THESIS

NITROGEN MINERALIZATION FROM BIOFERTILIZER AZOLLA MEXICANA COMPARED TO TRADITIONAL ORGANIC FERTILIZERS

Submitted by

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ABSTRACT

NITROGEN MINERALIZATION FROM BIOFERTILIZER AZOLLA MEXICANA COMPARED TO TRADITIONAL ORGANIC FERTILIZERS

Organic agriculture has become an essential approach to meeting the growing global food production demand and long-term soil sustainability, as well as addressing environmental problems connected with the use of synthetic agrochemicals. As a result, biofertilizers (biological fertilizers) have become promising resources to meet the growing demands for healthy and safe food production. Biofertlizers supply nutrients and take advantage of microorganisms that contribute to sustainable practices. One such biofertilizer is the aquatic pteridophyte *Azolla mexicana* which can be found in both tropical and temperate climates. *Azolla* can multiply rapidly, ensuring year-round biomass, and has also been found to have fast and high rate of N fixation. *Azolla* strains have been successfully exploited as effective biofertilizers in Asia but strains native to the Great Plains have not. There is no literature that assesses N mineralization (N_{min}) rates of *Azolla mexicana* compared to other organic fertilizers in Colorado soils. A laboratory soil incubation was conducted to determine the rates of N_{min}, N availability and total C and N of *Azolla mexicana* applied to soils compared to commonly-used organic fertilizers. Then, a greenhouse study was conducted to assess the organic fertilizer and urea treatment responses on kale growth and yield, leaf and petiole N percentage, total N uptake and percentage N recovery. In this study, we hypothesized that *Azolla* biofertilizer application will enhance soil inorganic nitrogen (soil ammonium-N and nitrate-N) concentrations and that soil amended with *Azolla* will also enhance

vegetable plant growth parameters (plant height, leaf and petiole N percentage, total N uptake and percentage N recovery).

In the incubation study, soil NH_4^+-N for all treatments tended to increase until day 56 where they all peaked then subsequently decreased until the end of the incubation period. Compost treatment recorded higher initial soil NH_4^+-N while Azolla + Watanabe treatment recorded higher soil NH_4^+-N concentration towards the end of the study. The soil NO^-_3-N concentrations in all treatments increased throughout the 140-day study. The Azolla + Watanabe treatment showed highest average soil NO^-_3-N concentration at day 140 while the Control treatment had the lowest soil NO^-_3-N concentration throughout the experiment. The decline in soil NH_4^+-N concentration formed during ammonification was followed by an increase in soil NO^-_3-N concentration because of nitrification.

In the greenhouse study, Azolla + Watanabe treatments had taller kale, significantly higher leaf fresh weight as well as significantly higher leaf dry weight. Both Azolla + Watanabe and Urea treatments recorded significantly higher yields compared to the other treatments. The Azolla + Watanabe and both Cyano treatments recorded significantly higher root dry weights compared other treatments. Control treatment had significantly higher root to shoot ratio. There were no significant differences in leaf N (%) among Azolla + Watanabe, Azolla, Cyano and Cyano + Moringa treatments. Azolla + Watanabe treatment also had significantly higher total N uptake among the organic fertilizers but was not significantly different from Azolla. Urea treatment recorded significantly higher N recovery and showed a similar pattern as the total N uptake whereby Azolla + Watanabe had significantly higher N recovery.

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INTRODUCTION

Organic agriculture has become an essential approach to meeting the growing demand for healthy and safe food production globally as well as ensuring long-term sustainability and addressing environmental problems connected with the use of agrochemicals (Mahdi et al., 2010). In parts of the world where soil fertility is progressively diminishing due to soil erosion, water logging, the buildup of salts and toxic materials, and loss of nutrients, organic residues and biological fertilizers have become alternative sources to chemical fertilizers to meet the nutrient demands of crops (Shehata and El-Khawas, 2003).

Biofertilizers are promising renewable resources for supplying nutrients to agricultural systems by providing organic making use of microorganisms that are a significant part of environmentally friendly sustainable practices (Ramazan et al., 1999; Bloemberg et al., 2000). In general, biofertilizers consist of microbial preparations that contain living cells of different microorganisms, which when applied to seeds, roots or soil, have the ability to mobilize plant nutrients in soil from unusable to usable forms through biological processes known as mineralization (Bhattacharjee and Dey, 2014). Nitrogen mineralization is the process by which organic N is converted to plant-available inorganic forms (Aber and Melill, 2001).

According to Goel et al. (1999), biofertilizers range from N fixing to phosphate solubilizing and plant growth-promoting microorganisms. Some examples of these biofertilizers that are beneficial to crop production include *Azotobacter*, *Azospirillium*, P-solubilizing microorganisms, mycorrhizae, *Sinorhizobium*, cyanobacteria (blue green algae) and *Azolla* (Hedge et al., 1999). The aquatic pteridophyte *Azolla* has excellent potential as a biofertilizer, green manure, poultry feed and cattle fodder with global distribution because of its rapid biomass production (Singh, 1978;

Yadav et al., 2014). *Azolla* can be found in both tropical and temperate climates and grows prolifically in fresh water ponds, paddy fields and ditches (Yadav et al., 2014). The ability of this fern to multiply rapidly primarily by vegetative reproduction ensures that year-round biomass can be sustained.

Azolla's ability to fix N is due to the presence of a heterocystous cyanobacterium *Anabaena azollae* that grows inside the dorsal leaf cavity of the fern (Peters and Meeks, 1989). The Azolla-Anabaena system's ability to fix atmospheric N makes it an outstanding agronomic choice for the cultivation of rice under tropical conditions (Yadav et al., 2014). The N fixation potential of the Azolla-Anabaena system has been estimated to be 1.1 kg N ha^{-1} day^{-1}, and one crop of *Azolla* was documented as providing 20-40 kg N ha^{-1} to a rice crop in about 20-25 days (Watanabe et al.,1977; Lumpkin and Plucknett, 1980).

Due to fast and high rates of N fixation combined with high biomass production, *Azolla* strains have been successfully exploited as an effective biofertilizer in Asia. In rice paddy fields, the application of *Azolla pinnata* has been seen to have a positive role in improving soil fertility (Yadav et al., 2014). For example, *Azolla pinnata* is reported to fix 75 mg N g^{-1} dry weight day^{-1} and generates 353 metric ton fresh weight ha^{-1} of biomass per year. The biomass contains 868 kg N that is equivalent to 1900 kg urea (Singh, 1988).

Prior to their dispersal by man, *Azolla mexicana* were endemic to northern South America through western North America (Lumpkin and Pluckett, 1980; Sculthorpe, 1967; Svenson, 1944). Like the other species of *Azolla*, *Azolla mexicana* can be collected from ponds and ditches and be applied directly to soils either as fresh or dried-crushed material. Since *Azolla mexicana* is native to the Great Plains, it has the potential to be grown in this region and used as a fertilizer. However, there is no literature that specifically evaluates N mineralization (N_{min}) rates of *Azolla mexicana* compared to other organic fertilizers on Colorado soils. Therefore, an evaluation of the application of *Azolla* on soils was conducted as an alternative to compost or cyanobacteria (*Anabaena* spp.). The objectives of the incubation study were to determine the rates of N_{min} of *Azolla mexicana* compared to commonly-used organic fertilizers, determine the N availability, and evaluate the total C and N applied from these fertilizers. The objective of the greenhouse study was to determine vegetable crop response to *Azolla mexicana* application as compared to other fertilizers. The hypotheses for this study are as follows: (1) *Azolla* biofertilizer application will enhance soil inorganic nitrogen (soil ammonium-N and nitrate-N) concentrations, and (2) *Azolla* utilization will also enhance vegetable plant growth parameters (plant height, leaf and petiole N percentage, total N uptake and percentage N recovery).

MATERIALS AND METHODS

Mineralization Experiment

Soil sampling

The soil used in the mineralization study was collected from the Agricultural Research Development and Education Center (ARDEC), Colorado State University (40°39'21.2"N, 104°59'45.2"W) and classified as a fine-loamy, mixed, mesic Aridic Haplustalf of Fort Collins series (NRCS, 1980). The soil was sieved through 8.0 mm and then 2.0mm sieves to obtain uniform soil particle size.

Soil analyses

Bulk density of the above soil series was determined using the cylindrical core method (Arshad et al., 1996). Chemical analyses consisted of electrical conductivity (EC) and soil pH measured in a supernatant suspension of 1:1 soil to water using a Mettler Toledo pH/EC meter (Thermo Fischer Scientific, Waltham, MA). The cation exchange capacity (CEC) of the soil was determined using a standard method used for soil surveys by the Natural Resources Conservation Service that uses 1 M ammonium acetate (NH_4OAc) at pH 7 (neutral NH_4OAc) (Burt, 2004; Carter et al., 2008). Loss by ignition method was used to determine organic matter content of the soil (Blume et al., 1990). The soil physical and chemical properties are presented in Table 1.

Fertilizer analysis

A liquid medium for *Azolla* culture was prepared in order to maintain *Azolla* in the vegetative state in preparation for the study (Watanabe et al., 1977). The liquid medium (to be referred to as Watanabe solution) was prepared in the laboratory before being added to a shallow, circular, open container that had a diameter of 111 cm, depth of 12.7 cm and volume of 122 L. The medium consisted of $CaCl_2 \cdot 2H_2O$ (40 mg/L), $NaH_2PO_4 \cdot H_2O$ (20 mg/L), $MgSO_4 \cdot 7H_2O$ (40 mg/L), and K_2SO_4 (40 mg/L) as macronutrients and $MnCl_2 \cdot 4H_2O$ (0.5 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (0.15 mg/L), H_3BO_3 (0.2 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.01 mg/L), $CuSO_4 \cdot 5H_2O$ (0.01 mg/L), $CoCl_2 \cdot 6H_2O$ (0.01 mg/L) and $FeSO_4 \cdot 7H_2O$ (0.50 mg/L) as micronutrients (Watanabe et al., 1977). These nutrients were then diluted with deionized H_2O to make a final volume of 55 L.

Azolla plants used in this study were *Azolla mexicana* obtained from a koi store in Fort Collins, CO. *Azolla* was grown in 68 L shallow, circular pools in either Watanabe solution (Azolla + Watanabe) or dechlorinated tap water (Azolla) (Yatazawa et al., 1980). Plant-based compost was obtained from Hageman Earth Cycle also located in Fort Collins. The cyanobacteria species used in this study was *Anabaena cylindrica,* and the cyanobacteria (Cyano) were cultured in a hoop house at ARDEC, settled by gravity, and then air-dried and ground to powder. The use of *Moringa oleifera* seeds as a coagulate or flocculate has given satisfactory results in reducing the number of cyanobacterial cells in water-treatment processes (Nishi et al., 2011). As a result, some cyanobacteria were flocculated in raceways by sprinkling ground *Moringa oleifera* seeds (Cyano + Moringa) on the surface of the nutrient media prior to settling, drying and grinding. In order to determine the C/N ratio of each fertilizer, the total C and N concentrations of the five different organic fertilizers were analyzed using a LECO Tru-SPEC elemental analyzer (Leco Corp., St. Joseph, MI) (Table 2) (Keeney and Nelson, 1982).

Soil incubation and sampling procedure

The soil incubation study was carried out at the Soil, Water and Plant Testing Laboratory, Colorado State University in an incubation room kept at a constant 25°C. The treatments in this study were control (no fertilizer), compost (Compost), Azolla grown in dechlorinated tap water (Azolla), Azolla grown in Watanabe solution (Azolla + Watanabe), cyanobacteria (Cyano) and cyanobacteria flocculated with moringa (Cyano + Moringa). All the fertilizers were applied at a rate of 50 kg N ha^{-1}. Fifty-gram sub-samples of air-dried soil were mixed with the fertilizers and placed in 1 L Mason jars according to the treatment assigned. All Mason jars were loosely lidded to prevent anaerobic conditions. Each treatment had four replications. The water content was adjusted once a week to 60% Water-Filled Pore Space (WFPS) (Griffin et al., 2002). Treatments were arranged in blocks to represent each sampling date. The treatments within the blocks were then randomized. The total experimental units were 192 jars.

The soils were incubated for 140 days, and inorganic N (NH_4^+-N and NO_3^- -N) was analyzed on the initial sampling date (t=0) after fertilizer application and on 7, 14, 28, 56, 84, 112, and 140 days after treatment. Twenty-four experimental units were destructively sampled on each sampling date throughout the incubation period.

Determination of inorganic nitrogen

Inorganic N for each sample was determined by extracting a 5 g sub-sample in 25 mL 2M KCl, shaking for 60 minutes on a reciprocating shaker at 180 cycles per minute and filtering to obtain a clear extract (Keeney and Nelson, 1982). Leachates were filtered using Whatman No. 42 filter paper. The extracts were analyzed immediately for NH_4^+ and NO_3^- using an Alpkem Flow

Solution IV Auto Analyzer (OI Analytical, College Station, TX). If immediate analysis was not possible, the extracts were frozen at -20^{o}C to prevent further microbial processes.

Nitrogen mineralization and N availability calculation

Nitrogen mineralization was calculated as follows:

Net N mineralization =

$$(NH_4^+\text{-}N_{\text{ Treatment}} + NO_3^-\text{-}N_{\text{ Treatment}}) - (NH_4^+\text{-}N_{\text{ Control}} + NO_3^-\text{-}N_{\text{ Control}}) \qquad \text{(Eqn. 1)}$$

$$\text{Nitrogen availability \%} = (\text{Net N mineralization/ amount N applied as fertilizer}) \times 100 \qquad \text{(Eqn. 2)}$$

Statistical analysis

Data were analyzed using R version 3.2.2 (The R Foundation for Statistical Computing). The experimental units were arranged in a factorial design with 6 treatments, 8 dates, and 4 replications. Boxplot procedures were used to evaluate the normality of data distribution. Interaction plots were generated to observe interaction effects when the effect of one variable depends on the value of another. Analysis of variance (ANOVA) was performed on the data using the linear models function since we had replicates for each Day and Treatment combination. Pairwise comparisons with Tukey adjustment were obtained to determine whether main effects or interactions were significant ($P < 0.05$).

Greenhouse Experiment

Site description

The pot experiment was carried out from November 2016 to January 2017 in a greenhouse at the Colorado State University Horticulture Center (40 ^{o}56'54"N, 105 ^{o}08'43"W). Certified organic kale seeds (*Brassica oleracea* 'Toscano') from Johnny's Selected Seeds (Waterville, ME) were grown in a greenhouse equipped with an evaporative cooling system in natural light during the day and LED top lighting for 8 hours during the night.

Potting mixture sample

A soilless potting mixture known as PRO-MIX® BX (Premier Horticulture Inc., Quakertown, PA) was used in this experiment. It is a general purpose, peat-based growing medium typically used in greenhouse and transplanting applications. Ingredients include: sphagnum peat moss (75-85%), horticultural grade perlite, horticultural grade vermiculite, calcitic limestone, dolomitic limestone, macronutrients, micronutrients and a wetting agent.

Potting mixture physical and chemical analyses

Bulk density was determined using a calibrated plastic milk carton container as described by Massey et al. (2001). The density was measured by filling the calibrated container with a well-mixed sample of the potting mix, measuring the mass of the mix and dividing the sample mass by the container volume. There were three replications performed, and the dry bulk density (g dry matter / cm^3) was calculated from the wet bulk density (g sample / cm^3) divided by the dry matter fraction (g dry matter/ g sample).

Other physical evaluations of the soil included air porosity and water-holding capacity using methods adapted from Gessert (1976) and Whitcomb (1988). Chemical analyses consisted of electrical conductivity (EC) and pH measured in a supernatant suspension of 1:1 soil to water using a Mettler Toledo pH/EC meter (Thermo Fischer Scientific, Waltham, MA). The soil physical and chemical properties are presented in Table 3.

Fertilizer analysis

The fertilizers used for the greenhouse experiment are the same as those used in the mineralization experiment with the addition of urea. The analyses are summarized in Table 2.

Experimental design

The experiment consisted of seven treatments (Azolla, Azolla +Watanabe, Cyano, Cyano + Moringa, Compost, Urea, and Control) with four replications. All treatments were applied at a rate of 56 N kg/ha. The pots used measured 15.24 cm by 15.24 cm and were watered to maintain an equal weight twice weekly on Wednesday and Sunday throughout the entire experiment. The treatments were arranged in a single bench in a Randomized Complete Block Design (RCBD).

Plant material

Seeds of 'Toscano' kale were planted in plastic trays containing 7.62 cm of well-mixed PRO-MIX® BX in the second week of November 2016. After four weeks, seedlings were transplanted into pots.

Pot experiment

Fertilizers were applied when seedlings were being transplanted into each pot. Each pot had one seedling. The pots were watered and maintained at equal weight throughout the experiment.

Parameters measured

Plant height was measured from the top of soil surface to the top of the leaf once a week from the time of transplanting to harvest. Harvesting was done 56 days after transplanting. At harvest, the leaf blade and stem and petiole samples were separated from each other and placed in labeled paper bags after measuring fresh weight. The roots were carefully separated from the potting mix and washed with deionized water. All plant material was then dried at 70^{o}C for 72 hours and then weighed to determine dry matter. Leaf blade and stem and petiole samples were then ground and sieved to pass an 80 mesh sieve prior to analysis. The root to shoot ratio was calculated from the dry material weight.

Leaf blade and stem and petiole N concentrations were measured using a LECO Tru-SPEC elemental analyzer (Leco Corp., St. Joseph, MI). The plant N uptake was calculated by multiplying the N concentration in leaf blades by the dry weight of the leaf blades and adding that to the N concentration in stems and petioles multiplied by the dry weight of the stems and petioles.

Calculation of percentage N recovery

Percentage N recovery was calculated by dividing N uptake of each treatment minus N uptake of control by the rate of N applied and multiplied by 100 as shown by the following equation.

$$N \text{ recovery } (\%) = (NL - NC)/ R * 100$$

where,

NL = N uptake by kale from fertilized treatments

NC = N uptake by kale from unfertilized treatments

R = Rate of fertilizer N applied

Statistical analysis

Data were analyzed using R version 3.2.2 (The R Foundation for Statistical Computing). Boxplot procedures were used to evaluate the normality of data distribution. Analysis of variance (ANOVA) was performed on the data using the linear models function. Pairwise comparisons with Tukey adjustment were obtained from mean square errors to determine whether main effects or interactions were significant ($P < 0.05$).

RESULTS

Mineralization Experiment

Soil inorganic nitrogen (Soil ammonium-N and nitrate-N)

Interactions between days of incubation and treatment were observed for soil NH_4^+-N ($P< 0.05$) and NO^-_3-N ($P< 0.05$). Soil NH_4^+-N concentration in all the treatments tended to increase until day 56 (Figure 1). Compost treatment recorded higher initial soil NH_4^+-N levels compared to all other treatments from day 0 to day 56 (Table 4 and Figure 1). Soil NH_4^+-N for all treatments peaked at day 56 and then subsequently decreased until the end of the incubation period. The Azolla + Watanabe treatment resulted in greater soil NH_4^+-N concentration towards the end of the study (day 84 to day 140) compared to all other treatments (Figure 1). There were no significant differences in soil NH_4^+-N mean estimates between all treatments at the end of the study (Table 4). The Control treatment produced significantly lower soil NH_4^+-N concentrations throughout the study (Table 4 and Figure 1).

Conversely, the soil NO^-_3-N concentrations in all treatments increased throughout the entire 140-day study (Figure 2). Soil NO^-_3-N concentrations significantly increased from day 14 until the end of the experiment in all treatments except the control treatment (Table 5 and Figure 2). Soil NO^-_3-N concentrations temporarily leveled off from day 56 to day 84 before they increased following day 84 until the end of the study (Table 5 and Figure 2). The Azolla + Watanabe treatment showed highest average soil NO^-_3-N concentrations at day 140 while the Control treatment had the lowest soil NO_3-N concentrations throughout the experiment (Figure 2). The Azolla + Watanabe treatment produced significantly higher soil NO_3-N mean estimates compared to all other treatments (Table 5). There were also no significant differences in soil NO_3-N mean estimates between Azolla and Cyano + Moringa treatments, and the Cyano and Compost treatments (Table 5).

Cumulative soil inorganic nitrogen (Soil ammonium-N and nitrate-N)

The cumulative soil N mineralized were calculated by summing the amount of NH_4^+-N and NO_3-N for each treatment throughout the 140-day incubation period. Cumulative soil N increased steadily for all the treatments throughout the study. However, between day 56 to day 84, temporary immobilization was seen to occur for all the treatments. All treatments remineralized after day 84. The Azolla + Watanabe treatment cumulative soil N was significantly different than all other treatments ($P<0.001$).

Soil inorganic N (Soil ammonium-N and nitrate-N) mineralization rates

Soil net N mineralization rates were determined by subtracting final (day 140) soil inorganic N from initial (day 0) soil inorganic N and dividing the resulting number with the number of incubation days. Azolla + Watanabe recorded significantly higher ($P<0.01$) soil N mineralization rates compared to all other treatments (Table 6). There were no significant differences in N mineralization rates of Cyano and Cyano +Moringa treatments.

Soil nitrogen availability

An interaction ($P< 0.05$) between treatment and days of incubation in N availability was observed. Initial N availability was observed to be the highest in compost treatment compared to all other treatments (Figure 4). Temporary immobilization was seen to occur on day 28 in the Compost and Azolla treatments (Figure 4). However, both treatments remineralized after day 56, and the N availability of all treatments stayed stable until day 84. After day 84, both Azolla and Azolla + Watanabe treatments continued to mineralize while Cyano, Cyano + Moringa and Compost experienced immobilization until the end of the 140-day study (Figure 4). At the end of the experiment, the Azolla + Watanabe treatment had the highest N availability while compost recorded the lowest (Figure 4).

Greenhouse Experiment

Height

Kale treated with Urea grew rapidly until day 28 and were the tallest plants from day 35 to day 56, while the Control treatment had the shortest plants throughout the experiment (Figure 5). Azolla + Watanabe treatments resulted in taller kale compared to the other organic fertilizers (Figure 5). On day 14, Azolla + Watanabe treatment recorded significantly shorter plants compared to plants treated with Urea ($P=0.002$).

Leaf Blade and Petiole and Stem Fresh Weight

The Azolla + Watanabe treatment recorded significantly greater leaf blade fresh weight compared to all other fertilizers (Figure 6). There were no significant differences observed between Azolla, Cyano, Cyano + Moringa and Urea fertilizer treatments in leaf blade fresh weight (Figure 6). The Urea fertilizer treatment experienced significantly greater petiole and stem fresh weight compared to all other treatments (Figure 7). There were no differences in petiole and stem fresh weight amongst the organic fertilizers, except for Azolla + Watanabe which was significantly greater than the other organic fertilizers.

Total yield

Treatments significantly affected yield (P< 0.05). Both Azolla + Watanabe and Urea treatments resulted in significantly greater yields compared to the other treatments (Figure 8). There were no significant differences in yield between the Azolla, Cyano and Cyano + Moringa treatments (Figure 8), but Compost resulted in the lowest organic treatment yield, although it was significantly greater than the Control.

Leaf Blade and Stem and Petiole Dry Weight

The Azolla + Watanabe treatment recorded significantly greater leaf blade dry weight than all other treatments, while there were no significant differences in leaf blade dry weight between Azolla, Cyano, Cyano + Moringa and Compost treatments (Figure 9). The urea treatment had significantly higher leaf blade dry weights than all other treatments except Azolla + Watanabe.

The petiole and stem dry weights of the Urea, Azolla + Watanabe and Azolla treatments were significantly greater than all other treatments (Figure 10). There were no significant differences in petiole and stem dry weights between the Azolla + Watanabe and Azolla treatments or between the Cyano and Cyano + Moringa treatments (Figure 10).

Root Dry Weight

All treatments resulted in significantly greater root to dry weights compared to control. There were no significant differences recorded in root dry weight of Azolla + Watanabe and both Cyano treatments (Figure 11). There were no significant differences in root dry weight between Cyano + Moringa and Azolla treatments. There were also no significant differences in root dry weight between Compost and Urea treatments, although both were significantly higher than Control.

Root: Shoot Ratio

Treatment significantly affected root to shoot ratio ($P = 0.032$). The Control treatment had significantly greater root to shoot ratio compared to all other treatments (Figure 12). There were no significant differences in root to shoot ratio among Cyano, Cyano + Moringa and Azolla + Watanabe treatments. Urea resulted in significantly lower root to shoot ratio except compared to the Compost treatment (Figure 12).

Leaf Blade and Stem and Petiole N Percentage

Treatments significantly affected leaf blade N (%) ($P = 0.013$) as well as stem and petiole N (%) ($P = 0.025$). Urea resulted in significantly greater leaf blade N (%) compared to all other treatments (Figure 13). There were no significant differences in leaf blade N (%) among Azolla + Watanabe, Azolla, Cyano and Cyano + Moringa treatments (Figure 13). Urea also had significantly greater stem and petiole N (%) compared to the other treatments (Figure 14).

Total N Uptake

N uptake by kale was significantly affected by fertilizer treatment ($P < 0.05$). The Urea treatment resulted in significantly greater total N uptake compared to all other treatments. The Azolla + Watanabe treatment had significantly greater total N uptake among the organic fertilizers but was not significantly different from Azolla and Cyano treatments (Figure 15).

Percentage N Recovery

Treatment significantly affected N recovery percentage ($P < 0.05$). The Urea treatment recorded significantly greater N recovery and showed a similar pattern as the total N uptake whereby Azolla + Watanabe had significantly greater N recovery followed by Azolla, Cyano, Cyano + Moringa and finally Compost (Figure 16).

DISCUSSION

Nitrogen availability is frequently limited in agricultural systems. Organic fertilizers that have a low C:N ratio with adequate N to support crop growth and microbial biomass are preferred. The addition of fertilizers which are usually N-rich to soils typically improves soil fertility and builds SOM. Nitrogen mineralization differed among the fertilizers applied during the 140-day incubation period, with the greatest soil N availability from the Azolla + Watanabe and the lowest N availability from the Compost treatment. The Cyano and Cyano + Moringa treatments have lower C:N ratios compared to the other fertilizers and were seen to have higher N availability than Compost which has the highest C:N ratio. In a study by Flavel and Murphy (2006), the greatest net N mineralization of organic amendments applied to soils was attributed to amendments with low C:N ratio and high N content.

In a similar mineralization study conducted by Sukor (2013) using Composted manure and two different forms of cyanobacteria as fertilizers, C:N ratio of Composted manure was higher than that of cyanobacteria. The Composted manure and control treatments both resulted in lower soil inorganic N (NH_4^+-N and NO^-_3-N) concentrations and lower soil N availability than the other treatments, proving similar results to this study.

A comparable incubation study on loam soil by Abbasi and Khaliq (2016) experienced immobilization between days 1 and 63, reaching highest immobilization on day 35 in the Composted Manure. Net mineralization was observed from days 84 to 140. In our study, the soil NH_4^+-N reached its peak on day 56 and then declined due to conversion to soil NO^-_3-N. Biosolid incubation experiments have shown similar trends, whereby NH_4^+ was the dominant form of soil inorganic N during the first half of the incubation and then NO^-_3 constituted more than 50% of the inorganic N during the latter half of the study (He et al., 2000). In addition, the results of this study, like the others described, show that when soil NH_4^+-N concentration declined, there was a corresponding increase in soil NO^-_3-N concentration demonstrating that NH_4^+-N formed during ammonification was then nitrified into the NO^-_3-N form (Sukor, 2013).

An interconnection exists between N availability of the organic fertilizers measured in the incubation study and leaf N concentrations found in the greenhouse study. This is seen on day 56 whereby the Azolla + Watanabe treatment resulted in both highest N availability (Figure 4) and leaf blade N concentration (Figure 13), excluding the urea treatment. This interconnection could perhaps be explained by Azolla-Anabaena symbiosis whereby *Azolla* harbors the domesticated cyanobacteria which provides it with unlimited C (the intracellular chloroplasts) and N (the extracellular cyanobiont) (Larson, 2001). In a study by Yanni (1992) on rice, inoculation with either *Azolla* or cyanobacteria, even with urea fertilizer at 144 kg N/ha led to the increase of N accumulation in grain and straw, but the effect of *Azolla* was superior to that of cyanobacteria. Furthermore, highest N availability and leaf blade N concentrations in Azolla +Watanabe could be attributed to addition of essential nutrients from the Watanabe solution. Essential nutrients availability influence the effectivity of N fixation (O'Hara, 2001). Essential nutrients such as Fe and Mo are also required to produce the nitrogenase enzyme to fix atmospheric N (Carithers et al., 1979).

In this study, Azolla + Watanabe fertilizer had lower C:N ratio than both Azolla and Compost fertilizers (Table 2). The quantity of N released to crops depends on the chemical composition of the organic matter (Calderón et al., 2005). N content, C:N ratio, lignin, and contents of cellulose and hemicelluloses, and polyphenols are some of the factors that affect amount N release to soils (Mohanty et al., 2011). Organic fertilizers with high N contents and low C:N ratios mineralize sufficient N to meet the demands of plant growth (Cordovil et al., 2005 and Seneviratne, 2000).

Comparatively, leaf blade N concentration in Azolla + Watanabe was still significantly lower than that of Urea. Inorganic fertilizers such as Urea are soluble (Cooke, 1982), and therefore, N is immediately available for uptake when the material is incorporated into the soil (Epstein, 1972). Although Urea treatment resulted in taller kale compared to all other treatments after day 35 (Figure 5), we noticed that kale in all the Urea treatment replications began to shrivel and turn yellow after day 48. Previous studies indicate that N leaching losses, occurring primarily as NO_3^-, are higher from quick-release-N sources such as Urea, and are enhanced by well-drained sandy soils (Volk and Bell, 1945; Bates and Tisdale, 1957; Reike and Ellis, 1974; Smika et al., 1977; Mitchell et al., 1978; Petrovic et al., 1986). Since this greenhouse study used a potting mixture which drained well because it contained vermiculite, we could expect N losses through leaching as well. As a result, there were not significant differences in kale height at the end to the study between the Urea treatment and Azolla +Watanabe treatment.

There is also some delay between incorporation of organic fertilizers and the release of available N (Bunt, 1976), but crop plants would be better established and able to utilize more of the applied available N than would be the case with an inorganic fertilizer (Smith et al., 1989).

Phosphorus is an essential macro element required for growth and development of plants while Potassium enhances plant growth and is effective on different processes like photosynthesis, translocation of food, cell extension and formation of proteins (Inam et al., 2011). All treatments except for Urea released both phosphorus and potassium (Table 2). However, the was no significant effect of P and K on height (Figure 5) and yield (Figure 8) in this study.

Compost and vermicompost release limited available nutrients even though they have produced good results for vegetable transplant production in many cases (Arancon et al., 2004; Ozores-Hampton et al., 1999; Raviv et al., 1998; Reis et al., 1998). The Compost treatment in this study showed both lower mineralization rates and smaller yields compared to the other organic fertilizers. Clark and Cavigelli (2005) explain that not all composts are the same and not all show potential as good potting media for transplant production, mainly because of the low N mineralization rate, N immobilization and high salinity.

With increasing oil prices resulting in increasing fertilizers prices since the 1970s, many concerns over the stability of world chemical fertilizer prices have been raised (Socolow, 1999). Concerns have also been raised over the long-term adverse effects of heavy use of chemical fertilizers on crop productivity, soil structure, and off- farm pollution. As a result, organic fertilizers and green manures are seen as having many agronomic and environmental advantages (Rosegrant and Roumasset, 1987). The economic feasibility of substituting Azolla for chemical fertilizers can be determined by comparing the price of N from chemical fertilizers to the cost of producing N through Azolla (Rosegrant and Roumasset, 1987). In a study conducted in the Philippines, where low-resource farmers are rarely able to purchase chemical fertilizers without government credit, the use of Azolla was relatively attractive especially in farms with poor irrigation systems. Ample water is critical to Azolla growth; the recommended water depth is 40-50mm throughout the growth period. Growth is delayed as water depth approached zero, and Azolla ordinarily will die if the field is fully drained for 1 to 2 days (Lumpkin and Plucknett, 1982). At \$0.55/kg N for ammonium sulfate, intercropping with Azolla during the wet seasons was considered to be more feasible by these farmers (Rosegrant and Roumasset, 1987).

High labor costs and high opportunity costs of land use are two major constraints to the economic feasibility of organic fertilizers produced on-farm, such as cyanobacteria or Azolla. Capital requirements for Azolla cultivation are quite small (Lumpkin and Plucknett, 1981). Kikuchi et al. (1984) pointed out that Azolla can be grown in small catchments, tanks, or canals of irrigation systems and can be floated onto fields and incorporated into the soil. These methods of growing Azolla could provide large savings in both cost of land and labor (Kikuchi et al., 1984). For most farmers, only a small amount of phosphorus fertilizers, often no more than would be required for the rice crop, and pesticides to protect the Azolla from pests (Lumpkin and Plucknett, 1981).

TABLES

Table 1. Chemical and physical properties of Fort Collins soil from ARDEC.

Texture	pH	EC	CEC	OM	Bulk Density
		--dSm^{-1}--	--cmol kg^{-1}--	--%--	--g cm^{-3}--
Fort Collins fine-loam	7.2	1.6	20.1	2.5	1.21

Table 2. Nutrient values and C/N ratio of fertilizers evaluated in the incubation and greenhouse studies.

Treatment	N	P	K	C	C/N ratio
	--%--	--%--	--%--	--%--	
Compost	1.53	0.630	0.52	20.06	13.1
Cyano	8.81	1.402	1.04	43.18	4.9
Cyano +Moringa	7.38	2.036	0.72	42.78	5.8
Azolla	3.27	0.347	0.96	45.44	13.9
Azolla +Watanabe	4.04	1.111	2.87	44.80	11.1
Urea	46	0	0	-	-

[1]Treaments consisted of Compost (Compost), Cyanobacteria (Cyano), Cyanobacteria and Moringa (Cyano +Moringa), Azolla (Azolla) and Azolla grown in Watanabe solution (Azolla +Watanabe).

Table 3. Chemical and physical properties of PRO-MIX® BX potting mix used in the greenhouse study.

Analyses	Result
pH	6.1
EC	1.5 dSm^{-1}
NO_3-N	93 mg/l
PO_4-P	28 mg/l
K	67 mg/l
Bulk Density	0.13 g/cm^3
Air Porosity	13%
Water-Holding Capacity	67 mg/l

Table 4. Soil NH_4^+-N mean estimates for 140 days of incubation. Parameters with a common letter are not significantly different from each other ($p < 0.05$) according to Tukey's test for mean separation.

Day	Treatments						
	Control	Azolla	Azolla + Watanabe	Cyano	Cyano + Moringa	Compost	$\bar{x}$
	-- mg kg^{-1} --						
0	4.1 DEc	6.7 Eb	8.5 Eab	8.3 Dab	6.4 Eb	16.9 CDa	8.48 CD
7	4.5 BCd	9.5 DEc	11.2 CDEbc	12.7 BCDb	10.0 DEbc	17.8 BCa	10.95 C
14	6.1 ABd	10.9 BCc	13.2 CDbc	14.4 Bb	12.4 Cbc	17.6 CDa	12.43 BC
28	10.1 ABd	13.2 ABCc	17.3 ABb	16.3 Abc	14.9 BCbc	18.4 Ba	15.03 AB
56	12.3 Ac	15.9 Ac	18.7 Ab	18.0 Abc	17.8 Abc	19.5 Aa	17.03 A
84	8.9 ABc	13.6 ABb	15.9 ABa	15.3 Bbc	16.4 ABbc	15.7 CDbc	14.30 B
112	4.3 CDc	10.1 CDbc	15.5 BCa	12.9 BCbc	11.9 CDbc	13.8 CDb	11.42 BC
140	1.8 Ec	7.7 DEbc	10.7 DEa	8.8 CDb	8.7 Eb	7.2 Dbc	7.48 CD
$\bar{x}$	6.51 d	10.95 c	13.88 b	13.34 bc	12.31 bc	15.86 a	

ABC Dates within treatment with a common capital letter are not significantly different ($p<0.05$) by Tukey's test for mean separation.
abc Treatments within dates with a common small letter are not significantly different ($p<0.05$) by Tukey's test for mean separation.

Table 5. Soil NO_3^--N mean estimates for 140 days of incubation. Parameters with a common letter are not significantly different from each other ($p < 0.05$) according to Tukey's test for mean separation.

Day	Control	Azolla	Azolla + Watanabe	Cyano	Cyano + Moringa	Compost	$\bar{x}$
	----------	----------	----------	mg kg^{-1}	----------	----------	---
0	7.7 Fa	8.6 Ga	8.5 Ha	7.6 Ha	7.7 Ga	8.2 Ha	8.05 F
7	18.2 Ea	23.2 Fb	25.9 Ga	20.8 Gb	24.2 Fa	18.7 Ga	21.83 E
14	19.9 Dd	29.1 Ec	31.9 Fa	23.3 Fc	28.0 Ec	29.3 Fb	26.92 D
28	31.4 Cd	38.1 Ebc	41.9 Ea	37.1 Ec	40.4 Eb	36.2 Ed	37.52 C
56	34.4 Be	51.6 Ccd	56.5 Ca	52.6 Cc	53.9 Cb	48.8 Cd	49.63 BC
84	31.5 Cd	49.9 Db	53.9 Da	47.9 Dc	52.1 Db	46.9 Dd	47.03 BC
112	44.5 Be	60.9 Bb	65.1 Ba	56.4 Bd	60.8 Bc	53.9 Bd	56.93 B
140	56.6 Af	82.5 Ab	88.9 Aa	62.9 Ad	67.9 Ac	58.9 Ae	69.62 A
$\bar{x}$	30.53 d	42.99 b	46.56 a	38.58 c	41.88 b	37.61 c	

ABC Dates within treatment with a common capital letter are not significantly different ($p<0.05$) by Tukey's test for mean separation.

abc Treatments within dates with a common small letter are not significantly different ($p<0.05$) by Tukey's test for mean separation.

Table 6. Soil N mineralization rates among fertilizers as a function of time during 140-day incubation study. Values followed by common small letter are not significantly different (P<0.05) by Tukey's test for mean separation.

Treatment	N mineralization rates
	mg N kg^{-1} day^{-1}
Control	0.333d
Azolla + Watanabe	0.589a
Azolla	0.535b
Cyano	0.399c
Cyano + Moringa	0.447bc
Compost	0.293e

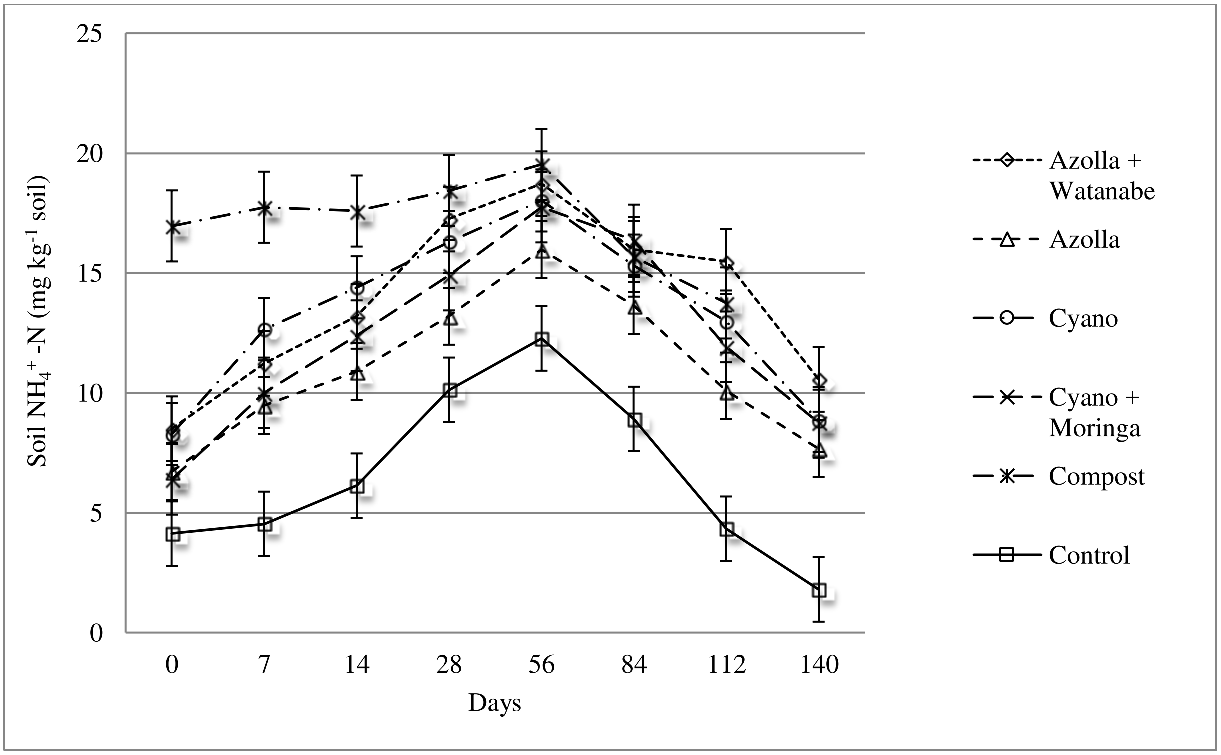

Figure 1. Soil NH_4^+-N concentration as a function of time during the 140-day incubation period. Bars represent standard errors of mean.

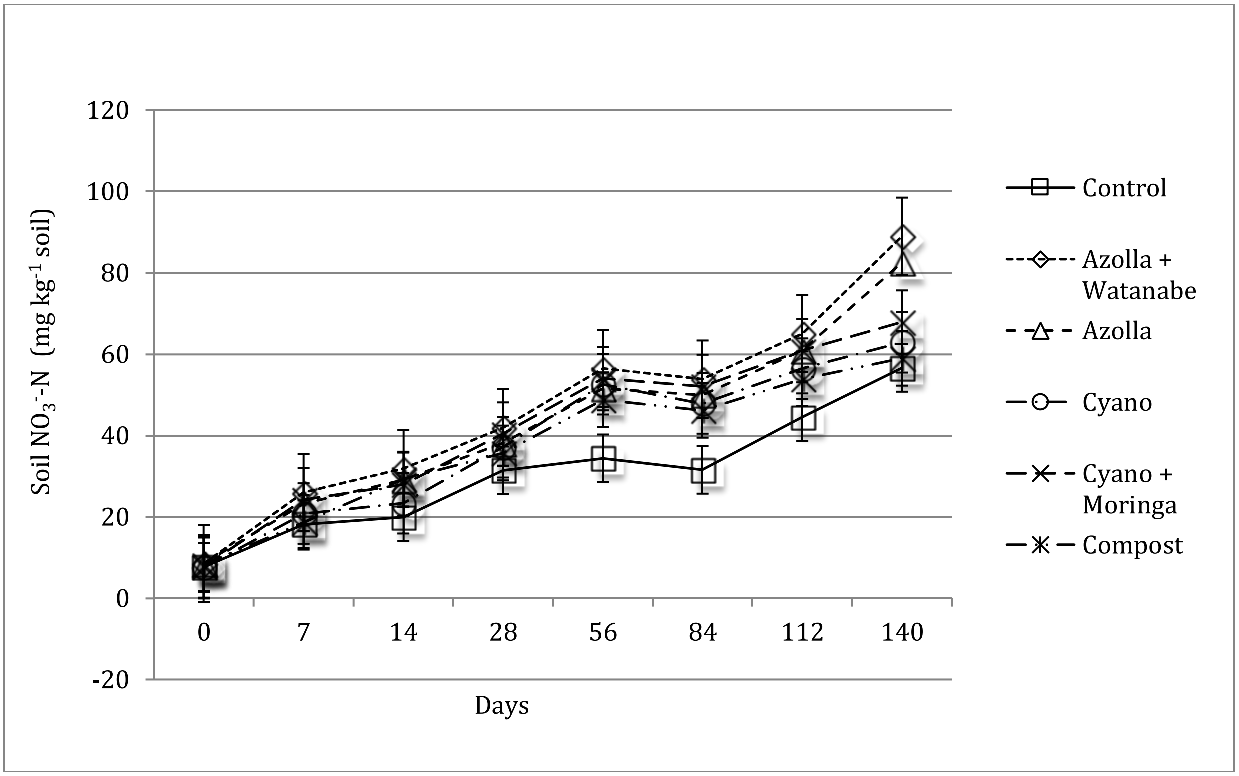

Figure 2. Soil NO_3^-N concentration as a function of time during the 140-day incubation period. Bars represent standard errors of mean.

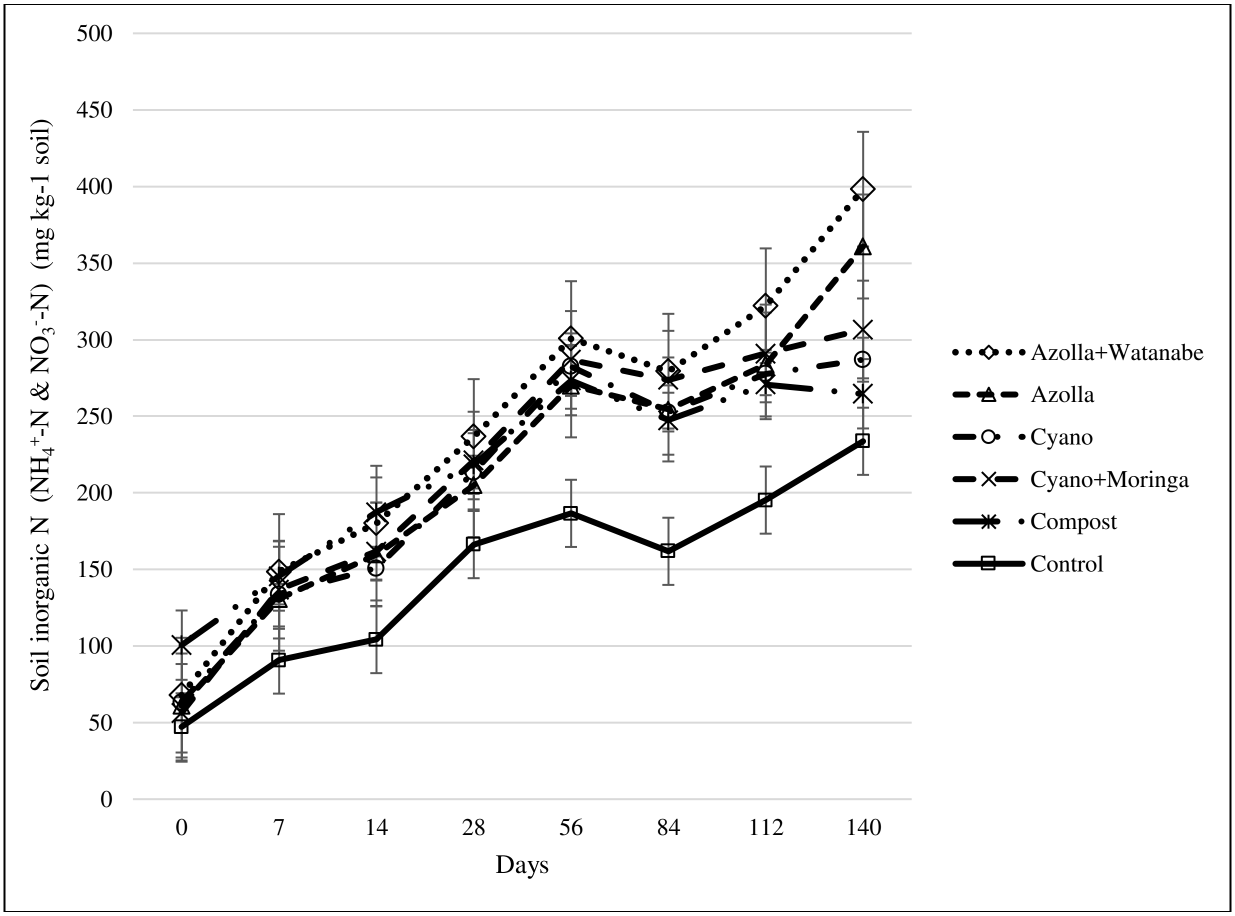

Figure 3. Cumulative N mineralized as a function of time during the 140-day incubation experiment. Bars represent standard errors of mean.

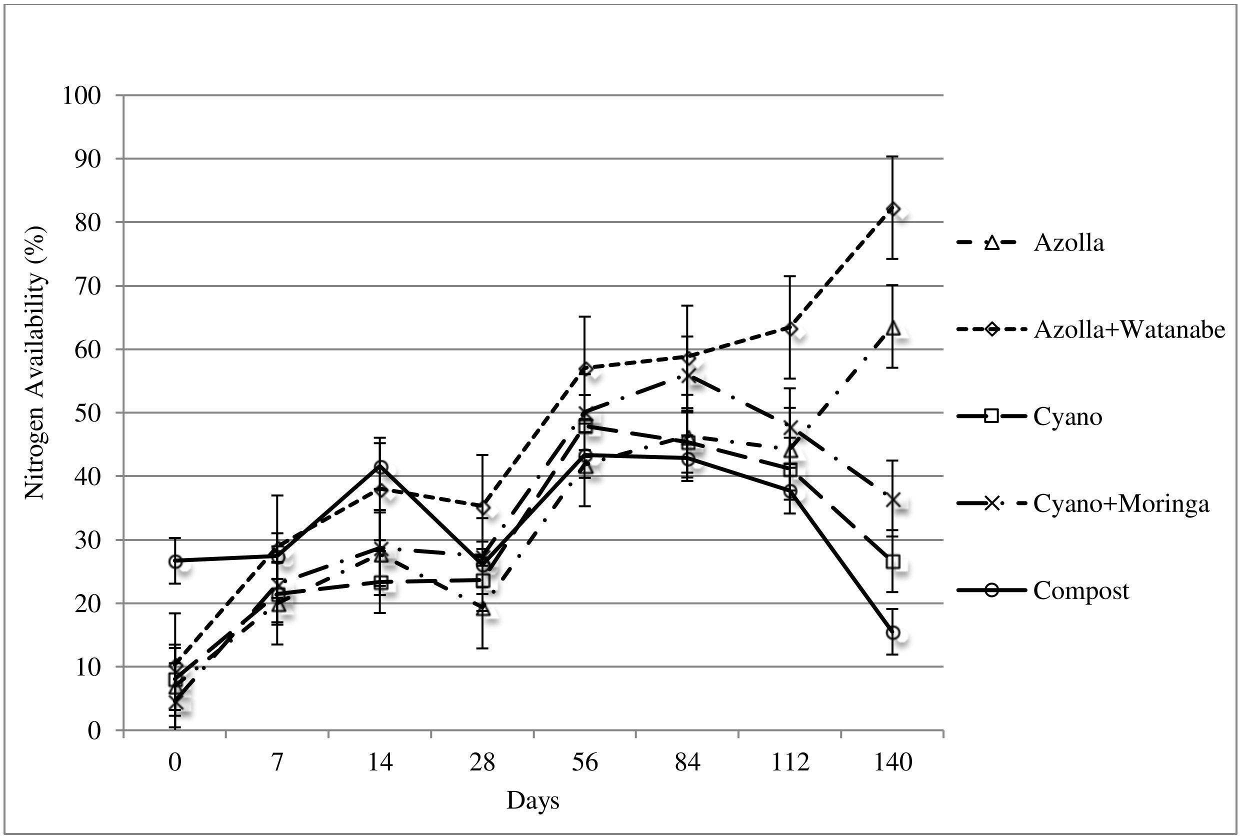

Figure 4. Nitrogen availability as a function of time during the 140-day incubation experiment. Bars represent standard errors of mean.

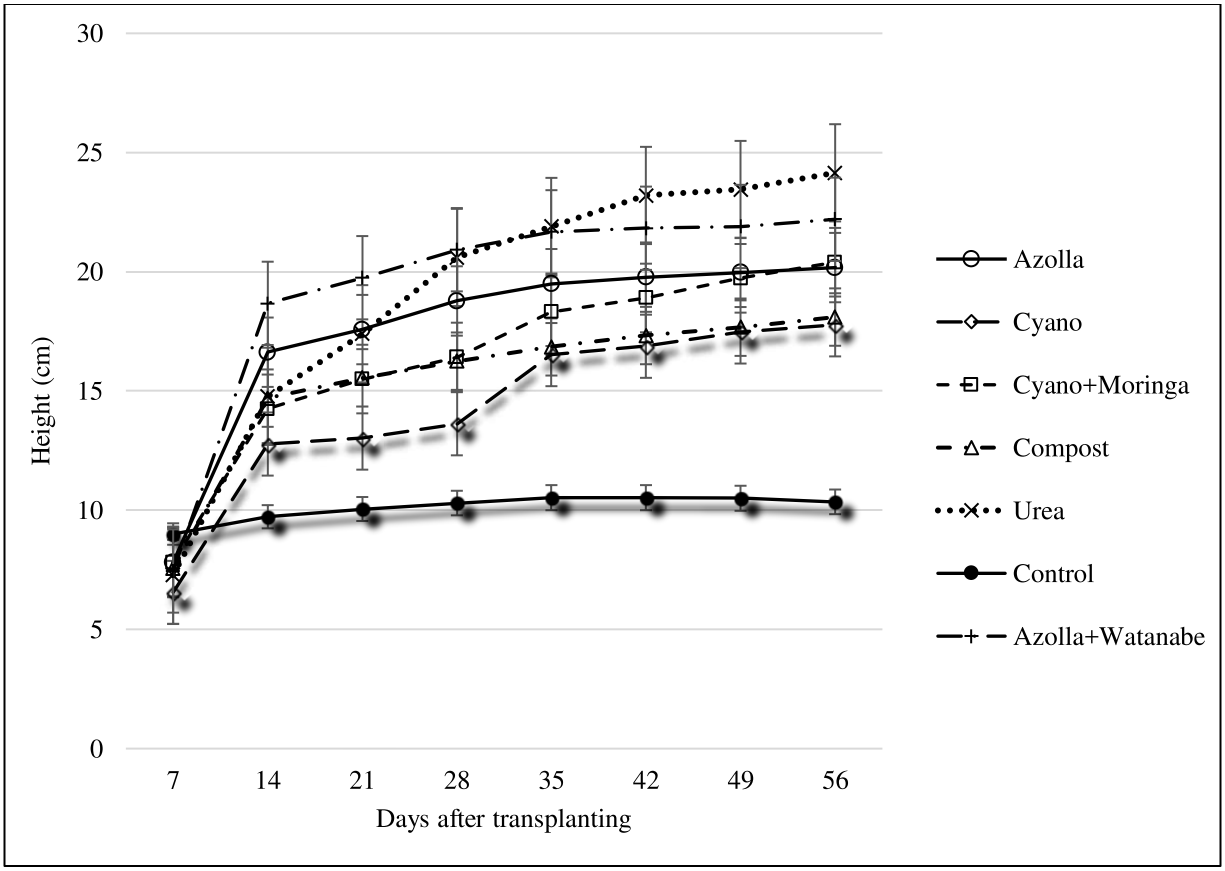

Figure 5. Height of kale plants throughout the 8-week experiment from one time fertilizer application into potting mix treatments at 56 N kg/ha. Bars represent standard errors of mean.

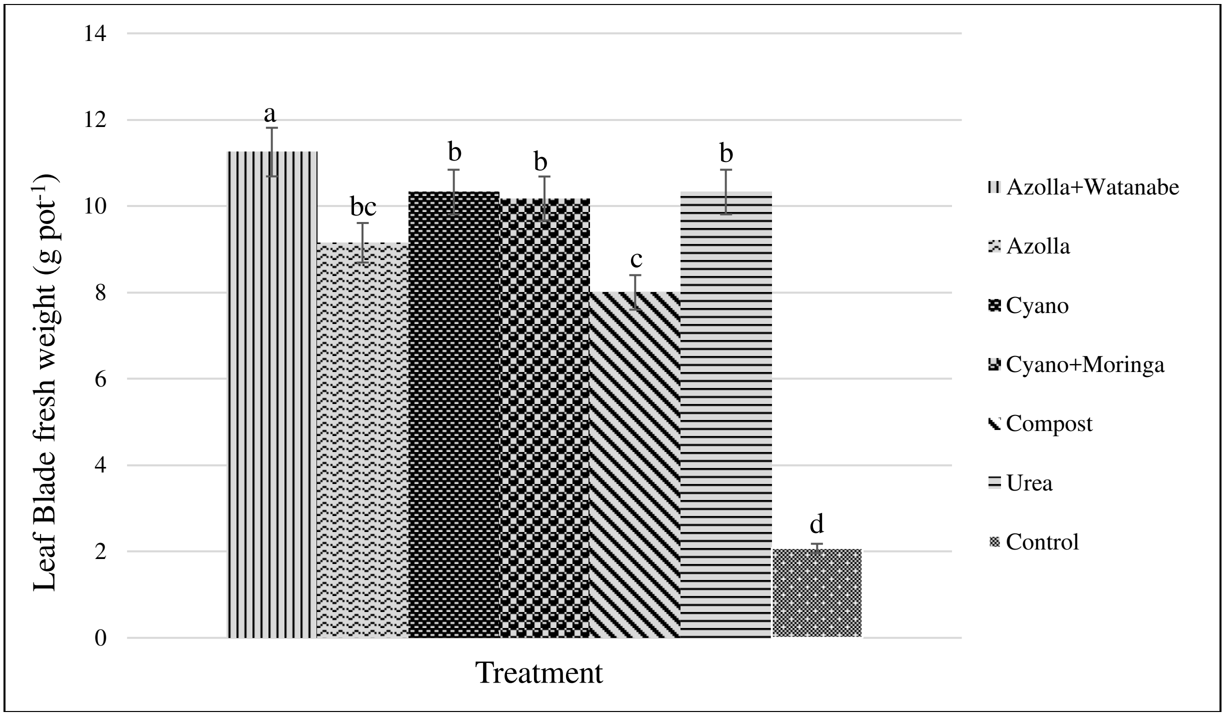

Figure 6. Leaf Blade fresh weight of kale tissue 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

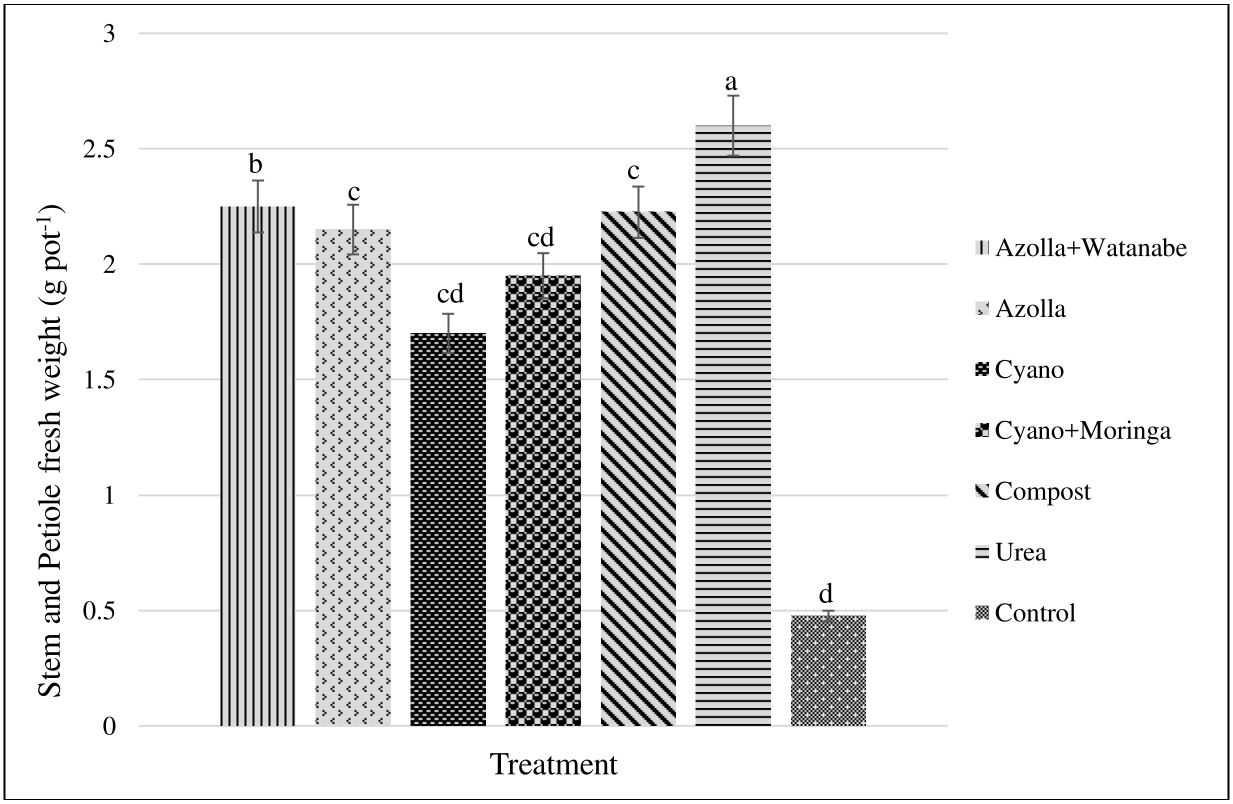

Figure 7. Stem and Petiole fresh weight of kale tissue 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

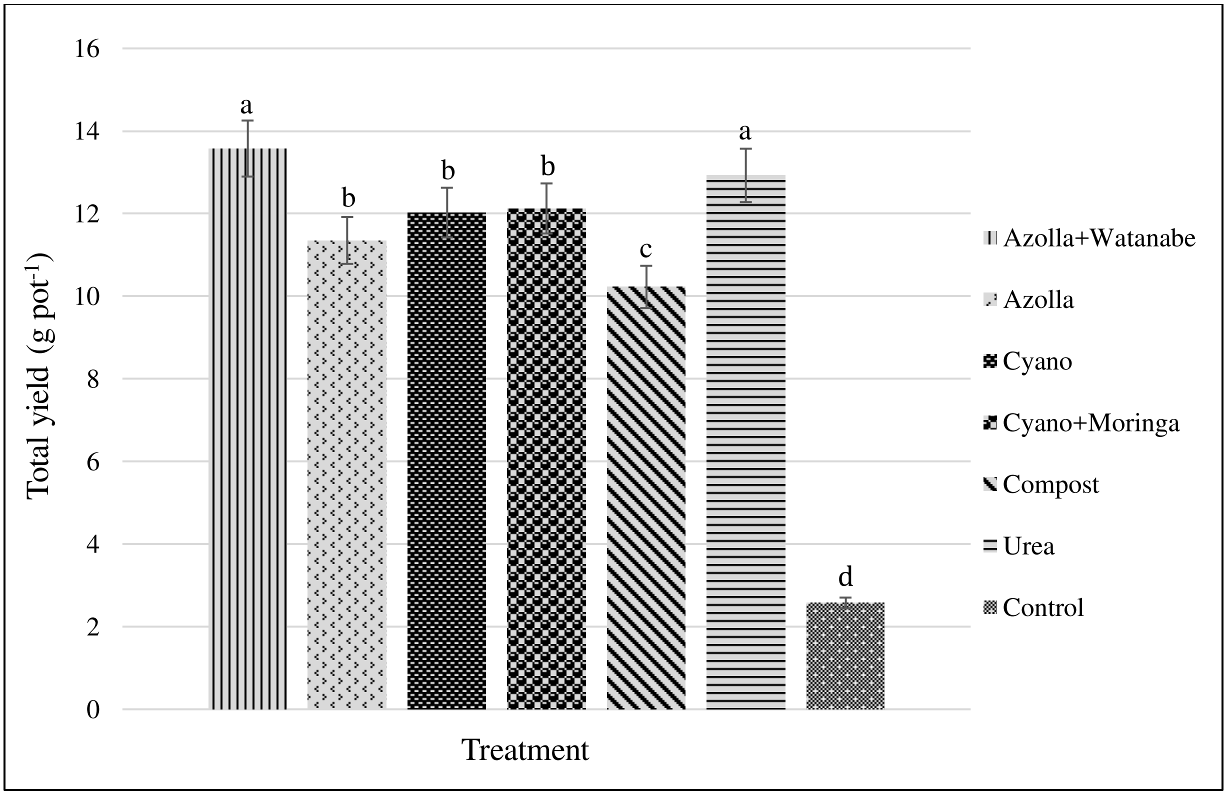

Figure 8. Total yield (leaf blade and stem and petiole weight) of kale tissue 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

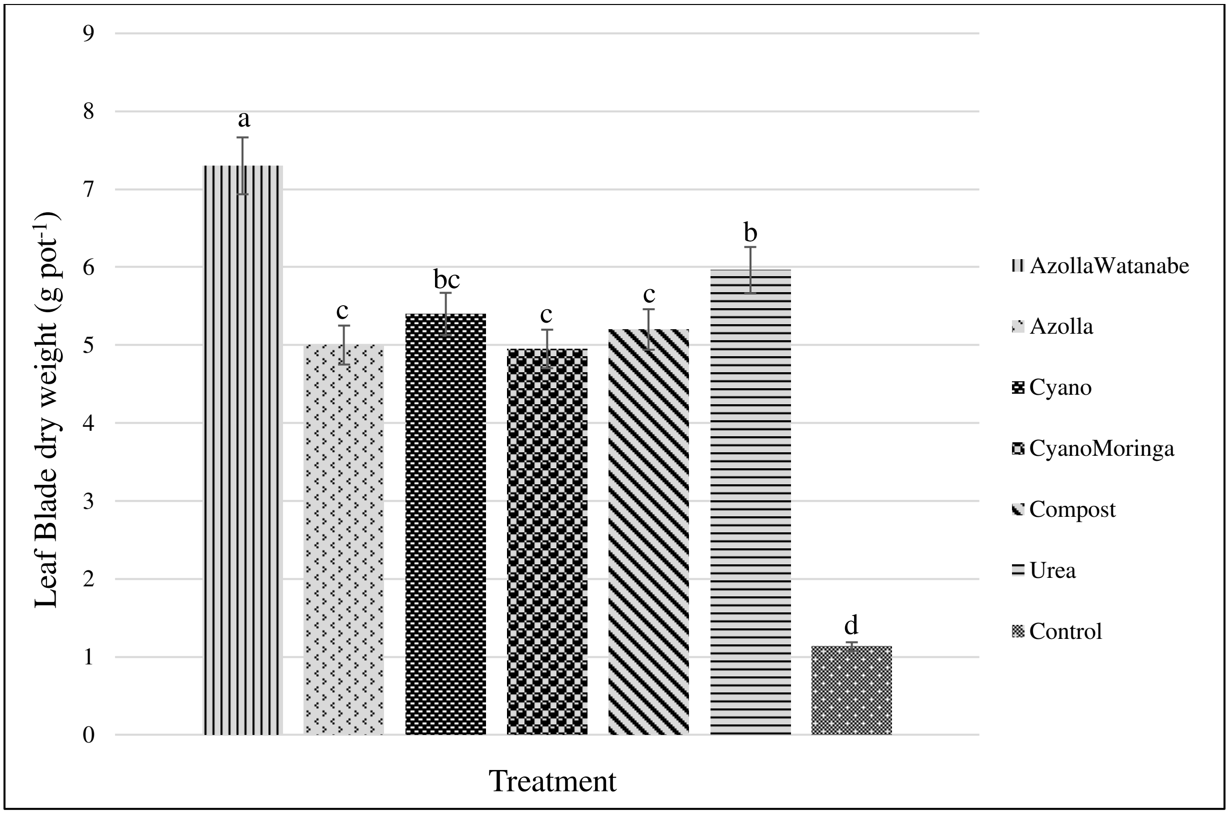

Figure 9. Leaf Blade dry weight of kale tissue 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

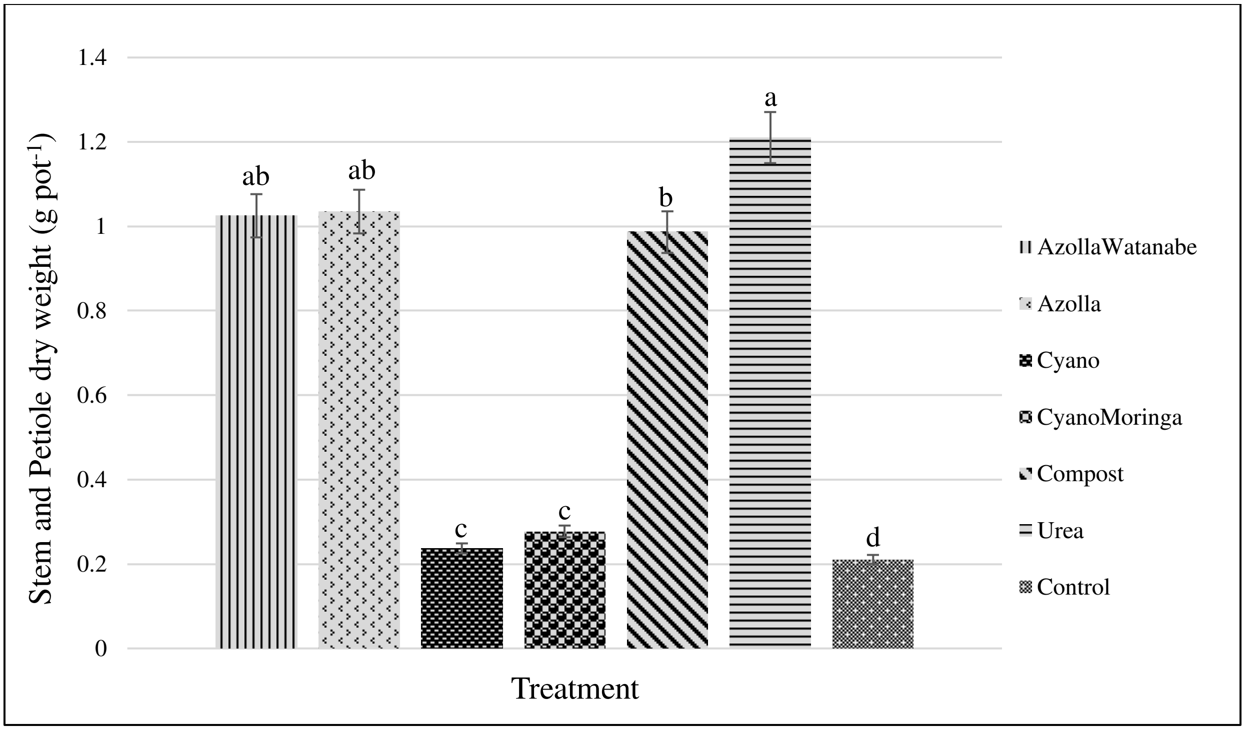

Figure 10. Stem and Petiole dry weight of kale tissue from a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

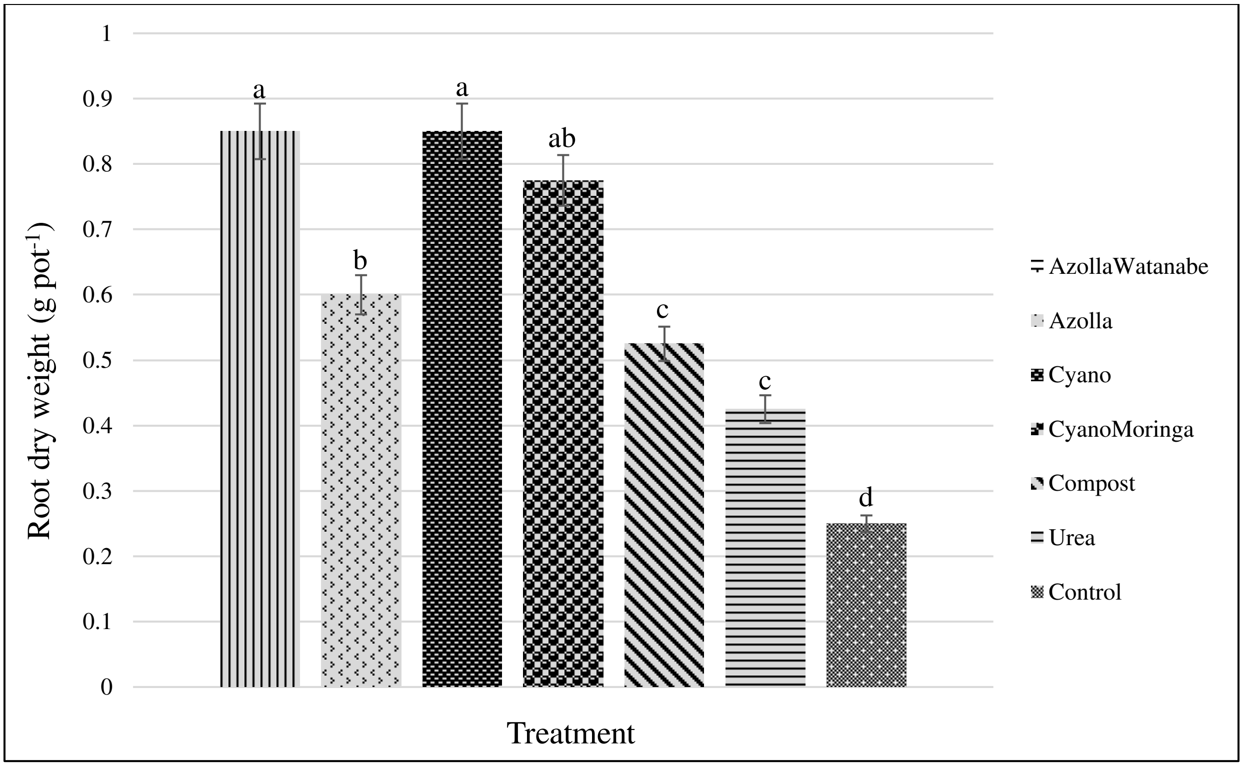

Figure 11. Root dry weight of kale tissue 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

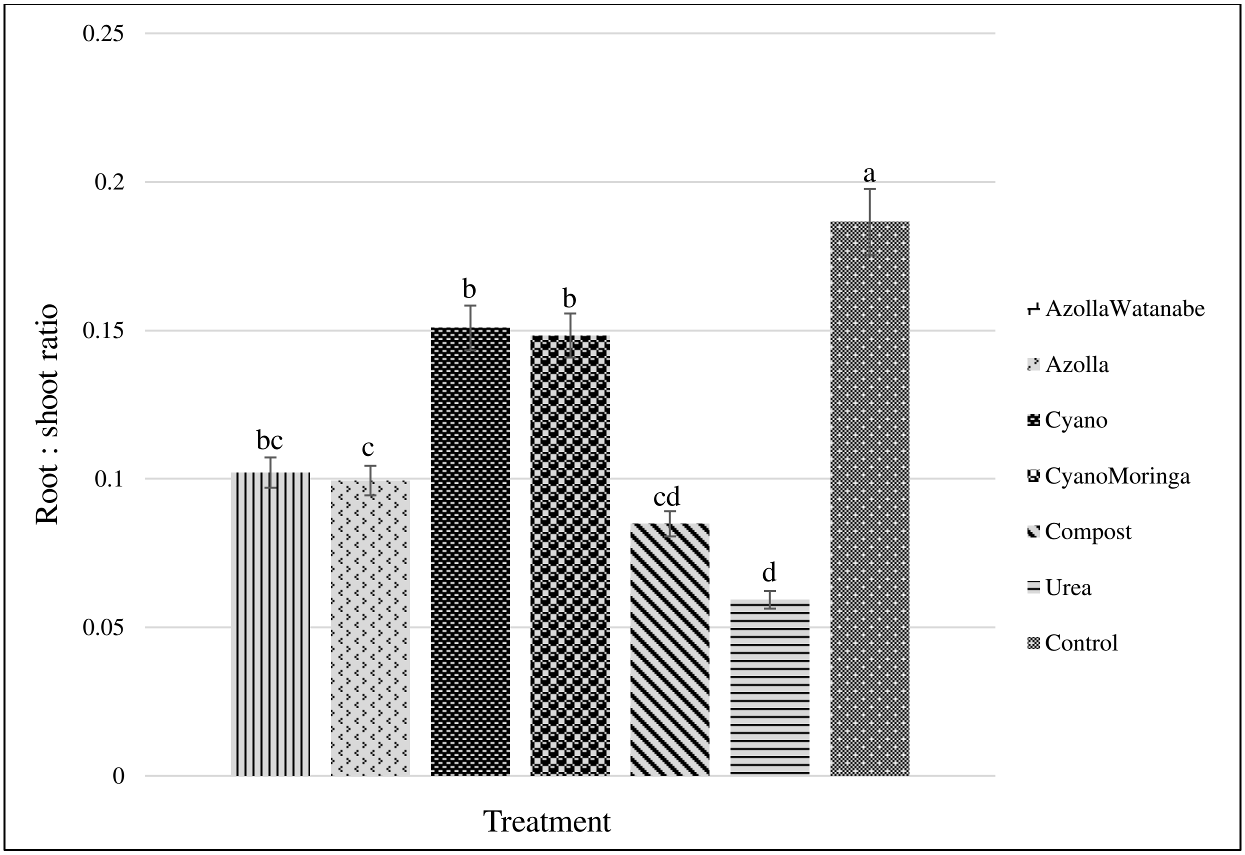

Figure 12. Root to shoot ratio of kale tissue 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

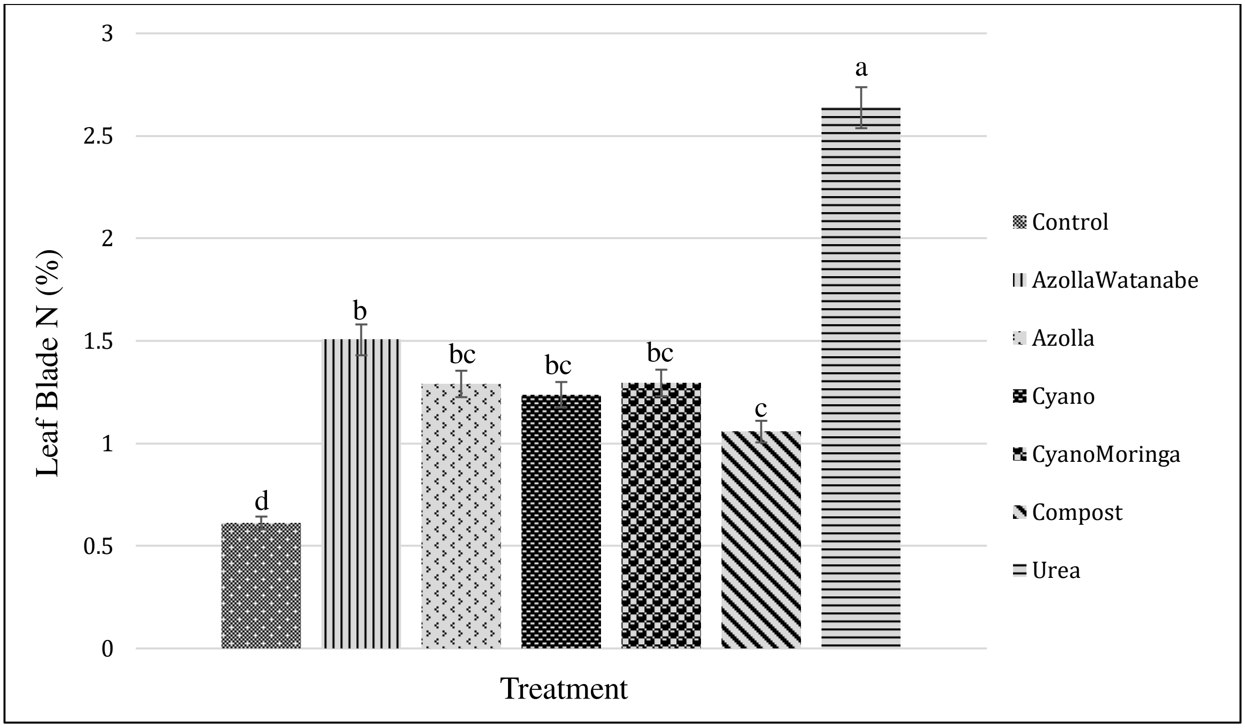

Figure 13. Leaf Blade N concentration of kale leaves 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

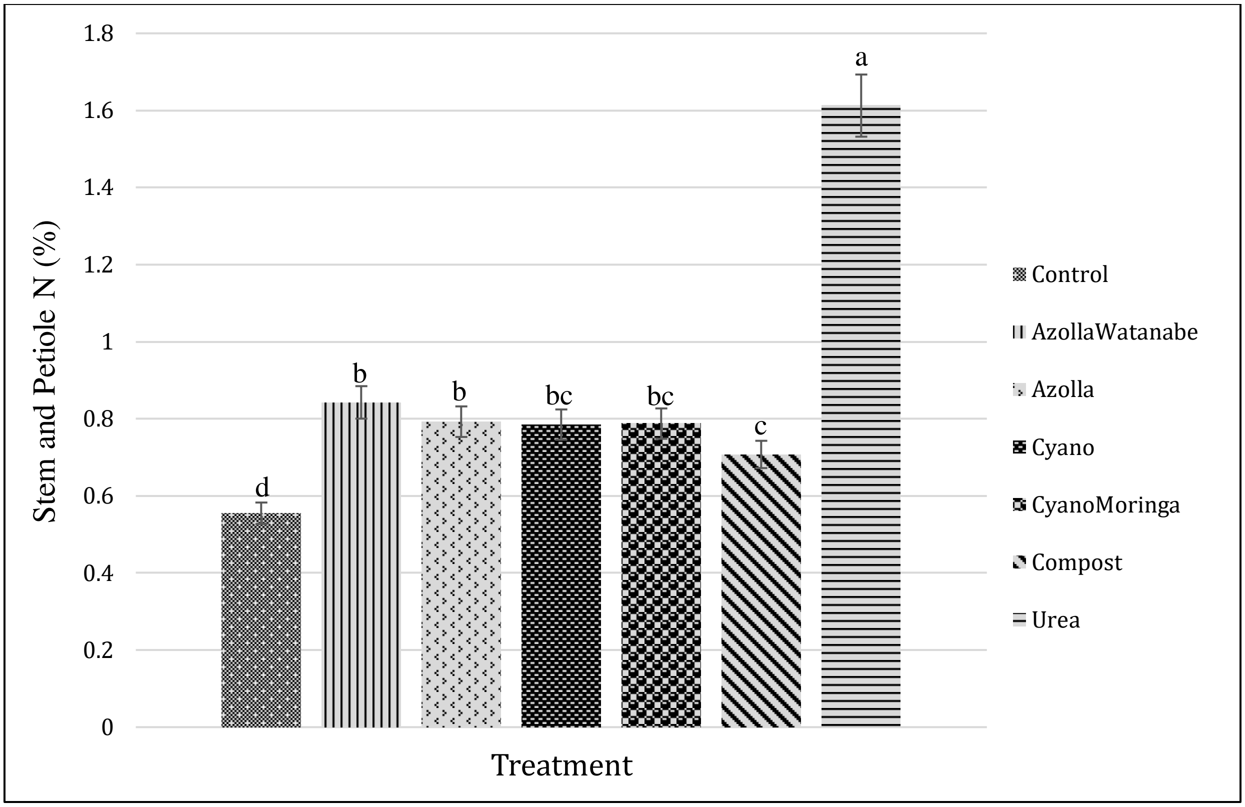

Figure 14. Stem and Petiole N concentration of kale stems 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

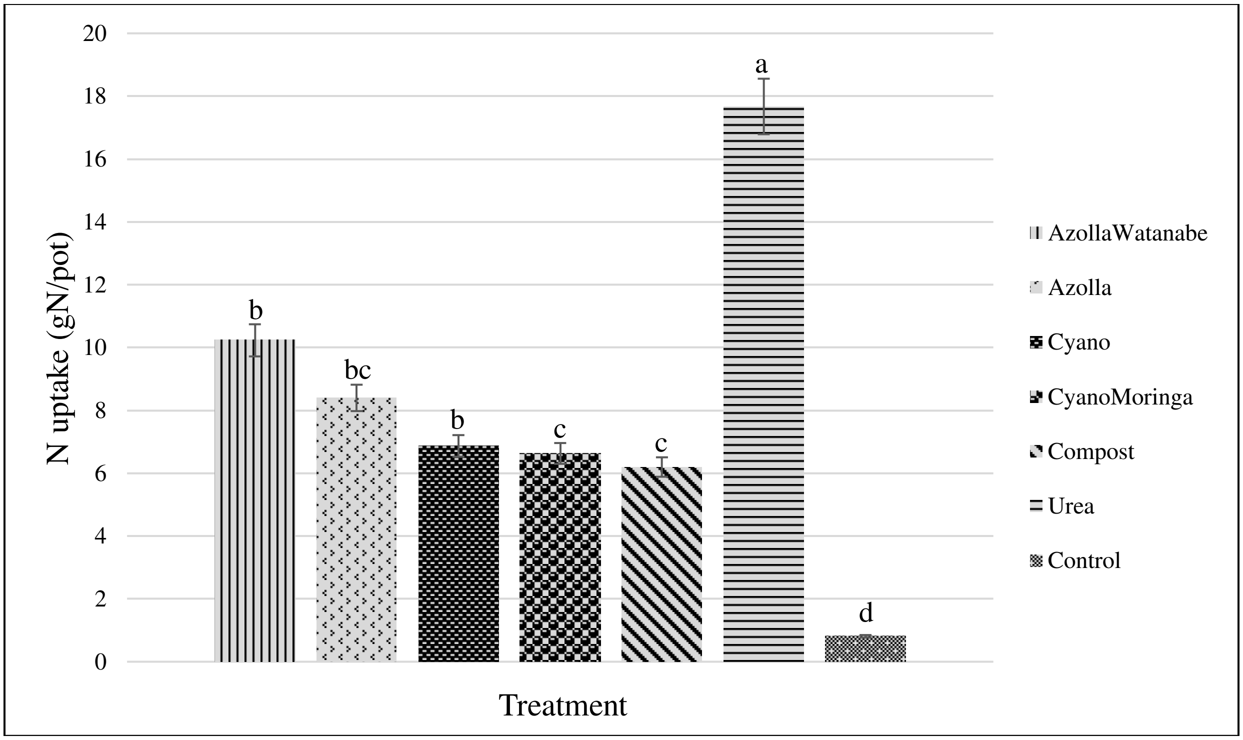

Figure 15. Total N uptake in kale tissue 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

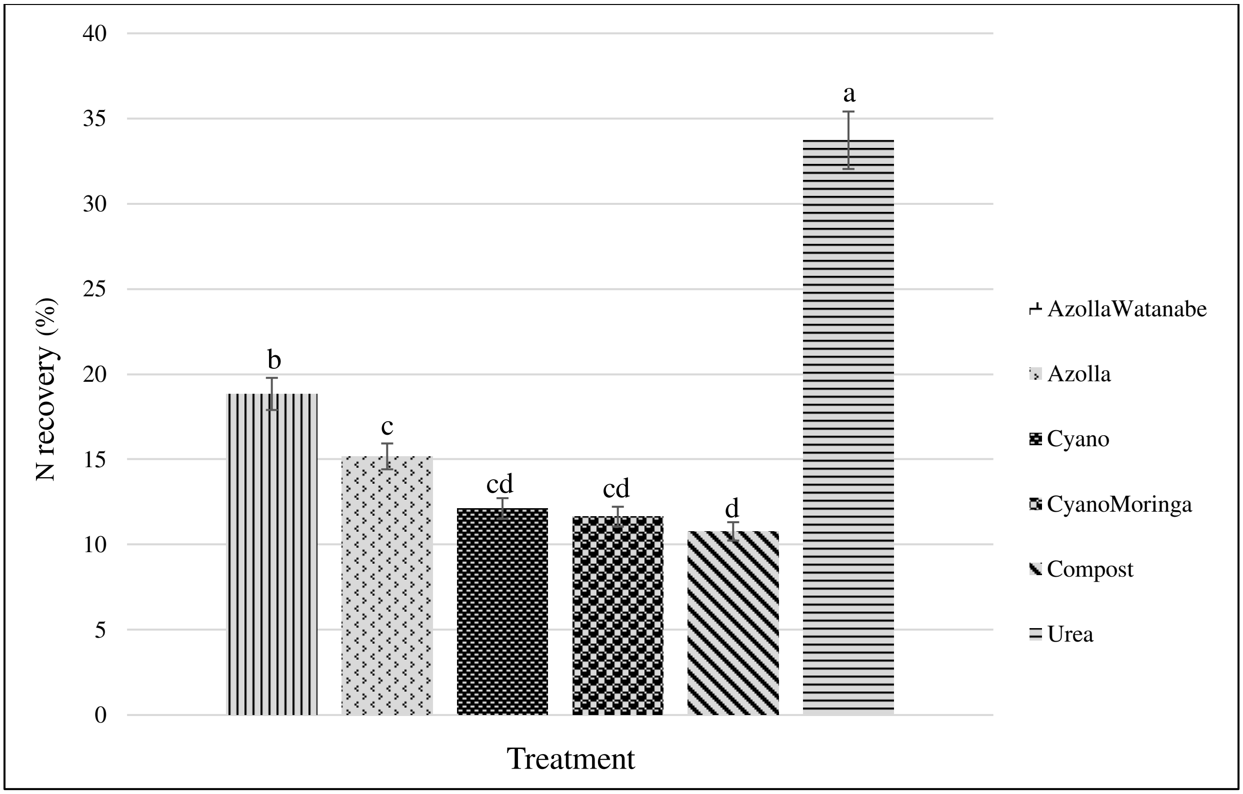

Figure 16. Nitrogen recovery percentage in above ground kale tissue 56 days after a one-time fertilizer application at 56 N kg/ha. Bars represent standard errors of mean.

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