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Invited Paper

The *Azolla-Anabaena*-Bacteria System as a Natural Microcosm

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ABSTRACT

Azolla is an aquatic fern that contains a permanent endosymbiotic prokaryotic community (cyanobacteria and bacteria) inside of the cavity in the leaf dorsal lobe of the pteridophyte. This is an unique situation and can be seen as a microcosm inside of an organism and also can be considered a good example of a living model for biological and environmental studies. These symbionts are specific of this symbiosis and lives immobilized in a mucilaginous fibrillar network, which fills part of the cavity. The symbionts works as immobilized organisms in a natural system that can be used as a model for biotechnological research and in biologically based life support systems. The nature and the complexity of this system is simultaneously a reference and a challenge for the research in the communication between the two levels of nature organization (microcosm and mesocosm), and can also be used as a reference for the design of new environmental engineered symbiotic systems that include man as a prelude to life in space.

Keywords: *Azolla*, *Anabaena*, bacteria, leaf cavity, microcosm, living model, space

1. INTRODUCTION

Natural microcosms can be defined as "small, partly isolated ecosystems…" that are present in nature[1]. These "natural containers" indicate that these systems have a self-organization and an ecological defined structure. The concept used here can be applied to the *Azolla* leaf cavity and its symbionts. Until recently, this pteridophyte was seen as a botanic curiosity by Western researchers, since the presence of cyanobacteria inside the leaf cavity allowed it to be considered as a classical example of a mutualistic symbiosis, earlier described by the German scientist Eduard Strasburger in 1873. This aquatic fern has been used as green manure for rice cultivation and animal feed in China and in Vietnam, during several centuries, and more recently in Africa[2]. The new advances in the investigation of this symbiosis have contributed to a more comprehensive perception of the complexity between the host and the symbionts[3]. This complexity has been translated into new models of knowledge and new areas of application. The biotechnology and environmental engineering are some of these main fields, which have and can still profit with this new data. New bioreactors can be developed if we consider these results, namely those related with the living conditions existing in the leaf cavity of the fern. The latter enable high performance for some specific metabolic reactions of the symbionts, namely nitrogen fixation, ammonium and hydrogen production by the immobilized cyanobiont[4]. Further, the use of *Azolla* as wastewater biofilter[5] and in biologically based life support systems (BLSS) incorporated in bioregenerative space devices are now in progress in several laboratories[6].

2. *AZOLLA-ANABAENA* SYMBIOSIS

Azolla is a small-leaf floating fern, whose genus was established by Lamarck in 1783. It contains an endosymbiotic community living in the dorsal lobe cavity of the pteridophyte leaves. This community is composed of two types of prokaryotic organisms: one species of nitrogen-fixing filamentous cyanobacteria - *Anabaena azollae* Strasb. - (first described by Strasburger as *Nostoc* in 1873 and re-named *A. azollae* in 1884)[7] and a variety of bacteria strains mainly identified as members of the genus *Arthrobacter* and *Agrobacterium*, associated with others species showing the presence of nitrogenase[3,8,13]. In this association, it is assumed that an exchange of metabolites, namely fixed nitrogen compounds, occurs from the cyanobiont to the host and photosynthate sugars from *Azolla* to the cyanobacteria[9,10]. In this exchange, a special type of trichomes, the simple hairs, is involved. These symbionts are specific of this symbiosis and live immobilized in a mucilaginous fibrillar network, which fills part of the cavity[3]. The symbionts work as an immobilized organism in a natural system that can be used as a model for biotechnological research. The prokaryotic community co-exists with the fern and it can be considered as a microcosm inside of a living organism when compared with the macrocosm, where the all-symbiotic system develops and interacts. In order to understand the relationship and the energetic flows between these two levels of organization, we must look at the *Azolla*-*Anabaena*-bacteria system with a more open mind, seeing this symbiosis in a dynamic and synergistic way.

One of the most interesting features is the role played by the cyanobacterium in this association. Filaments of *Anabaena azollae* are localized in a cavity of the dorsal lobe of the fern's leaves, where special conditions stimulate high heterocyst frequency and a vegetative cell differentiation during leaf development[9]. The existence of the two symbionts inside the *Azolla* leaf cavity and its relationship with the fern, namely the metabolites flowing between the host and the symbionts, can be seen as a unique micro-ecosystem with own well-established characteristics. This association is maintained during all the life cycle of the pteridophyte. The *Anabaena* apical colony is associated with shoot apex, which lacks heterocysts and, therefore, is unable to fix nitrogen. In mature leaves, the *Anabaena* filaments cease to grow and differentiate heterocysts, which are the site of N_2 fixation. Besides the cyanobacteria, a population of bacteria undergoes a pattern of infection identical to *Anabaena* and is probably the third partner of this symbiosis[3]. In the establishment of the *Azolla*-*Anabaena*-bacteria symbiosis several molecules are involved, namely carbohydrate-binding proteins, such as lectins, which may play a role in it[8]. The prokaryotic colony - cyanobacteria and bacteria - is also present in the sexual structures (sporocarps) of the fern[3]. The cyanobacterium is transferred from the sporophyte to the next generation via the megasporocarp. A cyanobacterium colony resides between the megasporocarp wall and the megasporagium and inoculates the newly emerging sporophyte plant. A colony of the symbiotic cyanobacteria is formed near the shoot apex and thus enables symbiosis to be established within the developing leaf cavities. The presence of bacteria in the megasporocarps in association with the cyanobacteria also suggests a behaviour pattern similar to that of the cyanobionts[3,11]. The presence of *Anabaena* throughout the life cycle of the fern favours the obligatory nature of the symbiosis and suggests a parallel phylogenetic evolution of both partners[11].

3. THE *AZOLLA* LEAF CAVITY

Within the *Azolla* dorsal leaf lobe exists a specialized cavity (Figure 1) that contains a permanent endosymbiotic prokaryotic community (cyanobacteria and bacteria). The latter is associated with trichomes, traditionally called hairs (simple and branched), which show an ultrastructure of transfer cells. This cavity can be considered as the basic physiological unit of the symbiotic association[12], and can also be seen as a special micro-ecosystem - a natural microcosm - revealing a self-organization and an ecological defined structure. Another interesting approach is to consider this micro-ecosystem as a natural photobioreactor, with millions of years of evolution, where the symbionts are immoblized and driven by the fern into increasing some of its own physiological and metabolic activities.

When mature, the cavities are ellipsoid in shape, measuring approximately 0.15 X 0.30 mm[13]. The interior of the cavity is lined by an outer and an inner envelope, creating a narrow space close to the periphery of the cavity where the bacteria, the

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cyanobacteria and the hairs are located. This, results in an intimate contact between all the partners[14]. In this narrow space all the endosymbionts are embedded in a mucilaginous network that immobilizes all of them, helping in the recognition process as well as in the exchange of metabolites[9]. The centre of the mature cavity is apparently empty, without any mucilage, cyanobacteria or bacteria but is probably occupied by liquid or gas[12]. The trichomes present in the cavity are involved in translocation of photosynthates and in the uptake of nitrogen compounds. Lipids, proteins, tannins and polysaccharide compounds were detected in the vacuoles of simple hairs. These compounds may play a role in the selection of the microorganisms that are not useful to the fern, in the control of the endosymbionts in the cavity and in the establishment and maintenance of the symbiosis. These data also suggest an exchange of chemical compounds between the host-endosymbionts-host, functioning as a biological and chemical communication language in this dynamic association[15]. In this sense, the *Azolla* cavity, working as the basic physiological unit of this symbiotic system can be considered as a good model for basic and applied research, namely in the biologically based life support systems, as it will be discussed in the next chapter.

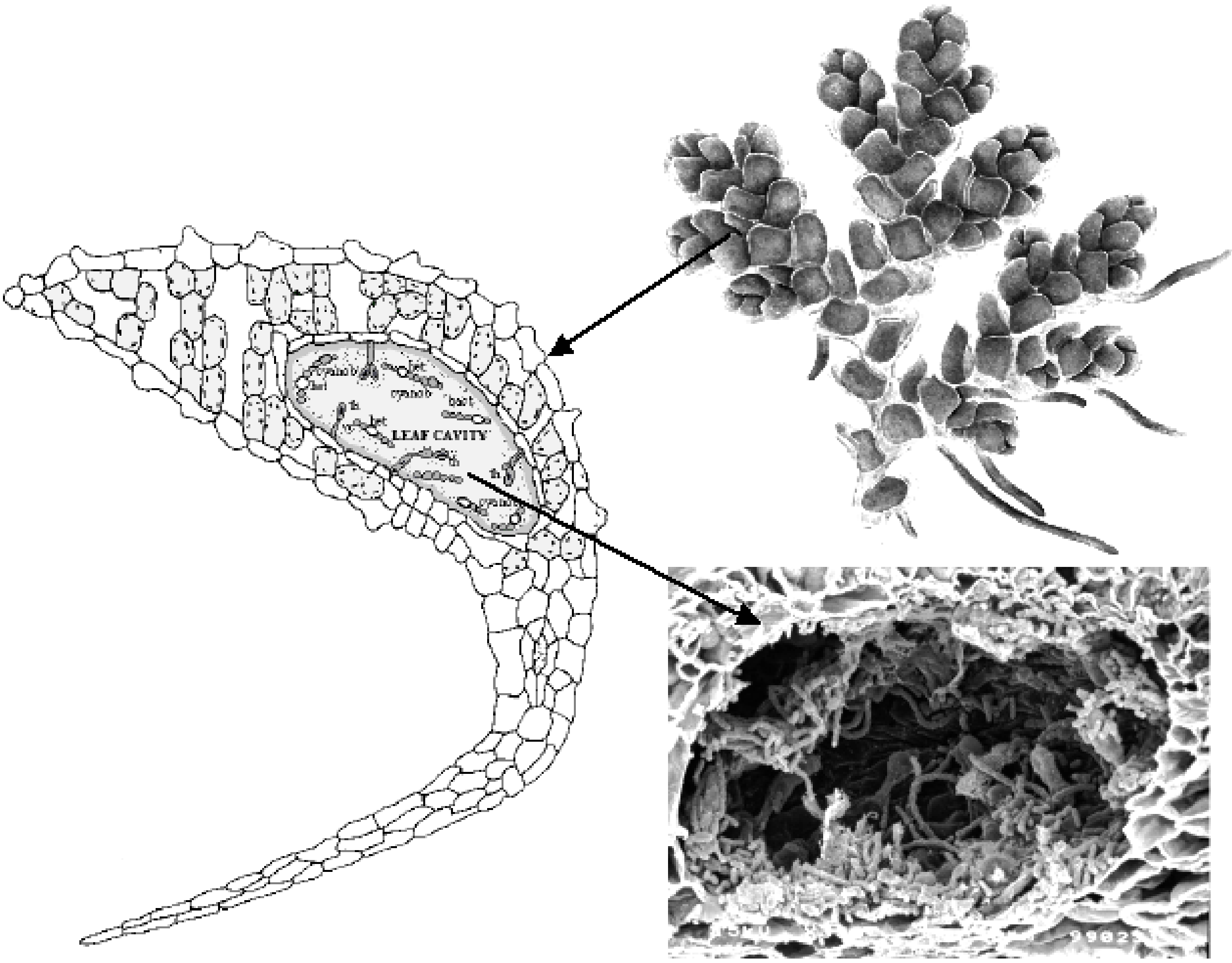

Figure 1: Location of the leaf cavity in *Azolla filiculoides.*

4. *AZOLLA* AND SPACE EXPLORATION

One of the main problems of long-duration space missions involving human presence, is trying to avoid demands for costly resource resupply through provision of the essential functions of food production, gas exchange, water reclamation, and nutrient recycling[6]. For that task we must implement the design, development, and operation of a biologically regenerative life support system (BLSS), that in conjunction with the adequate physico-chemical processes[6] will be capable of successfully sustaining the quality of human life in an hostile environment, such as space. The regenerative stabilization of the mass conservation of nitrogen in a BLSS is one of the most important challenges for engeneering. This task can be done through two ways: a physico-chemical or a biologically based process. The first one needs a great deal of energy, operating with temperatures of 550°C and pressures of 200 atmospheres. Even using ruthenium, instead of iron, in catalyzing ammonia synthesis at normal atmosferic pressure and at the temperature of 250°C, which is more manageable at industry level and spends less energy, these physico-chemical processes are still quite unfeasible for regenerative space applications[6]. On the other hand, the biological nitrogen fixation, which converts this gas into ammoniac, is catalyzed by nitrogenase with molybdemium as its cofactor, and proceeds at room temperature and pressure without the needs for complicated product recovery and recirculation maneuverings[6], with the exception of high ATP consuption.

The *Azolla-Anabaena* symbiotic association is the biocatalyst system with the highest nitrogen-fixing capacity in nature (400-500 Kg.N_2 fixed/hectare/year). It has been used as biofertiliser for rice cultivation and animal feed in China and in Vietnam, during several centuries, and more recently in Africa[2,6]. A new method of using nitrogen fixing cyanobacteria in a photobioreactor for ammonia production for rice crop was developed by Kannaiyan's team (Tamil Nadu Agricultural University, India), by means of immobilisation of *Anabaena azollae* and *Anabaena variabilis* in solid matrix of polyurethane and polyvinyl foam[2]. The latter excreted ammonia continuously with very positive results on rice culture, but with a life period of two weeks. For those reasons, *Azolla* is still the best option for biologically based life support systems on long-duration space missions. The first clear indication that *Azolla-Anabaena* system could be used with success in a BLSS was shown in the 1980 seven days space mission of the joint Soviet-Vietnamese cosmonauts crew Salyut 6 - Soyuz 37 station[16]. *Azolla pinnata* was exposed to the weightlessness of space during six days and the results were quite positive. The conditions of microgravity did not affect the main biological characteristics of both the fern and the cyanobacteria, as well as the subsequent generations of *Azolla* on Earth[6]. All these data strongly suggest that this symbiotic association should be used in a BLSS for space exploration involving men.

5. CONCLUDING REMARKS

The *Azolla-Anabaena*-bacteria symbiotic system is a good example of a living model for biological and environmental research. The presence of a prokaryotic community inside the leaf cavity of the fern is an unique situation that can be used to study the communication and relations between these two ecological worlds, especially if we consider that there are two different levels of cellular organization. Another of the main conclusions that we can draw from the study of this symbiotic system is that cooperation is also an important mainstream supporting evolution. In this sense, space exploration must be seen and developed in a cooperative and symbiogenic way. Symbiogenesis may be the main mechanism for the Terraforming process of planetary engineering. Perhaps, in the near future, we can have a new science called Symbiotica, a study of the social and biological relations of the symbiotic processes, applied to the research of the natural. It may also represent the main bridge between natural and artificial systems, which in our opinion could have a significant importance in the development of mankind.

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