Provisional chapter

The Unique Symbiotic System between a Fern and a Cyanobacterium, *Azolla-Anabaena azollae*: Their Potential as Biofertilizer, Feed, and Remediation

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Abstract

The free-floating aquatic fern *Azolla* is small and heterosporic, with a worldwide distribution in quiet waters (rivers, dams, creeks, etc.) and is considered an invasive species. This is the only known fern with a permanent symbiotic association with the heterocystic nitrogen-fixing cyanobacterium *Anabaena azollae* where the cyanobiont is transmitted through the *Azolla* generations without a *de novo* infection. The cyanobiont and other bacteria genera inhabit an ovoid cavity in each dorsal lobe of the leaf. The cyanobiont has a high rate of nitrogen fixation and thus this symbiosis was analyzed regarding its biofertilization (incorporated in soil or as manure). In addition, due to the amino acids and protein contents, this fern can also be used as food, and due to the high ability to uptake heavy metals and other pollutants, it can be used as phytoremediator. Since this fern is grown in tropical and subtropical zones where most of the countries have problems regarding the living conditions (health, sanitary, and food, among others) of people, this fern can be a very useful and cheap tool to cope with the severe problems that they face.

Keywords: *Azolla*, *Anabaena azollae*, symbiosis, fern, cyanobiont, bacteria, biofertilizer, food, phytoremediation

1. Introduction

The prokaryotic organisms (cyanobacteria and bacteria) appeared on Earth about 3 billion years ago, and during their evolution, both formed associations with other organisms. Such associations are a "combination of different organisms" living in a body that is developing throughout the life of another body [1], which should be named as symbiosis.

The cyanobacteria can form symbioses with several organisms such as sponges, amoebae, diatoms, fungi, and plants (bryophytes, ferns, gymnosperms, and angiosperms). The most common cyanobacteria (called cyanobionts) that form those symbioses belong to the genus *Anabaena* and *Nostoc*, which are able to fix the atmospheric nitrogen in specialized cells (called heterocysts) through the nitrogenase complex enzyme and thus convert it into the ammonium ion (NH_4^+). On the symbioses, both partners get benefits of the association. The prokaryote has a continuous supply of nutrients and protection against herbivores and desiccation, while the host has a provision of all or almost all of the nitrogen needed for its development, allowing the colonization of nitrogen-poor environments (aquatic or terrestrial) [2–4].

Although all the symbioses are very interesting and there is still no complete knowledge of all of them, this chapter focuses on the unique symbiosis between the aquatic fern *Azolla*, the cyanobacteria *Anabaena azollae,* and some bacteria with a description of the symbiotic morphology and the potential uses of this fern in agriculture, feeding, and remediation of contaminated wastewaters. Also, the challenges of their use will be addressed.

2. The partners *Azolla*, *Anabaena azollae*, and bacteria and their relationship

This fern is widespread in the world, from tropical to subtropical and temperate regions flourishing in calm waters (rivers, dams, creeks, and others), which shows their adaptability to several environmental conditions, from very hot regions to moderate temperature or even freezing temperatures [5–7]. This adaptability makes this fern to be considered as an invasive species in many countries especially in Europe such as Germany, Portugal, Spain, Great Britain, and others. When the environmental conditions (light, temperature, photoperiod, and nutrients) are optimal, *Azolla* overgrows forming a dense thick mat causing many problems such as the clogging of water pumps, the drowning of livestock, or the reduction of water flow in the irrigation channels [8]. However, the control of *Azolla* spread is difficult since this species can be bought online as a plant for a fish aquarium.

2.1. Genus *Azolla*

Priest Louis Fevillé made the first description of *Azolla* in botanical literature in 1725 from a Peruvian record, giving it the name of *Muscus squamosus aquaticus elegantissimus*, but the genus *Azolla* was described by Lamarck in 1783 [9]. The name *Azolla* derives from two Greek words, αξω (dry) and οδδνω (kill), which reflect the inability of this fern to survive in dry environments meaning that it has to always be in contact with water.

For many years, the genus *Azolla* was placed in the family Azollaceae, but recent research shows that this genus belongs to the family Salviniaceae [10]. The genus has seven species: (1) *A. pinnata* and *A. nilotica* belonging to the section *Rhizosperma* and (2) *A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla*, and *A. rubra* belonging to the section *Azolla*. The taxonomy of *Azolla* is mostly made by morphological characters (vegetative and reproductive), but in the last years, the gene sequencing contributed to the clarification of the relationships between the species although some controversies still remain about the number of species [11–14].

The oldest *Azolla* fossil is dated from the superior cretaceous period (Mesozoic Era), but the majority of the fossil records are dated from the Tertiary and Quaternary periods (Cenozoic Era) [15].

2.2. Morphology of the symbiosis

The multibranched sporophyte of *Azolla* has round (**Figure 1A**) or pinnate (**Figure 1B**) shape, and the main rhizome has alternate lateral ramifications fully covered with alternate, imbricate, and deeply bilobed leaves that form a dorsal (**Figure 1A**) and a ventral lobe (**Figure 1C**). The adventitious roots emerge from the ventral side of the rhizome at ramification points (**Figure 1C**) and hang down into the water. The dorsal lobe is aerial and chlorophyllous and has papillae and stomata (**Figure 1D**), and the ventral lobe is partially submerged and hyaline (**Figure 1C**). When environmental conditions are favorable, a red color develops in the leaves and rhizome due to the synthesis and accumulation of anthocyanins [5, 16–18].

This symbiotic association is permanent, meaning that it occurs at all stages of the life cycle of the pteridophyte, whether the propagation is made through vegetative fragmentation or by sexual reproduction. On the vegetative life cycle, there is a synchronous development of both symbionts (*Azolla* and *A*. *azollae*) from the apical meristem until the leaves become fully developed (**Figure 2A**). During this development, some cyanobiont filaments become entangled on primary branched hairs at the apical meristem (**Figure 2B**) and are partitioned into the forming cavities. On fully developed foliar cavities, the filaments of the cyanobiont occupy a very narrow space at the periphery of the leaf cavity (**Figure 2C**) and encircle the secondary branched hairs (**Figure 2D**) and simple hairs (**Figure 2E**) [5, 6, 18–20].

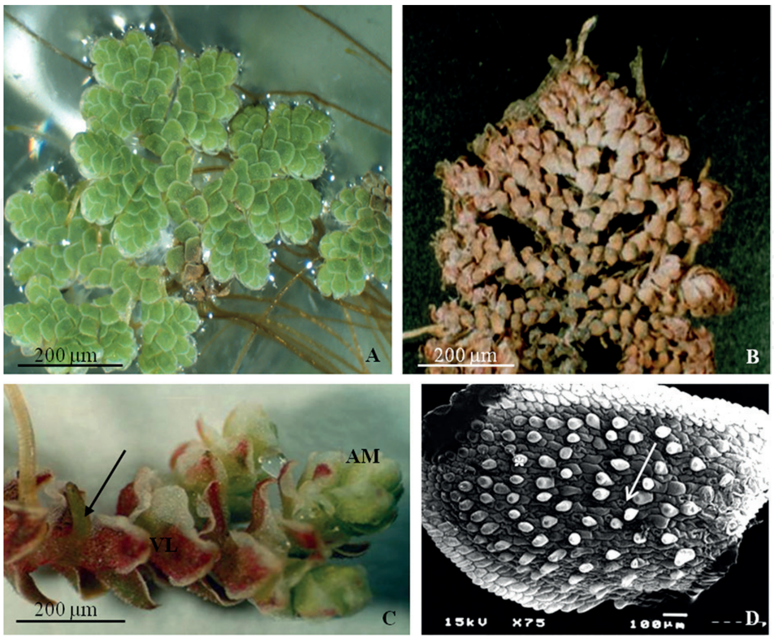

Figure 1. Morphology of the sporophytes of *Azolla*. (A) Round shape of *Azolla filiculoides* with chlorophyllous dorsal lobes. (B) Pinnate shape of *Azolla pinnata*. (C) Ventral view of the rhizome with the hyaline and ventral lobes (VL), curved apical meristem (AM), and adventitious root (arrow). (D) Upper surface of the dorsal lobe covered with papillae (*) and stomata (arrow).

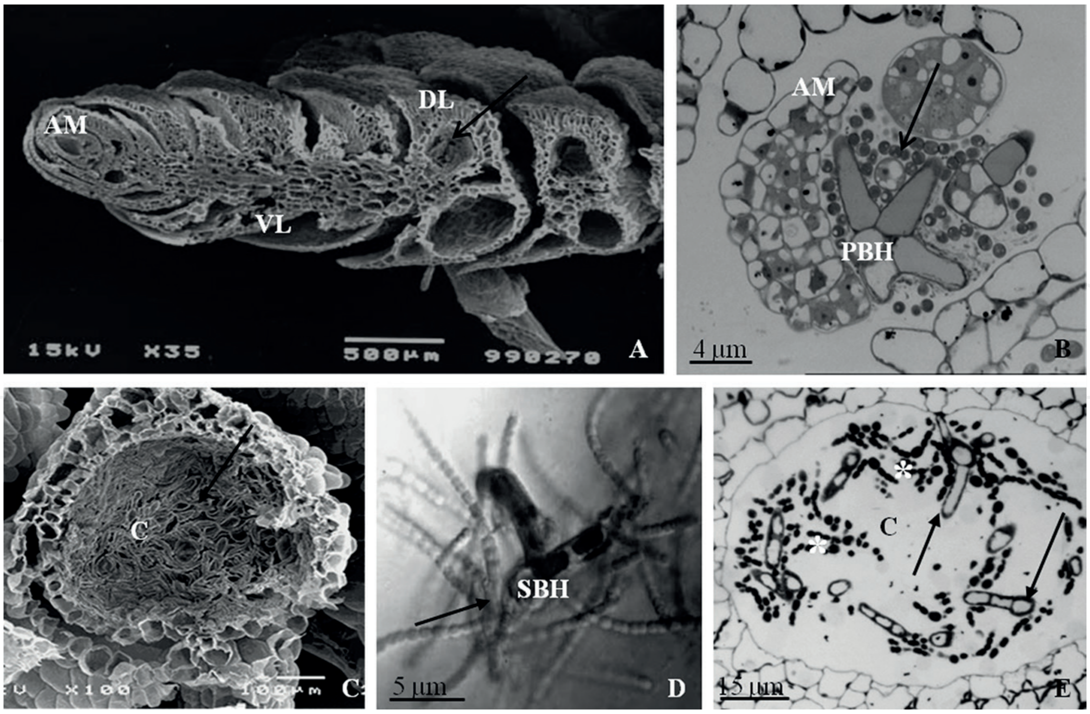

Figure 2. Morphology of the symbiosis between *Azolla* and the cyanobiont. (A) Cross section of the sporophyte showing the curved apical meristem (AM) until the fully developed dorsal lobe (DL) with an ovoid cavity (arrow) and the ventral lobes (VL). (B) Detail of an apical meristem (AM) with the cyanobiont (arrow) entangled in the primary branched hair (PBH). (C) Filaments of *A. azollae* (arrow) on the periphery of the leaf cavity (C). (D) Secondary branched hair (SBH) of the mature cavity surrounded by filaments of the cyanobiont (arrow). (E) Simple hairs (SH) of the mature cavity (C) surrounding with filaments of the *A. azollae* (*).

In the sexual reproduction, the sporocarps are differentiated on the ventral lobe when the environmental conditions are adverse to the vegetative propagation or survival of the fern. Since this fern is heterosporic, it forms a female sporocarp called macrosporocarp (**Figure 3A**) and a male sporocarp called microsporocarp (**Figure 3B**). The macrosporocarp has an indusium or cavity where it harbors a small inoculum of the cyanobiont (**Figure 3C**). When the environmental conditions become favorable, the fertilized macrosporocarp germinates and the inoculum of the cyanobiont is partitioned into the new leaves [21, 22].

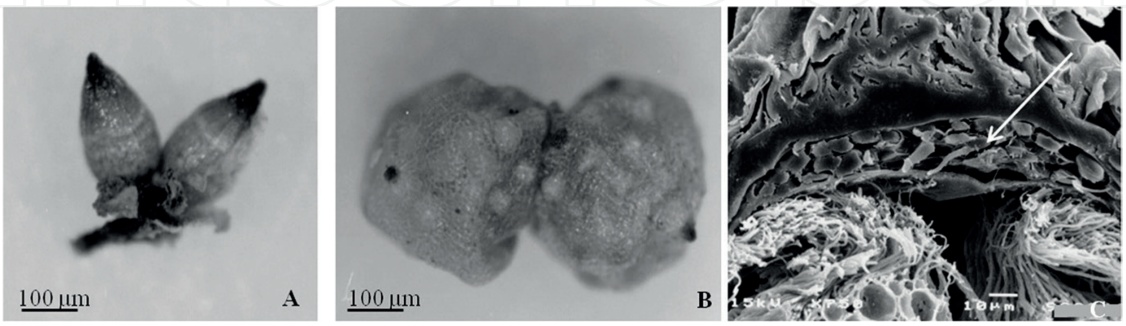

Figure 3. Sporocarps of *Azolla*. (A) Macrosporocarps. (B) Microsporocarps. (C) Indusium with cells of the cyanobiont *A. azollae* (arrow).

The cyanobiont of this symbiosis is Gram-negative, colonial, filamentous, and nitrogen-fixing having vegetative cells, heterocysts, and akinetes. Since the development of the cyanobiont and foliar cavities are synchronous, the filaments on the apical meristem only have vegetative cells (**Figure 4A**) without nitrogenase activity, while in the foliar cavities, the cyanobiont filaments have vegetative cells, heterocysts, and akinetes (**Figure 4B**) and can fix nitrogen [16, 23]. The fixed nitrogen is excreted into the leaf cavity as ammonium ions, which are assimilated by the hairs that exist in the foliar cavities and incorporated as amino acids in the fern [6, 17, 24, 25]. Although the cyanobiont is known as *A. azollae*, studies on the taxonomy of cyanobacteria indicated that it can be classified as *Anabaena*, *Nostoc*, or *Trichormus* [26, 27].

The bacteria found in the apical meristem and foliar cavities of the *Azolla* sporophyte (**Figure 5**) were identified by several researchers as *Aeromonas*, *Agrobacterium*, *Alcaligenes faecalis*, *Arthrobacter*,

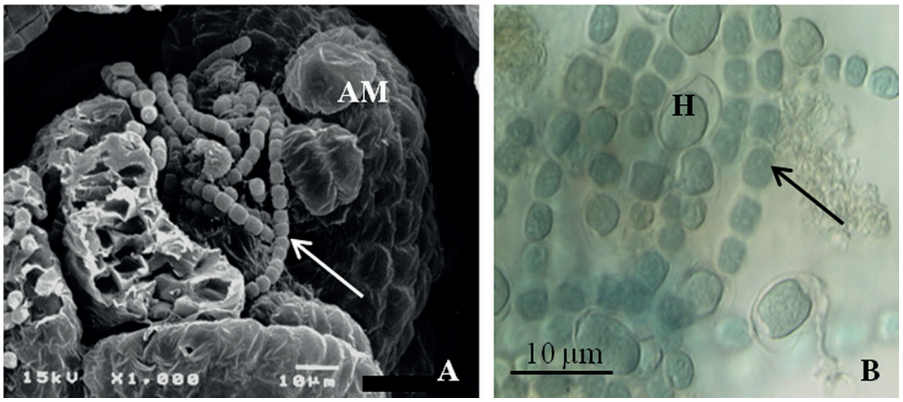

Figure 4. Filaments of the cyanobiont *A. azollae* in the sporophyte of *A. filiculoides*. (A) Filaments of vegetative cells (arrow) on the apical meristem (AM). (B) Filaments with vegetative cells (arrow) and heterocysts (H) in the mature leaf cavities.

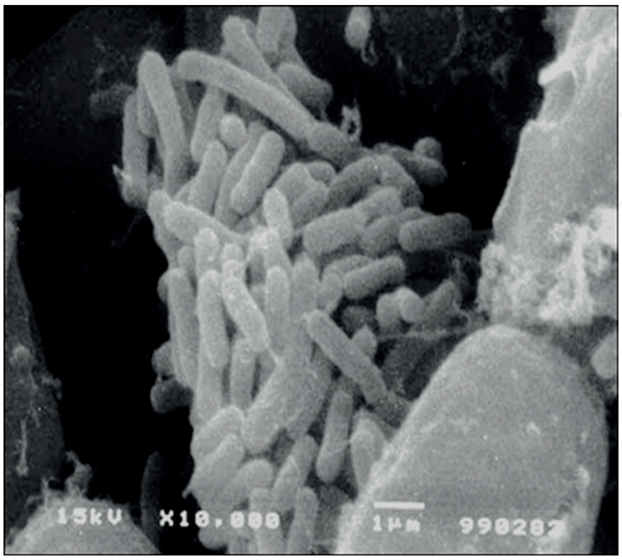

Figure 5. Bacteria inhabiting mature foliar cavity of the *Azolla* sporophyte.

Bradyrhizobium, *Caulobacter fusiformis*, *Flavobacterium ferrugineum*, *Pseudomonas*, and *Xanthomonas* [28–35]. At first, the bacteria that inhabit the foliar cavities were not considered as nitrogen-fixing, but the presence of nitrogenase was detected in some bacteria [36], but their role and importance in the symbiosis are not clear.

3. Applications

The world distribution, the resilience to several environmental conditions of light and temperature, and the formation of thick mats when the environmental conditions are optimal make the *Azolla* symbiosis a good candidate to be used in agriculture as biofertilizer, to the phytoremediation of contaminated water, and as nutritional supplement, among others.

3.1. Biofertilizer

In a world of increasing demand for biofertilizers especially nitrogen for a high productivity in agriculture, the use of industrial fertilizers increases not only the production costs but also the environmental impacts of the runoff with a high content of nitrogen to the water bodies and their eutrophication. Therefore, a system that reduces the costs and allows a more green agriculture without loss of production is desirable and welcome. Although the fuel crisis in 1970 of the twentieth century stimulates the finding of new alternatives and sources of fertilizers to be used in agriculture, nowadays only a few countries such as India and China still do research for the use of *Azolla* as biofertilizer.

The high rate of nitrogen fixation by the cyanobiont *A. azollae* and thus the content of nitrogen of the fern [5–7, 37] make this symbiosis suitable for use as biofertilizer, thus limiting the use of synthetic nitrogen.

Azolla is used for a long time in Asian countries as crop biofertilizer especially in paddy fields and also more recently in Africa [5–7, 37]. But, the use of *Azolla* can be limiting since large amounts of this fern are necessary. In controlled conditions of nutrients, light, and temperature, *Azolla* can grow and provide sufficient amount of biomass [38, 39]. Another way to have a large biomass is growing *Azolla* in treated domestic effluents, but the content of nitrogen, phosphorous, and heavy metals must be below the admitted limits. That way, the fern does not bioaccumulate those compounds above the limits and hence *Azolla* can be used to fertilize soils [40]. For large scale production of *Azolla* biomass, ponds, ditches, channels, small creeks, and tanks, among others can be used with costs of less than one dollar per week [7].

Azolla biomass can be used in rice fields as partial or total replacement of synthetic fertilizers because this fern can accumulate up to 2–3 Kg N/ha/day [41]. But, the sole use of *Azolla* biomass to fertilize soils is not enough to cover the high demands in nitrogen uptake by crops and thus it is always necessary to use chemical fertilizers [7, 42–46]. However, since it is necessary to use less amount of synthetic fertilizers, the production costs become lower.

When *Azolla* is applied in the soil of paddy fields, there is an increase in income of grain yield, caryopsis, straw, and dry matter [7, 42, 47]. However, the method of incorporation of the fern in rice fields seems to be important. So if *Azolla* is included in the soil as co-culture (dual crop), before transplanting rice, the biomass of rice grows faster in this stage, but it needs synthetic fertilizer for the grain production when the rice needs more nutrients [48, 49]. The amount of nitrogen in caryopsis increases after the application of *Azolla* in the intercropping system (between cultures) [50]. With the incorporation of 40 Kg N/ha as dried *A. filiculoides* into the soil, the rice grain yield was similar to the application of an equal quantity of sulfate of ammonia. However, it should be around 228 Kg N/ha of *Azolla* to equal the income obtained from 160 Kg N/ha of ammonium sulfate [37, 41]. Also, the geometry of rice fields is important [51]. So, the time and quantity of *Azolla* incorporation into the paddy fields depend on whether the farmer wants high nitrogen at the beginning of the culture or high amount of nitrogen when the rice blossoms and at the formation of the grain, thus having a higher grain yield.

Although research was mainly made in rice, this fern can also be used as biofertilizer on other plants such as water bamboo (*Zizania aquatica*), taro (*Colocasia esculenta*), and wheat (*Triticum aestivum*) [6, 7, 49, 52]. But, there are studies regarding other crops.

The incorporation of *Azolla* in the soil also improves the physical properties of soil such as the organic and nitrogen content, the availability of phosphorus, soil texture, pH, and porosity, among other soil properties [7, 46].

Yet, in a modern agriculture where a high crop yield is necessary, a great amount of *Azolla* that almost replaces the chemical fertilizer needed for the crop production is required. The traditional way to produce biomass, while valid, is not suitable. So, the domestication of this fern through the induction of sporulation, the collection of spores, storage, and their germination to have more biomass is needed. This is a not well-known process and difficult to obtain in controlled conditions, but the recent sequencing of the RNA of the sporocarps and sporophyte of *A. filiculoides* is a major breakthrough [53].

3.2. Phytoremediation

The continuous degradation of water bodies due to the continuous discharge of contaminants (heavy metals, nitrogen, dyes, cyanotoxins, and others) drove researchers to new remediation methods using plants.

The presence of heavy metals in the environment (soil or water) poses health treats since they cause intoxication and several diseases. The seven species of *Azolla* can remove by uptake and accumulate from 20 to 95% of a vast array of heavy metals such as arsenic [54], mercury [55, 56], zinc [57–62], lead [57, 61–64], chromium [56, 65, 66], copper [67–69], gold [70, 71], strontium [72], uranium [67], cadmium [61, 62, 67, 69], and nickel [61, 62].

The use of *Azolla* to the phytoremediation of soils and water is due to the high ability to uptake and chelate and due to biosorption of the compounds. In fact, the high biosorption capability of *Azolla* was used to make a biofilter with packed dried *Azolla* in order to provide an efficient tool to decontaminate industrial and domestic effluents [73, 74]. However, the efficient uptake of heavy metals by dried *Azolla* depends on time, the flux of the effluent, pH, and temperature, and *Azolla* species is turning difficult to make an optimized and efficient system to be applied in large-scale decontamination of wastewaters. Another application is the recovery of heavy metals, which can be made by electroplating or by using solvents and reusing them. This method could be used in small or large scale to diminish the depletion of the natural resources [58, 59, 62, 65, 68]. However, this technology was only tested in the laboratory.

The research on *Azolla* for the treatment of domestic wastewater has also been hypothesized. For instance, *A. filiculoides* can grow in a sewage pond of a wastewater treatment plant to remove nitrogen and phosphorous [75–77] and thus make the water suitable to be discharged into rivers, dams, and others or to be used as irrigation water. But again, as in the case of heavy metals, the application is hampered by the lack of large-scale research.

In fish aquaculture, it is usual to add sulfadimethoxine to prevent diseases in animals but the not absorbed fraction of this antibiotic is excreted through the fish feces and thus contaminates the water. The decontamination of water can be performed using *A. filiculoides* since it absorbs (until 88.5%) and degrades this antibiotic [78] and thus cleans the water, which can be used to irrigate farmland or can be dumped into the normal sewage, and the wastewater treatment plants will no longer need to remove this antibiotic from the water.

Another problem is the discharge of dyes from the textile industry, which contaminates water bodies such as rivers. In laboratory studies, the dyes Acid Red 88, Acid Green 3, Acid Orange 7 [79], and Basic Orange [80] can be removed from water up to 80% by dried *A. filiculoides*.

In most recent years, the water blooms of harmful cyanobacteria that synthesize cyanotoxins (microcystins, cylindrospermopsins, and others) have been increasing due mainly to water eutrophication. Some authors hypothesize that the *A. filiculoides* could uptake and remove those cyanotoxins from the water. However, the fern did not uptake nor accumulate microcystins [81] and cylindrospermopsin [82], making this fern not suitable to the phytoremediation of those environmental contaminants.

3.3. Food

Azolla has been studied for its possible use as feed not only for livestock but also for humans since it is rich in fibers, proteins, fatty acids, amino acids, polyphenols, vitamins, minerals, and others [83–90]. It can also be combined with other foods to create balanced diets. Although *Azolla* is rich in amino acids, methionine and cysteine are present in small amounts [83–86] meaning that the diet must include another source of these two essential amino acids.

Another aspect to consider is the digestibility and smell of *Azolla*, which can be a feeding deterrent. This fern has good digestibility [83, 85, 91, 92], but considering the smell, fresh *Azolla* has a green, mold odor due to some volatile compounds such as alcohols, ketones, and aldehydes that can contribute to its unpleasant smell [93].

Replacing 20% of the commercial feed by *Azolla* induced a weight gain in chickens and also gain in costs [94, 95]. When feeding laying hens with *A. pinnata* mixed with other food sources, there was an improvement in the production of eggs and the color of yolk [96].

Ducks fed with 20% of *A. microphylla* showed no reduction of growth but a lower cost production and higher profits [97].

Regarding fishes, the investigations have been focused on *Oreochromis*, *Tilapia*, and *Cichlasoma*. The fish *Cichlasoma* consumes preferably *A. microphylla* and *A. pinnata*, while *Oreochromis* consumes *A. filiculoides* [98, 99]. In the case of *Tilapia*, about 20% of the commercial feed can be replaced by dried *A. filiculoides* with fish having a weight gain similar to their feeding only with commercial fish feed [88]. However, *Oreochromis* can be fed with up to 40% of *Azolla* and still gained weight with lower production costs and higher profits [100]. Moreover, black tiger shrimps (*Penaeus monodon*) fed with meals with 40% of *Azolla* increase their weight, meaning that this can be an alternative to soybean meals [101].

Although *Azolla* can be useful for human consumption, there are very few reports about it. This fern can be integrated into soups and meatballs [6, 7] and pancakes [102], which proved to be acceptable in terms of taste and smell. A more extensive research with *Azolla* for human ingestion was made for space travels, which reveals that it would be beneficial to include steam sterilized leaves and roots in the space diet of astronauts to fulfill the human nutritional requirements [103, 104].

4. Future perspectives

The research on *Azolla* is vast and has been made for several decades, but only some countries such as India, China, and other Asian countries try to apply the outcomes of this research to benefit populations and especially agriculture practices. One of the problems is the use of *Azolla* collected from the environment for biofertilization and as a food supplement since if it can grow in polluted water bodies and since *Azolla* can accumulate contaminants such as heavy metals this would be a health problem due to human exposure to those contaminants. So, the strategy to follow depends on the ultimate use of *Azolla*.

If the fern is only used in the phytoremediation of water, the local populations can send this fern to an industry to recover the heavy metals and receive a monetary compensation or small villages can build a small recovery factory and sell the heavy metals to advanced factories. This would help in the economy of these small populations and also the environment. However, the technology to recover the heavy metals from this fern was only tested in the laboratory.

However, if the purpose is to include the fern as biofertilizer and/or as food for livestock or humans, there are two approaches:

(1) Since a continuous supply of *Azolla* devoid of contaminants is necessary, it will be better to grow *Azolla* in a small water pond. The water of the pond should be analyzed for the presence of harmful contaminants;

(2) If it is decided to use the *Azolla* that grow in water bodies, the water and fern must be analyzed in terms of contaminants since if it surpasses the legal amounts (provided by each country, WHO, FAO, or other) it cannot be used. For that reason and given that in many countries the populations are far away from cities or do not have scientific resources, it would be useful to discover a cheaper, simple, and rapid method to detect such compounds.

Another approach is the implementation of integrated farms with the cultivation of *Azolla*, fish in aquaculture, and agriculture. The water from fish tanks can be cleaned from any contaminants with *Azolla*, and in turn, this water can be used to grow *Azolla* or to irrigate crops, and *Azolla* can be used to feed fish or other animals and also humans. However, this means a good availability of water, which in many tropical countries especially in Africa is not possible due to severe dryness.

In conclusion, although many researches regarding the potential use of *Azolla* as biofertilizer, food, and phytoremediation and applications are made by farmers in India, China, or Vietnam, there are still many gaps and the research still does not really meet the needs of the populations of the countries that might benefit from this natural tool. For the effective application of this fern in the field, probably it will be beneficial to have a partnership with FAO, which has many people in the field of many countries and drives the research to fulfill specific demands from populations.

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