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Utilization of *Nostoc piscinale* as Potential Biofertilizer to the Growth and Development of *Oryza sativa* L.

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Abstract

Nostoc is a blue-green cyanobacteria that produce their food through photosynthesis and nitrogen fixation. These organisms undergo nitrogen fixation and provide a potential nitrogen source for growth and development. Since rice is known as one of the world's staple foods, especially in Asia, this study aims to determine the utilization of *Nostoc piscinale* as a potential biofertilizer for planting rice crops. *N. piscinale* was inoculated into three subcultures and incubated for 87 to 170 days, and then analyzed for nitrogen-fixing activity and rice plant development. Growth of cyanobacteria showed a significant increase in chlorophyll *a* starting from day 30 up to day 170 while nitrogen-fixing activity remained constant from day 4. On the other hand, the growth and development of rice treated with cyanobacteria showed correlated trends with commercial fertilizer (CSF) in terms of root and shoot (growth and fresh weight) and chlorophyll *a* content with no statistical differences (p-value ≥ 0.05). Nitrogen tests indicate the utilization of ammonia produced by *N. piscinale* and the change in soil pH. After harvesting the samples at 20 days and measuring the soil pH, the cyanobacterial samples were seen to lower the soil pH before planting, which is significantly different from the untreated and CSF-treated samples. The utilization of nitrogen for the growth and development of *Oryza sativa* subsp. *indica* proved that *N. piscinale* would be a positive alternative source of nitrogen due to the results obtained from the soil nitrogen composition and soil pH.

Keywords: biofertilizer; cyanobacteria; nitrogen fixation; nutrient availability; rice development

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INTRODUCTION

A sustainable agricultural system provides equitable attention to economic and social issues focusing not only on agriculture but also on the impact of the same system on the environment (Brodt et al., 2011). As the term system implies, addressing one of these issues directly or indirectly affects other sectors within the system. A new approach in agriculture, called agroecology, focuses on the natural environment and is centered on sustainable production, which integrates ecology and agriculture (Martínez-Castillo, 2016). This framework may help build a paradigm that could scientifically address agricultural issues and provide sustainable alternatives for crop and food production.

The food crisis in 2008 left several countries, including the Philippines, to double the price in the last eight years alone, making it difficult for families to make ends meet due to food scarcity and economic problems (Mittal, 2009; Vaughan, 2020; Rother et al., 2022; Ouko and Odiwuor, 2023). The United Nations Secretary-General, Ban Ki-Moon, has expressed his concern about the food crisis and since then, agricultural research has taken multiple steps to alleviate this problem (Mittal, 2009). However, with a growing

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population and decreasing amount of farmland utilized for crops, the risk of a food crisis is a great obstacle. To prevent this, farmers tend to utilize fertilizers and genetically modified seeds to increase crop production. Fertilizers provide crops the nutrition they need to develop more quickly, more healthily and with higher yields. In 2014, rice production in the Philippines increased by 2.87%, due to the higher usage of chemical fertilizers. However, the overutilization of chemical fertilizers can lead to low soil quality and therefore, reduced organic matter, promoting stunting in plants, and even acidifying the soil (Bisht and Chauhan, 2020).

Rice as a crop plant has two branches of major significance: first, as a global staple of food, and second, its effect on the global rice economy (Mittal, 2009; Pernil et al., 2010; Hasan et al., 2020; Zhao et al., 2020; Ikhajiagbe et al., 2021; Mohidem et al., 2022). Rice is one of the three leading food crops worldwide, along with corn and wheat. These three crops supply more than 50% of the calories consumed by the whole human population. Rice produces 15% of the protein per capita and about 21% of global human per capita energy. It also provides minerals and fiber, which are reduced upon milling.

One solution to higher yield production in rice is to introduce the means of biofertilizers that could enhance crop yield without damaging the soil quality. One such biofertilizer is cyanobacteria, blue-green algae found in wet areas, such as rice fields (Mishra and Pabbi, 2004). Heterocystous cyanobacteria like *Nostoc piscinale* contain organelles, called heterocysts, which carry out nitrogen fixation termed "CYANOFIX". The study by Chittapun et al. (2018) found that introducing *Nostoc* cyanobacteria improved rice seedling growth and yield compared to the control group and showed a significant increase in root length. The study conducted by Ördög et al. (2021) on the biostimulating effects of the cyanobacterium *N. piscinale* on maize (*Zea mays* L.) discovered that cyanobacterium-based biostimulants are beneficial to crop production that promoted increased yield of maize that is sustainable and environmentally safe. Another interesting investigation by Prasanna et al. (2013) proved that strains of *N. piscinale* in combination with strains of other cyanobacteria such as *Nostoc carneum*, *Anabaena torulosa*, *Anabaena doliolum* using vermicompost-based carrier revealed through microscopic examination of soil enrichment cultures that, inoculation of these combined cyanobacteria significantly enhanced soil condition, including microbial biomass carbon, humus content and nitrogen, phosphorous and potassium (NPK) content, which is statistically at par with the fertilizer treatment. Since the amount of nitrogen is one of the major limiting factors in rice development, nitrogen fixation increases the nitrogen in the plant in its growing conditions, thereby enhancing the growth development of rice, as well as retaining the original composition of soil attributes, such as pH (Bisht and Chauhan, 2020).

Using chemical fertilizers to enhance the growth development and production of rice is useful for short-term benefits, beneficial, but it has negative long-term effects on soil quality maintenance (Bisht and Chauhan, 2020). Chemical fertilizers are synthesized materials that aid in the productivity of crop growth and development. However, there can be negative impacts on the soil condition. Furthermore, using chemical fertilizers may destroy the natural fertility of the soil after some time (Bisht and Chauhan, 2020; Abebe et al., 2022), thus making the method unsustainable in improving rice production and yield. On the other hand, the agroecological paradigm may aid in discovering an alternative to chemical fertilizer to a sustainable and environmentally friendly method that will improve rice production and yield. Thus, this research is designed to utilize *N. piscinale* as a potential biofertilizer for the growth and development of *Oryza sativa* subsp. *indica* and compare the efficacy of *N. piscinale* with a commercially available nitrogen standard fertilizer.

MATERIALS AND METHOD

Sample acquisition

The cyanobacterial sample utilized in the experiment was a pure culture of *N. piscinale* from the Institute of Biological Sciences of the University of the Philippines, Los Banos through Prof. Milagros Goss, Ph.D. Meanwhile, the rice seed samples for the experiment were acquired from the International Rice Research Institute in the Philippines. The seeds were confirmed and authenticated to be *O. sativa* subsp. *indica* (Variety 436) by the GenBank and Genetic Resources Center of the International Rice Research Institute.

Soil sample

The soil sample used for the experiment was rice paddy soil (clay loam) gathered from a rice

plantation in Linang, Lopez, Quezon Province, Philippines. All soil used in the experiment was sterilized and dried in an oven at 50 ºC for 40 hours and manually ground into a fine powder before experimentation.

Propagation of *N. piscinale*

N. piscinale was subcultured using liquid BG11 medium without nitrogen (BG11–N) based on the study of Chaurasia and Apte (2011) and Takacs et al. (2019) but with some minor modifications. Liquid media were prepared by mixing NaCl (0.396 M), K_2HPO_4 (0.0229 M), $MgSO_4$ (0.0623 M), $CaCl_2$ (0.0326 M), citric acid (0.00056 M), ammonium ferric citrate (0.00203 M), Na_2-EDTA (0.00269 M) and Na_2CO_3 (0.0189 M) (Sigma-Aldrich, USA). Supplemental metals (A5 trace metal solution) were added in the medium containing H_3BO_3 (0.046 M), $MnCl_2$ (0.0144 M), $ZnSO_4$ (0.00136 M), $MaMoO_4$ (0.00189 M), $CuSO_4$ (0.00501 M) and $Co(NO_3)_2$ (0.000273 M) (Sigma-Aldrich, USA). The liquid medium (100 ml) was transferred into 250 ml Erlenmeyer flasks and autoclaved at 121 °C, 115 psi. Once the flasks were cooled to room temperature, 10 ml of *N. piscinale* stock culture was introduced into each Erlenmeyer flask. The subcultures and the stock culture were then incubated at 25±3 ºC, with a cycle of 8 hours of light and 16 hours of dark. The incubator retained a relative humidity of 60 to 70%.

Determination of chlorophyll *a* content

Chlorophyll *a* content determination was based on the study of Zavrel et al. (2015) and used to measure the concentration of *N. piscinale* in the growth medium. One milliliter of *N. piscinale* sample was centrifuged at 14,000 Xg at 15 ºC for 7 minutes. The supernatant liquid was discarded and the remaining pellet homogenized with 1 ml methanol and incubated for 20 minutes at 4 ºC. After incubation, the sample was centrifuged at 14,000 Xg for 7 minutes at 4 ºC. The supernatant was read at 470, 665 and 720 nm to compute the chlorophyll *a* content (Porra et al., 1989).

Nitrogen fixing activity through acetylene reduction assay (nitrogenase activity)

In this assay, cyanobacteria used its nitrogen-fixing ability to reduce acetylene to ethylene. The assay was based on the study of Chaurasia and Apte (2011) and Larue (1973). An oxidant solution was prepared by mixing 80 ml of 0.05 M $NaIO_4$, 10 ml of 0.005 M $KMnO_4$ and 10 ml of distilled water (Sigma-Aldrich, USA). Nash reagent was prepared by mixing 150 g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of distilled acetylacetone, then diluted to 1 l (Sigma-Aldrich, USA). Acetylene gas was prepared by adding 2 ml of distilled water in a pinch amount of calcium carbide; the gas generated in the set-up was collected and stored before use in the assay procedure.

N. piscinale (4 ml) samples were placed in a 10 ml tube with a rubber stopper and approximately 3 ml of acetylene gas was introduced into the tube and incubated for 10 days. After incubation, the gas from the sealed tube was collected and placed in another sealed tube containing 1.5 ml of oxidant solution. The solution was placed in a rotary shaker at 300 rpm for 90 minutes. Sodium arsenite (250 µl, 4 M) and sulfuric acid (250 µl, 4 N) (Sigma-Aldrich, USA) were added to the solution and mixed thoroughly. Nash reagent (1 ml) was added to the solution and read at 412 nm.

Seed germination of *O. sativa* subsp. *indica*

A 15 g sample of *O. sativa* seeds was placed in an oven at 50 ºC for 5 days to stimulate the germination process of the seeds. The seeds were washed in 10 ml 70% ethanol for 90 seconds and rinsed twice with 10 ml sterilized distilled water. After overnight germination, the seeds were germinated in Petri dishes with sterilized filter paper damped with 4 ml sterilized distilled water. Each petri dish held 30 seeds for germination set-up. The seeds were then incubated at 25±2 ºC for 2 days with 8 hours light and 16 hours dark cycle. After 2 days of incubation, the sample was checked and dormant seeds were discarded.

Effect of *N. piscinale* on the growth of *O. sativa* subsp. *indica*

The seeds (5 seeds per container) were then transplanted to a 150 ml beaker containing 60 g dried and homogenized soil sample and 80 ml distilled water. The samples were incubated at 25±2 ºC temperature with 12 hours of light and 12 hours of dark cycle and kept damp with distilled water by maintaining a 3 cm water level above the soil throughout the experiment. The experiment was done in two batches; the first batch was under observation for 10 days, and the second batch was under observation for 20 days. Each batch consisted of five categorical samples in triplicates: control (no added fertilizer and sample), treatment 1 (3 ml, 5.07 µm *N. piscinale*), treatment 2 (3 ml, 4.26 µm *N. piscinale*), treatment 3 (3 ml, 7.25 µm *N. piscinale*) and commercial fertilizer (0.6725±0.03 g 70 g^{-1} soil

sample). After incubation for 10 and 20 days, the samples were harvested by washing the rice plants in distilled water. The soil was then collected for soil analysis. The growth of rice samples was determined by measuring the root length, shoot length, fresh weight and chlorophyll *a* content of the rice shoots (Malam Issa et al., 2009; Maqubela et al., 2009).

Chlorophyll *a* content determination and soil analysis

Rice leaves and shoots were collected and washed with distilled water and then cut into small pieces. Samples (100 mg) were then subjected to 25 ml 80% buffered acetone extraction and incubated for 27 hours in a cool and dark environment. Samples were then filtered using Whatman filter paper no. 1 and the absorbance of the filtrate was read at 664 and 647 nm (Pramanik and Bera, 2013; Yepremian et al., 2016; Pagels et al., 2021; Sasadara et al., 2021). Soil from the set-up of the experiment was collected and placed in vials for soil analysis. Soil analysis specifically pH and nitrogen test (Kjeldhal method) were done by the Analytical Services Laboratory of the Department of Chemistry, University of the Philippines Los Baños.

Statistical analyses

Multivariate analysis of variance (MANOVA) was used as a statistical test for the presence of a significant difference between one or more characteristics between groups. The independent variables were the cyanobacterial strain used, the standard urea fertilizer and the control rice sample. The dependent variables were the root length, shoot length and the chlorophyll *a* content of the rice after 10-day incubation and 20-day incubation.

To test for the significance between variables throughout the experiment, a Spearman's rho correlation (two-tailed test) was subjected to the results. The significance within and between samples was subjected to an independent T-test to determine the significance of the results acquired at different harvest periods.

RESULTS AND DISCUSSION

Morphology and growth of *N. piscinale*

N. piscinale is a heterocystous strain of filamentous cyanobacteria that divides in only one plane and contains heterocysts locally found in tropical soils and naturally found in rice paddy environments. They are ubiquitous, mostly found in aquatic sites, though some extremophiles are also known to be found in areas of extreme temperature or salinity (Ananya and Ahmad, 2014). They make use of various accessory pigments, such as phycocyanin and allophycocyanin, which give most cyanobacteria their characteristic "blue-green" color due to their ability of nitrogen fixation and photosynthesis (Vincent, 2009; Ananya and Ahmad, 2014).

Since these organisms are pigmented and the cells are small and arranged in a chain-like projection, an alternative way to quantify the number of microalgal cells was through its chlorophyll *a* content, which is measurable under the UV spectrum. The growth curve of the cyanobacteria was observed for 170 days as part of the production before the incorporation of the cells into the soil for nitrogen fixation. The growth pattern of cyanobacteria and cellular density was observed through its chlorophyll *a* absorbance reading. The total chlorophyll concentration is used to measure filamentous cyanobacteria since the number of cells cannot be determined and counted (Li et al., 2014; Baracho and Lombardi, 2023). Figure 1 shows there is an observable latent period between 0 to 21 days, which suggests that the cyanobacterial subculture was still adjusting to its new medium and adjusting itself with the incubation light cycle time frame of 8 hours of light or 16 hours of dark at 25±2 °C with 60 to 70% relative humidity (Chaurasia and Apte, 2011; Takacs et al., 2019). At around 20 days after subculture, all three samples started to increase their cell density, before decreasing again for another slowly increasing period of 2 weeks. The decrease and slow progression in cell density after 30 days of incubation was because of the formation and differentiation of heterocysts and release of chlorophyll by dead cells (Aguilera et al., 2021). At day 87, the subculture with the highest chlorophyll concentration was 3SB with a concentration of 7.25 µm followed by 1SB with 5.07 µm and 2SB with 4.26 µm. The growth curve determined based on the concentration of chlorophyll was performed instead of determination by cell counting because it is difficult to perform cell counting (Baracho and Lombardi, 2023). It is shown in Figure 1 that there are no significant differences among the three subcultures based on the chlorophyll content that is associated with its growth. The produced cyanobacterial cells with that concentration were used for the nitrogen fixation analysis and the analyses of the growth and development of rice.

Nitrogen fixation activity of *N. piscinale* through nitrogenase activity

Nitrogenase, scientifically known as flavodoxin or dinitrogen oxidoreductase, is composed of two parts: the first is an iron metalloprotein, a dinitrogenase iron-molybdenum cofactor biosynthesis known as dinitrogen reductase, and the second is a Fe-Mo metalloprotein, with the cofactor dinitrogenase (Issa et al., 2014; Fulweiler et al., 2015). This mechanism, which is found in heterocysts, takes place in an aerobic environment. Various cyanobacterial species are known to possess nitrogen-fixing activity even without a heterocyst (Wiig et al., 2014).

The utilization of the nitrogen-fixing capacity of *N. piscinale* was measured through its nitrogenase activity, which reduced acetylene to ethylene. The concentration of chlorophyll *a* measured was noted and used as a reference for the nitrogen fixation activity of the cyanobacteria for 10 days.

Figure 2 shows the incubation period from 0 to 8 days shows the relatively stable activity of the cyanobacteria to fix the acetylene to ethylene. At day 4; however, subculture 2SB (4.26 µm) had peak activity, which then decreased by day 6. Subculture 1SB was noticed to exhibit peak activity at day 2 up to day 8, then a sudden decrease in activity at day 10 (Figure 2). Subculture 3SB was steady in its activity until it reached a slightly lower plateau from days 2 to 8, and then rose to its peak activity on day 10. Among the three subcultures, 1SB performed the best nitrogen-fixing abilities even though it had the best nitrogen-fixing ability. The three subcultures showed no significant differences in terms of their activities. This observed behavior of inconsistent results in the nitrogen fixation activity of the microorganism is attributed to the factor that when nitrogen fixation happens the product will then be used by the microorganism as part of its growth and differentiation. Another factor that also plays a role is nutrient starvation, wherein nutrients for nitrogen fixation are fully consumed during *in vitro* culturing (Aguilera et al., 2021). The subcultures of cyanobacteria with different concentrations showed abrupt changes in their nitrogen fixation activities due to the adaptation of the cells in the environment; nitrogen sufficiency and cell density may also affect the activity, as well as the presence of heterocysts where the nitrogen fixation activity happens (Thiel and Pratte, 2001).

The nitrogenase assay provides an illustration of the nitrogen fixing cyanobacteria at work in the atmosphere. Since cyanobacteria fix nitrogen in the same way, reducing acetylene into ethylene, this was an ideal assay to picture the dynamics of cyanobacteria making it suitable to be used as a biofertilizer and may affect the growth and development phase of rice, as well as in the soil nitrogen and pH during soil analysis (Hardy et al., 1968; Berman-Frank et al., 2003; Issa et al., 2014; Fulweiler et al., 2015).

Soil infertility is a common consequence of agricultural practices worldwide. Maintaining soil quality can help maintain the production of important food crops such as rice. Using biofertilizers as a possible solution for the

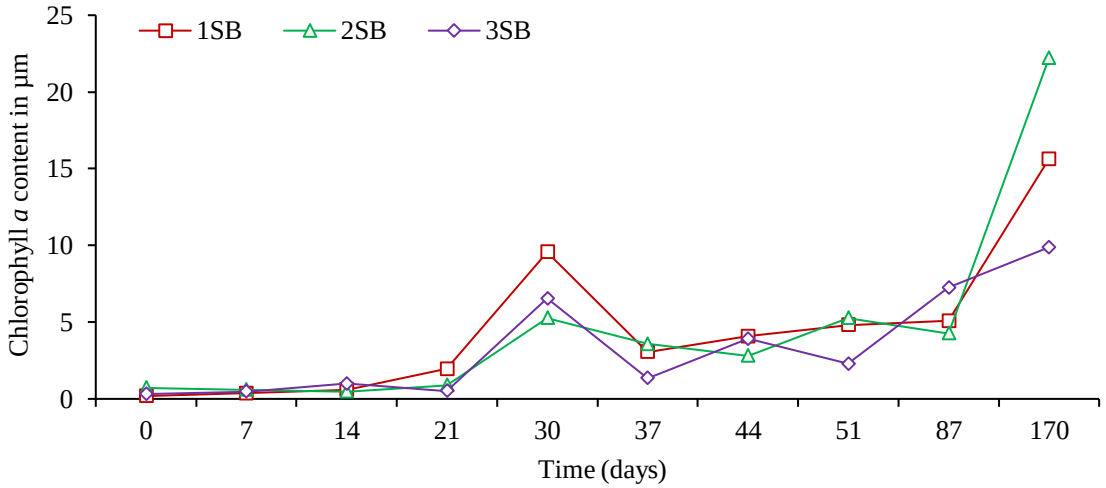

Figure 1. The growth curve of *N. piscinale* over a period of 170 days based on the chlorophyll *a* content of the cell density

Note: SB = Subculture of *N. piscinale* from a mother stock culture, 1SB = 5.07 µm, 2SB = 4.26 µm, 3SB = 7.25 µm

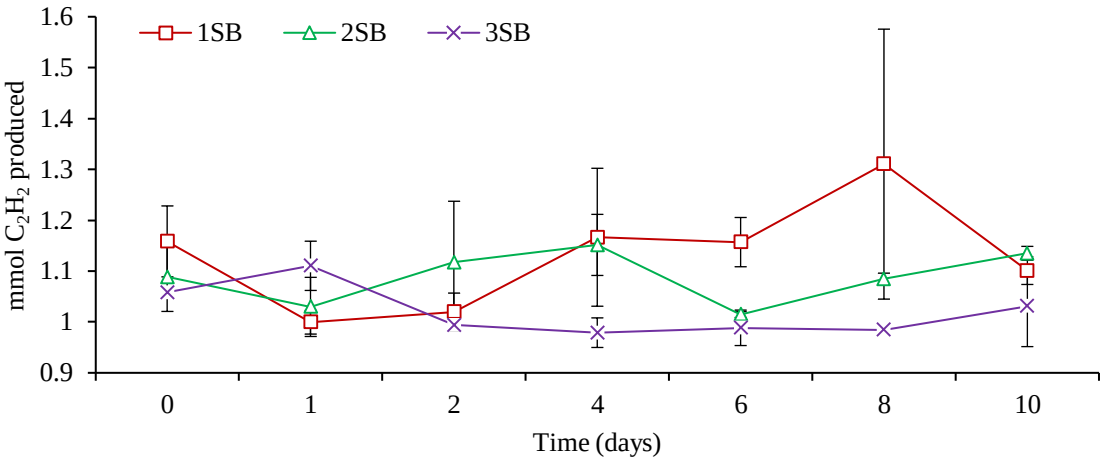

Figure 2. Nitrogen fixation activity of *N. piscinale* through its nitrogenase activities for 10 days
Note: SB = Subculture of *N. piscinale* from a mother stock culture, 1SB = 5.07 µm, 2SB = 4.26 µm, 3SB = 7.25 µm

decrease in soil conditions has led to the research of natural sources of nutrients for the environment (Prasanna et al., 2012). Heterocystous cyanobacteria have been researched to become one of the most prominent components of retaining soil conditions compared to chemical fertilizer (Anees et al., 2014). Soil-borne cyanobacteria, such as *N. piscinale*, are one of two commonly found heterocystous cyanobacteria found in rice paddies and are known for their high rate of nitrogen-fixing activity. They aim to mediate the soil conditions in which they are inoculated and have been researched to restore soil pH and combined nitrogen (Larue, 1973; Thiel and Pratte, 2001; Kumar et al., 2009).

Growth and development of *O. sativa* subsp. *indica*

The growth and development of rice can be classified under three main headings: the vegetative phase, the reproductive phase and the ripening stage. Its growth and development can be measured through its root and shoot length, and its refresh height of the root and shoot after harvest. The growth of *O. sativa* at the vegetative stage can be determined through the fresh weight and the length of the shoots and roots harvested. The root length denotes the nutrient and water availability for the crop, while the shoot corresponds to the food production process of *O. sativa* as it photosynthesizes its food through the chlorophyll present (Change and Bardenas, 1965; Pramanik and Bera, 2013).

Soil with rice planted was treated with three subcultures of *N. piscinale* having a chlorophyll *a* content of 5.07 µm (1SB), 4.26 µm (2SB) and 7.25 µm (3SB) and incubated for 10 and 20 days. Growth and development of rice were documented during the treatment for 10-day incubation and 20-day incubation (Figure 3).

A steady growth pace in root length was observed among samples until the 10th day. The sample 1CB (cyanobacterial treatment) maintained the longest length after 10 days and showed a significant difference with the rest of

Figure 3. Growth of *O. sativa* subsp. *indica* treated with cyanobacteria (*N. piscinale*) from 0-day to 20-day incubation (from left to right)

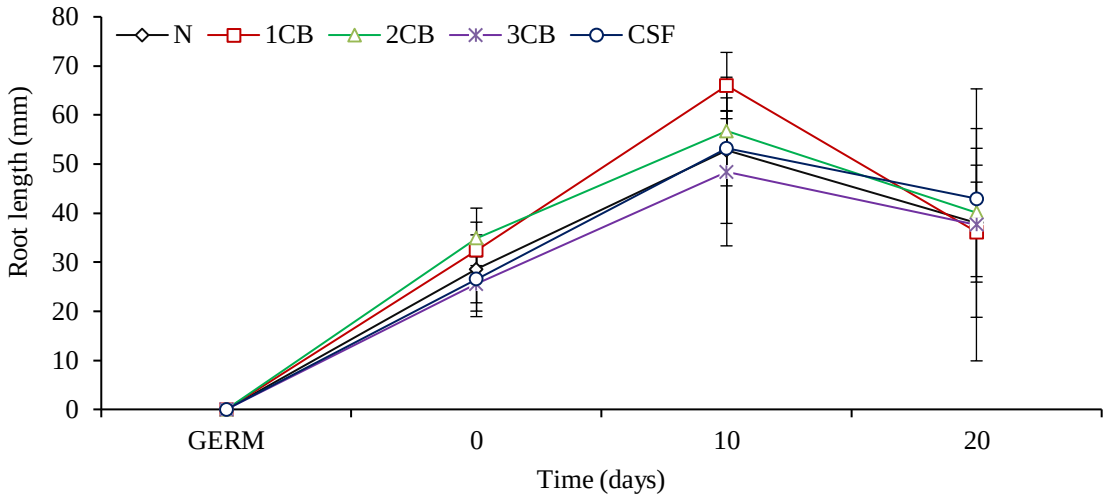

Figure 4. Root growth (length, mm) of *O. sativa* subsp. *indica* treated with cyanobacteria (*N. piscinale*, 1CB, 2CB, 3CB) from 0 to 20-day incubation
Note: N = Normal condition, CB = *N. piscinale* treatment, CSF = Commercial fertilizer

the group treatments (p-value of 0.011) as seen in Figure 4, but as seen also in the figure that there was no significant difference between the commercial fertilizer (CSF) treated and cyanobacteria treated rice plants ($p \geq 0.05$). According to El-Sheekh et al. (2018), the root growth of rice is enhanced when treated with cyanobacteria and when inoculation size increases.

Root elongation and development are necessary to procure water and nutrients from the soil to promote growth and production (Seo et al., 2020). The root hairs produced in plants can increase root surface area and are responsive to changes in water reduction and soil and nutrient availability (Seo et al., 2020). As the *O. sativa* subsp. *indica* plant grew from 0 to 10 days, an increase in the growth of the roots was observed and expected since the plant requires root length to elongate to procure water and nutrients. After the 10-day growth period, the roots harvested after 20 days were seen to have decreased in length (Figure 4), either suggesting a water reduction or a loss in soil nutrients, since the plants were watered *ad libitum* throughout the growth period to ensure at least 2 to 4 cm of water above the soil throughout the planting stage. Reduction in soil nutrients limits the nutrient uptake of roots and decreases the root's length aside from the abiotic stress (Rani and Sukumari, 2013). This decrease in root length can also be attributed to the microenvironment where the plant was introduced, which is significantly smaller than the traditional rice plot or paddy rice usually introduced to.

Another reason for the decrease in root length is the presence of root hairs; tiny tertiary roots that sprout after the primary and secondary roots of the plant. These roots are very fine and are used in nutrient uptake (Seo et al., 2020). However, the root hairs were too delicate and washed away during harvest, making the length of the roots appear less after 20 days.

The fresh weight of the root was gathered right after harvest and length measurement. It was seen that there was a direct relationship between the length of the roots and their fresh weight after the correlation test having a correlation coefficient of 0.851 (Sig. value of 0.00015). All of the rice samples significantly increased the weight of their roots (p-value ≤ 0.05) except for 3CB treatment after 10 days of incubation (Figure 5). The decrease in root length was also seen in the decrease of the fresh root weight after 20 days of incubation (Figure 4 and 5). It can also be seen in Figure 5 that there was no significant difference (p-value ≥ 0.05) in the fresh root weight of the untreated rice (normal) and treated rice (CB and CSF) after 20 days of incubation.

Figure 6 showed that the shoot length of the rice samples was measured after the specified growth periods, and a similar trend was seen from germination to 10 days with the root length with a correlation coefficient of 0.550 (sig. of 0.03415). All samples underwent a similar trend of increasing length from germination to 10 days, which is to be expected as the rich shoot develops. After the 10-day growth period, there was a variation in the length of the shoots, with CSF still maintaining its longest shoot length, followed

by 2CB and 3CB compared to the normal sample (p-value ≤ 0.05). Only the cyanobacterial subculture, 1CB, showcased a decrease in shoot length (p-value ≤ 0.05), which has a concentration of 5.07 µm chlorophyll *a* present with correlation coefficient of 0.680 (sig. of 0.00515). Although there is a sudden change in the shoot length of rice, based on Figure 6, there was no significant difference in the shoot length of rice when treated with CSF and CB. Same with root growth, shoot length increases significantly when treated with cyanobacteria (*Nostoc calcicola*, *Anabaena variabilis*, *Nostoc linkia*) for 40 days according to El-Sheekh et al. (2018).

The shoot of the rice plant is the source of food production for *O. sativa* subsp. *indica*. As the plant grows, the shoot length elongates, increasing its capacity to produce its own food. The common trend from 0 to 10 days was seen with the dramatic increase in shoot length across all samples. The variation in shoot length after the 10-day period was due to the nutrient availability of the soil and was a sign that the seedling was already maturing to the transplanting stage, in which it needed a larger environment to continue toward maturation.

Based on the weight of fresh shoots, 1CB and 2CB treatments were seen to have greater fresh weight after 10 days of incubation against the other treatments but are not statistically significant (p-value ≥ 0.05) (Figure 7). However, the fresh weight of the rice shoot was seen to be increasing in all samples since the plant is continuously growing. The shoot length directly

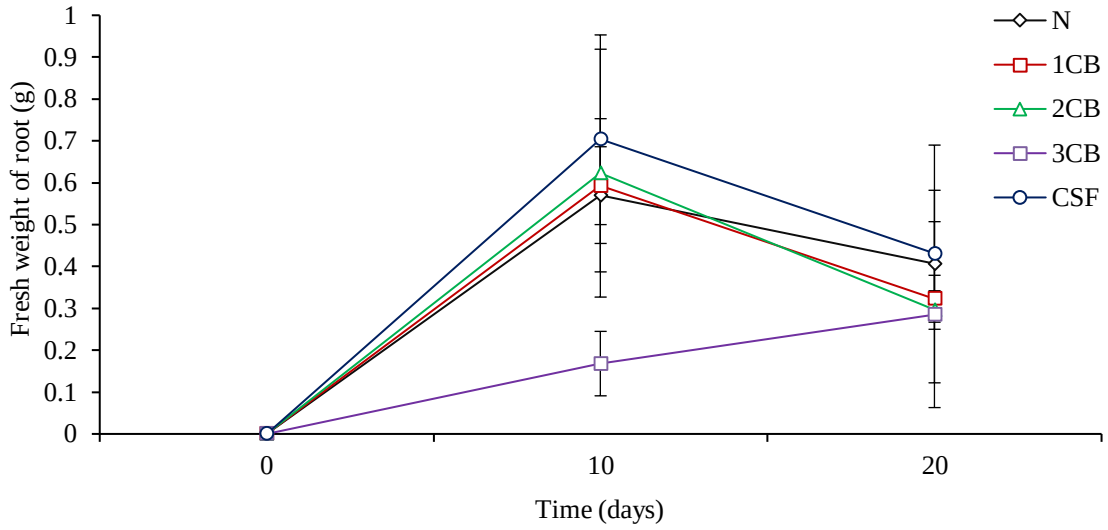

Figure 5. Root fresh weight (g) of *O. sativa* subsp. *indica* treated with cyanobacteria (*N. piscinale*, 1CB, 2CB, 3CB) from 0 to 20-day incubation
Note: N = Normal condition, CB = *N. piscinale* treatment, CSF = Commercial fertilizer

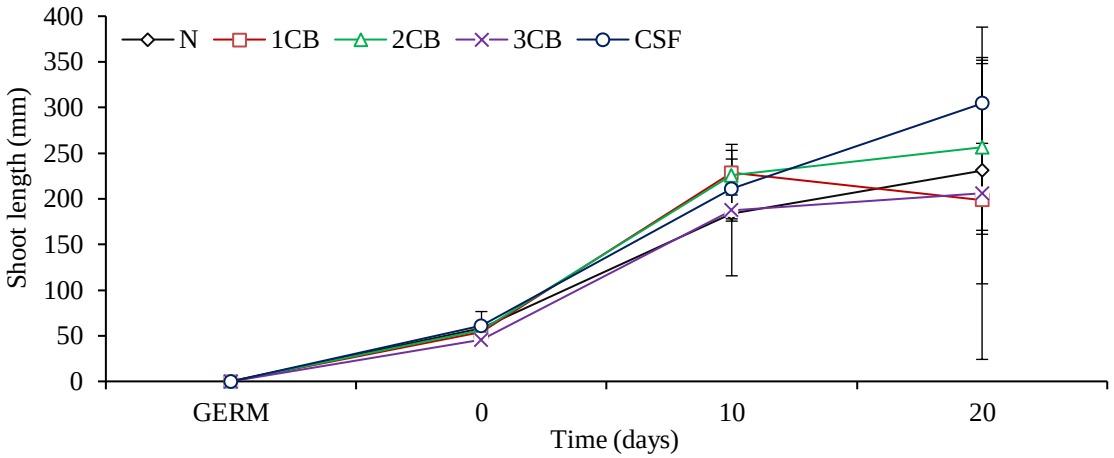

Figure 6. Shoot growth (length, mm) of *O. sativa* subsp. *indica* treated with cyanobacteria (*N. piscinale*, 1CB, 2CB, 3CB) from 0 to 20 days incubation
Note: N = Normal condition, CB = *N. piscinale* treatment, CSF = Commercial fertilizer

correlates with the fresh weight, in which the longer the shoot, the heavier the fresh weight of the shoots with a correlation coefficient of 0.764 (sig. of 0.00115). The decrease in shoot fresh weight is also a consequence of the seedling's development to the transplanting stage, where it will need a larger environment to continue its growth into a mature plant. The normal (untreated) and CSF samples are heavier than the CB-treated plants after 20 days of incubation. Although they showed heavier mass, this is not statistically significant with the CB-treated plants (p-value ≥ 0.05) (Figure 7).

Chlorophyll *a* content of *O. sativa* subsp. *indica* leaves and soil analyses

The development of rice was measured through its chlorophyll *a* content in its leaves after two growth periods of 10 and 20 days. Chlorophyll *a* content measures the development of the rice plant as it grows to advance into another stage of plant development (Li et al., 2014; El-Sheekh et al., 2018; Baracho and Lombardi, 2023). As the rice plant grows from germination, an increase in chlorophyll *a* content can be observed (Li et al., 2014; Baracho and Lombardi, 2023). This remains steady until it is ready for the next stage of development, when the leaves thin out and become longer, and lighter in color, resulting in a lighter green shoot color, and a decrease in chlorophyll *a* content. Previous studies show that a decrease in chlorophyll *a* content decreases as it readies for further development.

The development of *O. sativa* is commonly measured by the chlorophyll *a* content of the rice shoots. Several methods have been utilized in extracting chlorophyll *a* from the leaves and reading its absorbance from a set of wavelengths (nm). The method observed to give the highest percent quality extraction was the 80% buffered acetone extraction. A study extracted chlorophyll from rice leaves through several methods, and

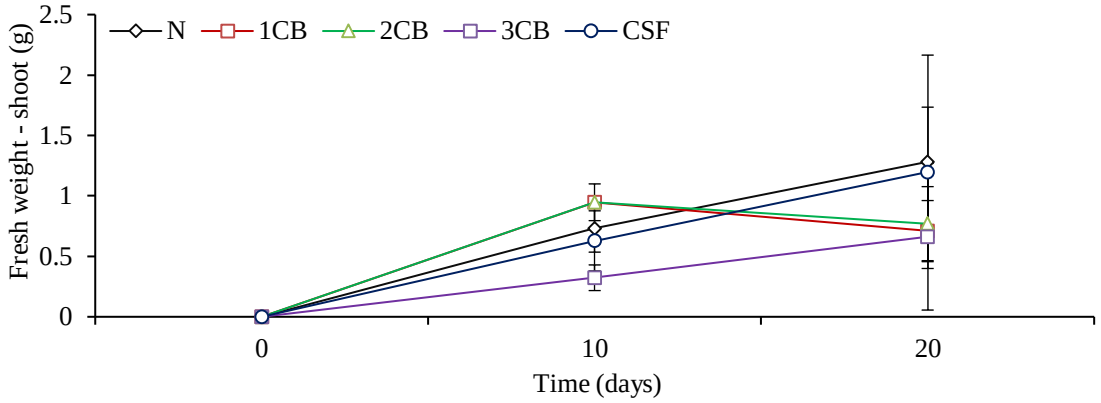

Figure 7. Shoot fresh weight (g) of *O. sativa* subsp. *indica* treated with cyanobacteria (*N. piscinale*, 1CB, 2CB, 3CB) from 0 to 20-day incubation
Note: N = Normal condition, CB = *N. piscinale* treatment, CSF = Commercial fertilizer

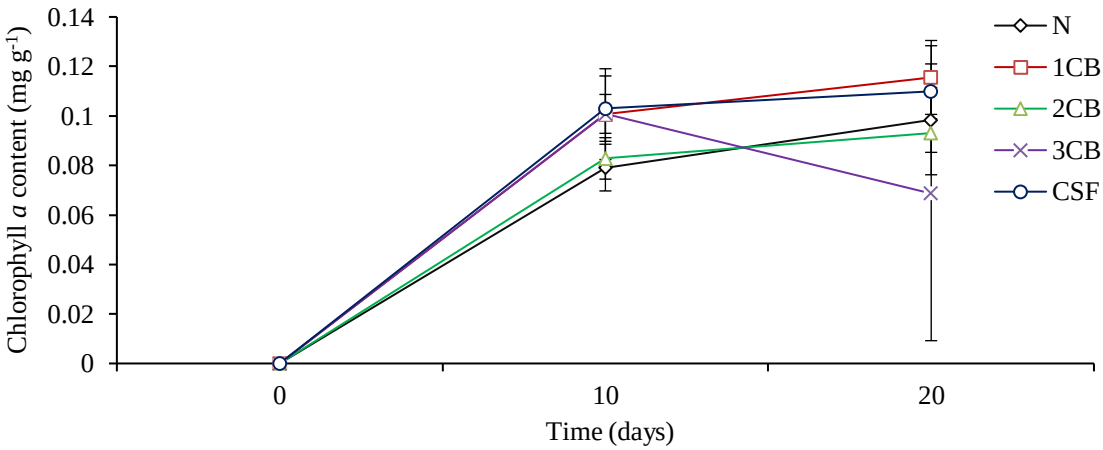

Figure 8. Chlorophyll *a* content (mg g^{-1}) of *O. sativa* subsp. *indica* treated with cyanobacteria (*N. piscinale*, 1CB, 2CB, 3CB) from 0 to 20 days incubation
Note: N = Normal condition, CB = *N. piscinale* treatment, CSF = Commercial fertilizer

the 80% acetone extraction gave a 24% higher yield (Porra et al., 1989; Pramanik and Bera, 2013).

As seen in Figure 8, there was an increasing trend in chlorophyll *a* content for all samples from germination to the first harvest at 10 days with a significant difference from the untreated sample (p-value ≤ 0.05). A change in chlorophyll content was seen after 20 days of incubation wherein the cyanobacterial subculture 3CB was seen to have a significant decrease (p-value ≤ 0.05) in chlorophyll *a* content while other treatments showed a continuous increase.

In this research, the study was lengthened to 20 days to provide a comparison between planting (day 0), its halfway point (day 10), and the end of experimentation (day 20). Hence, after 20 days, a decrease in the chlorophyll *a* content of the shoots of the rice sample was aimed as the primary observation. The sample which showed a decrease after the 10-day growth period was 1CB. Compared with this, the other 2 cyanobacterial samples increased their chlorophyll *a* content, while the CSF and normal sample have a trend of an increase in their chlorophyll *a* content even after 20 days, thus indicating the need for further maturation and still has room for development before the next stage of rice development and reaching the stage of decreasing chlorophyll *a* content.

The difference in chlorophyll *a* content of all treatments is not statistically significant after 20 days of incubation maybe because the plant is still in its early stage and is not fully matured to analyze its chlorophyll *a* content. Based on a previous study, the chlorophyll *a* content of rice seedlings decreases slightly after 15 days, indicating another step or maturation in its growing stage (Porra et al., 1989; Pramanik and Bera, 2013).

To determine the actual changes in the microenvironment as the rice seeds underwent growth and development during the two growth periods, the harvest of samples led to the question of the difference in soil nitrogen before and after the experiment. The Kjeldhal nitrogen of soil measures the total amount of nitrogen present in a sample. This was compared with the soil of the original environment before experimentation and with each sample that underwent inoculation of different concentrations of cyanobacteria and the inoculation of the commercial standard fertilizer.

Ideally, the more Kjeldhal nitrogen present in the soil, the less ethylene was utilized by the plant. This inverse relationship pertains to the utilization of the rice plant of the nitrogen present in the soil, provided by the cyanobacterial subculture through nitrogen fixation. In another sense, a high amount of ethylene produced and a high Kjeldhal nitrogen value can indicate the presence of nitrogen that still needs to be utilized by the plant (Thiel and Pratte, 2001; Wiig et al., 2014; Aguilera et al., 2021).

In Figure 9, subcultures 1CB and 2CB both relatively have higher amounts of ethylene

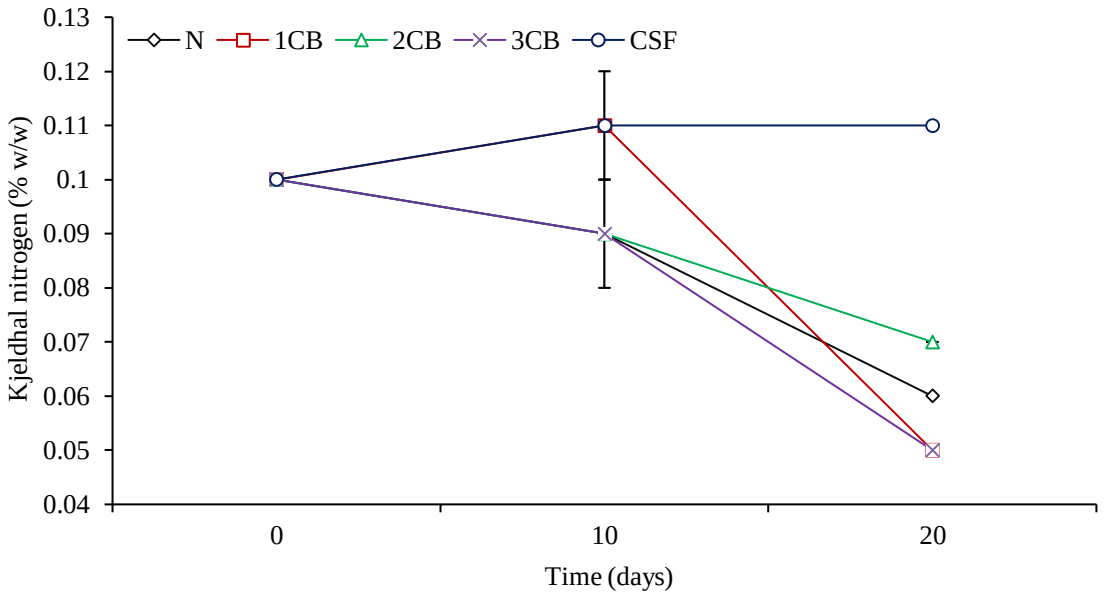

Figure 9. Nitrogen content of soil treated with cyanobacteria (*N. piscinale*, 1CB, 2CB, 3CB) from 0 to 20-day incubation

Note: N = Normal condition, CB = *N. piscinale* treatment, CSF = Commercial fertilizer

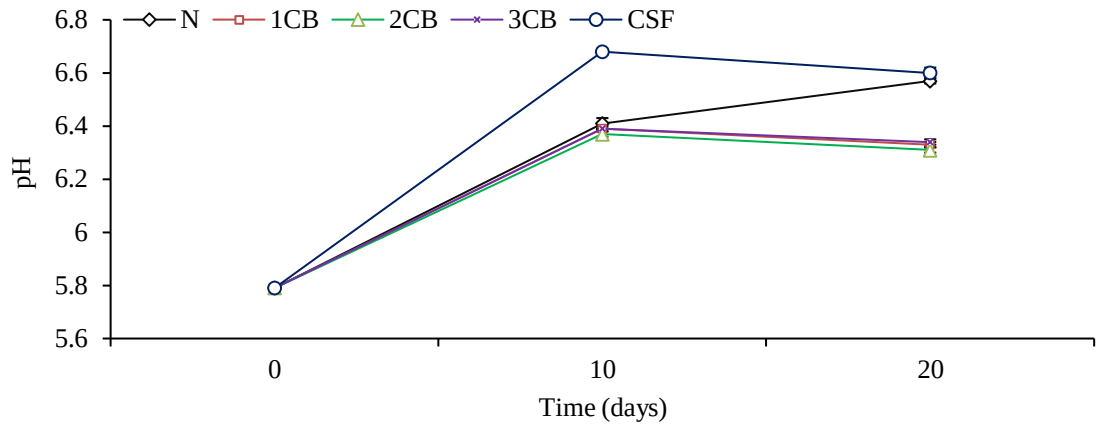

Figure 10. Soil pH before and during treatment with cyanobacteria (*N. piscinale*, 1CB, 2CB, 3CB) from 0 to 20-day incubation
Note: N = Normal condition, CB = *N. piscinale* treatment, CSF = Commercial fertilizer

produced at 0 days compared to 3CB, which had the least amount of ethylene produced at 0 days. After 10 days, there was a significant increase in the ethylene produced in 1CB and CSF (p-value ≤ 0.05), with a stable observation of the amount of ethylene produced in the 2CB sample, while the rest of the treatments showed lower soil nitrogen content at 10 days of incubation compared to 1CB and CSF (p-value ≤ 0.05). This indicates that the nitrogen being produced by the cyanobacteria is being used, causing a decrease in soil nitrogen after 10 days. However, the 1CB sample had an increase in both the Kjeldhal nitrogen and the ethylene produced after 10 days, indicating the presence of nitrogen still available for utilization after harvest.

The pH was analyzed at the same time as the test for Kjeldhal nitrogen of the soil samples. The pH of the soil increased from inoculation to harvest after 10 days (Figure 10). After the 20 days of harvest period, there was a decrease in the pH, which indicated the utilization of nitrogen and the soil restoring capacity of the cyanobacteria to bring back the soil to the original pH. The CSF sample still had the highest pH after 20 days, indicating the presence and steady utilization of the chemical fertilizer in the soil. The normal sample was also observed to have an increase in pH. The three cyanobacterial samples, however, all showcased a significant decrease in the pH of soil compared to untreated or normal samples and CSF-treated samples (p-value ≤ 0.05), heading back to its original pH after harvest (Figure 10). This indicates a potential capability of the cyanobacteria to restore soil to its original pH, or original state, as it was before harvest, unlike the CSF sample.

Basic compounds alkalize the environment in which the plant is located. Although soil alkalinity indicates ample supply of nitrogen which is necessary for the growth of *O. sativa*, however high alkalinity may affect soil pH, which may result to unsuitable condition for future planting.

After 20 days, a decrease in the pH was seen for the samples inoculated with the cyanobacterial subcultures (Figure 10). The pH of the soil may return to its original pH after a longer period of time, which is not included in this study. As a biofertilizer, the change in pH back to the value found at day 0 implicates that the *N. piscinale* is capable of reviving the soil to original (before planting) conditions, unlike the chemical fertilizer, which, though slightly decreased after 20 days, still remained at a higher

Table 1. Tests of between-subjects effects through MANOVA analysis

Dependent variable	Sig.	Noncent. parameter	Observed power
Days before harvest	.000	72.300	.999
Root length	.000	343.068	1.000
Shoot length	.011	29.767	.899
Fresh weight root	.017	25.626	.845
Fresh weight shoot	.001	55.525	.995
Chlorophyll *a* of rice	.000	666.069	1.000
pH of soil	.000	311.807	1.000

Table 2. Spearman's rho correlation analysis of subjects on the plants' root length, shoot length, fresh weight of root, fresh weight of shoot and its chlorophyll *a* content

		RL	SL	FWR	FWS	pH	CHL*a*	Days
RL	Correlation coefficient	1.000	0.550*	0.851**	0.829**.	0.729**	0.607*	0.567*
	Sig. (2 tailed)	.	0.034	0.000	0.000	0.002	0.016	0.28
	N	15	15	15	15	15	15	15
SL	Correlation coefficient	0.550*	1.000	0.560*	0.764**	0.548*	0.680**	0.718**
	Sig. (2 tailed)	0.034	.	0.030	0.001	0.034	0.005	0.003
	N	15	15	15	15	15	15	15
FWR	Correlation coefficient	0.851**	0.560*	1.000	0.789**	0.774**	0.615*	0.577*
	Sig. (2 tailed)	0.000	0.030	.	0.000	0.001	0.015	0.024
	N	15	15	15	15	15	15	15
FWS	Correlation coefficient	0.829**	0.764**	0.789**	1.0000	0.672**	0.726**	0.808**
	Sig. (2 tailed)	0.000	0.001	0.000	.	0.006	0.002	0.000
	N	15	15	15	15	15	15	15
pH	Correlation coefficient	0.729**	0.548*	0.774**	0.672**	1.000	0.724**	0.713**
	Sig. (2 tailed)	0.002	0.034	0.001	0.006	.	0.002	0.003
	N	15	15	15	15	15	15	15
CHL*a*	Correlation coefficient	0.607**	0.680**	0.615*	0.726**	0.724**	1.000	0.828**
	Sig. (2 tailed)	0.016	0.005	0.015	0.002	0.002	.	0.000
	N	15	15	15	15	15	15	15
Days	Correlation coefficient	0.567*	0.718**	0.577*	0.808**	0.713**	0.828**	1.000
	Sig. (2 tailed)	0.028	0.003	0.024	0.000	0.003	0.000	.
	N	15	15	15	15	15	15	15

Note: RL = root length, SL = shoot length, FWR = fresh weight of roots, FWS = fresh weight of shoot, CHL*a* = chlorophyll *a* content

value than the other samples. The biofertilizer capacity of heterocystous cyanobacteria can be seen in the effort of the sample to restore the soil conditions (Thiel and Pratte, 2001; Kumar et al., 2009; Banayo et al., 2012).

Previous studies have shown that nitrogen in fertilizer has been used to increase the rice yield during one harvest. In Mexico, the use of chemical fertilizers has led to a steady decrease in soil quality and ultimately to a lower yield. As an alternative solution, these Mexican farmers turned to biofertilizers as a main source of nitrogen, which cuts down the amount of chemical fertilizer utilized for a higher rice yield (Bhardwaj et al., 2014). This is in line with the growing need for fertilizer, which according to the Food and Agriculture Organization of the United Nations, would increase from 105.3 million to 112.9 million tons by 2015, an overall increase of 1.7%, with the largest demand from Asia (68%). Study conducted by Banayo et al. (2012) in collaboration with the International Rice Research Institute combined the use of cyanobacterial biofertilizer and inorganic fertilizer to come up with a higher rice crop yield. The increase in rice yield was then determined to be directly related to higher amounts of cyanobacterial fertilizer and low to medium rates of inorganic fertilizer applied (Kumar et al., 2009; Banayo et al., 2012; Bhardwaj et al., 2014).

The results of MANOVA in Table 1 show statistically significant differences in all the variables, e.g., root length, shoot length, fresh weight of roots, fresh weight of shoot, chlorophyll *a* content, all having of < 0.05 significance value in relation to the Kjeldhal nitrogen. Thus, proving that all these variables can be indicated by the amount of Kjeldhal nitrogen. On the other hand, Spearman's correlational analysis in Table 2 showed that all the above-mentioned variables,

where data had p values of ≤ 0.05 and ≤ 0.01 indicated by a single asterisk (*) and double asterisk (**) respectively indicated that these variables are significantly correlated to each other in relation to Kjeldhal nitrogen.

CONCLUSIONS

The use of a *N. piscinale* as a potential biofertilizer was highlighted in this experiment through its ability to provide an alternative nitrogen source for the growth and development of *O. sativa* subsp. *indica*. This production of nitrogen was utilized as seen through the increase of Kjeldhal nitrogen due to the production of nitrogen-containing compounds and its increase in soil pH after 10 days. This was further illustrated by a decrease (utilization) in Kjeldhal nitrogen and pH values after 20 days. The utilization of *N. piscinale* as a positive alternative source of nitrogen was seen through the growth and development of the plant and its measurement of its root and shoot length, as well as its chlorophyll *a* content of the shoot leaves. Independent sample T-tests disclosed no significance between the paired samples, though MANOVA analysis and Spearman's rho correlation analysis between dependent variables of the experiment showed that all variables involved were significant towards the growth and development of *O. sativa* subsp. *indica*.

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