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Rate of *Azolla microphylla* dry matter on nitrogen use efficiency and yield of Japonica rice

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Abstract The responses to the application of *Azolla microphylla* dry matter for nitrogen use efficiency for biomass (NUE) and nitrogen use efficiency for grain yield (NUEg) were investigated in two Japonica rice varieties. NUE and NUEg reduced with higher rated of Azolla application. The highest values were recorded at the rate of 37.5 kg N ha^{-1}, and the lowest values were recorded at 187.5 kg N ha^{-1}. DOA1 was higher than DOA2 for NUE, but they were similar for NUEg. Grain yields increased with higher rates of Azolla application. Grain yield was the lowest at the rate of 37.5 kg N ha^{-1} and the highest at the rate of 187.5 kg N ha^{-1}. DOA1 and DOA2 which were similar for grain yield.

Keywords: Azolla, Nitrogen use efficiency, Grain yield, Japonica rice

Introduction

Azolla (*Azolla* spp.) is a small aquatic plant commonly used as green manure and bio-fertilizer in rice fields in many Asian countries. Azolla has a symbiotic relationship with blue-green algae (*Anabaena azollae*) and can fix atmospheric nitrogen into a form useful to plants. *Azolla microphylla* has been recently developed by collaborative research of the Department of Agriculture and the International Rice Research Institute (IRRI). The newly developed species has a larger size, rapid propagation and high nitrogen fixation than the local species (*Azolla pinnata*) (Department of Agriculture, 2023). The farmers prefer to adopt new species, and the utilization of new species is expanded throughout the country.

Azolla can be used in fresh form and dry form. However, fresh Azolla is too bulky, and using fresh Azolla is not convenient although nitrogen contents are not different. Dry Azolla can be stored up to 3 years, but it should be

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incorporated into the soil before rice transplanting. Azolla decomposed in the soil faster than in water (Talley *et al*., 1977). Because Azolla has a low C/N ratio of about 10:1, it can be used immediately without transforming to the compost. In some flooded soils, ammonia was released rapidly within 2 weeks, and the release was slower after 2 weeks (Powlson and Addiscott, 2005). For effective use of Azolla, Azolla should not be applied at excessively high rates and phosphorus deficiency should be avoided.

As most soils in Thailand are low in organic matter and are at risk from nutrient loss through water runoff and leaching. Organic matter is a main source of micronutrients, and excessive and long-term use of chemical nitrogen fertilizer can cause micronutrient deficiency. Therefore, the use of fertilizers from organic sources is important for maintenance of soil fertility.

To improve soil fertility, organic matter from various organic sources is bulky, costly and difficult to manage. Green manure is another option for increasing organic matter in the soil in a relatively simple way. It will help add primary macronutrient elements such as nitrogen for plants. Nitrogen is an essential nutrient affecting growth, rice grain yield and the chemical quality of rice. Nitrogen use efficiency varies according to rice type, rice variety, soil fertility and other environmental factors in which the seedling stage has 30% nitrogen uptake (Wei *et al*., 2018). Application of excessive nitrogen is at risk for nitrogen loss and environmental pollution.

Nitrogen use efficiency (NUE) refers to the ability of plants to yield per unit of nitrogen in the uptake of nitrogen and convert it into yield. Application of nitrogen fertilizers before transplanting affected the increase in nitrogen use efficiency. Using Azolla as a nitrogen source can increase rice yield and nitrogen use efficiency. Because of its ability for biological nitrogen fixation, it can reduce nitrogen fertilizers. However, the information on the rate of *Azolla microphylla* dry matter is scant and not conclusive. The objective was to investigate the effects of *Azolla microphylla* dry matter on nitrogen use efficiency and yield of Japonica rice.

Materials and methods

Location and experimental design

The experiment was conducted during January, 2023 to April, 2023 at the Department of Plant Production Technology, School of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (Latitude: 13.7440 Longitude: 100.7920). Pot experiment was set up in a 2×6 factorial experiment in completely randomized design (CRD) with four replications in the open environment. Factor A had two Japonica rice varieties consisting of DOA1 and DOA2, and factor B had six rates of *Azolla microphylla* dry matter including 0.0, 37.5, 75.0, 112.5, 150.0 and 187.5 kg N ha^{-1} equivalent to 0.00, 1,136.31, 2,272.68, 3,409.06, 4,545.43 and 5,681.81 kg dry matter ha^{-1}. *Azolla microphylla* was harvested at 2 weeks after the pond was full and sun-dried for 2-3 days. Dry Azolla was then incorporated into the soil and incubated under soil capacity moisture content for 2 weeks prior to transplanting of rice crop.

Preparation of Azolla microphylla

Azolla microphylla was propagated in a pond on January 13, 2023, harvested at 14 days after the pond was full and sun-dried for 2-3 days. Sun-dried Azolla was weighted daily until the weight was constant. Total nitrogen concentration of 3.30% of dry matter *Azolla microphylla* (Suphanburi Land Development Station, 2014) was used as a reference for calculation of dry Azolla used in the treatments.

Crop management

Soil was obtained from a rice field, dried for a week under shade and crushed into small particles. The dry soil was loaded into rectangle cement pots with 30 cm in width, 100 cm in length and 30 cm in height. Soil was also sampled for analysis of the physical and chemical properties before planting. Soil samples with the same amount in all containers were collected, bulked into a pile and mixed thoroughly. The mixed soil sample was further divided into four piles, and one pile was used for soil analysis.

Two Japonica rice varieties consisting of DOA1 and DOA2 were used in this study. These varieties were released by the rice department, Thailand. Seeds were germinated by soaking in water for 24 hours and covered with wet cloth for 48 hours. Pre-germinated seeds were planted with 3 seeds per hill in seedling trays for 25 days, and the seedlings were transplanted into the rectangle cement pots on January 27, 2023. Twelve treatments including six rates of *Azolla microphylla* dry matter were prepared for two weeks before transplanting. In this experiment, no chemical fertilizers were used but six rates of Azolla dry matter were added according to the soil analysis value before transplanting.

Azolla microphylla green manure at the predetermined rates according to the treatments was mixed into the soils, and the mixed soils were loaded into the rectangle cement pots. The mixed soils were then incubated under field capacity condition for 2 weeks before transplanting. After incubation, the mixed soils in all pots were randomly sampled at the depth of 0-15 cm and analysed for ammonium ion (NH_4^+) by the Kjeldahl method (Bryson and Mills, 2015). Irrigation water was monitored and supplied at the controlled level at 10 mm above the soil surface throughout the experimental period, and the application of irrigation water was terminated at one week before harvest.

Data collection

The crop was harvested at 114 days after transplanting on January 27, 2023. Data were recorded at harvest for biomass and grain yield. The samples were oven-dried at 60-65 °C for 72 hours or until dry weights were constant, and the dry weights of the samples were recorded. The data were derived for nitrogen use efficiency (NUE) and nitrogen use efficiency for grain yield (NUEg) were calculated as follows:- NUE = Biomass (kg ha^{-1})/ N applied (kg ha^{-1}), and NUEg = Grain yield (kg ha^{-1})/ N applied (kg ha^{-1}).

Data analysis

The data were analyzed statistically according to a 2×6 factorial experiment in a completely randomized design. The differences among treatment means were compared by Duncan's New Multiple Range Test (DMRT) at 0.05 probability level. All statistical analyses were accomplished using M-STATC program from Michigan State University (Bricker, 1989).

Results

Nitrogen use efficiency for biomass (NUE)

Differences among the rates of Azolla application, rice varieties and the interaction between variety and the rate of Azolla application were significant ($P \leq 0.01$) for NUE at harvest (Table 1). Application of Azolla at the rate of 37.5 kg N ha^{-1} had the highest NUE (70.30 kg biomass/kg N applied), whereas application of Azolla at the rate of 187.5 kg N ha^{-1} had the lowest NUE (18.95 kg biomass/kg N applied). NUE reduced with the high rates of application and it was lowest at the highest rate (187.5 kg N ha^{-1}).

DOA1 had higher NUE (44.57 kg biomass/kg N applied) than DOA2 (28.68 kg biomass/kg N applied). Two Japonica rice varieties responded to Azolla application in a similar pattern, Application of Azolla at the rate of 37.5 kg N ha^{-1} for DOA1 had the highest NUE (88.54 kg biomass/kg N applied).

Table 1. Means for nitrogen use efficiency for biomass (NUE) of two Japonica rice varieties at harvest as affected by different rates of Azolla application

Rate of Azolla application (R) (kg N ha^{-1})	NUE (kg biomass/kg N applied)		Mean
	Variety (V)		
	DOA1	DOA2	
0.0	-	-	-
37.5	88.54^{a}	52.06^{b}	70.30^{A}
75.0	45.41^{c}	32.45^{e}	38.93^{B}
112.5	40.34^{d}	22.11^{f}	31.22^{BC}
150.0	28.05^{e}	19.43^{f}	23.74^{CD}
187.5	20.55^{f}	17.35^{f}	18.95^{D}
Mean	44.57^{A}	28.68^{B}	
F-test			
Variety (V)		**	
Rate of Azolla application (R)		**	
V x R		**	
C.V. (%)		20.70	

ns = non significant, ** = significantly different at $P \leq 0.01$, Means within the same column followed by the same letter are not significantly different by DMRT

Nitrogen use efficiency for grain yield (NUEg)

Significant differences ($P \leq 0.01$) among the rates of Azolla application were observed for NUEg at harvest, and difference between Japonica rice varieties was not significant (Table 2). Application of Azolla at the rate of 37.5 kg N ha^{-1} had the highest NUEg (57.86 kg biomass/kg N applied), whereas application of Azolla at the rate of 187.5 kg N ha^{-1} had the lowest NUEg (17.16 kg biomass/kg N applied). Similar to NUE, NUEg reduced with high rates of Azolla application, and it was lowest at the highest rate (187.5 kg N ha^{-1}).

DOA1 and DOA2 were not significantly different for NUEg (32.20 kg biomass/kg N applied for DOA1 and 30.14 kg biomass/kg N applied for DOA2). Two Japonica rice varieties responded to Azolla application in a similar pattern, and the interaction between variety and the rate of Azolla application was not significant.

Grain yield

Rates of Azolla application was significantly different ($P \leq 0.05$) for grain yield at harvest, whereas Japonica rice varieties were not significantly different (Table 3). Application of Azolla at the rate of 187.5 kg N ha^{-1} had the highest grain yield (3,217.2 kg ha^{-1}), and application of Azolla at the rate of 0.0 kg N ha^{-1} had the lowest grain yield (2,136.0 kg ha^{-1}). In contrast to NUE and NUEg, grain yield increased with high rates of Azolla application, and it was highest at the highest rate (187.5 kg N ha^{-1}).

Two Japonica rice varieties were not significantly different for grain yield (2,652.8 kg ha^{-1} for DOA1 and 2,683.9 kg ha^{-1} for DOA2). Two Japonica rice varieties responded to Azolla application in a similar pattern, and the interaction between variety and the rate of Azolla application was not significantly differed.

Table 2. Means for nitrogen use efficiency for grain yield (NUEg) of two Japonica rice varieties at harvest as affected by different rates of Azolla application

Rate of Azolla application (R) (kg N ha^{-1})	**NUEg (kg biomass/kg N applied)** Variety (V)		**Mean**
	DOA1	DOA2	
0.0	-	-	-
37.5	61.04	54.69	57.86^{a}
75.0	37.49	33.39	35.44^{b}
112.5	26.63	25.59	26.11^{bc}
150.0	20.35	18.21	19.28^{c}
187.5	15.50	18.82	17.16^{c}
Mean	32.20	30.14	
F-Test			
Variety (V)	ns		
Rate of Azolla application (R)	**		
V x R	ns		
C.V. (%)	24.33		

ns = non significant, ** = significantly different at $P \leq 0.01$, Means within the same column followed by the same letter are not significantly different by DMRT

Table 3. Means for grain yield of two Japonica rice varieties at harvest as affected by different rates of Azolla application

Rate of Azolla application (R) (kg N ha^{-1})	**Grain yield (kg ha^{-1})** Variety (V)		**Mean**
	DOA1	DOA2	
0.0	1,862.7	2,409.3	2,136.0^{b}
37.5	2,289.0	2,050.7	2,169.8^{b}
75.0	2,811.7	2,504.3	2,658.0^{ab}
112.5	2,996.0	2,879.0	2,937.5^{a}
150.0	3,052.3	2,731.0	2,891.7^{ab}
187.5	2,905.3	3,529.0	3,217.2^{a}
Mean	2,652.8	2,683.9	
F-Test			
Variety (V)	ns		
Rate of Azolla application (R)	*		
V x R	ns		
C.V. (%)	25.75		

ns = non significant, ** = significantly different at $P \leq 0.01$, Means within the same column followed by the same letter are not significantly different by DMRT

Discussion

Nowadays, Azolla is important as a natural source of nitrogen in rice cultivation in modern agriculture. The purposes of Azolla application are to reduce application of inorganic nitrogen fertilizer, to provide sufficient nitrogen to the crops and to increase soil organic matter. Application of Azolla in agriculture has benefits in reducing environmental pollution, improving soil fertility and increasing crop productivity in the long term. Incorporation of organic matter to the soil is therefore very important. Organic fertilizers can add nitrogen to rice plants at all growth stages and increase organic matter in the soil. Green manure, glucose and rice straw increased the organic acids in the waterlogging soil (Yoshida, 1981). The use of Azolla as a green manure is therefore an alternative means to increase yield for Japonica rice, to protect the environment and substitute nitrogen fertilizers to a certain extent. With proper management, approximately 50 kg ha^{-1} of nitrogen from Azolla could be applied to rice crops, and using Azolla alone increased rice yield by 50% (Adhikary and Shrestha, 2018). The use of Azolla at the rate of 10-12 t ha^{-1} could increase the amount of nitrogen in the soil by 50-60 kg ha^{-1} and could reduce the use of nitrogen fertilizers by 30-35 kg ha^{-1} (Kandel *et al*., 2020).

The soil used in this study is clayey soil collected from a rice field in the Central plain of Thailand. Soil analysis indicated that application of the highest Azolla rate (187.5 kg N ha^{-1}) considerably increased ammonium ion in the soil from 162.45 to 269.89 mg/kg in the plots planted with DOA1 and from 190.06 to 245.32 mg/kg in the plots planted with DOA2 (Data not shown). Under waterlogging conditions, ammonium is the main form of nitrogen. Nitrogen from Azolla dry matter under aerobic conditions was released rapidly within 2 weeks and Azolla rapidly decomposed in the soil at 3 to 8 weeks after application and released 56% to 80% of ammonia nitrogen (Khan, 1983). The rapid release of nitrogen from Azolla is due to low C/N ratios.

According to Chattha *et al*. (2022) legume green manure with a low C/N ratio promoted the mineralization of organic nitrogen, leading to higher NUE. Compact soil under waterlogged conditions caused have denitrification process, while coarse soils had more nitrogen leaching or evaporation. At higher temperatures, the soil nitrogen content is reduced by 5-10% due to increased mineralization (Govindasamy *et al*., 2023). This study compared the effects of six rates of Azolla dry matter on nitrogen use efficiency and yield of Japonica rice.

Nitrogen use efficiency (NUE) for biomass and nitrogen use efficiency for grain yield (NUEg)

The responses of Japonica rice varieties to the rates of Azolla application for NUE and NUEg were in a similar pattern. NUE and NUEg reduced with high rates of Azolla application, and they were highest at the highest rate of 187.5 kg N ha^{-1}. The results imply that the more application of Azolla dry matter the more reduction in nitrogen use efficiency.

Low NUE and NUEg at high rates of Azolla application would be due mainly to high nitrogen loss. When rice receives about 40% of the total nitrogen, nitrogen is taken up in the form of nitrate (Lee, 2021). Crops are not able to utilize more than half of the nitrogen applied into the soil, and NUE decreases with high rates of nitrogen application (Dobermann, 2005).

Application of Azolla biofertilizer has a tendency to increase NUE better than application of inorganic nitrogen fertilizer because of its potential for biological nitrogen fixation. Increasing NUE in low-nutrient areas can reduce the use of chemical fertilizers. In South America, Africa, and Asia, a decrease in NUE was reported because of the lack of crops with biological nitrogen fixation. The application of 25% urea fertilizer at the rate of 225 kg N ha^{-1} in place of Azolla was an interesting approach to increase NUE, rice yield and simultaneously reduce N loss (Yao, 2018). A fertilization rate of 250 kg ha^{-1} gave the highest grain yield and nitrogen accumulation (Hu *et al*., 2023). Wan *et al*. (2023) recommended to use of fertilizer at the rate of 180 kg ha^{-1} for high grain yield, NUE, and protein yield. Khemtong *et al*. (2023) reported that application of sunn hemp as green manure caused significantly higher NUE, tiller number, leaf area index, crop growth rate, biomass and grain yield than the application of urea, and it could promote the growth and yield of Japonica rice cv. DOA1.

DOA had significantly higher NUE than DOA2, but these Japonica rice varieties were similar for NUEg. The results indicated that DOA1 accumulated biomass in response to Azolla application better than DOA2, and DOA2 showed better performance in transforming biomass to grain yield. Therefore, NUEg values of these Japonica rice varieties were not significantly different.

NUE is under genetic control, and phenotypic difference can affect nitrogen uptake (Han *et al*., 2015). NUE also depends on environmental factors such as the cropping system and the rate of nitrogen used (Ortiz-Monasterio *et al*., 1997). Lee (2021) found that Indica rice and Japonica rice were different in NUE and nitrate absorption. High NUE indicates that high proportion of nitrogen applied to the crop is used for biomass production, and high NUEg indicated that high biomass produced is transformed to economic yield. Rice varieties with high

NUE are preferred because they need lower nitrogen rate to produce higher biomass, and production costs can be reduced.

The interaction between variety and rate of Azolla application was not significant for NUEg. Similar responses to the rates of Azolla application would be due to the fact that these Japonica rice varieties were selected for adaptation to growing environments in Thailand, and the number of varieties in this study was limited to only two varieties.

Grain yield

Grain yields increased with high rates of Azolla application and were highest at the highest rate 187.5 kg N ha^{-1}. Continuous nitrogen uptake is important for obtaining high crop yield. Compost and manure are good sources of nutrients like phosphorus and potassium. Because compost releases nitrogen slower than chemical fertilizers, the plants applied with organic nitrogen sources can obtain sufficient nitrogen in the latter growth stages (Yoshida, 1981).

For yield increase, compost can be applied to rice crop at the rates between 8 and 30 t ha^{-1}, and about 20 kg of nitrogen will produce 1 t ha^{-1} of rice. Therefore, application of high nitrogen rates increases rice yield. Zhu *et al*. (2017) reported that application of nitrogen fertilizer at the rate of 270 kg ha^{-1} caused the highest yields of two Japonica rice varieties, and high nitrogen rates also reduced nitrogen agronomic efficiency.

Yield increases as affected by nitrogen application is related to change in important yield components. Li *et al*. (2009) found that application of high nitrogen rated increased number of spikes and tiller number of two Japonica rice varieties. According to Wu *et al*. (2011) application of nitrogen rates higher than 300 kg ha^{-1} resulted in a large number of plants of transplanted Japonica rice and increased shading among plants causing yield reduction. Sun *et al*. (2020) reported that excessive nitrogen consumption doesn't always make the rice yield high, and nitrogen should be used at optimum level to avoid environmental pollution problems. The optimum nitrogen rate for the Jia 58 variety was 225 kg ha^{-1}. The average yields of Japonica and Indica hybrid rice were higher than that of Japonica rice because Japonica and Indica hybrid rice had a stronger root system than normal Japonica rice thus absorbing more nitrogen.

As high yield is a main target of crop production, the farmers can apply Azolla dry matter to Japonica rice as long as it does not cause yield reduction and it is profitable. Therefore, cost-benefit of Azolla application should be further studied and the optimum rates of application should be identified. Furthermore, investigations on application methods should also be carried out to identify the most appropriate method that provides the highest NUE.

DOA1 and DOA2 have a similar yield, and the farmers can select any varieties as their preference. The interaction between variety and rate of Azolla application was not significant for grain yield. The results indicated similar responses of these varieties to the rates of Azolla application.

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