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Effects of Organic Amendments on Phenol Oxidase, Peroxidase, Urease, and Nitrogen Mineralization: A Laboratory Incubation Study

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Abstract: Mitigating climate change and enhancing fertility in agricultural systems require the adoption of more sustainable fertilizer management practices. Applications of recycled organic materials, such as animal and green wastes, can promote soil carbon stabilization via changing extracellular enzyme activities while providing the necessary nitrogen (N) for plant growth. The goals of this study were to quantify the effects of compost type (cow manure, green manures, mixtures of green and cow manure at various proportions, and inorganic fertilizers) on (1) enzyme activity (phenol oxidase, peroxidase, and urease), and (2) mineralized N under laboratory incubation at 30 °C over an eight-week period. The lowest oxidative enzyme activities (phenol oxidase and peroxidase) were found in the soil treated with a mixture of 50% cow manure and 50% green manure ($2.45~\mu mol~h^{-1}~g^{-1}$) and a mixture of 30% cow manure and 70% green manure ($3.21~\mu mol~h^{-1}~g^{-1}$) compared to all other fertilizer treatments. The highest phenol oxidase activity was found in soils amended with green manures ($3.52~\mu mol~h^{-1}~g^{-1}$), while the highest peroxidase activity was found in soils amended with a mixture of 70% cow manure and 30% green manure ($5.68~\mu mol~h^{-1}~g^{-1}$). No significant differences were found in total net mineralized N content among all organic fertilizer treatments, but these were significantly lower than total net mineralized N in soil treated with inorganic fertilizers. These results indicate similar effects of organic amendments and mixtures in providing plant-available N, but different effects on lignin-degrading enzyme activities, which may lead to differences in soil organic carbon cycling and long-term C storage depending on which organic amendment is utilized.

Keywords: cow manure; green manures; inorganic fertilizers; enzyme; mineralized N

1. Introduction

The intensification of commercial agriculture combined with the increasingly negative effects of global climate change requires the adoption of more sustainable soil management practices [1,2]. Potential solutions for the mitigation of climate change and improvement of soil quality include the recovery of natural resources lost in the food supply chain [3–5]. Diverting organic matter from landfills into green manures can provide a suitable soil amendment capable of enhancing soil fertility [6,7].

Organic composts can positively affect soil's physical, chemical, and biological properties compared to standard practices of inorganic fertilizer application [8–11]. However, the use of animal manure-based fertilizers can present concerns regarding environmental contamination [12–14]. Additionally, animal manure commonly contains antibiotic-resistant bacteria and other pathogens that can be harmful to human health [13,14].

Combinations of organic fertilizers may be more efficient in providing plant-available nutrients and promoting soil organic carbon stabilization than the sole application of a single type of compost material. Soil amended with food waste-based composts (green manures) or combinations of green and animal manure was shown to be more beneficial for plant productivity and environmental health than soil amended with cow manure [15],

producing less CO_2 emissions than cow manure-based compost in laboratory incubation studies [6,16]. Applications of plant manures are also suitable for providing long-term N supplies to crops [17]. Additionally, mixed manure treatments and integrated fertilizer regimes have also shown success in increasing crop maize yield and N availability as well as increasing soil organic C and N [7,18,19].

Nitrogen (N) availability in soil and composts can vary considerably. Organic N sources are only available for plant uptake after they are mineralized to inorganic N [20]. Mineralization is mediated by the production of extracellular enzymes by soil microbial communities [21,22]. Phenol oxidase and peroxidase are involved in the degradation of lignin polyphenols commonly found in green manure inputs [23,24]. These lignin-degrading enzymes are particularly important in soil environments with plant litter inputs, such as forest ecosystems, or in green manures in agricultural operations [25]. They are effective indicators of soil organic matter (SOM) dynamics and nutrient availability [26,27]. Repression of the activity of these enzymes was correlated with the accumulation of organic matter in soils [23]. For example, large accumulations of SOM and C in peatlands were attributed to their low rates of phenol oxidase activity [28]. Inhibiting these oxidative enzymes may be beneficial for the long-term storage of SOC in agricultural systems. In soils amended with inorganic fertilizers and animal manures, urease catalyzes the hydrolysis of urea into ammonia and carbon dioxide [29–32]. While urease activities are important for transforming N inputs into plant-available N, increased risks of ammonia volatilization and N losses following high urease activity can be problematic [29]. Inhibiting urease can be helpful in reducing these losses and organic amendments that will lower urease activities should be investigated [33]. Finding organic manures that inhibit oxidative enzymes and lower urease responses can be beneficial in agricultural systems.

The production and activities of phenol oxidase and peroxidase are sensitive to changes in soil fertilizer management practices and various environmental factors, such as nitrogen inputs, spatiotemporal microbial community structure, lignin inputs, soil pH, water content, and oxygen availability [23,34–36]. Phenol oxidase and peroxidase are sensitive to N fertilizer inputs due to the effects of N fertilizer on the soil microbial community composition [25,37]. Microorganisms release phenol oxidase and peroxidase to degrade lignin for the acquisition of nitrogen under microbial N limitation; these processes are inhibited when more readily available N sources are applied [23,38]. Iyyemperumal and Shi [26] reported that phenol oxidase and peroxidase activities decreased after inorganic fertilizer application and increased after subsequent applications of swine effluent in bermudagrass and tall fescue forage systems due to the change in soil microbial community structure. Waldrop and Zak [39] found that forest ecosystems containing leaf litter high in lignin and soils with diverse fungal and bacterial communities responded negatively to NO_3^- inputs by decreasing phenol oxidase activity. In contrast, forest ecosystems containing leaf litter low in lignin with a lower index of fungal and bacterial diversity responded positively to NO_3^- by increasing phenol oxidase activity [25].

Our knowledge of the effects of commercially available green manures on N supply and soil enzyme activity is limited, and results from published studies are variable [26,40,41]. For example, Dong et al. [41] reported decreased phenol oxidase activities following green manure applications in walnut orchards. In contrast, Hassan et al. [40], who investigated rice straw and peanut plant-based green manures, found increased phenol oxidase activity in alkaline soils treated with green manures. These variations in phenol oxidase activity following green manure applications demonstrate that soil amendments can affect enzyme expression and activity, and thus nutrient availability, in different ways. This ambiguity may be a barrier for producers interested in utilizing organic fertilizer regimes [42].

The goals of this study were to determine and compare the effects of commercial organic composts (green manure, animal manure, and mixtures of cow manures and green manures) and inorganic fertilizers on (1) N mineralization and (2) extracellular enzymes

involved in C and N mineralization processes (phenol oxidase, peroxidase, and urease) using a laboratory incubation approach.

2. Materials and Methods

2.1. Soil Collection and Preparation

Surface soil (0–15 cm) was collected from the campus community garden plot at Sweet Briar College in Amherst, VA, USA (37°33′02.0″ N, 79°05′06.3″ W). The soil was mapped as 4C (Clifford clay loam) with 7 to 15 percent slopes. According to the record reported by WeatherSpark (accessed on 23 March 2022), the high and low average monthly temperatures are 19.7 °C and 9.3 °C. The average monthly precipitation is 10.6 cm. The site had a long history of cropping. Plant debris was removed from the soil sample. The soil was air-dried at room temperature until it reached constant mass. The air-dried soil sample was then ground and sieved through a 2 mm sieve for analysis and incubation.

2.2. Physical and Chemical Determination of Soil Samples

The air-dried soil samples were used to determine soil pH, macronutrient content (determined by the Virginia Tech Soil Testing Laboratory, Blacksburg, VA, USA), and total C and N (determined by the Stable Isotope Mass Spectrometer Laboratory, University of Florida, Gainesville, FL, USA). Soil pH was measured with a LabFit pH analyzer (Bayswater, UK) by adding 10 mL DI water to 10 cm^3 soil for a 1:1 (v/v) ratio. Macronutrient determination was performed using Mehlich 1 extraction methods [43] by inductively coupled plasma atomic emission spectrometer (SPECTRO Analytical Instruments, Kleve, Germany). Total C and N were determined by dry combustion using an elemental analyzer (N.C. Technologies, Charlotte, NC, USA).

Soil particle size was determined using methods outlined by Kettler et al. [44]. Concentrations of soil NH_4^+ and NO_3^- were determined using 1M KCl extraction and colorimetry at 697 nm and the difference between 220 nm and 275 nm, respectively, using a Cary ultraviolet spectrophotometer (Agilent, Santa Clara, CA, USA) [45,46]. Soil organic matter was determined by loss of ignition [47]. The physical and chemical properties of the original unamended topsoil are presented in Table 1.

Table 1. Physical and chemical properties of unamended soil.

Properties	Value
pH	7.5
TC (%)	3.88
TN (%)	0.31
C:N	12.5
NO_3^- (mg kg^{-1})	3.48
NH_4^+ (mg kg^{-1})	5.53
CEC (meq/100 g)	16.4
P (mg kg^{-1})	142
K (mg kg^{-1})	380
Mg (mg kg^{-1})	561
Ca (mg kg^{-1})	5541
SOM (%)	8.77
Sand (%)	27.08
Silt (%)	47.7
Clay (%)	25.21

Total carbon (TC), total nitrogen (TN), C:N, nitrate (NO_3^-), ammonium (NH_4^+), cation exchange capacity (CEC), and Mehlich I extractable phosphorus (P), potassium (K), magnesium (Mg), and calcium (Ca), and soil organic matter (SOM). The data were generated by analyzing three subsamples of one composited soil sample.

2.3. Chemical Property Determination of Soil Amendments

Commercially available cow (Black Kow, Oxford, FL, USA) and green manures (Blue Ribbon Organics, Caledonia, WI, USA) were used as soil organic amendments. A commer-

cially available inorganic fertilizer (13-13-13) was used as an inorganic soil amendment for comparison (Winston Weaver Co., Winston Salem, NC, USA). Organic amendments were dried in an oven at 105 °C for 24 h and lightly ground before incorporation into the soil. The inorganic fertilizer was directly applied to soil samples. A subsample of oven-dried organic amendments was used to analyze total C and N through dry combustion using an elemental analyzer (N.C. Technologies, Charlotte, NC, USA). Concentrations of NH_4^+ and NO_3^- in each amendment were determined as described above. The chemical properties of soil amendments are presented in Table 2.

Table 2. Chemical properties of soil amendments.

Amendment	pH	TC (%)	TN (%)	C:N	NO_3^- (mg kg^{-1})	NH_4^+ (mg kg^{-1})
Black Kow (CM)	7.5	31.3	1.43	21.96	350.60	146.20
Blue Ribbon Organics (GM)	7.3	20.57	1.48	13.90	271.21	98.14

Total carbon (TC), total nitrogen (TN), carbon to nitrogen ratio (C:N), nitrate (NO_3^-), ammonium (NH_4^+). The data were generated by analyzing three subsamples of each soil amendment.

2.4. Laboratory Incubations

The treatments in this study included seven amendment applications: an unamended control (CON), Black Kow cow manure (CM), Blue Ribbon Organics organic compost (green manure GM), 50:50 mixture of cow manure and green manure (50CM50GM), 30:70 mixture of cow manure and green manure (30CM70GM), 70:30 mixture of cow manure and green manure (70CM30GM), and Winston Weaver 13-13-13 inorganic fertilizer (IN). The treatments were replicated three times. Data were collected at four weeks and eight weeks.

Amendments were thoroughly mixed with soil at a rate of 58 mg g^{-1} soil to be consistent with other lab incubation and field studies [6,7,48]. The soil moisture content during incubation was 28%, which represented 80% field capacity and was determined according to Grewal et al. [49] and Richards [50]. To aid in distributing the amended soils in each jar, large batches of soil and amendment mixture were made. There were seven batches, one for each treatment consisting of 1 kg of soil and the appropriate amounts of soil amendment (5.8 g cow manure in CM, 5.8 g green manure in GM, 2.9 g each cow manure, and green manure in 50CM50GM, 1.79 g cow manure 4.06 g green manure in 30CM70GM, 4.06 g cow manure and 1.79 g green manure in 70CM30GM, and 0.6 g inorganic fertilizer in IN). DI water was added at 280 mL for each batch and the mixtures were mixed to ensure the amendments were consistently applied to the soil. Then, 128 g (accounting for dry soil and the added water weight) of the homogenous treatment mixtures were applied to their corresponding polystyrene jars (250 mL). Each treatment was replicated three times. A single layer of gas permeable parafilm was secured over the jars. The jars were incubated in the dark in a temperature-controlled chamber at 30 °C. Water content was monitored every ten days by weighing the jars and distilled water was added as needed to maintain moisture content.

2.5. Accumulated Mineralized N Analysis

Inorganic N was extracted from moist soils using 4 g of soil and 40 mL 1M KCl (1:10 m/v ratio). Samples were shaken on a reciprocating shaker for 1 h at 200 oscillations per minute, centrifuged at 3000 rpm for 10 min, and filtered using Whatman NO. 42 filter paper before ultraviolet spectroscopy. Concentrations of NH_4^+ and NO_3^- were determined before incubation, after four-week incubation, and after 8-week incubation using the methods described above.

The net mineralized NH_4^+ after 4-week and 8-week incubation was determined by subtracting the NH_4^+ concentration before incubation from mineralized NH_4^+ after 4-week and 8-week incubation, respectively. Net mineralized NO_3^- after 4-week and 8-week incubation was determined by subtracting the NO_3^- concentration before incubation from mineralized NO_3^- after a 4-week and an 8-week incubation, respectively. Total mineralized

N after 4 weeks and 8 weeks was determined by adding net mineralized NH_4^+ and NO_3^- after 4-week and 8-week incubation, respectively. The change in total net mineralized N was determined by subtracting total net mineralized N after 4-week incubation from total net mineralized N after 8-week incubation.

2.6. Soil Enzyme Analysis

Enzyme assays for phenol oxidase, peroxidase, and amidohydrolase (urease) were performed at eight weeks. Following the methods outlined by Wallenstein et al. [51] and Sinsabaugh et al. [35], 2 g of moist soil subsamples from each incubation jar were homogenized with 200 mL of 50 mM, pH 5.0, acetate buffer. Phenol oxidase and peroxidase were measured spectrophotometrically using L-3,4,-dihydroxyphenylalanine (DOPA) as the substrate. Phenol oxidase assays were prepared using 2 mL of 5 mM L-DOPA solution and 2 mL of soil solution in 15 mL centrifuge tubes. Controls were prepared using 2 mL of soil solution and 2 mL of acetate buffer. Peroxidase samples and controls were prepared similarly, with the addition of 0.2 mL of 0.3% hydrogen peroxide. There were five replicate samples and three replicate controls for each incubation jar. The centrifuge tubes were incubated at 20 °C for 1 h and centrifuged for 10 min at 3000 rpm. Absorbance was measured at 450 nm using a spectrophotometer. The activity was calculated according to Sinsabaugh et al. [35] and expressed as μmol g^{-1} h^{-1}. Urease activity was measured using urea as the substrate [52]. The preparation of this assay was similar to the methods described for the phenol oxidase assay with 2 mL of 20 mM urea added to all samples and control replicates. Absorbance was measured at 697 nm. Enzymatic activity of urease was calculated as the amount of μg NH_3 catalyzed in the sample homogenate per hour of incubation and expressed as μg NH_4^+ g^{-1} h^{-1} released during the reaction.

2.7. Data Analysis

Analysis of variance (ANOVA) was used to evaluate the effects of soil amendments on net mineralized NO_3^-, net mineralized NH_4^+, and total net mineralized N after four weeks of incubation; and phenol oxidase, peroxidase, urease, net mineralized NO_3^-, net mineralized NH_4^+, and total net mineralized N after eight weeks incubation, using JMP (version 10. SAS Institute Inc., Cary, NC, USA). Prior to the ANOVA test, the normality of data was evaluated using a Shapiro-Wilk test. Non-normal data were transformed using a log transformation. If the *p*-value generated from the ANOVA was less than 0.05, the difference was considered statistically significant and then multiple comparisons were conducted using the Tukey-Kramer Test.

3. Results

3.1. Soil Enzyme Activity

A significant effect of soil amendments on phenol oxidase activity was found ($p < 0.001$). The highest phenol oxidase activity was 3.52 ± 0.29 μmol h^{-1} g^{-1}, observed in soil with green manure amendments (Figure 1a). The lowest phenol oxidase activity was 0.42 ± 0.02 μmol h^{-1} g^{-1} in soils with inorganic fertilizer application (Figure 1a). No significant differences were observed among soil amended with CON, CM, 50CM50GM, 30CM70GM, and 70CM30GM (Figure 1a).

A significant effect of soil amendments on peroxidase activity was found ($p = 0.001$). Different mixtures of green manure and cow manure displayed different effects on peroxidase activity. The highest peroxidase activity was 5.68 ± 0.37 μmol h^{-1} g^{-1}, observed in soils with 70CM30GM amendments (Figure 1b). The lowest peroxidase activity was 0.80 ± 0.35 μmol h^{-1} g^{-1} in soils with 50CM50GM amendments (Figure 1b). No significant differences were observed between CM and IN, which have significantly higher peroxide activity than CON and 50CM50GM (Figure 1b). No significant differences were found among treatments with a high proportion of GM including GM, 50CM50GM, and 30CM70GM (Figure 1b).

No significant effect of soil amendments on urease activity was found ($p = 0.17$, Figure 2).
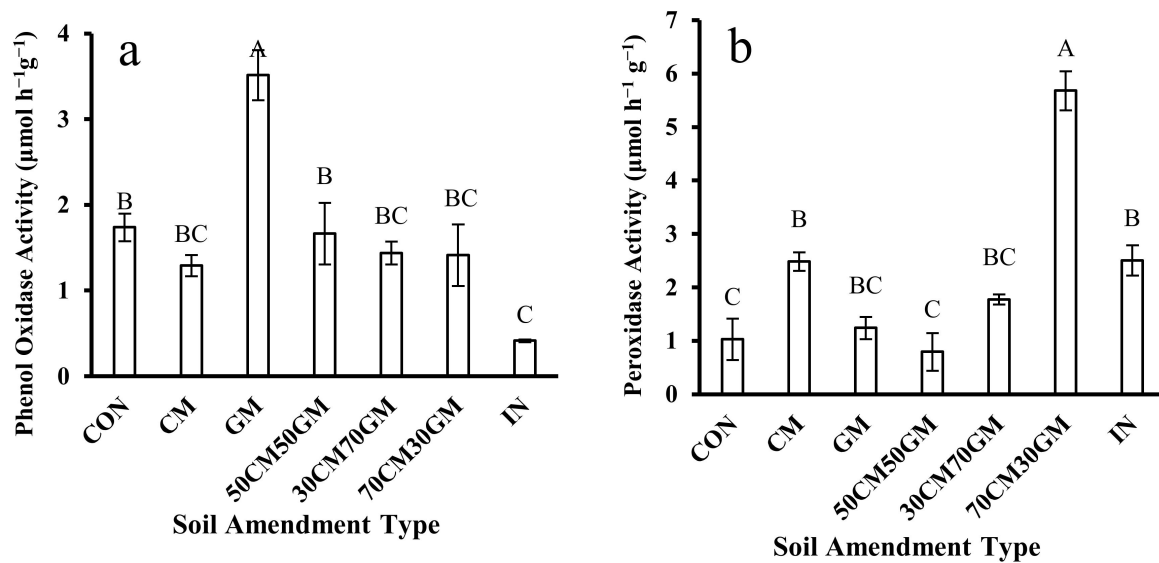

Figure 1. Phenol oxidase (**a**) and peroxidase (**b**) activity after eight weeks in soil treatments with six types of amendments expressed in $\mu mol\ h^{-1}\ g^{-1}$ for the unamended soil (CON) and soils amended with cow manure (CM), green manure (GM), 50:50 ratio of cow manure and green manure (50CM50GM), 30:70 ratio of cow manure and green manure (30CM70GM), 70:30 ratio of cow manure and green manure (70CM30GM), and inorganic fertilizer (IN). Different letters indicate significant differences at $p < 0.05$. The error bars represent standard error ($n = 3$). Peroxidase data were log-transformed.
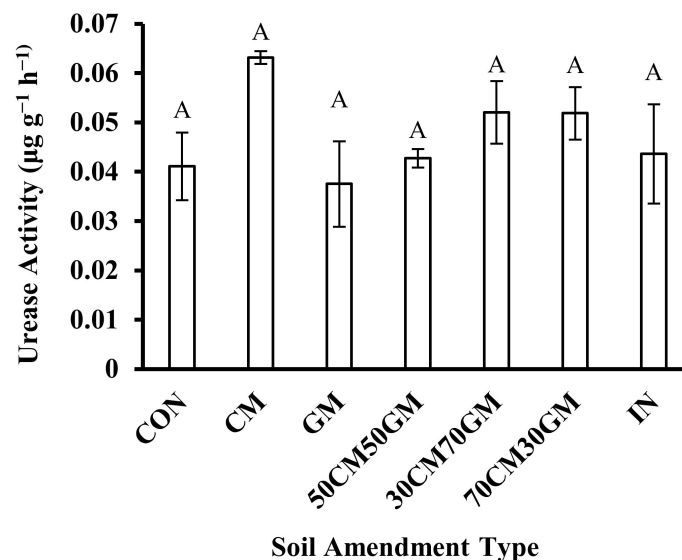

Figure 2. Urease activity in soil treatments with six types of amendments after eight weeks of incubation expressed as $\mu g\ NH_4^+\ g^{-1}\ h^{-1}$ in unamended soil (CON), soils amended with cow manure (CM), green manure (GM), 50:50 ratio of cow manure, and green manure (50CM50GM), 30:70 ratio of cow manure and green manure (30CM70GM), 70:30 ratio of cow manure and green manure (70CM30GM), and inorganic fertilizer (IN). Different letters indicate significant differences at $p < 0.05$. The error bars represent standard error ($n = 3$).

3.2. Net Mineralized NO_3^- and NH_4^+ Content

Significant effects of soil amendments on net mineralized NO_3^- were found after four-week ($p = 0.001$) and eight-week incubation ($p < 0.001$). The highest net mineralized NO_3^- was observed in soils treated with IN amendments (161 ± 14 mg kg^{-1} after a four-week incubation, and 214 ± 4 mg kg^{-1} after an eight-week incubation), which was significantly higher than all organic treatments and CON (Figure 3). Unamended control soils displayed the lowest NO_3^- (68 ± 2 mg kg^{-1} at four weeks and 86 ± 4 mg kg^{-1} at eight weeks (Figure 3). No significant differences were found between all organic treatments and CON after four weeks (Figure 3a). At eight weeks, all organic treatments did not have significant differences, but all organic treatments had significantly higher amounts of net mineralized NO_3^- than CON (Figure 3b).
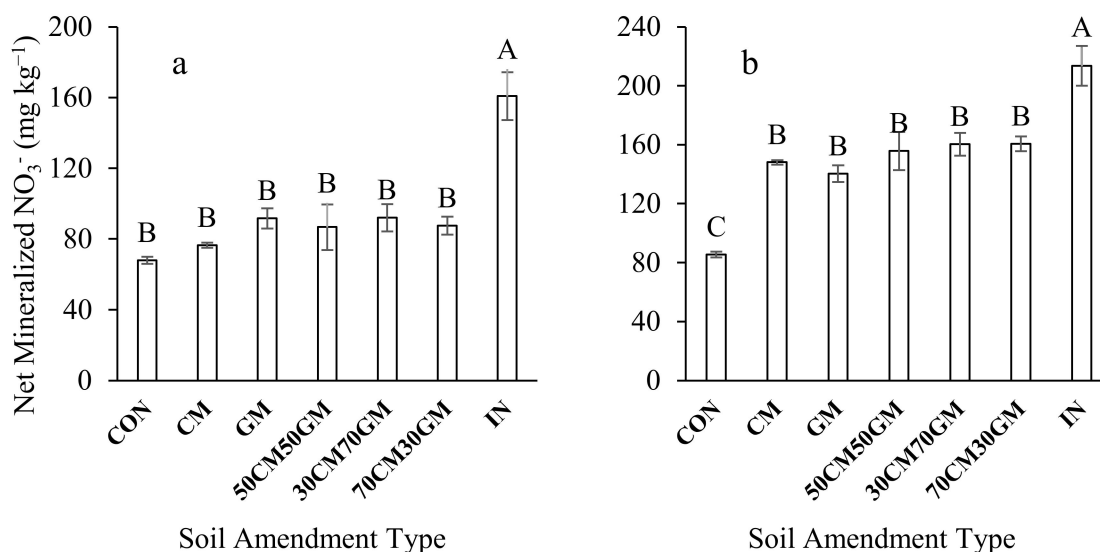

Figure 3. Mineralized NO_3^- at four (**a**) and eight (**b**) weeks in soil treatments with six types of amendments expressed as mg kg^{-1} An unamended soil (CON), soils amended with cow manure (CM), green manure (GM), 50:50 ratio of cow manure and green manure (50CM50GM), 30:70 ratio of cow manure and green manure (30CM70GM), 70:30 ratio of cow manure and green manure (70CM30GM), and inorganic fertilizer (IN). Different letters indicate significant differences at $p < 0.05$. The error bars represent standard error ($n = 3$). For four weeks mineralized NO_3^- data were log-transformed.

Significant treatment differences in net mineralized NH_4^+ were found after four-week ($p = 0.023$) and eight-week incubation ($p = 0.001$). At four weeks, no significant differences were found between CON and IN treatment, which were significantly higher than all organic amendment treatments (Figure 4a). Among all organic treatments, GM and 50CM50GM had significantly higher net mineralized NH_4^+ than 30CM70GM and 70CM/30CM.

There was a decrease in net mineralized NH_4^+ in all treatments at eight weeks compared to the four-week incubation (Figure 4). At eight weeks, the soils treated with IN amendments had the highest NH_4^+ content (2 ± 0.03 mg kg^{-1}), which was significantly higher than all other treatments (Figure 4b). Net mineralized NH_4^+ content observed in soils treated with GM amendments was significantly higher than all other organic treatments (Figure 4b). Soil treated with CM and 70CM30GM had the lowest net mineralized NH_4^+ (0.2 ± 0.06 mg kg^{-1} and 0.09 ± 0.02 mg kg^{-1}, respectively) (Figure 4b).

3.3. Net Mineralized N

Significant effects of all soil amendments on total net mineralized N were found after four-week ($p < 0.001$) and eight-week incubation ($p < 0.001$). The highest net mineralized N content was observed in soils treated with IN amendments (164 ± 13.50 mg kg^{-1} after a four-week incubation, and 215 ± 4 mg kg^{-1} after an eight-week incubation), which was

significantly higher than all organic treatments and CON (Figure 5). Unamended control soils displayed the lowest N content (72 ± 2 mg kg^{-1} at four weeks and 87 ± 4 mg kg^{-1} at eight weeks (Figure 5). No significant differences were found between all organic treatments and CON after four weeks (Figure 5a). At eight weeks, all organic treatments did not have significant differences, but all organic treatments had significantly higher net mineralized N than CON (Figure 5b). In terms of changes in total mineralized N, significant effects of soil amendments were observed ($p = 0.023$). The CM incorporated treatments and IN showed significantly higher increases in total mineralized N than CON (Figure 6). The GM treatments showed lower total net mineralized N than all CM incorporated treatments and higher total net mineralized N than CON (Figure 6).
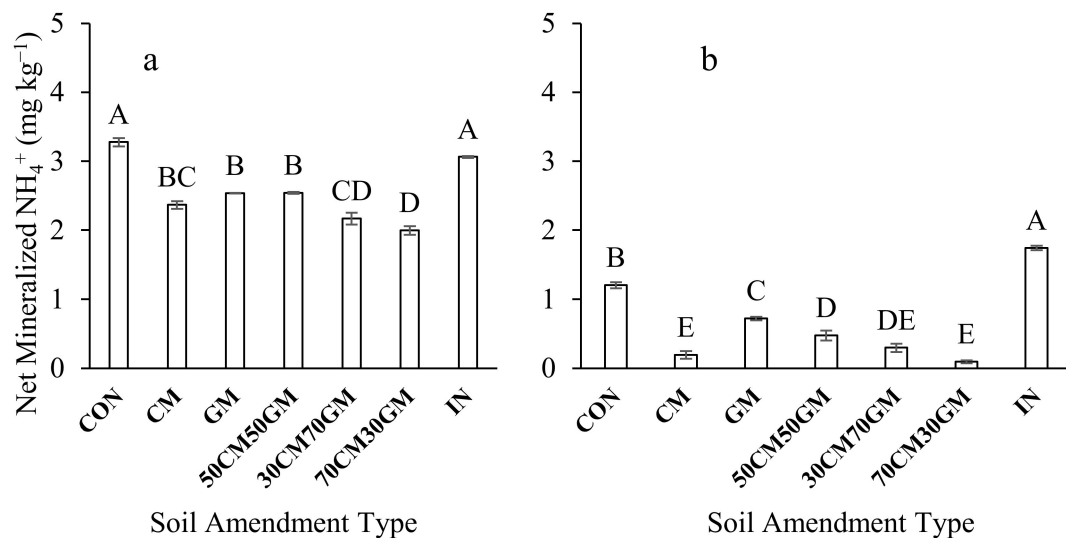

Figure 4. Mineralized NH$_4^+$ at four (**a**) weeks and 8 weeks (**b**) in soil treatments with six types of amendments expressed as mg kg^{-1} in an unamended soil (CON), soils amended with cow manure (CM), green manure (GM), 50:50 ratio of cow manure and green manure (50CM50GM), 30:70 ratio of cow manure and green manure (30CM70GM), 70:30 ratio of cow manure and green manure (70CM30GM), and inorganic fertilizer (IN). Different letters indicate significant differences at $p < 0.05$. The error bars represent standard error ($n = 3$). Eight weeks of mineralized NH$_4^+$ data were log-transformed.
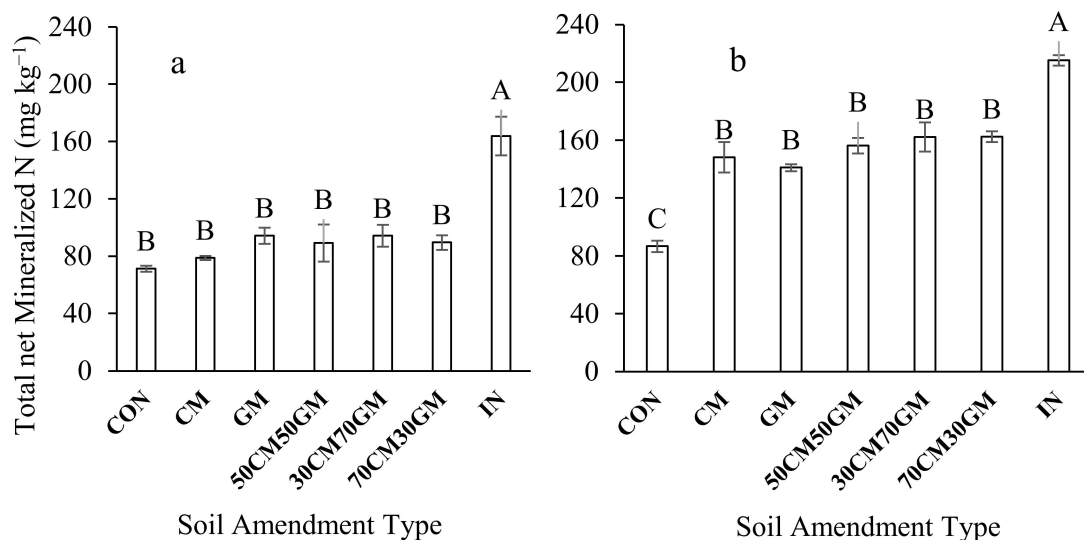

Figure 5. Total net mineralized N at four (**a**) weeks and eight weeks (**b**) in soil treatments with six types of amendments. Expressed as mg kg^{-1} in an unamended soil (CON), soils amended with cow

manure (CM), green manure (GM), 50:50 ratio of cow manure and green manure (50CM50GM), 30:70 ratio of cow manure and green manure (30CM70GM), 70:30 ratio of cow manure and green manure (70CM30GM), and inorganic fertilizer (IN). Different letters indicate significant differences at $p < 0.05$. The error bars represent standard error ($n = 3$).
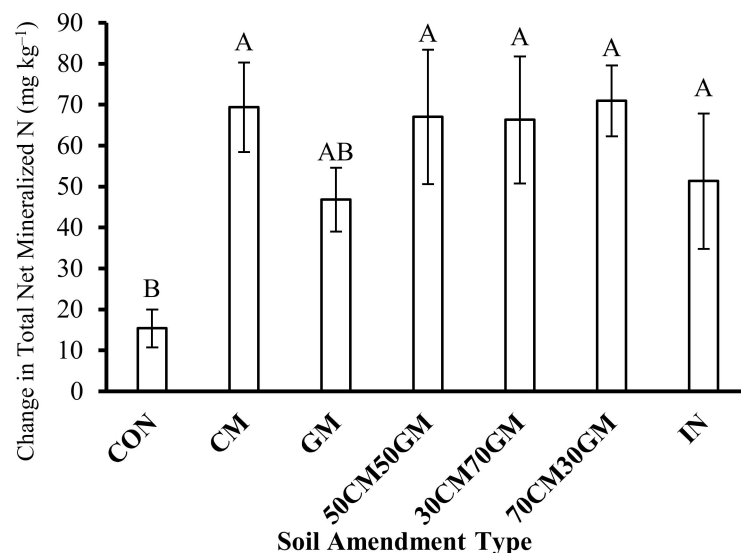

Figure 6. Change in total net mineralized N between four and eight weeks in soil treatments with six types of amendments expressed as mg kg^{-1} in an unamended soil (CON), soils amended with cow manure (CM), green manure (GM), 50:50 ratio of cow manure and green manure (50CM50GM), 30:70 ratio of cow manure and green manure (30CM70GM), 70:30 ratio of cow manure and green manure (70CM30GM), and inorganic fertilizer (IN). Different letters indicate significant differences at $p < 0.05$. The error bars represent standard error ($n = 3$).

4. Discussion

4.1. Effects of Soil Amendment Type on Enzyme Activity

In the present study, we did find significant differences among all the treatments in peroxidase and phenol oxidase activities. Significantly higher phenol oxidase activity was found in soils treated with green manure compared to all other amendment applications (Figure 1a). Compared to green manure, the addition of cow manure to soil amendments at various ratios inhibited phenol oxidase activities, consistent with Saiya-Cork et al. [25] and Nyiraneza et al. [53]. This may be due to increased inputs of lignin in the green manures derived from plant litter materials, which was shown to increase phenol oxidase activities [25]. Conversely, Dong et al. [41] reported a decrease in phenol oxidase activities after applications of green manure compared to an unamended control, and Khan et al. [54] reported a decrease in phenol oxidase activities and an increase in hydrolytic enzymes in response to green manure amendments. These differences in changes in soil enzyme activities might be explained by differences in the sources of organic material between green manure and amendments containing cow manure, such as the relative amounts of complex versus easily decomposable organic C compounds, and soluble carbon content [26,55].

We found the lowest phenol oxidase activity in soils amended with inorganic fertilizers (Figure 3), which indicates that inorganic fertilizer treatments promote inorganic N availability more than organic amendments. This is due to the increased availability of inorganic N input decreasing the expression of phenol oxidase [56,57]. No differences in phenol oxidase were found between cow manure and cow-green manure treatments, which were significantly lower than sole green manure treatment (Figure 3). This indicates that adding cow manure to green manure may be helpful in reducing phenol oxidase activity since cow manure was shown to inhibit phenol oxidase activity [26,53].

Compared to phenol oxidase activity, we observed different and larger variations in peroxidase activity. Significantly higher peroxidase activity was found in soil with the 70CM30GM treatment than in all other organic and inorganic treatments, even though they displayed similar NO_3^- and total mineralized N content (Figures 1b, 3 and 5). However, no significant differences among all high ratios of green manure treatments (green manure, 50% green manure 50% cow manure, and 70% green manure 30% cow manure) were observed (Figure 1b). This may be due to a high proportion of plant-based materials, such as plant litter amendment, which was shown to lead to lower peroxidase activity [25,58].

The different amendments in our studies may lead to changes in different organic carbon pools, for example, higher concentrations of labile organic carbon, and complex organic substances with carboxylic and phenolic-OH groups [53,57,59]. Thus, our study indicates that the addition of a high proportion of cow manure into green manure may change significantly the quantity and quality of biochemical conditions, which may be optimal and promote the activity of certain microorganisms and thus the biochemical processes [55]. For example, higher peroxidase activity was found in soils amended with *Azospirillum + Pseudomonas* biological fertilizers and ligninolytic microorganisms than in the control [60,61]. However, this needs future research for support.

Urease in soil derived from plant residues, animal waste, or soil microbes is responsible for the hydrolysis of urea to NH_3 and CO_2 [29]. Unlike the variations in phenol oxidase and peroxidase activity among treatments, significant differences were not observed among all treatments (Figure 2). Compared to the cow manure treatment, all green manure incorporated treatments showed lower urease activity (Figure 2). This indicates that cow manure may produce greater urea products, which promote urease activity more than green manures. Additionally, our study indicates that adding green manures into the animal manure may reduce urease activity and thus N loss, which is beneficial to agricultural management practice in reducing N loss.

4.2. Effects of Soil Amendment Type on Accumulated Inorganic N

Compared to CON, all fertilizer amendments showed significantly higher net accumulated nitrate and total mineralized N (Figures 3 and 5). Inorganic treatments had the highest inorganic N after four and eight weeks of incubation, which was significantly higher than all organic treatments (Figures 3–5). This is expected since inorganic fertilizers release inorganic N faster than organic manures [62]. However, the rate of inorganic N release slowed down from week 4 to week 8 (Figure 6), which was similar to GM treatment and lower (not statistically significant) than all CM-involved treatments. This may indicate the GM was slower in N mineralization rate than all CM-involved treatments, which would be consistent with Flavel and Murphy [63] and Agehara and Warncke [62]. Thus, GM shows advantages for crops at the earlier growth stage when less N is needed, and for crops that have a slower N uptake rate, such as high tunnel crops and greenhouse crops to reduce loss [63]. However, high application rates of plant waste may be needed to achieve suitable N availability [63].

No significant differences in mineralized nitrate and ammonium among all organic treatments were found after four weeks of incubation as well as eight-week incubation. This indicates that all organic amendments may show similar initial labile C and N, which may lead to similar biochemical N processes at the earlier stage of mineralization [62]. This trend may be different and differentiated among organic treatments after a long time of incubation due to changes in the labile C and N pool in each treatment [64]. Our study indicates the similar efficiency of inorganic N supply among inorganic fertilizer, cow manure, and green manure, as well as combinations of cow manure and green manure for short life-cycle crops, such as high tunnel and greenhouse crops. However, different amendments may lead to changes in long-term soil productivity and nutrient supply due to the changes in oxidative enzyme activity observed in this study (Figure 1).

4.3. The Implications of Soil Amendment Applications for Sustainable Agriculture

Soils currently act as potent carbon sinks and have the potential to store more carbon under proper management practices [65,66]. Efficient utilization of soil organic amendments manures can sustain SOC storage, reduce CO_2 emission, and mitigate climate change, especially in agricultural systems [67,68]. Agriculture typically leads to the loss of soil organic matter, which is due to increased phenol oxidase activity leading to faster recalcitrant SOC turnover [69]. Repressing or activating these enzymes through applications of soil amendments can influence an ecosystem's ability to act as a carbon sink or a net source of carbon to water bodies and the atmosphere [65,66]. Inhibiting these enzymes could be beneficial in promoting SOM accumulation and C storage in agricultural systems.

Fertilization significantly affects the production and activity of soil extracellular oxidases, which are responsible for soil organic matter turnover and stabilization as well as long-term nutrient cycling and soil carbon sequestration capacity [69]. While inorganic fertilizer amendments also reduced phenol oxidase and peroxidase activities and displayed increased N mineralization indicating increased C storage and N supply, applications of inorganic fertilizers are not beneficial for long-term C storage [70]. Additionally, inorganic fertilizer applications are associated with losses of N through leaching which can negatively impact surrounding ecosystems [71]. Therefore, inorganic fertilizers would not be optimal in promoting soil amendment management practices that meet the multiple ecological functions of providing sufficient nutrients for crops, reducing nutrient loss, and promoting long-term SOC stabilization [72].

Integrated fertilizer applications are promising alternatives for sustainable agriculture. We found similar total net accumulated inorganic N among all organic and inorganic treatments while 50CM50GM and 30CM70GM treatments displayed lower oxidative enzyme activity after eight weeks of incubation compared to cow manure, green manure, and inorganic fertilizer treatments (Figures 1 and 6). This indicates a higher potential of 50CM50GM and 30CM70GM fertilizer application in SOM accumulation and C storage than other treatments while providing similar N availability. Similarly, combinations of green and cow manure or inorganic fertilizers maintain soil fertility while providing the necessary N inputs for plant productivity [15,18,19]. In addition, animal manure application in agriculture led to antibiotics and pathogen issues [14]. Thus, lowering the proportion of cow manure might be helpful in mitigating these issues. Thus, combinations of organic fertilizers may be more efficient in providing plant-available nutrients and SOC storage than the sole application of a single type of compost material. However, SOC decomposition and stabilization are influenced by diverse physical environmental factors, such as variations in soil type, temperature, and water content, which were kept consistent in our study. The efficiency of integrated amendments applications needs further long-term field investigations.

5. Conclusions

Although green manures showed similar inorganic N availability to all other organic manure treatments, higher phenol oxidase enzyme activity was observed. This indicates enhanced decomposition of recalcitrant C sources in a short time following amendment application. In contrast, mixed manure amendments containing equal or lower proportions of cow manures compared to green manures displayed similar amounts of accumulated inorganic N and reduced oxidative enzyme activity after eight weeks of incubation. Therefore, mixed manure amendments at 50:50 ratios or 30:70 ratios of cow manure and green manure may be promising in promoting C accumulation in agricultural soils while also providing mineralized N content similar to other applications of manures. However, all these effects need to be further studied under field conditions for their long-term effects.

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