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COMPARISON OF SOIL- AND NUTRIENT-BASED MEDIUM FOR MAINTENANCE OF *AZOLLA* CULTURES

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ABSTRACT

Soil- and nutrient medium-based cultures of *Azolla* were compared in terms of growth, nitrogen (N) fixation and chlorophyll content. Soil-based cultures show similar growth in terms of fresh weight, as media-based cultures. Acetylene reduction activity and chlorophyll content were found to be very high in soil-based cultures. Studies on soil N, phosphorus (P), potassium (K), and carbon (C) contents reveal that N and K contents in soil increase after inoculation of *Azolla* plants in soil-based cultures, while P content of soil decreases. In *Azolla* plants available N, P, and K contents were high in nutrient media-based cultures, initially, but N and P content of soil-based cultures kept for long period (>2 months) were significantly higher than media-based cultures. Nutrient media-based cultures require an early transferring in to fresh media as compared to soil-based

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cultures. The results indicate that soil-based cultures of *Azolla* are as good as nutrient media-based cultures of *Azolla*.

Key Words: *Azolla*; Nutrient medium; Soil medium

INTRODUCTION

Azolla is a fern of agronomic importance due to its ability to fix atmospheric nitrogen (N).[1,2] The presence of the algal symbiont *Anabaena–Azollae* in its leaf cavities helps in N fixation and in turn increases soil organic content in terms of total N after death of the *Azolla* plant.[3,4] Reports are available to support its role as an animal feed,[5] in hydrogen production,[6] and as a biofertilizer.[1,7,8] Use of *Azolla* as green manure is also reported to improve soil physical properties by increasing the porosity (3.7–4.2%) and decreasing the bulk density of soils.[9] Due to its multiple uses efforts are on to optimize its growth[10–12] while maintaining N_2-fixation. Soil-based as well as media-based cultures have been proposed for the growth of the fern.[13,14] Espinas and Watanabe's medium (EWM), Hoagland medium, and IRRI (International Rice Research Institute) medium are some of the commonly used media for its growth. Through various reports it is an undisputed fact that major macronutrient required for *Azolla* growth is phosphorus (P).[15] *Azolla* can even absorb P from the soil and accumulate in its body, this P gets released in to the soil after the plant's death. *Azolla*, therefore, not only supplies N to the rice crop, but simultaneously increases the soil organic matter and fertility too.

Azolla propagation is normally carried out in soil-based nurseries while *Azolla* germplasm is maintained as media-based cultures. Though it is being maintained as soil medium (SM)-based culture at CRRI (Central Rice Research Institute), Cuttack, Collections in India. The intent of this work was to compare the media-based cultures of *Azolla* with the soil-based cultures of *Azolla* for general maintenance. Growth (in terms of biomass increase), N fixation (acetylene reduction activity), chlorophyll content, available N, P, potassium (K), and carbon (C) content in soil- and media-based cultures of five different species of *Azolla* were analyzed. The pH of soil- and media-based cultures was also checked during growth.

MATERIAL AND METHODS

The five *Azolla* species chosen for the experiments, *Azolla pinnata*, *Azolla microphylla*, *Azolla filiculoides*, *Azolla mexicana*, and *Azolla rubra* belong to two different sections of *Azolla*, i.e., *Azolla* and *Rhizosperma*. *Azolla pinnata* being

the sole member from *Rhizosperma* section and rest are from *Azolla*. These *Azolla* cultures are from *Azolla* culture collections of CRRI (Cuttack) India, maintained in the polyhouse of our center for the last six years. *Azolla microphylla* is highly adapted to high temperature (>35°C), whereas *A. pinnata* is native with high sporulation ability. *Azolla rubra*, *A. filiculoides*, and *A. mexicana* have high growth capacity.

Wide-mouthed glass beakers (15 × 12 cm) were used for both soil and nutrient medium for growth of *Azolla* plants. For nutrient medium grown plants, EWM was taken. The medium consisted of $CaCl_2$ (40 ppm), KH_2PO_4 (20 ppm), $MgSO_4$ (40 ppm), and KCl (40 ppm) as macronutrients and $MnSO_4$ (0.5 ppm), Na_2MoO_4 (0.15 ppm), H_3BO_3 (0.2 ppm), $ZnSO_4$ (0.01 ppm), $CuSO_4$ (0.01 ppm), and Ferric citrate (2 ppm) as micronutrients at pH 6.5. Two and a half (2.5) g fresh wt. of washed *Azolla* plants were taken as initial inoculum. In SM cultures 150 g of autoclaved soil was mixed with tap water. After incubation for 2 h upper clear layer of water establishes, and the soil settles down, it was taken as soil medium. A known amount of each *Azolla* culture (2.5 g fresh wt.) was added. Each experiment was kept in triplicates and the mean of three individual readings was plotted. *Azolla* uninoculated SM served as control. Plants were kept in polyhouse for growth where diurnal temperatures of 25°C during a 16 h day and 19 + 1°C at 8 h night were maintained. Light was provided by cool white fluorescent lamps of normal, incandescent bulbs (16,000 lux). Intensities were measured using a lux meter.

Biomass Measurement

Prior to initial measurements, the plant material was washed in distilled water and gently blotted several times to pieces of absorbent paper to remove adhering liquid. The growth rate was expressed as the biomass increase (mg fresh wt./m^2) for a week period.

Acetylene Reduction Assay and Chlorophyll Determination

Nitrogenase activity was determined in terms of acetylene reduction assay[16] using gas liquid chromatograph (Nucon model EC 5700) with porapak R columns. One-half (0.5) g *Azolla* plants were incubated for 1 h in the polyhouse under an atmosphere of 10% C_2H_2, 0.03% CO_2 in argon in sealed vials using the media at pH and temperature employed for their growth. The light intensity for incubation was 16,000 lux. One milliliter of the gas sample after incubation was injected in to the column and peak height noted on the

recorder. Ethylene was measured as *n* mole ethylene/mg chl/h with a FID detector.

Chlorophyll was determined according to Wintermans and Demots.[17] One-half (0.5) g culture of *Azolla* with 5 mL of 95% ethanol was finely grounded in a small polytron. The extract was centrifuged at 4000 *g* for 5 min and the supernatant was taken for the reading at 665 nm and 649 nm.

Available P and K of SM based and EWM based plant samples after two weeks of growth were also determined[18] to see if the depletion of nutrients from soil is directly proportional to the increase of the same nutrients in plant material.

Available C, N, P, K of soil were measured following the methods in.[18] Available P was determined by Olsen's method,[19] C by Walkley and Black method,[20] and N by Kjeldahl method.[21]

RESULTS

Biomass measured in terms of fresh weight was found to be a little higher for EWM grown plants initially, but in the 3rd week of inoculation it was almost equal to SM grown plants (Fig. 1). Soil medium based cultures could sustain for two months and more, while in EWM based cultures growth diminished after three weeks. *Azolla pinnata* showed the highest growth amongst the five species, in both SM as well as EWM grown plants. *Azolla microphylla* too showed good growth but it was 10% less than *A. pinnata*.

In contrast, chlorophyll content, was high in *A. pinnata* and *A. microphylla* [Fig. 2(a)] in SM cultures. *Azolla mexicana* and *A. rubra* too showed very high chlorophyll content in SM cultures. Accumulation of more pigment in *Azolla* in SM cultures compared to EWM cultures is an interesting result. It has been observed that if the EWM based cultures are not transferred to fresh EW medium, after 15 days the fronds become yellow. Since Mg is the main ion linked to chlorophyll, it may be due to early uptake of the cation (Mg^{2+}) leading to deficiency in the later stages of growth.

Acetylene reduction activity (ARA) measuring N fixation was found to be very high in *Azolla* grown as SM cultures [Fig. 2(b)]. Among different species of *Azolla*, SM based *A. microphylla* showed maximum ARA. Again, good ARA in the SM cultures is an indication of better adaptability of the plants in soil based medium than the EWM based cultures.

The P content of *Azolla* plants after two week's growth in EWM showed higher P content than the SM based *Azolla* plants (Table 1), indicating that plants have harvested more P as EWM grown cultures. Soil P content decreased a little by growing *Azolla* for two months as SM based cultures (Table 2). Since EWM has a high concentration of P, plants may have taken up more P as EWM based

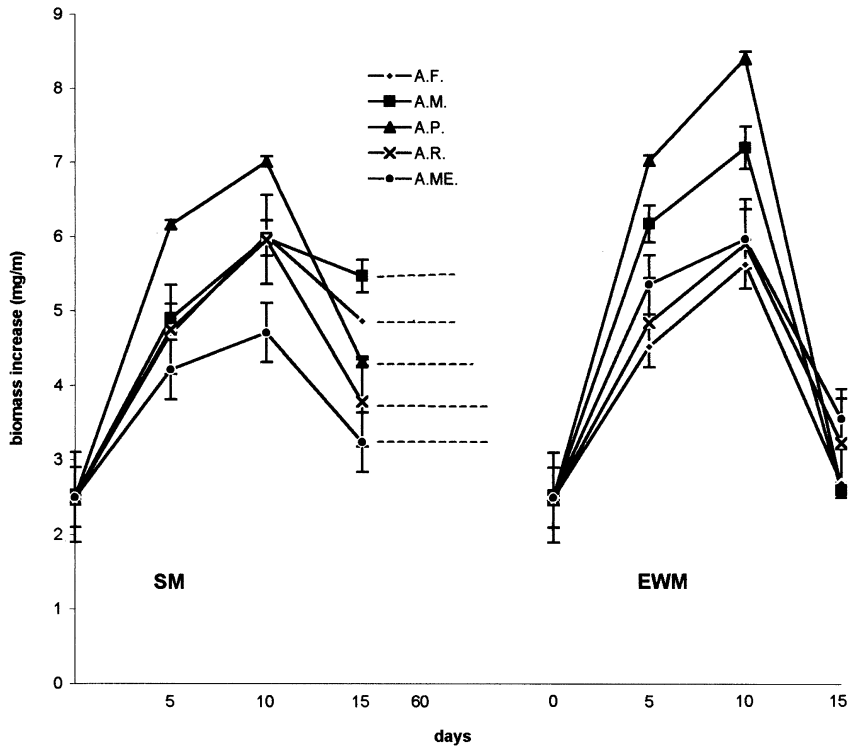

Figure 1. Biomass increase in terms of fresh weight in different *Azolla* species in SM-based and EWM-based cultures. Mean of three different sets is plotted and SE is marked.

cultures, this also explains the better biomass in EWM grown plants than the SM grown plants up to 15 days incubation.

Percent N and % K of *Azolla* plants were found to be almost double in EWM grown plants as compared to SM grown plants (Fig. 3) after two week's growth. *Azolla pinnata* had highest N and K content. *Azolla microphylla* too had high % of N and K. Among SM grown plants, *A. filiculoides* had very high % of N and K than other species. Available P (kg/ha) and available K (kg/ha) of the soil in SM *Azolla* cultures after two months of growth showed increase in K content while a decrease in the P content was noticed (Table 2).

Available C in soil was also calculated did not change and found to be 0.5–0.6% in the control (without *Azolla*) and *Azolla* grown plants.

The pH of the soil before and after growth of the *Azolla* plants did not change and was found to be 6.7.

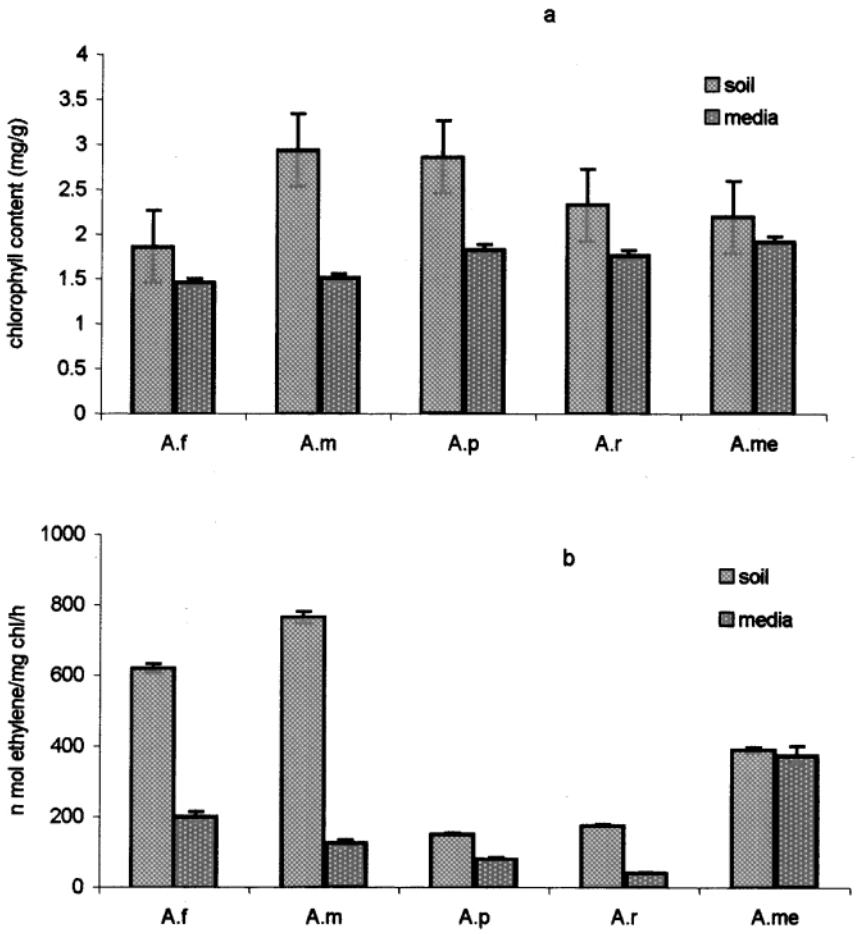

Figure 2. Chlorophyll content (a) and acetylene reduction activity (b) of SM- and EWM-based cultures of different *Azolla* species. Values represent mean of three separate experiments. Mean of three different sets is plotted and SE is marked.

DISCUSSION

With the upsurge in the use of chemical fertilizers for increasing productivity the soil is adversely affected thus soil health is an area of great concern to the farmers now. Research in the past decades was focused at maximizing agricultural yields, the present international aim is to combine increased productivity with environmentally sustainable agricultural practices.[22]

Table 1. Phosphorus Content (%) of Different Species of *Azolla* Plants Grown in SM- and EWM-Based Cultures; Values Represent Mean of Three Separate Experiments

	Phosphorus(%)	
Species	Soil-Based Culture	Media-Based Culture
A. filiculoides	0.53 ± 0.014	1.60 ± 0.0072
A. microphylla	0.37 ± 0.023	1.67 ± 0.081
A. pinnata	0.33 ± 0.052	1.40 ± 0.002
A. rubra	0.48 ± 0.002	1.25 ± 0.01
A. mexicana	0.61 ± 0.001	2.08 ± 0.0162

Rice is a staple food of half of the world's population and blue-green algae and *Azolla* are the common biofertilizers employed, supplying an additional 30–40 kg of N/hectare into the soil.[23] Due to very high multiplication rate of *Azolla*, biomass produced is multifold in two–three weeks. It not only saves the expensive chemical fertilizer, but also adds organic matter to the soil after decaying, since fixed N is available to the soil only after *Azolla* decays, rest of the plant part adds to soil nutrition.

Azolla is an aquatic fern and it requires water and a little P for its growth. Rice-field conditions are suitable for its multiplication, due to waterlogging in the fields and with supply of a little P it grows very well along with the rice as a dual crop. The primary method of propagation in *Azolla* is vegetative, though sporulation too occurs but many constraints hamper the spore to become a propagule for *Azolla*.[24] It is maintained both as a soil based as well as a nutrient medium based culture. It was found during our studies that it can be well maintained in the laboratories in soil based medium, and its growth rate, N

Table 2. Available P and K Content of Soil After Growing Different *Azolla* Species in SM and EWM; Control Is Uninoculated Soil; Values Represent Mean of Three Separate Experiments; SE Is Marked

Species	Phosphorus ($kg\,ha^{-1}$)	Potassium ($kg\,ha^{-1}$)
Control	94.08 ± 0.023	227.2 ± 0.012
A. microphylla	87.81 ± 0.0342	232.8 ± 0.0756
A. filiculoides	89.52 ± 0.004	236.5 ± 0.052
A. pinnata	83.21 ± 0.0865	237.6 ± 0.0121
A. rubra	88.14 ± 0.004	231.4 ± 0.0067
A. mexicana	85.48 ± 0.001	238.2 ± 0.0021

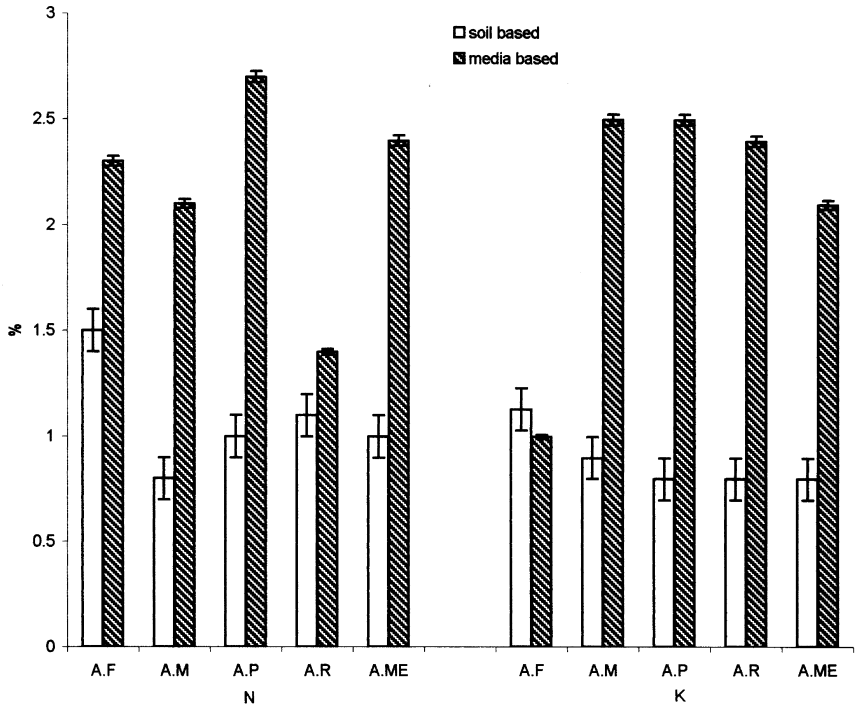

Figure 3. Percent N and % K content of different *Azolla* species in soil- and media-based cultures. Mean of three different sets is plotted and SE is marked.

fixation, % P, K, N, C content remain equal or more than the nutrient medium based culture, if kept for long duration (Figs. 1–3). It is also reported that another benefit of applying *Azolla* as a biofertilizer is that in low K soil it has greater ability to accumulate K than does rice. Thus, when the fern decomposes, it acts indirectly as a K fertilizer.[25] Our studies also support this finding (Table 2). Kushari and Taheruzzaman[26] reported that productivity of *Azolla* and its symbiotic N fixation were regulated by the nutrient status of the medium and were influenced more by orthophosphate P of a particular medium. They found that ditch and pond water was quite significant to influence the productivity and suitable for luxurious consumption of P in excess of metabolic demand. Jeschke and Simonis[27] have shown that the main source of P for aquatic plants is in the form of inorganic phosphates. This could be the reason of high P content in EWM grown cultures, and P loading capacity is also different among the various species of *Azolla* (Table 1). This finding is also supported by Talley and Rains[28] and Watanabe et al.[29]

Our work suggests that *Azolla* grown on soil based medium as soil + water also works well for maintenance of *Azolla* plants. The biomass of the nutrient medium based cultures show increase till two weeks period, when the concentration of all salts N, K, P, and C is high in media to support good plant growth, after which the growth rate stagnates and plants require transfer in fresh media. In soil based cultures, however, the growth is steady for about two months, without any decay. Plants could harvest a little P from the soil and can survive well (Table 2). Soil acts as good buffering agent too, while in media based cultures, pH of the medium rises after sometime due to accumulated debris of decayed plant material and roots.[30] In soil based media this debris gets added in to the soil as soil component and pH of water above soil is not adversely affected. So the SM based cultures can be stored for a longer period and transferring in to the fresh soil is required only after 2–3 months time. This saves the labor on one hand and cost of expensive medium components too. Though the cultures should not be kept for very long durations as incorporation of organic matter in soil decreases the total bacterial count of the soil and increases the total fungal count.[31] As the period of incubation in soil increases the total population of these fungi too increases, in all the species of *Azolla* except *A. microphylla*. Due to fungal population cellulolytic and hydrolytic activity of *Azolla* too increases and it enhances the decomposition of *Azolla*. Soil based medium for *Azolla* with a constant level of water and a little P (not more than 300 ppm) would serve as a good system for healthy *Azolla* maintenance.

REFERENCES

1. Singh, P.K. Multiplication and Utilisation of Fern *Azolla* Containing Nitrogen Fixing Symbiont as Green Manure in Rice Cultivation. Riso **1977**, *26*, 125–136.
2. Moore, A.W. *Azolla*: Biology and Agronomic Significance. Bot. Rev. **1969**, *35*, 17–35.
3. Watanabe, I.; Espinas, C.R.; Berja, N.S.; Alimagno, B.V. Utilisation of the *Azolla–Anabaena* Complex as a Nitrogen Fertilizer for Rice. IRRI Paper Series **1977**, *11*, 1–15.
4. Singh, P.K. Symbiotic Algal N_2 Fixation and Crop Productivity. In *Annual Review of Plant Sciences*; Mallick, C.P., Ed.; Narendra Publications: New Delhi, India, 1979; 37–65.
5. Subudhi, B.P.R.; Singh, P.K. Nutritive Value of Water Fern *Azolla* for Chicks. Poultry J. **1978**, *57*, 378–380.
6. Peters, G.A.; Toia, R.E.; Lough, S.M. *Azolla–Anabaena* Relationship. Plant Physiol. **1977**, *59*, 1021–1025.

7. Singh, P.K. Biofertilisation of Rice Crop. In *Biofertilizers: Potentialities and Problems*; Sen, S.P., Palit, P., Eds.; Plant Physiology Forum: Calcutta, India, 1988; 109–114.
8. Singh, P.K. Use of *Azolla* in Asian Agriculture. Appl. Agric. Res. **1989**, *4*, 149–161.
9. Ventura, W.; Watanabe, I. Green Manure Production of *Azolla microphylla* and *Sesbania rostrata* and Their Long-Term Effects on Rice Yields and Soil Fertility. Biol. Fert. Soils. **1993**, *15*, 241–248.
10. Peters, G.A.; Toia, R.E.; Evans, W.R.; Crist, D.R.; Mayne, B.C.; Polle, R.E. Characterization and Comparisons of Five *Azolla–Anabaena* Associations. Plant Cell Environ. **1980**, *3*, 261–269.
11. Kitoh, S.; Shiomi, N. Effects of Mineral N nutrients and Combined Nitrogen Sources on Growth and Nitrogen Fixation of *Azolla–Anabaena* Association . Soil Sci. Plant Nutr. **1991**, *37*, 419–426.
12. Yatazawa, M.; Tommatsu, N.; Mosada, N.; Nunome, K. Nitrogen Fixation in *Azolla–Anabaena* as Affected by Mineral Nutrient Status. Soil Sci. Plant Nutr. **1980**, *26* (3), 415–426.
13. Nickell, L.G. Physiological Studies with *Azolla* Under Aseptic Conditions. II. Nutritional Studies and the Effects of Chemicals on Growth. Phyton **1961**, *17*, 49–54.
14. Malavolta, E.; Acorsi, W.R.; Ruschel, A.P.; Krug, L.J.; Nakayama, L.I.; Fiomi, I. Mineral Nutrition and Nitrogen Fixation in *Azolla.* In *Associative Nitrogen Fixation*; Vose, P.B., Ruschell, A.P., Eds.; CRC Press: Boca Raton, FL, 1981; Vol. II, 229–231.
15. Watanabe, I.; Berja, N.S.; Del Rosario, D.C. Growth of *Azolla* in Paddy Field Soils as Effected by Phosphorus Fertilizer. Soil Sci. Plant Nutr. **1980**, *26*, 301–307.
16. Jewell, W.J.; Kulasooriya, S.A. The Relation of Acetylene Reduction to Heterocyst Frequency in Blue-Green Algae. J. Exp. Bot. **1970**, *21*, 881–886.
17. Wintermans, J.F.G.M.; Demots, A. Spectrophotometric Characterisation of Chlorophylls a and b and Their Pheophytins in Ethanol. BBA **1965**, *109*, 448–450.
18. Walinga, I.; Vanwork, W.; Hauba, V.J.G.; Vanderke, J.J. Plant Analysis Procedure (Part 7). In *Soil and Plant Analysis*; Kluwer Academic Publishers: Dordrecht, Netherlands, 1995; 12–138.
19. Olsen, S.R.; Cole, C.V.; Watanabe, F.S.; Dean, L.A. *Estimation of Available Phosphorus in Soils by Extraction with Sodium Biocarbonate*; USDA, U.S. Government Printing Office: Washington, DC, 1954; 939.
20. Walkley, A.J.; Black, I.A. Estimation of Soil Organic Carbon by the Chromic Acid Titration Method. Soil Sci. **1934**, *37*, 29–38.
21. Keeney, D.R.; Bremner, J.M. Chemical Index of Soil Nitrogen Availability. Nature **1966**, *211*, 892–893.

22. Wagner, G.M. *Azolla*: A Review of Its Biology and Utilization. Bot. Rev. **1997**, *63* (1), 1–26.
23. Singh, P.K. Nitrogen Economy of Rice Soils in Relation to Nitrogen Fixation by Blue-Green Algae and *Azolla.* In *Increasing Rice Yield in Kharif*; Central Rice Research Institute: Cuttack, India, 1978; 221–239.
24. Dawar, S.; Dey, T.; Saxena, S.; Singh, P.K. Biodiversity of *Azolla* and Prospects of Its Genetic Improvement in Relation to Temperature, Salinity and Pest Infestation. In *Recent Advances in the Exploitation of Blue-Green Algae and Azolla*; Singh, P.K., Dhar, D.W., Pabbi, S., Prasanna, R., Arora, A., Eds.; NCCUBGA, IARI: New Delhi, India, 2001; 95–107.
25. Van Hove, C. *Azolla and Its Multiple Uses with Emphasis on Africa*; FAO: Rome, Italy, 1989.
26. Kushari, D.P.; Taheruzzaman, Q. Multiplication of *Azolla pinnata* in Primary Production Units. In *Proc. National Symposium on Cyanobacterial Nitrogen Fixation*; Kaushik, B.D., Ed.; NFBGAC, IARI: New Delhi, India, 1990; 353–358.
27. Jeschke, W.D.; Simonis, W. Uber die Aufnahove von Phosphatund Sulfatonen. Planta **1965**, *67*, 6–32.
28. Talley, S.N.; Rains, D.W. Potential Mechanisation of *Azolla* Cultivation in Rice Fields. In *Azolla as Green Manure*; Lumpkin, T.A., Plucknett, D.L., Eds.; West View Press: Boulder, CO, 1982; 141–159.
29. Watanabe, I.; Lin, C.; Ramirez, C.; Lapis, M.T.; Ventura, S.T.; Liu, C.C. Physiology and Agronomy of *Azolla–Anabaena* Symbiosis. In *Nitrogen Fixation with Non-Legumes*; Skinner, F.A., Boddey, R.M., Fendrik, I., Eds.; Kluwer Academic Publishers: Dordrecht, Netherlands, 1989; 57–62.
30. Dawar, S.; Singh, P.K. Growth, Nitrogen Fixation and Occurrence of Epiphytic Algae at Different pH in the Cultures of Two Species of *Azolla.* Biol. Fert. Soils **2001**, *34*, 210–214.
31. Sahrawat, K.L. Mineralisation of Soil Organic Nitrogen Under Waterlogged Conditions in Relation to Other Properties of Rice Soil. Plant Soil **1983**, *42*, 305–308.