# ANABAENA AZOLLAE AND ITS HOST AZOLLA PINNATA[(1)(2)]

by

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Blue-green endophytes, belonging to the genus Anabaena, are found with considerable regularity as symbionts in Azolla. The genus Azolla, though a small one, has representatives in all the divisions of the globe (Campbell 1893). This small floating water fern forms a magenta blanket which covers the ponds and ditches in my native land each fall and winter. The plants which grow in Taiwan are similar to those found in the Lower Yangtze Valley (Eastern China), but these seldom turn red.

Although Azolla is distributed all over the world and its endophytic Anabaena always lives in symbiotic relation with it, yet its morphology, taxonomy and relationship is still not clear. Tilden, J., 1910, in her Minnesota Algae in describing *Anabaena azollae* makes the brief statment that "gonidia (are) unknown". Prescott, G. W. 1951, in his "Algae of the Western Great Lake Area", under *Anabaena azollae* Strasburger, stated that "lack of gonidia in these plants makes their identification questionable". Geitler, L., 1932, did not mention the structure of the spore in his description of *Anabaena azollae* Strasb.

It is true that the Azolla found growing in paddy fields and ponds of Taiwan is usually non-fruiting. but fruiting speciments have been found on Taiwan in January and February and in Eastern Central China fruiting occurs in the late fall and early winter. Because of the small size of this fern most people are not familiar with its detail structures. In 1935 when the writer was a student in Soochow University, he once collected some Azolla and found several sporocarps. It was his first chance to examinate them, but unfortunately he did not make a detail study of them. Because of his interest in alga, last year his attention was again turned to Azolla since Azolla always contains Anabaena. The study of the Azolla common in Taiwan shows it is morphologically similar to the Azolla he had seen in Soochow, twenty five years before.

The Azolla plant fragments easily, and its vegetative multiplication is rapid. Therefore wherever we find Azolla growing, its growth is always abundant. Although Azolla is widely distributed all over the world only a few species have been studied carefully.

The species which is found in China and Formosa has never been studied carefully

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(1) Acknowledgment is made to Dr. Charles E. DeVol for his suggestions and for supplying some specimens for this investigation.

(2) Aided in part by a grant from the National Council of Long Range Plan for Science Development in China.

by botanists. After Nakai (1925) identified this species as *Azolla imbricata* (Roxb.) Nakai, botanists accepted it and have been using this name for more than thirty years.

## AN INVESTIGATION OF THE VEGETATIVE ORGANS OF AZOLLA

Our local species is triangular in shape (pl. I fig. 4; pl. III fig. 1). Branching is free and dense; its rhizome are easily broken. On the lower side of the plant are simple roots, which are solitary, and extend a short distance down in the water.

The roots develop from the prostrate shoot of Azolla in acropetal succession at the points of branching. They are from 8 to 15 mm in length, undivided, and delicate, and provided with long spreading hairs (pl. I figs. 1, 2). The root is enveloped with a sheath and cap when young (pl. I fig. 3). When the root is fully mature the sheath is sloughed off and the root hairs spread out (pl. 1 fig. 2). Usually aquatic plants are without a root cap and do not bear root hairs like those of the terrestrial plants but that is not the case with this plant.

From the root initial a pyramidal apical cell is organized. A single cap cell is cut off, which afterward, according to Strasburger, it divides but once periclinally, a two-layered adherent cap is thus formed (pl. I fig. 6; pl. III fig. 4).

A medium longitudinal section of a primordium of a root is shown in photograph in plate V fig. 5. It is enveloped by a sheath composed of a single layer of cells. The cap is composed of two very similar layers. In roots slightly more advanced a sharp differentiation of these layers is seen to have taken place.

The young stage of the root thus presents a state of things analogous to that in Nicotiana, a typical dicotyledon, since the superficial layer of the body of the root is derived from the calyptrogen, the original cap segment. The mature root is exposed and unprotected by the cap and sheath.

The origin of the root hair in Azolla is peculiar, but this kind of root hair formation has been reported in Lycopodium (De Bary) and Isoetes (Brunchmann) (Leavitt 1902). The initials of the root hair in the root of Azolla arise within a belt of actively dividing cells, lying immediately under the inner root cap not far from the apex, the actual distance varying with the rate of growth of the terminal region. Special hair initial cells are cut on the peripheral layer of the root; these cells never elongate much in a direction parallel to the length of the root. The tube begins to grow out toward the root apex. As the hairs lengthen they at first lie appressed to the root and may be seen confined by the inner cap, which is now distended and pushed away from the root trunk (pl. I fig. 4). The whole cap and sheath structure is finally sloughed off through the growth of the basal hairs, and the hairs themselves stand out strongly (pl. I fig. 2). The hairs being arranged in whorls, and are nonseptate (pl. 1 fig. 1). They often attain a length of about 2 mm.

In the transverse section of a root, the mature tissues are differentiated as follows:

On the outside the root is enveloped by a sheath or root cap. The outermost layer of the root proper is the epidermis. Internal to this are two layers of cortical cells, the outer layer with nine cells and the inner layer with six cells. Successively within the inner cortical layer and derived from the same mother-cell layer are a six-celled endodermal layer and a six-celled pericyclic layer. The stele is diarch and the phloem is radiately arranged with the xylem (pl. V fig. 6).

The leaves are alternate and stand on the dorsal surface of the rhizome in two rows. As the leaf primordium develops and becomes a young leaf, it soon becomes differentiated into a dorsal or upper aerial and a ventral or lower submersed lobe (pl. I fig. 7). The dorsal lobe, which stands oblique and touches the water only by one edge, is several cells thick in the central region and is photosynthetic, with papillae on the upper surface (pl. III fig. 7). The ventral lobe is thin, of one cell layer thick through most of its extent, and is non-photosynthetic. The shape of the leaf lobe is broader near the apex being a trapezoid, 1.2 to 1.4 mm long.

Early in development of the dorsal lobe, there is the formation of a cavity on its ventral side and near the leaf base (pl. III fig. 5). The cavity opens externally by a large circular pore. Within this cavity filaments of Anabaena grow permanently. A mature dorsal lobe has an upper and a small vein, the remaining tissue is a palisade parcenchyma with large intercellular spaces (pl. III fig. 5).

## MORPHOLOGY OF THE REPRODUCTIVE ORGANS OF AZOLLA

The sporangia of this genus are enclosed by an indusium and form a special structure called a sporocarp. Sporocarps are borne on the ventral lobe of the first leaf of a lateral branch. In these fertile leaves the submersed lobe is reduced to two divisions, on each of which a sporocarp is borne terminally and is nearly sessile. The sporocarps contain either microsporangia or megasporangia and are different in size and shape: Those bearing the microsporangia are large and globular; those bearing megasporangia are smaller and ovoid in shape (pl. II fig. 6). Usually a microsporocarp and a megasporocarp develop together but less frequently two of the same kind of sporocarps may develop together. The cells of the sporocarp wall develop anthocyanin pigment just as leaf cells do but may even deeper and brighter and in color.

The wall of the sporocarps is two cell in thickness, with an opening at the apical end, where a cluster of Anabaena can always be found. Within the globular microsporocarp are many long stalked spherical microsporangia borne laterally on a columella (pl. IV figs. 1, 3), and the order of development of the sporangia within the sorus is gradate. The stalked microsporangia (pl. II. fig. 5) are released when the microsporocarp wall breaks.

There are four or more "massulae" in each microsporangia (pl. IV figs. 1, 3). The yellow massulae (pl. V figs. 7, 8, 9) are released when the microsporangium wall cracks open. The cells of the massulae are globular and vary considerably in size. The lamon yellow microspores are imbedded in the body of the massula. The massulae of our local species is not isodiametric. Their shape is almost like a hat with a dorsal and ventral face. Three to eight trichomes hang from ventral side. The trichomes are not glochidia-like (appendages with hooks) (pl. II fig. 2; pl. V fig. 7). The contents of the trichomes are highly vacuolate thus it may appear to be septate, but they are not branching and not anastomosing. The microspores are 13–18 $\mu$ in diameter.

The ovoid megasporocarp has a similar ontogeny to that of the microsporocarp. Its subsequent history depends upon the behavior of the megasporangium. If a single young megaspore in a megasporangium begins to enlarge and form a big functional megaspore; then there is no further development of the juvenile microsporangia below the megasporangium.

If the megasorocarp wall is carefully dissected away it will be seen that at the base of the megasporocarp is a spherical megasporangium, above which are nine floats arranged in two tiers, with three floats above and six floats below. Above the floats there is a tuft of long hairs (pl. II fig. 4). A single large megaspore is formed within the megasporangium (pl. II fig. 3; pl. IV fig. 4)

The wall of the megasporangium shows various types of markings in different species. In our species the megasporangium wall has two layers, the outer is finely tuberculate and covered with scattered vermiform papillae; the inner layer is non-cellular and appears homogeneous. The megaspore wall has very close striations. Our sections were stained with Safranin 0 and Fast Green FCF. The megasporocarp wall stained green, the outer layer of megasporangial wall stained reddish orange, the non-cellular layer was lavender, and the megaspore wall stained yellow (pl. II fig. 7; pl. III fig. 3)

## ANABAENA IN THE LEAF CAVITY OF AZOLLA

As previously stated the dorsal lobe of the vegetative leaf has a cavity on its ventral side near the base of the leaf. This cavity opens externally by a large circular pore. Inside of this cavity filaments of Anabaena grow vigorously (pl. V figs. 1, 2). Anabaena grows also inside the indusium (pl. IV fig. 2) of the micro- and megasporocarp; they are nearly always found just below its apical opening. These algae grow well permanently imprisoned within these cavities. They are always to be found in specimens of Azolla growing on Taiwan and are reported universally present in all species of Azolla from different localities of the world, with the exception that Fremy (1930: 373) stated that he had examined freshly collected specimens of Azolla from French Equatorial Africa and these did not contain Anabaena. The alga symbiont has been considered as the same species no matter what species the host was, and has always been named ***Anabaena azollae*** Strasburger, 1884.

The trichomes of ***Anabaena azollae*** are straight or coiled, without a sheath, often in small clusters but more frequently solitary, inhabiting the leaf cavity and sporocarps of Azolla; cells are subglobose to ellipsoidal with granular contents, 4–5 $\mu$ in diameter, 5–7 $\mu$ long; heterocysts are ellipsoidal 6–7.5 $\mu$ in diameter, 7.5–8.5 $\mu$ long. The heterocysts usually contains cell contents and are not entirely empty but they can be easily recognized by their transparent contents and the presence of polar nodules ("cellulose buttons"). The lack of spores in the natural condition makes the identification of this species difficult. Fritsch (1904) reported the reproduction of ***Anabena azollae*** and described in detail the way its spores germinated but he did not mention under what condition he observed these spores or their germination. He did not state whether the spores occurred singly or in a series, but his illustration would indicate they occur in a series.

Several methods have been attempted in the hope of inducing the formation of spores in this algae. Fritsch in his "The Structure and Reproduction of the Algae" makes the statement: "One factor leading to the formation of akinetes appears to be nitrogen-deficiency" (Fritsch 1952 Vol. II: 808). Therefore the writer tried to cultivate Azolla in a nitrogen free medium for half a month. Observations were made at different intervals, but no akinetes ever observed. He tried to induce akinetes by low temperature treatment; and also by light stimulation, first by ultraviolet irradiation and then by placing the plant under dark conditions. None of these methods induced the formation of akinetes in the alga. But finally the writer ran on to an efficent method inducing the formation of spores in this alga. It is simple but has proved effective. The way he did it was just to put some of the living Azolla in a glass tube with both ends open but covered with cheese cloth fastened by rubber bands. Then we put this tube in a jar and let running tap-water continually flow through it for two weeks. When we examined the Anabaena we found that spores has been formed. This simple method can be repeated although we do not know why the spores were formed. Since there is chlorine in the tap-water, it may have stimulated their formation. Secondly we placed the material where it had no direct sunlight and this made it unfavorable for photosynthesis and thirdly being under running water the air supply was abundant. The size of the spore is 6.25 to 7.5 $\mu$ in diameter and 9 to 13 $\mu$ long. Its wall is smooth and yellowish; spores are solitary and do not grow near the heterocyst (pl. II fig. 1 pl. V fig. 3).

The germination of spores has been observed. The sporemembrane itself becomes mucilaginous and greatly swells up (pl. II fig. 1; pl. V fig. 4), this is what Fritsch (1904) called the second type of germination. The contents of the spore divides and forms a small thread of a few cells before rupturing the spore wall and coming out (pl. II fig. 1).

## DISCUSSION AND CONCLUSION

The relation between this alga and its host is, however, not yet clear, while some (Geitler, L.; Wtanable, K.) regard the former as a pure "space-parasite", perhaps leading an heterotrophic existence within the host (Harder, R.), others believe there is a true symbiotic relationship in which the activities of the associated bacteria (Takesige, T.) or even the alga itself (Molisch, H.) (Fritsch 1952, II: 808) is mutually beneficial to alga and fern. The writer agrees with Molisch's conception of the symbiosis between alga and the fern. The alga always grows very well inside of the leaf cavity of the Azolla, but it grows very slowly when isolated out from its host and grown in culture media. This indicates that the alga is benifited by its host, Azolla. On the other hand when we examine the growth of the alga within its host, we never find an algal cell growing into the tissue of the fern, and the fern leaves always grow normally. The alga never destroys the tissue of its host. There is never any symptoms of pathogenesis. Therefore the alga is not parasitic on the fern. At the same time the alga does show an ability of fixing free nitrogen, this benefits the living fern Azolla. They do no harm to each other and both benefit from association with the other.

The discovery of the spores in the *Anabaena azollae* adds much to our knowledge of this alga. It seems closely related to *Anabaena variabilis* Kuetzing, which is a free living species of very wide distribution. Its vegetative cells, heterocysts, and spores are of about the same size and shape, but there is one striking difference, in *Anabaena variabilis* the spores are numerous and grow in a catenate series, while in *Anabaena azollae* all spores thus far observed by us have been solitary.

We have called this aquatic fern on Taiwan *Azolla imbricata*. It is similar to *Azolla africana* Desvaux, which has been reduced (Christenson 1906) to *Azolla pinnata* R. Br. (1810). The detailed structure of the sporocarps of *Azolla africana* and *Azolla imbricata* have not been previously reported. After a carefully study on its vegetative and reproductive structures of our local specimens, we find them to be similar to Strasburger's drawing of *Azolla pinnata*. They both have the same shaped massulae with trichomes attached on only one surface and are without glochidia. They have the same type of ciliated apex, floats, and structure of megasporangial wall. They both lack a collar between the floats and megasporangium. Hence the older name *azolla pinnata* R. Br. is accepted here as the correct name for our species.

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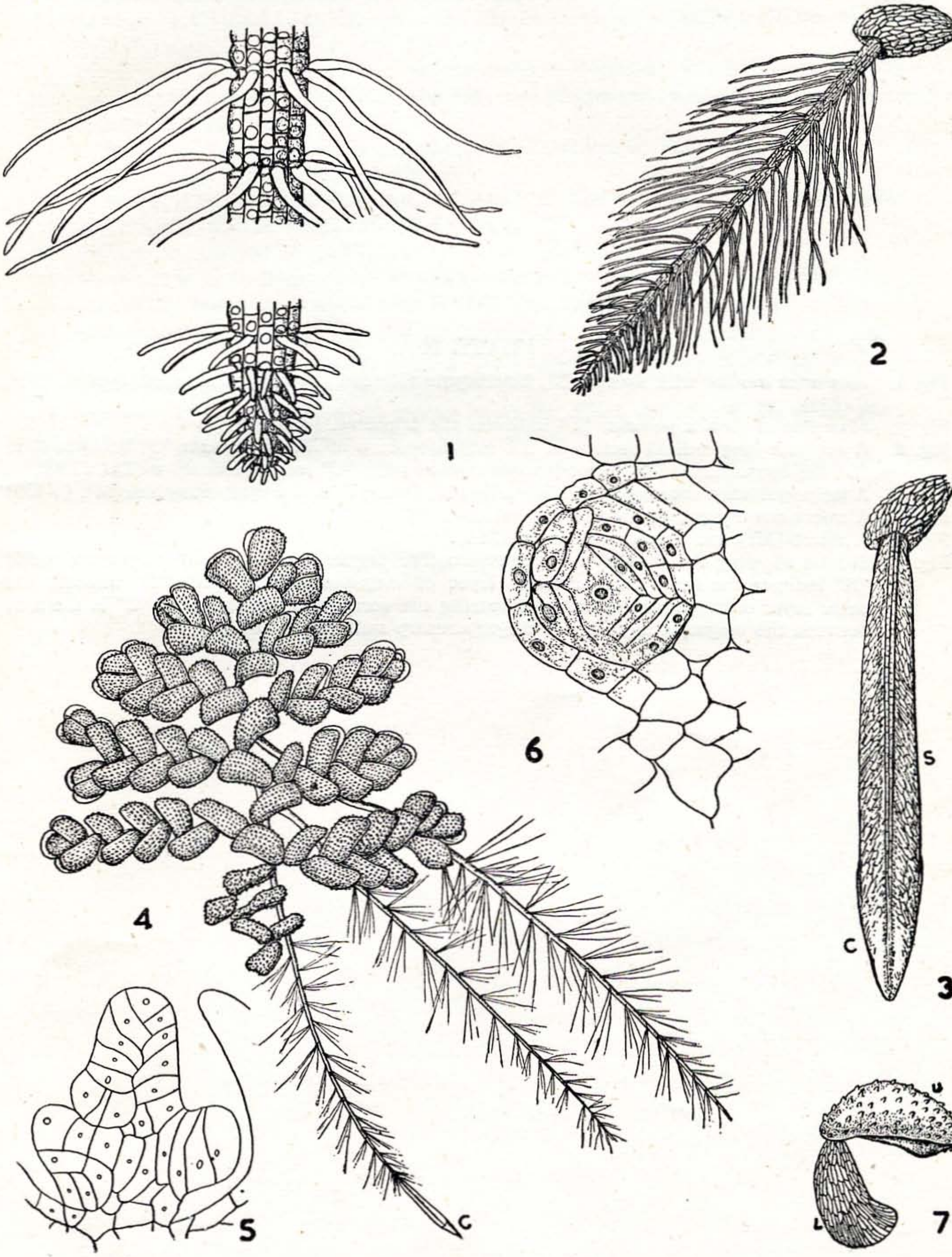
## PLATE I

Fig. 1. A portion of root with root hairs (enlarged fig. 2) (×60)
Fig. 2. A root with root hairs spread after its sheath and cap is sloughed off. (×20)
Fig. 3. A young root of Azolla with root cap (c) and sheath (s). (×20)
Fig. 4. A habit sketch of *Azolla pinnata;* c, root cap. (×10)
Fig. 5. Sagitall section of the shoot apex of *Azolla pinnata.* (×500)
Fig. 6. A young root tip showing the initials of sheath, cap and apical cell. (×600)
Fig. 7. The dorsal (U) and lower (L) lobes of an Azolla leaf. Note the papilla on the upper surface of the dorsal lobe (×30)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## PLATE II

Fig. 1. *Anabaena azollae* with spores (S), heterocysts (H) and several germinated spores (SG). ( $\times$650)

Fig. 2. Massulae of *Azolla pinnata*, "T" indicates the trichome. ( $\times$60)

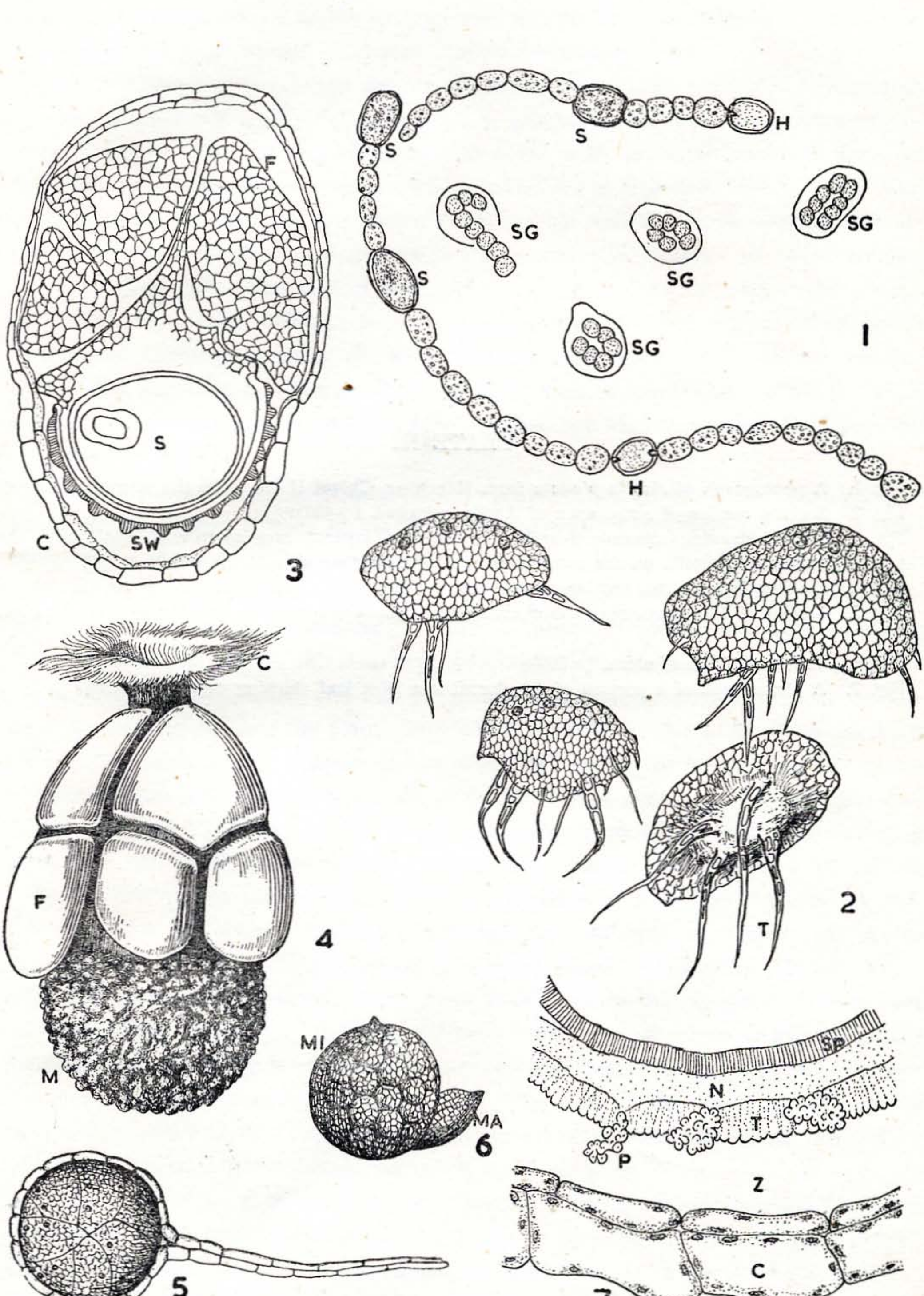
Fig. 3. A medium longitudinal section of the megasporocarp of *Azolla pinnata*, "F" indicate float, "S" the megaspore, "SW" the megasporangium wall, "C" megasporocarp wall. ( $\times$150)

Fig. 4. A megasporocarp showing furnal-like cilia (C), floats (F) and megasporangium (M). ( $\times$150)

Fig. 5. A microsporangium, containing massulae. ( $\times$100)

Fig. 6. A micro-(MI) and a mega-sporocarp (MA). ( $\times$15)

Fig. 7. Section showing the wall of the megasporocarp, megasporangium and megaspore wall; "N" indicate the inner none cellular layer of megasporangium wall, "T" indicate the outer layer of megasporangium wall bearing the vermiform papillae (P); "Z" is the zone between the megasporangium and megasporocarp wall (C). ( $\times$600)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## PLATE III

Fig. 1. A photograph of *Azolla pinnata* from Nanching, Chiayi Hsien. Note the sporocarps. ( ×4)

Fig. 2. Segittal section of shoot apex of Azolla enlarged. ( ×450)

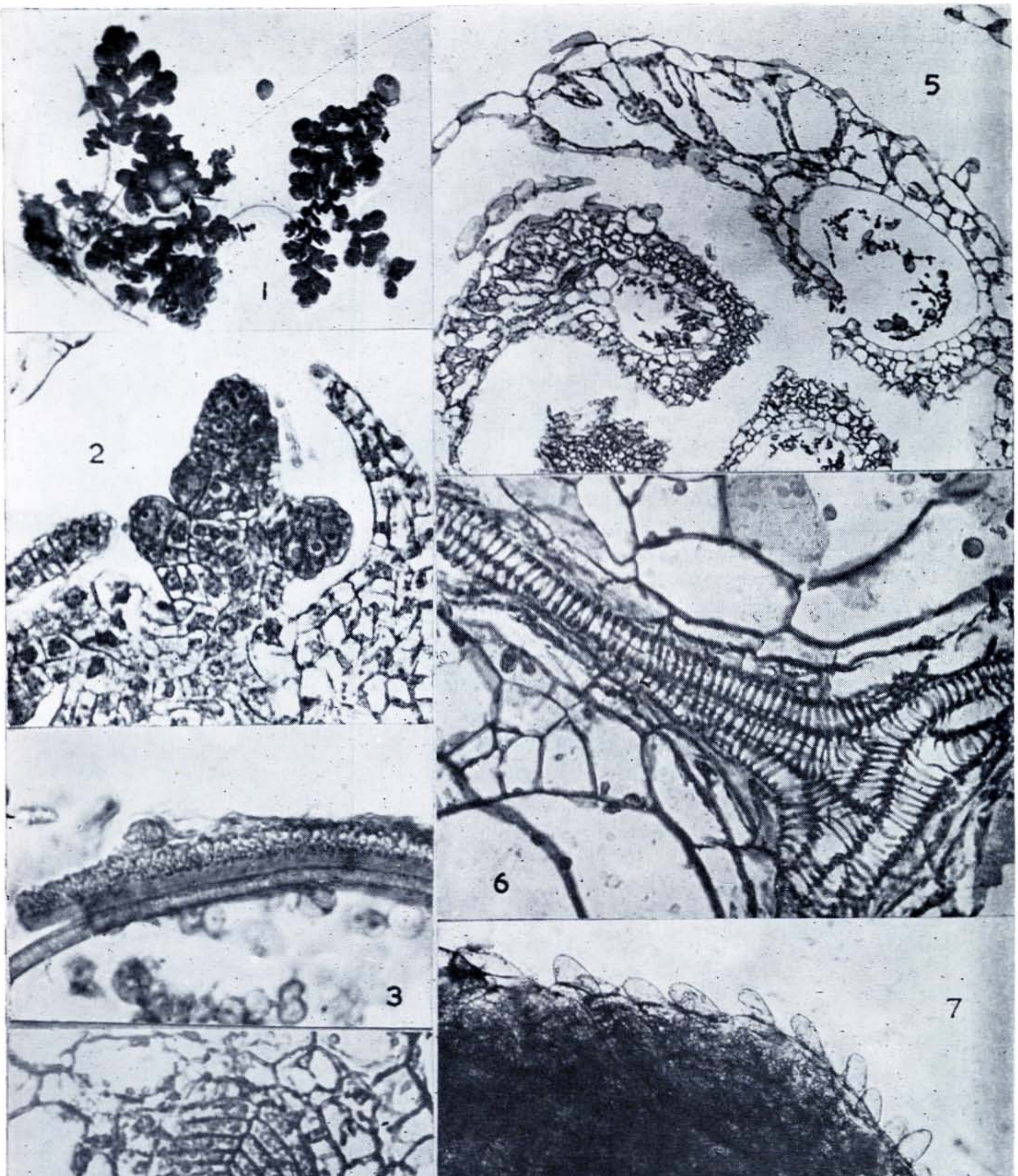
Fig. 3. Section showing megaspore wall and the two layered megasporangial wall, note the vermiform papilla on the outer surface of the sporangial wall. ( ×650)

Fig. 4. Medium longitudinal section of root tip. ( ×500)

Fig. 5. Section of leaves showing the chamber with the algae and an opening of the chamber. ( ×120)

Fig. 6. Vascular tissue of stem. ( ×500)

Fig. 7. A photograph of a portion of the dorsal lobe of a leaf showing papillae. ( ×200)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## PLATE IV

Fig. 1. A sagittal section of the contents of a microsporocarp showing the microsporangia in various stages of maturation, attached to the columella. ( ×100)

Fig. 2. Section showing the apical opening of the microsporocarp and the endophytic Anabaena within the sporocarp cavity near the mouth. The sporangia contain mesullae with imbedded microspores. ( ×120)

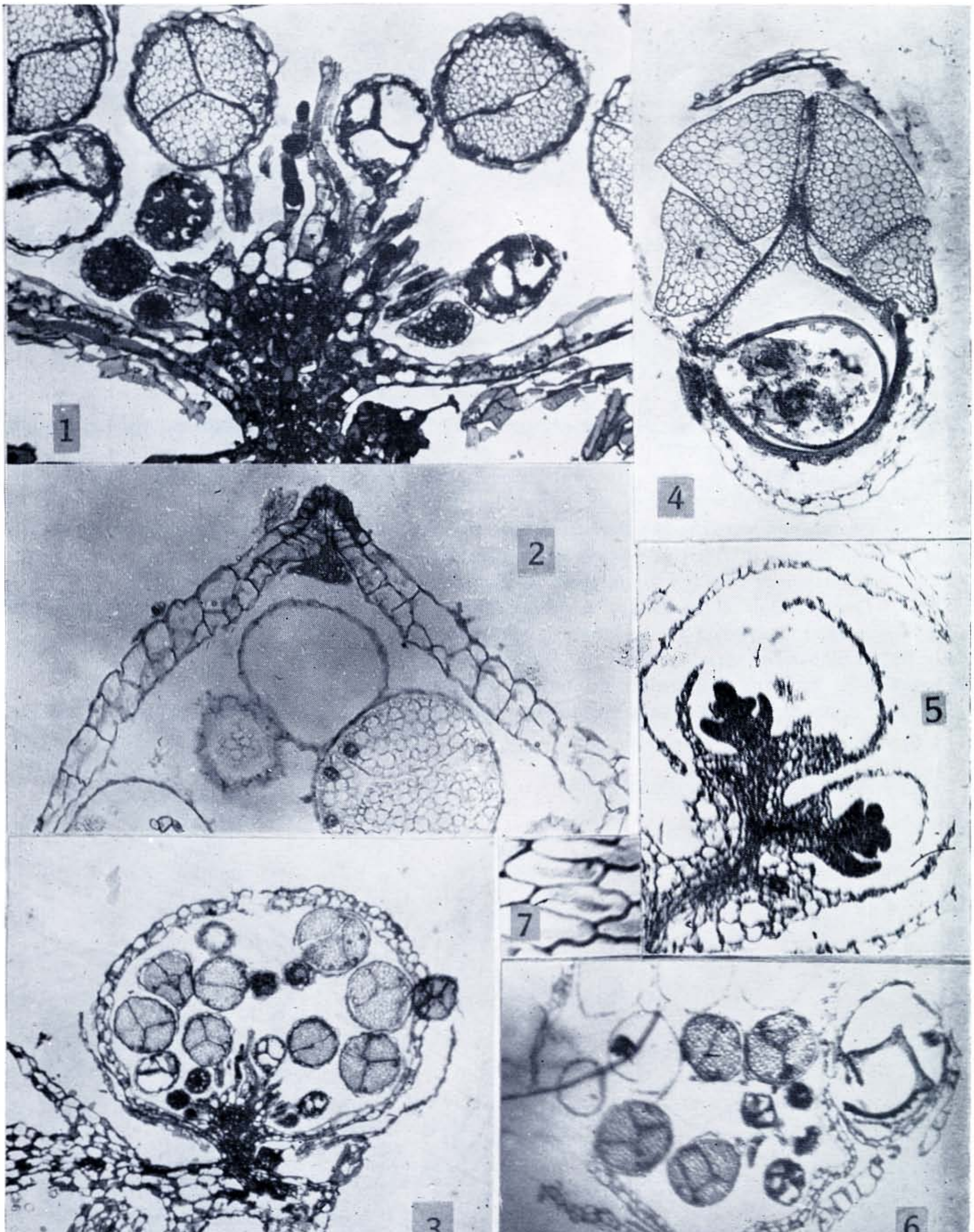
Fig. 3. A medium longitudinal section of a microsporocarp showing the arrangement of the sporangia on the columella.( ×40)

Fig. 4. Longitudinal section of a megasporocarp. ( ×120)

Fig. 5. Sagittal section of shoot apex of Azolla. ( ×100)

Fig. 6. Section of a part of the microsporocarp and a longitudinal view of a megasporocarp. ( ×50)

Fig. 7. A paradermal section of the leaf of Azolla, showing the leaf mis. ( ×250)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## PLATE V

Fig. 1. A portion of the leaf chamber enlarged showing the endophitic Anabaena and the opening of the chamber. ( × 250)

Fig. 2. Section of leaf chamber showing the endophytic Anabaena inside. ( × 100)

Fig. 3. *Anabaena azollae* showing spores and heterocysts. ( × 500)

Fig. 4. Photograph showing the germination of a spore, with spore wall gelatinized and swollen and content divided into small short filament. ( × 700)

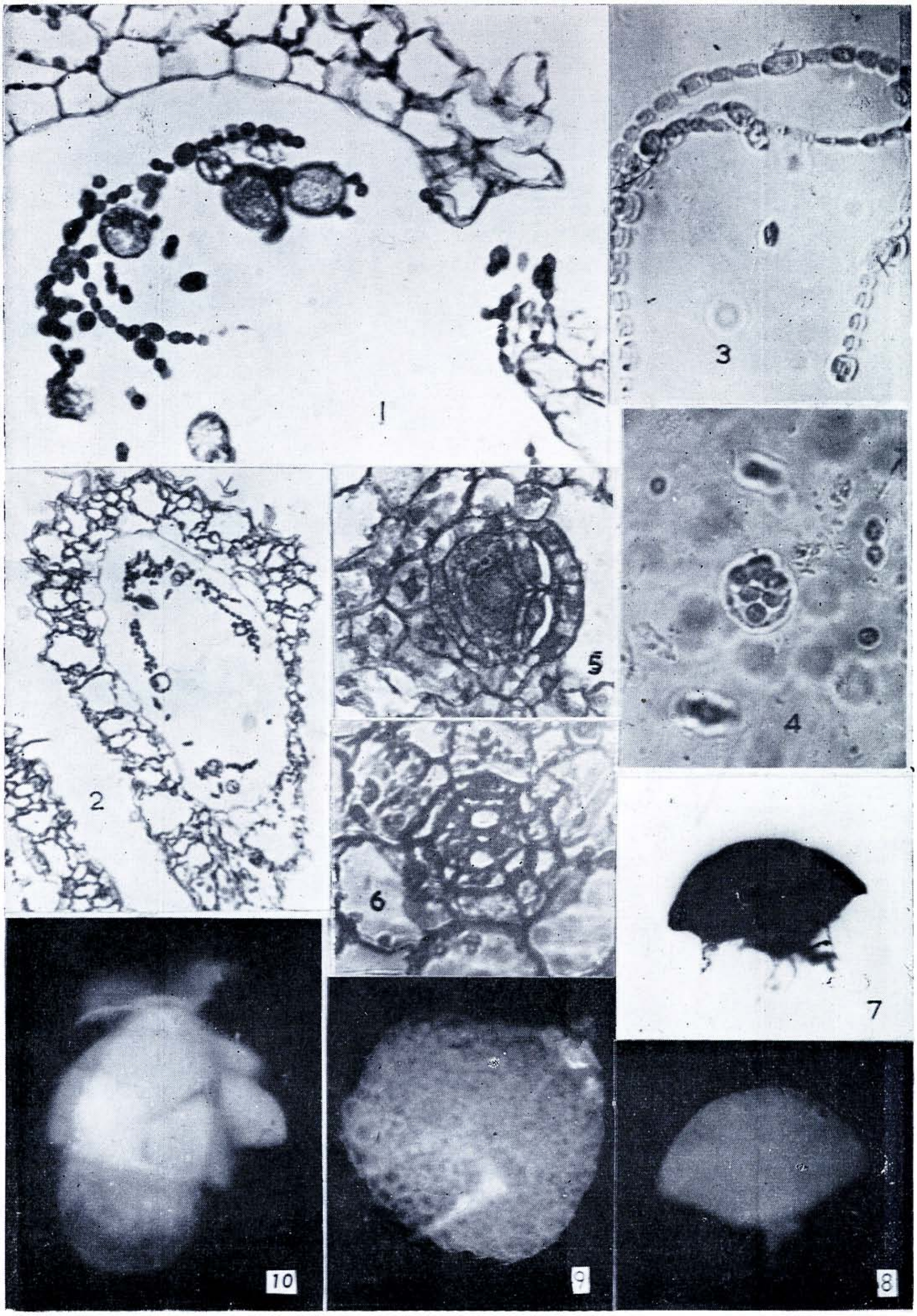
Fig. 5. A picture of medium longitudinal section of a primordium of a root. ( × 500)

Fig. 6. Cross section of the stele of a root showing the diarc arrangement of xylem and the number of cells of the endodermis and pericycle. ( × 500)

Fig. 7. A picture of massula taken under transmitted light. ( × 150)

Figs. 8 & 9. Pictures of massula taken under reflected light. ( × 150)

Fig. 10. A photograph of a megasporangium and its floats taken under reflected light. ( × 80)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