone was composed of fused or partially fused clavae, any spaces between adjacent clavae forming the small foveae seen on the surface (Fig.4.6lc). It was possible that many of these foveae penetrate the entire thickness of the exoperine 2 and probably exoperine 1.

(Fig. 4.61c). There was no evidence of endoperine extending upto the surface through these foveae, and in sections the globular bodies (described in the sculpturing) were not seen. The exoperine 2 was ca 6 $\mu$ m thick. Extensions of the exoperine 2 clavae were found to form the exoperine 3 of the protuberances. During this brief part of the investigation it could not be clearly demonstrated whether or not the endoperine intruded into the protuberances. If it did they would be classified as excrescences. As in the *A.pinnata* type the exoperine 3 was limited to only the protuberances.

#### 4.8.2 Characters Associated with Microsporocarps

**Microsporocarps:** In both the *A.pinnata* and *A.nilotica* types the microsporocarp shape was usually spheroidal, although this shape was often elongated. Sporocarps in the *A.nilotica* type always occurred in fours, whereas in the *A.pinnata* type they occurred in pairs. From this brief part of the investigation the apparent ratio of megasporocarps to microsporocarps, although variable, did not appear to be correlated with either megaspore apparatus type.

**Microsporangia:** Although no quantitative data were collected, the number of microsporangia per microsporocarp appeared variable in each type. Similarly, the number of massulae per microsporangium was not quantified because only few populations of each type were examined.

Massulae: Surface sculpturing of the massulae in both megasporic apparatus types was disfigured by drying, and there appeared to be little to distinguish either type. However, in the *A.pinnata* type the massulae often had a distinctive shape. The external surface was convex, while the internal surface was concave. Massula trichomes in both the megasporic apparatus types were usually situated centrally, in a group of one to several on the internal massula surface. Only rarely were these trichomes absent in the *A.pinnata* type. The above features are illustrated in Figures 4.60d & 4.61e.

Massula trichomes: Those of the *A.pinnata* type were like long spines with broad bases and occasionally the trichomes branched or their bases appeared to be fused (Fig.4.60d). However, in the *A.nilotica* type the trichomes were filamentous and occasionally branched. The most distinctive feature of these trichomes were the rounded nodules on their surface (Fig.4.61e); these were consistently observed, as was the presence of massula trichomes in the *A.nilotica* type.

#### 4.8.3 Nomenclature Types of Taxa in Section Rhizosperma

##### 4.8.3.1 *A.pinnata* R.Br. Prodromus Florae Novae Hollandiae et Insulae van Dieman. J. Johnson & Co., London (1810).

Species Types were available, Meyen (1834) and Mettenius (1847) having accepted R.Brown's specimens as the Section Type. The *A.pinnata* Type material was located at the British Museum (Natural History) (BM). Four groups of specimens of which three were collected in Australia by Brown, were on one sheet

(Fig. 4.62a). Two groups possessed the same number (134) and were collected from near Richmond and Hawksbury (Fig. 4.62a). The other group was No. 135 and was collected from the Patterson River. These numbers were assigned by Bennett who distributed duplicates of Brown's herbarium (Stearn 1960). The protologue of *A.pinnata* indicated that the Type specimens were collected from the Port Jackson (Sydney) area. The Patterson River is a long way inland from Port Jackson whereas Richmond and Hawksbury are close to Port Jackson. Therefore a specimen from No. 134 should be selected as the lectotype.

Pinnate branching and trichomes on the stem clearly indicate that the Robert Brown Type material of *A.pinnata* belonged to Section *Rhizosperma*. Furthermore, the *A.pinnata* megaspore apparatus type was within the present understanding of *A.pinnata*. Other Type material that possessed features consistent with *A.pinnata* included *A.imbricata* Nakai, *A.africana* Desv. and *A.decomposita* Zoll.. The former was located at BR and the latter two at P. Type material of *A.guineensis* Schum. was not available for examination.

#### 4.8.3.2 *A.nilotica* Decsne ex Mett. In: *Plantae Tinneanae*, T. Kotschy and J. Peyritsch (eds.). Vienna, 51-54 (1867).

This Type material was located at the Museum National D'Histoire Naturelle, Paris (P). According to Mettenius (1867) an unpublished manuscript by Decaisne describing the specimens was at P, but it could not be found (Badre, pers.comm.). Type material was included in the description of the *A.nilotica*

megaspore apparatus type (section 4.8.1.2.). Because so few populations were examined in the present study inter- and intra-population variation appeared equal, therefore, separate description of the Type was unnecessary. Figure 4.63 illustrates the Type material of *A.nilotica* in P, from which a lectotype should be designated.

FIGURE 4.47

Megaspore apparatus abnormalities

(a) SEM illustrating a megaspore apparatus with a partially formed collar and unusually large holes in the float surface.

(b) SEM illustrating a double megaspore. Parts of two collars can be seen, and the floats have been displaced laterally.

(C = collar; Fl = float; SPd = distal sporoderm; Scale bar = 100 $\mu$ m)

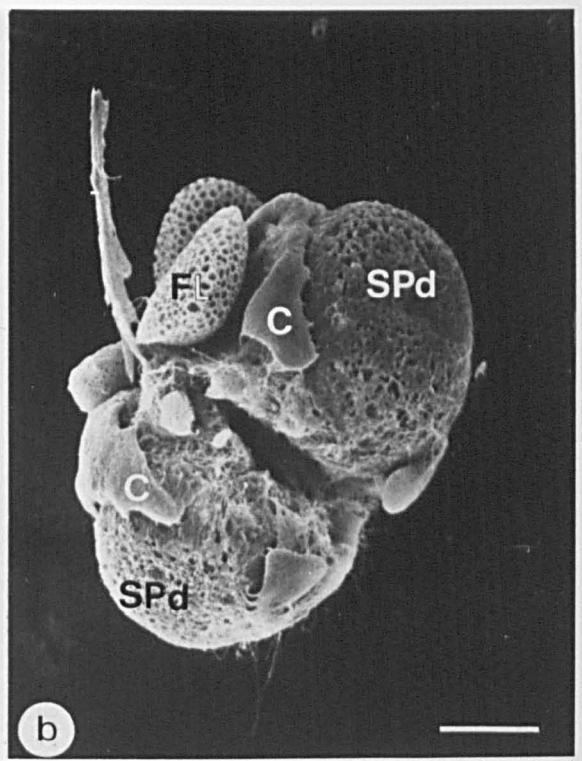
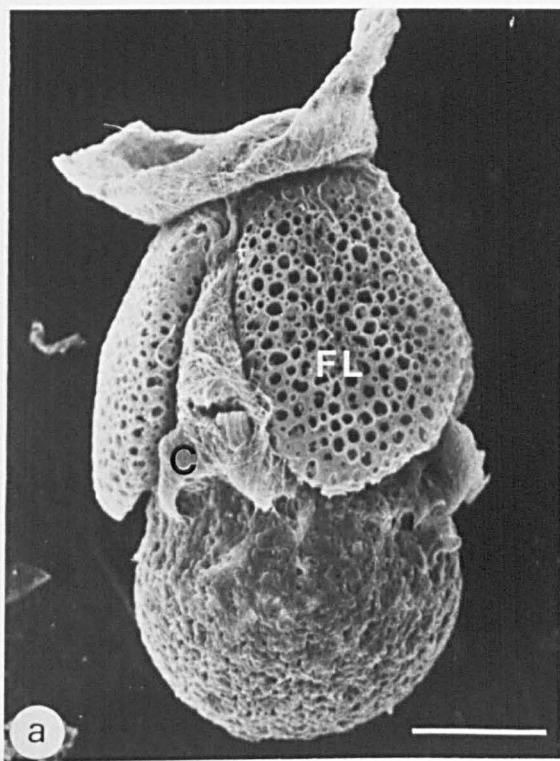
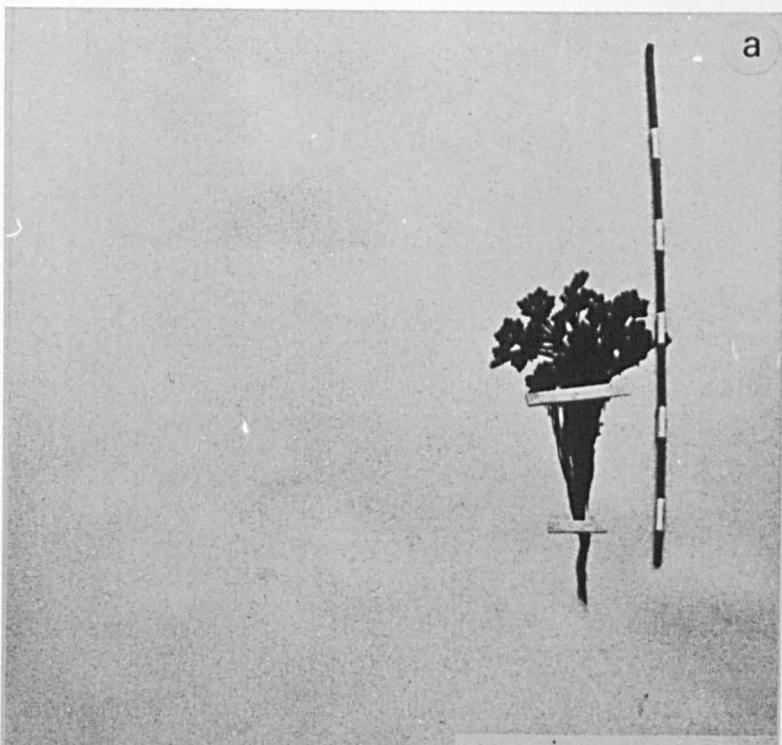


FIGURE 4.48

Nomenclatural Type material of *A.filiculoides* Lam.

- (a) Herbarium sheet of the Type specimens.
- (b) Annotation on the Type herbarium sheet in Lamarck's handwriting.
- (c) Annotation in A. Jussieu's hand, associated with syntypes of *A.filiculoides* (P-J 1601-B). The annotation casts doubt on the Type locality given by Lamarck.



HERB. MUS. PARIS.

pl. aquatique rapportée de  
Magellan par Cll<sup>r</sup> Commerson  
quid? [azolle, enc.]

TYPE

Herbier de LAMARCK,  
lequel se Novembre 1806.

pl. aquatique rapportée de b  
Magellan par Cll<sup>r</sup> Commerson  
quid? [ azolle, enc.]

c

M. Lamarck l'a annoncé dans le Journal de Magellan, par erreur.

Buenos Ayres et Montevideo - Herb. Dr. Commerson - Jany nom.

FIGURE 4.49

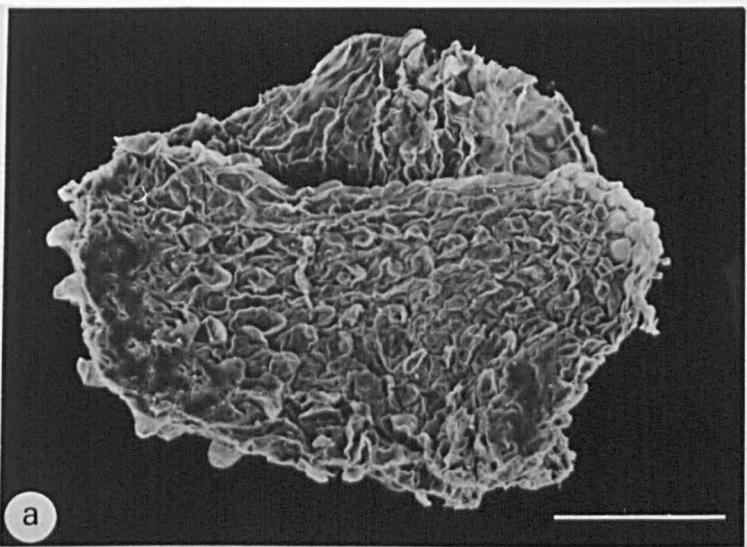
Nomenclatural Type material of *A.filiculoides* Lam.

(a) SEM of a contorted dorsal leaf lobe (abaxial surface). (Scale bar = 25 $\mu$ m).

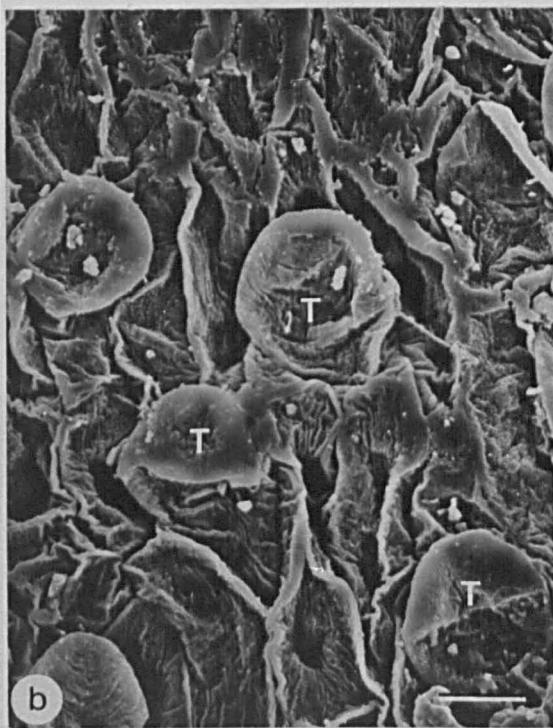
(b) SEM illustrating one-celled trichomes on the abaxial surface of the dorsal leaf lobe. (Scale bar = 25 $\mu$ m).

(c) SEM of a one-celled trichome and surrounding epidermal cells. (Scale bar = 2.5 $\mu$ m).

(T = trichome)



a



b



c

FIGURE 4.50

Nomenclatural Type material of *A. rubra* R.Br.

- (a) SEM of a megaspore apparatus. (Scale bar = 100 $\mu$ m).
- (b) SEM of sporoderm sculpturing. (Scale bar = 50 $\mu$ m).
- (c) SEM of L.S. collar near the cusp region. (Scale bar = 50 $\mu$ m).
- (d) SEM of L.S. collar in the mid-float region. (Scale bar = 50 $\mu$ m).
- (e) SEM of the radially external exospore surface. (Scale bar = 5 $\mu$ m).  
(bar = equatorial axis)

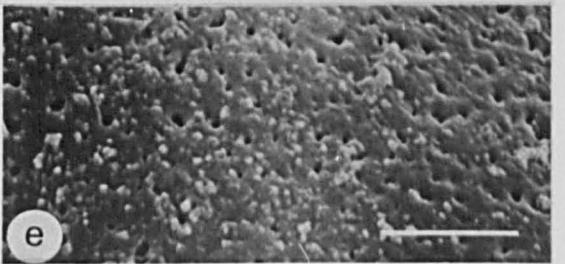
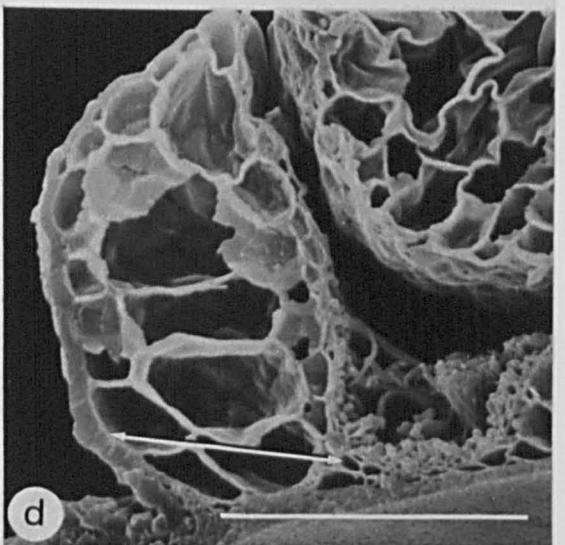
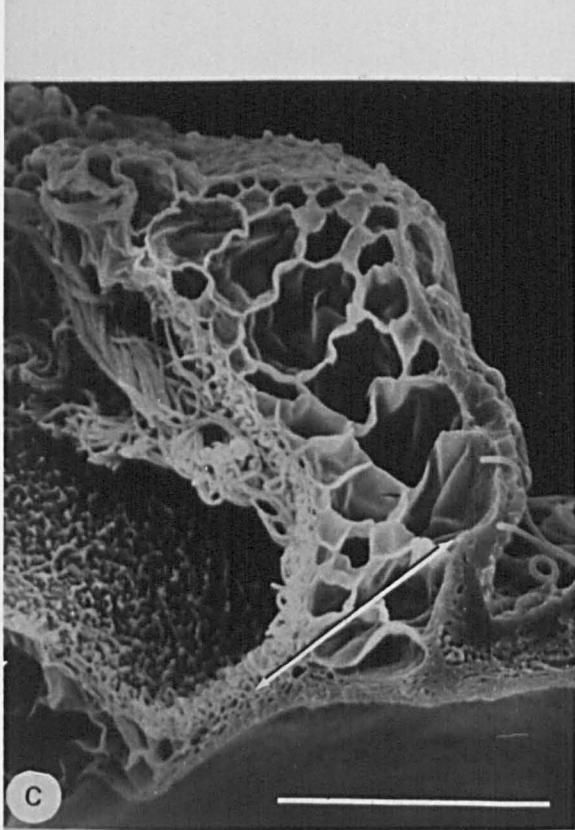
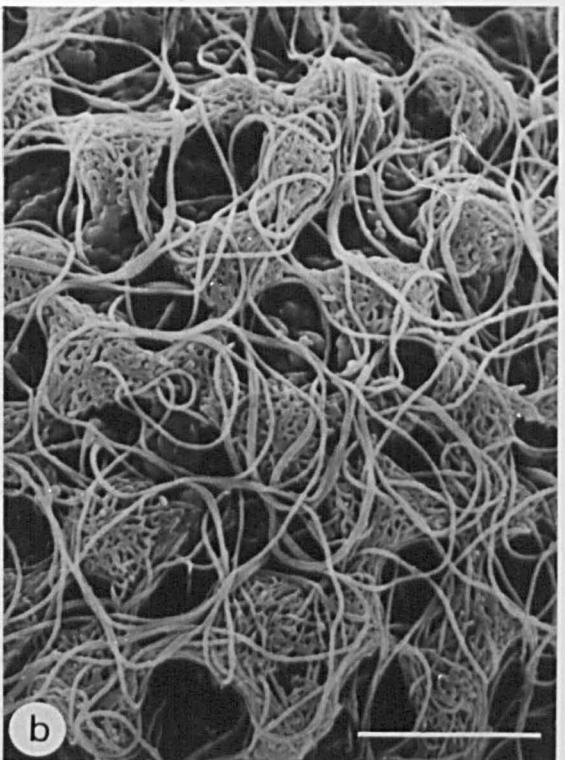
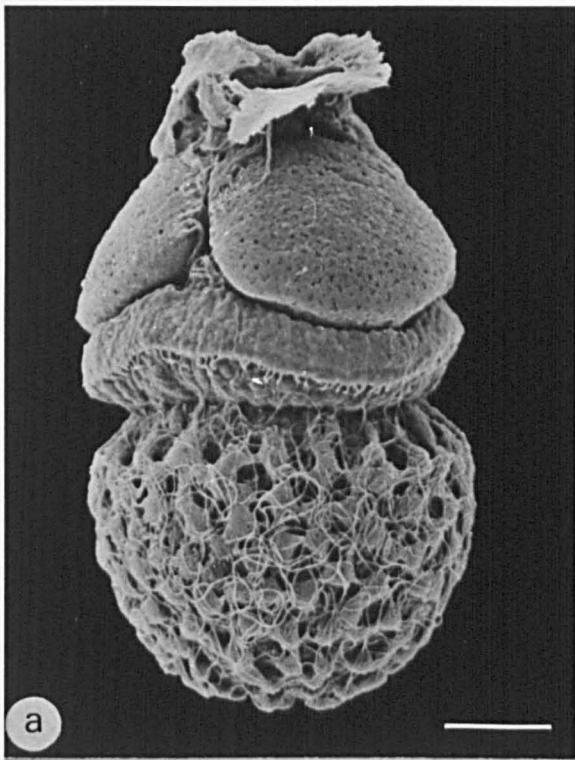


FIGURE 4.51

Nomenclatural Type material of *A. rubra* R.Br.

(a) SEM of perine structure viewed at low magnification. (Scale bar = 25 $\mu$ m).

(b) SEM of perine structure viewed at high magnification. (Scale bar = 5 $\mu$ m).

(c) SEM of the abaxial surface of the dorsal leaf lobe with one-celled trichomes and stomates. (Scale bar = 25 $\mu$ m).

(en = endoperine; ep = exoperine; E = excrescence; T = trichome; S = stomate).

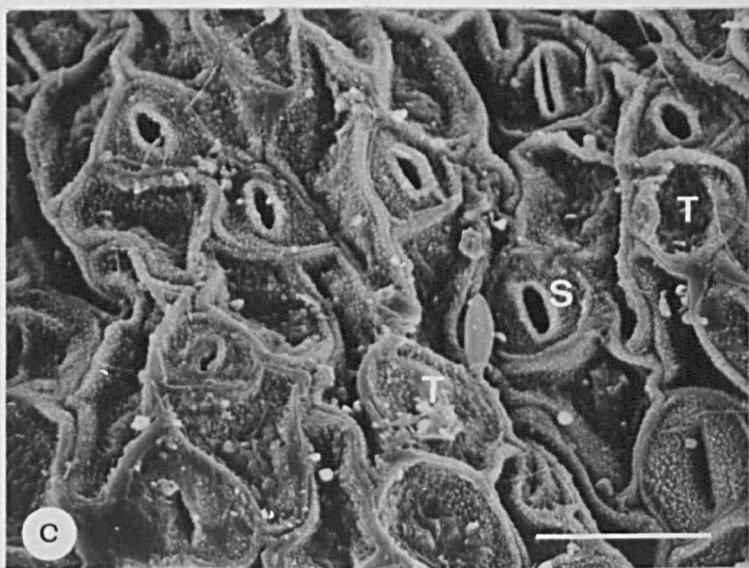
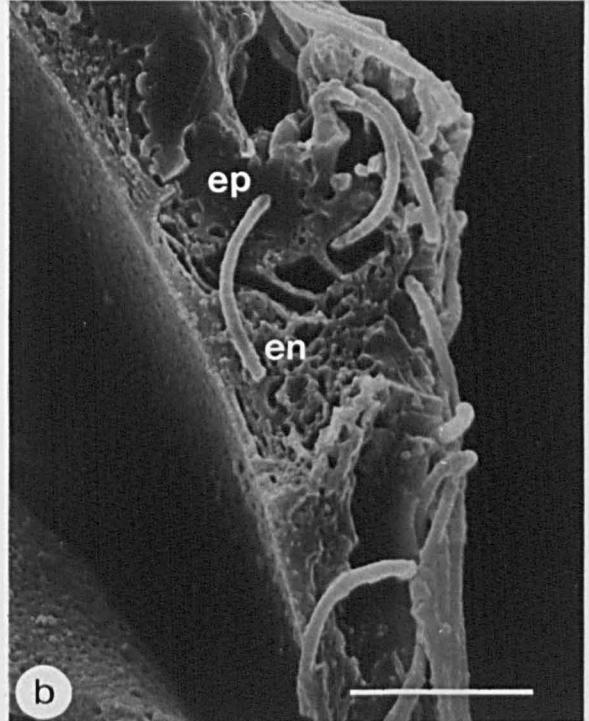
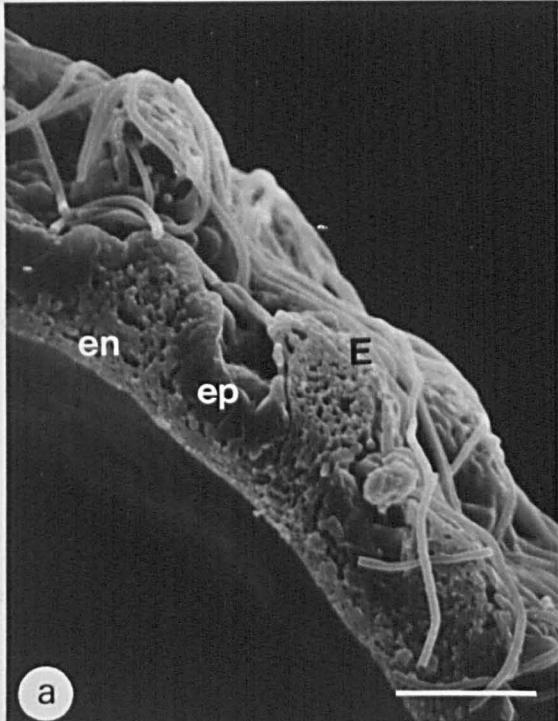
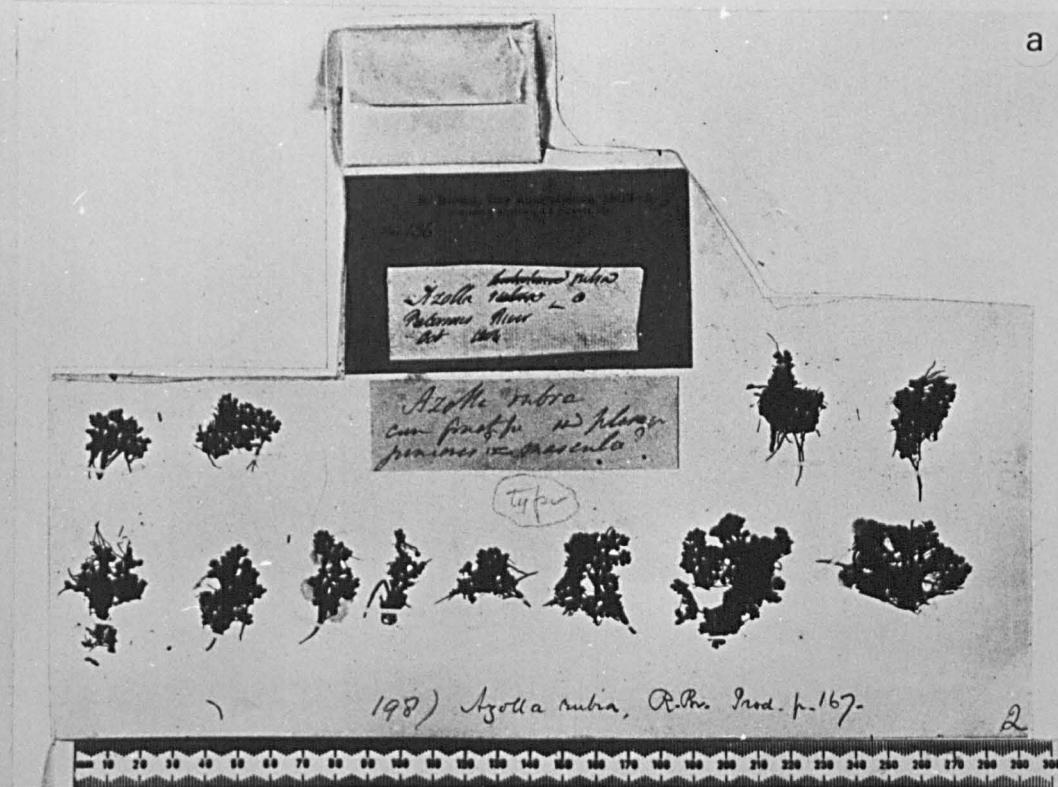


FIGURE 4.52

Nomenclatural Type material

- (a) The herbarium sheet of the Type specimens of *A.rubra* R.Br from BM.
- (b) The herbarium sheet of the Type specimens of *A.caroliniana* Willd. from B. (Herb.Willdenow No. 20260). Photograph courtesy of B.

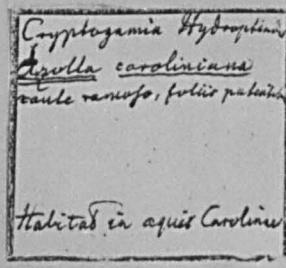
a



Glochidion  
nicht vorhanden!

det. S. Meyer, 8.4.59

b



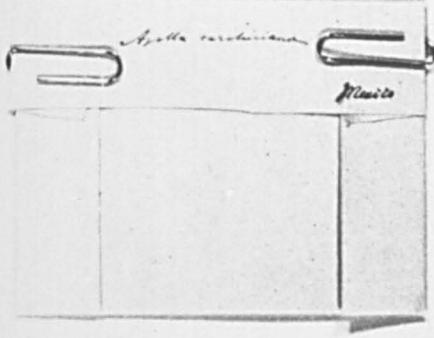
Neoglypta  
caroliniana  
*Agolla* sp. (Lamark) f. Richard

W

FIGURE 4.53

Nomenclatural Type material of *A.mexicana* Presl

The herbarium sheet of the Type specimens together with the annotation.



*Sabicea*  
in aqua et stagnis,  
Tibus inter al. spicis  
et lotos reg. acut.  
Pax. 29. C.J.W. Schiede

839. Azolla sp.  
Sabicea sp.  
Tibus spicis et  
Estero in aqua  
stagnantibus  
et lotos reg.  
Pax. 29. C.J.W. Schiede

Azolla caroliniana Willd.  
det.: M. Kuhn

Azolla sp. sp. ?  
Pax. No 839  
B.M. febr. 1911  
script: G. Kunze

839.  
Azolla sp.  
Mexico. inter horilla  
et lotos reg. C.J. Jones  
Dwyer & Schiede

scr. pali:  
A. v. Chemisee  
D. F. L. v. Schlechtendal

SEKTION BIOWISSENSCHAFTEN  
der Martin-Luther-Universität Halle-Wittenberg  
Azolla caroliniana Willd.  
= A. mexicana Schldl. & Cham.  
Linnaea 2 : 625, n. 839 (1830) nom. nud.  
Mexico  
Inter Serpillo et Esterio in aqua  
stagnantibus reg. calidae.

leg.: C.J.W. Schiede  
det.: am 1.1829  
am

FIGURE 4.54

Nomenclatural Type material of *A.mexicana* Presl.

- (a) SEM illustrating general morphology and sporoderm sculpturing at low magnification of a megaspore apparatus. (Scale bar = 100 $\mu$ m).
- (b) SEM illustrating sporoderm structure. (Scale bar = 10 $\mu$ m).
- (c) SEM illustrating sporoderm structure. (Scale bar = 10 $\mu$ m).
- (d) LM illustrating the glochidia on a massula. (Scale bar = 10 $\mu$ m).
- (e) SEM illustrating two-celled trichomes on the abaxial surface of the dorsal leaf lobe. (Scale bar = 25 $\mu$ m).

(ex = exospore; en = endoperine; ep = exoperine; T = trichome)

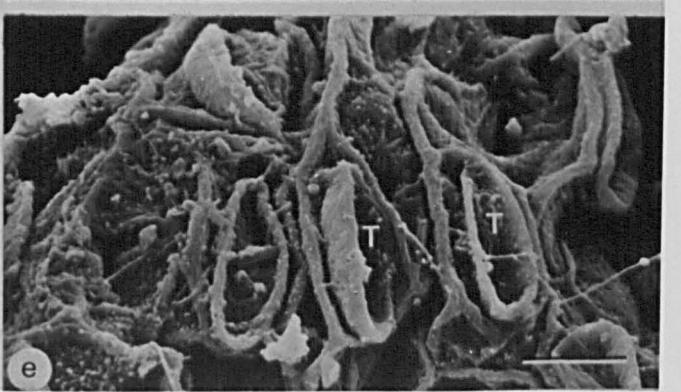
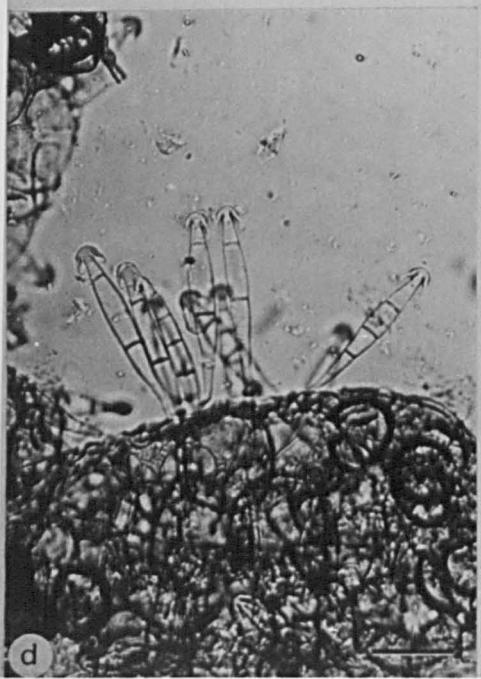
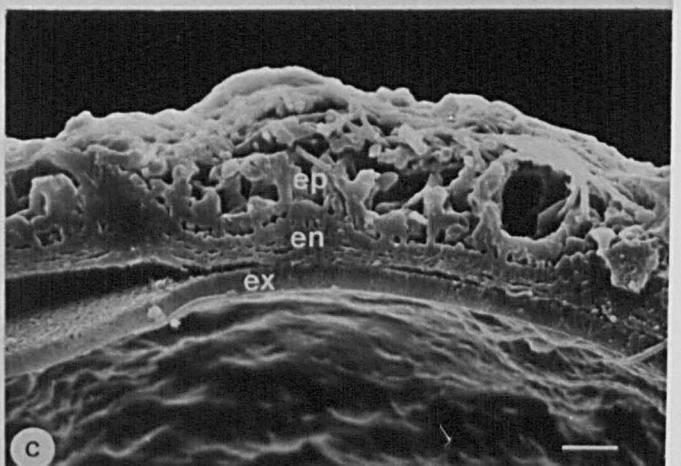
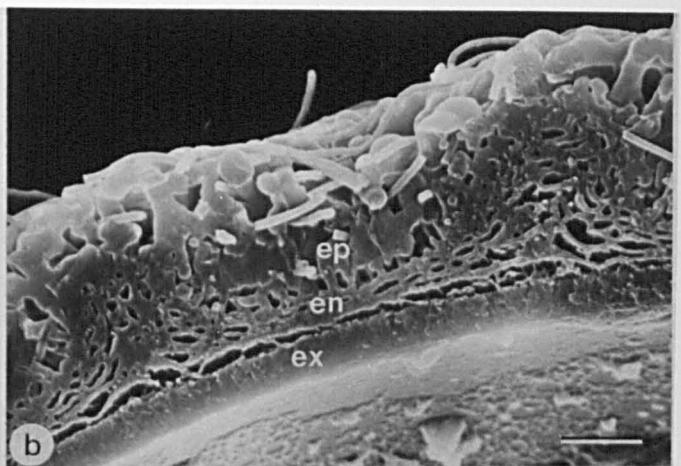
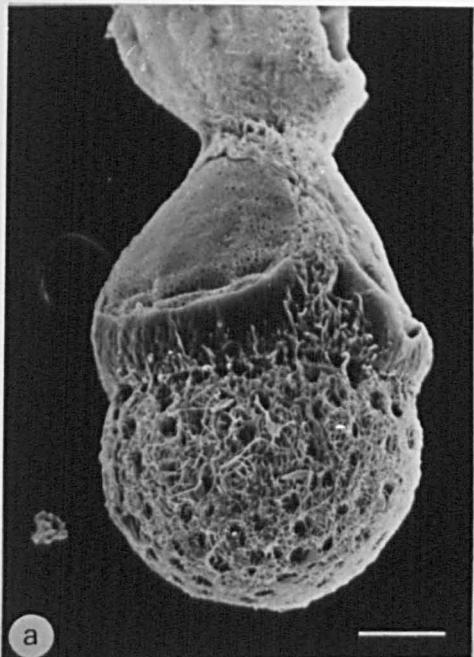


FIGURE 4.55

Nomenclatural Type material of *A.microphylla* Kaulf.

- (a) The herbarium sheet showing the right hand half attached to the sheet, but the annotation by A.H.G. Alston (1949) on the left clearly does not correspond to the right hand side.
- (b) The annotation from the right hand half of (a). The top packet with annotation contains the 'holotype' specimens. The annotation by A.H.G. Alston (2/56) clearly states that he considers the specimens to be *A.filiculoides*.

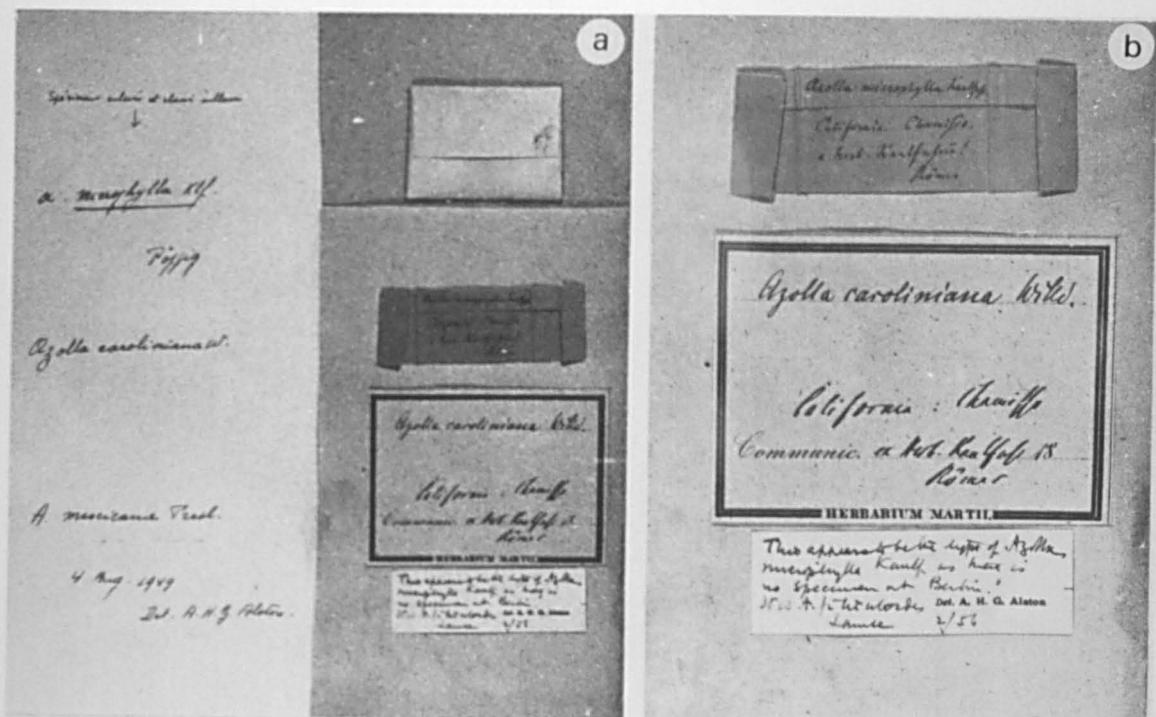


FIGURE 4.56

Nomenclatural Type material of *A. microphylla* Kaulf.

(a) SEM illustrating general morphology and sporoderm sculpturing of the megaspore apparatus. Its size suggests that it is immature. (Scale bar = 100 $\mu$ m).

(b) SEM illustrating sporoderm sculpturing at low magnification. (Scale bar = 50 $\mu$ m).

(c) SEM illustrating sporoderm sculpturing at high magnification. Note the large quantity of infrafilosum. (Scale bar = 25 $\mu$ m).

(d) SEM illustrating glochidia on a massula. (Scale bar = 50 $\mu$ m).

(e) SEM illustrating one-celled trichomes on the abaxial surface of the dorsal leaf lobe. (Scale bar = 50 $\mu$ m).

(g = glochidium; T = trichome)

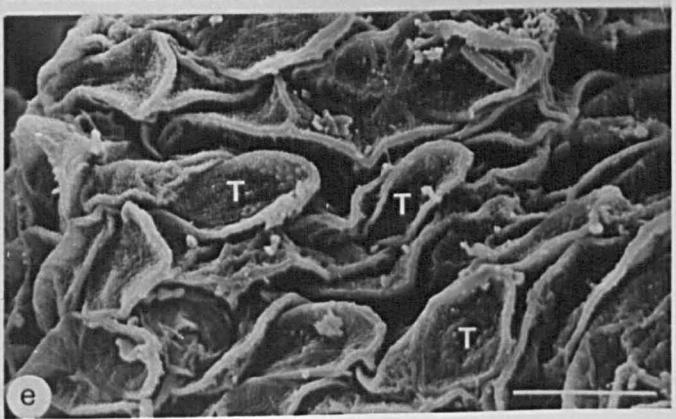
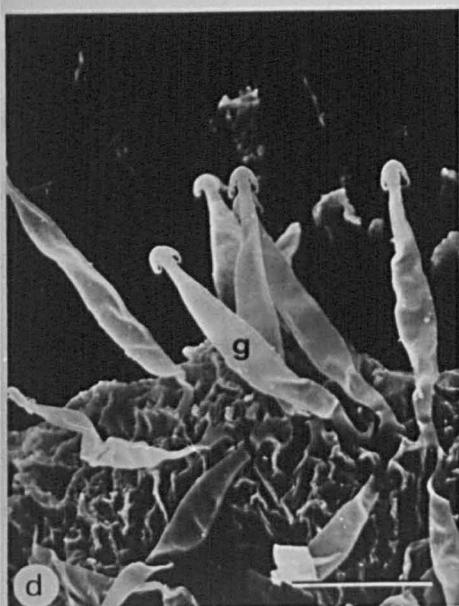
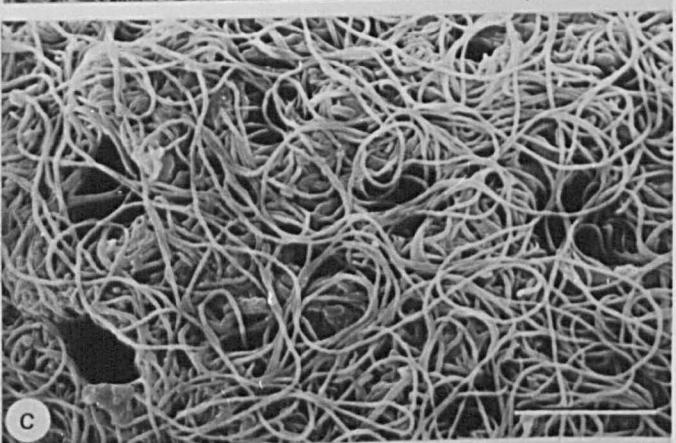
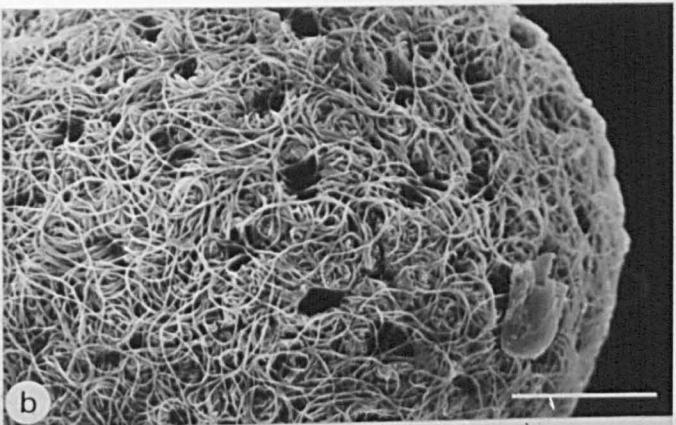
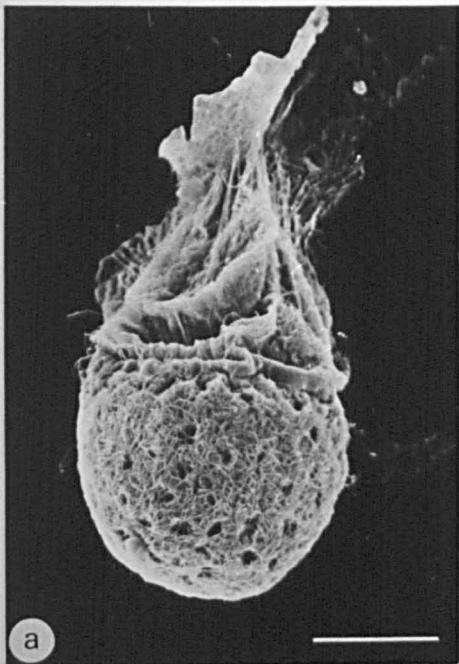


FIGURE 4.57

Nomenclatural Type material

- (a) L.M. of the herbarium sheet with the holotype of *A.arbuscula* Desv. in P.
- (b) L.M. of the herbarium sheet with the 'holotype' collection of *A.densa* Desv. in P.

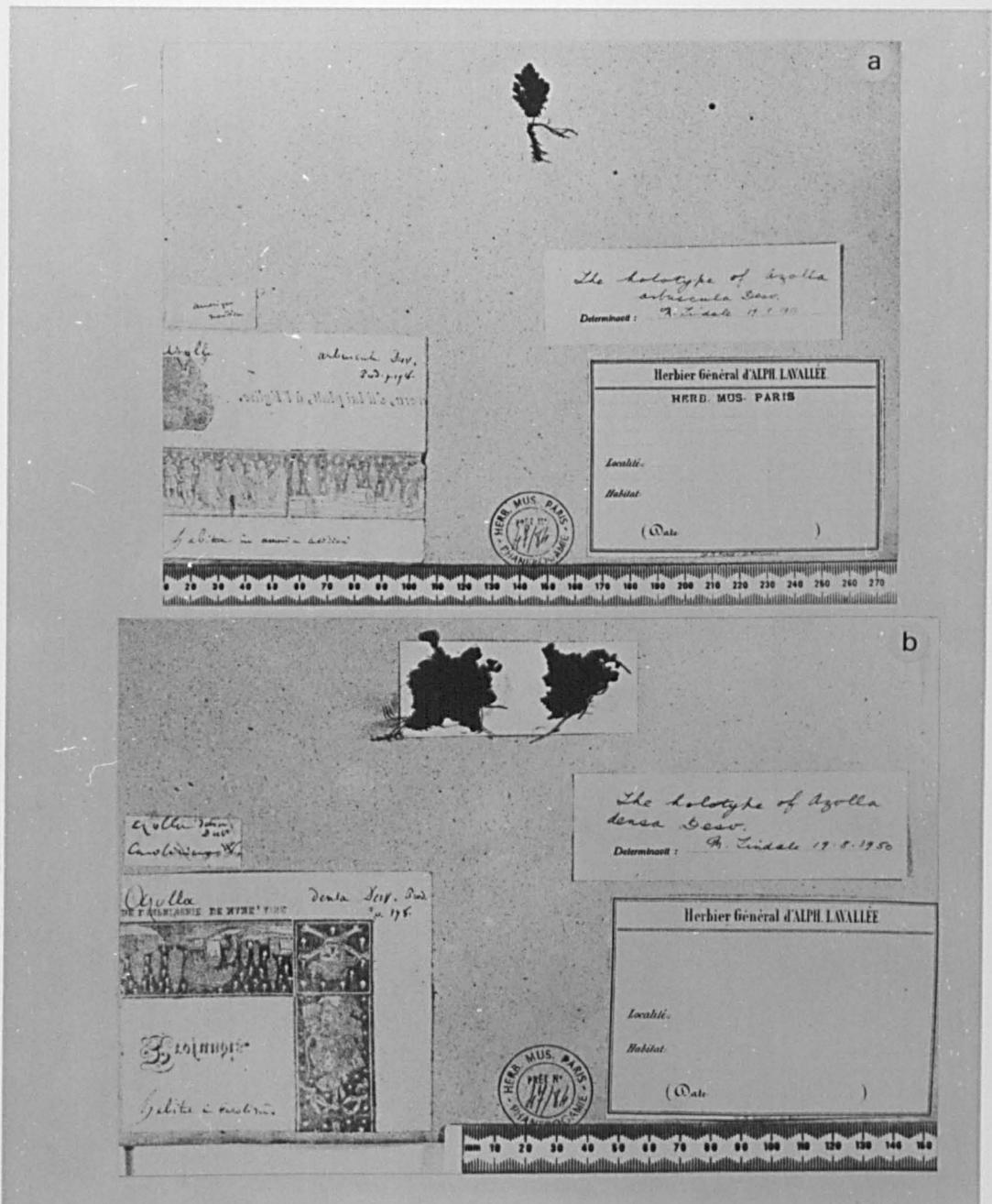


FIGURE 4.58

Nomenclatural Type Material

- (a) L.M. of the annotation associated with the Type material *A.cristata* Kaulf. from BR.
- (b) L.M. of the herbarium sheet of the Type material of *A.japonica* Franch. & Savat. from P.

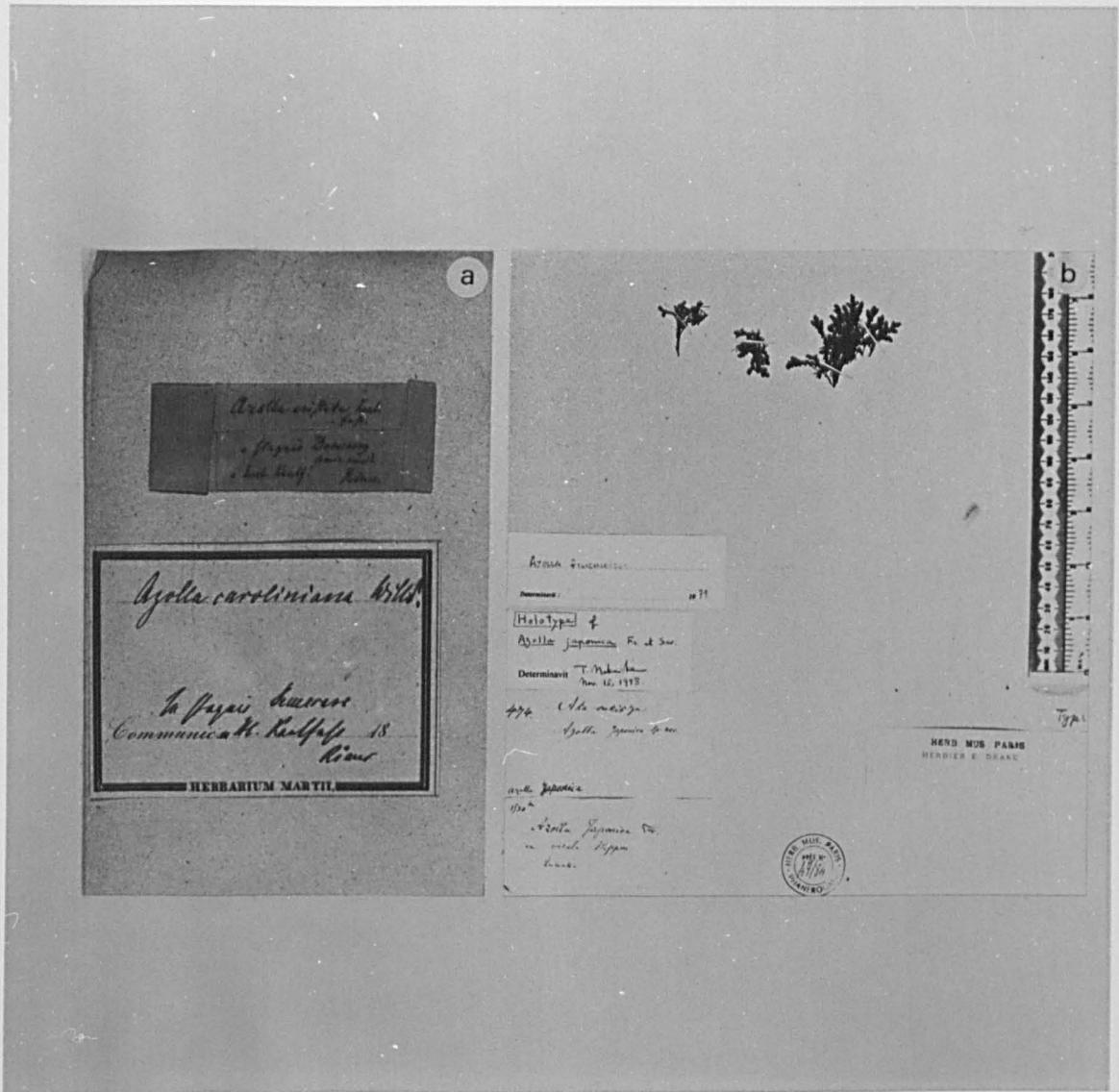


FIGURE 4.59

The A.pinnata type

- (a) SEM illustrating general morphology and sporoderm sculpturing of a mature megaspore apparatus at low magnification. (Scale bar = 100 $\mu$ m).
- (b) SEM illustrating general morphology and sporoderm sculpturing of an immature megaspore apparatus at low magnification. (Scale bar = 100 $\mu$ m).
- (c) SEM illustrating sporoderm sculpturing at high magnification. Note the finely sculptured surface with prostrate excrescences. (Scale bar = 10 $\mu$ m).
- (d) As (c) but side view to show prostrate excrescences.
- (E = excrescence)

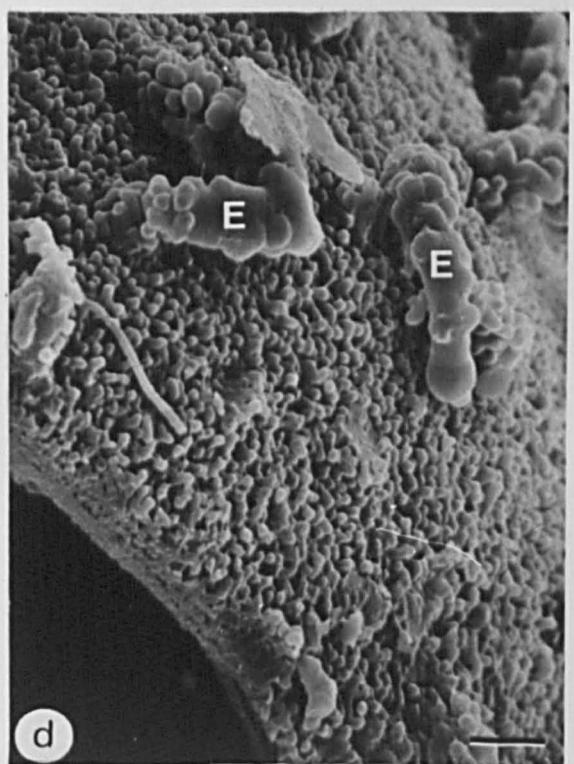
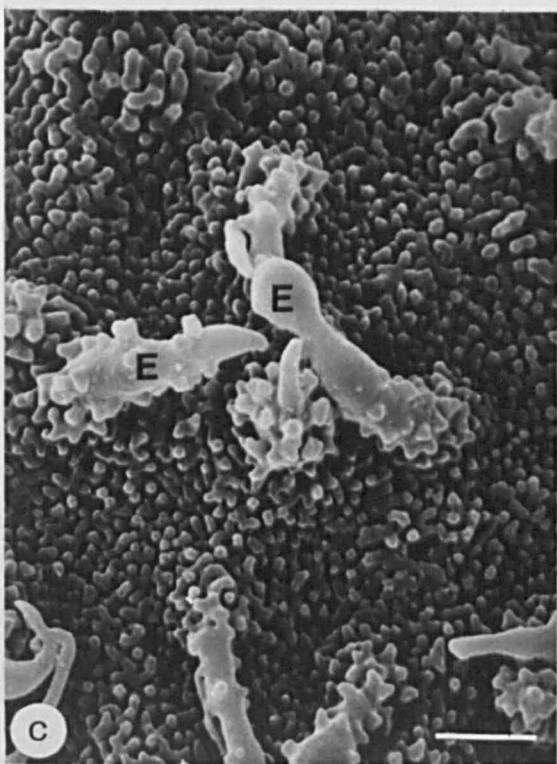
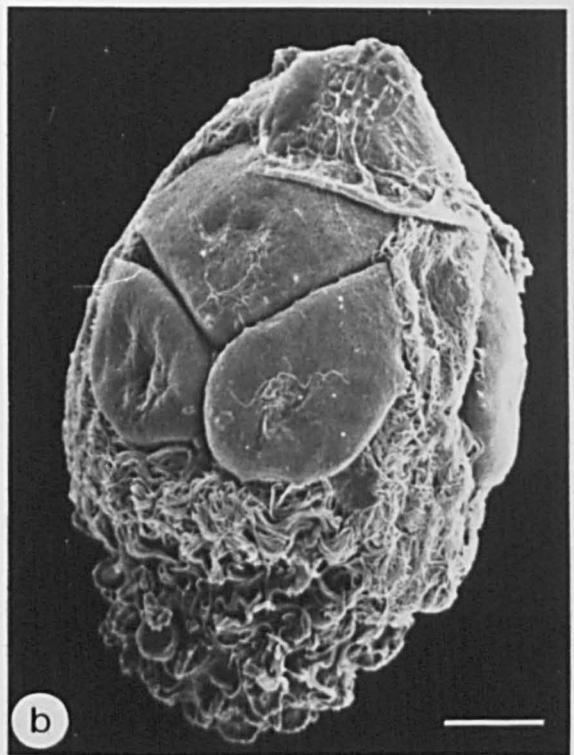
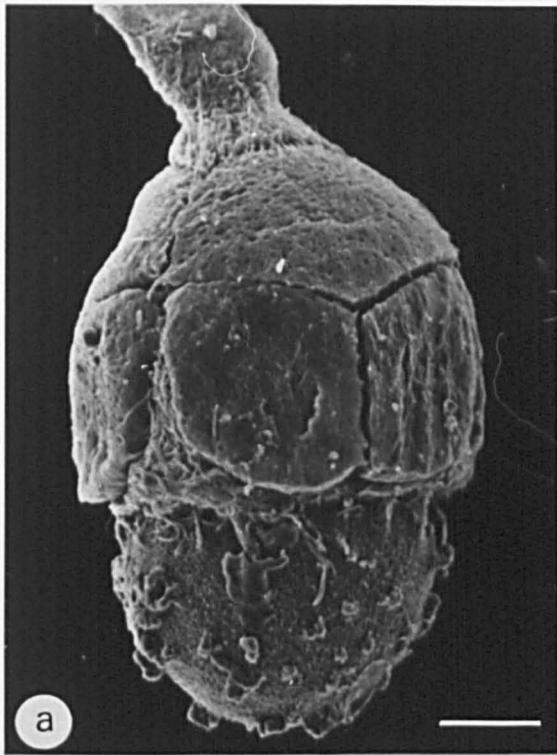


FIGURE 4.60

The A.pinnata type

- (a) SEM illustrating sporoderm structure. (Scale bar = 10 $\mu$ m).
- (b) SEM illustrating sporoderm ultrastructure, especially of the endoperine. (Scale bar = 10 $\mu$ m).
- (c) SEM illustrating an L.S. of the collar in the mid-float region. Note the filosum on the collar surface. (Scale bar = 25 $\mu$ m).
- (d) SEM illustrating spine-like trichomes on the internal massula surface. (Scale bar = 50 $\mu$ m).

(ex = exospore; en = endoperine; ep = exoperine; C = collar;  
F = filosum; MT = massula trichome; bar = equatorial axis)

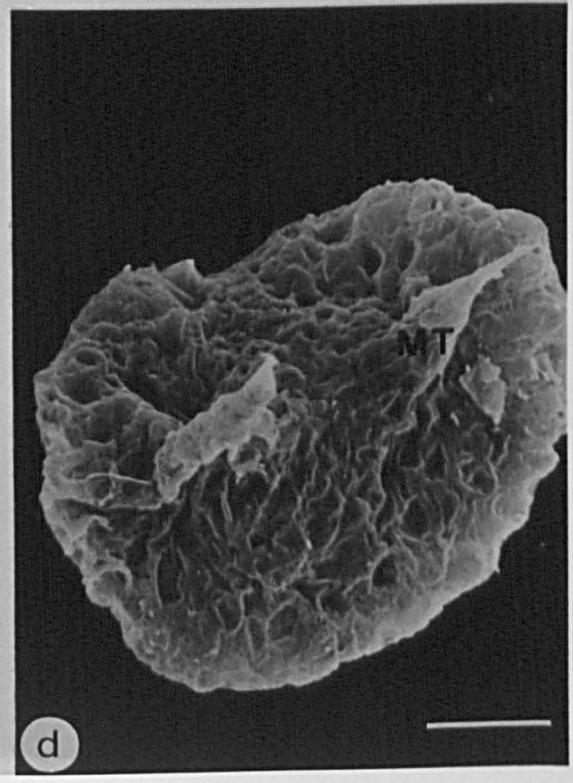
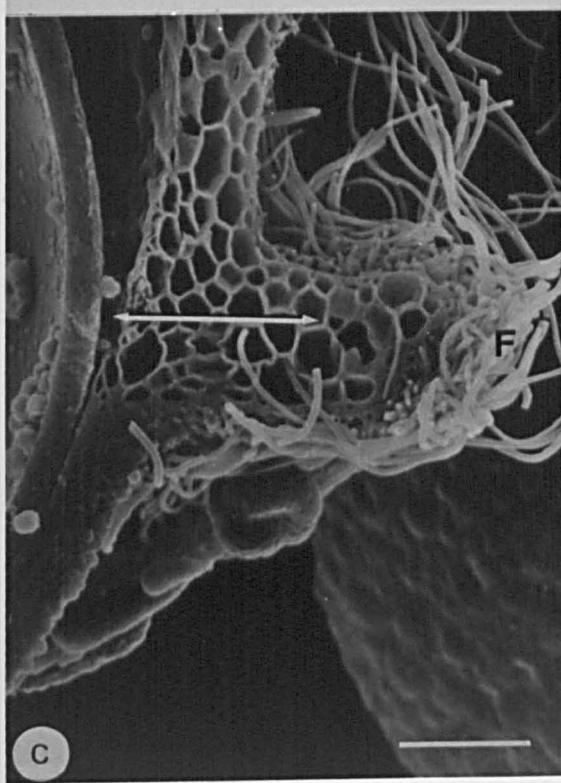
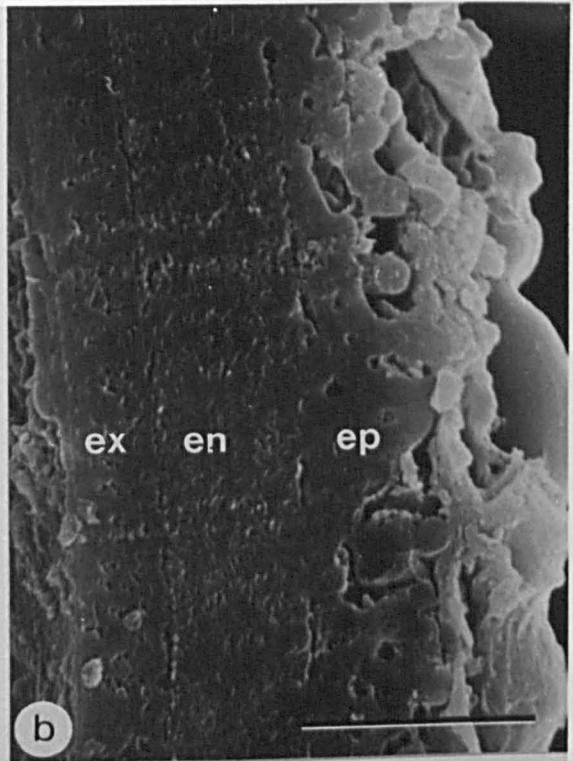
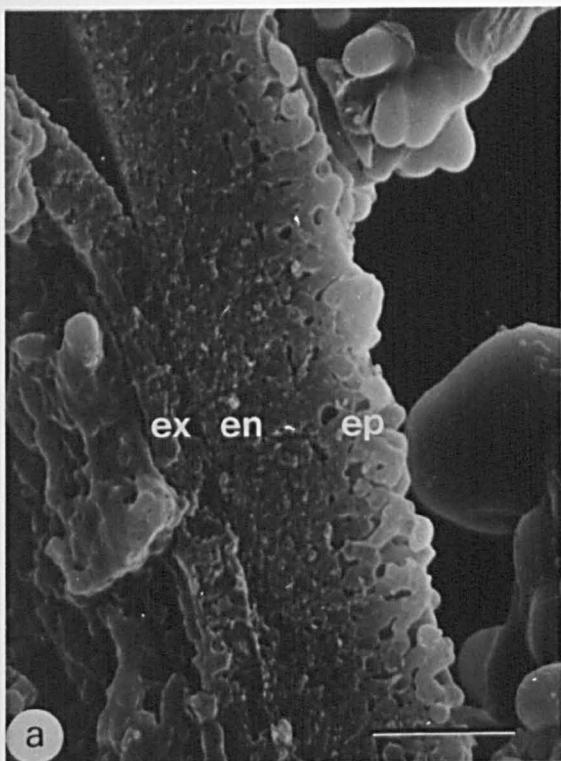


FIGURE 4.61

Nomenclatural Type material of *A.nilotica* Descne.ex Mett.

- (a) SEM illustrating general morphology and sporoderm sculpturing of a megaspore apparatus. (Scale bar = 100 $\mu$ m).
- (b) SEM illustrating sporoderm sculpturing at high magnification showing the spine-like exoperine protuberances. (Scale bar = 25 $\mu$ m).
- (c) SEM illustrating sporoderm structure. Note the exospore, thin endoperine and foveae between the exoperine claveae. (Scale bar = 10 $\mu$ m).
- (d) SEM illustrating an L.S. of the collar in the mid-float region. Note the exoperine extending onto the float surface. (Scale bar = 25 $\mu$ m).
- (e) SEM illustrating massula trichomes. (Scale bar = 25 $\mu$ m).
- (pr = exoperine protuberance; ex = exospore; en = endoperine; ep = exoperine; F = fovea; C = collar; FL = float; MT = massula trichome)

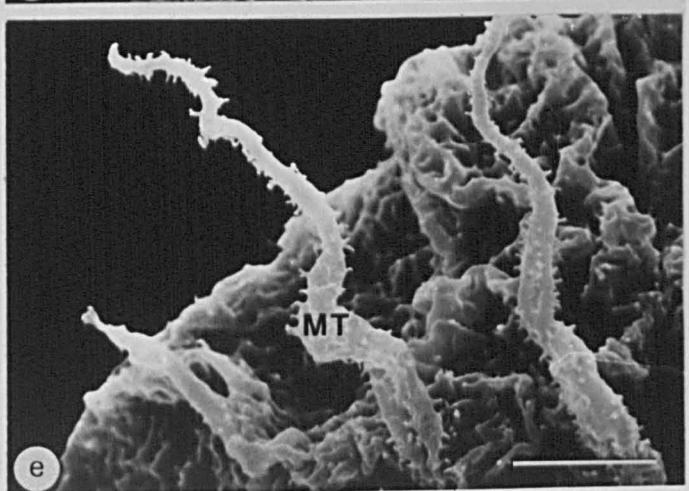
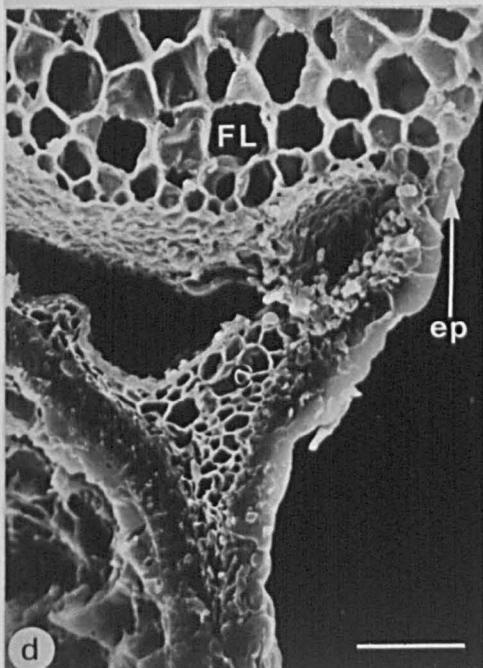
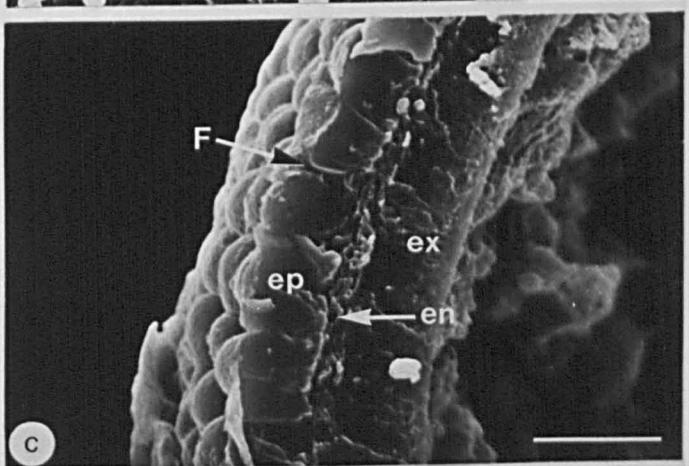
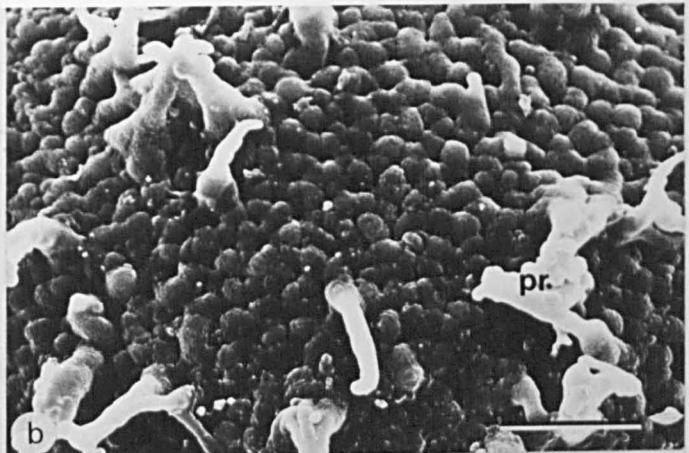
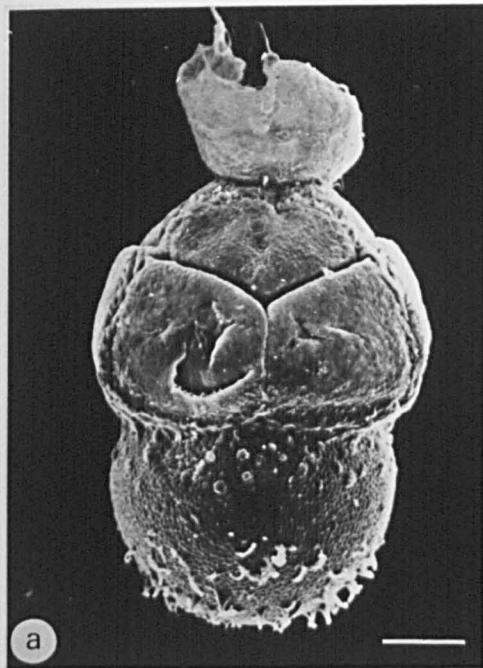


FIGURE 4.62

Nomenclatural Type material

- (a) L.M. of the herbarium sheet with the Type specimens of *A. pinnata* R.Br. from BM.
- (b) LM of R. Brown's specimens No 135 collected from Paterson's River. These are not to be confused with R. Brown's No 134, the lower of which in (a) is the lectotype and was collected from Richmond/Hawkesbury. (Scale bar = 5 cm).

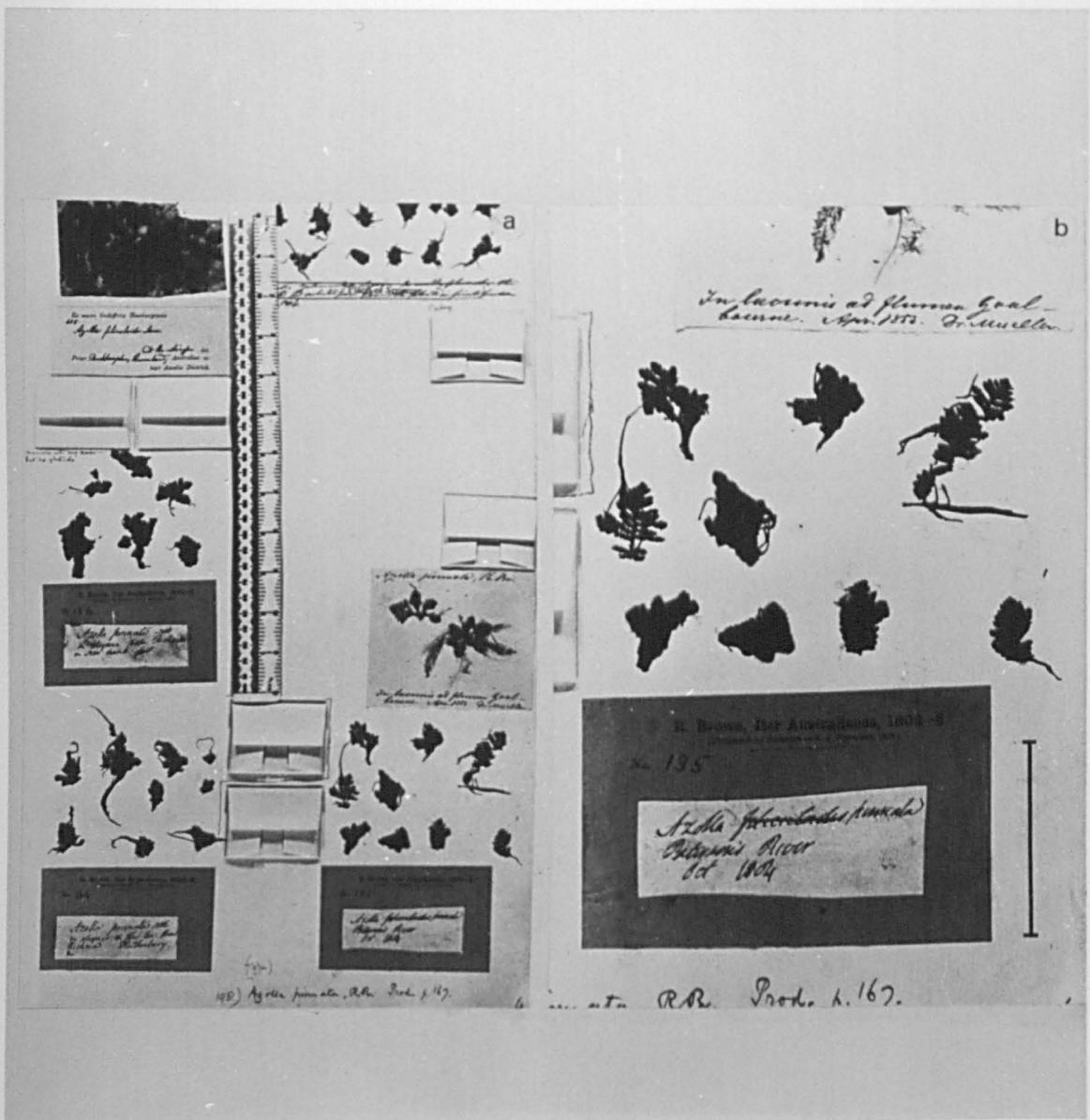


FIGURE 4.63

Nomenclatural Type material of *A.nilotica* Descne. ex Mett.

The herbarium sheet of the Type specimens together with the annotation.



Holotype of *Agyrtis reticulata*

Dominican Republic J. Schulte 22-4-1968

2000 000 0000  
Smithsonian Institution



2000 000 0000  
Smithsonian Institution

TAXONOMIC RE-EVALUATION AND SPECIES RECOGNITION  
OF **AZOLLA** LAM., WITH PARTICULAR REFERENCE TO  
SECTION **AZOLLA**.

DAVID G. DUNHAM.

This thesis is submitted in accordance with the regulations of the Council for National Academic Awards, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Sponsoring establishment:

School of Biological Sciences.

Portsmouth Polytechnic,

Portsmouth,

PO1 2DY

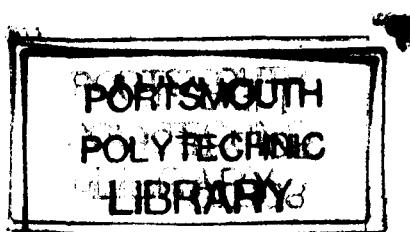
8606614

**AUGUST**

**1986**

Collaborating establishments:

The International Rice Research Institute. British Museum (Nat.Hist.).  
Los Baños, Philippines. London.



FOR KATHY... WITH ALL MY LOVE.

THANK YOU FOR YOUR  
PRESENCE IN MY LIFE...  
YOU ENCOURAGE ME  
TO GO BEYOND MYSELF,  
YOU HAVE THAT INVISIBLE TOUCH...

## ABSTRACT.

DAVID G. DUNHAM - TAXONOMIC RE-EVALUATION AND SPECIES RECOGNITION OF **AZOLLA** LAM., WITH PARTICULAR REFERENCE TO SECTION **AZOLLA**.

**PART A:** Lack of a stable taxonomic framework for **Azolla** Lam. hampers research on the **Azolla-Anabaena** symbiosis which has special economic importance in flooded agricultural systems.

Using material from twenty-one major world herbaria and some fresh material the present investigation aims to critically re-evaluate the taxonomy of Sect. **Azolla**. Scanning electron and light microscopy are employed and some characters quantified. Vegetative and reproductive characters, often poorly defined and undelimited, are evaluated to assess the extent of phenotypic variation; those specimens examined include nomenclatural Type material.

Some terminological confusion concerning megasporangium apparatus structure is revealed and recommendations are made. Extensive sampling and description of phenotypic variation provides a more rational approach to species recognition. The megasporangium apparatus, particularly sporoderm structure, is confirmed as the best taxonomic indicator. In contradiction to previous reports, massula characters are of some taxonomic use, particularly when quantified (eg. glochidial septation). Phenotypic polymorphisms render vegetative characters of little or no diagnostic value, except for the dorsal leaf lobe trichomes. The evolutionary significance of some features is reviewed in the context of fossil and extant **Azolla**.

The following taxonomic proposals are made:- **A.filiculoides** Lam. is a valid taxon, with two subspecies to account for phenotypic variation; **A.caroliniana** Willd. and **A.microphylla** Kaulf. are synonymous with **A.filiculoides**. However, the name **A.microphylla** has been used for a valid taxon which now requires typification and a new name; **A.mexicana** Presl is a valid taxon and intraspecific taxa are not fully substantiated; an un-named taxon requires typification, it has previously been called **A.caroliniana** by some workers, but is not **A.caroliniana** of Willdenow. A preliminary study of Sect. **Rhizosperma** (Meyen) Mett. is also made, with **A.pinnata** R.Br. and **A.nilotica** Descne. ex Mett. being recognised. Future taxonomic research is suggested, and should be more applied.

**PART B:** Aspects of the reproductive biology of **A.filiculoides** are investigated. Inoculation of the embryo and leaf cavity with **Anabaena azollae** Stras. are described, illustrating the subtlety of morphogenesis in maintaining the symbiosis.

DECLARATION

I hereby certify that the work submitted in this thesis  
is my own and has not been presented previously, or  
separately, for any other degree.

Signed

D.G. Dunham.

#### ACKNOWLEDGEMENTS

I gratefully acknowledge and thank my supervisor, Dr. Keith Fowler, for his enthusiastic guidance and patience. My gratitude extends to the Trustees of the British Museum (Nat. Hist.) for providing facilities, but especially to Mr. A.C. Jermy (Head of the Fern Section) for help in obtaining herbarium specimens, and for encouragement and invaluable discussion.

I am indebted to Portsmouth Polytechnic for providing the Research Assistantship and funding, especially the School of Biological Sciences, for providing research facilities. Thanks go to the International Rice Research Institute, Los Baños, Philippines for making funds and facilities available, in particular Dr. I. Watanabe for supplying material, assistance and for his hospitality during a period of study at IRRI. I also thank all those Keepers of Herbaria who loaned material; without them this investigation would not have been possible.

Thanks extend to all those who have given technical advice, especially Colin Derek and the late Eddie Hawton for their willing assistance with photography.

Many thanks to Jane Forbes for typing this thesis from an often illegible handwritten manuscript; errors and omissions are therefore of my own making.

Finally, very special thanks are given to Kathy for her patience and encouragement through the frustrations of preparing this thesis.

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**VOLUME II**

**PART A**

5. DISCUSSION AND CONCLUSIONS

5. DISCUSSION

5.1 STRUCTURE AND TERMINOLOGY

5.1.1 Sporocarps

The structure of the two types of sporocarp is well understood and this investigation supports most recent accounts including Konar & Kapoor (1974) and Becking (1978). A microsporocarp lacks a demarcated dehiscence zone, whereas the megasporocarp usually dehisces by splitting equatorially at the level of the collar. Although this zone is where the thin fragile sporocarp cells become thickened robust cells, there is apparently no specialisation of cells. A microsporocarp contains many microsporangia, the walls of which are simple compared with many other ferns. Absence of an annulus or dehiscence zone was rarely mentioned by previous authors (Campbell, 1893; Rao, 1935; Becking, 1978; Calvert *et al.*, 1983). However, absence was implied by reports that the microsporangial wall disintegrated or sloughed off. In contrast, the single megasporangium within a megasporocarp usually splits equatorially at the same level as the split in the sporocarp wall. The significance of the megasporangial wall appears to have been underestimated by previous authors. In the apical region it is important for partitioning the endosymbiont within the megasporocarp and during embryo development; these aspects will be discussed at greater length in Part B. Collapse of the megasporangial wall cells and their fusion to the funnel perhaps render the wall somewhat insignificant, but it has been preserved in some fossil specimens (Trivedi & Verma, 1971; Bertelsen, 1974; Fowler, 1975) and was termed the apical membrane (Fowler & Stennett-Willson,

1978). This term is quite acceptable for **Azolla** providing it is appreciated that the apical membrane is a remnant of the megasporangial wall which is fused to the funnel, and is therefore not strictly part of the megaspore apparatus; the essence of this was appreciated by Martin (1976). However, Rao (1935) interpreted the fused funnel and apical membrane as being part of the megasporangial wall. Capping the apical region of a released megaspore apparatus is part of the megasporocarp wall which is conveniently termed here the indusial cap. Although first used by Rao (1935), it reaffirms the modified nature of the indusium and describes the nature and position of this persistent part of the megasporocarp wall. Furthermore, the term prevents confusion with the term 'apical cap', which was applied to the apical membrane (Calvert et al., 1983).

### 5.1.2 Megaspore apparatus

Considerable controversy and confusion surround the structure and terminology applied to the megaspore apparatus. The terms 'infraspore' and 'supraspore' were first used by Sweet & Hills (1971). The prefixes 'infra' and 'supra' were presumably used to express the positions of components relative to the laesurae, and therefore, did not compromise any structural terms, while maintaining the palynological practice of describing positions relative to the laesurae. Other terms such as epispose (Campbell, 1893; Roa, 1935, Sweet & Hills, 1976), perispore (Martin, 1976) have structural affinities, and therefore cause confusion. "Capture mechanism" used by Lucas & Duckett (1980) has functional and non-palynological affinities, thereby avoiding

any confusion with structural terms. However, the present author considers "capture mechanism" inappropriate. Sweet & Hills (1971) delimited the infra- and supraspore at the collar. However, it may not be possible to observe a collar, even in extant taxa, while some fossil taxa ~~may~~ not possess one. It should also be emphasised that these terms, 'infraspore' and 'supraspore', are only useful in respect to the external morphology of the megaspore apparatus; they do not reflect its structure, and in this respect, they are not useful except perhaps for measurement. Furthermore these terms can only be applied to a megaspore apparatus with polarity, therefore, some fossil taxa are excluded. The terminology proposed by Fowler & Stennett-Willson (1978) for the megaspore apparatus was based mainly on structural features and was confirmed by the present investigation as the most useful scheme. Their scheme recognised the complex arrangement of megaspore apparatus components while previous schemes have taken an over simplistic view in the absence of detailed investigation. Furthermore, the terms defined by Fowler & Stennett-Willson (1978) can be applied to both fossil and extant *Azolla*. The present study adopts most of their terms and definitions, although modifications are made because a more critical and wider investigation has now been made. Terms used in the present study are defined in Section 3, and it is necessary to compare them with previous terminologies.

The apical region of a megaspore apparatus with polarity is usually dominated by the floats. Strasburger (1873) called this region the "Schwimmkörper" composed of "Schwimmapparat" because

the alveolate float structure was thought to endow buoyancy to the megasporangium apparatus. Strasburger's terms have subsequently been interpreted as meaning swimming apparatus, floating structures and floats, which have been widely applied to both fossil and extant *Azolla* (eg. Rao, 1935; Hall & Bergad, 1971; Sweet & Hills, 1971, 1976; Bertelsen, 1974; Fowler, 1975; Martin, 1976a, 1976b; Fowler & Stennett-Willson, 1978; Calvert et al., 1983 and others). However, as pointed out as early as 1893 by Campbell, but later erroneously contradicted by Rao (1935), the floats do not endow buoyancy. Campbell (1893) also suggested that use of the term 'float' should discontinue. It appears that this suggestion has not been followed; however, Becking (1978) used "massulae" or "alveolar bodies". The term is in such wide and unambiguous use that changing it would create confusion. The most correct term would be 'megasporangial massula', which reflects the homology of a float and its filosum to a microsporangial massula and its appendages (Campbell, 1893; Martin, 1976a and others). Furthermore, the perine, including its elaborations, is also homologous to massulae (Martin, 1976a). These homologies have been substantiated through developmental investigations by Duncan (1940), Bonnet (1957), Konar & Kapoor (1974), Herd et al. (1985) and Cutter & Herd (in press). It is to these elaborations that the discussion now turns.

#### 5.1.3 The Megaspore

Many megasporangia of heterosporous ferns have a proximal tripartite prolongation of the perine which has been variously named and interpreted (Hall, 1975). In respect to these ferns, it has been

called a 'trifolium', 'gula' and 'acrolamella' (Hills, 1967; Potonie, 1956 and Tschudy, 1966 respectively). However, Eames (1936) used the term 'columella' in *Azolla*, which Campbell (1893) and others had used for the megasporangial stalk. This dual usage lead Kempf (1969a) to use 'gula' for this megaspore apparatus feature. Subsequently, the term gula has come into wide use along with columella (e.g. Fowler, 1975; Fowler & Stennett-Willson, 1978; Lucas & Duckett, 1980; Calvert et al., 1983 and others). Although Martin (1976a) used the term 'column', he correctly argued that it was not a float column because floats have a cytologically different origin to the column, and therefore did not give rise to them. The term 'acrolamella' is used in the present study because Tschudy (1966) originally intended to distinguish perinous and exinous prolongations of the proximal sporoderm (Hall, 1975). An acrolamella is composed of perine, whereas 'gula' is a palynological term applied to elaborations of laesurae of trilete spores (Potonie, 1956) and is therefore part of the exospore. Furthermore, 'acrolamella' can be applied to many heterosporous ferns, but in particular can be recognised even in ancestral taxa of *Azolla* and *Salvinia* (Hall, 1975).

Regardless of the term used for the acrolamella, most previous authors have included the superstructure of filosum originating from its surface. However, the present investigation follows Fowler & Stennett-Willson (1978) by considering the superstructure of filosum (= suprafilosum) separately from the acrolamella. The suprafilosum forms a hairy tube from the

acrolamella up between the floats and terminates with the funnel that caps the floats (Fowler, 1975). Furthermore, the tube of suprafilosum invaginates into three equal sectors to accommodate the floats; therefore forming two-layered to ~~mentose~~ septa which separate the floats in each sector (Fowler, 1975). This lead to the erection of the fossil Section **Trisepta** Fowler containing **A.prisca** Reid & Chandler. In doing so Fowler (1975) recognised the structural and evolutionary significance of the triseptate acrolamella and the suprafilosum. In the present investigation of extant species only the **A.pinnata** type was clearly seen to have a complete tube of suprafilosum between the acrolamella and funnel, with double layered to ~~mentose~~ septa dividing the float sectors. In all the other types of Sections **Azolla** and **Rhizosperma**, the septa are perhaps less obvious compared to the **A.pinnata** type, with the tube of suprafilosum being incomplete, or absent, as in the **A.nilotica** type. These observations in taxa of Section **Azolla** support the view that reduction of the suprafilosum is of evolutionary significance (Fowler, 1981). The funnel, although in continuity with the suprafilosum tube is mainly attached to filosum of the float apices.

Filosum originating from the floats should not be considered part of the suprafilosum, because it probably has different cytological and structural origins. Although important for attaching the floats to the megasporangium by entanglement, the present investigation has shown that float filosum is limited to certain areas of the floats, with apparently much less in extant taxa than fossil taxa; this supports the results of Martin

(1976a). Also supporting him, the floats of the extant types of Section *Rhizosperma* have nodules and hook-like appendages which may attach floats to each other and also to the suprafilosum. Therefore, in this Section only, there are two means of attaching the floats to the megaspore. For *A.nilotica*, Martin (1976a) suggested that the hooks or nodules are ~~supplemental~~<sup>ary</sup> attachment because the acrolamella and suprafilosum are reduced. But as the present study has shown suprafilosum is lacking in the *A.nilotica* type, with float retention on the megaspore being mostly by exoperine which extends from the distal sporoderm. However, this does not explain the presence of hooks in *A.pinnata* where, compared with *A.nilotica*, the acrolamella is large and there is much suprafilosum.

The acrolamella in extant *Azolla* is clearly tripartite and composed of highly vacuolate endoperine. The sutures of the acrolamella directly overlie the laesurae as described by Fowler (1975). The three acrolamella septa in effect form the bases of the suprafilosum septa, and both extend out to the collar. Therefore, the present study endorses the findings of Fowler (1975), Martin (1976a), Fowler & Stennett-Willson (1978) and others, that together the acrolamella and collar offer support for the floats, and may explain why there is a reduction in suprafilosum in extant taxa compared with fossil taxa, i.e. elaboration of proximal perine associated with a reduction of suprafilosum, while maintaining support for floats.

**Collar:** This has been termed the 'girdle' by Bates (1980), Calvert et al. (1983) and others. However, 'collar' is the most

widely used term, and is adopted here together with the definition by Fowler & Stennett-Willson (1978). Additionally the collar appears to offer some taxonomic information. There are clear differences in the collar of extant Section **Azolla** and **Rhizosperma** namely the presence of prominent cusps in the former, and their absence in the latter. A cusp is a localised tricorn-shaped upward extension of the collar that is in continuity with the acrolamella septa. Although these findings confirm those of Fowler & Stennett-Willson (1978), Martin (1976a) reported six cusps in **A.pinnata** and three in taxa of Section **Azolla**. Zhou (1983) made no reference to cusps in his detailed study of **A.pinnata**, and the present author suggests that Martin (1976a) was in error in respect to cusps in **A.pinnata**. It appears that cusps have not been reported in fossil taxa, and their development might be another example of evolutionary elaboration of the proximal perine. A further elaboration of the collar in extant Section **Azolla** is the downwardly projecting flange, the position of which may represent an evolutionary sequence (Fowler, 1981, unpub.) (see section 5.2.1).

Only in the **A.nilotica** type does exoperine consistently extend from the distal sporoderm up over the collar on to the lower float tier surface. In the other types exoperine may extend over the collar, but does not extend on to the float surface. Filosum may originate on or cascade over the external collar surface, but appears to be of little or no structural significance. The collar appears to be a transition zone between the proximal and distal sporoderm because the highly alveolate collar endoperine

gradually changes to more dense granular endoperine towards the distal sporoderm.

Distal sporoderm: This is the basal, more or less spheroid region of the megaspore apparatus which houses the megaspore proper. Although early observation techniques led to a simplified concept of wall stratification, recently considerable confusion has been created by applying pollen wall terminology. This confusion is illustrated in Figure 1.1. Kempf (1969a, 1969b) recognised the major sub-divisions of the sporoderm, as did Fowler (1975). However, it was not until the terminological proposals by Fowler & Stennett-Willson (1978) that a workable and stable scheme emerged; this scheme was found to be wholly usable in the present investigation with only minor modifications. The delimitation and terms used for the strata are completely homologous from one taxon to another, unlike the scheme used by Calvert et al. (1983). However, the present author believes that they followed Martin's (1976a) terminology for the perine, but misinterpreted terms and strata. Whereas poor quality of the sections used by Lucas & Duckett (1980) may have lead them to question the proposals of Fowler & Stennett-Willson (1978).

Although 'sporoderm' can apply to only the spore wall proper (see Kremp, 1965) and was used in this way by Martin (1976a, 1976b), this term is generally used for the whole spore wall which can be divided into two principle layers. In *Azolla* the innermost layer, the megaspore wall proper, was originally termed the 'exospore' (Campbell, 1893). A thin basement layer was later

recognised (Demalsey, 1953 and others) and has been variously named (see Fig.1.1). This layer was rarely and not always convincingly observed in the present study using the scanning electron microscope and it is possible that the basement layer is an artefact of preparation in light and transmission electron microscopy. Alternatively, it may be a developmental layer (Lugardon, 1978b) and is not always discernible. It is equally likely that this basement layer was not distinguishable using the techniques employed here. The term 'exine' was originally used by Kempf (1969a) for the whole exospore of **Azolla**, in an attempt to equate it to pollen walls. Subsequently, this term has been widely used (see Fig.1.1). However, Lugardon (1978) has shown that, although structurally homologous, the spore walls of ferns (*sensu lato*) and angiosperms develop differently. In recognition of this it was proposed that 'exospore' and 'exine' be used for fern and angiosperm spore walls respectively (Lugardon 1978b). This proposal has been followed by the present author, and replaces 'exine' *sensu* Kempf (1969a) and others.

It appears that the terms 'exospore' and 'exine' as used by Hall & Swanson (1968), Jain & Hall (1969) and Jain (1971) represents most of the sporoderm and appears unacceptable even within palynological definitions. Comparison of exospore descriptions in both fossil and extant **Azolla** reveals that they are essentially similar to those in the present study. The striated appearance under the light microscope was probably caused by more or less radially arranged components and sinuous cavities observed using the scanning electron microscope. Interestingly, cytochemical

analysis of the exospore revealed non-cellulosic carbohydrates and sporopollenin, whereas the perine is composed of mainly the latter with a little lipid (Lucas & Duckett, 1980). The exospore is loosely attached to the radially external perine by means of irregularly arranged filaments on the exospore surface. These observations endorse those by Zhou (1983) for *A.pinnata* and Fowler (1981) for *A.prisca*.

As stated by Fowler (1981), the term perine has become acceptable following the proposals of Kempf (1969a, 1969b), however, the sub-divisions of this layer have received various names (see Fig.1.1). As stated previously, the terms used here follow those proposed by Fowler & Stennett-Willson (1978) because the strata are positionally defined. The innermost perine layer, the endoperine, appears to have a more or less common structure in all extant taxa of *Azolla*, and indeed fossil taxa (Kempf 1969a, 1969b, 1974 ; Fowler 1975, 1981). Ultrastructurally the present investigation supports the findings of Zhou (1983), who used transmission electron microscopy to show that this layer is composed of closely packed tiny rod-to-thread-like, simple or branched, irregularly arranged vermiform elements. However, unlike Zhou (1983) it was not possible to show that the elements were coiled more or less perpendicular to the long axis of the megaspore apparatus. This may have been caused by the elements being more closely packed and apparently fused in Section *Azolla* compared with *A.pinnata*. Indeed, in all specimens where the endoperine was alveolate the individual elements were rarely discernible. The variable degrees of endoperine alveolation,

seen at low magnification, may have taxonomic significance and will be discussed later.

A comparison of fossil taxa (e.g. Kempf 1969a, 1969b; Fowler 1975; Martin 1976a; Sweet & Hills 1976) suggests that only in extant Section *Azolla* does the endoperine become alveolate, and within these taxa there is an evolutionary sequence which appears to corresponds to the one proposed by Fowler (1981) for the collar. Also through a comparison of fossil and extant taxa, endoperine in the basal megaspore region has a uniform thickness in fossil taxa. However, in extant *Azolla* this erupts to varying degrees into the outer exoperine layer. These eruptions may or may not be responsible for sculptural features (i.e. raised areas). If these features include endoperine they are termed 'excrescences' (Fowler & Stennett-Willson, 1978) whereas they should be termed 'exoperine protuberances, if they comprise exclusively of exoperine. For example, the *A.filiculoides* type always possesses excrescences whereas the *A.mexicana* type may possess exoperine protuberances. Excrescences and protuberances can only be distinguished by their structure because the present investigation has shown that sculpturally they may be similar. Furthermore, the regularity of occurrence and size of excrescences in Section *Azolla*, found during the present investigation, tentatively suggests an evolutionary sequence similar to that of flange position.

Radially external to the endoperine is the exoperine which, according to Fowler & Stennett-Willson (1978), and endorsed by

*A.microphylla* type). In extant Section *Azolla* the exoperine 3 is composed of more or less tangentially arranged elements, whereas in extant Section *Rhizosperma* this zone forms the solid covering of an excrescence or protuberance. The same definitions can be applied to an exoperine 3 in fossil taxa. The present study revealed that an exoperine 3 was occasionally indistinguishable from the exoperine 2 because the component elements were fused. In such instances, which rarely affected every specimen sampled in a single population, only two exoperine zones (1 and 2) can be recognised. The cause of this might be developmental in respect to maturity or excess exoperine deposition. Where the latter occurred there appeared to be no reduction in any other layer. Whatever the cause, there is an obvious need for a reasonable sample size when describing taxa.

It can be argued that the exoperine 3 zone is not a true stratum because it occasionally cannot be distinguished, whereas all other zones can always be distinguished. The present author believes that delimitation of an exoperine 3 is useful for relating structural and sculptural features. Furthermore, tangentially arranged elements are considered sufficiently different from radially arranged elements to warrant separate classification. A similar argument for recognising an exoperine 3 in Section *Rhizosperma* can be forwarded, except that in these taxa it is a solid zone.

Taking exoperine as a whole layer, there is often variation, within a single specimen, in respect to its thickness. This is seen in a thinning of this layer towards the collar and is

associated with loss of distinct zones, particularly exoperine 2 and 3. In Section **Azolla** the exoperine appears to terminate or die out in the groove of the collar. However, in some specimens a thin layer of exoperine extends up on to the collar surface, but there is no evidence for any zonation of the exoperine. Fowler (1981) illustrated this in his section on the significance of collar morphology. This evidence leads the present author to suggest that exoperine may extend over the external collar surface in all specimens, but the lack of zonation and thinness of the exoperine renders it indistinguishable from the collar endoperine. Further evidence is found in the **A.nilotica** type where, as already stated, exoperine consistently extends over the collar surface.

Extending radially beyond the exoperine is infrafilosum. This term was introduced by Fowler & Stennett-Willson (1978) for filosum (hair-like filaments) originating in the distal sporoderm; 'infra' implying its <sup>place of</sup> origin rather than any morphological or structural differences from other types of filosum. Those authors noted that infrafilosum appeared to arise from the exoperine 1 zone, however, did not exclude the possibility that it arose from the endoperine (Fowler, 1981). Origin of infrafilosum from the exoperine 1 is confirmed by the present study, but was only very rarely observed even in specimens with much infrafilosum. Similarity in the exoperine zones, through branching of their respective elements, suggests that it is not unlikely that infrafilosum originates from all three zones. Infrafilosum is lacking in extant Section **Rhizosperma** but

is present in varying quantities in all taxa of extant Section **Azolla**. In fossil **Azolla** there were also taxa with and without infrafilosum (Jain, 1971; Bertelsen, 1974; Fowler, 1975; Sweet & Hills, 1976 and others), while taxa considered to be ancestral to the Salviniaceae apparently possessed much infrafilosum (Sweet & Hill, 1974; Hall, 1975). The function of infrafilosum appears to be for entanglement with glochidia (Campbell, 1893; Becking, 1978) or massula trichomes. However, in the absence of infrafilosum, attachment of massulae seems less likely. It is interesting to postulate that glochidia would be better able to attach to a surface lacking infrafilosum than massula trichomes. In taxa with massula trichomes the number of trichomes is considerably less than the number of glochidia in other taxa and infrafilosum is lacking (e.g. the **A.pinnata** and **A.nilotica** types). It would appear that a reduction of, or complete loss of, infrafilosum was not associated with the type and number of massula appendages. However, infrafilosum and massula appendages appear developmentally and chemically homologous. (Bonnet, 1957; Konar & Kapoor, 1974; Lucas & Duckett, 1980).

Although transmission electron microscopy techniques have the potential to provide superior ultrastructural resolution (Kempf, 1969a and Zhou, 1983) the procedures are lengthy, including the reconstruction of 3-dimensional structure from 2-dimensional images. The scanning electron microscopy techniques employed in the present investigation, and by Calvert *et al.* (1983), are clearly appropriate, particularly in respect to the time available. Furthermore, the present study has shown that detail

comparable to that seen under the transmission electron microscope can be obtained under the scanning electron microscope. This has lead to the confirmation that megaspore apparatus structure is elaborate and complex requiring, in many instances, specialised and often unique terminology to accurately describe it. Most previous attempts at providing both descriptions and terminology have not succeeded, and have caused confusion by using poorly defined and inappropriate pollen wall terms. However, through the present critical investigation it has been possible to confirm and modify, where necessary, the terminology proposed by Fowler & Stennett-Willson (1978). This was achieved by much wider sampling of specimens, and further establishes and enhances a unified terminology, together with the understanding of megaspore apparatus structure in both extant and fossil *Azolla*. Structural and sculptural features appear to be extremely useful means of taxonomic separation (Fowler & Stennett-Willson 1978; Zhou 1983) and their importance is confirmed by the present study.

## 5.2 EVALUATION AND TAXONOMIC SIGNIFICANCE OF CHARACTERS

### 5.2.1 The Megaspore Apparatus

The present investigation uses megaspore apparatus characters to define the types. These types then form the basis for evaluating other characters. In defining the types there is delimitation of variation and evaluation of megaspore apparatus characters. Part of this process involved discussing the structure and terminology of the megaspore apparatus (see Section 5.1) which naturally pre-empts some discussion of taxonomically useful characters.

However, it was considered necessary, to discuss megasporangium characters separately and after structure and terminology.

Size of the megasporangium appears to be of no taxonomic use. This supports the findings that can be extrapolated from previous studies (see Table 5.1). Although these pertain mainly to fossil taxa, there has been no real change in megasporangium dimensions through time. However, *A.pinnata* may have become slightly smaller (Zhou, 1983).

Variation in dimension data for fossil specimens may be attributable to unidentifiable developmental variation, preservation and deformation during fossilisation. Additionally, previous authors rarely state where diameter (width) measurements were made. This may introduce variation if one investigator measures median diameter, while another measures maximum diameter. The present study has provided perhaps the most detailed data on dimensions for extant Section *Azolla* indicating that dimensions do not always reflect shape as is evident where there is often a distinct waist. Furthermore, the number of floats may influence the overall megasporangium shape, in so far as nine floats renders the apical region somewhat spheroid; this was particularly evident in the *A.nilotica* type. A literature survey reveals that shape has received little attention, and if described, it is usually said to be ovoid (e.g. Mettenius, 1847; Strasburger, 1873). This indicates that shape is of no diagnostic value, and is supported by the present investigation.

TABLE 5.1

<u>TAXA</u>	<u>LENGTH <math>\mu\text{m}</math></u>	<u>WIDTH <math>\mu\text{m}</math></u>
<u>A.extincta</u>	460-540	280-360
<u>A.montana</u>	390-530 (450)	270-360 (310)
<u>A.bulbosa</u>	385-555 (455)	230-350 (270)
<u>A.elegans</u>	430	350
<u>A.fragilis</u>	450-520 (500)	350-410 (390)
<u>A.stanleyi</u>	350-640 (480)	220-400 (370)
<u>A.velus</u>	350-570 (450)	340-430 (380)
<u>A.teschiana</u>	425	250
<u>A.colwellensis</u>	377-550	180-462
<u>A.areolata</u>	346-595 (479)	215-380 (324)
<u>A.angularis</u>	446-599 (531)	-
<u>A.prisca</u>	446-475 (455)	237-270 (255)
<u>A.capricornica</u>	410-560	340-400
<u>A.roemoeensis</u>	445-570	292-368
<u>Azolla</u> sp sensu Bertelsen 1974	381-496	292-343
<u>A.pinnata*</u>	425-660 (560)	225-463 (310)
<u>A.nilotica*</u>	480	300
<u>Azolla</u> sp.*	437-543 (485)	220-341 (321)
<u>A.microphylla*</u>	462-600 (511)	269-512 (338)
<u>A.mexicana*</u>	475-560 (518)	312-387 (343)
<u>A.filiculoides*</u>	490-607 (546)	304-412 (364)

Table to show megaspore apparatus dimensions of fossil and extant (\*) taxa.

Data from:- Hall & Swanson (1968); Jain & Hall (1969); Hall & Bergad (1971); Jain (1971); Sweet & Hills (1971, 1976); Bertelsen (1974); Fowler (1975); Martin (1976b); Collinson (1980); Foster & Harris (1981); Zhou (1983) and the present study. Numbers in brackets are mean values.

Although the *A.filiculoides* and *A.microphylla* types possess similar float puncturing, the *A.mexicana* and *Azolla* sp. types appear more or less distinct in respect to the float surface. Deformation of the floats may obscure the true nature of this feature, and some caution is required when examining fossil and air dried specimens. Although limited, the nature of the float surface does have some diagnostic value. Furthermore, this feature has not previously received scrutiny. Although not investigated in the present study, this feature may be related to the size and nature of the sub-surface alveolae. Other characters that appear to be related to each other are float number and shape. This renders shape to be of no diagnostic value, but where there are more than three floats per megasporangium apparatus the floats are described as being in tiers and groups (Konar & Kapoor, 1974; Sweet & Hills, 1976 and Rao, 1935; Sweet & Hills, 1971 respectively). Consideration of the floats occupying three real or apparent sectors created by the acrolamella and suprafilosum may be more appropriate. This may lead to a recognition of an evolutionary sequence of progressive sectorisation of the apical megasporangium apparatus region which is associated with elaboration of the proximal perine.

Although shape and number of floats appear to be related, the latter character has received the greatest attention. The present investigation suggests that float shape and number in extant *Azolla*, and probably fossil *Azolla*, are diagnostically useful at the Section or Subgenus level only.

Although Meyen (1836) proposed two genera base primarily on the

number of floats on the megaspore apparatus, subsequent authors have used float numbers, at least in part, to define subgeneric taxa (see section 1), which is supported by the present investigation. However, the number is almost invariably divisible by three in both fossil and extant *Azolla*. The presence of supernumerary floats (equally or unequally divided) has only occasionally been observed (Sweet & Hills, 1971), which is supported by the present study. Furthermore, supernumerary floats are of no diagnostic value. Although unequally divided floats may be readily recognised, equally divided ones may not. To prevent misinterpretation, particularly in multi-floated fossil taxa, this should be noted for future reference. Indeed Hills & Gopal (1967) reported that Florschutz (1945) observed supernumerary floats in *A.teschiana* Florschutz (which had a basic number of twenty-four floats) and that these may have been unequally divided into three or four. Apart from the three and nine floated extant taxa, the fossil record reveals multi-floated taxa with fifteen and twenty-four floats (Fowler, 1975; Martin, 1976a). The number of floats was considered to be of evolutionary importance by many authors and has been used to construct phylogenies for *Azolla* (Hills & Gopal, 1967). Although such evolutionary considerations are not necessarily the purpose of the present investigation, it is interesting to briefly outline current views as described by Fowler (1975, 1981, unpub.) and Martin (1976a).

Early suggestions concluded that the three floated condition was more primitive than the nine-floated condition (Hills & Gopal,

1967). However, this was based upon misinterpreted fossil material, and later suggestions indicated that in fossil taxa the to ~~mentose~~ acrolamella lacked floats. Advancement included organisation of the acrolamella with the development of float-like structures of the multi-floated condition; however, the floats were not alveolate. Subsequently, they became alveolate and then reduced to nine and three in number. This was accompanied by elaboration of the proximal perine (Fowler, 1975; 1981; unpub.). Therefore, the assumption was that one form gave rise to another. However, Martin (1976a) questioned the validity of using float numbers in phylogenies and suggested that certain numbers of floats may have arisen more than once. Instead, he suggested that the method of float retention on the megaspore apparatus is phylogenetically important. This retention involved the elaboration of proximal perine and hairs and hooked appendages on the floats (Martin, 1976a). The common features between this and the outline by Fowler (1975) are the elaboration of the proximal perine to form the acrolamella and the suprafilosum as a means of retaining the floats. Consequently float number may not be as phylogenetically important as once thought.

Unlike float number and shape, the suprafilosum and acrolamella appear to be of no diagnostic value. However, as stated in section 5.1 the latter two features may be of evolutionary significance. Coupled with this is the development of a collar which is, <sup>in fact</sup>, further elaboration of proximal perine. Fowler (1981, unpub.) examined only a small number of specimens but suggested that variation in sectional morphology of the collar

may be accounted for by an evolutionary sequence. His hypothesis suggested that through the course of evolution a collar has developed, was initially more or less horizontal and flangeless; this condition is illustrated by some fossil taxa (e.g. *A.prisca*) and extant Section *Rhizosperma*. In the more advanced Section *Azolla*, the collar became more erect and developed a flange; this distinguishes the extant sections. In the more primitive condition the flange was inserted towards the lower collar edge, and advancement has lead to the flange being inserted higher up the collar. In addition to this Fowler suggested that in Section *Azolla*, the collar became less massive in the more advanced types. The present investigation generally supports this hypothesis in extant *Azolla*, but with the addition of the *A.mexicana* and *Azolla* sp. types to Fowler's scheme, the sequence, from relatively primitive to advanced, appears to be the *A.microphylla* and *A.mexicana* to *Azolla* sp. to *A.filiculoides* types. The difference between the *Azolla* sp. and *A.filiculoides* types was not clearly demarcated but can be accounted for by Australian populations of the latter type. This sequence clearly illustrated the diagnostic value of sectional collar morphology. However, there is overlap between the types in respect to robustness, erectness and flange position. The wider sampling in the present study revealed some mergers of the collar forms which was not described by Fowler (1981).

In Section *Azolla* some intratype variation in collar sections within one specimen can be attributed to morphological differences between the cusp regions. Consequently, the present author

recommends that the collar is observed from sections made midway between the cusps. Fowler & Stennett-Willson (1978) indicated that prominent cusps were unique to extant Section **Azolla** and by implication suggested the presence of smaller cusps in other taxa. It is therefore surprising that previous authors, including Calvert *et al.* (1983), have completely overlooked collar structure. It was only the investigation by Fowler (1981, unpub.), supported by the present one, that have realised the diagnostic and possible evolutionary importance of the collar. Where the collar was considered by previous authors it was only in respect to its surface features (e.g. Bates, 1980). The present investigation has revealed that these are of little or no diagnostic value. In Section **Azolla** only the **Azolla** sp. type is usually distinctive by virtue of the collar being at least partly obscured by filosum. Although collar sculpturing is variable, the variation is not diagnostically useful.

As previously stated, megaspore apparatus shape is not taxonomically useful, and this can be extended to shape of the basal region, any variation in this being attributed to perine distribution and developmental stage. Infrafilosum is commonly found in variable quantities on the basal megaspore region. In extant **Azolla** the quantity of infrafilosum has been used in descriptions of certain taxa (Bates, 1980; Lumpkin & Plucknett, 1982). However, the present investigation has shown that it is of extremely limited diagnostic use, with only the **Azolla** sp. type consistently possessing copious quantities of infrafilosum. The present author believes that not only is there inherent

variation in all the types which cannot be explained, but removal of the megasporangial wall may also be responsible for removing infrafilosum. The fossil record indicates that the quantity of infrafilosum is variable between taxa (Fowler, 1975; Hall, 1975; Sweet & Hills, 1976). However, possibly closely related ancestral taxa consistently possessed large quantities of filosum (Sweet & Hills, 1974; Hall, 1975). These taxa exhibited little megasporangial apparatus polarity and the filosum probably aided float retention. The present study found no significant differences in the diameter of infra- and suprafilosum elements, either within or between the types. Furthermore, the range of variation in this character in extant *Azolla* appears to be within the variation recorded by previous authors (Fowler, 1975; Martin, 1976b; Sweet & Hills, 1976; Fowler & Stennett-Willson, 1978; Bates, 1980; Calvert et al., 1983; Zhou, 1983) in both extant and fossil *Azolla*. Infrafilosum often obscures the perine surface and causes difficulties in observing the sculpturing, particularly using a light microscope. This method of observation is not recommended by the present author, especially with the current availability of scanning electron microscopes.

Kempf (1969a, 1969b) was followed by Fowler & Stennett-Willson (1978), Calvert et al. (1983) and Zhou (1983) in suggesting that the sporoderm (including its sculpturing) offered the best means of taxonomic separation of species. This is clearly supported by the present investigation. All of the above studies employed transmission and/or scanning electron microscopy which should have provided more critical descriptions compared with light

microscopy. Unfortunately, descriptions derived from the latter technique cannot readily be compared with descriptions from the former because of differences in the observed detail. This is clearly illustrated by a comparison of the present descriptions with those by, for example Svenson (1944) or Lumpkin & Plucknett (1982). In the latter two, sculptural descriptions were no more detailed than indicating that the surface was smooth, pitted and with islands or pads. Such descriptions are of little assistance in critically separating taxa. By using scanning electron microscopy as in the present study, descriptions are more critical and reveal the real extent of variation. The present study records intratype variation which may cause resemblance between types in respect to sculpturing. Therefore, although this character is diagnostically useful, it does have some limitations. This is illustrated by the description of two of the present types as one species in Bates (1980). These limitations can be seen at both high and low magnification, and may be influenced by the nature of the exoperine 2 and 3 as well as the quantity of infrafilosum. The present investigation has examined many more populations of each type from a variety of geographically and environmentally diverse situations and is therefore, more likely to have delimited variation; unlike most previous studies.

The present results show that perine sculpturing is, in many respects, determined by perine structure and that similar sculptural features may have different structure. This has not specifically been reported by previous authors, possibly because

structural variation has not been delimited. However, it obviously has consequences in the description of taxa from limited material and/or from only sculptural features. Following the initial suggestion by Kempf (1969a, 1969b), Fowler (1975, 1981) and Fowler & Stennett-Willson (1978) illustrated the importance of examining sporoderm stratification for species determination; the present investigation also supports this. Although the importance of sporoderm stratification has been highlighted, few studies have attempted to evaluate the taxonomic value of the various strata. Zhou (1983) was the first to evaluate the strata from many populations of one species (*A.pinnata*), but the present investigation represents the most critical evaluation of sporoderm strata in more than one taxon.

Exospore structure, thickness and sculpturing, although variable, appear essentially similar in all extant types and fossil taxa. Therefore, these characters are of no taxonomic value. However, it should be pointed out that descriptions of most fossil taxa are poor, and it is often difficult to relate them to the present observations. In contrast to the exospore, the endoperine appears to offer some taxonomically useful characteristics. Endoperine structure at high magnification is probably similar in all the types, and it is the packing and fusion of these elements that appears to vary. The present investigation reveals that the endoperine elements in Section *Azolla* appear more closely packed and fused than in Section *Rhizosperma*. Furthermore, in the former Section there appears to be a general increase in the degree of packing and fusion of the endoperine elements from the

*A.microphylla* and *A.mexicana* types to the *Azolla* sp. and *A.filiculoides* types. It is probably no coincidence that this sequence follows the evolutionary sequence of collar morphology, which was described previously. Furthermore, Australian populations of the *A.filiculoides* type usually possess less closely packed and fused endoperine elements than other populations of this type. Zhou (1983) reported structural differences similar to this between African and other populations of *A.pinnata*. The *A.filiculoides* type is similar to *A.pinnata* in that it is widely distributed. Therefore, it is not necessarily surprising to find subtle differences in the endoperine of these two ubiquitous taxa. Endoperine structure, as seen at high magnification, is not always an employable diagnostic character because it relies heavily on comparative features which are only apparent in general terms. Of greater diagnostic value are endoperine structural characters seen at low magnification. One of these is the variation in thickness of the endoperine. The literature reveals that thickness is more or less uniform in specimens of fossil taxa. However, in extant *Azolla* thickness may vary and give rise to intrusions and excrescences; this occurs in, and is distinctive to both Sections. Therefore, it appears that variation in thickness is a recent evolutionary development manifested in endoperine elaboration. Evidence for this comes from the present observations that the *A.microphylla* type may possess only a few distal intrusions or excrescences, while the *A.mexicana* type commonly possesses endoperine intrusions. The *Azolla* sp. type may be considered intermediate between this latter type and the *A.filiculoides* type, which is

the most elaborate; once again this sequence follows that of collar morphology. The degree of endoperine alveolation also appears to follow this sequence. However, the occurrence of intrusions or excrescences with few and small alveolae in the *A.microphylla* and *A.mexicana* type suggests that excrescences did not develop to accommodate alveolae, particularly large ones. Further evidence for this is found in the *Azolla* sp. type where the alveolae may be uniformly sized and numerous, but the endoperine does not markedly intrude into the exoperine. Australian populations of the *A.filiculoides* type may not always conform to this type in respect to alveolation, but they consistently possess excrescences comparable in size to other populations of the *A.filiculoides* type.

Assuming endoperine alveolation is a more recent evolutionary development, it can be postulated that the lack of it in Section *Rhizosperma* indicates that this Section is more primitive than Section *Azolla*. In the former Section excrescences appear to be less massive, and morphologically different from those in Section *Azolla* which may suggest different origins of the Sections; this was suggested by Martin (1976a), but based upon other features. These two tentative evolutionary hypotheses pertaining to alveolation and excrescences may suggest common and different origins, (respectively), for the two Sections, and are therefore contradictory. Unfortunately, many studies of fossil material lack critical and detailed descriptions of endoperine structure which limits the present discussion. Clearly, further critical examination of endoperine structure in fossil taxa is required.

because , as illustrated by the present investigation, it is taxonomically useful. Furthermore, the present investigation has shown that the endoperine in extant **Azolla** can be an elaborate layer and is highly variable. The exoperine may be considered equally variable, and some of this variation can be explained by recognising two or three zones.

The exoperine 1 is common in structure and thickness in all extant taxa. This agrees with previous investigations by Kempf (1969a), Fowler & Stennett-Willson (1978), Calvert et al. (1983) and Zhou (1983). Examination of illustrations of fossil taxa (Kempf, 1969a; Hall & Bergad, 1971; Jain, 1971; Fowler, 1975, 1981; Martin, 1976a, 1976b; Collinson, 1980; Foster & Harris, 1981) indicate that a similar layer can be found in fossil taxa. However, in Hall (1969) and Jain & Hall (1969) the illustrations are poor which may explain why an exoperine 1 cannot be distinguished; however, it is expected to be present. Therefore, the exoperine 1 appears to be of no taxonomic use in **Azolla**. However, it may be characteristic of the genus because the literature (Sweet & Hills, 1974; Hall, 1975) indicates that this zone was absent in closely related fossil genera.

In contrast to the exoperine 1, the present investigation reveals that the exoperine 2 is highly variable; some of this variation being diagnostically useful in extant **Azolla**. This might be expected from a comparison of previous studies (e.g. di Fulvio, 1957, 1961 ; Kempf, 1969a, 1969b ; Fowler, 1975 ; Martin, 1976a ; Fowler & Stennett-Willson, 1978 ; Calvert et al., 1983

and Zhou, 1983). However, unlike these studies, the present one elucidated variation within the types attributable to Section **Azolla**. This variation is in respect to thickness and the nature of the zone. The exoperine 2 zone in the **A.filiculoides** type is consistently solid (except usually at excrescence apices) and in the **A.mexicana** type some specimens possess both solid and columellate exoperine 2 or only the latter, but never all the former. The other types possess columellate exoperine 2; the columellae being fused to each other in varying degrees. It is important to appreciate the variability in the degree of fusion because it occurs at both the population and type level. Not only does the degree of fusion influence structure of the exoperine 2, it may have considerable influence on the sculpturing; this was discussed earlier in this section. Just as the exoperine 2 usually contributes to perine sculpturing the exoperine 3 usually has a considerable contribution to it, particularly the sculpturing seen at high magnification.

The present investigation has revealed considerable variation in the degree of fusion of exoperine 3 elements which is not diagnostically significant. Some of this variation is illustrated by the observation that the exoperine 3 may be indistinguishable from the exoperine 2; this also illustrates variation in exoperine structure. In the **A.filiculoides** type exoperine 3 is somewhat localised and confined to excrescence apices. In extant Section **Rhizosperma** the exoperine 3 is confined to a surface covering of excrescences or protuberances and is always, therefore, distinct because it is raised above the exoperine 2. Morphologically these excrescences and protuberanc-

-es are diagnostically significant and the exoperine 3 contributes to their morphology. This is despite this zone being solid in the *A.pinnata* and *A.nilotica* types. In the former, variation is in respect to excrescence sculpturing (Zhou, 1983). In fossil taxa, such as *A.prisca*, the exoperine 3 appears not unlike that of extant Section *Azolla* in its composition (see Fowler, 1975, Text Fig 1(c), Plate 61(3)), although it is localised towards excrescence apices. Fowler (1975; 1981) and others describe little intraspecific variation of the exoperine 3, whereas Zhou (1983) and the present investigation, with wider sampling, recognise much more intraspecific variation. The present author suggests that taxonomically insignificant variation of exoperine 3 may have an environmentally induced developmental cause because the selection, in the present investigation, attempted to minimise age-related developmental variation. This was by selecting obviously full megasporocarps from older regions of fronds. Experimentation aimed at elucidating any environmental and developmental influences on sporoderm structure and sculpturing are lacking and clearly required.

Having discussed the taxonomic significance of individual sporoderm strata it is important to appreciate that together these strata can be considered as taxonomically useful. This is particularly evident where there is positional interaction between layers (e.g. in an excrescence) and where there is little or no interaction between layers (e.g. in a protuberance). Excrescences and exoperine protuberances are indistinguishable in surface view, therefore, it is important to examine perine

structure, particularly if the researcher is not familiar with the extent of variation in sculpturing.

It is reported that most sculptural types observed in extant *Azolla* have been found in geologically recent fossil specimens (Fowler, 1981). Despite this, these fossils have not been assigned to extant taxa (Bertelsen, 1974). Although Bertelsen did not examine sporoderm structure, the erection of new fossil taxa, might be unjustified because sculpturing can be used to quite accurately determine taxa. Examination of sporoderm structure in taxa described by Bertelsen (1974) may show how closely they resemble extant taxa. Structural differences similar to those described here between Australian and other populations of the *A.filiculoides* type and, as shown by Zhou (1983) in *A.pinnata*, may be found. The taxonomic significance of characters discussed in this section is summarised in Table 5.3.

## 5.2.2 Other Reproductive Characters

### 5.2.2.1 SPOROCARPS

The occurrence of sporocarps in pairs in all taxa, except the **A.nilotica** type, where they occur in fours, is wholly in agreement with previous authors from the time of Meyen (1834) to the most recent studies by Calvert et al. (1983). Although there appears to be little information on this from fossil **Azolla**, probably because megasporocarps are dispersed in the rock, Trivedi & Verma (1971) suggest that sporocarps arose in pairs in **A.indica** Trivedi & Verma. The occurrence of groups of four sporocarps, as in **A.nilotica**, would be of great interest because it is only represented in one extant taxon, and might possibly be of phylogenetic importance. Bonnet (1957) and Konar & Kapoor (1974) reported that the sporocarps replace the ventral lobe of the first leaf of a branch, and that the involucre develops from the dorsal leaf lobe; this was confirmed in the present study. Sporocarp development was not the subject of this investigation, but it is interesting to briefly outline our current knowledge to which Campbell (1893), Pfieffer (1907), Duncan (1940), Demalsey (1953), Bonnet (1957), Konar & Kapoor (1974), Becking (1978), Herd et al. (1985) and Cutter & Herd (in press) have contributed, because it does have some influence on taxonomic investigations.

Each type of sporocarp begins development as a megasporocarp (Strasburger (1873) Becking (1978) and others). A pair of single celled megasporangial initials are produced which develop in a

typically leptosporangiate manner (see Foster & Gifford, 1974, p.73 - 76). Accompanying their development is growth of the enclosing indusium (= sporocarp wall), and the enshrouding involucre. During megasporangial development the central cell undergoes repeated divisions producing a tapetum surrounding a primary sporocyte which gives rise to eight megaspore mother cells. A cytoplasmic tapetal syncytium forms and meiosis takes place producing thirty-two haploid nuclei from the eight spore mother nuclei. Usually only one of the thirty-two nuclei survive, the others aborting into the tapetal syncytium. At this point it is interesting to speculate on the origin of 'double megaspores' which were occasionally found during the present investigation (see section 4.6.2) and by Strasburger (1873) and Sweet & Hills (1971). It is reasonable to assume that such specimens arise when only thirty nuclei abort. If this is the case, then it is likely that surviving haploid megaspore nuclei are responsible for the control and co-ordination of sporoderm development. Furthermore, such specimens may represent a link to ancestral taxa in which megasporangia contained more than one megaspore apparatus. Another megaspore apparatus abnormality found in the present investigation was the inverted development of a megaspore apparatus (see section 4.6.2). Such a specimen may indicate polarity in the abortion of the thirty-one nuclei; this has not been commented upon by previous authors. The surviving nucleus forms a wall and develops into the mature megaspore apparatus. The surrounding tapetal syncytium (= periplasmodium) forms the megaspore apparatus, while the aborted nuclei have been observed in the floats and collar (Campbell,

1893; Bonnet, 1957; Martin, 1976a). The periplasmodium divides into four (Sect. *Azolla*) or ten (Sect. *Rhizosperma*) and, at this stage, is highly vacuolate. The part immediately surrounding the developing megaspore proper forms the perine of the megaspore, while the three or nine remaining parts of periplasmodium form the floats (Konar & Kapoor, 1974; Martin, 1976a). Although the present knowledge implies simple developmental sequences, the control of development is not understood. One question that is currently being investigated is what causes all thirty-two megaspore nuclei to abort. When this happens the megasporangium senesces and microsporangial initials develop at the base of the placenta (or columella).

Senescence of the megasporangium is associated with senescence of the enclosed endosymbiont, therefore it only passes through the megaspore line of the sexual life cycle. The present study indicates, via non-developmental means, that sixty-four microspores per microsporangium develop in Section *Azolla*. This confirms previous reports from developmental investigations of both Sections in which sixteen microspore mother cells undergo meiosis, all resulting microspores being functional (Campbell, 1893; Rao, 1935; Duncan, 1940; Konar & Kapoor, 1974). The periplasmodium, formed from the tapetum, divides and becomes vacuolate to form the massulae (Herd et al., 1985); as this investigation has shown, the periplasmodium may only divide into two parts, or as many as eleven. (see section 4.3.2). The microspore tetrads become segregated into the massulae prior to polymerisation of the sporopollenin (Herd et al., 1985) which

forms the substance of a massula. Herd **et al.** (1985) have confirmed Fowler's (1975) observations of the spermatozoid escape mechanism during their developmental study; however, they do not describe glochidial formation.

Many questions still remain in respect to sporogenesis. For example, what induces sporulation? At present, all investigations requiring reproductive structures are severely limited by <sup>lack of</sup> a consistent source of fruiting material. Another question is what determines whether thirty-one or thirty-two megasporule nuclei abort? The present investigation has found populations of all the types with only one type of sporocarp or with predominantly one type. The former case supposedly prevailed in *A.caroliniana* (*apud* Svenson, 1944) where only microsporocarps were found (Svenson, 1944; Godfrey **et al.**, 1961 and others). This has also been found in cultured populations of *A.pinnata* (Than and Puyawal, pers.comm.). These two unanswered questions, together with the variability of sporocarp ratios observed here in all the types, completely undermines the use of ratios and frequency of sporulation as distinguishing features. Such features have been used by Svenson (1944) and Lumpkin & Plucknett (1982). Bates (1980) suggests that sporulation is suppressed in subtropical climates, thereby explaining the lack of fruiting material from south eastern USA. The present investigation does not support Bates (1980) because many sporulating populations from subtropical and tropical regions of the Americas were found. Furthermore, Cutter & Herd (in press) report that temperature, even diurnal variations, do not appear

to induce sporulation. However, Ashton (1977) concludes that sporulation is regulated by interacting effects of light, temperature, pH and N<sub>2</sub>. The need for collecting fertile **Azolla** (Bates, 1980) is reiterated by the present author because only ca 15% of herbarium populations obtained were fertile.

Although distinction of two types of sporocarp and sporangia has been recognised since 1810 (Brown 1810), there are no reports of differences between taxa in respect to size and shape of the respective sporocarps and sporangia and nature of their walls; the lack of differences is supported by the present investigation. Therefore, these characters, together with ratios of mega- and microsporocarps, are of no taxonomic use. It is interesting to note that few authors refer to the lack of an annulus in both mega- and microsporangia. However, the lack of an annulus is expected, and due to the aquatic habit (Campbell, 1893). It would be interesting to know if ancestral taxa possess even a rudimentary annulus, which may link them to homosporous leptosporangiate ferns and the terrestrial habit.

#### 5.2.2.2 MICROSPOANGIA

The gradate nature of microsporocarps made scoring shape, size and number of microsporangia unreliable. Furthermore, in herbarium specimens deformation had obviously occurred. Therefore, these characters were not numerically scored in the present study. However, in fresh material, mature microsporangia were spherical, and attached to the placenta by long stalks. A literature search reveals that numbers of microsporangia per

microsporocarp varies from eight to sixty-six (Martius, 1834, 1884; Svenson, 1944; Godfrey *et al.*, 1961). The present study, although without numerical data, supports the conclusion that number of microsporangia per microsporocarp is an unreliable distinguishing feature; intratype variation being large and overlapping within all types. The report of two to eight microsporangia in fossil *A.indica* (Trivedi & Verma, 1971) is considered unreliable because the counts were made from sections. The contents of microsporangia in both fossil and extant *Azolla* have received much scrutiny, and appear to have some interesting features.

#### 5.2.2.3 MASSULAE

There is more information concerning the number of massulae per microsporangium in extant *Azolla* than fossil ones. In the former, numbers ranged from two to nine, however, three to eight appears to be commonly reported for *A.filiculoides*, *A.caroliniana*, *A.mexicana* and *A.microphylla* (Martius, 1834, 1884; Strasburger, 1873; Svenson, 1944; van Ooststroom, 1948; Godfrey *et al.*, 1961; Calvert *et al.*, 1983). It is quite clear from these reports that variation in the number of massulae negates the taxonomic use of this character, and is in agreement with the present investigation. Although the t-Test indicated a significant difference between North American and European populations of the *A.filiculoides* type and the *A.mexicana*, *A.microphylla* and Australian *A.filiculoides* types, the data suggests that the t-Test was not appropriate in this instance. This is because, as Fig. 4.3la and Table 4.1 show, intratype

variation is not equal at the intertype level. Calvert et al. (1983) clearly did not find intrapopulation variation in their study of one population of *A.mexicana*, which, according to them, usually has four massulae per microsporangium. The present investigation has shown that information must be gathered from many populations. The nature of fossil *Azolla* usually means that intact microsporangia are rarely found, and palaeobotanists have rarely ventured into speculating how many massulae might have been in a microsporangium. However, the present investigation provides some information which is relevant to this problem.

The number of massulae per microsporangium and the number of microspores per massula are correlated, with the product of their numbers being approximately sixty-four; this is the number of microspores per microsporangium (Campbell, 1893; Konar & Kapoor, 1974 and others). In the present investigation data were collected so as to randomise it as much as possible (see section 2). This yielded results for which 68% of the variation was accounted for by the correlation. Furthermore the method simulated the situation found in fossil samples where massulae are dispersed. An alternative method of preparing massulae from extant *Azolla* would involve scoring numbers of massulae and microspores from the same microsporangium, and may have provided a better correlation. However, results from the present study are comparable with results from fossil taxa. Table 5.2 lists some fossil taxa together with number<sup>of</sup> microspores per massula. In very general terms, and assuming that fossil taxa also produce sixty-four microspores, it can be seen that older taxa

(e.g. *A.distincta* Snead and *A.gigantea* Bergad & Hall) may have only one or two massulae per microsporangium. In the late Cretaceous/early Palaeocene there were approximately nine, whereas in extant *Azolla* the number of massulae per microsporangium has reduced to approximately five. Alternatively, late Cretaceous/early Palaeocene taxa may have only produced thirty-two microspores per microsporangium, suggesting two major evolutionary changes in respect to the number of microspore mother cells; one in the late Cretaceous and one possibly between the Miocene or Pliocene. Such changes are very unlikely, and it is more probable that *Azolla* is like *Azollopsis*. Information from Sweet & Hills (1974) suggests that *Azollopsis* contains taxa which produce thirty-two microspores while others give rise to sixty-four (see Table 5.2.); these taxa are represented in the late Cretaceous early Palaeocene (Sweet & Hills, 1974). However, in *Azolla* only those that produce sixty-four microspores have persisted today, and this may reflect the need for higher potential fecundity. Further speculation is beyond the scope of the present investigation. However, there is a need for more information from fossil taxa in respect to the number of microspores per massula and per microsporangium; it may then be possible to incorporate such information into an evolutionary scheme. For the present however, the number of microspores per massula cannot be used to distinguish extant taxa, which is not surprising because it is related to the number of massulae per microsporangium; a previously discredited feature. Other features of massulae appear to offer better prospects for distinguishing taxa.

TABLE 5.2.

	No. microspores/ massula	No. massulae/ microsporangium
<u>Azolopsis</u> ( <u>A.coccoides</u> , <u>A.to<sup>*</sup>mentosa</u> , <u>A.pusilla</u> , <u>A.intermedia</u> *)	1-4, 1-5, 1-7 1*	7-10, *33 - 45+
<u>Azolla</u>		
<u>A.distincta</u>	-	1
<u>A.gigantea</u>	43 & 55	-
<u>A.bulbosa</u>	4 - 10	-
<u>A.stanleyi</u>	6 - 12	-
<u>A.aerolata</u>	2 - 8	-
<u>A.indica</u>	2 - 8 (3 or 4)	-
<u>A.prisca</u>	3 - 9 (6)	-
extant <u>Azolla</u>	(13)	2 - 11 (5)

Table to show number of microspores per massula and, where known, number of massulae per microsporangium. () = mean number.

Although descriptions of fossil taxa often include shape, size and surface ornamentation of massulae (Bergad & Hall, 1971; Sweet & Hills, 1974, 1976; Fowler, 1975), these features have been consistently neglected in extant Azolla. The present study found that shape and size were related to the number of massulae per microsporangium, and are therefore, not useful distinguishing features. However, for descriptive purposes, surfaces were defined by the present author. Surface ornamentation of massulae usually distinguishes the A.mexicana type from all other types. Sweet & Hills (1974) indicate that massula alveolae influence

surface ornamentation in *Azolopsis* but not in fossil *Azolla*. Although not investigated here, the sub-surface massula alveolae may influence surface ornamentation in extant *Azolla*, thereby causing distinctive ovoid depressions in the *A.mexicana* type. The descriptions of fossil *Azolla* massula surfaces by Sweet & Hills (1976) appear to be similar to the *A.filiculoides*, *A.microphylla*, *Azolla* sp., *A.pinnata* and *A.nilotica* types. However, it is possible that drying and fossilisation may have induced similar artefacts. The presence of short blunt processes on the external massula surface in most, but not all, Australian populations of the *A.filiculoides* type clearly distinguishes them from other populations and types, and is not recorded in the literature, even in fossil taxa. The "granular to psilate" surface of massulae (Sweet & Hills, 1976) is not considered here to be equivalent to the massula surface in the *A.filiculoides* type. However, large surface processes, termed trichomes and glochidia, have been recognised as distinguishing features since the investigation by Meyen (1834).

#### 5.2.2.4 GLOCHIDIA

In extant *Azolla* the nature of the large processes was used to distinguish two genera (Meyen, 1834, 1836) and later infrageneric taxa (Mettenius, 1847; Strasburger, 1873 and others). Glochidiate and trichome-like processes are found in extant *Azolla*, and in addition to these, circinate ones are found in fossil *Azolla* (Fowler, 1975); with other features, massula processes have been used to define infrageneric taxa (Fowler, 1975, 1981; Sweet & Hills, 1976). In the present investigation, which mainly

concerns extant *Azolla*, glochidiate processes (or glochidia) were found on massula surfaces. They are composed of sporopollenin, as are massulae (Lucas & Duckett, 1980), and their name expresses their fluke-shaped apex. For this reason the term "glochidium" should not be used for any other type of massula process. Unfortunately, many authors use the term "glochidium" for trichome-like and circinate processes (e.g. Rao, 1935; Konar & Kapoor, 1974; Sweet & Hills, 1974; Lumpkin & Plucknett, 1982 and others). Most previous authors indicate that glochidia occur on the massula surface. However, Campbell (1893) states that glochidia develop on surfaces where there are spaces between the massulae. This is confirmed by the present investigation, but glochidia also form in groups on the external massula surface in Section *Azolla*. However, in Section *Rhizosperma* trichome-like processes only form in one or two groups on the internal surface. The formation of massula processes, in apparently available spaces, is typical of sporopollenin deposition in many other ferns (Jermy, pers.comm.). Sweet & Hills (1976) noted unequal distribution of glochidia in two early Tertiary species of *Azolla*, and state that it was unknown in Cretaceous species. The present study indicates that localisation of glochidia has continued in recent species. Other glochidial and trichome characters have received quantification by palaeobotanists (Sweet & Hills, 1974, 1976), but to the present author's knowledge, no attempts have been made to quantify such characters in extant *Azolla* and, compared with fossil taxa, glochidia in recent taxa have been neglected. The present investigation attempted to rectify this.

In some fossil taxa the glochidial shaft tapers slightly towards its base, is parallel sided or is basally and apically tapered (Srivastava, 1968; Bergad & Hall, 1971; Fowler, 1975; Sweet & Hills, 1976). The present investigation revealed that while all these tapers (shapes) were represented in extant *Azolla* they were not necessarily exclusive to one particular type. However, each does possess a predominant shaft shape, but the variation observed limits use of this character on its own. The present investigation shows that when used in conjunction with septation, shape can be used with greater certainty. Previously, septation was inadequately defined as non-septate, one or two and multi-septate (Mettenius, 1847, 1867; Strasburger, 1873; Svenson, 1944; Pieterse et al., 1977 and others). However, Bates (1980) and Bernard (1984) observed variation of septation within a single massula, and it has subsequently been reported by Godfrey et al. (1961). This type of variation is confirmed by the present investigation, and is quite extensive. With inadequate definition septation has been neglected as a taxonomic character, (e.g. Lumpkin & Plucknett, 1982; Calvert et al., 1983), but it seems unwarranted to continue using loose definitions of septation as seen in Pieterse et al. (1977) and Tryon & Tryon (1982). The present investigation has shown that, when quantified, the variation in septation is a useful taxonomic feature in respect to indicating types and sub-types (see Figs. 4.34-4.39). Although there is overlap in septation of the types, distribution of variation appears characteristic of each type, and comparison of these and the sub-types (using the t-Test) also indicates differences in respect to septation.

Furthermore, the sampling procedure employed appears to be adequate. Although Table 4.10 suggests that the **A.filiculoides** type is significantly different from all the other types, partitioning into sub-types reveals a much better separation of the types and sub-types (see Table 4.11). This further enhances the need to account for variation using sub-types, which are supported by other features. Sub-types in the **A.filiculoides** type are convincingly supported by sporoderm structure, massula ornamentation and biogeography. However, in the **A.mexicana** and **Azolla** sp. types the variation in septation is more weakly accounted for by biogeography. The variation in the **A.microphylla** type cannot be correlated with other features that were studied. Therefore, sub-types cannot be justified at present. The predominant position of septa has not previously been investigated except where there are few septa, when they are reported as being apically located (Strasburger, 1873; Svenson, 1944; Godfrey et al., 1961 and others). In the present investigation this character was studied. Where there are few septa per glochidium (i.e. 1-4) they are usually apically located. However, in all the types of Section **Azolla**, except the **A.filiculoides** type, there may be at least one basal septum. It should be noted that basal septa are often overlooked, particularly when only part of a glochidium is visible. As might be expected the more septa per glochidium, the more likely they are to be uniformly distributed. It appears therefore, that the position of the septa is not diagnostically useful, except perhaps in respect to the presence of one or two basal ones which excludes the **A.filiculoides** type in a diagnosis. Clearly, for

the first time, the present investigation has delimited variation of glochidial septation in extant *Azolla*, and accounted for much of this variation. Even in fossil *Azolla*, this type of treatment for septation has not been performed, and in the future may provide fruitful results. This could be extended to using shaft shape and septation which appear to be complementary characters because together they may be used to distinguish taxa. For example, both the *A.mexicana* and *A.microphylla* types may have three or four septa, however, the latter type has distinct basal and apical (B-A) shaft tapering, whereas the former usually has parallel sided or weakly B-A tapered.

Glochidial length is not unlike glochidial septation, in as much as it is a continuous character in extant *Azolla*. Other data of glochidial length come from fossil *Azolla* only, and it is suggested that variation in glochidial length may have phylogenetic significance (Sweet & Hills, 1976). Although the length increased from the Cretaceous to the Eocene, with anticipated intermediate lengths in the Palaeocene (Sweet & Hills, 1976), the present investigation reveals that since then there has not been a significant increase in glochidial length. However, the length attained in the Eocene may have been the maximum. The range of the type means in extant *Azolla* is only ca 26um, and there is considerable overlap between the types. However, each type appears to have a distinctive and apparently different variance. The only type that is obviously distinctive is the *A.mexicana* type because there is considerable variation in glochidial length compared with the other types. The intra-population and intra-

and intertype variation of this character could not be accounted for by correlating with any other characters. Initially, it was thought that longer glochidia would tend to have more septa. However, this is not proven, and is illustrated by the *A.micropheylla* and *Azolla* sp. types possessing, on average, shorter glochidia and more septa than the *A.filiculoides* type. It appears therefore, that glochidial length is not taxonomically useful. Although differences were indicated by the t-Test, the data variation and skewness indicate that this test was not suitable for the analysis. Although some variation may be attributed to sampling error, it is highly unlikely that such unequal intertype variation could be introduced by this alone. The sample number for each population and the procedure used are considered here to be adequate to elucidate any differences that might have been present; the intratype variance being evidence of this.

Like glochidial length, shape of the glochidial apex was also found to be of no use in distinguishing extant *Azolla*. The literature indicates that this character has not previously been investigated in both fossil and extant *Azolla*. However, in the former, plates indicate that, like extant taxa, fossil ones possess glochidia with mainly rounded apices (Srivastava, 1968; Bergad & Hall, 1971; Fowler, 1975; Sweet & Hills, 1976). In extant *Azolla* illustrations are too poor for accurate assessment of shape; however, the present investigation has shown that in some populations massulae may bear glochidia with obtuse and acute apices.

Immediately behind the fluked apex of a glochidium all extant taxa possess a dilation (or distal dilation and neck swelling, Fowler (1975) and Sweet & Hills (1976) respectively). However, it was more obvious in some glochidia than others, particularly in immature specimens but was not specific to certain taxa. In the fossil record this dilation was reported in **A.simplex** Hall from the Cretaceous and was absent until the Eocene (Fowler, 1975). However, it was present in at least three taxa from the Palaeocene (Sweet & Hills, 1976). The function of the dilation is unknown and appears to have little evolutionary significance.

The taxonomic significance of characters discussed in this section is summarised in Table 5.3.

### 5.2.3 Vegetative Characters

The protogues together with many other publications consistently use vegetative characters to distinguish taxa. As indicated by the recent use of only reproductive characters, vegetative ones have fallen into disrepute. However, Lumpkin & Plucknett (1982) attempted to provide a key, albeit tentatively, based mainly on vegetative features. A careful search of the literature reveals that all features used by these authors have previously been used. Therefore, this key appears to represent a coalescence of dispersed published observations reinforced by limited personal observation. The present investigation has shown that vegetative characters are of little use in accurate identification of taxa at species level.

#### 5.2.3.1 FRONDS

Frond habit - During a five year study of a wild population of **A.filiculoides**, only in August 1939 were erect fronds reported (Dallman & Williams, 1940; Williams, 1943, 1944), and under prolonged glasshouse and field conditions Pieterse et al. (1977) did not record erect fronds in two species. Although Lumpkin & Plucknett (1982) recognised that all their species may have horizontal fronds, they also found that **A.filiculoides** and **A.microphylla** became erect or nearly vertical. In these two species the vertical habit was linked with maturity which appears to be defined by their ability to sporulate (Lumpkin & Plucknett, 1982). The present investigation supports these observations except that sporulation also occurred in the **A.filiculoides** and **A.microphylla** types when the fronds were horizontal. This was

more common in the *A.microphylla* and Australian populations of the *A.filiculoides* types. Therefore frond habit is not a consistent feature for distinguishing taxa. However, if specimens are vertical they are either the *A.filiculoides* or the *A.microphylla* type. However, care is required because disturbance of the water surface can cause fronds to assume a vertical orientation.

Frond shape is rarely commented upon in the literature except perhaps in some protalogues. This character, as shown by the present investigation, is influenced primarily by branching pattern and secondarily by fragmentation. Branching pattern has figured highly in distinguishing taxa (Martius, 1834, 1884; Mettenius, 1847, 1867; Strasburger, 1873; Svenson, 1944; Bonnet, 1957; Tryon & Tryon, 1982). In these publications the branching is usually described as dichotomous, sub-dichotomous, lateral and pinnate. The present investigation indicates that, in extant Section *Azolla*, the branching is based on a flabellate dichotomous system. This is a flattened dorsiventral dichotomous system as opposed to the 3 dimensional cruciate dichotomous system (Troll, 1937). In the flabellate system each dichotomy may produce approximately equally developed branches, and is termed 'isotomous' (Troll, 1937) and gives rise to a highly symmetrical system. However, if one branch of a dichotomy develops less than the other, often alternating left and right, it is termed 'anisotomous' (Troll, 1937). Figures 3.11a & b diagrammatically illustrate these two dichotomous forms. In respect to anisotomous forms, Broch (1962) believes that it may have given rise to lateral branching in vascular plants.

It appears, purely from the morphology, that **Azolla** illustrates this. The pinnate branching in Section **Azolla** (Tryon & Tryon, 1982) is believed, by the present author, to be more accurately described as sub-pinnate, with pinnate being reserved for one branching pattern found in the **A.pinnata** type. The sub-pinnate form is envisaged as a highly modified anisotomous form where there is a distinct main axis. What are considered here to be minor modifications to the anisotomous form create the deltoid and elongated frond shapes which may appear laterally branched. These modifications include developmental suppression and promotion of certain branches. The isotomous form gives rise to the rounded and elliptically shaped fronds. Furthermore, the present study has shown that variation in the branching pattern is continuous and a population may have isotomous elliptical fronds as well as anisotomous deltoid ones. Therefore, in **Azolla** there is considerable plasticity in respect to branching, and this may be observed in one type. As with frond habit, the present study has revealed that although the **A.filiculoides** and **A.microphylla** types may be distinct from the other types in respect to branching and shape, all the types may be similar. Interestingly these differences and similarities are associated with variation in frond habit. Bonnet (1957) investigated whether or not successive orders of branches were left or right, and suggests a difference between **A.filiculoides** and **A.caroliniana**. This aspect of branching was not investigated during the present study. However, it is believed that at least some specimens (those from Toscane, France) examined by Bonnet (1957) were incorrectly identified as **A.caroliniana** and were in fact

**A.filiculoides** because the present study has only recorded the latter from Toscane. Therefore, branching pattern and frond shape, like frond habit cannot be consistently used to separate types (taxa). Frond size also appears to be associated and interactive with these characters.

Martius (1884) and van Ooststroom (1948) carefully delimit frond size in **A.filiculoides** and **A.caroliniana**, whereas Nakai (1925) uses relative size (i.e. small, large, etc.) in his decidedly inferior descriptions of taxa. Although Svenson (1944) quotes size in his prospectus, it is clear that there is no real separation of the taxa. However, subsequent workers have followed Svenson, but apparently misinterpreted his descriptions by stating that **A.caroliniana** and **A.filiculoides** are the smallest and largest taxa respectively. Therefore, small fronded sterile populations are often designated as **A.caroliniana**. However, it is recognised that herbarium and cultured populations of **Azolla** may possess fronds of widely varying size (Dallman & Williams, 1940; Williams, 1943, 1944; Correll & Correll, 1972; Ott & Petrik Ott, 1973; Pieterse et al., 1977). This is supported by the present investigation, with the **A.filiculoides** and the **A.microphylla** types often attaining the larger sizes. Furthermore, this is associated with the vertical habit and deltoid to elongated fronds. However, these two types may be found as small plants similar to the other types. Although not clearly stated, this may be deduced from the key by Lumpkin & Plucknett (1982). The present investigation found that frond size was greatly influenced by the tendency of the frond

population to fragment. Therefore, both size and definition of an individual frond are extremely difficult to delimit. This and the inherent variation in size renders this character unsuitable for distinguishing taxa.

Although frond habit, branching and size have been considered independently, the present study consistently found that these characters are interactively associated. This has not previously been stated, but is implied in the study by Pieterse et al. (1977). Furthermore, the present investigation indicates that the *A.filiculoides* and *A.microphylla* types are polymorphic. Observations of living populations at Portsmouth and IRRI suggest that frond characters are environmentally influenced. This is supported by various physiological investigations which show that a variety of environmental factors that influence *Azolla* growth (Peters et al. 1980; Lumpkin & Plucknett, 1982; Watanabe & Berja, 1983). The physiological variation appears to be at the population level, and may be, in part manifested morphologically.

#### 5.2.3.2 LEAVES

The bilobed leaves of *Azolla* are inserted laterally and alternately which gives a dorsi-ventral appearance to fronds. The number of leaves between branches only appears variable when branches fragment, but is <sup>↔</sup> in fact two or three in all the types. This feature cannot therefore be used to distinguish taxa. These findings are in agreement with those of Bonnet (1957), but he found that *A.pinnata* possessed more leaves per interbranch; for the *A.pinnata* type, this feature was not investigated in the

present study. The dorsal and ventral leaf lobes are very different in their nature, the former being robust and with a cavity that usually contains the endosymbiont *Anabaena azollae*. The ventral lobe however is mostly achlorophyllous, one cell thick and very fragile. This fragility lead to it being damaged and irreversibly distorted during drying. This lobe could not therefore be measured accurately enough to obtain sufficient data. However, it was possible to observe the shape of one or two lobes in most populations. It was found that all the types possessed a similarly shaped ventral lobe which renders it unsuitable for distinguishing taxa. The broadly ovate shape of this lobe in each type compared to the narrower dorsal lobe may cause an apparent difference in size of the lobes. This was observed in *A.mexicana* (Svenson, 1944), but there is no indication of it in other taxa. Furthermore, when the ventral lobe is contorted it appears smaller than the dorsal lobe. There are various notes in the literature pertaining to the position of the ventral leaf lobe to the water surface. It has been stated that the ventral lobe is submerged (Campbell, 1893; Eames, 1936). However, Rao (1935), Bates (1980) and others indicate that it is on the water surface; this is supported here. Unlike the ventral leaf lobe the dorsal leaf lobe is more suitable for obtaining numerical data.

The ranges in size of the dorsal leaf lobe found in the present study correspond to the sizes quoted by Martius (1884), Svenson (1944), van Ooststroom (1948) and Pieterse et al. (1977) for their various taxa; these include all the types of the present study.

In all those studies *A.filiculoides* generally has the longer dorsal leaf lobes. However, it appears that distinction of their taxa is tenuous using this character. The present investigation clearly shows that dorsal leaf lobe dimensions, including length-width ratio and width of the hyaline margin, are not useful distinguishing characters. This is despite differences being indicated by the t-Test. The data reveals considerable overlap in the ranges (Fig.4.42a-c). The groups within the *A.filiculoides* type not only suggest differences between Australian and other populations of this type, but also reflect the morphological changes associated with the frond polymorphism of this type. However, in the *A.microphylla* type, which is also polymorphic, this is not evident from the dorsal leaf lobe data. This may be due to this type exhibiting frond polymorphism to a lesser degree than the *A.filiculoides* type. A positive correlation of dorsal lobe width and hyaline margin width is expected in all types because the latter is a component of the former. Only the *A.microphylla* type did not have this correlation, and the poor negative value in this type cannot be taken as significant; however, it may suggest a difference in this type. Pieterse et al. (1977) reported that luxuriantly growing *A.filiculoides* had a wider hyaline margin than *A. cf caroliniana*. These results appear consistent with the present investigation. Obviousness and nature of the hyaline margin and shape of the dorsal leaf lobe apex are of no use for distinguishing types. However, the *Azolla* sp. type possesses, on average, the narrowest and consistently least obvious hyaline margin. Pieterse et al. (1977) noted differences in this latter

character, although they associated it with frond morphology in *A.filiculoides*. The present study supports this; furthermore, these observations illustrate the wide ranging effects of the polymorphism. A literature search reveals that the present investigation represents the most critical and comprehensive assessment of dorsal leaf lobe characters. Also investigated were trichomes on the abaxial surface of the dorsal leaf lobe.

#### 5.2.3.3 TRICHOMES

Results of the present investigation show that the most useful distinguishing vegetative feature is the nature and distribution of epidermal trichomes. In Section *Azolla* these trichomes are confined to the abaxial surface of the dorsal leaf lobe, whereas in Section *Rhizosperma* they also extend to the stem surfaces. As previously stated in section 1 differences in the trichomes were noted by Strasburger (1873), however, it appears that Mettenius (1867) was the first to use trichomes as distinguishing features. It is perhaps surprising that use of trichomes was not popularised until recently (Lumpkin & Plucknett, 1982).

The statement that it would be difficult to study dorsal lobe trichomes in dried and hard pressed material (Bates, 1980) is refuted by the present author. Following the examinations of both fresh and dried specimens from the same population, (employing a scanning electron microscope) it was possible to determine the nature of these trichomes in herbarium specimens. Furthermore, the reflation technique developed during this study aided more accurate description of trichomes where the nature was

not readily discernible. Although trichomes are best studied under the scanning electron microscope, it is possible, with experience, to quite accurately determine the number of cells comprising them using the light microscope in both fresh and herbarium material. Following Mettenius's (1867) prospectus for *A.filiculoides* and *A.caroliniana* they are respectively described as having one-and two-celled trichomes (van Ooststroom, 1948; di Fulvio, 1957; Pieterse et al., 1977 and others). The present investigation has shown that these dorsal lobe trichomes are one celled in the *A.filiculoides* type, while the other types possess two celled trichomes. Furthermore, the prolongation of the apical trichome cell in the *Azolla* sp. type may be a third cell. However, evidence for this third cell is perhaps tenuous. Clearly, this feature requires investigation using fresh material. In their descriptions of *A.microphylla* and *A.mexicana*, Lumpkin & Plucknett (1982) state "Trichomes with one pedicel cell and one or two apical cells" and "Trichomes occassionally of three cells" respectively. This indicates that both species may possess two and three-celled trichomes, and are therefore essentially similar. Additionally, the descriptions of trichomes in *A.microphylla* is ambiguous because it can be interpreted as all trichomes on a dorsal lobe are two-celled or three-celled, alternatively there is a mixture of two and three-celled trichomes on a dorsal lobe. Clearly the wording in the key by Lumpkin & Plucknett (1982) is somewhat misleading. Furthermore, the present investigation only tentatively indicates three-celled trichomes in one taxon; as will be shown later this taxon does not correspond to either *A.microphylla* or *A.mexicana*. Barely

discernible one-celled trichomes in *A.rubra* (Lumpkin & Plucknett, 1982), which corresponds to Australian populations of the *A.filiculoides* type, is not completely endorsed by the present investigation because some of these populations possess well marked trichomes. Another feature of these trichomes is their orientation relative to the dorsal lobe. Although Lumpkin & Plucknett (1982) considered this important for separating *A.mexicana* and *A.caroliniana* from the other taxa of Section *Azolla*, the present investigation does not support this. In all the types, towards the proximal dorsal lobe region, trichomes are usually more erect compared to trichomes in the distal region. All these discrepancies between the present investigation and the observations of Lumpkin & Plucknett (1982) indicate that these workers have failed to delimit trichome characters. Indeed they have observed features (e.g. three-celled trichomes) which may not exist. Other discrepancies also exist in descriptions of trichomes in taxa of Section *Rhizosperma* which indicate that Lumpkin & Plucknett (1982) have misinterpreted trichome nature. Single-celled trichomes were reported by them on the leaf lobes and ventral rhizome surface of *A.pinnata*. Firstly, the dorsal leaf lobe possesses two-celled trichomes which are illustrated by Lumpkin & Plucknett (1982 , p.27, Fig.11). Secondly, the present study found that stem trichomes, although appearing one-celled, are on most surfaces. The number of cells comprising stem trichomes in the *A.nilotica* type appeared to be two, however, only one fresh population was investigated; Lumpkin & Plucknett (1982) state that these trichomes are five or more cells long. These authors did not record the nature of dorsal lobe trichomes

in *A.nilotica* which are clearly two-celled. Other descriptions of the dorsal lobe surface in extant *Azolla* commonly state that it is relatively smooth or *laevibus* or *papillose* (most protogues; Mettenius (1847, 1867); Dallman & Williams (1940); Svenson (1944); Ashton & Walmsley (1984) and others). Such descriptions are clearly inaccurate because the present study found that populations of all the types could have been described as one or the other. In herbarium specimens this appeared to be determined by the degree of pressing.

Although orientation and shape of dorsal lobe trichomes appear not to be useful distinguishing features, it is interesting to speculate upon the significance of one- and two- or more celled conditions. The function of dorsal lobe trichomes may be to repel water. However, because each is associated with a stoma, it is conceivable that trichomes reduce air movement over stomata, reducing water loss. From an evolutionary point of view epidermal trichomes may possibly be in tandem with the trend described by Fowler (1981) for elaboration of the megasporangium apparatus collar. *A.filiculoides* is considered more advanced than *A.microphylla* and Section *Rhizosperma* (the latter being considered more primitive than Section *Azolla*) (Fowler 1981). The general trend in respect to epidermal trichomes appears to be reduction. Section *Azolla* illustrates a reduction in numbers compared to Section *Rhizosperma*, while the *A.filiculoides* type illustrates a reduction in the number of cells comprising trichomes. This may explain why some specimens of the latter occasionally possess a few two-celled trichomes; an expression

of the more primitive form. However, from the trend suggested by Fowler (1981) it might be expected that Australian populations of the *A.filiculoides* type would have more two-celled trichomes than populations from other regions. This was not found in the present investigation and commonly trichomes of the former populations were more reduced (barely discernible) than the latter populations. If the reduction of epidermal trichomes is of evolutionary significance, fossil material should possess several to many-celled trichomes on leaves and stems. Unfortunately, this information is not available and the present suggestion is tentative and speculative. Fossil vegetative structures may be recognised not only by the generically distinctive frond morphology, but also by the presence of distinctive trichomes. These are generically characteristic by each being associated with a stoma. This feature is rarely reported in descriptions of the abaxial surface of the dorsal leaf lobe.

A more commonly reported vegetative feature is that of frond colour (Benedict, 1923; Dallman & Williams, 1940; Williams, 1941, 1943; Svenson, 1944; Cohn & Renlund, 1953; Pieterse *et al.*, 1977; Lumpkin & Plucknett, 1982). All taxa, except *A.nilotica*, are reported as being various shades of green to red-browns (Lumpkin 1981); the red colouration being caused by anthocyanidins (Pieterse *et al.*, 1977). The present study, together with other investigations link colour with environmental variables such as temperature and water chemistry (Benedict, 1923; Svenson, 1944; Dubois, 1967; Pieterse *et al.*, 1977). All previous investigations,

with the exception of Pieterse et al. (1977), have crudely assessed colour by eye. This is a very inaccurate method because different people see and describe the same shade differently. Therefore, Lumpkin & Plucknett (1982) use of colour in their key to distinguish species seems unwarranted. Chromatographic and colour chart techniques employed by Pieterse et al. (1977) are more accurate, objective and discriminating methods, and they revealed consistent differences in colour, but not pigmentation, between two taxa. Consequently colour was dismissed as a useful distinguishing feature, in the field at least (Pieterse et al., 1977), which is supported by the present investigation.

#### 5.2.3.4 ROOTS

In extant *Azolla* roots arise at stem branch points. Taxa of Section *Azolla* produce one or two roots at these points whereas *A.pinnata* produces one to six roots, and *A.nilotica* produces many roots in distinct bundles or groups. This was also noted by Mettenius (1867), Strasburger (1873) and most subsequent authors. Baker (1887) states that Section *Rhizosperma* is characterised by having "fascicled" roots; this description is more appropriate for *A.nilotica*. Root length is rarely considered as a diagnostic feature and the present investigation has shown that it should be dismissed. Observations of cultured and field populations indicate that root length is probably environmentally influenced; roots tend to be lost quite frequently under culture conditions as seen by the accumulation of roots in the bottom of the culture containers. Rao (1935) describes roots in *A.pinnata* as "deciduous", with a definite

abscission zone. Deposition of dark coloured material and loss of root hairs in all the types appeared to be environmentally influenced. Another environmentally influenced feature was the coiling of root tips. Tan (unpub. work) indicated that only *A.filiculoides* possess this feature. Although, most obvious in the *A.filiculoides* type, root tip coiling can be observed in all other types. It appears likely that this coiling anchors fronds to each other or submerged bodies, thereby stabilising the fronds and promoting mat formation. This is supported by observations of root coiling not being found in matted populations. Therefore, this feature is probably light induced. Although studied here in cultured material it is possible to find coiled root tips in many herbarium specimens. However, root hairs were more difficult to study in such material. The IRRI culture collection revealed that prominence of root hairs is not a diagnostic feature. Generally, root hairs arise in whorls (Queva, 1910; Chauveaud, 1911; di Fulvio, 1957; Bonnet, 1957; Konar & Kapoor, 1972) in all the types and, although are particularly obvious in the *A.pinnata* type, the whorls and numbers of root hairs appear not to be useful diagnostic features. Tan (unpub. work) suggests that transverse root structure is a diagnostic feature; the number of epidermal, inner cortical and outer cortical cells providing a species specific ratio. Unfortunately, there is little indication of the sample size. Furthermore, the populations used by Tan are known to the present worker, and are of uncertain identity. Consequently, little weight can be attached to Tan's suggestions without further corroboration through extensive study of fresh material of certain identity. It was hoped to investigate root

anatomy during the present study, but time did not permit it; this feature should certainly be included in future work. Interestingly, Rao (1935) indicated that root anatomy is trimerous; the number of cells in each tissue layer being based on three. Tan's (unpub work) results support this, as does a scrutiny of various other studies (Queva, 1910; Konar & Kapoor, 1972) and limited observations during the present investigation.

The taxonomic significance of characters discussed in this section is summarised in Table 5.3.

#### 5.2.4 Biogeography

The most striking feature of global biogeography of extant **Azolla** is the geographical separation of the two Sections (see Fig.5.7), the types of Section **Rhizosperma** being restricted to the African and Austral-Asian continents and Section **Azolla** being restricted to the Americas, Europe, Australia, New Zealand and Japan. Reports of **A.filiculoides** in China and South Africa (Lumpkin & Plucknett, 1982; Ashton & Walmsley, 1984) have probably resulted by introduction. In Japan and Australia the <sup>types</sup> **A.pinnata** and **A.filiculoides** are both found. Although Lumpkin & Plucknett (1982) state that when two or more species occur within the same vicinity each occupies different parts of the environment, the present author found one herbarium collection containing the **A.pinnata** and **A.filiculoides** types clearly mixed when they were collected. However, it is probable that the former and latter types usually inhabit tropical to sub-tropical climatic regions and temperate to sub-tropical regions respectively.

Furthermore it is likely that climate limits the distribution of *A.pinnata* to the South island of Japan. It should be pointed out that in the present study the biogeographical information obtained for types of Section *Rhizosperma* is considerably less than that for types of Section *Azolla*. In general the present investigation has shown that previous biogeographical distributions of taxa attributable to Section *Azolla* are not too unreliable. However, the distributions given by Mettenius (1847, 1867), Strasburger (1873), Svenson (1944), Lumpkin & Plucknett (1982) and others are clearly limited by incorrectly determined specimens. In Europe, *A.filiculoides* and *A.caroliniana* are widely distributed (Lumpkin & Plucknett, 1982), but the present investigation shows that one taxon (the *Azolla* sp. type) is apparently limited to the Netherlands, whereas the *A.filiculoides* type is distributed as previously described. Dallman & Williams (1940) report that two species of *Azolla* were apparently introduced into Europe in 1872, and subsequently escaped from Botanic Gardens. It appears that these species had reached England by 1880 (Marsh, 1914); however, the present investigation has only found evidence of the *A.filiculoides* type being present. *A.filiculoides* has spread in Europe (Dallman & Williams, 1940) while *A.caroliniana* is restricted to north eastern Holland (van Ooststroom, 1948; Pieterse et al., 1977). The present study supports the observations for two types in Europe. Prior to the late nineteenth century *Azolla* was not reported in Europe (Pieterse et al. 1977). However, *A.filiculoides* is known to occur in the interglacial deposits in England (West, 1953) Holland, Germany and Russia (Duigan & Cookson, 1956). Although lack of reports is not

sufficient evidence, the absence of **Azolla** in Europe may indicate that **Azolla** became extinct during the last ice age or at least restricted to warmer regions (Duigan & Cookson, 1956). This theory of extinction is most feasible for the British Isles and northern Europe which ~~were~~ mostly covered by the ice sheet. Whatever the origin of **Azolla** in Europe (i.e. indigenous or introduced) the similarities between the two types and their counterparts in northern America may favour the hypothesis that they were introduced. The present author believes that there may be a combination of native and introduced taxa and suggests that a detailed search for, and examination of, more sporulating populations may eventually reveal differences in sporoderm structure. Alternatively, chemotaxonomic investigation may elucidate differences.

With the wide global distribution of the **A.filiculoides** type it is not surprising to find a morphological cline related to geography in this type. Results of the present study clearly indicate that Australian populations appear to form a coherent group within the **A.filiculoides** type. This group is reported as being present for at least 8,700 years (Duigan & Cookson, 1956). However, the evidence comes from glochidial septation and sporoderm sculpturing and not the more taxonomically reliable perine structure. It would be interesting to examine this fossil **A.filiculoides** from Australia to determine whether or not differences exist between them and American populations.

The occurrence of more than two types in certain areas of the

Americas is interesting because certain inferences can be made. In North America the **Azolla** sp. type usually inhabits a region where the **A.filiculoides** and **A.mexicana** types also occur (i.e. central eastern states). Although no mixed collections of the latter two types were found it is conceivable that they are genetically sympatric. The common and unique features of **Azolla** sp. type and the other two sympatric types may suggest that the **Azolla** sp. type has arisen through hybridity. The same indication is evidenced for the **Azolla** sp. type in South America with the 'parents' being the **A.filiculoides** and **A.microphylla** types. This hypothesis may explain the disjunct distribution of the **Azolla** sp. type. However, the character similarity of the two disjunctions may be caused by the **A.filiculoides** type being the common parent. The evidence for the hybrid origin of the **Azolla** sp. type is tenuous, and would require chromosome doubling after hybridisation. Therefore, this hypothesis could be tested by cytological and electrophoresic methods. Possibly cross-breeding could also be used to resolve this question of hybridity. Evidence against hybridity may come from the sympatric occurrence of the **A.filiculoides** and **A.mexicana** types on the western seaboard of North America and in Mexico, and the absence of the **Azolla** sp. type in these regions. However, it can be argued that perhaps the polyploid hybrid has only occurred once in North America and its distribution is therefore limited. It is also possible that the **Azolla** sp. type does occur in Mexico and the western USA seaboard, but is not represented in herbarium collections. Therefore, the present author recommends that more collections are made, and like Bates (1980), urges sporulating

specimens should be collected if possible.

Collections of specimens from North America appear to be more numerous than from South America (including meso-america). The *A.microphylla* type is represented in tropical and sub-tropical regions of this continent. This type occurs in the Galapagos Islands, and it was present during the Quaternary (Kempf, 1974) and the most plausible explanation for its occurrence is that it was introduced by avian agents. Isolation of the *A.microphylla* type in the Galapagos Islands is perhaps reflected in the often observed differences in vegetative morphology compared to mainland populations. However, the present investigation did not find any difference in respect to perine structure.

It is clear that the *A.microphylla* type and types of Section *Rhizosperma* are predominantly tropical and sub-tropical taxa whereas other extant types occur in continental temperate to tropical climates. Therefore, there is potential for alien types to be introduced to most climatic regions either accidentally via field trials or as an ornamental plant. With the intense research on *Azolla* and the establishment of large culture collections, the introduction of alien types is inevitable. The present worker believes that it will soon be impossible to describe the indigenous distribution of *Azolla* taxa except through herbarium collections, which are not always comprehensive. The consequences of introducing taxa has not been evaluated, but it is conceivable that, like its close relative *Salvinia*, *Azolla* could be a serious weed. Indeed this threat prompted the study by Ashton (1977).

### 5.2.5 Anabaena-free Azolla

This is rarely reported as occurring naturally (Moore, 1969) and may be attributed to lack of observation. However, the present investigation found that naturally occurring **Anabaena**-free populations are indeed rare. Furthermore, it appears from the present study, together with Huneke (1933) and Shen (1961), that absence of the endosymbiont is not confined to one taxon or Section. The occurrence of **Anabaena**-free **Azolla** contradicts the suggestion that **Azolla** cannot exist naturally without **Anabaena** (Watanabe & Ladha, pers.comm.). The **Anabaena**-free populations found at Greywell and Fordingbridge in England, and monitored during the present study, are interesting in several respects. Firstly, the populations regularly fruited and grew vigorously which contradicts the findings of Huneke (1933). Secondly, Marsh (1914) reports<sup>an</sup> **Anabaena**-free population in the Reading area, which is not too far from Greywell. The now redundant canal system in the area may well have been the vector of distribution for this population. The occurrence of **Anabaena**-free **Azolla** in the 'wild' clearly opens speculation as to how this condition may arise (see Part B). Furthermore, such populations provide material for physiological and structural investigations which aim to elucidate the complexities of the **Anabaena-Azolla** association. Additionally, it may be possible to use such material for introducing **Anabaena** into **Azolla**.

TABLE 5.3.

Synopsis of characters investigated and their taxonomic value.

VEGETATIVE CHARACTERS	Usefulness
FROND	
Habit	-
Shape	-
Branching	-
Size	-
LEAVES	
No. per interbranch	-
Imbrication	-
Ventral lobe shape	-
Ventral lobe nature	-
Dorsal lobe length	-
Dorsal lobe width	-
Dorsal lobe length: width ratio	-
Dorsal lobe apical shape	-
Hyaline margin width	-
Hyaline margin obviousness	-
Hyaline margin nature	-
Trichome distribution	+
No. cells per trichome	+
Trichome orientation	-
COLOUR	-
ROOTS	
Length	-

TABLE 5.3 cont.

	Usefulness
ROOTS cont.	
Root hairs	-
Tip coiling	-
REPRODUCTIVE CHARACTERS	
SPOROCARPS	
Occurrence <sup>e</sup>	-
Ratio of mega:microsporocarps	-
Shape	-
MICROSPOROCARP WALL	-
MICROSPOANGIA	
Shape	-
Size	-
No. per microsporocarp	-
MASSULAE	
No. per microsporangium	-
Shape	-
Ornamentation	+
Type of process	+
MICROSPORES	
No. per microsporangium	-
No. per massula	-
GLOCHIDIA	
Shaft shape	+
Apical shape	-
No. septa	+

TABLE 5.3 cont.

	Usefulness
GLOCHIDIA cont.	
Position of septa	-
Length ( $\bar{x}$ & $S^2$ )	-
Shaft shape and No. septa	+
Distribution	● ?
MEGASPOROCARP	
Size	-
Shape	-
Wall nature	-
MEGASPORANGIUM	
Size	-
Wall nature	-
MEGASPORE APPARATUS	
Size	-
Shape	-
APICAL REGION	
No. of floats	●
Float shape	-
Float puncturing	+
Suprafilosum	●
Acrolamella	●
COLLAR	
Morphology, surface aspect	-
Morphology, sectional view	+ *
BASAL REGION	
Shape	+
Perine sculpturing	+

TABLE 5.3 cont.

BASAL REGION cont.	Usefulness
Infrafilosum	+
Sporoderm structure	+ *
STRUCTURE	
Exospore structure	-
Exospore thickness	-
Endoperine structure	+
Endoperine thickness variation	+
Exoperine 1 structure	-
Exoperine 1 thickness	-
Exoperine 2 structure	+
Exoperine 2 thickness	-
Exoperine 3 structure	+
Exoperine 3 thickness	-

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+ = Useful; - = not useful; \* = Useful at Section level only; ● = of possible evolutionary significance only.

5.3.1 Infrageneric taxa

Although it may appear ironical that the subgeneric taxonomy of **Azolla** is so difficult, it is not necessarily surprising in such a coherent and distinct group of ferns; this is recognised by treating **Azolla** as a monogeneric family (i.e. Azollaceae) rather than including it within the Salviniaceae.

The nomenclatural history of **Azolla** is outlined in section 1, and it is clear that since Mettenius (1847) reduced the two genera (**Rhizosperma** and **Azolla**) to sectional status there has been a somewhat arbitrary use of subgeneric and sectional ranks, based on essentially similar characters (i.e. float numbers and type of massula appendages). The proposed dual hierarchy for Subgenera and Sections (Sweet & Hills, 1976) may appear attractive because there are only three types of massula appendage (glochidiate, simple trichomes and coiled (circinate) trichomes) and therefore, only three Subgenera. However, in respect to fossil taxa, a possible difficulty arises in that massulae and a megaspore apparatus cannot always be conclusively correlated. Although this cannot be overcome, a dual hierarchy would have severely limited use where only the massula or megaspore apparatus are known. Furthermore, some criteria upon which the Sections are defined have been shown, by the present investigation, to be inappropriate. Size characters are not taxonomically useful, whereas perinal zonation and sculpturing are only insignificant at the sectional level. Furthermore, megaspore apparatus

structure is now understood, and the interpretation by Sweet & Hills (1976) is somewhat in error. With the dismissal of these characters only float number is left, which is considered, by the present worker, to provide inadequate definition of Sections in a dual hierarchy. Therefore, it appears appropriate to reject such a hierarchy, and propose a single one based on both massula appendages and the megaspore apparatus. In respect to the latter, megaspore apparatus structure (i.e. number of floats, the acrolamella and suprafilosum) and the collar (if present) are the most significant.

With the present proposal of one rank between genus and species, it is considered most appropriate and convenient for it to be the rank of Section. This allows greater flexibility than Subgenus for any future findings, which may elucidate as yet unrecognised taxa that cannot be encompassed by another Section or genus; this would probably apply to fossil taxa. Furthermore, it is generally recognised that Subgenus is the only workable rank between genus and Section, whereas between Section and species there are three ranks (i.e. Subsection, Series and Subseries) (Staflau, 1978). These three ranks should enable future findings to be incorporated into the framework without a change in rank of the currently proposed Sections. The present author anticipates that ranks between Section and species would be erected, if necessary and appropriate, from results of cyto- and chemotaxonomic investigations.

Four fossil Sections were outlined, together with a proposed

fifth Section, by Fowler (1975, 1981) based on features of the megaspore apparatus and massula appendages. The essential characteristics of each of the five Sections are given below together with suggested members of each. The two final Sections, making up seven altogether, were initially proposed for ~~ext~~<sup>t</sup> **Azolla**, but subsequently fossil taxa have been included.

Section SIMPLICISPORA Hall 1970: Float-like acrolamella or single float; massulae with glochidia. Fossil species:- **A.geneseana**, **A.primaeva**, **A.simplex**. This Section may have been established on wrongly interpreted specimens, and it is doubtful whether the above species should be included.

Section KREMASTOSPORA Jain & Hall 1969: Megaspore apparatus with more than nine floats; massulae with glochidia. Fossil species **A.distincta**, **A.montana**, **A.schopfi**, **A.stanleyi**, **A.teschiana**, **A.velus**.

Section FILIFERA Hall 1968: Megaspore apparatus with more than nine floats; massulae with simple trichomes or coiled trichomes. Fossil species:- **A.barbata**.

Section ANTIQUA Dorofeev 1959: Megaspore apparatus with six to nine floats in two tiers; massulae with glochidia. Fossil species:- **A.antiqua**, **A.aspera**, **A.nikitinii**, **A.sibirica**.

Section TRISEPTA Fowler 1975: Megaspore apparatus with nine floats in two tiers; massulae with glochidia. Fossil species:- **A.prisca**.

Amended circumscriptions of Sect. **Rhizosperma** and Sect. **Azolla** are given below. The features used are some of those that have been evaluated during this investigation (see Table 5.3).

Section **RHIZOSPERMA** (Meyen) Mettenius, 1847: Sporocarps arising in two's or four's; suprafilosum and/or acrolamella divide the apical region of the megaspore apparatus into three equal sectors, each sector accommodating three floats. Attached to the suprafilosum by float filosum, the nine floats are arranged in two tiers, the lower of six smaller floats and the upper of three larger floats. Collar always visible in sectional view; more or less horizontal, but is a diminutive elaboration of the proximal perine; perine surface lacking infrafilosum, although filosum may extend down from the collar region; perine always with distinctive excrescences or prolongations; endoperine with little or no alveolation. Massulae usually with a few simple and/or branched trichome-like appendages limited to one or two groups on the internal massula surface. Epidermal trichomes arising on the stem as well as the abaxial surface of the dorsal leaf lobe.

Extant species: **A.pinnata**. **A.nilotica**. Fossil species require re-examination to confirm their inclusion in this Section.

Section **AZOLLA** Lam. (1783): Sporocarps arising in two's; Suprafilosum and the acrolamella divide the apical region of the megaspore apparatus into three equal sectors, each sector accommodating one float; a tricuspid collar usually visible in sectional view, the cusps joining the acrolamella septa; the

collar angle (in sectional view) between horizontal and erect; may possess a more or less distinct downwardly directed flange. Distal perine surface with at least some infrafilosum; perine with prolongations, endoperine intrusions or excrescences; endoperine little to highly alveolate. Massulae with glochidia on the internal and external surfaces; glochidia may or may not arise in groups. Glochidial shaft may possess septa. Epidermal trichomes confined to the abaxial surface of the dorsal leaf lobe.

Extant species:- *A.filiculoides*, *A.mexicana*, '*A.microphylla*',

**Azolla** sp. Fossil species:- probably unknown.

*A.to<sub>4</sub>mentosa* Nikitin & Dorofeev, a late Tertiary species as described by Bertelsen (1974), appears similar to '*A.microphylla*' or **Azolla** sp. Other late Tertiary and Quaternary specimens, although not named as extant taxa, may indeed be synonymous with them.

The aim of the present investigation is to re-evaluate the taxonomy of **Azolla** Sect. **Azolla**, providing both a taxonomic framework and species recognition. The characters have been re-evaluated in respect to their taxonomic usefulness. So far these characters have been used for differentiating the two extant Sections. The following accounts provide a proposed taxonomic framework for **Azolla** species, with particular emphasis on Section **Azolla**, together with amended and new diagnoses for the taxa that are recognised by the present author. Table 5.4 provides a synopsis of the proposed framework (see p. 318).

### 5.3.2 Specific taxa

#### 5.3.2.1 **A.filiculoides** Lam. 1783

Synonymy:- **A.caroliniana** Willd. 1810; **A.magellanica** Willd. 1810;  
**A.rubra** R.Br. 1810; **A.microphylla** Kaulf. 1824; **A.cristata**  
Kaulf. 1824; **Salvinia azolla** Raddi 1825; **A.arbuscula** Desv. 1827;  
**A.densa** Desv. 1827; **A.japonica** Franch. & Savat. 1876.

Type material at P-LAM. Syntypes at P-JUS and BM.

This species is referred to as the **A.filiculoides** type in the present investigation.

#### **Protologue (Lamarck 1783)**

"AZOLLE filiculoides, AZOLLA filiculoides. C'est une petite plante aquatique, qui paroît flotter à la surface des eaux à la manière des Lenticules, (*Lemna*) avec lesquelles elle semble avoir beaucoup de rapports, & qui a néanmoins l'aspect d'une très-petite fougère. Cette plante consiste en quantité de petites feuilles ovoides, longues d'une demi-ligne, ponctuées sur une de leur faces, qui paroissent vésiculeuses, font embrigées & ferrées ces unes contre les autres, & ferment de petites ramifications disposée par bouquets ou rosettes filiciformes, sous lesquels on remarque de longues racines simples & capillaires. Cette plante a été rapportée de Magellan par M. de Commerson. (v.f.) Les petits points rousseâtres qui couvrent entièrement un des côtes de la plupart de ses feuilles, lui donnent en quelque sorte l'apparence d'une espèce d'Acrostique; mais ces mêmes feuille vésiculeuses, membraneuses & embrigées comme dans certaines espèces de Jongermanes, & les longues

racines capillaires dont leurs bouquets sont munis, la rapprochent davantage des Lenticules, & nous sont présumer qu'elle constitue un nouveau genre de la famille des Naiades".

It appears widely accepted that the **A.filiculoides** Type specimens were collected from the Magellan area of South America by Philibert Commerson, who was the naturalist on Bougainville's circumnavigation of the World (1766-1769) (Svenson, 1944; Stafleu & Cowens, 1976). However, the present investigation has revealed evidence which casts doubt on the Type locality (see section 4.7). It cannot be conclusively shown who, out of Jussieu and Lamarck, was correct because early collectors kept very poor records (Jermy, pers.comm.). Furthermore, Commerson died in Mauritius in 1773, and his specimens did not arrive in Paris until 1774 and were not given in to Jussieu's custody until 1776; Jussieu eventually received Commerson's field notes in 1784 (Stafleu, 1964). It is unlikely that Lamarck was in possession of full details of the material when he published the protologue in 1783. Therefore, it can only be stated that the specimens were collected from South America.

**A.filiculoides** has, through the taxonomic history of **Azolla**, remained the most easily distinguished taxon of Section **Azolla**. This might be a consequence of its distinctive reproductive characters, and often more or less distinctive vegetative form. However, the present investigation has shown **A.filiculoides** to be very similar to the other taxa in respect to some reproductive, but especially vegetative features. The latter

have undoubtedly led to the establishment of new specific epithets which are considered, by the present author, synonymous with *A.filiculoides*. One such 'species' is *A.caroliniana*, which was described by Willdenow (1810) (see Appendix V for protologue).

Syntype material of *A.caroliniana* can be found in the Richard Herbarium which is at P. According to Svenson (1944) these specimens were probably collected by Michaux. Morten (1967) confirms that Michaux did collect plant material from the Carolinas in the USA. Although there are only two fronds in the Willdenow Type collection of *A.caroliniana*, there is much more material dispersed within the General and Jussieu herbaria at P.

The protologue is hardly sufficient to distinguish *A.caroliniana* from other species of Section *Azolla*. A further complication is that the Type specimens lack reproductive structures which offer the most useful diagnostic characters. The only useful diagnostic vegetative feature is nature of the dorsal leaf lobe trichomes. Based on this, the Type specimens of *A.caroliniana* are clearly *A.filiculoides* because they possess one-celled trichomes on the abaxial surface of the dorsal leaf lobe. Therefore, the present worker recommends that *A.caroliniana* be considered synonymous with *A.filiculoides* in accordance with the ICBN articles (Stafleu, 1978).

*A.caroliniana* apud Mettenius (1867) probably encompasses the *A.mexicana*, *A.microphylla* and *Azolla* sp. types recognised by the

present worker, and clearly excludes the Type specimens of *A.caroliniana*. Examination of material studied by Svenson indicates that *A.caroliniana apud* Svenson (1944) is probably similar to the Type material. Recently the trivial epithet has been applied, by Lumpkin & Plucknett (1982), to specimens that possess two or more cells comprising the leaf trichomes; clearly unlike the *A.caroliniana* Type specimens. This error, which has introduced more confusion into the diagnosis and taxonomy of *Azolla*, appears to be caused by authors neglecting Type material.

Like the protologue for *A.caroliniana*, the one for *A.microphylla* could apply to any species within Section *Azolla* (see Appendix V). Although Bates (1980) states that the Type specimens were collected from British Guiana, Kaulfuss (1824) clearly states that Chamisso collected them from California. Alston (a former keeper of Botany at BM) examined the Type material at BR, and in his annotation states that no Types are to be found at B. However, the present investigation contradicts this and recommends that the Type collection at BR is used to select a lectotype because of the uncertain location of holotype material. The sheet found at BR by Alston originally held two different populations. Subsequently, the sheet has been divided and the more recent annotation, for the respective halves, has been mixed up. Once this is appreciated it is possible to recognise the Type material of *A.microphylla*. Furthermore, Alston's annotation indicates that, based on non-septate glochidia, this Type is *A.filiculoides*.

Although this is confirmed by the present investigation, more convincing evidence is furnished by one-celled leaf trichomes and the only megasporocarp which possesses **A.filiculoides** type of sculpturing; albeit somewhat obscured by infrafilosum (see Fig.4.56). Using this convincing evidence, the present author considers **A.microphylla** synonymous with **A.filiculoides**. Unfortunately there is a species that is distinct from **A.filiculoides** and which is currently recognised as **A.microphylla** (Svenson, 1944; Fowler & Stennett-Willson, 1978; Lumpkin & Plucknett, 1982) this will be discussed later.

Like **A.microphylla**, **A.cristata** is synonymous with **A.filiculoides** based on the dorsal leaf lobe trichomes. The protologue of **A.cristata** is decidedly uncertain compared with that of **A.microphylla** (see Appendix V), and there is no indication of who collected the specimens, only that they were collected from Demerary in South America. The herbarium sheet, now in the Kaulfuss herbarium (BR), possesses a sequential number to the syntype of **A.microphylla** Chamisso at P and indicates that the material was collected by Ossenbaul. Therefore, the possible Type material, so far examined, of **A.cristata** is of doubtful significance. There appears to be some confusion concerning the Kaulfuss herbarium. After his death in 1830, his pteridophytes were apparently sold to Römer (Stafleu & Cowens, 1979). Although some ferns are at PH, and some labelled as Kaulfuss material are from the J.S. Kaulfuss herbarium (Stafleu & Cowens, 1979), the specimens examined during the present study, from BR, B and P, were originally from the G.F. Kaulfuss herbarium; his specimens

were therefore widely distributed throughout Europe.

Mettenius (1847) considered *A.cristata* a distinct species but later placed it in synonymy with *A.caroliniana* (Mettenius, 1867). In respect to *A.arbuscula* and *A.densa*, Mettenius (1847) placed them in synonymy with *A.filiculoides* and *A.caroliniana* respectively, while Svenson (1944) dismissed them in his discussion of *A.mexicana*. However, the present study revealed that both *A.arbuscula* and *A.densa* are synonymous with *A.filiculoides* on the basis of one-celled trichomes on the dorsal leaf lobe. The present study found *Azolla* collections from the Desvaux herbarium dispersed in the General herbarium at P. Information in the protogues corresponding to the annotation, and the latter being in Desvaux's own hand, lead the present worker to consider these specimens to be Type specimens. The present author was unable to gather much information on *Salvinia azolla*. The only specimens with this combination, and possibly the authors signature (Raddi), were at B. Therefore, these specimens may not be Type specimens. However, they are clearly *Azolla* as opposed to *Salvinia* and, on grounds of priority, the combination must be rejected. The present investigation clearly demonstrates that these specimens belong to *A.filiculoides*.

*A.japonica*, established by Franchet & Savatier (1876), is here considered synonymous with *A.filiculoides*. The protologue (see Appendix V) is very brief and uninformative with the trivial epithet apparently derived from the country of origin (Japan). Few authors have recognised *A.japonica* as a distinct taxon while some researchers have considered populations from Japan to be

**A.rubra** (e.g. Lumpkin & Plucknett, 1982).

**A.rubra** was collected and described by Brown (1810) (for protologue see Appendix V); however, his lack of detail, when cataloguing specimens, probably led him to state that **A.rubra** was collected from Port Jackson (= Sydney, Australia) and Insula van Diemen (= Tasmania). Stearn (1960) states that localities given by Brown (1810) cover vast areas and suggested that Port Jackson may mean a coastal zone from Sydney northwards to Newcastle. Brown's collections were given into J.J. Bennett's custody who later distributed duplicates to various herbaria (Stafleu & Cowens, 1976). In respect to this distribution, duplicates of **A.rubra** Types located at E and P are consistent with accounts by Stafleu & Cowens (1976). Brown (1810) did not designate a holotype, and the present worker suggests that the isotypes at BM should be selected as lectotypes.

As indicated by Table 1.1 **A.rubra** has been the subject of much nomenclatural change in rank. Mettenius (1847) considered it to be a distinct species, but later in 1867 combined it with **A.filiculoides**. Strasburger (1873) recognised **A.rubra** as a variety of **A.filiculoides** based on the presence of an apical glochidial septum. This feature is clearly very poor delimitation of a taxon. Svenson (1944) suggested that this feature occurred throughout the range of **A.filiculoides** in the americas, and dismissed **A.rubra** even as a variety. However, based mainly on the possession of two, three and four glochidial septa, **A.rubra** has been recognised as a variety of **A.filiculoides** in

Quaternary deposits from Australia (Duigan & Cookson, 1956). More recently however, Lumpkin & Plucknett (1982) have extended the tentative suggestion by Fowler & Stennett-Willson (1978) and have recognised **A.rubra** as a distinct species.

The present investigation has demonstrated that **A.rubra** should be considered synonymous with **A.filiculoides**, but it was also found that Australian populations of this species possess certain character states that distinguish them from other populations. These 'unique' character states are considered sufficient, by the present worker, to propose the establishment of a subspecies of **A.filiculoides** called subspecies **rubra** based on Brown's Type specimens from BM. In view of this, it is also proposed that another subspecies be established, called **A.filiculoides** subsp.**f.filiculoides**, to encompass all those populations not conforming to **A.filiculoides** subsp.**rubra**. These subspecies illustrate an appreciation of the considerable phenotypic variation of reproductive structures in **A.filiculoides**. Subspecific rank is considered more appropriate because glochidial and, more importantly, perine strata provide the diagnostic characters for the taxa.

In conclusion the following is the amended diagnosis of **A.filiculoides** together with the diagnoses of the proposed subspecies.

**A.filiculoides** Lam. (= the **A.filiculoides** type)

Megaspore apparatus (also see section 4.2.1)

FLOATS:- puncturing usually of small (ca 3um) decidedly

irregularly shaped holes (Fig. 4.7b & c).

Collar:- often giving a distinct waist to the megaspore apparatus; in sectional view it is at a relatively high angle to the equatorial axis; the flange inserted half to two thirds up the collar (Figs. 4.8,4.9).

Sporoderm sculpturing:- a mosaic of raised, often angular areas, and often deep and rounded depressions; raised areas interconnected by ridges of exoperine material; density of the raised areas variable; a thin weft of infrafiloseum is usually present, but variable in quantity - large amounts obscuring the raised areas (Figs. 4.5,4.6,4.7a). At high magnification sculpturing on the apex of a raised area is verrucate to rugulate although infrafiloseum may obscure it; in the depression areas sculpturing is smooth to undulating, sometimes with verrucose bodies (Fig.4.10).

Sporoderm structure:- raised areas are excrescences. Endoperine composed of closely packed tiny irregular vermiform rods to apparently fused elements. Beneath a depression the endoperine with a few to many alveolae, may be uniform or variable in size in any one specimen; in an excrescence one, two or a few large to many smaller alveolae, or alveolae more or less absent; sometimes there is a dense plug of endoperine at the base of an excrescence. Exoperine 2 solid, usually becoming baculate towards an excrescence apex where exoperine 2 and 3 may exhibit variable degrees of fusion (Figs. 4.14a-c). Idealised sporoderm structure is illustrated in Figure 5.1,5.2

Massulae (also see sections 4.3.4 and 4.3.5)

Surface relatively smooth, sometimes with small blunt

protuberances at least on the external surface. Glochidia with 0 to 12 septa, (commonly 1 to 3 septa). When few septa present they are always apically located, but become uniformly distributed with more septa. Glochidial shaft strongly tapered at proximal and distal ends to almost parallel sided (Fig. 4. 32a & b).

Dorsal leaf lobe:- one-celled trichomes on the abaxial surface, prominent to almost indiscernible.

**A.filiculoides Lam. subsp.*filiculoides*.**

Collar:- flange commonly inserted in distal third of sectional length (Figs. 4.8d, 4.9a).

Perine structure:- endoperine elements fused and almost indiscernible, general appearance of endoperine is alveolate. Excrescences always with one to four large alveolae, may also have smaller ones. Outer edge of exoperine 2 in a depression usually smooth to undulating (Fig. 4.13). Idealised sporoderm structure is illustrated in Figure 5.1.

Massulae:- surface without small blunt protuberances. Glochidia with 0 to 6 septa, commonly with 0, 1 and 2 apical septa, rarely more; glochidial shaft usually with distinct apical an tapering (Fig. 4.32a).

Biogeography:- USA, Mexico, South America, Europe, Japan, New Zealand.

**A.filiculoides subsp.*rubra* (R.Br.) Dunham subsp. et stat.nov.**

(**A.rubra** R.Br. basionym: Prod.Nov.Holl.:278.(1810)).

Collar:- flange commonly inserted half way along sectional length (Fig.4.9c & e).

Perine structure:- endoperine elements usually not fused and more or less discernible; general appearance of endoperine rarely highly alveolate, usually quite dense. Excrescence endoperine usually with small alveolae, commonly lacking large alveolae, size of alveolae may be variable. Where few or no alveolae, there is often a dense plug of endoperine at the base of an excrescence. Outer edge of the exoperine 2 often with verrucose bodies (Figs. 4.11, 4.12,). Idealised sporoderm structure is illustrated in Figure 5.2.

Massulae:- surface usually with small blunt protuberances at least on external surface. Glochidia with 0 to 12 septa, commonly with 2 to 5 septa, apically and uniformly distributed; glochidial shaft with weak proximal and distal tapering to more or less parallel sided (Fig. 32b).

Biogeography:- East and South Eastern Australia (see section 5.2.4).

### 5.3.2.2 *A.mexicana* Presl. 1845

Synonymy: No epithets have been considered synonymous with *A.mexicana* but it has been considered synonymous with *A. caroliniana* (Mettenius, 1847, 1867; Bates, 1980). A later homonym by Schaffner appears to be *A.filiculoides*; however, the present author could not find a protologue or Type material for it.

Holotype collection at PR. Syntypes at HAL, B, BM and P.

This species is referred to as the *A.mexicana* type in the present investigation.

Protologue (Presl. 1845)

"Nova Azollae species est: *Azolla mexicana*; fronde pinnata, foliolis imbricatis laevibus subrotundis coloratis, radicibus capillaribus. Habitat in Mexico, ubi legit clar. Schiede.

Affinis videtur *A. portoricensi*, differt foliolis margin non hyalinis".

The name *A.mexicana* was first published without description or illustration by Schlechtendal & Chamisso (1830) ("839. AZOLLA species ut videtur nova *mexicana*, quam in vivis recognoscat ipse inventor, nos e siccis mancam desumere diagnosin noluimus. \_\_\_\_\_

\_\_\_\_\_ Inter Serpillo et Estero in aquis stagnantibus reg. Calidæ. Jan."). This has led to *A.mexicana* being attributed to Schlechtendal & Chamisso by some researchers. But such citation is invalid under the rules of the ICBN. It appears that Schlechtendal & Chamisso (1830) and Presl (1845) examined material from the same source, namely specimens collected by Schiede and Deppe from Mexico, but Presl (1845) makes no reference to Schlechtendal & Chamisso (1830). The protologue appears to be rarely found (Svenson, 1944) because it is in a supplement dated 1844 which is separately paginated<sup>d</sup> from the journal which was published in 1845; the present worker, in conjunction with A.C. Jermy, discovered this by accident.

Although Mettenius (1847) apparently examined Schiede's material (i.e. Type material), he placed *A.mexicana* in synonymy with *A.caroliniana*. It was not until nearly 100 years later that *A.mexicana* was recognised as a separate taxon by Svenson (1944).

Subsequently, most authors followed this proposal. However, Bates (1980) tentatively concluded that *A.mexicana* is synonymous with *A.caroliniana*. The present investigation found several duplicate collections of Schiede's material which is readily recognised by the number 839 and locality. The material examined during the present study is from HAL, and will be designated as the lectotype collection, because the holotype collection is presumably at Prague. The Type material is particularly important because it possesses many micro- and megasporocarps; this was not noted by Presl (1845). Most recent studies, including the present one, recognise *A.mexicana* as a distinct species (e.g. di Fulvio, 1956, 1961; Lumpkin & Plucknett, 1982; Calvert et al., 1983 and others). Where comparable, these descriptions appear to agree, in general, with the results of the present investigation. However, the present study found previously unrecognised variation which leads to the tentative suggestions of infraspecific (or subtype) taxa. However, the present worker believes that evidence for such taxa is weak. With further morphological, but certainly chemotaxonomic investigation it may be necessary to establish infraspecific taxa in order to express and account for the observed variation. An amended diagnosis of *A.mexicana* is given below.

*A.mexicana* Presl (= the *A.mexicana* type)

Megaspore apparatus (also see section 4.2.2)

Floats:- puncturing usually of minute relatively smooth edged holes, often appearing absent (Fig. 4.16b & c).

Collar:- in surface view often appearing somewhat large; in

sectional view relatively robust and at a relatively low angle to the equatorial axis, flange inserted in the lower third (Fig. 4.1-6d & e) ..

Sporoderm sculpturing:- general appearance usually finely sculptured surface with large foveae. At high magnification sculpturing verrucate to rugulate often with many small foveae, this surface usually extends down into the large foveae, sometimes lining them; if not, the base may be smooth to undulating surface. Variable quantities of infrafilosum may variably obscure the sporoderm surface (Fig. 4.17).

Sporoderm structure:- endoperine composed of tiny closely packed vermiform rods which are often discernible. Alveolae variable in number - none to often few, but usually small and of uniform size; endoperine sometimes intruding into the exoperine, very rarely forming an excrescence. Sculpturally raised areas created by endoperine intrusion or localised variation in exoperine 2 thickness. Exoperine 2 solid to shortly columellate in large foveae, otherwise irregularly arranged columellae, although with variable degrees of fusion, but dividing then anastamosing towards their apices (Fig. 4.15, 4.17, 4.18, 4.20, 5.3)

Massulae (also see sections 4.3.4 and 4.3.5)

Surface usually with ovoid depressions. Glochidia with 0 to 12 septa, commonly 2 to 6, apically, basally and uniformly distributed; shaft usually possessing more or less parallel sides and short stalk, rarely with basal and apical tapering shaft and relatively long stalk (Fig. 4.32c-e).

Dorsal leaf lobe.

Two-celled trichomes on the abaxial surface.

Biogeography:- USA, Mexico, meso- and N. south America (see section 5.2.4 and Figures 4.45, 4.46 & 5.7).

5.3.2.3 **Azolla** nomen novum (= the **A.microphylla** type)

Synonymy: No epithets have been considered synonymous with **A.microphylla**, except **Salvinia azolla** (Mettenius, 1847). However, **A.microphylla** has been considered synonymous with **A.caroliniana** (Mettenius, 1867; di Fulvio, 1957).

The present investigation revealed that the Type material of **A.microphylla** Kaulfuss (1824) is **A.filiculoides**. This requires that the epithet 'microphylla' be rejected, and the use here of '**Azolla** nomen novum' is in recognition of this rejection, prior to the valid proposal of a new epithet.

The name **A.microphylla** was widely used until Mettenius (1867) combined it with **A.caroliniana** (Martius, 1834; Meyen, 1836; Mettenius, 1847). Martius (1834) appears to have been responsible for much of the early morphological information and illustrations based on Brasilian specimens. But, with the reinstatement of **A.microphylla** it was suggested that its Type specimens, when examined, would either be **A.filiculoides** or **A.mexicana** (Svenson, 1944). Subsequently, most workers recognise **A.mic<sup>o</sup>rophylla** which, from the literature, appears to be similar to the **A.microphylla** type of the present study (see Kempf (1974), Fowler & Stennett-Willson (1978) and Lumpkin & Plucknett (1982)). However, according to di Fulvio (1957); **A.microphylla** sensu Svenson is equivalent to **A.caroliniana** sensu Mettenius (1867) but not to **A.microphylla** sensu Kaulfuss.

In conclusion, the taxon called *A.microphylla* *sensu* Svenson (1944), Fowler & Stennett-Willson (1978), Lumpkin & Plucknett (1982) and others is indeed a distinct species, but is known by an illegitimate specific epithet under the ICBN Articles. Therefore, a new epithet will have to be proposed and published in accordance with the ICBN. It is anticipated that the new name will reflect the essence of this taxon's geographical distribution. Furthermore, Type material will be designated and deposited at B.M.

The doubtful Type material of *A.portoricensis* (B74) cannot be positively attributed to either *Azolla* *nomen novum* or *A.mexicana* because it is sterile. The material is a duplicate of Mettenius' herbarium, his original herbarium being destroyed during World War II. Although Mettenius (1847) states that he examined some of Sprengel's specimens, it cannot be determined with certainty whether or not B74 is genuine Type material. Therefore, it appears prudent to disregard B74 as Type material because it could cause nomenclatural uncertainty in respect to *Azolla* *nomen novum*. The diagnosis of *Azolla* *nomen novum* is as follows:-

***Azolla* *nomen novum* (= the *A.microphylla* type).**

**Megaspore apparatus** (also see section 4.2.3)

**FLOATS**:- puncturing usually of irregularly shaped holes (ca 4-7 µm diameter) or almost indiscernible (Figs. 4.21, 4.22a).

**COLLAR**:- in surface view usually appearing somewhat large; in sectional view rather robust and at a low angle to equatorial axis, flange inserted in the proximal third, often proximal quarter (Fig. 4.22c & d).

Sporoderm sculpturing:- general appearance of a smooth surface often with shallow foveae (depressions) particularly evident towards the distal sporoderm (Fig. 4.21). At high magnification rugulate-verrucate to mostly free-ended elements, often with numerous irregular small foveae even in the large foveae; variable quantities of infrafilosum which variably obscures the sculpturing (Fig. 4.23).

Sporoderm structure:- endoperine composed of tiny closely packed, almost indiscernible vermiform rods; endoperine uniformly thick or undulating, rarely with intrusions, if so then towards the distal sporoderm; excrescences very rare. Exoperine 2 of irregularly arranged radial columellae which tend to fuse towards their apices varying to columellae which may be more or less fused along their length; exoperine usually obviously thinning towards the collar, where it is usually more solid; shallow foveae created by variation in thickness of the endoperine and/or exoperine 2 (Fig. 4.24). Idealised sporoderm structure is illustrated in Figure 5.4.

Massulae (also see sections 4.3.4 and 4.3.5)

Surface relatively smooth. Glochidia with 0 to 10 septa, commonly 2 to 5; apically and basally distributed, although some uniformly so; glochidial shaft apically and basally tapered, sometimes with obvious glochidial stalk (Fig. 4.32f & g).

Dorsal leaf lobe

Two celled trichomes on the abaxial surface.

Biogeography:- temperate to tropical meso- and south America (see section 5.2.4 and Figures 4.45, 4.46 & 5.7).

#### 5.3.2.4 *Azolla* sp.nov. (= the *Azolla* sp.type)

This taxon has so far been referred to as '*Azolla* sp.' in the present study. It is believed, by the present worker, to be synonymous with *A.caroliniana* *sensu* Lumpkin & Plucknett (1982). The present investigation has clearly shown that *A.caroliniana* *sensu* Willdenow (1810) is synonymous with *A.filiculoides*, and use of the epithet '*caroliniana*' is invalid. Descriptions and illustrations by Mettenius (1847, 1867) and Strasburger (1873) are not detailed enough to determine whether *Azolla* sp.nov. was recognised. However, there is reference to a specimen lacking a collar (Mettenius, 1847), and the present worker believes that this might be *Azolla* sp.nov. because the collar may be completely obscured by filosum. There is evidence to suggest that Svenson (1944) saw one population of *Azolla* sp.nov. The herbarium sheet of GH11 is annotated by Svenson with the note, "apparently *A.mexicana*: glochidia septate, megaspore obviously punctate but I have seen no other material with similar narrow falcate leaves". However, he apparently ignored this population in his 1944 publication; GH11 is attributed to *Azolla* sp.nov. by the present worker. Through examination of Type material it was possible to demonstrate that *Azolla* sp.nov. appears not to have been formally described. Therefore, a name will be proposed and validly published in the future, and the Type material will be deposited at BM. The diagnosis of *Azolla* sp.nov. is given below:-

*Azolla* sp.nov. (= the *Azolla* sp. type).

Megaspore apparatus (also see section 4.2.4)

Floats:- puncturing rare, if so then minute holes; float surface rugulate-verrucate; dimpled or with vermiform furrows (Fig. 4.27e & f).

Collar:- surface view usually more or less completely obscured by filosum; in sectional view the flange inserted more or less half way along collar length, rarely in the lower quarter, may be obscured by filosum (Figs. 4.25, 4.26a-c).

Sporoderm sculpturing:- general appearance relatively smooth, covered by infrafilosum, deep large foveae may be seen (Fig. 4.2-5). At high magnification rugulate-verrucate to mostly free ended elements or occasionally vermiculate, with small foveae, usually obscured by infrafilosum (Fig. 4.27a-d).

Sporoderm structure:- endoperine composed of tiny closely packed vermiform rods, usually fused and almost indiscernible; no or few alveolae to highly alveolate, or few small alveolae with occasional larger alveolae often in groups, the latter creating excrescences, intrusions and excrescences may lack alveolae. Exoperine 2 of irregularly arranged columellae tending to fuse at their apices, although they may also tend to fuse along their length, local areas of short columellae and/or intrusions cause deep large foveae (Figs. 4.28, 4.29). Idealised sporoderm structure is illustrated in Figure 5.5.

Massulae (also see sections 4.3.4 and 4.3.5)

Relatively smooth surface; glochidia 0 - 15 septa, commonly with 2 to 5 septa, which are apically, basally and/or uniformly distributed; glochidial shaft with weak apical and basal tapering to parallel sided (Fig. 4.32h & i).

Dorsal leaf lobes

Two or three-celled trichomes on the abaxial surface, the apical cell often appearing as a prolongation of the adjacent cell; herbarium specimens may or may not have phosphorous-containing

crystals on the prolongation.

Biogeography:-USA, meso- and south America, Holland (see section 5.2.4 and Figures 4.45, 4.46 & 5.7).

#### 5.3.2.5 **A.pinnata** R.Br. (= the **A.pinnata** type)

Iter Australiense, 1802-05, R. Brown 134 (presented by direction of J.J. Bennett 1876) (BM) (see Fig. 4.62).

Synonymy: \***A.africana** Desv. 1827; **A.guineensis** Schum. 1827; **Salvinia imbricata** Roxb.~~ex~~ Griff. 1844; \***A.decomposita** Zollinger 1854; **A.pinnata** var. **africana** (Desv.) Baker 1887; **A.pinnata** var. **imbricata** (Roxb.~~ex~~ Griff.) Bonap. 1918; \***A.imbricata** (Roxb.~~ex~~ Griff.) Nakai 1925; **A.pinnata** var. **pinnata** Sweet & Hills 1971.

This species is referred to as the **A.pinnata** type in the present investigation. (\*= Type material examined, all sterile but clearly belonging to **A.pinnata**).

#### Protologue (Brown 1810)

"1. **A.pinnata**, fronde circumscriptione triangulari pinnata, foliolis superioribus papulosis, radicibus longitudinaliter plumosis (J.) v.v."

The Type specimens were collected from Port Jackson (= Sydney, Australia), and similar comments apply to these as to the Types of **A.rubra** in respect to locality and distribution of specimens by Bennett. The holotype collection is at BM with a syntype collection at E (Sweet & Hills, 1971).

**A.pinnata** appears to be readily recognised, although the various proposed varieties have proved difficult to distinguish. Currently two varieties are recognised from leaf-branch

morphology (Sweet & Hills, 1971; Lumpkin & Plucknett, 1982), namely **A.pinnata** var. **pinnata** and **A.pinnata** var. **imbricata**. Recently, differences in the perine between African and other populations were found, but taxonomic inferences were not made because only a small number of populations was examined (Zhou, 1983). If these differences are real, they do not entirely conform to the biogeography given by Sweet & Hills (1971) for the two varieties. Clearly, much more ultrastructural observation of **A.pinnata** is required. This may eventually lead to the recognition and delimitation of infraspecific taxa. The small number of populations examined during the present investigation does not permit meaningful suggestions to be made in respect to infraspecific taxa.

The following is a description of **A.pinnata** (the Type specimen is excluded in respect to reproductive structures because it is sterile):-

Sporocarps arising in pairs at branch points.

Megaspore apparatus (see section 4.8.1.1).

Floats:- minutely punctured; usually with hooks on some edges of floats.

Collar:- in surface view covered by filosum; in sectional view more or less horizontal to the equatorial plane, club shaped (Figs. 4.59a & b, 4.60c).

Sporoderm sculpturing:- surface devoid of infrafilosum, although filosum cascades down from the collar; smooth granular surface with conspicuous prostrate tuberculate, elongated bodies with irregular constrictions, these bodies are narrower immediately below the collar. At high magnification sculpturing rugulate to pilate (Fig. 4.59c & d).

Sporoderm structure:- endoperine composed of tiny closely packed, irregularly vermicular rods, without alveolae; endoperine extending into the surface bodies (= excrescences); exoperine 2 composed of branched anastomosing columellae/bacula which tend to fuse towards their apices forming a more solid zone which give rise to branches which terminate in round, mostly free apices; exoperine 3 on excrescences only, mostly solid (Fig. 4.60). Idealised sporoderm structure is illustrated in Figure 5.6a.

#### Massulae

External and internal surfaces convex and concave respectively; surface relatively smooth; simple and/or branched trichomes arising from a more or less central position on the internal surface; trichomes usually straight with more or less acute apices (e.g. spine-like) (Fig. 4. 60d).

#### Epidermal trichomes

Found on the abaxial surface of the dorsal leaf lobe and on the rhizome, clearly two-celled on the former, becoming echinate towards the leaf base, echinate on the rhizome.

#### Biogeography:- see Figure 5.7.

5.3.2.6 **A.nilotica** Decaisne ex Mettenius 1867. (= the **A.nilotica** type)  
"2<sup>me</sup> voyage aux sources de Nil-Blanc 1840. 25 Decembre. Donne par M. D'Arnaud commandant de l'expedition". (Recognised as holotype 22-8-1966) (Fig. 4.63).

Synonymy:- this species appears not to have been confused with other taxa or been given any other name.

Protologue (in Mettenius, 1867)

A lengthy comparative description is given, but there is a short diagnostic synopsis:-

"Cuticula macrosporae aequaliter minute tuberculata et inter tubercula regulariter perforato-punctata, massulis microsporarum duabus".

The Type specimens were probably collected by Binder during the second voyage to the source of the White Nile. However, D'Arnaud, the leader of the expedition, apparently gave the specimens to P. Decaisne, a Belgian botanist attached to P, but his Types and herbarium are in a private herbarium at BR. It is likely that the material at P, although designated as the holotype, is in fact a syntype, and is considered by the present author to be a lectotype collection. If specimens of *A.nilotica* are present at BR, they were not available for the present investigation.

Decaisne described and named the specimens in a manuscript that Mettenius (1867) states is at P. He used this manuscript as a basis for the first valid publication of this species; the manuscript by Decaisne cannot be considered as a valid publication. The present author was unable to find this manuscript at P.

*A.nilotica* is perhaps the most distinctive of all extant species, particularly in respect to frond morphology; which is quite unlike any other taxon. However, Peters (pers.comm.) states that it may be similar to other species under certain culture conditions. There are very few investigations of *A.nilotica*, but Demalsey (1953) and Lumpkin (1981) provide morpho-anatomical and

potential use (agronomically) type accounts respectively, while Fowler & Stennett-Willson (1978) provide a detailed account of megaspore apparatus sculpturing and structure. The present investigation examined only four populations of **A.nilotica** and, therefore, may not have sampled much phenotypic variation. It remains to be seen whether **A.nilotica**, with its relatively limited geographical range, exhibits much variation in sporoderm sculpture and structure. Examination of sporoderm ultrastructure in many more populations should reveal the extent of variation in **A.nilotica**.

The following is a description of **A.nilotica** and includes the Type specimens from P.

Sporocarps arising in fours at branch points.

Megaspore apparatus (see section 4.8.1.2)

Floats:- minutely punctured; exoperine extending from the distal perine on to the lower float tier; usually hooks on some edges of floats (Fig. 4.61d).

Collar:- not obvious in surface view, obscured by exoperine; in sectional view diminutive, horizontal to equatorial plane, but turned upwards distally (Fig. 4.61d).

Sporoderm sculpturing:- general appearance - surface devoid of infrafilosum, conspicuous spine-like projections on a smooth granular surface, projections more numerous distally, appearing as one to several fused elongated clavae (or columellae) usually recurved at their apices; granular surface composed of closely packed, regularly sized clavae, small foveae may be seen between clavae; below the collar, the surface of the clavae may be granular (Fig. 4.61a & b).

Sporoderm structure:- endoperine composed of closely packed tiny vermiform rods, a few small alveolae present; exoperine 2 composed of clavae with variable degrees of fusion; spine-like surface projections appear to be extensions of the exoperine 2 clavae (Fig. 4.6lc). Idealised sporoderm structure is illustrated in Figure 5.6b.

#### Massulae

Surface usually relatively smooth; simple and/or branched trichomes originating from a more or less central region of the internal surface; trichomes not always obvious, may appear vermiform, with small blunt appendages on their surface (Fig. 4.-6le).

#### Epidermal trichomes

Found on the abaxial surface of the dorsal leaf lobe and on the rhizome; clearly two-celled on the former; on older regions of the rhizome the trichomes senesce and persist rendering the rhizome tomentose.

Biogeography:- see Figure 5.7.

TABLE 5.4

PROPOSED TAXONOMIC FRAMEWORK FOR EXTANT TAXA

AZOLLACEAE C.Chr.

AZOLLA Lam.

\*Sect. *Rhizosperma* (Meyen) Mett.

\*Sect. *Azolla*

*A.pinnata* R.Br.

\**Azolla* nomen novum (*A.microphylla* auctores non Kaulf.)

*A.nilotica* Decne. ex Mett.

\**A.mexicana* Presl

*Azolla* sp.nov.

\**A.filiculoides* Lam.

subsp. *filiculoides*

(nov.subsp.)

subsp. *rubra* (R.Br.)

Dunham subsp. et stat.nov.

(\* = Amended diagnosis provided by the present investigation)

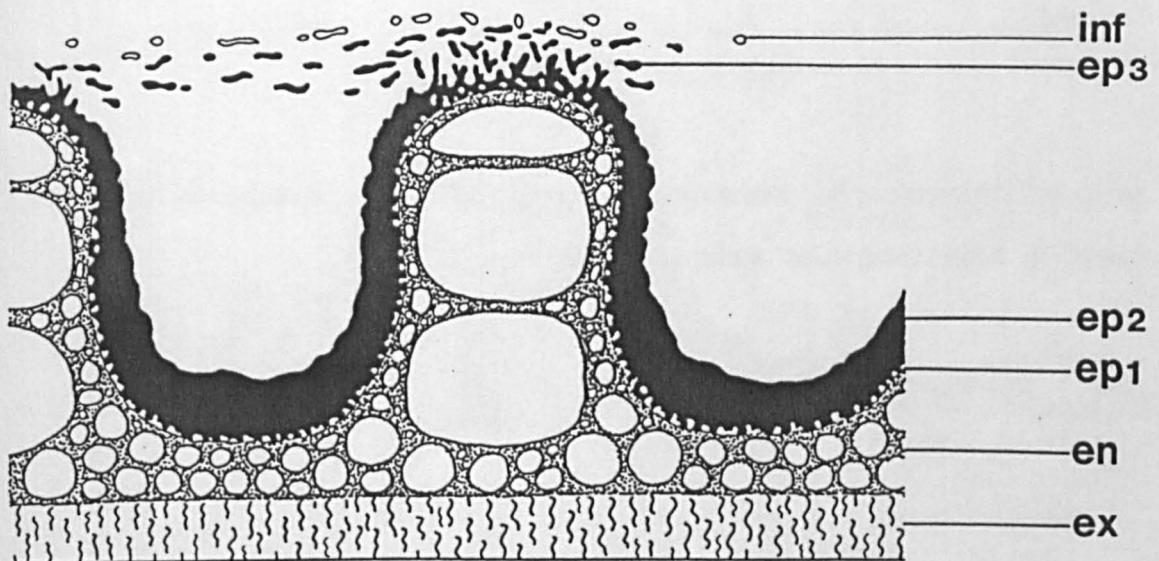
FIGURE 5.1

A.filiculoides subsp. filiculoides

(a & b) Diagrammatic representations of sporoderm structure typically found in **A.filiculoides** subsp. **filiculoides**.

(inf = infrafilosum; ep.3 = exoperine 3; ep.2 = exoperine 2;  
ep.1 = exoperine 1; en = endoperine; ex = exospore)

**a**



**b**

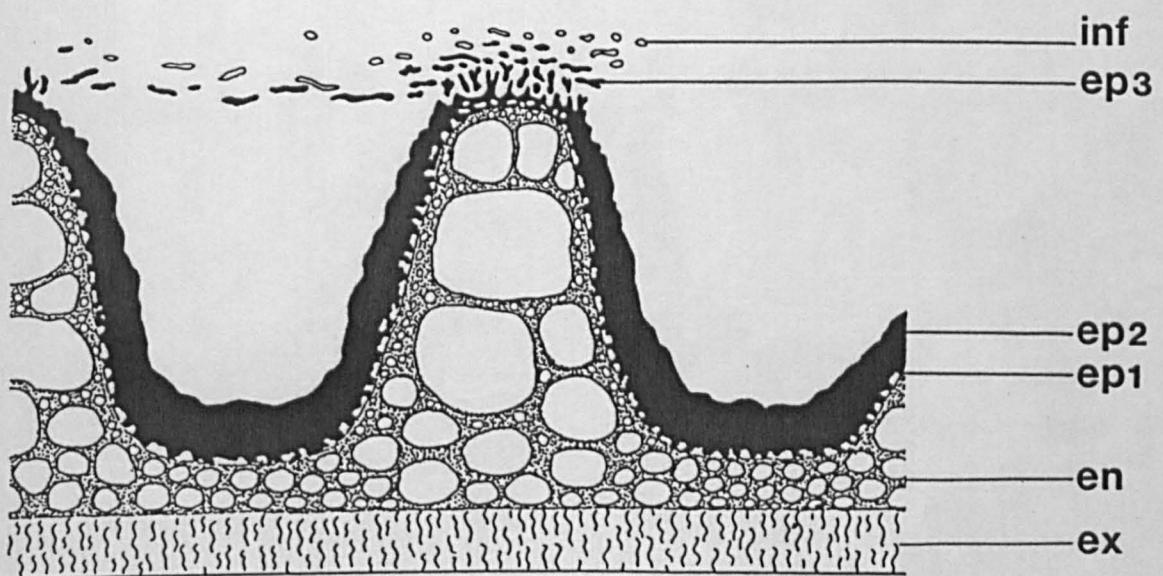


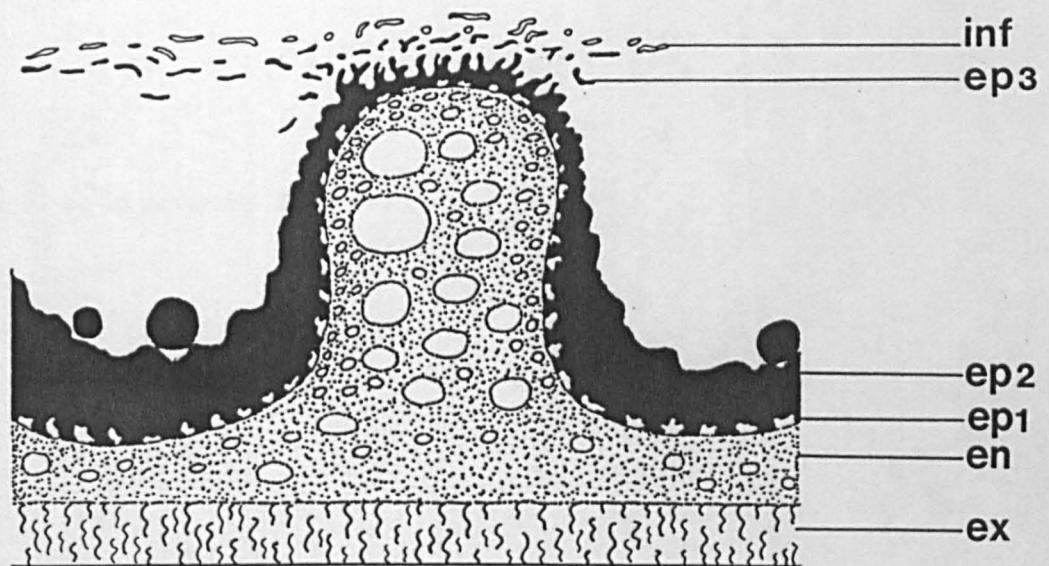
FIGURE 5.2

A.filiculoides subsp. rubra

(a & b) Diagrammatic representations of sporoderm structure typically found in **A.filiculoides** subsp. **rubra**.

(key - see Figure 5.1)

a



b

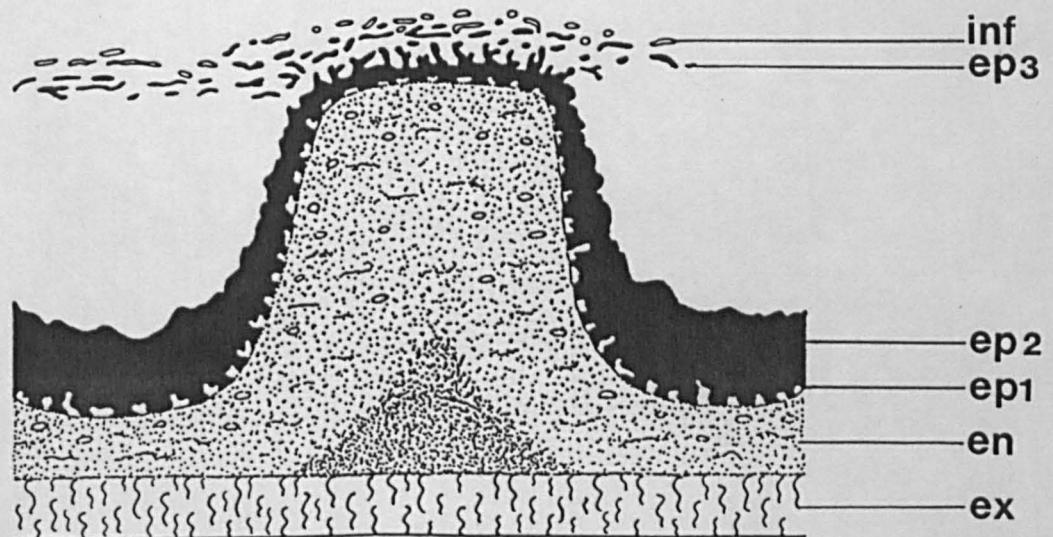


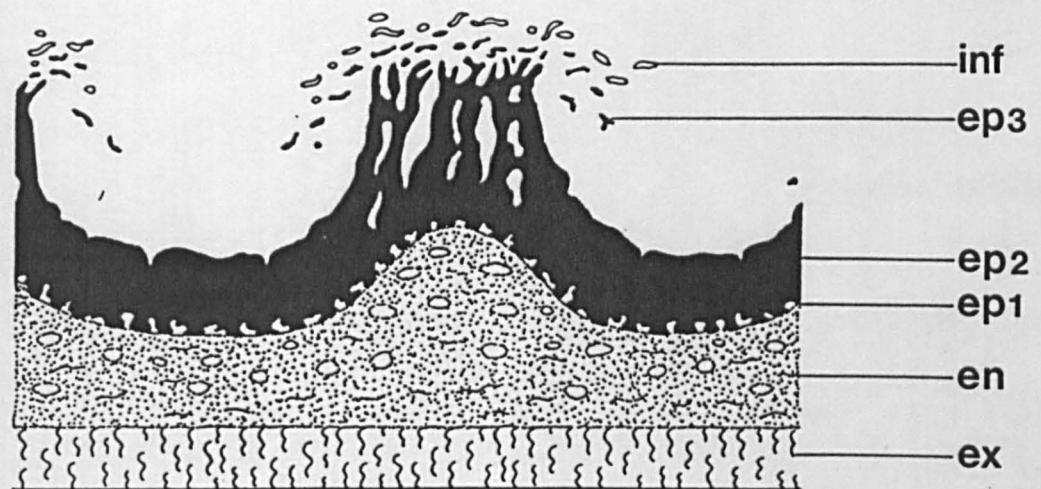
FIGURE 5.3

A.mexicana

(a & b) Diagrammatic representations of sporoderm structure typically found in A.mexicana.

(key - see Figure 5.1)

a



b

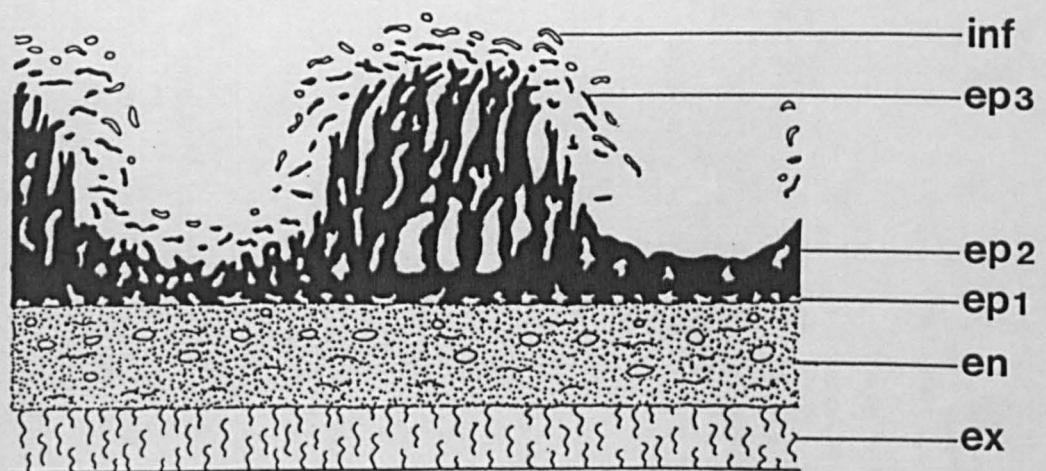


FIGURE 5.4

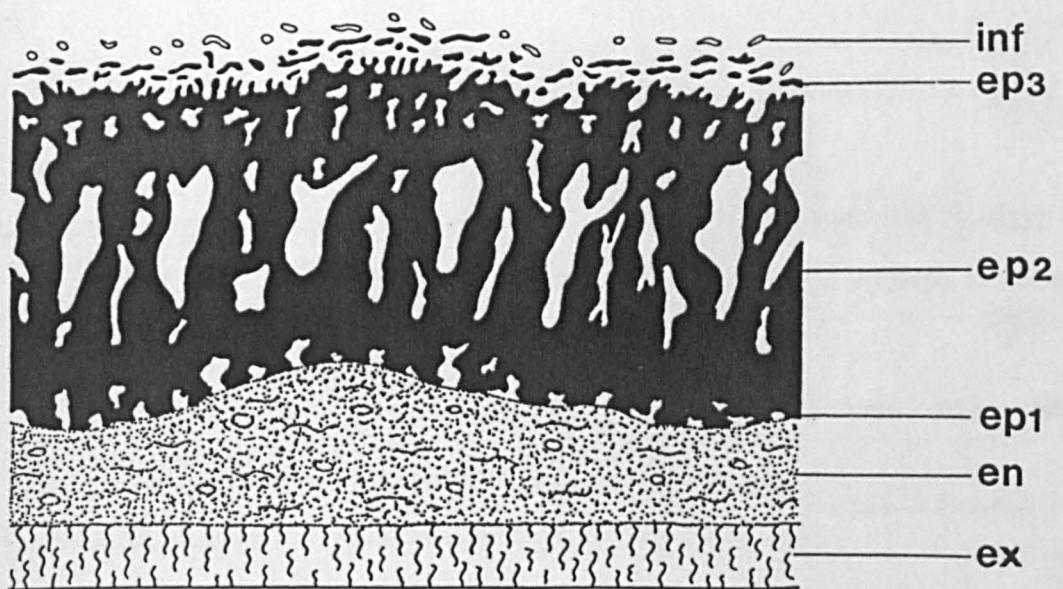
Azolla nomen novum

(=the *A.microphylla* type)

(a & b) Diagrammatic representations of sporoderm structure typically found in **Azolla** nomen novum.

(key - see Figure 5.1)

a



b

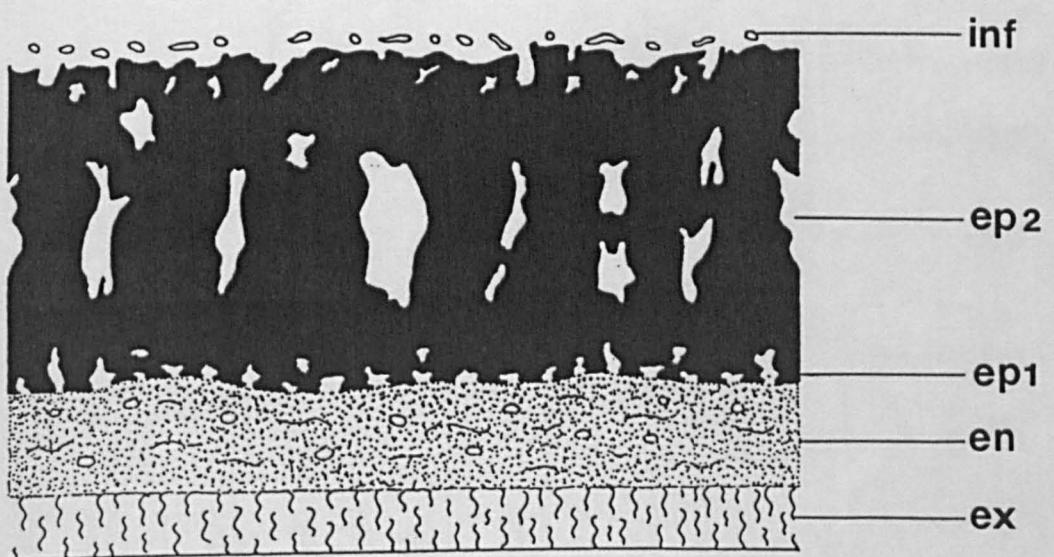


FIGURE 5.5

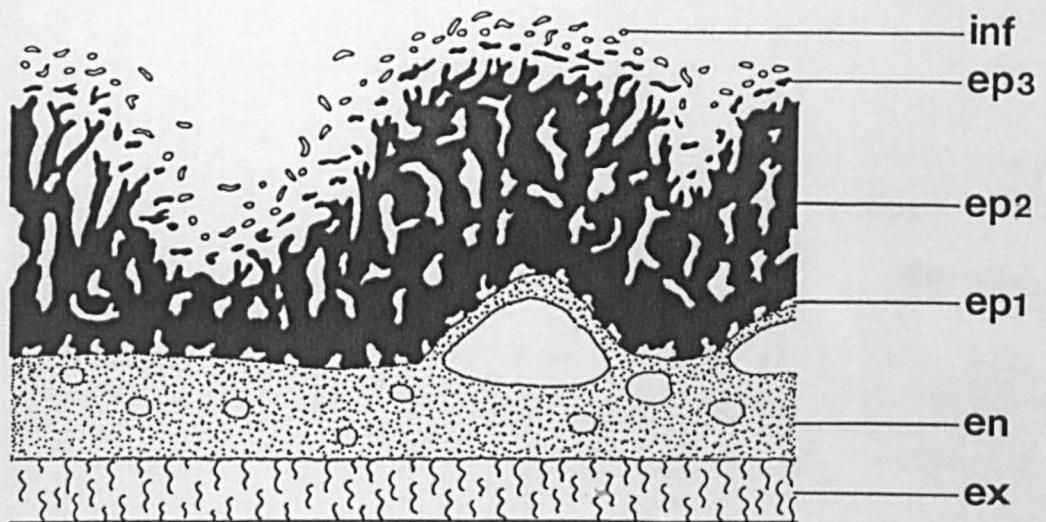
Azolla sp.nov.

(= the **Azolla** sp. type)

(a & b) Diagrammatic representations of sporoderm structure typically found in **Azolla** sp. nov.

(key - see Figure 5.1)

a



b

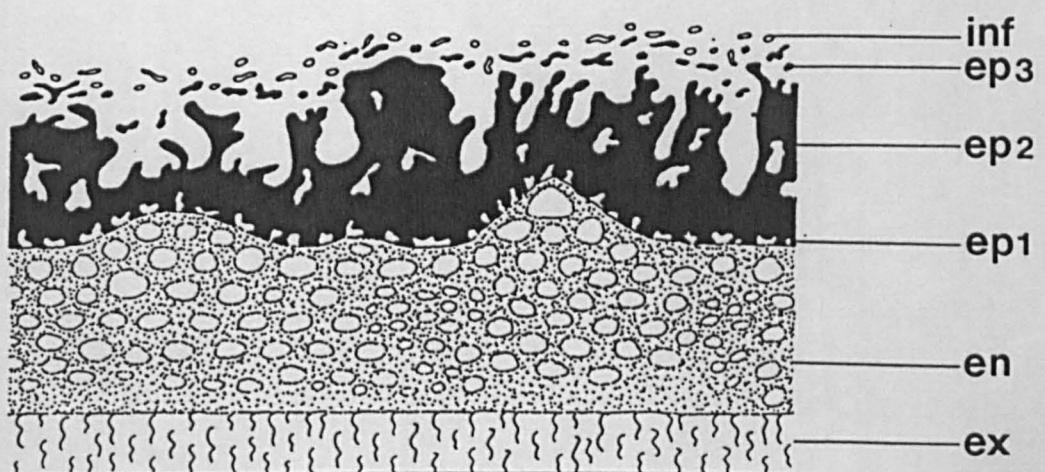


FIGURE 5.6

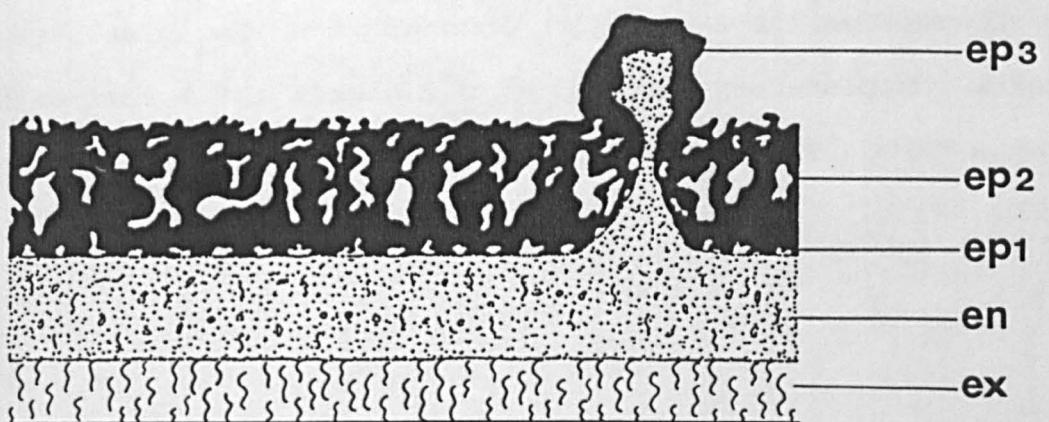
Sect. Rhizosperma

(a) Diagrammatic representation of sporoderm structure typically found in *A.pinnata*.

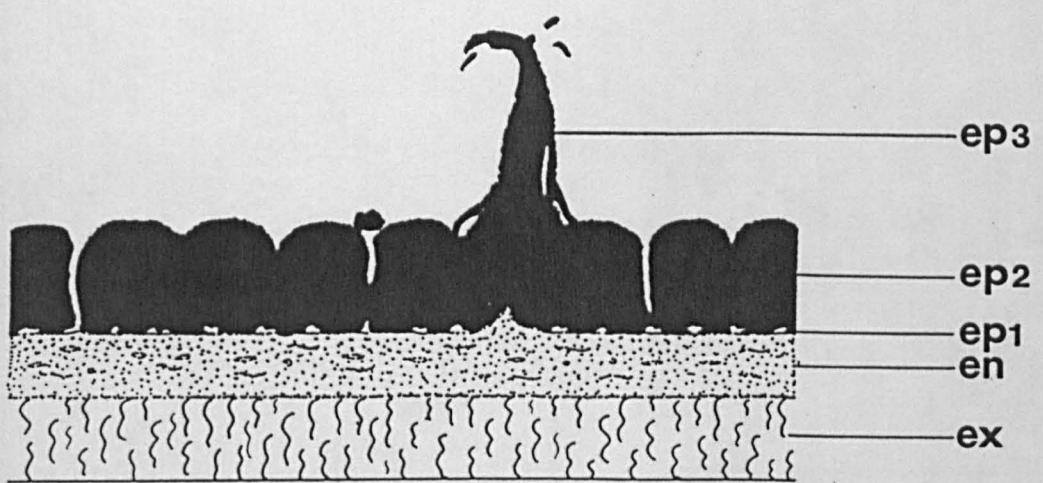
(b) Diagrammatic representation of sporoderm structure typically found in *A.nilotica*.

(key - see Figure 5.1)

a



b



**FIGURE 5.7**

Map illustrating the geographical distribution of the extant taxa of *Azolla*. Supplementary information on *A.pinnata* and *A.nilotica* from Sweet & Hills (1971); Lumpkin & Plucknett (1982) and Ashton & Walmsley (1985).



FUTURE TAXONOMIC RESEARCH

The present investigation has offered a taxonomic framework using fundamental practices, namely evaluation of variation in characters and reference to Type material. Such basic research, as stated in section 1, has not previously been undertaken. However, having offered a meaningful framework it is important to continue building upon it. This would involve amending and improving it as new data become available through future investigations. An important aspect of the present study is to indicate possible avenues of future research.

One obvious area of research is the re-examination of fossil taxa using techniques employed in the present investigation; these have proved more than adequate for diagnosing taxa. Such a re-examination would up-date and improve descriptions of fossil taxa. This would possibly lead to a reduction in the number of taxa through synonymy and perhaps provide a realistic phylogeny for *Azolla*. To this end, not only should taxonomically significant characters be scrutinised in fossil taxa, but the terminology used here should also be adopted. It appears that few fossil taxa are known from vegetative remains; this is not surprising in view of their fragility. The characteristic association of a dorsal leaf lobe trichome with a stoma would appear sufficient to recognise vegetative fossil remains.

Vegetative features offer little or no assistance in distinguishing extant taxa. This, coupled with the present lack of knowledge of sporocarp induction and morphogenesis, is a

continual hindrance to many researchers, and, to increase the present understanding of these aspects, the 1st International Workshop on *Azolla* use at Fuzhou, China (1985) gave a high priority to research into them, this is endorsed by the present study. In an attempt to identify individual strains in culture collections it would be desirable to 'finger-print' strains using isoenzymes and secondary metabolites. Such investigations may complement or contradict the present proposals. If undertaken using these, any results might be a way of building upon the present framework.

Yet another method of testing the present proposals would be through a cytological approach. At present reports of chromosome numbers are confused (see Litardière 1921; Duncan 1940; Loyal 1958; Love *et al.* 1977; Loyal *et al.* 1982). However, Than (pers.comm.) indicates the presence of a polyploid series within *Azolla*. His results are rather unreliable and more extensive study of karyotypes is required. Such an investigation, in conjunction with a breeding programme, may elucidate the origin of taxa and the recognition of any hybrids. A breeding programme involving attempts to cross different species would phylogenetically test the validity of the present proposals. Apart from the taxonomic implications of such a study, it may produce novel 'strains' with unique and agriculturally useful attributes. Unfortunately, a breeding programme may be limited by the present lack of knowledge of inducing and controlling sporulation. However, a starting point would be with those strains that regularly sporulate.

Although, in the present investigation, there was extensive acquisition of herbarium material, much of it was sterile and therefore of little use. It was suggested that collectors should endeavour to collect sporulating material in larger quantities (Bates, 1980); this is reiterated by the present author. In respect to culture collections, it is recommended that the original material should be fertile, some of which should be preserved as herbarium specimens for future reference. In addition to this, there should be an international codification of 'strains' held in culture collections. At present, 'strains' are being exchanged and there is no continuity in the codes used. This is already leading to the loss of information, duplication and confusion. An international code would require a central register, but would reduce duplication, remove confusion, hopefully ease the exchange of specimens and create better collaborative links between institutions and individual researchers involved in a multitude of projects aiming to exploit **Azolla** in a large number of countries.

As previously stated in section 4.5 biogeographical results are dependant upon where collections were made, and are therefore not necessarily representative of the true distribution of the taxa. The present author recommends that attempts should be made to provide representative and accurate distribution information on **Azolla** by collecting from what appear to be remote or unrepresentative regions (e.g. South America and Central USA respectively). Fertile material of **Azolla** sp.nov. is poorly represented in herbaria, thus limiting the present investigation

in respect to this taxon. The comparatively large amount of variation, even in a small sample size, in this species, indicates that phenotypic variation may not have been fully delimited. Furthermore, the present study tentatively indicates that this variation, together with disjunct biogeography, might be better explained by more than one species or by infraspecific taxa. Therefore, it would be desirable to examine more fertile populations of *Azolla* sp.nov. from Holland, USA and South America. Another tentative suggestion was that this species has hybrid origins; this could be confirmed or rejected through breeding experiments. Clearly, further investigation, both phenetic and phylogenetic, of *Azolla* sp.nov. are required. In respect to the latter taxonomic approach, all Section *Azolla* taxa require further investigation.

In contrast, Section *Rhizosperma* first requires extensive phenetic study similar to the present study on Section *Azolla*. This would bring the understanding of both Sections up to the same level. Based on vegetative characters two varieties of *A.pinnata* are currently recognised (Sweet & Hills, 1971; Lumpkin & Plucknett, 1982). However, Zhou (1983) reports geographically associated differences in sporoderm structure, but no taxonomic inferences were made because only a small number of populations were studied. These differences were not entirely consistent with the distribution of the two varieties recognised by Sweet & Hills (1971) and Lumpkin & Plucknett (1982). The present investigation did not aim to extensively examine *A.pinnata* and it appears that this is now required. Such a phenetic investigation

should be extended to include *A.nilotica* which has received little taxonomic scrutiny. This, of course, should be complimented by cytological and breeding studies and 'fingerprinting'.

During the course of the present investigation it came to this workers's attention that root anatomy may be diagnostically useful (Tan, pers.comm.). It is not possible to examine this character in herbarium specimens, and time did not permit the acquisition of sufficient fresh/fixed material to provide a meaningful sample size. Root anatomy should be investigated in all taxa because it may provide another much needed diagnostic vegetative feature.

With the exception of root anatomy and the phenetic investigation of Section *Rhizosperma*, the approach of future taxonomic investigations is clearly more applied. Justification for the present investigation was the lack of basic taxonomic information which has led to considerable confusion. Having proposed a taxonomic prospectus which fulfils the objectives of the present investigation it is not surprising that future research should be more applied. The proposed framework, species recognition and terminology can be applied to future taxonomic investigations on extant and fossil *Azolla* in addition to other types of *Azolla* research (e.g. morphological, physiological and biochemical) by being unified reference points. Future applied taxonomic approaches will hopefully not only build upon the present proposals, but also provide information for 'mainstream' research

programmes being undertaken in various institutions around the World. These programmes being aimed at exploiting the potential use of **Azolla** in agricultural practices.

PART B

PART B

1. ASPECTS OF THE REPRODUCTIVE BIOLOGY OF AZOLLA:

The maintenance of the symbiosis

1.1 INTRODUCTION

The exploitation of **Azolla** as a source of nitrogen is dependent upon the maintenance of the symbiosis between the **Azolla** host and its cyanophycean endosymbiont, **Anabaena azollae** throughout the complete life cycle. There is inadequate knowledge concerning the way in which the symbiosis is maintained during the sexual phase of the life cycle. Such information might aid the development of useful strains of **Azolla** for use in a variety of agricultural practices. Most previous investigations relate mainly to ontogeny of germination and embryology (Berggren, 1879-80, 1882; Rozé, 1883; Campbell, 1893; Rao, 1935; Bonnet, 1957; Konar & Kapoor, 1974). Becking (1978) made the first serious attempt to follow **Anabaena** through the life cycle of **Azolla**. However, all of these authors failed to recognise the intimacy of the symbiosis in relation to the structure of the megasporangium, gametophyte and embryo. Furthermore, no satisfactory mechanism of inoculation of the embryo has emerged. The development and morphology of spermatozoids is also poorly understood (Rozé, 1883, 1888; Campbell, 1893; Bonnet, 1957).

**Anabaena** is almost invariably present (part A section 5.2.5) at the stem apex of the sporophyte from where it becomes enclosed in a cavity in each dorsal leaf lobe and within the developing

sporocarps (Peters & Calvert, 1983; Calvert et al., 1983). Within the leaf cavity are two types of trichome which are distinguished by their morphology and development. Furthermore, both types of trichome exhibit transfer cell characteristics (Duckett et al., 1975; Peters et al., 1978). The presence of trichomes has also been reported just behind the stem apex of the 'mature' sporophyte (Rao, 1935; Konar & Kapoor, 1974; Peters et al., 1978; Calvert et al., 1980, 1981) and the embryo (Berggren, 1882; Campbell, 1893). These trichomes just behind the stem apex of the 'mature' sporophyte are thought to be responsible for carrying filaments of **Anabaena** from the colony at the stem apex into the developing dorsal lobe cavity (Peters & Calvert, 1983). This trichome has been termed the primary branched hair by Peters & Calvert (1983) and is described as developing on the leaf lobe initial, entangling **Anabaena** and then becoming wholly enclosed in the dorsal lobe cavity as it develops around the primary branched hair; this sequence is likened to inoculation with a needle (Peters & Calvert, 1983).

The presence of **Anabaena** as 'resting cells' (Becking, 1978) or 'akinetes' (Ashton & Walmsley, 1976) located immediately within the distal megasporocarp wall is reported by Rao (1935), Konar & Kapoor (1974), Ashton & Walmsley (1976), Calvert et al. (1983) and others. However, only remnants of cells of **Anabaena** are reported from the microsporocarp (Becking, 1978). The development of sporocarps is reviewed in part A, section 5.2.2.1. Initially, a sporocarp contains a developing megasporangium which may abort, for reasons as yet unknown, to continue development as a micro-

-porocarp containing microsporangia (Rao, 1935; Konar & Kapoor, 1974; Becking, 1978). Incorporation of *Anabaena* into the developing sporocarp is thought to be not unlike the inoculation of the dorsal lobe cavity. Evidence for this being the presence of a fringe of trichomes at the base of a megasporocarp (Calvert et al., 1983). Inoculation of the developing embryo with *Anabaena* occurs when the embryo passes the indusial cap as it emerges from the megaspore apparatus (Campbell, 1893; Becking, 1978), but information on inoculation is sparse and inadequately illustrated.

During the taxonomic re-evaluation of *Azolla* reported in part A the opportunity arose to employ scanning electron microscopy and thin-sectioning to investigate germination and embryology in *Azolla filiculoides*. Particular emphasis is placed on the maintenance of the *Azolla-Anabaena* symbiosis in relation to the structure of the megaspore apparatus and that of the developing gametophyte and embryo. An abstract outlining the intimacy of the symbiosis at the gametophyte-sporophyte interface leading to the successful inoculation of the embryo with *Anabaena* has been published (Dunham & Fowler, 1985). In the present account the work is fully described and illustrated. Furthermore, it forms the basis of a publication currently in preparation.

## 1.2 MATERIALS AND METHODS

Part A Sections 2.2.1., 2.3.2.2 and Appendix I Methods F provide full details of materials and methods.

## 1.3 RESULTS AND DISCUSSIONS

The mature megasporangium was consistently observed to be released from the floating sporophyte by the splitting of the two layered megasporocarp and single layered megasporangial walls in an equatorial zone. However, this zone cannot be likened to an annulus, the lack of which was commented upon in part A section 5.2.2.1. The two-layered microsporocarp wall also splits to release microsporangia but, unlike the megasporocarp, there appears to be no designated zone of dehiscence. Previous reports that mature sporocarps are released intact (Rozé, 1883; Campbell, 1893; Rao, 1935; Konar & Kapoor, 1974) are not corroborated here. The present author suggests that these reports are not usual in nature, and are the result of physical manipulation of the material. The present investigation indicates that the reproductive structures (megasporangium and microsporangium) sink when released; this is further evidence that the so-called 'floats' do not endow buoyancy. However, use of the term 'float' is justified in part A section 5.1.3.

### 1.3.1 Megasporangium and gametophyte

The released megasporangium is capped by the remains of the distal part of the megasporocarp wall which is here referred to as the *indusial cap* (see part A section 5.1.1) (Figs.Bla & b,B2a). The megasporangial wall closely adheres to both the inner surface of the *indusial cap* and the funnel of the *suprafilosum* (see part A section 3 and 5.1.3 for terminology), which is folded back over the floats. Within the megasporangium *Anabaena* cells are found distally, in the apical region, situated between the

megasporangial wall and indusial cap (Figs.B1b & c,B2a). The **Anabaena** cells lack the thick walls which are characteristic of akinetes. Additionally, they apparently do not shed their outer wall when they begin to develop. However, they are unlike vegetative cells in both shape and size. Herd & Cutter (1985) describe the **Anabaena** cells as akinetes, while Ladha (pers.comm.)— contradicts this. Clearly there is some controversy in respect to the nature and term applied to these cells. The present author follows Becking (1978) by regarding them simply as resting cells. Although it was once thought that such algal cells entered the megasporangium underneath the indusial cap (Berggren, 1882), the existence of a symbiotic association is now appreciated and also that **Anabaena** becomes incorporated during sporocarp development (Becking, 1978; Calvert et al., 1983 and others). In **Azolla filiculoides** the sporoderm surface bearing infrafilosum is exposed when the megasporangium is released; the infrafilosum appears to facilitate the attachment of massulae by means of their glochidia (Fig.B1a).

The female gametophyte develops between the floats enclosed within the incomplete cylinder of suprafilosum having emerged through the acrolamella. In **A.pinnata** this cylinder is complete and continuous with the funnel of suprafilosum, however, in other extant species, the cylinder is not complete (see part A section 5.1.3). Expansion of the chlorophyllous trilobed gametophyte moves the floats aside causing the indusial cap to be lifted upwards. Archegonia develop from the superficial cells of the gametophyte and have four tiers of neck cells (Figs.B1d,B2b,c & d).

By the time the first archegonium is mature, the indusial cap has been raised to expose approximately one third of the float region. This is contrary to the observations of Becking (1978) who states that the first external sign of germination of the megasporangium occurs only after the embryo has developed the cotyledonary leaf. Absence of fertilisation results in further development of archegonia on an enlarging gametophyte which becomes distorted with the development of secondary lobes. However, under normal conditions, further float displacement is caused by embryo growth. As the indusial cap is raised there is increased separation of the indusial cap and megasporangial wall, the latter remaining intact, and resting cells of *Anabaena* lying in the cavity created by the separation of the walls (Fig.B2c & d).

### 1.3.2 The spermatozoid

Spermatozoid development and release is poorly understood and it was not possible to make a critical study of these aspects during the present investigation. However, the short-lived spermatozoids were occasionally observed in the culture medium and provided the opportunity to obtain the only photographic illustration to date of this structure (Fig.B1e). As previous reports indicate, the spermatozoid in *Azolla filiculoides* is multiflagellate with approximately two gyres (Rozé, 1883, 1888; Bonnet, 1957). Flask-shaped cavities linking the microspore-containing cavity with the exterior of the massula were first described by Fowler (1975) who also suggested that these flask-shaped cavities probably formed an escape mechanism for

spermatozoids. During the present study antheridia produced by microspores were seen to occupy the flask-shaped cavities as suggested by Fowler (1975). Examination of sectioned material indicates that eight spherical cells are produced within each antheridium, confirming previous observations of *A.filiculoides* (Campbell, 1893; Bonnet, 1957). However, in *A.pinnata*, Konar & Kapoor (1974) report four, rarely eight, spermatozoids per antheridium. The present author considers that such a variation in the number of gametes is unexpected and may have a considerable influence on fecundity. The relatively low number of spermatozoids per antheridium suggests that the attachment of massulae to the megasporangium apparatus is a quite efficient method of ensuring fertilisation occurs. If it were not, large numbers of spermatozoids per antheridium might be expected. Similar spherical cells showing motility were observed both within the flask-shaped cavities and the culture medium. This motility appeared to be provided by free flagella on the maturing spermatozoid. The present observations suggest that the spermatozoids are released in an immature state, perhaps explaining the 'types' of spermatozoid illustrated by Rozé (1883, 1888). Scanning electron microscope examination of massula surfaces revealed that the flask-shaped cavities were sealed prior to release of the immature spermatozoid.

Development of the female gametophyte displaces the floats and raises the indusial cap; this could provide access to the archegonia for the spermatozoids. According to Rozé (1888), the spermatozoids swim under the indusial cap and travel distally to

reach the archegonia via the passage between the floats. However, the present study indicates that the access route is more probably through the suprafilosum behind the displaced floats, because the megasporangial wall remains intact providing a barrier preventing access for spermatozoids to the distal part of the float region. The present investigation clearly shows that little is known about spermatogenesis. Further critical study is required because, as indicated by Calvert *et al.* (1983) and confirmed by the present study, *Azolla* is not parthenogenetic. Therefore, the male gamete is reproductively as important as the female gamete.

### 1.3.3 Embryo development and maintenance of the symbiosis

The present study has shown that early embryo development is closely associated with the 'collection' of *Anabaena* to perpetuate the symbiotic association. The embryo emerges from the archegonial chamber and develops between the floats; at an early stage the stem apex is seen to be partially surrounded by the cotyledon leaf (Fig.B3a & b) which later develops into a funnel shape with a prominent cleft along one side and enclosing the stem apex in the centre. Immediately behind the curved stem apex, what appear to be four trichomes arise (Fig.B3a & b). However, in the mature sporophyte the present study indicates that initially there is one simple trichome which becomes branched; this agreed with previous descriptions of the mature sporophyte (Peters & Calvert, 1983). Therefore, it appears that in an embryo there may be a branched trichome or several trichomes just behind the stem apex; these are here called sub-apical trichomes (or primary branched trichomes (Calvert *et al.*, 1980)). They appear

morphologically similar to some of those found in the dorsal leaf lobe cavities.

Growth of the cotyledonary leaf pushes apart the apical regions of the floats which ruptures the apical membrane (or megasporangial wall) covering the funnel of suprafilosum. This development of the cotyledon leaf provides access to the **Anabaena** in its apical location (Fig.B3e), and a channelled route between the endosymbiont and the stem apex of the embryo. Subsequent leaf development severely restricts such access. Although there is some morphological evidence to indicate that **Anabaena** resting cells become active at this stage, it appears that growth of the cotyledon and first true leaves are instrumental in collecting the **Anabaena**. The cotyledon leaf extends upwards and touches the indusial cap before its margins grow inwards. This dislodges some or all of the **Anabaena** cells (Fig.B3e). At the same time the first leaf grows upwards within the cotyledon leaf, gradually arching over to envelop the stem apex. This scoops and entraps the now actively growing **Anabaena** colony in the vicinity of the stem apex. Filaments of **Anabaena** are retained in this region by entanglement around the sub-apical trichome (Figs.B2e & f,B3c). It can be seen that inoculation with **Anabaena** is a subtly timed sequence during morphogenesis of the embryo in which the **Azolla** host plays the leading rôle.

The process of leaf cavity inoculation has been investigated in the mature sporophyte with the assumption that it is similar in the embryo plant. Associated with each leaf primordium is an

apparently axillary sub-apical trichome which is initially simple. Its development is rapid compared with the primordium, and the trichome acts like a 'peg' which entangles and retains **Anabaena** filaments in a sub-apical position (Fig.B3e). Subsequently the sub-apical trichome forms branches and during leaf development it is carried from its axillary position to an adaxial position on the periphery, but axillary side of the developing dorsal leaf lobe cavity. This trichome retains some entangled filaments of **Anabaena** which become incorporated into the leaf cavity as it closes over (Fig.B3f); this happens when the leaf has become the second or third leaf. The present study observed that the branched trichome either becomes completely enclosed in the dorsal lobe cavity or it projects from the cavity and is probably pinched off on closure of the cavity. This process of dorsal leaf lobe cavity inoculation generally agrees with the same process described by Peters & Calvert (1983). However, these workers suggest that the sub-apical trichome grows into the apical **Anabaena** colony, entangles **Anabaena** filaments and then carries them into the leaf cavity. The present study suggests that the sub-apical trichome has a two-fold function of positional maintenance of the apical **Anabaena** colony followed by retension of some **Anabaena** filaments for their incorporation into the dorsal leaf lobe cavity.

Returning to the embryo, elongation of the cotyledonary leaf eventually pushes off the indusial cap which usually remains attached to the edge of the funnel of suprafilosum. This latter structure has by now become inverted, assuming as its name

suggests, a funnel shape (Fig.B3d). The developing sporophyte, its apex enclosed by the first and subsequent leaves, rises to the water surface to lie in a vertical position still attached to the remains of the gametophyte and megasporangium apparatus (Fig.B3d). The present study confirms the observations by Roze (1888) and Campbell (1893), indicating that it is the accumulation of gases in inter-cellular spaces of the cotyledon leaf which causes the embryo to float to the surface. No buoyancy is provided by the remains of the megasporangium apparatus which sinks if detached from the embryo. The shoot emerges from the confines of the cotyledonary leaf to assume the horizontal habit of the 'mature' sporophyte. All leaves except the cotyledon leaf develop cavities containing apically derived *Anabaena*. Only the first two true leaves are entire, as indicated by Roze (1888), subsequent leaves being bi-lobed.

#### 1.3.4 Miscellaneous observations

Various, possibly significant, observations were made during the present study. Firstly, when fertilisation did not occur or was prevented by excluding massulae from the culture vial, the female gametophyte produced secondary lobes which eventually pushed out through the filosum between the floats. Archegonia were seen on these secondary lobes. If the oosphere within one of these archegonia was then fertilised an embryo often developed, however, it emerged from between the floats leaving the indusial cap in place. Therefore, *Anabaena* was not collected and the resulting sporophyte was *Anabaena*-free. Such observations have not previously been reported, and are significant by being a potential cause

of an *Anabaena*-free population. Indeed, Watanabe (pers.comm.) reported that a population established from sporocarps collected from Portsmouth lacks *Anabaena*. However, the parent population of the material used by Watanabe was also used in the present investigation and contains *Anabaena*. It is possible that Watanabe's *Anabaena*-free population may have come about by the above method.

Another observation was the development of two embryos from one female gametophyte. Both embryos emerged in the usual way or one emerged via the usual way while the other emerged between the floats. In respect to the former emergence of two embryos, their symmetry may suggest that they were homozygotic. However, this is the only evidence for such an origin. It is, of course, more likely that two oospheres from two different, but adjacent archegonia were simultaneously fertilised. It could not be established whether one or both embryos had collected *Anabaena*. Where one embryo emerged laterally between the floats it is assumed that it was *Anabaena*-free (this is another potential cause of such a population). The other embryo was presumably able to collect the endosymbiont.

Development of *Anabaena*-free embryos indicates that the endosymbiont has little or no influence on embryo development. Further evidence for this comes from a naturally occurring *Anabaena*-free population that readily produces healthy embryos. However, should the indusial cap together with the resting cells of *Anabaena* be removed at the gametophyte or early embryo stage

i.e. prior to inoculation) further development ceases. This may be due to infection by fungi and/or bacteria. If the cultures were axenic it might be possible to exchange indusial caps between different species and/or 'strains' of **Azolla** and hence **Anabaena** strains. Therefore it may be possible to improve the nitrogen-fixing ability of an association by changing the 'strain' of endosymbiont. To date there are no reports of successful re-introduction of **Anabaena**, in the 'mature' sporophyte, and the embryo stage; changing the indusial cap now appears to be a particularly attractive method.

The present investigation of immature megasporocarps indicates that the trichomes first reported by Calvert et al. (1983), at the base of the sporocarps may not be involved in the inoculation of young sporocarps. The mechanism of sporocarp inoculation with **Anabaena** is therefore still a mystery, and requires critical investigation.

#### 1.4

#### CONCLUSIONS

This small secondary investigation undertaken during the 'main-stream' taxonomic study has shown how structure and morphology within a temporally organised developmental sequence ensures that the **Azolla-Anabaena** association is maintained through the sexual phase of the life cycle; this is through the female or megasporocarp line. The **Azolla** host appears to be more active than the endosymbiont in ensuring the association is maintained. The development of sub-apical trichomes at an early stage in both the embryo and 'mature' sporophyte reflects the importance of these

trichomes in the physical maintenance of the association; this includes maintaining an apical colony and inoculation of the dorsal leaf lobe cavity. The results of the present investigation illustrate the intimacy of this symbiosis. Inoculation of a young sporocarp has still not been elucidated and requires critical investigation. Similarly the cytology of the mega- and micro gametophyte phases of the life cycle together with the ultra-structure of gametes and fertilisation require critical description.

FIGURE B1

Scanning electron micrographs

- (a) Megaspore apparatus before germination. The indusial cap covers the distal part of the floats, and the massulae are attached by glochidia to the sporoderm. Scale bar = 100 $\mu$ m.
- (b) Distal region of a megaspore apparatus cut longitudinally to show 'resting' cells of *Anabaena azollae* located between the indusial cap and megasporangial wall. Scale bar = 50 $\mu$ m.
- (c) Interior of a detached indusial cap showing *Anabaena azollae*. Scale bar = 50 $\mu$ m.
- (d) Indusial cap, floats and suprafilosum removed to show the fully developed female gametophyte with archegonia. Scale bar = 100 $\mu$ m.
- (e) Mature multiflagellate spermatozoid. Scale bar = 5 $\mu$ m.

Key

C = indusial cap; m = massula; Aa = *Anabaena azollae*; mw = megasporangial wall; fg = female gametophyte; a = archegonia; f = float; sc = 'cylinder' of suprafilosum; sa = stem apex; sf = funnel of suprafilosum; Ll = first leaf; t = trichome; Lc = cotyledon leaf.

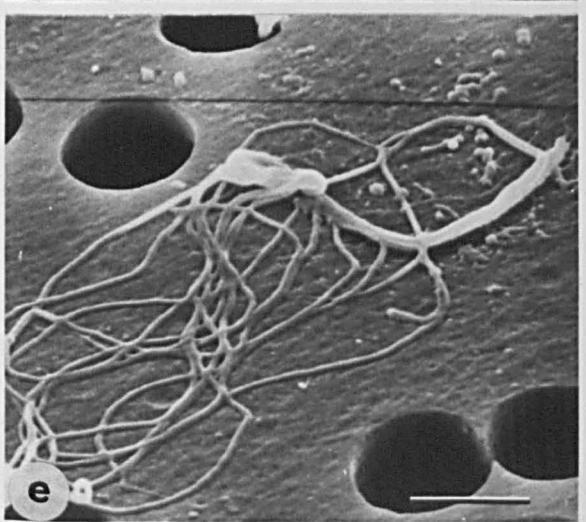
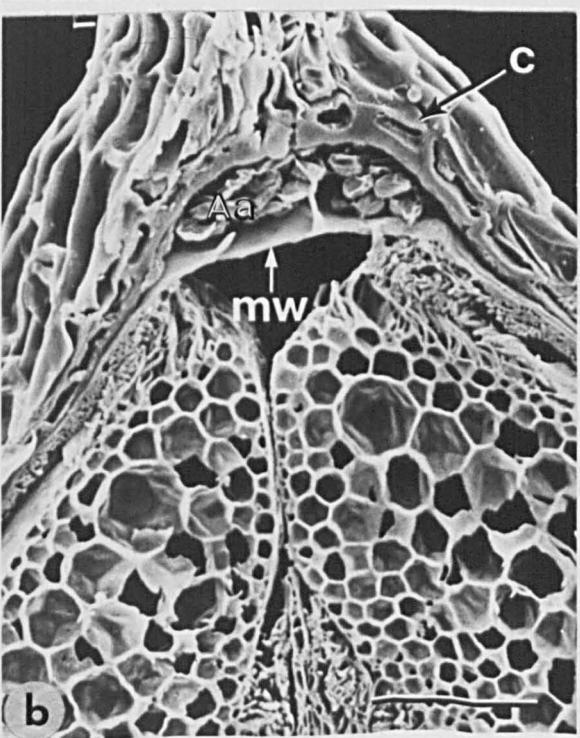
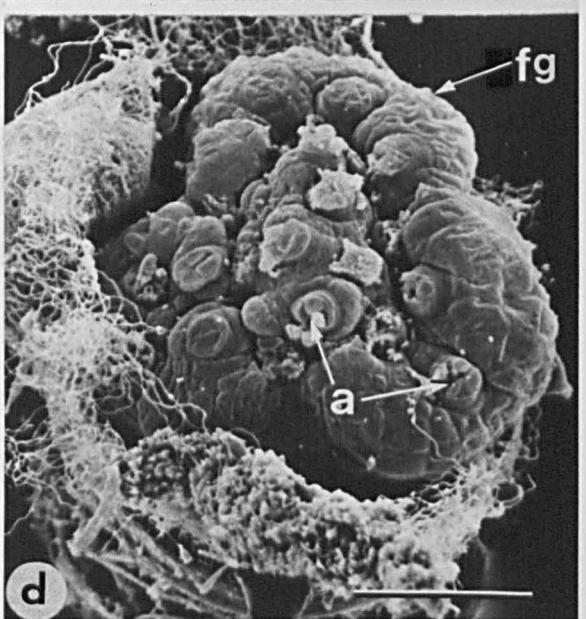
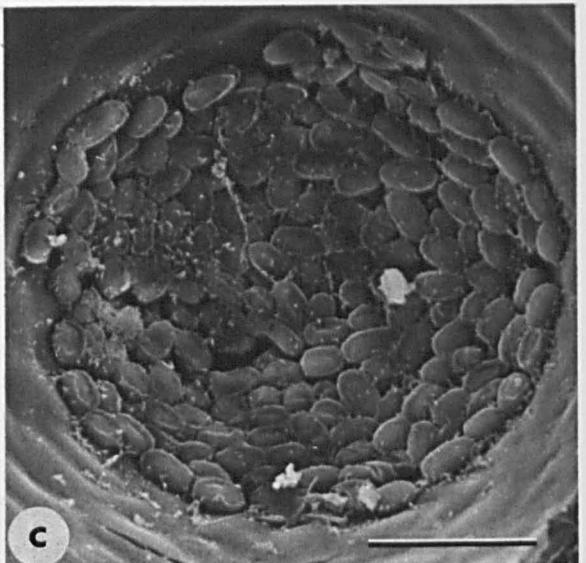
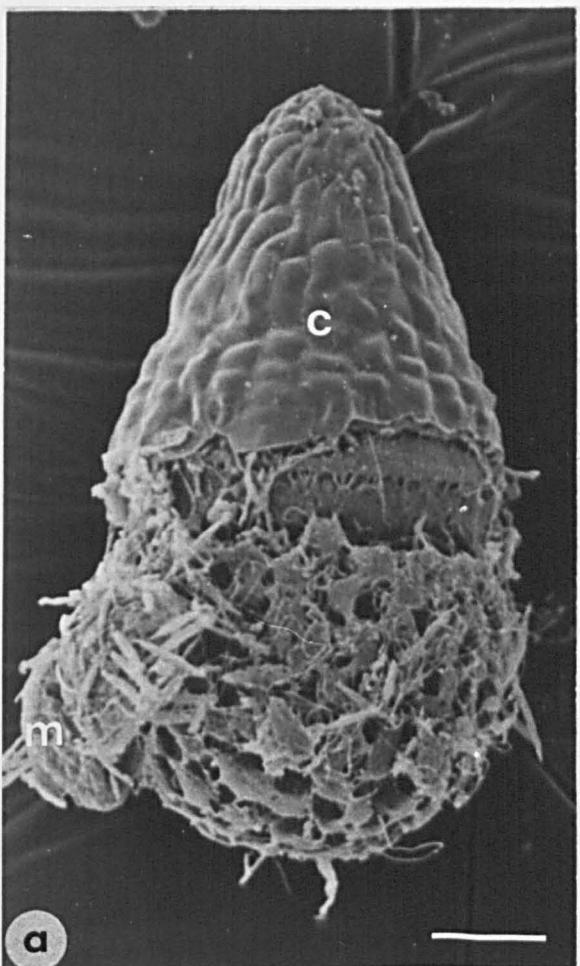


FIGURE B2

Light micrographs

- (a) L.S. distal region of an ungerminated megasporangium to show *Anabaena azollae* between the indusial cap and the megasporangial wall. Scale bar = 100 $\mu$ m.
- (b) L.S. of a female gametophyte to show a mature archegonium. Scale bar = 10 $\mu$ m.
- (c) L.S. of a germinated megasporangium, showing the female gametophyte with archegonia, developing between the floats and enclosed within the 'cylinder' of suprafiliolum. Note that the megasporangial wall forms a seal across the float apices and is fused to the funnel of suprafiliolum which is folded back over the floats. Scale bar = 50 $\mu$ m.
- (d) Line drawing labelling structures shown in Fig.B2c.
- (e) L.S. of an embryo at a stage when *Anabaena azollae* comes into contact with the shoot apex; further development of the first leaf prevents access. Scale bar = 50 $\mu$ m.
- (f) L.S. showing the indusial cap pushed aside with inversion of the funnel of suprafiliolum. The first leaf over-arches the shoot apex, near which is a trichome and the *Anabaena* colony. Scale bar = 50 $\mu$ m.

For key see Fig.B1.

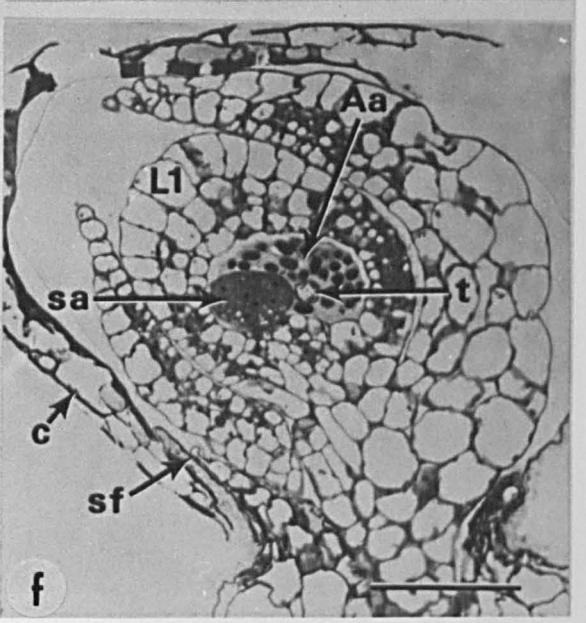
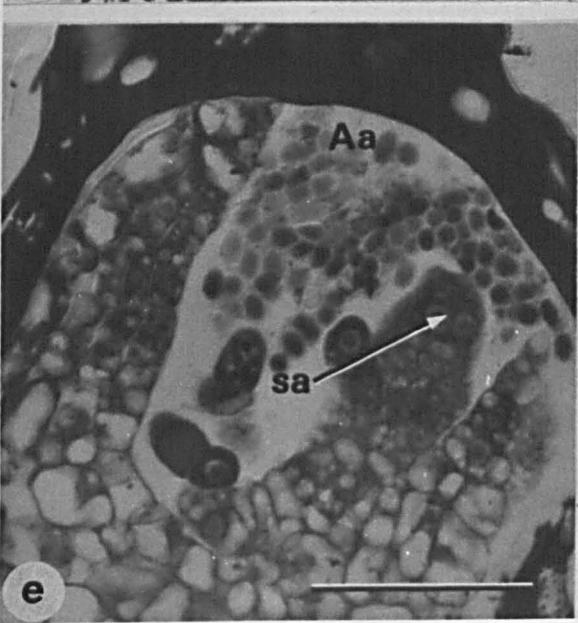
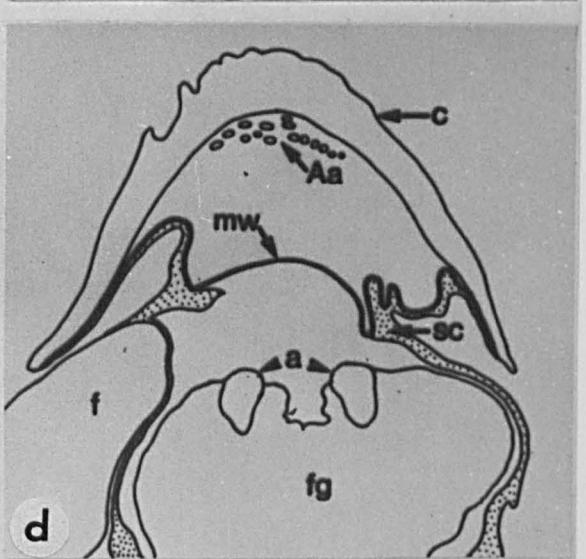
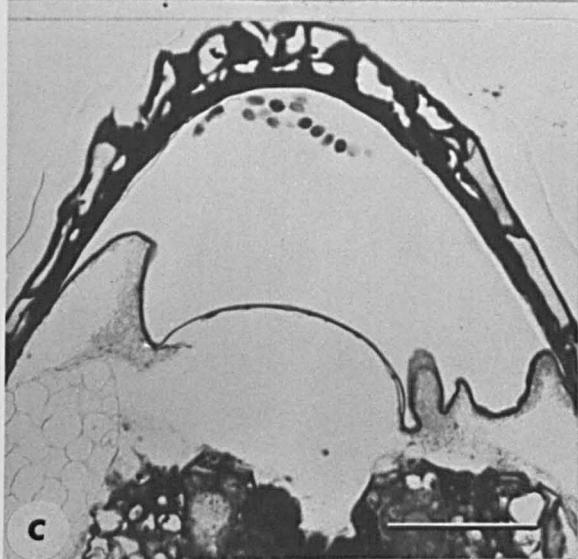
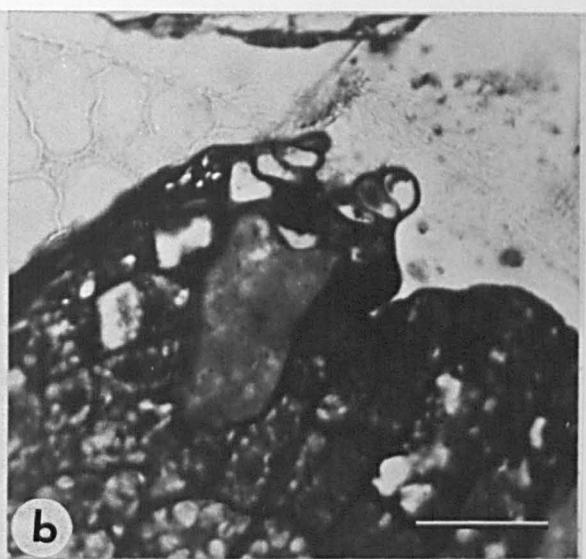
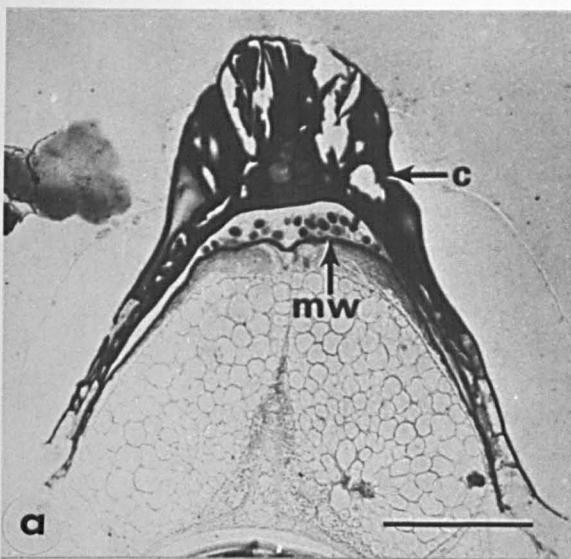
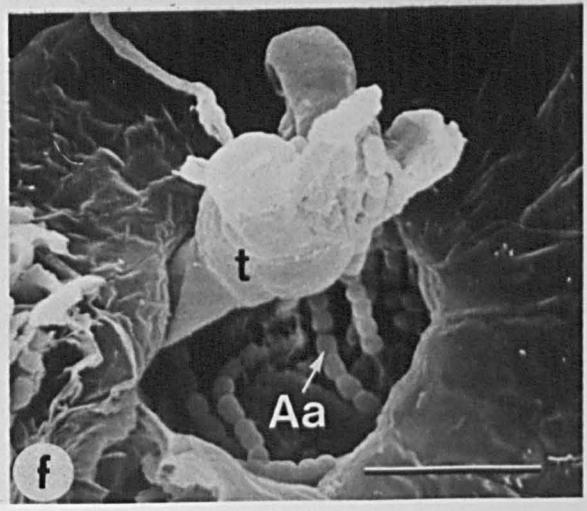
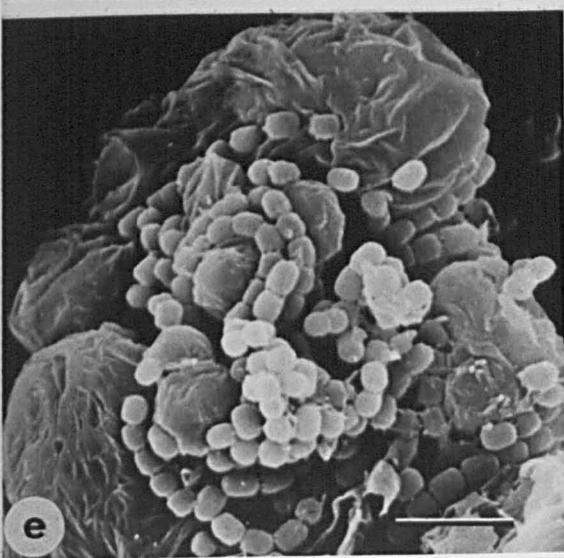
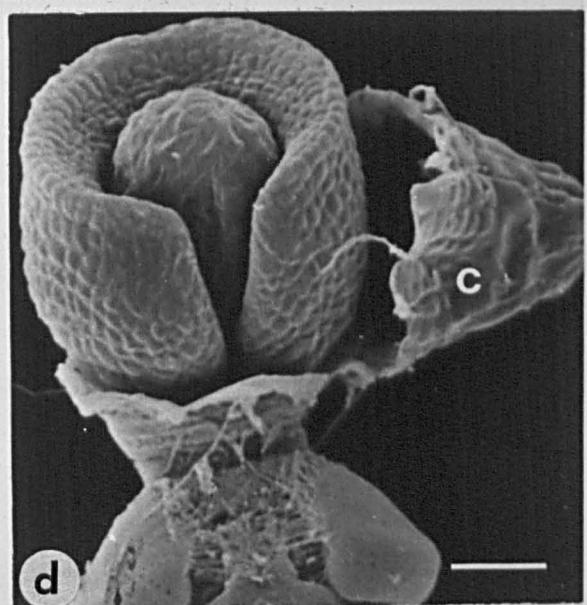
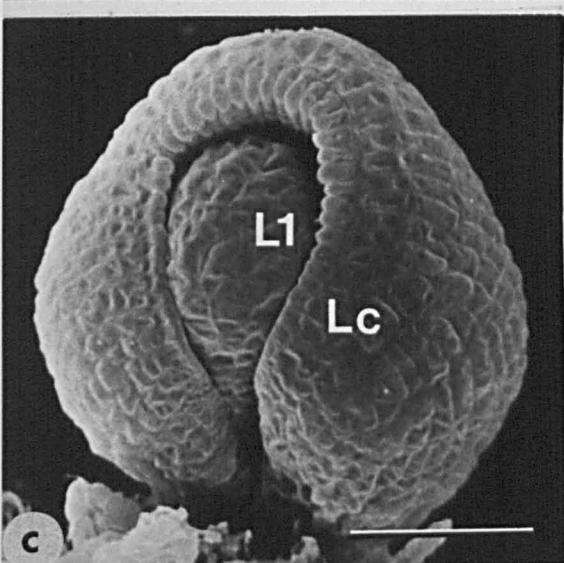
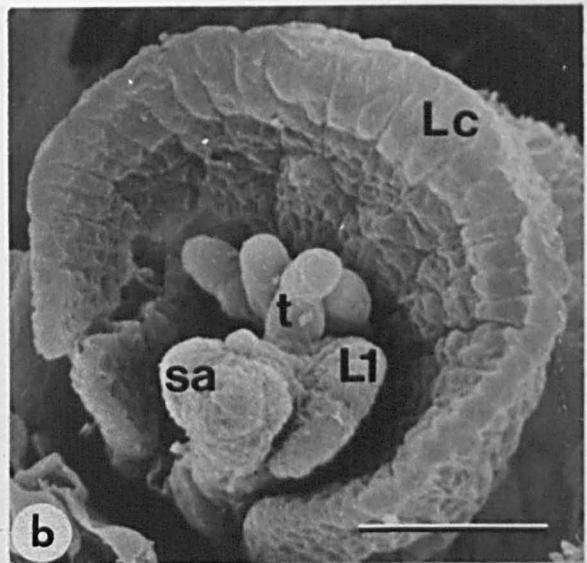
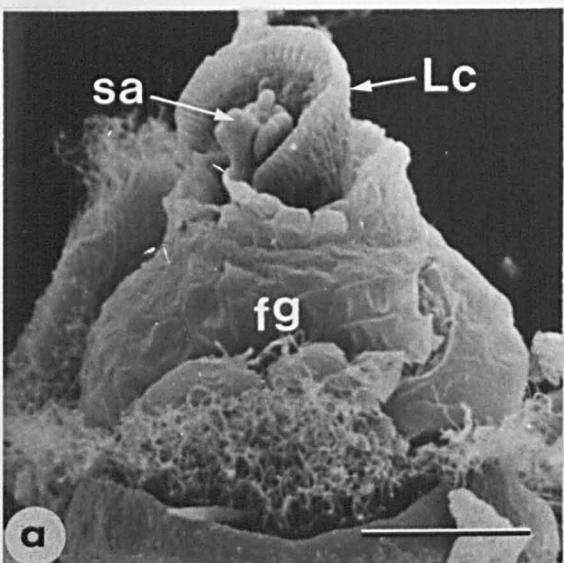


FIGURE B3

Scanning electron micrographs

- (a) Embryo growing from the female gametophyte. The funnel-shaped cotyledon leaf surrounds the shoot apex. Scale bar = 100 $\mu$ m.
- (b) Four trichomes below the shoot apex of an embryo; the first leaf is just discernible. Scale bar = 50 $\mu$ m.
- (c) Developing embryo showing the first leaf within the enclosing cotyledon leaf, completely covering the shoot apex, indicating that the *Anabaena* must contact the shoot apex prior to this stage. Scale bar = 100 $\mu$ m.
- (d) Floating embryo stage. Note that the embryo has emerged from the megasporangium by pushing aside the indusial cap. Scale bar = 100 $\mu$ m.
- (e) Colony of actively growing *Anabaena* at the stem apex of an embryo plant. The cotyledon and younger leaves have been removed. Scale bar = 12 $\mu$ m.
- (f) Cavity of a dorsal leaf lobe closing over following 'inoculation' with *Anabaena*. The large branched trichome has not been fully incorporated in to the cavity, its distal part projecting up through the cavity pore. Scale bar = 25 $\mu$ m.

Figures (a) to (c) the indusial cap, floats and suprafilosum have been removed to show the developing embryo. For key see Fig. B1.



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## APPENDIX I

### LISTING OF MATERIAL EXAMINED.

The code number letter refers to the International Herbarium code in Holmgren *et al* (1978) unless otherwise stated.

(M = megasporocarps and contents examined; m = microsporocarps and contents examined; v = vegetative features examined; T = nomenclatureal Type material; ( ) = duplicate).

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
A1	ASTERN,H.I. (485)	NE Victoria.	96014105 mv
B1	SCHICKENDANTZ,F (365)	Guebrade de Belen, Argentina.	187
B2(B18)	OSTEN,C (3265)	Paranas,Uruguay.	001135 Mmv Mmv
B3	VIEDERLEIN,S	Rio Allo,Pasana, Brasil.	001126 Mv
B4	LORENTZ,P.G.(1766)	Laguna Culaenas, Uruguay.	001134 Mv
B5	LORENTZ,G & HIERONYMUS,G.(577)	Dragones Gran Charo, Argentina.	001139 Mv
B6	Anon.	Boruwulleg ?	001142 v
B7	HIERONYMUS,G & LORENTZ,P.G.(473)	Rio del Tala, Argentina.	001138 Mmv
B8	ARERHAVALET A	Montevideo,Uruguay.	001133 Mm
B9/B10	Anon. (471)	Montevideo,Uruguay.	001130 v
B11	ARERHAVALET A (2055)	Montevideo,Uruguay.	001132 Mmv
B12	Anon. [(37)6549]	Chandy,Ecuador.	001014 v
B13	ULE,E (311)	Santa Catherina, Brasil.	001043 Mmv
B14	STEINBACH (8738)	Cochabamba,Bolivia.	001028 Mm
B15	METTENIUS,G.(817) Herb.	Brasil.	001050 v
B16	ROSE,L.S.	San Francisco,USA.	001002(42246) v
B17	REITZ & KLEIN (3.876)	Ibirama,Brasil.	001042 Mmv
B19	OSTEN,C. (100)	Buenos Aires, Argentina.	001087 Mv
B20	OSTEN,C (25)	Buenos Aires, Argentina.	001088 v
B21	MEYER,T & SLEUMER,H (15.706)	Cordoba,Argentina.	001073 Mm
B22	MEYEN	Rio de Janero, Brasil.	001047 v
B23	MEYEN	St.Fernando,Chile.	001095 Mmv
B24	LUSTUATH	Rio de Janero, Brasil.	001046 Mm
B25	LOSSE,W. (248)	Cordoba,Argentina.	001080 v
B26	LORENTZ,P.G. (1664)	Concepcion,Uruguay.	001066 v
B27	LORENTZ,P.G.	Lachen,Uruguay.	001067 Mmv
B28	LORENTZ,P.G. (22)	Cordoba,Argentina.	001078 Mmv

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
B29	KUNTZE,O	Buenos Aires, Argentina.	001082 Mnv
B30	KARSTEN	Porto Cabello, Colombia.	001006 Mnv
B31	KARSTEN	Porto cabello, Colombia.	001007 v
B32	HOLLERMAYER,R.A. & MISSIONAIR,K.(1031)	Monuma, Chile.	001113 Mnv
B33	HERTER,W. (11188)	Maidonado,Uruguay.	001057 v
B34	HERTER,W. (9098b)	Toledo,Uruguay.	001052 v
B35 (M15)	HERTER,G [11D(79125)]	Montevideo, Uruguay.	001064 Mnv
B36 (M17)	HERTER,G [11C(76280)]	Montevideo, Uruguay.	001062 v
B37 (M16, M014,M015)	HERTER,G [11(76839)]	Montevideo, Uruguay.	001063 v
B38	HEIHRICHS,E. (513)	Rio Yasuni, Ecuador	001013 mv
B39	HARTMAN,C.V. (614)	St.Diego,Chihuahua, NW Mexico.	000053 Mv
B40	EICHLER,A.W. (14416)	Rio de Janero, Brasil.	001048 Mn
B41	GAUTCHUAD (40)	Valsparuso,Chile.	001102 Mnv
B42	D'ARBIGUY (63)	Montevideo, Uruguay.	001068 Mv
B43	COURBON (51)	Montevideo, Uruguay.	001070 v
B44	CHAMISSO,de	Chile.	001094 M
B45	BEYRICH	Amazonenotron, Brasil.	001049 Mnv
B46	BETTFREUND,C (1024)	Buenis Aires, Argentina.	001084 m
B47 (L51)	BALANSA,B.	Buenos Aires, Argentina.	001083 v
B48	BALANSA,B. (1121)	L'Asomption, Paraguay.	001051 Mnv
B49	Anon.	Rio de San Franci- sco,Argentina.	000923 v
B50	Anon.	Rio de San Franci- sco,Argentina.	000922 mv
B51	HAND,wen H.	San jose,Costa Rica.	000941 Mn
B52 (BKL2, M034)	TRYON,R.M. & GODFREY,R.K. (907)	Clarendon Co.,S. Carolina,USA.	----- v
B53 (M23)	SPRUCE,R. (596)	Santarem,Brasil.	000986 v

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
B54	illegible! (661)	Paramaribo, Surinam.	000969 Mv
B55 (HAL2)	SCHIEDE (839)	Inter Serpillo et Estero, Mexico.	000936
B56	RUTIO	Rio de Janero, Brasil.	000979
B57	BIEDEL	Rio de Janero,	000980 v
B58	PÖEPPIG	Macuripes, Cuba.	000961 Mmv
B59	PÖEPPIG	Brasil.	000990 Mmv
B60 (GH7, L48)	PATTERSON, H. N.	Oquawka, Illinois, USA.	000907 mv
B61	MARTIUS	Brasil.	000988 v
B62	LEGRICUR, M (384)	Guyana.	000968 v
B63	LEGRICUR, M	Guyana.	000967 v
B64	KAOPEN	Venezuela.	000976 v
B65	ROSENSTOCK, E (229)	Rio Grande du Sul, Brasil.	000977 v
B66	HOWARD, R.A. & HOWARD, E.S. (8790)	San Juan, Dominican Republic.	000944 Mmv
B67	HAHN, L.	Mexico.	000926 m
B67a	HOFFMANN, C. (510)	St. Jose, Costa Rica.	000942 v
B68	GLATION, A (16649)	Rio de Janero, Brasil.	000984 v
B69	FEURTES, M. (714b)	St. Domingo, de la Vega.	000946 v
B70 (MO52)	BOLANDER	San Bernadino, California, USA.	000921
B71	METTENIUS, G. (768)	Copucabana, Brasil.	001045 Mm
B72	CHAMISSO, Av.	California, USA.	000916 vT
B73	MARTIUS	Brasil.	000996 v
B74	METTENIUS, G.	Porto Rico?	000950 v
B75	HOOKER von.	Demerary, S. America.	000880 vT?
B76	RADDI	?	000994 vT?
B100	?	?	003163 Mv
B101	?	?	006671 Mv

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
BKL1(M012, M019)	SVENSON,H.K.S. (86)	Indefatigable Is., Galapagos Is.	Mmv
BKL3	PARISH,S.B. (5278)	San Bernadino, California,USA.	mv
BKL4	HOTCHKIS & SOOTER (6387)	San Bernadino, California,USA.	mv
BKL5	LEVINE,C.O. (207)	Canton, China.	v
BM1	BROWN,R.	Patterson River. Oct. 1804.	Mmvt
BM2	Anon.	Wagga Wagga, Australia.	P8310 Mmv
BM3	MEULLER von B.F.	Murrambridge R., NSW,Australia.	M
BM4	DRUMMOND,J.	Australia.	Mmv
BM5	MASON,R. (744)	Lake Ellesmere, New Zealand.	Mmv
BM6	MEULLER von D.	Gippsland,Victoria, Australia.	Mmv
BM7	DRUMMOND,J.	West Australia.	Mmv
BM8	CONSTABLE,E.F. (7136)	Warilda,NSW, Australia.	P9249 Mmv
BM9	illegible	Otill Waters, S. Carolina,USA.	v
BM10	BROWN,R.	Richmond,Hawksbury, Australia.	134 vT
BM11	BROWN,R.	Patterson River, Oct.1804.Australia.	135 v
BR0	CHAMISSO von A	St.Francisco, California.	'Romer'
BR0a	RÖMER	Demerary,Amer.merid.	Mmvt vT
BR1	POPPIG	?	v
BR2	CALLE,J.	Gippsland,Australia.	M
BR3	Anon.	Tokyo, Japan.	30683 M
BR4	MARUYAMA,N & OKAMATO,K	Honshu, Japan.	1606 v
BR4a	PATTERSON,T.M. (1357)	Corrientes, Argentina.	v
BR5	TOGASI,M.	Honshu, Japan.	v
BR5a	LUSCHNATH	Rio de Janero,Brasil.	v
BR6	PITTIER,M. (9997)	Rio goto? Costa Rica?	v
BR7	EVARD,C. (8052bis)	Argentina.	v

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
BR8	BALANSA,B.	L'Assumption, Paraguay.	v
ENCB1	FISCHER,P.,PITMAN,M. & HEVLY,R.M.(2125)	San Vincente,Mexico.	v
ENCB2	SALDANA,H.C.	Sinaloa, Mexico.	M
ENCB3	CRUZ,R.C.	Atlacomulco, Mexico.	M
ENCB4	WIGGINS,I.L.(20,793)	Monterey Co., California, USA.	v
GH1	STEWART,A	Charles Is., Galapagos Is.	3441
GH2	MEYER,T.	Chaco,Argentina.	2.337
GH3	MEYER,T.	Chaco,Argentina.	2.338
GH4	ULE,E.	Santa Catherina, Brasil.	m
GH5	LEHMANNIANAE,F.C.	?	6363 v
GH6	EYERDAM,W.J. & BEETLE,A.A.	Lagunas de Yale,	22193 v
GH8 (NY9)	MACOUN,J.	Sicamous,Brit.Colu- mbia,Canada.	14,205 Mm v
GH10	LOWELL,J.A.	Santa Barbara, California,USA.	668 Mm v
GH11	Anon.	Morrels,Ohio?,USA.	Mm v
GH12(MO48, NY2)	BOLANDER,H.N.	San Francisco, California,USA.	v
GH13	OBERHOLSER,H.C.	Brownsville,Texas, USA.	Mm v
GH14	BROOK,R. (5293)	Douglas Co., Kansas, USA.	Mm
GH15	MOLINA,A.R.	Llano, Lizapa.	3962 Mm
GH16	PRUELT,E.A.	Ada Co., Idaho, USA.	v
GH17	HOOKER,J.D. (62)	Collenso, New Zealand.	v
GH18	SINCLAIR	Waikaho, New Zealand.	v
GH19	NALSON,J.C. (2761)	Clachamas Co., Oregon, USA.	v
HAL2(B55)	SCHIEDE (839)	Inter Serpillo et Estero,Mexico.	Mm vT
HAL3	Anon.	?	v

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
HAL4	Anon.	Chile.	v
HAL5	Anon.	Cuba.	v
HAL6	POEPPIG, E.F. (700)	Chile	v
L3	JANSEN & WATCHER	Schiedam, Holland.	934-83-183 Mv
L5	HENRARD & DANSER	Rotterdam, Holland.	921-262-450 M
L8	SOEST van.	Welft, Holland.	12927 M
L12	BARKMANN, J.J. (922)	Leiden, Holland.	M
L22	Anon.	Leiden, Holland.	927-364-420 M
L25	HATTUM van H.J. (321)	Wassenaar, Holland.	M
L26	HOOGLAND, R.D. (398)	Voorschoten, Holland. 1402	v
L34	JANSEN, M.T. & WEEDA, E.J. (408237)	Holle, Holland.	32.14.42 v
L36	WAKKER, J.H.	Holland.	941.314.644 Mmv
L40	?	Holland.	Mmv
L41 (L42)	J.N.S.	Boskoop, Holland.	Mmv
L43	WEEVERS, Th.	Utrecht, Holland.	M
L44	KALKMAN, C.	Meranke, S. New Guinae. BW 3743	Mmv
L45	SCHODDE, R. (2673)	Papua, New Guinae. 963.234.444	Mmv
L46	DIETRICH (2270)	Rockhampton, Australia. 249319	v
L49	SWEET, A.R. (1000)	Sicamous, Canada. 71071	Mmv
L52	POEPPIG	Andes, Chile 909.30.153	M
L53	HIERONYMUS	Cordoba, Argentina. 924.320.310	v
L55	SMITH, C.	Georgia. 924.320.125	v
L56	DIETRICH, A.	Brisbane, Australia. 384239	v
L58	STRUCK, P.	Hamburg, W. Germany. 961.97.363	Mv
L59	NEYRAUT, E.J.	Bordeaux, France.	v
L60	CLAVAUD, A. T SANDWICH, N.Y.	Bordeaux, France.	v
L61		Oxford, England.	v

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
L63	Anon. comm. Martius (1829)	?	909.27.363 Mv
L65	KAULFUSS, J.	Nurnburg, W. Germany.	v
L100	POSTHUMUS, O. (3972)	Java.	Mv
L101	OOSTSTROOM, van, S.I. (13000)	Java.	Mv
M1	Anon.	Liegetsberg, W. Germany.	v
M6	MERXMULLER, H. & WIEDMANN, W.	Philipsburg, New Jersey, USA.	v
M7	SCHULZ, P.F.F.	Berlin, W. Germany.	53573 v
M8	MERXMULLER, H. & GRAU, J.	Portugal.	21621 v
M9	FERNANDES, A. PANA, J. & MATOS, J. (7821)	Portugal.	v
M11	GORBIERE, L.	Cherburg, France.	v
M19	SWEET, A.R., SWEET, A.L. & YOUNG, C. (1001)	Sicamous, Canada.	Mmv
M20	MARTIUS	Brasil.	v
M22	MARTIUS	Bahiens, Brasil.	9 v
M24	STORK, H.E. & HORTON, C.B. (8908)	Boca Barranca, Costa Rica.	m
M25	HEINRICHS, E. (513)	Rio Yasum, Ecuador.	Mmv
MIL1 (M017)	DEGENER, O.	Kawaihapai, Oahu, Hawaii, USA.	(12,913) Mmv
MIL2	TAYLOR, W.C. (2863)	Crittenden Co., Arkansas, USA.	125052 Mmv
MIL3 (M02)	HOLZINGER, J.M.	Winona Co., Minnesota, USA.	109000 Mmv
MIL4	FASSETT, N.C. (20349)	Pierce Co., Wisconsin, USA.	103181 Mv
MIL5	SHINNERS, L.H.	Crawford Co., Wisconsin, USA.	122819 Mv
MIL6	PECK, J.H. (81-669)	Monroe Co., Wisconsin, USA.	127637 Mv
MIL10	CORRELL, D.S. (5323)	Citrus Co., Florida, USA.	99438 v
M01	KIENER, W. (22552)	Fillmore Co., Nebraska, USA.	1634723 Mmv
M03	WILLIAMS, L.O. & MOLINA, A.R. (10619)	El Sauce, Ecuador.	1308335 mv
M04	ARSENE, B.G. (8177)	Michoacan, Mexico.	v

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
M05	KIENER,W. (21535)	Guttenberg,Iowa, USA.	1634734
M06	? (178/97)	Kennet? Missouri, USA.	1855833
M07	EGGERT,H.	Pands (Mexico).	1855834
M08	BUSH,B.F.	R.Kennet,Missouri, USA.	1855836
M09	BUSH,B.F. (828)	Kennet,Missouri,USA.	1855840
M010	TRELEASE,W.	Dunklin,Missouri, USA.	1855841
M011	STEYERMARK,J.A. (56006)	Merida,Venezuela.	1621337
M013	MEYER,T. (7289)	Rio Negro,Argentina.	1624543
M016	SOLOMON,J. & SOLOMON,A. (4028)	Buenos Aires, Argentina.	2737997
M018	MARTELLI,U.	Pisa,Italy.	1855855
M020	RENOIZE,S.A. (3588)	San Fernando, Argentina.	2987750
M021	SOLOMON,J. & SOLOMON,A. (4333)	Curico,Chile.	2738011
M022	PETERSON,T.M. (1357)	Corrientes, Argentina.	1700874
M023	SEIBERT,R.J. (1134)	St.Clair Co., Illinois,USA.	1244825
M026	TRYON,R.M. (336) (2,795)	Knox Co.,Indiana, USA>	1855800
M027	FAGERLIND,F. & WIBOM,G. (122)	Guayaquil,Guayana.	1765300
M028	LECTAE,A.M.B. (1032 & 1033)	Cochabamba,Bolivia.	185585
M029	WRIGHT,C. (1797)	Cuba.	1855848
M030	ARSENE,B.G. (2363)	Puebla,Mexico.	844969
M031	WILLIAMS,L.O. & MOLINA,A.R. (12192)	Yeguare,Honduras.	1307832
M032	LIESNER,R. & GONZALEZ,A. (12698)	Portuguesa, Venezuela.	3017105
M033	STEYERMARK,J.A. & LIESNER,R. & ESPINOSA,V.C. (12140)	Sucre,Venezuela.	2804020
M037	MACKENZIE,K.K. (385)	Clay Co.,Missouri, USA.	146259
M038	HALE	louisiana,USA.	v
M039	TRYON,R.M. (335)	Warren Co.,Indiana, USA.	(2,682) 2416444
M040	FERRIS,R.S. (2102)	San Mateo Co., California,USA.	1855823

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
MO41	REVERCHON, J.	Dallas, Texas, USA.	1855815 Mv
MO42	WILKINSON, E.H.	San Antonio, Texas, USA.	1855817 MnV
MO43	SUKSDORF, W.N.	W.Klickitat Co., Washington, USA.	1855819 mv
MO44 (BKL6)	EGGERT, H.	St.Louis, Missouri, USA.	766746 v
MO46	PARISH, S.B. & PARISH, W.F. (2731) (915)	San Bernardino, USA.	120245 m
MO49	BOLANDER, H.N.	San Francisco, California, USA.	1855825 mv
M050	ABRAMS, L. (1833)	Santa Cruz Co., California, USA.	1855826 MnV
M051	RIMBALL, L.	San Diego Co., California, USA.	1855828 MnV
M055	WHEELER, L.C. (4013)	Madoc Co., California, USA.	1174555 MnV
M057	HOLZINGER, J.W.	Winona Co., Minnesota, USA.	2117620 MnV
NSW1	McBARRON, E.J. (2808)	Albury, NSW, Australia.	MnV
NSW2	MCOLL, S.R.	Bourke, NSW, Australia.	P5632 MnV
NSW3	DAINES, D.G.	Paterson, NSW, Australia.	MnV
NSW4	WALTER, C.	Warrnambool, Victoria, Australia.	P3715 MnV
NSW5	MELVILLE, R. 2113)	Quail Is., Victoria, Australia.	MnV
NSW6	McBARRON, E.J. (2809)	Albury, NSW, Australia.	P5723 MnV
NSW7	MCOLL, S.R.	Bourke, NSW, Australia.	P5256 MnV
NSW8	VICKERY, J.	R.Darling, NSW, Australia.	P6243 (2) MnV
NWS9	VICKERY, J.	R.Darling, NSW, Australia,	P6243 (1) MnV
NSW10	MUELLER, F.	Gippsland, Victoria, Australia.	P5684 MnV
NSW11	BRUMBY	Coonamble, NSW, Australia.	P5685 MnV
NSW12	JACOBS, S. (412)	Lachlan R., NSW, Australia.	MnV
NSW13	CONSTABLE, E.J.	Unumgar State Forest, NSW, Australia.	P6485 MnV
NSW14	CHISHOLM, T.	Goulburn, NSW, Australia.	MnV
NSW15	DIETRCH, A.	Rockhampton, Queensland, Australia.	MnV

CODE No.	COLLECTOR (No.)	LOCATION	SHEET/OTHER No.
NY1	HOLZINGER, J.M.	Mississippi R. sloughs, USA.	MmV
NY3 (MO35)	HALL, E. (698)	Oregon, USA.	Mv
NY4	VASEY, G.R. (694)	San Diego Co., California, USA.	Mv
NY5	PRINGLE, C.G.	Rillita, Arizona, USA.	Mv
NY6	GREEN, H.A.	Willauette R., Oregon, USA.	Mv
NY7	GODFREY, R.K.	Taylor Co., Florida, 60394 USA.	Mv
NY8 (MO56)	THOMPSON, J.W.	Mason Co., Washington, USA. 9958	Mv
NY10	McGREGOR, R.L. (5198)	Barton Co., Kansas, USA.	Mv
NY11	CORRELL, D.S. & CORRELL, H.B.	Union Co., Illinois, 31367 USA.	MmV
NY12	TIEHM, A. (3944)	Reno, Nevada, USA.	MmV
NY13	AUSTIN, R.M.	N. California, USA.	m
NY14 (MO36)	BUSH, B.F.	Indian Territory, Missouri, USA. 837	MmV
NY15	CORRELL, D.S.	Jefferson Co., Texas, USA. 13559	MmV
NY16	MUENSCHER, W.C. & CURTIS, O.F.	Suffolk Co., NY, USA. 6647	MmV
NY17	NOBS, M.A. & GALEN-SMITH, S. (380)	Oispo Co., California, USA.	m
NY18	HOWE, M.A.	San Francisco, California, USA.	m
P1	COMMERSON, P	Rapportee de Magellan, Argentina.	P-LAM
P2	Anon.	Brasil.	P-LAM v
P3	COMMERSON, P	Monte Video to Buenos Aires, Argentina.	P-JUS.1601-B v
P4	JUSSIEU, A.	Africa.	P-JUS.1601-B v
P5	COMMERSON, P	Buenos Aires to Monte, Argentina.	P-JUSS.1601-B vT
P6	COMMERSON, P.	Buenos Aires to Monte Video, Argentina.	P-JUSS.1601-B v
P7	VAHL	Africa	P-JUSS.1602 v
P8	MICHAUX	S. Carolina, USA.	P-JUSS.1602 v
P9	DESVAUX, A.N.	Carolina, USA.	vT?
P10	DESVAUX, A.N.	America Calidiori	vT
P11	DESVAUX, A.N.	?	v

CODE No.	COLLECTOR	LOCATION	SHEET/OTHER No.	
P12	FRANCHET,A. (1530bis) (474)	?	vT	
P13	ZOLLINGER (408)	Java.	vT	
P14	Anon. (408)	?	v	
P15	DECAISNE	White Nile,Africa.	MnvT	
P16	DESVAUX,A.N.	America Calidiore et ?	v	
P17	DESVAUX,A.N.	?	v	
P18	KARSTEN	Cabello,Venezuela.	2903	Mnv
P19	GLAZIOU,A.	Brasil.	16649	Mnv
P20	REIECH,E.M.	Porto Algre,Brasil.	Mv	
P21	FUERTES,M.P. (1714b)	De la Vega,Dominican Republic.	Mnv	
P22	Anon.	Tonyo Allg,Japan.	Mnv	
P23	WALTER,E.(155)	Drusenheim W.Germany.	Mnv	
P24	LESPRIEUR,M.	Guyana.	Mnv	
P25	BRUCE,G.G.	Madoc Co.,California, USA.	Mnv	
P26	REMY,M.J. (voyage of)	Sacramento City.	Mnv	
P27	MICHAUX	Carolina.	v	
P28	RICHARD	Carolina.	v	
P30	OSSENBAUL	Demerary,America meridioa.	herb.Kaulf. 6483	v
P31	SAVATIER,L. (1530)	?	v	
P32	CHAMISSO (6484)	St.Francisco,California.	vT	
PE1	LING,Y.X.	in Chinese.Type of var. <b>prolifera</b>	Mnv	
PE2	LING,Y.X.	in Chinese.Type of var. <b>sempervirens</b>	v	
U1	DIETRICH,A.	Brisbane,Queensland, Australia.	Mnv	
U2	TAGAWA,M.	Matugasaki,Kyoto, Japan.	3839	v
U3	TAGAWA,M.	Takaraga-iko,Kyoto, Japan.	3844	v
U4	TAGAWA,A.	Honshu,Japan.	8683	v

CODE No.	COLLECTOR	LOCATION	SHEET/OTHER No.
U5	GONZALEZ,A. (408)	Limones, Soledad.	18456B v
UNA1	DAVENPORT,L.J. (1675)	Guarico, Venezuela.	Mv
US1	FOSBERG,F.R.	Lake Taupo, New Zealand.	30423 Mv
US2	CABSEIA,D.L.	Jujuy, Argentina.	20776 v
US3	BURKART,A.	Corrientes, Argentina.	19.368 v
US4	DUNCAN,W.H.	McIntosh Co., Gorgia, USA.	19969 v
US5	HESS,W. (3201)	Eddy Co., Texas, USA.	275 3809 v

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The following populations were obtained from culture collections or collected from the field. The codes used for these populations are therefore not related to the codes used in Holmgren *et al* (1981-82)

CODE No.	COLLECTOR (date)	LOCATION	
H1	THAN,D.	From culture collection, Hanoi.	M
H2	THAN,D.	From culture collection, Hanoi.	M
H3	THAN,D.	From culture collection, Hanoi.	v
H4	THAN,D.	From culture collection, Hanoi.	M
IRRI5	T.L. (1977)	Bangkok, Thailand.	v
IRRI7	THAI,D.A. (1978)	Bangkok, Thailand.	Mmv
IRRI9	WATANABE,I. (1978)	Sampatong, Chieng Mai, Thailand.	v
IRRI22	F.A.A.S. (1979)	Tancheng, Shandong, China.	v
IRRI26	O.R.S.T.O.M. (1980)	Senegal.	v
IRRI101	CHINA,I.B. (1979)	East Germany.	Mv
IRRI106	SCHARFENSEEL (1980)	Hamburg, W.Germany.	Mmv
IRRI108	RAINS	Cranmore Rd., Sutter Co., USA.	Mv

CODE No.	COLLECTOR (date)	LOCATION	
IRRI110	C.I.A.T. (1982)	PUFFI., Lima, Peru.	v
IRRI111	YAMAGUCHI (1982)	Tsuzuki-gun, Kyoto-fu, Japan.	v
IRRI201	RAINS (1978)	Graylodge, California, USA.	Mmv
IRRI203	RAINS (1981)	Guyana.	v
IRRI301*	RAINS (1978)	Ohio, USA.	mv
IRRI305	F.A.A.S. (1982)	Fuzhou, Fujian, China.	v
IRRI401	RAINS (1981)	Paraguay.	v
IRRI414	RAINS (1981)	Paraguay.	Mv
IRRI417*	RAINS (1981)	Paraguay.	Mmv
IIRI418	RAINS (1981)	Paraguay.	Mmv
IRRI421	LUMPKIN (1982)	Santa Cruz Is. Galapagos Is.	v
IRRI501	LUMPKIN (1982)	Kosti, Sudan.	v
IRRI601	LUMPKIN (1982)	Osaka, Japan.	v
IRRI701	YATAZAWA (1982)	Kakadu Northern Park, N.Ter., Australia.	v
IRRI704	IRRI (1982)	Fog Dam, Australia.	v
KET1	MEEKS, R.	Port Clinton, USA. (RM-PC-CF)	Mm
KET2	WARNE, T.	Sherman Is. California, (ST-SI-CF) USA.	Mm
Q1	FOWLER, K.	Bromfield Swamp, N. Queensland, Australia.	Mmv
SWT1	SWEET, A. R.	Shushup Lake, Brit. Columbia, Canada.	Mmv
---	DUNHAM, D.G.	Greywell, Hants., England.	Mmv
---	DUNHAM, D.G.	Portsmouth Polytechnic, England.	Mmv
---	DUNHAM, D.G.	From UCNW Botanic Gardens, Wales.	v
---	FOWLER, K.	Fordingbridge, Hants., England.	Mmv

IRRI = from the IRRI culture collection; KET = from C.F. Kettering Laboratories, USA.; \* = also grown at Dept. of Botany, Manchester University.

The following sheets were examined late in the investigation.

CODE No.	COLLECTOR (No)	LOCATION	SHEET/OTHER No.
BM12	Commerson, P	Buenos Aires-Monte Video, S.America	_____ VT
PR1	Schiede	Mexico.	_____ MmvT

## APPENDIX II

NITROGEN-FREE CULTURE MEDIUM

ELEMENT (ppm in culture medium)	REAGENT (Molecular Weight)*	WEIGHT OF REAGENT in stock solution (g/l)
P (20)	NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O (156.01)	100.45
K (40)	K <sub>2</sub> SO <sub>4</sub> (174.30)	71.32
Ca (40)	CaCl <sub>2</sub> .2H <sub>2</sub> O (147.02)	117.36
Mg (40)	MgSO <sub>4</sub> .7H <sub>2</sub> O (246.39)	324.00

Make and store the above stock solutions in separate stoppered bottles.

## TRACE ELEMENTS

Mn (0.50)	MnCl <sub>2</sub> .4H <sub>2</sub> O (197.91)	1.441
Mo (0.15)	NaMoO <sub>4</sub> .2H <sub>2</sub> O (241.96)	0.303
B (0.20)	H <sub>3</sub> BO <sub>3</sub> (61.83)	0.915
Zn (0.01)	ZnSO <sub>4</sub> .7H <sub>2</sub> O (287.56)	0.035
Cu (0.01)	CuSO <sub>4</sub> .7H <sub>2</sub> O (249.68)	0.031
Fe (2.00)	FeCl <sub>3</sub> .6H <sub>2</sub> O (270.03)	7.736
	Citric acid monohydrate	11.900
	H <sub>2</sub> SO <sub>4</sub> concentrated	50 ml

Dissolve trace elements separately in the minimum amount of demineralised water; warming the water may be required. Then add the solutions in the above order to approximately 200 ml of demineralised water. Care is required when adding the H<sub>2</sub>SO<sub>4</sub> (conc.). Then make up to volume with demineralised water.

Autoclaving the stock solutions and storing at 4°C prolonged the 'shelf-life' of the solutions by preventing microbial growth.

### Preparation of culture medium:-

Add 5 ml of each stock solution to 4 l of demineralised water. Add each in the order P, K, Ca, Mg, trace elements. Then adjust pH to 5.5 with 1N or 2N NaOH.

### Growth room cultures

A 250 ml beaker was three-quarters filled with culture medium and then five to ten fronds (depending upon their size) were selected and placed on the culture medium surface. A petri dish bottom was inverted and placed on top of the beaker; this reduced fungal infestation. If the parent population was contaminated with algae and fungi the fronds were surface sterilised for fifteen minutes. (The sterilant was a 1% solution of sodium hypochlorite containing 5 drops of 'Tween 80'). Then washed five times in demineralised water to remove the sterilant. The culture medium was replenished as required, with sub-culturing approximately every ten days; this method was labour intensive.

### Greenhouse cultures

A plastic tray (ca 25 cmL x 15 cmW x 10 cmD) was filled to a depth of ca 3 cm with autoclaved potting compost (John Innes No. 1). Culture solution was added until the tray was three quarters filled. Approximately thirty healthy fronds were placed on the culture medium surface. Liquid culture medium was replenished as required, each culture being left for up to a month without harvesting or sub-culturing. This method required little maintenance and was not labour intensive.

### APPENDIX III METHODS A

#### PREPARATION OF FRESH SPECIMENS FOR EXAMINATION BY SCANNING ELECTRON MICROSCOPE

Living specimens were placed in stoppered vials containing 2.5% buffered (pH 7.2) glutaraldehyde solution to 'fix' the material. In this condition, 20 strains in 1982 and 80 strains in 1985 from the IRRI culture collection were transported to Portsmouth. Living specimens at Portsmouth were fixed as above at 4°C for 8 hours. Fixed specimens were washed in distilled water for 30 minutes with two changes of the water, followed by dehydration in a graded series of ethanol (10%-100%), with 10 minutes between each increment (10%-90% = 10% increments, 90%-100% = 5% increments). The specimens remained in 100% ethanol for 30 minutes during which time it was changed twice. The 100% ethanol was then replaced by 100% acetone through a graded series (ethanol:acetone mixtures = 3:1, 2:1, 1:1, 1:2, 1:3) over a period of 60 minutes, followed by the same time in 100% acetone. The specimens were subsequently critical-point dried in CO<sub>2</sub>, mounted on stubs with 'Scotch' double sided sellotape. The mounted specimens were then sputter coated with gold for 2 minutes at 1.2kv in a Polaron E5000 unit. Examination employed a JOEL T20 scanning electron microscope fitted with a medium format (6x7) camera. It was found that 'charging' under the electron beam was considerably reduced when the stub with sellotape on it was 'ringed' with carbon adhesive. This method was also used in the study of the reproductive biology in *A.filiculoides*.

### APPENDIX III METHODS B

#### PREPARATION OF HERBARIUM MATERIAL FOR EXAMINATION BY SCANNING ELECTRON MICROSCOPE

##### (i) Megaspore apparatus

Megasporocarps judged to be mature by their size and position on the fronds were removed from the specimens and soaked in distilled water for at least 10 minutes. After soaking the sporocarp and sporangial walls were removed, using fine tungsten needles, to release the megaspore apparatus. The indusial cap (thickened, distal region of the megasporocarp wall) was left in place on at least one specimen in order that when it was halved the presence-absence of *Anabaena azolla* could be determined. The megaspore apparatus was then placed in 50% acetone for at least 4 hours, and subsequently transferred to 100% acetone for at least 24 hours. The megaspore apparatus was then mounted on a stub with double-sided sellotape ('Scotch tape') either whole or halved (cut longitudinally in half using a new grease-free razor blade and mounted with the cut surface uppermost). The best results were obtained when cutting was carried out under acetone which disperses any remaining lipid which may foul the cut surfaces. The mounted specimens were air dried in a desiccator for 24 hours.

The above method was compared with the standard method of fixation, dehydration and critical point drying (see Appendix III Methods A), with cutting prior to mounting on stubs, and was found not to induce artefacts.

Where possible, three megasporocarps, one for mounting whole and two for cutting, were selected from different fronds. If abnormalities (e.g. supernumerary floats, collar absent, etc.) were visible under the light microscope, more megasporocarps were selected. If the specimens had few sporocarps and were considered important (geographically and/or historically) fewer megasporocarps were selected. When only one megasporocarp was available it was cut and the two halves mounted so that both the sporoderm sculpturing and structure could be viewed.

(ii) Massulae

Several microsporangia were removed from different microsporocarps and mounted on a stub with double-sided sellotape. The massulae were then teased from the microsporangia using fine tungsten needles.

(iii) Vegetative material

A small branch of a frond was selected and mounted on a stub, using carbon adhesive, with the dorsal leaf lobes facing uppermost. When the nature of the trichomes of the dorsal leaf lobe could not be discerned the following method for 'reflating' material was used. Herbarium material was soaked in 10%  $\text{Na}_2\text{PO}_4$  solution for 30 minutes to soften the tissues, washed in distilled water and impregnated with a 10% hydrogen peroxide solution for 8 hours at 0°C. Subsequently a 5% potassium permanganate solution (acidified) was added drop-wise until effervescing had ceased and a pale purple coloured solution remained. The addition of potassium permanganate solution liberated oxygen

from the hydrogen peroxide and reflated much of the tissue. This tissue was then dehydrated, critical point dried and mounted on stubs as described in Appendix III Methods A.

By careful arrangement it was possible to mount megaspore apparatuses, massulae and vegetative material for each population on one stub. All mounted material was stored in a vacuum desiccator for 24 hours prior to sputter-coating with gold for 2 minutes at 1.2kv (with repetition if necessary) using a Polaron E5000 unit, and examination using a JEOL T20 scanning electron microscope fitted with a medium format (6x7) camera.

### APPENDIX III METHODS C

#### EXAMINATION OF MATERIAL BY LIGHT MICROSCOPE : SAMPLING AND RECORDING (See also Appendix III Methods B (ii))

##### (i) Sampling and recording

At least four microsporocarps from at least four different fronds in a sample were selected on the basis of their maturity (i.e. large, full microsporangia within large full microsporocarps). Microsporangia were removed from the microsporocarps and placed in a drop of water on a glass slide. In some instances the microsporocarp wall had been ruptured and microsporangia may have broken open whilst still contained within the sporocarp; such specimens were used. Only in the event of the sample having few microsporocarps, but of known geographical or historical importance were loose microsporangia and/or massulae used.

The sampled microsporangia were stirred in the drop of water to aid 'wetting-up', and also to homogenise the samples from the microsporocarps. Fine tungsten needles were used to tease open 20 microsporangia, and the number of massulae in each was recorded. The wetted structures were then tapped with a metal rod to release all the massulae from all the microsporangia. The water was allowed to evaporate until the massulae were damp. Then a permanent preparation was made with 'Hydromount' (National Diagnostics, U.S.A.) containing 0.01% sodium azide (this prevented microbial growth). The cover glass was sealed

with 'DPX' mountant.

Ten massulae with ten well displayed glochidia were scored in respect to the number of septa per glochidium (giving a total of 100 scores) and the whole length of 50 glochidia. The taper (shape) of the glochidial shaft and typical shape of the glochidial apex were also recorded for each massula. The number of microspores per massula was recorded from at least 30 massulae.

The sampling was made as random as possible, and any departures from the procedure were noted on the record sheet for consideration during analysis. The minimum number of microsporocarps and microsporangia for sampling was four; however, it was more practical to remove many of the latter from the former.

Because the massulae were randomly mixed when released, those that were scored were selected on a first-found, first-scored basis when viewing across and down the preparation; thus no one massula was scored twice. Each glochidium was selected, for scoring septation, on the basis of being able to view the length of its vacuole, and for scoring length, by being able to view the entire length. Selection of the glochidium nearest to the 12 o'clock position, and then scoring every other one in a clockwise direction until ten glochidia had been scored, prevented double scoring of glochidia.

The method of scoring the number of microspores per massulae was performed so as to test the hypothesis that the mean number of microspores per massula is dependent upon the mean number of massulae per microsporangium. Accepting this hypothesis would indicate that the number of microspores per massula is logically correlated to the number of massulae per microsporangium, and is therefore not a useful character.

Observations were made using Wild M5 and M20 light microscopes fitted with eye-piece graticules and photoautomat cameras. All data were recorded on a record sheet.

### APPENDIX III METHODS D

#### EXAMINATION OF VEGETATIVE FEATURES : SAMPLING AND RECORDING

##### (i) Preparation of material

The following is a standard method for preparing herbarium specimens for detailed study; it returns tissues to a more life-like condition. It was employed in this study for collecting quantitative and qualitative data on some vegetative characters.

The required number of fronds from a herbarium sample were wetted and softened in a watch-glass containing a 10%  $\text{Na}_2\text{PO}_4$  solution for at least 30 minutes. If specimens were attached (glued) to the herbarium sheet careful soaking was performed without dislodging the specimens. Care was always taken to wash the specimens in water and to air dry them.

##### (ii) Sampling and recording

Two dorsal and ventral leaf lobes from the region between the third and fourth branches from at least five fronds were scored in respect to maximum length and width. It was rarely possible to measure both lobes of the same leaf; this was accommodated by preparing more than five fronds. The maximum width of the hyaline margin of the dorsal leaf lobe was also measured. The number of leaves between the third and fourth branches of a

frond was recorded from ten different fronds; however, subtending leaves were excluded from the score. Other features recorded were qualitatively scored and were:-

- a) nature of the hyaline margin (e.g. smooth, serrate, toothed, etc.)
- b) shape of the apex of the dorsal and ventral leaf lobes
- c) nature of the trichomes on the abaxial surface of the dorsal leaf lobes and stem, i.e. distribution, number of cells, shape, orientation, etc.
- d) general appearance of the fronds, (e.g. shape, branching, robustness, size, colour, etc.)
- e) presence/absence and abundance of mega- and microsporocarps.

When the nature of the sample did not permit measurement or scoring from a designated region, the next nearest region was used, and noted on the record sheet. Similarly, characters that could not be scored accurately were not scored at all. Observations of (c) above were usually made using a JEOL T20 scanning electron microscope fitted with a medium format (6x7) camera, whereas all other observations were made using a Wild M5 light microscope fitted with an eye-piece graticule.

### APPENDIX III METHOD E

#### PREPARATION OF FRESH MATERIAL FOR EXAMINATION USING THE LIGHT MICROSCOPE

##### (i) Wax embedding

The material used was collected from the IRRI culture collection in 1982 and 1985 and from Portsmouth Polytechnic. The fresh specimens were fixed in 2.5% buffered (pH 7.2) glutaraldehyde solution. Material from IRRI was transported to Portsmouth in this fixed state in small vials. Specimens were washed in distilled water for 30 minutes with two changes of water. The subsequent dehydration method was modified from the procedure described by Johansen (1940); in this publication tertiary butyl alcohol (TBA) is equivalent to what is now called 2 methylpropan-2-ol. The washed specimens were dehydrated in a graded series of 2 methylpropan-2-ol (30%-100%) with 15 minutes at each 10% increment, followed by 8 hours in 100% 2 methylpropan-2-ol at 35°C, with two changes. The specimens were transferred to a 1:1 mixture of 2 methylpropan-2-ol and liquid paraffin at 40°C for 8 hours, followed by 8 hours in absolute liquid paraffin at 40°C. Regular changes of the liquid paraffin enhanced clearing of the specimens. When cleared, the specimens were transferred to molten 'Fibrowax' (Raymond-Lamb, U.K. suppliers) in an oven for 1 hour, followed by vacuum embedding in fresh molten 'Fibrowax' (see Johansen, (1940, pp 134-138) for general wax embedding techniques). When the 'Fibrowax' had hardened, sections were

cut at 8 $\mu$ m thickness using a Cambridge or Jung Rotary microtome fitted with steel knives. The sections were stained with haematoxylin and eosin (Smith & Bruton, 1977) or safranin and fast green (Johansen, 1940), prior to being permanently mounted on glass slides in 'DPX' mountant. Xylene was used to remove excess embedding medium. Observations were made using a Wild M20 light microscope fitted with Photoautomat.

(ii) Methacrylate Resin Embedding

In the investigation of the reproductive biology of *A.filiculoides*, reproductive structures for light microscopical observation were embedded in resin. Selected megasporangia were fixed in 2.5% buffered (pH 7.2) glutaraldehyde solution at 4°C for 8 hours. This was followed by washing in distilled water and dehydration as described in Appendix III Methods A. The dehydrated structures were then placed in an uncatalysed mixture of JB-4 resin for 8 hours, then transferred to a catalysed mixture of JB-4 resin. The quantities of the resin components followed the manufacturers (Polysciences Inc. USA) instructions. Before the resin 'gelled', the impregnated structures and resin were transferred to 'Beem' capsules. Polymerisation of the resin was complete within 24 hours, after which thin sections were cut at 4 $\mu$ m on a LBK Pyramitome fitted with glass knives. The sections were floated on to glass slides, air dried and stained with 1% aqueous toluidine blue, washed in distilled water, air dried and finally mounted in 'DPX' mountant. Observations were made utilising a Wild M20 light microscope fitted with Photoautomat.

### APPENDIX III METHOD F

#### COLLECTION AND CULTURE OF MATERIAL FOR THE STUDY OF THE REPRODUCTIVE BIOLOGY OF *A.FILICULOIDES* LAM.

A population of *A.filiculoides* was cultured at Portsmouth Polytechnic, which regularly sporulated in outdoor tanks between April and November each year. Sporulating fronds were collected and cultured in plastic trays containing tap water in the laboratory. Released reproductive structures were collected daily from the bottom of the trays, and placed in stoppered vials containing tap water. These vials were left in the light in the laboratory. Daily observations were made to follow the morphological changes during germination and embryo development. As necessary, reproductive structures were removed to prepare them for examination employing scanning electron and light microscopes.

Spermatozoids were obtained by culturing (as above) large numbers of massulae in a vial. Daily observations, using a Wild M20 microscope enable the optimum time for 'fixation' to be determined.

Compared with conventional nitrogen free media, the use of tap water as the culture medium in this study considerably reduced algal, fungal and bacterial growth. Structures cultured by the above method were prepared for scanning electron microscopical examination utilising the method described in Appendix III Method A and for light microscopical examination Appendix III Method E.

APPENDIX IV

POPULATION DATA OF THE NUMBER OF SEPTA PER GLOCHIDIUM

(100 glochidia scored from each population)

Code No.	No. of septa														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
B2	28	43	28	1											
B7	19	51	30												
B8	70	29	1												
B11	78	21	1												
B13	76	24													
B14	26	74													
B17	96	14													
B21	80	20													
B23	17	65	17	1											
B24	18	50	23	9											
B27	46	54													
B28	79	21													
B29	62	30	8												
B30	78	19	2	1											
B32	69	31													
B35	65	34	1												
B38	14	42	33	7	4										
B40	-	23	44	27	5	1									
B41	4	41	41	14											
B45	-	3	16	35	31	12	3								

No. of septa

Code No.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
B46	54	45	1													
B48	1	8	20	38	25	7	1									
B50	8	32	41	16	3											
B51	-	1	11	34	28	20	5	1								
B58	-	-	3	21	33	28	13	2								
B59	-	-	8	24	29	24	15									
B64	55	36	8	1												
B66	-	-	-	1	2	12	29	20	23	11	2					
B67	3	8	20	25	19	11	8	3	1	2						
B70	60	32	8													
BKL1	-	3	6	21	29	20	16	3	1	1						
BM2	-	-	7	29	30	28	4	2								
BM3	-	2	41	52	4	1										
BM4	5	7	35	33	16	3	1									
BM5	9	50	37	4												
BM6	2	17	40	26	14	1										
BM7	-	3	33	42	21	1										
BM8	3	36	37	15	9											
BR2	6	26	44	19	5											
BR3	10	23	49	13	5											
GH3	1	4	22	44	21	5	3									
GH13	9	34	43	12	2											
GH14	-	-	4	13	33	27	13	9	1							
GH15	2	1	15	28	27	15	7	4	1							
HAL2	2	9	38	33	14	3	1									

**No. of septa**

<b>Code No.</b>	<b>0</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>	<b>13</b>	<b>14</b>	<b>15</b>
L36	-	-	-	1	3	5	5	11	17	12	6	8	7	3	1	1
L40	-	3	8	20	23	15	15	11	2	2	-	-	1			
L41	-	-	1	16	23	24	12	14	5	2	2	1				
L50	35	55	10													
M4	81	19														
M19	2	19	41	24	13	1										
M24	10	22	20	26	13	7	2									
M25	-	1	17	38	27	12	4	1								
MILL	97	3														
MIL2	47	24	19	8	-	2										
MIL3	-	-	-	17	36	8	19	13	4	1	1	1				
MIL4	-	-	-	1	10	17	22	21	18	10	1					
MIL6	66	34														
M01	-	15	36	34	12	3										
M03	6	27	51	13	3											
M05	-	1	-	3	15	23	23	18	13	4	1					
M06	-	3	20	36	22	9	8	2								
M07	-	-	-	-	3	9	30	24	16	9	6	1				
NSW2	4	14	48	21	9	4										
NSW3	2	13	52	13	14	3	3									
NSW4	11	50	33	5	-	1										
NSW5	-	-	1	1	2	10	14	16	15	18	9	9	5			
NSW6	7	20	39	21	11	2										
NSW7	2	9	40	35	13	1										
NSW8	1	20	39	27	10	2	1									

Code No.	No. of septa															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
NSW9	17	21	35	16	9	2										
NSW11	-	-	7	29	37	17	8	2								
NSW12	2	15	34	31	15	3										
NSW13	4	13	47	30	6											
NSW14	1	10	36	39	13	1										
NSW15	3	11	34	33	15	3	1									
P18	48	50	2													
P19	28	49	23													
P21	-	-	-	1	10	33	29	21	6							
P22	40	39	20	1												
P23	70	29	1													
P24	2	17	62	18	1											
P25	-	-	22	40	34	5										
P26	7	22	36	21	14	3										
U1	4	13	49	28	6											
US1	5	28	48	15	4											
IRRI201	38	34	18	10												
IRRI301	96	4														
IRRI417M	-	2	17	30	19	21	9	1	1							
IRRI417	-	-	3	5	31	38	16	7								
IRRI418	-	3	12	31	26	26	1	1								
KET1	-	2	2	7	19	23	24	12	7	1	2	1				
M09	-	4	10	21	35	18	9	3								
M019	-	3	11	26	28	20	10	2								
M023	1	15	22	31	12	9	5	2	1	1	1					

No. of septa

Code No.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MO24	-	-	-	1	6	14	29	27	11	8	2	2				
MO29	-	10	23	36	27	3	1									
MO31	2	10	15	17	25	9	9	8	3	1	1					
MO42	-	-	4	11	18	33	19	9	6							
MO43	-	-	9	34	32	14	8	2	1							
MO45	-	-	-	-	-	4	26	27	20	8	3	1				
MO46	97	3														
MO50	98	2														
MO55	15	26	30	22	6	1										
MO57	-	2	2	14	25	29	16	7	3	2	1					
NY1	-	-	-	-	-	6	6	17	21	18	17	10	6			
NY2	54	43	3													
NY3	-	12	36	37	14											
NY4	51	49														
NY5	86	14														
NY6	-	2	33	54	8	3										
NY7	60	36	3	1												
NY8	94	6														
NY9	-	13	31	38	14											
NY10	-	-	-	3	17	26	23	20	7	3	1					
NY11	11	61	14	10	2	2										
NY12	98	2														
NY13	88	12														
NY14	64	20	9	8												
NY15	2	24	38	30	4	1	1									

## No. of septa

Code No.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
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NY16	93	7														
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NY17	59	26	9	6												
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NY18	55	43	2													
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KET1	18	24	27	19	11	1										
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KET2	-	2	12	29	42	14	1									
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POLY	24	68	8													
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APPENDIX V

PROTOLOGUES

**A.magellanica** (Willdenow, 1810)

"Caulis radiatim divisus natans alternatim ramosus radiculas longas capillares simplices fusco-nigricantes exserens.

Folia oblonga obtusa margine membranacea undique caulem et ramos arcte oblegant. Capsulam imperfectam subrotundam examinavi, sed de charactere non certus sum.W."

"Habitat in aquis regni Peruviani, Chilensis et freti Magellanici (v.s.)"

**A.caroliniana** (Willdenow, 1810)

"Caulis ut in praecedente. Folia ovato-oblonga obtusa margine non membranacea supra viridia subtus rubra, patentia.W."

"Habitat in aquis Carolinae (v.s,) Richard"

**A.rubra** (Brown, 1810)

"A.rubra, fronde circumscriptione orbiculata, lobis palmatis, lobulis indivisis bifidisve, foliolis superioribus laevibus, radicibus extra medium plumosis. (J.D.) v.v."

**A.microphylla** (Kaulfuss, 1824)

"A.frondibus orbiculatis semipinnatis pinnis trilobis, foliolis imbricatis adpressis minutis.

Habitat in California. Chamisso.

Frondes tri-quadrilineares orbiculatae subradiatae, folia papillosa arcte adpressa minutissima hyalina. Capsulae globuliferae femine papaveris fere duplo maiores"

**A.cristata** (Kaulfuss, 1824)

"Species Azollae statu sicco difficillime distinguende. Ex America meridionali (Demerary) plantulam vidi sterilem quidem, sed foliis ovatis obtusis papillosis squarrosis incurvis, satis diversam, Azollæ cristatae nomine insigniendam"

**A.densa** (Desvaux, 1827)

"A.densa N. Frondis circonscriptione lobata, frondellis dense imbricatis, oblongis, obtusinsculis non emarginatis, Crescit in aquis Carolinæ at Virginiae"

**A.arbuscula** (Desvaux, 1827)

"A.arbuscula N. Circonscriptione frondis oblonga: divisuiris elongatis ramosis; frondellis ovatis, latis nec marginatis. Habitat in America Calidiore"

**A.bonariensis** (Bertoloni in Schlechtendal 1861)

"-6. Azolla bonariensis, fol. ovali-oblongis, late marginatis, quadrifariam, imbricatis, conceptaculis subrotundis granulatis, Tab. v.f.2.a.b, von Buenos Ayres durch den im taurischen Kriege getodteten FOX-STRANGWAIS erhalten"

**A.japonica** (Franchet & Savatier, 1876) Enum. Pl.Jap.II p.195

"2294 JAPONICA Franch. et Sav. Sp.nov.

Hab. in Japonia, probabiliter in insula Nippon ex Tanaka  
(Savatier, n 1530<sup>bis</sup>)

Japonice - Aka oukikouza (Tanaka)

Observ. - Cette espèce, qui rappelle certaines formes de l'**A.caroliniana** à feuilles laches, paraît bien distincte de l'**A.pinnata** par ses feuilles très-écartées, distinques presque complètement membraneuses, surtout les inférieures; par ses dimensions elle est intermédiaire entre l'**A.caroliniana** et l'**A.pinnata**."

**A.africana** (Desvaux, 1827)

"**A.africana** N. Circonscriptione frondis pinnatae pyramidata: divisuris linearibus, simplicibus; frondellis basi pressis, acuminatis, laxe imbricatis. Crescit in aquis Africæ"

**A.decomposita** (Zollinger, 1854)

"l A.decomposita zoll. n.sp. H.408. Cum praeced. Etiam in fossis pr. Tjipannas 3500. (**A.pinnata** R.Br. Hassm. in Cat.) (2)"

**A.imbricata** (Roxburgh ex Griff.) Nakai, 1925)

"AZOLLA IMBRICATA Nakai, comb, nov.

**Salvinia imbricata** Roxburgh apud Griffith in Calcutta Journ.

Nat. Hist. IV. p.469 (1844).

A species resembling to **Azolla africana**, differs from that by the shorter papillae of the leaves and less pointed apex. Distributed over Ceylon, East Indies, Tonking, Mekon, China (Kantung, Hongkong, Yunnan, Chusan)"

