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Title: Growth Performance of *Azolla pinnata* in Different Nutrient Media Containing Nitrogenous and Phosphorous Fertilizer

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ABSTRACT

An experiment was conducted at the net house of Plant Breeding and Biotechnology Laboratory of Agrotechnology Discipline, Khulna University, Bangladesh from March to May, 2019 to study the growth performance of *Azolla pinnata* in different nutrient media. The experiment was conducted using Completely Randomized Design (CRD) with three replications which consisted of seven treatments namely 20 g TSP, 30 g TSP, 40 g TSP, 50 g TSP, 20 g TSP + 1 Urea Super Granule, 1 Urea Super Granule and control (fresh water). Each treatment was set in a 35 L bowl where 20 L water and 3 kg soil were added. Data were collected in weekly basis (from 1st to 4th week) for the experiment. No significant variation in plant number was observed at 1st and 2nd week whereas at 4th week, the maximum plant numbers (1629.33) were observed in 50 g TSP and the minimum plant numbers (145) were recorded in 1 Urea Super Granule. In case of plant biomass, no significant variation was observed only at 1st week. At 4th week, the maximum plant biomass (43.62 g) was observed in 50 g TSP and the minimum plant biomass (5.51 g) was recorded in 1 Urea Super Granule. The total nitrogen of plant was maximum (9.6%) in 1 Urea Super Granule and the minimum (5.96%) was in 50g TSP. Considering above parameters, growth of *Azolla pinnata* was found better when it was grown in media containing 50 g TSP with 20 L water and 3 kg soil.

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INTRODUCTION

Azolla pinnata belongs to family Azollaceae, is a small free-floating aquatic pteridophyte with bilobed leaves (dorsal and ventral lobes). The dorsal lobe has an ovoid cavity in which inhabits the nitrogen-fixing cyanobacterium *Anabaena azollae* Strasburger. This unique association is important as rice culture biofertilizer, animal feed or wastewater purifier (Carrapico and Pereira, 2000).

Azolla grows in waterways in dense patches, which can look like a green or red carpet. Generally, it grows in stagnated water, streams, canals, ponds etc. where water is present for longer period under sunlight or shade of tree is widely distributed throughout the world (Masoodi and Khan, 2012). Roots of *Azolla*

plants remain suspended in water. *Azolla* contains around 23-27% crude protein and 10% carbohydrates on a dry weight basis (Kathirvelan et al., 2015).

According to Santos et al. (2009), high ammonium concentrations had no effect on nitrogen fixation dynamics. Phosphorus is the most important and often limiting nutrient for *Azolla* growth (Dawar et al., 2001). Productivity of *Azolla* was regulated by the nutrient status of the medium and was influenced more by orthophosphate P of a particular medium. Increasing phosphorus supply and/or plant density led to increased sporulation. If there is enough phosphorus in the aquatic environment, *Azolla* will be able to grow without the need to provide combined nitrogen such as NH_4NO_3 (Costa et al., 1999). When the concentration of all salts N, P, K and C is high in media, they support good plant growth (Dawar and Singh, 2002).

While *Azolla* is widely recognized for its capacity to fix atmospheric nitrogen through its symbiotic relationship with *Anabaena azollae*, most existing studies focus on its role as a biofertilizer or green manure in rice cultivation. However, there is limited understanding of how varying concentrations of externally supplied nitrogen and phosphorus fertilizers affect *Azolla*'s growth and nitrogen-fixing ability under controlled conditions as it is important for the production of large amounts of biomass and for ensuring the good quality of *Azolla*.

Azolla possesses the ability to utilize the atmospheric N_2 for their biomass production due to a symbiotic association with the blue-green algae *Anabaena azollae*. It is capable of fixing nitrogen at higher rates than legumes and is able to grow successfully in waterlogged habitats having low level of nitrogen. It can fix over 1 kg N ha^{-1} day^{-1}, providing sufficient nitrogen to allow sustainable rice cultivation. Increase of 14 to 40% in grain yield of rice has been reported with *Azolla* used as dual crop (Kannaiyan, 2002). The use of *Azolla* also increases organic matter and potassium contents of the soil. Being bio-fertilizer, it can substitute for chemical fertilizers to certain extent and increases the nutrient utilization efficiency which increases the crop yield as well as quality of product (Bhuvaneshwari and Singh, 2015).

Azolla became progressively more popular as animal feedstock because of its capability to grow on artificial water pools, fast growth in nitrogen deficient habitats, production of enormous green and dry fodder with high nutrient content and above all cost of production is very low (Shukla et al., 2018; Immanuel et al., 2019).

Considering the above circumstances, the present study was undertaken

- to study the growth performance and production of biomass of *Azolla* growing in different nutrient media containing different concentrations of nitrogenous and phosphorous fertilizer.

MATERIALS AND METHODS

An experiment on *Azolla pinnata* growth performance in different nutrient media was conducted at net house of Plant Breeding and Biotechnology Laboratory, Khulna University, Khulna- 9208, Bangladesh from March to May, 2019. The experiment was laid out in Completely Randomized Design (CRD) with 3 replications. Here, 7 treatments were used and different nutrient medium were considered as treatment. Treatment combinations of the experiment were T_0 Control (fresh water), T_1 (20 g TSP), T_2 (30 g TSP), T_3 (40 g TSP), T_4 (50 g TSP), T_5 (20 g TSP + 1 Urea Super Granule) and T_6 (1 Urea Super Granule). Treatment 'T_0' is used as control to evaluate the growth performance of *Azolla pinnata* in different nutrient content and Urea Super Granule of 2.6 g was used.

At first the bowls (35 L capacity having 18 cm^2 surface area) were taken, cleaned properly and kept them treatment wise in rows and replication wise in column. Then 3 kg soil (Sandy-loam soil, pH= 6.87, N= 0.15%) and 20 L water in each bowls were taken at same day. The next day, nutrients in these bowls except T_0 were added. After that all the nutrients were stirred to make sure that they are fully dipped down to the water.

Then *Azolla* was placed on absorbent paper to remove excess water. After absorption of extra water, fresh weight of *Azolla* was measured and each fragment of *Azolla* was counted very carefully that the roots of *Azolla* might not be injured. After that, these weighted *Azolla* (1g fresh weight) was placed in each bowl at the same day and kept for next four weeks to determine the growth performance of *Azolla*. The temperature of net house was varied from 30-33° C and humidity from 45-50%.

Fresh weight (g) of *Azolla* was measured and manually counted the number of *Azolla* at weekly interval. Each fragment of *Azolla* was counted as individual plant.

Total nitrogen percentage estimation of plant and soil samples

After completion of four weeks, *Azolla* were collected treatment wise and dried them in oven (WTC-Binder) at 70 °C for 48 hours. After that dry weight (g) of *Azolla* from each bowl was measured using balance. Total nitrogen percentage was estimated from 1 g of oven dried *Azolla* through digestion and distillation process by using Kjeldahl methods where the total N% was determined using the following formula by Kjeldahl, 1983-

(1000 ml 1N H_2SO_4≡14 g N)

$$N\ (\%) = \frac{[(T - B) * 14 * S * fv * 100]}{(1000 * E * W)}$$

Where,

T = Amount of H_2SO_4 required in sample titration, B = Amount of H_2SO_4 required in blank titration, S = Strength of H_2SO_4, fv = Final volume of the digest, E = Volume of digest taken for distillation, W = Weight of oven dried plant sample

Recorded data were analyzed with the help of computer package program STAR (Statistical Tool for Agricultural Research), Microsoft Excel and the mean differences were separated at 5% level of significance with Tukey's Honest Significant Difference (HSD) Test (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Effect of different nutrient media on plant number of *Azolla*

Up to 2nd week, no significant difference was observed among the treatments. At 3rd week, significant difference was observed where the highest plant number (325) was recorded from 50 g TSP. Statistically similar results were recorded from 40 g TSP (284.33) followed by 30 g TSP (274.33), 20 g TSP (260.67), control (236.67) and 20 g TSP + 1 Urea Super Granule (222.67). The lowest plant number (141) was obtained from 1 Urea Super Granule which was statistically different from other treatments (Table 1).

At 4th week, the maximum plant number (1629.33) was recorded in 50 g TSP which was statistically different from other treatments. The 2nd highest plant number was observed in 40 g TSP (811). 30 g TSP (542.33), 20 g TSP (467.67), control (439.67) and 20 g TSP + 1 Urea Super Granule (360.33) showed statistically similar result. The minimum plant number (145) was obtained in 1 Urea Super Granule which was statistically different from others (Table 1).

Effect of different nutrient media on fresh weight (g) of *Azolla*

At 1st week, significant differences ($p<0.05$) were observed among treatments. The highest plant weight (6.6) was observed in 30 g TSP which was statistically similar with 20 g TSP (5.95) followed by 50 g TSP (5.26), control (4.15) and 40 g TSP (3.71). The lowest plant weight (2.32) was recorded in 1 Urea Super Granule followed by 20g TSP + 1 Urea Super Granule (3.3) (Table 2).

At 2nd week, no significant variation was observed among treatments. At 3rd week, significant differences ($p<0.05$) were recorded. The highest plant weight was observed in 50g TSP (17.62) and the lowest plant weight was in 1 Urea Super Granule (1.19). All other treatments showed statistically similar results (Table 2).

At 4th week, the highest plant weight (43.62) was observed in 50 g TSP followed by 40 g

TSP (32.13). The lowest plant weight (5.51) was recorded in 1 Urea Super Granule and 20 g TSP + 1 Urea Super Granule (6.89) (Table 2).

Phosphorus is the most important and often the limiting nutrient for *Azolla* growth. Its deficiency is indicated by smaller, less vigorous plants and may cause the plants to become pink to deep red and fragile and develop very long roots. Soil based medium for *Azolla* with a constant level of water and a little phosphorous would serve as a good system for healthy *Azolla* maintenance.

Table 1. Effect of different nutrient media on plant number of *Azolla pinnata*

Treatment	Initial	1st week	2nd week	3rd week	4th week
Control	20.33	91.67	132.67	236.67 ab	439.67 bc
20 g TSP	19.33	83.67	128.33	260.67 ab	467.67 bc
30 g TSP	26.33	86.33	137.67	274.33 ab	542.33 bc
40 g TSP	21.33	68	160	284.33 ab	811 b
50 g TSP	22	79.33	190.67	325a	1629.33 a
20 g TSP+1 Urea Super Granule	19.67	86.67	165.67	222.67 ab	360.33b c
1 Urea Super Granule	22.33	96	112	141 b	145 c
Level of Significance	NS	NS	NS	*	*

NS= Non-significant; *= In a column, values followed by the different letter (s) are significantly different from each other at 5% level of significance by Tukey's Honest Significant Difference (HSD) Test.

Total nitrogen (%) of soil at different nutrient media

Total nitrogen (%) in soil varied from 0.07 to 0.12 due to different nutrient medium level. The highest total N% of soil (0.12) was obtained from 50 g TSP and the lowest total N% (0.07) was obtained from control (Figure 1).

Total nitrogen (%) of plant at different nutrient media

Total nitrogen (%) of plant was varied from 5.96% to 9.8%. The highest N% (9.8) was obtained from 1 Urea Super Granule and the lowest N% (5.96) was obtained from 50 g TSP. N% was gradually decreased from control to 50 g TSP and then gradually decreased (Figure 2).

Table 2. Effect of different nutrient media on fresh weight (g) of *Azolla*

Treatment	1st week	2nd week	3rd week	4th week
Control	4.15 abc	5.84	16.96 ab	19.89 abc
20 g TSP	5.95 a	8.48	14.07 ab	15.19bc
30 g TSP	6.6 a	7.90	15.06 ab	15.16bc
40 g TSP	3.71 abc	6.10	13.56 ab	32.13ab
50 g TSP	5.26 ab	8.87	17.62 a	43.62 a
20 g TSP+1 Urea Super Granule	3.30 bc	6.47	8.04 ab	6.89 c
1 Urea Super Granule	2.32 c	6.06	7.74 b	5.51 c
Level of Significance	*	NS	*	*

NS= non-significant; *= In a column, values followed by the different letter (s) are significantly different from each other at 5% level of significance by Tukey's Honest Significant Difference (HSD) Test.

Better growth performance was occurred with high readily available nitrite, nitrate and with the presence of phosphate. In this experiment, the highest growth in respect of plant number and plant biomass of *Azolla pinnata* was observed in 50 g TSP followed by 40 g TSP (Table 1 and 2). Similar observation was reported by Dawar et al. (2001) and recommend that, being a nitrogen fixing plant *Azolla* requires water and a little phosphorous for its growth. Phosphorus would help in growing vigorous and healthy *Azolla* and also would help in developing long root growth. Rice-field conditions are suitable for its multiplication, due to water logging in the

fields and with supply of a little phosphorous it grows very well along with the rice as a dual crop. In this study, initial increase of TSP (upto 30 g TSP) in water and soil media showed statistical similar results with control. It may be because upto 30g TSP might not supplied enough phosphorous for optimal plant growth. Costa et al. (1999) observed that, if there is enough phosphorus in the aquatic environment, *Azolla* will be able to grow without the need to provide combined nitrogen such as NH_4NO_3.

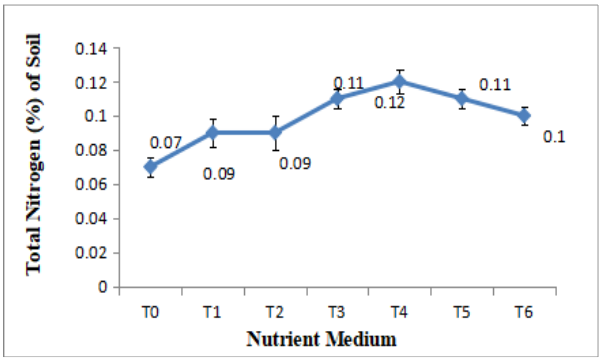

Figure 1. Total nitrogen (%) of soil at different nutrient media

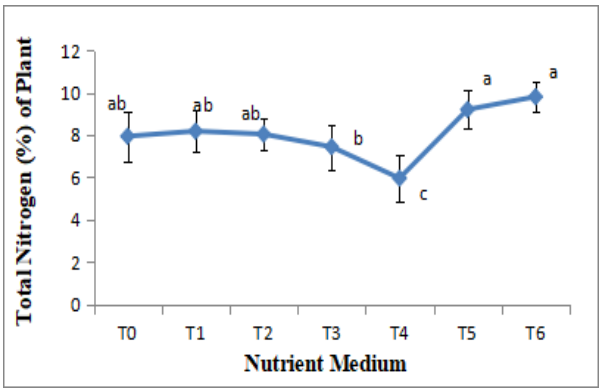

Figure 2. Total nitrogen (%) of plant at different nutrient media

They also said that, optimum growth of *Azolla* species responds to different concentrations of phosphorus.

From Table 1 and 2 of this study, the lowest plant growth was observed in 1 Urea Super Granule followed by 20 g TSP +1 Urea Super Granule because the extra added N affects the growth of *Azolla* and N_2 fixation rates. The excess N_2 was uptaken by plants and may be became toxic to plant growth resulting minimum number and biomass of plants. This result was found similar with Singh et al. (1992). They observed that the level of nitrogen affects the growth of *Azolla* and nitrogen fixation rates because nitrogen was assimilated by *Azolla* from the atmosphere and water respectively. *Azolla* growth rate was higher at a nitrate level of 5 mM than in a nitrogen free medium, but higher levels of nitrate reduced growth.

Nordiah et al. (2012) found that the growth of *A. pinnata* in each treatment of waste water, drain water, paddy field water and distilled water varied with respect to the number of plants produced, morphology and color as the treatments differed in nutrient content especially phosphorous. They also found that combination of more than one nutrient or multiple nutrient contents explained the observed increased in biomass and plant weight of *A. pinnata* grown in the different water sources. The increased in number of plants did not necessarily cause high increased in biomass, since it is influenced by the plant's size. It was also observed when less fragmentation occurred, the plants were bigger in size and formed thick mat on water's surface and the resultant biomass produced was higher.

In this experiment, the highest nitrogen (%) in soil was recorded from 50 g TSP and the lowest was in control sample (Figure 1). In T_5 and T_6 extra nitrogen was added and from control to T_4, no extra nitrogen was added. That means nitrogen level was increased by plant fixation from control to T_4. In the contrary, total nitrogen (%) in plant was highest in 1 Urea Super Granule and the lowest was obtained from 50 g TSP (Figure 2). This is because *Azolla* itself a nitrogen fixing plant. When 1 Urea Super Granule was the treatment, then excess nitrogen was added to the media and plant uptake excess nitrogen from media which might be harmful for optimal plant growth. That's why total nitrogen (%) was maximum in plant in case of 1 Super Granule Urea treatment but show minimum plant biomass. Similar observations were found by Sadeghi et al., (2012) and recorded that an increase in nitrate concentration in soil might result in a low coverage of *Azolla*. Maejima et al. (2002) also reported that low levels of combined nitrogen in the medium increased the specific growth rate of *Azolla pinnata* strains whereas rapidly increased nitrogen in soil decreased the plant

growth rate. Because by presence of NH_4^+, nitrogen uptake was exceeded and nitrogen fixation was gotten low. If nitrogen was increased rapidly about 18% growth of *Azolla* could be reduced. Korner and Vermaat (1998) demonstrated that, excess nitrogen uptake by plants was the cause of reduction of *Azolla pinnata* growth rate. They also suggested that, if the maximum ammonium removal efficiency was 99.7% and the ammonium removal rate coefficient was 51.9% then specific higher plant growth rate was obtained. Sah et al. (1989) observed that when nitrogen was available, plants were preferred to absorb nitrogen in NH_4^+ rather than in NO_3^-. In the presence of NH_4^+ or NO_3^- enabled *Azolla* in nitrogen uptake by exceeding the corresponding decreased in nitrogen fixation, resulting in increased of nitrogen concentration in plant and plant growth decreased.

CONCLUSION

Plant number as well as biomass of plant was increased with the increase of time in all treatments except in 20 g TSP +1 Urea Super Granule and 1 Urea Super Granule though no significant variation was observed initially. The highest plant number (1629.33) and plant weight (43.62) was recorded in 50 g TSP and the lowest plant number (145) and plant weight (5.51) was obtained in 1 Urea Super Granule at 4^{th} week. 50 g TSP is recommended for optimal growth of *Azolla pinnata* in 20 L water. Field study should be conducted to better understand the growth performance of *Azolla* in different nutrient media.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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