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Are bacteria the third partner of the *Azolla-Anabaena* symbiosis?

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Abstract

The development of the prokaryotic colony in *Azolla filiculoides* indicates that *Anabaena azollae* is maintained through the life cycle of the fern and present in the leaves and megasporocarps. The same biological pattern is applied to the bacteria that are also present in these structures and seems to follow a development pattern identical to the cyanobacteria and probably can be considered the third partner of this symbiotic association.

Introduction

The presence of bacteria in the *Azolla* leaves were previously described by Grilli (1964) and later referred by several authors, with some reserve, as an organism present in the leaf cavity of the fern (Forni and Grilli Caiola, 1986; Gates et al., 1980; Peters et al., 1978). In spite of this, organism having been identified by the first time by Bottomley in 1920 as *Pseudomonas*, the correct identification was done by Wallace and Gates (1986) and included in the genus *Arthrobacter*. Based on this identification Petro and Gates (1987) suggest that these bacteria may be the third component of the symbiosis. Using different methods Carrapiço and Tavares (1987, 1989) have shown, by electron microscopy studies, that these bacteria were always present as a permanent member of the prokaryotic colony at different stages of leaf development of *Azolla filiculoides* and based on these data they have also suggested that these bacteria were the third partner of the symbiosis. At the same time Forni et al. (1987, 1989) have identified by biochemical tests several species of *Arthrobacter* present in different species of *Azolla*, suggesting the same hypothesis. The first references of the presence of bacteria in sporocarps was made by Peters and Calvert (1982) and by Tavares and Carrapiço (1988). In this work, further research was made on this symbiotic association, trying to integrate and develop the understanding of the relationship between the bacteria and the life cycle of the fern.

Materials and methods

The *Azolla filiculoides* plants were collected in Alcochete (70 km from Lisbon) and maintained in cultivation chambers. The megasporocarps were collected in April of 1987 and 1988 directly from the wild and prepared for further studies. Light and electron microscopy research were made on dorsal lobe leaves and in megasporocarps. For electron microscopy studies, leaves were submitted to a double step fixation process by 4% glutaraldehyde in 0.05 *M* cacodylate buffer (pH 7.2) and in 1% O_sO_4 in 0.05 *M* cacodylate buffer (pH 7.4) with or without ruthenium red (Carrapiço and Tavares, 1989). The leaves were dehydrated in acetone series and embedded in Epon-Araldite (Mollenhauer, 1964). The megasporocarps were fixed in a fixative consisting of formaldehyde-glutaraldehyde (2%–2.5%) in 50 m*M* PIPES buffer (pH 7.4) for

3 h at room temperature and postfixed in 1% O_sO_4 in 50 m*M* cacodylate buffer (pH 6.8) for 2 h at room temperature. The formaldehyde was prepared fresh from paraformaldehyde immediately before use. The megasporocarps were dehydrated through an ethanol series and embedded for several days in LR White's resin. Thin sections (60–80 nm) of the specimens were cut with a glass knife and Porter-Blum MT-2 ultramicrotome and mounted on copper grids. The sections were post-stained in 2% (w/v) uranyl acetate and Reynold's lead citrate and the observations were made in a Hitachi-12 electron microscope operating at an accelerating voltage of 75 Kv. Some megasporocarps were prepared for scanning electron microscopy and observed at 10–15 Kv in a Jeol JSM-820 electron microscope. For the light microscope, semi-thin sections (800 nm) were made from the specimens prepared for the electron microscope and transferred to a small drop of distilled water on a microscope slide. The sections were stained with acqueous toluidine blue and photographed in a Dialux Leitz Wetzlar microscope.

Results

Bacteria which show a coryneform or rod morphology are found in the dorsal lobe of the leaf cavity in close association with the transfer hairs and *Anabaena* cells (Fig. 1). The bacteria and the cyanobacteria are immersed in a fibrillar network, revealed by the ruthenium red, that fills the leaf cavity and establish connections between *Azolla* and the partners of the association (Fig. 2). Megasporocarps, with an oval morphology, are about 1 mm in length and 0.5 mm in breadth (Fig. 3). Inside this structure, below the indusium exists a cavity that contains *Anabaena* cells (akinetes) (Figs. 4, 5), that are maintained through the life cycle of the fern. This cavity observed in transmission electron microscopy shows the simultaneous presence of *Anabaena* akinetes and bacteria cells (Figs. 6, 7). These bacteria are morphologically similar to the bacteria found on the leaf cavity (Figs. 2, 7, double arrows).

Discussion

The presence of bacteria in the *Azolla-Anabaena* symbiosis was referred for the first time by Grilli (1964) by means of transmission electron microscopy and observed in the leaf cavity of the fern. These bacteria show a coryneform or rod morphology typical of the genus *Arthrobacter* (Carrapiço and Tavares, 1989; Gates et al., 1980; Peters et al., 1978) and were taxonomically identified by Wallace and Gates (1986). Recently, Forni et al. (1989) provide information on the enzymatic activities and the nutritional characteristics of the bacteria isolated. These authors have isolated from five *Azolla* species (*A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla* and *A. pinnata*) different species of a bacterium of the genus *Arthrobacter*. The species isolated were *A. globiformis*, *A. nicotianae*, *A. aurescens* and *A. cristallopoietes* or *A. pascens*, suggesting that this prokaryotic organism may be the third member of the symbiotic association.

In our observations we have observed that the bacteria were always present in all stages of leaf development in close association with the primary branched hairs, simple hairs or *Azolla* epidermal cells, following a location identical to the cyanobacteria *Anabaena azollae* (Carrapiço and Tavares, 1989). To confirm the idea that *Arthrobacter* is the third member of the symbiosis it would be necessary to look for the sexual structures of the fern. The presence of the bacteria in these reproductive structures was previously referred by Peters and Calvert (1982) in the microsporocarps of *Azolla filiculoides* and afterwards, in preliminary observations, in the indusium cavity of the megasporocarps of the same species by Tavares and Carrapiço (1988). Further research by means of transmission electron microscopy was done in this structure, showing that the bacteria found in the megasporocarp were morphologically similar to those found in the different stages of leaf development and strongly suggesting that this prokaryotic organism was retained throughout the sexual cycle of the fern, as it happens with the *Anabaena* cells, and probably can be considered the third partner of this symbiosis. Further research is needed to determinate the exact role of the bacteria involved in the symbiosis and if the

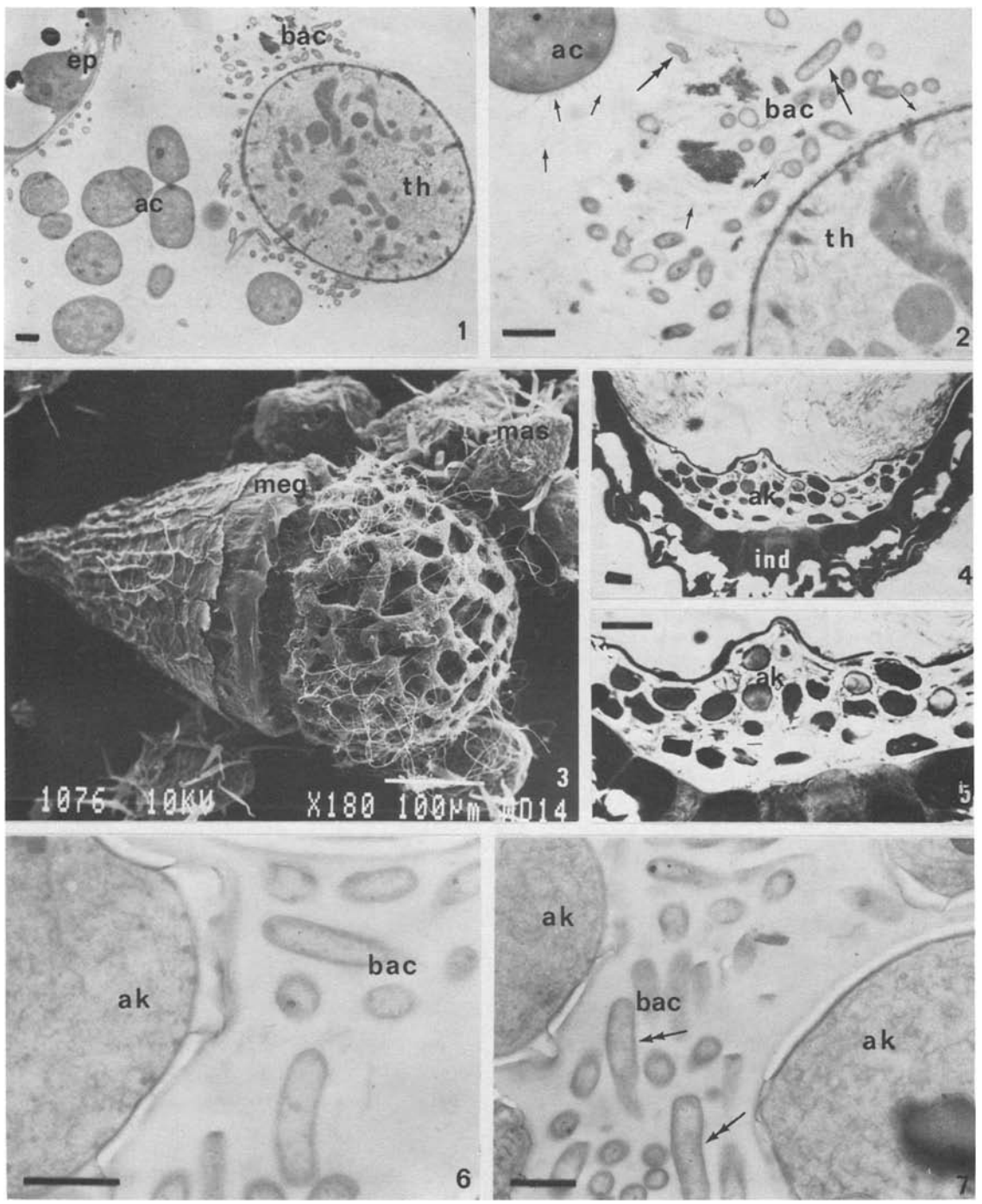

Fig. 1. Ultrastructural aspect of the leaf cavity. The transfer hair (th) is surrounded by a great number of bacteria (bac). *Anabaena* cells (ac) are close to these cells and *Azolla* epidermal cells (ep). *Bar* = 1 μm.

Fig. 2. Magnification of Figure 1, showing the fibrillar network (single arrows), where bacteria and *Anabaena* cells are immersed. *Bar* = 1 μm.

Fig. 3. A scanning electron microscopy micrograph of *Azolla filiculoides* megasporocarp (meg) surrounded by massulae (mas).

Figs. 4. and 5. Two images of the indusium zone of the megasporocarp, showing the *Anabaena* akinetes (ak) in the cavity below the indusium (ind). *Bar* = 10 μm.

Figs. 6 and 7. Micrographs of the indusium cavity showing *Anabaena* akinetes and bacteria cells. Some bacteria are morphologically similar to the bacteria found on the leaf cavity (double arrows, compare figure 2 with figure 7). *Bar* = 1 μm.

species found in the sporocarps are the same recently isolated from leaf symbiosis.

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