Symbiosis, 5 (1988) 199–208
Balaban Publishers, Rehovot/Philadelphia

Study on the Association Between *Anabaena azollae* and *Azolla microphylla* During the Germination of Megasporocarps

CHANG LIN* and IWAO WATANABE

The International Rice Research Institute
Los Baños, Laguna, Philippines
Tel. 742-0580; Telex 45365 RICE INST PM

Received August 9, 1987; Accepted May 1, 1988

Abstract

The early phase of association between *Azolla* and *Anabaena* cells was studied. In a mature megasporocarp, *Anabaena* cells cluster inside the inner portion of the indusium. Some scatter over the surface of the apical membrane of the megaspore apparatus. Most *Anabaena* cells occur singly but a filamentous form of *Anabaena* was also observed. Initial contact between *Anabaena* cells and *Azolla* plant takes place after the sporophyte breaks out through the apical membrane. *Anabaena* cells stayed on apical membrane and those residing in the inner portion of indusium became the sources of infection to sporophyte.

Keywords: *Azolla*, *Azolla microphylla*, germination of megasporocarp, *Anabaena-Azolla* association

1. Introduction

One of the most interesting subjects in *Anabaena-Azolla* symbiosis is the relationship between fern and alga. Many scientists studied this topic since the 1970s (Hill, 1975, 1977; Peters and Mayne, 1974; Peters, 1975, 1982; Calvert and Peters, 1981; Peters and Calvert, 1983;Ray et al., 1979;Sun et al., 1984). *Anabaena* is present in *Azolla* throughout the *Azolla* life cycle. During

*Present address: National Azolla Research Center, Fujian Academy of Agricultural Sciences, Fuzhou, Fujian, People's Republic of China

0334-5114/88/$03.00

the sporophyte stage, *Anabaena* filaments are located on the shoot apex and in leaf cavities. At the shoot apex, *Anabaena* filaments adhere to specialized epidermal hairs — called the primary branched hairs (PBH) (Calvert and Peters, 1981; Peters and Calvert, 1983). With the development of the leaf primordium, a PBH with adhering *Anabaena* is engulfed by the forming leaf cavity. *Anabaena* inside the leaf cavity grows rapidly and some cells differentiate into heterocyst which fix nitrogen. The *Anabaena* cells in *Azolla* undergo a developmental pattern of differentiation which is synchronized with the host (Hill, 1975, 1977). At the mature sexual stages, *Anabaena* cells are found in the apical portion of the megasporocarp (Konar and Kapoor, 1974; Herd et al., 1986; Calvert et al., 1983). They are separated from the *Azolla* tissues by the apical membrane of the megaspore apparatus. By removing the indusium and apical membrane, the megaspores germinated and developed into *Anabaena*-free *Azolla* plants (Lin and Watanabe, 1988).

This paper provides information on the development of the association between *Anabaena* and the germinating sporophyte and clarifies the morphological basis of this process.

2. Materials and Methods

Harvesting sporocarps and their germination

Azolla microphylla Kaulfuss (IRRI accession No. 4031) was used. The method of sporocarp harvesting and germination was as described by Lin and Watanabe (1988).

Scanning Electron Microscope (SEM)

Different stages of germinating megasporocarps were selected from the germinating material. They were prefixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), postfixed in 2% osmium tetraoxide in the same buffer for 1–2 hr, and dehydrated in a water-acetone series. Fixed and dehydrated sporocarps were critical-point dried. Dried megasporocarps were placed under a dissecting microscope to remove the indusium. Megasporocarps without the indusium were mounted on the stub with silver paste. Sections, 15–30 μm, from paraffin-embedded germinating megasporocarps were mounted on glass slips. After deparaffinization, specimens were treated with a series of xylene: ethanol, and ethanol: acetone solvent mixture, and then critical-point dried. Sections were coated with gold, using Polaron E 5000 Sputter coater and observed under SEM Philips model 505 at 15–25 kV.

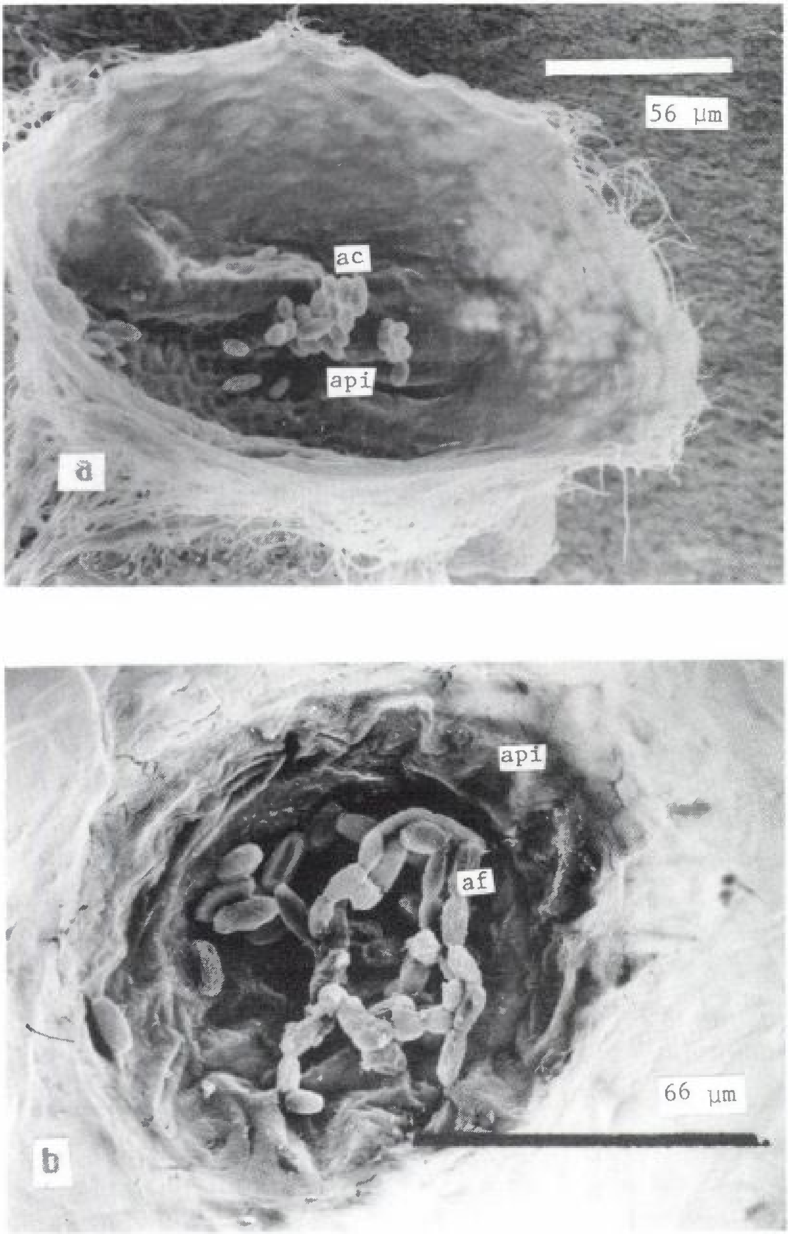


Figure 1. *Anabaena* cells clustered on the apical membrane.
(a) *Anabaena* cells on the surface of the apical membrane.
(b) *Anabaena* filaments on the apical membrane.
ac = *Anabaena* cells, api = apical membrane, af = *Anabaena* filament.

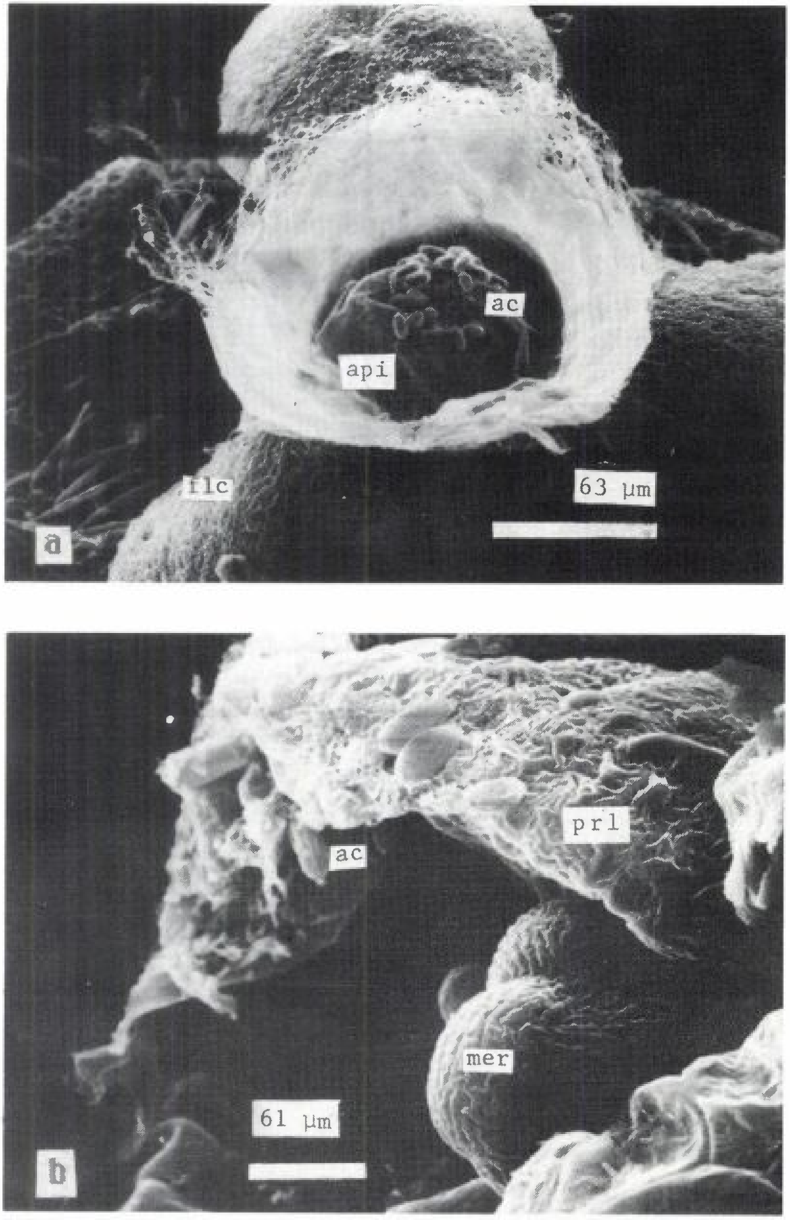
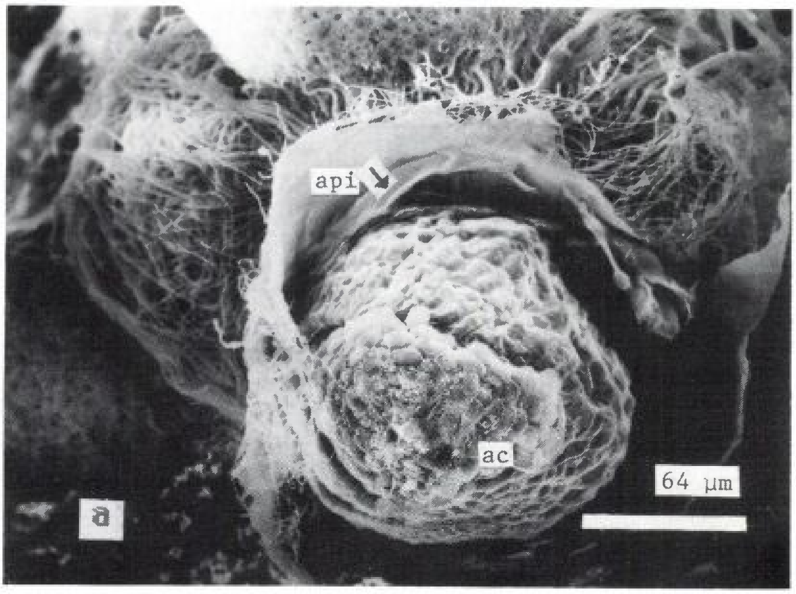


Figure 2. *Anabaena* cells on the apical membrane connected with sporeling, broken through the apical membrane.
(a) *Anabaena* cells on the surface of the apical membrane,
(b) *Anabaena* cells on the apical membrane attached to the surface of the primary leaf, flc = float corpuscle, api = apical membrane, ac = *Anabaena* cells, mer = meristem, prl = primary leaf



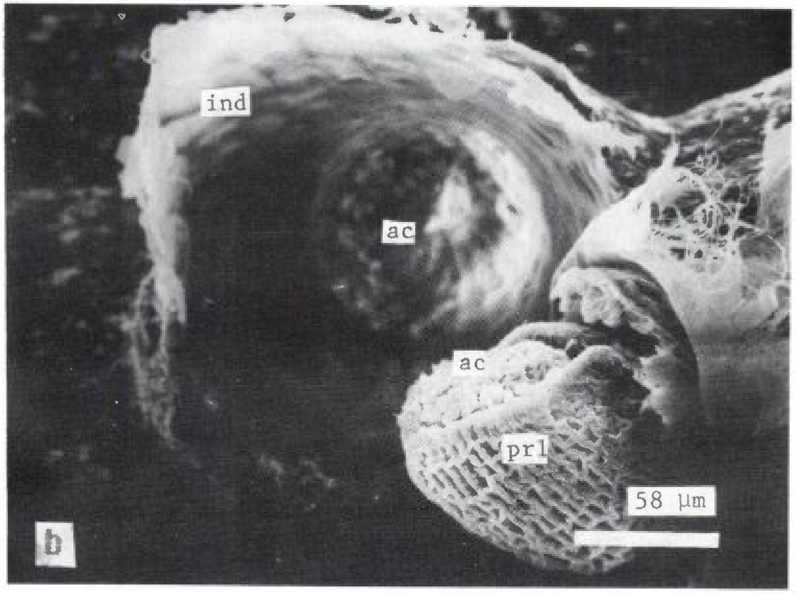


Figure 3. Sporeling connected with the different portions of the indusium.
(a) *Anabaena* cells clustered on top of sporophyte.
(b) *Anabaena* cells inside the indusium and on the surface of sporeling.
ind = indusium, ac = *Anabaena* cells, ind = indusium, ac = *Anabaena* cells,
ind = indusium, prl = primary leaf, api = apical membrane.

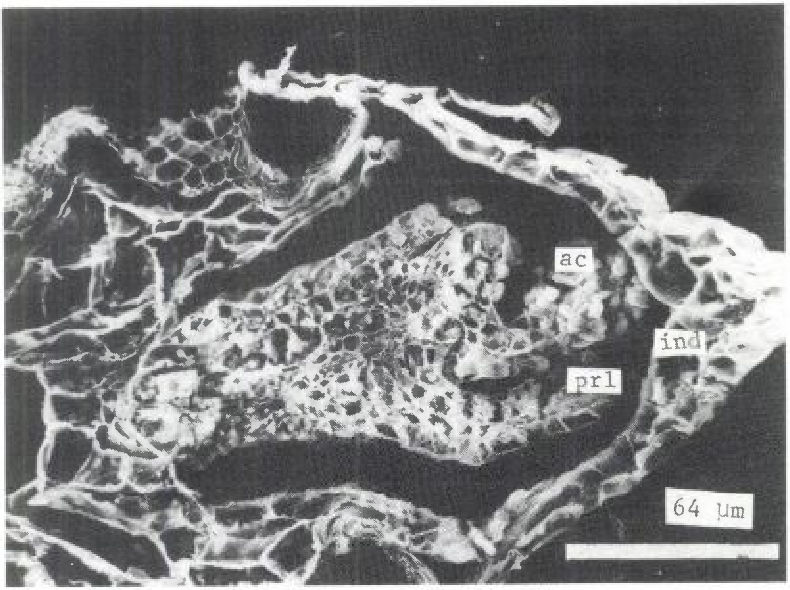


Figure 4. *Anabaena* cells present between the sporeling and the indusium. Vertical section of a germinating sporocarp at early shooting stage. ac = *Anabaena* cells, prl =primary leaf, ind = indusium.

3. Results

In the megasporocarps, some *Anabaena* cells from clusters inside the inner portion of the indusium cap and some lie on the apical membrane of the megaspore apparatus (Fig. 1a). The frequency of occurrence of *Anabaena* on the surface of the apical membrane of the megaspore apparatus was about 40% of 129 random samples. Most *Anabaena* cells were single. Filamentous forms of *Anabaena azolla* were also observed on the apical membrane, but at a much lower frequency (Fig. 1b). No size and shape difference of the cells in the filaments could be seen, indicating that there was no heterocyst formation.

Association of *Anabaena* cells with the young sporophyte took place after the sporophyte broke out through the apical membrane of the megaspore apparatus. There are probably two ways, by which the *Anabaena* cells could initiate the association with the young sporophyte.

(1) The apical membrane with *Anabaena* cells is pushed out (Fig. 2a) and finally broken by the developing sporophyte. *Anabaena* cells on the broken apical membrane may come in contact with the surface of the primary leaf

(Fig. 2b) or the apex of the sporeling.

(2) When *Anabaena* cells do not reside on the apical membrane, they may become associated with the young sporophyte in another way. The sporophyte, while breaking out through the apical membrane, contacts with different parts of the indusium, where *Anabaena* cells are present. Some *Anabaena* cells are already on the tip of the young sporophyte, transferred from the indusium of the sporeling (Fig. 3a), while others are still inside the indusium (Fig. 3b). This phenomen was observed even after the late shooting stage. *Anabaena* cells on the young sporophyte may become transferred to the primary leaf and onto the outer surface of the first young leaf and the shoot apex (Fig. 4).

4. Discussion

Ye (1983) reported that *Anabaena* cells entered the leaf cavity when the first leaf developed. Becking (1978, 1987) considered that the initial association between *Anabaena* and *Azolla* occurred when the cotyledon leaf passes the indusium. Morphological observation confirmed that *Anabaena* present on the surface of the apical membrane become associated with the young sporophyte. But the frequency of this case was low. This coincides with the low frequency of the success of association from the indusium-removed megaspocarps. Removal of both apical membrane and indusium was needed to assure 100% success of obtaining *Anabaena*-free *Azolla* (Lin and Watanabe, 1988). When young sporophytes fail to be associated with *Anabaena* after fracture of the apical membrane, *Anabaena* adhering to the inner side of indusium may become the source of infection. In the majority of cases, this happened. The possibility of a mechanical transfer of *Anabaena* cells onto the apical membrane during removal of indusium can be ruled out, since the indusium was removed after fixation of the samples for SEM.

Observations of our material also showed that the *Anabaena* cells, either single or filamentous, had a uniform morphology. It seems, therefore, more appropriate to consider *Anabaena* cells in megasporocarps as resting cells (Campbell, 1893) rather than as akinetes (Huneke, 1933, Ashton and Walmsley, 1976; Herd et al., 1986; Becking, 1987). Becking (1987) showed micrographs of *Anabaena* in megasporocarps. Neither Becking (1987) nor this paper showed the process of *Anabaena's* entry to the leaf cavity of the first and second leaves. The details of this process has been studied by the authors, and will be published later.

Acknowledgment

The authors thank Mr. Pei-ji Lu of the Fujian Academy of Agricultural Sciences for the sporocarps and Mrs. F. Sta. Cruz of IRRI, EM laboratory, for technical assistance. A part of the project was supported by the United Nations Development Programme Global Project.

REFERENCES

Ashton, P.J. and Walmsley, R.A. 1976. The aquatic fern *Azolla* and its *Anabaena* symbiont. *Endeavor* **35 (124)**: 39–43.

Becking, J.H. 1978. Ecology and physiological adaptation of *Anabaena* in the *Azolla-anabaena azollae* symbiosis. Environmental role of nitrogen-fixing blue-green algae and symbiotic bacteria. *Ecol. Bull.* (Stockholm) **26**: 266–281.

Becking, J.H. 1987. Endophyte transmission and activity in the *Anabaena-Azolla* association. *Plant Soil* **100**: 183–212.

Calvert, H.E., Perkins, S.K., and Peters, G.A. 1983. Sporocarp structure in the heterosporous water fern *Azolla microphylla* Presl. *Scanning Electron Micros.* **III**: 1499–1510.

Calvert, H.E. and Peters, G.A. 1981. The *Azolla-Anabaena* relationship IX. Morphological analysis of leaf cavity hair populations. *New Phytol.* **89**: 329–335.

Campbell, D.H. 1893. On the development of *Azolla filiculoides* Lam. *Ann. Bot.* **7**: 155–187.

Herd, Y.R., Cutter, E.G., and Watanabe, I. 1986. An ultrastructural study of postmeiotic development in the megasporocarp of *Azolla microphylla*. *Can. J. Bot.* **64**: 822–833.

Hill, D.J. 1975. The pattern of development of *Anabaena* in the *Azolla-Anabaena* symbiosis. *Planta* (Berl.) **122**: 179–184.

Hill, D.J. 1977. The role of *Anabaena* in the *Azolla-Anabaena* symbiosis. *New Phytol.* **78**: 611–616.

Huneke, A. 1933. Beiträge zur Kenntnis des Symbiose zwishen *Azolla* und *Anabaena*. *Beitr. Biol. Pflanz.* **20**: 315–341.

Konar, R.N. and Kapoor, R.K. 1974. Embryology of *Azolla pinnata*. *Phytomorphology* **24**: 228–261.

Lin, C. and Watanabe, I. 1988. A new method for obtaining *Anabaena*-free *Azolla*. *New Phytol.* **108:** 341–344.

Peters, G.A. 1975. The *Azolla-anabaena azollae* relationship III. Studies on metabolic capabilities and a further characterization of the symbiont. *Arch. Microbiol.* **103:** 113–122.

Peters, G.A. 1982. *Azolla-Anabaena* symbiosis: Basic biology, use and prospects for future. In: *Practical Application of Azolla for Rice Production.* W.S. Silva and E.C. Schröder, eds. Dordrecht, Martinus Nijhoff Publisher, pp. 1–14.

Peters, G.A. and Mayne, B.C. 1974. The *Azolla-Anabaena azollae* relationship. I. Initial characterization of the association. *Plant Physiol.* **53:** 813–819.

Peters, G.A. and Calvert, H.E. 1983. The *Azolla-anabaena azollae* symbiosis. I. *Algal Symbiosis.* C.J. Goff, ed. Cambridge University Press, pp. 109–145.

Ray, T.B., Mayne, B.C., Toia, R.E. Jr., and Peters, G.A. 1979. *Azolla-anabaena azollae* relationship VIII. Photosynthetic characteristic of the association and individual partners. *Plant Physiol.* **64:** 791–795.

Sun, J.S., Chen, W.L., Zhu, Z.O., and Zhu, Y.M. 1984. An autoradiographic study of the *Azolla-anabaena azollae* relationship. *Acta Bot. Sinica* **26(2):** 115–119.

Ye, X.Z. 1983. Morphological observations on the symbiosis of *Anabaena* and *Azolla* in development from megasporocarps to young sporophyte. *Acta Bot. Sinica* **25(2)** 192–194.