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COMPARATIVE BIOACCUMULATING CAPABILITIES OF N P K BY THREE SPECIES OF *AZOLLA* TO COMBAT THE EUTROPHICATION IN SUMMER

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ABSTRACT

Water eutrophication is a global environmental concern. *Azolla*, a N-fixing water fern, can be cultivated in eutrophicated water and the harvested biomass can be used as valuable green manure, as it has a remarkable ability to collect excess P and K in its body than they require, and can be used as NPK tablets in the crop field in order to maintain the sustainability.

So, the experiment was conducted in order to find out the efficient species with high biomass with high N, P, K, content, growing in different levels of media- P. Open area was more favourable than the partial shade for biomass production and tissue- N, P accumulation. All the species were found to have their capability to tolerate 60 ppm of media- P. With the exception of *A. filiculoides*, the optimum concentration of media- P for biomass production was found to be 20 ppm. But, for *A. filiculoides*, the optimum concentration was 15 ppm. However, at 15 ppm of media- P, it accumulated the highest amount of P, though the least amount of tissue-K, and the moderate tissue-N, in comparison to other species.

In contrast, the highest amount of tissue-N, tissue-K was found to be accumulated by *A. microphylla* at 20 ppm of media-P generated its highest amount of biomass.

So, in tropical climate, *A. filiculoides* can be considered as a low media-P utilizing species with better accumulation of Phosphorus and *A. microphylla* as a high media-P utilizing species with better accumulation of tissue-N, P, K, thus both being an effective bio-accumulator of P, K. Depending on the needs of the area, the suitable *Azolla* species can be applied to the crop field as green manure after being harvested from the eutrophicated water.

***Keywords*:** *Azolla* spp, Biofertilizer, green manure, Environmental Factors, Waste Water Management.

Introduction

Azolla sp and waste water

Most of the water-bodies 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. India lacks enough waste water treatment facilities. Domestic sewage is most likely to contain detergents, as most detergents and washing powders contain phosphates. Phosphates may be one of the major causes 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 phosphorous and nitrogen (Golzary *et al.* 2018).

Rice cultivation and *Azolla* spp.

On the other hand, a large proportion of the global population considers rice to be a very important staple food. Attention needs to be paid to rice productivity on an environmentally sustainable basis in order to meet the population needs. A crucial component in raising rice yield is nitrate fertilization. Nevertheless, because of their higher cost and the finite supply of rock phosphate, frequent and continuous application of N and P fertilizers would not be environmentally or economically feasible (Liu *et al.,* 2010, Jwaideh *et al.,* 2022). Lower use-efficiency of applied P-fertilizers by crop plants, and excessive application of N-& P-fertilizers lead to the environmental damage (Ahmed *et al.,* 2017, Elser, 2012, Tariq Bashir *et al.,* 2013, Sood *et al.,* 2012, Thomas & 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.

Importance of *Azolla* sp.

Azolla sp (an aquatic pteridophyte) has long been important to agriculturists and botanists because of its symbiotic relationship with *Anabaena azollae*, which provides an excellent source of organic matter and nitrogen for crops, is fed to a variety of farm animals, and suppresses weeds, ammonia volatilization, and 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). 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 11^{th} 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, Saunders and Fowlers, 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).

This experiment compared the productivity of three species of *Azolla* namely *A. filiculoides,* the Hybrid species of *Azolla* and *A. pinnata* at different concentrations of media-P, under two different light intensities (open area and partial shade) in summer season. Temperature ranged between 23.07 ºC to 35.65 ºC with an average relative humidity of 61.59%, and 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. The purpose of the experiment was to determine which specific species could be used locally in the field based on its ability to fix more N, accumulate more P and K, and tolerate both high and low media-P levels.

Materials and Methods

Plant Material

Three species of *Azolla* such as *A filiculoides,* the Hybrid species of *Azolla, A. microphylla* 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,* the Hybrid species of *Azolla* and *A. microphylla* 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-metre-high transparent polythene sheet to avoid rainfall.

Partial shade condition: The plants were cultured under a 2 m high coloured 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. 23019' N latitude and 87054' E longitude. The Seasonal conditions during the experimental procedure is presented in Table1

Productivity: The primary productivity (on the basis of dry wt.) was measured according to the following formula by harvest method (Misra, 1974).

$$\text{Productivity} = W_2 - W_1 / t_2 - t_1$$

Where W_2 = dry wt at time t_2,

W_1 = dry wt. at time t_1.

Phosphorus & 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.

Statistical Calculations: Analysis of Variance and co-variances were estimated followed by Singh and Chaudhary (1995) model.

Results and Discussion

The development and accumulation of NPK in three species of *Azolla* at two light intensities and different media-P concentrations over the summer was investigated. The meteorological data has been presented in Table 1.

Table 1 : Meteorological Data (MD) recorded by District Seed Farm, Kalna Road, Burdwan, Paschim Banga, India. O.A. Open Area, P.S. 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

Table 2 : Changes in productivity on the basis of dry weight (g m^{-2} d^{-1}) of *A. filiculoides*, ahned Hybrid species of *Azolla* and *A. microphylla* under two different light intensities, at different media- P concentrations during different seasons. (O.A. = Open area; P.S. = P.S.) $p < 0.05$

Name of the Species	Light intensities	Concentrations of media-P (ppm)					
		0	5	10	15	20	60
A. filiculoides	O.A.	0.926	1.542	1.615	1.841	1.621	1.446
	P.S.	0.836	1.435	1.548	1.615	1.508	1.344
Hybrid species of *Azolla*	O.A.	0.824	1.559	1.649	1.740	1.813	1.661
	P.S.	0.757	1.435	1.542	1.615	1.672	1.604
A. microphylla	O.A.	1.107	1.892	2.005	2.152	2.169	2.124
	P.S.	1.067	1.751	1.858	2.022	2.033	1.971

A general trend of increasing biomass was observed with increasing media-P concentration up to a certain point. *A. filiculoides* had a preference for 15 ppm of media-P for its maximum biomass (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) during summer, whereas the other two species were found to utilize 20 ppm of media-P for maximizing their biomass under both the light intensities. *A. microphylla* was found to be the most proficient species in terms of producing the highest amount of biomass, followed by *A. filiculoides* and the hybrid species of *Azolla.* At all concentrations of media-P, every species demonstrated peak productivity in an open region (Table 2), when compared with the partial shade conditions. Not so significant divergence in production of biomass at 60 ppm of media-P was noted in case of any species.

At 60 ppm of media-P, the productivity of *A.filiculoides* the Hybrid species of *Azolla* and *A. microphylla* was 1.446 g m^{-2} d^{-1} , 1.661 g m^{-2} d^{-1} and 2.124 g m^{-2} d^{-1} in open area and 1.344 g m^{-2} d^{-1} , 1.604 g m^{-2} d^{-1} and 1.971 g m^{-2} d^{-1} in partial shade respectively.

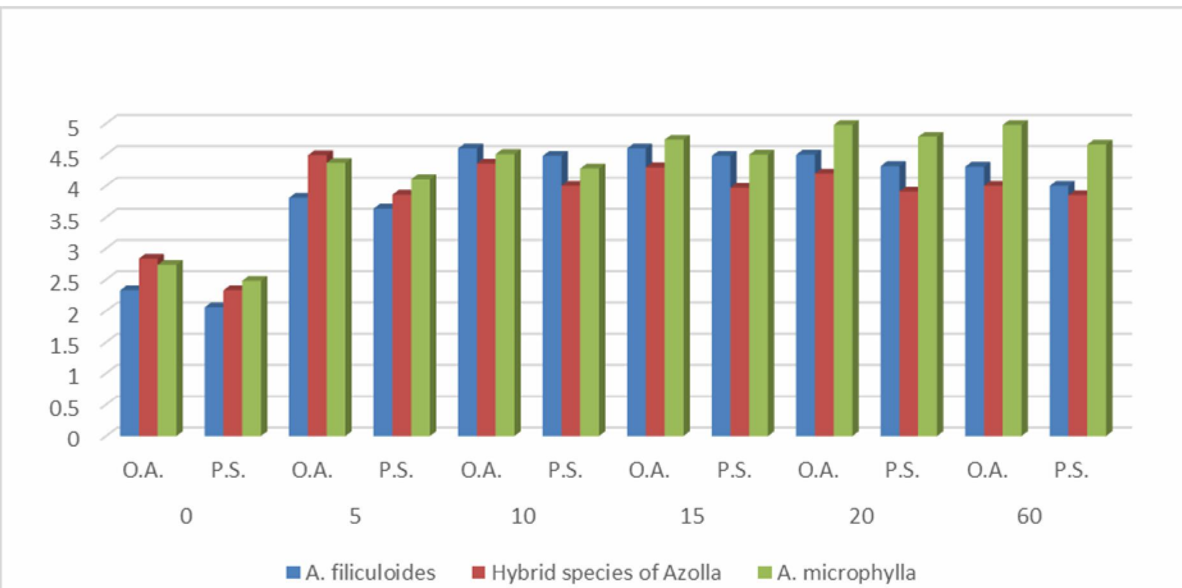

Fig. 1 : Tissue-N content (%) of different species of *Azolla* at different media-P concentrations (ppm) in open area (O.A.) and Partial Shade (P.S.) during Summer. $p < 0.05$.

In Summer, open area worked better for fixation of tissue-N, than the partial shade In an open area, the Hybrid species of *Azolla*, was found to be the most effective in fixing N (2.84%) at 0 ppm of media-P, followed by *A. microphylla* (2.74%) and *A.filiculoides* (2.33%). However, in a partially shaded environment, the highest amount of tissue-N was 2.48% in *A. microphylla* followed by the Hybrid species of *Azolla* (2.33%) and *A.filiculoides* (2.06%). The similar pattern was also observed when the media-P concentration was 5 ppm. However, at 10 ppm of media-P, *A.filiculoides* was found to have the highest accumulation of tissue-N (4.60% in open area, 4.48% in partial shade condition) followed by *A. microphylla* (4.51% in open area, 4.28% in partial shade condition) the Hybrid species of *Azolla* (4.36% in open area, 4.00% in partial shade condition). At 15 ppm to 60 ppm of media-P, *A. microphylla* was found to contain the highest amount of tissue-N, followed by *A.filiculoides* and the Hybrid species of *Azolla*. The largest amount of tissue-N was found to be recorded at 20 ppm of media-P by *A. microphylla* (4.98% in open area, 4.79% in partial shade condition), but in case of other two species it was 15 ppm of media-P. The maximum amount of tissue-N of *A.filiculoides* and the Hybrid species of *Azolla* was recorded to be 4.60%, 4.49% in open area, 4.48%, 4.00% in partial shade condition respectively (Fig. 1).

Phosphorus is an important nutrient to yield a successful and rapid growth of these species (El Katony *et al.*, 1996). Increasing NaH_2PO_4 concentration can improve the biomass, the total chlorophyll and beta-carotene content (Wongsnansilp *et al*., 2016)

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.

Table 3 : P-accumulation (%) by different species of *Azolla* at different media-P concentrations in open area during Summer $p < 0.05$

Concentrations of media-P (ppm)	Name of the species		
	A. filiculoides	Hybrid	*A. microphylla*
0	0.38	0.30	0.26
5	0.89	0.90	0.81
10	1.68	1.32	1.07
15	1.68	1.51	1.36
20	1.68	1.51	1.66
60	1.68	1.51	1.70

Tabe 3a: P-accumulation (%) vs different species of *Azolla* at different media-P concentrations (F values)

Concentrations of media-P (ppm)	**Name of the species**		
	A. filiculoides	**Hybrid**	***A. microphylla***
0	0.38	0.30	0.86
5	0.89	0.90	1.81
10	0.78	1.32	1.77
15	0.71	1.50	1.39
20	0.78	1.51	1.69
60	0.88	1.55	1.77

Table 4 P-accumulation (%) by different species of *Azolla* at different media-P concentrations in partial shade condition during Summer. $p < 0.05$.

Concentrations of media-P (ppm)	**Name of the species**		
	A. filiculoides	**Hybrid**	***A. microphylla***
0	0.30	0.25	0.22
5	0.74	0.78	0.74
10	1.49	1.12	0.86
15	1.48	1.32	1.17
20	1.49	1.43	1.43
60	1.48	1.43	1.58

Table 4a: P-accumulation (%) vs different species of *Azolla* (F values).

Concentrations of media-P (ppm)	**Name of the species**		
	A. filiculoides	**Hybrid**	***A. microphylla***
0	0.0033	1.25	3.21
5	0.0071	1.78	3.70
10	0.0045	0.12	2.86
15	0.0047	1.32	1.17
20	0.0049	1.43	2.43
60	0.0048	2.43	3.58

One of the most crucial macro-elements that has a big impact on plant metabolism and growth is P. It is evident from the present study, that 15-20 ppm of media-P was enough to enhance the growth of three species during the summer season, in open area condition. Even, the ideal concentration of media-P in order to achieve the maximum accumulation of tissue-P in *A.filiculoides* was recorded to be 10 ppm of media-P, both in open area and partial shade conditions 1.68%, 1.49%, respectively. But, for Hybrid species of *Azolla*, 15 ppm of media-P was favorable for maximum accumulation of tissue-P (1.51%) in open area condition but 20 ppm of media-P under partial shade conditions, accumulating 1.43% of tissue-P. On the other hand, 60 ppm was optimum for *A. microphylla*, both in open area and patial shade condition accumulating 1.70% and 1.58% respectively (Table 3, 4).

However, the maximum amount of tissue-P, found to be accumulated by *A.microphylla* at its optimum concentration of media-P was more than that of *A.filiculoides* and the Hybrid species of *Azolla* at their optimum concentrations indicating more capability of lixury composition of phosphorous of *A. microphylla* in comparison of those two species.

A. microphylla was found to be the most capable species in accumulation of tissue phosphorus (1.70% in open area and 1.58% in partial shade condition) followed by *A. filiculoides* accumulatig Tissue-P (1.68% in open area and 1.49% in partial shade condition) and the Hybrid species of *Azolla* (1.51% in open area and 1.43% in partial shade condition) throughout the summer based on the statistically highly significant findings. *A. filiculoides* tended to utilize lower concentration of media-P, i.e. 10 ppm of media-P for its maximum accumulation of tissue-P.

However, Hossain *et al*. 2021 observed that in the instance of *A. pinnata* tissue-P increased as media-P input increased, reaching as high as 15 ppm. Additionally, Oyange *et al*. (2019) have shown a positive correlation between supplementation and tissue P concentration.

Table 5 K-accumulation (%) by different species of *Azolla* at different media-P concentrations under two light intensities during the Summer season. (O.A. = Open Area, P.S. = P.S.). $p < 0.05$

Name of the Species	Light intensities	Concentrations of media-P (ppm)					
		0	5	10	15	20	60
A. filiculoides	O.A.	2.38	3.61	3.86	3.80	3.61	3.34
	P.S.	2.51	4.20	4.27	4.22	4.06	3.97
Hybrid species of *Azolla*	O.A.	2.02	3.86	4.25	4.32	4.28	4.10
	P.S.	2.26	4.16	4.62	4.78	4.60	4.50
A. microphylla	O.A.	1.93	3.98	4.22	4.33	4.44	4.40
	P.S.	2.16	4.33	4.51	4.78	4.82	4.67

Table 5a K accumulation (%) vs different species of *Azolla* (F values)

Name of the Species	Light intensities	Concentrations of media-P (ppm)					
		0	5	10	15	20	60
A. filiculoides	O.A.	0.38	1.61	1.86	2.80	3.61	4.30
	P.S.	0.51	1.20	1.27	2.22	3.06	4.90
Hybrid species of *Azolla*	O.A.	0.02	1.86	1.25	2.32	3.28	4.10
	P.S.	0.26	1.16	1.62	2.78	3.60	4.50
A. microphylla	O.A.	0.93	1.98	1.22	2.33	3.44	4.40
	P.S.	0.16	1.33	1.51	2.78	3.82	4.60

Result of potassium accumulation indicated that *A. microphylla* usually tended to accumulate more tissue-K (4.44% in open area and 4.82% in partial shade) than the Hybrid species of *Azolla* utilizing 15 ppm of media-P (4.32% in open area and 4.78% in partial shade) and *A.filiculoides* found to utilize 10 ppm of media-P (3.86% in open area and 4.27% in partial shade condition) for their maximum accumulation of Potassium in summer (Table 5).

Once again, all these data have been compared by Analysis of Covariance as followed by Singh and Chaudhary (1995). In this model the sum of square $\sum(X - \bar{X})^2$, can also be written as $\sum(X - \bar{X})$ $((X-\bar{X})$, where the latter presentation is in product form. Using two variables instead of one, say X_i and Y_i we, therefore, can have sum of products rather than sum of squares, i.e., $\sum(X-\bar{X})$ $(Y-\bar{Y})$. In the same process if there were more variables, sum of products of all can be obtained as desired.

Let us first consider in this experiment Light intensities verses Concentrations of media-P (ppm 5, 10, 15, 20 and 60) as shown in Tables.

Correction Factor = (Grand total for X_1) (Grand total for X_2) / Total number of observations. Accordingly, total sum of product, treatment sum of product, replication sum of product were calculated and put-forth in the ANCOVA table of each Character and calculated 'F-values' of respective trait and finally tabulated in the table of Components of Cavariances as shown in the table- 6 below:

Table 6 : Components of Covariances

Components	T-1 X T-2	T-1 X T-3	T-1 X T-4	T-1 X T-5	T-1 X T-6	T-1 X T-7
$\partial g_i g_j$ [OA]	0.338	1.671	1.876	2.810	3.651	4.302
$\partial g_i g_j$ [PS]	0.541	1.220	1.287	2.232	3.066	4.901
$\partial e_i e_j$ [OA]	0.012	1.896	1.235	2.332	3.278	4.101
$\partial e_i e_j$ [PS]	0.206	1.126	1.652	2.798	3.610	4.501
$\partial p_i p_j$ [OA]	0.993	1.978	1.252	2.353	3.445	4.402
$\partial p_i p_j$ [PS]	0.196	1.335	1.551	2.768	3.821	4.603

T-1 = Intensity of Light, T-2 to T-7 = Concentrations of media-P (ppm- 0, 5, 10, 15, 20 and 60 respectively).

From the statistically highly significant data, it can be concluded, light intensity, species variation have a profound effect on the production of biomass, as well as accumulation of tissue-NPK of the spp of *Azolla*.

A higher light intensity and high humidity resulted in higher growth rates of *A.pinnata*, being reported by da Silva Emelia Jesus *et al*. in the year 2022 also. Interestingly, Maejima Kazuhiro *et al*, in the year2003, 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.

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.

Conclusion

A. microphylla was the most efficient species in production of biomass at 20 ppm of media-P, followed by the Hybrid species of *Azolla* and *A.filiculoides* producing more or less equal amount of biomass at 20 ppm and 15 ppm of media-P respectively. All the three species had the ability to tolerate 60 ppm of media-P without experiencing adverse effects on their ability to produce biomass.

A. microphylla can be considered as the most suitable species for production of maximum biomass and maximum accumulation of tissue-N & K in Summer. *A.filiculoides* and *A.microphylla* can be used as a better tissue-P accumulator, in comparison to the Hybrid species of *Azolla*, at higher concentration of media-P, i.e. at 60 ppm of media-P, whereas, at lower concentration of media-P, i.e. 5ppm of media-P, all the three species were found to be more or less equally effective in P accumulation. At 20 ppm of media-P, *A. microphylla* can be considered as the best accumulator of K of all the species. But, at 15 ppm of media-P, in contrast to *A.microphylla*, the Hybrid species of *Azolla* accumulated the maximum tissue-K among the three species. On the contrary, *A.filiculoides* accumulated its maximum amount of tissue-K at 10 ppm of media-P. Open area was more favourable than the partial shade condition for biomass production N fixation and P accumulation, but partial shade was more favourable for K accumulation. Thus, in tropical countries, selected species of *Azolla* can be cultivated depending on the degree of eutrophication, under appropriate light intensities and after being harvested from the eutrophicated water, can be applied to the crop field as a green manure on the basis of the requirement of the field.

In general, Coheritability is estimated taking data from the table of Components of Covariances (in this case Table 6). But, in this experiment there were no variation of plant species or type. Only media treatments were variability. So, no Coheritability was estimated.

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