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Production of an Aquatic Fern –Azolla Use as a Bio-Fertilizer and Nutrient Source for Aquatic and Domestic Animals

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Abstract

Agriculture developed about 10,000 years ago in the Fertile Crescent of Iran and Palestine. Rice cultivation also began in central China near the Yangtze River over 8500 years ago. These early farmers used a natural biological system to fertilize the rice; they used Azolla's ability to draw down nitrogen from the atmosphere, which then bio-fertilized the growing rice plants. The use of Azolla in rice production use dates back at least a thousand years in Vietnam. Some legends say that its cultivation was introduced by the Buddhist Monk Khong Mirh Khong in the eleventh century. Azolla /mosquito fern/ duckweed fern/ fairy moss/ water fern is a free-floating aquatic fern and is genus which belongs to family Salviniaceae. It grows in fresh water and is naturally available mostly on moist soils, ditches marshy ponds and is widely distributed in tropical belts of India. Shape of Indian species is typically triangular measuring about 1.5 to 3.0 cm in length 1 to 2 cm in breadth. Roots emanating from growing branches remained suspended in water.

The dorsal lobe which remains exposed to air is having a specific cavity containing its symbiotic partner, a Blue Green Algae (BGA), the Anabaena azollae. The fern is capable of fixing atmospheric nitrogen in the soil in the form of NH4+ and becomes available as a soluble nitrogen for the cultured species. A pot experiment was conducted in the experimental field of the University of Ghana, Legon to observe the effectiveness of dry Azolla as N source in flooded rice field. The treatments consist of incorporating fresh Azolla (FA at 90 kg N/ha), dry Azolla (DA at 90 kg N/ha), dry Azolla + Ammonium sulphate (DA at 45 kg N/ha + AS at 45 kg N/ha), fresh Azolla + dry Azolla (FA at 45 kg N/ha + DA at 45 kg N/ha), ammonium sulphate (AS at 90 kg N/ha) and a control (C at 0 kgN/ha). The treatments were applied 8 days after transplanting rice. Results showed that the DA + AS treatment, that is, the treatment where dry Azolla + ammonium sulphate were used to fertilize the rice had the highest dry weight and total N yield followed by the treatment AS. Total N for the DA + AS treatment was 36.67% over the control whilst that for the AS was 25% over the control.

This experiment results stated that the Azolla is potential for the nitrogen fixation in the soil. There is ample scope for improving the productivity of livestock by better balancing of nutrients and optimizing the utilization of feed resources. Azolla has been reported as potential feed supplement for dairy animals, which have rich nutrient and mineral profile. An On Farm Trail was conducted to study the effect of Azolla as feed supplement in buffaloes. The control group (C) farmers practice was fed wheat straw and green fodder with cottonseed cake. In the treatment group (T) 1.5 kg fresh azolla/animal/day was supplemented over conventional ration. This experiment was stated that the dry Azolla is useful for cattle feed and the milk yield is gradually increased. An attempt was made to evaluate the proximate value of Azolla pinnata as a feed for poultry. Azolla was cultivated following the NARDEP method. After harvesting it was sundried and stored in polyethylene bags and was further analysed for proximate principles. The dry matter content was 90.03 %, 22.79 % crude protein, 3.59 % ether extract, 15.49 % crude fibre, 19.46% total ash, 38.67 % NFE, 1.93% calcium and 0.26% phosphorus, this article results explains the suitability of Azolla as a poultry feed. To evaluate the nutritive value and digestibility of Azolla in ruminants by in vitro techniques. The crude protein, crude fibre and ether extract contents were at a level of 21.37%, 12.5% and 2.3%, respectively. The neutral and acid detergent fibre levels were about 35.4 and 23.9%, respectively. The average in vitro dry matter digestibility, in vitro organic matter digestibility and metabolozable energy contents were 79.5%, 63.8 mg/200mg and 7.36 MJ/kg DM (1759 kcal/kg), respectively. The various protein fractions A, B1, B2, B3 and C estimated by Cornell net crude protein solubility system were 18.22, 42.56, 15.15, 7.47 and 16.61% of total protein, respectively. The Azolla contained significantly higher B1 fraction followed by A, B2 and C and lowest fraction of C. Thus in view of above, present study indicated Azolla to be a good source protein supplement with 21.37% crude protein with highest B protein fractions, moderate source of energy (1759 kcal ME/kg), high dry matter and organic matter digestibility and rich in trace minerals thus could be used as an alternate protein supplement or as supplementary protein supplement to ruminants.

Present investigation was carried out to study the influence of fresh Azolla when used as basal incorporation in soil and as dual cropped with rice variety Mahsoori separately and together with and without chemical nitrogen fertilizer in pots kept under net house conditions. Results showed that use of Azolla as basal or dual or basal plus dual influenced the rice crop positively where use of fern as basal plus dual was superior and served the nitrogen requirement of rice. There was marked increase in plant height, number of effective tillers, dry mass and nitrogen content of rice plants with the use of Azolla and N-fertilizers alone and other combinations. The use of Azolla also increased organic matter and potassium contents of the soil. The overall study explains about the Azolla is suitable for the alternative protein supplement feed for the cattle's, pigs, poultry etc… and the Azolla useful for the organic fertilizer to fix the nitrogen along with the anabaena.

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Introduction

Azolla is a genus of rapidly growing, nitrogen-fixing aquatic fern. Azolla biomass is particularly rich in protein with favorable amino acids. Therefore, it has been proposed as a novel protein source for feed in aquaculture and domestic animals, it is used as a bio-fertilizer in agriculture, it may also useful for human beings. Azolla Established by Lamarck in 1783, it belongs with the Salviniales. In 1980 Lumpkin and Plucknett considered this genus belongs to the family Salviniacae, this family has two subgenera with six living species (1980). In this subgenus Euazolla: A.filiculoides (Lam.,), A.caroliniana and subgenus Rhizosperma: A.pinnata (R.Br.,) are mainly important for Nitrogen fixation and protein production [1]. In 1986 Tan et.al replaced the Azolla family with Azollaceae, then the Azolla separated from the salviniacae family.

The Azolla production was heavily promoted in the early 1960s in China and Vietnam, then it attracted all other countries to the rapid expansion of Azolla and used as a potential replacement for agriculture to the extension of the Nitrogen fixation levels in soil and also give nutrients to promote the plant growth. In 1993 Saunders and Fowler also make some changes and developments in the Azolla taxonomy. In this Azolla is divided into two sections, one section Euazolla includes five species they are – A.filiculoides-Lam., A.rubra-R.Br., A.mexicana- Presl., A. caroliniana-auct. non Wild., and A.microphylla-auct. non Kaulf. And another section Rhizosperma contain only one species that is A.pinnata-R.Br.,

Azolla present in freshwater habitats in tropical, subtropical, and warm temperature regions trough out the world.A.pinnata widely available in most Asian countries and other species are available mostly in American and African countries. Azolla survived at a pH between 3.5 to 10. Azolla is a macrophyte called a frond, it may range from 1 cm to 15 cm or more. A.pinnata as 1 cm to 2.5 cm range(smallest) and A.nilotica as 15 cm or more (largest [2].

Azolla consists of unbranched, adventitious roots hand down into the water, the roots absorb nutrients directly from the water, it also contains the main rhizome, the small leaves are alternatively arranged. The roots may also touch the soil, derive nutrients from it. Azolla has leaves and each leaf consists of two lobes i.e Arial dorsal lobe, which is chlorophyllous, and the submerged ventral lobe which is uncolored. Azolla is also called mosquito fern, duckweed fern, fairy moss, and water fern.

Azolla requires nutrients that are essential for plant growth. They are Phosphorus, K, Ca, Mg, Fe, and Zn, etc… be necessary for Azolla growth and N-fixation. Azolla is rich in proteins, essential amino acids, vitamins, and minerals and this mostly contains 27% crude protein and 10% of carbohydrates on a dry weight basis. Azolla increases the dissolved oxygen concentration in the standing water and improves the soil's redox status.

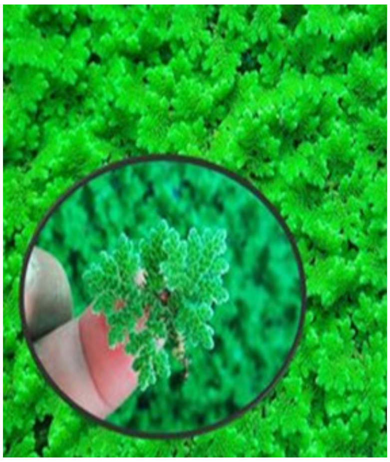


Figure 1: Image of Azolla

Azolla-Anabaena Association

Azolla and Anabaenaisthe best relation for Nitrogen fixation in plants. In this Cyanobacteria plays a vital role to fix Nitrogen in the presence of the nitrogenase enzyme. Cyanobacterium does not exist outside of the plant so symbiosis is always important for the ferns. However, there are several environmental conditions are occurred for the optimum function of the nitrogenase enzyme. The existence of available C, and the occurrence or lack of combined Nitrogen and molecular oxygen play a crucial role in controlling the synthesis and level of nitrogenase activity. Due to the injurious effect of Oxygen on Nitrogen fixation by cyanobacteria, Azollasupplies an oxygen-free environment for Anabaena. In return, Anabaena withdraws Nitrogen directly from the atmosphere, which is useful for Azolla growth.

Azolla and Anabaena combined able to perform photosynthesis. Photosynthesis is carried out by the presence of a low rate and it largely depends upon sugar derived from plants. Most of the energy that obtains from the sugar it takes from Azolla is provide into Nitrogen fixation. At the stage when Nitrogen fixation as its maximum level, the bacteria are no longer separate. The main product of photosynthesis in Azolla is sucrose.

The symbiotic relationship between Azolla and Anabaena has survived for approximately 70 million years (The Azolla shows the great impact in the Agriculture sector started in 2016). In the period of symbiosis, these two (Azolla-Anabaena) have influenced each other to increase efficiency and help each other beneficial to grow and supply nutrients to the plants.

In the bacteria, N fixation occurs in specialized cells, called heterocysts or heterocytes, which are more frequent in the symbiont than in related free-living Nostac species. Fixed N is supplied to Azolla, and about 40% of the N is exported from the bacteria and released in the form of ammonium into the leaf cavity.

Figure 2: Microscopic view of Anabaena Azolla

Azolla as Bio-Fertilizer

Organic fertilizers are plays a key role in the conservation of soil since they improve the organic matter level and increase long-term soil fertility then increase plant growth. The addition of organic fertilizer to water plays an effective role to strengthen the chemical, physical, and biological properties of water. According to the basis of organic fertilizer, both the stability and quality of fertilizer are affected based on the raw material composition. Since organic wastes can pollute the surrounding environment, its organization and management will benefit soil as a source of organic fertilizer that benefits the soil and water fertility, as well as keeping the environment hygienic its also beneficial to the farmers.

In bio-fertilizers, N:P (nitrogen: phosphorous) ratios play a vital role to enhance plant growth and yield. Azolla is a water fern used for the bio-fertilizer to reduce the carbon dioxide discharge from fossil fuels and produce inorganic fertilizers like ammonium nitrate or ammonium sulfate and urea. Azolla developed as a biological nitrogen fertilizer, particularly in tropical countries like India, Indonesia, Brazil, etc... Azolla may also release synthetic nitrogen which may cause environmental pollution. This problem may fix by the Azolla symbiosis with Anabaena. Hill and Henderson in 1999 stated that the Azolla is a renewable bio-fertilizer and this may also produce weed to reduce the potentiality.

Azolla is used as a bio-fertilizer in rice crops to increase the yield of productivity. Azolla mainly showed that the species A.filiculoides can release the nutrients to the soil to decrease the soil-specific gravity and increase the soil porosity and organic matter content. To make use of the Azolla fern as a workable bio-fertilizer even in the high parallels of the Rice Temperate in areas of Europe, research is required for identifying fern strains with good resistance to both low winter temperatures and alternating day/night temperatures period and to be capable to produce the needed quantities to be incorporated in the soil.

Moreover, to use the Azolla as a bio-fertilizer in conventional farming, not just in organic farming, for the resistance most popular herbicides also required. In other words, the fern should reach a high-quality growth rate during spring (the spring season – March to June), with a high gain of nitrogen, and it should be quite resistant to the most common herbicides used in the rice areas. Until now, most of the research on Azolla is up to date in rice and it has been carried out in tropical or subtropical environments.

Azolla as a Feed

Azolla was also used as a poultry feed in Peru in the early 18th century (Feuillée,1725). Azolla can be feed to livestock whichever in a fresh or dried form. It can be given directly or mixed diets to cattle, poultry, sheep, goats, pigs, and rabbits. In the dairy industry, Azolla increases milk production and in the poultry industry, it gradually increases the weight of the broilers and egg production of the layers. The waste comes from the dairy industries i.e dung is used as fertilizer in the Azolla ponds; the Azolla should be wash carefully with fresh water to eliminate the smell of the dung.

Trials in India indicate that the fresh or dried Azolla can be a preferential substitute to most of the conventional sources of protein such as groundnut meal. Most of the feeding trials with Azolla in dairy cattle, growing buffaloes, sheep, and goats have been conceded in India. Since the year 2000, Azolla has been promoted in India for the production of dairy.

This trails may useful for so many poor farmers because some of the farmers don't have a lot of money to buy the expensive feeds available in the market and this Azolla feed shows so much effect on the national markets then the expensive feed costs also reduced [3]. There have been several aquaria or field trials on the benefits of Azolla for fish culture. These trails increased the fish growth in many fishes but also shown some negative effects on the fed of fresh Azolla to the fingerlings like Nile tilapia finger lings (Oreochromis niloticus).

The present investigation aims to evaluate the impacts of organic and inorganic fertilizers on the water parameters and physiological behaviors of an aquatic plant (Azolla sp). The addition of fertilizers led to an increase in the tissue contents of N and P compared to the control. This increase was highest when Azolla sp. was fertilized with organic fertilizer. The atomic N:P ratio was low in tissues subjected to either treatment compared with the control [4].

Four different tests showed the effectiveness of Azolla pinnata plant extracts against Aedes aegyptiand Aedes albopictus mosquitoes. This clearly indicates the presence of bioactive compounds which are responsible in adulticidal and ovicidal activity in Aedes mosquitoes and at the same time inducing repellence towards the mosquitoes [5].

Now-a-days suitable biotechnological approaches are into practice which can be therefore used to improve the overall crop health and productivity. Biofertilizers also called as Bio-inoculants, are those organic preparations which contain microorganisms that are beneficial to agricultural production particularly with respect to Nitrogen and Phosphorous. When it is applied as seed treatment or as soil application, they have the tendency to multiply rapidly and develop a thick population in the rhizosphere. Biofertilizers can fix atmospheric N through the process of biological nitrogen fixation (BNF), Azolla can fix the nitrogen in plants and also enhance the growth promoting substances [6]. Azollais an aquatic fern that fixes atmospheric nitrogen in symbiotic association with the cyanobacterium Anabaena azollae [7].

The study was undertaken to evaluate the nutritive value and digestibility of Azolla in ruminants by in vitro techniques. The crude protein, crude fibre and ether extract contents were at a level of 21.37%, 12.5% and 2.3%, respectively. The neutral and acid detergent fibre levels were about 35.4 and 23.9%, respectively. The average in vitro dry matter digestibility, in vitro organic matter digestibility and metabolozable energy contents were 79.5%, 63.8 mg/200mg and 7.36 MJ/kg DM (1759 kcal/kg), respectively [8].

The water fern Azolla harbors nitrogen-fixing cyanobacterium Anabaena azollae as symbiont in its dorsal leaves and is known as potent N2 fixer. Present investigation was carried out to study the influence of fresh Azolla when used as basal incorporation in soil and as dual cropped with rice variety Mahsoori separately and together with and without chemical nitrogen fertilizer in pots kept under net house conditions. Results showed that use of Azolla as basal or dual or basal plus dual influenced the rice crop positively where use of fern as basal plus dual was superior and served the nitrogen requirement of rice. There was marked increase in plant height, number of effective tillers, dry mass and nitrogen content of rice plants with the use of Azolla and N-fertilizers alone and other combinations. The use of Azolla also increased organic matter and potassium contents of the soil [9].

This article states that the Azollaor the "green gold" is an aquatic nitrogen-fixing pteridophyte with a wide distribution in temperate and tropical freshwater ecosystems and paddy fields. Azollais an ideal candidate for food, feed, and fodder applications. It can be utilized as a natural plant-based antimicrobial and also as a water purifier in a laboratory or industrial wastewater treatment. Its feasibility as a source for the development of health supplements was tested by analyzing the antioxidant and antimicrobial properties of the fern. The DPPH antioxidant activity of the various extracts shows the good presence of antioxidants [10].

Materials and Methods

Methodology

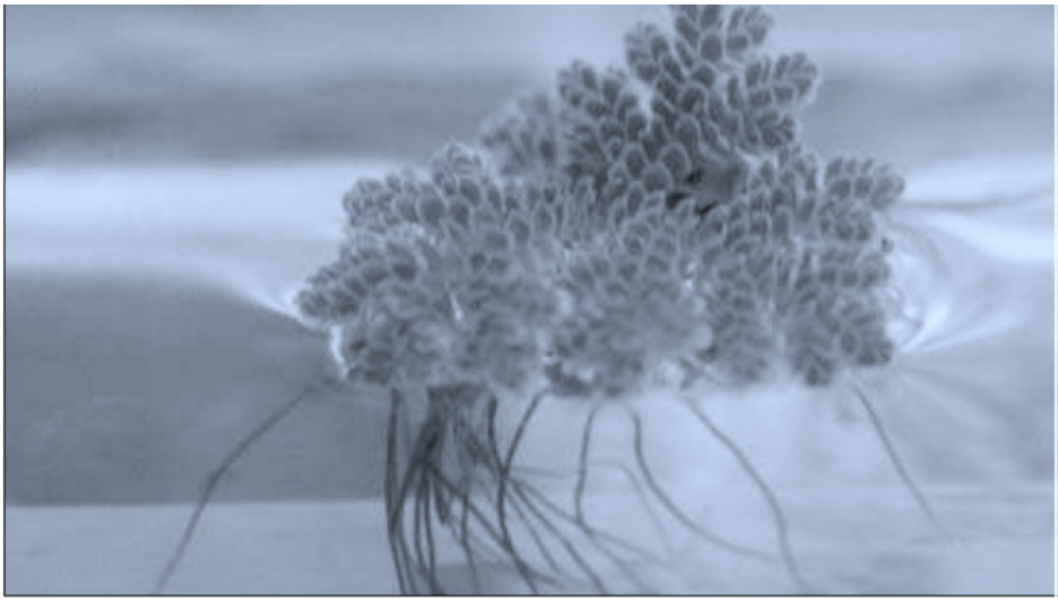


Figure 3: Floating Azolla Plant

Cultivation of Azolla

Figure 4: Naturally Growen Azolla Within Crop Field

Methods Involved in Azolla Bio-Fertilizer

Preparation of Azolla Bio-Fertilizers Extract

About one Kg of Azolla was boiled in 1 Liter distilled water for about 30-45 minutes, after filtration, filtrate was considered as 100% raw Azolla extract, from which different concentrations were made (5%, 10%, 20%, 30%, 40%, and 50%) by means of distilled water. Control solutions without Azolla bio-fertilizers extract was prepared. All Azolla bio-fertilizers and control solutions were kept refrigerated prior to use [11].

Soil Samples

Soil samples were taken from the plough layer (0 to 20 cm) of the Bumbi series from an uncultivated field at the Irrigation Development Authority (IDA), Ashaiman located on latitude 05° 41.400, and longitude 00°03.018. The area has an annual rainfall between 700 and 1000 mm. All samples were transported to the Department of

Soil Science Laboratory of the University of Ghana. The soil was air dried, crashed and passed through a 2-mm sieve to obtain the fine earth fraction that was used for pot experiments later. Part of the fine earth fraction was used for laboratory analysis [12].

Pot incubation experiment was conducted between June and October 2024 under net house condition at the Institute of Agricultural Sciences, Banaras Hindu University (B.H.U.), Varanasi, India. Soil samples used in this experiment were collected from Khaira, a village near Varanasi, India. All the samples were brought to the laboratory, where sieving was done with 2-mm sieve. Part of the sieved soil was air dried [9].

Soil Tests

The above two articles explains about the impact of the biofertilizer in the rice crop the authors used almost same methods for soil testing they are as follows –

pH Test

The pH of the soil was determined using a pH meter with glasscalomel combination in distilled water and 0.01 M $CaCl_2$ solution at a ratio of 1:2 soil: solution. Determination was done in duplicate.

Organic Matter Determination

The organic matter was determined using Walkley and Black method (Jackson 1967). Total nitrogen was determined by Kjeldahlmethod (Jackson 1973). Exchangeable K, CaandMg were extracted using ammonium acetate, K was determined on flame photometer and Ca and Mg by EDTA titration.

Kjeldhahl Method

The Kjeldahl method was used in the total nitrogen determination whereby soil (0.1 g) was weighed into Kjeldahl flask and selenium catalyst was added to accelerate the digestion process. This was followed by addition of 5 ml of concentrated H_2SO_4. The mixture was digested until the digest became clear. It was cooled and transferred into a volumetric flask and made to volume. An aliquot of 5 mL was taken into Markham distillation apparatus and 10 mL of 40% NaOH was added. The solution was distilled and the distillate was collected in 2% boric acid (H_3BO_3) solution which was then titrated with 0.01 M HCl from green to purplish endpoint and the percentage of N was calculated.

Statistical Analysis

Statistical analysis was performed by Tukey HSD test using the SPSS 16.0 (Statistical package for Social Sciences) software package [9]. The data on plant height, tiller number, dry weight and Total N were subjected to analysis of variance using Genstat software, 9th edition. The significance of treatment means was tested at the 5% level of probability and the least significant difference (LSD) was used to separate the means [12].

Green House Rice Experiment

Greenhouse to determine Azolla influence on rice growth Rice seeds (IR841) of 90% germination were nursed in pots and seedlings transplanted later. Basal phosphorus and potassium were applied to all treatments at the rate of 45 kg P_2O_5/ha and 40 kg K_2O/ha. The experimental design was completely randomized (CRD).

The experimental treatments were as follows: Table 1

Treatment	21 DAT*	28 DAT	35 DAT	42 DAT
AS	20.23	42.6	48.53	54.20
AS + DA	36.13	45.9	49.90	54.27
DA	35.73	45.59	49.43	54.07
DA + FA	31.17	44.6	49.99	55.17
FA	36.13	49.3	53.27	56.83
C	39.77	48.9	50.40	51.93
LSD < (0.05)	7.45	7.71	5.83	5.58

*DAT: Days after transplanting

The table shows the growth of the plant giving nitrogen treatment with different sampling times and the height of the rice plant

- Fresh Azolla (FA), 90 kg N ha was applied (that is, 45 kg N/ha FA was basally applied and 45 kg N/ha FA topdressed at booting stage);
- Dry Azolla (DA) 90 kgN/ha was applied (that is, 45 kgN/ha DA was basally applied and 45 kg N/ha DA topdressed at booting stage);
- Ammonium sulphate (AS) 90 kgN/ha (that is, 45 kgN/ha AS was basally applied and 45 kg N/ha AS topdressed at booting stage);
- Dry Azolla + sulphate of ammonia (DA + AS), 45 kg N/ha DA basal application + 45 kg N/ha AS top-dressing;
- Dry Azolla + Fresh Azolla (DA + FA that is, 45 kg N/ha FA basally applied + 45 kg N/ha DA topdressed;
- Control (C), no Azolla nor ammonium sulphate was added to soil sample.

Azolla species	Crop			Experimental Details	Reference
Used as			Total Nitrogen Fixed	Role	
A. pinnata	Rice	Dual crop	75.5%-98.2%	Azolla combined with reduced dose of urea suggested that it can substitute up to 25% of nitrogen	Yao et al., 2018
A. pinnata	Rice	Dual crop	0.71	Deep placement of urea combined with Azolla reduces N loss and improves fertilizer N recovery	Yao et al., 2018
A. pinnata	Rice	Manure	Not specified	Incorporation of fresh Azolla at 20 t ha^{-1} and 5 t ha^{-1} for compost, increased the soil available P, plant P content and tiller number	Setiawati et al., 2018
A. filiculoides	Rice	Compost	Not specified	Under water deficit conditions, application of Azolla compost provided grain yield of an average of (13.8%) higher than that of the nonamended control	Razavipour et al., 2018
All species	Coffee	Compost	Not specified	Azolla acts as a cheap source of nitrogen in coffee	Titus and Pereira, 2006

Each treatment was replicated three times. Each pot was filled with 3 kg of 2 mm sieved soil which was then flooded with water and left for a period of time for an equilibrium to be established between the soil and water. Three weeks after transplanting the rice (that is, 21 days), the plant height and tiller numbers were measured and recorded weekly. The plants were harvested 60 days after transplanting by cutting them just above the soil surface. The plants were washed with distilled water and ovendried at 68°C for analysis. The plant samples were then grinded.plant sample analysis also done in the laboratory [12].

Table 2: Role of Azolla Species in Nitrogen Fixation

Figure 5: Greenhouse Rice Farming

Methods involved in feed stock

Feeding to Fish (Azolla): Young Tilapia nilotica (30 fish), which preferentially feed on phytoplankton rather than on zooplankton, were used in the fish feeding test. In the experimental diets, dried Azolla powder cultured in the secondary effluent was included at levels of 20.7, 34.4, and 48.2% of the total weight of the diets. The percentage of weight gain of the fish «final weight ~ initial weight)/initial weight X 100) was recorded. Analysis of the nutrient composition and Tilapia feeding was conducted three times and the data were usually shown as an average.

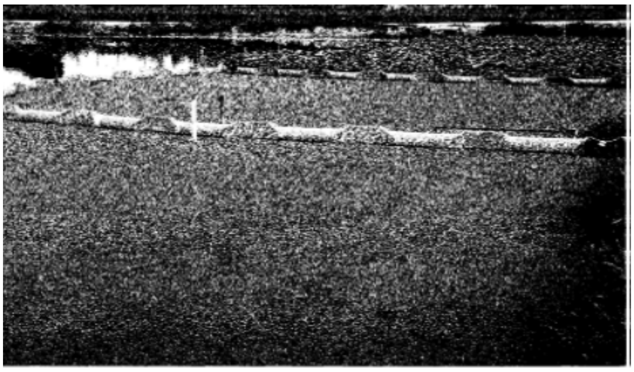


Figure 6: A filiciloides growing in pond (culture)

Figure 7: Image of Azolla Fish Feed

Feed Suplementary for Poultry

Azolla pinnata was cultivated by Nardep method (Pillai et al., 2002) with little modifications according to the availability of material. Azolla started to grow rapidly filled the pit within 15-20 days. A mixture of 20 g of Super Phosphate, 1.5 kg of cow dung and 5 g of mineral mixture (Ranmix) was added after every 10 days. This was done to keep the Azolla in rapid multiplication phase, to avoid any nutrient deficiency. Azolla was harvested manually using aluminium mesh nets. After harvesting Azolla was washed thoroughly and sun dried for 2-3 days under shade such that it become crispy and green colour retained. The dried Azolla was collected and packed in plastic bags for further use. A sample of 100 g of sun dried Azolla meal was further used for analysis.

Figure 8: Image of Azolla Poultry Feed

Figure 9: Buffalos are Feeding Azolla

Proximate Analysis of Azolla

Sun dried Azolla meal sample was analyzed for proximate composition (dry matter, crude protein, ether extract, total ash, crude fibre, calcium and phosphorous as per standards using AOAC, 2012 [3]. While as Nitrogen Free Extract (NFE) was calculated using the formula: NFE (%) = 100- (CP% +EE% +CF% +TA%) and calcium content was determined by the precipitation method.

Methods Involved in Buffalo Feed

The present study was carried out in 2015-16 and 16-17 by establishing azolla production units in adopted villages of District Bundi, Rajasthan by Scientists of Krishi Vigyan Kendra Bundi. The livestock owners were proper trend by on and off campus practical training on azolla production technology, according to Kamalasanana et al., (2002). Thereafter, a survey was conducted to assess the feeding, milk production and physical health status of animals in different villages where azolla production units were established and an On Farm Trail was conducted to study the effect of azolla on buffaloes milk yield in adopted villages Bagli, Neem Ka Khera, Hado Ka Pipalda and Ratabarda by Krishi Vigyan Kendra Bundi.

For this purpose, 20 lactating buffaloes of first to third lactation of 4 to 8 years old at mid lactation were selected for the study and randomly distributed into two groups of ten animals in each. The buffaloes were hand-milked twice daily. Daily milk yield of each animal was recorded for a period of 2 months. The control (C) group (n=10) farmers practice was fed wheat straw, paddy straw and berseem with cottonseed cake. Whereas, the treatment (T) group (n=10) was fed with same quantity of roughages and concentrate with supplementation of 1.5 Kg fresh green azolla (azolla pinnata). The feeding trial lasted for two months. During the trial daily milk yield were recorded and physical health status of animals were observed [13].

Figure 10: Azolla cattle feed seeds

Method Involved in Protein Analysis

The samples of Azolla (Azolla pinnata), harvested on 10-15 days of cultivation were procured from different localities in and around the Hyderabad, Andhra Pradesh. The fresh samples of Azolla were collected in two sets, one set for dry matter (DM) estimation and other for sun drying. The dried samples were ground separately to get 1 mm size. Later the ground samples were mixed to get homogeneous sample before subjecting to analysis.

The mixed samples were analyzed in triplicate for proximate principles (AOAC, 1997) and fibre fractions analysis (Van Soest et al., 1991). The calcium (Ca) and phosphorus (P) contents were estimated as per Talapatra method (Talapatra et al., 1940), while the trace minerals (Cu, Fe, Zn and Mn) were estimated using atomic absorption spectrometry (Arenza et al., 1977).

The samples were screened for in vitro DM digestibility (IVDMD) (Goering and Van Soest 1970) and in vitro gas production techniques (Menke et al., 1979) using buffalo rumen liquor. The in vitro organic matter digestibility (IVOMD) and metabolizable energy (ME) was estimated as per the formulas suggested by Krishnamoorthy et al. (2005) and Menke and Steingass (1988), respectively.

IVOMD (mg) = Gv x 2.2 ME (MJ/kg DM) = 2.2+ 0.136 x Gv + 0.0057 x CPDM/kg Where, Gv = Net gas volume at 24 hours incubation (ml/200 mg DM) CPDM = Crude protein on dry matter.

The protein fractionation was done according CNCP system (Licitra et al., 1996), where in the protein was sub divided in to 5 divisions (Fraction A, B1, B2, B3 and C) according to their degradabilities and passage rate in gastro intestinal tract (Pichard and Van Soest, 1977 and Van Soest, 1994). Fraction A (PA) constitutes non-protein nitrogen (NPN), was separated by using trichloroacetic acid (TCA) according to the method described by Licitra.et al (1996).

The fraction B of azolla protein considered as true protein (Pichard and Van Soest, 1977) was further divided into 3 parts namely, fraction B1 (rapidly degraded true protein), B2 (intermediately degraded true protein), B3 (slowly degraded true protein) (Van Soest et al., 1981 and Krishnamoorthy et al., 1983).

The fraction B1 (PB1) was expressed by estimating the true protein soluble in a borate-phosphate buffer at pH 6.7-6.8 (Krishnamoorthy et al., 1982) and the fraction B2 (PB2) known as neutral detergent soluble protein, was estimated as the difference between buffer insoluble protein (IP) and protein insoluble in neutral detergent (NDICP), and the latter was expressed by estimating the amount of protein recovered in the neutral detergent residue obtained upon standard fibre fraction analysis (Van Soest et al., 1991).

The fraction C (PC) referred as acid detergent insoluble protein (ADIP), measured by estimating nitrogen in ADF residue. The amount of soluble fibre-bound CP (Fraction B3; PB3) was calculated as CP in NDF minus ADIP. The data was subjected to one way analysis of variance as per the procedures of Snedecor and Cochran (1980) by using SPSS 17. The differences between the means were tested by significance using Duncan's multiple range test (Duncan, 1955) [8].

Discussion

Growth of Rice Crop

The height of rice plant increased for all treatments with time. In the first week of sampling, the least plant height was recorded by the treatment AS and the highest height by the control, whilst the different treatments (FA, DA, FA + DA, AS + DA) attained almost the same heights with no significance difference among the various heights of the various treatments. For the subsequent weeks, that is, week 2 through to week 4, there was no significant difference among treatments for a particular week. The rice variety IR 841 has been bred to medium height to avoid lodging. Tiller number increased with time and by the fourth week of sampling, that is, 42 DAT, the highest tiller number was produced by the AS > AS + DA > DA + FA > DA > FA > C. The application of ammonium sulphate produced more tillers than any other treatment and the least tiller number was produced by the control. Tiller number ranged from 11 to 30 by the fourth week of sampling. Ammonia sulphate being an inorganic fertilizer released the nitrogen early enough for the plant to use for tiller production as compared to the N released from Azolla which is organic N that had to be mineralized to release inorganic forms of N for rice plant to use.

The highest dry weight was observed for the AS + DA treatment followed by the AS treatment and there was no significant difference between them (Table 2). The treatment AS + DA was significantly different from all the other treatments. Even though the dry weight of DA + FA was higher than the individual dry weights of DA and FA, there was no significant difference between dry weight of DA + FA, DA and FA. This shows that the combined use of DA and FA has the potential to increase rice dry matter yield more than the use of dry Azolla alone and the use of fresh Azolla alone in growing lowland rice. The control had the lowest dry weight. Correlation of 0.879 and 0.853 existed between the dry weight of the rice plant and the tiller number at 35 DAT and 42 DAT implying that tiller number contributed immensely to the dry matter yield of the rice plant in this study.

The AS + DA treatment had the highest total N and this was significantly different from all other treatments (Table 2). The AS treatment had the next highest total N and it was significantly different from DA + FA treatment. The individual treatments of DA and FA had lower total N compared to the combined treatment of DA + FA. Similarly, the treatment AS + DA had higher total N yield than the individual treatments of AS and DA. Combining DA with AS, ensures prolonged N availablility for rice plant use. This is because AS releases N at the initial stage of rice plant growth and at a later stage, DA mineralizes and releases N making that combination asuperior treatment to use than DA alone. Similarly, the DA + FA treatment performed better than the DA and FA treatments because the rice plant had available N for the growth of rice over a longer span of time. There was no significant difference in the treatments for total N of DA and DA + FA. The lower total N recorded for the treatment FA as compared to the treatment DA is difficult to explain because one would have expected higher total N yield from the fresh Azolla than the dried Azolla since dried Azolla mineralizes and releases N more slowly (Ito and Watanabe, 1984) than fresh Azolla and that can retard the vegetative growth of rice plant. [9,12]

Graph 1 (below) shows the behavior of the rice plant height, influenced by Azolla incorporation and/or association as well as different nitrogen (N) rates. An increase for this character is seen in treatments where incorporated and/or associated Azolla was used. However, nitrogen dose and Azolla influence did not show significant differences.

Graph 2 shows the number of effective tillers per plant. Nitrogen doses applied together with Azolla in this experiment influenced this variable as well. Treatments where Azolla was incorporated and/or associated (T8, T12, T11, T16, and T7) stood out, for presenting the highest number of tillers per plant.

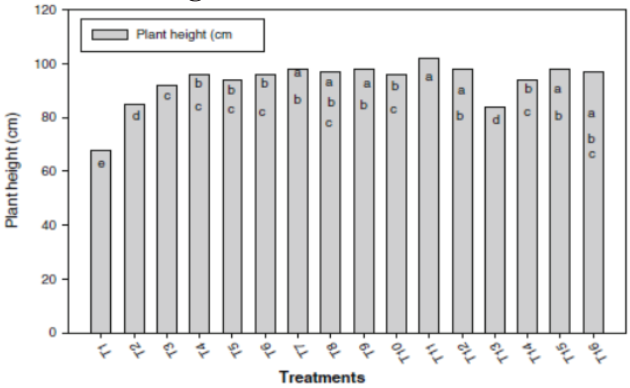
Graph 3 and 4 show nitrogen extracted by rice plants and plant dry mass at different variants, up to 100 % flowering. Like the number of tillers per plant and height nitrogenous fertilization along with Azolla influenced nitrogen extracted by plants and dry mass production.

Graph 5 and 6 show the influence of associated and/or incorporated Azolla on phosphorus and potassium contents in rice plants, with different nitrogen doses, where both nutrients presented similar behavior to that of nitrogen, positively influenced by N fertilization and Azolla. In general, combining both ways of using Azolla surpassed the remaining treatments, followed by variants where Azolla was associated to rice crop, and where fern was incorporated.

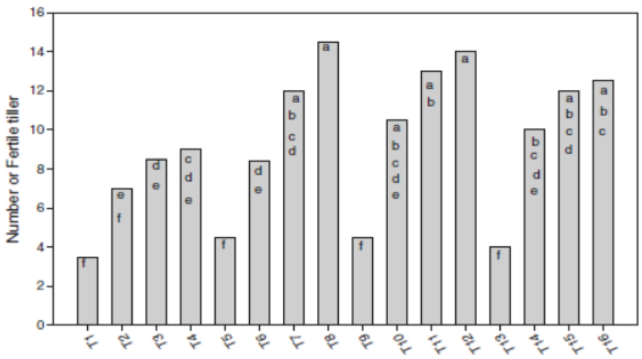
Regarding dry mass production and nitrogen content, in treatments where incorporated and associated Azolla were combined, value tended to be higher, compared to other treatments presenting the same nitrogen doses. This is caused by the influence of incorporated and associated Azolla on plant available nitrogen content in the soil, as a result of nitrogen release during fern decomposition, reduction in loss of nitrogen might have occured when applied as N fertilizer and that excreted into water by associated Azolla (de Macale et al. 2002).

The utilization of Azolla as a green manure benefits rice crop, obtaining the highest response when combining incorporation and association of this fern. It also favors nutrient absorption by rice and increases its yield, as well as organic matter content of the soil.

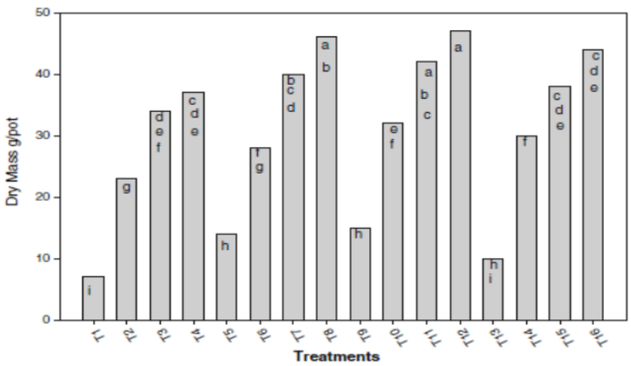
The Six figures are shown in below



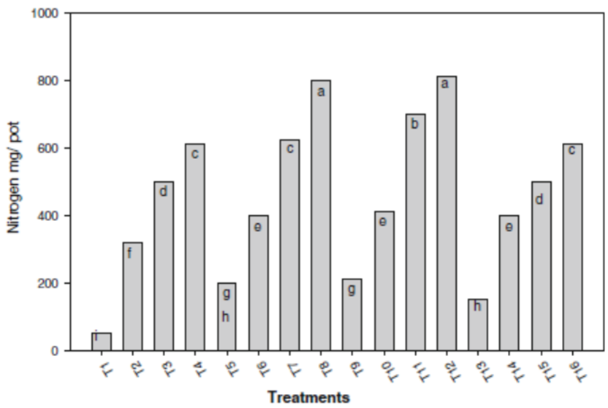
Graph 1



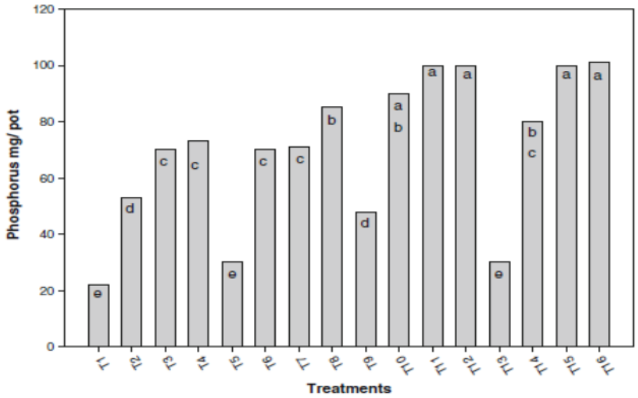
Graph 2



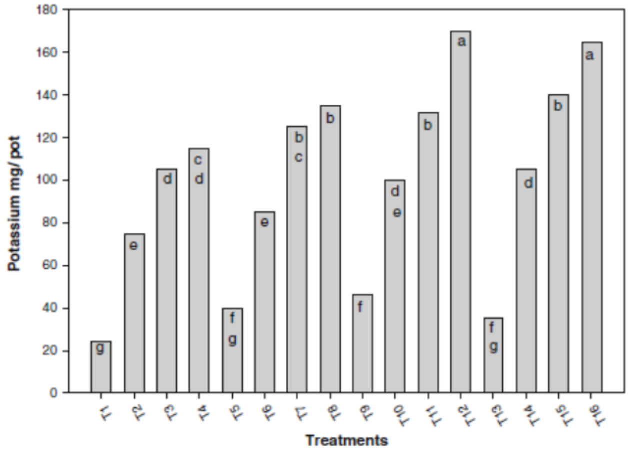
Graph 3



Graph 4



Graph 5



Graph 6

Feeding

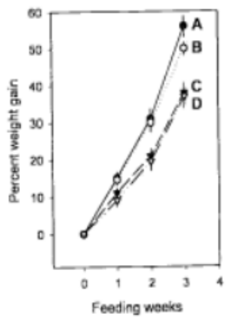
The experimental diets is shown in Table, The feeding results are expressed as the percentage of the weight gain of the fish. The control diet (A) containing 68.9% flour as filler exerted a slightly unfavorable effect on the percentage of weight gain (56.4%) in Tilapia for 3 weeks, compared with the diet containing a-starch and cellulose powder (Shiomi and Kitoh 1987b). Other diets with increasing concentrations of Azolla were not suitable. Diet B which contained 20.7% Azolla, displayed the same effect as the control for 2 weeks, then induced a 5.2% decrease in Tilapia weight after 3 weeks. Diets C and D exerte the same effect on the growth of Tilapia after 3 weeks of feeding with a 17% decrease of growth compared to the control. Diet A consisting of only flour as a filler would be ideal.

Component	Diet/composition (%)			
	A (control)	B	C	D
Fish meal	28.1	28.1	28.1	28.1
Flour	68.9	48.2	34.5	20.7
Azolla	0	20.7	34.5	48.2
Vitamin	2	2	2	2
Mineral	1	1	1	1

1 Vitamin mix for carps (Nihon Haigo Shiryo Co., Ltd.) was used. Mineral-SS (Tomita Pharmaceutical Co., Ltd.) was used.

Table 3: Compositions of Experimental Diets

Hence, the fact that the diet containing 20.7% of Azolla (B) induced the same effect on growth as that of the control is highly significant. Yada and Kawamura (1982) improved the food of Tilapia using various mixtures. They concluded that about 10% of commercial fish meal can be replaced by soybean refuse. From their standpoint, our present results show that Azolla can replace about 20% of Tilapia feed, which indicates the beneficial effect of the use of aquatic plants.



Graph 7: Shows the diet of Tilapia composition shown in the table 3.

Though Azolla could become a potential source of fish feed, factors such as nutritional value of various species or changes with aging, edibility, stable yield of Azolla, and storage techniques require further studies [14].

Nutrients Availability

Analysis of A.pinnata: The values of proximate composition of sun dried Azolla sample are presented in (Table No.4). The dry matter (DM) content of sun dried Azolla meal was 90.03 percent, Crude protein (CP) was 22.79 percent, ether extract (EE) was 3.59 percent, crude fibre (CF) was 15.49 percent, total ash was 19.46 percent, NFE was 38.67 percent, 2.03 percent calcium and 0.48 percent phosphorus.

S.No	Nutrient	%DM
1	Dry matter	90.3
2	Crude protein	22.79
3	Ether extract	3.59
4	Crude fibre	15.49
5	Total Ash	19.46
6	Calcium	2.03
7	Phosphorous	0.48

Table 4: Proximate Composition of Azolla Meal on DM Basis

Increase of milk yield: The present study was undertaken to know effect of feeding azolla on milk yield of lactating buffaloes under field conditions. The milk yield showed increasing trend and it increased to 9.30 L/day from 8.0 L/day after 60 days of feeding 1.5 Kg azolla per day with conventional feed cottonseed cake. (Table 5). Azolla fed group has not only increased the milk yield but also increased in physical health status and reproductive efficiency. It is concluded that feeding fresh green azolla with cottonseed cake enhanced the milk production and the animal starts showing excellent sign of health, such as improved hair coat condition, shining in skin, brightness in eyes, moist muzzle and always activeness.

Therefore, it can be used as a valuable green feed supplement for buffaloes, particularly under low input livestock production system, where livestock owners fed only cottonseed cake as concentrate or unbalanced concentrate ration, because the only single cake or unbalanced concentrate ration cannot fulfillthe nutrient requirement of animal. Azolla can fulfill the requirement of nutrients of animal with cottonseed cake. Azolla is important for feeding to buffaloes for milk production and health. So, we can produce more milk from indigenous buffaloes at low cost by feeding azolla with cottonseed cake [13].

Details of the Treatment

S. No.	Treatments	Milk Yield (Lit./Day)
1	T1 = As per farmers' practice (Wheat straw, paddy straw, grass, berseem and cotton seed cake)	8
2	T2= T1 + Deficiency of nutrients was fulfilled by providing green azolla meal (1.5Kg/day/animal)	9.3
3	Average Increase	1.3
4	% Increase	16.25

Table 5: Increasing Milk Yield and Treatments Given

Rich in Protein

Azolla contained 8.7% dry matter (DM). The proximate constituents and fibre fractions Azollla is given in Table. Azolla contained 21.37% crude protein (CP), 35.40% neutral detergent fibre (NDF) and 23.97% acid detergent fibre (ADF) on dry matter basis. (Table 6) The proximate composition of Azolla obtained in the present study was in similar range to values obtained by Ahirwar and Leela (2012). The concentration of calcium, phosphorus, copper, iron, zinc and manganese in Azolla were 0.58%, 0.44%, 17.15 ppm, 710.65 ppm, 77.30 ppm and 207.87 ppm, respectively, indicating to be a rich source of micro nutrients. The CP was comparable, while crude fibre (CF) content was lower in Azolla in comparison to Lucerne (16-25% CP and 20-30% CF, ICAR, 1998). Thus, it indicates that Azolla could be good source of protein having low fiber content compared to legume forages.

The *in vitro* dry matter digestibility, *in vitro* organic matter digestibility and metabolizable energy contents were 79.5%, 63.8 mg/200mg and 7.36 MJ/kg DM (1759 kcal/kg), respectively (Table 7). Several in vivo experiments indicated improvement in DM digestibility with replacement of 50 % of ground nut nitrogen in diets of buffalos (Indira et al., 2009) and 30 parts of ground nut in concentrate diet of Nellore Sheep (Ravindra reddy et al., 2011) [8].

Table: 6 Contents of the Nutrients

Nutrient	**%on DMB**
Dry matter	8.70±0.49
Crude protein	21.37±0.91
Crude fibre	12.73±0.50
Ether extract	2.36+0.21
Ether extract	2.36+0.21
Total ash	16.23±0.52
Nitrogen free extract	47.30±1.61
Neutral detergent fibre	35.40±0.64
Acid detergent fibre	23.97±1.01
Cellulose	12.15±1.47
Hemicellulose	11.43±1.47
Lignin	12.57±0.81
Calcium	0.58±0.07
Phosphorus	0.44±0.08
Trace minerals	**ppm**
Copper	17.15± 0.25
Iron	710.65± 23.31
Zinc	77.30± 2.95
Manganese	207.87± 22.46

Table 7: In Vitro Dry Matter Digistibility

Attribute	**Content**
In vitro dry matter digestibility (%)	79.55 +or- 0.26
In vitro organic matter digestability (mg/200mg)	63.8 +or- 3.36
Metabolizable energy (MJ/Kg)	7.36 +or- 0.21

Table 8: Protein Fractions of Azolla as per CNCPS System

	A	**b1**	**b2**	**b3**	**c**	**P value**
% protein	18.22b +or- 4.30	42.56a +or- 2.54	15.15b+or- 1.04	7.47 c+or- 0.48	16.61b +or- 2.32	0.001

Azolla being evaluated as protein supplement, its protein fractions were evaluated under CNCPS system The protein fraction B3 (PB3) having 80% Intestinal degradability in ruminants was 7.47% for Azolla. This finding is in accordance with who reported that protein supplements contain a small amount of PB3 which mainly included prolamine proteins such as zein protein in corn [15,16]. According to Van Soest metabolizable protein is defined as the amount of true protein or amino acids absorbed in the small intestine and specifically in ruminants, are represented by the amount of amino acids or protein of microbial or dietary origin absorbed from the intestine [17]. In this study, the metabolizable protein in Azolla was approx 84% of CP (PA+PB1+PB2+PB3) which implies the capability of Azolla as a protein supplement.(Table 8)

Conclusion

Azolla is useful for the bio-fertilizer to the agriculture and this azolla is so much helpful for the farmers who suffer to buy expensive fertilizers. Azolla bio-fertilizers are effective that the yield of the crop is increased. The utilization of Azolla as a green manure benefits rice crop, obtaining the highest response when combining incorporation and association of this fern. It also favors nutrient absorption by rice and increases its yield, as well as organic matter content of the soil.

Thus, dry Azolla can be used to supplement the nitrogen fertilizer supplement of lowland rice. Also, the full potential of Azolla can be realized when both fresh and dried forms of Azolla are considered for rice and other crop production. Azolla is a rich source of protein, minerals and other essential nutrients also the supplemental feeding of fresh green Azolla has positive effect on the milk production and economics. The low-cost supplementation of Azolla can also improve the mean economic returns from single cow through additional milk yield per month in the village. Azolla seems to be good replacer of protein from expensive sources such as fish meal depending on feeding habits of the species. This is due to proper corroboration between the activity pattern of the digestive enzymes in fish and the essential nutrients such as ω-6 fatty acids from Azolla diet. Also, the dietary Azolla supplementation shows to have a positive effect on growth performance of fish and reduce the cost of feeding from fish meal and fish oil diet. Azolla increases nutrient contents (N and P), as well as improves the biochemical composition and growth rate of Azolla sp. The N and P parameters were highest following organic fertilizer treatment compared with inorganic treatment. This fertilizer may be added to Azolla sp. grown in ponds to enhance its nutrition value, which enhances the protein content.

Therefore, Azolla pinnata can be used as an alternative feed to animal food. Azolla is a potential feed ingredient for livestock. Livestock easily digest it due to its low lignin content. Azolla can be mixed with concentrates or can be given directly to Livestock. So, Azolla is considered as the most economic and efficient feed substitute and sustainable feed for livestock Azolla easily grows in wild and can grow under controlled condition also. It can easily be produced in large quantity required as green manure in both the seasons – Kharif and Rabi. Azolla can fix atmospheric CO2 and nitrogen to form carbohydrates and ammonia respectively and after decomposition it adds available nitrogen for crop uptake and organic carbon content to the soil. The oxygen released due to oxygenic photosynthesis, helps the respiration of root system of the crops as well as other soil microorganisms. It solubilizes Zn, Fe and Mn and make them available to the rice. Azolla suppresses tender weeds such as Chara and Nitella in a paddy field. Azolla releases plant growth regulators and vitamins which enhance the growth of the rice plant. Azolla can be a substitute for chemical nitrogenous fertilizers to a certain extent (20 kg/ha) and it increases the crop yield and quality. Azolla increases the utilization efficiency of chemical fertilizers. It reduces evaporation rate from the irrigated rice field. Azollais a good bioremediation and can be used for the treatment of wastewater such as industrial effluents, sewage water etc. on a large scale, The wastewater treatment processes employing Azollaare advantageous over chemical methods, as the azolla treatment are eco-friendly and effective. Azolla can be used as an ideal feed substitute for cattle, fish, pig and poultry, apart from its utility as a bio-fertilizer for wetland paddy. It is popular and cultivated widely in other countries like China, Vietnam, and Philippines etc., and is yet to be taken up in India, in a big way. The production technology has to be standardized to the diverse and different agro-climatic zones of the country, to enable its wider spread. Plants are the basic source of medicines for the modern life sciences. The cheap cost, low incidences of adverse reactions when compared to modern pharmaceuticals are encouraging public and health care institutes to turn to plant medicines. Azolla pinnata was cultivated in tanks, and antibacterial activities were studied. A fair antibacterial activity was shown against the disease. This dissertation tells about the Azolla is the ultimate source of nutrients and minerals, this Azolla is the best feeding replacement for cattles, poultry, goats and other domestic animals. This Azolla feed shows the increase in the buffalo milk yield production. Azolla Bio-fertilizers are decrease the cost for farmers and gives financial support also and this bio-fertilizer increases the crop yield in rice, maize etc.. Azolla dry feed (pinnata) increases the Fish growth and useful for the supplement feeding. The Scientists may also plan to grow the Azolla plant in Mars planet because of the high protein value and digestibility. Azolla as Food for the Human beings are under trail period, by god's grace this may get successful results this feed can make to decrease the malnutrition in humans and every human being can stay healthy and may also decrease the hunger dies [18-39].

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