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Culture of the Cyanobiont *Anabaena azollae* Strasburger: Is it Possible?

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Abstract

The culture of the nitrogen-fixing cyanobacterium *Anabaena azollae*, the cyanobiont of the fern Azolla, is a long-time debate. Therefore, on the present research, the cyanobiont was cultured in three culture media (AA, AA1/8, BG-11_0) supplemented with an aqueous extract of *Azolla filiculoides*, three sugar sources (fructose, D-glucosamine, N-acetyl-glucosamine), KNO_3 and B_{12} vitamin and their fitness was assessed by the analysis of the Phycobiliproteins (PBP) content. The results of the present research showed that the addition of a fern aqueous extract to the culture media AA, AA 1/8 and BG-11_0 induced the loss of *A. azollae* cells. Also, in all culture media without any supplements and media complemented with N-acetyl-glucosamine, KNO_3, and B_{12} vitamin, the cyanobiont is formed by very short filaments or isolated vegetative cells and heterocysts. Regarding the PBP, the phycocyanin content almost disappeared from the cyanobiont followed by a sharp decrease in the allophycocyanin and phycoerythrocyanin in a lesser extent. The cyanobionts growing in the media AA, AA 1/8 and BG-11_0 without sugar source and supplements, medium AA 1/8+N-acetyl-glucosamine+B_{12} vitamin, and medium BG-11_0+N-acetyl-glucosamine+B_{12} vitamin has phycoerythrocyaninin a higher amount than the freshly isolated cyanobiont. These changes can be due to nutritional and/or light limitations that are not adequate to this cyanobiont. Also, the culture conditions probably do not imitate the existing environment in the foliar cavities of the fern. However, N-acetyl-glucosamine seems to hinder a drastic decrease of allophycocyanin and phycoerythrocyanin, pointing to that this amino sugar can be beneficial to the growth of *A. azollae*.

Keywords: *Anabaena azollae*; *Azolla filiculoides*; Aqueous extract; Sugar source (fructose, D-glucosamine, N-acetyl-glucosamine); Supplements (B_{12} vitamin, KNO_3)

Abbreviations

AA: Medium Allen and Arnon; **AA 1/8:** Medium Allen and Arnon at 1/8 strength; **APC:** Allophycocyanin; **BG-11_0:** Medium Blue-Green without Nitrogen; **H-40**: Medium Hoagland at 1/4 strength; **KNO_3**: Potassium Nitrate; **NaClO:** Sodium Hypochlorite; **PC:** Phycocyanin; **PEC:** Phycoerythrocyanin; **v/v:** volume/volume

Introduction

The nitrogen-fixing cyanobacteria can form symbioses with plants in which both partners get benefits from the association. The cyanobiont has a supply of nutrients, and protection against herbivores, while the plant obtains all or almost all of the nitrogen needed for its development [1].

The fern *Azolla* (Figure 1A) is a unique plant because it is the only fern that has a never lasting symbiosis throughout the entire life cycle (both sexual and asexual) with a heterocystous nitrogen-fixing cyanobacterium *Anabaena azollae* Strasburger which occupy a narrow space at the periphery of the foliar cavity leaving the centre empty (Figure 1B). The filaments of the cyanobiont contain vegetative cells, heterocysts and occasionally akinetes. The cyanobacterial cells have a Gram-negative cell wall type, inclusions in the cytoplasm (carboxysomes, cyanophycin granules, and others) and a rudimentary thylakoid system. The heterocysts are specialized cells for nitrogen fixation, with two polar nodes of cyanophycin, honeycomb-like thylakoids, thick cell wall [2] and a proteinaceous extra-sheath [3]. The high heterocyst frequency (30-40%) and high nitrogen fixation rate led several researchers to the isolation and growth of several presumptive cultivable cyanobionts from all *Azolla* species using different growing conditions (Table 1). The most widely used culture medium is BG-11_0, combined with a wide range of conditions such as light intensity (from 9 to 200 μmol/m^2.s), temperature (22-33ºC) and

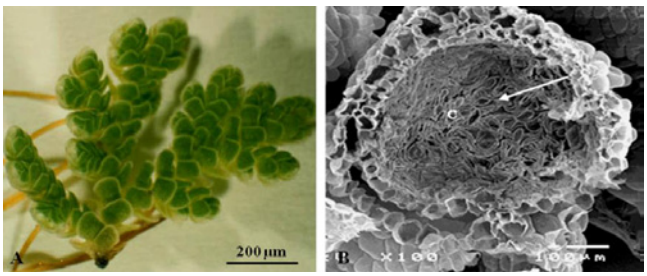

Figure 1: Morphological aspects of *A. filiculoides*. A) Round sporophyte with alternate ramifications with the chlorophyllous dorsal lobes. B) Cross-section of the cavity (C) of the dorsal lobes with filaments of the cyanobiont *A. azollae* (arrow).

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Table 1: Major findings of the cultivable cyanobiont *A. azollae* isolated from Azolla species and growth conditions.

Growth conditions	Cyanobiont	Reference
Culture medium: $BG\text{-}11_0$ Light intensity: 5 W/m^2 (9 µmol/m^2.s) Temp: 26°C Supplements: 1) nitrate at 125, 350 ppm 2) ammonium at 10, 20, 40 ppm 3) fructose, sucrose and glucose at 2, 5, 10, 30 mM	• Nitrate at 350 ppm stimulated growth, protein and chlorophyll contents; inhibition of phycobiliprotein content and heterocyst frequency • Ammonium at 20 ppm stimulated growth, protein, chlorophyll and phycobiliproteins contents • Fructose (2-30 mM) stimulated nitrogen fixation • Glucose and sucrose (2-30 mM) decreased nitrogen fixation	[4]
Culture medium: $BG\text{-}11_0$ Light intensity: 5 W/m^2 (9 µmol/m^2.s) Temp: 26°C Supplements: Fructose, sucrose and glucose at 8 mM	• Fructose stimulated higher growth than sucrose and glucose • Fructose increased chlorophyll *a* and glycogen content, heterocyst frequency, nitrogen fixation and glucose-6-phosphate dehydrogenase activity	[5]
Culture medium: $BG\text{-}11_0$ Light intensity: 200 µmol/m^2.s Temp: 33°C Supplements: 1) bubbled air and pH 9.3 2) air + 1.5% CO_2, pH 6.4 3) air + 1.5% CO_2 + 20 mM $NaHCO_3$, pH 8.2 4) air + 1.5% CO_2, 10 mM HEPES, pH 7.2 5) air + 1.5% CO_2 + 20 mM $NaHCO_3$, 15 mM $NaNO_3$, pH 8.3 6) air + 1.5% CO_2 + 20 mM $NaHCO_3$, 2.5 mM $NaNO_3$, pH 8.3 7) air + 1.5% CO_2 + 20 mM $NaHCO_3$, 5 mM NH_4Cl, pH 8.0	• Aeration with CO_2 and nitrogen source induced a little growth of *A. azollae* • Phycocyanin increased with the addition of HEPES and ammonium	[6]
Culture medium: $BG\text{-}11_0$ (pH 7.4) Light intensity: 135 µmol/m^2.s Temp: 22-25°C Agitation in a water bath at 30°C	• Colonial, globular, fimbriate and tubular growth • Vegetative cells and heterocysts size are not homogenous among cyanobiont • Low heterocyst frequency	[7]
Growth conditions: 1) $BG\text{-}11_0$, 0.1% agar, 30°C, 10000 lux (135 µmol/m^2.s), without initial darkness 2) $BG\text{-}11_0$ or AA, 30°C, 10000 lux (135 µmol/m^2.s), initial 6 days in darkness 3) AA + 2 mM NH_4Cl + 10 mM fructose, 4.5 days with agitation in darkness at room temperature; 3 days at 10000 lux (135 µmol/m^2.s) and 30°C 4) AA + 2 mM NH_4Cl + 10 mM fructose + 0.1% yeast extract (0.1% agar), pre-incubation in darkness; agitation at room temperature for 15 days; growth at 5000 lux (68 µmol/m^2.s) after transfer to semi-solid media 5) AA + 5mM NH_4Cl + 10 mM fructose + 0.1% agar, pre-treatment in darkness; transfer to 30°C and then increase radiation until 10000 lux (135 µmol/m^2.s) 6) AA + 10 mM fructose + 0.05% yeast extract + 0.05% casamino acid + 0.47 mM $NaNO_3$, pre-treatment in darkness; transfer to 30°C and then increase radiation until 10000 lux (135 µmol/m^2.s) 7) AA + 10 mM fructose + 0.05% yeast extract + 2% agar + 0.05% casamino acid + 0.47 mM $NaNO_3$, 7 days incubation in darkness; under 1% O_2 and 99% N_2, growth at 10000 lux (135 µmol/m^2.s) and 30°C 8) AA + 10 mM fructose + 0.05% yeast extract + 0.05% casamino acid + antibiotic mixture + 2% agar, 7 days incubation in darkness; under 1% O_2 and 99% N_2, growth at 10000 lux (135 µmol/m^2.s) and 30°C 9) AA + 10 mM fructose + 0.05% casamino acids + 0.05% yeast extract + 0.35 mM $NaNO_3$ + 2% agar, 7 days incubation in darkness; under 1% O_2 and 99% N_2, growth at 10000 lux (135 µmol/m^2.s) and 30°C in Petri dishes	• Photobleaching in cyanobiont cells • Green color maintenance depends on culture medium, supplementation and growth conditions -highest days (119 and 183 days) in experiment 7 and 9 • Cell division only in agar cultures	[8]
Culture medium: $BG\text{-}11_0$ Light intensity: 15 µmol/m^2.s Temp: 28°C Supplements: Fructose, glucose, sucrose, and maltose at 0.3 %	• Colonies flat and smooth • Filaments with cylindrical vegetative cells and ellipsoidal heterocysts • Presence of hormogonia • Photoheterotrophic growth with fructose • Cultured filaments did not show chromatic adaptation	[9]
Culture medium: N-free medium Supplements: 1) NO_3^- and NO_2^- at 5 mM 2) NH_4^+ at 1 mM	• Cyanobionts only grown with supplements	[10]
Culture medium: $BG\text{-}11_0$ Light intensity: 52 µmol/m^2.s Temp: 25°C Photoperiod: 16/8 h (day/night)	• Lower content of chlorophylls, phycobiliproteins, proteins, and sugars • Low heterocyst frequency • Filaments with small vegetative cells and heterocysts • High nitrogen fixation	[11]

Culture medium: BG-11_0 Light intensity: 170 and 250 µmol/m^2.s Temp: 29°C Photoperiod: 16/8 h (day/night) Supplements: Glucose and sucrose at 0.5%	• Dry mass increased with high light intensity • Protein, chlorophyll, carotenoids and phycobiliproteins content increased with high light intensity and supplements • Nitrogen fixation increased with high light intensity	[12]
Culture medium: BG-11_0 Light intensity: 52 µmol/m^2.s Temp: 25°C Photoperiod: 16/8 h (day/night)	• Low heterocyst frequency • Coiled and straight filaments • Small sized vegetative cells and heterocysts • Dull green color	[13]

supplements (different nitrogen sources, sugar sources, pH among others) turning the data comparison very difficult [4-13]. However, Tang et al. [8] using a broad array of culture conditions pointed to the difficulty in maintaining *A. azollae* in culture, especially due to photo bleaching and no cell multiplication. The maintenance of the green colour of the cyanobiont cells in complex culture conditions by 119 days (medium AA + 10 mM fructose + 0.05% yeast extract + 2% agar + 0.05% casamino acid + 0.47 mM $NaNO_3$; 7 days incubation in darkness; 1% O_2 and 99% N_2, 10000 lux, 30°C) and 183 days (medium AA + 10 mM fructose + 0.05% casamino acids + 0.05% yeast extract + 0.35 mM $NaNO_3$ + 2% agar; 7 days incubation in darkness; 1% O_2 and 99% N_2, 10000 lux, 30°C in Petri dishes) was used as a parameter to assess the viability of the cyanobiont cells [8] Compared to freshly cyanobiont isolated from *Azolla* cavities, the presumptive cultivable cyanobiont has low heterocyst frequency, small vegetative cells, and heterocysts, and dissimilar contents of chlorophyll *a*, proteins and phycobiliproteins [6-7,11-13]. The genome sequencing of *A. azollae* supported this difficulty due to the existence of pseudogenes or gene loss involved in the replication, repair, glycolysis and nutrient uptake [14] pointing that the presumptive cultivable cyanobiont was probably a resilient contaminant [15]. Given that the nitrogen fixation is a highly energy-demanding process and that the cyanobiont multiply inside the foliar cavities, the fern seems to be the main provider of carbohydrates for the cyanobiont [16]. But amino sugars as N-acetyl-glucosamine and D-glucosamine [17] can also be provided by the fern.

The aim of the present study was to culture the cyanobiont *A. azollae* in three media (BG-11_0, AA and AA 1/8) supplemented with an aqueous extract of *A. filiculoides*, Potassium Nitrate (KNO_3), B_{12} vitamin and three sugar sources (fructose, D-glucosamine, and N-acetyl-glucosamine). The cyanobiont fitness was assessed by quantification of the Phycobiliproteins Phycoerythrocyanin (PEC), Phycocyanin (PC) and Allophycocyanin (APC).

Material and Methods

Growth of *Azolla filiculoides*

The sporophytes of *A. filiculoides* (accession FI1001) obtained from the germplasm collection at International Rice Research Institute (IRRI) were grown in H-40 medium, pH 6.1-6.2, in controlled conditions [18].

Extraction of aqueous compounds from *Azolla filiculoides*

In a sterile flow hood, the sporophytes of *A. filiculoides* were washed in sterile tap water, immersed in sterile distilled water and ground with a sterile pestle. The homogenate was centrifuged at 4546 xg, for 30 min, at 4°C. The supernatant was filtered through filter paper (Whatman Nº 1) to eliminate remaining debris and stored at -20°C.

Isolation of the cyanobiont *Anabaena azollae*

In a sterile hood, roots were cut and the rootless sporophytes were disinfected twice in an aqueous solution of sodium hypochlorite (1 ml NaClO:10 ml distilled water, v/v) for 20 min, followed by three washes in sterile ultrapure water. The cyanobiont was isolated from sporophytes of *A. filiculoides* using the gentle roller method described by Rai and Rai [19]. The isolated cyanobiont was inoculated in each culture medium.

Experimental design

The cyanobionts were cultured in three media - AA, AA 1/8 fold strength [20] and BG-11_0 [21]. The added supplements were the aqueous extract from *A. filiculoides* (filtered through a sterile syringe filter 0.45 µm pore diameter prior to their adding to each culture media) in1:10 (v:v, extract: culture medium), 8 mM D-(-)-fructose [5], 5 mM D-(+)-glucosamine, 5 mM N-acetyl-D-glucosamine, 1 mg/L B_{12} vitamin [22] and 5 mM KNO_3 [9] (Figure 2). The addition of 100 mg/L of cycloheximide [23] to all the media inhibited the eukaryotic growth. The cyanobionts freshly isolated from the foliar cavities of *A. filiculoides* was regarded as the control. Due to a shortage of filaments of *A. azollae*, it was only made one replica for each medium and supplements. Following three days of darkness to prevent photobleaching [8], the cyanobionts were grown at 24°C, photoperiod of 16 h/8 h (day/night) and light intensity of 40µmol/

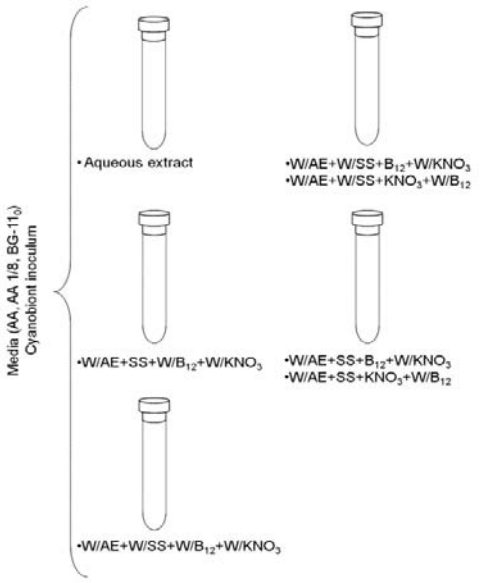

Figure 2: Diagram of the experimental design. Legend: W/AE - without aqueous extract; W/SS - without sugar source; W/B_{12} - without B_{12} vitamin; W/KNO_3 - without potassium nitrate; SS - sugar source (Fructose or D-glucosamine or N-acetyl-D-glucosamine); B_{12}- B_{12} vitamin; KNO_3 - potassium nitrate.

m2s provided by one fluorescent lamp Phillips Cool White TLD 36W. The medium was changed every 15-20 days. After 3 months, the medium was centrifuged at 4542 xg, for 5 min, at 4ºC and the pellet was stored at -20ºC. An aliquot of the non-frozen pellet was observed under an Olympus BX41 (Olympus, Lisbon, Portugal) light microscope.

Extraction and quantification of phycobiliproteins

The phycobiliproteins from each assay (except the assay supplemented by an aqueous extract of *A. filiculoides*) and a freshly isolated *A. Azollae* sample were extracted following Pereira et al. [24]. The absorbance was measured at 572, 612 and 647 nm in a Synergy HT spectrophotometer (BioTek, Madrid, Spain) equipped with Gen5 2.00 software. The content of PEC, PC, and APC was calculated according to Kaplan et al. [25].

Data analysis

Prior to the analysis of variance, data of the three phycobiliproteins were transformed as a percentage to control. This data was analyzed by two-way ANOVA without replication. The significance level (α) was 0.05. Statistical analyses were made using the Microsoft® Office Excell® 2007.

Results and Discussion

One of the particularities of the *Azolla-A. azollae* symbiosis is the synchronous development of both partners [15] indicating that probably *Azolla* might have a chemical or nutritional control regarding the development, differentiation, and multiplication of the cyanobiont. In the present research and after three months in culture conditions there were no filaments or isolated cyanobacterial cells in all the three media tested to which was added an aqueous extract of *A. filiculoides*. Probably water-soluble compound(s) from *A. filiculoides* can impair the multiplication of the cyanobiont or may induce its necrosis, but nothing is known about it.

A. azollae growing in artificial conditions showed straight filaments with cylindrical vegetative cells and ellipsoidal heterocysts [7,9,11-13]. However, at the present research, in all the media (AA, AA 1/8 and BG-11_0) lacking any sugar source and supplements (KNO_3 and B_{12} vitamin), the cyanobiont did not had green colour, did not grew and had very short filaments formed by cylindrical vegetative cells and ellipsoidal heterocysts with full-size inclusions (Figure 3A) or isolated vegetative cells and heterocysts (Figure 3B). The addition of a nitrate source stimulated the *A. azollae* growth [4,6,8,10], but in the present research, the addition of potassium nitrate as an additional nitrogen source did not induce growth of *A. azollae*. Moreover, since the nitrate transport gene in *A. azollae* is a pseudogene [13] the KNO_3 is not transported to the cyanobiont cells and thus not necessary for the cyanobiont growth. Regarding the B_{12} vitamin, there are no studies about their use for *A. azollae* growth, but since after 3 months very few filaments of *A. azollae* were observed probably their addition is not necessary.

The present research showed that the addition of fructose to the medium BG-11_0 without KNO_3 and B_{12} vitamin, medium BG-11_0+KNO_3 and medium AA+KNO_3 did not induce *A. azollae* growth. Instead, short filaments formed by vegetative cells and heterocysts with cytoplasm inclusions and without the typical green colour were observed (Figure 3C). On the remaining combinations of culture media with fructose, the cyanobiont cells were rare and contained many bacteria. These results are in disagreement with Rosen et al. [5] and Vagnoli et al. [9], which showed that the addition of fructose increased the photo heterotrophic growth of *A. azollae*. This dissimilarity could be due to the different temperatures and/or different light intensities used in the present research (40 µmol/m2.s and 24ºC) and by Rosen et al. [5] (9 µmol/m^2.s and 26ºC) and Vagnoli et al. [9] (15 µmol/m^2.s and 28ºC). On the other hand, Tel-Or and Sandovsky [4] showed that fructose had not induced an increase in *A. azollae* growth rate, which agrees with the results of the present research. In the assays made in the present research with the three media (AA, AA 1/8 and BG-11_0) to which was added D-glucosamine and with or without KNO_3 and B_{12} vitamin, the cyanobiont cells were rare, did not exists filaments of the cyanobiont pointing to its almost disappearance of culture media. Thus, D-glucosamine seems to be not useful for the cyanobiont growth. However, in all the assays at the present research with the N-acetyl-glucosamine with or without KNO_3 and B_{12} vitamin, the cyanobiont had a few short filaments formed by vegetative cells and heterocysts with inclusions and no green colour (Figure 3D), which may indicate that this amino sugar may be used as sugar source in more complex formulations of culture media.

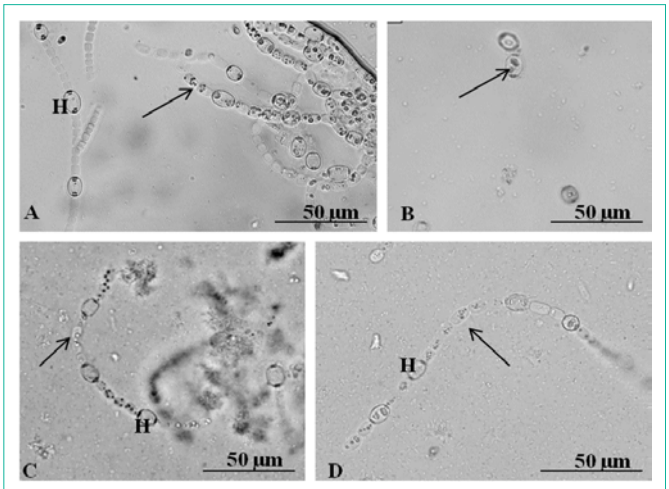

Figure 3: *A. azollae* growth under a light microscope. **A)** Filaments with heterocysts (H) and vegetative cells (arrow) grown in BG-11_0 withKNO_3 and without sugar source and B_{12} vitamin. **B)** Isolated cells of the cyanobiont (arrow) grown in medium AA without sugar source, KNO_3, and B_{12} vitamin. **C)** A short filament with heterocysts (H) and vegetative cells (arrow) of the cyanobiont grown in medium BG-11_0 supplemented with fructose and KNO_3 and without B_{12} vitamin. **D)** Filaments formed by vegetative cells (arrow) and heterocysts (H) grown in medium AA supplemented with N-acetyl-glucosamine and KNO_3 and without B_{12} vitamin.

The phycobiliproteins content can be related to chromatic adaptation or nutrient starvation such as a lower influx of carbohydrates or nitrogen [26]. In the present study, the cyanobionts growing in the medium AA with fructose and N-acetyl-glucosamine without supplements (KNO_3 and B_{12} vitamin), N-acetyl-glucosamine + B_{12} vitamin and N-acetyl-glucosamine + KNO_3 showed low levels of phycocyanin (Figure 4A). The most severe decrease or disappearing of phycocyanin occurred with media AA 1/8 (Figure 4B) and BG-11_0 (Figure 4C), but adding N-acetyl-glucosamine appeared to hinder the total loss of phycocyanin. Probably, this may indicate that

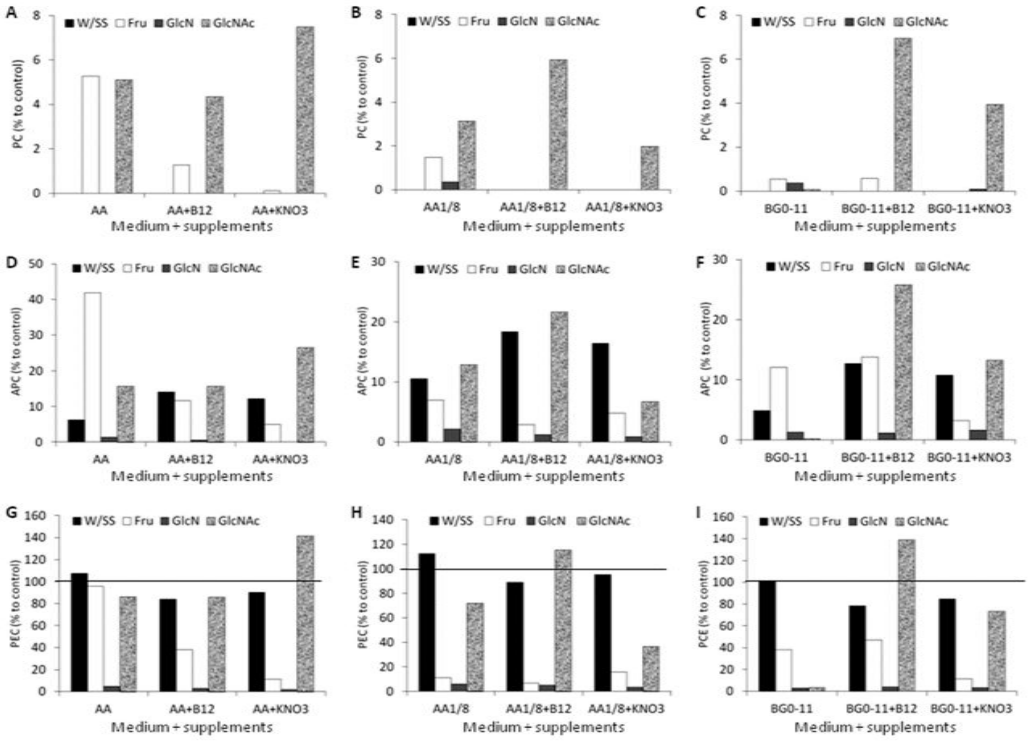

Figure 4: Phycobiliprotein content of *A. azollae* grown in three culture media with or without sugar source, KNO_3, and B_{12} vitamin. Data represent the percentage of PBP to control (fresh isolated *A. azollae*). **A)** Phycocyanin of *A. azollae* grown in medium AA. **B)** Phycocyanin of *A. azollae* grown in medium AA 1/8. **C)** Phycocyanin of *A. azollae* grown in medium BG-11_0. **D)** Allophycocyanin of *A. azollae* grown in medium AA. **E)** Allophycocyanin of *A. azollae* grown in medium AA 1/8. **F)** Allophycocyanin of *A. azollae* grown in medium BG-11_0. **G)** Phycoerythrocyanin of *A. azollae* grown in medium AA. **H)** Phycoerythrocyanin of *A. azollae* grown in medium AA 1/8. **I)** Phycoerythrocyanin of *A. azollae* grown in medium BG-11_0.

phycocyanin could be the first phycobiliprotein to be degraded or their synthesis repressed regardless medium, sugar source or other supplements added. But, the two-way ANOVA indicated that the sugar source added to the all the culture media induced statistical significant differences on the PC content ($F=17.44$, $P<0.05$) but not the type of culture media ($F=0.47$, $P>0.05$) pointing to the sugar source as an important parameter in the culture media formulation due to their possible influence on the phycocyanin content in the cyanobiont. Relating to allophycocyanin, the addition of fructose to medium AA seemed to protect against their loss from *A. azollae* (Figure 4D). The cyanobionts that grew in the media AA 1/8 without added sugar source and with N-acetyl-glucosamine showed a smaller loss of allophycocyanin in comparison to other growth media (Figure 4E). As to the medium BG-11_0, the content of allophycocyanin in *A. azollae* growing with N-acetyl-glucosamine without KNO_3 and B_{12} vitamin induced a more rapid loss of allophycocyanin (Figure 4F). The analysis of variance (two-way ANOVA) showed that the sugar source added to the media induced statistical significant differences on the APC content ($F=5.30$, $P<0.05$) but not the type of culture media ($F=0.80$, $P>0.05$) again pointing to the sugar source as an important constraint in the culture media formulation and in the APC content of the cyanobiont. The content of phycoerythrocyanin surpassed the 100% in six assays - medium AA without sugar source and supplements, medium AA +N-acetyl-glucosamine + KNO_3 (Figure 4G), medium AA 1/8 without sugar source and supplements, AA 1/8 + N-acetyl-glucosamine + B_{12} vitamin (Figure 4H), medium BG-11_0 without sugar source and supplements, BG-11_0 + N-acetyl-glucosamine and B_{12} vitamin (Figure 4I) - which means that the cultured cyanobionts have higher amounts of phycoerythrocyanin than the freshly isolates of *A. azollae*. The two-way ANOVA pointed to the sugar source as an important parameter in the culture media formulation and the maintenance of APC in the cyanobiont since it induced statistical significant differences on the PEC content ($F=21.18$, $P<0.05$) but not the type of culture media ($F=0.83$, $P>0.05$). The amino sugar D-glucosamine has induced almost the total loss of allophycocyanin (Figure 4D,4E,4F) and phycoerythrocyanin (Figure 4G,4H,4I) from the cyanobionts. Nitrogen starvation induced the degradation of phycobiliproteins in *A. azollae* [25], but Tel-Or and Sandovsky [4] showed that the phycobiliprotein content was inhibited by nitrate and Zimmerman [6] showed that phycocyanin was stimulated by the presence of ammonium. However, the phycobiliprotein data from the present research seems to indicate that the cyanobiont in culture probably suffered from a severe carbohydrate and/or nitrogen starvation. Also, the type of sugar source added to the medium and not the type of culture medium seems to have a major influence on the PBP content of the cyanobiont. Moreover, the data of the present research seems to point to that the amino sugar N-acetyl-glucosamine can be included in more complex formulations since it seems to hinder the total loss of phycocyanin and prevented a more drastic decrease of allophycocyanin and phycoerythrocyanin and also seems to favour the presence of cyanobiont cells but not the loss of the green colour.

Conclusion

In conclusion, following 3 months in culture 1) *A. azollae* fade away in media with the *A. filiculoides* aqueous extract; 2) cyanobiont filaments became very short, did not show hormogonia, and loss the blue-green colour; 3) fructose and D-glucosamine did not benefit *A. azollae* growth; 4) N-acetyl-glucosamine seems to favour the maintenance of some cyanobiont filaments; 5) KNO_3 and B_{12} vitamin are not essential for growth of *A. azollae*; 6) artificial growth conditions induced degradation or non-synthesis of the phycocyanin, but the allophycocyanin and phycoerythrocyanin remain in the cyanobiont; 7) the type of sugar source is more important to the PBP content than the culture media. These differences could be due to nutritional starvation and/or light intensity limitation which did not replicate the *in vivo* conditions inside the foliar cavities of *A. filiculoides*. So, knowing the metabolite exchange between the fern *Azolla* and the cyanobiont inside the foliar cavities should be crucial to grown *A. azollae in vitro*. Also, due to the high nitrogen fixation rate of *A. azollae* from *Azolla* species and other cyanobionts that form symbioses with *Gunnera*, cycads or lichens it is important to analyse how this is made to establish a culture in bioreactors and produce ammonium and probably a fertilizer which was not produced by chemical synthesis but by a microorganism.

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