DISSERTATION

AZOLLA BIOFERTILIZER GROWTH AND UTILIZATION FOR VEGETABLE PRODUCTION

Submitted by

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ABSTRACT

AZOLLA BIOFERTILIZER GROWTH AND UTILIZATION

FOR VEGETABLE PRODUCTION

Food security is a fundamental issue in Indonesia, in terms of how to provide nutritious and affordable food for the growing population in an era of climate change. One approach to resolve that issue is through strengthening national food security that starts from the household level. The Ministry of Agriculture in Indonesia developed the Sustainable Food-Reserved Garden Program that encourages every household to grow vegetables as a nutritious food source in their backyard.

In order to intensify vegetable production through the use sustainable fertilization, we utilized locally-grown fertilizer using *Azolla* as N source and biofertilizer in place of conventional fertilizers. *Azolla* is a biological N fertilizer that can be utilized and developed particularly in tropical countries. Besides, *Azolla* can also be utilized as livestock and poultry feed, food, or biofuel, and at the same time, it can also help to reduce the threat of climate change by fixing CO_2 from the atmosphere. Additionally, utilizing *Azolla* as biofertilizer can mitigate CO_2 emissions from fossil fuel that is used in producing inorganic fertilizers such as urea.

Azolla utilization can also address some issues such as synthetic N fertilizer scarcity and environmental pollution due to synthetic N fertilizer application, and most important is the ability of *Azolla* to naturally fix N and grow rapidly. *Azolla* is a promising biofertilizer that has proven agronomic value for paddy rice. Additionally, *Azolla* may improve soil properties and

vegetable plant nutrition. As a biofertilizer, there is also potential for *Azolla* to alter soil microbial communities.

A series of greenhouse studies were done to improve knowledge regarding *Azolla* production in natural or artificial ponds for field application. Field experiments were done to evaluate whether *Azolla* is feasible to be developed as biofertilizer compared to commonly-used N fertilizers (urea and chicken manure) in tropical countries such as Indonesia.

The greenhouse study aimed to identify the optimum nutrient concentrations in the growing medium, inoculation rate, and combined nutrient solutions that can maximize growth of *A. mexicana* and to identify the nutrient concentrations in *A. mexicana* as biofertilizer. *A. mexicana* was cultivated in nutrient solutions in the greenhouse to examine the impact of ten individual nutrients at four different concentrations using a randomized complete block (RCB) design with three replicates. In addition, inoculation rate and combined nutrient solution studies were conducted. I hypothesized that optimum concentrations of essential nutrients**,** inoculation rate, and combined nutrient solutions improve *Azolla* growth parameters (biomass, relative growth rate (RGR), doubling time, and percent greenness of *Azolla* plants), and also increase nutrient concentrations in the *Azolla* plant tissue. The parameters determined in these studies were *Azolla* growth (biomass, RGR, doubling time, and percent greenness of *Azolla* plants) and nutrient concentrations of the *Azolla*. Comparison of treatment means used the honestly significant difference Tukey adjusted post hoc test (n= 3, P <0.05).

There were no significant differences in *Azolla* growth parameters among nutrient concentrations with all other nutrients held constant, except for Zn which increased greenness percentages of *Azolla* plant. The inoculation rate of 100 g m^{-2} was optimum for the 14-day *Azolla* growing periods in the greenhouse. The inoculation rates altered doubling time, RGR, and *Azolla*

nutrient concentrations (K, Fe, Mn, and Zn). Whereas, combined nutrient solutions altered K, Fe, Mn, Mo, and Zn *Azolla* nutrient concentrations. *Azolla* nutrient concentrations were also influenced by several solution nutrient concentrations including P, K, Ca, Mg, Fe, Mo, B, and Cu. It is recommended to use Wd3 nutrient solution [10 mg P L^{-1} ($NaH_2PO_4.H_2O$), 20 mg K L^{-1} (K_2SO_4), 10 mg Ca L^{-1} ($CaCl_2.2H_2O$), 10 mg Mg L^{-1} ($MgSO_4.7H_2O$), 0.375 mg Mn L^{-1} ($MnCl_2.4H_2O$), 1 mg Fe L^{-1} ($C_6H_5FeO_7$), 0.075 mg Mo L^{-1} ($Na_2MoO_4.2H_2O$), 0.15 mg B L^{-1} (H_3BO_3), 0.01 mg Cu L^{-1} ($CuSO_4.5H_2O$), 0.01 mg Zn L^{-1} ($ZnSO_4.7H_2O$), and 0.01 mg Co L^{-1} ($CoCl_2.6H_2O$)], in order to obtain the highest *Azolla* biomass and the shortest growing period, at the least cost, due to lower nutrient concentrations used in Wd3, compared to Wt nutrient solution.

The purposes of the field study were to evaluate the contributions of *A. pinnata* as a biofertilizer compared to commonly-used fertilizers in enhancing vegetable crop yields and other agronomic parameters, soil chemical properties, plant nutrient concentrations, and soil microbial communities specially for red spinach and radish crops on Inceptisols and Histosols in West Kalimantan, Indonesia. The hypotheses of the field study were as follows: (1) *Azolla* as a biofertilizer will increase vegetable plant growth (plant height, leaf numbers, branch numbers, and soil plant analysis development (SPAD) reading), (2) Soil amended with *Azolla* will enhance vegetable yields and agronomic parameters related to N (N leaf or bulb contents and NUE), (3) *Azolla* as a biofertilizer will enhance soil chemical properties (pH, total N, P, K, Fe, and Zn concentrations, organic C, and C/N ratio) in alluvial and peat soils in West Kalimantan, Indonesia, comparable to commonly-used fertilizers, (4) *Azolla* utilization as a biofertilizer will enrich nutrient concentrations (N, P, K, Fe, and Zn) in vegetable plant tissues, and (5) *Azolla*

application will affect soil microbial community biomass and structure, primarily bacteria and fungi, in mineral soil (alluvial) and organic soil (peat).

There were two field studies; each set evaluated treatments effects on red spinach and radish grown on peat and alluvial soils. Both studies were arranged in the RCB design with four N fertilizer treatments, one control treatment, and three replications. First, a preliminary N rate study was carried out to determine the optimum N rate for urea and whether manure had an effect on increasing vegetable yield. The N study treatments were N0 (control or no N fertilizer), N1 (urea 23 kg N ha^{-1}), N2 (urea 46 kg N ha^{-1}), N3 (urea 69 kg N ha^{-1}), and N4 (urea 92 kg N ha^{-1}). The *Azolla* study had the following treatments: N0 (control or no N fertilizer), urea (23 kg N ha^{-1}), *Azolla*-U (*Azolla* applied at the same urea-N rate (23 kg N ha^{-1})), manure (108 kg N ha^{-1}), and *Azolla*-M (*Azolla* applied at the same manure-N rate (108 kg N ha^{-1})). Treatment means were then compared using the honestly significant difference Tukey adjusted post hoc test (n= 3, P <0.10).

The N rate study results suggested that the optimum N rate for increasing vegetable yields was 50 kg urea ha^{-1} (23 kg N ha^{-1}), and chicken manure was used as a commonly-used organic fertilizer. *Azolla* applied at the manure N rate and manure increased spinach yield and the agronomic parameters on the spinach–peat site, while manure only altered spinach yield on the alluvial site. Radish plant height was increased by manure treatment, in both alluvial and peat soils. Urea exhibited the highest N Use Efficiency (NUE) in the spinach–alluvial site. Manure and *Azolla* biofertilizer had similar NUE, in the order of higher NUE in manure, *Azolla*-U, then *Azolla*-M. Soil P concentration in the radish-alluvial and spinach-peat sites was enhanced by manure. In addition, K concentration in the radish crop was affected by manure in the alluvial soil, whereas manure and the *Azolla* applied at the manure N rate increased K concentration in

the radish and spinach crops in the peat soil. Vegetable yields was highly positive correlated with N content in both alluvial and peat soils. Furthermore, *Azolla*-M treatment resulted in a shift in the microbial community structure in peat soil, but not in alluvial soil. Microbial community biomass was greater in the alluvial soil than in the peat soil, and bacteria were dominant in both soil types, regardless of the N fertilizer treatment. Greater fungal community biomass was found in soils amended with *Azolla*-M and manure, compared to control soil and soils amended with urea or *Azolla*-U. A greater ratio of fatty acid stress biomarkers was indicated in control soil and urea-amended soil, as well as in the peat soil compared to alluvial soil. *Azolla*-M may possibly diminish stress encountered by the microbial community due to unfavorable environmental conditions.

Hence, *Azolla* could be utilized as a sustainable biofertilizer for vegetable production in dryland acidic tropical soils, in order to promote higher yields and maintain soil fertility. Moreover, *Azolla* biofertilizer and manure can be used to enhance yields and nutrient concentrations in radish and spinach crops, improve soil fertility in the alluvial and peat soils, and enhance soil microbial communities and reduce abiotic microbial stress.

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Far and away the best prize that life offers is the chance to work hard at work worth doing. ~Theodore Roosevelt~

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CHAPTER 1

BACKGROUND

Nitrogen (N) is an essential macronutrient that is needed by plants to form proteins. Protein is a compulsory element for plants, and chlorophyll is a protein that allows them to harvest sunlight. Unfortunately, although N_2 is the primary element in the atmosphere, i.e. 78 percent, N_2 gas is in an inert form that is not available to be used by plants. There are some organisms that have the ability to convert inert atmospheric N_2 to an available form for plants (ammonia). These organisms are blue-green alga (cyanobacteria), certain genus of bacteria, such as *Rhizobium* in legume crops, and *Azotobacter*.

Some N-fixing bacteria can live as free-living diazotrophs or in symbiotic relationships with plants. A common symbiotic relationship of cyanobacteria is with *Azolla*, a water fern. All *Azolla* species typically comprise an N_2-fixing cyanobacterium as an endophyte that inhabits special cavities in the dorsal leaf of the *Azolla* fern and can supply N to the *Azolla* by atmospheric N_2-fixation (Peters, 1984).

The symbiotic relationship between *Anabaena* (a genus of cyanobacteria) and *Azolla* is the foundation for N_2 fixation. Cyanobacteria can fix N_2 in the presence of the nitrogenase enzyme in specialized heterocyst cells. However, there are several environmental conditions that are needed for optimum function of the nitrogenase enzyme. The presence of available C, and occurrence or lack of combined N and molecular oxygen play a role in controlling the synthesis and level of nitrogenase activity (Sylvia et al., 1999). Due to the harmful effect of O_2 to N fixation by cyanobacteria, *Azolla* supplies an oxygen-free environment for *Anabaena.* In return, *Anabaena* sequesters N_2 directly from the atmosphere, which is utilized for *Azolla* growth.

The symbiotic relationship between *Azolla* and *Anabaena* has existed for approximately 70 million years (the *Azolla* Foundation, 2016). During that considerable period of time, the two partners have co-evolved numerous complementary characteristics that make them increasingly efficient. An estimated average rate of biological N_2 fixation for cyanobacteria as free-living microorganisms is 25 kg N ha^{-1} yr^{-1}; whereas, in *Azolla*–cyanobacterial associations, they can fix up to 313 kg N ha^{-1} yr^{-1} (Stevenson, 1982).

Azolla is unique due to the fact that it is the fastest-growing aquatic plant on Earth since it can double in two days with a relative growth rate of 0.34 g g^{-1} day^{-1} (Peters et al., 1980). However, there is variation in doubling time of *Azolla* depending on the species and the environmental conditions. *A. filiculoides* needs 5–8 days to double its biomass. Whereas, in homogenous cultures (KH_2PO_4 5.4 g L^{-1}, KCl 14.9 g L^{-1}, $MgSO_4.7H_2O$ 19.7 g L^{-1}, $CaCl_2.2H_2O$ 29.4 g L^{-1}, FeEDTA 0.0385 g L^{-1}, A_5 microelement solution 1 mL L^{-1}, agar 12%, distilled H_2O 1 L) of *A. nilotica, A. microphylla, A. mexicana, A. caroliniana,* and *A. pinnata,* 7–9, 9–11, 9.5–12.5, 10–12.5, and 11–34 days doubling time are needed, respectively (Bozzini et al., 1984). The *Azolla* growth rate under undisturbed environmental conditions can be exponential at 0.23 g g^{-1} day^{-1}; however, the growth rate will decline to 0.032 g g^{-1} day^{-1} under severely crowded *Azolla* populations (Becking, 1979). Salinity can inhibit *Azolla* growth, as described by Kannaiyan (1990), where in the control condition, the relative growth rate (RGR) of *A. pinnata* ranged from 0.115–0.116 g g^{-1} day^{-1} and 0.112 g g^{-1} day^{-1} for *A. filiculoides* and *A. microphylla*. RGR is defined as the daily increment in total biomass (Kannaiyan and Kumar, 2005). However, in NaCl medium solution (0.32% NaCl), the RGR declined to 0.098–0.100 g g^{-1} day^{-1} (*A. pinnata*), 0.097 g g^{-1} day^{-1} (*A. filiculoides*), and 0.099 g g^{-1} day^{-1} (*A. microphylla*)

(Kannaiyan, 1990). Saline medium solution (0.32% NaCl) also lowered nitrogenase activity, chlorophyll a:b ratio, and photosynthesis and respiration rates (Kannaiyan, 1990).

Lower light intensity also reduces *Azolla* growth rates. Under 180 and 380/μE m^2 s^{-1} light intensity, *A. pinnata* growth rate was 0.116–0.149 and 0.157–0.164 g g^{-1} day^{-1} with doubling time 4.66–6 and 4.23–4.4 days, respectively (Kannaiyan, 1988). Whereas, *A. microphylla* have RGRs of 0.154 and 0.165 g g^{-1} day^{-1} with 4.49 and 4.2 days doubling time under 180 and 380/μE m^2 s^{-1} light intensity (Kannaiyan, 1988). In concordance with the two-previous species, *A. caroliniana* also has a slower growth rate (0.14 g g^{-1} day^{-1} with 4.94 days doubling time) under lower light intensity than under higher light intensity (0.165 g g^{-1} day^{-1} with 4.19 days doubling time) (Kannaiyan, 1988). In a field experiment with temperature of 24.8 ^{o}C and relative humidity (RH) of 53.9% and a pot experiment with 23.2 ^{o}C heat and 67.4% RH, different performance was reported (Lumpkin and Plucknett, 1982). *A. caroliniana* had maximum RGR of 0.256 and 0.186 g g^{-1} day^{-1} under pot and field experiments (Lumpkin and Plucknett, 1982). In agreement with that, *A. filiculoides, A. microphylla, A. pinnata,* and *A. rubra* (japonica) had RGRs of 0.26, 0.254, 0.252, and 0.176 g g^{-1} day^{-1}, respectively, under pot culture and 0.186 and 0.185, 0.185, and 0.144 g g^{-1} day^{-1}, respectively, under field conditions. Whereas, RGR of *A. mexicana* and *A. nilotica* under pot culture was 0.243 and 0.22 g g^{-1} day^{-1}.

The maximum biomass, N, and N_2-fixation rate of the *Azolla–Anabaena* symbiosis varies among species and environmental factors such as temperature. According to Watanabe (1982), *A. pinnata* that was grown in a fallow paddy in the Philippines yielded 900–1200 kg biomass ha^{-1} which equaled 48 kg N ha^{-1} during 25–30 days with N_2-fixation rate of 1.6–1.9 kg ha^{-1} day^{-1}. Whereas, under controlled environmental conditions in a phytotron with 26 ^{o}C (day)/18 ^{o}C (night), the biomass of *A. pinnata* was 2170 kg ha^{-1} or 96 kg N ha^{-1} within 37 days,

and N_2-fixing rate was 2.6 kg N ha^{-1} day^{-1}. However, *A. filiculoides* and *A. caroliniana* under the same temperature conditions, yielded 3200 and 3190 kg biomass ha^{-1}, 126 and 146 kg N ha^{-1}, 2.5 and 3.6 kg N_2-fixation rate ha^{-1} day^{-1} during 51 and 41 days, respectively. High temperature conditions of 37 ^{o}C (day)/29 ^{o}C (night) reduced the biomass to 1120 kg ha^{-1} or 30 kg N ha^{-1} during 23 days with N_2-fixation rate of 1.3 kg N ha^{-1} day^{-1}. *A. pinnata* var. *africana* yielded only 640 kg dry matter ha^{-1} or 26 kg N ha^{-1} and N_2-fixation rate of 1.8 kg ha^{-1} day^{-1} within 15 days. Another *Azolla* species, i.e. *A. filiculoides*, that was grown in the United States also showed differences in performance when grown under different environmental conditions. In fallow paddy, *A. filiculoides* produced 1700–2300 kg ha^{-1} dry matter, equivalent to 52–93 kg N ha^{-1} with 1.5–2 kg ha^{-1} day^{-1} N_2-fixation rate within 35–46 days. In shallow ponds, it produced 1820 kg ha^{-1} biomass or 105 kg N ha^{-1}; while in pots of paddy soil, it yielded 5200 kg dry matter ha^{-1} or 128 kg N ha^{-1} with N_2-fixation rate of 2.6 kg N ha^{-1} day^{-1} within 50 days (Watanabe, 1982). In summary, environmental conditions such as temperature, light intensity, pH, salinity, and humidity play a role in enhancing the growth potential of *Azolla* (Kannaiyan, 1988), in addition to plant density that could also affect the *Azolla* growth rate (Becking, 1979).

Azolla can be utilized as biofertilizer, livestock and poultry feed, food, or biofuel, and at the same time, it can also help to reduce the threat of climate change by fixing CO_2 from the atmosphere. Additionally, utilizing *Azolla* as biofertilizer could mitigate CO_2 emissions from fossil fuels used to produce inorganic fertilizers such as urea, ammonium nitrate, or ammonium sulfate.

The growth of *Azolla* species may be stimulated by high *p*CO2 (Idso et al., 1989). In two years of experiments with *Azolla pinnata* var. *pinnata* in Phoenix, Arizona, when the mean air temperature rose above 30 °C, the *Azolla* growth rates first decreased, then stagnated, and finally

became negative. Based on the results of this study, based on both weekly biomass and periodic net photosynthesis determinations, it was demonstrated that atmospheric CO_2 enrichment may be capable of preventing the deaths of *Azolla pinnata* due to high temperatures (Idso et al., 1989).

Azolla significantly reduces CH_4 emissions from paddy rice fields as shown by the significantly negative correlation between Azolla and CH_4 emission (r= –0.57) in an organic rice experiment (Mujiyo et al., 2016). The average CH_4 emission produced by a rice paddy with *Azolla* was significantly reduced (4.54 kg ha^{-1} per growing season) compared to the treatment without *Azolla* (11.96 kg ha^{-1} per growing season). Indeed, *Azolla* application to paddy fields can significantly lower CH_4 emissions (Bharati et al., 2000; Prasanna et al., 2002; Sasa et al., 2003). Despite no effect of *Azolla* treatment on dry grain harvest of rice, the use of *Azolla* as biofertilizer enhanced the concentration of ammonium (NH_4^+) and nitrate (NO_3^-) in soil (Mujiyo et al., 2016). In addition, in another study of N_2O emissions from upland *kangkong* (water spinach) fertilized with *Azolla* compost and urea, the results showed that urea fertilizer increased N_2O emissions (Jumadi et al., 2014). Global warming potential was reduced by 98% from soil with *Azolla* over the 4-week incubation, compared to the urea treatment without *Azolla* (Jumadi et al., 2014). However, *Azolla*-amended soil had higher NO_3-N levels and lower NH_4-N levels compared to urea-fertilized soils. Composted *Azolla* and urea treatments had similar growth (plant height) and yields (dry weight) of upland *kangkong* receiving; therefore, the *Azolla* compost can substitute for urea fertilizer which could reduce N_2O emissions while maintaining plant growth (Jumadi et al., 2014).

The overall objectives of this dissertation were to identify the optimum nutrient concentrations for growing *A. mexicana* under greenhouse conditions, to compare the use of *A. pinnata* as a biofertilizer with commonly-used fertilizers for vegetable production, soil

properties on mineral (Inceptisols) and organic (Histosols) soils, and plant nutrient concentrations in West Kalimantan, Indonesia, and to evaluate the soil microbial community as impacted by the N fertilizer treatments. The hypotheses of this study were as follows:

(1) Optimum concentrations of essential nutrients such as P, K, Ca, Mg, Mn, Fe, Mo, B, Cu, and Zn will improve *Azolla* growth parameters.

(2) Inoculation rate and combined nutrient solutions influence *Azolla* growth parameters.

(3) Nutrient concentrations in the *Azolla* growing medium affect nutrient concentrations in the *Azolla* plant tissue.

(4) *Azolla* as a biofertilizer can increase vegetable plant growth (plant height, leaf numbers, branch numbers, and soil plant analysis development (SPAD) reading).

(5) Soil amended with *Azolla* can enhance vegetable yields and agronomic parameters related to N (N leaf or bulb contents and NUE) because *Azolla* is a biofertilizer and can supply N and other nutrients.

(6) *Azolla* as a biofertilizer will improve soil chemical properties (pH, total N, P, K, Fe, and Zn concentrations, organic C, and C/N ratio) in alluvial and peat soils in West Kalimantan, Indonesia, comparable to commonly-used fertilizers.

(7) *Azolla* utilization as a biofertilizer can enrich nutrient concentrations (N, P, K, Fe, and Zn) in vegetable plant tissues.

(8) *Azolla* used as a biofertilizer, will affect soil microbial community biomass and structure, in particular bacteria and fungi, in mineral soil (alluvial) and organic soil (peat).

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CHAPTER 2

OPTIMIZATION OF THE NUTRIENT GROWING SOLUTION FOR *Azolla mexicana* PRODUCTION AND AZOLLA NUTRIENT CONCENTRATIONS FOR USE AS FERTILIZER

Summary

Azolla is a water fern that can be utilized and developed as a biological N fertilizer, particularly in tropical countries. *Azolla* utilization can also address some issues such as synthetic N fertilizer scarcity and environmental pollution due to synthetic N fertilizer application, and most importantly, *Azolla* can fix atmospheric N in symbiosis with *Anabaena*. Thus, this greenhouse study focused on enhancing the growth of *Azolla*, in order to enhance knowledge regarding the *Azolla* production in natural or artificial ponds for field application. This study aimed to identify the optimum nutrient concentrations to maximize growth of *A. mexicana* and to identify the nutrient concentrations in *A. mexicana* for use as biofertilizer. *A. mexicana* was cultivated in nutrient solutions in a greenhouse, and ten individual nutrients were examined at four different concentrations using a randomized complete block design with three replicates. In addition, studies on inoculation rate and combined nutrient solutions were also conducted. *Azolla* growth parameters (biomass, relative growth rate (RGR), doubling time, and percent greenness of *Azolla* plants) and nutrient concentrations of the *Azolla* were measured. Only Zn concentrations significantly affected *Azolla* color. There were no significant differences in *Azolla* growth parameters among nutrient concentrations with all other nutrients held constant, except for Zn within affected the greenness percentages of *Azolla*. The inoculation rate of 100 g m^{-2} was optimum for 14-day *Azolla* growing periods in the greenhouse. The inoculation rates and

combined nutrient solutions altered doubling time, RGR, and *Azolla* nutrient concentrations (K, Fe, Mn, Mo, and Zn). *Azolla* nutrient concentrations were influenced by several nutrient concentrations in the media, i.e. P, K, Ca, Mg, Fe, Mo, B, and Cu. A nutrient solution (Wd3) was developed that resulted in the highest *Azolla* biomass and the shortest doubling period, while also being economical due to its lower nutrient concentrations.

Introduction

There are many varieties of *Azolla* species, including *A. caroliniana, A. filiculoides, A. microphylla, A. mexicana, A. nilotica,* and *A. pinnata.* They are widespread all over the world. Based on modern geographic distribution of *Azolla*, the most common species of *Azolla* in tropical or sub-tropical ecosystems is *A. pinnata* (Lumpkin and Plucknett, 1980; Small and Darbyshire, 2011).

In the United States, *A. pinnata* (mosquito fern, water velvet) is considered to be a prohibited federal noxious weed under United States Department of Agriculture (USDA)-Animal and Plant Health Inspection Service (APHIS) regulations. Thus, it is allowed to be imported or moved between states only using PPQ 526, a permit to move parasitic plants or noxious weeds (USDA-APHIS, 2016). Therefore, we cannot use *A. pinnata* in our study in the United States. Most *Azolla* species require similar environmental conditions, such as temperature, pH, and nutrients. In order to conduct a study to identify optimal conditions for *Azolla*, we used *A. mexicana* which is native to Colorado.

Several studies on growth of *Azolla* have been done in culture solutions. The culture solution is not species specific but for the *Azolla* genus in general. Watanabe et al. (1977) used a N-free nutrient solution with a pH adjusted to 5.5 to grow *Azolla*. The nutrient solution

contained 20 mg L^{-1} P ($NaH_2PO_4.2H_2O$); 40 mg L^{-1} K (K_2SO_4); 40 mg L^{-1} Ca ($CaCl_2$); 40 mg L^{-1} Mg ($MgSO_4.7H_2O$); 0.5 mg L^{-1} Mn ($MnCl_2.4H_2O$); 0.1 mg L^{-1} Mo ($Na_2MoO_4.5H_2O$); 0.2 mg L^{-1} B (H_3BO_3); 0.01 mg L^{-1} Zn ($ZnSO_4$); 0.01 mg L^{-1} Cu ($CuSO_4.5H_2O$); and 2 mg L^{-1} Fe ($C_6H_5FeO_7$). In addition, in later studies 0.01 mg L^{-1} Co ($CoCl_2.6H_2O$) was added to the nutrient solution (Watanabe et al., 1992). The *Azolla* was grown in 3 to 5 cm deep solutions.

Azolla requires all macro and micro nutrients which are essential for plant growth. Phosphorus, K, Ca, Mg, Fe, Mo, Co, and Zn have been shown to be essential for *Azolla* growth and N-fixation. Molybdenum is needed at higher concentrations than for most other plants (Kannaiyan, 1982).

If the P level drops in the *Azolla* growth medium, it will affect growth rate and N fixation. *Azolla* also has reduced growth in low concentrations of Fe, Ca, or P. In addition, Ca and P have influential roles in growth and N fixation (Kannaiyan et al., 1981).

According to Watanabe et al. (1977) and Peters et al. (1980), *Azolla* can double its weight in 3 to 5 days during its first week in a N-free solution. In two weeks, *Azolla* contains 3–5% N on a dry-weight basis; thus, the accumulation of N content in *Azolla* biomass can be equivalent to 22 to 36 kg N ha^{-1} (Watanabe et al., 1977). Under optimum conditions, Peters et al. (1981) also reported that *Azolla* obtained 5–6.5% N and 40–43% C. Whereas, Tally and Rains (1980) stated that *Azolla* could have 2.2–5.6% N. The maximum daily N_2-fixing rate of *A. filiculoides* and *A. pinnata* were reported to be 2.8 kg N ha^{-1} and 3.1 kg N ha^{-1}, respectively (Watanabe, 1982).

The concentration of NH_4^+ in N-free solution should be less than 1 mg L^{-1} to grow a dense population of *Azolla* (Watanabe et al., 1977). Kannaiyan and Kumar (2005) focused on the P requirement in nutrient solution to optimize *Azolla* growth. The biomass of *A. filiculoides* was greater at 20 mg L^{-1} of P; however, 5–10 mg L^{-1} P level was also sufficient for optimum growth

and multiplication with a 2–3 day doubling time (Subudhi and Watanabe, 1980; Kannaiyan, 1985). Reddish-brown discoloration of *Azolla* that spreads from the center to the tip of the leaf with a smaller frond size can be a reflection of P deficiency. In addition, roots may be reddish-brown, longer, and easily separated from the *Azolla* body. Similarly, Fe deficiency affects *Azolla* frond discoloration, since it reduces chlorophyll content and makes plants turn yellowish (Watanabe et al., 1977).

Azolla is a biological N fertilizer that has potential to be utilized and developed in tropical developing countries such as Indonesia due to the year-round solar intensity. *Azolla* can be grown in rice fields along with paddy rice or in natural or artificial ponds, and then applied as a N biofertilizer to any crop. The utilization of *Azolla* is also a good solution to address N fertilizer scarcity that sometimes happens in some regions in Indonesia. In addition, it can reduce environmental pollution that commonly occurs due to synthetic N fertilizer application. But most importantly, *Azolla* may play a significant role as biofertilizer due to its ability to fix N in symbiosis with *Azolla* and grow rapidly.

Essential nutrient availability influences the effectivity of N fixation (O'Hara, 2001). The growth of *Azolla* in a variety of soils and water bodies might be regulated by the P supply (Watanabe and Ramirez, 1984). In addition, two other essential nutrients that are required for nitrogenase activity to fix atmospheric N are Fe and Mo (Carithers et al., 1979).

Anabaena uses the nitrogenase enzyme to enhance N fixation. This enzyme is influenced by certain nutrients, such as P, Fe, and Mo (Carithers et al., 1979). The main macronutrients and other essential nutrients that are necessary for optimizing *Azolla* growth and N fixation are P, K, Ca, Mg, Fe, Mo, Co, and Zn (O'Hara, 2001; Carithers et al., 1979; Kannaiyan, 1982). In previous studies, Ca and P deficiencies had a considerable effect on *Azolla* growth and N fixation

compared to K and Mg deficiencies (Watanabe et al., 1977; Subudhi and Singh, 1978; Kannaiyan et al., 1981).

Enhancing growth rates of *Azolla* is important, in order to achieve high production of *Azolla* to be used as biofertilizer. Additionally, higher nutrient concentrations of *Azolla* are also essential since they reflect the quality of the biofertilizer itself. Thereby, the research activities in this study include growing *A. mexicana* in greenhouse and optimizing the growth condition of *A. mexicana* by changing nutrient concentrations in the media. The objective of this study was to identify the optimum nutrient concentrations to maximize growth of *A. mexicana* and to identify the nutrient concentrations of *A. mexicana* as biofertilizer. Based on this greenhouse study, we aim to improve *Azolla* growth in natural or artificial ponds for field application of *Azolla.*

The hypotheses of this study were

1. Optimizing concentrations of essential nutrients such as P, K, Ca, Mg, Mn, Fe, Mo, B, Cu, Zn is essential for improving *Azolla* growth parameters.
2. Inoculation rate and combined nutrient solutions influence *Azolla* growth parameters.
3. Nutrient concentrations in the *Azolla* growing medium affect nutrient concentrations in the *Azolla* plant tissue.

Materials and Methods

Azolla Nursery and Experimental Design

A series of greenhouse studies was conducted in 2013 and 2014 on the Colorado State University (CSU) campus. *Azolla mexicana*, a native *Azolla* of Colorado, was obtained from a pond store in Fort Collins, Colorado. Sometimes *A. mexicana* can be collected from the natural

environment in the South Platte River. However, in Spring 2014, it was not found in the common natural environment due to recent flooding.

Prior to the nutrient study, an *Azolla* nursery was prepared. The media for the *Azolla* nursery was tap water considered to be good irrigation quality water in Fort Collins, Colorado based on the essential nutrient content (Table 1). Then, 4 mL liquid plant fertilizer (Miracle Gro) was applied into 15 L tap water. The nutrient concentrations of that liquid fertilizer are 1% NH_4-N, 3% NO_3-N, 3% P_2O_5, 6% K_2O, 1% Ca, and 0.5% Mg.

Azolla is sensitive to heat and light; therefore, in order to prevent discoloration of *Azolla* seedlings, the nursery was shaded. In this study, Aluminet (Green-Tek Inc., Janesville, WI) reflective shade cloths that provided 70–74% shade and 45% diffused light transmission was used to reflect the sun's rays while preserving quality light transmission. Growing media (tap water) was replenished if there was discoloration of *Azolla*. It was not necessary to add liquid fertilizer into the *Azolla* nursery growing media, rather evaporated tap water was replenished within 4–6 weeks.

First, ten individual macro/micro nutrient studies (P, K, Ca, Mg, Mn, Fe, Mo, B, Cu, Zn) were carried out in which the concentration of one nutrient was varied while holding all the others constant at the Watanabe prescribed concentration (Watanabe et al., 1977). Each study used a Randomized Complete Block (RCB) Design with three replicates and four concentrations (Table 2). Each container was inoculated with 400 g m^{-2} *A. mexicana* seedlings from the *Azolla* nursery, and grown for 14 days in 10 L of nutrient solution in a 0.16-m^2 plastic container.

Next, an inoculation rate study was carried out to identify the optimum inoculation rate to achieve maximum growth. Four *Azolla* inoculation rates were evaluated, i.e., 50, 100, 200, and 400 g m^{-2}. Furthermore, the optimum inoculation rate determined in this study was then used in

the final combined nutrient solution study. The combined nutrient solution study had four treatments: Wd1 (the nutrient concentrations that had the highest relative growth rate (RGR) in the individual nutrient studies), Wd2 (the lowest nutrient concentrations which supported *Azolla* growth that was not significantly different from the maximum in the individual nutrient studies), Wd3 (Wd1 plus 0.01 mg Co L^{-1} ($CoCl_2.6H_2O$) that was later recommended by Watanabe et al., 1992), and Wt (nutrient concentrations formulated by Watanabe et al., 1977 without Co) (Table 3).

Data Collection and Analysis

The parameters measured in these studies were biomass, relative growth rate (RGR), doubling time, plant color, and nutrient concentration of *Azolla* biomass. *Azolla* biomass was harvested after 14-days growth. Then, the biomass production was calculated based on fresh weight of day 14 minus initial fresh weight on day 0. Relative growth rate was calculated as in Eqn. 1 (Pabby et al., 2001).

$$RGR = \frac{(\log W2 - \log W1)}{(t2 - t1)} \qquad \text{(Eqn. 1)}$$

Doubling time (Td) was calculated using the following Eqn. 2 (Kannaiyan and Kumar, 2005):

$$Td = \frac{(t2 - t1) \times \log 2}{\log (W2 / W1)} \qquad \text{(Eqn. 2)}$$

In both equations,

$t1$ = time initial (0 day)

$t2$ = time of harvest (in days)

$W1$ = fresh *Azolla* biomass at time initial (in grams)

$W2$ = fresh *Azolla* biomass at harvesting time (in grams)

Azolla samples were oven-dried, ground, and weighed before digestion. Total C and N were determined by the dry combustion method using a LECO CN analyzer (Leco Corp., St Joseph, MI) (Mulvaney, 1996). Nutrient concentrations of *Azolla* biomass were determined by digesting the samples with 6 mL concentrated nitric acid (HNO_3) and 3 mL concentrated hydrochloric acid (HCl) to burn off all the organic matter. Then 1 mL 30% hydrogen peroxide (H_2O_2) was added to dissolve any fats and oils to drive the reaction to completion by allowing for a higher boiling point. The samples were cooled under the hood, then brought to volume, mixed well, and filtered. Samples were diluted 5:1 by mixing 2 mL of sample and 8 mL of 2% HNO_3 (Campbell and Plank, 1991; Kovar, 2003; Wolf et al., 2003). Finally, Ca, Mg, K, Zn, Fe, Mn, Cu, P, S, Na, Mo, and B were analyzed by inductively coupled plasma atomic emission spectrometry (ICAP).

Azolla leaf color was observed using Munsell color charts for plant tissues. The criteria of plant color were determined by: (1) the darkest green *Azolla* leaf was indicated by a hue of 7.5 GY, (2) the moderately green leaf color was designated by a hue of 5 GY, and (3) the lightest green was signified by a 2.5 GY hue. The observation of *Azolla* leaves was based on percent coverage of the green color.

The percent coverage of greenness was performed using hues of 5 or 7.5 GY, based on the majority of *Azolla* plants. During the first week of *Azolla* growth, the *Azolla* had mostly hue 2.5 GY, and some parts were yellowish or reddish, although a few had a hue of 5 GY. After two weeks of growth, the *Azolla* became darker green with a hue of 5 or 7.5 GY. Overall, *Azolla* became darker green overtime within each 14-day growth period. Higher coverage (stated as a %) of darker green *Azolla* signified healthier plants.

Data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC). Analysis of variance (ANOVA) was performed on the data by using the Mixed procedure (Proc Mixed). The fixed-effects were four levels of nutrient rates in ten individual nutrient studies, four inoculation rates, and four combined nutrient solutions; whereas, the random variable was block as replication. Treatment means were compared using Tukey's honestly significant difference (Tukey's HSD) post hoc test (n= 3, P <0.05). Pearson correlation (PROC CORR procedure) was used to examine the relationships between growth parameters and *Azolla* nutrient concentrations.

Results

Nutrients Affecting *Azolla* Growth Parameters

In general, the ten individual nutrient studies, there was very little significant effect of individual nutrients on *Azolla* growth parameters (biomass, RGR, doubling time, and percent greenness of *Azolla* plant) (Table 4). Zn is the only nutrient that had a significant effect on percent greenness of *Azolla* 14 days after inoculation (Table 4; Fig. 1). The highest Zn concentration (0.01 mg Zn L^{-1}) demonstrated the highest % greenness although it was

statistically similar with the 0.0075 mg Zn L^{-1} concentration, and it was significantly greener than the 0.0025 and 0.005 mg Zn L^{-1} concentration (Fig. 1).

This study also showed that there were no significant differences in terms of *Azolla* growth parameters in biomass, RGR, or doubling time at various P, Fe, or Mo concentrations, although those are essential nutrients required for N fixation (Table 4). In addition, there were no significant effects of the other individual nutrients that were examined in these studies (Table 4).

Inoculation Rates and Combined Nutrient Solutions Affecting *Azolla* Growth Parameters

Azolla biomass and percent greenness was not significantly different among the inoculation rates; however, inoculation rate had significant effects on RGR and doubling time of *Azolla* (Table 4; Fig. 2). The highest inoculation rate (400 g m^{-2}) had the longest doubling time and lowest RGR. The shortest doubling time of *Azolla* growth (4.52 days) occurred in the 50-g m^{-2} inoculation rate, which was statistically similar with 100 (5.37 days) or 200 (6.58 days) g m^{-2} inoculation rates. Whereas, the RGR of *Azolla* was significantly higher in the 50 and 100 g m^{-2} inoculation rates, i.e. 0.153 and 0.129 g g^{-1} day^{-1}, respectively (Fig. 2).

There were no significant differences in *Azolla* color, biomass, doubling time, or RGR in the final combined nutrient solution study (Table 4). Nevertheless, the highest biomass and RGR were obtained in Wd3, i.e. 28.18 g and 0.0736 g g^{-1} day^{-1}. As a result, Wd3 combined nutrient solution had the lowest doubling time (9.42 day) with *Azolla* greenness of 99.66%.

Nutrient Concentrations in *Azolla*

In general, based on twelve greenhouse experiments, there were some effects of individual nutrient study on *Azolla* P, K, Mg, Mo, and S nutrient concentrations (Table 5). P concentrations in *Azolla* were highest at 10 mg Mg L^{-1}; whereas, Mg concentrations of 20–40 mg Mg L^{-1} in the *Azolla* growing medium resulted in the higher Mo concentrations in the *Azolla* plant (371–412 mg Mo kg^{-1}) (Fig. 3).

Azolla K concentrations were influenced by P and K levels. *Azolla* had significantly higher K concentration in the 5–10 mg P L^{-1}, i.e. 6.41 and 6.19% K, respectively (Fig. 4) and greater K concentrations in the 30–40 mg K L^{-1}, i.e. 5.65 and 5.75% K, respectively (Fig. 5).

Azolla S concentrations were affected by P, Mg, and Cu levels. Higher Mg concentrations in the growing medium (30–40 mg Mg L^{-1}) increased *Azolla* S concentrations (0.93–0.95% S) (Fig. 3). In contrast, lower P concentrations in the growing medium (5–15 mg P L^{-1}) increased S concentrations in *Azolla* (1.00–1.02% S) (Fig. 4). Similar to Mg, lower Cu concentrations (0.0025–0.0075 mg Cu L^{-1}) increased *Azolla* S concentrations, i.e. 0.86–0.87% S (Fig. 10).

The effect of Ca, Mg, Fe, Mo, B, and Cu concentrations in the growth solution for *Azolla* had significant impact on Ca, Mg, Fe, Mo, B, and Cu concentrations of *Azolla*, respectively (Table 5). Higher concentration of Mg (30–40 mg Mg L^{-1}), Ca (30–40 mg Ca L^{-1}), and Mo (0.1 mg Mo L^{-1}) resulted in significantly higher *Azolla* Mg, Ca, and Mo concentrations, i.e. 0.13–0.14% Mg, 0.42–0.44% Ca, and 291.46 mg Mo kg^{-1}, respectively (Figs. 3, 6, and 7). On the contrary, the lower Mo concentrations in the growing medium resulted in significantly higher *Azolla* Fe and Cu concentrations. Molybdenum concentration of 0.025–0.075 mg Mo L^{-1} induced 2064, 1834, and 1830 mg Fe kg^{-1}. While the lower concentration of Mo (0.025–0.05 mg

Mo L^{-1}) resulted in significantly higher *Azolla* Cu concentrations (44.10 and 42.77 mg Cu kg^{-1}) (Fig. 8).

The similar trends were observed in *Azolla* Fe, B, and Cu. Higher *Azolla* Fe (3536 mg Fe kg^{-1}), B (47.70–53 mg B kg^{-1}), and Cu (40.73 and 40.03 mg Cu kg^{-1}) concentrations were found in the higher concentrations of corresponding nutrients in the *Azolla* growing media, i.e. 2 mg Fe L^{-1}, 0.1–0.2 mg B L^{-1} and 0.0075–0.01 mg Cu L^{-1} (Figs. 8, 9, and 10).

Inoculation Rates and Combined Nutrient Solution Effects on *Azolla* Nutrient Concentrations

Inoculation rates significantly affected *Azolla* K, Fe, Mn, and Zn concentrations (Fig. 11). In general, the lower inoculation rate resulted in higher *Azolla* nutrient concentrations. The 50–100 g m^{-2} inoculation rate resulted in higher K concentrations in the *Azolla* plants (4.64–5.05% K). The higher Fe and Zn *Azolla* concentrations (2106–2455 mg Fe kg^{-1} and 94.67–108 mg Zn kg^{-1}) occurred under the 50–200 g m^{-2} inoculation rates. Likewise, the 50-g m^{-2} inoculation rate had the highest *Azolla* Mn concentration (1521 mg Mn kg^{-1}).

The combined nutrient solutions of Wd3 and Wt had the highest *Azolla* K (5.25 and 5.00% K) and Mn (1542.33 and 908.33 mg Mn kg^{-1}) concentrations (Figs. 12 and 13). Wt was the only combined nutrient solution that induced higher *Azolla* Fe and Mo concentrations, i.e. 1687 mg Fe kg^{-1} and 120 mg Mo kg^{-1}, respectively (Fig. 13). On the other hand, Wd3, Wd2, and Wd1 combined nutrient solutions had higher *Azolla* Zn concentrations (102.33 and 92 mg Zn kg^{-1}) than Wt (Fig. 13).

Correlations Among *Azolla* Growth Parameters and *Azolla* Nutrient Concentrations

There were some moderate and strong correlations among the *Azolla* growth parameters and *Azolla* nutrient concentrations (Table 6). However, the only significant *Azolla* growth parameters that were influenced by inoculation and nutrient rate treatments were percent greenness, relative growth rate, and doubling time (Figs. 1 and 2). The relationships among those *Azolla* growth parameters and *Azolla* nutrient concentrations could be explained in positive or negative trends in moderately to highly correlations (Table 6). There were also some moderate to strong correlations among the *Azolla* biomass and *Azolla* N concentrations with the other *Azolla* nutrient concentrations; however, biomass and *Azolla* N concentration were not affected by the ten nutrients, inoculation rates, or combined nutrient solution studies (Tables 4 and 5).

Discussion

Media Nutrient Concentrations Affecting *Azolla* Growth Parameters and *Azolla* Nutrient Concentrations

Growth parameters. The percent greenness of *Azolla* plant was correlated to biomass in four out of 12 experiments (Table 6). Conversely, the longer time needed by *Azolla* to double itself somehow represented the less percent green color of *Azolla* plant, as shown in six out of 12 experiments (Table 6). As a result, *Azolla* produced less biomass at a slower growth rate when the observed *Azolla* green color was in lower percent (Table 6). Based on the ten nutrient studies, there was no significant effect of those nutrient rates on *Azolla* growth parameters (biomass, RGR, and doubling time) (Table 4). Nevertheless, there was a moderately positive correlation

between *Azolla* N content and *Azolla* biomass (R= 0.65–0.68) in three out of 12 experiments; and conversely, a negative correlation occurred between *Azolla* P content and *Azolla* biomass (r= –0.67 to –0.72) in two out of 12 experiments (Table 6).

It is expected that biofertilizer which is higher in Zn will increase Zn concentration in crops which may be useful in addressing Zn insufficiency in about half of the world's population (Brown et al., 2001). The results of this study revealed that the only nutrient that significantly affected (and one out of 12 experiments had a positive correlation) percent *Azolla* greenness at 14 days after inoculation was Zn (Table 4). The 0.01 and 0.0075 mg Zn L^{-1} concentrations demonstrated the highest percent greenness (Fig. 1). The relationship between the Zn concentrations and the percent of *Azolla* greenness was indicated in a moderate correlation (r= 0.56, P= 0.06) (Table 6).

This study showed that there were no significant differences in terms of *Azolla* growth parameters in biomass, RGR, doubling time, or percent greenness of *Azolla* at various P, Fe, or Mo concentrations, although those are essential nutrients required for N-fixation (Table 4). However, the only effect of nutrients on the percent greenness of *Azolla* occurred in the Zn rate study.

Plant color is generated by chlorophyll, carotenoids, anthocyanins, and betalains (Yeap, 2014). The green and blue pigments are found naturally in the chloroplasts of plants and algae, including in the photosynthetic cyanobacteria such as spirulina and chlorella. Photosynthesis is conducted in chloroplasts that contains chlorophyll. This chlorophyll as the primary pigment in photosynthesis, reflects green light and absorbs red and blue light (Nature, 2014).

Zn application to plants that suffered from salinity stress triggered considerable improvement in photosynthesis, water use and mesophyll efficiency, and yield (Weisany et al.,

2011). In addition, lipid peroxidation and hydrogen peroxide concentration significantly diminished due to Zn utilization. Likewise, Havlin et al. (2014) asserted that Zn is essential for synthesis of some proteins and growth hormones e.g., indoleacetic acid, and is also involved in the synthesis of chlorophyll, cell membranes, and enzyme activation. Shukla and Yadav (1982) reported that Zn up to 19 mg L^{-1} and P up to 50 mg L^{-1} increased the number, dry matter, leghaemoglobin concentrations of nodules, and the amount of N-fixed. Balancing P and Zn improved nodulation and N-fixation due to enhanced leghaemoglobin, K, and Fe concentrations in nodules, in addition to increased plant growth.

In this study, Zn concentrations in the *Azolla* plant in all 12 experiments was in the sufficiency level (25 to 150 mg kg^{-1}) (Havlin et al., 2014). Thereby, there was no symptom of chlorosis in the *Azolla* plant due to Zn deficiency; however, Zn influenced the percent greenness displayed by the *Azolla* plant.

Macronutrients. The main purpose for growing *Azolla* as a biofertilizer is for its N content to be used as N fertilizer. In general, higher nutrient concentration in *Azolla* is presumed to enhance nutrient concentration in crop yields, that furthermore could improve human nutrition. The highest N, P, and Zn concentrations in *Azolla* were found in the B rate study, i.e., 4.08–4.28%, 1.82–1.92%, and 102–115 mg Zn kg^{-1}; respectively. The P rate study resulted in the highest K (5.8–6.41%) and Mo concentrations (422–479 mg Mo kg^{-1}). The highest Fe content in *Azolla* (3257–3690 mg Fe kg^{-1}) was obtained in Mn rate study.

Based on the twelve studies, there were not any significant effects of nutrient concentrations, inoculation rates, or nutrient solutions on the N concentration of *Azolla* (Table 5). Yet, 0.0025 mg Zn L^{-1} and 0.1 mg B L^{-1} resulted in higher N concentration of *Azolla* (Fig. 14). Phosphorus concentration of 15 mg P L^{-1} also increased N *Azolla* concentration (Fig. 14). The

higher inoculation rate (400 g m^{-2}) produced the highest N concentration (3.87%) (Fig. 14). Whereas, the Wd3 combined nutrient solution showed the highest N concentration compared to Wt solution, i.e. 3.97 and 3.71% N, respectively (Fig. 14). In accordance with that, Subudhi and Watanabe (1980) stated that 5–10 mg P L^{-1} was necessary for optimal growth rate.

There was a significant effect of Mg level in the nutrient solution on *Azolla* P concentration (Fig. 3). The correlation between P and Mg was slightly negative (r= –0.01) (Table 6), in which the lowest Mg level of 10 mg L^{-1} had the highest P concentration in the *Azolla* plant (1.65%) (Fig. 3). In spite of no significant effect of K rate on *Azolla* P concentration, the K concentration in K nutrient study indicated the higher amount of *Azolla* P concentration compared to the other nutrients (data not shown). The 10 mg K L^{-1} yielded the highest P concentration in *Azolla* plant tissue, then the *Azolla* P concentration decreased in the higher K concentrations. Surprisingly, P nutrient levels did not have any significant effect on the *Azolla* P concentration.

Azolla K concentrations were enhanced by P and K levels in the nutrient solution (Figs. 4 and 5). A weak negative correlation was revealed in the *Azolla* K concentration and P media concentration (r= –0.07) (Table 6). Thus, the lower P concentration in the media had higher K concentration in the *Azolla* plant (Fig. 4). Whereas, the higher levels of K in the growing medium (30–40 mg K L^{-1}) increased *Azolla* K concentration (Fig. 5).

Phosphorus coupled with K concentrations had significant effects on *Azolla* K concentration (Figs. 4 and 5). Increasing K concentration in the growing medium (30–40 mg K L^{-1}) increased *Azolla* K concentration (Fig. 5). There was no statistical difference in terms of *Azolla* K concentration in the 30 and 40 mg K L^{-1} treatments (Fig. 5). In contrast, a weak negative correlation (or almost no relationship) was revealed in the *Azolla* K concentration

and P nutrient levels (r= –0.07) (Table 6). Thus, the slightly lower P levels in the media had higher K concentration in the *Azolla* plant (Fig. 4). This result was in agreement with an extensive review by Adams (1980) who asserted that there was little evidence of a P–K interaction concept in the plant, aside from a part of the cation-anion balance in the plant system that was strongly influenced by organic acids. Furthermore, the absorption rates of P and K in the nutrient solution are independent (Edwards, 1968). Wagner (1979) asserted that in order to get a maximum crop response from the addition of P, the plants also need sufficient levels of K.

Iron. *Azolla* Fe concentration was influenced by Mo nutrient concentrations in the nutrient solution (Table 5; Fig. 8) in an inverse relationship (r= –0.59) (Table 6). The Fe concentration in *Azolla* plant tissue tended to decrease at the higher Mo concentrations in the nutrient solution.

Iron is required for the formation of several key enzymes of the nitrogenase complex as well as for the electron carrier ferredoxin and for some hydrogenases. In particular, high Fe concentration is needed for the heme component of hemoglobin in legumes that results in a greater amount of nodule formation (Tang et al., 1990).

The plant host makes leghaemoglobin, an Fe protein, in the nodule (O'Hara et al., 1988). Whereas in bacteria, nitrogenase and nitrogenase reductase contain FeS clusters and also has the FeMoCo cofactor at the active site for N_2 reduction. Reduced nitrogenase activity rates occur in limited Fe nodules (O'Hara et al., 1988). Hence, Fe deficiency has a possible effect on reduced nodule mass, leghemoglobin content, number of bacteroids, and nitrogenase activity.

Molybdenum. Molybdenum is a micronutrient needed for root nodule formation with nitrogen-fixing bacteria and also for a protein involved with N metabolism and uptake in plants that do not form nodules (Wiedenhoeft, 2006). Mo has a role in N_2 fixation as well, given that

the Mo in 'FeMoCo' cofactor is essential for the N reduction process and for most nitrogenases. Iron also plays a role in physiological processes such as photosynthesis, chloroplast development and chlorophyll biosynthesis. Moreover, Mehraban et al. (2008) asserted that Fe is the main component of the cell redox systems, such as heme proteins (cytochromes, catalase, peroxidase and leg-hemoglobin) and Fe-S proteins (ferredoxin, aconitase and superoxide dismutase).

The excess of Mo uptake may cause physiological disorders and metabolic pathway alterations in plants (Rout and Das, 2002). However, Mo deficiency reduces chlorophyll concentration in plant leaves (Das, 1977) which directly reduces photosynthetic efficiency. Iron and Mo are both part of the nitrogenase enzyme; consequently, the abundance or unavailability of these elements may influence the enzyme activity and the biological N_2-fixation. Havlin et al. (2014) added that even though it does not represent an antagonistic interaction, metal cations such as an excess of Mo can induce Fe deficiency.

Actually, the highest Mo concentration in *Azolla* plant was found in the P study, in spite of no statistical differences among P rates. This result was in agreement with Mortvedt and Cunningham (1971) who stated that Mo concentration in plants increased with soluble P. Additionally, Stout et al. (1951) showed that high P levels in solution culture could enhance Mo uptake by 10-fold.

Significantly higher Mg and Mo concentrations in solution contributed to higher Mo concentrations in *Azolla* plants (Figs. 3 and 7). A strong correlation between Mo and Mg was also shown in the Mg nutrient study (r= 0.87) (Table 6). Furthermore, the greatest *Azolla* Mo concentration (292 mg kg^{-1}) was indicated by the biggest slope of 0.1 mg L^{-1} Mo nutrient level (Fig. 7).

Molybdenum plays a role in metabolic function of the nitrate reductase enzyme that reduces nitrate to nitrite in plants (Marschner, 1995; Bambara and Ndakidemi, 2010). In addition, Mo is involved in biological N-fixation as a co-factor of nitrogenase enzyme. An increased Mo requirement is commonly found in crops with *Rhizobium* symbiosis (Jongruaysup et al., 1993). Foliar application of Mo can enhance nodulation and biological N-fixation, thus improving plant growth (Elkhatib, 2009; Chahal and Chahal, 1991).

Increased foliar application of Mo had positive effects on vegetative growth characteristics and cauliflower yields (Ahmed et al., 2011). The stimulatory effect of Mo is possibly due to increased metabolic pools required for saccharide synthesis and enhanced photosynthetic capacity (Mendel and Haensch, 2002). Hence, Mo enhanced plant development through its function in N-fixation enzyme and nitrate reduction. Under Mo insufficiency, plants are not able to fix adequate N or incorporate nitrate into their metabolic system; and thus, Mo deficiency can lead to N deficiency (Bambara and Ndakidemi, 2010). The results of experiments by Bambara and Ndakidemi (2010) were in agreement with those obtained by Elkhatib (2009) on common bean, Chahal and Chahal (1991) on pea, and Gupta (1997) on soybean.

Magnesium. Magnesium on the other hand, has a fundamental effect on plant physiological processes such as photosynthesis (Mg is the central element of the chlorophyll molecule), sugar synthesis, starch translocation, plant oils and fat formation, nutrient uptake control, increased Fe utilization, and N-fixation in legume nodules that results in increased plant growth (Marschner, 1995; Mengel and Kirkby, 1987; Hopkins, 1995; Allison et al., 2001). Mg has an effect similar to Mo, in terms of yield enhancement. This may be due to Mg functions in sugar and protein formation, as well as P uptake regulation that is involved in carbohydrate translocation and metabolism (Kiss, 1989). The foliar application of Mo and Mg significantly

enhanced cauliflower yields, N and P concentrations in leaves and curds, as well as total chlorophyll, Mg concentration of leaves and vitamin C contents of curds (Ahmed et al., 2011). Other research also reported the same findings (Allison et al., 2001; Awad and El-Ghamry, 2007; Shanmugasundaram and Nanjan, 1992).

A negative relationship between Mo concentration in the growing medium and Fe and Cu concentrations in the *Azolla* plants (r= –0.59 and –0.34) (Table 6) was found in this study. According to Hangar (1965), there is an inverse relationship between Fe and Mo, in which reduced Fe translocation from roots to shoots occurred when Mo in the growth medium was at higher levels. He also added that chelated Fe in culture solutions can reduce symptoms of Mo toxicity in red clover grown in culture solutions. Similarly, Mo was also reported to have antagonistic effects on Cu uptake (Havlin et al., 2014). Excess Mo triggers Cu deficiency, and vice versa, excess Cu leads to Mo deficiency (Clark, 1984). Additionally, Gupta and Mehla (1979) stated that C and Fe content in berseem plants in normal and reclaimed saline sodic soils were lowered by Mo application.

Sulfur. A moderate positive correlation between *Azolla* S concentration and Mg levels in the growing medium was found in this study (Table 6; Fig. 3). Significantly contrasting patterns were illustrated between the S *Azolla* content and the P and Cu rates in the nutrient solutions (Table 6; Figs. 4 and 10). The higher P and Cu rates resulted in the lower *Azolla* S concentrations (Figs. 4 and 10).

An interaction between S and Mg occurred in Chinese cabbage (Reich et al., 2016). S deficiency resulted in higher Mg concentration in fresh weight vegetable shoots. In contrast, when S applied as H_2S through fumigation, Mg concentration in the cabbage declined (Reich et al., 2016).

The interaction between S and P has been studied in a greenhouse experiment on mung bean (*Phaseolus aureus*). The grain yield and vegetative plant tissues increased with the individual application of S or P, yet decreased when they were both applied in different combinations (Aulakh and Pasricha, 1977). Sulfur application increased S concentration, but it reduced P in the vegetative plant tissue and grains. Whereas, P application increased total P concentration in mung bean plants. Additionally, protein content in the grains was greater with S application, but not in P application. In summary, yield, grain quality, concentration and total removal of S and P by mung bean crop were controlled by the significantly negative interaction between S and P (Aulakh and Pasricha, 1977). The S and P fertilizers that were applied together resulted in an antagonistic effect on plant uptake.

It was suggested to apply a balanced application of S with other kinds of fertilizer such as Mo and Cu, in particular for forages (Alcanada, 2016). Sulfur addition influences the availability of other nutrients, except for Mo. S and Mo have an inverse interaction (Alcanada, 2016; MacLeod et al., 1997; Reich et al., 2016). Since alfalfa is a legume, it responded favorably to Mo application. Thus, the alfalfa needed the recent soil and plant analysis information before Mo was applied, in order to achieve optimum S, Mo, and Cu nutrient composition in soil for alfalfa forages (Alcanada, 2016).

The immediate effects of Ca, Mg, Fe, Mo, B, and Cu nutrient levels in the nutrient solutions appeared in the *Azolla* concentrations of those corresponding nutrients. The correlations among the nutrient concentrations in the growing solution and the corresponding *Azolla* nutrient concentrations were positively correlated (Table 6; Figs. 5, 6, 7, 8, 9, and 10). Thus, the higher levels of Ca, Mg, Fe, Mo, B and Cu significantly contributed to the higher *Azolla* concentrations of those corresponding nutrients. Similar to the results of this study,

Khan et al. (2014) also revealed that Fe and N concentrations of chickpea leaves at the flowering stage were significantly increased with the higher levels of Mo (0.5 kg ha^{-1}) and Fe (2.0 kg ha^{-1}) applications in soil, in addition to the highest grain yield, yield parameters, and numbers of root nodules.

Inoculation rates and combined nutrient solutions affect *Azolla* growth parameters and *Azolla* nutrient concentrations

Inoculation rate altered the RGR and doubling time of *Azolla* (Table 4; Fig. 2). The lowest inoculation rate of 50-g m^{-2} had the shortest doubling time of *Azolla* growth (4.52 days). It was statistically equal to the 100–200 g m^{-2} inoculation rates, i.e. 5.37 and 6.58 days, respectively. Furthermore, the 50 and 100 g m^{-2} inoculation rates induced significantly higher *Azolla* RGR (Fig. 2).

These circumstances could be explained due to the fact that in the higher inoculation rate, there was a higher population density of *Azolla.* Therefore, the more competition among plants for nutrients and light intensity slowed the growth rate, and resulted in a longer doubling time.

Although the final combined nutrient solution study indicated no significant effects on the *Azolla* growth parameters such as percent greenness of *Azolla* color, biomass, doubling time, or RGR, the Wd3 combined nutrient solution resulted in the highest biomass and RGR and the lowest doubling time with 99.66% *Azolla* greenness.

Three kinds of nutrient deficiency that can have a negative effect on RGR of *Azolla* are Fe, Ca, and P (Kannaiyan et al., 1981). A higher RGR resulted at 1 mg L^{-1} Fe (level 2), 10 mg L^{-1} Ca (level 1), and 10 mg L^{-1} P (level 2), although there was no significant effect of those nutrient concentrations on RGR of *Azolla*.

Inoculation rates and combined nutrient solutions affected Fe and Mo concentrations in *Azolla* (Table 5). Iron concentration in *Azolla* plant tissue tended to decrease at higher inoculation rates (Fig. 11). The Wt solution gave the significantly highest *Azolla* Fe content compared to the other combined nutrient solutions (Fig. 13). Similarly, Mo concentration in the *Azolla* plants was also increased by Wt combined nutrient solution (Fig. 13).

K, Mn, and Zn concentrations in the *Azolla* plant tissue were significantly affected by the inoculation rates and combined nutrient solutions. The higher inoculation rates generated reduced concentrations of K, Mn, and Zn in the *Azolla* plant (Fig. 11). Wd3 combined nutrient solution tended to result in significantly higher *Azolla* K, Mn, and Zn concentrations (Figs. 12 and 13).

Based on the overall results of these greenhouse studies, it appears that the inoculation rates and combined nutrient studies have significant impacts on nutrient concentrations in *Azolla* (K, Fe, Mn, Mo, and Zn) (Table 5; Figs. 11, 12, and 13). There are two options of combined nutrient solutions that are suggested for *Azolla* optimum growth: Wt and Wd3 solutions. Based on significant differences of nutrient concentrations in *Azolla* plant tissue (Figs. 12 and 13), Wd3 and Wt nutrient solutions were similar in their effects on *Azolla* nutrient concentrations. However, based on *Azolla* growth parameters, it is suggested to use Wd3 in order to have the highest *Azolla* biomass and the shortest growing time. If economical perspective is also considered in *Azolla* culture, it is recommended to utilize Wd3 solution, because the Wd3 solution has lower nutrient concentrations (and, therefore, a lower cost) than the Watanabe solution.

Conclusions

In conclusion, this series of *Azolla* studies showed that:

1. The higher greenness percentages were shown in solutions with 0.01 and 0.0075 mg Zn L^{-1}.
2. The optimum *Azolla* inoculation rate was 100 g m^{-2} for 14-day growing periods under greenhouse conditions.
3. Changing nutrient concentrations in solution affected *Azolla* nutrient concentrations.
4. The recommended nutrient solution for *Azolla mexicana* cultivation in the greenhouse is the Wd3 solution.

TABLES

Table 1. Nutrient concentrations in tap water used for *Azolla* nursery.

Nutrient	Analysis[†]
pH	7.1
Ca	18.3 mg L^{-1}
Mg	2.4 mg L^{-1}
Na	3.27 mg L^{-1}
K	0.14 mg L^{-1}
CO_3^{2-}	< 0.1 mg L^{-1}
HCO_3^-	47 mg L^{-1}
Cl^-	4.7 mg L^{-1}
SO_4^{2-}	16.6 mg L^{-1}
NO_3^-	< 0.1 mg L^{-1}
NO_3-N	< 0.1 mg L^{-1}
B	< 0.01 mg L^{-1}
Sodium Adsorption Ratio	0.2
Salinity hazard	Low
Sodium hazard	Low
Electrical conductivity	79.7 dS m^{-1}

[†]Source: Colorado State University Soil, Water, and Plant Testing Laboratory, 2014.

Table 2. Nutrient concentrations used in a series of individual nutrient studies on *Azolla mexicana* in a greenhouse in 2014.

Nutrient	Source	Concentrations			
		Level 1	Level 2	Level 3	Level 4 (Watanabe)[†]
		---------- mg L^{-1} ----------			
P	$NaH_2PO_4.2H_2O$	5	10	15	20
K	K_2SO_4	10	20	30	40
Ca	$CaCl_2$	10	20	30	40
Mg	$MgSO_4.7H_2O$	10	20	30	40
Mn	$MnCl_2.4H_2O$	0.125	0.25	0.375	0.5
Fe	Fe-citrate	0.5	1	1.5	2
Mo	$Na_2MoO_4.2H_2O$	0.025	0.05	0.075	0.1
B	H_3BO_3	0.05	0.1	0.15	0.2
Cu	$CuSO_4.5H_2O$	0.0025	0.005	0.0075	0.01
Zn	$ZnSO_4$	0.0025	0.005	0.0075	0.01

[†]Source: Watanabe et al., 1977

Table 3. Nutrient concentrations used in the combined nutrient solution study in a greenhouse in 2014.

Nutrient	Source	Nutrient Solutions			
		Wd1	**Wd2**	**Wd3**	**Wt**[†] [‡]
		---------- mg L^{-1} ----------			
P	$NaH_2PO_4.2H_2O$	10	5	10	20
K	K_2SO_4	20	10	20	40
Ca	$CaCl_2$	10	10	10	40
Mg	$MgSO_4.7H_2O$	10	10	10	40
Mn	$MnCl_2.4H_2O$	0.375	0.125	0.375	0.5
Fe	Fe-citrate	1	0.5	1	2
Mo	$Na_2MoO_4.2H_2O$	0.075	0.025	0.075	0.1
B	H_3BO_3	0.15	0.05	0.15	0.2
Cu	$CuSO_4.5H_2O$	0.01	0.0025	0.01	0.01
Zn	$ZnSO_4$	0.01	0.0025	0.01	0.01
Co	$CoCl_2.6H_2O$	-	-	0.01	-

[†]Source: Watanabe et al., 1977
[‡]Level 4 from individual study

Table 4. Significance of *Azolla* growth parameters from twelve greenhouse experiments.

Experiment	*Azolla* parameters (*p*-value)[†]			
	Biomass	**Relative growth rate**	**Doubling time**	**% greenness**
P	0.235	0.240	0.175	0.320
K	0.806	0.774	0.689	0.738
Ca	0.824	0.839	0.894	0.272
Mg	0.613	0.616	0.618	0.457
Fe	0.235	0.237	0.236	0.347
Mn	0.427	0.438	0.452	0.684
Mo	0.651	0.636	0.592	0.387
Cu	0.916	0.922	0.911	0.332
Zn	0.623	0.623	0.647	0.009
B	0.742	0.758	0.782	0.064
Inoculation rate	0.152	0.0001	0.028	0.599
Nutrient solutions	0.366	0.351	0.360	0.967

[†]*p*-value <0.05 indicates significant difference across the treatment based on Tukey's honest significant difference (HSD) test.

Table 5. Significance of *Azolla* nutrient concentrations from twelve greenhouse experiments.

Experiment	*Azolla* nutrient concentrations (*p*-value)[†]											
	N	**P**	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Mo**	**Cu**	**Zn**	**B**	**S**
P	0.519	0.068	0.006	0.366	0.075	0.161	0.952	0.202	0.273	0.730	0.487	0.017
K	0.225	0.641	<0.0001	0.648	0.040	0.939	0.339	0.606	0.728	0.197	0.445	0.124
Ca	0.063	0.613	0.472	0.0001	0.247	0.111	0.159	0.850	0.306	0.367	0.181	0.170
Mg	0.513	0.062	0.723	0.338	0.001	0.071	0.311	0.042	0.079	0.708	0.098	0.007
Fe	0.707	0.327	0.872	0.561	0.585	<0.0001	0.257	0.230	0.062	0.607	0.344	0.246
Mn	0.457	0.699	0.639	0.783	0.716	0.496	0.253	0.127	0.952	0.398	0.739	0.222
Mo	0.648	0.249	0.630	0.960	0.765	0.027	0.636	<0.0001	0.053	0.415	0.806	0.132
Cu	0.265	0.108	0.457	0.395	0.953	0.988	0.750	0.104	0.005	0.660	0.177	0.023
Zn	0.900	0.373	0.126	0.424	0.530	0.623	0.379	0.568	0.342	0.337	0.275	0.056
B	0.520	0.191	0.266	0.692	0.842	0.347	0.707	0.079	0.618	0.165	0.053	0.057
Inoculation rate	0.111	0.164	0.064	0.132	0.100	0.002	0.0002	0.974	0.091	0.009	0.154	0.732
Nutrient solutions	0.274	0.224	0.008	0.943	0.220	0.013	0.011	0.001	0.146	0.039	0.762	0.258

[†]*p*-value <0.05 indicates significant difference across the treatment based on Tukey's honest significant difference (HSD) test.

Table 6. Correlation coefficients relating *Azolla* growth parameters and *Azolla* nutrient concentrations by experiment.

Parameters	Pearson correlation (r)[†] in greenhouse experiments										[£]IR	[‡]NS
	P	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Mo**	**Cu**	**Zn**	**B**		
[§]Bio vs. %greenness	¶	¶	¶	0.60 *P*<0.05	0.72 *P*<0.05	0.59 *P*<0.05	0.76 *P*<0.05	¶	¶	¶	¶	¶
Bio vs. N	¶	¶	¶	0.68 *P*<0.05	0.65 *P*<0.05	¶	¶	0.65 *P*<0.05	¶	¶	¶	¶
Bio vs. P	¶	¶	¶	¶	¶	¶	¶	–0.67 *P*<0.05	¶	¶	–0.72 *P*<0.05	0.76 *P*<0.05
Bio vs. K	¶	¶	¶	¶	¶	¶	0.67 *P*<0.05	¶	¶	¶	–0.82 *P*<0.05	¶
Bio vs. Ca	¶	¶	¶	¶	¶	¶	¶	¶	¶	¶	0.65 *P*<0.05	–0.63 *P*<0.05
Bio vs. Mg	¶	¶	0.67 *P*<0.05	¶	¶	¶	0.64 *P*<0.05	¶	¶	–0.60 *P*<0.05	¶	¶
Bio vs. Fe	¶	¶	¶	–0.65 *P*<0.05	¶	–0.69 *P*<0.05	¶	¶	–0.69 *P*<0.05	¶	¶	¶
Bio vs. Mn	–0.58 *P*<0.05	¶	¶	¶	¶	¶	¶	–0.70 *P*<0.05	¶	¶	–0.79 *P*<0.05	¶
Bio vs. Mo	¶	¶	¶	–0.74 *P*<0.05	–0.80 *P*<0.05	–0.73 *P*<0.05	¶	–0.68 *P*<0.05	¶	¶	¶	¶
Bio vs. Cu	0.60 *P*<0.05	¶	¶	¶	¶	¶	¶	¶	¶	¶	¶	¶
Bio vs. Zn	–0.65 *P*<0.05	–0.61 *P*<0.05	¶	¶	–0.60 *P*<0.05	¶	0.63 *P*<0.05	¶	¶	¶	¶	¶
Bio vs. B	–0.79 *P*<0.05	–0.69 *P*<0.05	¶	¶	–0.63 *P*<0.05	–0.59 *P*<0.05	¶	–0.76 *P*<0.05	¶	¶	0.79 *P*<0.05	¶
Bio vs. S	¶	¶	¶	¶	¶	¶	0.58 *P*<0.05	–0.73 *P*<0.05	¶	¶	¶	0.80 *P*<0.05
% greenness vs. [#]Td	¶	¶	–0.59 *P*<0.05	–0.61 *P*<0.05	–0.71 *P*<0.05	–0.60 *P*<0.05	–0.85 *P*<0.05	¶	¶	¶	¶	–0.60 *P*<0.05

Table 6. Correlation coefficients relating *Azolla* growth parameters and *Azolla* nutrient concentrations by experiment (continued).

Parameters	Pearson correlation (*r*) [†] in greenhouse experiments											
	P	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Mo**	**Cu**	**Zn**	**B**	**[£]IR**	**[‡]NS**
% greenness vs. [£]RGR	¶	¶	¶	0.60 *P*<0.05	0.72 *P*<0.05	0.59 *P*<0.05	0.76 *P*<0.05	¶	¶	¶	¶	¶
% greenness vs. Zn	¥	¥	¥	¥	¥	¥	¥	¥	0.56 *P*=0.06	¥	¥	¥
N vs. % greenness	0.96 *P*<0.05	0.85 *P*<0.05	0.88 *P*<0.05	0.88 *P*<0.05	0.87 *P*<0.05	¶	0.83 *P*<0.05	0.67 *P*<0.05	¶	¶	0.58 *P*<0.05	¶
N vs. P	¶	¶	0.63 *P*<0.05	¶	0.64 *P*<0.05	0.69 *P*<0.05	¶	¶	¶	¶	¶	¶
N vs. K	¶	–0.64 *P*<0.05	0.75 *P*<0.05	0.71 *P*<0.05	¶	0.61 *P*<0.05	0.62 *P*<0.05	0.91 *P*<0.05	¶	0.68 *P*<0.05	¶	¶
N vs. Ca	¶	¶	–0.73 *P*<0.05	¶	–0.64 *P*<0.05	¶	¶	–0.61 *P*<0.05	¶	¶	¶	¶
N vs. Mg	–0.60 *P*<0.05	¶	0.84 *P*<0.05	–0.64 *P*<0.05	¶	¶	¶	¶	¶	0.58 *P*<0.05	¶	¶
N vs. Fe	¶	¶	–0.68 *P*<0.05	–0.64 *P*<0.05	¶	¶	¶	¶	¶	¶	–0.61 *P*<0.05	–0.70 *P*<0.05
N vs. Mn	¶	¶	–0.86 *P*<0.05	¶	–0.72 *P*<0.05	¶	¶	–0.65 *P*<0.05	¶	¶	¶	¶
N vs. Mo	–0.64 *P*<0.05	¶	¶	–0.69 *P*<0.05	¶	¶	¶	–0.84 *P*<0.05	¶	¶	¶	–0.63 *P*<0.05
N vs. Cu	¶	¶	–0.63 *P*<0.05	¶	¶	¶	¶	¶	¶	¶	¶	¶
N vs. Zn	¶	¶	–0.60 *P*<0.05	¶	¶	¶	¶	¶	¶	¶	–0.65 *P*<0.05	¶
N vs. B	¶	¶	–0.94 *P*<0.05	¶	–0.73 *P*<0.05	¶	–0.76 *P*<0.05	–0.75 *P*<0.05	¶	¶	¶	–0.68 *P*<0.05

Table 6. Correlation coefficients relating *Azolla* growth parameters and *Azolla* nutrient concentrations by experiment (continued).

Parameters	Pearson correlation (*r*) † in greenhouse experiments										£IR	‡NS
	P	K	Ca	Mg	Fe	Mn	Mo	Cu	Zn	B		
N vs. S	¶	¶	¶	¶	¶	¶	¶	–0.83 *P*<0.05	-	0.60 *P*<0.05	¶	¶
P vs. K	–0.07 *P*=0.82	¥	¥	¥	¥	¥	¥	¥	¥	¥	¥	¥
P vs. Mg	¥	¥	¥	–0.01 *P*=0.99	¥	¥	¥	¥	¥	¥	¥	¥
P vs. S	–0.43 *P*=0.17	¥	¥	¥	¥	¥	¥	¥	¥	¥	¥	¥
Mg vs. Mo	¥	¥	¥	0.87 *P*<0.05	¥	¥	¥	¥	¥	¥	¥	¥
Mg vs. S	¥	¥	¥	0.65 *P*<0.05	¥	¥	¥	¥	¥	¥	¥	¥
Fe vs. Mo	¥	¥	¥	¥	¥	¥	–0.59 *P*<0.05	¥	¥	¥	¥	¥
Mo vs. Cu	¥	¥	¥	¥	¥	¥	–0.34 *P*=0.28	¥	¥	¥	¥	¥
Cu vs. S	¥	¥	¥	¥	¥	¥	¥	–0.21 *P*=0.51	¥	¥	¥	¥

£IR: Inoculation rate; ‡NS: nutrient solutions; §Bio: biomass; #Td: doubling time; £RGR: relative growth rate; ¶: no significant correlation found between *Azolla* parameters and/or *Azolla* nutrient concentrations; ¥: there are possible significant correlations between *Azolla* parameters and/or *Azolla* nutrient concentrations, however, it is not our particular interest.
†*P* <0.05 indicates significant correlation coefficient (*r*) based on Pearson correlation.

FIGURES

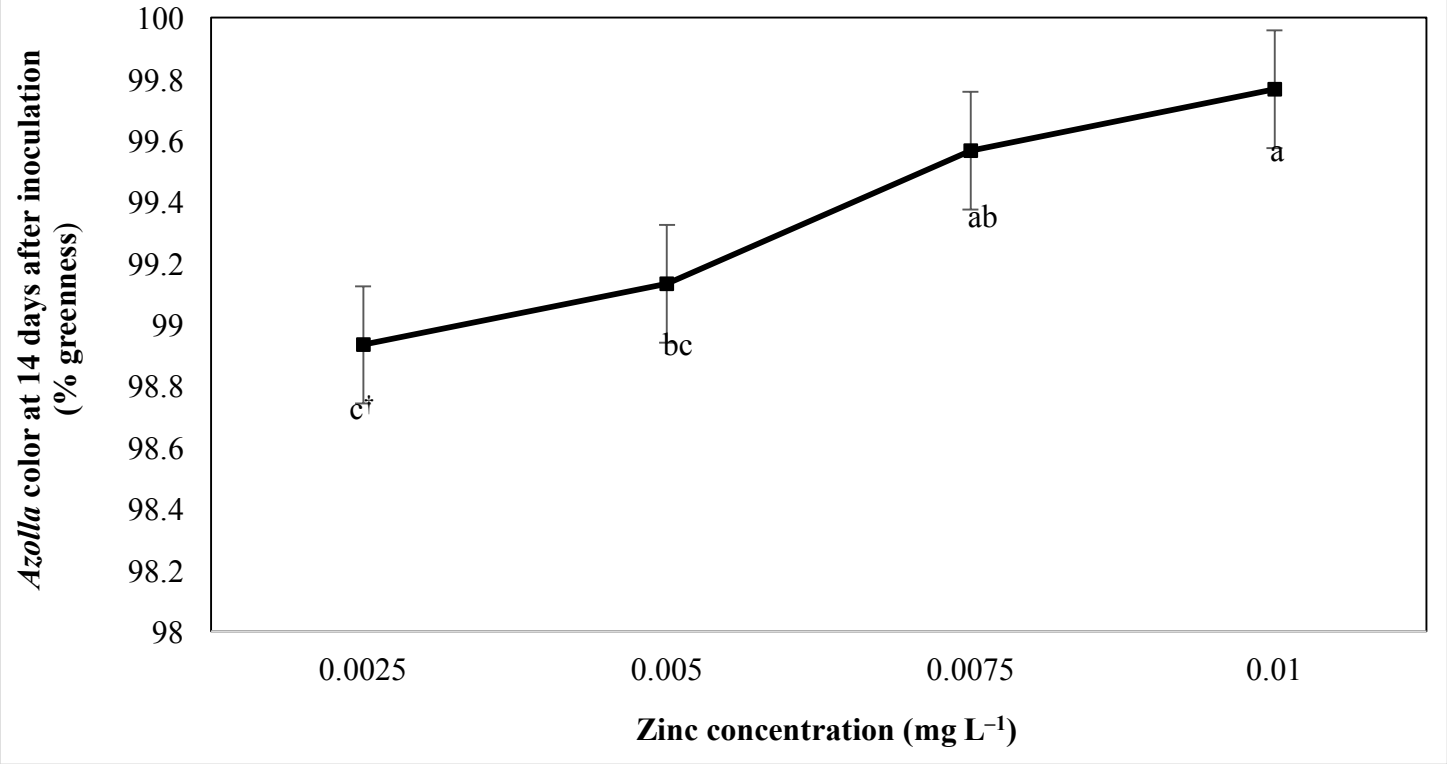
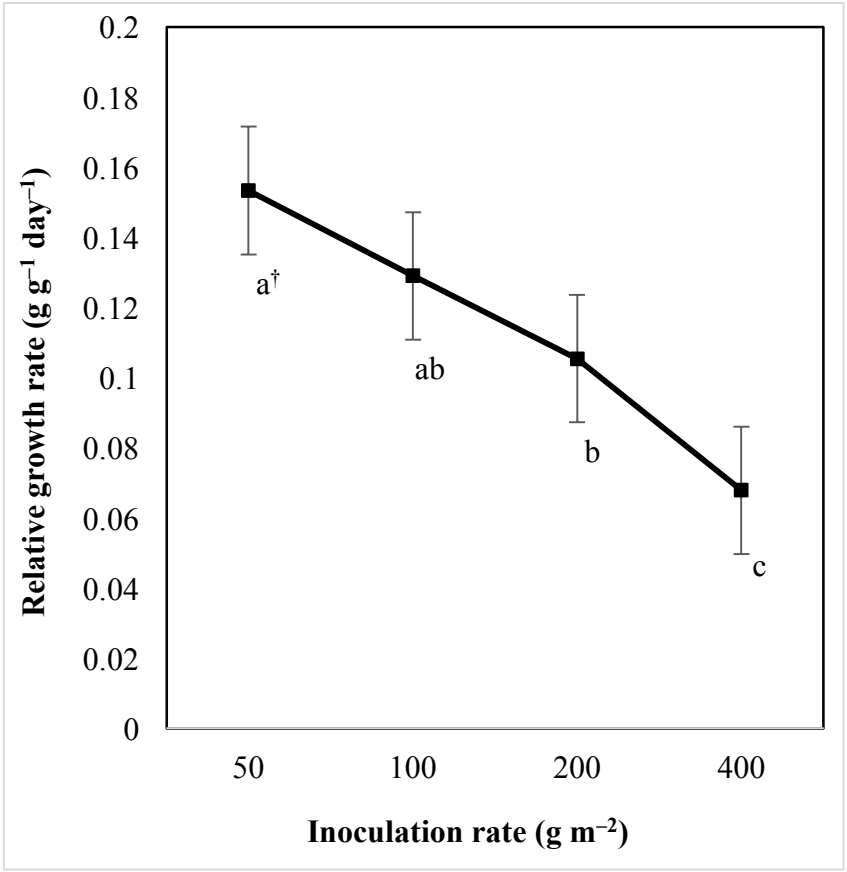
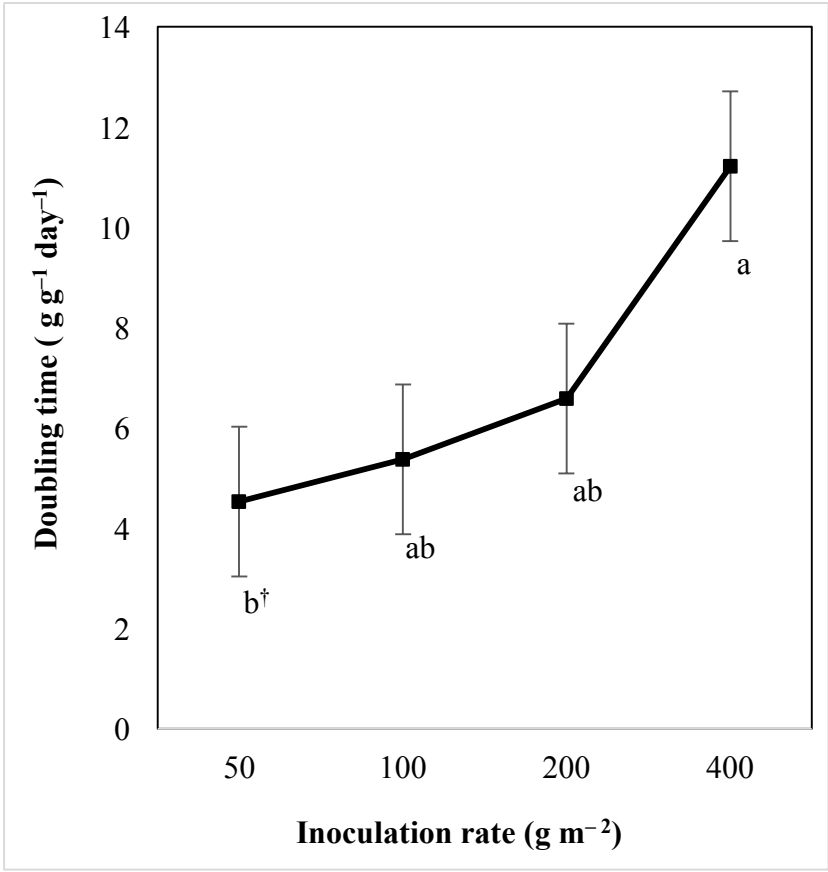


Fig. 1. Zn concentrations in the nutrient media affected *Azolla* color.
Azolla color was observed using Munsell color charts for plant tissues. The percent coverage of greenness was performed using the hue of 5. During the first week of *Azolla* growth, the *Azolla* had mostly hue 2.5, and some parts were yellowish or reddish. After two weeks of growth, the Azolla became darker green with a hue of 5. Higher coverage (stated as a %) of darker green *Azolla* signified healthier plants.
†Values followed by a common letter indicate no significant difference between Zn concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).



(A)



(B)

Fig. 2. Inoculation rates affected relative growth rate (A) and doubling time (B) of *Azolla.*
†Values followed by a common letter indicate no significant difference between inoculation rates based on Tukey's honest significant difference (HSD) test (P <0.05).

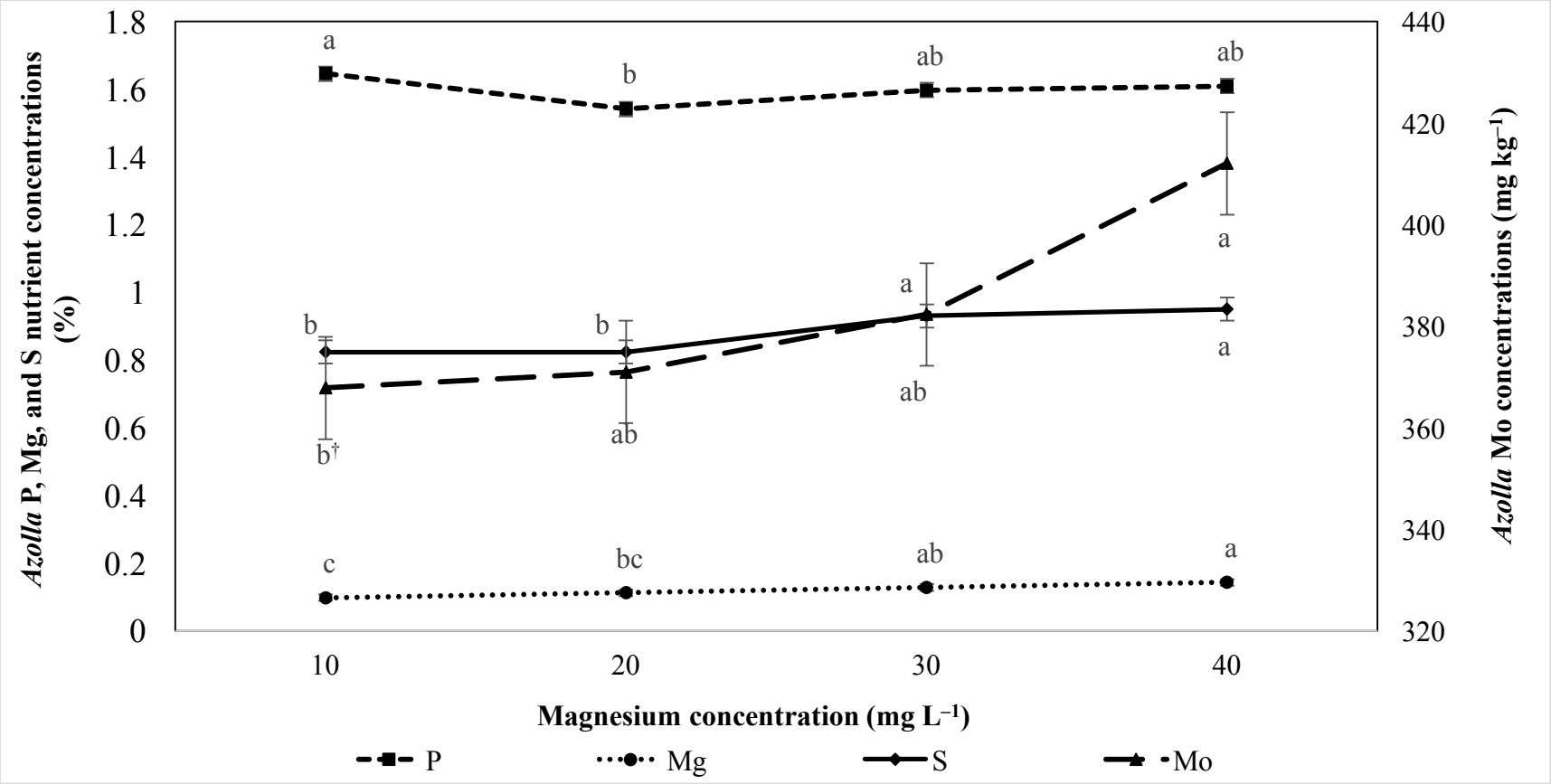


Fig. 3. P, Mg, S, and Mo concentrations in *Azolla* as affected by Mg concentration in solution.
†Values followed by a common letter indicate no significant difference between Mg concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).

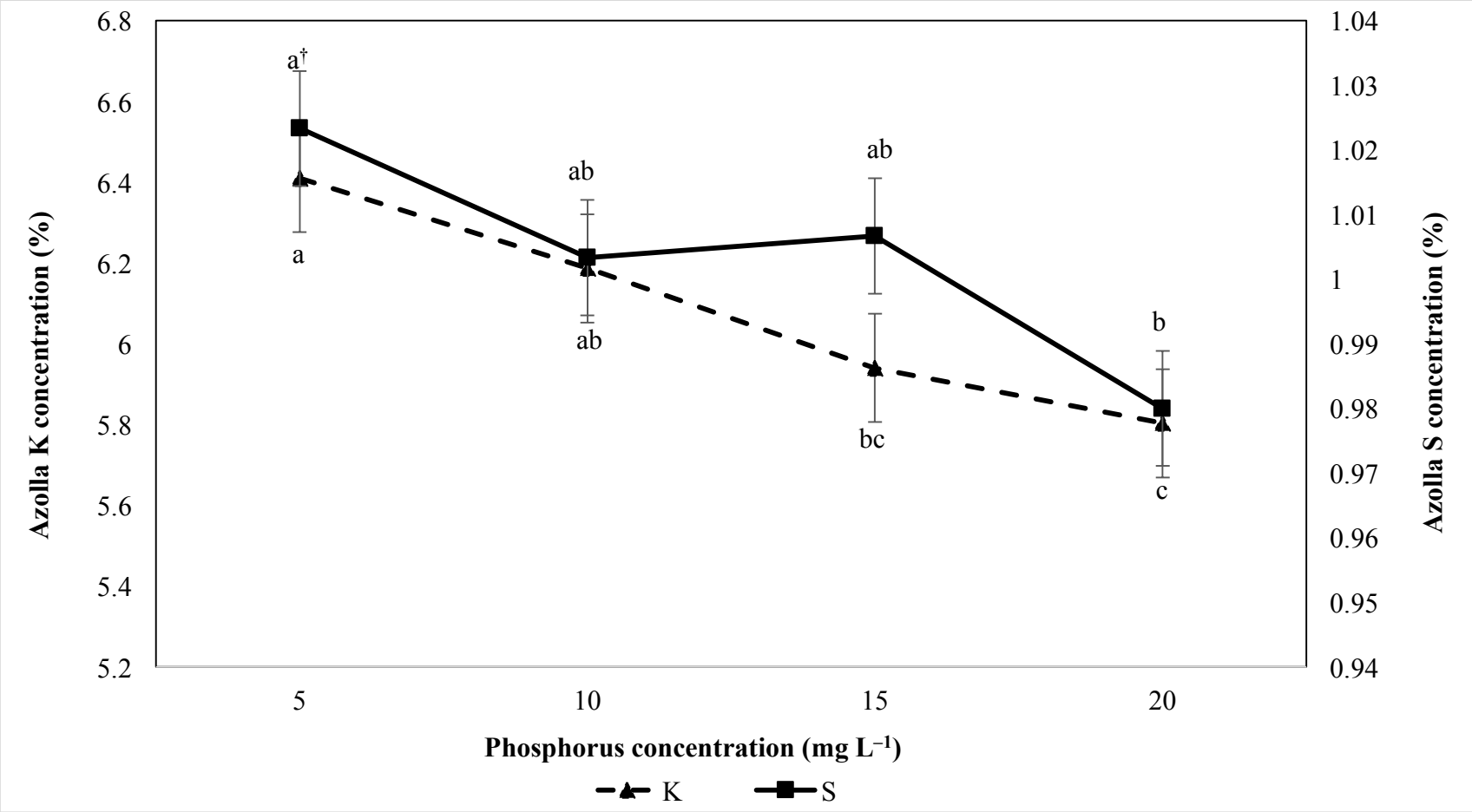


Fig. 4. K and S concentrations in *Azolla* as affected by P concentration in solution.
†Values followed by a common letter indicate no significant difference between P concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).

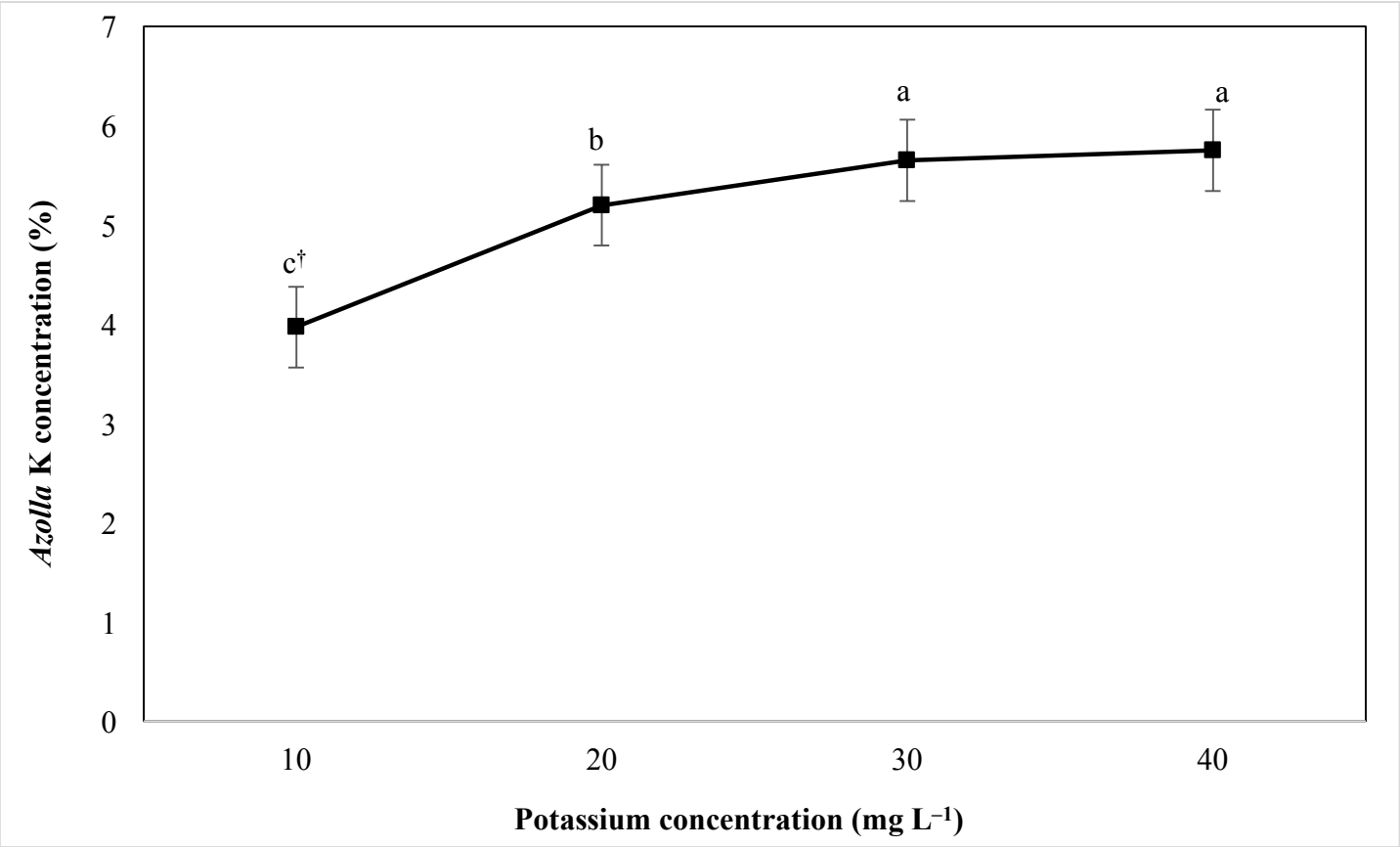


Fig. 5. K concentration in *Azolla* as affected by K concentration in solution.
†Values followed by a common letter indicate no significant difference between K concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).

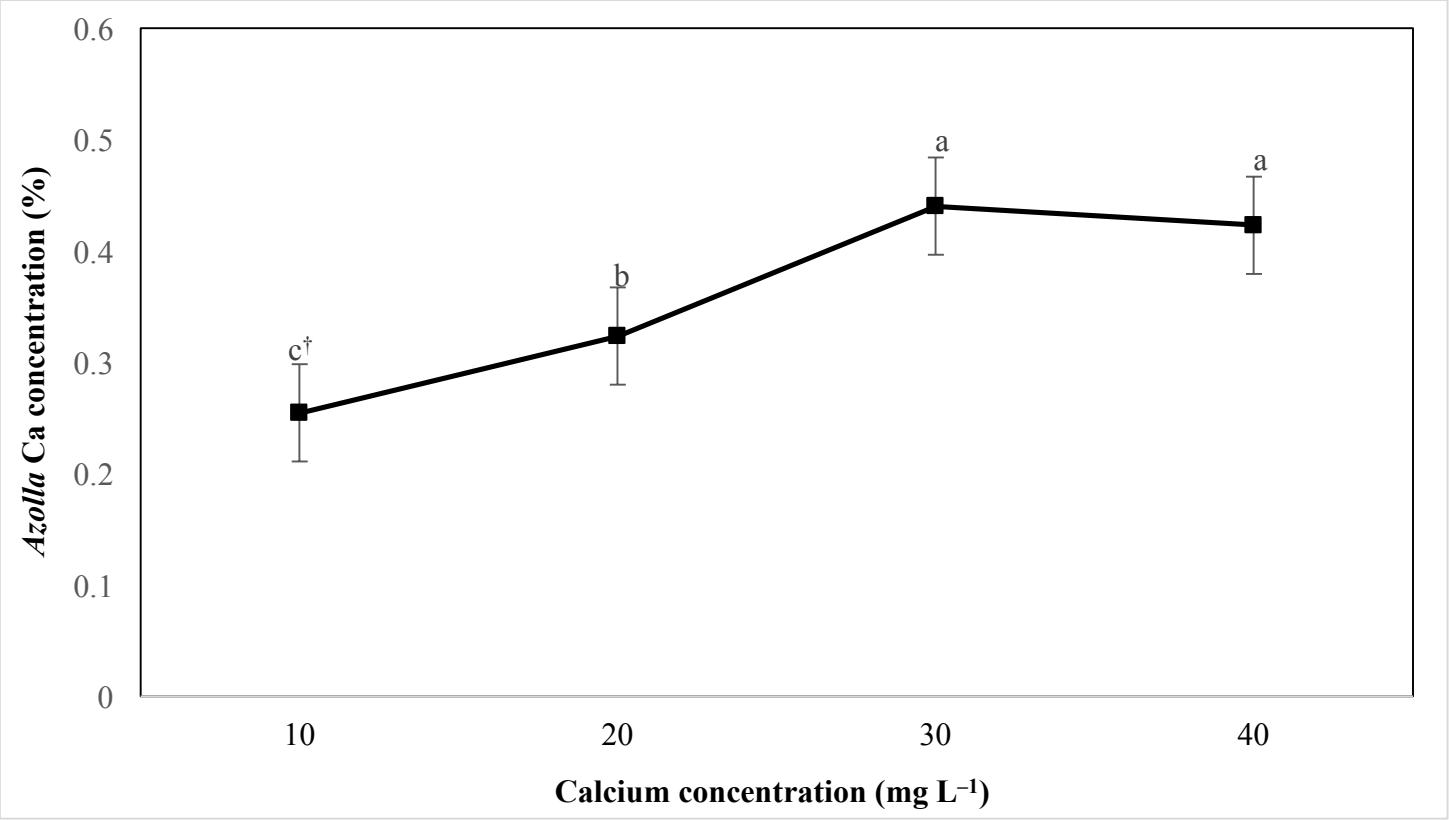


Fig. 6. Ca concentration in *Azolla* as affected by Ca concentration in solution.
†Values followed by a common letter indicate no significant difference between Ca concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).

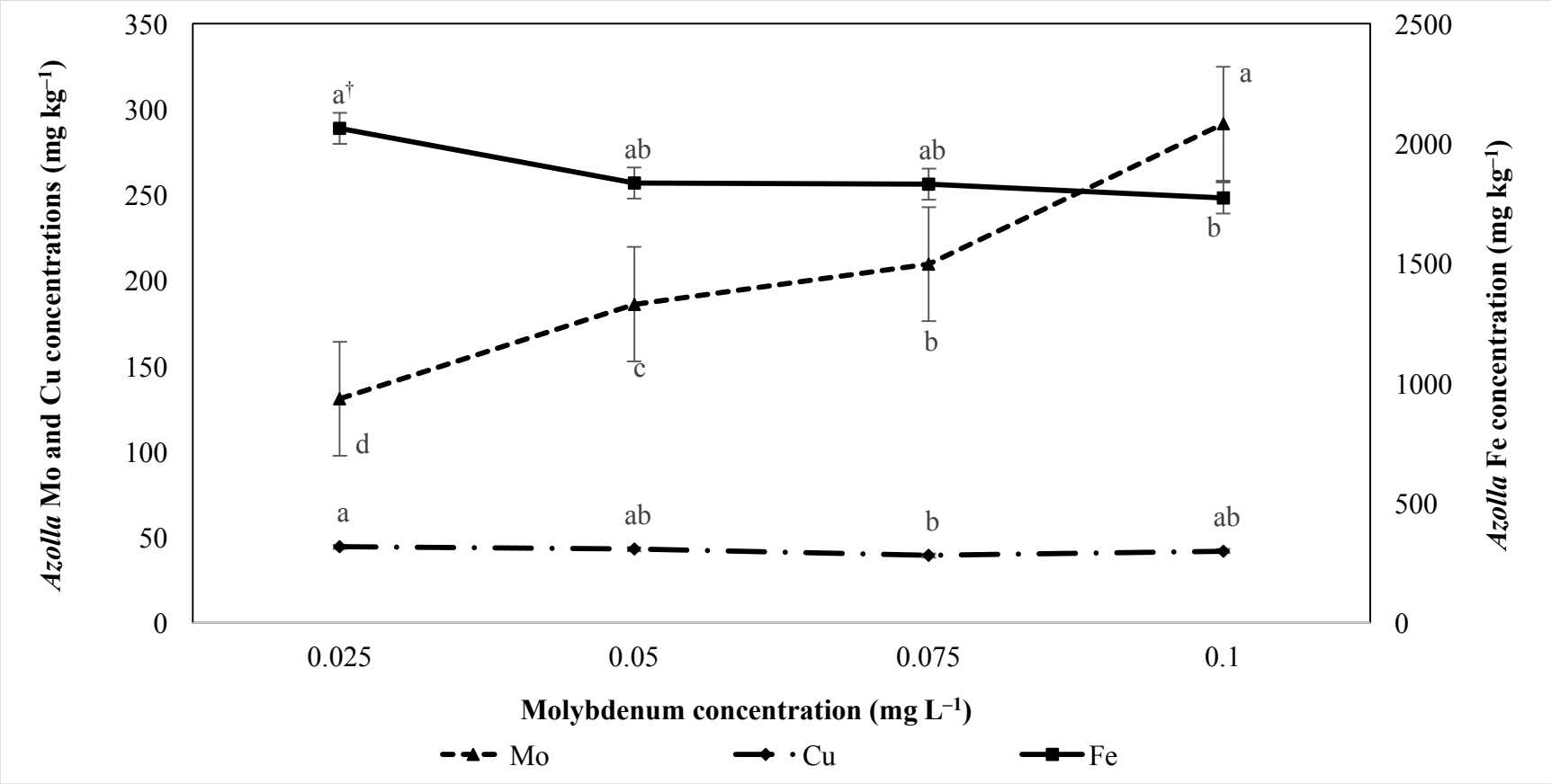


Fig. 7. Mo, Cu, and Fe concentrations in *Azolla* as affected by Mo concentration in solution.
†Values followed by a common letter indicate no significant difference between Mo concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).

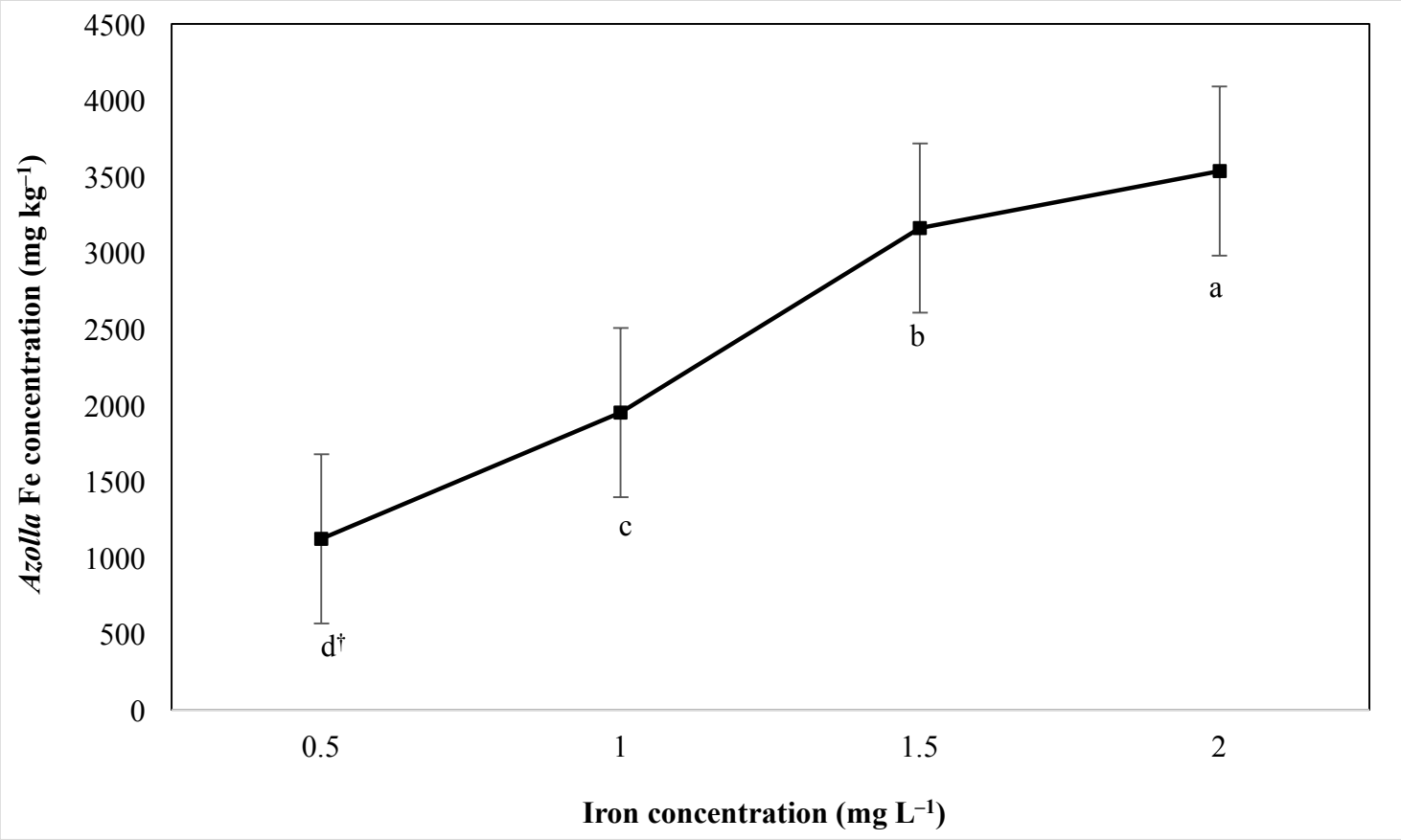


Fig. 8. Fe concentration in *Azolla* as affected by Fe concentration in solution.
†Values followed by a common letter indicate no significant difference between Fe concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).

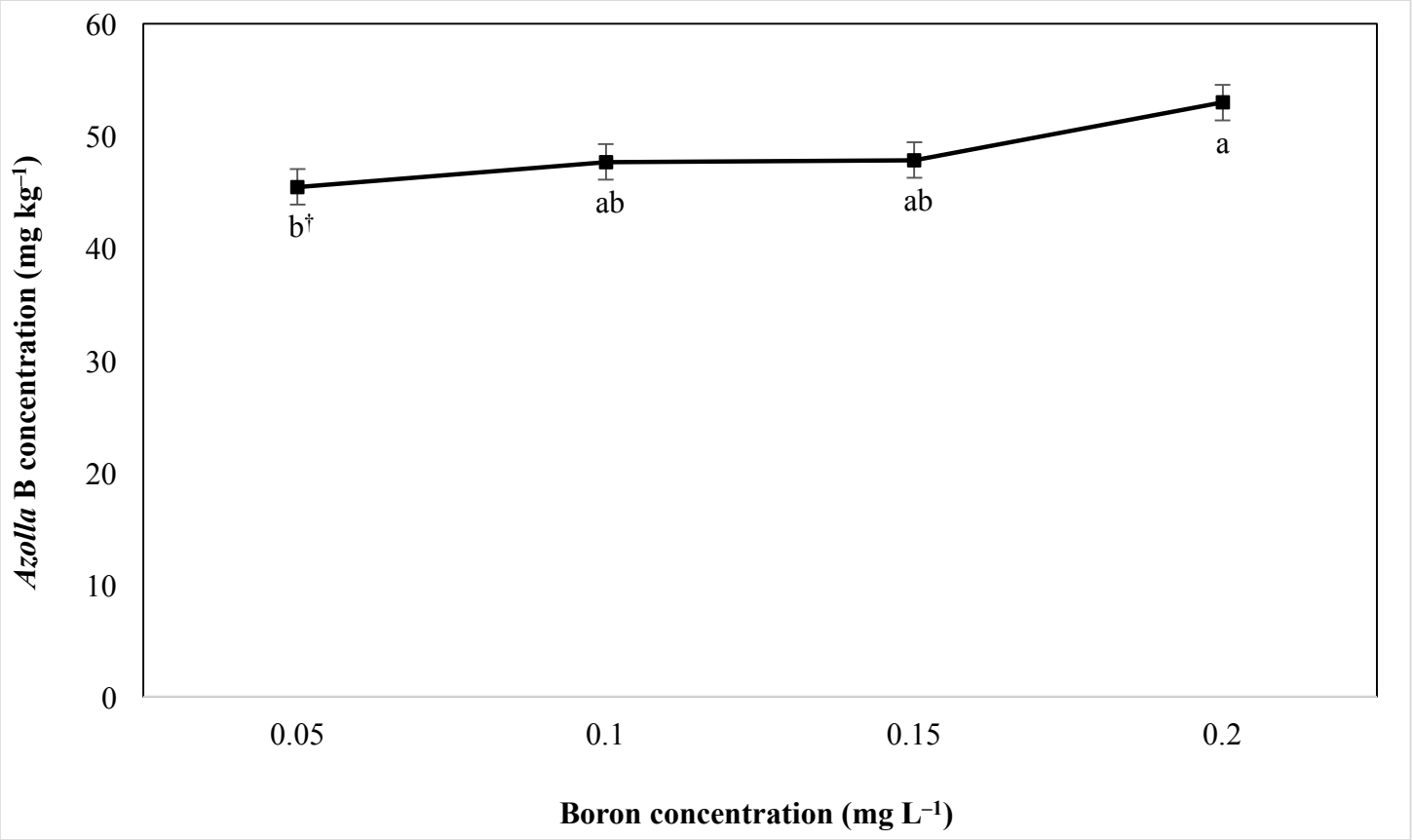


Fig. 9. B concentration in *Azolla* as affected by B concentration in solution.
†Values followed by a common letter indicate no significant difference between B concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).

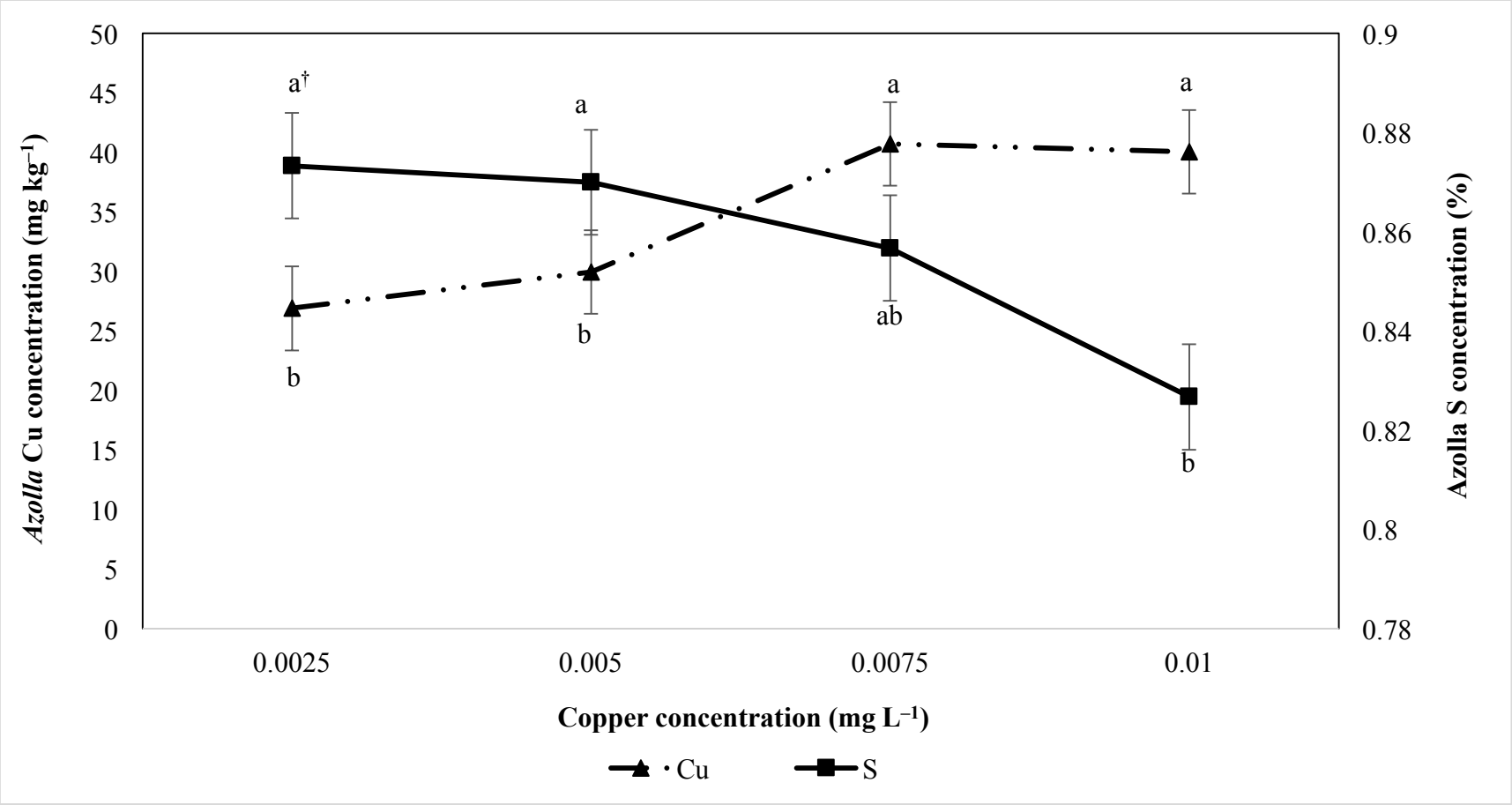


Fig. 10. Cu and S concentrations in *Azolla* as affected by Cu concentration in solution.
†Values followed by a common letter indicate no significant difference between Cu concentrations based on Tukey's honest significant difference (HSD) test (P <0.05).

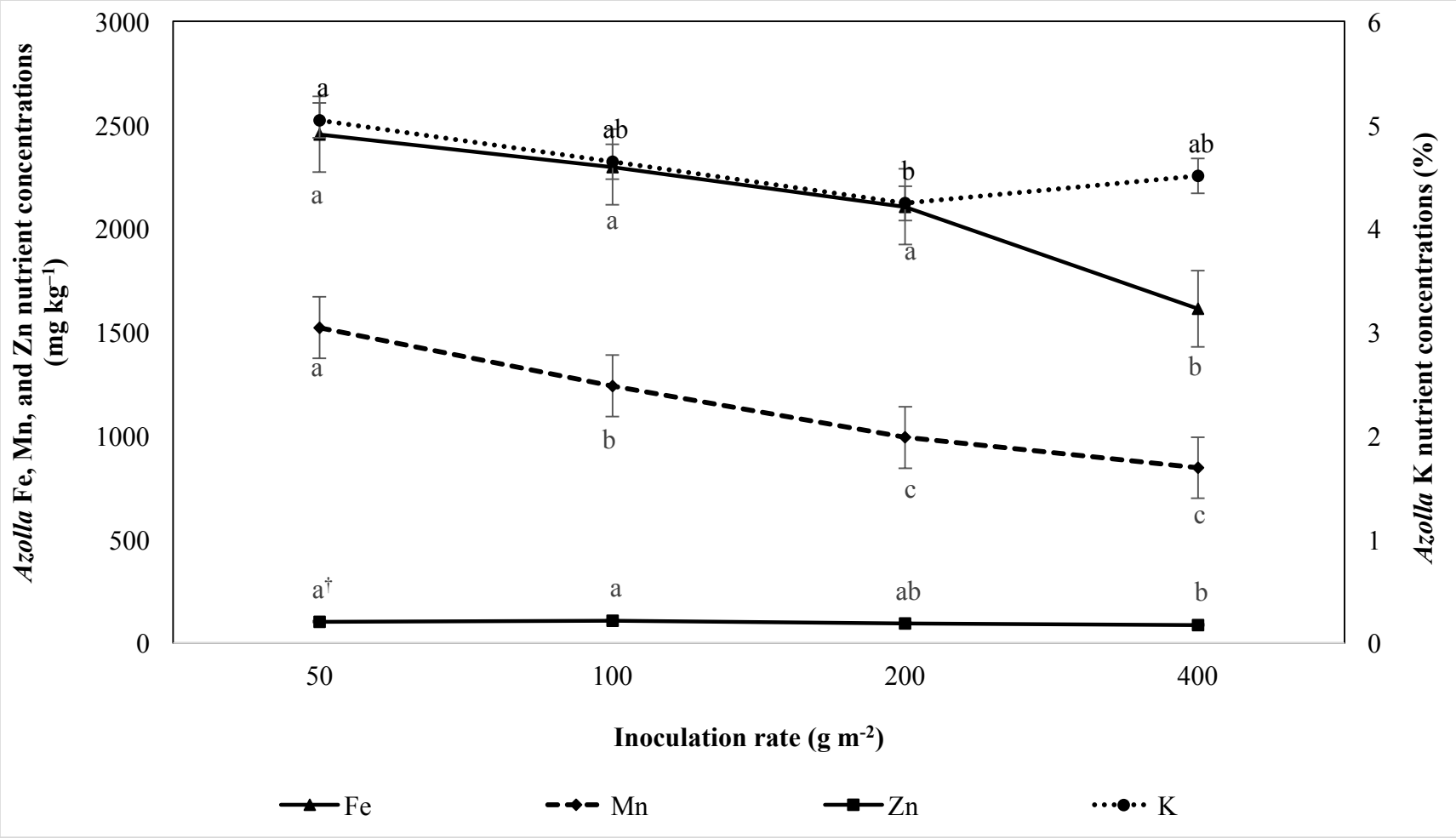


Fig. 11. Inoculation rates affected Fe, Mn, Zn, and K concentrations in *Azolla.*
†Values followed by a common letter indicate no significant difference between inoculation rates based on Tukey's honest significant difference (HSD) test (P <0.05).

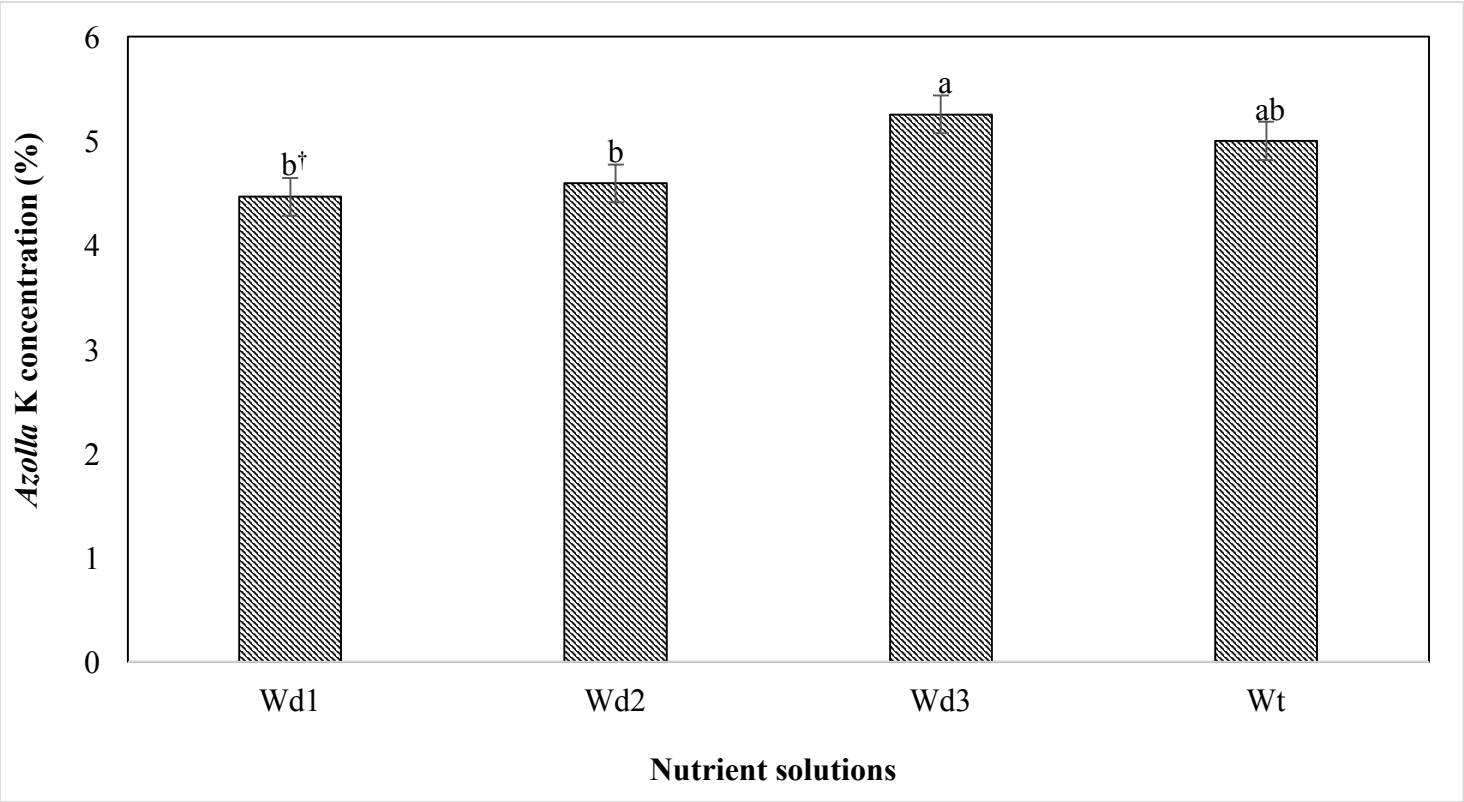


Fig. 12. Effect of nutrient solutions on K concentration in *Azolla*.
†Values followed by a common letter indicate no significant difference between nutrient solutions based on Tukey's honest significant difference (HSD) test (P <0.05).

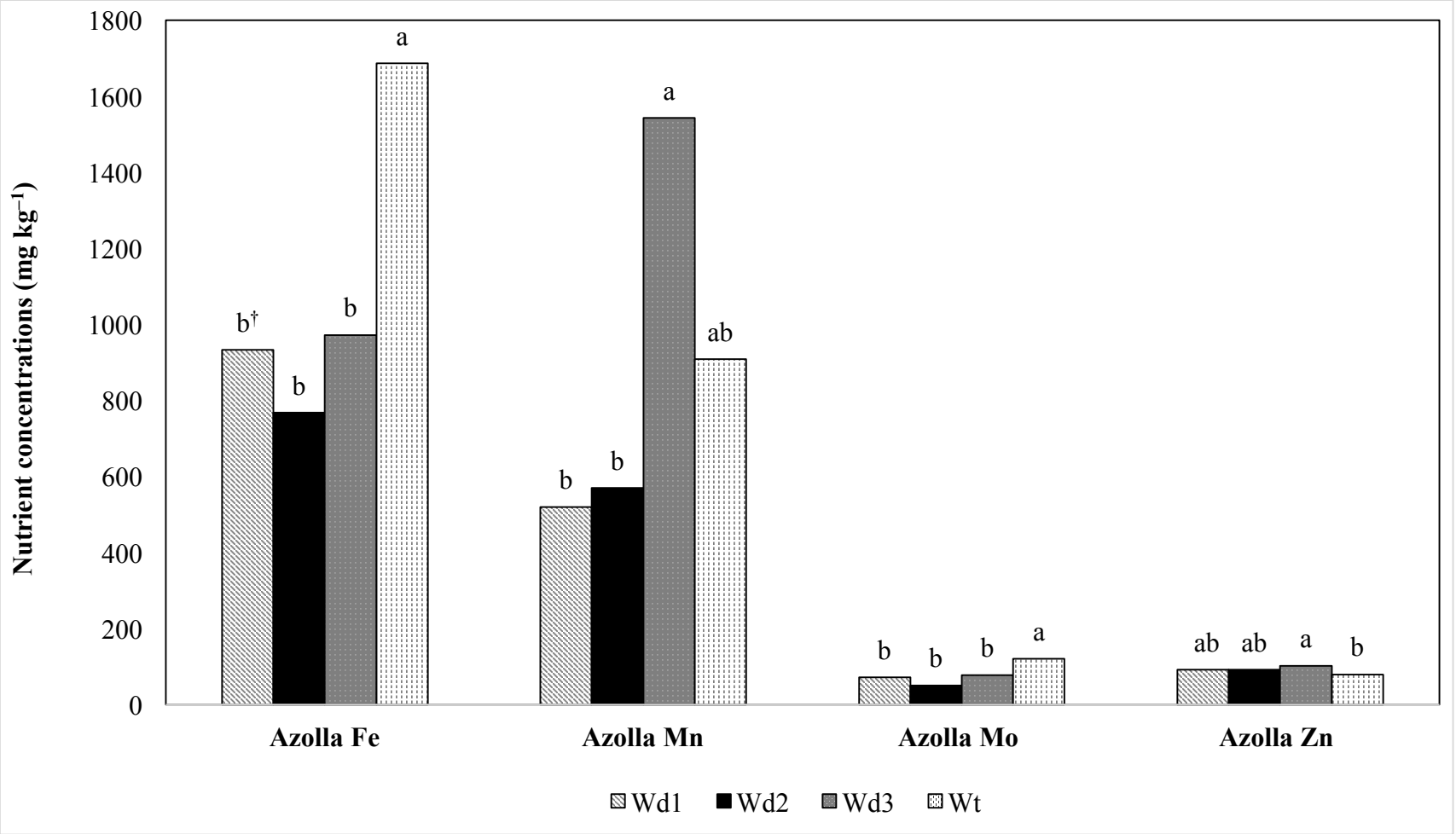


Fig. 13. Effect of nutrient solutions on Fe, Mn, Mo, and Zn concentrations in *Azolla*.
†Values followed by a common letter indicate no significant difference between nutrient solutions based on Tukey's honest significant difference (HSD) test (P <0.05).

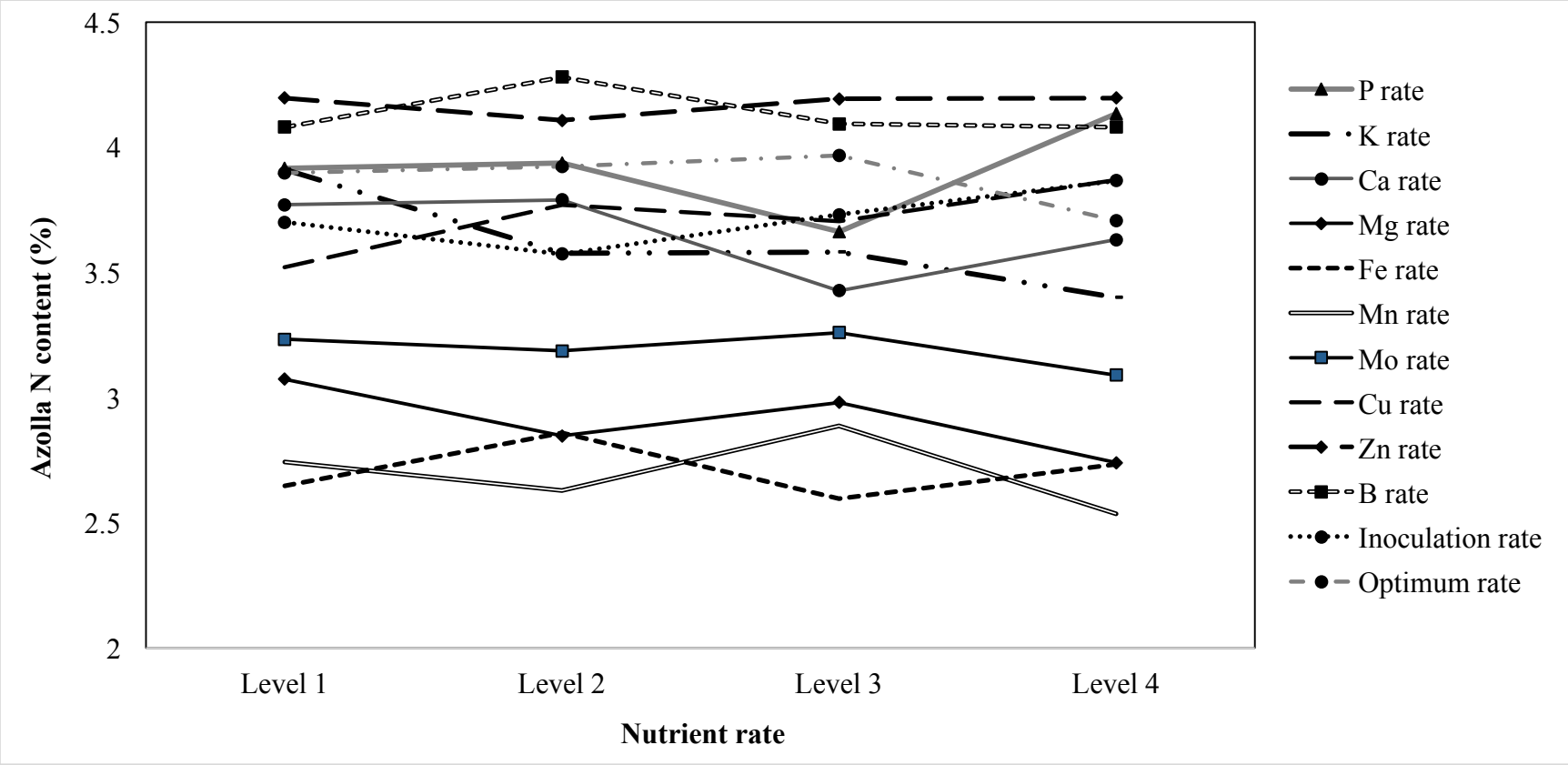


Fig. 14. ***Azolla*** **N concentrations as affected by ten individual nutrient studies, inoculation and optimum rates.**
Level 1–4 P: 5, 10, 15, 20 mg L^{-1} P; level 1–4 K: 10, 20, 30, 40 mg L^{-1} K; level 1–4 Ca: 10, 20, 30, 40 mg L^{-1} Ca; level 1–4 Mg: 10, 20, 30, 40 mg L^{-1} Mg; level 1–4 Fe: 0.5, 1, 1.5, 2 mg L^{-1} Fe; level 1–4 Mn: 0.125, 0.25, 0.375, 0.5 mg L^{-1} Mn; level 1–4 Mo: 0.025, 0.05, 0.075, 0.10 mg L^{-1} Mo; level 1–4 Cu: 0.0025, 0.005, 0.0075, 0.01 mg L^{-1} Cu; level 1–4 Zn: 0.0025, 0.005, 0.0075, 0.01 mg L^{-1} Zn; level 1–4 B: 0.05, 0.10, 0.15, 0.20 mg L^{-1} B; level 1–4 inoculation rate: 50, 100, 200, 400 g m^{-2}; level 1–4 optimum rate: Wd1, Wd2, Wd3, Wt.

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CHAPTER 3

EVALUATION OF *AZOLLA* UTILIZATION AS A BIOFERTILIZER IN SPINACH AND RADISH PRODUCTION SYSTEMS

Summary

Food security is a substantial goal for Indonesia, in particular to address the challenges of a growing population during a time of climate change. The Indonesian Ministry of Agriculture has launched a program known as "Sustainable Food-Reserved Garden" that promotes every household to grow food in their backyard. Thus, it serves to enhance food self-sufficiency starting at the household level. *Azolla* is a promising biofertilizer that has an important agronomic value for crops, in particular vegetable crops. This study was undertaken to evaluate the contributions of *A. pinnata* as a biofertilizer on red spinach and radish production on Inceptisols and Histosols in West Kalimantan, Indonesia compared to commonly-used fertilizers in enhancing vegetable crop yields and other agronomic parameters. A N rate study was performed as preliminary study to determine the optimum N rate for urea and whether manure had an effect on increasing vegetable yield, followed by the *Azolla* study. The N rate study was arranged in a randomized complete block design using a split-plot design. The whole plots were manure (10 t ha^{-1}) and no manure; whereas, the subplots were four N rate fertilizer (urea) treatments (N1 (23 kg N ha^{-1}), N2 (46 kg N ha^{-1}), N3 (69 kg N ha^{-1}), and N4 (92 kg N ha^{-1}) plus one control treatment (no N fertilizer). The *Azolla* study treatments were urea (23 kg N ha^{-1}), *Azolla*-U (*Azolla* applied at the urea N rate), manure (108 kg N ha^{-1}), and *Azolla*-M (*Azolla* applied at the manure N rate) plus one control treatment (no N fertilizer). Each treatment in either N rate or *Azolla* study was replicated three times. The N study revealed that N1 rate

(23 kg N ha^{-1} or urea applied at 50 kg ha^{-1}) or chicken manure was the optimum N rate for increasing vegetable yields. *Azolla* applied at the manure (*Azolla*-M) and urea (*Azolla*-U) N rates and manure increased spinach yield and the agronomic parameters in the spinach–peat site, while manure only improved spinach yield in the alluvial site. Radish plant height was shaped by manure treatment, in both alluvial and peat soils. Urea exhibited the most efficient use of N in the spinach–alluvial site. Manure and *Azolla* biofertilizer had similar NUE, in the order of higher NUE in manure, *Azolla*-U, then *Azolla*-M. Hence, *Azolla* could be utilized for vegetable production as a sustainable biofertilizer in dryland acidic tropical soils, to promote higher yields and maintain soil fertility.

Introduction

Malnutrition is a global fundamental concern that affects low to middle income countries. It manifests as a health problem that cannot be solved by health practitioners alone, but requires the expertise of agriculture professionals as well. In agriculture, we aim to provide sufficient, high-quality foods, that are widely available. Malnutrition can be associated with poor diets that is often the result of insufficient household food security.

Food security is a substantial goal for Indonesia, in particular to address the growing population in the face of climate change. Indonesia has the fourth largest population with 2.6 million people and faces some demographic issues due to the uneven distribution of population across regions (World Bank, 2017). The challenge of food security in Indonesia is how to supply sufficient, nutritious, and affordable food for the rising population.

The Indonesian Ministry of Agriculture was launched a program known as Sustainable Food-Reserved Garden that promotes every household to grow food in their backyard (IAARD, 2014). Thus, this program emphasizes that food self-sufficiency starts at the household level.

One of the nutritious food sources encouraged to be grown to improve national food security is vegetable crops. Spinach which has high Fe content (Dauthy, 1995; Yan, 2013) can be a good solution to tackle the Fe deficiency problem in Indonesia. Anemia affects 20.0–39.9% infants, children aged 6–59 months, and women of reproductive age (15–49 years) in Indonesia (WHO, 2015). Radish, on the other hand, may not serve as a vegetable rich in Fe; however, it still contributes to provide Fe for human beings.

One approach to intensify vegetable crop production and improve soil fertility is by using locally-grown fertilizer in vegetable crop fields. Sustainable fertilization could be accomplished through the process of biological nitrogen fixation.

Azolla is a biological N fertilizers which can fix atmospheric N_2 in a symbiotic relationship with *Anabaena azollae* (cyanobacteria) that occurs in the dorsal leaf cavities of the fronds (Peters and Meeks, 1989). Lumpkin and Plucknett (1980) estimated that *Azolla* N fixing capacity was 1.1 kg N ha^{-1} day^{-1} which is sufficient to supply the entire N need of rice. Moreover, this *Azolla* association may contribute to higher N-fixation compared to the legume–*Rhizobium* symbiotic relationship under favorable field conditions (Lumpkin and Plucknett, 1985).

There is some evidence that *Azolla* is an excellent source of N fertilizer for rice which can reduce or even substitute for the use of inorganic N fertilizers. *A. pinnata* can fix 75 mg N g^{-1} dry weight day^{-1}, equal to biomass production of 347 ton *Azolla* fresh weight ha^{-1} in a year. Depending on frequency and time of application, *Azolla* could provide 30–

60 kg of N ha^{-1} (Watanabe et al., 1989) or 40–60 kg N ha^{-1} per rice crop where *Azolla* is used as a dual crop grown along with rice (Kannaiyan, 1982). Additionally, Yadav et al. (2014) suggested that 347 tons of fresh *Azolla* biomass contained 868 kg N, equivalent to 1900 kg urea. Approximately 60–80% of N in *Azolla* mineralizes within two weeks when *Azolla* is incorporated into water-logged soils (Ito and Watanabe, 1985). In addition, Singh (1989) stated that *Azolla* performed better in dry season and with short duration rice varieties.

In many studies, *Azolla* has been successfully utilized as a green manure due to its N fixing potential, quick decomposition in the soil, and efficient N availability for rice (Kannaiyan, 1990). *Azolla* application increased nitrogen use efficiency (NUE) of urea by lowering NH_3 volatilization in flooded rice soil (Subedi and Shrestha, 2015). In rice, *Azolla* plays a role as a buffer for soil N availability and enhances NUE, since it captures excess N in early stages of rice growth and then releases N at later stages (Sisworo et al., 1995). The NUE was 32% when Azolla was applied alone, yet it increased to 43–53% when *Azolla* was applied in combination with urea (100 kg N ha^{-1}). The interaction between *Azolla* and inorganic N fertilizer resulted in increased NUE (Kumarasinghe and Eskew, 1993).

Azolla commonly has a low C/N ratio that allow it to mineralize faster than other organic fertilizers (Wang et al., 1987). As a result, *Azolla* will supply N to plants faster and possibly may increase plant N concentration.

In addition, other studies have revealed the potential use of *Azolla* for enhancing yield of other crops, such as corn (Ferrera-Cerrato and Romero, 1982; Kolhe and Mittra, 1990), wheat (Nain et al., 2010), and mungbeans (Ram et al., 1994), in addition to taro (*Colocasia esculenta*) in China (Wagner, 1997) and the Cook Islands (Teckle-Haimanot, 1995). In Senegal, *Azolla* is

harvested from ponds and incorporated into the soil in vegetable crop fields; whereas, on bananas, *Azolla* is used as a mulch surrounding the base of the plants (Van Hove, 1989).

In a long-term greenhouse experiment, *A. pinnata* as a biofertilizer showed higher grain and straw yields during the first rice crop, compared to NPK (80–60–40) fertilizer, decomposed cowdung, and compost (Zaman et al., 1995). Poultry manure, a commonly-used organic fertilizer, increased the length, breadth, and number of *Amaranthus* species leaves and crude fiber of *Amaranthus cruentus*. However, the growth parameters (plant height, number of branches, and stem girth), yield, and protein content performed better in NPK fertilizer (Oyedeji et al., 2014). The chicken manure was also more effective than other organic materials in enhancing plant height, dry weight of shoot and tuber of radish plants, and nutrient concentrations in leaves and tubers, except N content (Zeid et al., 2015).

Azolla is a promising biofertilizer that has an important agronomic value for rice and other crops. Yet, still little is known about utilizing *Azolla* as biofertilizer in dryland tropical vegetable cropping systems. Thereby, this study was undertaken to evaluate the contributions of *A. pinnata* as a biofertilizer on red spinach and radish production on Inceptisols and Histosols in West Kalimantan, Indonesia compared to commonly-used fertilizers in enhancing vegetable crop yields and other agronomic parameters.

Our hypotheses were as follows:

1. *Azolla* as a biofertilizer will increase vegetable plant growth (plant height, leaf numbers, branch numbers, and soil plant analysis development (SPAD) reading).
2. Soil amended with *Azolla* will enhance vegetable yields and agronomic parameters related to N (N leaf or bulb contents and NUE) because *Azolla* is a biofertilizer and can supply N and other nutrients.

Materials and Methods

Study Site Location

Field studies were located in West Kalimantan, Indonesia to represent the real condition of *Azolla* utilization in a tropical climate. For alluvial site, the field study was located at the Agricultural Research Station of the Assessment Institute for Agricultural Technology of West Kalimantan in Pal Sembilan Village, Sei Kakap, West Kalimantan (0^{o}03'32.5" S, latitude and 109^{o}15'27.9" E longitude); whereas, for the peat site, the study was located in a local farmer is field in Siantan, Pontianak, West Kalimantan (0^{o}00'57.2" N, latitude and 109^{o}20'13.6" E longitude). The elevation was approximately 0.1–1.5 m above sea level.

Based on USDA soil taxonomy, the soil type for the alluvial site is Sulfic Endoaquepts; and for the peat soil, it is Terric Sulfihemists (Hidayat et al., 2010) (Fig. 15). The climate is a tropical moist climate with III C and IV C classification based on the wet and dry months (Rejekiningrum et al., 2012). The average temperature is greater than 18 ^{o}C, and annual precipitation ranges from 2000–4000 mm with an average relative humidity of 80.8%.

Experimental Design

There were two types of field studies conducted in 2015. In order to determine the optimum dosage of N fertilizer, we arranged a preliminary study on both alluvial and peat soil sites. The N rate study was followed by a field study to evaluate the *Azolla* effect on vegetable production.

In the preliminary study, chicken manure was used as the commonly-used organic fertilizer and urea as the commonly-used inorganic N source. In order to determine the main effect of chicken manure and the optimum dosage of urea, we arranged a randomized complete block design (RCBD) using a split-plot design with three replications for each combination treatment, in the alluvial and peat soil sites. The treatments for whole plot were as follows:

1. M: manure (10 t ha^{-1}) (the commonly used dosage of chicken manure or 216 kg N ha^{-1})
2. NM: no manure

Whereas, the subplot treatments for the urea dosage were as follows:

1. N0: no urea control
2. N1: 50 kg urea ha^{-1} (23 kg N ha^{-1})
3. N2: 100 kg urea ha^{-1} (46 kg N ha^{-1})
4. N3: 150 kg urea ha^{-1} (69 kg N ha^{-1})
5. N4: 200 kg urea ha^{-1} (92 kg N ha^{-1})

In the alluvial and peat soil sites, the *Azolla* experimental design was arranged in a RCBD with N fertilizer as the treatment, as follows:

1. Control: no fertilizer control
2. Urea: urea applied at 50 kg ha^{-1} (23 kg N ha^{-1})
3. *Azolla*-U: dried *Azolla* applied at 1 t ha^{-1} (at the urea N rate of 23 kg ha^{-1}) (N content in *Azolla* was 2.88%)
4. Manure: chicken manure applied at 5 t ha^{-1} (108 kg N ha^{-1}) (N content in chicken manure was 3.19%)
5. *Azolla*-M: dried *Azolla* applied at 4.69 t ha^{-1} (at the chicken manure N rate of 108 kg N ha^{-1}) (N content in *Azolla* was 2.88%)

Each treatment was replicated three times. The dosage of urea (50 kg ha^{-1}) and chicken manure (5 t ha^{-1}) were determined based on the results of the preliminary N study.

Soil, Soil Amendment, and Biofertilizer Analysis

In order to identify soil properties of both study sites, soils at 0–20 cm depth were analyzed for their chemical and physical properties (Table 7). General soil properties analysis prior to the field study used air-dried and sieved (2 mm) soil for all analysis, including pH (soil:DI water extraction and soil:1 *N* KCl extraction with a ratio of 1:5) (Thomas, 1996; USDA-NRCS, 2005; Soil Survey Staff, 2014), soil organic matter (SOM) using Walkley–Black method (Nelson and Sommers, 1996), total N using Kjeldahl method (Bremmer, 1996), NH_4^+-N and NO_3^--N using 2 *M* KCl extraction and measuring with automated analyzer (Mulvaney, 1996), C/N ratio, available P using Bray-1 extraction and measuring with spectrophotometer (Kuo, 1996), exchangeable cations (K, Na, Ca, Mg) and cation exchange capacity (CEC) using ammonium acetate 1 *N* pH 7.0 extraction and measured with flame photometer for K and AAS for Na, Ca, and Mg (Helmke and Sparks, 1996). Base saturation was calculated based on total bases (exchangeable cations) and CEC (Havlin et al., 2014). Exchangeable H and Al were determined using 1 *N* KCl extraction and titrated with standardized 0.1 *M* NaOH (Sims, 1996), total Fe and Zn using nitric acid (HNO_3) and perchloric acid ($HClO_4$) extraction, and then measured with AAS (Loeppert and Inskeep, 1996; Reed and Martens, 1996), and soil texture using the pipette method (Olmstead et al., 1930).

In addition, prior to the field study, *A. pinnata* tissue was analyzed for its dry matter (Hoskins et al., 2003; Miller, 1998) and nutrient concentrations (Table 8). pH-H_2O was measured using a pH meter with dual electrode system (Wolf, 2003), organic C was determined by the

Walkley–Black method (Nelson and Sommers, 1996); whereas total N, $NH4^+$-N, and NO_3^--N were analyzed using the Kjeldahl and distillation methods (Watson et al., 2003; Peters et al., 2003). The other nutrients (P, K, Fe, and Zn) were determined following dry ashing and digestion of all organic matter with sulfuric acid, then reacted with hydrogen peroxide. P was measured with spectrophotometer and K was determined using flame photometer. Whereas, Fe and Zn was measured with atomic absorption spectrophotometer (AAS) (Lowther, 2008; Kovar, 2003).

Manure was analyzed for its moisture content (gravimetric) (Hoskins et al., 2003; Miller, 1998), pH (Wolf, 2003), organic C (Nelson and Sommers, 1996), total Kjeldahl N, NH_4^+-N, and NO_3^--N (Watson et al., 2003; Peters et al., 2003) (Table 8). Manure sample was dry-ashed and then extracted with 1 *N* HCl (Wolf et al., 2003). Spectrophotometer and flame photometer were used to determine P and K, while AAS was used for Ca, Mg, Fe, and Zn (Kovar, 2003) (Table 8).

The analysis of plant ash including pH (Thomas, 1996) and moisture content (Hoskins et al., 2003; Miller, 1998) (Table 8). Prior to further analysis for P and cations, plant ash was dried out in the oven at temperature 105 ^oC for 3 hours. Then it was extracted with 1 *N* HCl. Colorimetric method was used to measure P (spectrophotometer); whereas, flame photometer was used for measuring K, and AAS for Ca, Mg, Fe, and Zn (Wolf et al., 2003; Kovar, 2003) (Table 8). $CaCO_3$ equivalency was also determined after plant ash was extracted with 0.5 *N* HCl and titrated with 0.1 *N* NaOH (Sims, 1996) (Table 8).

Water was analyzed prior to the field study in order to identify the pre-existing conditions. Temperature, pH, salinity, PO_4^{2-}, NO_3^-, and Fe were measured (Motsara and Roy, 2008) (Table 9).

Cultivation

The spinach variety that was used for this study was Red "Giti" Spinach (Indonesian Vegetable Research Institute), and the radish variety was No. 22 short leaves (GL seeds, China). The spinach was transplanted 21 days after seeding, and the planting distance used for both spinach and radish was 20x15 cm.

Organic fertilizer was applied as a basal application, i.e. manure and *Azolla* was applied 3 days after transplanting (DAT) for spinach and 12 days after planting (DAP) for radish. Urea was applied on 3 and 14 DAT for spinach; whereas, for radish, it was applied on 12 and 27 DAP. There were additional P and K fertilizers that were used for radish, i.e. 250 kg SP-36 ha^{-1} and 180 kg KCl ha^{-1} were applied on 12 DAP. In order to increase pH of the peat soil, 3 t ha^{-1} of plant ash was applied following farmers common practice. Crops were harvested at 45 days for spinach and 49 days for radish.

Growing *Azolla* for Biofertilizer

Azolla pinnata was used for this field study, since this *Azolla* species is a native species in Indonesia. *Azolla* was grown in a natural pond at the alluvial site; whereas, in the peat site, it was grown in an artificial pond lined with polyethylene. *Azolla* is sensitive to heat and light. Therefore, when we grew *Azolla* in the natural or artificial ponds, we aimed to maintain the temperature of the growing media below 30 ^{o}C and protected the *Azolla* from high light intensity. The water resource for the *Azolla* ponds and the *Azolla* field study was from surface and rain water based on water availability at the alluvial soil site (Table 9). At the peat soil site, surface

water was used for the artificial *Azolla* pond and a mix of surface and rain water for the *Azolla* field study.

The inoculation rate of *Azolla* for the production ponds was 100–200 g m^{-2} based on the previous greenhouse study result. In the peat soil site, plant ash was applied into the *Azolla* pond at the rate of 2.68 t ha^{-1} to increase the water pH. *Azolla* was harvested 3–4 weeks after inoculation.

Data Collection and Analysis

The agronomic parameters measured in the preliminary N study and the *Azolla* field study were as follows:

Nitrogen study:

1. Yield
2. Plant height
3. Leaf numbers
4. Branch numbers (spinach)
5. Soil plant analysis development (SPAD) reading
6. Leaf area index (spinach)

Azolla study:

1. Yield
2. Plant height
3. Leaf numbers
4. Branch numbers (spinach)
5. SPAD reading (spinach)

6. Nitrogen use efficiency (NUE)

7. Leaf N content (spinach); bulb N content (radish)

SPAD reading was measured using a SPAD chlorophyll meter (SPAD 502 plus leaf chlorophyll meter, Konica Minolta, Japan). SPAD reading that was measured using SPAD-502 meter was related to the chlorophyll content in the leaf of *Arabidopsis thaliana* (Ling et al., 2011). There was a strong correlation between SPAD values into total chlorophyll per unit leaf area (R= 0.996). On average, it was around 6% the difference between the converted SPAD reading and photometric measurements of extracted chlorophyll (Ling et al., 2011). Whereas, leaf area index was measured using a portable leaf area meter (YMJ-A, Zhejiang Top Cloud-Agri Technology Co., Ltd, China).

Nitrogen use efficiency (NUE) was calculated using the following Eqn. 3 (Dobermann, 2005):

$$\text{Agronomic efficiency of applied N} = (Y_N - Y_0)/F_N \quad \text{(Eqn. 3)}$$

where:

Y_0 = yield in unfertilized N plot (kg)

Y_N = yield in N plot (kg)

F_N = kg N applied

All agronomic parameters were analyzed using SAS version 9.4 (SAS Institute, 2016). Analysis of variance (ANOVA) was performed on the data by using the Mixed procedure (Proc Mixed). Treatment means were compared using Tukey's honestly significant difference (Tukey's HSD) post hoc test (n= 3, P <0.10).

The relationships between yield and other agronomic parameters were assessed by Pearson correlation using the PROC CORR procedure. Model for predicting vegetable yield was determined using five methods as follows: backward, forward, and stepwise selections, and best subsets selection based on criteria of *Cp* and adjusted R^2. *Cp* and adjusted R^2 methods selected the model based on the lowest Akaike information criterion (*AIC*). Due to the more appropriate and accurate result, in which there was less variation in calculated yield data from the model selection compared to the average actual yield in the radish–spinach crops and alluvial–peat soils, backward elimination method was selected for yield estimation model of radish and spinach (Table 10).

Results

Agronomic Performance in N Rate Study

Overall, the only significant difference in agronomic parameters was found in yield and SPAD reading. Yield differences were only found in the spinach–alluvial site (Table 11; Fig. 16). There was no good yield result in the spinach–alluvial site that did not receive manure. Since N rate treatments did not affect spinach yield in the alluvial soil. The control treatment without manure resulted in the highest spinach yield, then it was followed by N3 (69 kg N ha^{-1}), N1 (23 kg N ha^{-1}), N4 (92 kg N ha^{-1}), and N2 (46 kg N ha^{-1}) (Fig. 16). N1, N2, or N4 treatment had significantly lower yield compared to control or N3. With manure application, the significantly highest yield was found in N3 (69 kg N ha^{-1}) treatment, and it had significantly greater yield compared to control. Nevertheless, the N3 treatment was not significantly higher

than N2 or N1. Furthermore, based on ANOVA (Table 11), the whole plots (manure and without manure applications) were similar (P= 0.42) in the spinach–alluvial site.

Another agronomic parameter that was statistically dissimilar was SPAD reading. In the whole plot without manure application, control or no urea fertilizer subplot had the highest SPAD readings in spinach–alluvial, radish–alluvial, and spinach–peat sites. In the spinach-peat site, N2 (46 kg N ha^{-1}) had the highest SPAD reading; however, it was not statistically different from control. In the radish–peat site, the manure whole plot displayed the highest SPAD reading in N4 subplot (92 kg N ha^{-1}), followed by N1 (23 kg N ha^{-1}). Yet, there were also no differences between those two N rates compared to control (Fig. 17).

Agronomic Performance in *Azolla* Study

N fertilizer affected spinach yield in both alluvial and peat soils (Table 12). Manure played a significant role in increasing spinach yield by 57.56% in the alluvial soil (Fig. 18). Whereas, in the peat soil, spinach receiving manure or *Azolla*-M treatments had significantly greater yield (231–233% higher) compared to control (Fig. 18). The *Azolla*-M treatment was also comparable to manure.

Manure treatment had an effect on radish height in the alluvial and peat soils (Fig. 19). It was the only N treatment that was significantly higher compared to control. Manure increased radish height by 11.05% in the alluvial soil and by 17.80% in the peat soil. Whereas, in the spinach–peat site, the *Azolla* applied at the manure N rate, manure, and *Azolla*-U increased the spinach height significantly compared to control, i.e. 66.39, 65.24, 34.49% (Fig. 19), and *Azolla*-M and manure were significantly greater than *Azolla*-U.

Leaf and branch numbers were significantly increased by the *Azolla* applied at the manure N rate in the spinach–peat site. The *Azolla*-M increased leaf number by 26.42% and branch number by 19.48% (Fig. 20).

N content was only significant in the spinach crop in both the alluvial and peat soils. In the spinach–alluvial site, manure had the highest N content followed by urea. Nevertheless, those two N treatments were not significantly higher in N content compared to control. In the peat soil, *Azolla*-M and manure treatments increased N content of spinach leaves by 98.54 and 63.60% compared to control (Fig. 21).

Urea was the most efficient N fertilizer compared to the other N fertilizer treatments in the spinach–alluvial study. Manure, *Azolla*-U, and *Azolla*-M treatments were statistically equal in NUE (Fig. 22).

Correlation Among Agronomic Parameters in N Rate and *Azolla* Studies

There were some highly significant moderate relationships (P <0.10) between yield and other agronomic parameters in the preliminary N study (Table 13). Those relationships were between yield and plant height in the radish–alluvial (r= 0.541), spinach–alluvial (r= 0.590), radish–peat (r= 0.489), and spinach–peat (r= 0.628). In addition, moderate correlations were found between yield and leaf number in the radish–alluvial site (r= 0.624), yield and branch number (r= 0.640) and yield and leaf area index (r= 0.698) in the spinach–peat site, and yield and SPAD reading (r= 0.520) in the spinach–alluvial site.

Plant height and leaf number was moderately correlated in the radish–peat site (r= 0.550) and strongly correlated (r= ≥0.700) in the spinach–peat site (r= 0.740). In addition, in the spinach–peat site, there were strong linear relationships between plant height and branch number

(r= 0.860) and plant height and leaf area index (r= 0.804). Leaf number and leaf area index demonstrated moderate correlation in the spinach–alluvial site (r= 0.636) and strong correlation in the spinach–peat site (r= 0.803). Branch number had strong relationships with leaf number (r= 0.769) and leaf area index (r= 0.707) in the spinach–peat site.

Similarly, in the *Azolla* study, there were some moderate and strong positive relationships between yield and plant height, i.e. radish–alluvial r= 0.611; spinach–alluvial r= 0.791; radish–peat r= 0.549; spinach–peat r= 0.860 (Table 14). Yield was also highly correlated with leaf number in the radish–peat site (r= 0.786). Some moderate correlations were discovered in the spinach–peat site, i.e. yield and branch number (r= 0.559) and yield and SPAD reading (r= 0.504). Correlations between yield and N content were found in all crops and soil types, i.e. radish–alluvial (r= 0.636, moderate), spinach–alluvial (r= 0.702, high), radish–peat (r= 0.863, high), and spinach–peat (r= 0.882, high) (Table 14).

Plant height was mostly in a moderate relationship with other agronomic parameters, except in the spinach–peat site (Table 14). Correlations were found between plant height and leaf number (radish–peat r= 0.662; spinach–peat r= 0.613), plant height and branch number (spinach–peat r= 0.675), and plant height and N content (spinach–alluvial r= 0.522; radish–peat r= 0.672; spinach–peat r= 0.804, strong).

Leaf number and branch number in the spinach crop were strongly correlated in the alluvial soil (r= 0.794) and in moderately correlated in the peat soil (r= 0.549). Furthermore, leaf number and N content was also shown to be highly correlated in the radish–peat site (r= 0.751). N content and SPAD reading in the spinach–peat site was moderately correlated (r= 0.509) (Table 14).

Model Prediction of Yield in N Rate and *Azolla* Studies

The selected model to estimate spinach yield in the alluvial soil in N rate study was yield = –28.95 + (0.53 x plant height) – (0.16 x leaf number) + (2.73 x branch number) (Table 10). In all other crops and soil types, based on ANOVA, the vegetable yields were not affected by N treatments (Table 11); therefore, we did not estimate the yield models for those corresponding crops or soils.

In the *Azolla* study, yield models were estimated for the spinach crop in both soil types (Table 10). In the spinach–alluvial site, the model was yield = –12.51 + (0.56 x plant height) + (5.62 x 10^{-2} x N leaf content). Whereas, in the spinach–peat soil, it was yield = –9.57 + (0.30 x plant height) + (0.11 x N leaf content). In both cases, spinach yield was best predicted from plant height and N leaf content.

Discussion

N Fertilizer Treatments Affect Agronomic Parameters of Vegetable Crops in N Rate and *Azolla* Studies

In manure whole plot, N rate treatment of N3 (69 kg N ha^{-1}) resulted in significantly higher yield than control (Fig. 16). Therefore, the N1 rate (23 kg N ha^{-1}), which was statistically equal to N3, was selected to be the N rate of urea, and the chicken manure dosage of 5 t ha^{-1} was used for the subsequent *Azolla* study. A reduced rate of chicken manure was used for the *Azolla* study based on efficiency, since there was no statistical difference between manure and no-manure whole plots (Table 11).

According to the ANOVA test (Table 11), there was no significant N treatment effect on SPAD reading in the no-manure whole plot or the manure application plot, except in the spinach–peat site. Control (no urea fertilizer) had the highest SPAD reading, even in the spinach–peat site that revealed higher but statistically equal SPAD readings in the N2 (46 kg N ha^{-1}) and control treatments in the no-manure plot (Fig. 17). Whereas, in the manure plot, N4 (92 kg N ha^{-1}) showed the highest SPAD reading in the radish–peat site; however, it was not statistically different from control, N1, or N2 treatments (Fig. 17).

Overall, manure resulted in the highest spinach yield in the *Azolla* study in both alluvial and peat soils. In the alluvial soil, manure had yield comparable to urea, while in the peat soil, manure was comparable with the *Azolla* applied at the manure N rate and greater than urea (Fig. 18). This result was in agreement with Oyedeji et al. (2014), in which NPK (15:15:15) contributed to the highest yield of *Amaranthus* species, followed by poultry manure treatment, and control. However, poultry manure was significantly higher than NPK and the control in terms of the proximate composition (protein, ash, crude fiber, lipid, and carbohydrate). Furthermore, Islam et al. (2011) also reported that poultry manure applied at 2.5 t ha^{-1} + 30% reduced recommended dose of chemical fertilizers produced the highest yield of stem amaranth and Indian spinach.

Azolla at the manure N rate (108 kg N ha^{-1}) enhanced fresh yield of spinach in the peat soil (Fig. 18). This result was in accordance with Jumadi et al. (2014) who stated that *Azolla* compost applied at a low rate (803 kg ha^{-1} or equal to 109 kg urea ha^{-1} or 50 kg N ha^{-1}), medium rate (1605 kg ha^{-1} or equal to 217 kg urea ha^{-1} or 100 kg N ha^{-1}), or high rate (2408 kg ha^{-1} or equal to 326 kg urea ha^{-1} or 150 kg N ha^{-1}) could increase the yield of upland water spinach grown in dryland, and it was statistically equal to urea (217 kg urea ha^{-1} or 100 kg N ha^{-1}). The

Azolla study revealed that manure or *Azolla*-M can be utilized as an N source to increase spinach yield in both alluvial and peat soil types. The C/N ratio of manure (10.11) and *Azolla* (16.08–16.36) indicated a good proportion of C and N which made N available for increasing yield (Table 8). This was confirmed by Kannaiyan (1990) who stated that *Azolla* decomposes rapidly in soil and thus, provided available N for rice plants.

In other studies, 75% recommended dose of N, P, and K fertilizers plus liquid foliar application of *Azolla* and mixed bacteria suspension (sprayed at 30 and 60 days after sowing) intensified yield and yield components of barley in saline soil, compared to the mixture of 75% recommended dose of N, P, K + *Azolla* dry + mixed bacteria suspension, 75% recommended dose of N, P, K + *Azolla* foliar, 75% recommended dose of N, P, K + mixed bacteria suspension, 75% recommended dose of N, P, K fertilizers + *Azolla* dry, and 75% recommended dose of N, P, K fertilizers (control) **(**El-Shahat et al., 2014**).** In addition, foliar application of *Chlorella ellipsoida* and *Spirulina maxima* tended to enhance growth and yield performance of wheat (El-Baky-Hanaa et al., 2008). Farmyard manure applied at 7.5 or 10 t ha^{-1}, or vermicompost applied at 1.5 t ha^{-1} was also equally effective to increase grain and straw yields of barley (Kumawat and Jat, 2005). Yield enhancement existed due to proper decomposition, mineralization, and available plant nutrients with manure application (Sinha et al., 1981).

Radish plant height was significantly influenced by manure treatment in both the alluvial and peat soils (Fig. 19). This was also discovered by Zeid et al. (2015) who reported that chicken manure was effective in enhancing radish plant height and dry weight of shoot and tuber, in addition to increased nutrients concentrations in leaves and tubers (except N concentration of radish grown in sandy soil). Increased spinach plant height in the peat site was affected by N treatments in the form of *Azolla* applied at the manure N rate, manure, and *Azolla* applied at the

urea N rate (Fig. 19). Jumadi et al. (2014) reported that plant height of upland water spinach was influenced by both *Azolla* compost and urea. *Azolla* incorporation coupled with or without addition of inorganic N fertilizer at different N rates affected rice plant height (Bhuvaneshwari and Singh, 2015). Additionally, Rivaie et al. (2013) stated that rice plant height was affected by the application of 5–10 t ha^{-1} *Azolla*.

Plant growth components such as leaf and branch numbers of spinach were substantially influenced by *Azolla* applied at the manure N rate in the peat soil (Fig. 20). This result was in accordance with Rivaie et al. (2013) who observed that the number of tillers per hill of rice crop was greater under 7.5–10 t ha^{-1} *Azolla* utilization. Similarly, Bhuvaneshwari and Singh (2015) found that *Azolla* application combined with inorganic N fertilizer or *Azolla* application alone enhanced the number of effective tillers per rice plant.

N content in spinach leaves was significantly higher in the *Azolla* applied at the manure N rate and manure treatments in the peat soil (Fig. 21). Whereas, in the alluvial site, the manure and urea treatments had similar N content in the spinach leaves as in control (Fig. 21). *Azolla* was comparable to manure in enhancing N content in spinach leaves in the peat soil. Spinach leaves that contains higher N content can be a good source of protein, as N is part of amino acids used as building blocks for proteins that are essential to good health. While the *Azolla* applied at the urea N rate and urea were similar in having lower N contents. This result was also reported by Manna and Singh (1990), Baker (2000), and Bhuvaneshwari and Singh (2012, 2015) showing that *Azolla* combined with inorganic N fertilizer increased N content in plants and yield. Combining *Azolla* with mineral N fertilizer enables higher N use efficiency to promote greater production. Moreover, Rivaie et al. (2013) recorded that different rates of *Azolla* application (2.5–10 t ha^{-1}) exhibited no statistically different N uptake by rice, and was similar to control.

Nitrogen use efficiency (NUE) in this study was represented as the agronomic efficiency of applied N (Dobermann, 2005). Overall, the highest NUE was indicated in the urea treatment, whereas, the manure, *Azolla*-U, and *Azolla*-M were similar to each other (Fig. 22). The higher agronomic efficiency for urea compared to *Azolla* was also reported by Watanabe et al. (1989); however, *Azolla* had higher physiological efficiency in rice. In contrast, another study that was conducted in wetland rice showed that urea and *Azolla* were statistically equal in both agronomic and physiological efficiency (Sisworo et al., 1990).

Correlation Among Agronomic Parameters in N Rate and *Azolla* Studies

In the N rate study, there were some observations of agronomic parameter data on radish and spinach, such as yield and yield components (plant height, number of leaves, number of branches (spinach), SPAD reading, and leaf area index (LAI) (spinach)). Based on correlation of some agronomic parameters in the N study, we decided which agronomic parameters would be collected in the subsequent *Azolla* study.

There were some moderate to strong relationships between yield and other agronomic parameters in the preliminary N rate study (Table 13). However, due to the limitations of the mobile LAI instrument, LAI data was not recorded in spinach in the *Azolla* study. Similarly, since there was no good relationship between SPAD reading and other agronomic parameters in the radish crop, the SPAD reading data was not observed for radish plants in the *Azolla* study.

The taller the spinach plants, the greater the branch number, leaf number, and LAI (Table 13). Likewise, there were some moderate and strong positive relationships among yield and other agronomic parameters in the *Azolla* study (Table 14). Interestingly, radish yield was also correlated with the number of leaves. Basically, marketable fresh yield for radish and

spinach was the same, i.e. from root to the plant shoot. Therefore, the more leaves or the taller radish plant affected the greater radish bulb fresh yield.

There were some highly prominent relationships between yield and N content in all crops and soil types, yield and SPAD readings in spinach, and also plant height and N content (Table 14). Furthermore, leaf number and N content were also shown to be highly correlated in the radish–peat site (r= 0.751). In the *Azolla* study, N content and SPAD readings in the spinach–peat site were moderately correlated (r= 0.509) (Table 14). There was a close relationship between chlorophyll and N content in wheat and soybean (Evans, 1983; Filed and Moony, 1986; Amaliotis et al., 2004; Bojović and Marković, 2007; Fritschi and Ray, 2007). This link is reasonable since N is a basic component of chlorophyll and protein molecules. Therefore, N content in spinach leaves or radish bulbs involves chloroplast structure and chlorophyll buildup (Tucker, 2004; Daughtry et al., 2000).

Conclusions

Overall, significant findings in this study are as follows:

1. *Azolla* and manure influenced yield and other agronomic parameters, in particular in the spinach crop on both soil types.
2. Manure affected radish plant height in both alluvial and peat soils.
3. Urea exhibited the most efficient use of N in the spinach–alluvial site. Manure as an organic fertilizer or *Azolla* biofertilizer were statistically similar in NUE.
4. Spinach and radish yields were positively correlated with N content, in both soil types.
5. *Azolla* biofertilizer or manure can enhance yield and other agronomic components, compared to the commonly-used N fertilizer (urea). Therefore, *Azolla* should be developed at a large

scale for vegetable production in dryland acidic tropical soils, to effectively promote higher yield utilizing sustainable fertilization.

6. *Azolla* utilization for dryland vegetable crop production could play a role in developing countries, in particular Indonesia, to enhance sustainable agriculture while reducing the negative environmental impacts of chemical fertilizer, and improving crop productivity and soil fertility.

TABLES

Table 7. Baseline soil properties of alluvial and peat soils.

Soil properties	N study		*Azolla* study	
	Alluvial	**Peat**	**Alluvial**	**Peat**
pH-H_2O	5.65	5.27	4.70	5.27
pH-KCl	4.62	4.65	3.89	4.65
Organic C (%)	1.80	50.27	2.15	50.27
NH_4^+-N (mg kg^{-1})	-	-	6.60	0.80
NO_3^--N (mg kg^{-1})	-	-	54.10	198.10
Total N (%)	0.24	2.26	0.25	2.29
C/N ratio	7.50	22.24	8.60	21.95
P-Bray 1 extraction (mg kg^{-1})	104.40	236.19	358.94	236.19
Exchangeable cations:				
- K (cmol(+) kg^{-1})	0.62	0.62	0.76	0.62
- Na (cmol(+) kg^{-1})	0.25	0.29	0.60	0.29
- Ca (cmol(+) kg^{-1})	7.03	58.36	1.80	58.36
- Mg (cmol(+) kg^{-1})	2.35	9.19	0.61	9.19
Cation exchange capacity (cmol(+) kg^{-1})	11.18	108.75	10.37	108.75
Base saturation (%)	91.68	62.95	36.37	62.95
Exchangeable H (cmol(+) kg^{-1})	0.19	0.13	0.15	0.13
Exchangeable Al (cmol(+) kg^{-1})	0.00	0.27	0.16	0.27
Fe (mg kg^{-1})	727.23	528.17	727.23	528.17
Cu (mg kg^{-1})	68.78	30.87	68.78	30.87
Zn (mg kg^{-1})	58.82	78.96	43.26	78.96
B (mg kg^{-1})	193.61	32.37	193.61	32.37
Texture:	Clay	-	Clay	NA
Sand (%)	5.32	-	5.32	NA
Silt (%)	41.52	-	41.52	NA
Clay (%)	53.16	-	53.16	NA

-: parameter was not analyzed in the samples.

Table 8. Soil amendment and biofertilizer analysis used in the field studies.

Parameter	Plant ash	Chicken manure	*Azolla*	
			Alluvial	**Peat**
pH-H_2O	9.13	6.88	6.75	7.15
Organic C (%)	-	32.25	45.14	48.07
NH_4^+-N (%)	-	0.33	0.25	0.25
NO_3^--N (%)	-	0.20	0.20	0.20
Total N (%)	-	3.19	2.76	2.99
C/N ratio	-	10.11	16.36	16.08
P (%)	1.28	0.74	0.23	0.31
P/N ratio	-	0.23	0.08	0.10
K (%)	5.49	5.08	3.91	4.10
K/N ratio	-	1.59	1.42	1.37
Ca (%)	5.51	3.27	-	-
Mg (%)	0.59	0.22	-	-
Fe (mg kg^{-1} for chicken manure and *Azolla*, and % for plant ash)	0.13	18.86	992.21	896.64
Zn (mg kg^{-1})	31.10	7.45	108.20	131.83
Moisture (%)	6.14	32.14	20.25	19.73
$CaCO_3$ equivalent (%)	38.51	-	-	-

-: sample was not analyzed for the parameter.

Table 9. Water analysis used for *Azolla* nursery.

Parameter	Rain water	Surface water	
		Alluvial	Peat
pH	4.790	6.410	2.790
Salinity (per-mille: parts per thousand, ‰)	0.000	0.100	0.100
PO_4^{2-} (mg L^{-1})	0.020	0.070	2.510
NO_3^- (mg L^{-1})	0.000	0.500	0.300
Fe (mg L^{-1})	0.058	0.000	0.046

Table 10. Models for yield estimation from the N rate and *Azolla* studies using backward elimination method.

Crop and site	Model selected	*p*-value[†]
N rate study:		
Spinach–alluvial	yield = –28.95 + (0.53 x height) – (0.16 x leaf#) + (2.73 x branch#)	0.0003
Azolla study:		
Spinach–alluvial	yield = –12.51 + (0.56 x height) + (5.62 x 10^{-2} x N content)	0.0003
Spinach–peat	yield = –9.57 + (0.30 x height) + (0.11 x N content)	<0.0001

Height: plant height; leaf#: leaf number; branch#: branch number; N content: N content in spinach leaf; SPAD reading: chlorophyll measurement using SPAD meter; LAI: leaf area index.

[†]*p*-value <0.10 indicates significant model based on backward elimination method.

Table 11. ANOVA (*p*-value) results showing the treatment effect (N fertilizer treatments) on agronomic parameters for radish and spinach on alluvial and peat soils in the N rate study.

Treatment effect	Alluvial		Peat	
	Radish	Spinach	Radish	Spinach
Yield				
Whole plot	0.1366	0.4193	0.0113†	0.0261†
Subplot (N fertilizer treatment)	0.9831	0.0106†	0.6650	0.1977
Whole plot * subplot	0.6003	0.0029†	0.8520	0.3702
Manure	0.8632	0.0057†	0.6860	0.3298
No manure	0.7500	0.0051†	0.8308	0.2219
Plant height				
Whole plot	0.1127	0.2568	0.1511	0.6440
Subplot (N fertilizer treatment)	0.7466	0.6323	0.1044	0.3433
Whole plot * subplot	0.8852	0.3064	0.6766	0.5230
Manure	0.9845	0.6257	0.1166	0.7477
No manure	0.6158	0.3099	0.6093	0.2314
Leaf number				
Whole plot	0.4741	0.9479	0.0271†	0.3955
Subplot (N fertilizer treatment)	0.3470	0.5377	0.4142	0.7442
Whole plot * subplot	0.1828	0.5323	0.7800	0.9800
Manure	0.4453	0.9992	0.5683	0.7895
No manure	0.1425	0.2208	0.5876	0.9518
Branch number				
Whole plot	-	0.6119	-	0.7315
Subplot (N fertilizer treatment)	-	0.8102	-	0.5116
Whole plot * subplot	-	0.0453†	-	0.8976
Manure	-	0.2179	-	0.8384
No manure	-	0.1692	-	0.5635

Table 11. ANOVA (*p*-value) results showing the treatment effect (N fertilizer treatments) on agronomic parameters for radish and spinach on alluvial and peat soils in the N rate study (continued).

Treatment effect	Alluvial		Peat	
	Radish	Spinach	Radish	Spinach
SPAD reading[¶]				
Whole plot	0.7997	0.2004	0.6713	0.0148[†]
Subplot (N fertilizer treatment)	0.3962	0.1652	0.2403	0.1013
Whole plot * subplot	0.0350[†]	0.0890[†]	0.1995	0.0273[†]
Manure	0.1343	0.3443	0.1107	0.1084
No manure	0.0951[†]	0.0449[†]	0.4397	0.0258[†]
Leaf area index				
Whole plot	-	0.8900	-	0.0132[†]
Subplot (N fertilizer treatment)	-	0.4009	-	0.2290
Whole plot * subplot	-	0.2508	-	0.8376
Manure	-	0.4668	-	0.5035
No manure	-	0.2147	-	0.4121

[†]*p*-value <0.10 indicates significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test.

[¶]SPAD reading: chlorophyll measurement using SPAD meter.

-: parameter was not measured for radish.

Table 12. ANOVA (*p*-value) results showing the treatment effect (N fertilizer treatments) on agronomic parameters for radish and spinach on alluvial and peat soils in the *Azolla* study.

Treatment effect	Alluvial		Peat	
	Radish	**Spinach**	**Radish**	**Spinach**
Yield	0.7853	0.0024†	0.7241	0.0017†
Plant height	0.0474†	0.3874	0.0092†	0.0002†
Leaf number	0.2690	0.3134	0.4007	0.1244
Branch number	-	0.4925	-	0.0548†
SPAD reading¶	-	0.3097	-	0.0677†
N content‡	0.9517	0.0367†	0.1042	0.0013†
NUE§	0.3162	0.0077†	0.5650	0.3372

†*p*-value <0.10 indicates significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test.

¶SPAD reading: chlorophyll measurement using SPAD meter.

‡ N content in spinach leaf and radish bulb.

§ NUE: nitrogen use efficiency.

-: parameter was not measured for radish.

Table 13. Correlation coefficients and *p*-values among agronomic parameters (*P* <0.10) across N fertilizer application treatments in the N rate study.

Agronomic parameters	Radish on alluvial		Spinach on alluvial		Radish on peat		Spinach on peat	
	r	***p*-value**[†]	***r***	***p*-value**	***r***	***p*-value**	***r***	***p*-value**
Yield vs. height	0.5410	0.0020	0.5895	0.0006	0.4889	0.0061	0.6280	0.0002
Yield vs. leaf#	0.6239	0.0002		NS		NS	0.4746	0.0080
Yield vs. branch#		NS	0.3465	0.0606		NS	0.6395	0.0001
Yield vs. SPAD reading		NS	0.5202	0.0032		NS		NS
Yield vs. LAI		NS		NS		NS	0.6981	<0.0001
Height vs. leaf#	0.4957	0.0053		NS	0.5496	0.0017	0.7400	<0.0001
Height vs. branch#		NS		NS		NS	0.8603	<0.0001
Height vs. SPAD reading	0.3359	0.0695	0.3252	0.0795		NS		NS
Height vs. LAI		NS		NS		NS	0.8044	<0.0001
Leaf# vs. branch#		NS		NS		NS	0.7685	<0.0001
Leaf# vs. SPAD reading	0.3571	0.0527	–0.4751	0.0080		NS	–0.4810	0.0071
Leaf# vs. LAI		NS	0.6359	0.0002		NS	0.8034	<0.0001
Branch# vs. SPAD reading		NS	0.4012	0.0280		NS	–0.4787	0.0075
Branch# vs. LAI		NS		NS		NS	0.7074	<0.0001
SPAD reading vs. LAI		NS		NS		NS	–0.4432	0.0142

[†]*p*-value <0.10 indicates significant correlation coefficient (*r*) based on Pearson correlation.
NS: no significant correlation found between agronomic parameters.
Height: plant height; leaf#: leaf number; branch#: branch number; SPAD reading: chlorophyll measurement using SPAD meter; LAI: leaf area index.

Table 14. Correlation coefficients and *p*-values among agronomic parameters (*P* <0.10) across N fertilizer application treatments in the *Azolla* study.

Agronomic parameters	Radish on alluvial		Spinach on alluvial		Radish on peat		Spinach on peat	
	r	***p*-value**[†]	***r***	***p*-value**	***r***	***p*-value**	***r***	***p*-value**
Yield vs. height	0.6113	0.0155	0.7911	0.0004	0.5486	0.0342	0.8595	<0.0001
Yield vs. leaf#		NS		NS	0.7856	0.0005		NS
Yield vs. branch#		NS		NS		NS	0.5592	0.0302
Yield vs. N content	0.6357	0.0109	0.7023	0.0035	0.8633	<0.0001	0.8821	<0.0001
Yield vs. SPAD reading		NS		NS		NS	0.5042	0.0553
Height vs. leaf#		NS		NS	0.6617	0.0072	0.6128	0.0151
Height vs. branch#		NS		NS		NS	0.6747	0.0058
Height vs. N content		NS	0.5218	0.0460	0.6722	0.0060	0.8041	0.0003
Height vs. SPAD reading		NS		NS		NS	0.5020	0.0565
Leaf# vs. branch#		NS	0.7943	0.0004		NS	0.5488	0.0341
Leaf# vs. N content		NS	–0.4589	0.0853	0.7508	0.0013		NS
Branch# vs. N content		NS		NS		NS	0.4909	0.0632
N content vs. SPAD reading		NS		NS		NS	0.5089	0.0527

[†] *p*-value <0.10 indicates significant correlation coefficient (*r*) within the same crop and soil based on Pearson correlation.

NS: no significant correlation found between agronomic parameters.

Height: plant height; leaf#: leaf number; branch#: branch number; N content: N content in spinach leaf or radish bulb; SPAD reading: chlorophyll measurement using SPAD meter.

FIGURES

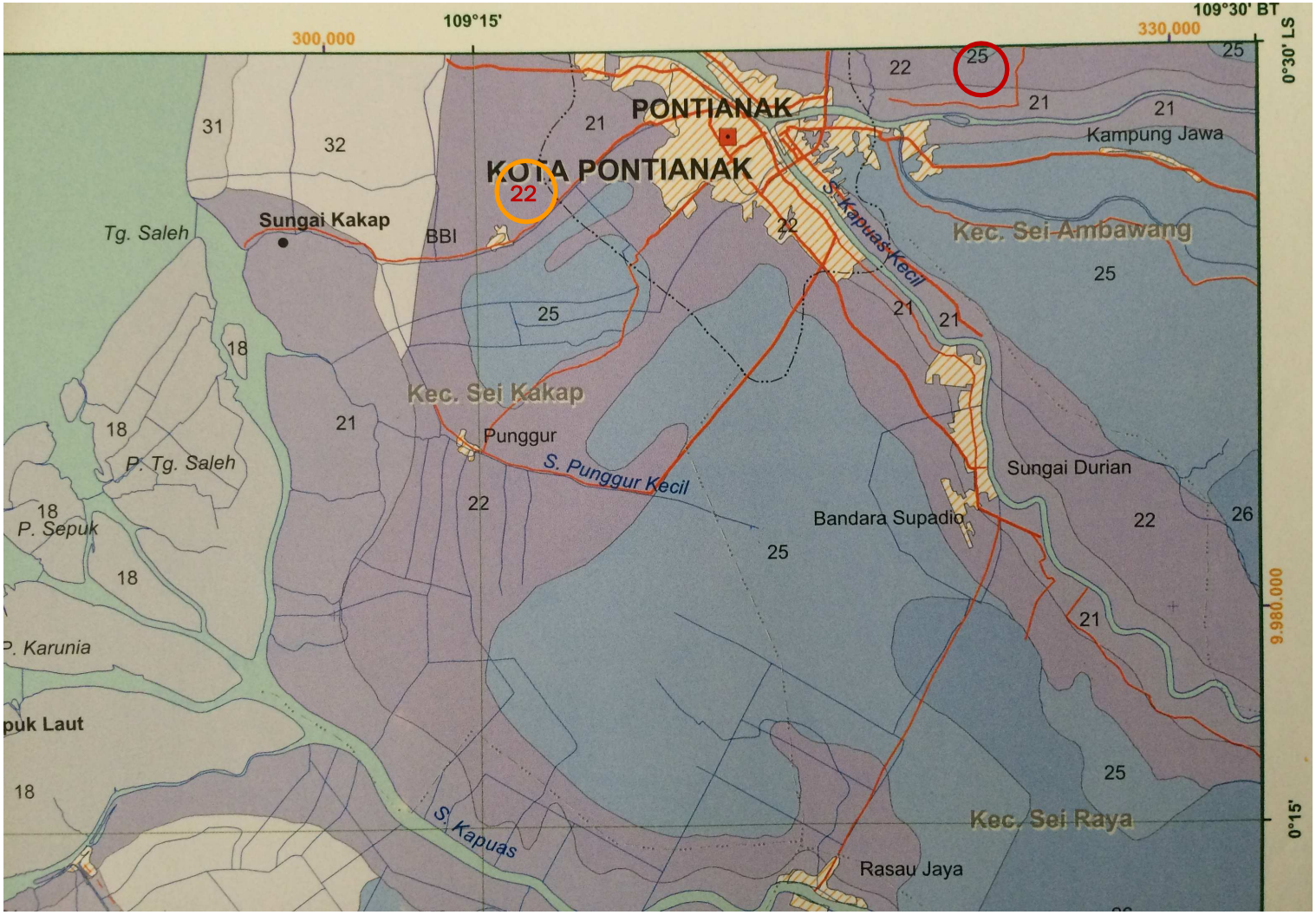

Fig. 15. Reconnaissance soil map of West Kalimantan Province, 1:250.000 scale (Hidayat et al., 2010).
25: Terric Sulfihemists; 22: Sulfic Endoaquepts

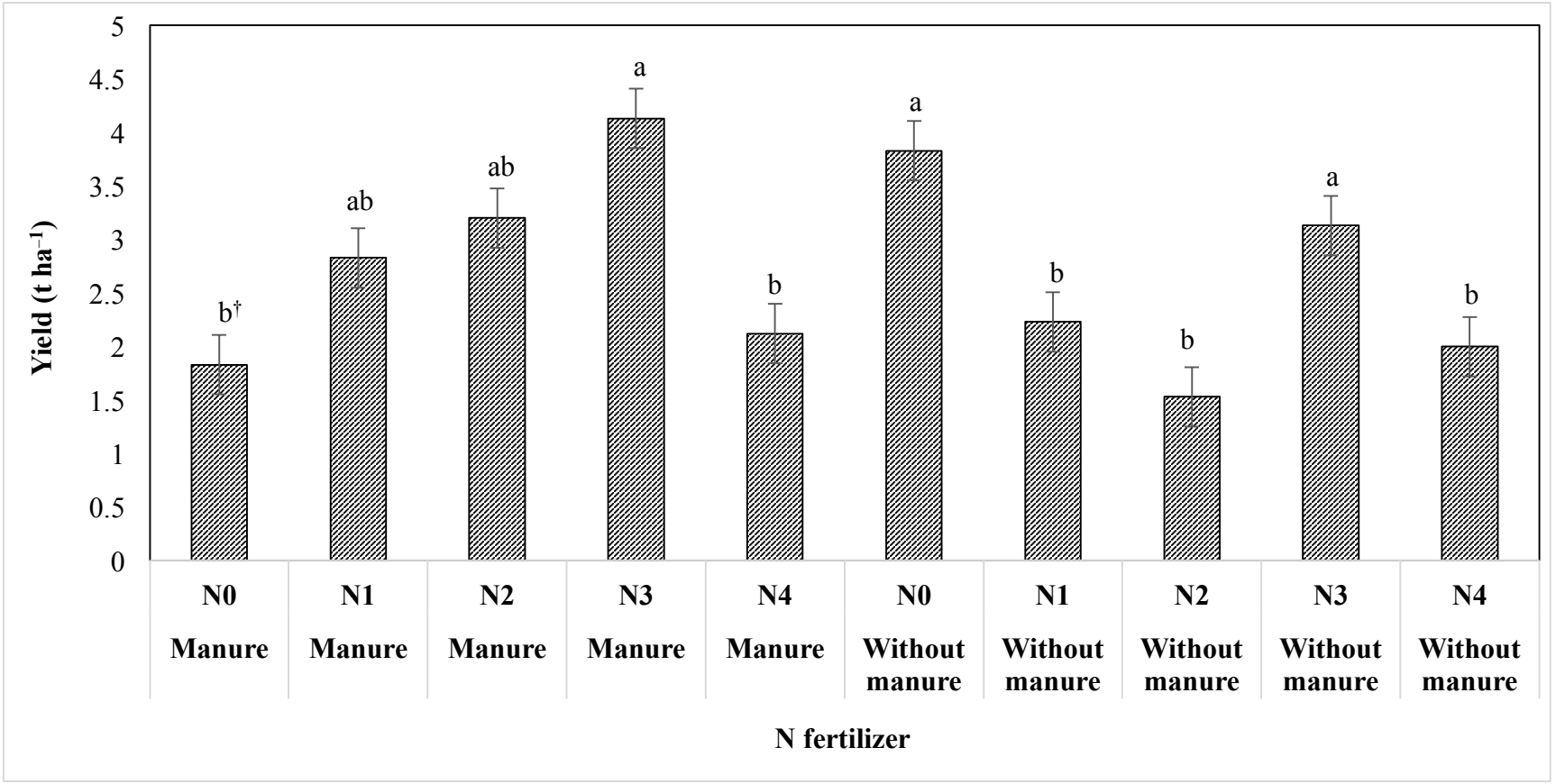

Fig. 16. Yield of spinach grown on alluvial soil in the N rate study.
There were no significant yield effects on spinach grown on the peat soil.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
N0: no urea control; N1: 23 kg N ha^{-1}; N2: 46 kg N ha^{-1}; N3: 69 kg N ha^{-1}; N4: 92 kg N ha^{-1}.
There was no significant difference between manure and no manure treatments (P= 0.42).

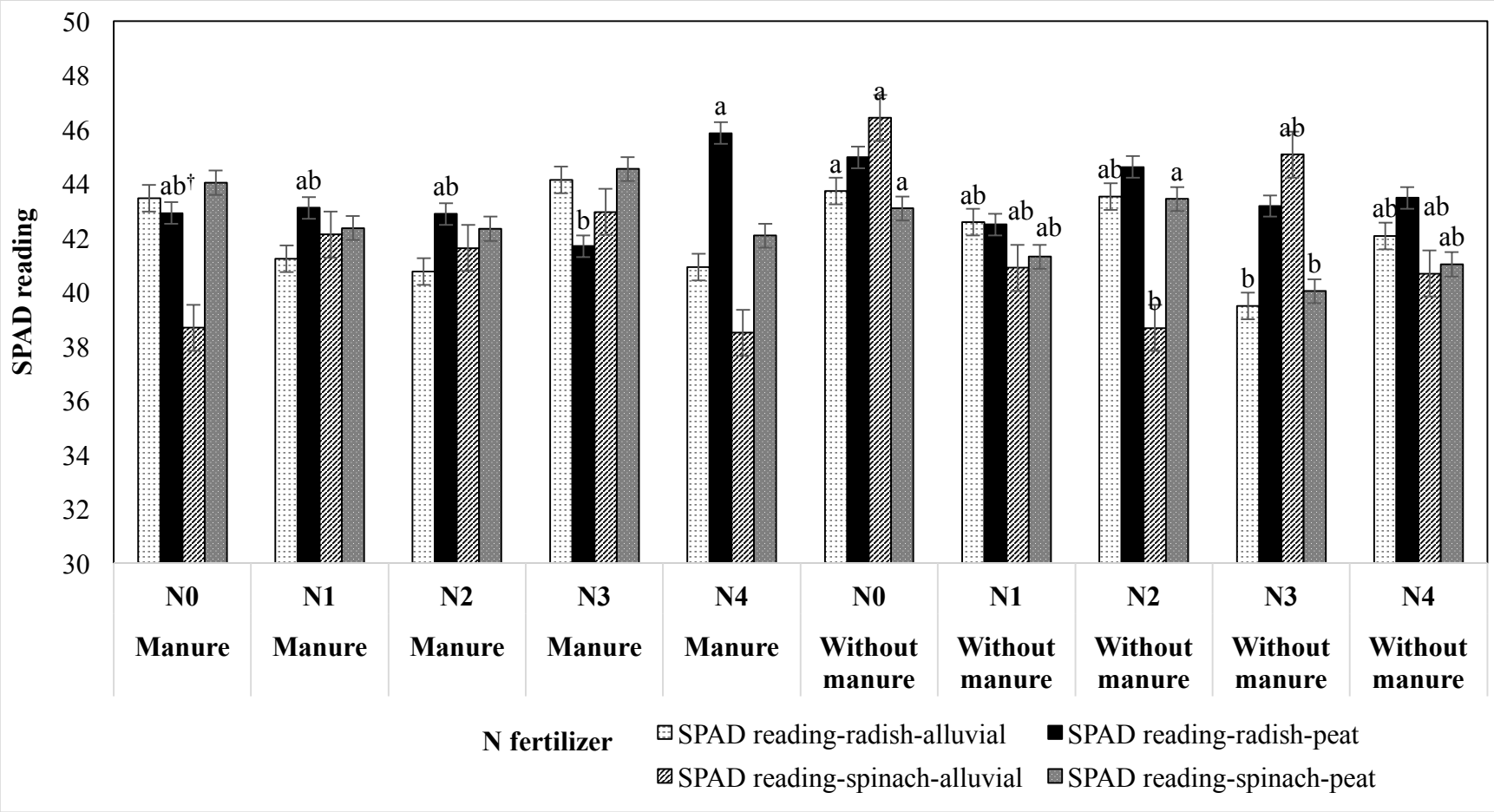

Fig. 17. SPAD reading of spinach and radish grown on alluvial and peat soils in the N rate study.
There were no significant SPAD reading effects on radish and spinach grown on the alluvial soil and spinach grown on the peat soil in the manure whole plot, and radish grown on the peat soil in the no-manure whole plot.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
N0: no urea control; N1: 23 kg N ha^{-1}; N2: 46 kg N ha^{-1}; N3: 69 kg N ha^{-1}; N4: 92 kg N ha^{-1}.

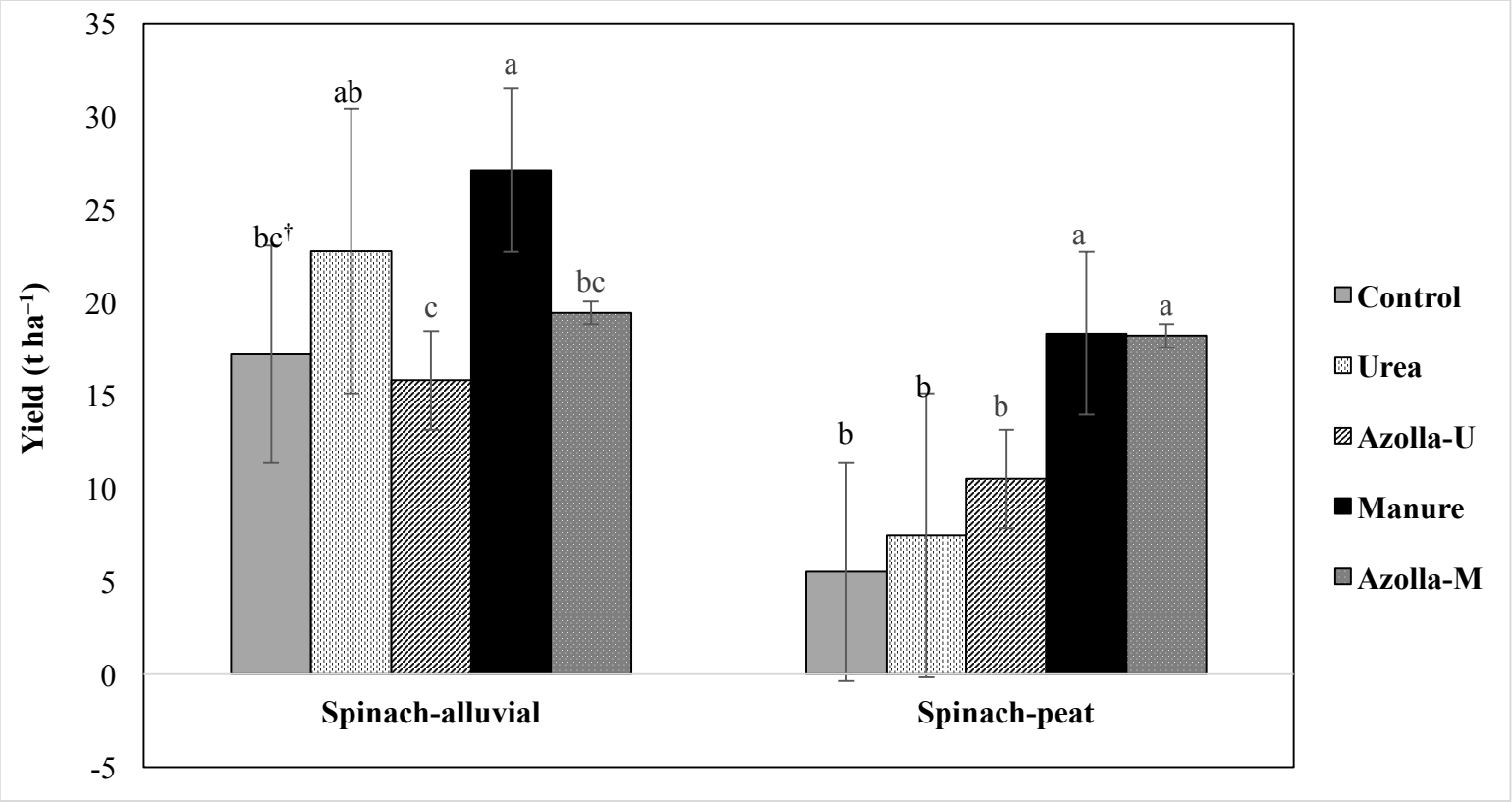

Fig. 18. Yield of spinach grown on alluvial and peat soils in the *Azolla* study.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

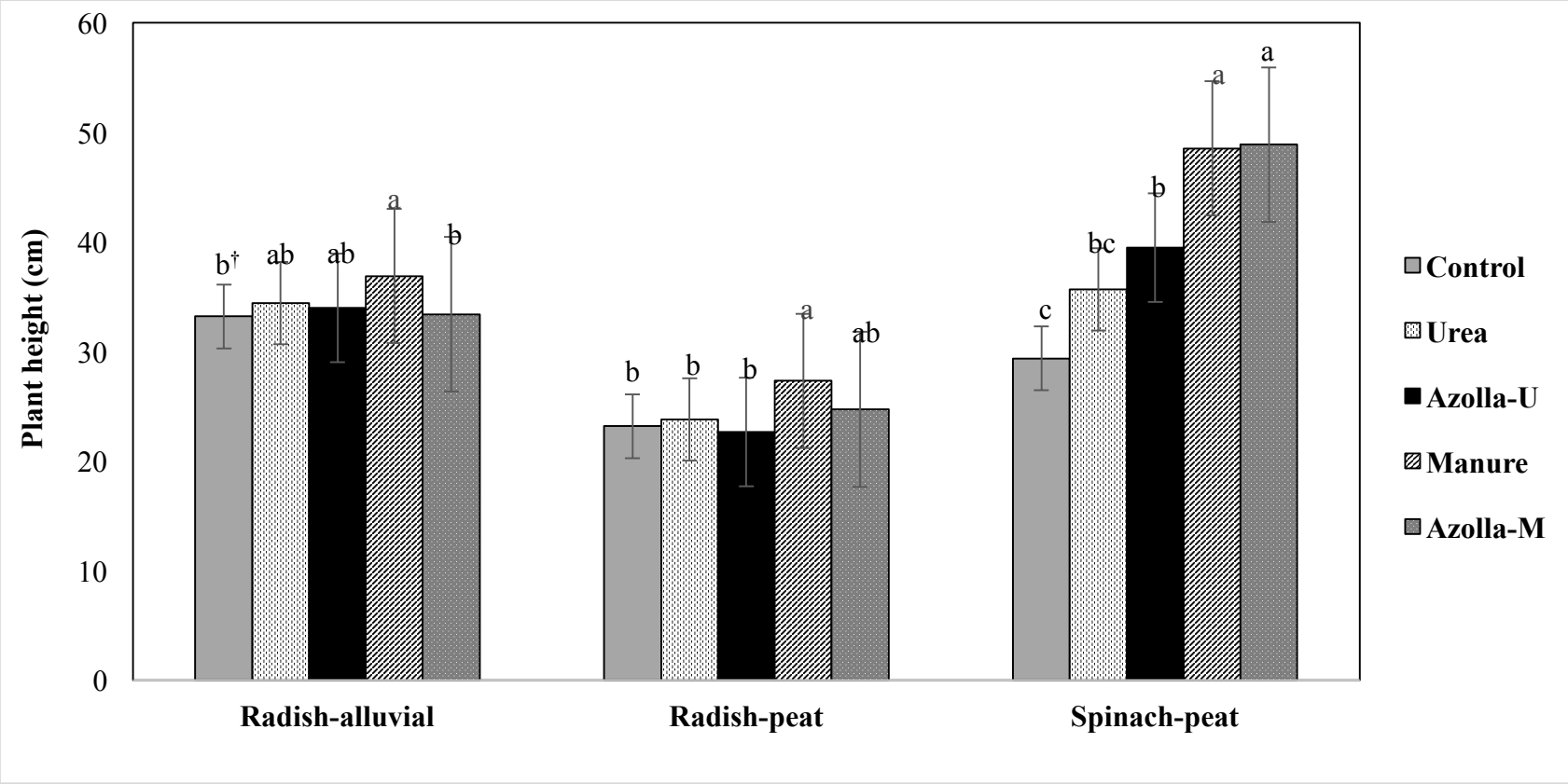

Fig. 19. Plant height of radish and spinach grown on alluvial and peat soils in the *Azolla* study.
There was no significant difference in the spinach-alluvial site.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

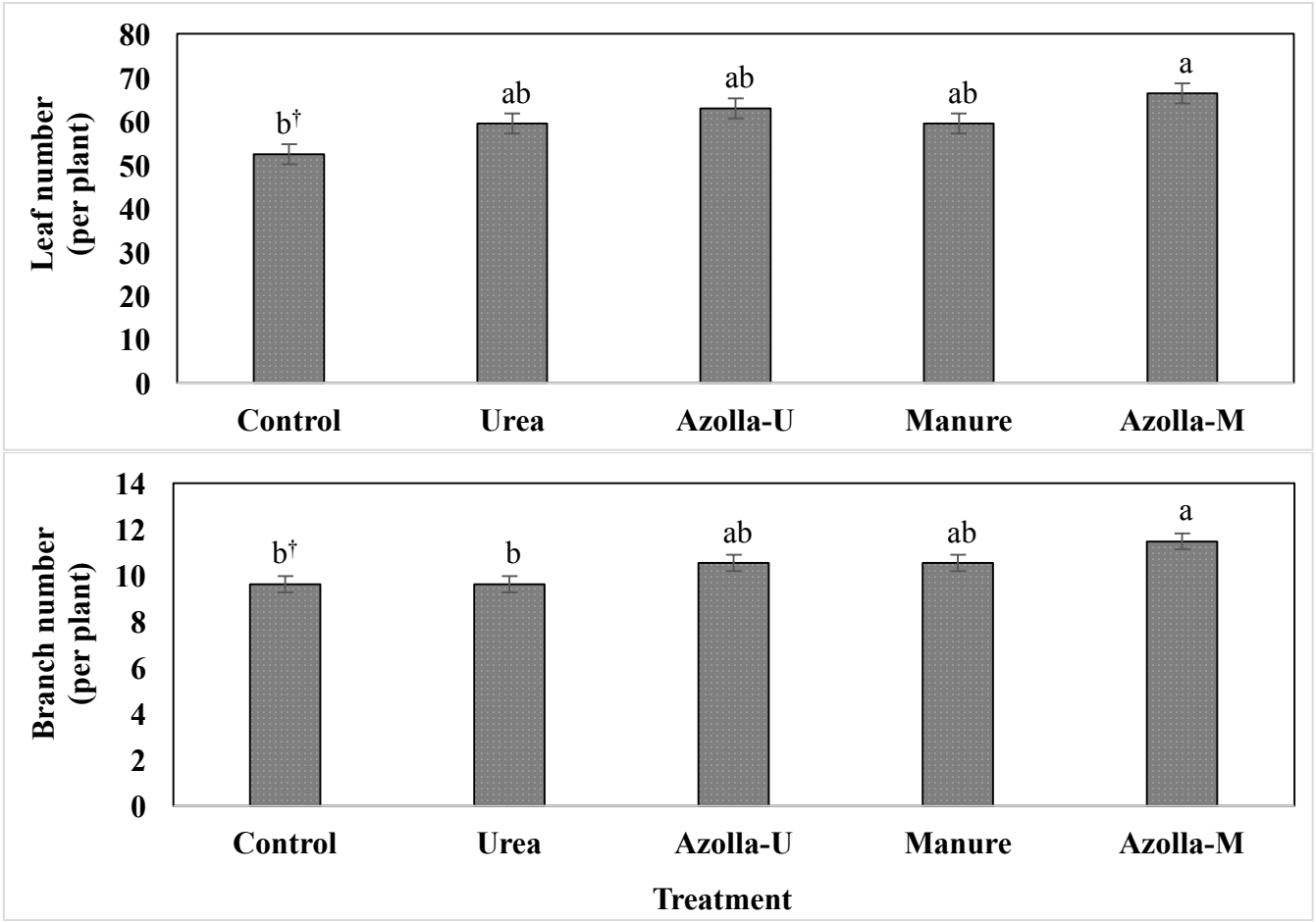

Fig. 20. Leaf and branch numbers of spinach grown on peat soil in the *Azolla* study.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

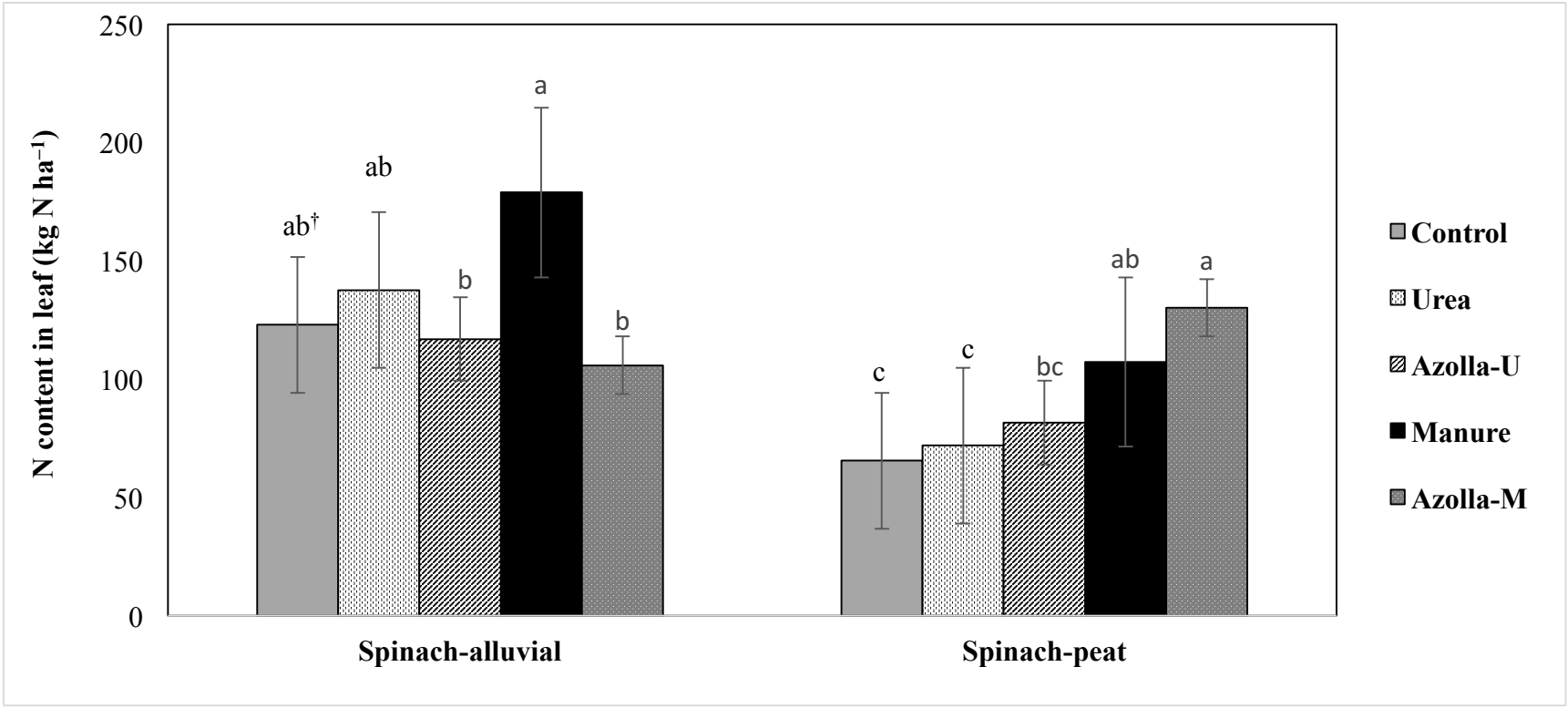

Fig. 21. N content of spinach leaf grown on alluvial and peat soils in the *Azolla* study.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

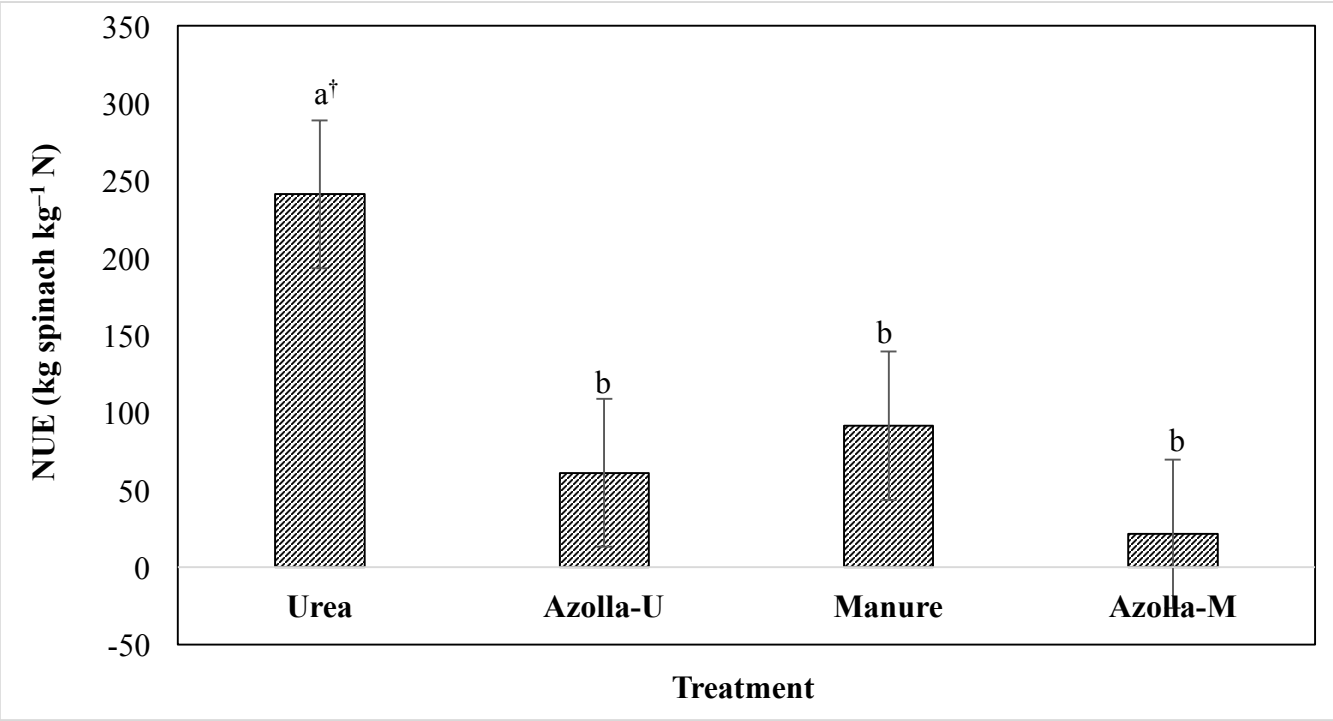

Fig. 22. Nitrogen use efficiency (NUE) of spinach grown on alluvial soil in the *Azolla* study.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

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CHAPTER 4

AZOLLA BIOFERTILIZER EFFECT ON SOIL PROPERTIES AND PLANT NUTRIENTS IN DRYLAND VEGETABLE PRODUCTION SYSTEMS

Summary

Food security is a critical issue in Indonesia, specifically the challenge of providing nutritious and affordable food for the growing population of Indonesia using sustainable agricultural practices. One approach to address this challenge is through a program to utilize backyards to grow vegetables to enhance food security at the household level. Intensifying vegetable production can be achieved through sustainable fertilization using biological nitrogen fixation using organisms such as *Azolla.* The objective of this study was to identify whether *Azolla* utilization could enhance soil chemical properties of Inceptisols and Histosols and plant nutrient concentrations of radish and spinach crops in West Kalimantan, Indonesia. The experimental design was a Randomized Complete Block Design with three replications. Five N fertilizer treatments were used: control (no N fertilizer), urea at 23 kg N ha^{-1}, *Azolla* at the urea N rate, chicken manure at 108 kg N ha^{-1}, and *Azolla* at the manure N rate. Treatment means were compared using the honestly significant difference Tukey adjusted post hoc test (n= 3, P <0.10). The manure treatment enhanced soil P concentration in the radish–alluvial and spinach–peat sites. K concentration in the radish crop was also increased by manure applied to the alluvial soil, whereas manure and *Azolla* applied at the manure N rate increased K concentration in the radish and spinach crops grown in the peat soil.

Therefore, manure or *Azolla* biofertilizer can be used to improve soil fertility in the alluvial and peat soils and increase nutrient concentrations in radish and spinach crops.

Introduction

National food security in Indonesia is a critical issue that the Indonesian government has prioritized. One of the food security challenges is how to provide nutritious and affordable food for the growing population in Indonesia using sustainable agriculture. In order to achieve this national food security goal, the Indonesian Ministry of Agriculture emphasizes that national food security has to be preceded by food self-sufficiency. Therefore, the Sustainable Food-Reserved Garden Program was launched so that food self-sufficiency could be initiated from the household level (IAARD, 2014). The nutritious food source that the government encourages people to grow in their backyards is vegetable crops. One key element of sustainable agriculture is sustainable fertilization. Through sustainable fertilizer practices, it is not only possible to enhance vegetable production but also to improve soil fertility.

Much interest has been generated for enhancing the nutrient status of paddy rice soils through enrichment with diazotrophic cyanobacteria and symbiotic systems such as *Azolla*, a water fern which harbors cyanobacteria in its leaf cavities (Prasanna et al., 2012; Vaishampayan et al., 2001). The ability of the endosymbiont *Anabaena azollae* to fix atmospheric N_2 inside leaf cavities of *Azolla* has made the association useful in rice ecosystems in several countries, such as Thailand (Moore, 1969), Indonesia (Brotonegoro et al., 1982), India (Singh, 1979), China (Lumpkin, 1982), the Philippines (Watanabe et al., 1977), Vietnam (Tuan and Thuyet, 1979), Taiwan (Lee and Lin, 1981), Brazil (Fiore and Ruschel, 1982), Italy

(Espinosa-Abarca et al., 1985), Mexico (Ferrera-Cerrato and Romero, 1982), and the region of West Africa (Reynaud, 1982).

In addition to their N-enrichment potential, the incorporation of 20 t ha^{-1} of fresh *Azolla* into paddy soil per season has been shown to increase water holding capacity by 19.9%, porosity by 22%, and cation exchange capacity by 8.6% following four successive rice crops (Mandal et al., 1992). *Azolla* also enhances soil fertility by increasing soil organic C, total N, available P, and exchangeable Ca, Mg, and Na (Satpathy, 1993; Thangaraju and Kannaiyan, 1993; Singh and Singh, 1995; Bhuvaneshwari and Kumar, 2013). Furthermore, Van Hove (1989) reported that *Azolla* improved soil structure and substituted K for fertilizer in a low K environment. *Azolla* additions have also been shown to improve mungbean yields in a dryland sandy loam (Ram et al., 1994).

Azolla has been proven to sustain soil fertility and crop productivity in paddy rice. *Azolla* has also elevated N mineralization of urea and N use efficiency (NUE) of urea in water-logged rice soil (Subedi and Shrestha, 2015). It also enhanced soil physicochemical properties in rice paddies (Subedi and Shrestha, 2015). On the other hand, the effects of organic residues such as *A. pinnata*, cowpea (*Vigna unguiculata*), dhaincha (*Sesbania aculeata*), decomposed cowdung, and compost on soil physicochemical properties may continue for three crop seasons as shown in long-term greenhouse experiments on water-logged paddy soil (Zaman et al., 1995).

A pot incubation experiment in a dark room at 25^{o}C using soil amended with *Azolla* at rates of 0, 20, 40, 60, and 80 g kg^{-1} also showed that *Azolla* influenced soil physicochemical properties (Awodun, 2008). The higher rates of *Azolla* increased soil pH, organic matter, and N, P, K, Ca, Mg, and Na concentrations. Porosity was increased and bulk density was reduced at greater *Azolla* application rates (Awodun, 2008).

Growing vegetables that concentrate Fe is a way to address Fe deficiency. Iron deficiency causes almost half of all anemia cases worldwide, and it commonly affects women more than men. Iron deficiency and anemia decrease the work capacity of entire populations which can cause serious economic and national development problems (WHO, 2017). Based on global estimates of the prevalence of anemia in 2011, Indonesia had 20.0–39.9 % prevalence of anemia in infants and children aged 6–59 months and 100% of women (pregnant and non-pregnant women) of reproductive age, 15–49 years (WHO, 2015). In addition, there is another nutrient that is commonly deficient in human populations in developing countries, i.e. Zn. The primary cause of Zn deficiency that threatens 25% of the world's population is low dietary intake. Zinc plays a fundamental role for human beings since it influences immune function, growth, and development (Brown et al., 2002; Shankar and Prasad, 1998). Furthermore, Zn deficiency can increase the prevalence of diarrhea, pneumonia, and malaria. More than 25% of the Indonesian population is classified as being at high risk of prevalence of inadequate Zn intake (Wessels and Brown, 2012). Enhancing Fe and Zn concentrations in soil and, thus, in crops is expected to be an effective preventative approach to alleviating Fe and Zn deficiency. According to Cakmak et al. (2010), Zn can be enriched in wheat whole grains and in each grain fraction through foliar application of Zn, in particular in soils with high N fertility. In Zn deficient soil, Cakmak et al. (2010) suggested combining $ZnSO_4$ applied both through foliar and soil applications.

One potential approach to improving Fe and Zn concentrations in plants is possible due to the fact that common alluvial and peat soils in Indonesia are acid soils that commonly have Fe toxicity problems (Havlin et al., 2014). Therefore, these kinds of soil are good growth media for

mining Fe and Zn by plants, so that the plants can then be consumed to tackle Fe and Zn deficiency in human populations.

Two vegetables were selected for this study, a leafy vegetable with typically high Fe concentration (red spinach) and a bulb vegetable (radish). In this study, we aimed to evaluate whether N treatments affected nutrient concentrations of red spinach and radish. Therefore, this study was undertaken to evaluate the contributions of *A. pinnata* as a biofertilizer in Inceptisols and Histosols in West Kalimantan, Indonesia compared to commonly-used fertilizers in enhancing soil chemical properties and nutrient value of vegetable crops.

Our hypotheses were

1. *Azolla* use as a biofertilizer will improve soil chemical properties (pH, total N, P, K, Fe, and Zn concentrations, organic C, and C/N ratio) in alluvial and peat soils in West Kalimantan, Indonesia, comparable to commonly-used fertilizers.
2. *Azolla* utilization as a biofertilizer can enrich nutrient concentrations (N, P, K, Fe, and Zn) in vegetable plant tissues.

Materials and Methods

Study Site

Field studies were undertaken in 2015 and were located in West Kalimantan, Indonesia. There were two field sites that represented two soil types. The alluvial site was located at the Agricultural Research Station of the Assessment Institute for Agricultural Technology of West Kalimantan in Pal Sembilan Village, Sei Kakap, West Kalimantan. The peat soil site was located in a farmer's field in Siantan, Pontianak, West Kalimantan.

According to USDA Soil Taxonomy, the alluvial soil was classified as Sulfic Endoaquepts; whereas, the peat soil was classified as Terric Sulfihemists (Hidayat et al., 2010) (Fig. 15). Sulfic Endoaquepts are characterized as soil in the suborder of Endoaquepts that have a slope of less than 25 percent and sulfidic materials. Soil suborder Sulfihemists that have a layer of mineral soil of 30 cm or greater thickness with its upper boundary within the control section, below the surface tier, are characterized as Terric Sulfihemists (Soil Survey Staff, 2014).

The climate of the study sites is a tropical moist climate with III C and IV C classification based on the number of wet and dry months (Rejekiningrum et al., 2012). The average temperature, annual precipitation, and relative humidity are more than 18 ^{o}C, 2000–4000 mm, and 80.8%, respectively.

Experimental Design

A set of preliminary studies were completed in order to identify the optimum N fertilizer dosage. The commonly-used N fertilizers for vegetable crops are chicken manure and urea. The dosage of urea (50 kg ha^{-1}) and chicken manure (5 t ha^{-1}) were determined based on preliminary studies.

The experimental design was arranged in a randomized complete block (RCB) with fertilizer type as the treatment and three replications for each crop–soil combination. Treatments were

1. Control: no fertilizer control
2. Urea: urea applied at 50 kg ha^{-1} (23 kg N ha^{-1})
3. *Azolla*-U: dried *Azolla* applied at 1 t ha^{-1} (at the urea N rate of 23 kg ha^{-1}) (N content in *Azolla* was 2.88%)

4. Manure: chicken manure applied at 5 t ha^{-1} (108 kg N ha^{-1}) (N content in chicken manure was 3.19%)
5. *Azolla*-M: dried *Azolla* applied at 4.69 t ha^{-1} (at the chicken manure N rate of 108 kg N ha^{-1}) (N content in *Azolla* was 2.88%)

Growing *Azolla* Biofertilizer

Azolla pinnata was used, since this *Azolla* species is a native species in Indonesia. *Azolla* was grown in a natural pond at the alluvial site; whereas, in the peat site, it was grown in an artificial pond lined with polyethylene. *Azolla* is sensitive to heat and light. Therefore, when we grew *Azolla*, we had to maintain the temperature of growing media below 30 ^{o}C, and it could not be exposed to high light intensity. The water resource for growing *Azolla* was from surface and rain water depending on availability at the alluvial site. At the peat site, surface water was used to grow *Azolla*.

The inoculation rate of *Azolla* for the production ponds was 100–200 g m^{-2} based on the previous greenhouse study results. In the peat soil site, plant ash was added to the *Azolla* pond at the rate of 2.68 t ha^{-1} to increase water pH. *Azolla* was harvested 3–4 weeks after inoculation.

Cultivation

The spinach variety that was used for this study was Red "Giti" Spinach (Indonesian Vegetable Research Institute), and the radish variety was No. 22 short leaves (GL seeds, China). The spinach was transplanted 21 days after seeding, while the radish was direct seeded. The planting distance used for both spinach and radish was 20x15 cm.

Organic fertilizer was applied as a basal application, i.e. manure and *Azolla* were applied 3 days after transplanting (DAT) for spinach and 12 days after planting (DAP) for radish. The urea application was split into two applications with 50% applied at each time. Urea was applied on 3 and 14 DAT for spinach; whereas, for radish, it was applied on 12 and 27 DAP. There were additional P and K fertilizers that were used for radish, i.e. SP-36 (superphosphate, 36% P_2O_5) applied at 250 kg ha^{-1} and KCl (potassium chloride, 60% K_2O) applied at 180 kg ha^{-1} on 12 DAP. In order to increase pH of the peat soil, plant ash was applied as a liming material at 3 t ha^{-1} based on the common rate that farmers use. Vegetable crops were harvested at 45 DAT for spinach and 49 DAP for radish.

Soil Analysis

A composite soil sample was taken from 0–20 cm depth on the alluvial and peat soils before the treatments were set up to identify the initial soil properties (Table 7). Soils were sampled again by plot after vegetable harvest, and the same analyses were conducted as the initial soil properties. Soil property analysis was conducted using air-dried and sieved (2 mm) soil for analysis including pH (soil:DI water extraction and soil:1 *N* KCl extraction with a ratio of 1:5) and measured using pH meter equipped with glass and reference electrode (Thomas, 1996; USDA-NRCS, 2005; Soil Survey Staff, 2014), organic C using Walkley–Black method (Nelson and Sommers, 1996), total N using Kjeldahl method (Bremmer, 1996), NH_4^+-N and NO_3^--N using 2 *M* KCl extraction method, then an automated analyzer was used for colorimetric determination (Flow Solution IV, O-I-Analytical) (Mulvaney, 1996), C/N ratio, available P using Bray-1 extraction method and measured with spectrophotometer (Kuo, 1996), exchangeable cations (K, Na, Ca, Mg) and cation exchange capacity (CEC) using ammonium acetate 1 *N* pH

7.0 extraction method and measured with flame photometer for K and atomic absorption spectrophotometer (AAS) for Na, Ca, and Mg (Helmke and Sparks, 1996), and base saturation by calculation with the following formula as described by Havlin et al. (2014):

$$\% \text{ base saturation} = \frac{\text{total bases}}{\text{CEC}} \times 100\% \qquad \text{(Eqn. 4)}$$

$$= \frac{K^{+} + Mg^{+2} + Ca^{+2} + Na^{+}}{\text{CEC}} \times 100\%$$

Exchangeable H and Al were extracted using 1 *N* KCl and then titrated with standardized 0.1 *M* NaOH (Sims, 1996), while total Fe, Cu, Zn, and B was determined by digestion using nitric acid (HNO_3) and perchloric acid ($HClO_4$) (Loeppert and Inskeep, 1996; Reed and Martens, 1996; Hou et al., 1996; Sah and Brown, 1997). Soils high in organic matter were pretreated with HNO_3 to minimize explosive reactions of organic matter with $HClO_4$. Then, total Fe, Cu, Zn, and B were measured by AAS. Soil texture was determined using the pipette method (Olmstead et al., 1930).

Plant Analysis

Plant nutrients were analyzed from the harvested vegetable crops (red spinach leaf and radish bulb) including:

- Total N using Kjeldahl method (Horneck and Miller, 1998).
- P, K, Fe, and Zn utilizing high-temperature oxidation (dry ashing) to digest organic matter followed by dissolution with 1 *N* HCl (Miller, 1998). P was measured using

spectrophotometer, whereas K was measured using flame photometer, and Fe and Zn were measured using AAS.

Manure and *Azolla* Analysis

Prior to the field study, manure, *A. pinnata*, and plant ash were analyzed in order to know the nutrient concentrations of the soil amendments. Parameter analysis for those soil amendments and biofertilizer are shown in Table 8.

Plant ash was analyzed for pH using 1:5 ratio with DI water and measured with a glass electrode (Thomas, 1996). Then, it was dried out in the oven at 105 ^{o}C for 3 hours before it was extracted with 1 *N* HCl for macronutrients and trace element analysis. P was measured using colorimetric method (spectrophotometer), whereas K was measured using flame photometer, and Ca, Mg, Fe, and Zn were measured using AAS. Moisture was determined using gravimetric method (Hoskins et al., 2003; Miller, 1998), and neutralization of $CaCO_3$ equivalency using 0.5 *N* HCl extraction followed by titration with 0.1 *N* NaOH (Sims, 1996).

Parameter analysis in chicken manure and *Azolla* included

- pH-H_2O using DI water extraction with a ratio of sample:DI water was 1:5 and then measured using pH/mV meter with a dual electrode system (Wolf, 2003).
- Organic C using Walkley–Black method (Nelson and Sommers, 1996).
- Total N, NH_4^+-N, and NO_3^--N using total Kjeldahl N and distillation methods (Watson et al., 2003; Peters et al., 2003).
- P, K, Ca, Mg, Fe, and Zn utilizing high-temperature oxidation (dry ashing) to digest organic matter followed by dissolution with 1 *N* HCl for manure sample (Wolf et al., 2003). Whereas, the *Azolla* sample was digested with sulfuric acid-hydrogen peroxide for

P, K, Fe, and Zn analysis (Lowther, 2008). P then was measured using colorimetric method (spectrophotometer), whereas K was measured using flame photometer, and Ca, Mg, Fe, and Zn were measured using AAS (Kovar, 2003).

- Moisture or dry matter analysis using gravimetric method (Hoskins et al., 2003; Miller, 1998).

Statistical Analysis

Data were analyzed using SAS version 9.4 (SAS Institute, 2016). Analysis of variance (ANOVA) was performed on the parameters by using the Proc Mixed procedure in separate analysis for every crop and soil combination (radish–alluvial, spinach–alluvial, radish–peat, and spinach–peat). Treatment means were compared using Tukey's honestly significant difference (HSD) post hoc test (n= 3, P <0.10). The relationships among soil chemical properties and plant nutrient variables were assessed by linear correlation using the PROC CORR procedure.

Results

Soil pH

In general, soil pH ranged between 4.52 to 5.75. Interestingly, soil pH in the spinach experimental plots was generally lower than in the radish plots.

Fertilizer treatments did not affect soil pH under the spinach crop in either soil type. *Azolla* did not increase soil pH on the alluvial soil in the radish experiment plot, compared to

control (Fig. 23). The significantly higher soil pH was found in the manure treatment (5.75), although it was not significantly different from control or urea treatments.

In contrast, *Azolla* had a reverse effect in the radish–peat site. All treatments significantly increased soil pH 11.25–17.62% compared to the control (Fig. 23).

Soil N

Interestingly, the higher total N content of the peat soil for both radish and spinach crops was mostly the same among the N fertilizer treatments, i.e. 2.24–2.27% N in the radish–peat soil and 2.25–2.28% N in the spinach–peat soil (Fig. 24). The peat soil on the radish site had significantly higher total N in the *Azolla* applied at the manure N rate compared to control and urea treatments. The lowest total N was found in the control (no N fertilizer) which was not significantly different from the urea, *Azolla*-U or *Azolla*-M treatments (Fig. 24).

The total N in the alluvial soil ranged from 0.21–0.31 % (radish) and 0.22–0.25% (spinach). In the alluvial soil with radish as the crop, it was obvious that the urea treatment had significantly higher total N, although there was no significantly difference with manure, control, or *Azolla* applied at the manure N rate (Fig. 24). Indeed, urea had significantly higher total soil N (47.62% higher) compared to *Azolla* applied at the urea N rate. The lowest soil total N concentration was found in the *Azolla* treatment applied at the urea N rate.

Soil P

The significantly highest soil P concentration in the spinach–peat, spinach–alluvial, and radish–alluvial sites was found in the manure treatment, while the rest of the treatments (control,

Azolla-M, *Azolla*-U, and urea) were not statistically different each other (Fig. 25). However, the manure treatment was not statistically different from control in the spinach–alluvial site. There were no significant differences across N treatments in the radish–peat site.

There was one outlier identified in the radish–peat dataset using the Benferroni test for outliers. The transformation to fix the data set did not work well; therefore, the outlier was deleted from the data set when it was analyzed statistically.

In the spinach–peat site, manure had 2-fold greater soil P, i.e. 123.1% compared to control, 113.8% compared to the *Azolla* applied at the manure N rate, and 100.8% compared to the *Azolla* applied at the urea N rate (Fig. 25). Additionally, soil P concentrations were not significantly different among manure, urea, and *Azolla* treatments applied at the urea N rate in the spinach–peat soil. Manure treatment also had almost 2-fold increased P compared to the *Azolla*-U in the radish–peat site. Interestingly, there were no statistical differences among N treatments in the radish–peat soil (Fig. 25).

Soil K

There was no effect of N fertilizer treatment on soil K concentration in the spinach–alluvial and the radish–peat sites. The manure treatment had the highest soil K for radish in the alluvial soil and in the spinach–peat site (Fig. 26). Manure treatment had 77.3% higher K than control in the radish–alluvial soil and 75% greater soil K compared to control in the spinach–peat site**.**

Soil Organic C (SOC)

The organic C in the peat soil for both crops was much higher than in the alluvial soil. Based on statistical analysis, there was no effect of N treatments on SOC in either of the vegetable crops and soils, except for radish–alluvial.

N fertilizer only had an effect on soil organic C in the alluvial soil for radish crop (Fig. 27). The significantly higher soil C was found in the urea treatment, although it was not statistically different from manure, control, and *Azolla* applied at the manure N rate (Fig. 27). Urea increased SOC by 62% as compared to *Azolla* applied at the urea N rate.

Soil C/N Ratio

The pattern of soil C/N ratio tends to follow the C and N contents in soil. The higher C and N in the peat soil affected the higher soil C/N ratio in both crops compared to the alluvial soil. *Azolla* treatments (*Azoll*a-M and *Azolla*-U) and manure had significantly higher soil C/N ratio, i.e. 22.8 compared to urea (22.5) and control (22.5) in the spinach–peat site (Fig. 28).

Soil Micronutrients (Fe and Zn)

There was no pattern of soil Fe in either soil or crop. However, the alluvial soil tended to have greater Fe, in particular in the radish plot. In spinach plot, there was significantly greater Fe in the alluvial soil in the control (no N treatment) and it was statistically comparable to the urea treatment. The alluvial soil in the spinach plot contained significantly lowest Fe in *Azolla*-U, manure, and *Azolla*-M treatments (Fig. 29).

Soil Zn concentration was altered by N fertilizer treatments only in the spinach–alluvial site (Fig. 30). Control treatment (no N fertilizer) contained the highest soil Zn concentration compared to the N fertilizer treatments (urea, manure, and *Azolla*). In general, there was a pattern of greater soil Zn in the alluvial soil (the insignificant data were not shown).

Plant Macronutrient Concentrations (N, P, and K)

N treatments had effects on N concentration in the radish plant tissue grown on the peat soil and P concentration in the spinach on the peat soil (Figs. 31 and 32). Manure increased plant N concentration by 29% compared to control in the radish–peat system (Fig. 31).

Manure was the only treatment that increased radish N concentration compared to control in the peat soil. Indeed, manure also raised leaf P concentration in spinach grown in the peat soil by 15.5% compared to control (Fig. 32).

N fertilizer treatments had a significant effect on K concentration in radish bulbs in both soil types and on K concentration of the spinach leaves in the peat soil (Fig. 33). The manure treatment consistently had significantly higher plant K in the radish bulbs and spinach leaves, except for the spinach–alluvial site.

Interestingly, in both crops grown in the peat soil, there was a similar pattern in plant K concentration among N fertilizer treatments with control, i.e. manure>*Azolla*-M>control (Fig. 33). The pattern slightly changed on the radish plant in the alluvial soil. Manure still displayed the highest K content, 13.7% higher than control. However, the *Azolla* and urea treatments did not contain significantly higher K concentration in the radish bulb compared to control (Fig. 33).

Plant Micronutrient Concentrations (Fe and Zn)

There was no N fertilizer effect on Fe concentration in the radish bulbs or spinach leaves. Nevertheless, in general, plants contained higher Fe in the alluvial soil regardless of plant type. In addition, spinach had higher Fe in its plant tissue than in the radish bulbs in both soil types. It is commonly known that spinach is a good source of Fe for human diets.

Basically, radish on the alluvial and peat soils and spinach on the peat soil contained almost the same plant Zn concentrations (data not shown since N treatments did not affect plant Zn concentrations). Nevertheless, N fertilizer treatment was only significant in radish on the peat soil. The *Azolla*-M treatment had significantly lower radish Zn concentration than in control on the peat soil (Fig. 34).

Discussion

Soil Properties Affected by N Treatment

In general, N treatment significantly influenced soil pH and total soil N in the radish crop in both alluvial and peat soils. This condition occurred probably because radish was able to adapt to the lower soil pH where the crops were grown. The optimum soil pH for radish growth is 5.5–6.5, the absolute minimum pH is 4.3, and the absolute maximum soil pH is 8.3; whereas, spinach requires optimum soil pH in the range of 6–7, and the absolute minimum and maximum soil pH levels are 5.3 and 8.3 (FAO, 2007a, 2007b; Roy et al., 2006). The initial pH levels for alluvial and peat soils in this study were 4.7 and 5.3 (Table 7).

Basically, soil pH was not the limiting factor for radish and spinach growth; however, the effect of N treatments on soil pH was only shown in radish plant in both soil types (Fig. 23). The N fertilizer forms used in this study were urea and organic fertilizers (manure and *Azolla*) that could induce soil acidification. The greater acidity will be produced if NH_4^+ fertilizer is combined with S and/or P fertilizer sources (Havlin et al., 2014). Furthermore, Havlin et al. (2014) asserted that P fertilizer releases phosphoric acid that may temporarily acidify small, localized zones at the site of application. Nevertheless, the effect of N (NH_4^+ or organic fertilizers) and P fertilizers has little long-term effect on bulk soil pH because the H^+ is produced in a small amount. In this study, P (superphosphate-36) and K (potassium chloride) fertilizers were also used only for radish. The ammonium-based N fertilizers undergo nitrification that convert ammonium to nitrate in soils by bacteria. Thus, H^+ is released, which can increase soil acidity. One molecule of H^+ is produced by urea for every molecule of NH_4^+ because one OH^- is released upon urea hydrolysis to form NH_4^+ (Havlin et al., 2014). Long-term effect (4 years) of urea acidification was shown in plot receiving 120 kg N ha^{-1} compared to the lower urea dosage (60 kg N ha^{-1}) or control (no urea) (Lungu and Dynoodt, 2008). The reduced 0.87 units of soil pH occurred in the plot receiving 180 kg N ha^{-1} at the fourth growing season. In this study, although it was still in the first growing season, soil pH in the radish plots had been affected by N treatments, as also reported by Lungu and Dynoodt (2008), that lower pH was detected starting from the second cropping season.

In the radish–alluvial site, the manure treatment had the highest soil pH, although it was not significantly different from control or urea treatments**.** This was due to the fact that chicken manure can increase soil pH, in particular in acidic soil, as found in a long-term study of manure amendment which showed that manure was able to sustain an optimum soil pH for most crops

(Zhang, 1998). Manure can have various pH levels depending on the animal feed; in this study, the manure pH was 6.88 (Table 8).

Azolla application significantly increased soil pH in the radish–peat site compared to control; however, the soil pH was not significantly different from the urea and manure treatments (Fig. 23). This indicated an improvement of soil fertility in an acidic soil. In addition, the result was in agreement with Awodun (2008) that found the higher soil pH occurred in soils amended with *Azolla* (6.56–6.63) compared to control (6.31). On the other hand, Ram et al. (1994) discovered that *Azolla* utilization in a dry land soil decreased soil pH from 7.8–7.9 (control) to 7.4–7.6 (*Azolla* treatments).

Overall, total N in the peat soil was about 10 times greater than in the alluvial soil. Peat soils store considerable amounts of N compared to alluvial soils. However, due to the lower bulk density of peat soil (0.1 g cm^{-3}), the average 2.2% N only contains 2200 kg N ha^{-1} in its upper 10 cm layer. In contrast, although the alluvial soil contained lower N concentration (0.2%), with the commonly higher bulk density for mineral soil of 1–1.2 g cm^{-3}, the alluvial soil contained 2000–2400 kg N ha^{-1}, roughly the same N content as the peat soil (Andriesse, 1988).

In the radish–alluvial site, the soil total N (TN) concentration found in the urea treatment, was significantly higher than TN of the *Azolla* treatment applied at the urea N rate (*Azolla*-U). The urea and *Azolla*-U had the same N rate; nevertheless, *Azolla*-U contains organic N that was mineralized over time that contributed to the lower overall total N in that treatment, compared to urea that had only mineral N. It is possible that N had not been all mineralized when *Azolla* was applied as biofertilizer. The drying process of *Azolla* after harvest was about 2–3 weeks before it was incorporated into the soil as biofertilizer. The decomposition of *Azolla* used for biofertilizer in this study can be predicted from the C/N ratio of *Azolla*, i.e. 16.1–16.4, compared to C/N ratio

of manure (10.1) (Table 8). Sisworo et al. (1990), Watanabe et al. (1989), Eskew (1987), Asuming-Brempong et al. (2008), and Bhuvaneshwari and Kumar (2013) suggested that *Azolla* might take 30–60 days in tropical climates or 60 days in temperate climates to mineralize the organic N to inorganic forms.

In the radish–peat site, the total N content in soil tended to be greater at the higher N rate. *Azolla*-M that had 108 kg N ha^{-1} contained greater soil total N (2.25–2.27% N) compared to urea (23 kg N ha^{-1}) that held 2.25% N (Fig. 3). This result was in agreement with Awodun (2008), Bhuvaneshwari and Kumar (2013), and Rivaie et al. (2013), who reported increased soil total N in soils receiving a higher *Azolla* rate or N rate.

N treatment had a significant effect on soil P concentration in both vegetable crops and soils, except in the radish–peat site (data not shown). Manure had the greatest soil P concentration in all four sites.

The fertilizer treatments were applied based on N rate. The ratio of P over N was highest in the chicken manure, i.e. 0.23, while the *Azolla* biofertilizer had 0.08–0.10 P/N ratio (Table 8). In addition, manure contained higher P (0.74%) than the other N fertilizers (*Azolla* (0.23–0.31% P) and urea (no P) (Table 8). Thus, this explains why soil P concentration was highest in the manure treatment. The result also showed that P had not been released optimally from the organic matter decomposition in the *Azolla* treatments (*Azolla*-U and *Azolla*-M) as implied by Nagarajah et al. (1989). In a soil–*Azolla* incubation experiment in dryland, Awodun (2008) found that the highest soil P concentration was found during the eighth week following fresh *Azolla* application. The *Azolla*-U was not different from control and contained the same soil P concentration as *Azolla*-M, despite the application rate being about ¼ of the *Azolla*-M rate. This result illustrates that the decomposition process had probably not been completed yet in the

Azolla treatments. Since Awodun (2008) in an eight-week laboratory incubation study using fresh *Azolla* in dryland soil, Bhuvaneshwari and Kumar (2013) in a sixty-day laboratory incubation study applying fresh *Azolla* in flooded soil, and Rivaie et al. (2013) incorporating fresh *Azolla* into a plowed paddy soil and letting it incubate for 21 days until the rice was harvested around 120 days, all reported that in higher *Azolla pinnata* application rates, there was higher soil available P concentration.

Peat soil contained greater exchangeable K than the alluvial soil possibly due to the fact that the peat soil was amended with plant ash that contained 5.49% K (Table 8). N treatment affected soil K concentration in the radish–alluvial and spinach–peat sites, in which manure showed 77.3% higher exchangeable K than control in the radish–alluvial soil. Perhaps 5.08% K in manure made this N treatment have the higher K (Table 8). Additionally, the ratio of K over N in the chicken manure was higher than the *Azolla* (Table 8). In the *Azolla* treatments, the lower K concentration (3.91–4.10% K) in addition to the decomposition time of only 2–3 weeks, resulted in the lower exchangeable soil K. Awodun (2008) reported that K release from *Azolla* incubation was highest in the eighth week.

The greater soil K in the higher N rate treatments such as manure and *Azolla*-M was in concordance with Rivaie et al. (2003) that found higher exchangeable K in higher *A. pinnata* application rates. In the radish–alluvial site only the manure treatment enhanced the soil K, as suggested by Dawar and Singh (2002). Furthermore, Van Hove (1989) and Dawar and Singh (2002) also described that *Azolla* was a good biofertilizer source for low K soil, since when it decomposes, *Azolla* provides K for plants, and thus it performed as a K fertilizer substitution (Van Hove, 1989; Dawar and Singh, 2002).

Soil organic C was only affected by the N treatment in the radish–alluvial site. However, all the treatments did not show any differences with control. It was interesting to observe that urea increased soil organic C compared to *Azolla*-U, and had 62.1% higher organic C than *Azolla*-U. This result was on the contrary with Ram et al. (1994) in dryland, Awodun (2008) in dryland, and Bhuvaneshwari and Kumar (2013) in flooded soil, who reported *Azolla* biofertilizer increased soil organic matter, in particular at the higher *Azolla* rates and longer decomposition times.

Christopher and Lal (2007) suggested that there may be greater root exudates and more crop residues in response to N fertilizer addition in the long-term, resulting in increased SOC sequestration. These were the main reasons why there was soil organic C (SOC) enrichment from mineral N fertilizer. Therefore, perhaps the higher SOC in urea treatment confirmed with the statement. However, in many studies such as in Canadian Chernozems, N addition, primarily as inorganic N fertilizer, did not enhance SOC concentration (Campbell et al., 1991a, 1991b; Huggins and Fuchs, 1997; Bélanger et al., 1999). Long-term N fertilization established for 29 and 31 years with varying N fertilizer rates (0, 50, 75 kg N ha^{-1}) also did not increase SOC concentration, even though the crop residue was returned to the soil. In fact, manure utilization in addition to enhanced crop rotation caused higher C sequestration, compared to inorganic N fertilizer with the same intensified crop rotation. This condition had been reported in many regions of the world, such as Ohio (Jarecki et al., 2005), Nepal (Matthews and Pilbeam, 2005), China (Wang et al., 2005; Su et al., 2006), Australia (Hati et al., 2006), and India (Rudrappa et al., 2006). Furthermore, manure (111 kg ha^{-1} yr^{-1}) was also reported to consistently result in the greatest yield and preserved higher SOC and N concentrations in the 0–30 cm soil depth, compared to the other N fertilizers, in a long-term experiment in Oregon (established since 1931)

(Rasmussen and Parton, 1994). Additionally, manure still maintained higher SOC over the 56-year period, even though the other N treatments showed reductions in SOC.

In this study at the radish–alluvial site, urea increased SOC more than the organic N fertilizers was probably due to short-term nature of the experiment. The other three sites did not show the same pattern. There were no statistically different SOC concentrations across the N treatments (data not shown) in the radish–peat, spinach–alluvial or spinach–peat sites. In a longer-term study, higher SOC concentrations may develop in the organic N fertilizer treatments.

Soil C/N ratio in the spinach–peat site was influenced by N treatment. The significantly higher soil C/N ratios were found in the *Azolla* treatments (*Azoll*a-M and *Azolla*-U) and manure (Fig. 28). It is obvious since those corresponding treatments that had higher C/N ratios were organic fertilizers that commonly contained higher organic C (51.23–51.68%) compared to urea (51.17%) and control (51.36%). Whereas, total N in the peat soil for spinach plot comprised almost the same amount among N treatments (2.25–2.28%). And more importantly, the *Azolla* biofertilizer and manure contained additional C, which urea did not (Table 8). Thus, those factors may contribute to the higher C/N ratio in organic fertilizers (*Azolla* and manure) compared to urea.

Soil Fe concentration was only affected by N treatment in the spinach–alluvial site (Fig. 29). However, there were no significant differences between control and urea treatments. The *Azolla* and manure treatments had lower soil Fe compared to control. Nonetheless, peat soil tended to have lower Fe concentration than alluvial soil. Andriesse (1988) stated that it is common that the oligothrophic peats in the tropics contain low Fe, especially in the center of the peat domes. Iron deficiency can occur with severe chlorosis symptoms in several kinds of crops, such as pepper, coffee, cassava, grasses, and legumes.

N fertilizer treatments decreased soil Zn concentration in the spinach–alluvial site (Fig. 30). However, there was a tendency that the alluvial soil contained greater Zn than the peat soil. Lower Zn concentration in the peat soil is related to the fact that peat soil is commonly prone to Zn deficiency (Dobermann and Fairhurst, 2000), although the Zn concentration in the initial analysis of alluvial soil was lower (43.3 mg kg^{-1}) than the peat soil (79.0 mg kg^{-1}) (Table 7).

Plant Nutrient Concentrations Affected by N Treatment

The only plant N concentration that was significantly influenced by N treatment was found in the radish–peat site. In general, the plant N concentration was within the sufficiency level, in the range of 2.50–4.50% (Munson, 1998). However, according to Hochmuth et al. (2012), the adequate level of N in harvested spinach leaves ranges from 3.0–4.0%. While Baker and Bryson (2007) only classified broadleaf vegetables generally was having a sufficiency level of 3.5–5.1% N.

The N concentrations in the radish bulb in peat soil were 3.5–4.7% (Fig. 31). According to Munson's (1998) criteria, N level in radish was still considered in the adequate range (2.50–4.50% N). Unfortunately, Hochmuth et al. (2012) only classified plant nutrient levels in radish leaves. A sufficiency levels for N in harvested radish leaves are 3.0–4.5% (Hochmuth et al., 2012). The significantly higher N concentration was found in the manure treatment which was comparable to the urea treatment. The *Azolla*-M and *Azolla*-U treatments had lower plant N concentrations, and they were not statistically different from control. Manure provided N at a higher level for radish in the peat soil, compared to *Azolla* applied at the same N rate.

It was interesting that total N in the radish plant tissue in the peat soil (Fig. 31) generally followed the pattern of soil total N in the alluvial–radish plot (Fig. 24). Yet, urea resulted in the

highest soil total N in the radish–alluvial site; on the contrary, manure contained the highest plant N in the radish–peat site. The same patterns occurred in the *Azolla* treatments where total N in the alluvial soil and the radish plant were not affected.

Plant P concentration was affected by N treatment in the spinach–peat site only (Fig. 32). Overall, P concentration in the radish bulb and spinach leaves (0.42–0.82%) was in the sufficiency level according to Munson (1998), i.e. 0.20–0.75%. Adequate P levels in mature spinach leaves are 0.30–0.50% (Campbell, 2000; Sanchez, 2007; Hochmuth et al., 2012). Manure had the highest P concentration in spinach in the peat soil (Fig. 36). The higher P concentration in manure (0.74%) in addition to its higher P:N ratio, compared to the other fertilizer treatments probably contributed to this finding.

Interestingly, in the spinach–peat site, the pattern of plant P (Fig. 32) was comparable to soil P (Fig. 25), in which manure treatment held the highest P concentration both in the soil and in the plant tissue across soil and crop types. The peat site generally contained higher soil and plant P than in the alluvial site (not all data shown).

Plant K concentration was significantly affected by N treatment in radish in both soil types, and in the spinach–peat site (Fig. 33). Overall, the K status in plant from either crop or soil types were in the range of 1.47–2.99%. According to Munson (1998), all N fertilizer treatments in this study were within the sufficiency range of plant K (1.50–5.50%) except for control (1.47%) in the spinach plant tissue on peat site. Whereas, the level of K adequacy varies, i.e. 2.5–3.5% K (Hochmuth et al., 2012), 3–8% K (Campbell, 2000), and 3.5–5.3% K (Mengel, 2007) for spinach, and 1.5–3.0% K for radish leaves (Hochmuth et al., 2012).

Manure had the highest plant K concentration in all cases. *Azolla* applied at the manure N rate increased plant K concentration in both crops grown on the peat soil. On the other hand, *Azolla*-U and urea had plant K concentrations comparable to control in all cases (Fig. 33).

The pattern of N fertilizer treatment effect on plant K (Fig. 33) generally follows the soil K pattern (Fig. 26). Manure and *Azolla* applied at the manure N rate had the highest K concentrations in soil and plant tissue, regardless of soil and crop types. In general, peat soil contained higher soil K than the alluvial soil. In contrast, vegetable plant tissue contained higher K in the alluvial site.

There was no N fertilizer effect on plant Fe concentration. Overall, radish and spinach plants contained higher Fe concentration in the alluvial soil (data not shown). It is common for a typical oligothropic tropical peat soil to have low plant Fe availability (Andriesse, 1998).

The influence of N fertilizer on plant Zn concentration was only significant in the radish plants grown on peat soil. The *Azolla*-M treatment reduced radish Zn concentration in the peat soil (Fig. 34). Nevertheless, the Zn concentration in spinach or radish in either alluvial or peat soil were in the sufficiency range, i.e. 27–100 mg kg^{-1} for both crops (Munson, 1998), 20–75 mg kg^{-1} for spinach (Campbell, 2000), 50–70 mg kg^{-1} for spinach and 30–50 mg kg^{-1} for radish leaves (Hochmuth et al., 2012), and 50–75 mg kg^{-1} for spinach (Storey, 2007). There was no correlation between Zn in the plant tissue and Zn in the soil.

Overall, macronutrient concentrations (N, P, and K) in either spinach leaves or radish bulbs in both soil types were highest in the manure treatment and in the *Azolla* treatment applied at the manure N rate (Figs. 31 to 33). The results corresponded with Zeid et al. (2015) who also found that chicken manure was more effective at enhancing radish plant height, dry weight of shoot and tuber, and nutrient concentrations in leaves and tubers compared to other organic

fertilizers and control. Additionally, the highest plant growth characteristics and nutrient concentrations of radish plants were observed in the mixture of chicken manure and 50% recommended rates of inorganic fertilizers (Zeid et al., 2015). Spinach grown with NPK fertilizers and poultry manure had higher protein levels (Adekayode, 2004; Oyedeji et al., 2014). The N taken up from those corresponding fertilizers caused higher protein concentration in spinach. Total N concentration is the basis used to determine protein content of foods (FAO, 2003). Thereby, based on nutrient concentrations in plants, manure and *Azolla* applied at the manure N rate are encouraged to be utilized as organic amendments in spinach and radish production.

Correlation Between Soil and Plant Nutrients

Total N of the peat soil had a strong negative relationship with soil P (r= –0.759) and strong positive correlations between soil total N with SOC in both soils (r= 0.996, 0.967, and 0.772) (Table 16). N fertilizer incorporated into the soil may enhance plant growth and in the long run, it may also result in more organic C added to soil that was derived from crop residues and root exudates (Christopher and Lal, 2007). Moreover, organic N fertilizer such as manure may also increase SOC (Jarecki et al., 2005; Matthews and Pilbeam, 2005; Wang et al., 2005; Su et al., 2006; Hati et al., 2006; Rudrappa et al., 2006; Rasmussen and Parton, 1994). Total N in the alluvial soil also had a positive relationship with C/N ratio (r= 0.916).

Likewise, soil P was also moderately negatively correlated with soil organic C (r= –0.644), while soil pH and C/N ratio showed moderately to strongly negative linear correlations (r= –0.547 and –0.703) (Table 16). The result of this study was in agreement with Wang et al. (2010), who found that soil pH increases with lower C/N ratio of plant residues.

Pocknee and Sumner (1997) stated that excess cations may be the main cause of plant residue capacity to neutralize soil pH. The excess cations induce the production of organic anions in which the decarboxylation of these organic anions utilized H^+ and thus, it would neutralize the soil acidity. Nevertheless, other factors that also controlled soil pH were the rate of plant residue decomposition, the pattern of anion and cation release, and microbial immobilization.

Soil organic C in both soil types was also strongly linear correlated with C/N ratio (r= 0.942 and 0.894) (Table 16). This relationship was very obvious since the higher SOC content would contribute to the higher C/N ratio. There was also an indication of moderate positive relationship between peat soil Zn and pH (r= 0.542) (Table 16). Sims (1985) asserted that positive pH interaction with Zn was detected under acidic conditions such as peat soil. Whereas, in the spinach–alluvial site, a strong positive correlation was found between soil Zn and Fe concentrations (r= 0.859) (Table 16).

Based on plant nutrient parameters, there were some moderate positive relationships between N and K concentrations in spinach grown in the alluvial soil (r= 0.588) and in radish in the peat soil (r= 0.534) (Table 17). Plant K concentration was also moderately correlated with plant P in both crops in the peat soil, i.e. r= 0.661 (radish) and 0.652 (spinach) (Table 17). Potassium had a positive correlation with N, as shown in the economically and environmentally interaction between these two nutrients in corn studies. In N fertilizer experiment, N uptake and utilization by plants is generally improved when the K level is adequate and results in higher yield (IPNI, 1998). Vice-versa, when N is sufficient, crops respond to higher K levels (IPNI, 1998). On the other hand, N form can also affect K absorption. N fertilizer in the form of NH_4^+-N reduced total K in tomato plants after 4 days (IPNI, 1998). Whereas, NO_3^--N fertilizer source kept the total K in plant constant (IPNI, 1998). Furthermore, other synergistic effects of P and K

were indicated in soybean and bermudagrass yields. The application of both P and K fertilizers enhanced yields of those crops. Spinach grown on the peat site indicated a moderate positive correlation between plant N and plant Fe (r= 0.509); whereas, plant K and plant Fe exhibited a positive relationship in the spinach grown in the alluvial site (r= 0.481) (Table 17).

In general, there was no significant correlation found between soil N and plant N in either alluvial or peat soils (Table 18). There were some correlations between soil properties of the alluvial soil with macro and micro nutrients in the radish crop.

Exchangeable K in the alluvial soil was moderately correlated with P concentration in the radish (r= 0.662) (Table 18). It was interesting to see that there were moderately negative correlations between soil pH with Zn concentrations in radish (r= –0.699 and –0.521) in both soils. Soil pH altered the Zn distribution (Sims, 1985). In acidic soils (pH below 5.2), the dominant nutrient is exchangeable Zn. The exchangeable cation fractions can influence plant Zn uptake. Indeed, plant Zn models could be predicted by soil pH (Sims, 1985).

K concentrations in radish had moderately positive relationships with K and pH in the alluvial soil, i.e. r= 0.488 and 0.467, respectively (Table 18). K is a major nutrient that is not influenced by soil pH; however, pH may have an indirect effect on K uptake to some extent (Jensen, 2010). According to McKenzie (2003), there is lower tendency of K fixation and entrapment between clay layers in acidic soils due to soluble Al occupying the binding sites. Liming also raises K fixation and lowers K availability for plant. Nonetheless, in the short term, K availability is enhanced through exchangeable K dislocation with Ca due to liming.

Soil Fe and P concentration in radish showed a moderately negative correlation (r= –0.469) (Table 18). Similarly, total N in the alluvial soil was moderately negative correlated with Fe concentration in the spinach (r= –0.500) (Table 18). It was interesting that the alluvial soil P

was strongly positively correlated with P concentration in the spinach (r= 0.885), but this was not true in the peat soil (Table 18). Negative relationship patterns were also found between exchangeable K in the alluvial soil and Zn concentration in spinach (r= –0.460) and SOC with spinach Fe concentration (r= –0.452) (Table 18).

Exchangeable K in the peat soil was highly correlated with K concentration of spinach crop (r= 0.754) and was moderately negatively correlated with plant N in spinach (r= –0.652) (Table 18). K concentration in the spinach crop also demonstrated a moderately positive correlation with P and C/N ratio of the peat soil, i.e. r= 0.630 and 0.580, respectively; moreover, the C/N ratio of peat soil illustrated a moderate relationship with P concentration in the spinach (r= 0.567) (Table 18). Negative correlations existed between soil pH and plant P (r= –0.480) and SOC and plant Zn (r= –0.463) (Table 18).

Total soil N of the peat soil was moderately correlated with K concentration of the radish crop (r= 0.631) (Table 18). Furthermore, positive correlations were also demonstrated in the relationships between N concentration in the radish plant with P in the peat soil (r= 0.694) and radish K concentration with peat soil Zn (r= 0.443) (Table 18).

Soil Zn concentration in the alluvial soil and N concentration in the spinach were negatively correlated (r= –0.612) (Table 18). Nitrogen fertilizer treatments (urea, manure, and *Azolla*) lowered soil Zn concentration in the spinach–alluvial site, compared to control (no N fertilizer) (Fig. 30).

Conclusions

In conclusion, there were some significant findings from this study, as follows:

1. Higher soil pH was found in all N treatments, in particular in the *Azolla* treatments in the radish–peat site.
2. Soil total N was influenced by *Azolla*-M in the radish–peat site.
3. Soil P concentration increased when the manure treatment was applied to the radish–alluvial and spinach–peat sites.
4. Soil K concentration was enhanced by the manure application on the radish–alluvial site.
5. N concentration in radish plants and P concentration in spinach plants increased when manure was applied to peat soil.
6. K concentration in the radish crop was increased by manure application to the alluvial soil, whereas manure and the *Azolla* applied at the manure N rate increased K concentration in the radish and spinach crops in the peat soil.
7. Soil organic C was positively correlated with soil total N and C/N ratio; in contrast, C/N ratio was negatively correlated to soil pH.
8. Positive correlations in plant nutrient concentrations occurred between N vs. K and P vs. K.
9. Soil K concentration and plant K concentration were positively correlated, and soil pH and plant Zn concentration were negatively correlated.

TABLES

Table 15. ANOVA (*p*-value) results showing the treatment effect (N fertilizer treatments) on soil properties and plant nutrient concentrations for radish and spinach on alluvial and peat soils.

Treatment effect	Alluvial		Peat	
	Radish	**Spinach**	**Radish**	**Spinach**
Soil properties:				
pH	0.0325†	0.2502	0.0021†	0.1855
Total N	0.1028§	0.2025	0.0219†	0.4309
P	0.0241†	0.0381†	0.4521	0.0586†
K	0.0301†	0.1809	0.8986	0.0427†
Organic C	0.1035§	0.3332	0.2661	0.8605
C/N	0.1350	0.7185	0.2888	0.0016†
Fe	0.9220	0.0379†	0.8776	0.7829
Zn	0.6610	0.0028†	0.0725‡	0.2627
Plant nutrients:				
N	0.1523	0.2888	0.0058†	0.4723
P	0.5118	0.1205	0.7273	0.1017§
K	0.0929†	0.9857	0.0075†	<0.0001†
Fe	0.7684	0.4029	0.6525	0.2875
Zn	0.8228	0.4144	0.0752†	0.3608

† *p*-value <0.10 indicates significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test.
‡ *p*-value <0.10 based on Tukey's HSD test; however, there was no significant difference across the treatments.
§ *p*-value >0.10 based on Tukey's HSD test; however, there was significant difference across the treatments.

Table 16. Correlation coefficients between soil properties (P <0.10) across N fertilizer treatments.

Soil properties	Radish on alluvial		Spinach on alluvial		Radish on peat		Spinach on peat	
	r	***p*-value**[†]	***r***	***p*-value**	***r***	***p*-value**	***r***	***p*-value**
N vs. P	-	-	-	-	-	-	–0.759	0.0010
N vs. pH	0.460	0.0846	-	-	-	-	-	-
N vs. SOC	0.996	<0.0001	0.967	<0.0001	0.480	0.0703	0.772	0.0007
N vs. C/N	0.916	<0.0001	-	-	-	-	-	-
N vs. Fe	0.487	0.0656	-	-	-	-	-	-
P vs. SOC	-	-	-	-	-	-	–0.644	0.0096
Fe vs. Zn	-	-	0.859	<0.0001	-	-	-	-
pH vs. SOC	0.463	0.0825	-	-	-	-	-	-
pH vs. C/N	-	-	-	-	–0.547	0.0347	–0.703	0.0035
SOC vs. C/N	0.942	<0.0001	0.475	0.0738	0.894	<0.0001	-	-
SOC vs. Fe	0.484	0.0674	-	-	-	-	-	-
C/N vs. Fe	0.445	0.0967	-	-	-	-	-	-
Zn vs. pH	-	-	-	-	0.542	0.0368	-	-

[†]*p*-value <0.10 indicates significant correlation coefficient (*r*) within the same crop and soil based on Pearson correlation.
-: no significant correlation found between soil properties.

Table 17. Correlation among plant nutrients (*P* <0.10) across N fertilizer treatments.

Plant nutrient	Spinach on alluvial		Radish on peat		Spinach on peat	
	r	***p*-value**[†]	***r***	***p*-value**	***r***	***p*-value**
N vs. K	0.588	0.0212	0.534	0.0405	-	-
N vs. Fe	-	-	-	-	0.509	0.0526
P vs. K	-	-	0.661	0.0073	0.652	0.0085
K vs. Fe	0.481	0.0699	-	-	-	-

[†]*p*-value <0.10 indicates significant correlation coefficient (*r*) within the same crop and soil based on Pearson correlation.
-: no significant correlation found between plant nutrient concentrations.

Table 18. Correlation coefficients between soil properties and plant nutrient concentrations (*P* <0.10) across N fertilizer treatments.

Soil properties	Plant nutrient	Radish on alluvial		Spinach on alluvial		Radish on peat		Spinach on peat	
		r	*p*-value[†]	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
N	K	-	-	-	-	0.631	0.0116	-	-
N	Fe	-	-	–0.500	0.0575	-	-	-	-
P	N	-	-	-	-	0.694	0.0041	-	-
P	P	-	-	0.885	<0.0001	-	-	-	-
P	K	-	-	-	-	-	-	0.630	0.0118
K	N	-	-	-	-	-	-	–0.652	0.0084
K	P	0.662	0.0071	-	-	-	-	-	-
K	K	0.488	0.0650	-	-	-	-	0.754	0.0012
K	Zn	-	-	–0.460	0.0843	-	-	-	-
Ph	P	-	-	-	-	-	-	–0.480	0.0705
Ph	K	0.467	0.0794	-	-	-	-	-	-
Ph	Zn	–0.699	0.0037	-	-	–0.521	0.0463	-	-
Fe	P	–0.469	0.0781	-	-	-	-	-	-
SOC	Fe	-	-	–0.452	0.0906	-	-	-	-
SOC	Zn	-	-	-	-	-	-	–0.463	0.0820
C/N	P	-	-	-	-	-	-	0.567	0.0274
C/N	K	-	-	-	-	-	-	0.580	0.0234
Zn	N	-	-	–0.612	0.0153	-	-	-	-
Zn	K	-	-	-	-	0.443	0.0985	-	-

[†]*p*-value <0.10 indicates significant correlation coefficient (*r*) within the same crop and soil based on Pearson correlation.
-: no significant correlation found between soil properties with plant nutrient concentrations.

FIGURES

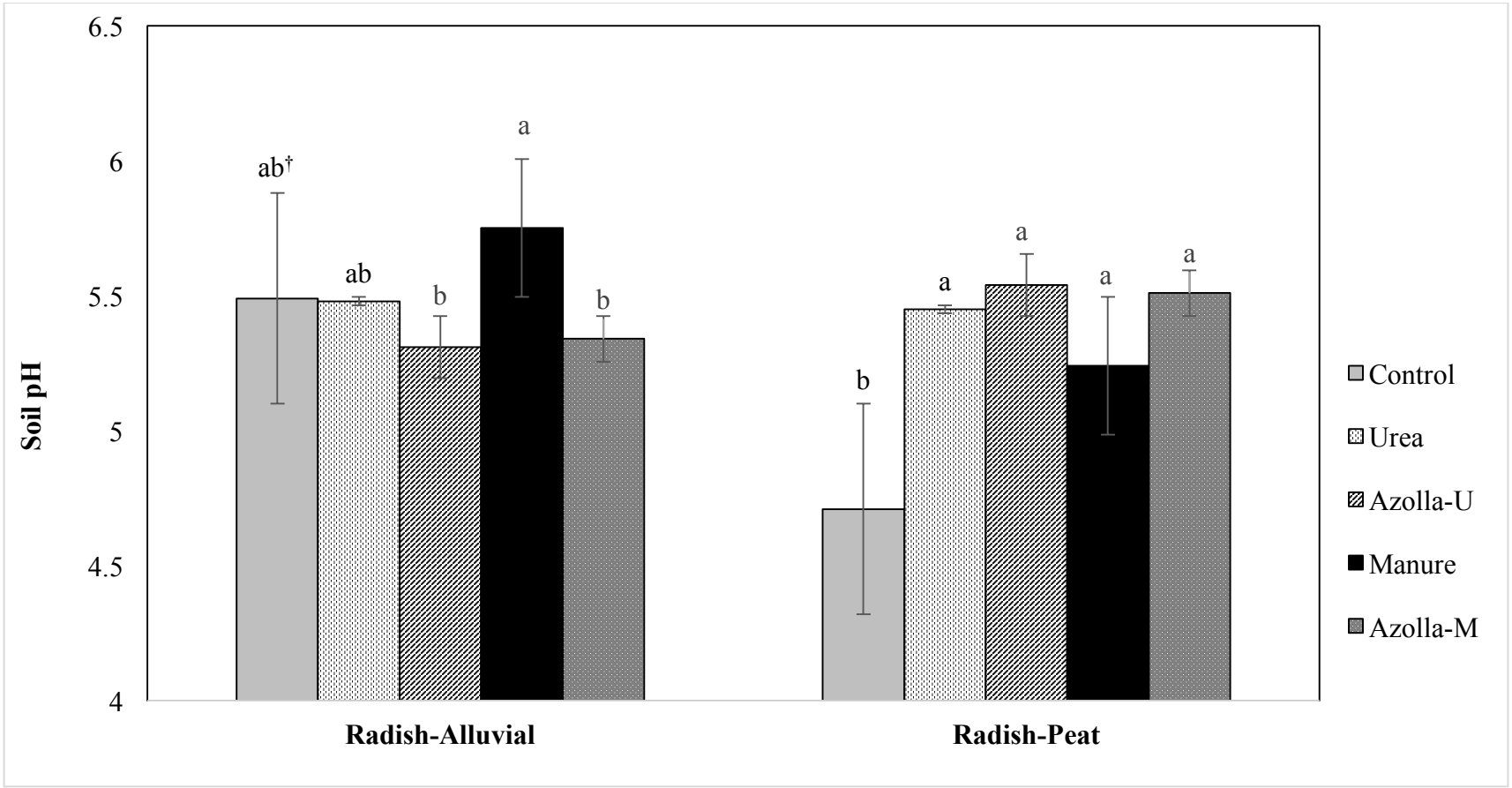

Fig. 23. Soil pH affected by N fertilizer treatments on radish crop.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

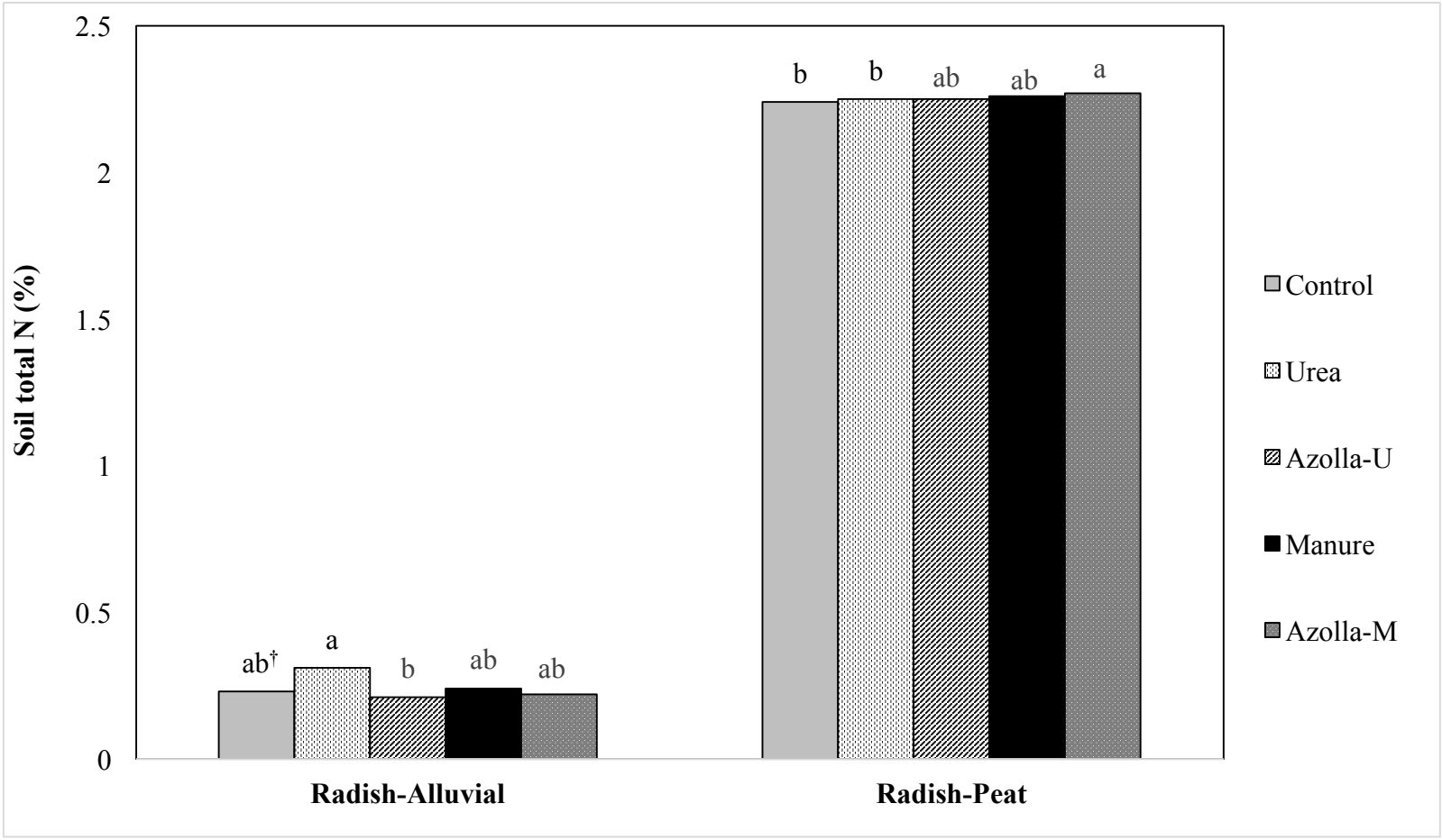

Fig. 24. Soil total N affected by N fertilizer treatments on radish crop.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

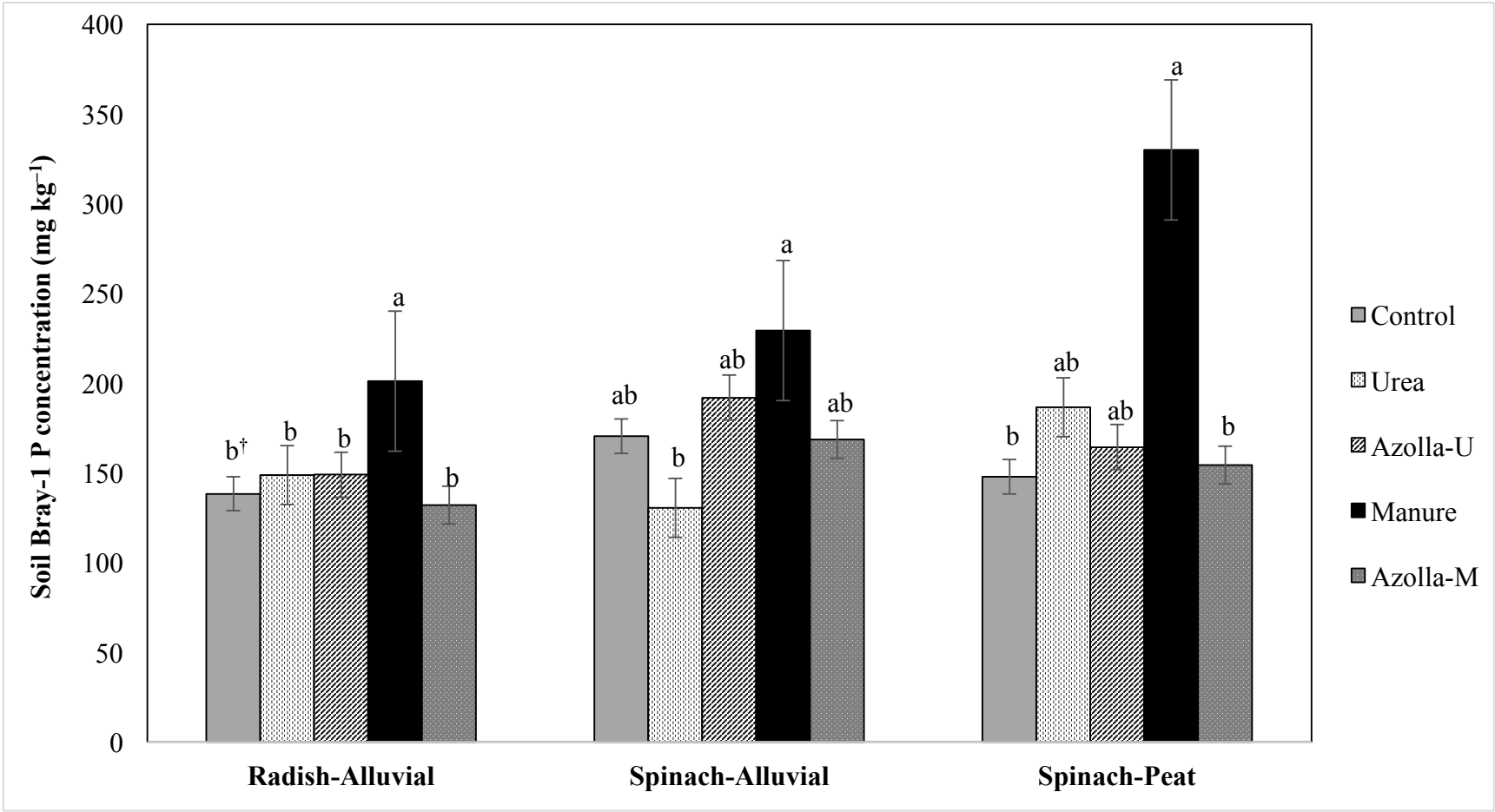

Fig. 25. Soil P concentration affected by N fertilizer treatments on radish and spinach crops.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

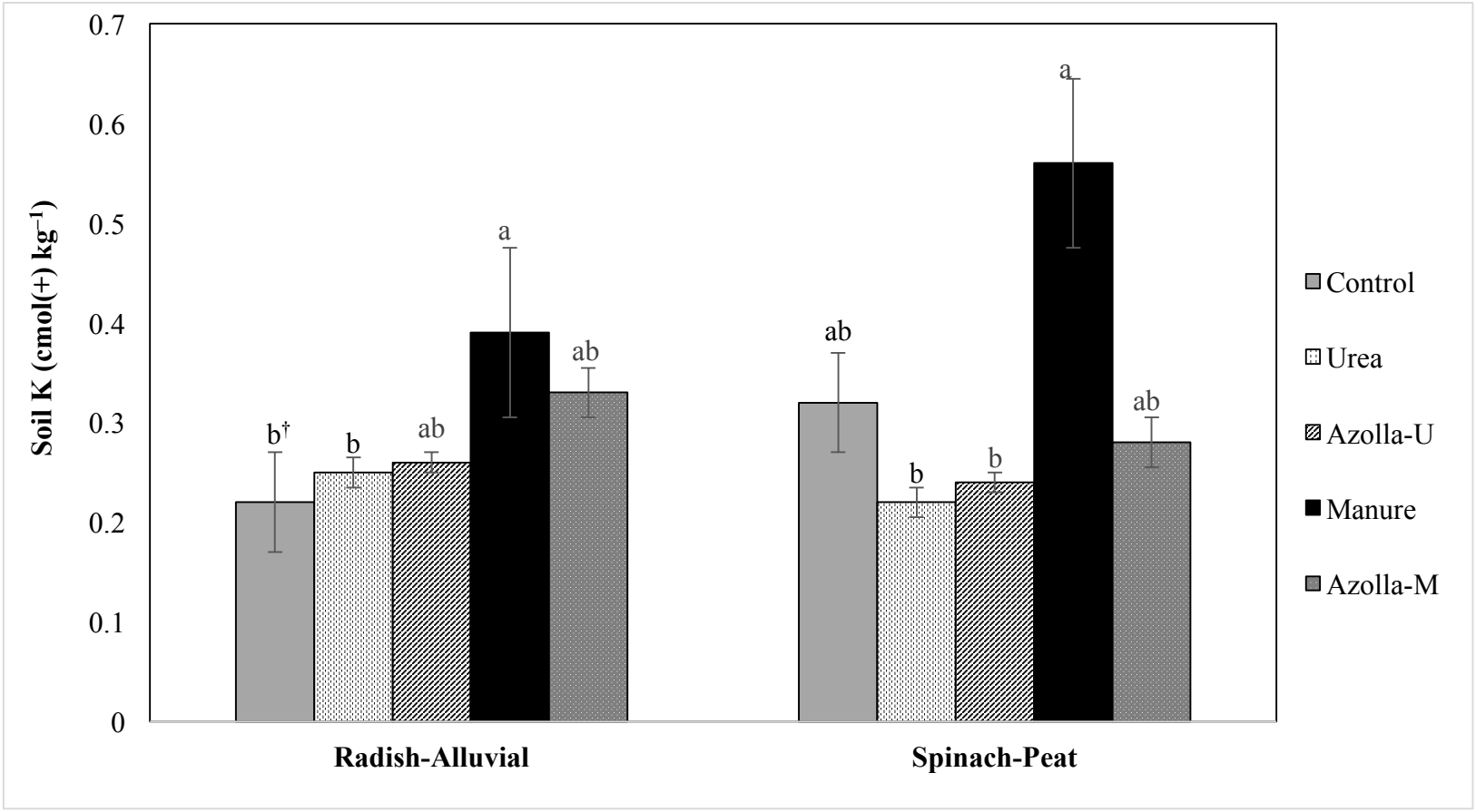

Fig. 26. Soil K concentration affected by N fertilizer treatments on radish and spinach crops.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

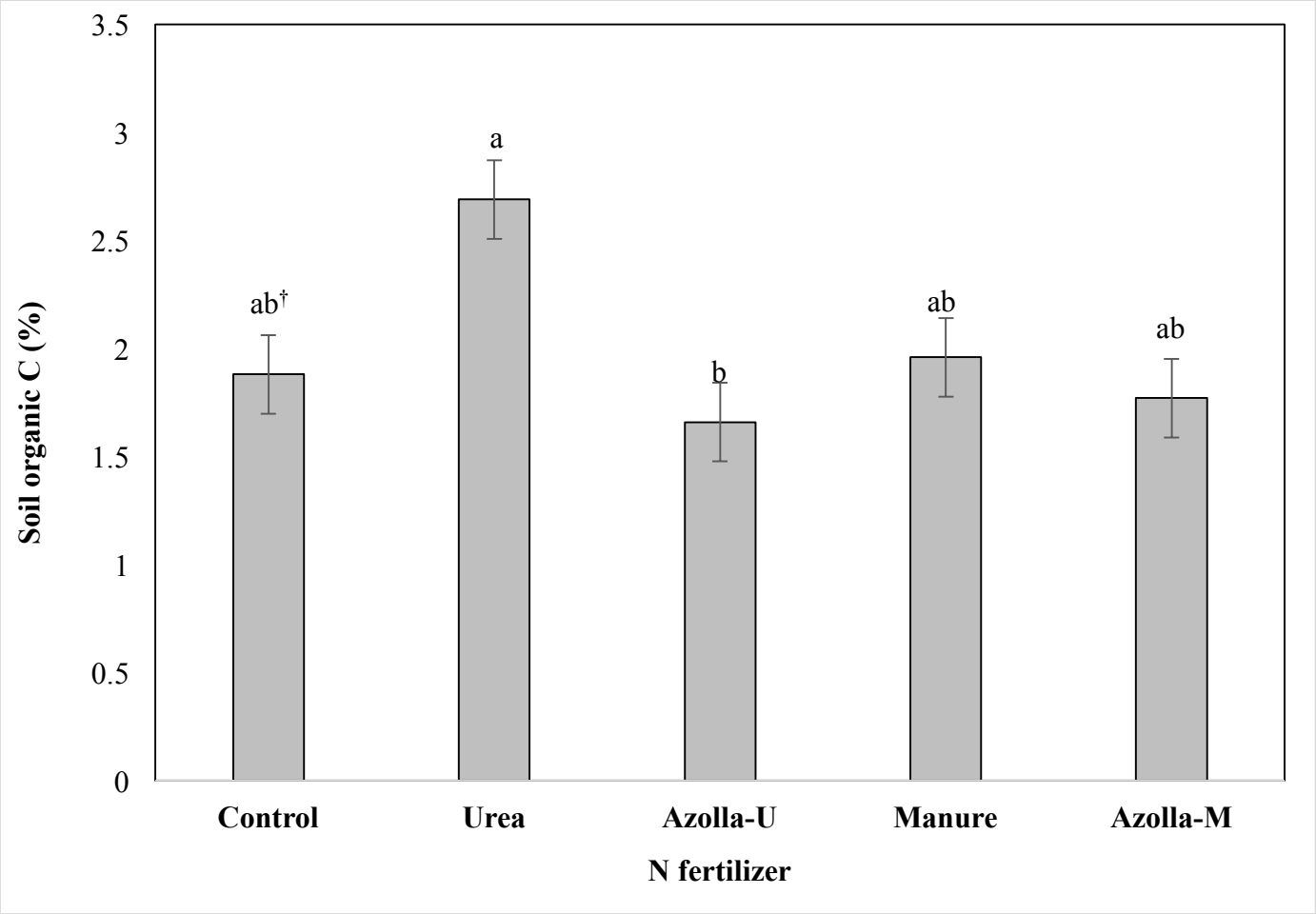

Fig. 27. Soil organic C concentration affected by N fertilizer treatments on radish in the alluvial soil.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

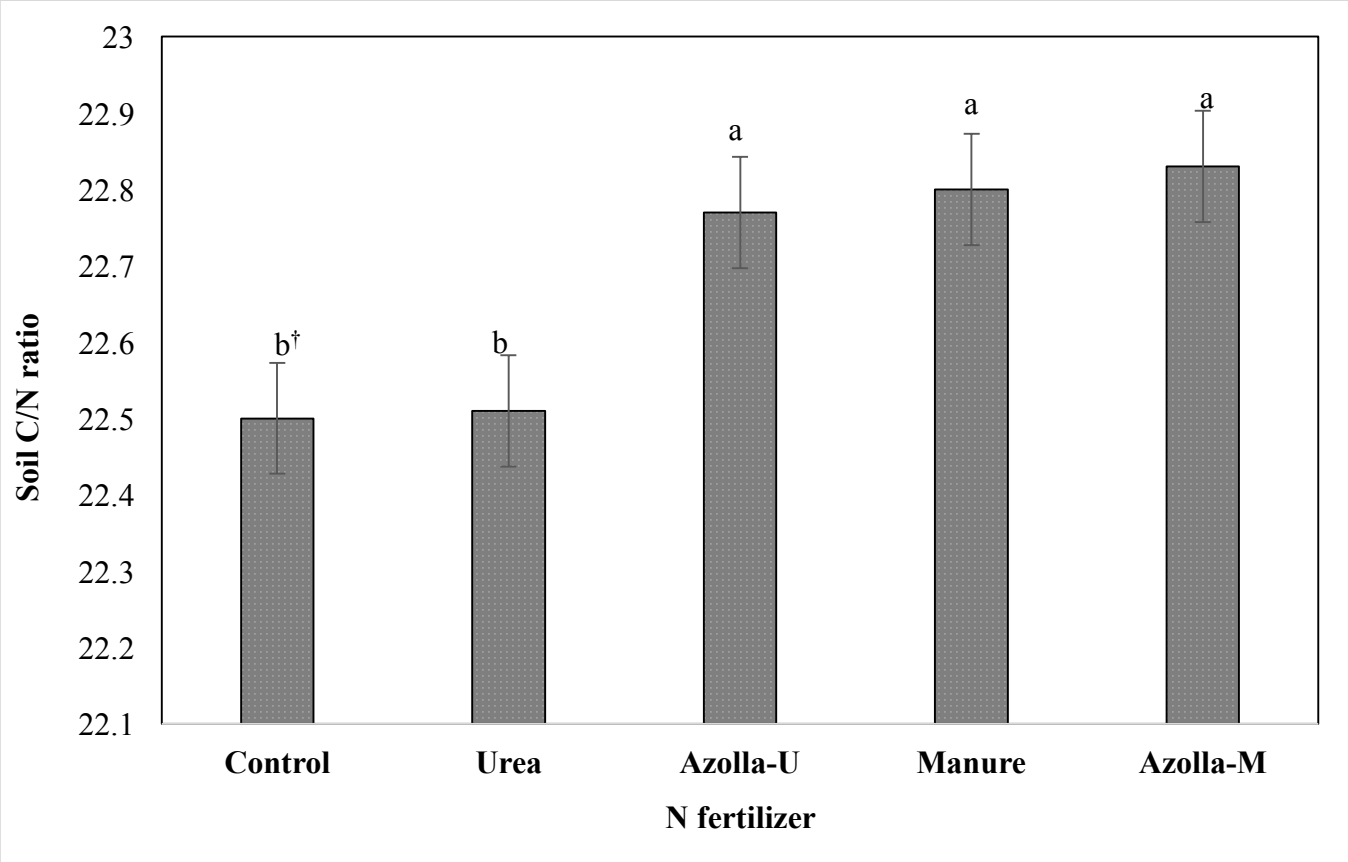

Fig. 28. Soil C/N ratio affected by N fertilizer treatments on spinach in the peat soil.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

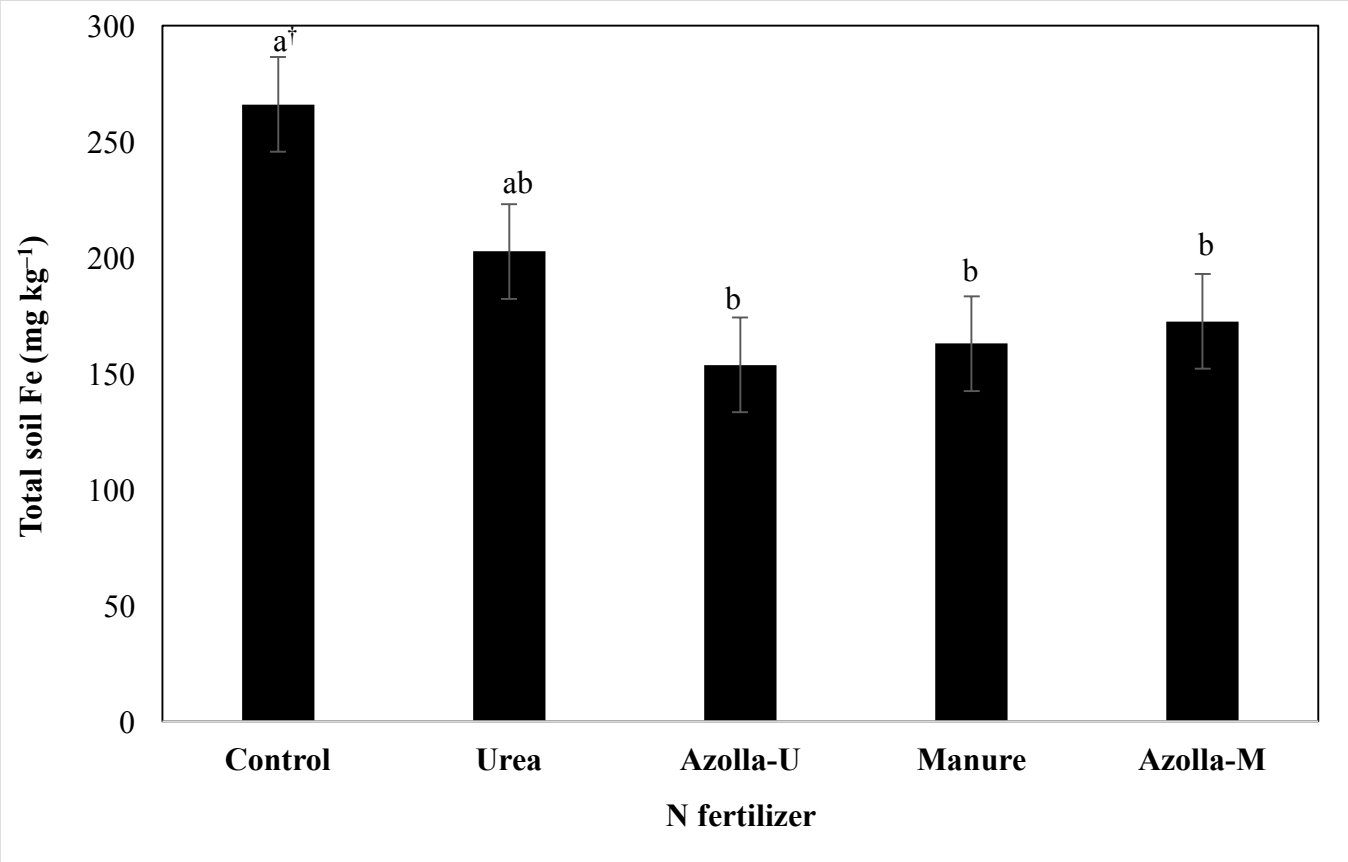

Fig. 29. Soil Fe affected by N fertilizer treatments on spinach in the alluvial soil.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

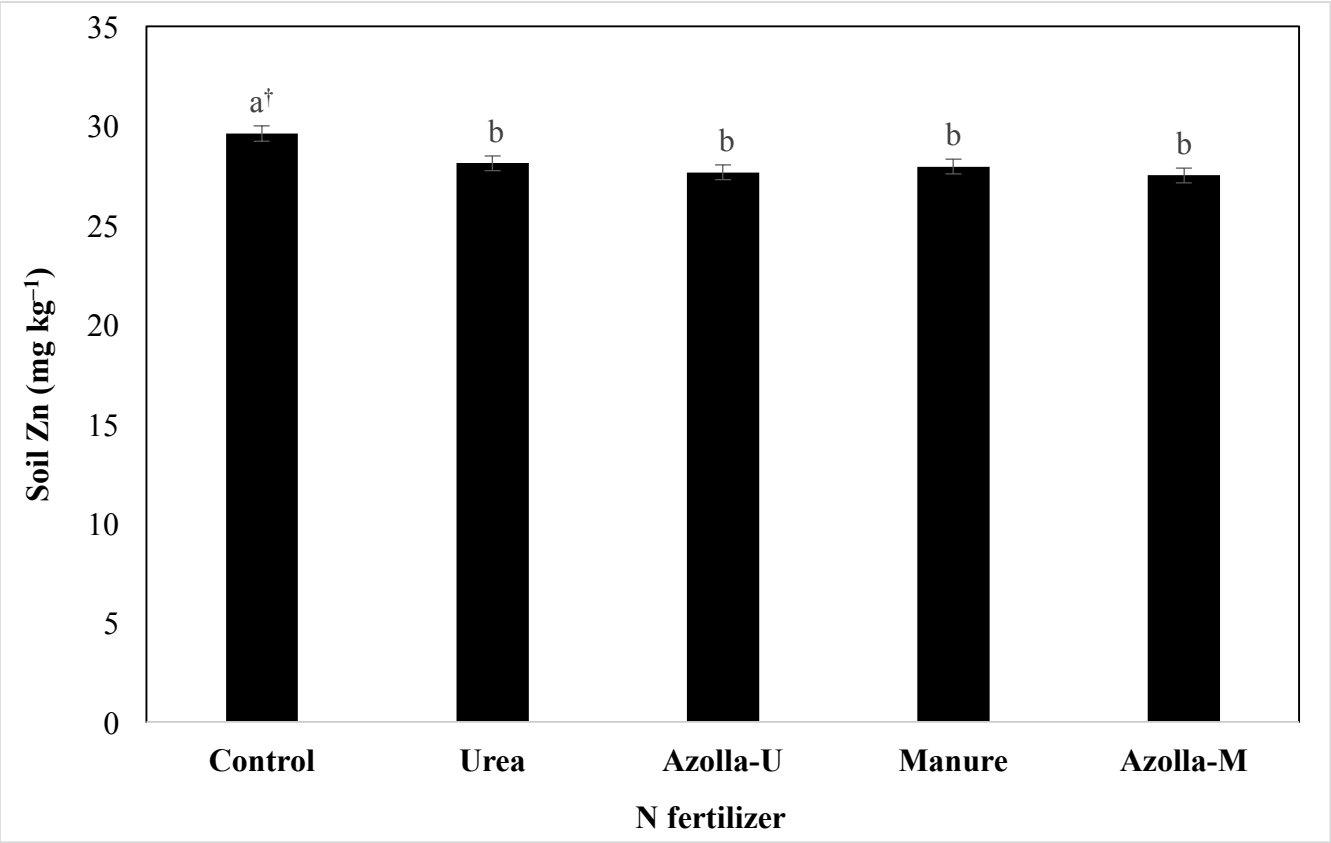

Fig. 30. Soil Zn affected by N fertilizer treatments on spinach in the alluvial soil.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

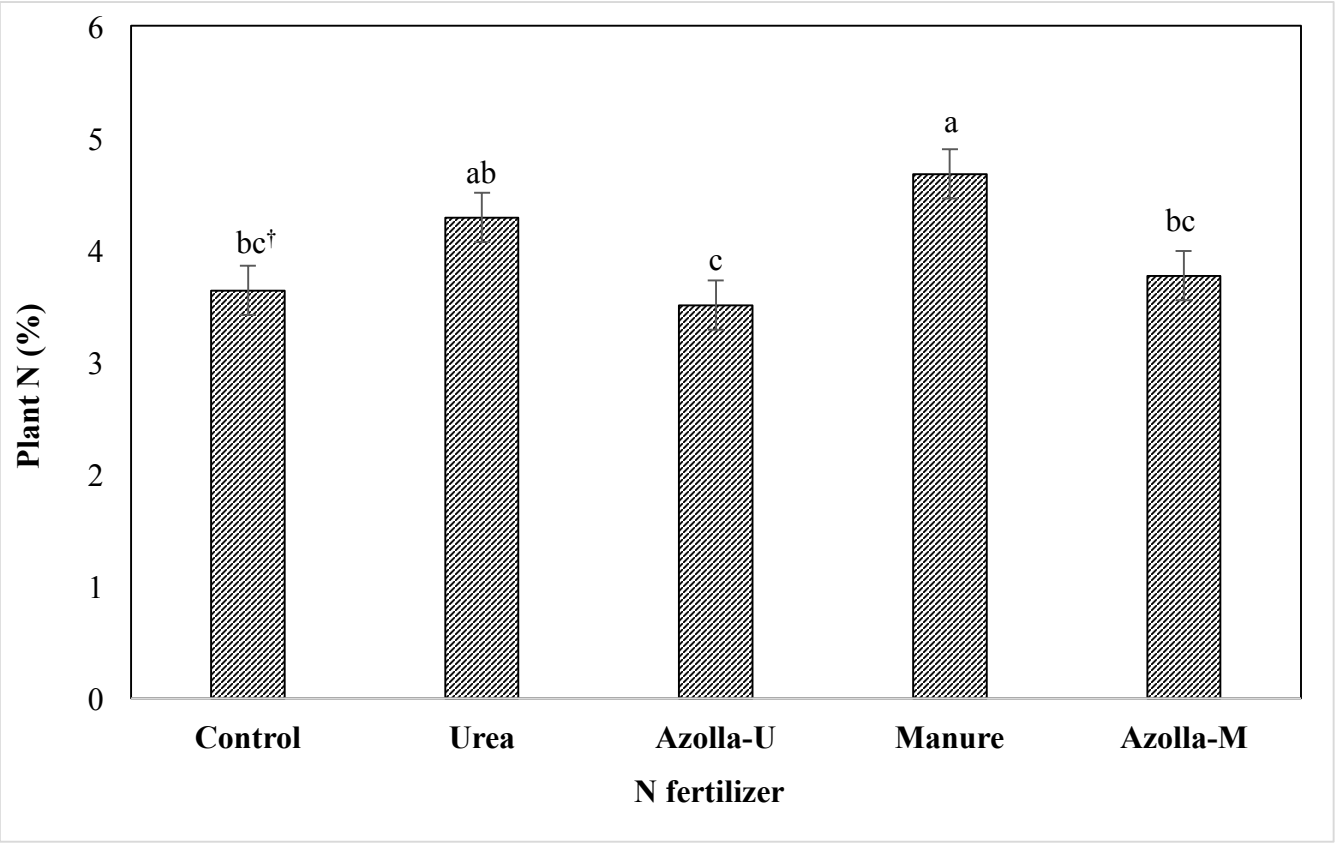

Fig. 31. Plant N concentration affected by N fertilizer treatments on radish in the peat soil.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

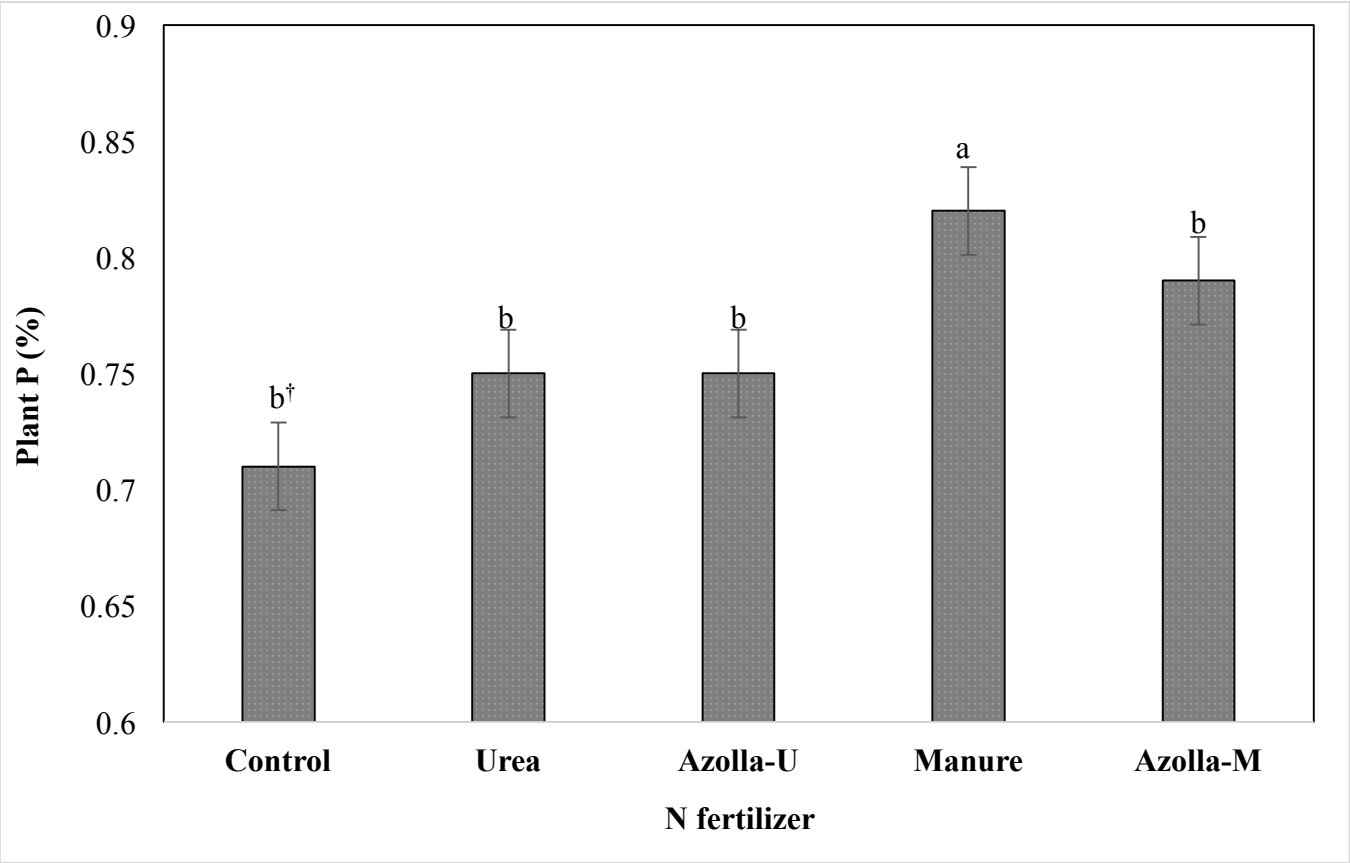

Fig. 32. Plant P concentration affected by N fertilizer treatments on spinach in the peat soil.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

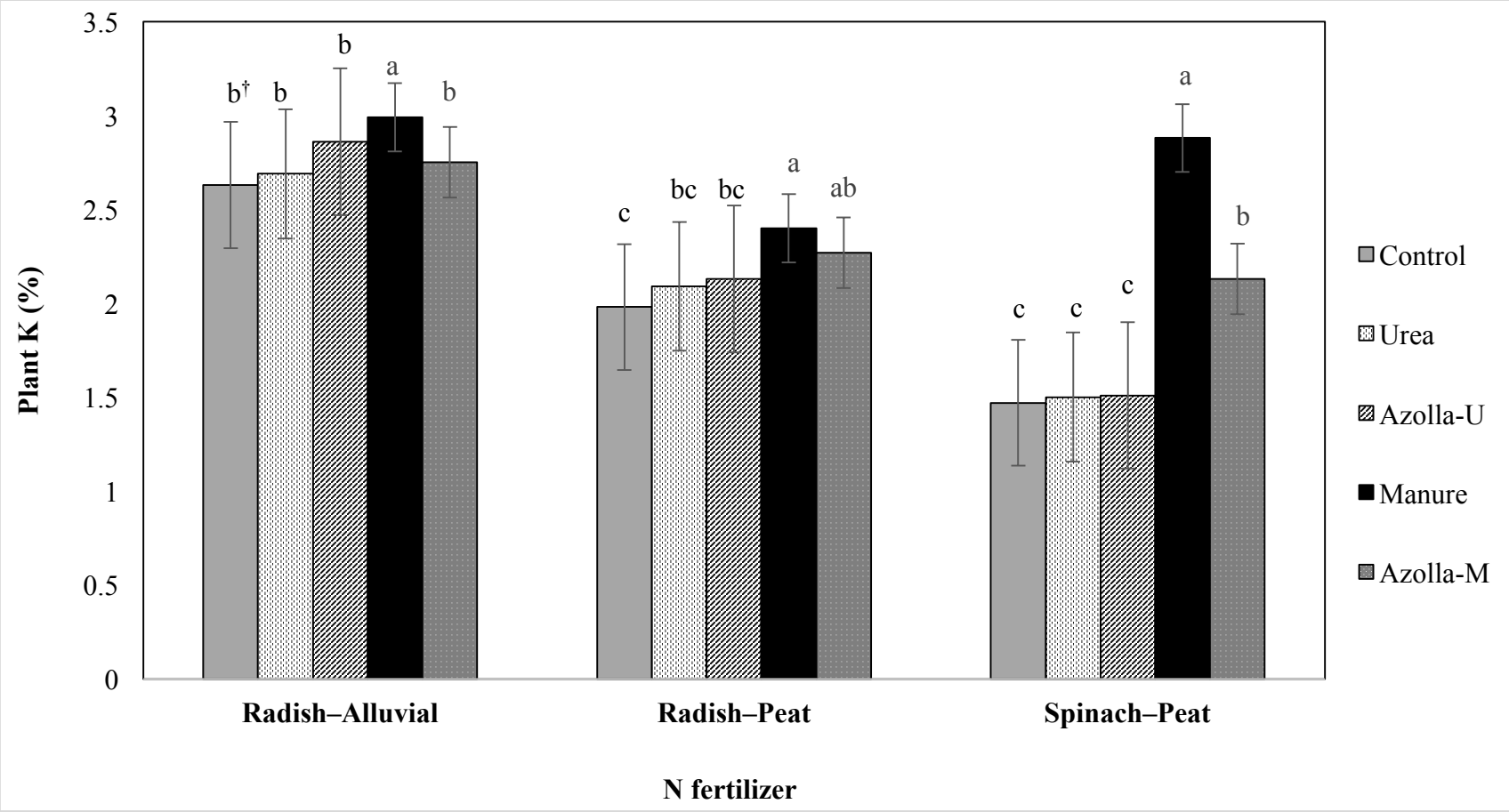

Fig. 33. Plant K concentration affected by N fertilizer treatments on radish and spinach crops in the alluvial and peat soils.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

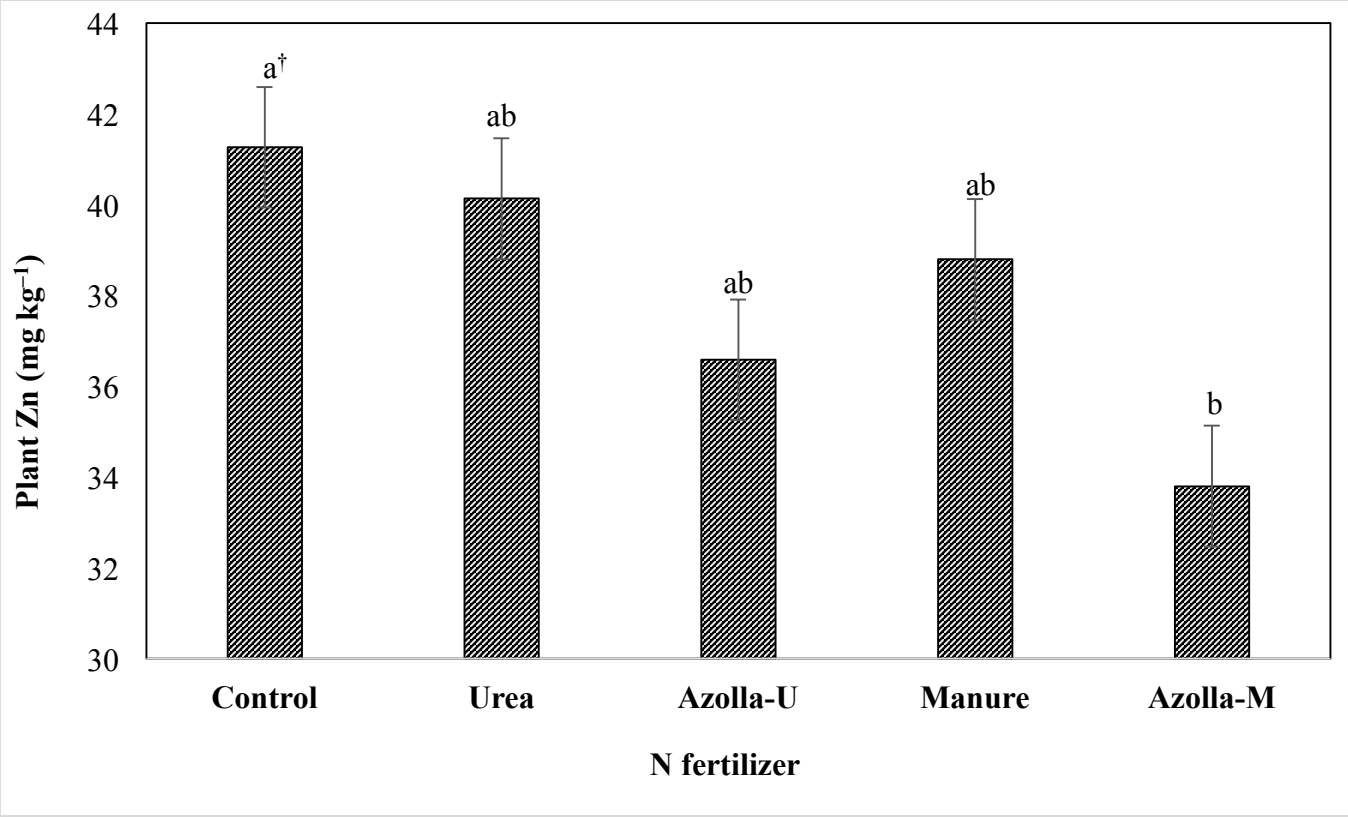

Fig. 34. Plant Zn concentration affected by N fertilizer treatments on radish in the peat soil.
†Values followed by a common letter indicate no significant difference within the same crop and soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea: 23 kg N ha^{-1}, *Azolla*-U: *Azolla* applied at the urea N rate (23 kg N ha^{-1}), Manure: 108 kg N ha^{-1}, *Azolla*-M: *Azolla* applied at the manure N rate (108 kg N ha^{-1}).

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CHAPTER 5

AZOLLA BIOFERTILIZER INFLUENCES THE SOIL MICROBIAL COMMUNITIES ON ALLUVIAL AND PEAT SOILS IN SPINACH PRODUCTION SYSTEM

Summary

Food security is an essential issue in Indonesia. The Ministry of Agriculture in Indonesia developed the Sustainable Food-Reserved Garden Program to improve national food security starting from the household level. In this program, every household is encouraged to grow vegetables as a nutritious food source in their backyard. One approach to intensify vegetable production is through using the locally-grown biofertilizer *Azolla* as a N source in place of conventional fertilizers. There is potential for *Azolla* biofertilizer to alter soil microbial communities. Therefore, the objectives of this study were to determine the effects of *Azolla*, compared to other commonly-used fertilizers, on soil microbial communities in the alluvial and peat soils in West Kalimantan, Indonesia. Five N fertilizer treatments were control (0 kg N ha^{-1}), urea (23 kg N ha^{-1}), *Azolla* applied at the same urea-N rate (*Azolla*-U, 23 kg N ha^{-1}), chicken manure (108 kg N ha^{-1}), and *Azolla* applied at the same manure-N rate (*Azolla*-M, 108 kg N ha^{-1}). *Azolla*-M and manure treatments increased red spinach yield and agronomic parameters in the peat soil; however, in the alluvial soil, the spinach yield was only affected by manure. *Azolla*-M treatment resulted in a shift in the microbial community structure in peat soil, but not in alluvial soil. Microbial community biomass was greater in the alluvial soil than in the peat soil, and bacteria were dominant in both soil types, regardless of the N fertilizer treatment. Greater fungal community biomass was found in soils amended with *Azolla*-M and manure, compared to control soil and soils amended with urea or *Azolla*-U. A greater ratio of fatty acid

stress biomarkers was indicated in control soil and urea-amended soil, as well as in the peat soil compared to alluvial soil. *Azolla*-M may possibly diminish stress encountered by the microbial community from unfavorable environmental conditions.

Introduction

Food security is an urgent global challenge, especially in relation to climate change and increasing world population. Developing countries are particularly sensitive to food insecurity, depending on their economic growth and resources. Indonesia is a developing country with one-fourth of the world's population (2.6 million in 2015; World Bank, 2017). Its population is unevenly distributed by regions, which presents additional food insecurity risks. One of the food security obstacles in Indonesia is how to provide sufficient, nutritious, and affordable food for the growing population. In order to accomplish its national food security goals, the Indonesian Ministry of Agriculture emphasizes that national food security has to be preceded by food self-sufficiency starting at the household level. Thus, a program called Sustainable Food-Reserved Garden was launched, so that every household would utilize their backyard to provide food for their family (IAARD, 2014).

Indonesia has 20.0-39.9 % prevalence of anemia in infants and children aged 6–59 months and 100% in women of reproductive age (15–49 years) (WHO, 2015). Iron as an element of hemoglobin plays a significant role in reducing the prevalence of anemia. One of the solutions for combating Fe deficiency is through nutritious food intake, either from animal protein or vegetables rich in Fe. In the Sustainable Food-Reserved Garden program, vegetables are a nutritious food that is encouraged to be grown in the backyard. Spinach is higher in nutritional value, and can be a good source of Fe (Dauthy, 1995; Yan, 2013). For one selected

serving, spinach contains 5% of the daily recommended value (DV) of Fe, in addition to the other nutrients such as Mn (13% DV), Mg (6% DV), K (5% DV), Ca (3% DV), Cu (2% D V), Zn (1% DV), P (1% DV), Na (1% DV), vitamins such as folate (15% DV), and total omega-3 and omega-6 fatty acids at 41.4 and 7.8 mg, respectively (Nutrition Data, 2014). Therefore, spinach was used as the crop of interest in this study.

One approach to enhance vegetable production and at the same time to improve soil fertility is through sustainable fertilization. This effort can be achieved by utilizing locally-produced fertilizers, in particular through biological N-fixing organisms. *Azolla* is a water fern that has a symbiotic mutual relationship with cyanobacteria. *Azolla* provides a suitable environment and the energy needed for the N fixation process carried out by cyanobacteria; in return, cyanobacteria furnish fixed N for *Azolla* growth. *Azolla* can supply 150–300 tons ha^{-1} yr^{-1} of green manure, which when amended to soil, stimulates microbial N mineralization activity, soil fertility, and subsequently, crop productivity (Kannaiyan, 1985). Furthermore, it considerably enhances soil physicochemical properties, microbial growth, and microbial activity overall (Subedi and Shrestha, 2015).

The soil microbial community plays a fundamental role in maintaining soil fertility (Luo et al., 2016). It regulates such functions as decomposition, N fixation, nutrient cycling, C sequestration, as well as disease suppression (Giller et al., 1997; Janvier et al., 2007). Soil microbial growth and activity are dynamic and are affected by a number of factors, including nutrient availability (Gilliam et al., 2011). For example, N fertilization can potentially affect soil microbial community composition due to the alteration of the competitive interface with plants (Harte and Kinzig 1993; Naeem et al. 2000; Bardgett et al. 1999; Lundquist et al. 1999). Effects of fertilization on the microbial community and soil properties have been demonstrated in rice

fields (Pascual et al., 2000); tropical forests (Cusack et al., 2011), and grassland ecosystems (Bradley et al., 2006). Additionally, soil type and crop management practices in intensive organic and conventional vegetable systems affect soil microbial community structure and activity (Moeskops et al., 2010, 2012).

Soil type is an influential factor in defining soil microbial community composition as found in some research (Bossio et al., 1998; Wieland et al., 2001). There are several kinds of soil types in the ecosystems of West Kalimantan, Indonesia. The common soil types for horticulture and vegetable production systems are peat, alluvial, red-yellow podzolic, and latosol. According to USDA soil taxonomy, these soils are classified as histosols, entisols, inceptisols, ultisols, and oxisols (Soil Survey Staff, 2014). These soil types may have some effect on soil microbial community structure due to their soil fertility status.

There are many methods to assess soil microbial community structure. The Ester-Linked Fatty Acid Methyl Ester (EL-FAME) method has been widely used to characterize microbial community composition according to the types and relative amounts of different fatty acid groups. Cell biomass is made up of a relatively constant fraction of fatty acids; and thus, signature fatty acids play a role in discrimination of major taxonomic groups within a microbial community (Ibekwe and Kennedy, 1998). Indeed, an alteration in the fatty acid profile would signify a change in the microbial population. Direct extraction of microbial fatty acids that is applied in the EL-FAME method provides a relatively simple and fast analysis capable of distinguishing microbial communities that vary in structure among different environments, soil types, and management practices (Schutter and Dick, 2000; Stromberger et al., 2007; Igalavithana et al., 2017). Indeed, the EL-FAME method is applicable for distinguishing changes in the microbial community as influenced by commercial organic fertilizer and cover crops in

organic vegetable systems in tropical soils (Negrete et al., 2015; Cusack et al., 2011) and boreal peatlands (Sundh et al., 1997; Halbritter and Mogyoróssy, 2002).

Despite the importance of soil microbial communities in regulating soil processes, including nutrient cycling, little is known about the microbial communities of tropical soils cropped to vegetables, and how communities are affected by agricultural management practices, including N fertilization. Given the concerns of food insecurity, particularly in developing countries, there is a need to better understand the key role of microbial communities in vegetable production ecosystems, in order to construct a sustainable and efficient tropical agriculture system. Therefore, the objective of this study was to evaluate soil microbial community biomass and structure under spinach grown in dominant soil types in West Kalimantan, Indonesia, as influenced by different forms of N fertilizer, including *Azolla*. Our hypothesis was that N fertilizer, in particular *Azolla* used as a biofertilizer, will affect soil microbial community biomass and structure, specifically bacteria and fungi, in mineral (alluvial) and organic (peat) soils.

Materials and Methods

Study Sites

This study was undertaken in 2015 on two soils in West Kalimantan, Indonesia. The alluvial soil (Inceptisols) in tidal lands agro-ecosystem was located on the Agricultural Research Station of the Assessment Institute for Agricultural Technology in Pal IX Village, Sei Kakap, Kubu Raya Regency, West Kalimantan. The peat soil (Histosols) was located in a farmer's field in Sei Selamat Village, Siantan, Pontianak, West Kalimantan. Both sites were utilized for

cropping, in particular staple (rice, corn) and vegetable crops at the Research Station and vegetable crops at the farmer's site.

The soil types are Sulfic Endoaquepts (alluvial soil) and Terric Sulfihemists (peat soil) according to USDA soil taxonomy (Hidayat et al., 2010). Sulfic Endoaquepts are characterized as soils in the suborder of Endoaquepts that have a slope less than 25% and contain sulfidic materials. Soil suborder Sulfihemists, on the other hand, have a mineral soil material layer thickness of 30 cm or more and if they have the upper boundary within the control section, below the surface tier, then they are characterized as Terric Sulfhihemists (Soil Survey Staff, 2014).

The climate is tropical moist (III C and IV C) with average temperatures above 18 ^{o}C year-round and average relative humidity of 80.8%. The annual precipitation ranges from 2000–4000 mm (Rejekiningrum et al., 2012).

Experimental Design

The experimental design was a randomized complete block design (RCB) with three replicates and 1x1 m plots. There were five treatments of N fertilizer: control (0 kg N ha^{-1}), urea at 50 kg ha^{-1} (23 kg N ha^{-1}), *Azolla* at the urea N rate of 23 kg N ha^{-1} (*Azolla*-U), chicken manure at 5 t ha^{-1} (108 kg N ha^{-1}), and *Azolla* at the manure N rate of 108 kg N ha^{-1} (*Azolla*-M). The N rates of urea and chicken manure were determined based on a preliminary study. The N fertilizer treatment was the fixed effect, and block was the random effect.

Azolla pinnata Nursery

Azolla pinnata, which is native to Indonesia, was used for this study. The *Azolla* nursery was grown in an artificial pond lined with polyethylene and in some natural ponds located close to the field sites. The water resource used for both the Azolla nursery and the irrigation water was from a mix of natural surface water and rain water at both sites. In order to increase the peat water pH, some ameliorant (plant ash) was applied at 2.68 t ha^{-1}. In the production ponds, the inoculation rate of *Azolla* was 100–200 g m^{-2}. Then, *Azolla* could be harvested three to four weeks after inoculation.

Spinach Cultivation

Manual tillage was utilized for both soil types. Plant ash as ameliorant was applied at 3 t ha^{-1} after tillage in the peat soil to increase soil pH. The spinach variety used for this study was Red "Giti" Spinach from the Indonesian Vegetable Research Institute. Spinach seedlings were transplanted 21 days after germination at a 20x15-cm planting distance. The vegetable crop was watered every two days or less depending on rainfall. Urea was applied in a split application (3 and 14 days after transplanting). Manure (3.19% N) and *Azolla* (2.88% N) were applied to the soil surface 3 days after transplanting. Spinach was harvested 24 days after transplanting.

Soil Sampling

Three representative soil subsamples (0–20 cm depth) were randomly collected per plot after crop harvest, using a soil auger that had drill bit length 20 cm and 6 cm diameter. Subsamples were mixed into one composite sample for each N fertilizer treatment plot and

placed into a plastic bag. A 100-g soil sample was transferred into two 50-mL centrifuge tubes and kept in a cooler while collecting soil samples and transporting them to the laboratory. Soil samples were frozen and stored at –18 °C. The remaining soils were air-dried for soil physicochemical analysis in the soil preparation laboratory. Frozen soils were freeze-dried and then stored at room temperature (26 ^{o}C) prior to EL-FAME extraction.

EL-FAME Extraction

Microbial community structure was determined from freeze-dried soil samples by EL-FAME as modified slightly from Schutter and Dick (2000). The whole cell fatty acids including phospholipids, glycolipids, and neutral lipids were extracted from the soil. To begin, 3-g of freeze-dried soil were placed into a 50-mL glass centrifuge or mild alkaline hydrolysis extraction in 15 mL 0.2 *N* KOH, prepared in methanol. Samples were extracted for 1 h in a 37-^{o}C water bath, during which samples were vortexed for 10 seconds every 10 minutes. Afterwards, samples were neutralized through the addition of 3 mL of 1 *M* acetic acid. Hexane (10 mL) was added to each tube to extract fatty acids into an organic (hexane) phase. Tubes were vortexed for 20 seconds, and then all tubes were centrifuged at 440 x g (Sorvall HS-4 swinging bucket motor) for 20 minutes at 4 ^{o}C to separate the hexane and aqueous phases. Using a Pasteur pipet, two-thirds of clean hexane supernatant was removed from each tube, and transferred into clean glass tubes (17 x 100 mm). Samples were prepared for gas chromatography (GC) by evaporating the hexane solvent under N_2 gas. The dried EL-FAME residue was then re-dissolved by adding 100 µL of hexane containing 0.10 µg µL^{-1} nonadecanoic acid methyl ester (C 19:0) as an internal standard. All samples were stored at –20 ^{o}C until analyzed (described below).

Samples were analyzed by gas chromatography-mass spectrometry (GCMS) with a Trace GC (TRACE 1310) coupled to a Thermo TSQ8000 Evo mass spectrometer (Thermo Fisher Scientific, 1400 North Point Blvd., West Palm Beach, FL 33407). It was equipped with a Phenomenex ZB-5HT Inferno GC column (30m x 0.25mm x 0.25 um). The gas chromatography inlet was set at 285 °C, and the oven temperature was programmed at 60 °C for 2 min, a ramp of 15 °C per min to 330 °C, and held at 330 °C for 10 min. EL-FAMEs were detected based on mass spectral and retention time matches to a 37 FAME mixture (Sigma) and a bacterial acid methyl ester mixture (Siga). Additional El-FAMEs were identified by comparing mass spectra to the NIST Mass Spectral Library (NIST, 2017).

EL-FAME biomarkers were assigned to the following microbial groups (Schutter and Dick, 2000; Stromberger et al., 2007): Gram-positive bacteria (i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0), Gram-negative bacteria (16:1ω7c, 17:1ω7, 17:0 cy, 18:1ω7c, 18:1ω8, and 19:0 cy), actinomycetes (10Me16:0, 10Me17:0, and 10Me18:0), fungi (18:2ω6,9c), and arbuscular mycorrhizal (AM) fungi (16:1ω5c). Total bacterial biomass was determined from the sum of biomarkers of Gram-positive and Gram-negative bacteria, actinomycetes, as well as the general bacterial fatty acids 15:0 and 17:0. Total microbial biomass was the sum of all bacterial and fungal EL-FAMEs. Stress indicators were signified by the ratio of 17:0 cy to its precursor 16:1ω7c (Stress 1), and by the ratio of 19:0 cy to its precursor 18:1ω7c (Stress 2) (Grogan and Cronan, 1997).

Statistical Analysis

Soil microbial community EL-FAME data were analyzed by principal components analysis (PCA), after normalizing the data from nmole g^{-1} soil to relative nmol %. Communities

were analyzed separately for each soil type, using the PC-ORD version 6 statistical package (McCune and Mefford, 2011). An outlier analysis conducted in PC-ORD revealed one outlier in the alluvial data set, which was removed from the PCA. Community structure comparisons were conducted by blocked multi-response permutation procedures (MRBP), also in PC-ORD (P <0.10).

Univariate data were analyzed by analysis of variance (ANOVA) tests with the PROC MIXED procedure using SAS version 9.4 (SAS Institute, 2016), separately for each soil. Multiple comparisons of soil microbial community groups across N fertilizer treatments were performed using HSD Tukey adjustment post hoc test if the ANOVA indicated significant N fertilization effects (P <0.10). The relationships between stress ratio and soil chemical properties were investigated using Pearson correlation (PROC CORR procedure).

Results

Microbial Community Structure

Soil microbial community structure was differentially affected by N fertilizer treatments under alluvial and peat soils. There was no difference among community structures based on the various N fertilizer treatments in the alluvial soil (Fig. 35A). In the peat soil, however, microbial community structure was different under the *Azolla*-M treatment than the other N fertilizer treatments, with the PCA analysis explaining 55.6% variance along PC 1 and 2 (Fig. 35B).

Ester-linked FAMEs with the highest positive eigenvector coefficients for PC 1 in the alluvial and peat soils were 10Me17:0 (actinomycetes) and 18:1ω8 (Gram-negative bacteria), respectively. Conversely, EL-FAMEs with the most negative eigenvector coefficients for PC 1

for both soil types were 16:1ω7c (Gram-negative bacteria) and i15:0 (Gram-positive bacteria). Along PC 2, Gram-positive bacteria (i14:0) and general bacterial (13:0) EL-FAMEs had the greatest eigenvector coefficients in the alluvial and peat soils, respectively. In contrast, EL-FAMES with the most negative eigenvector coefficients for PC 2 in both the alluvial and peat soils were biomarkers associated with AM fungi (16:1ω5c) and Gram-negative bacteria (17:1ω7) (Table 19).

Pairwise comparisons among the N fertilizer treatments by blocked multi-response permutation procedures (MRBP) analysis in PC-ORD showed that in the peat soil, the *Azolla* treatment with the same N rate as manure resulted in a soil microbial community structure significantly different from communities under the other N treatments. In contrast, microbial communities from the control, urea, manure, and *Azolla*-U treatments were not significantly different. There were no significant differences in microbial communities among the N fertilizer treatments in the alluvial soil.

Microbial Community Groups Under N Fertilizer Treatments on Alluvial and Peat Soils

In general, total microbial biomass was greater in the alluvial soil than in the peat soil regardless of the N treatments (Fig. 36). The total microbial biomass in the alluvial soil ranged from 314 to 437 nmol EL-FAMEs g^{-1} soil (Fig. 35); in contrast, microbial biomass was only about half this amount in the peat soil (163 to 259 nmol g^{-1}) (Fig. 36). *Azolla* applied at the manure N rate resulted in a 38.6% increase in total microbial biomass compared to microbial biomass in control and urea-amended alluvial soil. Furthermore, treatments of manure and *Azolla* applied at the urea N rate resulted in 16% and 14% greater total microbial biomass compared to control and urea-amended soil, respectively (Fig. 36). There was no effect of N fertilizer

treatment on the total biomass in the peat soil, although total microbial biomass tended to be lower in control soil compared to fertilizer-treated soil (Fig. 36).

Total bacteria did not show any response to N fertilizer on either soil type (data not shown). In the alluvial soil, there was no significant effect of N fertilizer treatments on Gram-positive bacterial biomass (data was not shown). However, in the peat soil, biomass of Gram-positive bacteria was 72% greater in the urea-treated soil than in the control soil, but it was not significantly different from control in the *Azolla*-U, *Azolla*-M, and manure treatments (Fig. 37). Nitrogen fertilizer treatments significantly affected biomass of Gram-negative bacterial biomass in the alluvial soil, where *Azolla* applied at the manure N rate increased Gram-negative bacterial biomass by 70% compared to control (Fig. 38). There was no effect of N fertilizers on Gram-negative bacteria in the peat soil (data not shown).

There was no N fertilizer effect on actinomycetes in the alluvial soil (data not shown). Biomass of actinomycetes exhibited the same pattern of fertilizer effects as did Gram-positive bacteria in the peat soil. Urea and manure treatments increased actinomycetes biomass by 102 and 68%, respectively, compared to control (Fig. 39). Although not statistically significant, *Azolla*-U and *Azolla*-M treatments tended to increase actinomycetes biomass by an average of 59% and 30%, respectively, compared to the control.

There were contrasting effects of N fertilizers on fungi and AM fungi in the two soil types. In the alluvial soil, N fertilizers significantly affected AM fungi (Fig. 40), whereas total fungi (not AM fungi) were affected by N fertilizer treatments in the peat soil (Fig. 41). In the alluvial soil, AM fungal biomass was lowest in urea-treated soil and greatest in *Azolla*-M soil (Fig. 40). In the peat soil, *Azolla*-M increased total fungal biomass by 97%, compared to the control (Fig. 41).

Neither the ratio of bacterial:fungal EL-FAMEs, nor the ratio of Gram-positive bacterial:Gram-negative bacterial EL-FAMEs, were affected by N fertilizers in the alluvial soil (data not shown). Both ratios, however, were affected by N fertilizers in the peat soil (Fig. 42). The bacterial:fungal ratio was lowest in soil amended with *Azolla*-M (20) and greatest in urea-treated soil (37) (Fig. 42). This result corresponds to the increase in fungal biomass in *Azolla*-M-amended peat soil (Fig. 41). The ratio of Gram-positive:Gram-negative bacteria followed a similar trend, with the lowest ratio under *Azolla*-M (0.9) and the greatest ratio under the urea treatment (1.3) and the control (1.3) (Fig. 41). This corresponds to increased Gram-positive bacterial biomass in urea-treated peat soil (Fig. 37).

The ratio of 17:0 cy to its precursor 16:1ω7c, Stress 1, was not affected by N fertilization in either the alluvial or peat soil (data not shown). However, the ratio of 19:0c cy-to-18:1 ω7c (Stress 2) was affected by N fertilization in the peat soil (Fig. 43). Specifically, the ratio was lowest in *Azolla*-M soil and greatest in control soil and soil treated with urea.

Discussion

Structure of Soil Microbial Community

In this study, microbial community structure was sensitive to different N fertilizers in an organic peat soil cropped to red spinach. In particular, *Azolla*-M resulted in a shift in the microbial community towards greater relative abundance of several Gram-negative bacterial EL-FAMEs (18:1ω8 and 18:1ω9c), lower ratio of Gram-positive:Gram-negative bacterial EL-FAMEs, lower ratio of Stress 2, and greater fungal biomass.

Azolla consisted of higher organic C content (45–48%) with a C/N ratio of 16.1–16.4, compared to the other organic N fertilizer (chicken manure) that contained 32.25% organic C with C/N of 10.11 (Table 8). In addition, the peat soil had greater organic C content (50.3%) with C/N ratio of 22.0. Whereas, the alluvial soil contained 2.15% organic C with C/N of 8.6 (Table 7). SOC and C/N ratio in soils were not affected by N fertilizer treatments. SOC ranged from 1.7 (*Azolla*-U) to 2.0 (manure) in the alluvial soil and 51.2 (urea) to 51.7 (*Azolla*-U) in the peat soil. Furthermore, the alluvial soil had C/N ratios from 7.8 (control) to 8.1 (manure), whereas, the peat soil contained C/N ratios of 22.5 (control) to 22.8 (*Azolla*-M).

Organic fertilizer and manure amendments significantly increased microbial biomass C and stimulated higher microbial activities, compared to inorganic N fertilizers (50 and 100% of recommended N rate) (Chaudhary et al., 2015). Furthermore, Chaudhary et al. (2015) discovered that organic fertilizers including manure caused substantial enhancement in the diversity and activity of soil microbes. This *Azolla* study suggests that organic N influenced the microbial community structure, in particular, *Azolla* biofertilizer on the peat soil.

Based on pairwise comparison across the N fertilizer treatments, the *Azolla* applied at the manure N rate (*Azolla*-M) contained the significantly greatest concentrations of EL-FAMEs in both soils. In the peat soil, the urea treatment also had greater biomass of EL-FAMEs; whereas, the rest of the N fertilizer treatments did not present any significantly different EL-FAMEs biomass in either soil type.

N Fertilizer Treatments Shaped Microbial Community Groups on Alluvial and Peat Soils

Overall, total microbial biomass and biomass of soil bacteria were greater in alluvial soil than in peat soil (Fig. 36). This finding is in agreement with Girvan et al. (2003) who found that total and active bacterial communities in arable soils were mainly influenced by soil type.

Azolla applied at the manure N rate had a significant effect on increasing total soil microbial biomass in the alluvial soil (Fig. 36). This result is in agreement with Gopalaswamy and Kannaiyan (2000a, 2000b); Kannaiyan and Subramani (1992); Krishnakumar et al. (2005); and Yadav et al. (2014) who reported that *Azolla* application in paddy soil combined with other kinds of organic fertilizer such as farmyard manure, neem cake, or *Sesbania* increased the microbial population, compared to recommended NPK fertilizer or control. In addition to *Azolla,* long-term organic fertilizer amendment, in particular farmyard manure, has been shown to contribute to microbial biomass, domain-specific biomass, microbial community structure and diversity under winter wheat crop rotation plots (Esperschütz et al., 2007). Greater total PLFA microbial biomass and bacterial PLFA communities (Gram-positive and Gram-negative bacteria, and actinomycetes) were discovered under organic fertilizers (*Jatropha* cake and farmyard manure) and 50% N rate of urea applications compared to 100% urea N rate in a tropical dry climate (Chaudhary et al., 2015).

Total microbial biomass under the peat soil was only about half of the total microbial biomass in the alluvial soil (Fig. 36). Although there were no differences across the N treatments, there was an indication that the enhancement of total soil biomass in high-organic carbon soil, as seen in the peat soil, is shaped by N fertilizers (Lin et al., 2014). The N treatments (*Azolla*-M, *Azolla*-U, manure, and urea) resulted in total microbial biomass ranging from 242–259 nmol g^{-1} compared to no N fertilizer (163 nmol g^{-1}) in the peat soil. N fertilization can affect microbial

enzyme activity (Carreiro et al., 2000; Sinsabaugh et al., 2002; Frey et al., 2004) in addition to soil microbial community composition (Bardgett et al., 1999; Lundquist et al., 1999).

According to Lv et al. (2017), total microbial biomass (PLFA) content increased with N application, particularly at the highest urea rate (12 g N m^{-2} yr^{-1} N rate). The model developed by Lv et al. (2017) showed that N addition strongly influenced total biomass by indirect effects on plant and soil properties. Furthermore, Chang et al. (2014) found that N treatments using compost (140 kg N ha^{-1}) and compost (140 kg N ha^{-1}) + 1/3 N (47 kg N ha^{-1} as urea) obtained the significantly highest total PLFA. Similarly, N fertilization increased total PLFA by 30, 12.9, and 6.8%, respectively, in the N fertilizer treatments of LNE (low doses of N-fertilizer + *P. liquidambari*), ON (high doses of N-fertilizer), and ONE (high doses of N-fertilizer + *P. liquidambari*), compared to control (unfertilized N) (Siddikee et al., 2016). On the contrary, Wu et al. (2013) found that the greatest total PLFA concentration occurred in the 12 g N m^{-2} yr^{-1} rate, and it was not significantly different from the lower N fertilizer treatment or control (6 and 0 g N m^{-2} yr^{-1}).

The microbial population (including total bacteria and cellulolytic, phosphate solubilizing and urea hydrolyzing bacteria, N_2 fixing *Azospirillum*, *Azotobacter*, fungi, and actinomycetes) and soil enzymes activities (L-asparaginase, urease, cellulose, dehydrogenase, and phosphatase) were significantly enhanced under *Azolla* incorporation in paddy soil over prilled urea (Gopalaswamy and Kannaiyan, 2000a, 2000b; Kannaiyan and Subramani, 1992; Thanikachalam et al., 1984) and in organic farming systems compared to conventional farming and the other organic fertilizers utilization (Krishnakumar et al., 2005). *Azolla* biomass decomposition has been shown to release humic substances that can improve soil fertility (Bhardwaj and Gaur, 1970). Yadav et al. (2014) asserted that biological activity and the micro

flora were built up due to soil nutrient availability from *Azolla* application, even after a short period of time for *Azolla* mineralization (Singh, 1977). Yadav et al. (2014) summarized that *Azolla* utilization improved rice yields and soil biological health. Thereby, in order to sustain crop productivity, ecological and soil conditions should be enhanced by taking into consideration the utilization of organic, inorganic, and biological inputs in an integrated manner (Yadav et al., 2014).

The ratio of bacteria:fungi revealed that the higher N rate treatments increased fungal biomass, relative to bacteria in peat soil (Fig. 42). The higher N rate of the *Azolla*-M treatment (108 kg N ha^{-1}) tended to result in smaller ratio of bacteria:fungi compared to urea (23 kg N ha^{-1}), i.e. 20 and 37, respectively. In contrast, urea and *Azolla*-U treatments at a lower N rate (23 kg ha^{-1}) compared to manure and *Azolla*-M, exhibited a greater ratio of bacteria to fungi, i.e. 37 and 34, respectively. Thus, the higher fertilizer N rate tended to enhance fungal biomass in peat soil, although there was no pattern of higher soil total N with the higher N addition from fertilizer application.

This result was in agreement with Lv et al., (2017) who found that fungal PLFA was increased by N addition, but then at higher N rates it tended to decrease. Similarly, Chang et al. (2014) evaluated long-term treatments (12 consecutive years) of urea and several kinds of organic fertilizer complemented with urea and found that compost (140 kg N ha^{-1}) + 1/3 N (47 kg N ha^{-1} as urea) and green manure (140 kg N ha^{-1}) + 1/3 N (47 kg N ha^{-1} as urea) obtained a greater fungal PLFA concentration, compared to compost (140 kg N ha^{-1}) or urea (140 kg N ha^{-1}) treatment alone. In addition, the soil microbial community composition may be influenced by the quality and quantity of substrate (Swift et al., 1998). In the higher substrate

supply, such as in the *Azolla*-M treatment, fungal community will be dominant over the bacterial community.

The result of this study was somewhat contrary to those of Bradley et al. (2006) for a grassland soil. They found that long-term N fertilizer applications at low or high rates increased bacterial FAMEs and reduced fungal FAMEs. The study site was established to assess plant species diversity, productivity, and dynamics as influenced by the 18-year long-term impacts of N deposition (Tilman, 1987). In addition, another study also concluded that fertilized N fields contained a smaller fungal component compared to unfertilized N fields (Bardgett and Shine, 1999). Högberg et al. (2003) and Myers et al. (2001) correspondingly revealed that intensified N availability and higher rates of N cycling were connected to a bacterial dominated community in the soil following by a reduced portion in the fungal community. In a short-term study, 40 days after transplanting 30-day old rice seedlings, the lower rate of inorganic N fertilizer + *P. liquidambari* increased fungal PLFA concentration in rhizosphere soil; in contrast, the higher doses of N fertilizer + *P. liquidambari* or high doses of inorganic N fertilizer alone had significantly lower fungal PLFA concentrations (Siddikee et al., 2016).

The positive response of fungi to *Azolla*-M in peat soil (Fig. 41) may be due to lower biomass of bacterial populations in the *Azolla* treatment applied at a relatively high N fertilizer rate (Figs. 36, 38, and 41). The ratio of SOC to total soil N in *Azolla*-M, manure, *Azolla*-U, urea, and control treatments were similar, i.e. 22.5 to 22.8. Yet, the mineralizable N in the form of ammonium was higher in chicken manure than in the *Azolla*, i.e. 0.33 and 0.25% NH_4^+-N, respectively; whereas, nitrate was present at the same concentration in both organic fertilizers (0.20% NO_3^--N) (Table 8). The bacterial and fungal communities in this study are similar to bacterial communities across long-term N fertilization gradients in two ecosystems, i.e. 27-year

N addition in a grassland and 8-year N addition in an agricultural field (Ramirez et al., 2010). The bacterial community structure was shaped by N and/or soil C availability that was related to the higher N rate (Ramirez et al., 2010).

Other studies have confirmed that N addition can enhance the fungal PLFAs in upper-elevation tropical forests (Cusack et al., 2011). In winter wheat that was grown under two different crop rotations (after potatoes and after maize), organic practices tended to result in the greatest fungal biomass, although the contribution of fungi to distinguish the community structure based on the management practice was relatively low (Esperschütz et al., 2007). Additionally, Chaudhary et al. (2015) found that soil amended with *Jatropha* cake enhanced the fungal PLFA community, compared to farmyard manure and inorganic N fertilizer (urea). Similar findings from another study also discovered that fungal community was increased in soil amended with organic fertilizer or biofertilizer (Smith et al., 2003). Early after farmyard manure application, bacterial PLFA biomass was decreased, but biomass was amplified later, and inorganic fertilizer diminished fungal PLFA biomass in a meadow grassland ecosystem (Smith et al., 2003).

AM fungi biomarker was indicated by the EL-FAMEs of 16:1ω5c which is a primary lipid element of AM fungi (Graham et al., 1995). That AM fungi biomarker has been utilized for soils and colonized roots (Olsson et al., 1995; Drijber et al., 2000; Larkin, 2003; Sullivan et al., 2006). The lowest biomass of AM fungi community that was assigned to El-FAMEs biomarker of 16:1ω5c was found in the urea treatment in the alluvial soil (Fig. 39**).** Whereas, the greatest biomass of AM fungi was detected in the *Azolla* treatment applied at the manure N rate. This was consistent with the result that AM fungi was found in greater amounts under grass plots because of the higher root biomass of grass plots (Stromberger et al., 2007). The *Azolla* treatment applied

at the manure N rate was also a source of greater C (45.14–48.07% organic C) in addition to serving as a N source. This additional C caused the *Azolla*-M treatment to presumably increase AM fungal biomass. While in the peat soil, fungi community from biomarker 18:2ω6,9c (Stromberger et al., 2007) was found in consistently greater amounts in *Azolla*-M treatment that had higher organic C. The result of this study revealed that fungal communities in the alluvial and peat soils were influenced by higher C and N content, as shown in the *Azolla*-M treatment.

A higher abundance of Gram-positive bacteria was found in the alluvial soil (data was not shown). Both the alluvial and peat soils were classified as acidic soils that contained soil pH of 4.6 to 5.3 (alluvial) and 4.5 to 4.8 (peat). Gram-positive bacteria have some mechanisms to tolerate acidic environments (Cotter and Hill, 2003). The 70.3–86.2 nmol g^{-1} Gram-positive bacteria was better adapted to the alluvial soil, despite the fact that there was no N treatment effect on that kind of bacteria. Although there was no N fertilizer effect on Gram-positive:Gram-negative bacteria in the peat soil, organic N fertilizer had a lower ratio compared to control and urea (Fig. 41). Similarly, Chang et al. (2014) reported a decreased Gram-positive:Gram-negative ratio due to organic matter application. The lowest Gram-positive to Gram-negative bacteria ratio occurred in green manure (140 kg N ha^{-1}) + 1/3 N urea (47 kg N ha^{-1}) and peat (140 kg N ha^{-1}) + 1/3 N urea (47 kg N ha^{-1}); whereas, significantly higher ratios were observed in control (without fertilizer) and chemical N fertilizer treatments. Additionally, control + *P. liquidambari* and low rate of synthetic N fertilizer + *P. liquidambari* treatments displayed the higher G+/G– ratio (Siddikee et al., 2016). The quality of organic amendment may alter the Gram-positive:Gram-negative ratio (Bastida et al., 2008). A higher ratio of Gram-positive:Gram-negative bacteria signified a modification from copiotrophic (higher nutritional intensity) to

more oligotrophic (low levels of nutrients) circumstances in soil (Borga et al., 1994; Yao et al., 2000; Koch, 2001; Fanin et al., 2014).

In contrast, N fertilizer treatment influenced the abundance of Gram-positive bacteria in the peat soil compared to no N fertilizer. There were no differences across organic and inorganic N fertilizers, despite the fact that urea increased Gram-positive bacteria compared to control (Fig. 37). Significantly higher Gram-negative bacteria was found under the *Azolla*-M fertilizer treatment compared to control; there were no differences among other N fertilizer treatments in the alluvial soil (Fig. 38). These results do not corroborate the Islam et al. (2009) study that found compost enhanced Gram-positive FAME profiles in long-term fertilization of a rice-based ecosystem. Another long-term fertilization study utilizing organic and mineral fertilizers also disclosed that microbial community structure was driven by soil pH and total organic C as the major drivers, while N and P fertilizers had minor influence (Francioli et al., 2016). Short-term utilization of *Azolla* as evaluated in this study may not have shown any considerable effect on Gram-positive or Gram-negative bacteria communities.

Understanding soil microbial community structure is important because microbial communities are sensitive to rapid changes in soil habitats in response to management and environment (Kennedy and Stubbs, 2006). Indeed, the alteration of microbial community structure could be an indicator whether an agricultural management practice, in particular N fertilization, has a positive or a negative impact on soil quality or soil health. This is due to the significant role of the soil microbial community in nutrient cycling, residue decomposition or soil organic matter dynamics, soil structure, plant growth, and plant health (Kennedy and Stubbs, 2006).

Greater total microbial biomass is usually interpreted as a positive indicator of a healthy soil. Furthermore, changes in the ratio of bacteria:fungi implies the transformation of soil microbial community composition due to the higher N fertilizer rate (Frostegård and Bååth, 1996). This result is not really a direct indicator of whether the management practice (N fertilization) has a positive or a negative effect on overall soil health, since there are some beneficial and potentially harmful effects of soil microorganisms. The positive influences of soil microbes (bacteria and fungi) are increasing mineral solubilization (Okon, 1982) and N_2-fixation (Albrecht et al., 1981), supplying hormones (Brown, 1972) and antibiotics, and controlling pathogens (Bruehl, 1987); while the harmful effects include plant diseases, production of plant-suppressive compounds, and loss of plant-available nutrients (Bruehl, 1987). This study investigates the soil microbial community structure; it does not evaluate the soil microbial diversity. Nevertheless, the greater AM fungi biomass is a strong marker of healthy soil and healthy plants, due to the prominent role of AM fungi in translocation of nutrients primarily soil P and enhancement of nutrient and water uptake (Ocampo, 1986; Tinker, 1976). A greater biomass of actinomycetes may increase plant materials (cellulose) decomposition and nutrient mineralization, and also release antibiotics (Kennedy and Stubbs, 2006). Unlike Gram-negative bacteria that are sensitive to drought and water stress, Gram-positive bacteria have a tendency to survive under water stress (Dick, 2009). Nevertheless, the distinguishing outer membrane of Gram-negative bacteria that contains phospholipids can make Gram-negative bacteria more hydrophobic and can develop further strategies to protect from unfavorable environments such as flooding (van Elsas et al., 2007). *Rhizobium,* a species of Gram-negative bacteria, forms N nodules in leguminous plant hosts to fix N for plant growth (Hoorman, 2016). In general,

Hoorman (2016) stated that plant communities are benefited by bacteria that can modify the soil environment when soil conditions change.

The higher the ratio of biomarkers 17:0 cy to 16:1ω7c (stress 1) or 19:0 cy to 18:1ω7c (stress 2) the greater the stress that the bacterial communities had to confront. These ratios were indicators of bacterial stress since the cyclopropyl fatty acids were detected under stress conditions, such as nutrient and oxygen reduction, low pH, and dehydration (Guckert et al., 1986; Petersen and Klug, 1994; Kieft et al., 1994; Grogan and Cronan, 1997; Bossio and Scow, 1998; Chang and Cronan, 1999; Mazumder et al., 2000; Boumahdi et al., 2001). Furthermore, it was found that the stress indicators were associated with more severe environmental conditions including high soil temperature (Petersen et al., 2002). This study revealed that urea and control treatments had more stress ratio 2 than manure and *Azolla* treatments under the peat soil (Fig. 43). The increased ratios of stress that occurred on Gram-negative bacteria demonstrated intensified environmental stress due to fertilizer application (Guckert et al., 1986; Heipieper et al., 1996; Kaur et al., 2005). In addition, C starvation in urea and control treatments may potentially increase stress ratio 2. There was no significant correlation (P= 0.38) between soil organic C and stress ratio 2 in the peat soil. However, soil C/N ratio and stress ratio 2 were negatively correlated (r= –0.54, P <0.10), providing some evidence that a lower soil C concentration (lower C/N ratio) may contribute to a higher stress ratio 2. According to Kjelleberg et al. (1993), C starvation promotes the enhancement of a starvation and stress resistant cell in bacteria. Carbon starvation can also hinder growth and cause physiological alteration of bacterial cells (Brauer et al., 2006; Klančnik et al., 2008). This study suggests that organic fertilizers (manure, *Azolla*-U, and *Azolla*-M treatments) reduced stress in the soil

microbial community. Furthermore, soil microbial communities in the peat soil were more vulnerable to stress.

The significantly highest bacterial stress 2 index (19:0 cy to 18:1ω7c) was obtained in the urea treatment (Fig. 43). Chang et al. (2014) reported on a long-term study that was established for 12 consecutive years in Fluvaquentic Dystroochrepts (alluvial soil). The results showed that green manure (140 kg N ha^{-1} for corn and 120 kg N ha^{-1} for rice) + 1/3 N (47 kg N ha^{-1} urea for corn and 40 kg N ha^{-1} ammonium sulfate for rice) contributed to the significantly lowest stress 2 ratio (19:0 cy to 18:1ω7c). Whereas, the other N treatments, i.e. synthetic N fertilizer (urea or ammonium sulfate), organic N fertilizer (compost or peat), or control (no fertilizer) that was applied in combination or the single fertilizer alone resulted in a higher ratio of bacterial stress 2. Furthermore, Chang et al. (2014) presented the significantly highest ratio of bacterial stress 1 (17:0 cy to 16:1ω7c) was obtained in the peat soil (applied as organic fertilizer with N rate of 140 kg N ha^{-1} for corn and 120 kg N ha^{-1} for rice) plus receiving 1/3 N (47 kg N ha^{-1} urea for corn and 40 kg N ha^{-1} ammonium sulfate for rice). Wu et a al. (2013) found that the higher rate of urea (24 g N m^{-2} $year^{-1}$) induced the highest stress 1 index in both 0–20 cm and 20–40 cm soil depth, compared to the lower N urea rate (6–12 g N m^{-2} $year^{-1}$) and control (no N urea).

Conclusions

EL-FAMEs profiles were studied to characterize soil microbial community composition, biomass, and stress response indicators in tropical alluvial and peat soils under vegetable production. By examining changes in microbial communities, the effect of N inputs could be tested in this study under two soil types.

Based on PCA analysis, the *Azolla* treatments, in particular *Azolla*-M and some *Azolla*-U, presented a significant effect on shifting the microbial community structure under peat soil. Total EL-FAMEs microbial biomass, Gram-negative bacteria, and AM fungi increased in alluvial soil with *Azolla*-M**;** whereas in the peat soil, *Azolla*-M also shifted the microbial community towards greater Gram-negative EL-FAMEs, a lower ratio of Gram-positive:Gram-negative bacteria, and greater fungal biomass.

This study indicated that bacterial and fungal communities across soil types were also associated with organic C in the N fertilizer treatments. *Azolla* applied at the manure N rate (*Azolla*-M) tended to increase fungal community. N fertilizer played a significant role in Gram-positive bacteria composition in the peat soil, whereas in the alluvial soil, N fertilizer had a greater effect on Gram-negative bacteria.

Urea fertilizer increased the amount of Gram-positive bacteria and actinomycetes in addition to the ratio of bacteria to fungi in the peat soil. Urea and no N fertilizer had higher ratios of stress biomarkers, and the peat soil had a higher ratio of stress indicator than the alluvial soil. However, the *Azolla* applied at the manure N rate could reduce the stress that microbes encountered from unfavorable environmental conditions under vegetable agroecosystem.

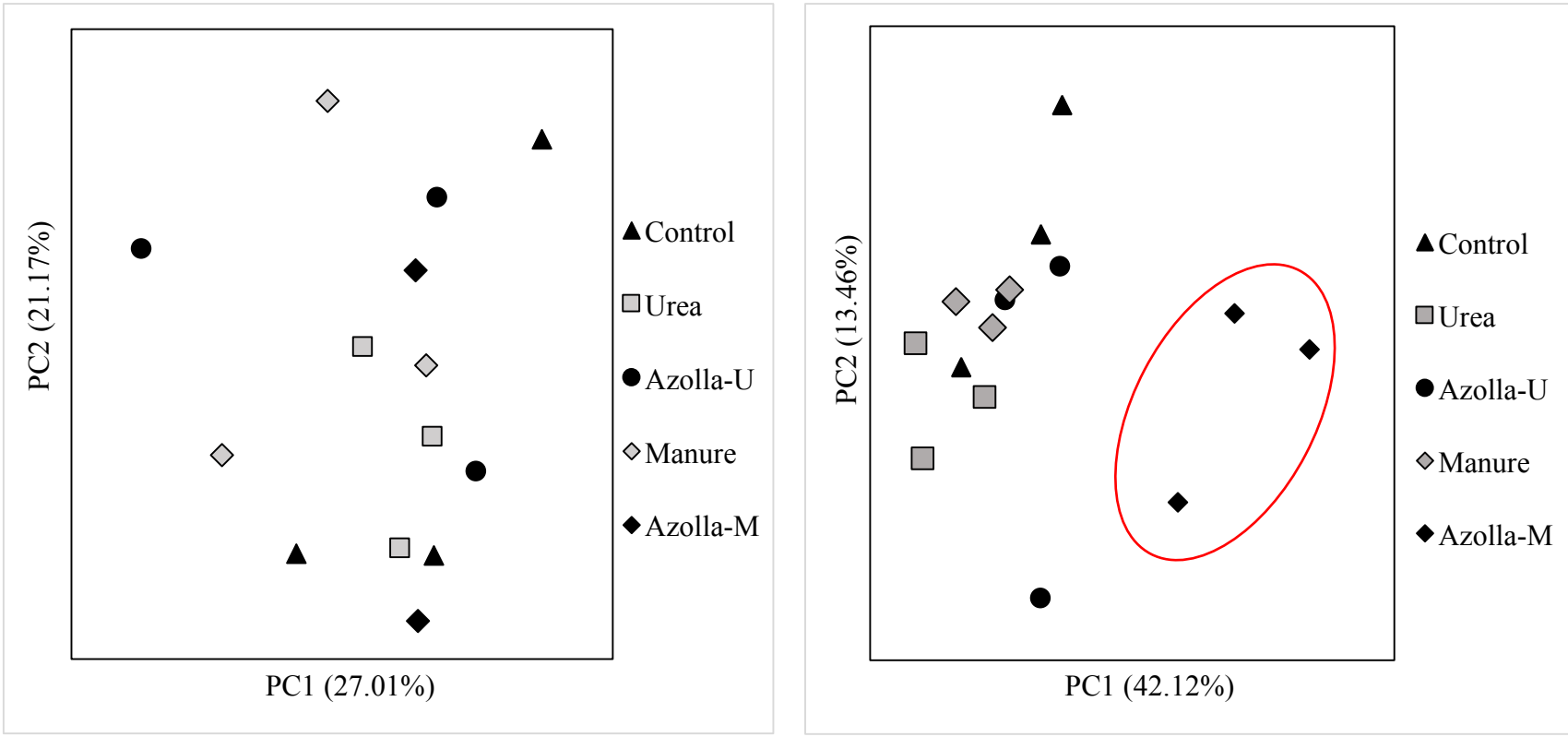
Soil microbial community plays a significant role in soil quality and structure, in addition to plant health and growth. Thus, alteration of soil microbial community structure could be an indicator of whether N fertilization has a positive or a negative impact on soil and plant health.

TABLE

Table 19. Eigenvector coefficients of microbial ester-linked fatty acid methyl esters (EL-FAMEs) for principal components (PC) axes 1 and 2, as shown in Figs. 35A and 35B.

EL-FAMEs	Alluvial		Peat	
	PC 1	PC 2	PC 1	PC 2
12:0	–0.55	0.61	–0.59	0.59
13:0	–0.32	0.27	–0.06	0.75
i14:0	–0.34	0.85	–0.06	0.61
14:0	–0.16	0.79	–0.60	0.62
i15:0	–0.18	0.53	–0.93	–0.06
a15:0	–0.63	0.25	–0.58	–0.22
15:0	0.66	0.68	–0.77	0.34
i16:0	0.77	0.29	–0.67	–0.14
16:1ω7c	–0.78	–0.04	0.09	–0.06
16:1ω5c	–0.06	–0.75	0.48	0.53
16:0	0.45	–0.41	0.89	0.18
17:1ω7	–0.76	0.19	–0.11	–0.64
10Me16:0	0.55	0.16	–0.53	–0.04
i17:0	0.77	0.43	–0.84	–0.19
a17:0	–0.64	0.58	–0.76	–0.34
17:0 cyclo	–0.53	0.42	–0.29	–0.01
17:0	–0.14	0.77	–0.55	0.47
10Me17:0	0.90	0.27	–0.90	–0.01
i18:0	0.26	0.20	-	-
18:2ω6,9c	–0.54	–0.47	0.41	0.43
18:1ω9cis	–0.31	–0.21	0.75	–0.01
18:1ω7cis	–0.20	–0.22	0.37	0.12
18:1ω8	0.37	0.34	0.92	–0.28
18:0	–0.48	–0.56	–0.89	–0.10
10Me18:0	–0.67	0.17	–0.72	0.26
i19:0	–0.42	–0.07	-	-
19:0 cyclo	–0.06	–0.26	–0.89	–0.27

FIGURES



(A) (B)

Fig. 35. Principal components analysis (PCA) of (A) alluvial and (B) peat soil microbial community ester-linked fatty acid methyl esters (EL-FAMEs) in plots receiving different N fertilizer treatments and planted to red spinach.
Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

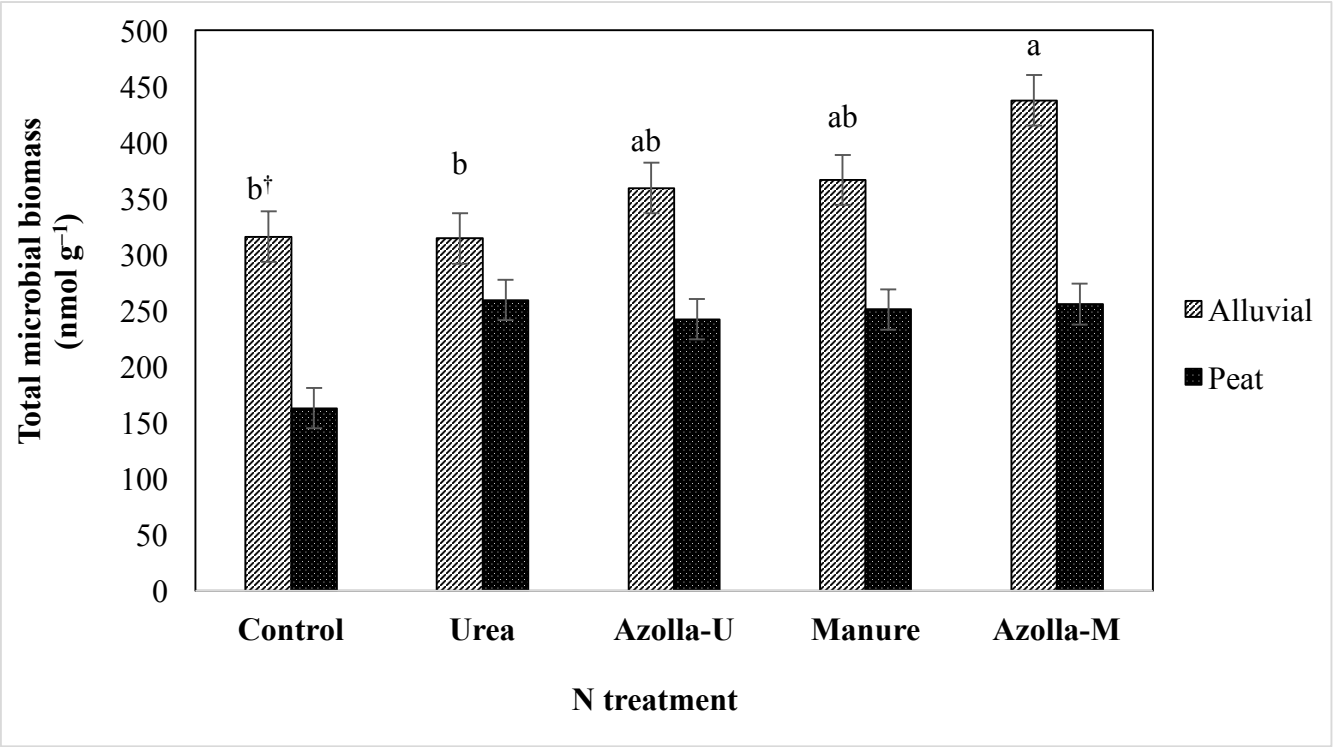


Fig. 36. Total microbial biomass by N fertilizer on alluvial soil and peat soil.
†Values followed by a different letter indicate significant difference within the same soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

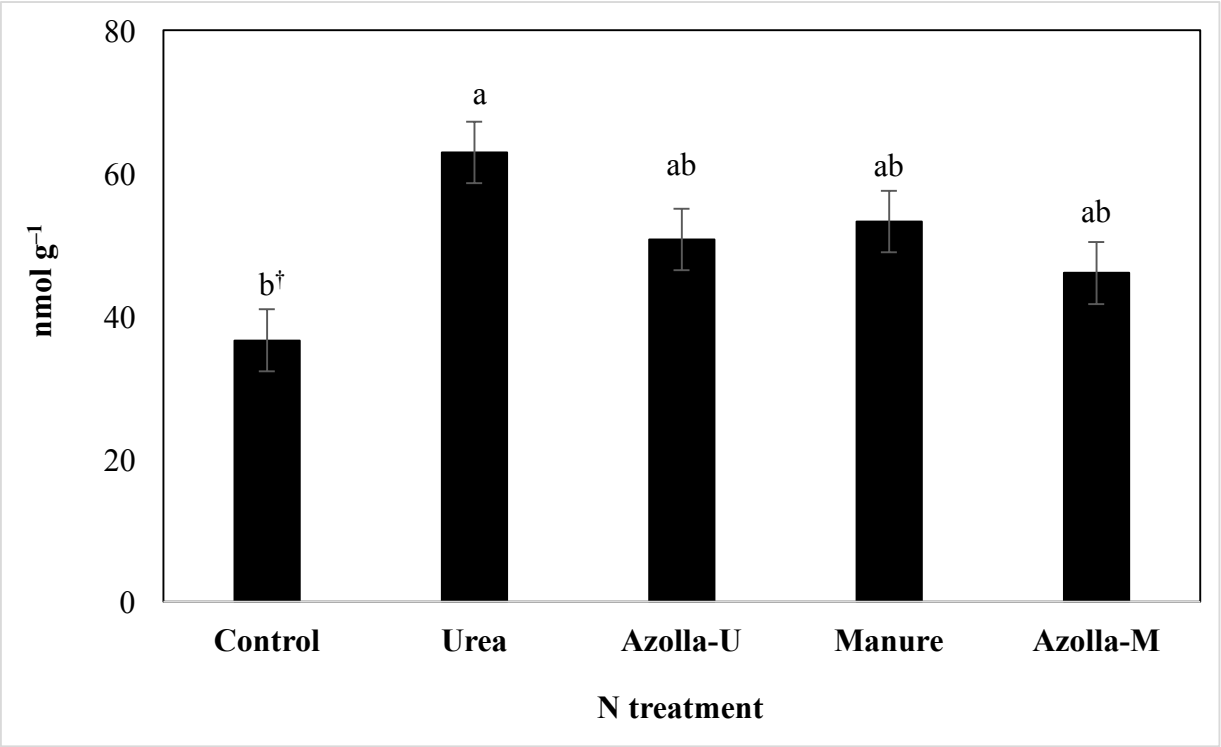


Fig. 37. Gram-positive bacteria community affected by N fertilizer on peat soil.
†Values followed by a different letter indicate significant difference within the same soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

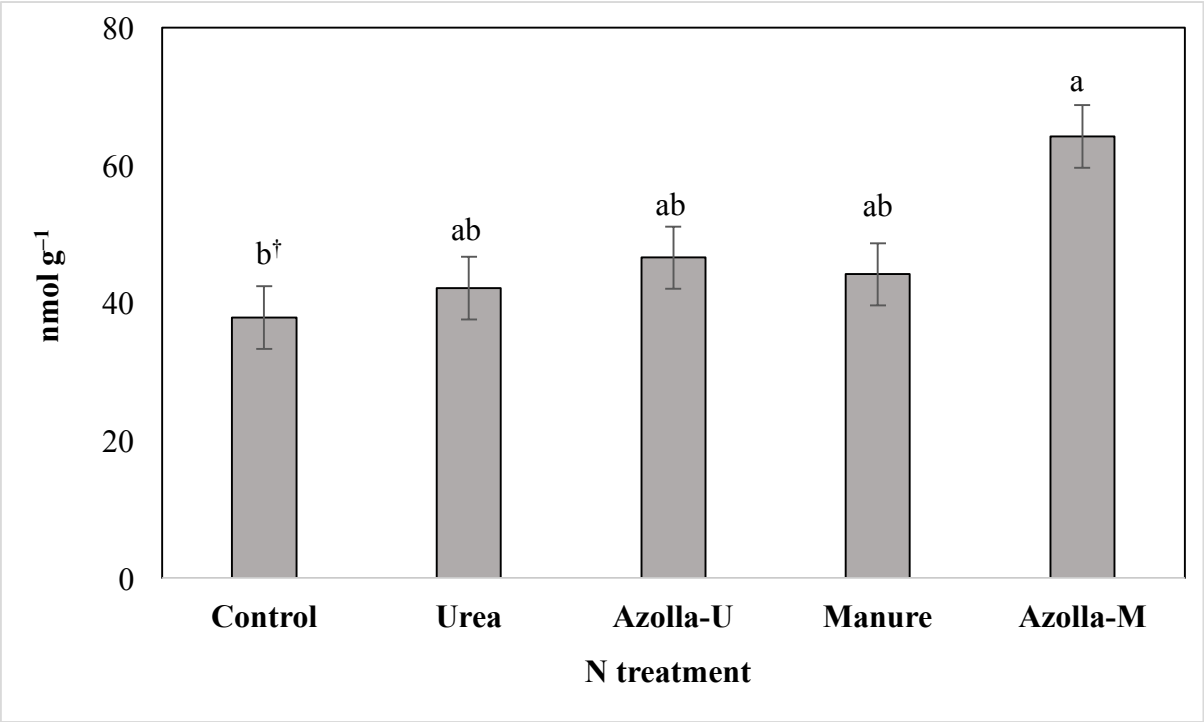


Fig. 38. Gram-negative bacteria community affected by N fertilizer on alluvial soil.
†Values followed by a different letter indicate significant difference within the same soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

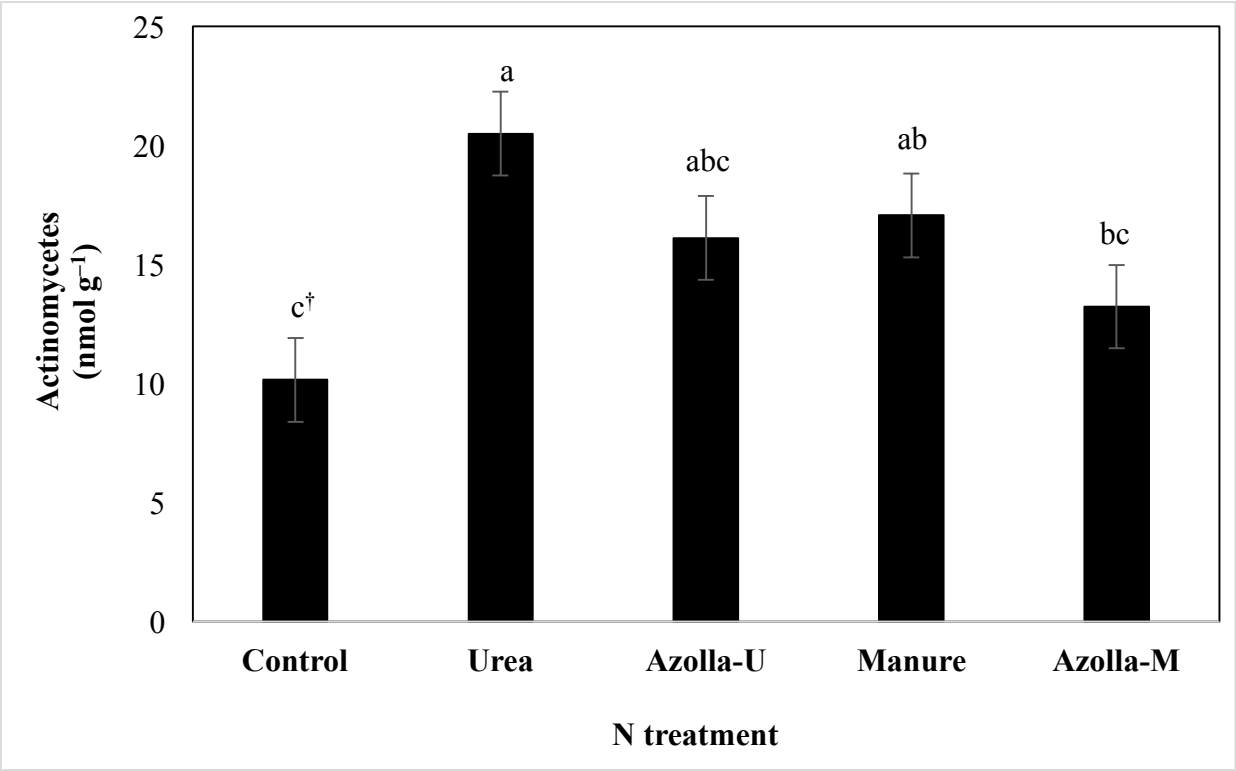


Fig. 39. Actinomycetes community affected by N fertilizer on peat soil.
†Values followed by a different letter indicate significant difference within the same soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

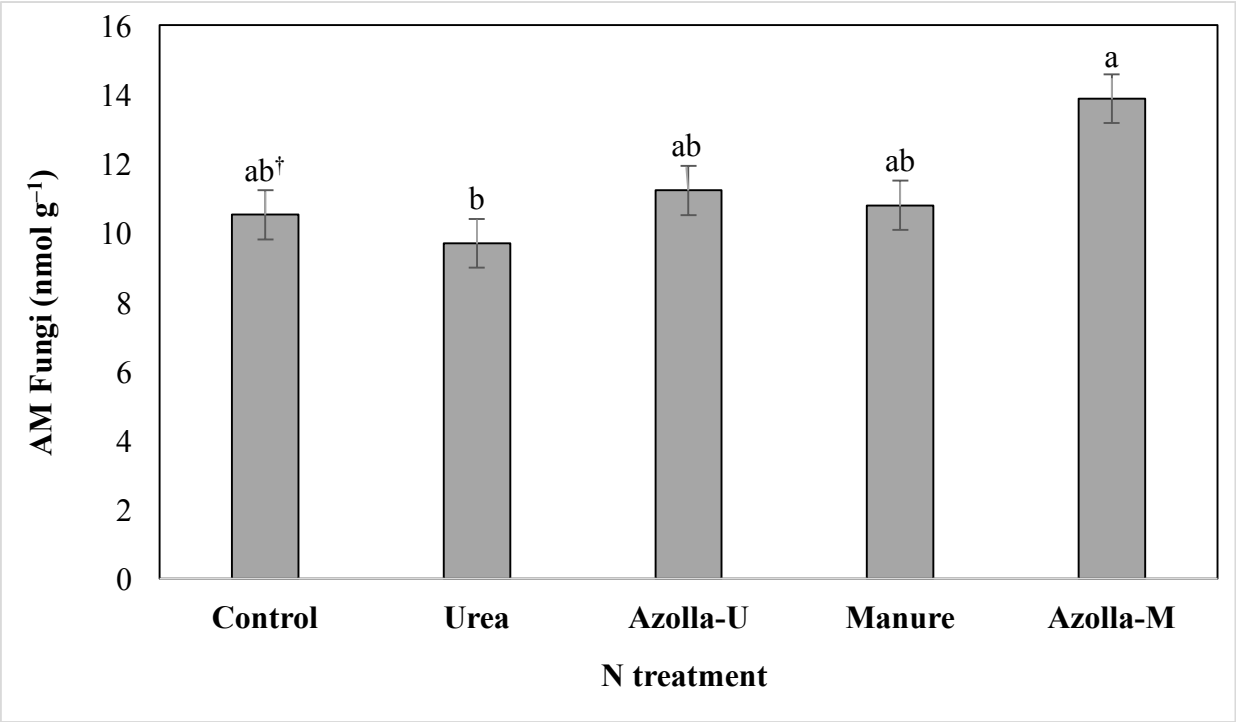


Fig. 40. Arbuscular mycorrhizae (AM) fungi community affected by N fertilizer on alluvial soil.
†Values followed by a different letter indicate significant difference within the same soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

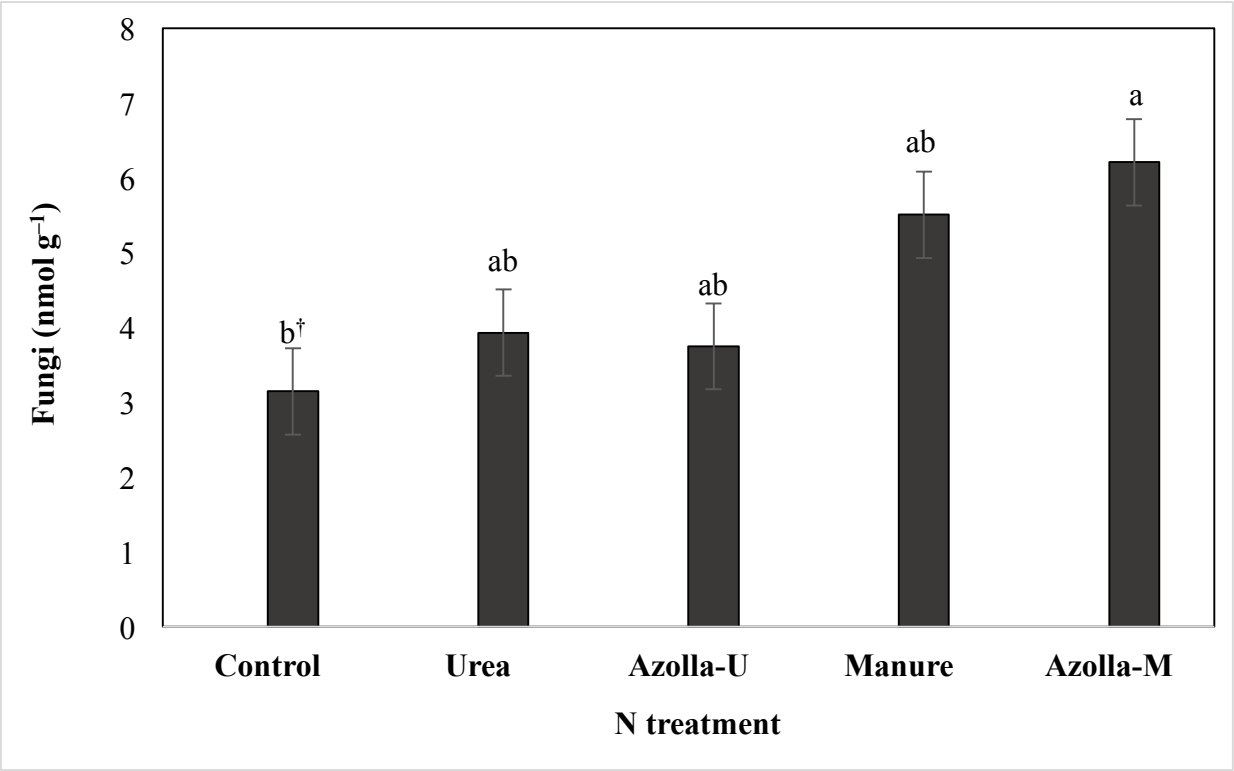


Fig. 41. Fungi community affected by N fertilizer on peat soil.
†Values followed by a different letter indicate significant difference within the same soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

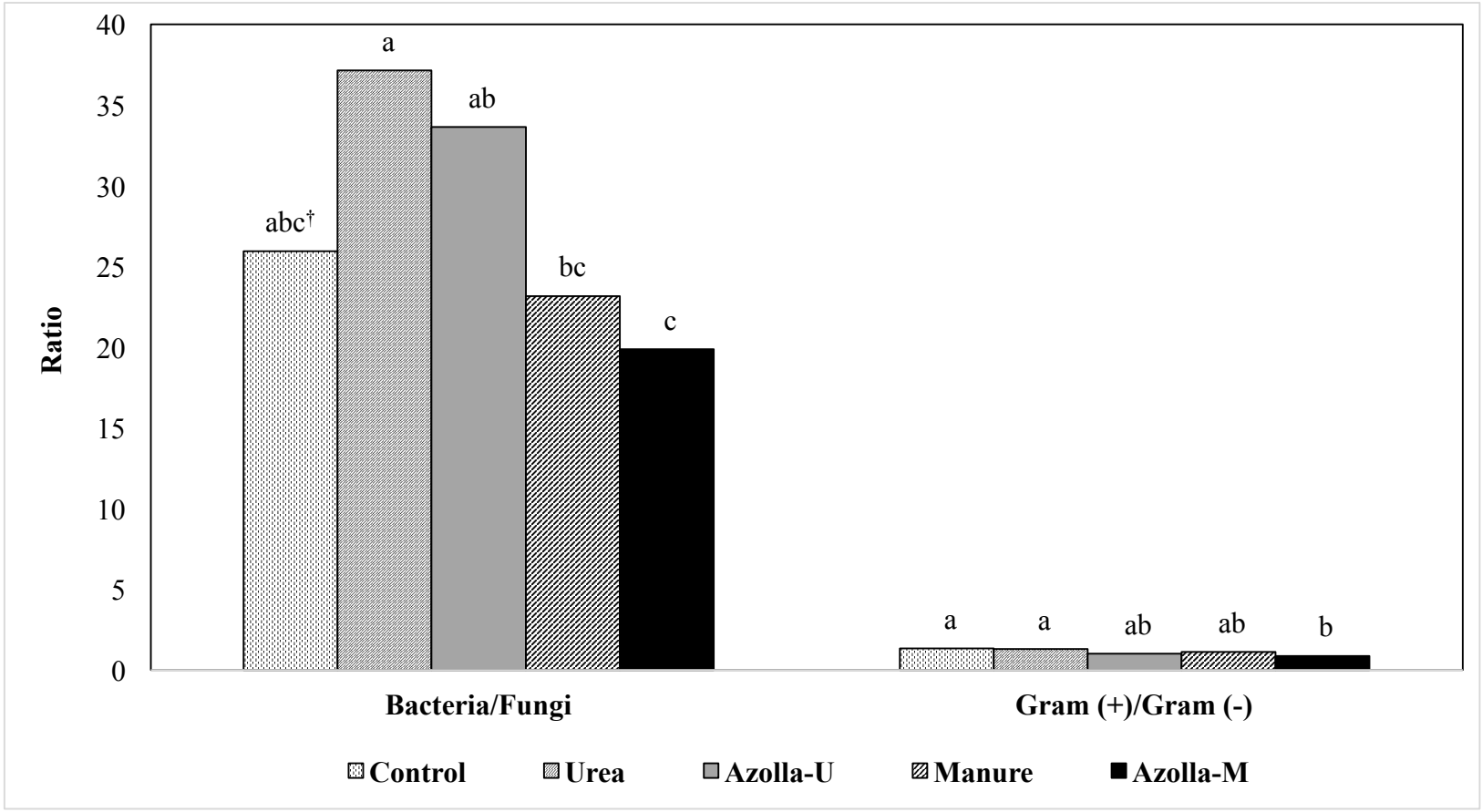


Fig. 42. Ratio of bacterial:fungal and Gram-positive:Gram-negative bacterial EL-FAMEs affected by N fertilizer treatment on peat soil.

†Values followed by a different letter indicate significant difference within the same soil based on Tukey's honest significant difference (HSD) test (P <0.10).

Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

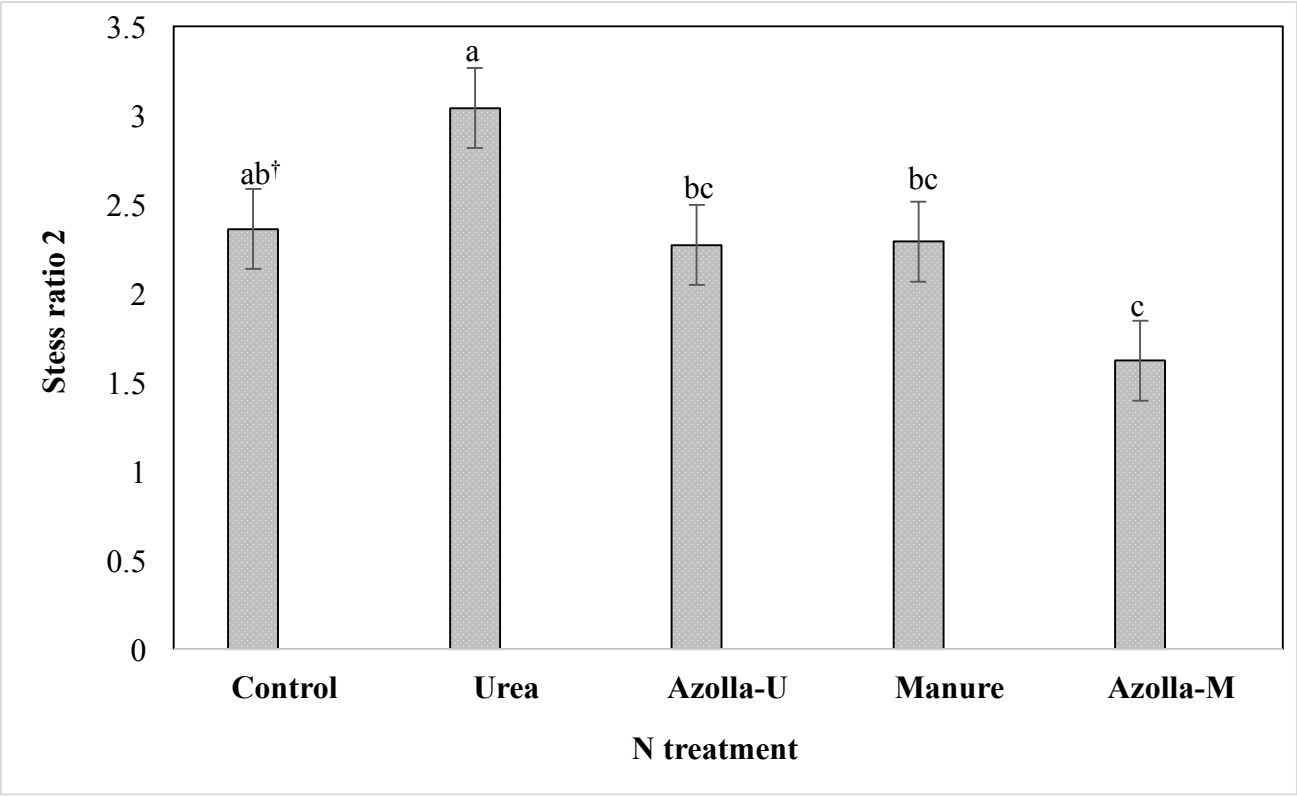


Fig. 43. Stress ratio 2 affected by N fertilizer on peat soil.
†Values followed by a different letter indicate significant difference within the same soil based on Tukey's honest significant difference (HSD) test (P <0.10).
Control: no N fertilizer, Urea, *Azolla*-U: *Azolla* applied at the urea N rate, Manure, *Azolla*-M: *Azolla* applied at the manure N rate.

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CHAPTER 6

RECOMMENDATIONS

Based on these *Azolla* growth utilization studies, there are some recommendations suggested as follows:

1. Some hypotheses about growing *Azolla* in the greenhouse were proven to be true, i.e. Zn concentrations in the *Azolla* growth solution, inoculation rates, and combined nutrient solutions improved *Azolla* growth parameters, and some nutrient concentrations in the *Azolla* growing medium affected nutrient concentrations in the *Azolla* plant tissue.
2. Inoculation rates play an important role in influencing doubling time, RGR, and *Azolla* nutrient concentrations of K, Fe, Mn, and Zn. Based on the inoculation rate and field studies, the optimum *Azolla* inoculation rate is 100 g m^{-2} for 14 day growing periods under greenhouse conditions; whereas for field nursery, the recommended inoculation rate is minimum of 200 g m^{-2}.
3. Nutrient concentrations in Azolla were affected by solution nutrient concentrations of P, K, Ca, Mg, Fe, Mo, B, and Cu.
4. The recommended nutrient solution for *Azolla mexicana* cultivation in the greenhouse is the Wd3 solution: P 10, K 20, Ca 10, Mg 10, Mn 0.375, Fe 1, Mo 0.075, B 0.15, Cu 0.01. Zn 0.01, and Co 0.01 mg L^{-1}.
5. Some hypotheses regarding *Azolla* effects on plant growth, yield, and N content were supported by the research findings.

6. *Azolla* biofertilizer applied at the manure N rate (108 kg N ha^{-1}) and manure (5 t ha^{-1} or 108 kg N ha^{-1}) can enhance vegetable yield and other agronomic components, compared to urea (application rate) as a commonly-used N fertilizer.
7. The hypotheses that *Azolla* as a biofertilizer will improve chemical properties (pH, total N, P, K, Fe, and Zn concentrations, organic C, and C/N ratio) of alluvial and peat soils and will enrich nutrient concentrations (N, P, K, Fe, and Zn) in spinach and radish plant tissues, were fulfilled by the study results.
8. Chicken manure and *Azolla* applied at the manure N rate are encouraged to be utilized in spinach and radish production due to the resulting higher nutrient concentrations (N, P, and K) in plants.
9. The hypothesis whether N fertilizer, in particular *Azolla* as a biofertilizer, affects soil microbial community biomass and structure, especially bacterial and fungal community, in mineral (alluvial) and organic (peat) soils, was verified by the study findings.
10. Another advantage of *Azolla*-M utilization is that it may reduce the stress that microbes encounter from unfavorable environmental conditions under vegetable agroecosystems.
11. Managing soil health is encouraged since healthy soil strengthens the functions of ecosystems by sustaining the health of plants, animals, and humans. A healthy microbial community is vital to fertility, productivity, and sustainability of an ecosystem. The recommended N fertilizer treatment is the *Azolla* application at the manure N rate, since this treatment altered the microbial community towards increased total EL-FAMEs microbial biomass, Gram-negative bacteria, and fungi communities in both alluvial and peat soils.

12. *Azolla* biofertilizer applied at the urea or manure N rate or chicken manure are recommended organic N fertilizers to improve vegetable yields and nutrient concentrations, soil chemical properties, and also microbial communities, in particular in acidic tropical soils.
13. *Azolla* can be used for sustainable fertilization and should be developed on a large scale for vegetable production in dryland acidic tropical soils. In an approximate calculation, *Azolla* grown in a 1000-m^2 natural pond for 7 months of productive yields per year can produce about 13.6–16.12 tonnes fresh *Azolla* or 2.72–4.12 tonnes air-dried Azolla.
14. *Azolla* utilization for dryland vegetable crops could facilitate developing countries, in particular Indonesia, to enhance agricultural sustainability while reducing the negative environmental impacts of chemical fertilizer and simultaneously improving crop productivity and nutrient concentrations, and maintaining soil fertility and soil microbial communities.
15. Future work is needed to ascertain long-term effects of *Azolla* application on agronomic characteristics, soil properties, plant nutrients, and soil microbial communities. *Azolla* is sensitive to heat and light intensity. However, the main challenge of growing *Azolla* in the tropical ecosystems is how to tackle the pests efficiently. If we can resolve this challenge, *Azolla* utilization, in particular for dryland application, will be a promising sustainable biofertilizer for tropical ecosystems. Future research can then be carried out to evaluate the long-term effects of *Azolla* for dryland application on agronomic characteristics, soil properties, plant nutrients, and soil microbial communities.