Symbiosis, 3 (1987) 41–48
Balaban Publishers, Philadelphia/Rehovot

Distribution of *Arthrobacter* sp. in the Leaf Cavities of Four Species of the N-fixing *Azolla* Fern

M.J. PETRO[1] and J.E. GATES[1,2*]

Department of Biology[1] and Department of Microbiology and Immunology[2], Virginia Commonwealth University, 816 Park Ave., Richmond, VA 23284, U.S.A.
Tel.(804) 257-1562

Received July 9, 1986; Accepted December 21, 1986

Abstract

Bacteria previously identified as *Arthrobacter* sp. (Wallace and Gates, 1986) were found in all *Azolla* specimens analyzed including fresh material collected from natural habitats and specimens obtained from another laboratory. There was a direct linear relationship between numbers of these bacteria and leaf age in *Azolla caroliniana*, *A. mexicana*, and *A. filiculoides* ($r^2 = 0.90$ or greater, $P < 0.01$), while no such relationship was found in *A. pinnata* which contained fewer numbers of bacteria. The ubiquity and constant population densities of these bacteria in any given leaf of a given fern species suggests a controlled situation and the possibility of a tripartite symbiosis. Whether their effect on the other symbionts is mutualistic or antagonistic is presently unknown.

Keywords: *Arthrobacter*, *Azolla*, tripartite symbiosis, nitrogen-fixation

Abbreviations: PCA (Plate Count Agar, Difco)

* Corresponding author

0334-5114/87/$03.00

1. Introduction

Previously, we reported that coryneform bacteria occurred in the leaf cavities of the nitrogen-fixing fern, *Azolla caroliniana* Willd., and that the numbers of these eubacteria exceeded those of the cyanobacteria responsible for the nitrogen-fixation process (Gates et al., 1980). We recently reported the presence of these microorganisms in other species of *Azolla* and identified these bacteria as *Arthrobacter* sp. (Wallace and Gates, 1986).

This study was undertaken to determine the distribution of these bacteria in leaves of different ages from 4 species of the fern, *Azolla caroliniana* Willd., *A. mexicana* Presl., *A. filiculoides* Lam., and *A. pinnata* R.Br.

2. Materials and Methods

Fern culture methods

Azollae were either obtained from D.W. Rains, University of California, Davis, CA, U.S.A., or field collected. These were either screened immediately for the eubacteria or cultured as reported previously (Wallace and Gates, 1986).

Bacterial enumeration

The dorsal lobes of several leaves of a given age from 0 (apex) to leaf 15 were removed by manual dissection and the rest of the fern discarded. These leaves were surface sterilized in 3% sodium hypochlorite, then washed with sterile 5% sodium thiosulfate to neutralize the bleach, and finally washed with sterile buffered water (American Public Health Association, 1971) to remove the sodium thiosulfate. These procedures were carried out on a 25 mm Nuclepore filter (0.4μm pore size) under vacuum filtration.

Ten surface sterilized leaves of each leaf age from each fern species were aseptically transferred to a sterile Ten Broeck glass tissue homogenizer (American Scientific Products, Columbia, MD, U.S.A.) containing 2 ml of sterile buffered water. The leaves were homogenized, and various dilutions of the homogenate were plated out on Difco Plate Count Agar (PCA) in triplicate using sterile buffered water as the diluent. Colonies were counted on a Quebec Colony Counter after 5 days at 30°C. Standard deviations between replicate plates never exceeded 10% of the means, so 3 replicate plates were considered to be adequate. Variations in colony morphology were noted, and over 100 single colony isolations were made. These assays were repeated 3 times over an 18 month time period. Relationships between leaf age and numbers of bacteria were determined using an IBM 370-150 computer under

Table 1. Numbers of bacteria in leaves of different ages in three species of *Azolla*

Leaf #	*A. caroliniana*	*A. mexicana*	*A. filiculoides*
0	1,000±80[a]	10±1	2,100±200
2	1,500±200	700±100	2,600±200
4	5,200±300	3,700±500	5,400±200
6	5,400±200	3,200±300	80,000±2,500
8	6,100±200	4,600±500	180,000±2,000
10	7,600±400	10,000±1,600	210,000±1,500
15	10,000±900	16,000±1,700	330,000±4,200

[a] Means and standard deviations from three experiments. Data rounded to two significant figures.

release 79.5 of the Statistical Analysis Systems Institute, Inc. (Barr et al., 1976).

To determine if any of the leaf cavities had ruptured during the sterilization procedure and to check the adequacy of the procedure for removing surface contaminants, the Nuclepore filters were routinely transferred to the surface of PCA plates and incubated for 5 days at 30°C. On only one occasion did bacterial growth occur. It was concluded that either some leaf cavities had ruptured or that surface sterilization was inadequate, and the experiments were repeated for that group of leaves. To further test the procedure, surface sterilized leaves were drawn across PCA plates. Absence of bacterial growth after 5 days indicated successful sterilization of leaf surfaces.

3. Results

The numbers of bacteria in leaves of different ages in *Azolla caroliniana*, *A. mexicana*, and *A. filiculoides* are shown in Table 1. Largest numbers were found in *A. filiculoides* which ranged from about 2000 cells associated with the leaf apex to over 300,000 cells in the oldest leaf studied. *A. caroliniana* and *A. mexicana* contained fewer bacteria, particularly in the older leaves, with about 1000 cells found in the youngest leaves and over 10,000 in leaf 15. The relatively low standard deviations from experiment to experiment indicates the stability of the bacterial populations within any given leaf in these 3 fern species.

Only 10–14 bacteria could be detected in the leaf cavities of *A. pinnata*. If the sodium hypochlorite treatments were omitted, large numbers of a variety of bacteria were found associated with the leaves of all fern species studied, including *A. pinnata*.

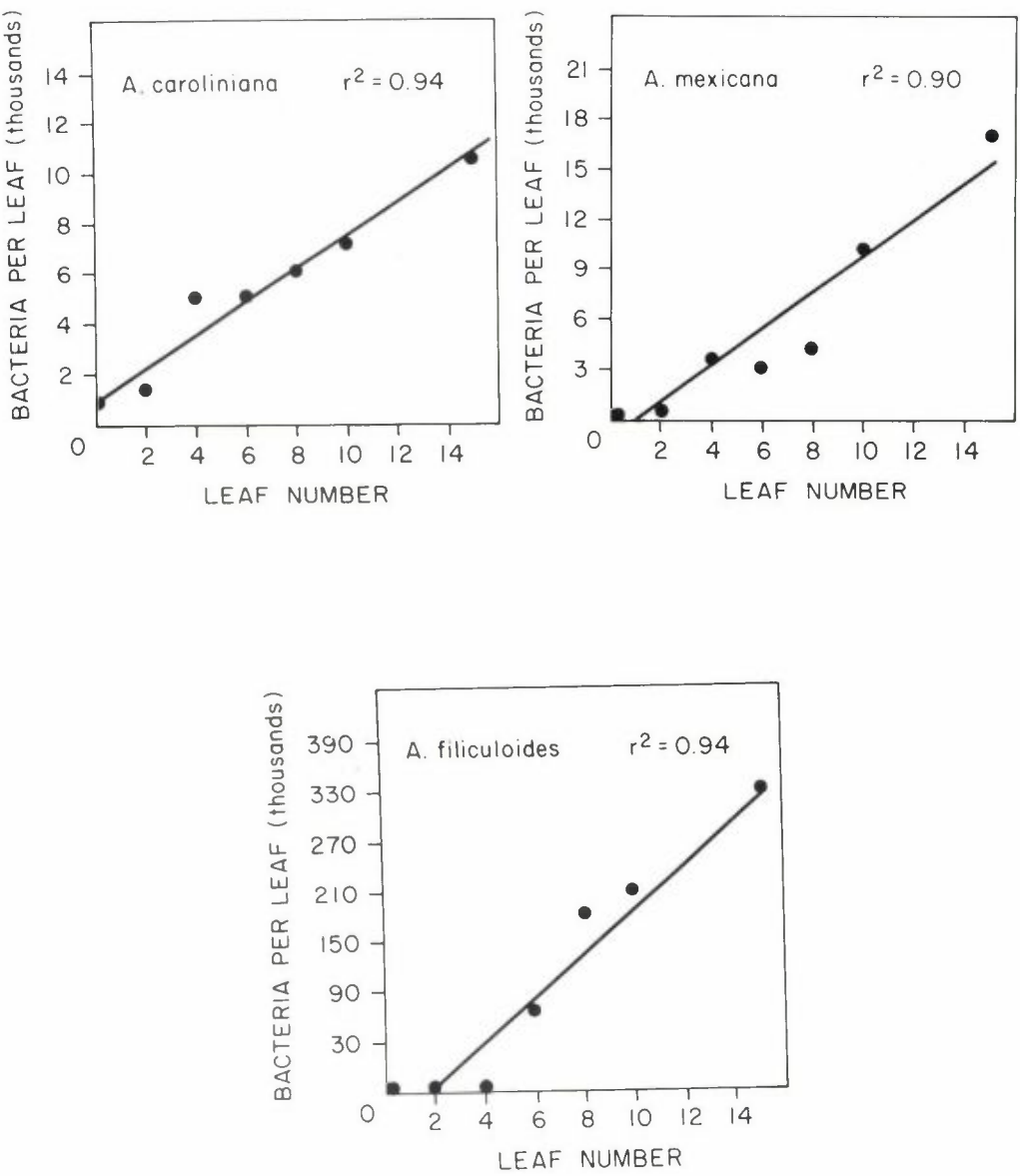

Figure 1. Distribution of eubacteria in *Azolla caroliniana*, *A. mexicana*, and *A. filiculoides*. Each data point represents the mean of 3 experiments. The r^2 value indicates the decimal fraction of the variation in the numbers of such bacteria from leaf to leaf that could be explained on the basis of leaf age.

With the exception of *A. pinnata*, a direct linear relationship significant at the 1% level was found to exist between fern leaf age and numbers of bacteria in the leaf cavity (Fig. 1). For *A. caroliniana*, $r^2 = 0.94$, indicating that 94% of the variation in numbers of bacteria could be attributed to leaf age, while *A. mexicana* and *A. filiculoides* had r^2's of 0.90 and 0.94. To examine the possibility that the relationship between leaf age and numbers of bacteria might be more similar to typical growth kinetics, the log of cell number was also regressed upon leaf age. Significant regressions resulted, but r^2's were somewhat lower, 0.78 ($P < 0.01$), 0.66 (< 0.05), and 0.83 ($P < 0.01$), respectively, for these 3 ferns. Therefore, the data had a better fit for a linear function.

The bacteria isolated from any given leaf cavity from all fern species generally produced a white colony with a slightly tan center on PCA. Occasionally, pigmented colonies occurred on the plates but these never exceeded 7% of the total bacterial cells from any leaf cavity. The pigments did not diffuse into the culture medium. One colony type was white initially but progressively darkened to brown and ranged from 0–4% of the cells in any given leaf cavity. Bright pink and bright yellow forms also occurred from 0 to less than 1% of the population from a leaf cavity. The morphological and metabolic characteristics of the pigmented bacteria were otherwise similar to the dominant white/tan colony type. All of these isolates were stable in culture if transferred to fresh PCA every 2–3 weeks and kept at 25–30°C. Pigmented forms maintained their pigmentation even after repeated transfers and did not arise spontaneously in cultures of the dominant white/tan isolates.

4. Discussion

The absence of significant numbers of these bacteria in *Azolla pinnata* is surprising as isolates from this fern species are very similar to those from the other 3 fern species analyzed. Perhaps the sodium hypochlorite more readily penetrates the leaf cavity killing the bacteria present. It may be significant that *A. pinnata* is taxonomically distinct from the other species of fern studied and has been placed in the subgenus *Rhizosperma*. The other species belong to the subgenus *Euazolla* (Moore, 1969).

The pigmented bacteria are of interest because of their low frequencies. No obvious relationship seemed to exist between their presence and any fern species or leaf age. Their metabolic and morphological characteristics were similar to the more dominant white/tan isolate, and we are assuming that they represent pigmented variants of one organism.

Previous investigators have noted the presence of aerobic rod-shaped bacteria in the *Azolla* fern leaf cavities. Bottomley (1920) suggested that *Pseudomonas* was always present in this fern. Peters et al. (1974, 1978) reported unidentified aerobic bacteria within the fern leaf cavities. Their electron micrographs clearly show these bacteria, and an interpretation of a coryneform morphology is possible from these micrographs. Newton and Herman (1979) found bacteria which they identified as *Alcaligenes faecalis* and *Caulobacter fusiformis* associated with cultures of *Anabaena azollae* isolated from surface sterilized fern leaves. It is likely that all of these reports may be about the same organism. As we reported earlier (Wallace and Gates, 1986), unless greater than normal care is taken to Gram stain cells from cultures of various ages, it is most likely that a Gram negative interpretation would be made. Most of the determinative results are consistent with either a *Pseudomonas* or *Alcaligenes* identification.

We recently (Wallace and Gates, 1986) found these bacteria isolates to belong to the genus *Arthrobacter* Conn and Dimmick. However, the actual identification of these bacteria is of less importance than the consistent observation by various investigators that O_2 consuming bacteria are prevalent in the *Azolla* leaf cavities. To insure that our culture conditions were not responsible for the presence of this particular microorganism, fern specimens from other investigators and from some natural sources including a California rice field and a North Carolina interdunal pond were assayed for these bacteria as soon as we received them. All were found to contain bacteria with these same characteristics. To date, we have assayed over twenty *Azolla* specimens, and every one contained these bacteria.

It is possible to obtain a rough estimate of the effect that these organisms might have upon O_2 and CO_2 tensions in fern leaf cavities. If we estimate the cavity volume in older leaves to be as large as 0.05μl, then the concentrations of these bacteria reach nearly 10^{10} cells/ml in these ferns. This is a very conservative estimate, because the cavity volume estimate is very liberal. Also, part of the cavity volume is occupied by cyanobacterial cells, fern hair cells, and possibly a gas bubble. Nevertheless, to reach even these conservatively estimated population densities, our growth studies (Wallace and Gates, 1986) would indicate that a substrate concentration comparable to over 5 mM of glucose would have to be oxidized generating over 30 mM of CO_2 and reducing over 30 mM of O_2.

We conclude that these bacteria are ubiquitous in the *Azolla* fern and have probably therefore co-evolved with the fern and cyanobiont over a long pe-

riod of time. That they reach such predictable population densities in the fern suggests a controlled situation and the possibility of a tripartite symbiosis between fern, cyanobacterium, and these eubacteria. Their effect on the other symbionts may be mutualistic by enhancing rates of photosynthesis and nitrogen-fixation as has been suggested by Lupton and Marshall (1981) and Paerl (1982) for cyanobacterial-eubacterial associations in general, or it may be antagonistic by competing with the cyanobacteria for available nutrients. Further study of the role of these eubacteria in the *Azolla-Anabaena* symbiosis is warranted.

Acknowledgement

This work was supported in part by U.S. Department of Agriculture Grant 5901-0410-9-0304-0 and in part by National Science Foundation Alternative Biological Resources Program Grant PCM 83-05202.

REFERENCES

American Public Health Association (APHA). 1971. *Standard Methods for the Examination of Water and Wastewater.* 13th ed., Washington, D.C., U.S.A.

Barr, A.J., Goodnight, J.H., Sall, J.P., and Helwig, J.T. 1976. *A User's Guide to SAS-76.* Sparks Press, Raleigh, NC.

Bottomley, W.B. 1920. The effect of organic matter on the growth of various water plants in culture solution. *Ann. Bot.* **39:** 353–365.

Gates, J.E., Fisher, R.W., Goggin, T.W., and Azrolan, N.I. 1980. The occurrence of coryneform bacteria in the leaf cavity of *Azolla*. *Arch. Microbiol.* **127:** 163–165.

Lupton, F.S. and Marshall, K.C. 1981. Specific adhesion of bacteria to heterocysts of *Anabaena* spp. and its ecological significance. *Appl. Environ. Microbiol.* **42:** 1085–1092.

Moore, A.W. 1969. *Azolla*: Biology and agronomic significance. *Bot. Rev.* **35:** 17–34.

Newton, J.W. and Herman, A.I. 1979. Isolation of cyanobacteria from the aquatic fern, *Azolla*. *Arch. Microbiol.* **120:** 161–165.

Paerl, H.W. 1982. Interactions with bacteria. In: *The Biology of Cyanobacteria.* N.G. Carr and B.A. Whitton, eds. *Botanical Monographs* **19:** 441–461.

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., Toia, Jr., R.E., Raveed, D., and Levine, N.J. 1978. The *Azolla-Anabaena azollae* relationship. VI. Morphological aspects of the association. *New Phytol.* **80:** 583–593.

Wallace, W.H. and Gates, J.E. 1986. Identification of eubacteria isolated from leaf cavities of four species of the N-fixing *Azolla* fern as *Arthrobacter* Conn and Dimmick. *Appl. Environ. Microbiol.* **52:** 425–429.