Development and evaluation of the formulation of a biofertilizer based on the Anabaena-Azolla complex

Kevin Wladimir Guanoluisa Salazar[1], Paula Daniela Arellano Zambrano[2], Ramiro Acurio[3]

Abstract — **Safety in agricultural production is one of the crucial parameters for fruits and vegetables, achieved through sustainable agriculture. In this study, the cyanobacterium Anabaena was evaluated in symbiosis with the plant Azolla sp, for the development of a biofertilizer. Samples were collected in Cusubamba, Pichincha, and seven culture media with different nutritional contributions were tested. In the Azolla study, the initial and final weights are analyzed to measure growth, the time to evaluate the development, and the cultivation area, which determines the area available for the determination of the biomass. These variables together allow the performance and sustainability of Azolla to be assessed. The best treatment was T2 (Nitrofoska 1g), achieving 66.59 g of fresh weight, a growth rate of 3.83 g/d, and a doubling time of 5.96 days. Dumas' method showed that T2 concentrated about 5% nitrogen. The biofertilizer complied with the characteristics and quality of the Phytosanitary and Zoosanitary Regulation and Control Agency (Agrocalidad-Ecuador), such as the absence of pathogenic microorganisms and the presence of macro and micronutrients such as nitrogen, which presented an average percentage of 1.54%, in addition to moderately optimal physical parameters such as a pH of 4.68 and a density of 0.016 g/mL.**

Keywords: **Bioinput, Anabaena, Azolla, Cyanobiont, Nitrogen.**

Resumen — **La seguridad en la producción agrícola es uno de los parámetros cruciales para las frutas y hortalizas, que se consigue a través de la agricultura sostenible. En este estudio se evaluó la cianobacteria Anabaena en simbiosis con la planta Azolla sp, para el desarrollo de un biofertilizante. Se recolectaron muestras en Cusubamba, Pichincha, y se probaron siete medios de cultivo con diferentes aportes nutricionales. En el estudio de Azolla se analiza el peso inicial y el peso final para medir el crecimiento, el tiempo para evaluar el desarrollo y el área de cultivo, que determina la superficie disponible para la determinación de biomasa. El conjunto de estas variables permite evaluar el rendimiento y la sostenibilidad de Azolla. El mejor tratamiento fue T2 (Nitrofoska 1g), alcanzando 66,59 g de peso fresco, una tasa de crecimiento de 3,83 g/d y un tiempo de duplicación de 5,96 días. El método Dumas mostró que el T2 concentraba aproximadamente un 5% de nitrógeno. El biofertilizante cumplió con las características y normas de calidad de la Agencia de Regulación y Control Fitosanitario y Zoosanitario (Agrocalidad-Ecuador), como la ausencia de microorganismos patógenos y la presencia de macro y micronutrientes como el nitrógeno, que presentó un porcentaje promedio de 1,54%, además de parámetros físicos moderadamente óptimos como un pH de 4,68 y una densidad de 0,016 g/mL.**

Palabras clave: **Bioinsumo, Anabaena, Azolla, Cianobionte, Nitrógeno.**

I. INTRODUCTION

In Ecuador, the agricultural sector is one of the most important productive axes because it occupies 24.29% of the country's total exports, in addition to contributing with participation the employment of around 26.8% of the economically active population (EAP). Agriculture, in turn, is a fundamental precursor of economic growth in developing countries such as Ecuador, so its contribution to the country's Gross Domestic Product (GDP) is 7.81%, which represents approximately 8,410.8 million dollars [1] [2].

Currently, agricultural systems in Ecuador present the need to improve their productivity and quality. Fertilization is a fundamental part of the production process, where the use of chemically synthesized fertilizers is common. Biofertilizers are an alternative to replace conventional fertilizers, as they allow the natural properties of crops to be maintained, without harming their cycles and preserving the fertility of ecosystems. In addition, biofertilizers facilitate the interaction between the existing microbiota in the crop and have the advantage of being made from microorganisms (bacteria, cyanobacteria, fungi, microalgae, or derivatives) which offer different benefits to crops, such as nitrogen fixation (N2), solubilization of phosphorus (P) and potassium (K), and assimilation of sulfur (S) [3] [4].

In agriculture, nitrogen is one of the macronutrients of greatest interest, it is used by the plant in the form of nitrate (NO3-) and ammonium (NH4+) for the optimal development of its vital processes. However, some agricultural producers use nitrogen fertilizers such as urea, excessively, forcing production without taking into account that the quality of the crop decreases due to poisoning by excess nitrogen. This also causes eutrophication, water toxicity, soil, and ecosystem degradation, contamination of groundwater flows, reduced biodiversity and biological imbalances [5].

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The *Azolla-Anabaena* partnership is presented as a viable alternative to reduce the adverse effects of the indiscriminate use of conventional agrochemicals, since it manages to fix atmospheric nitrogen (N2) and reduces it to ammonia, allowing the Azolla to assimilate nitrogen as a nutrient for its growth [4] *Azolla* spp. is a genus of floating aquatic ferns, which grow in bodies of fresh water, such as lagoons, lakes, or artificial reserves, and develop in tropical and warm areas, this fern can cover the entire surface of the body of water [6].

The leaves of the genus *Azolla* measure between 1 and 2 cm in diameter, and their color varies between reddish and greenish, with a circular or triangular shape. The adventitious roots can measure up to 30 mm in length and the stem, which is branched, is covered by small, alternate and lobed leaves, which consist of a submerged lower lobe (ventral) and an upper lobe (dorsal) that captures light energy, water and CO2 to carry out the photosynthesis process. In this upper lobe is a thin mesophyll filled with mucilaginous cavities that harbor the cyanobacterium *Anabaena azollae* [7].

Azolla reproduces in two ways: asexual and sexual; Asexual reproduction occurs through leaf fragments from lateral branches of the stem, where shoots grow spontaneously. Sexual reproduction is carried out using two types of floating spores: megaspores (female) and microspores (male), when fertilization occurs between the anterozoid that develops in the microspores (male gamete) and the egg (female gamete) [8].

Another organism that is part of the symbiosis is the filamentous cyanobacterium *Anabaena azollae,* whose hue is bluish-green and has chlorophyll a. This photosynthetic microorganism grows symbiotically extracellularly in the cavities of the dorsal lobes of aquatic ferns in the Azolla to harness photosynthetic energy and fix atmospheric nitrogen. In the filaments of Cyanobacteria, there are cells called heterocysts, which comply with the fixation of N2 [9].

Anabaena azollae is a photosynthetic cyanobiont that has vegetative cells in the apical meristem of the Azolla sporophyte, the differentiation of heterocysts occurs with the development of the leaf cavities. Cyanobiont has a gram-negative cell wall, cytoplasmic inclusions, and a simple thylakoid system. The heterocyst has cyanoffice nodes, a thick wall, and honeycomb thylakoids [10].

According to *to Anabaena* sp., it is a cyanobacterium that reproduces mainly by fragmentation by hormogonia. Hormones are specialized filaments or cell chains that separate from the parent colony and can mobilize to establish and colonize new areas, developing and forming new cyanobacteria [10].

In the symbiosis between the cyanobacterium Anabaena and the Azolla plant, contact is established when the Azolla sporophyte breaks the apical membrane, initiating the symbiosis. Anabaena concrete differentiates into akinetes in the megasporocarp, remaining dormant until the plant germinates, restarting the symbiotic cycle [11].

The growth conditions of the symbiosis can be categorized into two factors, as shown in Table I: physical and climatic factors, and chemical factors. It should be noted that in addition to the nutrients mentioned in the [10] [11], azolla also requires magnesium and sulfur, as well as minimal amounts of micronutrients such as zinc, manganese, copper, chlorine, and molybdenum.

TABLE I
GROWING CONDITIONS

Physical and climatic factors		**Chemical factors**	
Environmental Temperature	18 to 28 ºC	pH	5 a 7,5
		Nitrogen (N)	3 a 10 Kg N/ Ha
Water Temperature	5° to 35°C	Phosphorus (P)	2 to 4 ppm
Light	1500 lux	Calcium (Ca)	11 to 28 ppm
Relative humidity	70 to 75%	Potassium (K)	1 to 5 ppm
Water Depth	>3cm	Iron (Fe)	1 ppm

For the overcrowding of Azolla, it is important to bear in mind that, according to [12], this fern can double in weight in a period of 6 to 15 days. Starting from an initial amount of 0.16 kg, the biomass increased by 5.63 kg over a period of 19 days. Therefore, determining the appropriate culture medium is essential, since the amount of biomass obtained depends on the nutrients present in the medium.

For good massification and adequate development of symbiosis, the use of culture media that includes elements such as cow, horse or poultry manure (a source of nitrogen) is recommended. In addition, it is advisable to complement these with commercial media such as BG11, a culture medium for cyanobacteria that has macro and micronutrients or Nitrofoska which is a fertilizer used in agriculture rich in macronutrients [13].

The *Anabaena-Azolla* complex can fix about 200 kg of nitrogen (N) per hectare per year. This process is carried out thanks to the heterocysts of the Anabaena symbiont, where the nitrogen-fixing enzyme, nitrogenase, is located *Anabaena azollae* reduces the molecule from nitrogen (N2) to ammonia (NH3), as can be seen in Equation (1), proposed by [14].

$$N2 + ATP \rightarrow (nitrogenase) \rightarrow 2\,NH3 + 12ADP + 12Pi. \quad (1)$$

The FBN (Biological Nitrogen Fixation) in *Anabaena azollae* is intimately related to photosynthesis, which provides the ATP and reductant needed to convert nitrogen to ammonia in symbiosis. Three key enzymes in the assimilation of ammonium are glutamine synthetase, glutamate synthetase and glutamate dehydrogenase. Glutamine synthetase, present in the heterocyst, incorporates nitrogen fixed in glutamine, while glutamate synthetase acts in vegetative cells, transporting glutamate to heterocysts. The fixed nitrogen is transported to the host, where it is integrated into amino acids, which, together with the reductant and photosynthate, are supplied to the symbiont [15].

Anabaena azollae not only fixes nitrogen, but also excretes ammonia, cyanobacteria promote the use of N2 to determine the distribution of nitrogenous compounds produced by symbiosis with Azolla, activity that was evidenced in [16], where it was determined that N2 was distributed as extracellular ammonia (49.9%), intracellular ammonia (6.4%), extracellular organic nitrogen (5.6%), and intracellular organic nitrogen (38.1%).

It is important to mention that, in the nitrogen cycle, during the assimilation phase, plant roots take ammonia (NH3), ammonium (NH4+), and nitrate (NO3–) to integrate them into

the process of chlorophyll formation, leaf development, photosynthesis, and protein synthesis [17].

In Ecuador, this symbiotic complex has been used mainly in rice crops as an organic fertilizer. Due to the ability of the *Anabaena-Azollae* symbiotic complex to fix atmospheric nitrogen (N2), this partnership could be an alternative for the production of biofertilizers that meets the bio input control requirements of Ecuador regulated by the Agency for Phytosanitary and Zoosanitary Regulation and Control (Agrocalidad) under resolution 218, section X, specific requirements for the registration of biological inoculants for the registration of fertilizers an inoculum of nitrogen-fixing bacterial strains, whose relevance prevails in the control and registration of related products for agricultural use in Ecuadorian territory, by its requirements and procedures for correct control of fertilizers, soil amendments and related products for agricultural use [18].

This research was carried out in the laboratories of the Agency for Phytosanitary and Zoosanitary Regulation and Control (Agrocalidad), Tumbaco, for a period of ten months. The objective was to develop and evaluate the formulation of a biofertilizer made from the *Anabaena-Azolla* symbiotic activity, through massification in different Azolla culture media. This included identifying and characterizing *Anabaena–Azolla m*icroscopically, quantifying biomass and nitrogen content, analyzing growth rate, and evaluating the biofertilizer's physical, microbiological, and micro/macronutrient properties. The study ensured compliance with the quality control parameters outlined in resolution 218 of Agrocalidad for agricultural inputs and derivatives with nitrogen (N2) content.

II. MATERIAL AND METHODS

A. Harvesting of plant material for cultivation

The plant specimens of *Azolla* spp. were collected in Santa Rosa de Cusubamba (-0.028416, -78.283000), altitude: 2694 m.a.s.l., located in the province of Pichincha, Cayambe canton, with the help of 307.87 (L) plastic buckets and shovels for the collection of plant samples.

B. Suitability of culture media for the Azolla massification phase

The specimens were cultured in plastic containers 40 cm long by 25 cm wide, applying the A total of seven culture media, referred to as treatments (T), were tested along with one control (T8) Table II. The specimens were cultured in plastic containers measuring 40 cm in length and 25 cm in width. A total of seven culture media, referred to as treatments (T), were tested, along with one control (T8), as shown in Table II. Each of these contained 4 L of water with its respective components. 13 g of fresh Azolla was added as the initial inoculum in each of the treatments for massification; in these mass media, certain treatments containing commercial media were carried out such as Hoagland which is a hydroponic culture medium used for the growth of plant species, Nitrofoska whose nutritional content is nitrogen, phosphorus and potassium, BG11 which is a liquid culture medium for algae growth [17].

TABLE II
TREATMENTS FOR AZOLLA MASSIFICATION

Treatment	Description of compounds and quantities
T1	Soil (200g) + Manure (250g)
T2	Nitrofoska (1g)
T3	Soil (200g) + Manure (250g) + BG11 (100mL)
T4	Soil (200g)
T5	Soil (200g) + Manure (250g) + Nitrofoska (1g)
T6	Soil (200g) + Manure (250g) + Hoagland (100mL)
T7	BG11 (100mL)
T8	Irrigation water (control) (4L)

C. Identification of Anabaena azollae

Before massification, the cyanobacteria of interest were observed under a microscope. To do this, 1 gram of *Azolla was* weighed, macerated in 9 mL of distilled water, and a drop of the dilution was placed on a slide, which allowed the identification of cyanobacteria and their structures, such as heterocysts [18].

D. Experimental design of culture media

For the statistical analysis of the Azolla culture media, a Completely Randomized Design (DCA) was used with 3 blocks that were defined by the three different harvest times, with periodic evaluations of 14 days. At the end of each period, the biomass obtained was weighed and a 13 g inoculum was placed for a new reproduction of Azolla. The resulting biomass was dried in an oven for 24 h at temperatures ranging from 40 ºC to 50 ºC to later quantify the total nitrogen [18].

E. Azolla Biomass

To measure biomass growth in dry and fresh weight, the mean absolute growth rate (MAGR) (Equation 2), the average crop growth rate (ACGR), which is the growth of the plant per square meter per day (Equation 3), the average relative growth rate (ARGR), which represents that for each gram of Azolla base how many grams grow per day (Equation 4) and the doubling time (DT) (Equation 5). These quantitative analyses use data such as dry weight (W2), fresh weight (W1), time (T) and other variables such as the area of the container, which is determined by multiplying the length by the width (Equation 6), these data allow interpreting and describing the growth of plants in semi-natural environment conditions [19].

$$MAGR = \frac{W2-W1}{T} = g/day \quad (2)$$

$$ACGR = \frac{W2-W1}{Container\ area} = g\ m^2/day \quad (3)$$

$$ARGR = \frac{Log_{(e)}(W2)-Log_{(e)}(W1)}{T} = g * g/day \quad (4)$$

$$DT = \frac{ln_2}{ARGR} = days \quad (5)$$

$$Vessel\ area = Length\ (x)\ Width \quad (6)$$

F. Dumas Nitrogen Quantification

The percentage of total nitrogen (%N) at the Azolla plant was quantified using the Dumas combustion method. To do this, 1.5 mg of the dry and pulverized sample was weighed, placed on aluminum foil and fed into a nitrogen analyzer. The sample is burned at 1000 ºC in the presence of pure oxygen, generating a gaseous mixture. This mixture was conducted in a reduction chamber with hot copper, where the nitrogen oxides were converted into molecular nitrogen. Finally, water and carbon dioxide were removed, determining the percentage of total nitrogen using a thermal conductivity detector [20].

G. Biofertilizer formulation

The composition of the biofertilizer included *Azolla* spp. In addition, excipients were used. The biofertilizer was formulated in the liquid base from the harvest of azollas, they were drained in a sieve to remove excess water from the medium and then 50 g were weighed to mix them in 500 mL of boiled water, the solution was homogenized with a blender, then 1.5 g of citric acid was weighed. Which has preservative properties to improve the quality of the product by preventing contamination by microorganisms. For the other phase of the formulation, 1% carboxymethylcellulose (CMC) was placed in 500 mL of tap water, the function of the CMC was to retain the water [21] [22], then the solution was constantly stirred on a heating plate at 360 rpm until it dissolved completely and no lumps were observed, followed by this it was mixed with Azolla on the base solution (water and thickener) in constant agitation, until the bio preparation is homogenized [22].

H. Microbiological Testing

For the determination of the presence of microorganisms such as *E. coli, Staphylococcus aureus, Salmonella* Spp *Shigella* spp. and total coliforms, the proposed protocol was applied, where a dilution of 25 mL of the liquid biofertilizer was carried out in 225 mL of buffered peptone water, previously sterilized, and 15 minutes waited for the microorganisms to be incorporated into the environment. 100 uL of the previously prepared dilution was taken with the help of a micropipette and seeded in the following culture media: Titan Media brand methylene blue eosin agar (EMB) and BD brand Salmonella-Shigella selective culture medium. The dilution placed in each culture medium was dispersed with a sterile loop around the petri dish. 1 mL of the sample dilution was also placed in 3M Petrifilm ™ plates for Staph Express Counting and the culture media were incubated for 24 hours at 36 ºC. After this time, the presence of the corresponding microorganisms was determined, if they were present, they were quantified by determining the colony-forming units (CFU) obtained by multiplying the number of colonies by the dilution factor (Equation 7), and the interpretation of the results was made based on the Table III.

$$\frac{CFU}{g} = \frac{Number\ of\ colonies\ x\ dilution\ factor}{mL\ of\ the\ sown\ sample} \quad (7)$$

TABLE III
INTERPRETATION OF THE PRESENCE OR ABSENCE OF MICROORGANISMS IN THE CULTURE MEDIA

Culture medium	Microorganism	Colony	Presence or absence
Agar EMB	*E. Coli*	Bright green	Presence, LMP 1000 CFU ($^{w-1}$)
	Total coliforms	Purple	
Petrifilm Plates	*Staphylococcus aureus*	Blue dots or parts	Absence
Agar *SalmonellaShigella*	*Salmonella* spp.	The color is black due to hydrogen sulfide	
	Shigella spp.	Transparent with green center	

For microbiological analysis of microorganisms such as *Listeria monocytogenes* and *Salmonella* spp., 500 uL of the LMX SUPP supplement was placed in 225 mL of *Listeria monocytogenes* Xpress (LMX) broth to prevent the growth of other microorganisms, stirred and transferred 25 mL of the biofertilizer sample. The solution was incubated at 41.5 ºC.

In 25 mL of the sample, 225 mL of buffered peptone water was added and mixed. Subsequently, 1 mL of the 3M *brand Salmonella* supplement (SUPP) was added and incubated for 24 hours at 37 ºC. The two corresponding solutions were placed in wells of different stream plates, taken to a thermoblock at 95ºC and placed in the mini VIDAS equipment of the bioMérieux brand, which through the ELFA method (Enzyme-Linked Fluorescent Assay), antigens of bacterial microorganisms are detected. This equipment specifies that when the index value (VT) is <1 it is negative and when it is >1 it is positive [23].

I. Micronutrient and macronutrient analysis

Macronutrients such as potassium and phosphorus, and micronutrients such as zinc, sodium, iron, copper, and manganese, were quantified by atomic flame spectrophotometry. The methodologies used are endorsed by the Agency for Phytosanitary and Zoosanitary Regulation and Control (Agrocalidad): for macronutrients and for micronutrients under the document that defines the methodologies based on [23].

The methods established by the AOAC were considered to define the respective concentrations of each of the aforementioned elements for the reading of macro and micronutrients through the use of an atomic absorption spectrometer.

III. RESULTS AND DISCUSSION

A. Identification of Anabaena under the Microscope

Cyanobacteria *Anabaena azollae* They were recognized by the presence of heterocysts, which are yellow and round in shape, compared to vegetative cells that are oval-shaped [24] (Fig. 1).

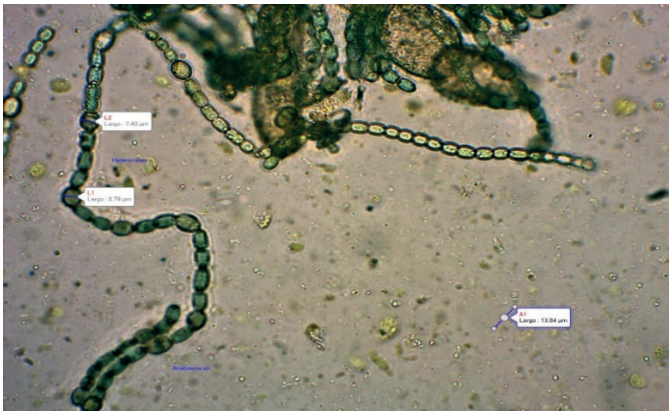

Fig. 1. Identification of *Anabena azollae*.

B. Massification of Azolla in culture media

From the initial inoculum, the first harvest was obtained after 15 days, it was determined that the Azollas of T4 (soil 200g) had long, reddish-brown roots and did not reproduce properly, since, according to the culture medium, it did not have enough supply of phosphorus, since the black soil did not contain enough of this nutrient [25].

In the second harvest, it was evident that the aquatic ferns adapted better to the treatments T1 (Earth (200g) + Manure (250g)), T2 (Nitrofoska (1g)), T3 (Earth (200g) + Manure (250g) + BG11 (100mL)), T5 (Earth (200g) + Manure (250g) + Nitrofoska (1g)) and T6 (Earth (200g) + Manure (250g) + Hoagland 100mL), since the massification time was faster unlike the first harvest, this is since studies such as the adaptation of the cyanobacteria to different conditions depend on components such as the production of redoxins in which glutarredoxins and thioredoxins are included, which are linked to the photosynthetic flow and nutrients that determine the expression of adaptability genes to the environment depending on the presence or absence of nitrogen in the environment [25]

In the third harvest, it was determined that the plants of the T1, T2, T3, T5 and T6 treatments have a complete adaptability to the culture media compared to other crops, as they obtain greater massification, with T2 being the one that generated a higher fresh weight as can be seen in Table IV.

C. Biomass and % nitrogen

The production of biomass and percentage (%) of nitrogen are positively correlated with the time of adaptability to the environment, since when observing the results concerning the blocks of time, the production of the aforementioned variables increases with respect to time, this is related to what was mentioned by those who describe that the Azolla together with the [26] *Anabaena azollae* it has the ability to adapt in the culture medium with respect to a period of time, as a consequence of the production of genes and proteins of the GS-GOGAT system (Glutamine synthetase-glutamate synthase) that when the nitrogenous conditions are rich activate all the nitrogen assimilation systems in the cells of the cyanobacteria, thus achieving a better assimilation of nitrogen and promoting the increase of biomass. Table V [27].

1. Biomass weights and percentage of Nitrogen

Fresh weight showed a significant difference with a p-value <0.0001, which means that at least one treatment is different. The T2 treatment (Nitrofoska 1g) obtained the highest average with a fresh weight of 66.59 g, as it can be interpreted that this fertilizer provides a base of selective nutrients to the medium so that the symbiotic association can increase its biomass [27]. On the other hand, the T8 treatment (control) obtained the lowest mean of 16.38 g. The T2, T3, T5, T1 and T6 treatments showed good biomass development because macronutrients such as K, N and P allowed biomass to double around 7 to 8 days (Table IV). Once the 3 harvests were concluded, it was determined that the Azollas can contain between 1 to 5% of the nitrogen. The nitrogen content can range from 1% to 5% in its dry matter, although some studies have reported figures that can vary between 2% and 7% depending on growing conditions and the specific species of Azolla [28].

The dry weight showed a significant difference with a p-value of 0.0034. The T3 treatment (soil + manure and BG11) obtained the highest mean of 2.03 g. This may be because the BG11 component contains Potassium Nitrate KNO3 as an active ingredient and its function is to allow the proliferation of cyanobacteria [29]. The T2 (Nitrofoska) and T5 (soil + manure + Nitrofoska) treatments also presented similar statistical results 1.88 to T3 for obtaining dry biomass by obtaining 1.91 g and g respectively [30]. The T8 treatment (control) had the lowest mean of 0.60 g.

In the analysis of treatments for the nitrogen percentage variable, using the DUMAS method, a significant difference was evidenced with a probability value p<0.05 of 0.0001. The T2 treatment (Nitrofoska), which showed 5.27% N, was the treatment with the highest percentage of nitrogen, this may be because Nitrofoska has essential nutrients such as nitrogen, phosphorus and potassium, to stimulate the growth of the azollas through the production of redox ins that increase the rate of photosynthetic electrons, which determines the expression of genes and regulation of different proteins to prioritize the absorption of nitrogen [31] On the other hand, the T4 treatment, which did not include additional supplements such as Nitrofoska or BG11, showed the lowest percentage of nitrogen, with 1.97%N.

TABLE IV
FRESH WEIGHT, DRY WEIGHT, NITROGEN PERCENTAGE OF TREATMENTS WITH TUKEY TEST (5%)

Treatment	Fresh weight	Dry Weight	% of N
T2	66,59 ± 5, 83 (a)	1,88 ± 0,81 (ab)	5,27 ± 0,10 (a)
T3	60,15 ±6,37 (ab)	2,03 ± 0,43 (a)	2,85 ± 0,12 (c)
T5	60,11 ± 6,67 (ab)	1,91 ± 0,39 (from)	3,17 ± 0,42 (bc)
T1	53,46 ± 15,78 (abc)	1,71 ± 0,17 (abc)	3,17 ± 0,28 (bc)
T6	46,81 ± 14,72 (abc)	1,53 ± 0,23 (abc)	2,88 ± 0,29 (c)
T4	36,97 ± 1,79 (bcd)	1,26 ± 0,35 (abc)	1,97 ± 0,34 (d)
T7	35,2 ± 1,16 (cd)	0,85 ± 0,27 (bc)	3,66 ± 0,12 (b)
T8	16,38 ± 2,07 (d)	0,6 ± 0,16 (c)	2,21 ± 0,44 (d)

TABLE V
TUKEY TEST (5%) FOR TIME LAPSES
IN PERCENT NITROGEN (%N) (BLOCKS)

Time	Mean ± D.E.	Rank
3	3,41 ± 0,94	a
2	3,03 ± 1,05	b
1	3,00 ± 1,08	b

D. Growth rate and doubling time analysis.

In this analysis, the data provided for the increase in biomass, time, area, length and width of the container is taken into account to develop the formulas for each of the treatments.

1. Mean absolute growth rate (MAGR)

In the absolute growth rate, the increase in Azolla biomass per day seem in the Fig. 2 (g/d).

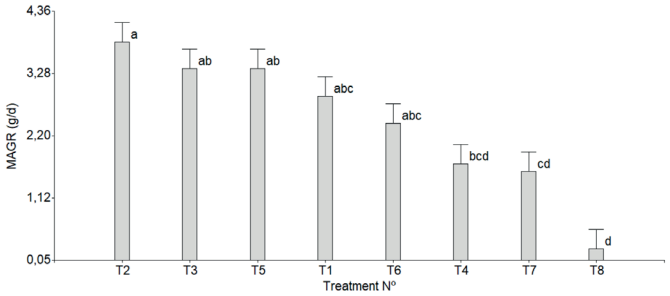

Fig. 2. Absolute growth rate of azollas.

It can be identified that treatments T2 (a), T3 (ab) and T5 (ab) are those with the highest growth rate with values corresponding to 3.83 (g/d), 3.37 (g/d) and 3.36 (g/d) respectively. The use of manure, such as goat and poultry manure, has shown positive results in the growth of Azolla, In addition, other elements such as Nitrofoska or BG11 provide essential nutrients that favor growth and nitrogen fixation by the symbiosis with Anabaena [30] [31].

2. Average Crop Growth Rate (ACGR)

As for the growth that was obtained per square meter per day (g.m2/d), in the Fig. 3 the bar graph for Tukey's test (5%) is presented.

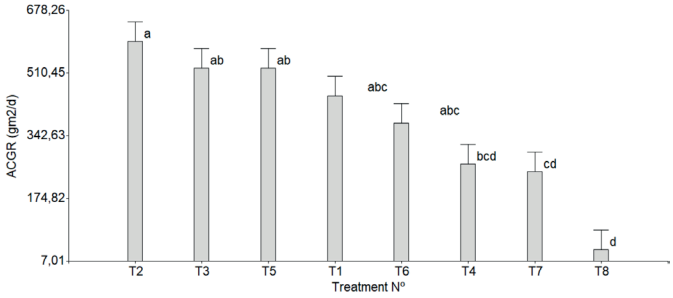

Fig. 3. Growth rate of azollas culture.

It can be observed that treatments T2 (a), T3 (ab) and T5 (ab) with means of 595 (gm2/d), 523.93 (gm2/d) and 523.40 (gm2/d) respectively, are the highest TMCC taking into account the short time in which biomass production is generated in the container area by the reason of partial shade is provided. especially to prevent the drying out of pteridophytes at midday [32].

3. Average Relative Growth Rate (ARGR)

In the TMCR, the growth is for each gram of base, how many grams the biomass of the symbiotic complex grows per day (gg/d) (Fig. 4).

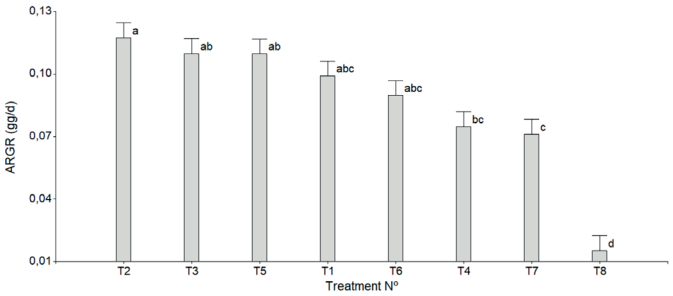

Fig. 4. Average relative growth rate of azollas

The treatments with the highest average are T2 (a), T3 (ab) and T5 (ab), with data corresponding to 0.12 (g.g/d) and 0.11 (g.g/d) for T3 and T5 treatments, since biomass production is distinctively high in 3 of the treatments, however, T8 shows a much lower growth [33].

4. Doubling Time (DT)

The variable (DT) allowed us to determine specifically in what time the biomass of the symbiotic complex begins to double in days (d) (Fig. 5).

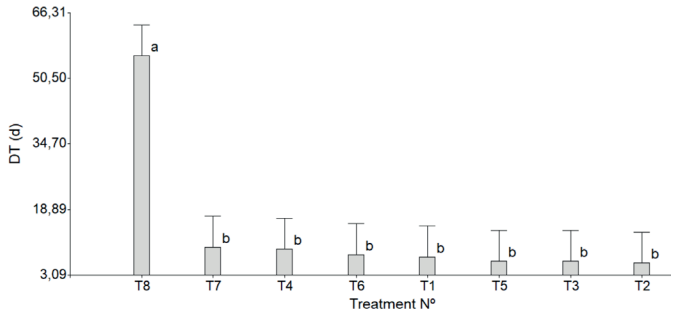

Fig. 5. Doubling time of the azollas

The treatment that requires the longest time to duplicate is T8, which in this case is precisely the control, while the treatments with the shortest doubling time are T2 (b) with 5.96 (d), T3 (b) and T5 (b) with 6.37 (d). It should be noted that the doubling time ranges from 5 days to 9 days in the case of treatments with the incorporation of culture media [34].

E. Physical analysis of the bioinput

1. Hydrogenic potential (pH)

The pH of the biofertilizer in its pure state was 4.68, however, when diluted in water in a ratio of 5 to 1, it tends to raise its pH to 6. The optimal pH present in a foliar input ranges from 5 to 6.5 [35].

2. Density

In the pycnometer a density of 1.016 g/mL was recorded, however, the most suitable density in a foliar fertilizer is around 1.20, the density obtained was low because there is more water in the fertilizer [36].

F. Microbiological analysis of the bioinput-

The bioinput complied with the quality control parameters in force in resolution 218 of Agrocalidad for organic fertilizers, since each of the microbiological tests yielded negative results. There was no presence of bacteria such as *E. coli,* total coliforms, *Shigella* spp., *Salmonella* spp. and *Staphylococcus aureus* [37].

For the bacteria *Salmonella* spp. and *Listeria monocytogenes*, the mini VIDAS team yielded negative results for the bioinput, for *Salmonella* spp. The result was a VT of 0.05 and in relation *to Listeria monocytogenes*, a VT of 0.00 was obtained.

G. Micro and macronutrient analysis

The analysis of macro and micronutrients using the Dumas method allowed us to determine their corresponding percentages. Three analyses of the bioformulation were carried out: at the time of obtaining the bioproduct with a difference of 15 days between each analysis, where T0, T1 and T2 correspond to each of the repetitions performed.

TABLE VI
PERCENTAGE OF LIQUID MACRO AND MICRONUTRIENTS PRESENT IN LIQUID BIOFERTILIZER

Macronutrients	**T0**	**T1**	**T2**
Nitrogen (N)	1,54	1,52	1,56
Phosphorus (P)	1,4939	1,4941	1,4942
Potassium (K)	1,107	1,103	1,109
Calcium (Ca)	1,5026	1,502	1,5021
Magnesio (Mg)	1,4981	1,4978	1,4983
Micronutrients	**T0**	**T1**	**T2**
Zinc (Zn)	1,035	1,035	1,035
Sodium (Na)	1,5219	1,5216	1,5219
Iron (Fe)	1,4904	1,4904	1,4902
Copper (Cu)	1,053	1,051	1,061
Manganeso (Mn)	1,098	1,10	1,096

Nitrogen, with an average of 1.54%, was identified as the most abundant element in the formulation of the bioinput like seem in the Table VI, thanks to the activity of the heterocysts of *Anabaena azollae*. It was evidenced that the amount of macronutrients and micronutrients did not vary with respect to time, if the formulation is left in conditions of humidity and extreme temperature, the nitrogen could volatilize [37].

III. CONCLUSIONS

The best treatments were T2 (Water (4L) + Nitrofoska (1g)), T3 ((Water (4L) + Soil (200g) + Manure (250g) + BG11 (100ml)) and T5 ((Water (4L) + Soil (200g) + Manure (250g) + Nitrofoska (1g)). The T2 treatment stood out for generating an average fresh biomass of 66.59 g and a dry weight of 1.88 g. In addition, it presented the highest percentage of nitrogen with 5.27%.

T2 also showed a high growth rate with a MAGR of 3.83 g/d, a ARGR of 0.12 g.g/d, and a ACGR of 595 g*m²/d. Its doubling time was 5.96 days, which favors a rapid production of biomass.

The control parameters were evaluated according to Agrocalidad regulation 218, determining that the physical parameters were moderately adequate such as a pH of 4.68 and a density of 1.016 g/mL. Microbiologically, the biofertilizer complied with the regulations, since the absence of pathogenic microorganisms such as total coliforms, *E. coli, Staphylococcus aureus, Salmonella* spp. and *Shigella spp. was observed* , which indicates the excellent state of the bioinput.

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REFERENCES

[1] J. Paladines, “Analysis of the income and trade balance of the agricultural sector in the Ecuadorian economy in the period 2019-2020,” 2021.

[2] J. Carrión, “Analysis of Ecuadorian Agricultural Gross Domestic Product,” *Scientific Journal*, vol. 6, no. 4, pp. 940-952, 2020.

[3] M. K. Bhatt, R. Labanya, and H. C. Joshi, “Influence of Long-term Chemical fertilizers and Organic Manures on Soil Fertility - A Review,” *J Agric Res Universe*, vol. 7, no. 5, pp. 177-188, Sep. 2019. https://doi.org/10.13189/ujar.2019.070502

[4] E. Vásquez, G. N. Abad-Calva, C. G. Angamarca-Angamarca and Y. A. Calle-Herrera, “Generation of nitrogen through the symbiotic production azolla–anabaena in the canton of Loja,” *Zero Latitude Forests*, vol. 13, no. 2, pp. 91-103, Jul. 2023. https://doi.org/10.54753/BLC.V13I2.1858

[5] M. Benimeli *et al.*, “Soil Nitrogen,” Tucumán, 2019. [Online]. Available: www.edafologia.com.arwww.edafologia.org

[6] A. Jama, D. P. Widiastuti, S. Gafur and J. G. Davis, “Azolla Biofertilizer Is an Effective Replacement for Urea Fertilizer in Vegetable Crops,” *Sustainability (Switzerland)*, vol. 15, no. 7, 2023. https://doi.org/10.3390/SU15076045

[7] S. Abin, D. Palengara and P. Majeti, “Azolla Farming for Sustainable Environmental Remediation,” *Handbook of Assisted and Amendment-Enhanced Sustainable Remediation Technology*, no. May, pp. 517-533, 2021. https://doi.org/10.1002/9781119670391.ch25

[8] B. Schneider, H. R. Hadad and E. E. Rodriguez, “Aquatic Plants of the Middle Paraná River,” 2021.

[9] A. L. Pereira and F. Carrapiço, "A fluorescence method to detect cyanophycin in the symbiotic cyanobacterium, Anabaena azollae," *Biotechnic & Histochemistry*, vol. 98, no. 4, pp. 291-295, May 2023. https://doi.org/10.1080/10520295.2023.2182452

[10] R. P. Rastogi, "Ecophysiology and Biochemistry of Cyanobacteria," *Ecophysiology and Biochemistry of Cyanobacteria*, pp. 1-478, Jan. 2022. https://doi.org/10.1007/978-981-16-4873-1/COVER

[11] N. Mishra, "Cyanobacterial symbiotic interaction in pteridophytes," *Microbial Symbionts: Functions and Molecular Interactions on Host*, pp. 3-13, Jan. 2023. https://doi.org/10.1016/B978-0-323-99334-0.00005-0

[12] H. J. Sánchez Sánchez, "Cultivation of microalgae in a residual aquaculture effluent for biomethane production," Sep. 2023, Accessed: Aug. 26, 2024. [Online]. Available: https://rinacional.tecnm.mx/jspui/handle/TecNM/6582

[13] Corporación Autónoma Regional de Cundinamarca CAR, "Plan for the Prevention, Control and Management of the species Azolla filiculoides L (Water fern) in the jurisdiction of the Corporación Autónoma Regional de Cundinamarca CAR," 2022, Accessed: Aug. 25, 2024. [Online]. Available: https://www.car.gov.co/uploads/files/64260164e4360.pdf

[14] E. J. Manrique, "Acceptability criteria for the commercialization of the organic fertilizer Azolla caroliniana willd 1810 prepared by the agricultural association of montubios San Vicente," Manabí, Feb. 2020.

[15] G. Verma *et al.*, "Implications and Future Prospects of Azolla as a Low-Cost Organic Input in Agriculture," *Agricolation*, vol. 1, no. 6, pp. 1-7, 2022, [Online]. Available: www.agricolation.com

[16] J. Guío Martínez, "Nuevas funciones de las proteínas Fur en el metabolismo del nitrógeno de Anabaena PCC7120 y su posible actuación como sensores del balance carbono/nitrógeno", M.S. thesis, Depto. de Bioquímica y Biología Molecular, Univ. de Zaragoza, Zaragoza, España, 2019. [Online]. Available: https://zaguan.unizar.es/record/86838/files/TAZ-TFM-2019-276.pdf

[17] K. Adhikari, S. Bhandari and S. Acharya, "An overview of azolla in rice production: A review," *Reviews in Food and Agriculture*, vol. 2, no. 1, pp. 04-08, Dec. 2020. https://doi.org/10.26480/RFNA.01.2021.04.08

[18] M. R. Aczel, "What Is the Nitrogen Cycle and Why Is It Key to Life?," *Front Young Minds*, vol. 7, no. March, pp. 1-9, 2019. https://doi.org/10.3389/frym.2019.00041

[19] L. García, "Importance of zeolite on the efficiency of nitrogen fertilizers, to increase yields in rice cultivation (Oryza sativaL.) in Ecuador," Los Ríos, 2020.

[20] H. Reyes Palma, "Azolla Anabaena as an alternative supplement to feed tilapia (oreochromisniloticus) and lower production costs," Babahoyo, 2021.

[21] G. E. Viteri Jiménez, "Evaluation of dose and time of application of azolla extract (Anabaena azollae) in the cultivation of broccoli (Brassica oleracea Var. italica)," 2023, Accessed: Aug. 24, 2024. [Online]. Available: https://repositorio.uta.edu.ec:8443/jspui/handle/123456789/39765

[22] B. L. Bianchini, G. L. de Lima and E. Gomes, "The TMCC in the Review of the Study of Function in an Engineering Problem," *Tecné, Episteme and Didaxis: TED*, no. 56, pp. 275-300, Jul. 2024. https://doi.org/10.17227/ted.num56-18773.

[23] Food and Agriculture Organization of the United Nations, "Standard Operating Procedure for Soil Nitrogen: Kjeldahl Method", Global Soil Laboratory Network (GLOSOLAN), Rome, Italy, Jan. 2021. [Online]. Available: https://openknowledge.fao.org/server/api/core/bitstreams/17a0e476-f796-4608-a869-295a367c0b56/content

[24] A. Cardozo, V. El Mujtar, V. Álvarez and S. Cáceres, "Manual for the Elaboration of Biofertilizer from Agricultural Waste." [Online]. Available: www.fontagro.org

[25] Y. Wang, M. Gao, H. Chen, Y. Chen, L. Wang and R. Wang, "Fertigation and Carboxymethyl Cellulose Applications Enhance Water-Use Efficiency, Improving Soil Available Nutrients and Maize Yield in Salt-Affected Soil," *Sustainability (Switzerland)*, vol. 15, no. 12, 2023. https://doi.org/10.3390/SU15129602

[26] M. Rea Naranjo, "Specific Test Procedure (Reference Paper)" Accessed: Nov. 16, 2024. [Online]. Available: https://www.agrocalidad.gob.ec/wp-content/uploads/2023/03/REFERENCIAS-DE-M%C3%89TODOS-AN%C3%81LISIS-FERTILIZANTES-2023.pdf

[27] S. Kailash, "Azolla Biofertilizer – The Nature's Miracle Gift for Sustainable Rice Production," *Journal Home*, vol. 2, no. 9, pp. 971-973, 2020, [Online]. Available: www.bioticainternational.com

[28] E. Morales, R. Martinez and G. Suárez, "View of Isolation, Culture, Viability and Evaluation of a Cyanobacteria-Microalgae Consortium as a Soil Conditioner," Biotechnology, ESPE, Quito, 2014. Accessed: Dec. 14, 2023. [Online]. Available: https://remcb-puce.edu.ec/remcb/article/view/249/196

[29] A. L. Pereira, B. Monteiro, J. Azevedo, A. Campos, H. Osório and V. Vasconcelos, "Effects of the naturally-occurring contaminant microcystins on the Azolla filiculoides–Anabaena azollae symbiosis," *Ecotoxicol Environ Saf*, vol. 118, pp. 11-20, Aug. 2015. https://doi.org/10.1016/J.ECOENV.2015.04.008

[30] D. Del Castillo *et al.*, "Development of a hybrid cell for energy production," *Nanotechnology*, vol. 32, no. 41, p. 415401, Jul. 2021. https://doi.org/10.1088/1361-6528/AC0C3E.

[31] D. Mishra, S. Rajvir, U. Mishra and S. Kumar, "Role of Bio-Fertilizer in Organic Agriculture: A Review," *Research Journal of Recent Sciences*, vol. 2, no. 2013, pp. 39-41, 2013, [Online]. Available: http://isca.in/rjrs/archive/special_issue2012/8.ISCA-ISC-2012-1AFS-32.pdf

[32] A. Bujak and J. Bujak, "Azolla's Use as a Biofertilizer and Livestock Feed," *Ferns: Biotechnology, Propagation, Medicinal Uses and Environmental Regulation*, pp. 671-695, Jan. 2022. https://doi.org/10.1007/978-981-16-6170-9_29/COVER.

[33] E. P. Valle Acaro, "Evaluation of the behavior of Azolla Anabaena (Azolla filiculoides Var. Cristata) under different doses of organic substrate in Santa Elena Ecuador," 2024. Accessed: Nov. 16, 2024. [Online]. Available: https://repositorio.upse.edu.ec/bitstream/46000/10887/1/UPSE-TIA-2024-0015.pdf

[34] E. Vásquez, G. N. Abad-Calva, C. G. Angamarca-Angamarca and Y. A. Calle-Herrera, "Generation of nitrogen through the symbiotic production azolla–anabaena in the canton of Loja," *Zero Latitude Forests*, vol. 13, no. 2, pp. 91-103, Jul. 2023. https://doi.org/10.54753/blc.v13i2.1858

[35] L. González, R. Félix, J. Sandoval, D. Escobedo and R. Longoria, "Characterization of biofertilizers used in the agricultural valley of Guasave, Sinaloa, Mexico," *Terra Latinoamericana Magazine*, vol. 39, pp. 1-14, 2021. https://doi.org/10.28940/terra.v39i0.859

[36] E. Pérez and D. Rodríguez, "Physico-chemical study for the formulation of a liquid fertilizer of complete composition," 2017. https://doi.org/10.15517/PA.V17I29.31551

[37] N. Ndraha, H. Y. Lin, S. K. Tsai, H. I. Hsiao and H. J. Lin, "The Rapid Detection of Salmonella enterica, Listeria monocytogenes, and Staphylococcus aureus via Polymerase Chain Reaction Combined with Magnetic Beads and Capillary Electrophoresis," *Foods 2023,* vol. 12, no. 21, p. 3895, Oct. 2023. d https://doi.org/10.3390/FOODS12213895