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Studies on biochemical profiling and bio-fertilizing efficacy of Azolla (*Azolla pinnata*) and Duckweed (*Spirodela polyrhiza*)

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Abstract
Aquatic plants have adapted to reside in water and extensively get used for human benefits. The nutritive value and bio-fertilizing efficiency of *Azolla pinnata* (Azolla) and *Spirodela polyrhiza* (Duckweed) were estimated in the present investigation. Both Azolla and Duckweed were observed to be high in nutritive values and rich in protein content, carotenoids, amino acids, anti-oxidants, starch and fibres. However, *Azolla pinnata* was observed to have prime nutritional qualities than *Spirodela polyrhiza.* Significant increase in growth of test plants (*Vigna unguiculata*) in terms of shoot and root length under the treatment with 10% and 20% of cellular extracts as compared to control condition confirmed their efficacy as bio-fertilizers even in low concentration of 20% extract. This study showed that both *Azolla pinnata* and *Spirodela polyrhiza* can be useful for quality nutrient supplement for humans as well as animals and can be applied as efficient environment-friendly and economic bio-fertilizers for plant growth.

Keywords: Azolla, duckweed, biochemical profiling, nutritive supplement, bio-fertilizer

Introduction
Azolla pinnata and *Spirodela polyrhiza* (Duckweed) are aquatic plants, Azolla being a pteridophyte and duckweed an angiosperm [30]. In this era, where the world is in continuous and never ending search for cheap and nutritional rich food supplement, these aquatic plants are emerging as a prominent source for nutrition rich supplements as well as for medical and agricultural benefits [11, 20]. Azolla is commonly referred as "Green Gold Mine" due to its nutrional value. Moreover, it is also known to be useful for biogas production, hydrogen rich fuel, biofertillizer production, water quality improvement, reduction to ammonia volatilization and as a controlling agent for mosquitos and weed [27, 72]. Several studies on Azolla have shown its great potential of nitrogen fixation in association with *Anabena azollae* [16]. Furthermore, it can be considered well for human consumption to enrich nutritional requirement for its nutritional value and ease of cultivation [73]. While, Duckweed has gained attention of many researchers because of its composition made up of large amount of essential amino acids along with macro and micro elements such as Ca and Cl [4, 36] with its tolerance to extreme conditions with high growth rate [28]. Even consumption of Duckweed is common with the local name 'Khai-Nam' in some regions of Southeast Asia, including Laos, Thailand and Myanmar [14]. These two plants are suitable to be used as bio-fertilizer, biofuel, livestock feed, nutrient supplement as useful for CO_2 sequestration, waste water treatment, rice production [7, 18, 33, 34, 44, 58, 70, 76, 77]. Several studies reported biochemical content and bio-fertilizing efficiency of Azolla and Spirodella. However, biochemical content and bio-fertilizing efficiency of different species of genus *Azolla* and *Spirodela* vary to a wide range. Hence, the present study was undertaken to to estimate the biochemical content of *Azolla pinnata* and *Spirodela polyrhiza* as well as effect of their cellular extract on plant growth in order to find their suitability for human/animal feed and bio-fertilizing efficiency.

Material and methods
Plant material
100 gm of *Azolla pinnata* was collected from K. K. Wagh college of Agricultural Biotechnology, Nashik and 200 gm *spirodella polyrhiza* was collected from Shri Swami

Samarth Pradhan Seva Kendra, Dindori, Nashik. Collected samples were subjected to multiplication in a tray with tap water for 25 days. After 25 days, sufficient amount of both plants was harvested for biochemical analysis and remaining plants were kept for multiplication.

Biochemical analysis

Estimation of total chlorophyll and carotenoid

Total chlorophyll content of Azolla and Duckweed was estimated according to procedure by Arnon, (1949) [9]. Where, Carotenoid contents were determined using method proposed by Duxbury and Yentach (1956) [24]. The absorbance of obtained solution was measured at 663 nm in the spectrophotometer for total chlorophyll content determination and for carotenoid estimation, pooled out supernatants was subjected to measure absorbance at 450 nm. The total chlorophyll and carotenoid content was calculated using following formula and output expressed in mg/ml.

Chlorophyll-a mg/gm tissue = 12.7 (A663) - 2.69(A645) × V/1000 x W
Chlorophyll-b mg/gm tissue = 22.9 (A645) - 4.65(A663) × V/1000 x W
Total chlorophyll mg/gm tissue = 20.2 (A645) + 8.02(A663) × V/l000 x W
Carotenoid content = D x V x f x 10/2500

Where,
A = absorbance at specific wave length
V = final volume of chlorophyll extract in 80% acetone
W = fresh weight of tissue extracted
D = absorption coefficient at 450 nm.
V = volume of extract in ml.
f = dilution factor.
2500 = average extinction coefficient of pigments

Estimation of total free amino acids and protein

Ninhydrin was utilized for estimation of total free amino acids from Azolla and Duckweed with reference to method of Moore and Stein, (1948) [43]. Three replicated trials were performed for each plant and the absorbance of obtained solution was observed at 570nm against blank reagent. Outcomes were determined in mg/gm of sample.

The protein content of Azolla and Duckweed ground samples were analyzed by method of Lowry *et al*, (1951) [41]. Folin reagent was added in three tubes of both plants containing initial solution and followed by mixing well. Blue color was developed in solutions and these solutions were analyzed in spectrophotometer at 660nm. Standard graph was prepared with bovine serum albumin and results were expressed in mg/ml

Estimation of total phenol, starch and cellulose content:

Total phenolic content was estimated using Folin-Ciocalteau reagent for Azolla and Duckweed with the reference to method proposed by Singleton and Rossi in 1965 [60]. Final extract solutions were analyzed in spectrophotometer at 650nm against blank reagent and further calculation were made on the basis of standard curve using different concentration of gallic acid as a check and results expressed in mg/ml. Whereas, Thayumanavan and Sadasivam's, (1982) [68] method was applied to determine starch concentration using anthrone reagent. After addition of anthrone reagent, initial solutions became red and intensity of red solution was measured in spectrophotometer at 630nm with blank reagent as standard. Standard graph was prepared for estimation of starch in mg/ml. Moreover, Cellulose content of both plants was estimated by method of Scott TA and Melvin EH, (1953) [55]. At the end of followed method, green color was observed and its intensity was measured at 620nm in spectrophotometer against blank reagent. Determination of cellulose concentration was done with standard graph in mg/ml.

Estimation of acid detergent fibre (ADF) and acid detergent lignin (ADL)

Both ADF and ADL were estimated by procedure reported by Goering HK and Van Soest PJ, (1975) [26]. Output of ADF was expressed in percentage (%) and total percentage of ADF was determined.

Plant growth analysis

The crop *Vigna unguiculata* was selected for study on effect of Azolla and Duckweeds biofertillizer extract in plant growth. For preparation of biofertillizer extract, 1kg of Azolla and Duckweed was boiled with 1 litter of distilled water for 15 minutes and then filtered out. The filtrate was considered as 100% concentration of the Azolla and Duckweed extract. Furthermore, 10% and 20% of extract was prepared from stock extract solution and stored under 0ºC - 4ºC.

Plant seedlings were supplemented with 10%, 20% of extract and normal tap water as a control. Growth parameters were analysed on 25th day after seed germination. Comparative study was carried out to check individual performance of Azolla extract and Duckweed extract with separate concentration of each, viz. 10% and 20%.

Results and Discussion

Azolla (*Azolla pinnata)* and Duckweed (*Spirodela polyrhiza*) have several versatile properties which make them suitable for nutritive supplement to humans, animal feeds, bio-fertilizer, biofuel, waste water treatment, bio-indicator, etc. [7, 8, 14, 52]. These are easy to grow and have short time period for doubling its biomass which make them suitable candidates for bio-production platforms [64]. Because of high nutritional properties, duckweed and Azolla are suitable for feeding a wide range of animals like broiler chicken, goats, sheeps, cattle, rams, buffalo calves, pigs, rabbits, horses, waterfowls, fishes, etc. [1, 6, 10, 12, 17, 21, 22, 23, 24, 28, 29, 36, 39, 52, 55, 56, 60, 64, 67]. Many duckweeds and Azolla have been suitable for human consumption as health food supplement [7, 8, 53]. Also, both these organisms have tremendous potential to be used as bio-fertilizers that enhance various crop plant growths [29, 48, 49, 61, 68, 71, 73, 77]. In the present study, we report the biochemical and nutritive value of Azolla and Duckweed for its suitability as human nutrient supplement and animal feed as well as their suitability to be used as bio-fertilizers for plant growth.

Biochemical and nutritive value of Azolla and Duckweed

The biochemical composition of Azolla and Duckweed was estimated in the present study. Both Azolla and Duckweed were observed to be rich in protein content, carotenoid, amino acids, phenolic antioxidants, starch, cellulose and lignin (Table 1). All these components confirm that these organisms can be good source of complete nutrition for humans and animals. There are many reports regarding the detailed biochemical properties of Azolla and Duckweed [2, 3, 5, 7, 8, 10, 13, 17, 31, 34, 37, 39, 41, 44, 46, 47, 58, 65, 66, 78]. The present investigation

also corroborates the earlier studies. Both Azolla and Duckweed were observed to be suitable for nutritive supplement. When the nutritive value and quality of both Azolla and Duckweed was compared, Azolla was observed to be better nutrient supplement than Duckweed as it contained more carotenoid, protein, phenolic antioxidants and fibre content.

Table 1: Biochemical Composition of Azolla and Duckweed

S. No.	Parameters	Azolla	Duckweed
1	Chlorophyll content (µg/g)	156.3±18.01	140.5±16.5
2	Carotenoid content (µg/g)	256.7±20.3	174.4±28.1
3	Amino nitrogen content (%)	5.804±1.2	4.858±0.73
4	Protein content (mg/g)	222.78±19.05	152.46±11.8
5	Total phenol content (mg/g)	73.02±9.1	70.47±11.3
6	Starch content (%)	4.91±0.8	3.736±0.48
7	Cellulose Content (%)	8.47±1.6	8.836±1.1
8	**Lignin content (%)**		
	A. Acid Detergent Fibre (ADF)	38±5.1	30±6.3
	B. Acid Detergent Lignin (ADL)	19.78±2.3	19.15±3.6

Efficiency of Azolla and duckweed as bio-fertilizer

The effect of Azolla and Duckweeds biofertillizer extract in plant growth was studied using the crop *Vigna unguiculata* at 10% and 20% diluted concentration of original extract (1kg FW/lit). This experiment showed that both Duckweed and Azolla have positive effect on plant growth (Table 2, Figure 1). Shoot length of Vigna plant increased significantly with 10% and 20% concentration of both Azolla and Duckweed bio-fertilizer extract as compared to the control (without any supplement), whereas root length increased only in 20% bio-fertilizer treatment of Azolla and Duckweed. There was no significant difference in root length under control and 10% bio-fertilizer treatment of azolla and duckweed. This showed enhanced root growth might not ensure better shoot growth.

When impact of different individual concentrations of Azolla extract on shoot and root length were compared, it was observed that there was statistically significant difference in root length between 10% and 20% bio-fertilizer extract, whereas such difference could not be obtained in case of shoot length of Vigna. Interestingly, shoot length under both the treatments with Azolla were significantly more as compared to control. However, in case of duckweed both root and shoot length were significantly different in case of both the treatments.

There are many earlier studies that reported positive impact of various bio-fertilizers including Azolla and Duckweed on plant growth [29, 48, 49, 61, 68, 71, 73, 77]. These studies reported the suitability of Azolla as well as Duckweed as bio-fertilizer. Our present investigation also confirms the finding of these earlier reports. In this study, there was an enhancement of shoot and root length upto 40% ans 71.4% with 20% Azolla biofertilizer and 50% and 42.8% with Duckweed biofertilzer, respectively (Figure 1B). Hence, it can be inferred that 20% diluted concentration of original extract (1kg FW/lit) of Azolla and Duckweed are suitable to be used bio-fertilizer for plant growth. The uniqueness of this study is that the diluted Azolla/Duckweed extract from fresh samples worked well at even low concentration i.e. 20% 1kg FW/lit extract. Also, the method of preparation that is used in this investigation is very easy and economic that needs no expertise, hence, can be used by any common man for application to their crop field along with irrigation.

Table 2: Effect of Azolla and Duckweed extract on growth of *Vigna unguiculate*

Treatment	Root length (cm)	Shoot length (cm)
Control (No supplement)	10.5 ± 1.2	15 ±1.8
Azolla extract 10%	11.5 ±1.8	18 ±3.7
Azolla extract 20%	18 ±1.9	21 ±3.9
Duckweed extract 10%	11 ±1.7	18 ±2.2
Duckweed extract 20%	15 ±1.6	22.5± 4.3

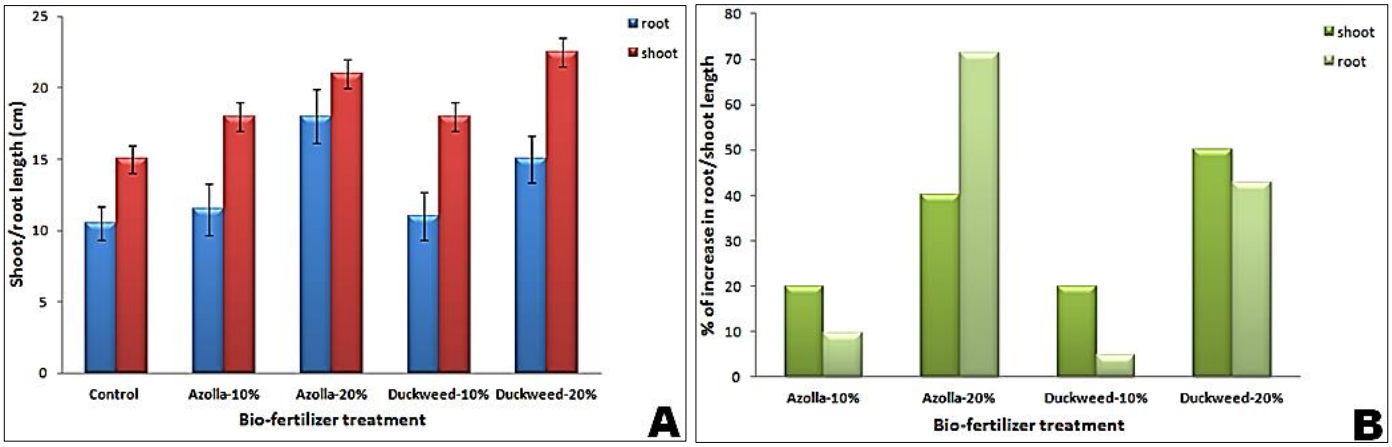

Fig 1: Effect of Azolla and Duckweed bio-fertilizers on growth of *Vigna unguiculata* in terms of root and shoot length. (A) Root and shoot length of *Vigna unguiculata* at 10% and 20% dilution of original bio-fertilizer extract. (B) Percentage of increase in root and shoot length with bio-fertilizer treatment as compared to control.

Conclusion

Azolla (*Azolla pinnata)* and Duckweed (*Spirodela polyrhiza*) are observed to be high in nutritive values and rich in protein content, carotenoids, amino acids and fibres. These can be useful source for quality nutrients for humans as well as can be used as animal feed. Also, both these organisms are potential, environment friendly and economic bio-fertilizers. The promotion of Azolla and Duckweed cultivation by small farmers and using them as bio-fertilizer in their own fields can be beneficial for developing countries like India.

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