Nano environment of *Azolla caroliniana*

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ABSTRACT

It´s known that the cyanobacteria Anabaena provides nitrogen species at the host fern *Azolla caroliniana* due to the enzymatic activity of the iron and the molybdenum proteins that fix the nitrogen into the cell. These enzymes could be inhibited by the elevated concentration of nitrogen species in the aqueous media being a stressing condition for Anabaena. The nitrogen is a constituent of the porphyrin ring of the chlorophylls, without this element the rings will have no shape and even exist. In the absence of chlorophylls the cell uses auxiliary pigments such as carotenoids and phycobiliproteins. For this reason is probably that the *Azolla c.* leaves become red before the proliferation of the nitrogen species in the aqueous media. By this, it has been proposed two analyses, *In vivo* and *In vitro*. These analyses evaluate the nitrogen species levels in the aqueous media, relating the red coloration in *Azolla c.* leaves. The results of the analyses from soil, water and leaves from (*Azolla c.* & rice) showed that the nitrogen chemical species inside the water of the Azollario (*Azolla c.* cultivated in water) doesn´t have significative variations that involves the coloration of *Azolla c.* In the Azorizario (paddy & *Azolla c.* cultivated in water) a deficit of potassium in soil was recorded, indicating a disproportional fertilization of soil. In other sample point the presence of green algae was found, which presumably would be the cause of the chlorosis and brown spots in rice leaves.

INTRODUCTION

Inside the Project: *"Develop of the Azolla Anabaena resource and applications in the agricultural, livestock and aquaculture sectors",* it has developed an investigation about the color change in *Azolla c.* leaves, being influenced by the presence of nitrogen chemical species in the aqueous media disturbing metabolic aspects of the symbiosis.

The host *Azolla* contains photosynthetic pigments such chlorophyll a, chlorophyll b and carotenoids associated to the chloroplasts whereas the filaments of Anabaena fern contains chlorophyll a, phycobiliproteins and carotenoids (these last two as accessories). The amount of photosynthetic pigments (main & accessories) allow the symbiotic organism expand the availability range of usable light energy.

Probably this feature is responsible for the change of color in *Azolla* leaves from green to red (Mosquera & Calderon, 2002).

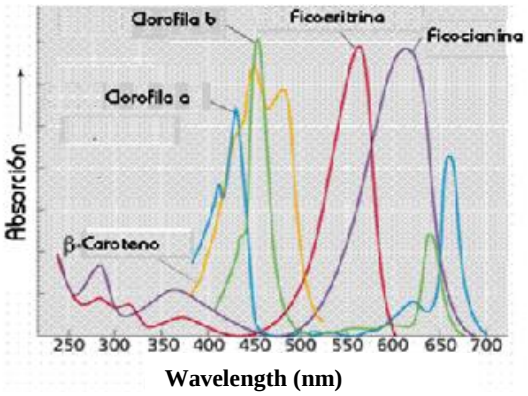

Figure 1. Comparison between the different wavelength absorb from the pigments of chlorophyll, phycobiliproteins and carotenoids

Photosynthetic pigments absorb specific wavelengths (λ = nm) of sunlight (Figure 1) so, the red coloration in *Azolla* can be expressed due to the absence of chlorophyll a and b.

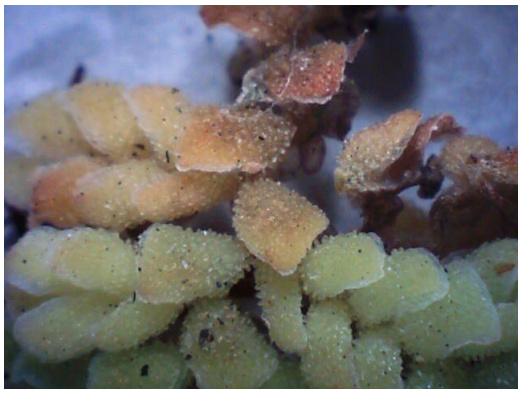

Figure 2. Change of color from green to red in *Azolla c*. 40x

When *Azolla* dies, it falls to the sediment releasing all the nitrogen fixed by *Anabaena* saturating the aqueous environment of nitrogen chemical species of easy assimilation, so *Azolla* takes this high availability and ignores the nitrogen fixed by *Anabaena* located in organic molecules and becomes part of pigments such as chlorophyll a in a lesser percentage. This event inhibits the enzymatic activity of the molibdenum-iron proteins of *Anabaena*. In addition, nitrogen fixation involves large energy expenditure for all cyanobacteria at a rate of 18-24 ATP's; the reasons for this energy "waste" are still unknown. Therefore, is probably that *Anabaena* look stressed when it host dispenses the nitrogen that is accumulated in itself (Prescott, 2002).

Chlorophyll need nitrogen to form the porphyrin ring, however, phycobiliproteins and carotenoids dispense it, being these pigments which absorb light (λ = 500-550 nm) and reflect the red color in *Azolla c.* leaves (Mosquera & Calderon, 2002).

ANALYSIS AND TREATMENTS

Azolla coloration could be influenced by the effects of the chemical species in nitrogen in water Anabaena enzyme inhibition, it is suggested that in vitro assays and in vivo, in terms of: Growth rate, chlorophyll and protein content; nitrogenase and nitrate reductase enzyme activity quantification and the amount of vegetative cells and heterocysts present in each plot (*In vivo* analysis) and treatment analysis (*In vitro* analysis) to determine the causes of coloration in *Azolla* (Mosquera & Calderon, 2002).

In vitro **analysis**

This analysis provides more consistent and reliable results, because the technique is developed under controlled conditions for temperature, light intensity, nutrient availability and specific wavelengths of light. The standardized culture media or growth media (IRRI) is applied for the symbiosis *Azolla-Anabaena* providing nitrogen as ions: NO_3^+ and NH_4^+, as detailed in Table 1.

Extracted from Mosquera & Calderón, 2002.

Medio IRRI +N		**Medio IRRI -N**	
Macronutrientes	(g / litro)	Macronutrientes	(g / litro)
NH_4NO_3	1.650	....	....
$CaCl_2$	0.333	$CaCl_2$	0.333
$MgSO_4$ $6H_2O$	0.492	$MgSO_4$ $6H_2O$	0.492
K_2SO_4	0.274	K_2SO_4	0.274
NaH_2PO_4	0.120	NaH_2PO_4	0.120
Micronutrientes	(mg / litro)	Micronutrientes	(mg / litro)
Fe	0.2	Fe	0.2
Mn	0.1	Mn	0.1
Zn	0.012	Zn	0.012
Cu	0.005	Cu	0.005
Mo	0.005	Mo	0.005
B	0.635	B	0.635

(International Rice Research Institute)

Table 1. Culture media for *Azolla* with and without nitrogen.

Materials and Methods:

Make use of twelve plastic containers (500 ml), separate 3 groups of 4 containers. In the 2 first groups, put the culture media (IRRI + N) and in the last group put the culture media (IRRI - N). In all containers seeded *Azolla c.* alike filling the entire space of the container. Each group would be exposed to a specific wavelength. The three groups will keep them under a lamp (Sylvania ® Tungsten-halogen DXM) with interference filters (Lee Filters ®) (Ray et al, 1973). Group 1 will use for blue light Lee filters 071 - Tokyo Blue (λ = 400-500 nm). Group 2 will use the filter Bright Red 026 for a (λ = 600-700 nm), and the last group will receive the white light without using a filter, this is the blank sample for this analysis. The photoperiod is about 12 hours of light and 12 hours of darkness, with temperatures ranging from (23 to 25) ° C for one month (Mosquera & Calderon, 2002).

In vivo **analysis**

The presence of chlorophyll depends on the amount of nitrate and ammonium ions in water, the nitrite is an intermediate, so their concentration is always very low. To establish a relationship of chemical species of nitrite, nitrate and ammonium, with red coloration in *Azolla*, analyzes were performed in water and soil when *Azolla* become green and when become red. Also, another analysis in leaf samples of *Azolla* and rice considering micro and macro elements that could be involved in the coloration of *Azolla* (Mosquera and Calderon, 2002). Samples of water, soil and leaves were taken from an Azollario (Pools with *Azolla* crops), and Azorizario (cultivation of paddy and Azolla) located in Guayas Province, Daule city, Parish: Boqueron.

Materials and Methods

In Azollario, samples of each of the numbered items 1 to 7 (Fig. 3) were taken. It was noted that sections 2 and 3 had no proliferation of *Azolla* as in other sample points, so that was omitted from the sample soil and water because both are found in the same condition and presumably have similar results.

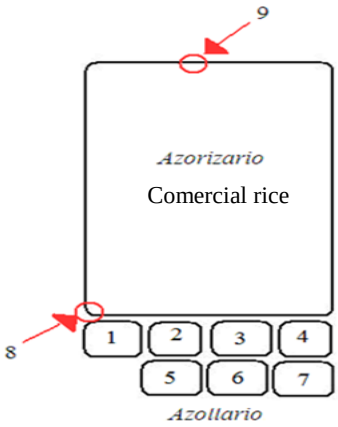

Figure 3. The numbers show the sample points.

The Azolla showed red only at points 1 and 5. The Azollario was covered with a "Zaran" (plastic mesh 50%), which reduces the intensity of light, mimicking the natural environment of *Azolla* in rice fields, being the rice which provides a natural shade in the plots.

The azorizario has a commercial rice cultivation (lNlAP 11), time of crop: 2 ½ months, missing for harvest month and a half. Two sample points were taken in azorizario (Fig. 3), point 8 and 9; sampling was performed on these two points because the crop had notable differences: In Section 8 we observed that the leaves of *Azolla* were red and very little, besides being in suspension with a lot of green algae, also rice plants showed chlorotic leaves with brown spots. In sample point 9 a proliferation of *Azolla* was observed with a bright green color, likewise the rice plants were green and healthy.

RESULTS AND DISCUSSION

Analyses were performed on all samples at the Laboratory of Agricultural Analysis of Dr. Jorge Fuentes (Guayaquil, Ecuador).

In the Azollario water nitrate levels are within normal ranges, NO_3^- (2.1 - 54.1) ppm, whereas for the ammonia, the data beyond the normal range NH_4^+ (2 - 18) ppm. In the sample point 5 the highest value (28 ppm) was obtained, remember that at this point the color of Azolla was red in contrast to the sample point 2, nonproliferation fern was recorded, giving a result of NH_4^+ (18) ppm (Espinoza & Gutierrez, 2003). In the sample points 2 and 3 is a notable absence of the acuatic fern, it is likely that this species of Azolla proliferate most successful higher than 18 ppm. Optimal levels of nitrate in water for growth of *Azolla pinnata* and *A. mycrophylla* are about is 2.5 mmol/l, while high levels of nitrate (15 mmol/l) a decrease in heterocystes production occurs (Pabby *et al* , 2001).

According to Rai and their collaborators (2000), at highest pH (> 7) there is increased activity of the nitrogenase enzyme in *Azolla* leaves, so that higher concentration of nitrogen should exist in the leaves.

The pH at all the sample points studied, both as in the azorizario or in the azollario was normal, ranking in a pH range 7.4-8.6; however, a sharp drop in *Azolla* proliferation was observed in the sample points 2 and 3 of azollario. In water point 8 of the azorizario was normal the phosphate concentrations (0.01 to 0.82 ppm) and calcium (11.6-28.6 ppm) were recorded. Also potassium levels were normal without revealing differences between points 2 and 7, respectively values of 5.083 and 2.346 ppm ppm, optimal range (1.27 - 6.5) ppm (Espinoza & Gutierrez, 2003).
According Rimache (2008) the optimum pH for a paddy soil is 6.6, an average acid helps to microbial release of nitrogen and phosphorous from the organic material, also the concentrations of substances that interfere with the absorption of nutrients, such as aluminum, manganese, iron, carbon dioxide and organic acids are below toxic levels.

The soil of sample point 8, a high ammonia level (60 ppm) is evidenced in comparison with point 9 (37 ppm). According Castilla (2003) in soils with low organic matter (OM) ammonia production decreases, while in others with average content - high organic matter, ammonia production is very high.
The OM content in point 9 (3.72 g / kg) does not show a great difference from the other points, indicating that this factor does not influence the high concentration of ammonium in the soil.

Phosphorus in the soil showed different values: In sample point 8 (3.9 ppm), point 9 (9.6 ppm), in paragraph 6 (9.8 ppm) and in 1 (4.8 ppm). The optimum range is (5.0 - 53.0) ppm. In a paddy phosphorus deficiency in soil has narrow small and erect a very dark green leaves, the stems are long and the growth is retarded. Young leaves appear healthy, but old take a brownish color and die (Rimache, 2008). These

characteristics are consistent with those observed in the azorizario in points 8 and 9. Likewise, phosphorus deficiency, is associated with other nutritional disorders such as iron toxicity at low pH, iron deficiency, salinity and alkalinity of the soil. These latter characteristics are not adjusted to the iron concentration recorded at points 8 (640 ppm) and 9 (143 ppm) (Rimache, 2008).

Foliar results showed homogeneous *Azolla* nitrogen percentages both azollario as in azorizario, with values of 5.06% N at point 9 and 4.57% N at point 3. A normal range is 2.6-5.7% N (Sanginga & Van Hove, 1989). P (Phosphorous) levels were homogeneous at all points corresponding to point 9 the highest value (5.06) ppm, and at point 3 the lowest value (4.57) ppm; so it is presumed that phosphorus does not affect the color of *Azolla*. *Azolla* leaves in an abnormally high concentration of cations (Ca^{++} , K^{+} and Na^{+}), while the concentration of chloride ions and the pH is less in older leaves than in another group of blades. The calcium concentration contributes to the aging of the plant and maintaining homogeneous the calcium values is inferred that it does not affect the color of *Azolla* (Rimache, 2008).

Data for rice leaf samples values are homogeneous, except in the phosphorous, however on the soil, this element has a deficiency in point 8.

There are damages that are produced by algae in rice fields, depending on the species and stage of rice cultivation, algae compete for the availability of light, nutrients and dissolved oxygen, producing chlorosis and wilting in the leaves of rice, accordingly algae difficult the feeding seedlings (Rimache, 2008). Being the the hoard as algae, are likely to be responsible for both chlorosis of rice leaves, and the absence of *Azolla* in point 8 (azorizario).

Extracted and modified from (Espinoza & Gutiérrez, 2003).

Característica	Suelo	Agua
	Rango	Rango
pH	5,2-7,4	7,4-8,6
*C.E. (mS/m)	0,03-0,4	0,14-11,9
**M.O (g/Kg)	1,9-46,8	-
P (ppm)	5,0-53,0	0,0-0,6
k(ppm)	24-272	1,27-6,5
Ca (ppm)	115-1395	11,6-286
Mg (ppm)	-	2,3-36-3
Fe (ppm)	-	1,53-11,9

Table 3. Concentration ranges of certain parameters of soil and water.

CONCLUSIONS

The levels of chemical species as ammonium and nitrate nitrogen, presented differences that don´t support the discoloration on *Azolla*, it is possible that this variation is more noticeable performing the *In vitro* analysis, by which is less probably to have external factors influencing on the final results.

In the azorizario highly variable final data were obtained regarding *Azolla* and their coloration. Possibly due to the rice plant that cover the fern in certain areas more than others, allowing the passage of certain wavelengths of light. The differences in certain nutrients such as phosphorus, suggest that the form of soil fertilization is not as homogeneous.

Green algae have a high proliferation rate and compete for food, which could influence the chlorosis and brown spots on the leaves of rice.

RECOMMENDATIONS

For the analysis *In vivo*, it is important to simultaneously measure chlorophyll levels (spectrophotometry according wavelengths) and the enzymatic activity of nitrate reductase and Nitrogenase (acetylene reduction), relating the results to their environment. Similarly, for the *In vitro* analysis, measure the concentration of molybdenum, due to its role in the nitrogen-fixing enzyme activity and determine a possible stress in Anabaena.

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