

On the Development of *Azolla filiculoides*, Lam.

BY

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With Plates VII, VIII, and IX.
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THE genus *Azolla*, though a small one, has representatives in all the divisions of the globe, except Europe, and here, according to Belajeff¹, one of the American species, *A. filiculoides*, has been introduced of late years. Of the four species given by Strasburger², two, *A. filiculoides* and *A. caroliniana*, are American; *A. pinnata* is found in Australasia, Asia, and Africa; and *A. nilotica* is exclusively African.

Of the American species, *A. filiculoides*, the subject of the present paper, is confined to the western part of America, being reported from as far south as Chile, and reaching to California at least, and probably beyond. Until very recently American botanists confounded this species with *A. caroliniana* of eastern America, and in the Botany of California³ only that species is mentioned. I have examined material from various parts of California, and in all cases the plants were undoubted specimens of *A. filiculoides*. Whether further

¹ Ueber das männliche Prothallium der Rhizocarpeen; Botanisches Centralblatt, 1892, No. 24.

² Strasburger: Ueber *Azolla*, Jena, 1873.

³ Geological Survey of California. Botany, vol. ii, p. 352.

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examination will show *A. caroliniana* to occur in California, remains to be seen, but its occurrence must at present be regarded as doubtful.

A. filiculoides is common in many parts of California, and often occurs in great quantity, so that the surface of a pond, or a quiet stretch of river, may be completely hidden. As the leaves are strongly tinged with purplish red, the plants are then very conspicuous and recognizable from some distance away.

Observations were begun in November, 1891, and continued, except during the summer, until October, 1892. Most of the material was taken from a pond about a dozen miles from Palo Alto, but specimens were also received from various points south. For some reason the plant disappeared completely from the pond mentioned during the past summer, and in September no trace of it was to be found except the fragments of the dead plants. As the plants were abundant and vigorous the year before at about the same time, it seems hardly likely that this is always the case. Ripe spores that germinated promptly were obtained from the decaying fragments of the plants, and probably will serve to stock the pond again, as the plants spread with great rapidity when once established.

Our knowledge of *Azolla* is based mainly upon the papers of Strasburger¹ and Berggren². Several earlier writers, Griffith, Mettenius, Meyen, and Martius, are referred to by Strasburger, but their works were not accessible. The results of their observations, however, are given by Strasburger in his admirable monograph of the genus.

Strasburger's work deals very exhaustively with the anatomy and histology of the mature sporophyte, and also of the full-grown sporangium and spores. The development of the latter he was not able to follow for lack of material. Berggren's paper deals with the female prothallium and embryo, and while valuable as the only contribution to our knowledge of

¹ loc. cit.

² Om Azollas prothallium och embryo. (Lund's Univ. Arsskrift. t. xvi.)

the subject, is very incomplete, especially with reference to the early stages of the former. Belajeff¹ has published an account of the male prothallium of *A. filiculoides* in a recent paper on the male prothallium of the Rhizocarpeae.

The same methods were used by me in the study of *Azolla*, that I have found most successful in the study of other delicate plant-tissues. The material was fixed with a 1 per cent. aqueous solution of chromic acid, stained *in toto* with alum-cochineal, and then imbedded in paraffin. After sectioning, the sections were stained on the slide with Bismarck-brown in 70 per cent. alcohol. As yet I have found no other method which gives such good results.

Before considering the development of the sporangia and spores it may be well to describe briefly the structure of the mature sporophyte. As Strasburger has treated this very exhaustively in the memoir referred to, it will not be worth while to go much into detail here. The plant is strongly dorsiventral; the leaves form two alternating rows completely concealing the stem. Each leaf is deeply two-lobed, and in the dorsal lobe is a large cavity in which is always found a colony of a *Nostoc*-like plant, *Anabaena Azollae*.

The growing-point of the stem is curved upward and backward, so that longitudinal sections parallel to the surface are very difficult to get. The stem grows from a single apical cell (Pl. VII, Figs. 2-4, ×), from which two series of segments are cut off with great regularity. Each segment now divides into a dorsal and ventral cell, so that a transverse section of the stem, just back of the apex, shows usually four cells arranged like quadrants of a circle (Fig. 5, I.). From the dorsal cells the leaves are developed: from the ventral, the lateral branches and roots. An examination of the growing-point of the stem shows that it is more or less surrounded by a tangle of *Anabaena*-filaments, and some of them creep into the cavities in the young leaves and form the beginning of the colony referred to.

The mother-cell of a leaf is distinguishable by its size and

¹ loc. cit.

position (Fig. 5, III. L.); and the first division-wall in it divides it into two nearly equal cells, which develop into the two lobes found in the full-grown leaf. No trace of an apical cell can be found even in the youngest leaves, and in this respect, as well as in the secondary divisions of the segments of the apical cell, *Azolla* differs from *Salvinia*, its nearest relative. Each leaf-lobe is next divided into an inner small cell, and an outer larger one, and the latter then is divided into two equal cells by a radial wall. This formation of alternate tangential and radial walls is repeated with great regularity in the ordinary leaf-lobes, and in their young stages can be traced for a long time.

The sporocarps or sori always arise, as Strasburger showed¹, from the ventral lobe of the first leaf of a branch. He was not able to get the earliest stages owing to lack of material, and based his conclusions upon a study of the later stages. According to his statement they arise from a part only of the ventral lobe, the rest giving rise to the enveloping involucre. From a careful study of the very young stages I have been led to a somewhat different conclusion. I find that the whole of the ventral lobe goes to form the sori, and that the involucre is derived from the whole of the dorsal lobe of the leaf. The latter, instead of developing as in the sterile leaves, remains but one cell thick, and forms a sort of hood arching over the sori. The latter are always found in pairs in *A. filiculoides*. These are sometimes of the same kind, or one may be male and the other female.

The leaf-lobe which is to develop into the sporocarps is distinguishable at an extremely early period. Its first divisions are like those in the sterile lobes, and like them it is divided into two very nearly equal parts. Each half now develops at once into a sporocarp. As soon as the first median wall is formed, each of the resulting cells becomes the initial cell of the future sporocarp. In it walls are formed that cut off three segments from its base, and these are followed by others following the same order, so that for some

¹ loc. cit. p. 52.

time each sporocarp-rudiment grows by a three-sided apical cell (Fig. 8). Next a slight outgrowth is observed near the base of the young sorus, which forms a ring-shaped projection around it (Fig. 9 *id.*); this is the beginning of the indusium or sporocarp-wall, and corresponds exactly to that of *Salvinia*¹.

From this point the two sorts of sporocarps differ. In the macrosporic ones the apical cell forms at once the body of the single sporangium; in the microsporic, it forms a columella, from which latter the microsporangia arise as lateral outgrowths. My own observations in these earlier stages were confined mainly to the female sorus, but there was nothing to indicate any difference in the development of the male, except in the sporangia themselves.

There has been more or less conjecture concerning the origin of the single macrosporangium, whether it was really the only one formed, or whether several were formed at first and one crowded out the others. A glance at a young sporocarp will show at once that only one sporangium is formed at first, and that this is formed directly from the apical cell of the sporocarp-rudiment. After a varying number of segments have been cut off, a periclinal wall is formed in the apical cell, which then proceeds at once to form the body of the sporangium. At this stage (Fig. 9) the sporangium has a very short pedicel, and the archesporium has the familiar tetrahedral form common to the other Leptosporangiatae. The next divisions follow closely those of the other Leptosporangiatae, and offer nothing new. The tapetum (Fig. 10 *t*), is formed as usual, and, coincident with its formation, radial walls are formed in the outer cells of the sporangium, whose wall, as usual, remains but one cell thick. The tapetum also divides only by radial walls, so that it too consists of but one layer of cells (Figs. 12-15). These cells, as well as the central cell, contain more granular protoplasm than do the cells of the sporangium-wall.

The divisions in the central cell correspond with those in other Leptosporangiatae. The first wall is nearly vertical, and this is followed by a transverse wall in each of the resulting

¹ Strasburger; Ueber *Azolla*, p. 54.

cells. Finally, in each of these four cells, another wall arises, so that eight cells in all are formed. While these divisions have been going on, numerous radial walls have arisen in both tapetum and sporangium-wall, the former being especially numerous.

Shortly after the divisions are completed in the central cell and tapetum, the cell-walls of the latter are dissolved. At first the group of spore-mother-cells remain together; but, finally, their walls are sufficiently dissolved to completely isolate the eight cells, which are then surrounded by the fused contents of the tapetal cells. Each of the eight spore-mother-cells, as usual, gives rise to four spores. In *Azolla* these are of the tetrahedral type.

At the time that the spore-mother-cells are about to separate, the nucleus is large, but contains but little chromatin and consequently does not stain deeply. The nucleolus is much less conspicuous than in the earlier stages, and after the cells are completely separated it becomes scarcely distinguishable.

The divisions of the nucleus can be traced without much trouble, but owing to the small amount of chromatin, and the correspondingly small size of the nuclear filaments, the karyokinetic figures are small, and the details difficult to follow. As there did not seem to be any deviation from the process as seen in the division of the spores of other plants, no special observations were made. The nuclear spindle is clearly defined, but small (Fig. 18 *b*); after the first nuclear division is completed, the daughter-nuclei divide again before any division in the protoplasm is to be seen. Then follows the simultaneous division of the protoplasm into the four young spores (Figs. 19, 20). Of the thirty-two young spores thus formed, only one comes to maturity, and the others are used up in its growth.

The spore which is to form the macrospore increases rapidly in size. It is at first a thin-walled oval cell, which lies free in the enlarged cavity of the sporangium. Examination shows that it is surrounded with a thick layer of densely granular protoplasm, in which may be plainly seen a number of nuclei,

evidently those of the tapetal cells (Fig. 21). It is evident at a glance that these nuclei are directly concerned in the growth of the spore, and that they play an important part in the formation of the extraordinary appendages of the ripe spore. Unfortunately, owing to my absence during the summer, I failed to get the later stages in the development of the macrospore, but to judge by analogy with the microspores, and also by what is known in the case of the Marsiliaceae, the outer spore-coverings are derived from the protoplasmic mass in which the macrospore is imbedded. This protoplasm is evidently active, as is shown by the presence of normal nuclei, as well as by its increase in bulk as the sporangium increases in size.

When the sporangium is about half grown, the outer cells of the very short stalk grow out into short papillae (Fig. 16 *sp.*), which apparently are abortive sporangia, as they show divisions which recall the earlier ones in the macrosporangium. Their position corresponds to that of the microsporangia, so that although formed much later than the macrosporangium, it is pretty safe to assume that *Azolla* is derived from some form in which, as in *Salvinia*, there were several macrosporangia in the sporocarp.

The indusium grows much faster than the sporangium and soon completely encloses it. It grows mainly by the activity of the marginal row of cells, in which divisions are cut off alternately upon the inner and outer sides. After reaching above the top of the sporangium the edges converge, and this, together with an enlargement of the marginal cells, finally completely closes the opening.

Before the opening closes, filaments of the *Anabaena* creep in and form a mass filling all the space between the top of the sporangium and the opening of the sporocarp. Here the cells separate completely, and the plant enters a resting-stage to resume its activity on the germination of the macrospore.

In studying the development of the macrosporangium, I could not but be struck by the extraordinary resemblance to that of an ovule. The close investment of the sporangium

by the indusium, and the origin of the latter from so near the base of the sporangium, at once suggest the possibility of a homology between the indusium and the first integument of the ovule. Prantl¹ has advanced the view that an ovule might be regarded as a monangic sorus, and the integument as homologous with the indusium. So far as I can find, *Azolla* has not been considered in the arguments for and against this view, but it certainly supports strongly the view held by Prantl². Of course this does not imply a direct relationship between *Azolla* and the Spermaphytes, as the sporangia themselves are widely different, *Azolla* being typically leptosporangiate, while the Spermaphytes are eusporangiate. Nevertheless, in view of all the facts bearing on the matter, it hardly seems necessary to regard the ovular integument as an entirely new structure, without any equivalent among Pteridophytes.

The ripe female sporocarp is about 1.5 mm. × 1 mm. in size, and strongly pointed at the top. Its outer cells become hardened, and those of the upper half very dark-coloured and lignified, so that after the lower part decays, these upper, dark-coloured cells remain as a little cap that covers the spore until it is thrown aside by the growth of the embryo. The inner cells of the wall remain thin, and become very much compressed by the growth of the sporangium which finally fills the sporocarp completely. The wall of the sporangium does not, however, as stated by Strasburger³, become absorbed. On the contrary, it can be plainly seen in sections of the full-grown sporocarp (see Pl. VIII, Fig. 38), but the cells are much flattened, and unless carefully examined might be taken for the inner layer of the indusium, which is compressed so as to be almost obliterated.

A longitudinal section of the full-grown sporocarp (Fig. 35) shows that the spore⁴ with its curious appendages fills the

¹ Untersuchungen zur Morphologie der Gefässkryptogamen: Die Schizaeaceen, p. 154.

² Since the above was written, I find that Eichler has called attention to the resemblance between the sorus of *Azolla* and an ovule: see Engler and Prantl, Die nat. Pflanzenfam. II. Theil, 1. Abth. p. 16.

³ loc. cit. p. 71.

⁴ Sometimes two spores are found in one sporangium.

sporangium completely, and that the latter is in close contact with the indusium except at the top, where there is a space between filled with the resting cells of the *Anabaena* (*n*). The sporangium-wall at this point (Fig. 35 *sp.*) is perfectly plain, but the cells have collapsed so that the separate cells are not very easily distinguishable. Near the base of the sporangium, however, they may generally be very easily seen, especially when, as often happens in sectioning, the wall is pulled away from the indusium. (See Fig. 38 *sp.*)

The curious episporic appendages of the macrospore have been frequently described, but the homologies of the parts have not been entirely understood. The ripe spore is perfectly globular and surrounded by a firm yellowish exospore, which appears almost perfectly homogeneous in section. Upon this is deposited a thick episporic of most peculiar form. This is covered with cylindrical papillae from the top of which numerous curious threadlike filaments extend. In section the episporic shows two distinct portions, an inner mass resembling exactly the substance of the massulae, and a denser part that covers the outside except the tips of the papillae. This outer part is solid and nearly homogeneous, in places densely granular. The episporic covering the top of the spore is developed in a most extraordinary manner. It consists here of four parts; a central conical part, and three somewhat pear-shaped masses that are partly sunk in shallow cavities in the sides of the central portion. To these Strasburger applied the name 'Schwimmapparat,' supposing them to be filled with air, and to thus raise the spore to the surface of the water. Repeated experiments with perfectly ripe spores, both before and after they had been freed from the indusium, resulted invariably in the spores sinking immediately, as was the case with the ripe massulae. This being true, the name must be abandoned as misleading. From the conical mass, as well as from near the apex of the others, the filaments, like those growing from the papillae of the lower part of the spore, are produced in great numbers. In the spaces between the masses, even in the full-grown

sporangium, the remains of the tapetal nuclei (Fig. 36 *n*) may be seen occupying much the same position with reference to these that those of the microsporangium do to the massulae. This, together with the similarity in the structure of the epispore of the macrospore, and the massulae, warrants the conclusion that the two are homologous structures. The threads attached to the epispore may morphologically as well as physiologically be compared to the glochidia.

Sometimes, but not always, a cleft may be seen extending upward part way through the central conical mass, but in no cases is there such an open canal as described and figured by Berggren¹ for *A. caroliniana*.

THE MICROSPORANGIA.

In the male sorus, as we have already seen, the apical cell of the young rudiment does not form a sporangium, but gives rise to a central columella or placenta, from which the microsporangia arise laterally, while the end projects as a cylindrical body (Fig. 22 *col.*). This latter was observed by Meyen², but Strasburger seems for some reason to have overlooked it. I found it in all my sections of the male sorus. As in the female sorus, the indusium is two cells in thickness, but the cells have their walls more uniformly thickened, and the inner layer is not compressed as in the female sorus; as in the latter, the opening at the top becomes completely closed, and the cells about it are thicker walled than the lower cells, and reddish brown, as in the upper cells of the indusium of the female sporocarp. Like that, too, the top is pointed, but the point is short and abrupt, and the body of the sporocarp is globular. They are more than twice as large as the female sporocarp. Both sorts of sporocarp have a very short stalk, into which a fibrovascular bundle extends for a short distance.

The development of the microsporangium corresponds closely to that of the macrosporangium, but differs in some

¹ loc. cit. p. 1; also, Fig. 1.

² See Strasburger, loc. cit. p. 57.

respects, especially in the later stages. While the macrosporangium has a very short massive pedicel, that of the microsporangium is long and slender, usually composed of two rows of cells, but not infrequently showing three. Up to the third division of the central cell of the sporangium the divisions are exactly as in the macrosporangium; but in the microsporangium there is one more division and consequently sixteen spore-mother-cells. The sixty-four spores that result from the division of these, all develop more or less completely, and about each is formed a smooth, thin, yellowish exospore. The ripe spore is about .035mm. in diameter, and its apex shows plainly the usual three radiating lines. The full-grown microsporangium (Fig. ^{*}26) is globular, its walls formed of tabular cells all about alike. I was unable to detect anything that looked like the annulus of the homosporous leptosporangiate Ferns. The complete disappearance of the annulus is no doubt attributable to the aquatic habit of *Azolla*.

When the spores are nearly mature, the formation of the massulae or masses of hardened protoplasmic matter in which the ripe spores are imbedded, begins. The spores collect in several groups (usually about five), and about these the protoplasm lying between them becomes aggregated. Apparently vacuoles are formed in this giving it a foamy appearance, and finally these become so large as to give the massula the appearance of a cellular tissue (Figs. 24, 27, 28). In *Salvinia*, where there is no division into massulae, according to Strasburger's¹ account, the nuclei of the tapetal cells are scattered uniformly through the protoplasm lying between the spores; but in *Azolla*, they are confined to the outside of the massulae, where they can be readily detected almost up to the time of the ripening of the sporangium. As the massulae mature there are formed upon the outside the *glochidia*, curious hair-like appendages with anchor-like tips. They are formed in the spaces between the massulae, and their flattened form is due to the narrowness of these spaces. The presence of the unchanged tapetal nuclei about the forming *glochidia*

¹ Bau und Wachstum der Zellhäute, p. 133.

(Fig. 24 *n*), indicates that, like the appendages of the macrospore, these too are formed, in part, at least, by the activity of the tapetal nuclei. In *A. filiculoides* the glochidia are undivided, except occasionally toward the tip, where one or two septa may sometimes be detected.

When the sporocarp of *Azolla* is compared with that of the other Hydropterideae, its nearest approach is found in *Salvinia*, with which it agrees closely in its origin and structure. Each sporocarp is a single sorus with a cup-shaped, completely closed indusium, while in the Marsiliaceae the sporocarp represents a whole leaf-segment with several sori. Among the homosporous Ferns, some of the Cyatheaceae and Hymenophyllaceae (especially *Trichomanes*), show very marked resemblance to the Salviniaceae in the position and form of the indusium.

GERMINATION OF THE MICROSPORES.

The study of the germinating spores offers great difficulties, as they are completely imbedded in the massulae, and cannot be freed in the living state. Belajeff¹, who has recently published some observations upon the male prothallium of *A. filiculoides*, treated the massulae with chromic acid which rendered them brittle enough to be broken in fragments, thus setting free the prothallium. My own observations were made mostly from sections. As the massulae adhere to the macrospores, in making sections of the latter, the massulae, with their enclosed spores, were also sectioned. Of course in this way it is impossible to regulate the direction in which the sections are made, but enough straight sections were obtained to give a clear idea of the development of the antheridium. In the material used by me, only a small percentage of the microspores seemed perfectly developed, and in consequence, the number of male prothallia found was very small compared to the number of massulae sectioned. As the germinating spores are completely buried in the massula, it is very difficult to judge of the state of develop-

¹ loc. cit.

ment, and I was unable to find fresh specimens with ripe antheridia and so failed to see the free spermatozoids; otherwise my observations on the antheridium were fairly complete.

The indusium decays slowly and the sporangia are thus set free, after which the wall of the latter also decays, and the massulae escape into the water. Contrary to the statements usually made, it was found that the ripe sporangia, and also the massulae themselves, sink at once to the bottom when placed in clear water. When entangled in the remains of the plant, it is true that they float, but this is not due to their own buoyancy, but to the air in the tissues of the stem and leaves.

As soon as the sporangium-wall is decayed, the glochidia stand out straight from the surface of the massulae. As they come in contact with the macrospore, they fasten themselves to it by means of the anchor-like ends of the glochidia.

The contents of the microspores are not very dense, and they contain but little granular matter. The nearly central nucleus, which is not very rich in chromatin, shows a more or less conspicuous nucleolus. The first indication of germination is the rupturing of the exospore along three radiating lines at the top, and the protrusion of a papilla through this (Fig. 29). This papilla is then cut off by a wall near the top of the spore-cavity, and forms at once the mother-cell of the single antheridium. The next divisions were not satisfactorily made out. According to Belajeff¹, the next divisions are nearly parallel to the first and divide the antheridium into three cells, one above another, and of these only the middle one undergoes further division. For some reason which is not clear from his account, Belajeff does not regard the whole of the upper cell as an antheridium, but states that the latter is only formed after five vegetative cells are cut off. It seems much more in accordance with what obtains in the related homosporous forms to regard the whole of the upper part of the prothallium as an antheridium. In spite of his statement

¹ loc. cit. p. 329.

that the development of the male prothallium has little in common with that of the true Filices, the figure of the prothallium of *Azolla* given¹, bears a very striking resemblance to the simple male prothallium of many Polypodiaceae, for instance. The small cell, cut off subsequently from the large basal one, as described by him, I failed to see in any of my sections. No indication of marked dorsiventrality, as he states, was noticed either. This may possibly have been due to slight shrinkage in imbedding, by which the central part of the prothallium was a good deal more constricted than it probably is in life. My own conclusion, reached after a careful study of a large number of prothallia, is that there is but a single vegetative cell formed (from which possibly later a small cell may be cut off), and that the rest of the prothallium forms at once a single terminal antheridium.

The subsequent divisions, as observed by me, correspond essentially with those given by Belajeff. In the middle cell of the antheridium two nearly vertical walls are formed, and with the upper cell (Fig. 31 *o*) completely enclosing the central cell of the antheridium. The cell (*o*) recalls in form and position the opercular cell of the antheridium of the Polypodiaceae, but apparently is formed here before the central cell is cut off. In one of the lateral cells a horizontal division is usually (perhaps always) formed, so that the central cell is surrounded by five parietal cells, one basal (*b*), one apical (*o*), and three lateral ones. The central cell now divides by an approximately vertical wall, and these cells divide twice by walls at right angles to each other, so that eight sperm-cells are formed. From the nucleus of each cell, in the usual way, the body of the spermatozoid is formed. In some cases it looked as if only four sperm-cells were formed; but this was not certain. The dehiscence of the antheridium and the free spermatozoids were not seen, but probably the latter resemble those of *Salvinia*. To judge from the appearance in the nearly ripe antheridium, they do not possess

¹ loc. cit. Fig. 2.

more than two complete coils. Their small size renders them unfavourable for a study of the details of development, and no attempt was made to study these.

The ripe prothallium remains completely imbedded in the substance of the massula (Fig. 28), and probably the spermatozoids escape by a softening of the outer surface of the massula which has a corroded appearance in microtome-sections, quite different from the distinct outline of the younger ones.

A comparison of the antheridium with that of other forms does not show a very close resemblance to any. From *Salvinia* it differs in the complete surrounding of the sperm-cells by the parietal cells, and in the separation of the sperm-cells into two groups in the latter. Among the homosporous Ferns, the antheridium of the Hymenophyllaceae, perhaps, resembles it most nearly, especially in regard to the arrangement of the parietal cells. In some cases a triangular opercular cell was observed which, from its position, looked as if it had been formed subsequent to the formation of the vertical walls, much as in *Osmunda*.

GERMINATION OF THE MACROSPORES.

The study of the germinating macrospores involves various difficulties. First, to collect a sufficient number it is necessary to collect a large number of plants, as each fertile one furnishes usually only one or two spores, and only a comparatively small number of plants have them at all. The spores only germinate after they have been set free by the decay of the indusium, and the best way to get a supply is to collect a number of fruiting plants and allow them to remain in water until the fertile branches die. These will then finally sink to the bottom of the vessel, and may be picked to pieces and the spores separated. Spores secured in this way will usually germinate promptly, but there is considerable difference in this respect; and, as there is nothing to indicate whether or not germination has begun, it was only by making repeated sowings and sectioning a very

large number of spores that, finally, it was possible to get all of the stages. There is a good deal of difficulty in satisfactorily sectioning the youngest stages, too; but a sufficient number of successful preparations were finally secured to make out clearly the earliest divisions in the prothallium, which Berggren, who alone has studied the female prothallium, failed to get. Berggren's account of the prothallium is extremely imperfect, and is confined entirely to the later stages, and it was largely to determine the early stages that the work was first undertaken.

The ripe macrospore does not become entirely free from the sporangium, the upper part of which remains covered by the cap-shaped upper part of the indusium. The more delicate lower part of the indusium, and the sporangium-wall, rots away and leaves the episporium exposed. From this the filamentous appendages stand out, very much as the glochidia do from the massulae; and when the latter come in contact with the macrospore, the anchor-like ends of the glochidia become entangled in the filaments, and the massulae remain thus firmly attached to the macrospore. Of course this brings the germinating microspores close to the macrospore and facilitates fertilization.

The most prompt germination of the macrospores was found in material gathered in early autumn, which is probably the ordinary time for germination. Spores collected late in November germinated, but less promptly. In all cases there is a good deal of variation, so that it is impossible to state positively just how long is required. In a few cases, within eleven days from the time that the spores were freed from the plants and placed in fresh water, the young plants had already broken through the prothallium, and usually within two weeks this was the case. Probably the first divisions of the prothallium may occur within two or three days, and the whole development be completed within a week, but this is only an approximation, as there is no means of telling the stage of development without killing the prothallium.

A section of the ripe spore, while still within the sporangium, shows its contents to be nearly uniform. The granular protoplasm is arranged somewhat reticulately, and in the living spore there is a good deal of oil, which is dissolved out in the process of imbedding. Besides this there are numerous albuminoid bodies of varying sizes, that stain deeply with alum-cochineal. The nucleus (Fig. 40 *n*) is at the top of the spore-cavity, and is not at all conspicuous. It is somewhat elongated and quite uniform in structure, apparently having very little chromatin, and scarcely staining at all. No nucleolus can be seen.

The first indication of germination is an increase in the size of the nucleus, which becomes nearly globular, and, at the same time, it shows more coarsely granular contents, and a small nucleolus becomes evident (Fig. 41). At this time the cytoplasm in the vicinity of the nucleus becomes free from large granules, and this is the first indication of the position of the mother-cell of the prothallium.

While we have no observations on the first divisions in the prothallium of *Azolla*, there have been some partial observations on that of the related *Salvinia*. Prantl¹ succeeded in demonstrating that the first division was by means of a distinct cell-wall, and that there was no formation of free cells such as Juranyi² describes. Prantl, however, failed to get the immediately following stages, although from a study of the older prothallia he was able to tell with considerable accuracy the succession in which the earlier walls were formed.

The earliest stage obtained by me showed the very recent separation of the prothallium mother-cell (Fig. 42). This was a small lenticular cell, whose contents were more uniformly granular than that of the body of the spore. The division-wall was delicate, but easily seen. The nucleus stained deeply, much more so than that of the undivided spore, and had a much larger and more distinct nucleolus. The other nucleus was

¹ Zur Entwicklungsgeschichte des Prothalliums von *Salvinia natans*, Bot. Zeit. 1879, p. 425.

² Ueber die Entwicklung der Sporangien u. Sporen der *Salvinia natans*, p. 18.

quite as large and closely resembled it. In this respect *Azolla* offers a strong contrast to *Marsilia*¹, where the prothallial nucleus is much larger than that of the spore. How it compares with *Salvinia* in this respect can only be known after stained sections of the latter have been studied. Berggren² failed to demonstrate the presence of a primary division-wall, and figures the earlier stages of the prothallium as having the lower cells of very indefinite form with no distinct wall separating them from the spore-cavity. The first division-wall in the prothallium seems to correspond with that in *Salvinia*. This is a vertical wall (Fig. 44 I-I), and divides the cell into two cells of unequal size. In *Salvinia*, according to Prantl³, the former cell remains sterile, while in *Azolla* it also may produce archegonia, although later than the rest of the prothallium. In a very young prothallium, having but three cells (Fig. 43), the next wall was also nearly vertical, but in other cases (Fig. 46) it looked as if this were not always the case, but that, as in *Salvinia*⁴, the second wall was horizontal and divided the larger cell into two nearly equal ones. From the upper one the first archegonium is developed at a very early stage. Its position varies a good deal, depending apparently upon the position of the first division-wall in the prothallium, and also upon the time when the first horizontal wall is formed. If the latter is formed early, the first archegonium is nearly central, but if this is not formed until after two vertical walls have been produced, the archegonium is nearer the side opposite the first cell cut off. In the few cases where successful cross-sections of very young prothallia were made, the archegonium mother-cell was decidedly triangular in outline, indicating that it is cut off by the walls meeting at nearly equal angles (Fig. 52). It is easily distinguished in the very young prothallium by its denser contents that stain more strongly than those of the surrounding cells. The archegonium-mother-cell divides now into two by a transverse wall, the lower of the

¹ Campbell. On the Prothallium and Embryo of *Marsilia vestita*, Proc. Cal. Acad. Sci. 1892, p. 196.

² loc. cit. p. 2.

³ Prantl, loc. cit. p. 427.

⁴ loc. cit. p. 429.

two cells giving rise directly to the egg and the canal-cells, the upper to the neck, no basal cell being formed. In this it agrees with the other heterosporous Pteridophytes.

Up to the time that the first division in the archegonium is completed, the whole prothallium has increased somewhat in size, but this has been entirely at the expense of the spore-cavity, and the exospore has remained intact. The central cell of the archegonium is separated by a single layer of cells only, from the spore-cavity. The young prothallium at this stage (Fig. 47) recalls quite strongly that of *Pilularia* at a similar stage, but also agrees closely with Prantl's account of *Salvinia*. Berggren's figures¹ of prothallia of *A. caroliniana*, at a stage presumably about the same, are too diagrammatic to allow of a satisfactory comparison. They represent the prothallium as composed of perfectly uniform cells arranged in rows converging at the top, where a very small archegonium mother-cell is shown. The whole is totally different from anything observed in *A. filiculoides*.

Shortly after the first division in the archegonium, a rapid increase takes place in the size of all the cells of the prothallium, by which it expands and ruptures the exospore, which breaks open at the top into three lobes corresponding to the three converging lines that mark it at that point.

The most remarkable difference observed between *Azolla* and the other Hydropterideae is the history of the lower of the two nuclei resulting from the division of the primary nucleus of the macrospore. In the Marsiliaceae this remains undivided, and in the later stages seems to become more or less completely disorganized. In *Azolla*, however, where it is quite equal in size to the nucleus of the prothallium mother-cell, it undergoes repeated division, the resulting nuclei remaining imbedded in the protoplasm of the upper part of the spore-cavity, in close proximity to the cells of the under side of the prothallium (Fig. 47 *n*). While the albuminous granules become larger in the lower part of the cavity, the upper nucleated protoplasm loses them almost entirely, and in

¹ loc. cit. Figs. 7 and 9.

stained sections contains only very small colourless granules. The amount of this finely granular protoplasm increases very much in quantity as the prothallium grows. This nucleated protoplasm is evidently concerned in the elaboration of the reserve food-stuff in the spore, in order to facilitate its absorption by the growing prothallium, and later by the embryo. These nuclei have a small nucleolus, and are quite rich in chromatin. The nuclei usually remain free in the protoplasm, but in exceptional cases indications of cell-formation were seen, resembling closely the secondary 'endosperm' in the macrospore of *Selaginella*, and no doubt homologous with it (Fig. 53 *en*). Nothing of a similar kind is known to exist elsewhere; although, in all probability, a careful examination will show the same state of things to obtain in *Salvinia*. That a similar behaviour of the nucleus is not found in the Marsiliaceae, may perhaps be explained by the extremely rapid development and small size of the prothallium in the latter, and the more intimate connexion of the embryo with the cavity of the spore.

The base of the prothallium, which at first is strongly convex, gradually becomes straight as the basal cells expand laterally (Fig. 49), and later, with the vertical growth of the cells, becomes strongly concave, this being especially marked in the older prothallia that have remained unfertilized (Fig. 60).

At the time that the first archegonium is ripe, the prothallium seen in longitudinal section appears nearly hemispherical, but somewhat narrower at the base owing to the lateral growth of the middle cells (Fig. 51). The central cell of the archegonium is separated by but one (or occasionally two) layers of cells from the spore-cavity, and the neck projects considerably above the upper surface of the prothallium. But very little chlorophyll is to be seen at this stage, and even in the older prothallia but very little is found as compared with *Salvinia*, or with the old unfertilized prothallia of the Marsiliaceae.

As the growing prothallium pushes up, it penetrates the

central conical mass of the epispore at the top of the spore, which here seems to become soft; Fig. 50 shows a cross-section of this mass with the top of the archegonium showing through the ragged triangular opening in the epispore. In this way the opening of the archegonium is left free for the entrance of the spermatozoids.

Cross-sections of the prothallium are more or less decidedly triangular, with one angle longer than the others (Fig. 54). This longer angle corresponds to the 'sterile third' of the prothallium of *Salvinia*, and represents the first cell cut off from the prothallium-mother-cell.

In case the first archegonium is fertilized at once, no others seem to be formed; but in the great majority of cases examined by me, the first archegonium was not fertilized, and a varying number of secondary ones had been formed. The first of these arises close to the primary one; indeed, its central cell is generally separated from that of the primary one by but a single layer of cells. The third arises near the base of the larger lobe (Fig. 54 a 3). In case all of these remain unfertilized, others arise between them, apparently without any regularity, as any superficial cell apparently can give rise to an archegonium. Nothing resembling the regular meristem of *Salvinia* could be detected, and after the first three, the other archegonia seemed to arise indifferently at any point in the upper surface of the prothallium.

THE ARCHEGONIUM.

The archegonium-mother-cell becomes early distinguished by its larger size, denser contents, and larger nucleus, from its neighbouring cells. It varies a good deal in size and shape, and the later-formed ones are decidedly smaller than the first and second. Sometimes the mother-cell is short and square at the bottom, and sometimes deep and narrow with a pointed lower end. Its development corresponds closely to that of the other Filicineae, especially *Salvinia* to which it bears a very strong resemblance. As in the primary archegonium, no basal

cell is formed, but the first division separates the neck from the central cell (Fig. 56). The neck-canal-cell is formed much earlier than given by Pringsheim for *Salvinia*¹, and is cut off from the central cell about the time that the vertical walls in the neck-cell are formed. The wall by which the neck-canal-cell is separated from the central cell, is concave, but becomes nearly straight as the neck lengthens. Each of the four primary neck-cells divides into four as in *Salvinia*, and like it, the divisions are completed before any marked elongation of the neck is noticeable. The ventral canal-cell is cut off by a very strongly curved wall, and sometimes, if not always, before the divisions in the neck-cells are completed (Fig. 58 *b*). The nucleus of the neck-canal-cell may undergo division, very much as in the homosporous Filicineae and *Isoetes* (Fig. 58). This has not been recorded for *Salvinia*. Whether it always occurs in *Azolla* it is impossible now to say. After the divisions are completed the neck rapidly lengthens and projects strongly above the surface of the prothallium, resembling much more that of the homosporous Filicineae than it does that of the Marsiliaceae.

A curious fact noted was the retention for a long time of an apparently normal structure of the protoplasm and nucleus of the central cell of the unfertilized archegonia. Instead of shriveling up, and the walls of the central cell turning brown as is usually the case, the cell remained turgid, and the protoplasm and nucleus retained much the same structure as in the freshly opened archegonium. Indeed it was often impossible to tell whether an archegonium had been fertilized or not.

The prothallium seems to have very little power of independent existence, and develops but little chlorophyll even in the older unfertilized ones. No root-hairs were observed in any case, and the limit of its growth is probably determined by the amount of food material in the spore. The number of archegonia may occasionally exceed twelve, and from

¹ Goebel : Outlines, p. 232.

eight to ten is not at all uncommon. Sometimes, in very old prothallia little elevations are formed projecting upward between the older archegonia, and upon these small archegonia are developed.

To judge from Berggren's¹ figures of *A. caroliniana*, the prothallium in that species is decidedly larger than in *A. filiculoides*, but the archegonia are much less numerous.

THE EMBRYO.

Berggren made out correctly the first divisions in the embryo, but did not trace in detail any but the very earliest ones, and his figures, as in the case of the prothallium are too schematic to show the cell-arrangement with any degree of accuracy.

The fertilized egg, previous to its first division, elongates vertically. The first, or basal wall, is usually horizontal², instead of vertical as in all the other Leptosporangiateae yet investigated. In a very few cases (Fig. 65), the basal wall was nearly vertical, but this was exceptional. From the upper cell the cotyledon and stem arise; from the lower, the foot and first root. Thus the position of the primary organs of the embryo is the same with reference to the basal wall, as in the other Leptosporangiateae, but different as regards the archegonium.

The next divisions, as in other cases, divide the embryo into four nearly equal cells. These quadrant-walls (*II*) do not always arise simultaneously. In the only case where a three-celled embryo was found by me, the upper cell was divided. Berggren³ states that in *A. caroliniana*, it is the lower cell that first divides. Probably it is not always the same one. The formation of the quadrant-walls determines the primary organs. In the upper cell one quadrant gives rise to the stem, and one to the cotyledon; and of the two

¹ loc. cit. Figs. 4-16.

² That is, assuming the axis of the archegonium to be vertical.

³ loc. cit. p. 4.

lower, the one next the leaf-quadrant forms the root, the other the foot ; so that these organs have the same relative positions as in the embryo of the ordinary Ferns. Berggren¹ states that the root does not arise until later, and is derived from the foot ; but in sections it is plainly recognizable from the very first, and corresponds in position exactly with that of other Ferns.

In regard to the next walls there is not always absolute regularity. In all but the stem-quadrant, the octant-walls divide the quadrants into exactly equal parts, and this may be true also of the stem-quadrant. In the latter, however, (Fig. 64 *a*), the octant-wall may make an acute angle with the quadrant-wall, and the larger cell of the two thus formed functions at once as the apical cell of the stem, and divides from now on by walls directed alternately right and left. When the stem-quadrant is divided into equal cells by the octant-wall it is probable, although this was not positively proved, that for a time the apical cell of the stem forms three sets of segments instead of two. This seems probable from the fact that often when seen from the side (see Fig. 71 *a*), two series of segments can be seen, which could hardly be true were there but two series cut off from the apical cell.

THE COTYLEDON.

The first divisions of the cotyledon are extremely regular, and resemble those in the later leaves. The cotyledon, however, as well as the other leaves of the young plant, is not divided into the lobes found in the leaves of the older plants. Following the median octant-wall, a vertical wall is formed in each of the two octant-cells (Fig. 66 *b*), forming two cells that seen in section appear triangular, and two which appear to be four-sided. The two former, which have larger nuclei than the other cells, divide for some time in much the same way that we saw in the formation of the sporocarps in the fertile lobes of the sporophylls, and may, perhaps, be equally

¹ loc. cit. p. 4.

well designated apical cells. The other cell in each octant divides by tangential and radial walls arranged with a good deal of regularity. By the growth of the two initial cells (\times , \times') the young cotyledon rapidly grows at the lateral margins, and it bends forward so as to partly include the stem-apex. At the same time the upper marginal cells divide rapidly by oblique walls alternately on the inner and outer side, so that the cotyledon also grows in height. By this time the cotyledon has become about four cells thick.

THE STEM-QUADRANT.

As we have seen, the divisions in the stem-quadrant are not perfectly uniform. In case a two-sided apical cell is established at once, it divides from this time very much as in the mature plant. Each segment divides into a ventral and dorsal half, and each of these again into an acroscopic and basisopic cell. In case the first division in the stem-quadrant divides it equally, it is not possible to say which of the cells will become the apical cell of the stem, but this is determined by the first division in each cell. One of the cells divides by a vertical wall into equal parts, and becomes the second leaf; the other, as already indicated, forms regular segments. When the octant-cells are unequal, the smaller of the two, which may be considered as the first segment of the apical cell of the stem, becomes the mother-cell of the second leaf. At the base of the first leaf, between it and the stem, a group of short hairs (Fig. 71 *h*) is formed at an early stage.

THE ROOT.

The primary root in *Azolla* arises in exactly the same way as in the other Leptosporangiateae. After the first division of the root-quadrant, one of the resulting octants becomes at once the apical cell. The first segment is usually cut off parallel to the basal wall of the embryo, and the next strikes it and the octant-wall (Fig. 66 *c*) so that the apical cell lies at this stage close to the octant wall. In the other octant of the

root-quadrant, the divisions are irregular, and its limits are soon merged in those of the foot. From the first the divisions in the root take place with great regularity. After one complete set of lateral segments has been cut off, a cell is cut off from the outer face of the apical cell, forming the root-cap. Unlike other Ferns, this is the only cap-cell cut off, and all the other segments are lateral. The cap-cell divides later into two (Fig. 71 *b*), and in these cells divisions continue to form where they join the rest of the epidermis, so that the young root is enclosed in a sheath formed of two layers of cells (Fig. 73). The lateral segments of the apical cell are shallow and arranged very symmetrically. The first wall in each divides it into an inner and outer cell, and from the latter is later developed the fibro-vascular bundle.

THE FOOT.

The divisions in the foot are more regular than is usually the case. This is especially noticed when sections are made parallel to the quadrant-wall (see Figs. 66, 72). The general arrangement of the cells is much like that of the cotyledon, but the divisions are much less numerous, especially the transverse walls, and the cells are therefore larger and more elongated. Corresponding with the upward growth of the cotyledon, there is an elongation of the foot, so that the base of the foot extends downward much beyond the base of the root-quadrant, which thus comes in the older embryo to appear lateral, and no doubt led to Berggren's mistake of supposing that the root originated from the foot.

THE SUBSEQUENT GROWTH OF THE EMBRYO.

The second leaf arises practically at the first segment of the apical cell of the stem, and each succeeding segment gives rise to a leaf. The longitudinal growth of the root is slow, although a large number of segments may be cut off from the apical cell; but these remain flattened, and only elongate at a late stage in the development of the embryo.

The fibro-vascular bundles are poorly developed and arise

comparatively late. No trace of them can be seen until the second leaf is pretty well advanced. There is nothing peculiar about their development. Simultaneously in the axis of the root and stem, and extending into the centre of the cotyledon, a series of longitudinal walls arise that give rise to a thin procambium-cylinder. In the axis of this the cells become transformed into tracheids with close spiral thickenings in their walls. At the point of junction of the primary bundles the tracheids are as usual irregular and connect the tracheary tissue of the three bundles. No trace of a bundle could be detected in the foot. The development of the fibro-vascular bundles does not take place until some time after the embryo has broken through the prothallium.

The second root arises close to the base of the second leaf, and originates from single epidermal cell in the same way that the later ones do (Fig. 70 *r'*). A rapid growth takes place now in all the cells of the embryo, and in the cotyledon intercellular spaces are formed, which filling with gases, soon cause the young plant to rise to the surface of the water. As the embryo breaks through the episporic appendages at the top of the spore, these are forced apart, and the top of the indusium, which has covered it up to this time, is thrown off. The young plant at this stage becomes very easily separated from the prothallium, and is often found floating free. At this stage the cotyledon forms a sort of bell-shaped sheath, opening only in one side by a narrow cleft, and completely surrounding the growing-point within. The root is still inconspicuous, and forms merely a slight protuberance on one side of the foot which has the form of a short cylindrical stalk. The stem is at right angles to the foot, and the succeeding leaves form, as in the mature plant, two ranks, overlapping and completely hiding the stem.

The growth of the first root is limited, and it is distinguished from the later ones by peculiar short root-hairs which stand out stiffly from it (Fig. 75). The succeeding roots, except the second, are formed considerably later, and there does not seem to be any determined point at which they arise.

The mesophyll of the cotyledons and the leaves immediately following does not show the peculiar elongated cells found in the later leaves, and these first appear in about the fourth leaf, but are not as well developed as in the later ones. In all of the leaves, however, with the exception of the cotyledon, the peculiar cavity, filled with a colony of *Anabaena*, is to be seen.

The *Anabaena* begins to grow almost as soon as the first divisions in the embryo are completed. If a young embryo is dissected out, it will be found that in the space between the cotyledon and the stem, that a number of very short *Anabaena*-filaments are present. As the embryo pushes up through the space between the archegonium and the indusium, the *Anabaena*-cells collected there are carried up with it, and then begin to grow. They assume the blue-green colour of the active cells, elongate and divide rapidly by a series of transverse walls into short filaments that at first look like an *Oscillaria*. Very soon, however, the cells round off, heterocysts are formed, and the typical form of the ordinary filaments is attained. Some of these remain tangled about the growing point of the stem, while others creep into the cavities at the base of the leaves.

No branches are formed in the young plant before about eight or ten leaves are produced. Whether the position of the first branch is constant, I cannot say, as the point was not critically examined.

CONCLUSION.

A comparison of the development of *Azolla* with other forms does not show a very close resemblance to any one, and indicates a somewhat isolated position for the genus. Its nearest ally is unquestionably *Salvinia*, with which it agrees in the general plan of its growth, the two-sided apical cell of the stem, and especially the development of the sporocarp. However, as Strasburger has shown, except the first divisions of the apical cell, the resemblance is not very close, and in the decidedly apical growth of the leaves and the absence of

bipartition, *Salvinia* offers a strong contrast to *Azolla*, as of course it does in the absence of roots. This latter is, however, probably of secondary importance, as the roots are replaced by the peculiar submerged leaves.

The origin and development of the sporocarp, as well as the structure of the spores, is too similar to be accounted for except by the assumption of a relationship. The massulae and the episporic appendages of the macrospore differ only in degree from those of *Salvinia*, where all the microspores are held together in one mass, and where the episporic of the macrospore is less developed. As indicated in the account of the macrosporangium of *Azolla*, there is every reason to believe that it is derived from some form with more than one macrosporangium in the sorus. In regard to the embryo, that of *Azolla* in its younger stages corresponds very closely to those of *Salvinia*¹. Indeed, so close is the correspondence that one is inclined to regard the embryo of the former as not really rootless, as the very first divisions in what corresponds to the root-quadrant of *Azolla* are very like. A further investigation of this point, by careful microtome-sections, is very much to be desired.

The prothallium and sexual organs of *Azolla* are also extremely like those of *Salvinia*, the most noticeable difference being the much greater size and regular meristem in the female prothallium of the latter. Whether the endosperm-nuclei are present in *Salvinia* remains to be seen.

Compared with the Marsiliaceae there is much less resemblance. The sporocarps are very different, being in the Salviniaceae single sori, while in the Marsiliaceae, each fruit is a whole leaf-segment with several sori. The prothallium is much less reduced, especially the female one, and the archegonia more nearly resemble those of the homosporous Ferns, both in the length of the neck and the division of the nucleus of the neck-canal-cell. A very noticeable difference, and one which must be regarded probably as an important one, is the presence of the endosperm-nuclei.

¹ Sadebeck in Schenk's 'Handbuch,' vol. i. p. 217.

When *Azolla* is compared with the other Pteridophytes, it is evident enough that its nearest affinities are with the homosporous Filices. We have already indicated the very clear resemblance to these in the development of the sporangia and the indusial nature of the sporocarp wall. When a nearer comparison is made it seems probable that it is with the lower members of the Leptosporangiate series that its affinities are most marked. The form of the indusium recalls some of the Cyatheaceae and Hymenophyllaceae, and the leaves in their earlier stages resemble those of some of the simpler forms in the latter family.

We may conclude, then, that the two families of the Hydropterideae represent the ends of two different lines of development. Of these the Salviniaceae have been derived from the lower members of the Leptosporangiate series, possibly from near the Hymenophyllaceae, and that the Marsiliaceae have arisen from forms more like the Polypodiaceae. Of the two families, the Salviniaceae have departed less from the parent stock in regard to the reduction of the sexual generation, but the sporophyte is much less like that of the ordinary homosporous forms than that of the Marsiliaceae.

The two genera of the Salviniaceae differ much more from each other than do those of the Marsiliaceae, and it is not at all likely that one form has been derived from the other, but that the two genera diverged at an early stage in the development of the line.