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Screening Potential Nitrification Inhibitors through a Structure–Activity Relationship Study—The Case of Cinnamic Acid Derivatives

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Abstract: Using a nitrification inhibitor to decrease nitrification rates in soil represents a promising strategy to improve nitrogen fertilizer use efficiency. Nonetheless, rapid screening of nitrification inhibitors remains challenging. In this study, we propose a strategy to screen potential nitrification inhibitors through a structure–activity relationship (SAR) study based on a rapid determination of nitrification inhibition. To demonstrate this, the nitrification inhibition potentials of cinnamic acid derivatives against Nitrosomonas europaea growth were evaluated in a liquid culture. The SAR study showed that hydroxyl and fluoride groups were the favorable substituents on the benzene ring, and the ester group and double bond in the side chain were essential for maintaining high inhibition efficacy. Three compounds with notable inhibitory efficacy (EC_{50} = 8–25 μM) were further assessed in agricultural soil, and they displayed a noteworthy reduction in nitrification rate and bacterial amoA gene numbers. Based on the results, we identified methyl cinnamate, methyl 4-hydroxycinnamate, and methyl 4-fluorocinnamate as promising candidates for nitrification inhibition.

Keywords: nitrification; nitrification inhibitor; structure–activity relationship; ammonia-oxidizing bacterium; cinnamic acid

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1. Introduction

The use of synthetic fertilizers in agriculture has become vital in order to sustain the food demand of the increasing human population. Nitrification, one of the central processes in the global nitrogen cycle [1,2], can lead to NO_3^--N leaching and greenhouse gas (such as N_2O) emissions, which not only reduce the nitrogen fertilizer use efficiency but also pose a threat to the environment [3,4]. The nitrification primarily occurs through the activities of ammonia-oxidizing bacteria (AOB) and archaea (AOA), nitrite-oxidizing bacteria, and complete ammonia-oxidizing (comammox) bacteria [5,6]. Generally, AOA's preference for oligotrophic conditions in acidic soils and nitrogen-limited environments is facilitated by their highly efficient metabolism [7–9]. Meanwhile, AOB communities are better adapted to high nutrient availability conditions [10], especially in soil used to grow vegetables [11]. It is necessary to apply nitrification inhibitors to reduce the nitrification rates in soil only when the application amount of N fertilizer is relatively large. Thus, most inhibitors target the AOB, especially their effect on the first step in nitrification, the enzymatic oxidation of ammonia to hydroxylamine by ammonia monooxygenase (AMO) [12]. The core mechanism of nitrification inhibition is the inactivation of AMO through copper chelation [11,13].

The use of nitrification inhibitors has been implemented in agriculture to slow the soil nitrification process in recent years [14]. Though several nitrification inhibitors have been developed, only a few compounds, including nitrapyrin, dicyandiamide (DCD), and

3,4-dimethyl pyrazole phosphate (DMPP), are proposed for use in production agriculture [15]. However, the synthetic chemical nitrification inhibitors are not able to be widely used in production agriculture due to their limited biological stability, low mobility, and cost increase [16]. Thus, the other nitrification inhibitors derived from plant roots, termed as "biological nitrification inhibition (BNI)", have attracted more and more attention [17]. Under natural ecosystems, nitrification can potentially lead to nitrogen starvation, which in turn, forces plants to develop strategies, such as releasing nitrification inhibitors from plant roots, to protect available nitrogen from loss [18]. For example, Sorgoleone releasing from sorghum roots was found to exhibit BNI activity [18]. The 1,9-decanediol releasing from rice roots also showed high BNI activity [19]. Although applying BNIs is a promising way to replace synthetic chemical nitrification inhibitors, there is still a lack of large-scale application of BNIs. Thus, finding efficient, eco-friendly, and low-cost nitrification inhibitors remains challenging, which calls for a rapid method to screen potential nitrification inhibitors.

A structure–activity relationship (SAR) study is a widely used method to discover drug and pesticide candidates form natural products [20–22]. In this study, we aim to provide a method to screen nitrification inhibitors through a SAR study based on the natural compounds with BNI activity. Two natural compounds, methyl-p-coumarate (**13**, Figure 1) and methyl ferulate (**17**, Figure 1), extracted from the root tissue of *Brachiaria humidicola*, were found to have high nitrification inhibitory efficacy against *Nitrosomonas europaea* [23]. These two compounds belong to cinnamic acid derivatives, which are widely distributed in the plant kingdom and are found to have diverse biological activities such as insecticidal activity [24] and antioxidant properties [25]. Based on the high nitrification inhibitory efficacy of methyl-p-coumarate and methyl ferulate, we hypothesize that other cinnamic acid derivatives may also display high nitrification inhibition. Thus, it is likely that potential nitrification inhibitors will be identified from these cinnamic acid derivatives through a structure–activity relationship (SAR) study of these compounds against AOB growth. In this study, a series of cinnamic acid derivatives (Figure 1) were selected considering structural changes in different regions of the molecular skeleton of cinnamic acid. The nitrification inhibition rates of these compounds were tested against a typical ammonia-oxidizing bacterium, *Nitrosomonas europaea*, in a liquid batch cultures [19]. After the rapid screening and SAR study, the compounds with high nitrification inhibition rates were further selected to study their performance in inhibiting nitrification in soil used to grow vegetables. Our study thus provides a strategy to screen compounds with excellent nitrification inhibition based on a case of cinnamic acid derivatives. This approach will help nitrogen management in sustainable agriculture.

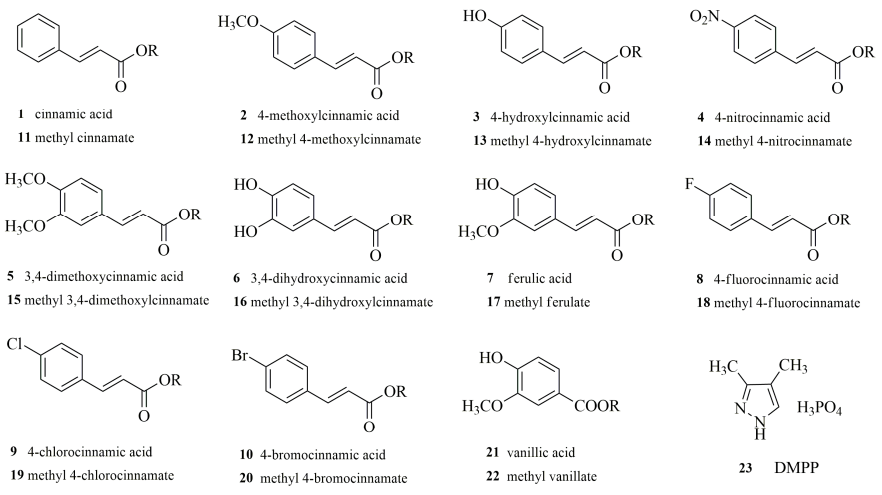


Figure 1. The names and chemical structures of cinnamic acid derivatives **1**–**20**, vanillic acid (**21**), methyl vanillate (**22**), and DMPP.

2. Materials and Methods

2.1. Chemicals

All of the names and chemical structures of compounds **1**–**23** mentioned in this study are shown in Figure 1. The *trans*-cinnamic acid derivatives **1**–**10**, the methyl esters **11**–**20**, vanillic acid (**21**), methyl vanillate (**22**), and DMPP (**23**) (Figure 1) are commercially available.

2.2. Soil Sampling

The vegetable field is located in Xinzhuang Town, Changsu City, Jiangsu Province, China (31°33′ N, 120°38′ E). The climate is a subtropical monsoon climate with about 240 frost-free days. The annual mean temperature is 15.5 °C, and the accumulated precipitation is 1038 mm. The topsoil was randomly collected at a depth of 20 cm and mixed to give a composite sample and then air dried. The soil is classified as an anthrosol, derived from lacustrine deposits. The physicochemical parameters (Table 1) of the soil was determined by standard procedures [26].

Table 1. Physicochemical properties the soil.

pH	SOC (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	TK (g kg^{-1})	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})
7.11	20.7	2.45	1.32	17.1	16.2	27.9

2.3. Nitrification Inhibition Rates in Liquid Batch Cultures

The ammonia-oxidizing bacterium *Nitrosomonas europaea* (ATCC 19718) was purchased from NITE Biological Resource Center (NBRC), Japan. The strain was grown aerobically in an autotrophic growth medium as recommended by NBRC at 28 °C. This medium contained (per liter of sterile water) 2.5 g of $(NH_4)_2SO_4$ (equal to 19 mM), 0.5 g of KH_2PO_4, 5 mg of $CaCl_2.2H_2O$, 0.1 g of $MgSO_4.7H_2O$, 0.5 g of $NaHCO_3$, 1 g of NaCl, 1 mL of 75 mg FeNaEDTA, and 11.92 g of HEPES [22]. The pH of the medium was adjusted to 7.5 with HEPES buffer. The medium and all flasks were heat sterilized by autoclaving at 121 °C at 103 kPa for 20 min prior to the beginning of each experiment. The strain was cultured in flasks (500 mL) that contain 200 mL of the HEPES medium using an orbital shaker at 28 °C and 150 rpm. As some of the compounds **1**–**23** have poor solubility in water, they were dissolved in an organic solvent and then added to the cultures. The effect of many organic solvents such as methanol, ethanol, acetone, *N*,*N*-dimethylformamide, and dimethyl sulfoxide (DMSO) on the growth of *N. europaea* were tested. It turned out that DMSO showed the minimum effect on the nitrite accumulation and all the compounds **1**–**23** also have excellent solubility in DMSO. Thus, DMSO was chosen to prepare the stock solutions of compounds **1**–**23** with different concentrations.

For each experiment, an exponential-phase culture (about 7 d old) mix was centrifuged and resuspended in fresh HEPES medium, and adjusted to an optical density at 600 nm (OD600) of 1.0 using a spectrophotometer (SmartSpec plus; Bio-Rad, Hercules, CA, USA). In the assay, a mixture of 200 µL of resuspended cells, 100 µL of HEPES medium, 195 µL of sterilized Milli-Q water, and 5 µL of compounds **1**–**23** (dissolved in DMSO) with a final concentration of 1000 µM were added to a 1.5 mL tube, and then they were incubated at 28 °C for 2 h [19]. The nitrite concentration at the beginning of each experiment was measured and normalized to ensure consistency between experiments. Cultures without the addition of a test compound and started with the same inoculum were used as negative controls in parallel. Each compound experiment was conducted in triplicate. After 2 h, the reaction was quenched by the addition of 20 µL of 0.1 mM allylthiourea [19]. The NO_2^- production was immediately determined using a modified Griess nitrite test method [27,28], with a microplate spectrophotometer (Biotech Epoch, Santa Clara, CA, USA) at 540 nm.

Nitrite production correlates with increasing ammonia-oxidizing bacterium cell densities; thus, this parameter has been widely used to approximate the growth of ammonia-

oxidizing organisms [19,29]. The nitrite production inhibition was calculated using the following equation:

$$\%\ \text{Nitrite production inhibition (NPI)} = (1 - (NO_2{}^-{}_{\text{sample}} - NO_2{}^-{}_{\text{initial}})/(NO_2{}^-{}_{\text{control}} - NO_2{}^-{}_{\text{initial}})) \times 100\%$$

The compounds with high NPI at 1000 μM were further tested at lower concentrations (0.1 μM–500 μM) to calculate the effective concentration causing a 50% inhibition rate (EC_{50}). Each EC_{50} was calculated based on at least five NPIs in different compound concentrations.

2.4. Nitrification Inhibition Test in Soil

Soil samples with a dry weight of 15 g were added to Erlenmeyer flasks (100 mL) and were treated with distilled water to a water holding capacity (WHC) of 40%. The samples were incubated at 25 °C with this WHC in the dark for 7 days to stabilize the microbial activities. Then, a 28-day incubation experiment was conducted to study the response of soil nitrification to the addition of different compounds. NH_4Cl solution was added to each bottle to provide a concentration of 400 mg N kg^{-1} of dry soil. The potential inhibitors **11**, **13**, **18**, and DMPP were added to the soil with a concentration of 50 μmol kg^{-1} dry soil (2.03–2.43% of the N addition for the four compounds) and the treatment without any inhibitor added was treated as the control (CK). Each treatment was replicated three times and the moisture level was maintained at 60% WHC throughout the incubation period. The flasks were then covered with polyethylene film and incubated in the dark at 25 °C. To maintain aerobic conditions during this process, the films were punctured with a needle.

Three flasks per treatment were destructively sampled at 3, 7, 14, and 28-day intervals, respectively. A total of 0.5 g of fresh soil was used to extract soil DNA and the remaining soil samples were treated with 75 mL 2 M KCl solution to extract $NH_4{}^+$ and $NO_3{}^-$. The concentrations of $NH_4{}^+$ and $NO_3{}^-$ in the KCl-extracted soil solution were measured using a Continuous Flow Analyzer (Skalar, Netherlands). As nitrite concentrations over the incubation were negligible because nitrite can be quickly oxidized to nitrate by nitrite-oxidizing bacteria in soil, the nitrification inhibition by adding compounds in soil was calculated based on the changes in nitrate, as reported by Majumdar et al. [30], as follows:

$$\text{Nitrification rate} = NO_3{}^-\text{-N}/(NH_4{}^+\text{-N} + NO_3{}^-\text{-N}) \times 100\%$$

$$\%\ \text{Nitrification inhibition (NI)} = (\text{nitrification rate in control} - \text{nitrification rate in sample})/\text{nitrification rate in control} \times 100\%$$

2.5. Soil DNA Extraction and Quantitative Polymerase Chain Reaction

Soil DNA was extracted from about 0.5 g of fresh soil using a FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instruction. The quality and quantity of DNA were checked using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Quantitative Polymerase Chain Reaction (qPCR), with three replicates for each sample, was performed to determine the copy number of the bacterial *amoA* gene using primer sets *amoA*-1F/*amoA*-2R-GG [30,31] with a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The qPCR standard was generated using plasmid DNA from representative clones containing the bacterial *amoA* gene. A standard template dilution series from 4.67×10^3 to 4.67×10^9 for the bacterial *amoA* gene per assay was used. The blank samples were run with water instead of soil DNA extract. The 25 μL reaction mixture contained 12.5 μL of SYBR Premix Ex Taq (TaKaRa Biotech, Dalian, China), 0.25 μmol L^{-1} of each primer, and 12.0 to 21.6 ng of DNA. Thermal cycling for the PCR consisted of 95 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 72 °C for 30 s, 72 °C for 8 min, and data collection at 95 °C for 10 s. A polymerase chain reaction amplification efficiency of 96.5% with an R^2 value of 0.993 was obtained for the *amoA* gene. The specific amplification of the *amoA* gene was checked by a melting curve analysis, which always resulted in a single peak.

2.6. Statistical Analysis

Microsoft Excel Software (2016) was used to calculate the means and standard deviations (SD) of triplicate samples. The difference between each sample was evaluated by using a one-way ANOVA with Duncan's test, and $p < 0.05$ was considered to be significant using SPSS 20.0 software (IBM). The EC_{50} values (effective concentration causing 50% inhibition of nitrite accumulation) were determined by log-transformed dose–response curves based on the % nitrite production inhibition using GraphPad Prism v.5.0 (GraphPad Software, San Diego, CA, USA).

3. Results and Discussion

3.1. SAR Study of Cinnamic Acid Derivatives Inhibiting the Growth of Ammonia-Oxidizing Bacteria

N. europaea, an autotrophic ammonia-oxidizing bacterium, was widely used as a model ammonia-oxidizing bacterium to test the nitrification inhibitory efficacy of special compounds [17,23,32]. Investigating its growth in pure bacterial cell cultures could rapidly conduct the structure–activity relationship (SAR) study of cinnamic acid derivatives inhibiting AOB growth. To study the SAR, based on the chemical structure characteristics of cinnamic acid (**1**, Figure 1), the effects of structural changes in different regions of the molecule were considered: change in the substituent to give the acids **2**–**10**, esterifying to give the structures of **11**–**20**, and elimination of the double bond of the side chain to give the structures of **21** and **22** (Figure 1). DMPP (**23**), probably the most effective commercial nitrification inhibitor [33], was tested as a control to make a judgment of the nitrification inhibition potency of the cinnamic acid derivatives.

To investigate the effects of compounds **1**–**22** (Figure 1) on the growth of *N. europaea*, a concentration of 1000 μM was first chosen to generally estimate the inhibition efficacy of all the compounds **1**–**23** in liquid batch cultures. The result showed that compounds **4**, **5**, **7**, **8**, **9**, **10**, **15**, **21**, and **22** inhibited the growth of *N. europaea* with NPI lower than 50% in this concentration, which meant their EC_{50} (effective concentration causing 50% NPI) values were higher than 1000 μM (Table 2). Thus, these nine compounds were discarded in the subsequent experiment, and the remaining fourteen compounds were further investigated with lower concentrations until their EC_{50} values could be calculated.

Table 2. Half-maximal effective concentration (EC_{50}) for the cinnamic acid derivatives to inhibit AOB growth.

Compounds	EC_{50} (μM)	Compounds	EC_{50} (μM)
1	828	**11**	25
2	721	**12**	230
3	742	**13**	20
4	>1000	**14**	850
5	>1000	**15**	>1000
6	893	**16**	260
7	>1000	**17**	75
8	>1000	**18**	8
9	>1000	**19**	100
10	>1000	**20**	760
21	>1000	**22**	>1000
		23	18

Based on the EC_{50} values (Table 2), the substituted cinnamic acids **1**–**10** generally exhibited a significantly lower ($p < 0.05$) inhibition efficacy than their corresponding substituted methyl cinnamates **11**–**20**, indicating the critical role of methyl ester in the side chain of the benzene ring. Some previous work also reports that the methyl ester compounds showed a higher inhibition efficacy than their corresponding acids. For example, the inhibitory effect of methyl linoleneate on soil nitrification was greater than that of linolenic acid [34]. Methyl ferulate (**17**) (EC_{50} = 75 μM) displayed a much greater inhibitory effect than methyl

vanillate (**22**) (EC_{50} > 1000 μM), which indicated that the double bond of the side chain is very important for maintaining the high inhibition efficacy of substituted methyl cinnamates. Similarly, previous work also found that the double bond in the side chain was very important for maintaining some biological activities for cinnamic acid derivatives [35]. These findings indicate that the strong inhibition is not solely determined by the phenolic acid's structure, but rather the presence of a side chain with a double bond, and methyl ether is crucial in maintaining the high inhibitory effect.

Different substituent groups usually lead to different biological activities for particular derivatives [35,36]. In this study, the different substituents in the benzene ring also imposed very different effects on the NPI. Compared with methyl cinnamate (**11**), introducing a hydroxyl (**13**) or fluoride (**18**) to C-4 on the benzene ring could slightly improve the inhibition efficacy, while introducing a methoxyl (**12**), nitro (**14**), chloride (**19**), and bromide (**20**) significantly decreased the inhibition efficacy. For the C-4, C-5 bisubstituted compounds **15**, **16**, and **17**, two methoxyl groups or hydroxyl groups showed a negative effect on inhibition efficacy, and only methyl ferulate (**17**) showed good inhibition efficacy. Generally, single nitro and methoxyl obviously decreased the inhibitory efficacy, while hydroxyl and fluoride were the favorable substituent groups. But, more hydroxyl groups also displayed a negative effect on inhibition efficacy. The negative effect of a polar carboxyl group and more hydroxyl groups indicated that high molecular polarity goes against the nitrification inhibition for cinnamic acid derivatives. Gopalakrishnan et al. [23] also indicated that methyl esters may have more suitable polarity for penetrating the bacterial cell, while a polar carboxyl group in the free acids might make their permeability too low to approach the action point.

For substituted methyl cinnamates, the high inhibitory efficacy of methyl cinnamate (**11**) indicated that the substituent group was not essential for high nitrification inhibition, but a hydroxyl group or fluoride at C-4 is the optimum substituent group for substituted cinnamic acids to maintain high nitrification inhibition. In general, the compounds methyl cinnamate (**11**), methyl 4-hydroxycinnamate (**13**), and methyl 4-fluorocinnamate (**18**), which exhibited the greatest inhibitory efficacy, are recommended for potential utilization as nitrification inhibitors.

3.2. The Effect of Cinnamic Acid Derivatives on Soil Nitrification

In pure bacterial cell cultures, the compounds **11**, **13,** and **18** showed very high inhibitory efficacies against *N. europaea* growth, and they were further selected to investigate their effects on soil nitrification. In the treatment of CK (without test compounds), the initial NH_4^+–N concentration (about 410 mg kg^{-1}) significantly ($p < 0.05$) decreased with time. At end of the experiment, little NH_4^+–N (62.9 mg kg^{-1} soil) was left in the N pool (Figure 2A). With the loss of NH_4^+–N, the concentration of NO_3^-–N increased to a maximum of 231.6 mg kg^{-1} at day 28, indicating that nitrification was strongly occurring. The significant reduction in NH_4^+–N concentration and the accumulation of NO_3^-–N were also observed in soils treated with **11**, **13**, **18**, and DMPP, but the rate and amount of NH_4^+–N depletion and NO_3^-–N accumulation were much slower than CK (Figure 2A,B). From day 3 to day 28, the treatments with **11**, **13**, **18**, and DMPP exhibited significantly higher NH_4^+–N and lower NO_3^-–N than CK ($p < 0.05$). The nitrification rate was much higher in CK (from 13.8% to 78.6%) compared to **11** (form 12.1% to 65.0%), **13** (form 11.5% to 58.7%), **18** (form 10.3% to 39.2%), and DMPP (form 9.5% to 30.0%) during the 28-day incubation (Figure 2C), confirming the inhibitory effects of the tested compounds on soil nitrification.

Percentage nitrification inhibition (NI) was used to clearly show the effect of the tested compounds in this study. The NI of these compounds showed a tendency of **11** < **13** < **18** < DMPP during the 28-day incubation (Figure 2D), which was in accordance with the results in liquid batch cultures. The results indicated compounds **11**, **13,** and **18** had much higher inhibitory efficacies than other cinnamic acid derivatives in a liquid culture and also displayed obvious nitrification inhibition in soil used to grow vegetables. In particular, compound **18** displayed a significantly ($p < 0.05$) higher NI value than

compounds **11** and **13**, showing a nitrification inhibition effect that was comparable to that of DMPP.

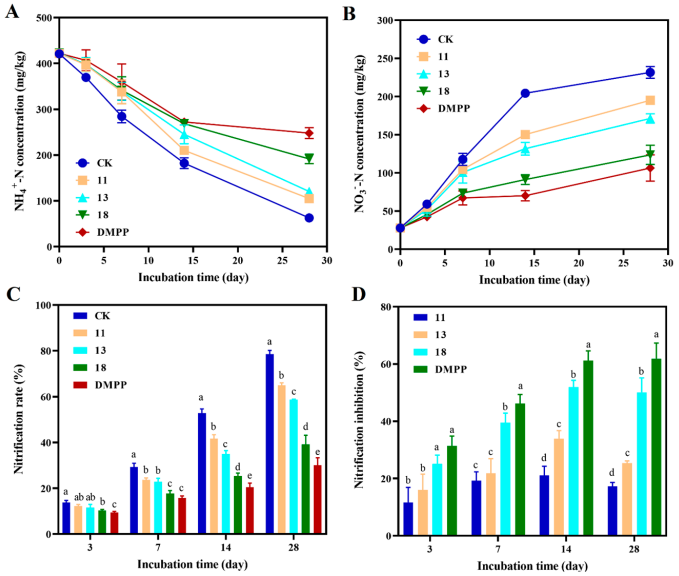


Figure 2. Changes in the concentration of NH_4^+–N (**A**) and accumulation of NO_3^-–N (**B**) in soil used to grow vegetables, the variation in nitrification rate (**C**), and nitrification inhibition (**D**) during the incubation. Error bars indicate the standard deviation of the mean (n = 3); means followed by different letters are significantly different at the 0.05 probability level according to Duncan's multiple comparison test.

To reveal the nitrification inhibition mechanism, the effect of different compounds on the AOB population abundance in terms of the *amoA* copy number per gram of soil were evaluated during the 28-day incubation. The *amoA* copy number of CK increased from 6.3×10^7 at day 3 to 22.9×10^7 at day 14 and decreased to low level of 9.1×10^7 copies g^{-1} dry soil at day 28 (Figure 3). The *amoA* copy numbers in treatments with **11**, **13**, **18**, and DMPP were lower than that in CK during the incubation. Especially at day 7 and day 14, the *amoA* copy numbers were significantly ($p < 0.05$) reduced to 19–32%, 40%–45%, 70–75%, 85%–88%, respectively for **11**, **13**, **18**, and DMPP as compared to CK. Like the liquid culture experiment, the qPCR results indicated that compounds **11**, **13**, and **18** could also significantly inhibit AOB growth in soil. This evidence indicated that the observed nitrification inhibition effects were probably due to a direct inhibition of compounds **11**, **13**, and **18** on AOB growth.

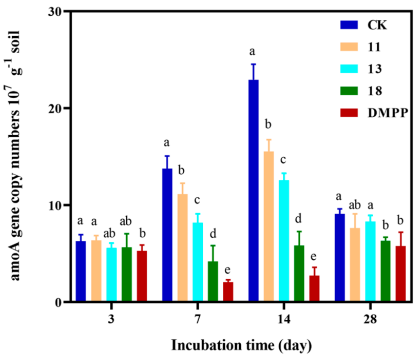


Figure 3. The change in copy numbers of ammonia-oxidizing bacteria (AOB) and *amoA* gene in soil during the incubation. Error bars indicate the standard deviation of the mean (n = 3); means followed by different letters are significantly different at the 0.05 probability level according to Duncan's multiple comparison test.

3.3. Implications and Insights of This Study

Nitrification inhibition is a very old topic and there have been many reports about the nitrification effects of many compounds; however, the need to find suitable nitrification inhibitors is still urgent. This study demonstrated a case of rapidly screening compounds with notable nitrification inhibition activity through a structure–activity relationship study, followed by verification in soil. In the future, when finding a compound displaying high nitrification inhibition activity, we can use the above method to evaluate a series of compounds with similar chemical structures and maybe we could find excellent nitrification inhibitors through a SAR study. In this study, we found three compounds showing notable nitrification inhibition activity, of which, compounds **11** and **13** are found to be widely distributed in some herbaceous and medicinal plants. For example, methyl cinnamate (**11**) was isolated from the fresh flowers of the silver wattle (*Acacia dealbata*) [37], and methyl 4-hydroxycinnamate (**13**) existed in extracts of the rhizomes and roots of black cohosh [38]. Although compounds **11** and **13** displayed weaker nitrification inhibition than DMPP, they are environmentally friendly and could be continuously supplied by plants in nature; thus, they could be treated as potential biological nitrification inhibitors. Methyl 4-fluorocinnamate (**18**) showed the highest nitrification inhibition among the tested cinnamic acid derivatives. However, it is a chemically synthesized compound. Thus, its cost and environmental effects should be considered before it could be used as a potential nitrification inhibitor. However, the applied dose and the stability of the compounds in soils should also be considered overall. This study demonstrates the feasibility of utilizing a SAR study to screen for nitrification inhibitors, and this approach could make contribution to the nitrogen management in sustainable agriculture.

4. Conclusions

Our findings demonstrate that cinnamic acid derivatives, especially ester derivatives, show promise as nitrification inhibitors against ammonia-oxidizing bacteria (AOB). Among the compounds tested, methyl cinnamate (EC_{50} = 25 μM), methyl 4-hydroxycinnamate (EC_{50} = 20 μM), and methyl 4-fluorocinnamate (EC_{50} = 8 μM) exhibit significant inhibition of nitrification against the growth of *N. europaea* in a liquid culture. Combining this method with structure–activity relationships (SARs) provides a practical strategy for screening potential nitrification inhibitors. These compounds also demonstrate high effectiveness in inhibiting nitrification in soil used to grow vegetables, highlighting their potential applications.

Author Contributions: G.L. and M.W. designed the experiments. J.L. completed the field sampling. G.L. and J.Z. performed the data analysis and prepared the figures. J.Z. wrote the manuscript. M.W. contributed to the revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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