RESEARCH ARTICLE

Effect of *Azolla caroliniana*: As Promising Biofertilizer for Improving the Growth and Yield of Rice

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Abstract

Biofertilizers play an important role in improving crop yield and quality. The uses of biofertilizer over chemical fertilizer have an attractive approach for developing sustainable goals and improving soil fertility without affecting its nutrient level. *Azolla* has proven to be an effective nitrogen-containing biofertilizer for rice yield because of having symbiotic association with cyanobacteria. The present study was conducted to determine the in vitro effect of *Azolla caroliniana* on rice yield. Experiments were conducted in triplicate under a completely randomized design and all the data were and analyzed by using ANOVA with statistically significant differences. The parameters used in this study were root height, shoot height, seed germination percentage, dry weight, fresh weight and pigment content. The result shows that rice seedlings treated with *A. caroliniana* showed increased plant height, grain weight, seed germination percentage and chlorophyll content. However, root height was reduced. Thus, the study concluded that the application of *A. caroliniana* led to the improvement of the growth traits of rice.

Highlights

- *Azolla caroliniana* is a small aquatic free-floating fern found mainly in stagnant water bodies.
- *A. caroliniana* has a symbiotic association with cyanobacteria and is used as an effective biofertilizer.
- Rice is an important staple food and dietary meal for people.
- Therefore can be used together without any difficulty and has the same growth condition as the Fern.

Keywords: Biofertilizer, Rice, *Azolla caroliniana,* cyanobacteria, nitrogen fixing bacteria, symbiotic association.

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Introduction

Rice is an important staple food and about half of the world's population consumes rice (*Oryza sativa* L.) in their dietary meal, more than half of people eat rice as a primary diet. Asia consumes 90% of the total rice production in the world (Khan, 2018). In India, more than 110,000 kinds of rice varieties are reported (Seckbach, 2006). The rise in the living standard of people demands high quality of rice. As of now, the increased use of chemical fertilizer at a geometric rate is linked to the rising productivity of rice; therefore, it is crucial to create environment-friendly alternative resources of nitrogen fertilizer (Khan, 2018). Bio-fertilizers, defined as mixtures of friendly microorganisms, help to support plant growth by providing the availability of nutrients. Some formulations comprise active or dormant cells of effective species of microorganisms that aid agricultural plants in absorbing nutrients through interactions with the rhizosphere. In sustainable farming, bio-fertilizers are primarily used to increase crop productivity and soil fertility (Adhikari *et al.,* 2020). The most common growth-promoting bacteria are phosphorus solubilizing bacteria (PSB) like *Pseudomonas, Bacillus,* mycorrhizal fungi, and cyanobacteria, used as biofertilizers. *Azotobacter* and *Azospirillum* are nitrogen fixers (Yasin *et al.,* 2012). Oppositely to synthetic fertilizers, biofertilizers are more beneficial and affordable because they produce the capability to fix atmospheric nitrogen and reproduce very fast and, strengthening soil structures; therefore, bio-fertilizers are viewed as an alternative to chemical fertilizers (Yasin *et al.,* 2012). India is a large producer of the cereal crop rice (Yasin *et al.,* 2012). Nitrogen-containing chemical fertilizers urea or ammonium sulfate, are commonly used by farmers for crop growth, but its effectiveness is less than 40% and also not good for human health. The use of pesticides and chemical fertilizers in agriculture practices is now a great concern due to their adverse effect on human health and the environment. To overcome such problems, countries are now focusing on bioorganic farming. A*zolla* could be used as a biofertilizer (Adhikari *et al.,* 2020), which can help in fixing the atmospheric nitrogen due to its symbiotic association with cyanobacteria which can reside in the leaf cavity of fern shown in Fig.1. *Azolla,* a free-floating, a widely dispersed aquatic fern has a lot of potential as a supply of nitrogen for rice farming and was first established in China, North Vietnam in 1957 as organic manure for rice. *Azolla* has been widely used as a biofertilizer due to its cheap source, rapid reproduction and high nitrogen content (3-6% N by dry weight) (Savaner *et al.,* 2024). Azolla, an aquatic fern, fixes atmospheric nitrogen in association with cyanobacteria. Its triangular-shaped fern has anabaena in the leaf pore cavity in association (Vanhove and Lejeune, 2002). *Azolla* double-weight within 3 to 5 days and fixes nitrogen as

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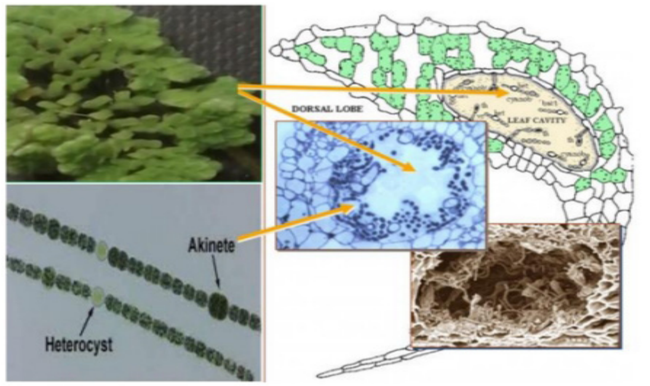


Fig. 1: *Azolla* dorsal leaf cavity shelter for Anabaena. (Figure modified from Carrapico, 2010)

high as 3 to 5 kg per day (Nurigevrek, 2000). Fern's high rate of reproduction and high rate of nitrogen fixation made it an excellent agronomic choice to meet the whole nitrogen needs of the rice crop in a matter of weeks (Ghadimi *et al.,* 2021). However, several other factors also affect the growth and nitrogen-fixing ability, like temperature, light, nutrient availability and time of inoculation (Oyange *et al.,* 2020). *Azolla* is supposed to be the best fertilizer for rice production because they both require the same growth and environmental conditions, like the need for a shallow, watery habitat, rapid growth, high nitrogen capacity for survival (Oyange *et al.,* 2020). The crop plant receives nitrogen from *Azolla,* which readily decomposes in the soil has improved the physical and chemical characteristics of the soil, especially nitrogen, organic matter, and other cations released into the soil, such as magnesium, calcium, and sodium, mineralization also raised the soil's microbiological status by enhancing the biological health of the soil and have been proven to be greatly improved by the application of fern (Bhuvaneshwari and Singh, 2015a). Keeping in the above view present publication includes the role of fern *Azolla caroliniana* on rice growth and yield.

Material and Method

Experimental Design

The experimental study was conducted at ISR, IPS Academy Indore. For the experiment design, the plastic pots were filled with 1 kg of vermiculite in triplicate. *A. caroliniana* collected from forest in the Mhow Region of Madhya Pradesh and maintained under laboratory conditions was used as a biofertilizer and promoter of rice growth and mixed with vermiculite before transplanting the seed. The treatments used for this study are shown in Table 1. The plants were grown in normal light to imitate field conditions.

Seed culture

The uniform seedling of rice was collected and used in study. Hundred healthy and uniform seed were placed into petri dishes containing a layer of cotton moistened with water (time to time) and kept for 10 days at 37℃ in the dark to promote germination. To ensure reproducibility, three replicates were employed; all experiments were conducted under the same conditions. The number of seed germinations was noticed and the germination percentage was calculated by the procedure given by (Abdulwehab *et al.,* 2015).

Table 1: Treatments of *A. caroliniana* as biofertilizers T1, T2 and T3 represent the test and C1, C2 and C3 represents the control

Experiments pots					
T1	*T2*	*T3*	*C1*	*C2*	*C3*
A. caroliniana + rice seed + vermiculites			Control + rice seed + vermiculites)		

Germinated seeds were incubated in a growth chamber for 20 days. Plants are monitored regularly and irrigated as per required in order to grow under carefully regulated conditions of temperature, light, and relative humidity. After 20 days of incubation, the morphological parameters such as root length, plant height, and pigment content, fresh and dry weight were calculated.

Shoot height of plant

The effect of *Azolla* on the shoot height of rice was studied under a glasshouse complex in a pot culture experiment. Ten plants were chosen from each pot and their lengths from top to bottom were measured by the method (Saud *et al.,* 2022).

Estimation of primary pigment and carotenoid content

The total chlorophyll pigment of plantlets was examined by the (Arnon, 1949) method. For estimation of chlorophyll pigment, 0.5 gm fresh leaves of rice plantlet were squashed in 80% acetone and supernatant was obtained after centrifugation at 5000 rpm for 12 minutes. The absorbance of the sample was recorded at a wavelength of 645, 663 and 420 nm, while 80% (v/v) acetone was used as a blank solution. The chlorophyll content was expressed as mg/gm of fresh weight. For carotenoids measurement the same plant extract is used to measure.

Root length of plant

The effect of *A. caroliniana* on the root length of rice was studied under a glasshouse complex in a pot culture experiment. Ten plants were chosen from each pot and their root lengths from top to bottom were measured by the method given (Saud *et al*., 2022). The average root length of the ten plants was then calculated.

Fresh and dry weight of plants

The effect of *A. caroliniana* on fresh and dry weight of rice plants was studied. After 20 days of incubation, fresh and dry weights were measured. Randomly ten plants from each pot (test and control) were selected for estimation of dry and fresh weight of both root and shoot after oven drying at 70^0C for 72 hrs to get constant weight (Adachi *et al.,* 2013).

Statistical analysis

Using ANOVA, parameters were statistically analyzed. The treated and control plants were correlated using the graph prism. All the experiments were conducted in a synchronized way in triplicates. Mean value and standard error for each experiment was calculated and data were shown as mean ± SE. Two-way analysis of variance (ANOVA) and GraphPad Prism

9 software with a significant difference P ≤ 0.005 was used to compare the means of different treatments.

Results

Effect of *A. caroliniana* on seed germination percentage

A. caroliniana significantly increased the seed germination percentage of rice, as evident in Fig.2. Results clearly show that a significant seed germination percentage was obtained in T1 and C1. Germination rate increases with time duration from 5 to 20 days of sowing.

Effect of *A. caroliniana* on shoot height of rice plant

The average height of ten plants was calculated, the results in Fig. 3 and images of the experiment in Fig.4 clearly show that *Azolla* treatments had a vigor effect on the shoot height of rice plants as compared to control plants. The maximum shoot height recorded in the treated plant was 27.4 cm and the control 22.7 cm after 20 days. However continuous increase in shoot height was observed in both the treatments and control plant from 10 to 20 days of plant cycle. Results show that all the treatments were able to increase the growth of plants over control.

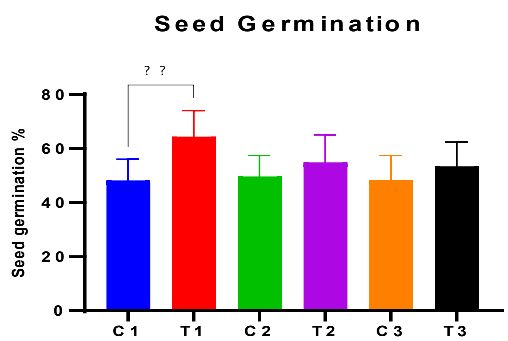


Fig. 2: Effect of *Azolla* treatments on seed germination of rice T1, T2 and T3 represent the test and C1, C2 and C3 represent the control. (*denotes significant differences among different cultivators)

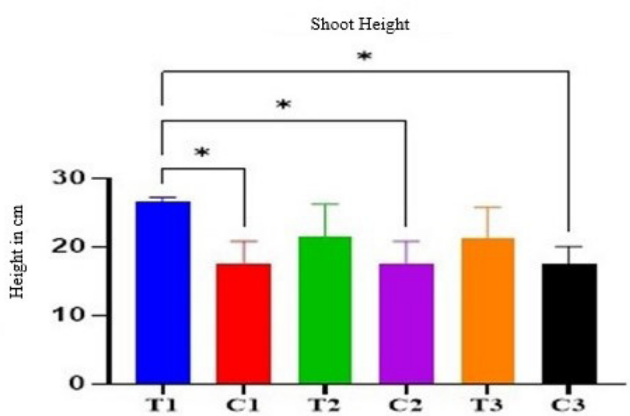


Fig. 3: Effect of *Azolla caroliniana* on shoot height of rice plant. *Denotes significant differences among different cultivators. T (1, 2, 3 replicates) represent test and C (1, 2, 3 replicates) represent control)

Effect of *A. caroliniana* on root length of plant

The average root length of the ten plants was calculated in the experiment. The results in Fig.5 clearly showed that in treated plants average root length was 10.7, 10.4 and 9.3 cm and the control was 14, 12.3 and 12.6 cm. The highest root length was observed in the control plant. *Azolla* does not show any significant impact on the root length of the test plant.

Effect of *A. caroliniana* on pigment content of plant

A. caroliniana as a biofertilizer was studied to assay the effect on pigment content. It is evident from data that treatments of *A. caroliniana* have significantly impacted the primary pigment of rice plants. The 20-day treated plants had the highest levels of pigments (*Chl a, Chl b*, total chlorophyll, and carotenoids) compared to the control. Results showed that maximum chlorophyll and carotenoid pigment was obtained in the test plant (Fig. 6).

Effect of *A. caroliniana* on fresh and dry weight

Effect of A. caroliniana on fresh and dry weight was also studied in the experiment. Results show that treatment plants have

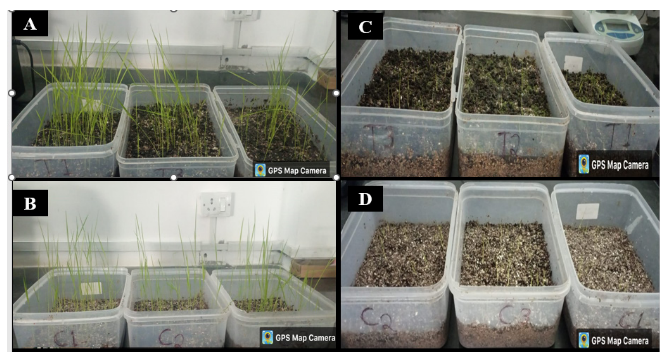
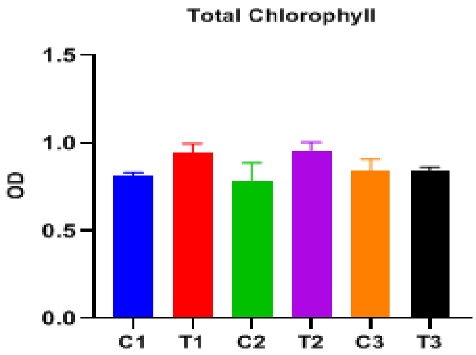


Fig. 4: Images of shoot height (cm) of rice plant. A (test) and B (control) after 20 days of sowing seed while C (Test) and D (control) after 6 days of sowing seed

Fig. 5: Image of root and shoot height of rice plant C is control and T is test after 20 days of seed sowing



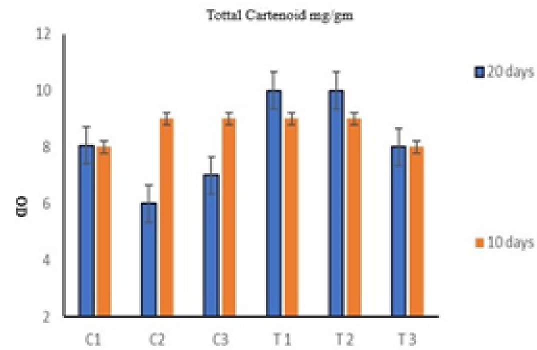


Fig. 6: Effect of *Azolla caroliniana* on total chlorophyll and cartenoid content of rice plant (mg/ gm- FW) (*denotes significant differences among different cultivators. Values are triplicates, mean± standard deviation. T1, T2 and T3 represent the test and C1, C2 and C3 represent the control

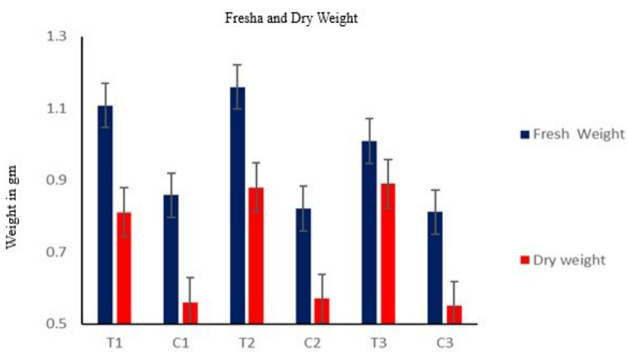


Fig. 7: Effect of *Azolla caroliniana* on fresh and dry weight of rice T (1, 2, 3 replicates) represent test and C (1, 2, 3) replicates represent control

recorded the highest fresh and dry weight when compared with control as shown in Fig. 7.

Discussion

It is possible to use minimal chemical fertilizers and pesticides while maintaining productivity. Numerous studies demonstrated that using biofertilizers can significantly decrease the use of chemical pesticides and fertilizers (Liu *et al.,* 2022). According to research, found that biofertilizers can replace 25% of chemical fertilizers without affecting the crop's quality and yield. Other studies show that applying biofertilizer with chemical N and P fertilizers that have been lowered by 50% can increase rice output (Jia *et al.,* 2020). The addition of biological fertilizer to chemical fertilizer raised the levels of grain protein and leaf chlorophyll while also increasing grain production (Naher, 2018). There is a lot of potential for the *Azolla* water fern to be utilized as organic fertilizer and the genera *Azolla* are well known for their potential to enhance plant growth. According to a comparable study conducted in Indonesia an application of *Azolla* on maize plants at a rate of 5 tons per hectare, the weight of the cobs might be raised by 20% (Prayoga *et al.,* 2020). Similar to this, applying *Azolla* to rice fields at a rate of 10 tonnes ha-1 increases the crop quantity by 9.06% (Simarmata *et al.,* 2021). *Azolla* fertilizer improved the quality of rice plants in terms of plant height root length, chlorophyll pigment, seed germination, and fresh and dry weight per plant (Bocchi & Malgioglio, 2010).

Past research looked at the impact of *Azolla* as a biofertilizer on the germination of rice seeds and other related processes. Growth metrics were seen to have greatly risen when compared to controls. Similarly, use of *A. caroliniana* in this study had a significant impact on a plant growth metric. Maximum plant height was observed on T1- 27.4 cm and T2- 27.3 cm, respectively. This is may be due to the N-fixing ability of *Azolla* and the release of growth-promoting chemicals like indole-3-acetic acid, gibberellins, and cytokinin, all are essential phytohormones are essential for growth and development (El-Shanshoury, 1995). Similar results were obtained by (Akhatar *et al.,* 2002). (Ghimire *et al.,* 2021), reported how rice plants responded to being inoculated with AMF and PGPB in both field and greenhouse settings. Our results are in agreement with (Bhuvaneshwari and Singh; 2015a) observed that the addition of *Azolla* to a dual-cropped system with rice also improved growth indicators like plant height, the number of tillers, and dry matter output when compared to the control. The nitrogen requirements of rice plants appear to be met by this treatment. According to earlier information, nitrogen content in the medium, which may be given in a variety of ways and methods, generally influences rice height (Bhuvaneshwari and Singh, 2015a). The photosynthetic capacity of the plant defines the yield and quality of a plant. The current study revealed that the *Azolla* inoculated plants had significantly higher chlorophyll content compared with the control. According to reports, rice plants that have received *Azolla* inoculation perform better in terms of photosynthetic rate, stomatal conductance, and specific relative chlorophyll concentrations (Doni *et al.,* 2018). In our study, the highest amount of chlorophyll was obtained in all treated plants T2 followed by T2 and T1. The reason of the rise may be associated with the function of *azolla* in enhancing leaf nitrogen content because it participates in the formation of porphyrin rings, which are essential for the production of basic chlorophylls for photosynthesis and respiration, as well as its involvement in the formation of the enzymes involved in photosynthesis, which act as cofactors for enzymes (Al-Bdairi & Kamal, 2021).

This explanation could be linked to how applying nitrogen fertilizer increased the amount of nitrogen in the plant. These findings are in line with those of (Barker & Pilbeam, 2015) (Thapa *et al.,* 2019), who claimed that the administration of nitrogen fertilizer resulted in a large increase in the amount of chlorophyll in the plant's leaves. In our study, the result shows a significant effect on seed germination in plants treated with *Azolla* as a biofertilizer. The highest germination percentage was obtained in T1 (78%) and the lowest germination percentage was obtained in control (59%).

All the treatments in the present study with biofertilizers registered higher germination percentages compared to control samples. These results are parallel with the findings of (Rajasekaran *et al.,* 2015) (Mohan and Rajendran, 2020) (Al-Bdairi and Kamal, 2021). This may be attributable to the action of biofertilizers in boosting the availability of nitrogen and phosphorus in the soil and making them available to the developing seed, which results in increased metabolic activity and seed germination (Mohan and Rajendran, 2020). Thus, applying biofertilizer to seeds not only promotes seedling growth but also significantly enhances overall seed germination.

In the present study effect of biofertilizer on root length was studied. There was no definite pattern of root height noted in response to biofertilizer treatments. The highest root length was observed in the control plant, which shows that *A. caroliniana* does not show a significant impact on the root length of a plant; a variable response of root biomass on applying biofertilizers was noted in the study. This might be possible because easily the availability of nutrients in the test plant needs to decrease in root length.

The fresh and dry weights are majorly affected by the biofertilizer applied. The highest mean of fresh weight was also found in treatment T1 and T2 followed by T3. Our results are in agreement with (Noraida and Hisyamuddin, 2021). Similar results were obtained by (Azmat *et al.,* 2020) on the application of bio-fertilizer on wheat under drought stress.

Conclusion

Azolla is free-floating magic fern rich in protein due to the wonder property of the symbiotic association and could be the best natural fertilizer used in controlled environmental conditions. Therefore, in our research, we observe the effect of *A. caroliniana on* rice growth in an *in-vitro* system. *The results* found *A. caroliniana* positively affected the physiological characteristics such as plant height, root height, seed germination, and the chlorophyll pigment in rice plantlets, as well as notably improved the development. The study suggested that the application of *A. caroliniana* can be a cheap and sustainable option for rice farmers to increase crop quality and production.

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Conflict of Interest

There is no conflict of interest among the authors or institution.

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