OPTIMATION OF NITROGEN AND PHOSPHORUS IN AZOLLA GROWTH AS BIOFERTILIZER

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Abstract

Nitrogen is one of the most important minerals for living organisms. Azolla is known as a small water fern which can fix nitrogen through the symbiotic association with the blue green algae Anabaena. Experimental studies were carried out in the glass house, investigating the growth of Azolla using different levels of nitrogen and phosphorus in the media. The experiment used 12 treatments with 3 levels of nitrogen (0, 5, and 10 mg atom/L) and 4 levels of phosphorus (0, 10, 20, and 30 mg atom/l) in a factorial design with 3 replicates. The results show that the highest biomass for fresh weight (13.28 g), dry weight (1,126 g), and the fastest doubling time (7.71 days) were found in combination of 10 mg atom/L N and 30 mg atom/L P. Meanwhile, the highest protein content was found in combination of 5 mg atom/L N and 20 mg atom/L P. After the third day heterocyst cells in Anabaena could only be found in those two combinations, but the highest heterocyst cells was found in the lower N and P combination similar to the highest protein content. Further experiment is suggested to introduce Azolla as bio-fertilizer for acid water system, such as peat land water.

Keywords: Azolla microphylla, Anabaena azolae, bio-fertilizer, protein content

1. Introduction

Nitrogen is one of the most important elements as the main component composed of living organism besides the other elements, such as hydrogen, oxygen, and carbon. The main nitrogen source which is used by living organism is appeared in the form of N gases, most of which are presented in the atmosphere. Nitrogen fixation has been observed at 139 to 170 x 1065 ton/year [1]. In fact, nitrogen in the element form could not be used by plants directly. Therefore, it has to be converted first into nitrate and ammonium form through any certain processes.

Based on their independence level performing the nitrogen fixation process, it is generally categorized into two kinds of groups, which are: a) nitrogen fixation process independently, and b) nitrogen fixation process in a viable state association/symbiosis with plants. There are only 4 kinds of microorganisms having ability to fix the nitrogen gas which is symbiosis with plants, and *Rhizobium* sp. is species having the most hosts followed by Frankia, Nostoc, and Anabaena, respectively [2].

In the other study, total nitrogen has been observed from plant tissue which showed a range from 3.9% to 5.4% of dry weight [3], and this plant is potential to be used as natural nitrogen source. This could be observed from a study about the addition of *Azolla* sp., even in fresh condition or compos, in various kinds of plant commodities. It was observed from the study that it was able to increase the growth of the plant [4]. Nitrogen fixation produced by *Azolla* sp. reaches 1.1 kg N_2/day. Therefore, it was potential to be used as green manure and soil fertilizer [5].

Based on the explanation above, it is necessary to perform a study about the association of *Azolla* sp. with microsimbion *Anabaena azollae* which is correlated with the availability of nutrients in the media. N and P in various concentration was used in order to fulfill *Azolla* sp., *A. azollae* requirement in the growth. The requirement of *Azolla* sp. – *A. azollae* to the nitrogen element is very low because *A. azollae* could fixate N from the air directly, while phosphorus element is the main nutrient limiting the growth and development of *Azolla* sp. – *A. azollae* [6] and the process of nitrogen fixation.

The purpose of this study is to determine the influence of N and P concentration to the production of biomass, cell number, and protein content of plants heterocyst *Azolla microphylla*. The study, was expected to find the best combination of fertilizers (especially N and P) on plant growing medium *A. microphylla* with

microsimbion *A. azollae*. Therefore, it could produce high levels of protein in plant *A. microphylla* with microsimbion *A. azollae.*

2. Methods

Materials used in this study includes organism, *A. microphylla*, liquid artificial media (MAC), ZA, and TSP fertilizer. Equipment used in this study was including aquarium, a set of protein analysis tools, pH meters, lux meters, microscopes, hemocytometer, objects glass, analytical scales, ovens, magnifiers, hand tally counters, and various kinds of glass equipment in various sizes.

The method used in this study was experimental and performed in factorial complete randomized design. Concentration of N (ZA) and P (TSP) expressed in mg atom/L was used as variables. Concentration of N used in this study was 0, 5, and 10 mg atom/L, while concentration of P used in this study was 0, 10, 20, and 30 mg atom/L [7]. Therefore, there were 12 combinations of treatment (control including) and each treatment was performed in triplicate. Overall treatment observed in this study was 36 treatments. Position of each treatment was placed randomized [8].

Growing medium of *A. microphylla* and *A. azollae* was prepared before *A. microphylla* seeds were planted. It used composition of liquid artificial media modification with various concentrations of N and P elements (in form of ZA and TSP). Plantation was performed by inoculating of *A. microphylla* 2 g/aquarium seeds [6] and inoculating *A. Azollae* seeds in equal addition (57.500/mL heterocyst cells and vegetative cells 1.332.500/mL). During maintenance, water levels were maintained constantly at 5 cm, while water volume was maintained at 6 litres.

A. microphylla was maintained for 21 days. Observation of the leaf *A. microphylla* to identify the appearance of *A. azollae*, heterocyst cell number, and the number of vegetative cells of *A. azollae* on leaves of *A. microphylla* was performed every day during the first 5 days and 1 day ago (2 days) for the next observation. Observation of pH and light intensity was performed every two days. At the end of the experiment (21 days), harvesting could be performed by collect overall *A. microphylla* in the aquarium. The growing medium, then filtered by using a filter with 30 of mesh size. Then, plant biomass and dry weight of plant biomass were measured by using weight balance. Plant protein concentration and growing medium protein concentration were measured by using Lowry Method [9].

The experimental variables are 1) fresh weight of *A. microphylla*, 2) dry weight of *A. microphylla*, 3) doubling time, 4) media pH, 5) analysis of *A. microphylla* protein content.

3. Results and Discussion

Biomass Plants *A. microphylla*. Result of the study showed an interaction between treatments using N and P to the fresh weight, weight of leering, and doubling time of plant *A. microphylla*. Average fresh weight, dry weight, and doubling time of *A. microphylla* were evaluated using Duncan test ($p = 0.05$; Table 1). It could be observed from the study that combination treatment N2P3 (10 mg atom/L N + 30 mg atom/L P) accelerated the doubling time, which was 13.28 g, 1,126 g, and 7.71 days. On the other hand, treatment combinations N2P0 (10 mg atom/L N + 0 mg atom/L P) obtained the lower result for fresh weight, dry weight, and the longest of the doubling time, which was 3,426 g, 0.29 g, and 35.3 days.

Based on the result of the study, addition of combination N (ZA) abd P (TSP) concentration could increase the yield of *A. microphylla* fresh and dry weight and accelerate the doubling time of *A. microphylla*. The highest result of fresh and dry weight was shown by treatment using N2P3 at 10 mg atom/L N and 30 mg atom/L P.

It might be caused by N as one of macronutrients required for cell biosynthesis during photosynthesis to yield energy. It is, then, utilized for the formation of plant tissues *A. microphylla*. Therefore, the availability of N elements will affect the biomass production [10]. The similar case appears by the availability of S element inside of ZA where S is involved in a low energy and protein synthesis.

Sulfur formed analog energetically with peptida bond. Sulfur is a cystine, cystein, and metionin amino acid composer [11]. It was clear from the study that N (ZA) and P (TSP) were supplemented to *A. microphylla* in order to stimulate their growth and development, so it could increase the fresh and dry weight of *A. microphylla*.

Table 1. Fresh Weight, Dry Weight, and Doubling Time of *A. microphylla* in Various Treatments

Treatments (mg atom/L)	Fresh weight (g)	Dry weight (g)	Doubling time (days)
N0P0 (N:0;P:0)	3.622^{a}	0.320^{a}	19.01^{c}
N0P1 (N:0;P:10)	3.895^{a}	0.319^{a}	17.23^{c}
N0P2 (N:0;P:20)	4.734^{b}	0.399^{ab}	18.02^{c}
N0P3 (N:0;P:30)	5.484^{bc}	0.463^{b}	15.98^{c}
N1P0 (N:5;P:0)	5.400^{b}	0.459^{b}	12.41^{b}
N1P1 (N:5;P:10)	6.307^{bc}	0.511^{b}	16.00^{c}
N1P2 (N:5;P:20)	7.400^{c}	0.634^{c}	9.60^{ab}
N1P3 (N:5;P:30)	3.925^{ab}	0.327^{a}	17.53^{c}
N2P0 (N:10;P:0)	3.426^{a}	0.290^{a}	35.30^{d}
N2P1 (N:10;P:10)	8.525^{d}	0.754^{d}	11.14^{b}
N2P2 (N:10;P:20)	4.750^{b}	0.423^{ab}	14.12^{bc}
N2P3 (N:10;P:30)	13.280^{e}	1.126^{e}	7.71^{a}

Note: Different letter in a column indicate a significant difference at ($p = 0.05$)

Total number of *A. microphylla* Leaf. Results of the study showed that there was an interaction between treatments N and P on the number of leaves of *A. microphylla* only at the end of observation (19th and 21st day), while from the first day to the 17th day it showed no interaction and did not show a significant difference. On the contrary, on the 11th day, treatment N had showed a significant difference among treatments. Total number of *A. microphylla* leaves at the concentration of 10 mg which was observed on 11th, 19th, and 21st days and analyzed using Duncan test shown on Table 2.

Table 2. Total Number of Leaves of *A. microphylla* at the Concentration of 10 mg in Various Observations of Time

	Observation Time (days)		
	11	19	21
N0P0	287.7	218.0 ^{a}	150.0 ^{a}
N0P1	247.3	312.3 ^{c}	296.7 ^{b}
N0P2	222.3	400.0 ^{e}	383.3 ^{d}
N0P3	208.3	291.7 ^{bc}	355.7 ^{cd}
N1P0	189.0	275.0 ^{b}	308.3 ^{bc}
N1P1	227.7	283.3 ^{e}	500.0 ^{f}
N1P2	197.0	366.7 ^{d}	450.0 ^{e}
N1P3	215.3	289.0 ^{bc}	366.7 ^{cd}
N2P0	200.0	394.3 ^{e}	461.0 ^{e}
N2P1	211.0	316.7 ^{c}	366.7 ^{cd}
N2P2	182.7	300.0 ^{c}	408.3 ^{d}
N2P3	178.0	291.7 ^{bc}	333.3 ^{c}

Note: Different letter in a column indicate a significant different at (p = 0.05)

Based on the data on Table 2, it could be observed that treatment with no nitrogen on the 11th day showed the highest result compared the other treatments (N1 and N2) althougs no statistics differences is found. On the 19th and 21st days, treatment showed an interaction between combination of treatment N and P to the total number of *A. microphylla* leaves. The highest number of leaves was shown by treatment using N1P1 (5 mg atom/l N + 10 mg atom/L P).

Association effectiveness between *A. microphylla* and *A. azollae* could occur if the only growing medium contain the nutrition required by *A. microphylla* and *A. azollae*. Addition of ammonium and nitrate in a trace amount could increase the effectiveness of association between *A. microphylla* and *A. azollae*. In common, addition of N in a high number will couse a negative effect on the association [12]. It could be, also, influenced by the availability of P element, which is the main nutrient limiting the growth and development of *A. azollae* and also the addition of N.

Total cells of Heterocyst *A. azollae*. Based on the observation, results showed that there was not any presence of heterocyst at the beginning of 3 days. Observation during day 4 to the end of observation period (day 21) resulted that there was interaction among treatment using concentration N and P to the total number of *A. azollae* heterocyst cells.

From day 4 to day 21, treatment using N1P2 dominated as the highest number almost at all of observation periods (days 4, 5, 7, 11, day 15, and 21). Treatment using N1P3 showed the highest result on days 9 and 13 (Figure 1).

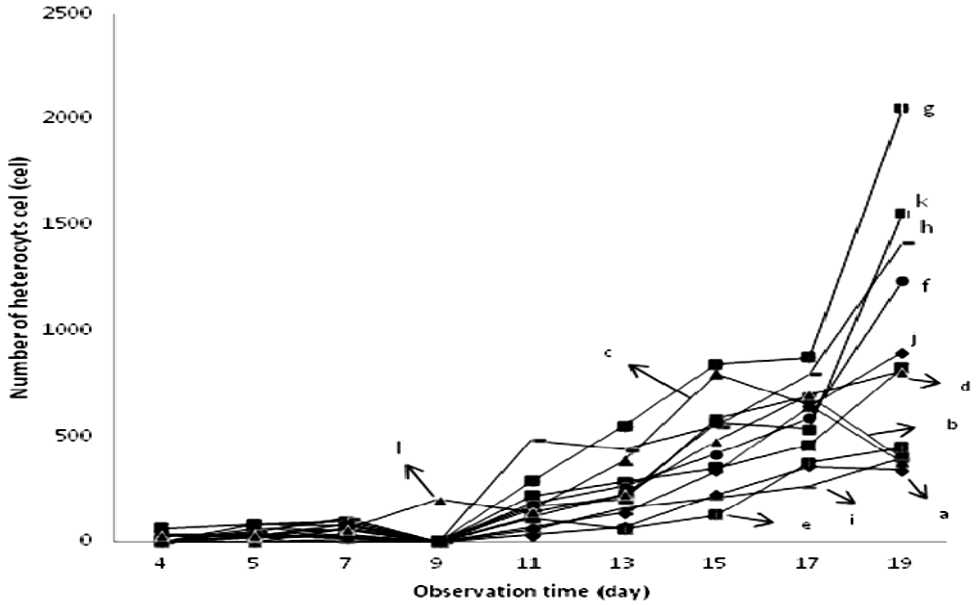

Figure 1. Anabaena Heterocyts Cell Number at Various Observations (a: N0P0, b: N0P1, c: N0P2, d: N0P3, e: N1P0, f: N1P1, g: N1P2, h: N1P3, i: N2P0, j: N2P1, k: N2P2, l: N2P3)

Nitrogenase activity is strongly influenced by growing medium condition, such as composition and content of each nutrient [6]**.** The lack of these factors in certain times still could be allowed by this symbiosis process, and its effect is not directly influenced the nitrogen fixation process [13]. However, it will influence the photosynthesis directly [14]. It was correlated with the availability of carbohydrate in sufficient amount which is required by microsimbion as energy resource.

In a growing medium containing low level of N, nitrogenase process that occurred on *A. azollae* will be more active so that it will be followed by the increase in heterocyst cell as the N2 fixation facilities. This could cause the decrease of energy which is used for development of other organs. On the contrary, on the media containing high N concentration, N2 fixation process will ensure the availability of nitrogen compound for *Azolla* sp. and other plants. Even though nitrogen is presented in a high number in biosphere, it could not be used directly by living organisms.

Protein Levels. Protein levels were observed from each treatment combination which can be seen on Table 3 and protein levels chart on *A. microphylla* and growing medium as shown at Figure 2.

Based on the data mentioned on Table 3 and at Figure 2, it could be concluded that protein content on *A. microphylla* and the highest growing medium were yielded on the treatment using N1P2. Total protein content in *A. microphylla* in N1P2 treatment was 39.62%, while in the growing medium was 21.21%. The lowest protein content observed in *A. microphylla* was observed on in the N0P1 treatment 13.1%, and growing medium was found in N1P1 treatment, amounting to 3.56%.

Azolla sp. plants were proven to be able to synthesize N compounds and transform them into protein form even though the growing medium contain low level of N [15]**.** This means that the source of N to be used for N compounds synthesis is molecular nitrogen originating from atmosphere in the presence of nitrogenase process performed by *A. azollae*. Level of protein content on the growing medium of *A. microphylla* is a balance between the release of N to the medium of fixation performing by microsymbion *A. azollae* and nitrogen placed on the media as treatment. In addition, analyzed proteins from other microalgae live in a growing medium of *A. microphylla*.

pH Media. Data of growing medium pH in a different observation time period were shown on Table 4. pH of the growing medium tends to decline by the observation time. pH of growing medium was ranging from 3.3 to 8.5. The highest pH was found on N1P0 treatment 7.5,

Table 3. Levels of Protein *A. microphylla* and Growing in Various Medium Treatments

	Levels protein (%)	
	A. microphylla plant	Growing medium
N0P0	18.53	7.11
N0P1	13.1	20.17
N0P2	28.12	7.11
N0P3	32.59	7.43
N1P0	20.45	8.39
N1P1	21.41	3.56
N1P2	39.62	21.21
N1P3	24.6	7.47
N2P0	30.99	4.51
N2P1	32.91	15.34
N2P2	33.07	21.09
N2P3	15.98	12.7

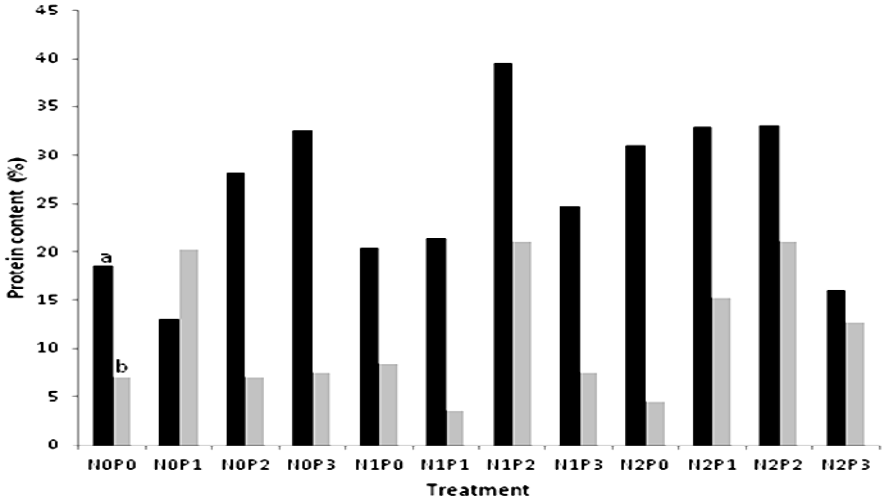

Figure 2. Protein Content in *A. microphylla* (a) and in the Growing Medium (b) on Various Treatments

Table 4. pH of Growing medium in Different Time Observations

	Observations Time (day)										
	1	3	5	7	9	11	13	15	17	19	21
N0P0	6.8	6.8	6.5	6.6	6.8	6.8	6.6	6.9	6.7	7.2	6.6
N0P1	6.8	6.7	6.6	6.7	7.2	6.7	6.7	6.7	6.7	7.2	5.9
N0P2	6.8	6.7	6.7	6.5	6.8	6.6	6.4	6.8	6.9	6.8	6.2
N0P3	7	6.7	6.3	6.3	6.4	6.5	6.4	6.7	6.6	6.4	5.9
N1P0	7.5	7.1	6.6	6.2	6.7	6.5	6.6	6.5	6.5	6.7	5.8
N1P1	7	6.9	6.6	6.4	6.6	6.4	6.3	6.7	6.7	6.6	5.7
N1P2	6.9	7	6.7	6.1	6.8	6.3	5.5	6	5.6	6.8	5
N1P3	6.9	7	6.6	6.9	6.9	6.6	6.6	6.8	6.6	6.9	6.4
N2P0	6.7	7.1	6.8	6.8	7.1	6.8	6.3	7	6.7	7.1	5.8
N2P1	7.4	7	6.6	6.3	6.6	6.3	5.7	6.4	5.8	6.6	4.5
N2P2	6.7	6.7	6.6	6.5	6.6	6.5	6.2	6.5	6.2	6.6	4.8
N2P3	6.6	6.6	6.4	6	6.6	6.5	6	6	5.9	6.6	4.3

which occurred on the first day, while the lowest pH was found at N2P3, treatment 4.3 which occurred on the 21th day.

It might be caused by absorption of NH_4 + (NH_4) $2SO_4$ (ZA), that caused it to retained SO_4^{2-} and caused the decrease in pH level. It was proven from the treatment using N2P3. The optimal pH range for the growth of *Azolla* sp. is 4-7, but *Azolla* sp. could live in a pH level of 3.5-10 [5].

4. Conclusion

The results show that the highest biomass for fresh weight (13,28 g), dry weight (1.126 g), and fastest doubling time (7.71 days) was found in combination of 10 mg atom/L N and 30 mg atom/L P. Meanwhile, the highest protein content was found in combination of 5 mg atom/L N and 20 mg atom/L P. After the third day hererocyst cells in Anabaena could only be found in those two combinations, but the highest heterocyst cells found in the lower N and P combination were similar to the highest protein content. The observation of the ecological factors shows that *Azolla* sp. grows in low pH. Further experiment is suggested to introduce Azolla as biofertilizer for acid water system such as peat land water.

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