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Effects of neem leaf extract on inorganic nitrogen transformation in sandy soil

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Abstract

The inhibitory effects of neem leaf extract on fertilizer nitrogen (N) transformation in soil have not been fully recognized. This study therefore aimed to evaluate the effects of five nitrification inhibitors on the status of soil inorganic N, urea hydrolysis, and nitrification: i) no inhibitor (control); ii) nitrapyrin; and three rates of neem leaf extract based on the dry weight of the raw material: iii) 1 g kg^{-1} soil; iv) 2 g kg^{-1} soil; v) 4 g kg^{-1} soil. Neem leaf extract in all rates increased urea hydrolysis rate on days 5–15. In contrast, nitrapyrin decreased urea hydrolysis on days 5–10, compared to the control. As for nitrification, neem leaf extract showed both stimulatory and inhibitory effects. The stimulation appeared on day 3, whereas inhibition occurred and peaked on days 5–15. Nitrapyrin showed inhibitory effects on days 10–15, reaching its peak on day 15. Increasing rates of neem leaf extract brought about increases in stimulation and inhibition of urea hydrolysis and nitrification. The results conclusively stated that the neem leaf extract had generally faster, stronger, and longer nitrification inhibition than nitrapyrin. The stimulation in the early phase and the inhibition in the later phase of the incubation of neem leaf extract were more pronounced with higher application rates.

1. Introduction

Chemical fertilizers, particularly nitrogenous fertilizers, represent an essential tool in improving crop productivity in modern agriculture. However, today's very expensive nitrogen (N) fertilizers drive up the cost of the input resource in crop production. According to Abbasi et al. (2011), 45−60% of applied N was lost, posing concerns to environmental and human health risks. As N fertilizers are mostly ammonium (NH_4^+)-producing compounds, NH_4^+ is rapidly transformed to nitrate (NO_3^-) in nitrification. Nitrate is vulnerable to being leached into surface and ground waters as pollution, and denitrified into the atmosphere as a greenhouse gas. Increasing N-use efficiency is acknowledged as a managing approach to minimizing fertilizer input, manifesting cost savings, and providing environmental protection (IPCC, 2007). Therefore, retarding the nitrification rate is widely recognized to improve the efficiency of N fertilizers (Konwar et al., 2016; Xi et al., 2017).

Chemical nitrification inhibitors come in a wide variety of nitrification inhibitors, with nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine] considered a favorite, due to its practical usage (Mohanty et al., 2008). Nevertheless, chemical inhibitors are very costly and difficult to access in traditional markets (Mohanty et al., 2008; Kumar et al., 2010). Additionally, due to environmental and financial reasons shifting toward minimizing the use of chemicals in agriculture, an effective nitrification inhibitor ought to be achieved from natural, locally available resources (Sabir et al., 2020).

Neem (*Azadirachta indica*) extract is a promising natural nitrification inhibitor concomitant with enhanced N use efficiency (Sharma and Prasad, 1995), reported to inhibit soil nitrifiers (Santhi et al., 1986; Mweetwa et al., 2016). In addition to nitrification, urea hydrolysis, which converts urea to NH_4^+, is seen to be inhibited by neem extract (Sahrawat, 1980; Prasad and Power, 1995; Varel, 1997). Therefore, the slowing of urea hydrolysis decreases N loss and improves N use efficiency (Mohanty et al., 2008; Patra et al., 2009).

The neem tree is commonly found in tropical and semi-tropical areas, including Thailand (Orwa et al., 2009). The wide distribution of neem trees signifies that the readily available leaves are well suited for use as a natural nitrification inhibitor. Even though some inhibition properties of neem seed extracts on nitrification were reported (Gnanavelrajah and Kumaragamage, 1998; Arafat et al., 1999; Majumdar, 2005; Solomon et al., 2008; Kumar et al., 2010), those of neem leaves is very limited. Santhi et al. (1986) and Ruanpan and Mala (2016) found that 0.2% of the raw material-basis of neem leaf extract inhibited nitrifying bacteria in paddy soils. Meanwhile, Mweetwa et al. (2016) reported that neem leaf extract in water (greater than 10% w/v) inhibited

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microbial biomass and activity in slightly acidic soil. However, these studies did not report on the effects of varied application rates of neem leaf extract on nitrification and urea hydrolysis. Additionally, a comparison to the standard chemical nitrification inhibitor was not determined.

The current study addresses the hypothesis that nitrification inhibition would increase with rising rates of the neem leaf extract. The objective of this study, therefore, was to estimate the effects of varied rates of neem leaf extract on soil inorganic N concentrations and the inhibitions of nitrification and urea hydrolysis within coarsely textured soil.

2. Materials and methods

2.1. Soil and neem leaf extract

The soil used in this study was a Roi-et series (isohyperthermic Aeric Kandiaquults, or Luvisols in the World Reference Base for Soil Resources nomenclature), identified in the location of its collection using the 1:25,000 soil map developed by Thailand's Land Development Department (2022). The soil was collected at depths of 0–15 cm from the Research Field Facility of the Plant Science Section, Sakon Nakhon Rajabhat University, Sakon Nakhon, Thailand (17° 11' 08.8" N; 104° 05' 18.5" E). The soil was air-dried and passed through a 2 mm mesh sieve before use in the experiment. The initial soil properties are presented in Table 1.

A natural nitrification inhibitor was achieved by extracting neem leaves, modified from the method described by Ruanpan and Mala (2016), which was locally available in the Sakon Nakhon province of Northeast Thailand. Briefly, neem leaves were cleaned and dried under a drying house at 60°C for five days before being crushed into segments approximately 1 mm in size. Then, 5 kg of the crushed neem leaf was mixed thoroughly into 15 L of 95% ethanol, equivalent to 1:3 w/v, and kept in a 20-liter polyethylene tank for a couple of days. Mixed using an electric blender, the filtrate was then filtered through Whatman No. 1, dried using a rotary evaporator at 40°C, and stored at 4°C until employed in the experiment. The neem leaf extract yielded 10.9% of the raw material. Carbon (C) and N contents of the extract were 200 and 0.90 g kg^{-1}, respectively, and the azadirachtin content was 0.40 mg mg^{-1} extract.

Table 1
Initial physicochemical properties of the soil used in this study.

Soil property	Value
Soil particle distribution	
Sand (%)	81.8
Silt (%)	14.15
Clay (%)	4.05
Soil texture	Loamy sand
Bulk density (g cm^{-3})	1.55
pH (soil to water = 1:1)	5.60
Electrical conductivity (mS cm^{-1})	0.033
Cation exchange capacity (cmol kg^{-1})	3.50
Organic C (g kg^{-1})	3.31
Total N (g kg^{-1})	0.31
NH_4^+–N (mg kg^{-1})	5.51
NO_3^-–N (mg kg^{-1})	2.89

2.2. Incubation experiment

A microcosm incubation experiment was conducted in a greenhouse equipped with an evaporative cooling system from January to March 2022. The greenhouse's mean air temperature and humidity were 30.9°C and 42.5%, respectively. The experiment was arranged in a completely randomized design and replicated three times. Five treatments of nitrification inhibitors were studied: i) no inhibitor (control); ii) nitrapyrin; and three rates of neem leaf extract based on the dry weight of the raw material: iii) 1 g kg^{-1} soil (Neem1); iv) 2 g kg^{-1} soil (Neem2); and v) 4 g kg^{-1} soil (Neem4). The experimental unit consisted of a glass jar (d = 10 cm, h = 15 cm, v = 1000 cm^3) filled with 500 g of air-dried soil. Chemical fertilizer grades 46-0-0 ($CO(NH_2)_2$), 0-46-0 ($Ca(H_2PO_4)_2{\cdot}H_2O$), and 0-0-60 (KCl) were applied at the beginning of the experiment to each jar to achieve a fertilizer rate of 110 mg N kg^{-1} soil, 37.8 mg P kg^{-1}, and 49.8 mg K kg^{-1} soil (Yu et al., 2016). Nitrification inhibitors were applied in a solution at the start of the experiment to each jar according to their treatments. A recommended rate of 0.14 mg nitrapyrin jar^{-1}, equivalent to 0.25 g nitrapyrin 100 g^{-1} urea N (Sun et al., 2015), was applied to each respective jar. Neem leaf extracts at the rates of 0.055, 0.11, and 0.22 g extract jar^{-1}, which were equivalent to the dry-weight basis of the raw materials at 1, 2, and 4 g kg^{-1} soil, were added to the appropriate jars. Each jar was watered every other day throughout the experiment with distilled water to a predetermined moisture content of 22.4% w/w, equivalent to 65% of the water holding capacity. Sampling intervals for determinations of soil microbiochemical properties denoted pH, inorganic nitrogen (NH_4^+–N and NO_3^-–N), microbial biomass C and N, and CO_2 evolution; undertaken at 1, 3, 5, 10, 15, 30, and 45 days after incubation (DAI).

2.3. Laboratory analysis

Soil particle size distribution and texture were determined according to the pipette method of Kroetsch and Wang (2008). Soil bulk density was obtained following the core method (Pansu and Gautheyrou, 2006), and the soil water holding capacity was measured according to the maximum water holding capacity (Wilke, 2005).

Soil pH was determined using the soil-to-suspension ratio of 1:1 w/v, while electrical conductivity was measured in 1:5 w/v. The cation exchange capacity of the soil was achieved following the procedure described by Pansu and Gautheyrou (2006). Organic C of the soil and neem leaf extract were determined by the Walkley and Black method (Nelson and Sommers, 1982), while total N was analyzed through the micro-Kjeldahl method (Bremner and Mulvaney, 1982). NH_4^+–N and NO_3^-–N were extracted in 2 *M* KCl using fresh soil and determined via the distillation method (Stevenson, 1982) on a micro-Kjeldahl distillator (Pro-Nitro S 4002851, JP Selecta, Barcelona, Spain). The azadirachtin content of the neem leaf extract was determined using high-per-

formance liquid chromatography following the technique developed by Stark and Walter (1995).

Microbial biomass C and N determinations were performed using fresh soil following the chloroform fumigation-extraction technique described in Amato and Ladd (1988). Briefly, 10 g of fumigated and unfumigated soils were extracted in 50 ml of 0.5 *M* K_2SO_4 for microbial biomass C, and 50 ml of 1 *M* KCl for microbial biomass N. Subsequently, the C in the extract oxidized with 0.07 *N* $K_2Cr_2O_7$ was determined by titration with 0.01 *N* $Fe(NH_4)_2SO_4$, while N reacted with ninhydrin and was colorimetrically measured on a UV−Vis spectrophotometer (Hitachi U-5100, Hitachi High-Tech Corporation, Tokyo, Japan) using a wavelength of 570 nm. Both microbial biomass C and N were calculated using the differences between values derived from fumigated and unfumigated soils. In addition, the k_{EC} factor of 0.33 (Sparling and West, 1988) and k_{EN} of 0.31 (Amato and Ladd, 1988) were employed to convert the extracted organic C and N to microbial biomass C and N, respectively.

Carbon dioxide evolution, which indicates microbial activity, was assessed using the alkaline trap method following Anderson (1982). Briefly, a small glass bottle (d = 4 cm, h = 4 cm, and v = 35 cm^3) containing 20 ml of 1 *M* NaOH was placed on a plastic tripod on soil within each experimental unit (jar). Alkaline traps were left in the airtight jars for 24 hours. Subsequently, carbonate in the alkaline solution was precipitated using an excess 0.5 *M* $BaCl_2$. The evolved CO_2 was then measured through back titration using 0.125 *M* HCl.

2.4. Data calculations and statistical analyses

Net urea hydrolysis rate (*UHR*) (Equation 1) and net nitrification rate (*NNR*) (Equation 2) were computed by modifying the procedure described by Bi et al. (2017), as follows:

$$UHR\ (\text{mg N kg}^{-1}\text{ soil day}^{-1}) = [(NH_4^+\text{–}N)_{t2} - (NH_4^+\text{–}N)_{t1}]/t \qquad \text{Equation 1}$$

where t is a day interval length between a current sampling ($t2$) and a previous sampling ($t1$), and $(NH_4^+\text{–}N)_{t2}$ and $(NH_4^+\text{–}N)_{t1}$ are NH_4^+–N concentrations (mg kg^{-1}) of $t2$ and $t1$, respectively.

$$NNR\ (\text{mg N kg}^{-1}\text{ soil day}^{-1}) = [(NO_3^-\text{–}N)_{t2} - (NO_3^-\text{–}N)_{t1}]/t \qquad \text{Equation 2}$$

where t is a day length interval between a current sampling ($t2$) and a previous sampling ($t1$), and $(NO_3^-\text{–}N)_{t2}$ and $(NO_3^-\text{–}N)_{t1}$ are NO_3^-–N concentrations (mg kg^{-1}) of $t2$ and $t1$, respectively.

Urea hydrolysis inhibition (*UHI*) (Equation 3) and nitrification inhibition (*NI*) (Equation 4) were calculated by modifying a method of Aspelin and Ekholm (2017) as follows:

$$UHI\ (\%) = [(UHR_{control} - UHR_{inhibitor})/|UHR_{control}|] \times 100 \qquad \text{Equation 3}$$

where $UHR_{control}$ and $UHR_{inhibitor}$ are net urea hydrolysis rates of the control and an inhibitor, respectively, while $|UHR_{control}|$ is an absolute value of $UHR_{control}$.

$$NI\ (\%) = [(NNR_{control} - NNR_{inhibitor})/|NNR_{control}|] \times 100 \qquad \text{Equation 4}$$

where $NNR_{control}$ and $NNR_{inhibitor}$ are net nitrification rates of the control and an inhibitor, respectively, while $|NNR_{control}|$ is an absolute value of $NNR_{control}$.

Evolved CO_2−C was calculated according to the Equation 5 described by Anderson (1982):

$$CO_2\text{–}C\ (\text{mg } CO_2\text{–C day}^{-1}\text{ kg}^{-1}) = (B–S)/NE \qquad \text{Equation 5}$$

where B and S are the volumes (ml) of acid (HCl) used to titrate alkali (NaOH) of a blank and a sample, respectively; and N is the normality of the acid, and E is the factor 6, which is the equivalent weight of CO_2−C.

One-way analysis of variance based on a completely randomized design using the PROC ANOVA procedure following SAS Institute Inc. (2004) was performed to evaluate the effects of the inhibitors on soil microbiochemical properties; consisting of pH, concentrations of NH_4^+−N and NO_3^-−N, net urea hydrolysis rate, net nitrification rate, urea hydrolysis inhibition, nitrification inhibition, microbial biomass C and N, and CO_2−C evolution. Multiple comparisons were conducted according to Fisher's least significant difference test. Significant differences were at $p \leq 0.05$.

3. Results and discussion

3.1. Urea hydrolysis

Urea hydrolysis, which is triggered by aerobic ureolytic bacteria (e.g., *Sporosarcina pasteurii*, *Lysinibacillus sphaericus*, and *Bacillus sphaericus*) (Sigurdarson et al., 2018; Jiang et al., 2021), drives the transformation of urea to NH_4^+ in soil (Vlek et al., 1980; Svane et al., 2020). Neem leaf extract used in the current study proved to be a stimulator of urea hydrolysis, in contrast to other studies whose findings indicated inhibitory effects (Sahrawat, 1980; Prasad and Power, 1995; Varel, 1997). Significantly increased NH_4^+–N concentrations throughout the experimental length (Fig. 1A) and significantly higher net urea hydrolysis rates at 5–15 DAI in all rates of neem leaf extract (Fig. 1B) led to significantly lower and concomitantly with negative valued urea hydrolysis inhibition during 5–15 DAI compared to the control (Fig. 1C). The stimulatory effects of neem leaf extract on urea hydrolysis were found to be in line with the former studies of Mohanty et al. (2008), Kizilkaya et al. (2015), and Sridharan et al. (2017). Urea hydrolysis stimulation was explained by Kizilkaya et al. (2015), who stated that neem extract acted as a substrate providing C, N, and energy for soil microorganisms. This mechanism was supported by the significant increases in microbial biomass C in all neem extract rates, except Neem1 at 10 DAI (Fig. 2A), and in microbial biomass N under Neem2 and Neem4 (Fig. 2B) from 1–10 DAI relative to the control.

In contrast to the natural neem product, nitrapyrin showed an inhibitory effect on urea hydrolysis as demonstrated by significantly decreased net urea hydrolysis rate (Fig. 1B) and increased (positively valued) urea hydrolysis inhibition (Fig. 1C), compared to the control, during 5–10 DAI. Certain nitrifying bacteria, *Nitrosomonas* and *Nitrosospira*, were not only able to produce ammonia monooxygenase for ammonium oxidation in nitrification (He et al., 2018) but also urease for urea hydrolysis (Hasan, 2000). However, these bacteria played only a small role in urea hydrolysis in acidic soil (Allison and Prosser, 1991). The inhibitory effect of nitrapyrin on urea hydrolysis hence appeared only within 5–10 DAI.

(A) NH_4^+-N

Inhibitor	Days after incubation						
	1	3	5	10	15	30	45
Control	d	d	e	d	e	b	b
Nitrapyrin	a	a	d	d	d	b	b
Neem1	a	a	a	c	c	a	a
Neem2	b	b	b	b	b	a	a
Neem4	c	c	c	a	a	a	a

(B) Net urea hydrolysis rate

Inhibitor	Days after incubation					
	3	5	10	15	30	45
Control	ns(-)	c(-)	d(-)	d(-)	a(-)	ns(-)
Nitrapyrin	ns(-)	d(-)	e(-)	c(-)	ab(-)	ns(-)
Neem1	ns(-)	b(-)	c(-)	b(-)	a(-)	ns(+)
Neem2	ns(-)	a(-)	b(-)	a(+)	ab(-)	ns(+)
Neem4	ns(-)	a(-)	a(+)	a(+)	b(-)	ns(+)

(C) Urea hydrolysis inhibition

Inhibitor	Days after incubation					
	3	5	10	15	30	45
Nitrapyrin	ns(-)	a(+)	a(+)	a(-)	ns(+)	ns(+)
Neem1	ns(-)	b(-)	b(-)	b(-)	ns(+)	ns(-)
Neem2	ns(-)	c(-)	c(-)	c(-)	ns(+)	ns(-)
Neem4	ns(-)	c(-)	d(-)	c(-)	ns(+)	ns(-)

(D) NO_3^--N

Inhibitor	Days after incubation						
	1	3	5	10	15	30	45
Control	a	a	a	a	a	a	a
Nitrapyrin	d	d	e	b	b	a	a
Neem1	c	c	c	c	c	b	b
Neem2	c	c	b	c	d	b	b
Neem4	b	b	a	d	e	b	b

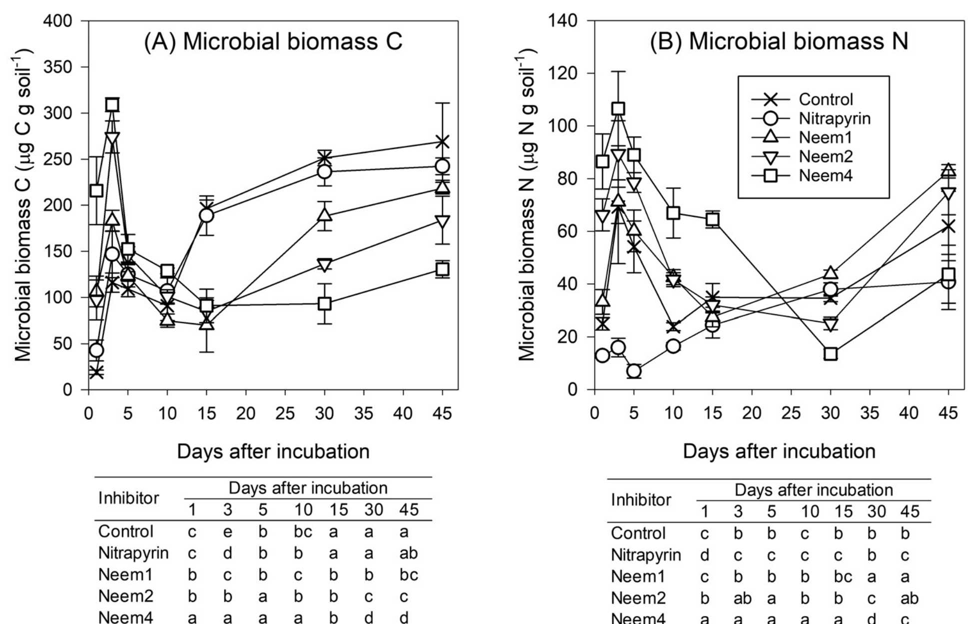
(E) Net nitrification rate

Inhibitor	Days after incubation					
	3	5	10	15	30	45
Control	e(+)	b(-)	a(+)	a(+)	c(-)	b(-)
Nitrapyrin	d(+)	a(-)	b(+)	c(+)	a(+)	b(-)
Neem1	c(+)	c(-)	c(+)	b(+)	b(-)	a(-)
Neem2	b(+)	d(-)	d(+)	c(+)	bc(-)	a(+)
Neem4	a(+)	e(-)	e(-)	d(-)	b(+)	a(+)

(F) Nitrification inhibition

Inhibitor	Days after incubation					
	3	5	10	15	30	45
Nitrapyrin	a(-)	d(-)	d(+)	b(+)	c(-)	a(+)
Neem1	b(-)	c(+)	c(+)	c(+)	ab(-)	b(-)
Neem2	c(-)	b(+)	b(+)	b(+)	a(-)	b(-)
Neem4	d(-)	a(+)	a(+)	a(+)	b(-)	b(-)

Fig. 1. The effects of different nitrification inhibitors on: (A) NH_4^+–N concentration; (B) net urea hydrolysis rate; (C) urea hydrolysis inhibition; (D) NO_3^-–N concentration; (E) net nitrification inhibition rate; (F) nitrification inhibition. The table accompanying each figure illustrates the comparisons of nitrification inhibitors at each time interval (period of day after incubation, DAI). Similar letters within a DAI are not significantly different ($p \leq 0.05$; Fisher's least significant difference test). Symbols (+) and (-) following letters indicate positive and negative values, respectively. Vertical bars represent standard deviations.



(A) Microbial biomass C

Inhibitor	Days after incubation						
	1	3	5	10	15	30	45
Control	c	e	b	bc	a	a	a
Nitrapyrin	c	d	b	b	a	a	ab
Neem1	b	c	b	c	b	b	bc
Neem2	b	b	a	b	b	c	c
Neem4	a	a	a	a	b	d	d

(B) Microbial biomass N

Inhibitor	Days after incubation						
	1	3	5	10	15	30	45
Control	c	b	b	c	b	b	b
Nitrapyrin	d	c	c	c	c	b	c
Neem1	c	b	b	b	bc	a	a
Neem2	b	ab	a	b	b	c	ab
Neem4	a	a	a	a	a	d	c

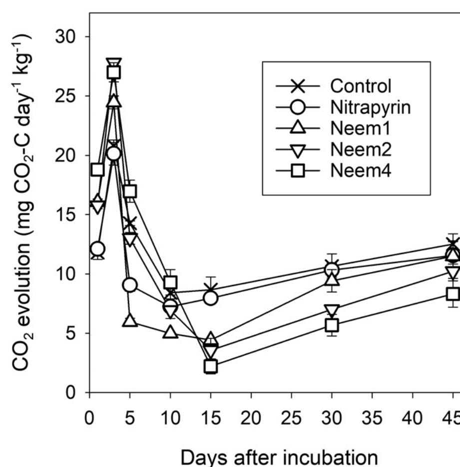
Fig. 2. The effects of different nitrification inhibitors on: (A) microbial biomass carbon; and (B) microbial biomass nitrogen. The table accompanying each figure demonstrates comparisons of nitrification inhibitors at each time interval (period of day after incubation, DAI). Similar letters within a DAI are not significantly different ($p \leq 0.05$; Fisher's least significant difference test). Vertical bars represent standard deviations.

3.2. Nitrification

In this investigation, all rates of neem leaf extract generally produced significantly higher NH_4^+–N concentrations (Fig. 1A) and significantly lower NO_3^-–N concentrations (Fig. 1D) compared to the control. Meanwhile, nitrapyrin yielded significantly higher NH_4^+–N concentrations at 1, 3, 5, and 15 DAI (Fig. 1A) and significantly lower NO_3^-–N concentrations at 1–15 DAI (Fig. 1D). Relative to nitrapyrin, all rates of neem leaf extract posed significantly higher NH_4^+–N concentrations at 5–45 DAI and significantly lower NO_3^-–N concentrations at 10–45 DAI. Overall, the results demonstrated that neem leaf extract inhibited nitrification, which was faster, longer, and much stronger than nitrapyrin. The long-lasting and high NH_4^+–N concentrations in the soil, attributed to the inhibitory effects of neem leaf extract (Fig. 1A), may become toxic to the plant (Guo et al., 2022).

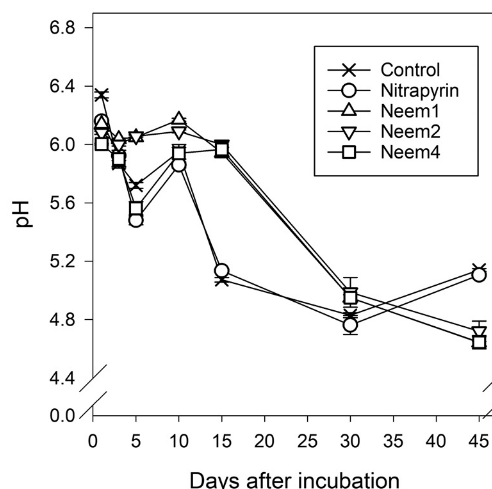
Nitrapyrin and the varied rates of neem leaf extract did not prompt an inhibiting nitrification function; but instead, acted immediately as stimulators. Their positive values of net nitrification rate (Fig. 1E) and negative values of nitrification inhibition (Fig. 1F) during the first 3 DAI supported the assertion. Azadirachtin, an active ingredient of the neem extract, may not have contacted the nitrifiers during that earlier period. Consistent with this assumption, previous studies have demonstrated that azadirachtin was not easily mobile in soil, due to its oily property (Kilani-Morakchi et al., 2021). Nitrapyrin, however, exhibited adsorption onto soil colloids (Wolt, 2000), contributing to its immediate non-function as a nitrification inhibitor. Additionally, higher neem leaf extract rates brought about greater nitrification stimulation on 3 DAI (Fig. 1E). Neem leaf extract stimulated nitrification by providing C and N, as well as energy for microorganisms (Kizilkaya et al., 2015), including nitrifiers. The high content (200 g kg^{-1}) of C and a portion of N (0.90 g kg^{-1}) contributed to the substrate-supporting properties of the neem leaf extract during such an early period. Furthermore, increases in microbial biomass C (Fig. 2A), microbial biomass N (Fig. 2B), and microbial activity (CO_2–C) (Fig. 3) related to increased extract rates, further demonstrating that nitrifiers received C and N from neem leaf extract.

Nitrification inhibitions abruptly increased under all rates of neem leaf extract at 5 DAI and leveled out during 5−15 DAI (Fig. 1F). Inhibition levels increased with increasing rates of neem leaf extract, revealing that azadirachtin, an active ingredient, plays a significant role in nitrification inhibition. Ammonia-oxidizing bacteria (i.e., *Nitrosospira*, *Nitrosomonas*, and *Nitrosococcus*) and types of ammonia-oxidizing archaea (i.e., *Nitrosopumilus* and *Nitrososphaera*) were nitrifiers that acted as key drivers in transforming NH_4^+ to NO_3^- in the acidic soil (He et al., 2018). Xi et al. (2017) found that nitrification inhibitors negatively affected nitrifiers directly by inhibiting ammonia-oxidizing bacteria, targeting ammonia monooxygenase that catalyst NH_3 to NH_2OH; as well as indirectly by raising soil pH, which was not preferred by ammonia-oxidizing archaea that are dominant nitrifiers in acidic soil (He et al., 2018). The indirect inhibitory effect of neem leaf extract was validated by higher soil pH under all rates of neem leaf extract at 3, 5, 15, and 30 DAI, compared to the control (Fig. 4).



Inhibitor	Days after incubation						
	1	3	5	10	15	30	45
Control	c	c	b	ab	a	a	a
Nitrapyrin	c	c	d	bc	a	a	ab
Neem1	b	b	e	d	b	a	ab
Neem2	b	a	c	c	b	b	b
Neem4	a	a	a	a	c	b	c

Fig. 3. The effects of different nitrification inhibitors on CO_2 evolution. The table accompanying each figure provides comparisons of nitrification inhibitors at each time interval (period of day after incubation, DAI). Similar letters within a DAI are not significantly different ($p \leq 0.05$; Fisher's least significant difference test). Vertical bars represent standard deviations.



Inhibitor	Days after incubation						
	1	3	5	10	15	30	45
Control	a	c	b	c	c	b	a
Nitrapyrin	b	b	d	d	b	b	a
Neem1	c	a	a	a	a	a	c
Neem2	d	a	a	b	a	a	b
Neem4	e	bc	c	c	a	a	bc

Fig. 4. The effects of different nitrification inhibitors on soil pH. The table accompanying each figure demonstrates comparisons of nitrification inhibitors at each time interval (period of day after incubation, DAI). Similar letters within a DAI are not significantly different ($p \leq 0.05$; Fisher's least significant difference test). Vertical bars represent standard deviations.

At 15−45 DAI, nitrification inhibition of all inhibitors went convexly downward (Fig. 1F). This change was consistent with decreased NH_4^+−N concentrations from 15 DAI onward for the neem treatments, and 3 DAI forward for the control and nitrapyrin treatments (Fig. 1A). Decreased NO_3^-−N concentrations from 15 DAI onward for all rates of neem leaf extract and the control, as well as nitrapyrin at 30 DAI onward also followed such chronological changes (Fig. 1D). These decreasing trends in soil inorganic N status were responsible for the declined urea that was the primary substrate for nitrification, corresponding to the first-order kinetic (Mohanty et al., 2008).

4. Conclusions

Overall, the results of this study demonstrated clearly that neem leaf extract produced faster, stronger, and longer-lasting inhibited nitrification, manifesting greater NH_4^+ and less NO_3^- concentrations than nitrapyrin. The neem leaf extracts exhibited both nitrification stimulation occurring on day 3, and inhibition behaviors that peaked over days 5 to 15. These effects of neem leaf extract were more pronounced with higher application rates. Nitrapyrin, conversely, limited its effects exclusively on days 10 to 15, reaching its peak on day 15. We would also like to recommend the further investigation of plant responses to inorganic nitrogen status, which are a consequence of the varied rates of neem leaf extract.

Acknowledgments

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