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Effects of Neem Leaf Extract on the Soil Properties, Growth, Yield, and Inorganic Nitrogen Contents of Lettuce

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Abstract: While lettuce offers essential human nutrients, it also contains anti-nutrients, particularly nitrate (NO_3^-). The use of neem leaf extract as a natural nitrification inhibitor has proven itself promising to remediate lettuce tissue NO_3^- content. This study evaluated the effects of neem leaf extract on soil properties, soil nitrification, lettuce growth, yield, and NO_3^- content. Five nitrification inhibitor treatments were evaluated: (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). Neem leaf extract generally increased soil concentrations: P (47.6–55.8 mg kg^{-1}), K (45.8–62.7 mg kg^{-1}), Ca (129–164 mg kg^{-1}), and Mg (29.0–35.7 mg kg^{-1}) compared with the control (50.6 mg P kg^{-1}, 35.3 mg K kg^{-1}, 123 mg Ca kg^{-1}, and 24.8 mg Mg kg^{-1}). Neem leaf extracts significantly increased soil NH_4^+–N concentrations (13.9–30.2 mg kg^{-1}) and nitrification inhibition (12.5–70.5%), but significantly decreased soil NO_3^-–N concentrations (6.4–13.2 mg kg^{-1}) and net nitrification rates (0.08–0.23 mg N kg^{-1} day^{-1}) relative to the control (6.6 mg NH_4^+–N kg^{-1}, 14.7 mg NO_3^-–N kg^{-1}, 0.26 mg N kg^{-1} day^{-1}, and 0% nitrification inhibition). The neem leaf extracts significantly decreased shoot fresh weight (13.5–43.1 g $plant^{-1}$), shoot dry weight (0.84–3.91 g $plant^{-1}$), and root dry weight (0.14–0.27 g $plant^{-1}$) compared with the control (52.3 g shoot fresh weight $plant^{-1}$, 5.36 g shoot dry weight $plant^{-1}$, and 0.35 g root dry weight $plant^{-1}$). The significant decreases in the lettuce biomass in the neem extract treatments paralleled the significant decreases in the shoot's tissue NO_3^-–N contents and significant increases in tissue NH_4^+–N content and soil Al concentrations.

Keywords: aluminum toxicity; ammonium injury; nitrification inhibitor; tissue nitrate; vegetable

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1. Introduction

Vegetables are globally consumed, accounting for 22% of the total worldwide diet [1] and 27% in Thailand [2]. Vegetables offer essential nutrients, such as vitamins (vitamins A, B, C, and E), beneficial substances (carotenoids, thiamin, riboflavin, and phenolic compounds), and essential elements [calcium (Ca), potassium (K), and sodium (Na)] [3,4].

Vegetables contain essential nutrients yet have high anti-nutrient contents, particularly nitrate (NO_3^-), which endangers human health. Human NO_3^- consumption from leafy vegetables accounted for 80–90% of the total NO_3^- intake [5,6]. High contents of NO_3^- were observed in lettuce (907–4674 mg NO_3^- kg^{-1} fresh weight), spinach (390–3383 mg NO_3^- kg^{-1}), and cabbage (150–1600 mg NO_3^- kg^{-1}) [7]. Nitrate is a precursor of *N*–nitroso compounds, resulting in many severe health risks, such as cancer, methemoglobinemia, hyperthyroidism, and diabetes [8].

A primary factor regulating plant tissue NO_3^- content is the cultivation practices in vegetable production [9]. Zandvakili et al. [10] demonstrated that plant NO_3^- content

increased through chemical fertilizer application relative to that under cow manure. It is indisputable that modern agriculture mainly relies on chemical fertilizers, particularly nitrogenous fertilizers, to increase crop productivity. Most N fertilizers primarily produce ammonium nitrogen (NH_4^+–N), which is immediately transformed to nitrate (NO_3^-) during the nitrification process, which further accumulates in the plant [11]. In addition to human health risks, soil NO_3^- is vulnerable to being leached into surface and ground waters, bringing about water pollution, or denitrified to greenhouse gas escaping into the atmosphere [12]. Retarding the nitrification rate using nitrification inhibitors is recognized to not only remediate NO_3^- production in soil and accumulation in the plant but also to minimize N losses to the environment [13,14]. A variety of chemical nitrification inhibitors is recognized, and nitrapyrin [2–chloro–6–(trichloromethyl)–pyridine: $C_6H_3Cl_4N$] has become a favorite in the academic community due to its effective use [15]. However, it is not practically favored, as it can be expensive and difficult to access in traditional markets [15,16]. Additionally, the economic and environmental concerns of chemicals used in agriculture have prompted the usage of locally available natural resources as nitrification inhibitors [17], such as neem extract. Neem extract represents a promising natural inhibitor due to its inhibitory properties on soil-nitrifying microorganisms, this is due to the functions of the most active ingredients in neem: azadirachtin and nimbolide [18,19].

Generally, neem trees are found in tropic and sub-tropic regions and are widely distributed throughout Thailand [20]. Neem seed extract has been used as a nitrification inhibitor in several studies [16,21–24]; however, investigations on the leaf extract are rather limited. The inhibitory property of neem leaf extract at 0.2% of the raw material basis in hindering nitrification bacteria in paddy soils was reported by Santhi et al. [19] and Ruanpan and Mala [25]. In addition, the water extract of neem leaves at rates higher than 10% w/v inhibiting microbial biomass and microbial activity in an acidic soil was demonstrated by Mweetwa et al. [18]. However, such reports did not show different application rates of neem leaf extract nor the influence on plant growth and yield, as well as tissue NO_3^- content.

This study hypothesized that neem leaf extract would inhibit the nitrification rate and affect vegetable growth and yield and remediated tissue NO_3^- concentration. Therefore, the objective of the study was to evaluate the effects of the application rates of neem leaf extract on soil properties and nitrification, as well as the growth, yield, and tissue NO_3^- content of a vegetable.

2. Materials and Methods

2.1. Soil and Neem Leaf Extract

The soil utilized in the current study was collected at a depth of 0 to 15 cm from the Research Field Facility of the Plant Science Section, Sakon Nakhon Rajabhat University, Thailand (17°11′08.8″ N; 104°05′18.5″ E). It was identified as a Roi Et series (isohyperthermic Aeric Kandiaquults) using the 1:25,000 soil map developed by Thailand's Land Development Department [26]. The soil was air-dried, crushed, and sieved to pass through a 2-mm mesh before being employed in the plant bioassay experiment. The initial soil physicochemical properties are shown in Table 1.

Neem leaf extract was obtained by extracting neem leaves using the method modified by Ruanpan and Mala [25]. Neem leaves, locally available in the Sakon Nakhon province, were cleaned and dried in a drying house at 60 °C for five days and later crushed into approximately 1 mm in size. The crushed neem leaves were immersed in 15 L of 95% ethanol in a 20-L polyethylene gallon for two days. The mixture was thoroughly mixed again with an electronic blender and then filtered through Whatman No.1. The filtrate was dried using a rotary evaporator at 40 °C and kept under 4 °C until used in the experiment. The characteristics of the neem leaf extract are presented in Table 2.

Table 1. Initial soil properties.

Property †	Value
Soil particle distribution	
Sand (%)	83.1
Silt (%)	11.9
Clay (%)	5.0
Soil texture	Loamy sand
BD (g cm^{-3})	1.54
Water holding capacity (%)	34.4
pH (1:1)	5.72
EC (mS cm^{-1})	0.034
CEC (cmol kg^{-1})	2.72
Organic C (g kg^{-1})	4.24
Total N (g kg^{-1})	0.13
NH_4^+–N (mg kg^{-1})	5.78
NO_3^-–N (mg kg^{-1})	2.98
P (mg kg^{-1})	18.3
K (mg kg^{-1})	29.3
Ca (mg kg^{-1})	108.1
Mg (mg kg^{-1})	28.0
Al (mg kg^{-1})	3.45

† BD = bulk density, EC = electrical conductivity, CEC = cation exchange capacity.

Table 2. Characteristics of the neem leaf extract.

Characteristic	Value
Extract yield (g kg^{-1} raw material)	109
Organic C (g kg^{-1} extract)	200
Total N (mg kg^{-1} extract)	900
NH_4^+–N (mg kg^{-1} extract)	452
NO_3^-–N (mg kg^{-1} extract)	75.3
P (mg kg^{-1} extract)	300
K (mg kg^{-1} extract)	3900
Ca (mg kg^{-1} extract)	200
Mg (mg kg^{-1} extract)	900
Azadirachtin (g kg^{-1} extract)	392
Nimbolide (g kg^{-1} extract)	544

2.2. Plant Bioassay Experiment

A plant bioassay experiment was conducted under greenhouse conditions equipped with an evaporative cooling system from January to March 2022. The mean air temperature of the greenhouse over the experiment was 30.9 °C, and the humidity was 42.5%. The experiment was arranged in a completely randomized design with three replications, each containing four pots. There were five nitrification inhibitor treatments: (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).

A pot (h = 14.3 cm, top d = 18 cm, bottom d = 13.5 cm, V = 2805 cm^3) was filled with 3 kg of air-dried soil. Furthermore, the pot was incubated with distilled water to a predetermined moisture content of 22.4% w/w or 671.1 mL pot^{-1}, equivalent to 65% of the water holding capacity for 15 days before transplanting lettuce.

A commercial variety of red coral lettuce (*Lactuca sativa* L.) was used as the test vegetable due to its high NO_3^- content [27]. The lettuce was seeded and nursed in a nursery tray for 15 days. A healthy seedling of single homogeneity was transplanted into each pot. Chemical fertilizer grades [46–0–0 (urea), 0–46–0 (triple superphosphate), and 0–0–50 (potassium sulfate)] were equally applied, twice, at 18 and 30 days after planting; or 3 and

15 days after transplanting to achieve the desired fertilizer rates [110 mg N kg^{-1} soil (0.717 g urea pot^{-1}), 85 mg P_2O_5 kg^{-1} (0.554 g triple superphosphate pot^{-1}), and 60 mg K_2O kg^{-1} soil (0.360 g potassium sulfate pot^{-1})] [28]. A recommended rate of 0.825 mg nitrapyrin pot^{-1}, equivalent to 0.25 g nitrapyrin 100 g^{-1} urea N [29], was applied to each pot accordingly. Neem leaf extracts at the amounts of 0.327, 0.654, and 1.308 g extract pot^{-1}—which were equivalent to the dry-weight basis of the raw materials for dried leaves (109 g extract kg^{-1} raw material, Table 2) at 1, 2, and 4 g kg^{-1} soil—were added to the respective pots. These nitrification inhibitors were split once into two applications and through dissolved in 1% dimethyl sulfoxide solution in each respective pot at 18 and 30 days after planting. Chemical fertilizer was simultaneously added. The soil moisture content of each pot was maintained at 65% of the water-holding capacity by weighing the pots daily throughout the experiment.

Lettuce growth parameters, including height, canopy size (diameter), and leaf number, were measured every three days, whereas leaf chlorophyll content was collected on the harvest date using a SPAD chlorophyll meter (SPAD 502 Plus, Spectrum Technologies, Inc., Aurora, IL, USA). Shoot biomass of the lettuce was harvested 45 days after planting for shoot fresh weight examination and then oven-dried at 65 °C until we obtained the constant shoot dry weight. Soil bulk density was measured on the same day. Fresh soil was immediately sampled for inorganic N (NH_4^+–N and NO_3^-–N) determination and then allowed to air dry. Roots were carefully separated and collected from the dried soil using a 1-mm mesh sieve. The roots were washed with distilled water and then oven-dried at 65 °C to achieve a constant root dry weight. The oven-dried shoot biomass of lettuce was ground and sieved through a 1 mm mesh, while the air-dried soil was sieved through a 2 mm mesh for further laboratory analysis.

2.3. Laboratory Analyses

Soil particle size distribution and texture were determined using the pipette method [30]. Soil bulk density was established by the core method [31]. Soil water holding capacity was assessed using the maximum water holding capacity following Wilke [32].

Soil pH and electrical conductivity (EC) were assessed using the soil-to-distilled water ratio of 1:1 *w*/*v* and 1:5 *w*/*v*, respectively. Organic carbon (C) of soil and neem leaf extract were determined according to the Walkley and Black method [33], while total nitrogen (N) determinations were performed following the micro-Kjeldahl method [34]. Inorganic N of the soil and the neem extract were determined by extraction in 2 M KCl and measured using the stream distillation method [35] on a micro-Kjeldahl distillation apparatus (Pro–Nitro S 4002851, JP Selecta, Barcelona, Spain). Phosphorus (P) of soil was extracted in Bray-2 solution, while P of the neem extract was provided through nitric-perchloric acid solution [36], and then determined on a UV–Vis spectrophotometer (Specord250 plus, Analytik Jena, Germany) using a wavelength of 820 nm [37]. The extraction of cations, i.e., potassium (K), calcium (Ca), and magnesium (Mg), in the soil were performed using 1 N NH_4OAc at pH 7 [31], while those of the neem leaf extract were extracted through nitric-perchloric acid solution [36]. The cations were then determined on a flame atomic absorption spectrometer (Flame AAS novAA® 350, Analytik Jena, Germany). The cation exchange capacity (CEC) was determined by saturating the negative surface charges of soil with NH_4^+ derived from 1 N NH_4OAc at pH 7. Ammonium ions were extracted from the adsorption sites with 10% acidified NaCl and determined using the distillation method for further CEC calculation [31]. The extraction of soil exchangeable Al was completed through 1 M KCl and measured by the titrimetry method following Pansu and Gautheyrou [31], which was modified using phenol red as an indicator, rather than the commonly used phenolphthalein [38]. The determination of azadirachtin and nimbolide in the extract was assessed using high-performance liquid chromatography following Stark and Walter [39].

Lettuce shoot tissue N, P, K, Ca, and Mg contents were extracted with nitric-perchloric wet digestion [36]. Tissue N content was then determined using the micro-Kjeldahl method [40]. P was measured on a UV–Vis spectrophotometer, while the K, Ca, and Mg

contents were measured via a flame atomic absorption spectrometer. Lettuce tissue NH_4^+–N content was extracted with 2% acetic acid solution and determined using the stream distillation method following Ali and Lovatt [41]. Tissue NO_3^-–N content was assessed through the salicylic acid assay of Cataldo et al. [42].

2.4. Data Calculation

The net nitrification rates were calculated following Equation (1), modified from Bi et al. [43]:

$$\text{Net nitrification rate}\left(\text{mg N kg}^{-1}\text{ soil day}^{-1}\right) = \frac{[NO_3^- \text{–N}]_{t2} - [NO_3^- \text{–N}]_{t1}}{t} \quad (1)$$

where $[NO_3^-\text{–N}]_{t2}$ and $[NO_3^-\text{–N}]_{t1}$ are soil NO_3^-–N concentrations in the harvest and the start of the experiment, respectively.

The nitrification inhibition was computed using a procedure modified from Aspelin and Ekholm [44], as shown in Equation (2):

$$\text{Nitrification inhibition (\%)} = \frac{(\text{Net nitrification rate})_{\text{Control}} - (\text{Net nitrification rate})_{\text{Inhibitor}}}{(\text{Net nitrification rate})_{\text{Control}}} \quad (2)$$

where $(\text{Net nitrification rate})_{\text{Control}}$ is the net nitrification rate of the control treatment, and $(\text{Net nitrification rate})_{\text{Inhibitor}}$ are the treatments of nitrapyrin, Neem1, Neem2, and Neem4.

The ammonium toxicity ratio was determined according to Song et al., 2022; as shown in Equation (3):

$$\text{Ammonium toxicity ratio (\%)} = \frac{\text{Number of plants deveoped ammonium toxicity symptom}}{\text{Number of plants in each experimental unit}} \times 100 \quad (3)$$

2.5. Statistical Analysis

The effects of different nitrification inhibitors on soil and lettuce were evaluated using an analysis of variance (ANOVA) based on a completely randomized design following the PROC ANOVA procedure [45]. Multiple comparisons were determined using Fisher's least significant difference at $p \leq 0.05$.

3. Results and Discussion

Neem leaf extract increased soil K, Ca, and Mg concentrations relative to the control, while soil P increased in only Neem4 (Table 3). All nitrification inhibitors produced soil total N (0.35–0.38 g N kg^{-1}), P (45.7–55.8 mg P kg^{-1}), Ca (123–164 mg Ca kg^{-1}), and Mg (25.9–35.7 mg Mg kg^{-1}) concentrations lower than what annual leafy crops required, i.e., 0.45 g N kg^{-1} [46], 115 mg P kg^{-1} [47], 240.5 mg Ca kg^{-1} [48], and 53.5 mg Mg kg^{-1} [49]. Meanwhile, these inhibitors produced soil K concentrations (41.4–62.7 mg K kg^{-1}) within the adequate level, i.e., 40 mg K kg^{-1} [50]. While generally lower than the adequate levels of leafy vegetable requirements, neem leaf extract increased P, K, Ca, and Mg concentrations in the soil (Table 3) due to the high contents of these macronutrients in the extract (Table 2). Additionally, given the very high K content of neem leaf extract (3900 mg kg^{-1}) (Table 2), only soil K concentrations fell within the established adequate level for annual leafy crops (Table 3).

Increases in soil macronutrient concentrations did not eventually raise their lettuce tissue contents, as seen in lower tissue N, P, K, and Ca contents in Neem1, than in the control (Table 4). Increases in these macronutrients in lettuce tissue under Neem2 and Neem4 may be due to the concentration effect [51]. Decreased soil P concentrations (Table 3), decreased lettuce tissue P, K, and Ca contents (Table 4), and a decreased uptake of P, K, Ca, and Mg (Table 5) were found in the nitrapyrin treatment relative to the control. These observations were earlier described by Luo et al. [52] that speculated that decreases in P and cations (K, Ca, and Mg herein) in soil treated with nitrapyrin were because of the precipitation of P with the cations.

Table 3. Soil macronutrient concentrations at lettuce harvest as affected by different nitrification inhibitors.

Inhibitor †	Total N (g kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})
Control	0.36	50.6 b ‡	35.3 c	123 c	24.8 c
Nitrapyrin	0.35	45.7 c	41.4 bc	123 c	25.9 c
Neem1	0.36	47.6 bc	45.8 b	129 bc	29.5 b
Neem2	0.37	47.9 bc	46.6 b	134 b	29.0 b
Neem4	0.38	55.8 a	62.7 a	164 a	35.7 a
p-value	0.296	0.008	<0.001	<0.001	<0.001
F test	ns	**	***	***	***
CV (%)	5.07	5.39	9.65	3.85	4.61

** = $p \leq 0.01$; *** = $p \leq 0.001$; ns = not significant; CV = coefficient of variation. † Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg^{-1} soil. ‡ Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Fisher's least significant difference test).

Table 4. Lettuce shoots tissue macronutrients and ammonium and nitrate contents as well as ammonium toxicity ratio as affected by different nitrification inhibitors.

Inhibitor †	N (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	NH_4^+–N (g kg^{-1})	NO_3^-–N (g kg^{-1})	Ammonium Toxicity Ratio (%)
Control	34.3 a ‡	6.57 ab	35.4 a	4.35 b	4.90 b	0.069 d	0.94 b	0
Nitrapyrin	26.0 b	3.97 c	20.5 b	3.55 c	3.97 b	0.070 d	1.06 b	0
Neem1	26.5 b	5.20 bc	23.5 b	3.47 c	4.35 b	0.106 c	0.41 d	100
Neem2	33.0 a	6.10 ab	33.6 a	4.60 b	4.55 b	0.117 b	0.72 c	100
Neem4	35.7 a	6.80 a	38.4 a	5.00 a	6.40 a	0.135 a	1.48 a	100
p-value	<0.001	0.01	<0.001	<0.001	0.002	<0.001	<0.001	–
F test	***	**	***	***	**	***	***	–
CV (%)	6.58	14.29	10.17	3.67	10.98	4.27	8.63	–

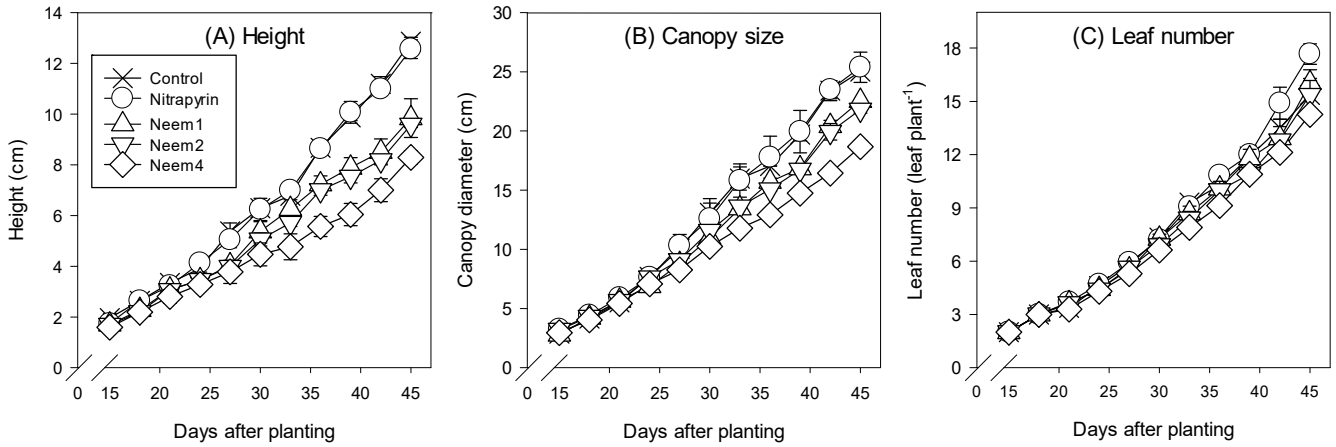
** = $p \leq 0.01$; *** = $p \leq 0.001$; CV = coefficient of variation. † Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg^{-1} soil. ‡ Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Fisher's least significant difference test).

Table 5. Macronutrient uptakes of lettuce as affected by different nitrification inhibitors.

Inhibitor †	N (mg Plant^{-1})	P (mg Plant^{-1})	K (mg Plant^{-1})	Ca (mg Plant^{-1})	Mg (mg Plant^{-1})
Control	18.4 a ‡	35.2 a	190 a	23.3 a	26.3 a
Nitrapyrin	16.0 b	24.4 b	126 b	21.8 b	24.4 b
Neem1	10.4 c	20.3 b	92 c	13.5 d	17.0 c
Neem2	10.9 c	20.1 b	111 bc	15.2 c	15.0 d
Neem4	3.0 d	5.7 c	32 d	4.2 e	5.4 e
p-value	<0.001	<0.001	<0.001	<0.001	<0.001
F test	***	***	***	***	***
CV (%)	10.12	16.46	13.73	3.27	5.66

*** = $p \leq 0.001$; CV = coefficient of variation. † Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg^{-1} soil. ‡ Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Fisher's least significant difference test).

The enhanced soil macronutrient concentrations through the neem extract application did not improve lettuce growth (Figure 1) or yields (Figure 2). Contrastingly, the neem leaf extract of all rates generally decreased the lettuce's growth, i.e., height (Figure 1A), canopy size (Figure 1B), and leaf number (Figure 1C), compared with the control. Yields, i.e., shoot fresh weight (Figure 2A), shoot dry weight (Figure 2B), and root dry weight (Figure 2C) also decreased relative to the control. Additionally, the growth (Figure 1A–C) and yields (Figure 2A–C) of lettuce decreased with increasing rates of neem leaf extract, whereas nitrapyrin significantly increased those parameters (Figure 2A–C).



Inhibitor	Days after planting										
	15	18	21	24	27	30	33	36	39	42	45
Control	ns	a	a	b	a	a	a	a	a	a	a
Nitrapyrin	ns	a	ab	a	a	a	a	a	a	a	a
Neem1	ns	b	a-c	cd	b	b	b	b	b	b	b
Neem2	ns	b	bc	c	b	bc	c	b	b	b	b
Neem4	ns	b	c	d	b	c	d	c	c	c	c

Inhibitor	Days after planting										
	15	18	21	24	27	30	33	36	39	42	45
Control	ns	ns	ns	ns	a	a	a	ab	a	a	a
Nitrapyrin	ns	ns	ns	ns	a	a	a	a	a	a	a
Neem1	ns	ns	ns	ns	b	b	b	bc	b	b	b
Neem2	ns	ns	ns	ns	b	ab	b	c	b	b	b
Neem4	ns	ns	ns	ns	b	b	c	d	c	c	c

Inhibitor	Days after planting										
	15	18	21	24	27	30	33	36	39	42	45
Control	ns	ns	b	ab	ab	a	a	a	a	b	bc
Nitrapyrin	ns	ns	a	a	a	a	ab	a	a	a	a
Neem1	ns	ns	a	bc	ab	a	bc	a	a	bc	b
Neem2	ns	ns	a	c	ab	ab	cd	a	b	bc	bc
Neem4	ns	ns	b	c	b	b	d	b	b	c	c

Figure 1. Lettuce growth: (**A**) height; (**B**) canopy size; and (**C**) leaf number as affected by different nitrification inhibitors. The table accompanying each sub-figure shows the comparisons of the effects of nitrification inhibitors at each time interval (days after lettuce planting). Similar letters within each time interval are not significantly different ($p \leq 0.05$; Fisher's least significant difference test). Vertical bars represent standard deviation. Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg $^{-1}$ soil.

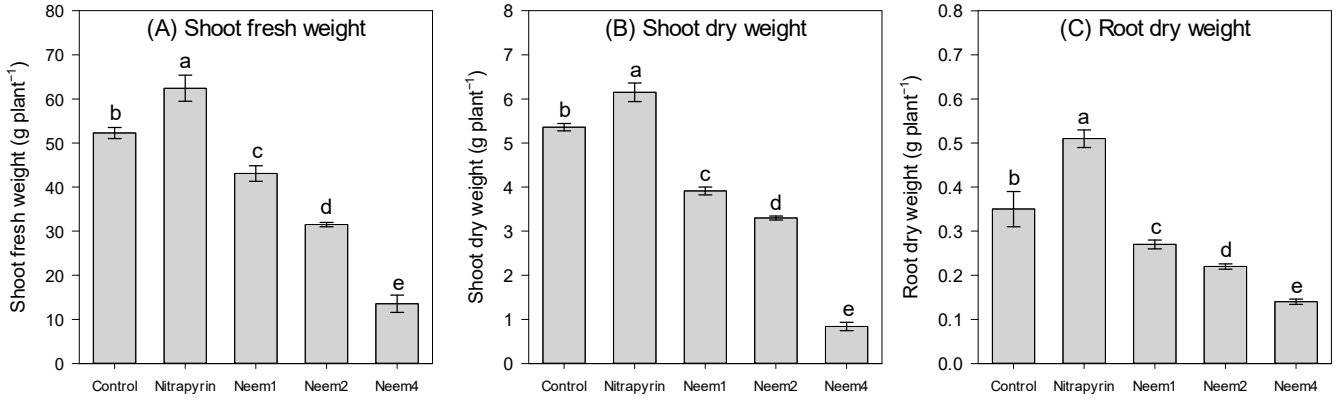


Figure 2. Lettuce yields: (**A**) shoot fresh weight, (**B**) shoot dry weight, and (**C**) root dry weight, as affected by different nitrification inhibitors. Bars with different letters indicate statistical differences ($p \leq 0.05$; Fisher's least significant difference test). Error bars represent the standard deviation. Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg $^{-1}$ soil.

Low soil NO_3^- availability and NH_4^+ toxicity could be the primary drivers of the decreased lettuce growth and yield. The adequate concentration of soil NO_3^-–N for a leafy vegetable is 45 mg kg^{-1} [46]; however, in the current study, soil NO_3^-–N concentrations were recorded at 4.3–14.7 mg kg^{-1} (Table 6). Moreover, increasing the neem extract rates brought about significantly decreased soil NO_3^-–N concentrations.

Table 6. Soil ammonium and nitrate nitrogen concentrations at the lettuce harvest as affected by different nitrification inhibitors.

Inhibitor †	NH_4^+–N (mg kg^{-1})	NO_3^-–N (mg kg^{-1})	Net Nitrification Rate (mg N kg^{-1} day^{-1})	Nitrification Inhibition (%)
Control	6.6 d ‡	14.7 a	0.26 a	0
Nitrapyrin	6.7 d	4.3 e	0.03 e	88.4 a
Neem1	13.9 c	13.2 b	0.23 b	12.5 d
Neem2	17.2 b	9.4 c	0.14 c	44.9 c
Neem4	30.2 a	6.4 d	0.08 d	70.5 b
p-value	<0.001	<0.001	<0.001	<0.001
F test	***	***	***	***
CV (%)	6.16	7.90	11.43	12.92

*** = $p \leq 0.001$; CV = coefficient of variation. † Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg^{-1} soil. ‡ Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Fisher's least significant difference test).

In addition to low soil NO_3^- availability, soil NH_4^+–N concentrations (Table 6), tissue NH_4^+–N content, and ammonium toxicity ratio (Table 4) in all rates of neem leaf extract increased. It is, therefore, evident that NH_4^+ toxicity was a factor in the lower lettuce growth (Figure 1) and yields (Figure 2). Theoretically, under high soil NH_4^+ concentration, horticultural crops rapidly uptake NH_4^+ due to the lesser energy requirement for its assimilation to organic nitrogen in plant cells [51,53,54]. Nevertheless, excessive NH_4^+ supply leads to cell acidification and is harmful to plants [55]. High soil NH_4^+ concentrations were shown to be toxic to lettuce [56]. According to Hawkesford et al. [54] and Song et al. [57], NH_4^+ poisoning signs include leaf chlorosis and necrosis, as well as eventually stunted growth. Figure 3 presents an illustration of the lettuce's responses to different nitrification inhibitors, thereby verifying the observation that lettuce suffers from NH_4^+ toxicity due to the treatments with neem leaf extract. Hawkesford et al. [54] and Song et al. [57] argued that NH_4^+ toxicity leads to a decrease in the uptake of essential cations. This is in line with the results of the study herein that witnessed a lower uptake of K, Ca, and Mg in neem leaf extract (Table 5) and lower tissue contents of these nutrients in Neem1 (Table 4).

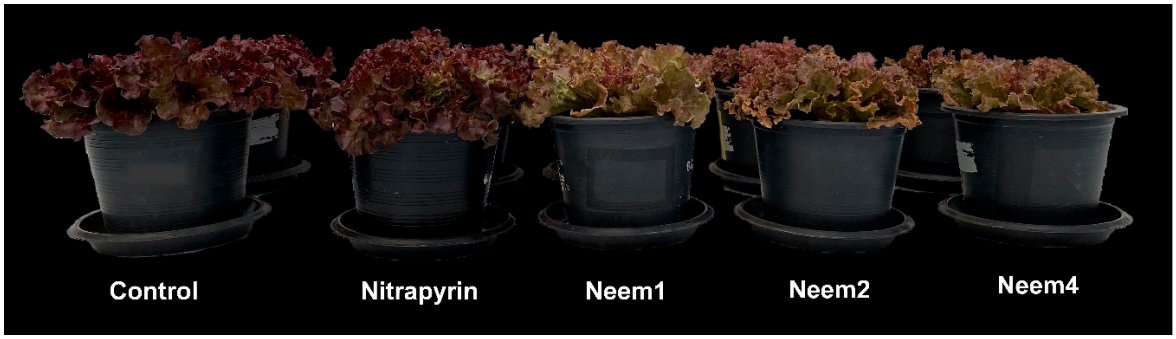


Figure 3. Lettuce responses to different nitrification inhibitors. Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg^{-1} soil.

Hawkesford et al. [54] claimed that NH_4^+ toxicity brought about the efflux of H^+ to the soil solution, rendering soil acidity as a consequence of Al toxicity. The current study's findings, which showed that the neem leaf extract treatments significantly decreased soil pH and increased soil Al concentrations (Table 7), validated this assertion. Furthermore, the photosynthesis interference resulting from NH_4^+ toxicity was suggested by Song et al. [57], who stated that to achieve NH_4^+ detoxification, the carbon skeleton must be withdrawn. A decrease in the carbon skeleton may affect chlorophyll biosynthesis, as seen in the significantly lower chlorophyll content of lettuce in the neem leaf extract treatments (Table 8). Furthermore, it was reported by Gopal et al. [58] that azadirachtin,

the most toxic neem-derived compound, was highly harmful to fungi, notably arbuscular mycorrhiza, which improves plant growth through increased available P in soil.

Table 7. Selected soil properties at lettuce harvest as affected by different nitrification inhibitors.

Inhibitor †	BD (g cm^{-3})	pH (1:1)	EC (mS cm^{-1})	CEC (cmol kg^{-1})	OC (g kg^{-1})	Al (mg kg^{-1})
Control	1.59	4.64 a ‡	0.102 d	3.05	3.58	2.75 d
Nitrapyrin	1.57	4.71 a	0.096 d	3.06	3.36	2.70 d
Neem1	1.56	4.12 b	0.271 c	3.08	3.31	5.63 c
Neem2	1.60	4.12 b	0.322 b	3.09	3.60	7.35 b
Neem4	1.60	4.16 b	0.525 a	2.98	3.54	8.70 a
p-value	0.012	<0.001	<0.001	0.998	0.466	<0.001
F test	ns	***	***	ns	ns	***
CV (%)	0.89	1.49	7.61	13.45	6.69	3.58

*** = $p \leq 0.001$; ns = not significant; CV = coefficient of variation. † Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg^{-1} soil. ‡ Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Fisher's least significant difference test).

Table 8. Chlorophyll contents of lettuce leaves as affected by different nitrification inhibitors.

Inhibitor †	Chlorophyll Content (SPAD Unit)
Control	30.4 a ‡
Nitrapyrin	30.0 a
Neem1	22.6 b
Neem2	18.3 c
Neem4	17.4 c
p-value	<0.001
F test	***
CV (%)	4.83

*** = $p \leq 0.001$; CV = coefficient of variation. † Neem1, Neem2, and Neem4 = neem leaf extract based on the dry weight of the raw material at rates of 1, 2, and 4 g kg^{-1} soil. ‡ Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Fisher's least significant difference test).

The inhibitory effect of the neem leaf extract on nitrification created NH_4^+ toxicity. This was proven by the significant increases in soil NH_4^+–N concentrations and significant decreases in soil NO_3^-–N concentrations, net nitrification rates, and the positive nitrification inhibition values seen in the neem leaf extract treatments (Table 6). The neem active ingredients, in particular azadirachtin and nimbolide (Table 2), were shown to hinder nitrifying microorganisms [18,59]. He et al. [60] determined that nitrifying bacteria, such as *Nitrosospira*, *Nitrosomonas*, and *Nitrosococcus*; as well as nitrifying archaea, such as *Nitrosopumilus* and *Nitrososphaera* are what transform NH_4^+ to NO_3^- in soil. Xi et al. [13] further revealed that a nitrification inhibitor interfered with ammonia monooxygenase, the enzyme that catalyzes the transformation of NH_3 to NH_2OH, thereby inhibiting the nitrifying microorganisms.

The inhibitory effect of neem leaf extract consequently decreased lettuce NO_3^-, which was validated by the significantly decreased NO_3^-–N tissue contents in Neem1 and Neem2, relative to the control (Table 4). The concentration effect of decreased lettuce biomass (Figure 2) and the overring effect of the high N supply of neem leaf extract (Table 2) may have contributed to the significantly higher tissue NO_3^-–N content in Neem4 (Table 4).

The inhibitory effect of nitrapyrin on soil nitrification (Table 6) did not result in a decrease in tissue NO_3^-–N contents (Table 4). Luo et al. [52] observed the inhibitory effects of nitrapyrin on nitrification only within the first seven days after application. The short length of the inhibitory effect of nitrapyrin on nitrification might be not able to remediate the NO_3^- uptake of plants.

4. Conclusions

The results of this study demonstrate that neem leaf extract could act as a natural nitrification inhibitor, and simultaneously improve soil concentrations of P, K, Ca, and Mg. The neem extract, nevertheless, could not improve the growth and yields of lettuce, but imposed detrimental effects on the lettuce. Nitrogen deficiency accompanied by NH_4^+ and Al toxicities drove the deleterious effects of the neem leaf extract on the lettuce.

Neem leaf extract of 1 and 2 g kg^{-1} decreased lettuce NO_3^- contents, yet not within the application of higher rates (4 g kg^{-1} soil). Further investigation will be necessary to utilize lower rates of neem leaf extract as a natural nitrification inhibitor for improving vegetable yield and remediating NO_3^- contents.

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