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COMPARATIVE STUDIES ON NUTRITIVE ANALYSIS AND CULTIVATION OF FOUR AZOLLA SPECIES

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ABSTRACT

In the present study estimation of proximate and nutritive analysis of four Azolla cultures were collected from Amravati research station, Guntur, Andhra Pradesh. The four species are *A. circinata*, *A. pinnata*, *A. rubra* and *A. Mexicana* were optimized for the estimation of biomass production, lipid, phenol, protein and amino acid content. The proximate and analysis reveals the production ranges are as follows Biomass production (11,500 to 18,000 kg⁻¹ DW), amino acid content (17.2±0.5 to 114.7±0.6), phenol (85.4±0.7 to 110.5±0.6) and lipid content (20.4±0.5 to 120.2±0.9) of Azolla species were studied. Growing of these Azolla species maximum yields was showed by *A. circinata* with 18000 kg⁻¹ DW after 90 days. The average production of amino acid concentration (114.9±0.6) was observed in *A. pinnata* species. *A. Mexicana* species containing the highest Protein (154±0.9), Lipid (120.2±0.9), Nitrogen (136.7±0.1) and Phenol (110.5±0.1) contents. Due to the high nutritive analysis of Azolla the green gold mine is useful for treatment of sewage, Biogas production, bio fertilizer and is also used as poultry feed and fish feed.

Keywords: Amino acids, Azolla, Lipid, Nitrogen, Protein.

Introduction

Azolla is a fastest growing, unique aquatic fern which floats freely on fresh water surface. As a small, flat, compact mass Azolla floats on water. It grows rapidly by doubling its biomass under optimum conditions for every three days. Worldwide there are nearly eight species of Azolla namely, *Azolla caroliniana*, *Azolla circinata*, *Azolla japonica*, *Azolla Mexicana*, *Azolla microphylla*, *Azolla nilotica*, *Azolla pinnata* and *Azolla rubra*. *Azolla pinnata* is a common species in India. It yields excellent quality of protein more than 4 to 5 times when compared to lucern and hybrid napier (Radhakrishnan *et al.*, 2014). Moreover, the production of biomass is 4 to 5 times more in comparison with hybrid napier and lucern.

Azolla is popularly known as Companion plant, because of its symbiosis with nitrogen fixing cyanobacteria (anabaena azolla) for nearly one thousand years in the fields of paddy. There is no other symbiotic relationship between cyanobacteria and a plant together during reproduction from generation to generation. Azolla-Anabaena super organism is unique, as they never depart for almost hundred million of years. It is used as bio fertilizer by rice farmers (Lumpkin & Plucknett, 1980). Nutritive analysis of dry and organic matter, ash content, crude fiber, cellulose, hemicellulose, lignin, calcium and magnesium were performed according to AOAC (1990) for the potential uses of Azolla (Mahadeva Rao, Nigusie Tesema, 2011).

Recent research studies revealed that the protein content of Azolla plant is 23% to

30% and also 55% of the amino acid (Gokcinar and Bekcan, 2015). The nutrient requirement for the optimum growth of tilapia depends on the quality of source, size or age of fish and the energy content of the diet. The average protein requirement varies between 45 and 50, proved by Stickney (1997). In tilapia diets, the minimum requirement of dietary lipid is 5% but by improved growth and efficiency to utilize protein has been reported that for diet with 10% to

15% (Ng and Chong, 2004). Azolla, as a valuable protein supplement for many species, which includes ruminants, poultry, fish and pig (Hasan, 2009). The wide distribution of Azolla from the regions of tropical and temperate, there are seven extant species. Some research studies suggested that the Azolla plant has crude protein content of 23% - 30% and

55% of amino acid (Gokeinar and Bekcan, 2015). Due to the high nutritional values of Azolla may be used in biotechnological and pharmaceutical industries.

Dry Azolla power is utilized as a feed ingredient from various species like, tilapia (Antonie *et al.*, 1986, Abou *et al.*, 2007; Bag and Mahapatra, 2011). The other forms of Azolla as fermented Azolla (Utomo *et al.*, 2001), Azolla protein concentrate (Sundarano, 2006), utilized as feed ingredients in fish diets. High nutritive content and lipid nano particles based drugs are also important (Prashant *et al.*, 2017; Manikandan and Sarumathy 2018; Mohd. Yaqub, 2014).

However the present study mainly focused on low cost cultivation methods and high nutritive values of Azolla species in our area. A number of fish ponds, poultry farms and farming organizations were located in and around Krishna and Guntur districts, Andhra Pradesh. This is

why we are selecting locally available Azolla species and were analysed and it is benefit to local farmers. The present study Azolla cultivation and nutritive values of the selected species needs to give more information to fish farmers, poultry industries and researchers.

Materials and Methods

Sample collection

In the present study the four different fresh Azolla species namely *A. circinata*, *A. pinnata*, *A. rubra* and *A. Mexicana* were collected from Amravati research station, Guntur, Andhra Pradesh. These species were further propagated in ideal ponds to provide natural day light, temperature conditions were monitored.

Cultivation of Azolla

Azolla was cultured in plastic trays (21.7 X 15.6 X 8.4 cm, surface area = 340 cm²) containing one litre of various media. The day/night temperature was 18±27°C and the light intensity was 15 klx with 16 h light periods. The standard medium (composition of synthetic nutrient solution) contained the following macronutrients: CaCl₂ ·2H₂O 1.00, MgSO₄ ·7H₂O 1.65, K₂SO₄ 0.50, and NaH₂PO₄ ·2H₂O 0.65 (in mmol L⁻¹). It also contained the following micronutrients: FeSO₄ ·7H₂O 27.0, MnCl₂ ·4H₂O 1.13, CuSO₄ ·5H₂O 0.08, ZnSO₄ ·7H₂O 0.19, Na₂MoO₄ ·2H₂O 0.05, and H₃B₃O₃ 5.77. The final pH of the medium was adjusted to neutral for further reaction.

Bio mass and chemical evaluation of Azolla

For the estimation of biomass, freshly collected samples were weighed and it was measured by Kg DW ha⁻¹. Sun dried Azolla sample was analyzed for proximate principles viz., dry matter, crude protein, ether extract, total ash, crude fibre and nitrogen free extracts as per the methods described by AOAC (2005).

Harvesting and feeding of Azolla

On the basis of initial quantity of culture added, environmental conditions and nutrition, Azolla's growth in the pond will be complete in about fifteen to thirty days period. Azolla can be harvested daily after the full growth. Plastic sieves can be used to harvest the biomass yield from the pond's surface. About 800 to 900 grams of fresh Azolla (mean yield per day in a season) can be produced from an area of 6 X 4 feet. Azolla can be fed to the livestock either in fresh or dried form.

Estimation of Amino acid concentration

For the estimation of amino acids fresh Azolla samples were grinded by using mortar and pestle with 6N HCl and transferred the contents to a test tube which was sealed latter. Later it was kept in an oven at 120°C for 24 hrs. The contents were filtered, flash evaporated and made up to volume with 0.05N HCl. The formed solution was filtered through a 0.45 µm pore size filter (Whatman, UK) and injected into an HPLC (Shimadzu-LC 10 AS). In this experiment amino acids were separated in a sodium ion exchange column (Shimadzu-CTO-6A) fitted with an oven maintained at 60°C. The post column derivatized amino acids were detected using a Shimadzu-FLD-6A-type fluorescence detector (Ishida *et al.*, 1981). Tryptophan was determined separately by a spectrophotometric method (sastry and Tammuru,1985).

Estimation of Total phenol and lipid content

One gram of material was used for dry weight determination after further oven drying at 105°C for 24 h of incubation, followed by 1 h cooling in a desiccator with activated silica. The material was Soxhlet extracted with a 7.5:1 dichloromethane-methanol solution

for 24 h with a water bath temperature of 80 °C. Then collected extracts were dried using a rotary evaporator and weighed to determine the amount of 'crude lipids'. After Soxhlet extraction plant residues were collected and dried. Total phenol was determined in triplicate after extraction of 100mg biomass with 10mL water–acetone (30:70, v/v) for 24 h at 20 °C using the reagent Folin–Ciocalteu assay in two technical replicates. (Waterman and Mole, 1994) Tannic acid was used as the calibration standard. Literature data, used for comparison, presented in gallic acid equivalents, were converted to tannic acid equivalents according to the relative reactivity with the Folin–Ciocalteu reagent.

Estimation of nitrogen and protein content

The oven-dried plant material was frozen in liquid nitrogen, ground and subsequently freeze dried. Total biomass nitrogen was analyzed in all growth replicates using the Dumas method (according to ISO 16634-1 analytical standard) and total amino acid analyses were performed according to the Ph. EUR 2.2.56 analytical standard (Silliker, Merieux Nutri Sciences, Ede, Netherlands).

Statistical analysis

Statistical analyses were performed by using SPSS 20 statistics software package. For determining the protein, Nitrogen and amino acid content in between the Azolla species and their effect was analysed by using a two-way analysis of variance (ANOVA). Differences were deemed significant for a *P*-value below 0.05.

Results and Discussion

In this study on the basis of growth yield and nutritive analyses for four Azolla species i.e. *A. circinata*, *A. pinnata*, *A. rubra* and *A. Mexicana*, a small production system was designed. By regular harvesting a part of the cultivation area, plants were kept within the linear growth phase to maximize productivity at 90 days of cultivation period. Azolla species were showed a high percentage of biomass content ranging from 11,500 to 18,000 Kg DW ha⁻¹. Biomass production was observed in 15 to 90 days of incubation periods represented in (Table-1). Various other studies are also reported that the important nutritional parameters like nitrogen and amino acid productions in (Morowat and Ghasemi, 2018; Kalita *et al.*, 2013)

Azolla circinata has highest biomass yield of 18,000 Kg DW ha⁻¹ followed by *A. pinnata* 13,000 Kg DW ha⁻¹. This concept was also tested by maintaining cultures of *A. filiculoides* and *A. pinnata* for over 100 days, while harvesting 33% of the surface area twice a week. *Azolla filiculoides* cultures equilibrated at around 1000 kg ha⁻¹ before harvest and 670 kg ha⁻¹ after harvest (Bhaskaran and kannapan, 2015). The present results with agreement of about 1,200 kg/100 m² of fresh *Azolla filiculoides* was harvested after culture for 97 days of the plant during cultivation, the density markedly affected the growth rate (Shiomi and Kitoh, 2001). Relatively Brouwer *et al.*, (2018) reported that the Azolla biomass production was high for 154 days of incubation period.

Table-1: Biomass yield of four Azolla species (Kg⁻¹ DW)

Days	<i>A. circinata</i>	<i>A. pinnata</i>	<i>A. rubra</i>	<i>A. mexicana</i>
15	750	550	450	500
30	6000	3700	4000	3200
45	12000	9000	7000	7300
60	16000	12000	9000	9600
75	17000	12500	10000	10500
90	18000	13000	11000	11500

*Each data is an average of three replicates. A considerable variation in the moisture content of four Azolla species was recorded i.e. *A. circinata* 96.44±0.25, *A. pinnata* 92.33±1.21, *A. rubra* 91.29±0.45, *A. mexicana* 98.77±0.34. The nitrogen content varied from 0.65 to 1.21 mg/10g for all the species studied. The total ash content ranged from 2 to 4 mg/10g was recorded. Similar reports were also reported by (Bhaskaran and kannapan, 2015).

Table-2: Proximate composition of Azolla species (g/100 g)

Azolla species	Biomass (90 days)	Moisture	Nitrogen content	Ash content
A. circinata	18000	96.44±0.25	1.21±0.01	4±0.03
A. pinnata	13000	92.33±1.21	0.78±0.05	3±0.05
A. rubra	11000	91.29±0.45	0.65±0.03	2±0.07
A. mexicana	11500	98.77±0.34	0.89±0.01	4±0.04

* The overall model is significant with p<0.05

Amino acid content was determined by the four species of *Azolla* was represented in Table 3. *A. pinnata* showed essential amino acid cysteine ranged from 17.2 ± 0.5 to 114.7 ± 0.6 . Maximum alanine production 70.2 ± 5.1 was recorded by *A. circinata*. There are twelve essential amino acids were represented in the study. Much variation was observed in the amino acid production by all the species studied. The tryptophan ranges from 39.7 ± 0.3 to 62.6 ± 0.8 . *A. mexicana* contain high proportion of glutamic acid (95.4 ± 0.1). Another species *A. rubra* contain high phenyl alanine content 47.6 ± 0.5 . The ideal pattern of adult plants was analyzed, compared to favourable and well exceeded with the except for phenylalanine and tryptophan was reported.

From the results it was tested for total phenols and lipids content of four *Azolla* species showed much variation. Maximum levels of phenol ($110.5 \pm 0.1 \text{ Kg}^{-1} \text{ DW}$), lipids ($120.2 \pm 0.9 \text{ Kg}^{-1} \text{ DW}$) was observed in *Azolla mexicana* (Table-4). The rest of the species also showed less quantity of phenols and lipids. The dry weight of *Azolla* species biomass content of lipids ($79-100 \text{ Kg}^{-1} \text{ DW}$) and phenols ($21- 69 \text{ Kg}^{-1} \text{ DW}$) showed maximum levels was reported by Brouwer *et al.*, 2018.

Table-3: Amino acid concentration in biomass of Azolla species

Amino acid	A. circinata	A. pinnata	A. rubra	A. mexicana
Alanine	65.7±0.1	70.2±5.1	59.2±0.1	62.4±0.5
Arginine	70.6±0.3	71.4±0.9	70.03±0.7	65.4±0.6
Cystine	102.3±5.2	114.7±0.6	103.4±0.4	98.0±0.8
Glycine	48.4±1.4	42.9±0.3	45.4±0.5	42.5±0.2
Glutamic acid	94.3±0.5	90.2±0.4	88.4±0.2	95.4±0.1
Isoleucine	39.4±0.3	36.6±0.7	32.0±0.6	39.3±0.7
Methionine	18.2±0.9	17.2±0.5	22.0±0.1	23.0±0.8
Proline	33.2±0.5	33.4±0.3	36.9±0.6	40.9±0.5
Phenyl alanine	40.6±0.2	45.7±0.5	47.6±0.5	48.3±0.3
Serine	76.0±0.1	50.4±0.5	72.4±0.2	69.5±0.2
Threonine	55.4±0.3	65.3±0.7	68.4±0.1	40.6±0.3
Tryptophan	62.6±0.8	39.7±0.3	49.4±0.4	55.7±0.7

* The overall model is significant with $p < 0.05$

Table-4: Total phenol and lipid content (Kg⁻¹ DW) of Azolla species

Estimation of phenol& lipid	A. circinata	A. pinnata	A. rubra	A. Mexicana
Phenol	85.4± 0.7	101.6±0.3	94.5±0.7	110.5±0.1
Lipid	20.4±0.5	50.5±0.2	60.5±0.8	120.2±0.9

* The overall model is significant with $p < 0.05$. From the results nitrogen and protein content of four Azolla species showed variations. Maximum nitrogen (136.7 ± 0.1) and protein (154.3 ± 0.3) was showed by A. mexicana (Table-5). Similarly the crude protein content determined was considerably high when compared with crude protein content of water hyacinth (*Eichhornia crassipes*) and *Azolla Mexicana* (Brouwer *et al.*, 2018). The major advantage of *Azolla* over established crops derives from the combination of nitrogen and high yields may expect. Considerable nitrogen and protein content was produced by all the four species studied.

Table-5: Nitrogen and protein concentrations Azolla species

Estimation of Nitrogen& Protein	A. circinata	A. pinnata	A. rubra	A. mexicana
Nitrogen	98.6±0.6	111.05±0.3	123.4±0.9	136.7± 0.1
Protein	132.2±0.7	120.7±0.5	115.7±0.8	154. 3±0.3

* The overall model is significant with $p < 0.05$

Conclusion

Results reveal that the cultivated four *Azolla* species showed much variation in their biomass production. Nutritive analysis of *A. mexicana* produced maximum content of nitrogen, phenols, Proteins and lipids. Due to high nutritional value of this *A. mexicana* useful for fresh feed stock and recommend for poultry and fish farms for better yield.

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