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The Potential Capabilities of Two Species of *Azolla* as a Bioaccumulator in Summer

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ABSTRACT

Now a days, worldwide excessive use of chemical fertilizer, can have many undesirable effects on human health and the environment, which can be reduced or eliminated by adopting new agricultural practices such as use of organic inputs, namely manure, biofertilizer. On the other hand, water eutrophication has been a challenging global environmental problem recently. Plants can be grown in eutrophicated water, and after consumption from eutrophicated water, the harvested biomass of this plant can play important role as a useful green manure, rich in N P K. The use of *Azolla*, a N fixing water-fern can be a novel idea in this regard. *Azolla* spp. have a unique capability to accumulate more P, K within its body than its requirement and can be considered as NPK tablets. So, the experiment was conducted in order to find out the efficient species with regard to high biomass production with high accumulation of P, K, and tolerating higher concentration of media-P in summer. Light intensity and concentrations of media-P have a remarkable influence on the productivity and NPK accumulation of the species. Open area was more favorable than the partial shade for biomass production. Both the species have the capability to tolerate 60 ppm of media-P and produce their maximum biomass at only 15 ppm of media-P. *A. filiculoides* was found to produce more biomass, accumulate more tissue-P, contain more or less the same amount of N, and less K when compared with *A. pinnata.* Thus, in hot climate, *A. filiculoides* can be considered better accumulator of Phosphorus and *A.pinnata* can be considered better accumulator of Potassium being an effective scavenger of P, K.

Keywords *Azolla* spp., Biofertilizer, Green manure, Environmental factors, Waste water management.

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INTRODUCTION

Rice cultivation and *Azolla* spp.

Rice is an enormously important staple food for a large number of the world's human population. In order to meet the needs of the populations, attention must be given to the productivity of Rice on an ecologically sustainable basis. N fertilization is a key input in increasing rice production. However, continuous and extensive use of N- and P-fertilizers would not be economically and ecologically sustainable because of the higher cost of those fertilizers, limited stocks of the rock phosphate (Li *et al.* 2014) lower use-efficiency of applied P-fertilizers by crop plants, and excessive application of N-and P-fertilizers lead to the environmental damage (Ahmed *et al.* 2017, Elser 2012, Tariq Bashir *et al.* 2013, Sood *et al.* 2012, Thomas and Singh 2019).

Excessive nitrogen fertilizer application also lead to pest problems by increasing the birth rate, longevity and overall fitness of certain pests (Jahn *et al.* 2005). Therefore, the need of the day is to improve N, P-use efficiency of crop plants, and to explore the possibility of utilizing the naturally available N, P in the soil. In recent years, many countries have been encouraged to use biofertilizers as a substitute for chemical fertilizers (Guo *et al.* 2021). It is known from the literature, *Azolla* spp. has been used for thousands of years as a "green" nitrogen fertilizer, to increase the rice production.

Importances of *Azolla* sp.

Azolla sp. (an aquatic pteridophyte) has been of immense importance for years, to agriculturists and botanists, because of its symbiotic relationship with *Anabaena azollae,* providing an excellent source of organic matter and nitrogen for crops, being used as a feed to a variety of farm animals and also being a suppressor of weeds, ammonia volatilization, water evaporation. It is a promising candidate to improve water quality of polluted water and has long been used all the world over for its diversified applications (Lumpkin and Plucknett 1980, Ahluwalia *et al.* 2002). However, Fjerdingstad (1976) claimed that the symbiont is actually an ecoform of *Anabaena variabilis* and should therefore be called *A. variabilis* status *azollae.* Use of DNA-DNA hybridization, DNA amplification fingerprinting (Eskew *et al.* 1993) and monoclonal antibodies show that the cyanobacterial partner is not uniform throughout the genus *Azolla* and seems promising for strain identification (Plazinski *et al.* 1988). The exact period when *Azolla* cultivation began is not found in the literature but it is reported to date back to the 11th century in Vietnam.

The genus *Azolla*

Lamarck in 1783 firstly established the genus *Azolla* (Svenson 1944) and it was included under the family Salviniaceae. The familly Salviniaceae was supported by many eminent systematists (Sadebeck 1902, Benson 1957, Ashton and Walmsley 1984). The first to consider *Azolla* in the monotypic family *Azollaceae* was Wettstein in 1903. But only with Reed in (1954), such proposal had begun to be accepted and followed (Ashton and Walmsley 1984). Subsequently, it is separated into a monotypic family Azollaceae (Konar and Kapoor 1974, Tan *et al.* 1986, Saunders and Flower 1993).

Azolla sp. has been used as an excellent bio fertilizer in rice field, for a long time. Under ideal conditions, it grows exponentially, doubling its biomass every 2 to 5 days (Kathirvelan *et al.* 2015). The application of *Azolla* has a tremendous potential to improve soil health and boost yield sustainability (Akhtar *et al.* 2020).

***Azolla* sp. and waste water**

Most of the waterbodies are getting enriched with urban and rural run-off containing domestic wastes, wastes from agricultural practices and industries, now-a-days. Consequently, the increasing severity of water eutrophication has been brought to the attention of both the governments and the public in recent years. Conventional methods of wastewater treatment are not only cost prohibitive, but also require regular and expensive maintenance and adequate technical manpower. Untreated sewage may have serious impacts on the quality of an environment and on the health of people. The facilities to treat waste water are not adequate in India. Domestic sewage is very likely to contain detergents, most of the detergents and washing powders contain phosphates. Phosphates may be one of the major factors of water eutrophication. *Azolla* has a unique ability to thrive well in partially treated domestic wastewater and in effluents from wastewater stabilization ponds despite the high ammonium content of the medium. This confirms the use as biofilter for the removal of both phosphorus and nitrogen (Golzary *et al.* 2018).

The objective of this experiment, was to compare the productivity of two species of *Azolla* namely *A. filiculoides* and *A. pinnata* at different concentrations of media-P, under two different light intensities, namely open area and partial shade conditions, in summer season, when temperature ranged between 23.07 ºC to 35.65 ºC and the mean average relative humidity being 61.59%, with no rainfall. The solar radiation was found to be 532.98µ mol $m^{-2}s^{-1}$ in open area, 252.62 µ mol $m^{-2}s^{-1}$ in partial shade during the season) and to select the particular species, capable of fixing more N, accumulating higher amount of P and K, tolerating low media-P and high media-P, so that they can be applied to the field regionally accordingly.

MATERIALS AND METHODS

Plant material

Two species of *Azolla* such as *A filiculoides, A. pinnata* were taken for the present experiment. The genus possesses intrinsic interest in that its members are capable of assimilating atmospheric nitrogen with the help of a symbiont within the cavities of their leaves.

Experimental procedure

Prior to inoculation of plant materials collected from the concrete tanks two species of *Azolla* were allowed to grow in distilled water for 5-6 days for starvation. Then 2 g of two species of *Azolla* each was washed several times in tap water to eliminate the disinfectant and other soil particles, if any. The plants were blotted off carefully to remove superficial water by keeping on absorbent paper and then weighed carefully and accurately by an electrically operated balance. Then 2 g of *Azolla filiculoides* and *A. pinnata* each was inoculated separately in different plastic vessels having the diameter of 15 cm, containing 250 ml of IRRI's nitrogen free (Watanabe *et al.* 1977) media alongwith a control set.

Open area condition: The experiment was conducted in open sunlight for the purpose and kept under a two meter high transparent polythene sheet to avoid rainfall.

Partial shade condition: The plants were cultured under a 2 m high colored polythene sheets to mimic the environment of tree canopy, so that the plants can receive approximately 50% of the sunlight along with normal temperature and humidity.

The experiment was performed using original IRRI's medium having 20 ppm of phosphorus concentration. Different other concentrations of phosphorus such as 5 ppm, 10 ppm, 15 ppm and 60 ppm, were prepared and two species of *Azolla* were allowed to grow in it to find out the different parameters after 10 days of incubation, both in open area and partial shade conditions.

Preparation of IRRI's growth medium for the culture of *Azolla*

The IRRI's medium has been introduced by Watanabe *et al.* (1977). For the preparation of IRRI's medium, the following reagents are used.

(a) NaH_2PO_4 - 20 ppm
(b) K_2SO_4 - 40 ppm
(c) $CaCl_2$ - 40 ppm
(d) $MgSO_4$ - 40 ppm
(e) $FeSO_4$, $7H_2O$ - 0.5 ppm
(f) EDTA - 26.1 g
(g) Trace element component includes the following elements:
(i) Mn - 0.5 ppm
(ii) Mo - 0.15ppm
(iii) B - 20 ppm
(iv) Zn - 0.01 ppm
(v) Cu - 0.01 ppm
(vi) Co - 0.01 ppm

To prepare 1000 ml of IRRI's medium 1 ml of each reagent but 0.1 ml of Fe EDTA was used and the final volume was made with distilled water. The experiment was performed in the net house of the Department of Botany, The University of Burdwan, Paschim Banga, India. Burdwan is a district town of West Bengal and is situated at $23^0$19' N latitude and $87^0$54' E longitude . The seasonal conditions during the experimental procedure is presented in Table 1.

Productivity: The primary productivity (Fresh/dry) was measured according to the following formula by harvest method (Misra 1974). Productivity = W2- W1 /t2 – t1

Table 1. Meteorological data(MD) recorded by district seed farm, Kalna Road, Burdwan, Paschim Banga, India. OA Open area, PS Partial shade.

Seasons	Atmospheric temperature (^{0}C)			Solar radiation (µmol $m^{-2}s^{-1}$)	Relative humidity			Total rainfall (mm)
	Mean maximum	Mean minimum	Mean average		Mean maximum	Mean minimum	Mean average	
Mar-Apr	35.65	23.07	29.36	532.98 (O.A.) 252.62 (P.S.)	91	32.18	61.59	0

Where W2 = dry wt at time t2,
W1 = dry wt at time t1.

Estimation of chlorophyll was done following (Arnon 1949). Heterocyst frequency was done by counting the number of heterocyst per number of vegetative cells counted × 100. Phosphorus and Potassium in plant tissue was estimated following ascorbic acid method (Murphy and Riley 1962) and Yoshida *et al.* (1976) respectively. Vogel *et. al.* (1961) was followed to estimate total nitrogen in plant tissue.

RESULTS AND DISCUSSION

A study was conducted on the growth and accumulation of NPK by two species of *Azolla* at different concentrations of media-P, under two light intensities in summer season. A general trend of increasing biomass yield was observed with increasing media-P concentration upto a certain limit. In summer, 15 ppm of media-P was preferred, as an optimum concentration for maximum production of biomass, under two light intensities by both the species and with no such a remarkable deviation in amount of biomass at 60 ppm of media-P.

Table 2. Changes in productivity on the basis of dry weight (g m^{-2} d^{-1}) of *A. filiculoides* under two different light intensities, at different media-P concentrations during Summer season (OA = Open area, PS = Partial shade) $p < 0.05$.

Season	Light intensities	0	5	10	15	20	60
		Concentrations of media-P (ppm)					
Summer	OA	0.926	1.542	1.615	1.841	1.621	1.446
	PS	0.836	1.435	1.548	1.615	1.508	1.344

Table 3. Changes in productivity on the basis of dry weight (g m^{-2} d^{-1}) of *A. pinnata* under two different light intensities, at different media-P concentrations during summer season (OA = Open area, PS = Partial shade) $p < 0.05$.

Season	Light intensities	0	5	10	15	20	60
		Concentrations of media-P (ppm)					
Summer	OA	0.949	1.451	1.514	1.610	1.587	1.457
	PS	0.864	1.322	1.389	1.485	1.463	1.338

In case of both the species, at all concentrations of media-P, it was observed that, the productivity was always more in open area than the partial shade (Tables 2 - 3). Table 2 shows that, 15 ppm was the optimum concentration for *A. filiculoides* for production of maximum biomass during summer (1.841g m^{-2} d^{-1} in open area and 1.615 g m^{-2} d^{-1} in partial shade on the basis of dry weight), even, the production of biomass at 15 ppm of media-P was more than those obtained by *A. pinnata* as its maximum biomass production in summer at the same concentration of media-P (Tables 2 - 3). At 0 ppm of media-P, *A. pinnata* was capable of producing more biomass than *A. filiculoides*, although at any other concentration of media-P, i.e. 5 ppm to 20 ppm of media-P, biomass production was more in *A. filiculoides* than *A. pinnata.* In case of *A. pinnata,* for its maximum biomass production, 15 ppm of media-P was found to be the optimum concentration (1.610 g m^{-2} d^{-1} in open area, 1.485 g m^{-2} d^{-1} in partial shade condition on the basis of dry weight) (Table 3).

It was observed that, at 60 ppm of media-P, the productivity of *A. filiculoides* and *A. pinnata* (1.446 g m^{-2} d^{-1}, 1.457 g m^{-2} d^{-1} in open area and 1.344 g m^{-2} d^{-1}, 1.338 g m^{-2} d^{-1} in partial shade respectively) remained more or less same.

Chlorophyll content was found to be more in partial shade condition than the open area at all concentrations of media-P, in case of both the species (Fig. 1) . Except 0 ppm of media-P only, *A. filiculoides* was found to contain more chlorophyll than *A. pinnata.* 15 ppm of media-P was found to be the optimum concentration for chlorophyll production in case of both the species under both open area and partial shade conditions. However, *A. filiculoides* tended to produce

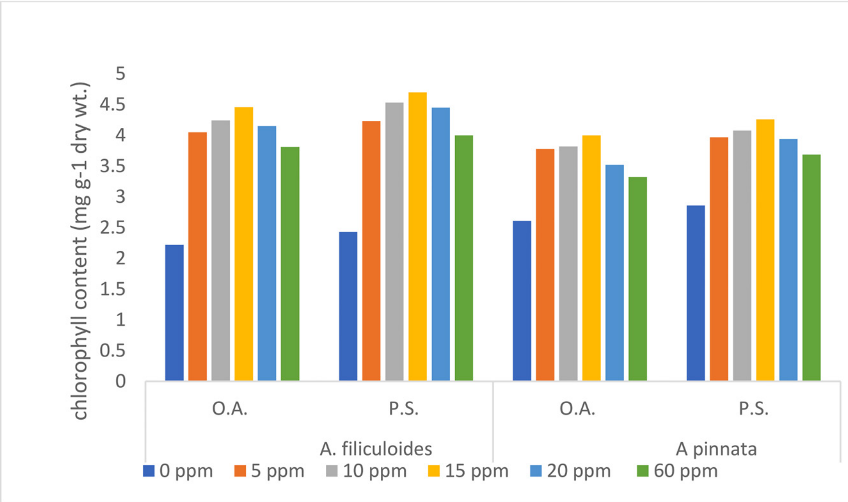

Fig. 1. Chlorophyll content (mg g[-1] dry wt) of two species of *Azolla* at different media P concentrations under two light intensities, during Summer Season (OA = Open area; PS = Partial shade).

the chlorophyll at an almost steady state from 20 ppm of media-P to 60 ppm of media-P in summer under both light intensities, but, the amount was found to be gradually decreased in case of *A. pinnata*, with the increase in concentration of media-P, from 20 ppm to 60 ppm of media-P under both the light intensities. Report says that the phosphorus can significantly affect the pigment formation. Effect of phosphorus on the growth and pigments accumulations was studied. In case of *Dunaliella salina*, increasing NaH_2PO_4 concentration can improve the biomass, the total chlorophyll and beta-carotene content (Wongsnansilp *et al.* 2016).

The P uptake in *Azolla pinnata* in mineral medium increased linearly with increasing phosphate

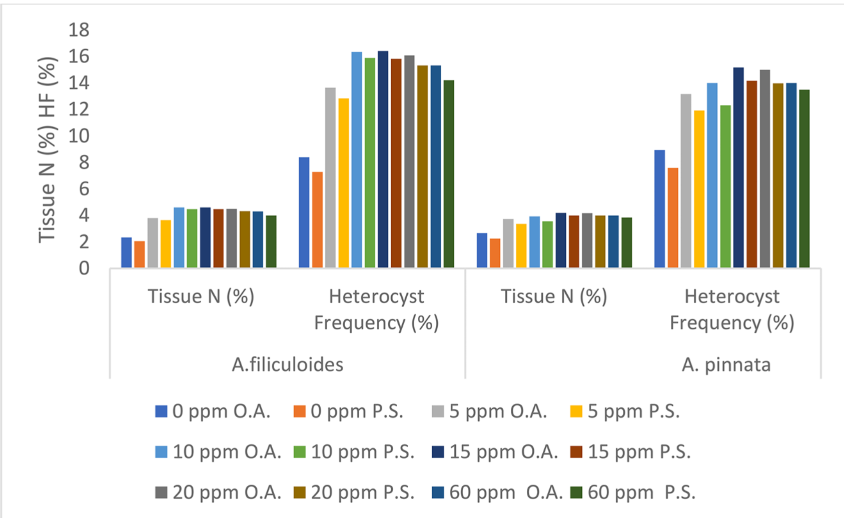

Fig. 2. Effect of different phosphorus concentrations of media-P on N content (%) of *Azolla* species and HF (%) of the symbiont within, under two light intensities, during Summer Season (HF = Heterocyst frequency (OA = Open area; PS = Partial shade).

Table 4. P and K -accumulation (%) by two species of *Azolla* at different media-P concentrations under two light intensities in Summer (OA = Open area, PS = Partial shade) $p < 0.05$.

Concentrations of media-P (ppm)	Light intensities	Name of the species			
		A. filiculoides		*A. pinnata*	
		P	K	P	K
0	OA	0.38	2.38	0.20	1.91
	PS	0.30	2.56	0.17	2.12
5	OA	0.89	3.61	0.59	3.97
	PS	0.74	4.20	0.47	4.26
10	OA	1.68	3.86	0.88	4.01
	PS	1.49	4.27	0.61	4.38
15	OA	1.68	3.80	1.45	4.18
	PS	1.48	4.22	0.82	4.58
20	OA	1.68	3.61	1.45	4.30
	PS	1.49	4.06	1.28	4.62
60	OA	1.68	3.34	1.45	4.09
	PS	1.48	3.97	1.28	4.44

doses and optimum growth and chlorophyll content of fern were recorded at 1.2 mM of K_2HPO_4. The higher doses of phosphate reduced growth and chlorophyll (Subudhi and Singh 1979).

Tissue-N content and the heterocyst frequency was found to be more or less proportional, open area was more suitable for fixation of tissue-N, than the partial shade during summer season. *A. pinnata,* was found to be more effective in N fixation only at 0 ppm of media-P, but, at all concentrations of media-P i,e at 5 to 60 ppm of media-P, either *A. filiculoides* was found to fix a little bit more N than *A. pinnata* or both the species were found to be more or less same efficient in N fixation (Fig. 2).

Again, the nitrogen fixing activity was more in open area than the partial shade in all the cases. The N-fixation activity of *Azolla -Anabaena* depends mainly on the number of heterocysts in the leaves, reported by Maejima *et al.* (2003). Interestingly, they have also noticed that the *Azolla* sp. when grown at high light intensity, if transferred to a low light intensity, the number of cyanobionts and and heterocysts gradually decreased in the mature region (Table 4).

Phosphorus is an important nutrient to yield a successful and rapid growth of these species (El Katony *et al*. 1996). The effect of this important nutrient on the growth of *Azolla* has also been confirmed in the Anzali wetland (Sadeghi *et al.* 2013). *A. filiculoides* has been proved to contain more nitrogen than *A. pinnata* (Brouwer *et al.* 2018).

Costa *et al.* (1999) reported that, if there is enough phosphorus in the aquatic environment, *Azolla* would be able to grow without the need to provide combined nitrogen such as NH_4NO_3.

P is one of the most important macro elements that significantly affect plant growth and metabolism. In the present study, it is evident, that 15 ppm of media-P enhanced the growth of both the species in summer. During summer season, in open area condition, 15 ppm of media-P was found to be the optimum concentration for maximum accumulation of tissue-P also, for both the species, but in partial shade condition 15 ppm of media-P was optimum for *A. filiculoides* and 20 ppm for *A. pinnata.*

However, the maximum amount of tissue-P, found to be accumulated by *A. filiculoides* at its optimum concentration of media-P was more than that of *A. pinnata* at its optimum concentration indicating more capability of luxury consumption of phosphorus of *A. filiculoides* in comparison to that of *A. pinnata.* The result indicates that, to achieve the maximum amount of tissue-P, both the species have the ability to tolerate and to utilize the higher concentration of media-P in summer, 15 ppm of media-P was optimum in case of *A. filiculoides*, whereas, 15-20 ppm of media-P was optimum in case of *A. pinnata* to obtain more or less the same amount of tissue-P, despite both having the ability to tolerate 60 ppm of media-P for accumulation of Phosphorus.

From the statistically highly significant data, it can be said, that *A. filiculoides* has the more capability to accumulate more Tissue-P (1.68% in open area, 1.49% in partial shade condition) than *A. pinnata* (1.45% in open area, 1.28% in partial shade condition in summer. *A. pinnata* had the capacity to accumulate a good quantity of tissue-P, within its body at higher concentration of media-P, but in open area only.

However, Hossain *et al.* (2021) reported upto 15 ppm of supplemental P, the tissue-P increased with the increase in supply of media-P in case of *A. pinnata*. Supplementation affected tissue P content, and there was also a positive correlation, it has also been evidenced by Oyange *et al.* (2019).

Regarding potassium accumulation, the result indicated that *A. pinnata* usually tended to accumulate more tissue-K (4.30% in open area and 4.62% in partial shade) than *A. filiculoides* and was found to utilize 20 ppm of media-P for maximum accumulation of Potassium in summer. On the other hand, *A. filiculoides* tended to utilize lower concentration of media-P, i.e. 10 ppm of media-P, for its maximum tissue-K (3.86% in open area and 4.27% in partial shade condition) in summer (Table 4).

Tissue potassium accumulation was usually higher in *A. pinnata* than *A. filiculoides* at all concentrations of media-P, except 0 ppm of media-P. 10 and 20 ppm of media-P was the optimum in case of *A. filiculoides, A. pinnata* respectively, under both the light intensities, for maximum accumulation of K during summer season. From the satistically highly significant data, it can be concluded, light intensity, species variation have a profound effect on the capability of biomass production, as well as accumulation of tissue-NPK, by spp. of *Azolla.*

Light intensity was found to have an effective role in increasing biomass accumulation and nitrogen fixation of *Anabaena* sp. culture (El-Bahbohy *et al.* 2014). A higher light intensity and high humidity resulted in higher growth rates of *A. pinnata* has been reported by da Silva *et al.* (2022) also.

Humidity has not received serious attention, even though many studies have stated the importance of a high humidity for the growth of the *Azolla* species. Wagner (1997), Kösesakal and Yildiz (2019), Goala *et al*. (2021) reported humidity values.

The results indicate the more efficiency in production of biomass of *A. filiculoides* in the utilization of 15 ppm of media-P during the season than *A. pinnata* and the tolerability of both the species to 60 ppm of media-P satisfactorily for its biomass production.

Both the species can be considered more or less suitable for production of biomass and accumulation of Tissue Nitrogen in summer. *A. filiculoides* can be utilized as better accumulator of tissue-P even at 60 ppm of media-P and *A. pinnata* can be utilzed as better accumulator of tissue-K even at 20 ppm of media-P than the other. Open area was more favorable than the partial shade condition for biomass production N fixation and P accumulation, but partial shade was more favorable for K accumulation than the open area condition. Thus two species of *Azolla* can be applied in the field according to the requirement of the field.

On behalf of the author, the corresponding author states that there is no conflict of interest.

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