EFFECT OF FERTIGROE®N NANOFERTILIZER APPLICATION ON BACTERIAL POPULATION, ENZYMATIC ACTIVITIES, AND MICROBIAL BIOMASS IN TWO SOIL TYPES

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ABSTRACT

Nanomaterials have various beneficial applications, however, studies on their effects on the environment are still lacking and need to be assessed. Microorganisms are considered as the most sensitive indicators of environmental stresses. This study was conducted to assess the application effects of a commercialized Nanofertilizer, FertiGroe®N, on the total culturable soil bacterial population, enzymatic activities namely dehydrogenase (DHA) and urease (UA), and microbial biomass (MCB) using Philippine soils. The factors included the two soil types, Lipa clay loam (LCL) and Sariaya sandy loam (SSL), and four treatments, namely, nanofertilizer, commercial fertilizer (urea), nanocarrier (treated zeolite), and untreated control. The experiment was laid out in split-plot completely randomized design (CRD) over 75 days incubation period under laboratory conditions. Results showed that treatments had no significant effect on the culturable bacterial population in both soil types. FertiGroe®N, urea, and the nanocarrier had no significant effect on the culturable bacterial population and DHA. At 1 day after amendment (DAA), FertiGroe®N significantly increased UA in LCL but was reduced in SSL. Urease activity in SSL was significantly reduced by urea up to 75 DAA. FertiGroe®N had no adverse effect on the microbial biomass of both soil types. On the other hand, urea significantly reduced MCB in both soil types at 35 DAA. The effect of FertiGroe®N and the non-nanomaterials on the parameters measured was influenced by soil type. FertiGroe®N seems to be less detrimental to the microbial biomass than urea. These results indicated that the FertiGroe®N Nanofertilizer can be applied in both soil types to enhance crop production without any adverse effect on soil microorganisms.

Key words: nanotechnology, nanomaterial, soil enzyme, fertilizer

INTRODUCTION

Nanotechnology is the most innovative aspect of the 21st century and has the potential to play a crucial role in food security, safety, and production (Sabir et al. 2014; Servin et al. 2015). Recent focus has been given to the optimization and development of NMs for application in the agricultural industry (Ioannou et al. 2020). Agricultural sector is heeding attention towards nanomaterial-based product in the form of nanofertilizers to resolve the scarcity of food (Pandey et al. 2021). The use of the nanofertilizers in crop production increases the efficiency of fertilizer application through target

delivery while reducing the risk of environmental pollution, such as nitrogen leaching, and minimizing the potential negative effects of overdosage as well as the frequency of the application. (Naderi and Danesh-Shahraki 2013; Srilatha 2011; Nbada et al. 2021). A nanoporous zeolite-based N nanofertilizer (nanozeourea) can be used as an alternate strategy to improve fertilizer use efficiency in crop production (Kaushik et al. 2014). Thus, employing these nanoparticles in agriculture will be beneficial to the crops (Srilatha 2011).

Nanotechnology, having an immense potential in various area of research and our daily life, has become a boon to the society (Rai et al. 2012; Ingle et al. 2014) and the production and use of NMs are increasing rapidly (Gottschalk et al. 2013). Since there is an increase in the manufacture, use, and disposal of nanomaterial-based products, their release in the soil and the environment also increases (Rousk et al. 2012; Rizwan et al. 2016; Holden et al. 2014). In fact, as fertilizer, NMs can be directly added to soil (Batley et al. 2011). The extensive production and excessive application of NMs poses a serious threat associated with nanotoxicity, especially the unintentional exposure of non-target environmentally beneficial bacteria, threatening the native soil inhabitants (Santimano and Kowshik 2013; Pandey et al. 2021).

Soil organisms are critically important since they directly influence soil ecosystem processes and soil quality. Thus, they serve as biological indicators and any factor that affect soil microbial biomass, activity, and populations would greatly affect soil quality, sustainability, and plant productivity (Dinesh et al. 2012; Hill et al. 2000). Soil microbial biomass plays an important role in maintaining the soil structure, facilitating microbial metabolic processes, and biogeochemical cycling of essential macro and micronutrients (Zhang et al. 2016; Chaudhary et al. 2021). Strongly associated with the microorganisms are soil enzymes which are closely related to the biophysicochemical characteristics of the soil and are important for the regulation of the formation of soil fertility, including nutrient cycling in nature (Purev et al. 2012; Makoi et al., 2008; Shiyin et al. 2004). Soil dehydrogenase, an intracellular enzyme, is highly correlated with the microbial biomass and its activity and can be considered as a good measure of soil microbial oxidative activity (Von Mersi and Schinner 1991; Camiña et al. 1998). Urease catalyzes the hydrolysis of urea into CO_2 and NH_3, hence dictating the fate of urea (Maddela and Venkateswarlu 2018). Research on the effect of nanomaterials on the biological activity in the soil is very important as well as to understand the impact on the environment (Handy et al. 2008).

FertiGroe® is a nanofertilizer containing the macronutrients N, P and K developed by the University of the Philippines Los Baños (Fernando et al. 2017; Fernando et al. 2019). FertiGroe® has been tested in various crops and has shown to increase uptake efficiency of nutrients in banana, rice, corn, sugarcane, coffee, cacao, and vegetables. However, the effects of FertiGroe®N Nanofertilizer on soil microorganisms have not been evaluated. In this study, the impact of using FertiGroe®N Nanofertilizer on the soil bacterial population, microbial biomass, and enzyme activities was investigated. These indicators could serve as important criteria for the commercialization of FertiGroe®N Nanofertilizer.

MATERIALS AND METHODS

Soil collection and characterization. Two soil types were used for the experiments: Lipa Clay Loam (LCL) and Sariaya Sandy Loam (SSL). LCL soil samples were collected from the Central Experiment Station, UPLB. On the other hand, the SSL soil samples were obtained from Brgy. Canda, Sariaya, Quezon. Soil sampling was done by collecting the top 20 cm of the surface soil. Prior to analysis and set-up of experiments, visible debris were removed, and the soil was air-dried and passed through a 2-mm sieve. A portion of the collected soil was characterized chemically by quantitatively determining the pH in water (1:2.5), organic matter (OM) (Walkley and Black 1934), total N (Kjeldahl Method), exchangeable K (Flame Photometer Method), CEC (Ammonium Acetate Method), and available P (Bray No. 2 Method). Physical characteristics, such as texture (Hydrometer Method) and field capacity

(FC) and moisture content (Gravimetric Method), were also determined. Selected physico-chemical characteristics of the two soils are detailed in Table 1.

Table 1. Selected physico-chemical properties of Sariaya and Lipa soil.

	Sariaya Sandy Loam	**Lipa Clay Loam**
pH (1:2.5 soil:water)	5.80	5.40
OM, %	1.40	3.11
CEC, $cmol_c\,kg^{-1}$	14.09	40.06
Total N, %	0.08	0.19
Available P, ppm	348.50	10.50
Exchangeable K, $cmol_c\,kg^{-1}$	1.99	2.05
Texture	6.48% clay, 28.94% silt, 64.58% sand	33.16% clay, 39.61% silt, 27.23% sand
% Field Capacity	33.1	50.8

Experimental design. The experiment was conducted at the Division of Soil Science (DSS)-Agricultural Systems Institute (ASI), College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB) (121°14'40.84" E 14°09'34.55" N) from July 2018 to September 2018. The nanofertilizer (FertiGroe®N) and nanocarrier (treated zeolite) were obtained from UPLB-Biotech (National Institute of Molecular Biology and Biotechnology). An incubation experiment was performed in a sterile 10" by 12" polypropylene containers each filled with 500-g sieved moist soil (100% moisture content at field capacity). Moist soil was pre-incubated for a week prior to addition of treatments. A 2 x 4 factorial experiment, which included two types of soils (LCL and SSL) and four types of treatments ((FertiGroe®N, (150 kg N/ha), commercial fertilizer, urea (150 kg N/ha), nanocarrier (150 kg/ha), and untreated soil as control, was carried out in the Soil Microbiology Laboratory of the Division of Soil Science at room temperature. The treatments were replicated three times and laid out in split-plot completely randomized design (CRD) (Gomez and Gomez 1984). Samples were collected at 1, 7, and 14 day(s) after treatments (DAT) for culture-dependent analysis of soil bacteria. Simultaneously, assessment of dehydrogenase and urease (modified non-buffer method) activities, and microbial biomass were also performed at 1, 35, and 75 DAT.

Enumeration of soil culturable bacteria. Indirect viable plate count was performed to determine the culturable soil bacterial population by pour plating in Asparagine Mannitol Agar medium (Thorton 1922) at 10^{-4}, 10^{-5}, and 10^{-6} dilution. Nystatin was added prior to pouring of the medium at a rate of 50 mg/L. Each dilution was plated in duplicate, allowed to solidify, inverted, wrapped with paper, and incubated at room temperature (~29 °C) for 5 days. After 5 days, the bacterial colonies were counted. Valid count is between 25 to 250 colonies for bacteria (Breed and Dotterer 1916). Bacterial colonies were expressed as colony forming units per gram of dry soil (CFU/g dry soil).

Dehydrogenase activity (DHA) assay. Soil dehydrogenase activity assay was performed according to the modified procedure of Tabatabai (1982). About 1-g soil was placed in a screw-capped tube, to which 1-mL 3% (w/v) 2,3,5-triphenyl tetrazolium chloride (TTC) was added, stirred, and incubated for 96 h at 27 °C. After incubation, 10-mL ethanol (EtOH) was added to the mixture and vortexed for 30 s. The tube was incubated for 1 h to allow the suspended soil to settle. About 5-mL of the supernatant was transferred to a clean test tube, and the absorbance was measured at 485 nm using a spectrophotometer (MultiSkan™ Go Microplate, Thermo Scientific). The result was reported in μg triphenyl formazan (TF) g^{-1} dry soil 96 h^{-1}.

Urease activity (UA) assay. Urease activity assay was performed using the modified non-buffer method (Kandeler and Gerber, 1988). A 5-g soil sample was mixed with 2.5 mL of 720-mM urea, respectively, and incubated for 2 h at 37 °C (Dash et al.1981). After incubation, the control sample was treated with 2.5-mL of 720 mM urea and all samples with 30-mL of acidified 2 M KCl. The samples were shaken for 30 min on a rotary shaker and filtered using Whatman Filter Paper No. 41. A 1.5-mL aliquot was taken and placed in an Eppendorf tube and centrifuged at 10,000 x g for 5 min. A 1-mL supernatant solution was mixed with 9-mL sterile distilled water, 5-mL sodium salicylate/NaOH solution, and 2-mL dichloroisocyanuric acid. The solution was incubated at room temperature for 30 min and the absorbance determined using a spectrophotometer (MultiSkan™ Go Microplate, Thermo Scientific) at 660 nm. The urease activity was calculated and expressed in μg NH_4–N g^{-1} 2 h^{-1}.

Microbial biomass (MCB) by substrate-induced respiration (SIR). Standardization of the amount of glucose, the substrate to be used, and the maximum duration for the incubation of samples were determined based on the procedure by Swain et al. (1978). Each soil type was standardized prior to laboratory experiments involving nanomaterials. Standardization was performed by mixing 10 mg/1 g to 70 mg/1 g glucose to 10 g of soil. The glass wire, which served as stand, along with plastic cup containing 10-mL of standard 0.04-N NaOH was immediately placed inside the jar, covered, sealed and incubated for 5 h. The amount of carbon dioxide (CO_2) released was determined by titrimetric method using standard 0.04 N of hydrochloric acid (HCl) with barium chloride ($BaCl_2$) and phenolphthalein as indicators. The maximum amount of glucose consumed was used for observing the microbial biomass throughout the experiments involving nanomaterials. The D-glucose used were 500 and 100 mg for every 10 g of soil samples for SSL and LCL, respectively. Using the maximum amount of glucose used, the time for initial biomass emission (z), median biomass emission at first generation (A), and median biomass emission generation at second generation (B) were determined within a 10-h incubation with 1 h of sampling interval. Equations were generated depending on the curve plotted and the value of proportional unit, z, signifying the volume (in mL) of standard 0.04 HCl used for titration. Using the value of z, the value of microbial biomass was computed. For LCL, z=0.714B-1.714A while for SSL, z=4.348B-5.348A. The standardized SIR procedure was used to measure microbial biomass all throughout the experiment. The calculations were performed using the formula below:

$$\text{mg } CO_2/10 \text{ g soil} = \frac{(b \times N \times 88) \text{ mg } CO_2}{10 \text{ g soil}}$$

$$\text{Microbial biomass } (\mu g \text{ C}/10 \text{ g soil}) = \frac{z \times N \times 12 \times 10^6}{10 \text{ g soil}}$$

where b is the difference between the delivered volume of HCl of the control and sample; N as the concentration of HCl in normality; and z as the proportional unit.

Statistical analysis. Two-way analysis of variance (ANOVA) (Gomez and Gomez 1984) was performed to assess the effects of treatments on culturable soil bacteria, DHA assay and UA assay, and microbial biomass determination. The Least Significant Difference (LSD) test at α=5% was used to determine whether means differed significantly.

RESULTS AND DISCUSSION

Effect on soil culturable bacteria. The treatments did not show significant effects on the soil culturable bacterial population at α=5% (Table 2). This may suggest that the FertiGroe®N, urea, and nanocarrier did not pose any harm to the culturable bacterial population in both LCL and SSL despite the possibility that nanomaterials may affect the bacteria by interfering in their biological processes such as behavior of cell membranes, biochemical pathways in cells, as well as their genetic code (Klaine et al. 2012). In fact, at 1 DAT, the FertiGroe®N- and urea-treated LCL and the urea-treated SSL had the highest total

bacterial counts relative to the control. At 7 DAT, the urea-treated LCL and the FertiGroe®N-treated SSL had the highest total bacterial counts. Perhaps, the increase in the concentration of nitrogen in the soil encouraged luxury consumption of N by soil microorganisms, which might have increased bacterial population (de Mazancourt and Schwartz 2012).

At 14 DAT, the nanocarrier-treated LCL had the highest total bacterial count, and this could be related to the high surface area of the nanocarrier which increased the cation-exchange capacity of the LCL. Addition of porous material in the soil, for example zeolite, can increase the nutrient preserving capacity and water status of the soil (Li et al. 2000; Ippolito et al. 2011).

Table 2. Effects of FertiGroe®N on the culturable bacterial population of Lipa clay loam and Sariaya sandy loam.

Soil Type	Treatment	Total Bacterial Count, log (CFU g^{-1} soil)		
		1 DAT	7 DAT	14 DAT
Lipa Clay Loam	control	6.04^{ns}	6.15^{ns}	5.40^{ns}
	FertiGroe®N	6.21	6.03	5.46
	urea	6.20	6.32	5.72
	nanocarrier	6.06	5.86	5.98
Sariaya Sandy Loam	control	5.71^{ns}	5.89^{ns}	5.18^{ns}
	FertiGroe®N	6.04	6.27	4.87
	urea	6.48	6.07	5.13
	nanocarrier	5.89	6.02	4.80

^{ns} not significant

Nanocompounds, such as nanoclay, nanochitosan, and nanozeolite, support microbial population due to high level of phosphorus, organic carbon, and ammoniacal nitrogen (Khati et al. 2017). But, the nanocarrier-treated SSL had the lowest total culturable bacteria which could be due to the physical property of sandy soils. Sandy soils have limited capacity to retain water and nutrients (Baghbani-Arani et al. 2020). However, the addition of nanocarrier could have improved the water retention of SSL since addition of high surface-area and porous nanocarrier can increase the soil moisture content of sandy soil (Ippolito et al. 2011). Zeolite also triggered a microbial mineralization process of soil organic carbon stocks and higher extent of decomposition of the SOM more stable pool.

In SSL, despite the possible increase in the availability of moisture and nutrient, culturable bacteria was still lower than the control. This could be due to the inaccessibility of microorganisms to the nutrient-rich soil solutions as affected by nanocarrier. Instead of the soil solution flowing through the interconnected pores of the sandy loam in a process called mass flow, the soil solution had likewise been retained in the pores and interconnected voids of the nanocarrier. The entrapment of soil solution by nanocarrier cut or reduced the water and nutrient access of bacteria present in the soil macropores, that could otherwise be supplied with growth factors by mass flow. The size and distribution of pores in soil are among the most important factors determining microbial life and water retention (Hattori et al. 1976). Bacteria located in the macropores are therefore subjected to more intense variation between desiccation and wetting (Ranjard and Richaume 2001).

Effect on dehydrogenase activity (DHA). There was no significant interaction effect between soil type; soil type and treatments; and among treatments in both LCL (Fig. 1a) and SSL (Fig. 1b) at α=0.05 except at 35 DAT in SSL in which significant interaction effect among treatments was observed. The nanocarrier-treated SSL had higher DHA while that of the urea-treated SSL had significantly lower DHA relative to the control. Perhaps, the mixing of nanocarrier in sandy loam soil improved its moisture retention, thus, enhanced microbial growth (Ippolito et al. 2011). Nanocompounds (nanoclay, nanochitosan, and nanozeolite) support microbial activity and, hence, the soil health (Khati et al. 2017) .

However, the decrease in soil DHA in urea-treated soil might be due to the decrease in soil organic matter (SOM) upon application of urea and that, at 35 DAT, the depleted carbon source was not enough to support microbial growth compared to that of the control. An increase in N concentration in soil accelerated the loss of SOM through its mineralization (Singh 2018).

A significant main effect on soil DHA was observed at 1 and 35 DAT as influenced by the type of soil (Fig. 1c). LCL exhibited higher DHA than SSL which may be attributed to the physicochemical properties, due to its finer particle size, that affected the microbial community (Sessitsch et al. 2001), Thus, a decrease in microbial community structure, as affected by soil texture, might cause a decrease in soil DHA which is related to the quantitative changes in the microorganism population and connected with living microbial cells (Wolińska and Stepniewska 2012).
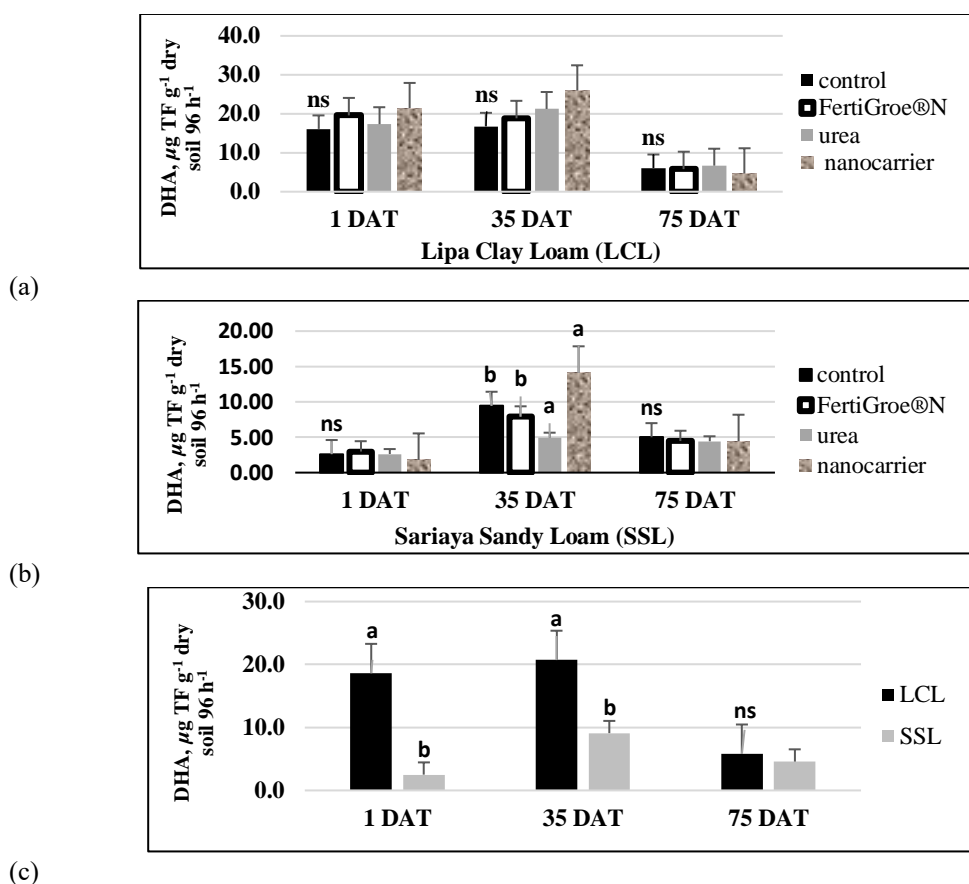
(a)

(b)

(c)

Fig. 1. Effect of treatments on the dehydrogenase activity of (a) Lipa clay loam and (b) Sariaya sandy loam and (c) interaction effect between soil types . Error bars represent the standard error of the mean (n=3). Different letters indicate significant difference among means (P<0.05) according to LSD test.

Dehydrogenase, an intracellular enzyme, can truly reflect microbial activity Mineralization of DHA by other enzymes with respect to the degradation processes of extracellular soil enzymes was possible unless they are either adsorbed by clays or immobilized by humic molecules. Another factor for the higher DHA of LCL than SSL is the level of organic matter. DHA reaches higher values in soils with higher total organic carbon content (Wolińska and Stepniewska 2012).

Effect on urease activity (UA). A significant interaction effect on UA between soil type and treatments at 1, 35, and 75 DAT at 95 % level of significance. Hence, an examination of simple effects of treatments on UA for each soil type is shown in Figure 2. At 1 DAT, significant increase in the UA was observed in LCL treated with FertiGroe®N and nanocarrier compared to control. In contrast, a significant decrease in SSL treated with FertiGroe®N and urea was observed at 1 DAT. At 35 DAT, nanocarrier-treated SSL and LCL were significantly increased compared to other treatments. The UA of nanocarier-treated SSL and LCL, however, had significantly decreased at 75 DAT relative to the control. The increase in the urease activity at 1 DAT might possibly be due to the introduction of porous FertiGroe®N and nanocarrier which caused an increase in the CEC of LCL. UA was found to be strongly correlated with the clay content and CEC, but not significantly correlated with organic C content (Kizilkaya et al. 2004). However, for SSL at 1 DAT, a decrease in UA could be due to the increase in the microbial population considering that nitrogen was introduced to both the LCL treated with FertiGroe®N and urea. SSL contained high phosphate concentration which, along with nitrogen, stimulated the growth of soil microorganisms. However, an increase in the microbial population led to the decrease in the UA of the soil. Previous studies under steady state conditions, showed 79-89 % of the urease activity of the soil was earlier determined to be due to urease adsorbed on soil colloids and an increase in microbial population reduced this percentage temporarily until a new steady state was reached (Paulson and Kurtz 1969). It is possible that the nanocarrier protected the enzyme from degradation by proteases while maintaining the enzyme's function at 35 DAT in both soil types. Surface micropores significantly influenced the protein-nanoporous material interaction (such as the case of nanozeolite). Cyto-c and *Candida antarctica* Lipase B (CALB) showed strong affinity to the porous nZeolite than to nonporous one and larger extent of protein unfolding induced by porous nZeolite was positive for the enzyme activity of Cyto-c while negative for that of CALB (Wu et al. 2013).
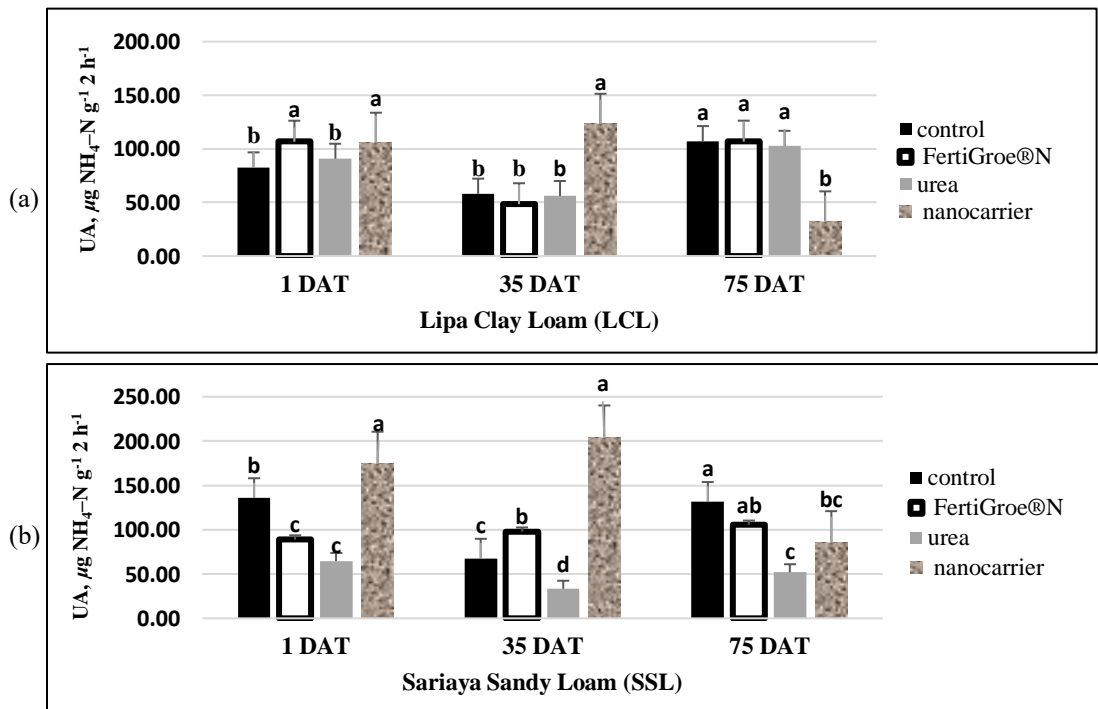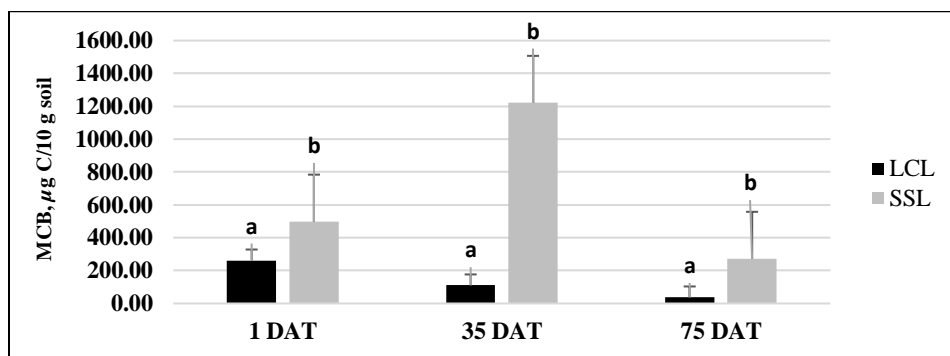

Fig. 2. Effect of treatments on the urease activity of (a) Lipa clay loam and (b) Sariaya sandy loam between soil types. Error bars represent the standard error of the mean (n=3). Different letters indicate significant difference among means (P<0.05) according to LSD test .

FertiGroe®N nanofertilizer effect on soil microbial biomass (MCB). A significant main effect on MCB between soil types at 1, 35 and 75 DAT was observed (Fig. 3a) at 95 % level of significance. The significant main effect among treatments was observed at 1 and 35 DAT for both LCL (Fig. 3b) and SSL (Fig. 3c). At 1 DAT, a significant increase in nanocarrier-treated soil was observed in both LCL and SSL relative to the control. Perhaps, this could be due to the adsorptive capacity of the nanocarrier. Upon addition, the nanocarrier had been immediately inoculated by the microorganisms in the soil and with the nanocarrier's capacity to adsorb nutrients, it was able to sustain the exponential growth of microorganisms.

The microbial activity, and hence the soil health could be improved by the introduction of nanocompounds such as nanoclay, nanochitosan, and nanozeolite (Khati et al. 2017). Nanozeolite, with a 99% purity, was able to support microbial population. However, at 35 DAT, the MCB of urea-treated SSL was significantly reduced compared to the control. This suggests that the introduction of urea increased the utilization of organic matter in the soil between the period of 1 DAT and 35 DAT, thus, reducing the availability of organic carbon at 35 DAT for microbial utilization as growth factor. Similarly, a significant reduction in soil MCB was observed in the nanocarrier-treated SSL. The nanocarrier has been found to decrease the distribution of nutrients, and consequently, the utilization of these nutrients by soil microorganisms by reducing the permeability of water (Tállai et al. 2017).

The amount of macropores was responsible for significant differences in the MCB between LCL and SSL and its interaction effect at 35 DAT. It is possible that the substrate added during the experiment was able to reach the micropores of the LCL which protected it from the utilization of microorganisms present in the macropores. The pore size distribution could give an indication of the accessibility of substrates to decomposition by microorganisms, with finer, clayey soils on average, show slower decomposition rates and higher retention of OM than coarse, sandy soils (Van Veen and Kuikman 1990). The mixing of substrates and microbes as found in the laboratory systems is very much restricted in soil due to the limited water flow and microbial movement. Therefore, the substrates even at distances of micrometers or less might not be readily available for microorganisms. Aside from the entrapment in pores, entrapment in aggregates rendered the substrate inaccessible by microorganisms (Van Veen and Kuikman 1990).

(a)
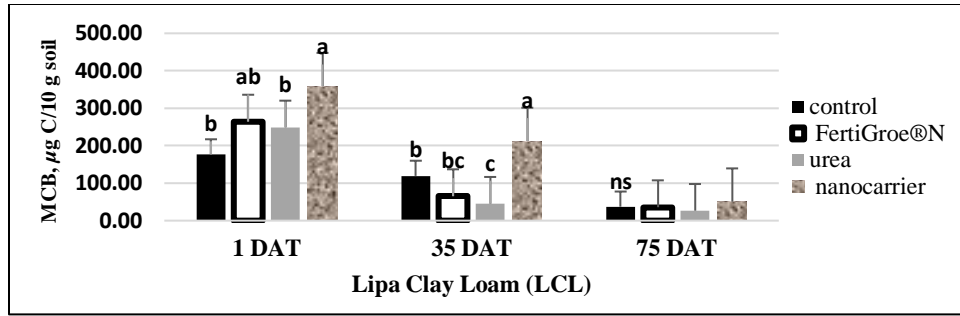
(b)
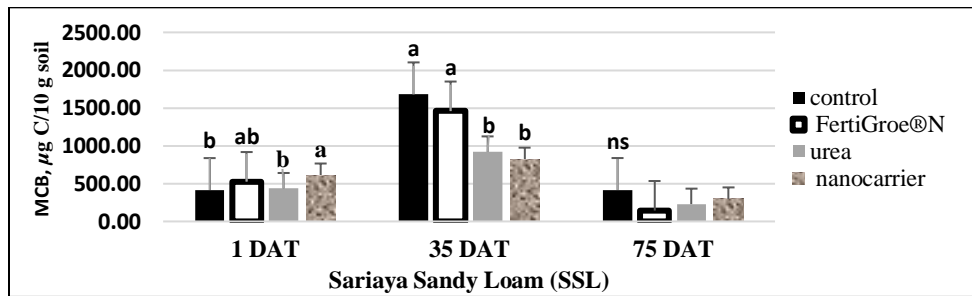
(c)

Fig. 3. (a) Differences in soil MCB between soil types; effect of treatments on soil microbial biomass of (b) Lipa clay loam and (c) Sariaya sandy loam. Error bars represent the standard error of the mean (n=3). Different letters indicate significant difference among means (P<0.05) according to LSD test.

CONCLUSION

An incubation experiment using two soil types was conducted to assess the effects of FertiGroe®N nanofertilizer on the culturable bacterial population, microbial biomass, and dehydrogenase (DHA) and urease activities (UA) prior to its extensive use in crop production and commercialization. The nanofertilizer, FertiGroe®N, was better in increasing both microbial activity and biomass than the commercial fertilizer, urea. FertiGroe®N can be safely applied in crop production. Since the effect of nanofertilizer on soil microorganisms may differ in laboratory conditions and the natural environment, there is a need to investigate the effects of FertiGroe®N on soil microorganisms in pot and field trials.

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