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Effect of Fresh Azolla (*Azolla pinnata*) on Growth and Yield of TR8 Rice Variety under Reduced Nitrogen Rates

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ABSTRACT

Nitrogen is required practically throughout the vegetative phase of rice especially during tillering and panicle initiation stage. Therefore, many synthetic fertilizers are required in rice cultivation. However, synthetic chemical fertilizers are extremely expensive. In addition, the use of chemical fertilizers has detrimental effects on the environment and human health over time. Thus, the use of Azolla as a biofertilizer can replenish nitrogen in the rice field. However, the effect of fresh Azolla in promoting TR8 rice variety growth and yield has not been reported. This experiment was conducted in the insect-proof rain shelter at the Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan Campus, Sabah, from August until December 2022. The experiment was laid in a completely randomized design (CRD) for six treatments with five replications. The treatments used in this study were: T1: 60 kg ha^{-1} N, 30 kg ha^{-1} P_2O_5, 30 kg ha^{-1} K_2O + 0% Azolla, T2: 50 kg ha^{-1} N, 30 kg ha^{-1} P_2O_5, 30 kg ha^{-1} K_2O + 10% Azolla, T3: 40 kg ha^{-1} N, 30 kg ha^{-1} P_2O_5, 30 kg ha^{-1} K_2O + 20% Azolla, T4: 30 kg ha^{-1} N, 30 kg ha^{-1} P_2O_5, 30 kg ha^{-1} K_2O + 30% Azolla, T5: 20 kg ha^{-1} N, 30 kg ha^{-1} P_2O_5, 30 kg ha^{-1} K_2O + 40% Azolla, T6: 100% Azolla (50 g). The rice plants were cultivated on the same planting medium of topsoil and goat dung with a ratio of 3:1. The plant height, number of tillers, panicle number per plant, spikelet number per panicle, grain weight per panicle and 1000 grain weight showed significant differences ($p<0.05$) among the treatments. Results revealed T4 recorded the highest number of tillers (41) compared to T6 (27, negative control) and T1 (32, positive control). There were 51% and 28% differences between T4 and T6 and T1, respectively. In addition, the T3 and T4 produced higher panicle number per plant (47%), spikelet number per panicle (9.4%), grain weight per panicle (27.1%) and 1000 grain weight (9%) compared to T6 (negative control), but showed a similar performance as T1 (positive control). Therefore, T4 is recommended for TR8 rice production with lower chemical fertilizer application with the addition of Azolla.

Keywords: Biofertilizer, Azolla, Chemical fertilizer, Rice growth, Rice yield

INTRODUCTION

Rice is the staple food for around half of the world's population, and it is grown primarily in Asia, which produces approximately 90% of the world's rice. But, roughly only 7% of rice produced in the country of origin is exported (Radin Firdaus, 2020). In 2020, Malaysia imported rice worth $ 620 million a year, primarily from Vietnam, India, Myanmar, Pakistan, and Thailand (The Observatory of Economic Complexity, 2020). Hence, the Ministry of Agriculture and Food Industries (MAFI) targeted in raising Malaysia's self-sufficiency level (SSL) target for rice production to 75% by 2025, an objective outlined in the 12th Malaysia Plan (12MP) to secure our food security. However, in 2020, rice SSL in Sabah was only at 22.81%, and by 2030, Sabah is also estimated to produce an average rice crop of 5 tons/ha/season with rice production of 234 000 tons/year or 60% SSL. To achieve the aim of SSL and higher yield in rice production, one of the initiatives that need to be done by the agro-food sector is to adopt more sustainable practices that protect the balance of the environment and ecosystem by ensuring that the industry's growth does not come at the cost of polluting and destroying the environment (National Agrofood Policy, 2021). Million tons of expensive synthetic fertilizers are required to cultivate such large regions and provide high crop yields for fast-rising populations and fulfil food requirements (Mahmoud *et al.*, 2022). In the long run, this excessive nitrogen fertilizer use can cause soil degradation and contamination (Thapa and Poudel, 2021).

Rice is a semi-aquatic annual grass that can thrive in a variety of soil types and water regimes, including irrigated systems, rain-fed lowland, upland, and flood-prone locations (Verheye, 2010). According to climatic conditions, it is separated into two subspecies which are Indica and Japonica. In addition, African rice (*Oryza glaberrima*) and Asian or common rice (*Oryza sativa* L.) are two cultigens (cultivated species) that developed separately from ancestral forms in West Africa and South and Southeast Asia (Supanit *et al.*, 2020). The rice plant has a main stem and several tillers when fully mature. A flowering head or panicle grows at the end of each productive tiller. Plant height varies depending on variety and environmental conditions, ranging from 0.4 m to over 5 m in some floating rice grains. Rice life cycle is separated into vegetative which is germination, seedling, and tillering stages, and reproductive phases including panicle initiation and heading stages (Mohapatra, 2021).

Azolla is an aquatic fern in the Salviniaceae family that grows in a symbiotic relationship with the blue-green algae Anabaena azollae. The name Azolla is derived from the Greek words azo (to dry) and allyo (to kill), implying that the plant will die if allowed to dry. Azolla contains about seven or eight living species and forty extinct species (Small *et al.*, 2011) (Figure 1). Thus, green manure rich in nitrogen, such as Azolla, can be used to replenish nitrogen in rice fields. Azolla is a unique plant that can help reduce man-made climate change and provide biofertilizer, livestock feed, food, and renewable energy anywhere in the world (Bujak, 2020). Azolla is a rich source of protein and essential amino acids (nitrogen 4% – 5%, phosphorous 0.5% – 0.9%) that contain several vitamins such as vitamin-A, vitamin B-12, and beta carotene. It is also rich in macronutrients and micronutrients such as calcium, phosphorus, potassium, magnesium, copper, zinc, others. Moreover, on a dry weight basis, the Azolla protein composition is around 25% - 35%. Nitrogen fixation potential of the Azolla-Anabaena system has been estimated to be 1.1 kg N ha^{-1} day^{-1} and one crop of Azolla provided 20-40 kg N ha^{-1} to the rice crop in about 20-25 days (Setiawati *et al.*, 2018).

Figure 1: Matured cultivated *Azolla pinnata*

Mineralization of organic nitrogen to ammonia is an important process in low-land rice cultivation. Azolla contributes to the long-term sustainability of soil nitrogen supplies by returning nitrogen to the soil in amounts equivalent to those absorbed by rice plants. Organic nitrogen content is made accessible to rice plants after decomposition. Field trials demonstrated the use of Azolla enhanced crop yield and crop N uptake significantly as compared to treatments without Azolla. Application of *Azolla pinnata* in the form of fresh and powder form results in high content of nitrogen up to 3 – 5% in rice production (Setiawati *et al*., 2018). Azolla helps to sustain soil nitrogen supply by returning N to the soil in quantities estimated equal to those extracted from soil by rice plants. The grain yield of rice under the incorporation of Azolla alone as well as in combination with inorganic fertilizer was significantly high (Khair *et al*., 2021). Chemical fertilizers and organic manure alone will not solve the critical challenge of sustaining soil fertility in tropical regions; biological inputs such as Azolla, particularly in rice cropping systems, must also be considered (Gaur, 2006). With that, this study was conducted to evaluate the effects of fresh *Azolla pinnata* in enhancing rice growth and grain yield.

MATERIALS AND METHODS

Study site and period of study

The planting was conducted in the insect-proof net house at the Faculty of Sustainable Agriculture (FSA) (38°31'52"N; 8º01'05"W), Universiti Malaysia Sabah (UMS), Malaysia. This study was conducted from 16th August 2022 until 23rd December 2022. A total of 100 seeds of the variety TR8 rice seeds were prepared for direct sowing. Then, three seeds were sown for each pot. After seven days of sowing (DAS), the thinning was performed and one plant for each pot for this study. The pots used were measured 34 cm in diameter and 30 cm in height.

Preparation of planting medium

There were 30 pots used in this study. All the pots were filled with a total of 11 kg of soil mixture. The planting medium used was mixture of topsoil and goat dung (3:1). The holes under the pots were covered with plastic to prevent water loss because lowland rice needed stagnant water.

Preparation of Azolla

Azolla pinnata was cultivated in a four-tank measuring 120 cm × 62 cm, where the depth of water was maintained at 50 cm in the insect-proof net house. The water in tank was fertilized with 500 g of goat dung. Then, 50 g of fresh Azolla was spread across the water surface in the tank within three weeks. Fresh Azolla was harvested using plastic sieves and was washed with clean water to remove excess nutrient water before being applied to the soil in the pot directly. The amount of fresh *Azolla pinnata* applied to each treatment on week 4 (28 DAS) were: T1, 0 g; T2, 5 g; T3, 10 g; T4, 15 g, and T5, 20 g.

Experimental design

The experimental design used in this study was a completely randomized design (CRD) arrangement of 5 replicates for each treatment. There were 6 treatments in this study (Table 1). The pots were placed at the spacing of 0.35 m × 0.65 m.

Table 1. Fertilizer treatments in the study

Treatment	Fertilizer rate
T1	60 kg ha^{-1} Nitrogen, 30 kg ha^{-1} Phosphorus (P_2O_5), 30 kg ha^{-1} Potassium (K_2O) + 0% Azolla
T2	50 kg ha^{-1} Nitrogen, 30 kg ha^{-1} Phosphorus (P_2O_5), 30 kg ha^{-1} Potassium (K_2O) + 10% Azolla
T3	40 kg ha^{-1} Nitrogen, 30 kg ha^{-1} Phosphorus (P_2O_5), 30 kg ha^{-1} Potassium (K_2O) + 20% Azolla

T4	30 kg ha^{-1} Nitrogen, 30 kg ha^{-1} Phosphorus (P_2O_5), 30 kg ha^{-1} Potassium (K_2O) + 30% Azolla
T5	20 kg ha^{-1} Nitrogen, 30 kg ha^{-1} Phosphorus (P_2O_5), 30 kg ha^{-1} Potassium (K_2O) + 40% Azolla
T6	100% Azolla (50 g)

Agronomic practices

Urea (N), Triple Super Phosphate (P), and Muriate of Potash (K) were used as fertilizers in this study. The recommended fertilizer rate by the Department of Agriculture, Sabah (2016) for the TR8 variety is 60 kg ha^{-1} N: 30 kg ha^{-1} P_2O_5: 30 kg ha^{-1} K_2O was used as a control (T1). The fertilizer was applied as a split application. The first application of fertilizer was on week 4 (26 DAS) and the second application was on week 10 (64 DAS). Azolla was applied one time at week 4 (28 DAS) with different rates according to treatments. Then, watering was carried out twice a day since the topsoil has low water holding capacity with 3 L amount of water per pot. During the reproductive stage of rice, watering was 2 L per pot once a day. The weeding activity was done manually by uprooting the weeds when needed. The rice grains were harvested at week 16 (107 DAS) at the physiological maturity stage.

Parameters of study

The vegetative growth parameters and yield components were recorded in this study. The plant height was measured from the soil surface until the highest point of the tips of tillers with the longest leaf using a measuring tape. The measurement of plant height was carried out once a week from week 2 (11 DAS) until the maximum height of rice can grow which was week 14 (95 DAS). Measurements of leaf length and width were recorded during the growth phase until the reproductive phase every week by using a measuring tape. Then leaf area was calculated using the data from the leaf length and leaf width. The number of tillers was taken every week by counting manually all culms that have three or more leaves. Productive tillers were manually counted and recorded as the percentage of productive tillers on a single panicle.

Rice grains were harvested at the stage of physiological maturity (107 DAS). Parameters of the rice yield components were recorded after harvest. The rice panicles (with spikelets) per plant were counted manually and collected from every pot. The panicle length was measured from the panicle neck to the apex. The spikelet was counted from the spikelet meristem manually. Grain numbers per panicle were recorded by threshing the rice panicle and then manually counting the grains. Then, the percentage of filled and unfilled grains was calculated from the collected grains. The grains per panicle weight, 1000-grain weight and grain yield per pot were recorded using an electronic weighing balance.

Statistical Analysis

The rice vegetative growth data was analyzed at week 14 while rice yield was analyzed after harvest with one-way analysis of variance (ANOVA) using Statistical Analysis System (SAS 9.4). Any significant differences between treatment means were then compared using the Least Significant Difference (LSD) test at $p < 0.05$ and presented in mean.

RESULTS AND DISCUSSION

Effect of fresh *Azolla pinnata* on the vegetative growth of TR8 rice variety

The efficacy of the fresh *A. pinnata*, a nitrogen-fixing cyanobacteria-associated floating fern, in substituting inorganic fertilizer (urea) on rice growth and yield was conducted. There were 6 treatments in this study each supplemented with different rates of urea and fresh *A. pinnata*, and a fixed amount of triple super phosphate (TSP) and Murate of potash (MOP). The growth of rice until harvested was shown in Figure 2.

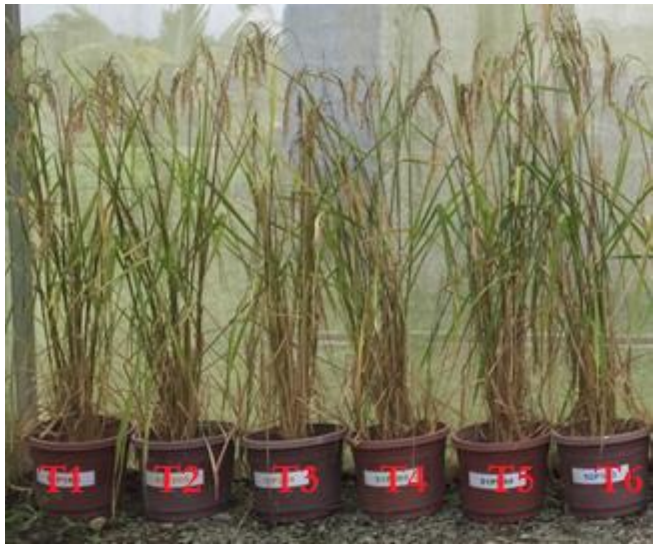

Figure 2: Rice plants reach physiological maturity stage on 107 DAS (From left: T1 until T6)

There was a significant difference in plant height between treatments ($p<0.05$). Treatment 1 (T1), treatment 2 (T2), and Treatment 6 (T6) showed a higher plant height ranged from 130.2-132.6 cm, while Treatment 3 (T3),Treatment 4 (T4) and Treatment 5 (T5) showed the lowest plant height of 123.0 cm. T6 showed comparable plant height as T1 and T2 (Table 2). Azolla has the necessary elements for plant growth which lead to the processes of cell division, elongation, and stimulating vegetative growth, including plant height (Al-Bdairi and Kamal, 2021). Plant height is an important agronomic trait of rice that directly affects crop yield. Plant height is strongly correlated with life span, seed mass, and time to maturity. However, increases in plant height after the vegetative phase commonly lead to postponed flowering time and ripening, thus will affecting crop yield (Zhang *et al.*, 2021). With the increase of nitrogen fertilizer application, the shortening degree decreased, and the plant height increased under high nitrogen fertilizer treatment, which may be related to the transport of stem sheath materials in the later stage of rice (Zhang *et al.*, 2020).

Table 2. Effect of fresh *Azolla pinnata* on the vegetative growth of TR8 rice variety

Treatment	Plant Height (cm)	Number of Tillers	Productive Tiller (%)	Leaf Area (cm^2)
T1	130.80^{ab}	32.20^{cd}	94.00^{ns}	117.16^{ns}
T2	130.20^{abc}	35.80^{bc}	89.04^{ns}	105.94^{ns}
T3	123.00^{d}	37.00^{b}	93.50^{ns}	121.95^{ns}
T4	125.20^{cd}	41.00^{a}	91.00^{ns}	107.16^{ns}
T5	125.60^{bcd}	31.00^{d}	93.30^{ns}	116.20^{ns}
T6	132.60^{a}	26.60^{e}	94.80^{ns}	102.46^{ns}

means with the same letter in each column are not statistically different at $p<0.05$. ns denotes non-significant at $p<0.05$

There was a significant difference ($p<0.05$) in the number of tillers between treatments (Table 2). T4 showed the highest number of tillers (41), while T6 shows the lowest (26.6). There was a 51% difference between T4 and T6. In contrast, there was no significant difference between T1 and T5, and between T2 and T3. Besides, T4 showed a significant difference between control treatments (T1 and T6). A rice tiller is a specialized grain-bearing branch that is formed on the un-elongated basal internode and grows independently of the mother stem called culm utilizing its adventitious roots (Li *et al.*, 2003). The production of rice tillers is affected by the phyllochrons (Veeramani *et al.*, 2012). A higher number of tillers can be achieved by incorporating Azolla either between nitrogen-potassium, or nitrogen-phosphorus showed comparable to NPK as additional fertilizers. This shows that without N, the number of tillers is significantly lower (Khair *et al.*, 2021). Rice absorbs nitrogen to improve its growth, development, yield, and grain quality. Azolla helps to sustain soil nitrogen supply by returning N to the soil in quantities estimated equal to those extracted from soil by rice plants. However, the rate of mineralization in Azolla is gradual. The slow rate of mineralization is due to the existence of lignified tissues, which make decomposition to be slow, leading to gradual availability. Therefore, T6 recorded the lowest number of tillers because of the low rate of available N in Azolla.

There was no significant difference in the percentage of productive tillers between treatments (p>0.05). All treatments were having more than 85% productive tillers (Table 2). The highly productive tillering ability can have a remarkable effect on rice production (Miller *et al*., 1991) because productive tillers are linked to the number of panicles. A higher panicle number per plant will result better in grain yield.

There was no significant difference in the leaf area between treatments (p>0.05) (Table 2). Leaf area growth determines light interception and is an important parameter in determining plant productivity (Koester *et al*., 2014) and biomass accumulation in rice plants. Therefore, an increase in leaf area means photosynthesis will also increase which will result in good vegetative growth. Azolla cultivation in rice fields increased plant height, number of effective tillers, leaf area, and dry biomass accumulation (Bhuvaneshawari and Pawan, 2014).

Effect of fresh *Azolla pinnata* on the yield components of TR8 rice variety

There was a significant difference (p<0.05) in the panicle number per plant (Table 3). The higher panicle number per plant was T4 and T3 with a mean of 37.2, and 34.75 respectively, while the lowest panicle number per plant was T6 with a mean of 25.2. There was a 47% difference between T4 and T6. Besides, T3 and T4 showed comparable panicle numbers per plant between the control treatments. Incorporated Azolla alone into rice cultivation had no improvement in the number of panicles (Khair *et al*., 2021). Therefore, T6 has a lower panicle number in this study. The rice panicle is closely associated with yield, given that it directly regulates the grain number (Duan *et al*., 2015). Oyange *et al.* (2019) revealed the incorporation time of Azolla in the soil at 21 DAT significantly increased the number of panicles and grain weight, which is similar to this study. Moreover, inorganic N application has been reported to enhance the growth, tillers, panicles number, and yield of rice (Yesuf and Balcha, 2014). Therefore, the balance rate of inorganic N and Azolla in T4 and T3 has been shown to produce positive outcomes in yield components of lowland rice of the TR8 variety.

Table 3. Effect of fresh *Azolla pinnata* on the yield components of TR8 rice variety

Treatment	Panicle Number per Plant	Panicle Length (cm)	Spikelet Numbers per Panicle	Grain Numbers per Panicle	Grain Weight per Panicle (g)	1000-grain weight (g)	Yield per Pot (g)	Filled Grains per Panicle (%)	Unfilled Grains per Panicle (%)
T1	30.20cd	35.06ns	15.00a	212.60ns	5.48a	26.48cd	133.48ns	91.60ns	8.40ns
T2	32.00bc	36.14ns	15.20a	224.40ns	5.80a	27.82a	140.58ns	893.80ns	10.20ns
T3	34.75ab	35.60ns	15.00a	215.75ns	5.70a	27.75a	148.40ns	89.00ns	11.00ns
T4	37.20a	35.14ns	14.60a	207.60ns	5.44a	27.68ab	144.90ns	91.40ns	8.60ns
T5	27.30de	37.96ns	14.30a	220.33ns	5.56a	26.56bc	138.80ns	93.30ns	6.67ns
T6	25.20e	35.56ns	12.80b	183.80ns	4.28b	25.40d	116.62ns	89.00ns	11.00ns

means with the same letter in each column are not statistically different at p<0.05. ns denotes non-significant at p<0.05

There was no significant difference in the panicle length between treatments (p>0.05) (Table 3). The panicle length primarily depends on how quickly the ranches get transformed into spikelets. Although the development of the rice varieties bearing numerous spikelets as panicle length increase leads to an increase in the sink size, it does not lead to any benefit in term of effective yield in rice cultivation (Sekhar *et al*., 2015). In this study, the application of fresh Azolla does not show a significant difference among treatments.

There was a significant difference (p<0.05) in the spikelet numbers per plant between treatments (Table 3). Treatments 1 – 6 showed significantly higher spikelet numbers per plant compared to T6 with only 12 spikelet numbers per plant. Spikelet number increases with increasing crop biomass and N concentration, and spikelet production efficiency per plant N decline with increasing plant N (Kamiji *et al*., 2011). In addition, spikelet number is also proportional to biomass production during the period from panicle initiation to heading. Consequently, it is important to provide sufficient N fertilizer to the rice plants which has a major effect on spikelet production in rice cultivation.

There was no significant difference in the grain numbers per panicle between treatments (p>0.05) (Table 3). Grain number is mainly determined by the panicle structure and branch differentiation. There is no additional N application was applied during the panicle initiation stage which makes no significant difference between mean grain numbers per panicle as shown in this study (Zhou *et al.* (2017).

There was a significant difference (p<0.05) in the grain weight per panicle between treatments (Table 3). The higher grain weight per panicle was 5.8 g for T1, T2, T3, T4, and T5 and the lowest was T6 with 4.28 g. There was a 35% difference between T4 and T6. All treatments show a comparable grain weight per panicle with T6 (100% Azolla, negative control). Moreover, the grain weight per panicle of T1 showed similar results to other treatments incorporated with Azolla. Grain weight per panicle is a varietal trait of secondary importance in determining rice yield. Grain weight is determined by the source capacity (photosynthetic leaves) to supply assimilate during the ripening period, and by sink capacity (developing grain) to accumulate the imported assimilate (Zhang *et al*., 2007). Cultivars with larger grain weights tend to have higher grain filling rates, resulting in higher assimilate accumulation and heavier grain weight. The increase in grain weight might be due to the efficient absorption of nitrogen and possibly other nutrients with Azolla, which increase the production and translocation of assimilates from soil (Teimour *et al*., 2018) as shown in this study.

There was a significant difference (p<0.05) in the 1000-grain weight between treatments (Table 3). The higher 1000-grain weight was T2, T3, and T4 with 27.75 g and the lowest was T6 with 25.4 g. This study showed comparable levels of 1000-grain weight between the control treatments. There was a 9% difference between T4 and T6. However, most of the rice varieties produced similar 1000-grain weight (Khair *et al*., 2021) between the treatments of fully NPK, treatment incorporated with Azolla and inorganic P+K, and fully Azolla treatment. Rice grown using the combination of Azolla and inorganic fertilizers can have better N use efficiency as a result of reducing N loss and enhancing N uptake by rice plants. Sufficient N availability can preserve green leaf area after heading, and consequently can enhance photosynthesis and improve grain yields (Mahmoud *et al*., 2022).

There was no significant difference in the percentage of filled and unfilled grain per panicle between treatments (p>0.05). The percentage of filled and unfilled grains has an average of 89 – 93% and 6 – 11%, respectively (Table 3). In this study, unfilled grain (%) was very low for all treatments, which shows a high percentage of filled grain. The application of Azolla in this study does not influence the percentage of filled and unfilled grains per panicle. Grain filling is the final stage in rice growth at which the fertilized ovaries develop into caryopses. The filing rate and extent determine the final weight of the rice grain. Generally, the upper or superior spikelet, flower earlier, fill faster and produce larger and heavier grains. On the other hand, lower spikelet flowers later, are either sterile or fill slowly to produce grains (Zhang *et al*., 2021). Hormone levels in the grain regulate rice grain filling (Zhang *et al*., 2016). Furthermore, the percentage of filled grains is controlled by temperature but not by fertilizers (Oyange *et al*., 2019).

There was no significant difference in the yield per pot between treatments and controls (p>0.05) (Table 3). Grain yield is largely determined by three characteristics which were grain weight, the number of grains per panicle, and the number of panicles per plant (Li *et al*., 2019). Although no difference among the treatments in this study, the Azolla can improve the efficiency of N usage in rice crops, increasing grain yield (Yao *et al*., 2018). The study showed that Azolla can partially replace the use of N inorganic fertilizer.

CONCLUSION

T4 (30 kg ha^{-1} N, 30 kg ha^{-1} P_2O_5, 30 kg ha^{-1} K_2O + 30% Azolla) produced the highest number of tillers (41) compared to T6 (27, negative control) and T1 (32, positive control). In addition, T4 also recorded higher panicle number per plant (47%), spikelet number per panicle (9.4%), grain weight per panicle (27.1%) and 1000-grain weight (9%) compared to T6 (negative control), but showed a similar performance as T1 (positive control). Therefore, T4 is recommended for TR8 rice production with lower chemical fertilizer application with the addition of Azolla. T4 was shown to have a balanced rate of inorganic fertilizer and Azolla was incorporated to increase a high yield in TR8 rice production. Azolla application can be viable for rice farmers to achieve environmentally friendly rice cropping systems, which helps reduce N chemical fertilizer usage. It is recommended to conduct an open field trial for fresh *Azolla pinnata* on the growth and yield of the TR8 rice variety. Due to the presence of other environmental variables, the performance of rice in the field study may vary.

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