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Bacteria in the *Azolla-Anabaena* Association

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Abstract

Leaf cavity of *Azolla caroliniana* Willd. contains the phycobiont *Anabaena azollae* Strasb. and bacteria. A study was carried out on the bacteria living in the *Azolla* leaf cavities, the bacteria present in non axenic cultures of *Anabaena azollae* and the bacteria isolated from the fern leaf cavities. SEM and TEM observations of the bacteria in the leaf cavities revealed a heterogenous population consisting of long rods and cocci. The bacteria, isolated from the fern, were aerobic, Gram-positive, non nitrogen-fixers. Based on morphological and biochemical data, they were identified as *Arthrobacter* sp. Conn and Dimmick.

Keywords: *Azolla*, *Anabaena azollae*, symbiosis, nitrogen fixation, *Arthrobacter* sp.

1. Introduction

The presence of bacteria in the fern leaf cavities of *Azolla* has been reported by many authors (Grilli, 1964; Duckett et al., 1975; Newton and Cavins, 1976; Hill, 1977; Peters et al., 1978; Gates et al., 1980; Wallace and Gates, 1986; Forni and Grilli Caiola, 1986; Grilli Caiola and Albertano, 1987). However, there is no common opinion about the nature of this microbial population and its role in symbiosis. In fact, some authors have identified these bacteria as *Pseudomonas* (Bottomley, 1920), or as *Caulobacter* and *Alcaligenes* spp. (Newton and Herman, 1979), or as coryneform bacteria (Gates et al., 1980). But only recently Wallace and Gates (1986) have reported the presence of *Arthrobacter* sp. Conn and Dimmick in 4 species of *Azolla*.

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The present study deals with the relationship between the bacteria, the phycobiont and the host, and particularly describes by SEM and TEM observations, the bacteria in the leaf cavities of *Azolla caroliniana* Willd. and the identification of the isolated bacteria as *Arthrobacter* sp.

Some assays of acetylene reduction (ARA) were made in order to determine nitrogen fixation activity of the isolated bacteria. This study also provides information on some biochemical characteristics of the bacteria.

2. Materials and Methods

Azolla caroliniana Willd. from the Botanical Garden of the University of Naples (Italy), algal packets, freshly isolated from the leaves by manual dissection, non axenic cultures of *Anabaena azollae* Strasb., isolated from *A. caroliniana* and cultures of bacteria, isolated from the algal packets were used for this study.

Scanning electron microscopy (SEM)

For SEM observations, leaves of *A. caroliniana* Willd., algal packets, non axenic cultures of *Anabaena azollae* and bacteria were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 6.9) at 4°C for 2 hr. The samples were postfixed in 2% buffered osmium tetroxide, dehydrated in acetone, air dried and coated with colloidal gold. Before the coating, the air dried leaves were opened in order to expose the algal packets. The observations were made by means of a Scannosan electron microscope.

Transmission electron microscopy (TEM)

For the TEM observations, apical (from 2nd to 4th) and basal (from 12th to 14th) leaves of *Azolla* and non axenic *Anabaena azollae* cultures were routinely fixed in glutaraldehyde and osmium tetroxide as described in the SEM section. A set of the same materials were also fixed and stained with 0.1% ruthenium red (Grilli Caiola and De Vecchi, 1985). The samples fixed in glutaraldehyde were postfixed in 2% osmium tetroxide, then all the samples were dehydrated in a series of graded alcohols and embedded in araldite.

Ultrathin sections, cut with a diamond knife, were observed either stained with uranyl acetate and lead citrate (Reynolds, 1963) or without staining using a TEM Philips 400.

Anabaena azollae cultures

Culture of *A. Azollae* was kindly provided by Dr. Moretti, University of Naples (Italy) (Grilli Caiola and Moretti, 1985). The endophyte was isolated from *A. caroliniana* following the method of Newton and Herman (1979).

Axenic and non axenic *Anabaena* cultures were maintained in nitrogen-free medium BD (Baslerova and Dvorakova, 1962) or in nitrate medium BG11 (Stanier et al., 1971) at 25°C and illuminated by Sylvania cool white lamps (PAR 50 $\mu E\ m^{-2}s^{-1}$) with a 16/8 light/dark cycle. Axenic *Anabaena* cultures were obtained by washing with 0.9% NaCl and subculturing in nitrogen-free medium.

Isolation and growth of bacteria

Algal packets were isolated from the fern leaf cavities (from the 5th to the 10th) by manual dissection. The bacteria from the algal packets were isolated following Gates et al. method (1980), except that sterile, distilled water was used instead of buffered water (APHA, 1971).

Various dilutions of the sterilized algal packets were plated on TRN medium containing ($g\ l^{-1}$):tryptone (Difco) 10; NaCl (Merck) 5; agar (Oxoid) 15; the pH was 7. The cultures were incubated at 30°C. Four-five days later the first bacteria colonies were visible. Anaerobic bacteria growth was determined on thioglycollate broth (Difco) solified with 1.5% agar. The agar plates were incubated in a Brewer chamber with a CO_2 and H_2 generating GasPak (Baltimore Biological Laboratories). *Bergey's Manual of Bacteriology* (1986) was used for bacterial identification.

Bacteria utilization of different carbon and nitrogen sources was determined on M 9 medium ($g\ l^{-1}$): KH_2PO_4 3; $MgSO_4$ 0.2; Na_2HPO_4 6; pH 7. Either 20 mM NH_4Cl or $NaNO_3$ was added where necessary. Solutions of single carbohydrate or organic acid (the acids were neutralized with NaOH to pH 7) were sterilized separately and added to the medium to give the final concentrations of 1% (w/v) or 0.5% (w/v).

Growth factor solutions (biotin or riboflavin 10 mg 100 ml^{-1}) were filter-sterilized (Millipore filter, 0.45 μm pore size) and added when needed (0.1 $\mu g\ ml^{-1}$) (Forni et al., 1987).

Enzymatic assays

Nitrogenase activity was determined by acetylene reduction assays (ARA). ARA was measured under 10% acetylene in aerobic, microaerobic (2% or 5% O_2 in argon) and anaerobic conditions in 7 day old cultures, grown in

semisolid N-free M 9 medium to which 1% glucose as carbon source was added. The cultures were incubated at 30°C.

Ethylene formation was followed by Perkin-Elmer gas-chromatography provided with a silica gel column. Catalase test was made according to Smibert and Krieg (1981); *Staphylococcus epidermidis* was used as positive control. Oxidase test was made by means of the Oxidase Sticks purchased from Oxoid. Gas and H_2S productions were determined on TSI medium (Triple Sugar iron), purchased from Baltimore Biological Laboratories.

3. Results

Bacteria in the Azolla leaf cavities

The bacteria observed by SEM in the leaf cavities of *Azolla caroliniana* appear to adhere to the envelope and to the hair cells of *Azolla* (Fig. 1). The algal packets, isolated from the leaves and opened after glutaraldehyde fixation, appear in a mucilagous matrix (Fig. 2).

The bacteria were rods, variable in size, ranging from 0.5 μm to more than 10 μm in length and 0.5–0.6 μm in diameter. Cocci (0.3–0.5 μm in diameter) are detectable, single or in different size colonial complexes (Fig. 3).

TEM observations showed also, that in the young leaves, as well as in the old ones, the bacteria appear either close to the hair cells (Fig. 4) or localized nearby the epidermal cells of *Azolla*. Rod was the more common bacteria shape (Figs. 5–6), sometimes being very long (Fig. 7).

Many bacteria adhered polarly to the fern hairs, but no particular adjoining structure could be distinguished between the bacteria end and the host cells (Fig. 5).

The bacteria cell wall had a peptidoglycan layer of variable thickness. The different staining and cytochemical reactions used showed no evidence of any structure external to the cell wall comparable to a glycocalyx. A large electron dense fibrillar area was often visible in the nucleoplasm, especially after ruthenium red staining (Fig. 8). Numerous electrondense granules, visible in the cytoplasm after Thiéry reaction (1967), were interpreted as glycogen (not shown).

Bacteria in non azenic culture of A. azollae

In non axenic cultures of *A. azollae* obtained from *A. caroliniana*, bacteria did not seem to be different from those present in the fern leaf cavities (Figs. 9–10). In the same cultures a mucilage, to which both *Anabaena* and bacteria adhere, was found (Fig. 11). Under SEM, this film was similar to the

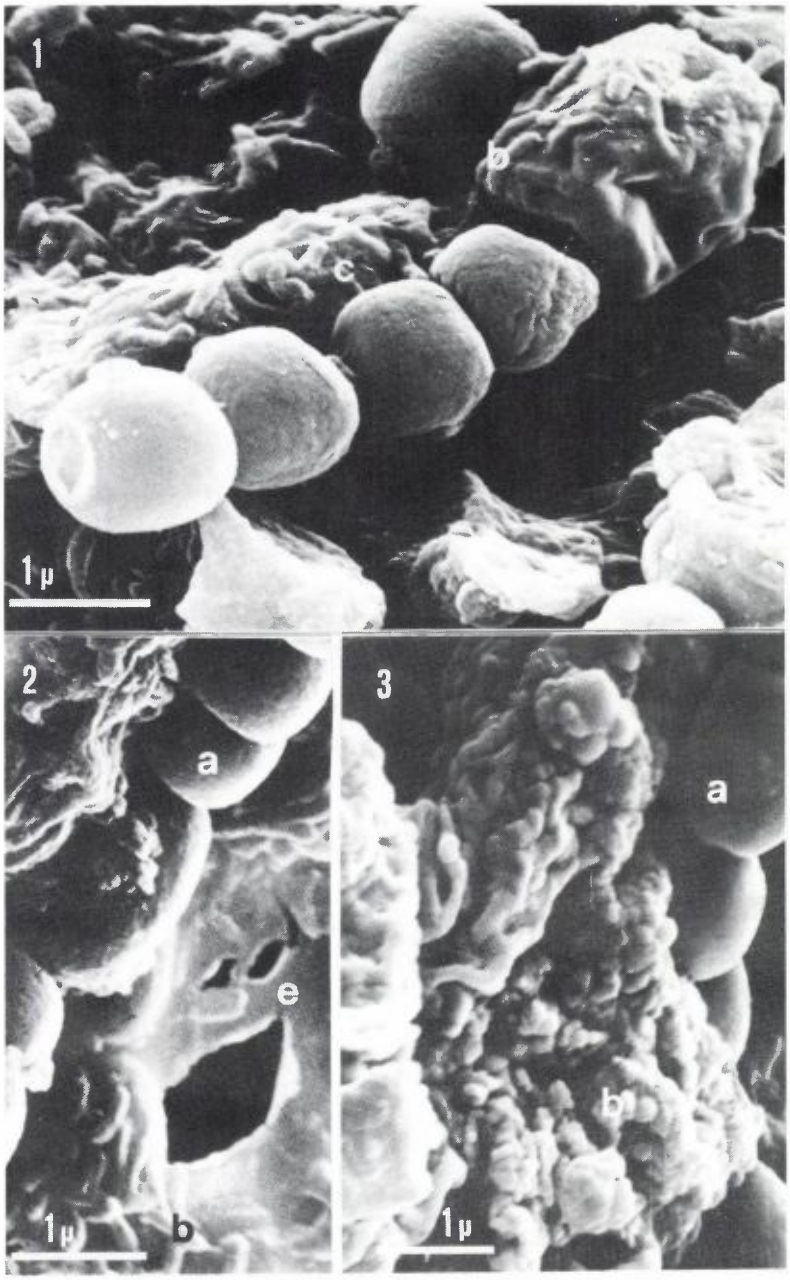

Plate I. SEM micrographs of *Azolla caroliniana* leaf cavities.
Figure 1. Algal packets and bacteria (b) adhering to hair cells (c).
Figure 2. Algae (a), bacteria (b) and the envelope (e).
Figure 3. Algae (a) and roundish and elongated bacteria (b).

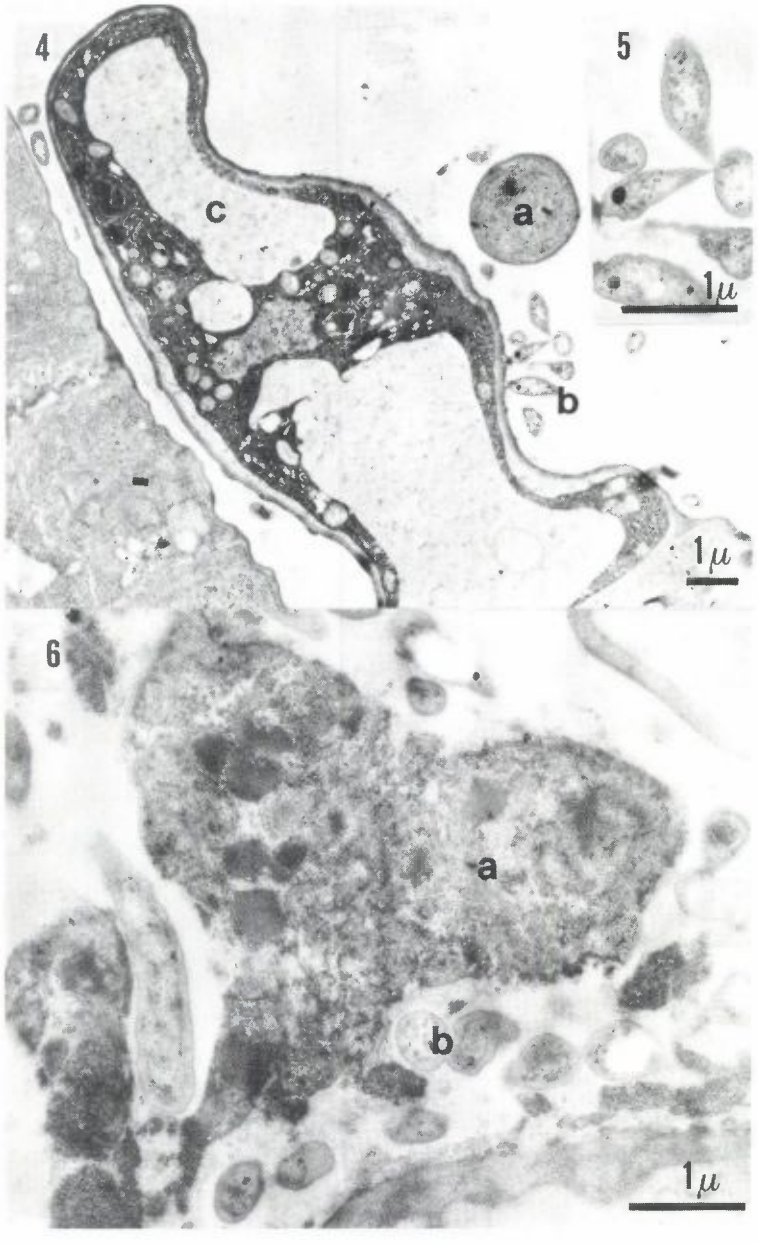

Plate II. TEM micrographs of leaf cavities

Figure 4. Apical leaf cavities with algae (a) and bacteria (b) adhering to hair cells (c). Glutaraldehyde osmium fixation.

Figure 5. Bacteria of Fig. 4.

Figure 6. Basal leaf cavity with bacteria (b) surrounding degenerated cell of *Anabaena* (a). Glutaraldehyde osmium fixation.

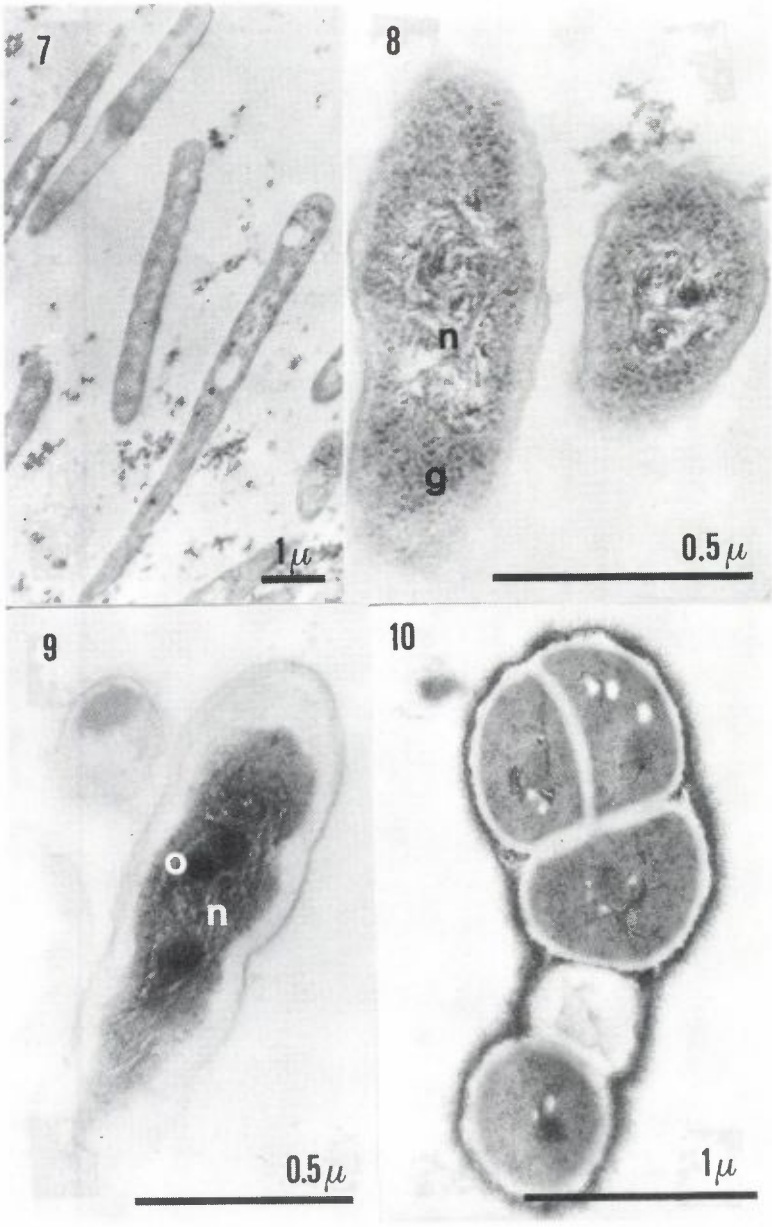

Plate III.

Figure 7. Long rod-shaped bacteria in the leaf cavity $KMnO_4$ fixation.

Figure 8. Bacteria with fibrillar area (n) and many glycogen granules (g) after glutaraldehyde ruthenium red fixation.

Figure 9. Bacteria in non axenic cultures of *A. azollae* growing in nitrogen-free medium. In the cytoplasm fibrillar area (n) and osmiophilic bodies (o) are visible. Glutaraldehyde ruthenium red fixation.

Figure 10. Cocci in non axenic *A. azollae* culture growing in nitrate medium BG 11.

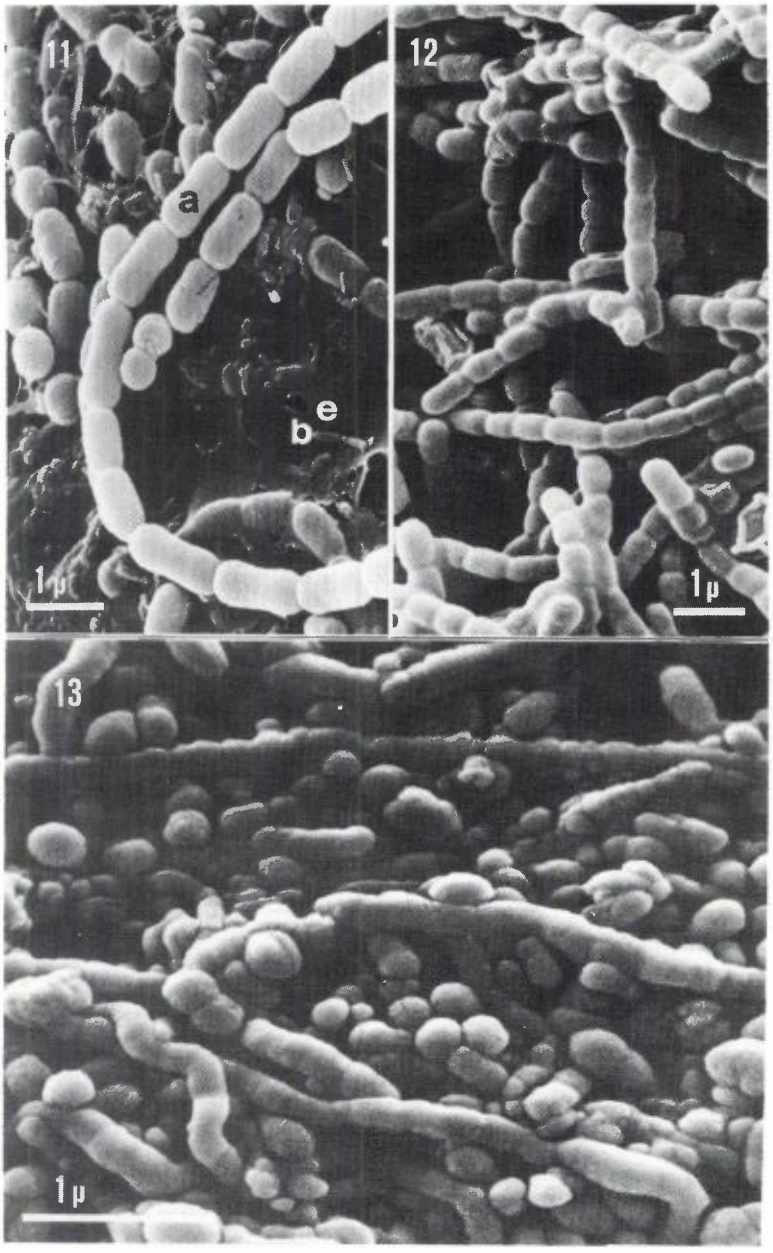

Plate IV. SEM micrographs

Figure 11. Non axenic culture of *A. azollae* showing an envelope-like production (e), algae (a) and bacteria (b).

Figure 12. Axenic culture of *A. azollae*.

Figure 13. Bacteria isolated from the previous non axenic culture of *A. azollae*.

mucilagous matrix observed in *Azolla* leaf cavities. There was no such mucilage production in the axenic cultures of the cyanobacterium (Fig. 12). In *Anabaena* cultures grown in nitrate medium (BG-11), the number of coccal forms increased (Fig. 10). Bacteria (rods or cocci) isolated from non axenic *A. azollae* cultures are shown in Fig. 13.

Bacteria isolation and determination

Bacteria were isolated from numerous fern leaf cavities (Forni et al., 1987). Two types of colonies were produced on TRN medium: bright-lemon yellow colonies and white colonies. Six colony isolates were obtained when using routine bacteriological procedures: i.e. two isolates produced bright-lemon yellow colonies, while the others produced white colonies.

The bacteria were Gram-positive, but were readily decolorized depending on the growth phase. A rod-coccus life cycle occurred in culture of all the bacteria isolated. None of them fixed nitrogen. They did not require vitamin (biotin or riboflavin) as growth factor. When ammonia or nitrate was present as nitrogen source, they could grow in M 9 medium containing different carbon sources, i.e. glucose, fructose, mannitol, galactose, sucrose, sorbitol, glycerol, ribose, acetate, succinate, and citrate.

All the bacteria isolated were catalase positive. The bacteria producing white colonies were oxidase positive, while the others were oxidase negative. One isolate formed acid from glucose, but only after 2 days or more of growth on TSI medium. No production of gas, H_2S or indole have been detected. Mucilage was produced by the 4 isolates forming white colonies.

Based on Gram reaction, life cycle, i.e. rod-coccus cycle with rods prevailing during the log phase and gradual shortening of these rods until cocci predominate in the death phase, and the biochemical characteristics given above, the isolates have been identified as *Arthrobacter* sp. Conn and Dimmick (Bergey's, 1986).

No anaerobic bacteria were detected.

4. Discussion

In the leaf cavities of *Azolla caroliniana* numerous bacteria are present, some of which are very close to the cell wall of the hair cells protruding into the cavity. These observations confirm the data of Peters et al. (1978) and Calvert et al. (1985).

In many symbiotic or parasitic associations, structural interactions (glycocalyx, fimbriae or pili) between the cells have been found (Dick and Stewart,

1980; Grilli Caiola and Pellegrini, 1984). The lack of such structures in the *A. azollae* or bacteria might mean the absence of structural mechanisms for recognition. This could be correlated to the unnecessary recognition process between the host and the phycobiont (Ladha and Watanabe, 1982; 1984), since the *Anabaena* is retained throughout the life cycle of the fern (Peters et al., 1982).

Many bacteria have been found in our non axenic cultures of *Anabaena azollae*, most of which were similar to those living in the leaf cavities. However an increase of morphologically different bacteria have been noted in *Anabaena* cultures grown in the presence of nitrogen compounds. Since *Arthrobacter* have been found not only in *A. caroliniana*, but also in other *Azolla* species (Petro and Gates, 1987; Forni et al., 1987), these bacteria seem to be a constant component of the *Azolla-Anabena* association. Based on the data of Petro and Gates (1987) and Forni et al. (1987), it is very likely that *Arthrobacter* is the only bacterial genus living in the fern, but perhaps as different species of it (Forni et al., 1987).

Concerning the significance of these bacteria in symbiosis, we can only put forward some hypotheses. Since no nitrogenase activity has been detected in the bacteria isolates, it is likely that the presence of the bacteria *in vivo* is correlated to the availability of nitrogen and carbon. Calvert et al. (1985) reported that in the fern apex the endophyte utilizes carbon and nitrogen compounds supplied by the host. These compounds may also be used by the bacteria. Furthermore, the number of the bacteria increases with the leaf age (Petro and Gates, 1987; Grilli Caiola et al., 1988); this might be due to the greater availability of carbon and nitrogen in the basal leaves. However, the relationship among the components of the symbiosis may be rather complex; it is likely that the bacteria can provide some substances, the role of which is still unknown (work in progress). The mucilagous matrix discerned by cytochemical reactions may have a role in the adherence of the bacteria and/or the endophyte to the envelope (Grilli Caiola and Albertano, 1987). The origin of this mucilagous matrix is still unknown. Peters et al. (1978) were unable to digest this matrix by different enzymes (i.e. cellulase, pectinase, lipase and protease). One or more organisms might be involved in the production of the envelope, i.e. the endophyte (Duckett et al., 1975), the host and/or the bacteria. Recently Robins et al. (1986) suggested that the mucilagous matrix is produced by the *Anabaena*, but the lack of similar production in axenic *A. azollae* cultures would rather suggest that *in vivo* the presence of the matrix is due to the bacteria, the isolates of which produce

mucilage on TRN medium. Further work on this subject is in progress.

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