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RESEARCH ARTICLE

Evaluating the impact of an organic nitrification inhibitor on nitrogen availability and its influence on microbial population in paddy soil

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Abstract

An incubation study was conducted to estimate the potential inhibition rates of nitrification using phytonim-coated fertilizers like urea and ammonium sulfate (AS) at different concentrations viz., 100 % and 65 % of the recommended dosage (RD) and additionally ammonifying bacteria (AB) was added with urea as one of the treatments. Samples were taken at 13 different intervals in a 45-day incubation experiment. Applying phytonim-coated fertilizers delayed the nitrification process for 35 days, improving the soils' available N. Ammonia oxidation and nitrite oxidation rates were reduced by 14 % and 35 % in phytonim-coated urea, 11 % and 23 % in phytonim-coated urea + AB and 24 % and 45 % in phytonim-coated AS, respectively over uncoated fertilizers. Nitrate reductase activity was reduced by 17 %, 16 % and 21 % in phytonim-coated fertilizers like urea, AS and urea + AB compared to uncoated fertilizers. Soil urease activity was inhibited in phytonim-coated urea (100 % RD and 65 % RD) of 9.6 % and 7.4 %, respectively, whereas 5.7 % and 6.7 % increased urease activity were observed in phytonim-coated urea + AB (100 % RD) and coated urea + AB (65 % RD). Increased soil dehydrogenase activity and FDA hydrolysis of 20 % and 15 % were observed in phytonim-coated urea, 15 % and 18 % in phytonim-coated urea + AB (100 % RD) and 17 % and 16 % in phytonim-coated AS over uncoated fertilizers. Results show that phytonim-coated AS (100 % RD) (T_8) has inhibited nitrification most, followed by phytonim-coated urea (100 % RD) (T_4) during the incubation period. This study concludes that applying organic nitrification inhibitors inhibited the nitrification and denitrification rates, affected the urea hydrolysis and positively affected the microbial population.

Keywords

ammonia oxidation; microbial population; nitrification; organic nitrification inhibitor; soil enzymes; soil incubation.

Introduction

Most nitrogen (N) fertilizers applied in the soil are rapidly oxidized to nitrate due to nitrification. Nitrification is a two-step process carried out by a group of bacteria and archaea, namely ammonia-oxidizing bacteria (AOB)/ammonia-oxidizing archaea (AOA) that converts ammonia into nitrite and nitrite-

oxidizing bacteria (NOB) that converts nitrite into nitrate (1). Nevertheless, nitrate can enter surface runoff, seep into groundwater, or through denitrification and release greenhouse gases into the atmosphere (2). Agricultural soils are the primary source of atmospheric N_2O emission due to denitrification caused by increased fertilizer use, accounting for over 69 % of all N_2O worldwide (3). Agriculture contributes around 20 % of the worlds' anthropogenic greenhouse gas (GHG) emissions. In contrast, nitrous oxide (N_2O) and methane (CH_4) are the two leading greenhouse gases released by agriculture and their respective global warming potentials (GWP) are 25 and 298 times more than those of CO_2 (4).

Globally, nitrogen inputs for crop production, including synthetic and organic fertilizers, biological fixation and atmospheric deposition, have roughly quadrupled since the 1960s at 40 (38-54) Tg N yr^{-1}, reaching 161 (139-192) Tg N yr^{-1} in 2010 (5). Synthetic N fertilizer consumption increased from 11.3 Tg N yr^{-1} (0.9 g N m^{-2} cropland/yr) in 1961 to 107.6 Tg N yr^{-1} (7.4 g N m^{-2} cropland/yr on average) in 2013 (6). The alarming increase in synthetic fertilizers in agriculture pollutes the ecosystem. About half of the fertilizer applied is lost due to NH_3 volatilization, nitrate leaching and nitrous oxide. To overcome this loss and to increase productivity, the farmers dump excessive fertilizers in the field, causing water, air and soil pollution. Nitrogen pollution causes eutrophication in lakes by algal blooms and higher nitrate concentration in drinking water causes methemoglobinemia in infants (7). Nitrification inhibitors (NI) are proven to reduce nitrification loss and fertilizer input and increase the N-uptake of the plants (8). Nitrification is a microbe-mediated process carried out by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The nitrifying bacteria produce enzymes such as ammonia-monooxygenase and nitrite-oxidoreductase that oxidize the ammonia into nitrite and nitrate. NIs can directly act upon these enzymes and competitively inhibit the process, thus increasing ammonia concentrations in the soil. Most commercially available NIs, such as Dicyandiamide (DCD), 3,4 Dimethyl Pyrazole Phosphate (DMPP) and Nitrapyrin, are of synthetic origin and expensive (9). Several plant products and extracts can rival synthetic NI to suppress nitrification (10). Compared to synthetic NI, some plant-based compounds are more readily available, less costly and readily decompose in the soil. It has been reported that neem (*Azadiracta indica*), karanja (*Pongamia glabra*) and tea (*Camellia sinensis*) exhibited nitrification inhibitory capabilities (11, 12).

Several indicators and enzymes were studied to study the effect of NIs on nitrification enzymes and microbial metabolism in the soil. Potential ammonia oxidation (PAO) acts as an indicator for the ammonia-monooxygenase enzyme produced by autotrophic ammonia-oxidizing bacteria (AOB) of the β- and γ- subgroups of Proteobacteria (13) and PNO acts as an indicator for the nitrite-oxidoreductase enzyme which is solely responsible for nitrification in soil. Nitrate reductase enzyme in soil indicates anaerobic nitrate reduction, which causes denitrification of nitrate and the production of nitrous oxide. Urea hydrolysis in the soil is measured using the soil urease enzyme activity. It is essential to observe urease activity as urea is the most abundant fertilizer used in agricultural systems and the urease enzyme transforms urea into ammonia rapidly in the soil (14). Dehydrogenase is regarded as an indication of oxidative metabolism in soils and microbiology. It depicts the intercellular flow of electrons to oxygen caused by the activity of many intercellular enzymes that catalyze the transfer of hydrogen and electrons from one substance to another (15). FDA hydrolysis indicates the total microbial activity of the soil as many enzymes, including lipases, esterases and proteases, may hydrolyze the FDA, indicating a broad spectrum of soil enzyme activity (16).

Rice (*Oryza sativa* L; 2n=24) is a wetland crop that generally requires standing water for better growth and productivity. Rice belongs to the Poaceae family and has a fibrous root system, with panicle type of inflorescence and caryopsis type of fruit (grain). Common high-yielding cultivars of rice grown across the globe were IR 64, NERICA, Swarna, sharbati, WITA 4, cypress and PSB RC 82. More than 80 % of the worlds' total rice area (164.12 Mha) is under wetland conditions. On a global scale, rice covers more than 21 % of human calorie needs and supports food security and community welfare. Global rice production in 2021 is about 787 million tons (17). India is the second most rice-producing country, followed by China, with a production of 122.27 million tonnes of rice and an average productivity of 2713 kg/hectare in 2020-21 (18). The standing water in the rice field creates anaerobic conditions in the soil, facilitating the methanogenesis and denitrification process (19). Both nitrification and denitrification enhance the loss of N fertilizer in the rice ecosystem, thereby creating economic and ecosystem loss. Applying phytonim, a plant extract from neem formulated as a nitrification inhibitor reduces nitrogen (N) fertilizer losses in rice habitats by delaying nitrification processes, thereby minimizing economic and ecological losses. Phytonim-coated fertilizers will effectively inhibit soil nitrification enzymes without adversely affecting the non-target soil microbiome, contributing to sustainable rice cultivation. In this study, we evaluated the application of organic NI phytonim on soil enzymes and its role in inhibiting the nitrification enzymes and non-target microbiome in the rice ecosystem.

Materials and Methods

Chemicals and cultures

All the chemicals used for laboratory assays are of analytical grade and acquired from Sigma-Aldrich®. Ammonifying bacteria *Bacillus altitudinis* FD48 was acquired from the Biocatalysts laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore (20). Phytonim and phytonim-coated fertilizers were acquired from Phytotron Agro Products Pvt Ltd, Bangalore.

Site description and experimental setup

Soil samples were collected from the Agricultural Research Station, Bhavanisagar, Erode, Tamil Nadu, India (11°29'1" latitude and 77°8'5" longitude). The soil sample was collected at the top layer (0 to 30 cm) following sampling

regulations, packed in a polythene bag and transported to the laboratory for an incubation experiment. Exactly 5 kg of soil was weighed, transferred to transparent polythene pots, flooded with water and mixed thoroughly to mimic wetland conditions. Twelve treatments include; T_1- Control (Soil alone), T_2- Soil + Phytonim, T_3- Urea(100 % RD), T_4- Phytonim coated Urea (100 % RD), T_5- Urea (65 % RD), T_6- Phytonim coated Urea (65 % RD), T_7 -Ammonium sulphate (AS) (100 % RD), T_8- Phytonim coated AS (100 % RD), T_9- AS (65 % RD), T_{10} - Phytonim coated AS (65 % RD), T_{11}- T_4 + Ammonifying bacteria (AB), T_{12}- T_6 + AB. Dose of fertilizers used in the incubation study: Urea at 100 % RD (164 mg kg^{-1} of soil) and urea at 65 % RD (106 mg kg^{-1} of soil), AS at 100 % RD (356 mg kg^{-1} of soil) and AS at 65 % of RD (230 mg kg^{-1} of soil). (RD - Recommended Dosage @ 150kg of N ha^{-1})

Soil sampling and analysis

During the 45-day incubation experiment, the soil samples were taken at 13 different intervals (0 hr, 6 hr, 12 hr, 18 hr, 24 hr, 2 days, 4 days, 6 days, 8 days, 16 days, 24 days, 35 days, 45 days) from the experimental setup. The collected soil samples were air-dried to remove excess moisture and passed through a 2 mm sieve to remove the coarse particles and plant debris. These samples were stored in an airtight polythene cover and the experiments were done within 24 hr of sampling. Soil pH was determined using a pH meter ratio 1:2.5 (w/v, soil/water). Total N was estimated using the distillation-alkaline permanganate method, total P was calculated using a photoelectric colourimeter and total K was estimated using a flame photometer. Table 1 represents the initial characteristics of the soil.

Table 1. Initial characteristics of the experimental soil

Initial parameters of experimental soil	
pH	7.85
EC	0.13 dSm^{-1}
Bulk density	1.3 Mg m^{-3}
Available N	250 kg ha^{-1}
Available P	15.2 kg ha^{-1}
Available K	347.2 kg ha^{-1}
Soil organic carbon	0.40 %

Determination of potential ammonia oxidation

Potential ammonia oxidation was estimated by following the chlorate inhibition method (21). To the 5 g of soil, 20 mL of phosphate buffer solution and 10 of potassium chlorate were added to inhibit nitrite oxidation and incubated in the dark at 25 °C for 24 hr and the nitrite was extracted using 2 M KCl solution and the filtrate was subjected to determination of potential ammonia oxidation spectrophotometrically at 540 nm using Greiss-Ilosvay reagent.

Determination of potential nitrite oxidation

Potential nitrite oxidation was estimated by following the protocol (22). To the 3 g of soil, 30 mL phosphate buffer with 0.15 mM $NaNO_2$ was added and incubated at 30 °C for 24 hr by covering the tubes with aluminium foil under shaking (200 rpm). The contents were filtered and the nitrite concentration in the filtrate was analyzed spectrophotometrically at 540 nm using the Griess reagent.

Nitrate reductase activity

Nitrate reductase (23) was measured by adding 2 mL of DNP and 10 mL of 5 mM KNO_3 to 5 g of soil and incubated at 25 °C for 24 h in the dark. The nitrite is then extracted using 2 M KCl solution and filtered through Whatman no 2 filter paper. The filtrate was then measured in a spectrophotometer at 540 nm with the help of Greiss-Ilosvay reagent.

Soil dehydrogenase activity

Dehydrogenase activity was determined using the protocol (24). Exactly 5g of soil sample was added with 0.05 g of calcium carbonate, followed by adding 1 mL of 3 % TTC (2,3,5-triphenyl tetrazolium chloride) and 2.5 mL of distilled water and mixed thoroughly and then incubated for 24 h at 37 °C. Methanol is added to the contents till a bright red colour appears. The contents were filtered and the intensity of the red color was measured spectrophotometrically at 485 nm.

Urease activity

Soil urease activity was measured using the phenolate-hypochlorite extraction method in which 1 mL toluene is added to 2 g of soil and allowed to stand for 15 min, followed by adding 2 mL potassium citrate-citric acid buffer (pH 6.7) and 1 mL of 10 % urea solution is then added and incubated for 6 hr at 37 °C (25). The contents were then filtered, and the ammonia released was extracted using a phenolate-hypochlorite solution that was read spectrophotometrically at 630 nm.

Fluorescein diacetate hydrolysis

FDA hydrolysis was determined by hydrolysis of the substrate Fluorescein Diacetate by the soil microbes into fluorescein (26). To the 1 g of sieved soil, 50 mL of THAM buffer (0.1M, pH 7.6) and 0.5 mL of FDA were added as substrate. The contents were incubated for 3 hr and 2 mL of acetone was added at the end of incubation. The contents were filtered and measured at 490 nm for colour intensity.

Statistical analysis

The mean value of triplicates was presented as data in graphs. Analysis of variance (ANOVA) was conducted to test the effect of treatments during each sampling of incubation. Duncans' Multiple Range Test (DMRT) is used to analyze the significant differences between the means at a 0.05 probability level. All statistical analyses were done using SPSS statistical software and graphs were plotted using GraphPad PRISM.

Results

Potential ammonia oxidation (PAO)

Applying phytonim-coated fertilizers significantly inhibited the potential ammonia oxidation rate (PAO) in paddy soil compared to uncoated fertilizers (Fig 1 and Table S1). The highest PAO, 28.46 µg NO_2^- g^{-1} day^{-1}, was observed on the 8th day of incubation in T_3, while the lowest PAO, 1.01 µg NO_2^- g^{-1} day^{-1}, was recorded on the 45th day in T_2. Treatments that received urea as a fertilizer (T_3 - T_6) showed a slower but steady increase in PAO. In contrast, treatments that received ammonium sulfate (AS) as a fertilizer (T_7 - T_{10}) exhibited rapid growth, followed by a decline in PAO compared to the urea treatments. However, T_{11} and T_{12} showed a steady increase and decline pattern but did not reach a peak like the other

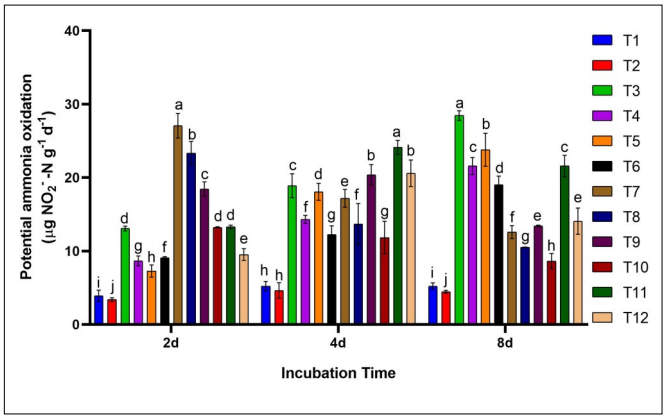

Fig 1. Effect of phytonim on potential ammonia oxidation in paddy soil during 45-day incubation studies. Error bars represent standard error (n=3). Different letters indicate significant differences between the treatments.

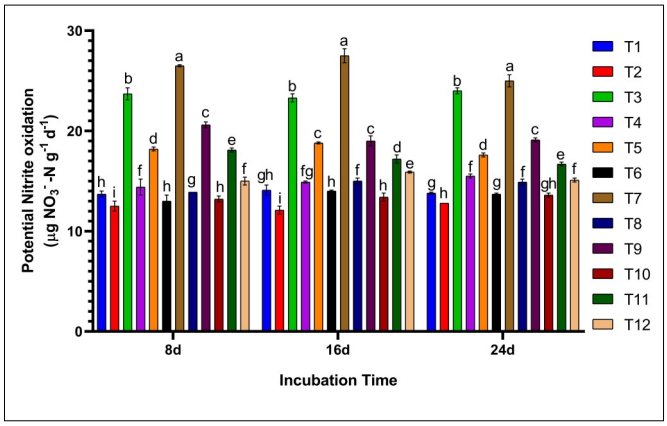

Fig 2. Effect of phytonim on potential nitrite oxidation in paddy soil during 45-day incubation studies. Error bars represent standard error (n=3). Different alphabets indicate significant differences between the treatments.

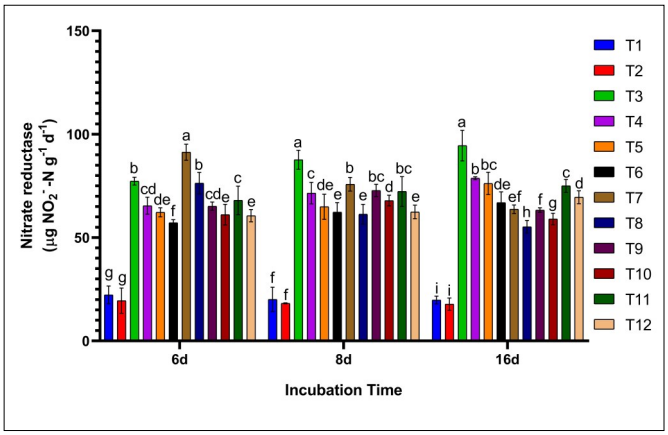

Fig 3. Effect of phytonim on nitrate reductase activity in paddy soil during 45-day incubation studies. Error bars represent standard error (n=3). Different alphabets indicate significant differences between the treatments.

treatments. Treatments T_3- T_6 peaked on the 8th day of incubation and then rapidly declined by the 16th day, while treatments T_7- T_{10} peaked on the 2nd day and gradually declined. PAO was inhibited by 14 % in T_4 compared to T_3 and 24 % in T_8 compared to T_7. Additionally, 20 % inhibition was observed in T_6 compared to T_5 and 28 % in T_{10} compared to T_9. A significant difference ($p \leq 0.05$) was noted between phytonim-coated fertilizer treatments and uncoated fertilizer treatments from the 2nd day to the 35th day of incubation. The inhibitory effect had no inhibition on the 45th day.

3.2. Potential nitrite oxidation

Applying phytonim-coated fertilizers significantly reduced paddy soil's potential nitrite oxidation rate (PNO). Initially, PNO increased in treatments following the application of urea, ammonium sulfate (AS) alone and phytonim-coated urea and AS (Fig 2a and Table S2). However, the increase in PNO was higher in treatments with uncoated fertilizers than those with phytonim-coated fertilizers. The PNO increase was more consistent in T_{11} and T_{12} than in other treatments. The highest PNO (27.5 µg g^{-1} day^{-1}) was recorded in the T_7, while the lowest activity of (12.1 µg g^{-1} day^{-1}) was observed in T_2. All treatments showed a uniform increase in PNO after the 2nd day of incubation, followed by a slight decline by the end of the 45th day. Significant differences between conventional fertilizers and phytonim-coated fertilizers were observed across all treatments, regardless of the dosage or nitrogen source (urea or AS). A maximum of 32 % reduction was observed in T_6 on 4th day of incubation and 28 % inhibition was observed in T_{10} on 6th day of incubation. PNO was reduced by 35 % in T_4 and 45 % in T_8 compared to T_3 and T_7, respectively.

Nitrate reductase

The application of phytonim-coated fertilizers resulted in significant differences compared to uncoated fertilizers. Nitrate reductase activity initially showed an increasing trend but decreased significantly across all treatments (Fig 3 and Table S3). In the treatments that received urea (T_3 - T_6), activity peaked on the 16th day before declining, while the treatments that received ammonium sulfate (AS) (T_7 - T_{10}) peaked earlier on the 6th day of incubation and then gradually declined. T_{11} and T_{12} showed a steady increase in activity up to the 16th day, followed by a gradual decline. T_1 and T_2 exhibited a linear trend throughout the incubation period without significant changes during the study. The highest nitrate reductase activity, 94.5 mg NO_2^- -N g^{-1} d^{-1}, was observed in T_3 on the 16th day, while the lowest activity, 17.8 mg NO_2^- -N g^{-1} d^{-1}, was recorded in T_2 on the same day. Nitrate reductase activity decreased by 17 % in T_4 and 16 % in T_8 compared to T_3 and T_7, respectively. Additionally, 12 % and 7 % inhibition were observed in T_6 and T_{10} on the 16th and 8th days, respectively, while a 20.6 % inhibition was noted in T_{12} compared to T_3 and a 12.2 % inhibition was noted in T_{12} compared to T_5.

Soil urease activity

Applying phytonim significantly impacted urease activity in treatments that received urea as the nitrogen source (T_3 - T_6, T_{11} and T_{12}). No noticeable difference in urease activity was observed in treatments that received ammonium sulfate (AS) as the nitrogen source (T_7 - T_{10}). Urease activity in T_3 - T_8, T_{11} and T_{12} showed an increasing trend up to the 6th day of incubation, followed by a decline until the 45th day (Fig 4, Supplementary Table S4). However, when ammonifying bacteria were added to the urea treatments, urease activity significantly increased compared to conventional fertilizers. The highest urease activity was recorded in T_{11} (45.3 µg g^{-1} h^{-1}) on the 6th day of incubation. Treatment T_4 exhibited lower urease activity compared to T_3 throughout the incubation period. The lowest activity was observed in T_2, with a 19.4 mg Kg^{-1} h^{-1} at 0 hours. Urease activity decreased by 9.6 % in T_4 compared to T_3 and 7.4 % in T6 compared to T_5 on the 6th day. In contrast, urease activity increased by 14.7 % in T_{11} compared to T_4 and 13 % in T_{12} compared to T_6 due to ammonifying bacteria. The AS treatments followed a linear

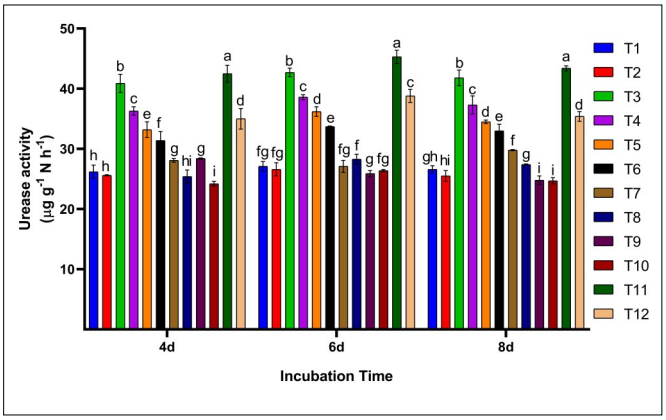

Fig 4. Effect of phytonim on soil urease activity in paddy soil during 45-day incubation studies. Error bars represent standard error (n=3). Different alphabets indicate significant differences between the treatments.

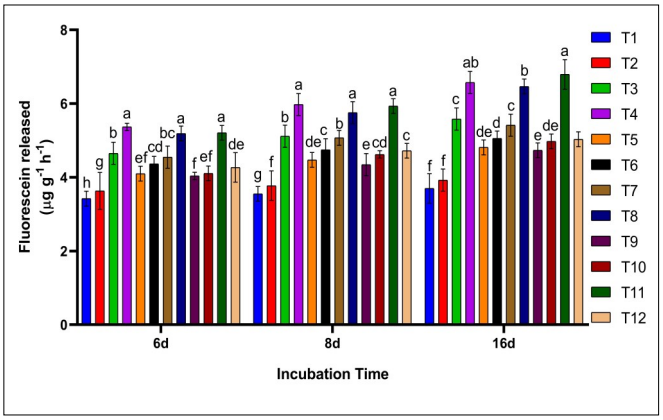

Fig 6. Effect of phytonim on FDA hydrolysis in paddy soil during 45-day incubation studies between treatments. Error bars represent standard error (n=3). Different alphabets indicate significant differences between the treatments.

trend throughout the incubation period, with no significant difference in urease activity. Soil urease activity peaked on the 6th day of incubation, declined by the 16th day and remained stable until the 45th day.

Dehydrogenase activity (DHA)

Applying phytonim-coated fertilizers increased DHA activity in soils compared to conventional fertilizers. DHA activity increased across all the treatments by the 4th day of incubation, followed by a gradual decline up to the 45th day. Throughout the incubation period, phytonim-coated fertilizers consistently showed a significant difference compared to conventional fertilizers. As shown in Fig 5, DHA activity peaked on the 4th day and gradually decreased. The highest DHA activity was observed in T_4 (28.9 µg g^{-1} hr^{-1}) and the lowest in T_1 (5.9 µg g^{-1} hr^{-1}) on the 16th day of incubation. DHA activity increased by 20 % in T_4 and 17 % in T_8 compared to T_3 and T_7, respectively. Additionally, a 9.4 % increase was seen in T_6 and a 5 % increase in T_{10} compared to T_5 and T_9, respectively. Furthermore, DHA activity increased by 15 % in T_{11} compared to T_3 and by 11 % in T_{12} compared to T_5 (Supplementary Table S5).

Fluorescein diacetate hydrolysis

The effect of phytonim as a botanical nitrification inhibitor on FDA hydrolysis across different treatments is presented in Fig 6 (Supplementary Table S6). During the 45-day incubation study, FDA hydrolysis in soils ranged from 2.4 to 6.7 µg g^{-1} hr^{-1}. FDA hydrolysis gradually increased until the 16th day, followed by a slight decrease. T_{11} consistently showed higher FDA hydrolysis compared to other treatments throughout the incubation period, with the highest value of 6.7 µg g^{-1} hr^{-1} recorded in T_{11} and the lowest value of 2.4 µg g^{-1} hr^{-1} observed in the control. Overall, phytonim-coated treatments exhibited better FDA hydrolysis compared to uncoated treatments. Specifically, FDA hydrolysis increased by 15 % in T_4 and 16 % in T_8 compared to T_3 and T_7, respectively. Additionally, a 4 % increase in FDA hydrolysis was observed in T_6 and T_{10} compared to T_5 and T_9. T_{11} and T_{12} showed an 18 % and 4 % increase compared to T_3 and T_5, respectively.

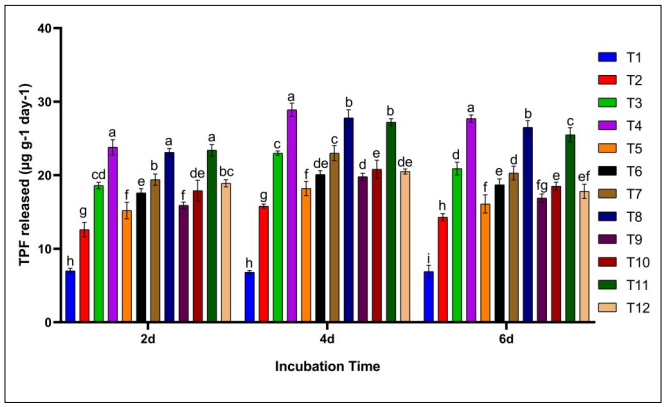

Fig 5. Effect of phytonim on soil dehydrogenase activity in paddy soil during 45-day incubation studies. Error bars represent standard error (n=3). Different letter indicate significant differences between the treatments.

Discussion

Potential ammonia oxidation

Potential ammonia oxidation (PAO) has been considered as a proxy for the population of ammonia-oxidizing bacteria and the enzyme ammonia monooxygenase (AMO) is regarded as an indicator for possible nitrification activity in soil (27). The high soil moisture content results in lower ammonia oxidation rates than dry soils. The present study shows a clear difference between the control and treatments that received fertilizer, indicating that fertilization has greatly enhanced PAO activity. After 35 days of incubation, the inhibitory effect that might be due to the degradation or dilution of phytonim present in the soil has decreased. Similarly, a biological nitrification inhibitor named 1,9- decanediol produced by rice root exudate showed similar ammonia oxidation at a 1.4 mg $NO3^-$-N kg^{-1} hr^{-1} rate after 14 days of paddy soil incubation (28). A similar inhibitory trend in nitrification potential between the NI-treated and untreated soil was also reported (29).

Potential nitrite oxidation

The nitrite-oxidizing bacteria (NOB) population and the nitrite oxidoreductase enzyme, which converts nitrite to nitrate and is a crucial stage in nitrification, are indicated by nitrite oxidation activity. Nitrite oxidation decreases along with nitrite levels as a result of ammonia oxidation inhibition, which reduces denitrification loss and increases N available for plant uptake. Because soil pH can affect the mobility and rate of NI degradation in soils, it has been thought to be one of the most significant elements influencing NI efficacy (30). In neutral and acidic soils, NI efficiency was higher and vice versa in alkaline soils (28). It has been reported that nitrate production is reduced in soils treated with four different NIs in a 28-day incubation

experiment (31). A study with biochar and NIs in a sugarcane field revealed that the nitrate concentrations were lower in the NI-treated soil (212.00 mg kg^{-1}) compared to biochar (279.25 mg kg^{-1}) or untreated soil (344.25 mg kg^{-1}), which shows that inhibition of ammonia oxidation leads to reduced nitrite oxidations in the soils (31, 32).

Nitrate reductase

Anaerobic nitrate reduction in soil is indicated by nitrate reductase activity. Dissimilatory nitrate reductase catalyzes the initial stage in the denitrification process by converting NO_3^- to NO_2^- (14). NIs can also reduce nitrogen loss through denitrification and decrease the nitrate concentration in the soil leachate and runoff (33,34). In this study, phytonim inhibited the nitrate reductase (NaR) activity and is one of the essential denitrifying enzymes. Reducing nitrate reductase is crucial in productive agriculture as it reduces the denitrification rate, thereby reducing the nitrous oxide emission and enhancing the N uptake by plants (35). Nimin, a similar organic nitrification inhibitor extracted from neem, has shown a better reduction of NaR activity than synthetic NIs (36). The NI (DMPP) application reduced the nitrate reductase activity at increased concentrations compared to untreated soils. Application of DMPP at a concentration of 0.025 g kg^{-1} of dry soil has more inhibitory effect on soil nitrate reductase activity (37). Another study reported that DMPP as NI inhibited nitrate reductase activity in rice soil by 43.5 % and 42.5 % in 2004 and 2005, respectively (38).

Urease activity

Soil urease is an essential enzyme in N cycling in soil and is a good quality indicator of the soil due to its sensitive nature (39). Urease enzyme helps break down urea into NH4+ -N, an available form of N for paddy. The urease is a sensitive enzyme whose effects are short-lived (40). The urease activity peaked on the 6th day after fertilizer application and declined after the 8^{th} day. The addition of ammonifying bacteria has shown a positive effect on urease as it actively breaks down the urea into ammonia. Applying phytonim has shown little inhibitory effect on urease compared to uncoated fertilizers. A similar inhibition of urease activity was seen in DMPP, a synthetic NI (37). DMPP effectively inhibited the urease activity up to 35 days, which showed a similar trend for urease inhibition with phytonim.

Dehydrogenase activity

The effect of phytonim on non-target microbes was observed by dehydrogenase activity and FDA hydrolysis of the soil microbes. Dehydrogenase is an intracellular enzyme that indicates the oxidative metabolism of viable microorganisms in the soil (41) and indicates carbon availability in the soil (42). In the present study, higher DHA activity was observed in phytonim treatments than in controls, suggesting that it helps increase the soil's microbial metabolism. An increase of 20 % in DHA activity was observed in phytonim-coated urea, which positively relates to plant growth and soil health. Dehydrogenase activity has been proven to be increased by adding NIs, which supports the positive effect of phytonim on soil dehydrogenase activity. It has been reported that applying biochar as a nitrification inhibitor increased the dehydrogenase activity compared to soil alone and treated with urea (43). NIs at their recommended dosage don't have any side effects on the soil microflora and soil metabolism. However, increasing the concentration of NIs beyond the recommended dosage may reduce the dehydrogenase activity, affecting soil metabolism due to the ecotoxicity of synthetic NIs (44).

4.6. FDA hydrolysis

The hydrolysis of FDA into fluorescein, catalyzed by enzymes such as proteases, lipases and esterases, is present in almost all fungi and bacteria in the soil. This shows that FDA hydrolysis is a sensitive indicator of the total hydrolytic activity of the soil microbes (45). Previously, FDA hydrolysis assessed the side effects of herbicides, heavy metals and organic wastes in different soils (46, 47, 48). In this study, the fluorescein released per hour is between 2.4 to 6.7 µg g^{-1} of soil. Initially, there was no significant difference between the treatments in the first few days, but phytonim-coated fertilizers showed better results than the controls. Similarly, in the present study, FDA hydrolysis has remained unchanged between treatments and the time may vary based on the sampling time (49). A 14-day incubation study conducted using DMPP has shown that FDA hydrolysis remains unchanged between 55.0-57.6 mg fluorescein released g^{-1} dry soil hr^{-1}) following the application of DMPP which is in contrast with current and this may be due to a shorter period of incubation and ecotoxicity of the DMPP applied (50).

Conclusion

Applying phytonim as an NI significantly decreased the nitrification rates in paddy soil. Phytonim inhibited the ammonia oxidation rate, which is a rate-limiting step of nitrification, by 14 % and 24 % when coated with urea and AS and decreased the nitrite oxidation massively by 35 % and 45 % when coated with urea and AS. Phytonim inhibited nitrate reductase activity by 17 % and 16 % when coated with urea and AS. Phytonim has no adverse effect on total microbial activity and instead has a positive impact as it improved dehydrogenase by 20 % and 17 % in coated urea and AS and improved FDA hydrolysis in soil by 15 % and 16 % in coated urea and AS, respectively. Phytonim, when coated with fertilizers, effectively inhibits nitrification enzymes at 100 % and 65 % of the recommended dosage. Phytonim-coated fertilizers at 65 % of the recommended dosage have also effectively inhibited the nitrification enzymes and improved microbial metabolism, which may support low input of fertilizers in the long run. Further, phytonim is an organic NI that enhances soil microflora and may positively affect soil health and crop yield. In conclusion, phytonim has proved to be a potent nitrification inhibitor and positively affects soil microflora and metabolism.

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Authors' contributions

SL has carried out the experiments and statistical analysis, US has conceptualised the research and revised the manuscript, TS has carried out the editing and review of the manuscript, MM has contributed to designing the experiments, SKR has coordinated the research and revised the manuscript, AR has conceptualised the study, designed the experiments and reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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