Pak. J. Bot., 39(1): 161-167, 2007.

DIAGNOSIS OF NUTRITIONAL CONSTRAINTS OF *AZOLLA* SPP. TO ENHANCE THEIR GROWTH UNDER FLOODED CONDITIONS OF SALT AFFECTED SOILS

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Abstract

A green house experiment was conducted to know the nutritional constraints of *Azolla* for enhancing its growth under flooded conditions and select the best ones for its use as biofertilizer in rice-wheat cropping system. Among the tested nutrients viz; phosphorus, iron and zinc, phosphorus was found to be the major limiting nutrient for plant growth. *A. pinnata* var. *pinnata* and hybrid *Azolla Rong Ping* gave better growth, hence can be used as biofertilizer in rice-wheat cropping system.

Introduction

Azolla is an aquatic pteridophyte that forms a regular permanent symbiosis, with a heterocyst forming nitrogen fixing, cyanobacterium, *Anabaena azollae*. It has been used traditionally as green manure for rice production in South East Asia and is considered an important biofertilizer for rice crop. The application of *Azolla* has been reported to increase rice yield by 0.4-1.5 t/ha over the control, in most of the experimental sites in China, Vietnam, India, Thailand, Philippines and USA. (Kikuchi *et al*., 1984). This association has gained attention in recent decades because of its potential use as an alternative or partial substitute to chemical nitrogen fertilizers and as feed for animals. In addition, the presence of an *Azolla* mat on the surface of the water body has been shown to significantly reduce weed development, limit evapotranspiration and reduce volatilization of applied N fertilizers (Lumpkin & Plucknett 1982).

The application of nitrogenous fertilizers has become an essential practice to increase crop yield. But the continuous use of only chemical fertilizers may inflict deleterious effects on soil organic matter reserves, essential for soil health. Therefore, global attention has been drawn to find out the alternatives and supplements to chemical nitrogenous fertilizers. The addition of bio-fertilizers and organic manure could be a priority to address this problem. The use of *Azolla* as bio-fertilizer for irrigated rice cultivation has already been found successful in many countries of the world (Lumpkin & Plucknett 1982; Mian 1993).

The benefits of enriching soil organic matter status by incorporated *Azolla* biomass has also been reported Singh & Singh (1987b); Hoque (1998), Mamun (2000). Peoples *et al*., (1995) estimated that *Azolla* can fix 22-40 kg N/ha per month, while driving 52-99% of its nitrogen from the atmosphere. It has been reported that *Azolla* grown dual with rice could fulfill the entire nitrogen requirement of rice crop through biological nitrogen fixation (BNF). Singh & Singh (1995), Singh (1998a, 1998b). Its growth rate is very high (3-5 days under optimal conditions), and its long-term use not only increased rice yield but also improved soil fertility (Ventura & Watanabe 1993). Recently Hassian *et al*.,

(2001) have shown that use of *Azolla* (incorporation of its two layers) as bio-fertilizer produced highest paddy as well as straw yield.

About 14 million acres of land are salt-affected in Pakistan. Since rice can grow under varying degree of flooding, and has shown some salt-tolerance, most of the salt-affected soils , whether reclaimed through chemical or biological means are invariably sown to rice as the first crop. These soils are usually saline sodic and due to high floodwater pH there is a significant loss of applied fertilizer –N through ammonia volatilization. (Hussain & Malik, 1983).

Recently Hassian *et al*., (2001) have shown that use of *Azolla* (incorporation of its two layers) as bio-fertilizer produced highest paddy as well as straw yield. Ali *et al*., (1998a) found that use of different biofertilizers including *Azolla*, alongwith a low input of chemical-N fertilizer, was useful for increasing rice yield, fertilizer-N use efficiency and BNF in rice, grown in flooded saline soils.

Hence considering the above said benefit of *Azolla* and local conditions of soil, studies were conducted to know the nutritional constraints viz., phosphorus, iron and zinc and alleviate them to have better growth of *Azolla* under flooded conditions of saline soils.

Materials and Methods

To diagnose nutritional constraints among *Azolla* species, 6 *Azolla* species viz. *A. filiculoides, A. caroliniana,* hybrid *Azolla* Rongping, *A. pinnata var pinnata*, *A. microphylla* and *A. pinnata* (local) were grown in plastic pots (surface area $56cm^{2)}$. The experiment was two factorial and pots were arranged in a completely randomized design. Using nutrient missing technique, with the following five treatments:

T_1 = -(P, Zn, Fe) control
T_2 = -P ,(+ Zn, +Fe)
T_3 = -Zn ,(+P, +Fe)
T_4 = -Fe ,(+P,+ Zn)
T_5 = +(P, Zn, Fe)

Soil was collected from Biosaline Research Station, Lahore (Table 1). It was mixed with distilled water at 1:5 ratios so that a water layer was formed on soil. Initial electrical conductivity (EC) of floodwater was 714 µS/cm and pH was 8.5.

Table 1. Chemical properties of the experimental soil.

EC (saturation extract)	4.87dS/m
PH (soil paste)	7.8
K (saturation extract)	0.15 meq/L
Na (saturation extract)	66.0 meq/L
Ca (saturation extract)	4.1 meq/L
Total N	40 mg/Kg
Available NH^{+}_{4}-N	11.5mg/Kg
Available NO^{-}_{3}-N	13.4mg/Kg

All the above said species of *Azolla* were inoculated @ 0.2 g fresh wt (average number of plant was two) per pot. After 4 days of *Azolla* inoculation, nutrients zinc, phosphorus and iron were added into floodwater on area basis. Zinc was applied in the form of zinc sulphate @ 2kg Zn/ha, (4.94mg $ZnSO_4.7H_2O$/ per pot 56cm^2), iron as $FeSO_4.7H_2O$@0.5kg Fe/ha (1.39 mg$FeSO_4.7H_2O$/ per pot), and phosphorus was applied in the form of superphosphate, @ 20kgP_2O_5/ha (11.2 mg P_2O_5/ per pot).

Plant number and frond size were recorded weekly, while electrical conductivity and pH of floodwater were noted twice a week. When plants reached to full cover in a pot, then *Azolla* plants were assayed for nitrogenase activity. Plants were picked and were then incubated in long glass tubes for 2 hour, 5 mL of gas sample were withdrawn in 13 mL vacutainer tubes. One ml of gas sample was taken from vacutainer and was injected by gas tight Hamilton (USA) syringe into the gas chromatograph (Gasukuro Kagyo, model 370) fitted with 0.75mx2 mm stainless steel column, packed with porapack R (80-100 mesh) and attached to a hydrogen flame ionization detector (FID). Column temperature of injection port was set at 250^{o}C. Nitrogen was used as a carrier gas at the flow rate of 30 ml per min. The peaks of acetylene and the ethylene produced by *Azolla* were recorded. The nitrogenase activity was expressed as n mole C_2H_4 produced/h/g dry weight.

When plants reached to full cover in the pots, after 30 days *Azolla* plants were harvested and fresh weight was noted. Plants were dried at 70°C and dry biomass was recorded. Analysis of variance table were constructed and least significant difference test to compare different treatments and different species were applied to assess the data statistically.

Results and Discussions

Effect of *Azolla* on flood water EC and pH: The growth of *Azolla* species affected EC of floodwater and pH differently. Electrical conductivity (Table 2) measured for different *Azolla* spp., for different treatment showed that EC was minimum (651µS/cm) for treatment –(P, Zn, Fe) and was maximum (823 µS/cm) for –Fe(+P+Zn), due to addition of phosphorus and zinc salts as nutrients in this treatment.

The pH of flood water (Table 3) for different treatment and *Azolla* spp. indicated that maximum pH (9.5) was observed for treatment –P(+Zn,Fe) and minimum 8.5 for +(P,Zn,Fe). The change in pH to lower side has been reported due to addition of superphosphate in fertilizers. A lower pH of floodwater due to *Azolla* growth has been reported by Ali *et al* (1995). This suggested that use of *Azolla* species in rice fields will keep the floodwater pH low and hence this led to fewer losses of applied fertilizers as reported by Norton (2004).

Effect of nutrients on *Azolla* growth: At the start of experiment, two *Azolla* plants were inoculated, which gradually increased in number. Table 4, shows that the increase in number was minimum (13) for –P(+Zn,+Fe) while maximum (24) for –Fe(+P+Zn). In the absence of phosphorus, increase in number of plants was less, whereas the addition of phosphorus increased plant number. It seemed that phosphorus was major limiting nutrient for *Azolla* growth. Increase in number of plant was more for *A.filiculoides*, for *A.pinnata var.pinnata* and for *Rong ping*, indicating that these species were more responsive to P fertilizer application.

Table 2. Average electrical conductivity (uS/cm) of floodwater during *Azolla* cultivation.

Azolla spp.	-(P Zn Fe)	-P(+Zn +Fe)	-Zn(+P +Fe)	-Fe(+P+Zn)	+(P Zn Fe)	Average
A. filiculoides	647	767	782	797	790	756 A
A. caroliniana	684	756	782	812	758	758 A
A. microphylla 418	640	776	803	866	778	773 A
A. pinnata var. *pinnata*	639	752	774	829	802	759 A
A. pinnata	658	778	757	828	715	754 A
Rong ping	638	787	737	806	714	736 A
Average	651 B	769 A	773 A	823 A	760 A	

LSD value 69.99 at alpha 0.05 for treatments.
LSD value 77.79 at alpha 0.05 for species.

Table 3. Average pH of floodwater during *Azolla* growth.

Azolla spp.	-(P Zn Fe)	-P(+Zn +Fe)	-Zn(+P+Fe)	-Fe(+P+Zn)	+(P Zn Fe)	Average
A. filiculoides	8.45	9.21	8.54	9.51	8.32	8.8 A
A. caroliniana	8.60	9.24	8.71	8.65	8.51	8.7 A
A. microphylla 418	8.79	9.93	8.94	9.24	8.81	9.1 A
A. pinnata var. *pinnata*	8.58	9.78	8.51	8.82	8.41	8.8 A
A. pinnata	8.92	9.3	9.0	9.28	8.77	9.0 A
Rong ping	8.64	9.40	8.79	8.74	8.38	8.8 A
Average	8.6 BC	9.5 A	8.7 BC	9.0 B	8.5 C	

LSD value 0.4113 at alpha 0.05 for treatments.
LSD value 0.4571 at alpha 0.05 for species.

Table 4. Total number of plants at the time of harvest (after 30 days).

Azolla spp.	-(P Zn Fe)	-P(+Zn +Fe)	-Zn(+P+Fe)	-Fe(+P+Zn)	+(P Zn Fe)	Average
A. filiculoides	17	16	23	25	26	21 A
A. caroliniana	16	14	25	29	25	22 A
A. microphylla 418	11	10	19	16	17	15 B
A. pinnata var. *pinnata*	17	11	27	24	27	21 A
A. pinnata	15	13	16	22	20	17 B
Rong ping	21	13	23	30	27	23 A
Average	16 B	13 C	22 A	24 A	22 A	

Note: At the time of inoculation, initial no. of plant was 2 per pot.
LSD value 2.932 at alpha 0.05 for treatments.
LSD value 3.259 at alpha 0.05 for species.

Plant growth was graded as shown in Table 5, and *A.pinnata var pinnata* seemed best for its growth followed by *Rong Ping* Ta at the time of harvest, minimum (fresh as well as dry) biomass was for –(P,Zn,Fe) and for –P(+Zn,+Fe), whereas *Azolla* plants produced significant biomass in –Zn(+P,+Fe), -Fe(+P,+Zn) and +(P,Zn,Fe), suggested that Zn and Fe were not the nutrient constraint, but P was the major limiting nutrient for *Azolla* growth in the tested soil. As *A.pinnata var pinnata*, *A.caroliniana* and *Rongping* produced 2-4 times more biomass in + P treatments as compared to – P treatments, than the rest of the species hence these species can be grown to obtain higher *Azolla* biomass, by applying P-fertilizer (Tables 6 & 7).

Table 5. Grading of *Azolla* growth with respect to appearance.

Azolla spp.	-(P Zn Fe)	-P(+Zn +Fe)	-Zn(+P+Fe)	-Fe(+P+Zn)	+(P Zn Fe)	Average
A. filiculoides	6	6	4	2	6	4.8
A. caroliniana	6	6	6	6	6	6
A. microphylla 418	1	1	1	1	1	1
A. pinnata var. *pinnata*	10	10	10	10	10	10
A. pinnata	6	6	4	4	2	4.4
Rong ping	10	10	8	10	10	9.6
Average	6.5	6.5	5.5	5.5	5.8	

Note: 8-10 means best growth, 7-4 means moderate growth. 3-1 means poor growth.

Table 6. Fresh biomass (g/pot) of *Azolla* spp. after 30 days.

Azolla spp.	-(P Zn Fe)	-P(+Zn +Fe)	-Zn(+P+Fe)	-Fe(+P+Zn)	+(P Zn Fe)	Average
A. filiculoides	4.9	8.1	25	15.1	25.74	15.8 B
A. caroliniana	4.6	7.99	23.79	27.2	25.00	18.0 AB
A. microphylla 418	6.1	5.47	15.56	10.0	12.86	10.0 C
A. pinnata var. *pinnata*	11.0	7.44	29.12	25.2	24.14	19.38 AB
A. pinnata	9.5	6.13	13.75	11.9	14.43	11.16 C
Rong ping	15.0	8.53	23.4	26.5	25.13	19.7 A
Average	8.55B	7.2B	21.8A	19.3A	21.0A	

LSD value 3.325 at alpha 0.05 for treatments.
LSD value 3.696 at alpha 0.05 for species.

Table 7. Dry biomass (mg/pot) of *Azolla* spp. after 30 days.

Azolla spp.	-(P Zn Fe)	-P(+Zn +Fe)	-Zn(+P+Fe)	-Fe(+P+Zn)	+(P Zn Fe)	Average
A. filiculoides	410	505	1635	1046	1486	1016 BC
A. caroliniana	459	624	1523	1618	1683	1181 AB
A. microphylla 418	497	372	1022	425	754	614 D
A. pinnata var. *pinnata*	722	609	2089	1577	1603	1320 A
A. pinnata	568	454	1113	748	867	750 CD
Rong ping	1080	347	1791	1649	1701	1312 A
Average	623 C	485 C	1529 A	1177 B	1349 AB	

LSD value 228.8 at alpha 0.05 for treatments.
LSD value 254.2 at alpha 0.05 for species

Table 8. Nitrogenase activity (n mol C_2H_4/ g dry wt./hr) of *Azolla* at time of harvest.

Azolla spp.	-(P Zn Fe)	-P(+Zn +Fe)	-Zn(+P+Fe)	-Fe(+P+Zn)	+(P Zn Fe)	Average
A. filiculoides	152	422	1126	1248	1215	833 B
A. caroliniana	161	79	1197	1225	1047	742 B
A. microphylla 418	141	499	1690	914	4947	1638 A
A. pinnata var. *pinnata*	285	141	1153	1301	724	721 B
A. pinnata	220	100	614	2809	988	946 B
Rong ping	168	431	745	1130	526	598 B
Average	188 B	279 B	1087 A	1438 A	1575 A	

LSD value 568.6 at alpha 0.05 for treatments.
LSD value 631.9 at alpha 0.05 for species

Effect of nutrients on nitrogen fixation of *Azolla*: Nitrogenase activity (Table 8) of the *Azolla* plants showed that activity was minimum (188 n mol C_2H_4 /g dry wt./h) for –(P,Zn,Fe) , followed by -P(+Zn,+Fe) treatment (279 n mol C_2H_4 /g dry wt./h) respectively, while plants gave sufficient activity in the rest of the three treatments. The highest nitrogenase activity (1575 n mol C_2H_4 /g dry wt./h) in +(P,Zn,Fe) indicated that presence of all three nutrients helped in this activity. However P was more important than others for this activity as it was low in –P treatments. Stal (2003) also reported the significance of P in nitrogen fixation in cyanobacteria.

Effect of nutrients on *Azolla* morphology: Data from fronds appearance indicated that *A.filiculoides* and *A.caroliniana* appeared healthy in all treatments, while *A.microphylla* did not grow well in any treatment, indicating that it did not grow at high pH of our soils. *A.pinnata var pinnata* grew well in all treatments. *A.pinnata* local appeared healthy in phosphorus deficient treatment, and appeared normal in rest of the four treatments. *Rong ping* appeared healthy in all the treatments. The better morphological appearance of the last three species (mentioned above) indicated that they were relatively adapted to low P conditions of soils.

Conclusion

Phosphorus was found to be the major limiting nutrient for plant growth. *A.pinnata var pinnata* and *Rong Ping* were the best species to be used as an inoculum for the saline soils in rice wheat cropping system. Iron and Zinc were not found the major limiting factors in the tested soil for *Azolla* growth. The 2-4 time growth of some *Azolla* species due to P application, indicated that significantly higher *Azolla* biomass can be produced in saline soils, by just application of P fertilizer in saline soils.

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(Received for publication 29 December 2005)