LAB ACTIVITY
FOR SYMBIOSIS TEACHING

DISCOVERING *AZOLLA*

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INTRODUCTION

What is *Azolla*?

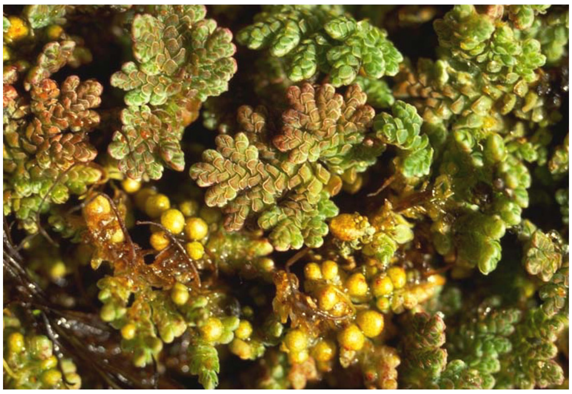


Figure 1. *Azolla filiculoides*

Taxonomy (Saunders and Fowler, 1993):

Division: Pteridophyta
Class: Filicopsida
Order: Salviniales
Family: Azollaceae
Genus: *Azolla*
Subgenus: *Azolla*
Section: *Azolla*
Species: *Azolla filiculoides*

Azolla is a small aquatic floating fern, whose genus was established by Lamarck in 1783 with a fossil record dating back to the mid-Cretaceous, that presents a very peculiar structure:

- The stems (rhizomes) are thin, ramified, horizontaly distributed and covered with leaves.
- The fern has numerous and simple roots that emerge in the ramification points of the ventral side of the stem.
- The fern's leaves are small, alternated, imbricated (as the tiles of a roof), bilobed and distributed in two rows on the backside of the stems. Each leaf is divided in two lobes: a thick, aerial, papillae and chlorophyllous dorsal lobe, which has an extracellular cavity with a permanent prokaryotic community, and a very thin, submerged and hyaline ventral lobe (figure 2). Usually the plant shows green colour, but in stress conditions the leaves acquire a red coloration due to the presence of anthocyanins (pigments) in epidermal cell vacuoles, giving the fern a reddish-brown colour.

Figure 2. *Azolla filiculoides*: bilobed leaves.

Why *Azolla*?

Although traditionally considered as a lower vascular plant (e.g. presence of a protostelic stem), *Azolla* exhibits symbiotic characteristics more evolved than the other vascular plant-cyanobacterial symbioses - cycads (Cycadophyta) and *Gunnera* (Anthophyta). There appears to be no direct correspondence between the fern's evolutionary phylogeny and the complexity of the symbosis. This unique symbiosis is sustained throughout the fern's life cycle, where the cyanobacteria and bacteria are always present, either in the dorsal leaf cavities or in the sporocarps, in contrast to the more evolved vascular plant groups that associate with cyanobacteria (Carrapiço, 2006). In fact, the *Azolla* plants are never infected *de novo*, since the cyanobiont is transferred between generations as akinete inocula. 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 (Carrapiço, 2006).

Azolla is in permanent symbiosis with the nitrogen fixing cyanobacterium *Anabaena azollae*. The leaf cavities of the pteridophyte can be considered as the basic physiological units of the symbiotic association and contains a prokaryote community of symbionts, the cyanobacterium *Anabaena azollae* (cyanobiont) (figure 3), and several bacterionts population (i.e. *Arthrobacter*, *Agrobacterium*). It is also the location of two types of epidermal hair cells (trichomes), the simple and branched hairs, that extend into the leaf cavities (figure 3). Trichomes are responsable for the exchange of metabolites between *Azolla* (host) and *Anabaena* (cyanobiont) and vice-versa.

Using the leaves of this fern for light microscopy, it is possible to observe in the same sample the presence of cyanobacteria and *Azolla* cells. In this sense, we can easily make the distinction between prokariotic and eukariotic cells, define in a correct way symbiosis, integrate it into the symbiogenic theory of evolution and establish the concept of symbiome as the structural and physiological unit of selection.

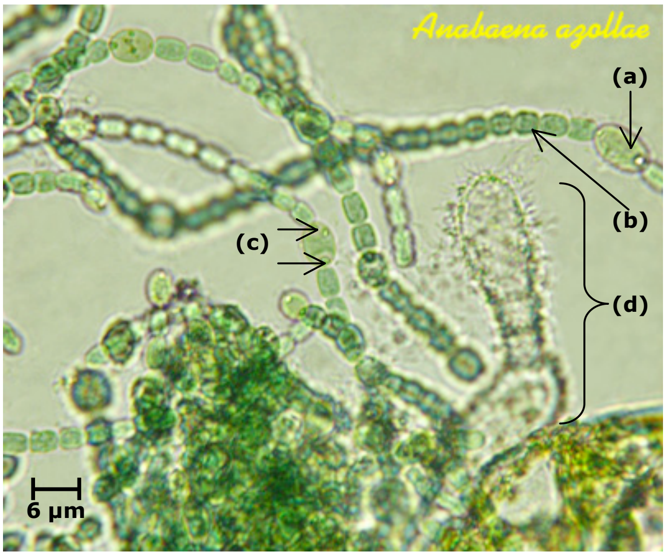


Figure 3. *Anabaena azollae*: heterocyst (a); vegetative cell (b); polar nodules (c). *Azolla filiculoides*: trichomes (d).

Anabaena azollae is a filamentous cyanobacterium, which presents vegetative cells and heterocysts – larger cells with a thickened wall, two punctuations in the extremities named polar nodules and capacity of nitrogen fixation (figure 3). This nitrogen fixation is made in the absence of oxygen by the nitrogenase complex present in hererocysts, which converts nitrogen present in the air into ammoniac.

This pteridophyte encloses a wide range of interests in different fields, namely in simbiomics, cell biology, taxonomy, ecology, self-sufficient agriculture and economy. Thus, the *Azolla-Anabaena* system is a good example of a living model for practical symbiosis teaching, able to be applied in several levels.

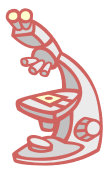
MATERIAL

- *Azolla filiculoides*
- coverslips
- dissection needle
- distilled water
- Pasteur pipettes
- immersion oil
- light microscope
- Petri dishes
- slides
- stereomicroscope
- tweezers
- filter paper

METHODOLOGY

Observation of *Azolla* with stereomicroscope

1. Place one or two specimens of *Azolla filiculoides* in a Petri dish.
2. Observe the sample with a stereomicroscope.
3. Make a diagram of the roots, rhizome and leaves (identify the dorsal and ventral lobe of the *Azolla* leaf and observe the characteristics of each lobe). Label them.



Microscopic observation of *Azolla*

1. Remove some *Azolla* rhizomes, with the respective leaves.
2. Put a drop of distilled water on a slide, followed by the sample.
3. Gently, squeeze the leaves with a dissection needle and cover it with a coverslip.
4. Put the slide in the stage of the light microscope and observe.
5. Make a diagram and label the main aspects of your observation:

- the epidermal cells of *Azolla* leaf: observe the presence of anthocyanins, a red pigment present in vacuoles (figure 4);
- the mesophyll cells of *Azolla* leaf: identify the dorsal lobe cells, which contain chloroplasts (figure 4);
- the transfer hairs (trichomes) (figure 3);
- the cyanobacteria: identify the typical filaments of this prokaryotic organism; observe the cell wall, the vegetative cells and the heterocysts (figure 3).

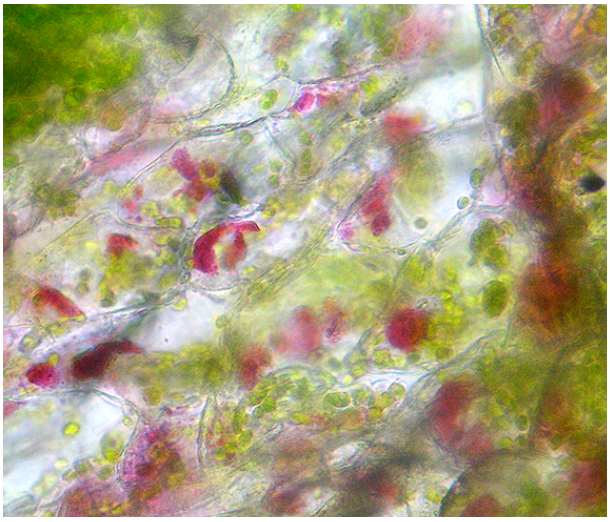


Figure 4. *Azolla filiculoides*: epidermal cells with anthocyanins and mesophyll cells with chloroplasts.

REGISTRY OF OBSERVATIONS

THINK ABOUT... / REFLECT...

Prokaryotic and Eukaryotic Cells - the Differences

The cell is the basic unit of life, common to all living beings, with a chemical composition based on carbon (C), hydrogen (H), oxygen (O), nitrogen (N), sulphur (S), phosphorus (P) and water (H_2O). The cell has its own metabolism, obtains matter and energy from the environment, maintains an internal balance, reproduces, develops and adapts to the environment. In spite of the morphological and functional cellular diversity, cells present two main types of cellular organization: prokaryotic and eukaryotic (Figure 5).

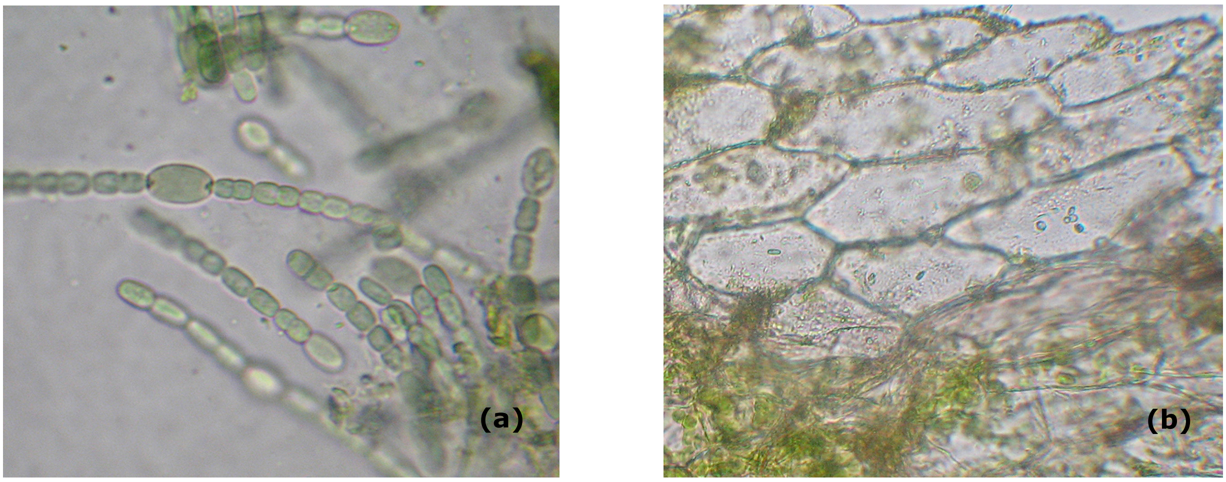


Figure 5. Cell structure: (a) prokaryotic cells - cyanobacteria; (b) eukaryotic plant cells.

The following table (table 1) establishes the comparison between prokaryotic and eukaryotic cells:

	Prokaryotic Cell	**Eukaryotic Cell**
Living beings	Mycoplasmas, bacteria and cianobacteria	Protists, fungi, plants and animals
Dimensions	1 – 10 µm	10 – 100 µm
Shapes	Spherical, rod-shaped or spiral	Several
Cell wall	Present	Present or Absent
Plasma membrane	Present	Present
Nucleus	Absent (Nucleoid)	Present (5 µm)
Organelles*/Structures	Absent or a few (ribosomes and/or photosynthetic membranes)	Nucleus, mitochondria, chloroplats (in plants), lysosomes, peroxisomes, vacuoles, endoplasmic reticulum (Smooth and Rough), Golgi apparatus, cytoskeleton
DNA	One circular DNA molecule, not associated with histones	Long DNA molecule, associated with histones and organized in chromosomes
Cell Division	Binary fission	Mitosis or meiosis
Life Way	Unicellular or Colonial	Unicellular or Multicellular

*The organelles provide compartments in which different metabolic activities are locatted.

Table 1. Comparison between prokaryotes and eukaryotes
(adapted from Figueiredo *et al*, 2003).

1. Taking in consideration the observations made in the activity, establish the differences between eukaryotic and prokaryotic cells.

***Azolla-Anabaena*-bacteria as a simbiotic system**

This simbiotic association is an example of mutualistic symbiosis, where *Azolla* as well as cyanobacteria benefit from this association. In a simple way, *Azolla* produces and supplies photosynthate sugars to the endosymbionts, while cyanobacteria supply the host with essential nitrogen compounds for its development. This exchange of matter between these two organisms is made by the fern's trichomes.

However, the existence of mutual benefit should not be considered, as the plus or common denominator of the symbiotic process. As it was adequately referred by Dubos and Kessler, in 1963, during the 1st International Conference on Symbiosis, in London, "the nutritional effects of symbiosis are not its most interesting manifestation. More remarkable is the fact that many symbiotic systems produce substances and structures that neither one of the two components produces when

growing alone" (Carrapiço & Rodrigues, 2005). We belive that this concept can also be applied to the *Azolla-Anabaena* symbiotic system.

1. How can the *Azolla-Anabaena* simbiotic system be an example of exchange of matter and energy?
2. Discuss the following statement: "The *Azolla-Anabaena* simbiotic system can be compared to an ecosystem."

Azolla as a symbiome

In 2003, Jan Sapp (Sapp, 2003) introduces the concept of symbiome, referring that "*every eukaryote is a superorganism, a symbiome composed of chromosomal genes, organellar genes, and often other bacterial symbionts as well as viruses. The symbiome, the limit of the multicellular organism, extends beyond the activities of its own cells. All plants and animals involve complex ecological communities of microbes, some of which function as commensals, some as mutualists, and others as parasites, depending on their nature and context*". In the same sense, we believe that this idea can be applied to the *Azolla-Anabaena*-bacteria symbiosis. The *Azolla* leaf cavity can be considered as the basic physiological unit of the symbiotic association (Grilli Caiola and Forni, 1999), where complex ecological communities of permanent microorganisms co-exist with the fern to maintain the whole. New novel metabolic and organic capabilities are acquired and developed by the partners to establish a new level of organization, extending beyond the capability of each individual forming the association (Carrapiço, 2006).

1. Why can we consider *Azolla* as a superorganism?

Symbiosis and evolution

Symbiomics is a new term created by Jan Sapp (2003) and defined, through personal communication between him and Francisco Carrapiço (2004), as a field that studies the biochemistry, physiology, genetics, ecology and evolution of the simbiotic systems, as well as their dynamic interfaces. This science considers every plant and animal as a symbiome - a poligenomic entity constituted by chromosomal genes, organellar genes, viral genes and other microbial symbionts – in which multiple

species function to maintain the whole system. Many protists, plants and animals harbor symbiotic bacteria, that are transmitted hereditarily from one generation to another. In this case, the symbiosis present in *Azolla-Anabaena* system is sustained throughout the whole fern´s life cycle, where the cyanobacteria and bacteria are always present, showing a syncronic cycle with the host (Selosse, 2005). This fact favours the obligatory nature of the symbiosis and suggests a parallel phylogenetic evolution of both partners – a successful co-evolved system (Carrapiço, 2006). Thus, symbiosis can be recognized as an evolutionary process which has an important role in evolution.

Even the eukaryotic cell can also be seen as a symbiome. According to the contemporary conceptual consensus, all eukaryotes emerged from mergers between different kinds of bacteria. The mitochondria of eukaryotic cells and the chloroplasts of plants and protists were once free-living bacteria that became incorporated in a primitive host cell (Sagan, 1967) and, in the course of evolution, some of their bacterial genes were lost and others transfered to the cell nucleus. In this sense, the horizontal gene transfer is a powerful mechanism of novelty allowing symbiosis can be recognized as an evolutionary mechanism with an important role in evolution. It is at the symbiome level, composed by an integrate multigenomic genetic pool, in which natural selection acts, constituting the unit of selection.

1. Explain how symbiosis contributes for the evolutionary process.
2. Why is the symbiome a poligenomic entity?
3. Which are the main reasons to consider the symbiome as the unit of selection?

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