Chemical and Biological Technologies in Agriculture

RESEARCH

Open Access

Evidences towards deciphering the mode of action of dimethylpyrazole-based nitrification inhibitors in soil and pure cultures of *Nitrosomonas europaea*

Adrián Bozal-Leorri[1*], Mario Corrochano-Monsalve[2], Izargi Vega-Mas[2], Pedro M. Aparicio-Tejo[1], Carmen González-Murua[2] and Daniel Marino[2,3*]

Abstract

Background: Agriculture relies on the intensive use of synthetic nitrogen (N) fertilizers to maximize crop yields, which has led to the transformation of agricultural soils into high-nitrifying environments. Nevertheless, nitrification inhibitors (NIs) have been developed to suppress soil-nitrifier activity and decrease N losses. The NIs 3,4-dimethylpyrazole phosphate (DMPP) and 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA) are able to reduce N_2O emissions and maintain soil NH_4^+ for a longer time. Although both NIs have been proven to be effective to inhibit soil nitrification, their exact mode of action has not been confirmed. We aimed to provide novel insights to further understand the mode of action of DMP-based NIs. We evaluated the performance of DMPP and DMPSA in soil and pure cultures of nitrifying bacteria *Nitrosomonas europaea*.

Results: DMPSA did not inhibit nitrification in pure cultures of *N. europaea*. In the soil, we evidenced that DMPSA needs to be broken into DMP to achieve the inhibition of nitrification, which is mediated by a soil biological process that remains to be identified. Moreover, both DMPP and DMPSA are thought to inhibit nitrification due to their ability to chelate the Cu^{2+} cations that the ammonia monooxygenase enzyme (AMO) needs to carry on the first step of NH_4^+ oxidation. However, the efficiency of DMPP was not altered regardless the Cu^{2+} concentration in the medium. In addition, we also showed that DMPP targets AMO but not hydroxylamine oxidoreductase enzyme (HAO).

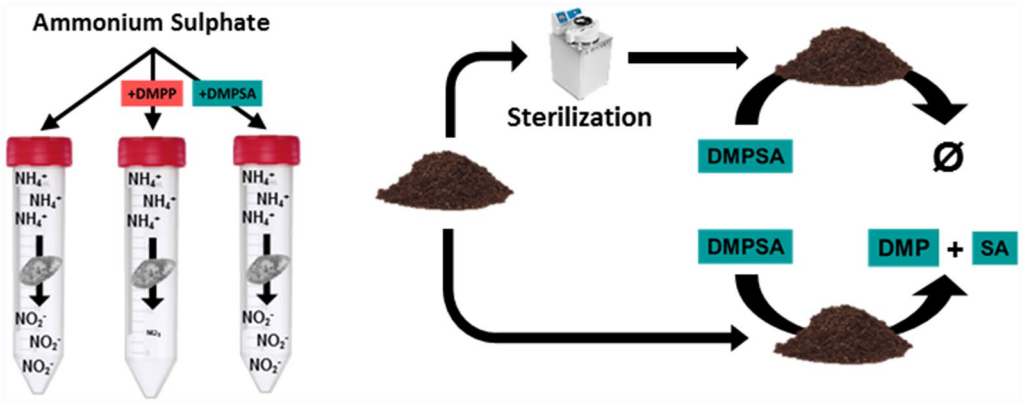
Conclusions: The inability of DMPSA to inhibit nitrification in pure cultures together with the high efficiency of DMPP to inhibit nitrification even in presence of toxic Cu^{2+} concentration in the medium, suggest that the mode of action of DMP-based NIs does not rely on their capacity as metal chelators.

Keywords: AMO, Copper, DMPP, DMPSA, Nitrification inhibitor, *Nitrosomonas europaea*

*Correspondence: adrian.bozal@unavarra.es; daniel.marino@ehu.eus

[1] Institute for Multidisciplinary Research in Applied Biology (IMAB), Public University of Navarre-UPNa, 31006 Pamplona, Spain
[2] Department of Plant Biology and Ecology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Bilbao, Spain
Full list of author information is available at the end of the article

Graphical Abstract



Background

Nitrogen (N) availability is a major limiting factor for crops growth [1]. Hence, agriculture relies on the use of N fertilizers to maximize crop yields. The application of synthetic N fertilizers has been increasing in the last decades and it is expected to reach 300 Tg N $year^{-1}$ by 2050 [2]. However, only 30% to 50% of the N applied as fertilizer is used by the crops and, consequently, great amount of N is lost to the environment as reactive N [3]. Indeed, agriculture is responsible for the production of more reactive N than all terrestrial natural processes [4]. N can be lost through ammonia (NH_3) volatilization, nitrate (NO_3^-) leaching, and the emissions of nitrogenous gases such as nitric oxide (NO) and nitrous oxide (N_2O) [5]. Main biological pathways for N losses are nitrification and denitrification. Ammonium (NH_4^+) in the soil can be aerobically oxidized to NO_3^- by nitrifiers, a process in which N_2O can be generated as a secondary product. Conversely, under low-oxygen conditions, NO_3^- can be reduced up to molecular N (N_2) by denitrifiers, where several intermediates, such as NO and N_2O, are also set free. NO_3^- leaching causes eutrophication and contamination of groundwater supplies. In addition, N_2O, derived from the use of N fertilizers, is the main greenhouse gas (GHG) generated in agriculture [6], with a global warming potential (GWP) between 265 and 298 times higher than that of CO_2 in a 100-year time horizon [7]. Indeed, it is estimated that agriculture is responsible for the emission of more than 1.15 Tg N_2O-N $year^{-1}$, which accounts for 19% of total N_2O global source and 49% of anthropogenic N_2O emissions [5]. Furthermore, N_2O is the single most ozone-depleting molecule [8].

One of the practices to improve crops' nitrogen use efficiency (NUE) and to reduce the risk of N losses both through NO_3^- leaching and the emissions of nitrogenous gases is the use of nitrification inhibitors (NIs) when applying ammonium-based fertilizers. NIs suppress soil-nitrifier activity, maintaining the NH_4^+ content for longer in soil, which reduces the formation of NO_3^- and its subsequent denitrification [9, 10]. Currently, the most worldwide used NIs are nitrapyrin (2-chloro-6-(tri-chloromethyl)-pyridine), dicyandiamide (DCD), and 3,4-dimethylpyrazole phosphate (DMPP) [11, 12]. However, the high volatility of nitrapyrin makes it necessary to be incorporated into the soil. In contrast, DCD is a cheaper NI and its non-volatility makes it more suitable to be used as a coating on solid fertilizers [13]. However, the DCD high water solubility may cause its leaching out of the action zone [14] or even to enter into the trophic chain [15, 16]. Alternatively, DMPP has lower volatility than nitrapyrin and lower mobility than DCD, also presenting similar efficiency to DCD with a 10 times lower application rate [17]. More recently, another dimethylpyrazole-based NI, 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA), has been developed. DMPSA presents a succinic acid covalently bond to the dimethylpyrazole (DMP) ring, instead of a phosphate group as in the case of DMPP, increasing its stability, which allows its use with other fertilizers such as calcium ammonium nitrate or diammonium phosphate that cannot be combined with DMPP [18]. In field conditions, both DMPP and DMPSA show a similar efficiency at inhibiting nitrification, reducing N_2O emissions of around 50% while maintaining NH_4^+ stability in the soil [19, 20].

Research concerning DMP-based NIs is extensive, but has mainly been focused on the effect of agricultural practices and environmental conditions on their

efficiency to inhibit nitrification [10]. Nonetheless, few works have studied the chemical and molecular aspects of their function. Indeed, in order to make a more efficient use of NIs, it is highly desirable to decipher their mode of action. Ruser and Schulz [10] indicated that the mode of action of DMP-based NIs may be related to their attributed ability to chelate the Cu^{2+} cations that ammonium monoxygenase (AMO) enzyme needs as co-factor [21]. Importantly, Corrochano-Monsalve et al. [22] recently demonstrated that effectively both DMPP and DMPSA are able to chelate Cu^{2+}. In addition, they reported that four DMPP molecules are needed to chelate one atom of Cu^{2+}, whilst DMPSA only needs two. In principle, this observation would make DMPSA a more efficient Cu^{2+} chelator than DMPP [22]. However, it remains to be confirmed whether the Cu^{2+}-chelating capacity of these compounds is responsible of their nitrification inhibition capacity.

In this context, we aimed to further understand the nitrification inhibition capacity of DMP-based NIs combining experiments performed in pure cultures of *Nitrosomonas europaea* and soil microcosms. Among others, we have tackled DMPSA break dynamic in soils and the importance of Cu^{2+} availability for DMP-based NIs mode of action.

Methods

Nitrosomonas europaea growth

Pure cultures of *Nitrosomonas europaea* ammonia-oxidizing bacteria (strain ATCC 19718) were cultivated on growth medium that contained Hepes buffer (pH 8) 11.9 g L^{-1}, $(NH_4)_2SO_4$ 2.5 g L^{-1}, KH_2PO_4 0.5 g L^{-1}, $NaHCO_3$ 0.5 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.1 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.005 g L^{-1}, NaFe–EDTA 0.004 g L^{-1}, 1 mL L^{-1} of trace elements solution, and 0.5 mL L^{-1} of phenol red solution (1:1000 p:v in water). The trace elements solution was composed of $MnSO_4{\cdot}4H_2O$ 0.045 g L^{-1}, H_3BO_3 0.049 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.043 g L^{-1}, $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$ 0.037 g L^{-1}, and $CuSO_4{\cdot}5H_2O$ 0.05 g L^{-1}. The culture was grown at 28 °C and 150 rpm. Every 2 days the medium pH was corrected by adding 10% $NaHCO_3$ until the medium colour was restored. Growth of the culture was monitored through NH_4^+ disappearance, quantified by the Berthelot method [23]; and NO_2^- apparition, quantified by the Griess reaction [24].

To assess the efficiency of DMPP and DMPSA inhibiting *N. europaea* growth, 100 µL from a 7-day-old *N. europaea* culture was added to 20 mL of fresh growth medium with or without NIs. Thus, treatments were (i) control conditions with ammonium sulphate (AS), (ii) AS + DMPP, and (iii) AS + DMPSA. Except for Fig. 3, where different DMPP doses were used, NIs were applied in a concentration of 5 mg L^{-1}, which is in the range 0.8–1% of the present NH_4^+-N, following the recommendation of manufacturers for field application. Cultures were maintained for 7 days following NH_4^+ and NO_2^- evolution on days 0, 1, 3, 5, and 7. At the end of the experiment, the OD_{600} was measured.

To address the inhibition capacity of DMPP, 100 µL from a 7-day-old *N. europaea* culture were added to 20 mL of fresh growth medium holding eight different DMPP concentration. Values of DMPP concentration were 0, 0.5, 1.0, 1.5, 2.0, 2.6, 5.2, 10.3, and 26 µM. NO_2^- was determined on day 7. The inhibition capacity was calculated as the percentage of reduction in the nitrification rate in each treatment (measured as NO_2^- production) relative to the control without inhibitor, following the formula: nitrification inhibition (%) = (1—($rate_{inhib}$/$rate_{control}$)) × 100.

For experiments with increased Cu^{2+} and Zn^{2+} concentration, 100 µL from a 7-day-old *N. europaea* culture were added to 20 mL of three different mediums containing different Cu^{2+} and Zn^{2+} concentrations added as $ZnSO_4{\cdot}7H_2O$ and $CuSO_4{\cdot}5H_2O$. The mediums were (i) control: growth medium with 0.01 mg L^{-1} Cu^{2+} and Zn^{2+}, (ii) + Cu: growth medium with 2.50 mg L^{-1} Cu^{2+} and 0.01 mg L^{-1} Zn^{2+}, and (iii) + Zn: growth medium with 0.01 mg L^{-1} Cu^{2+} and 2.50 mg L^{-1} Zn^{2+}. Each condition was studied in presence or absence of DMPP. NH_4^+ and NO_2^- determinations and OD_{600} measurement were carried out at day 7.

To evaluate whether DMPP targets AMO or hydroxylamine oxidoreductase (HAO) enzyme an actively growing *N. europaea* culture was gently centrifuged and the pellet washed twice with N-free growth medium to remove the possible residual NH_4^+ and NO_2^-. Final pellet was resuspended and maintained 24 h in N-free growth medium and washed again twice with N-free medium. Finally, pellets were resuspended in growth medium with $(NH_4)_2SO_4$ or NH_2OH at 1 mg N L^{-1} concentration. For each medium, two different treatments were performed; (i) control: growth medium without inhibitor, and (ii) DMPP: growth medium with 26 µM DMPP. After 6 h, NO_2^- production was determined.

Soil experiments

Soil experiments were carried out in microcosms under controlled greenhouse conditions, with a day/night cycle of 25/18 °C average temperature, and 50/60% relative humidity. Soil was collected from a 0–30 cm layer of a hypercalcic Kastanozem soil [25] and prepared as described in Bozal-Leorri et al. [26]. Before the start of the experiments, soil was supplied with 3.5 mg N kg^{-1} dry soil in the form of ammonium nitrate (NH_4NO_3; equivalent to 10 kg N ha^{-1}) and 500 mg C kg^{-1} dry soil to reactivate soil microorganisms [27]. In addition, in order to

optimize conditions for nitrification, soil was rehydrated with deionized water up to 50% water-filled pore space (WFPS) calculated as (soil gravimetric water content x bulk density) x (1—(bulk density/particle density))$^{-1}$ [28]. Soil bulk density was determined in the laboratory, with a value of 1310 kg m^{-3}, while particle density was assumed 2650 kg m^{-3}. To maintain the WFPS soil was watered every 2 days during the 14 days of activation.

To study DMP content evolution in soil (Fig. 2), six pots (12.5 cm diameter × 7 cm height) were filled with 250 g of dry soil. After soil activation, pots were divided into two groups: (i) AS + DMPP and (ii) AS + DMPSA. 63 mg N kg^{-1} dry soil was added to soil surface as $(NH_4)_2SO_4$, equivalent to 200 kg N ha^{-1}, mixed with DMPP or DMPSA at a rate of 0.8% of the applied NH_4^+-N. Pots were watered every 2 days to maintain the WFPS at 50% during the 30 days of experiment. To quantify the presence of DMP, three soil subsamples were taken from every pot with a hollow sampler (1.5 cm diameter × 5 cm depth) at 0, 2, 4, 8, 15, and 30 days post-fertilization.

To assess whether DMPSA breakdown occurs due to abiotic or biotic processes (Fig. 4), 16 pots (2.5 cm diameter × 6 cm height) were prepared with 35 g of dry soil and divided into two groups (i) sterile soil, and (ii) non-sterile soil. To sterilize the soil, soil were autoclaved three successive times (121 °C for 30 min) and later, dried at 80 °C in a circulation oven for 48 h. Soil from all pots was activated, and 3 mg cycloheximide g^{-1} dry soil was added to the sterile soil to further avoid fungal growth [29]. After soil activation, sterile and non-sterile soils were then divided in two groups (i) AS and (ii) AS + DMPSA. N was applied as previously stated. WFPS was maintained at to 50% during the whole experiment. Soil was destructively sampled for soil NH_4^+ and NO_3^- content measurement and DMP determination on day 8 post-fertilization.

To quantify soil NH_4^+ and NO_3^- contents, 20 g of fresh soil were mixed with 40 mL 1 M KCl and shaken at 165 rpm for 1 h. The soil solution was filtered firstly through Whatman n°1 filter paper (GE Healthcare) and secondly through Sep-Pak Classic C18 Cartridges 125 Å pore size (Waters) to remove particles and organic matter, respectively. The Berthelot method [23] was followed to quantify the NH_4^+ content. The NO_3^- content was determined according to Cawse [30].

Extraction of DMP was carried out from 10 g of fresh soil following Benckiser et al. [31] and quantified as described in [32]. Briefly, DMP from soil extracts was quantified by HPLC (Waters 2690 separation module with a Waters 2487 dual λ absorbance detector) using a 5 µm; 25 cm × 4 mm Tracer Excel column and a TR-C-160-1 pre-column (Teknokroma).

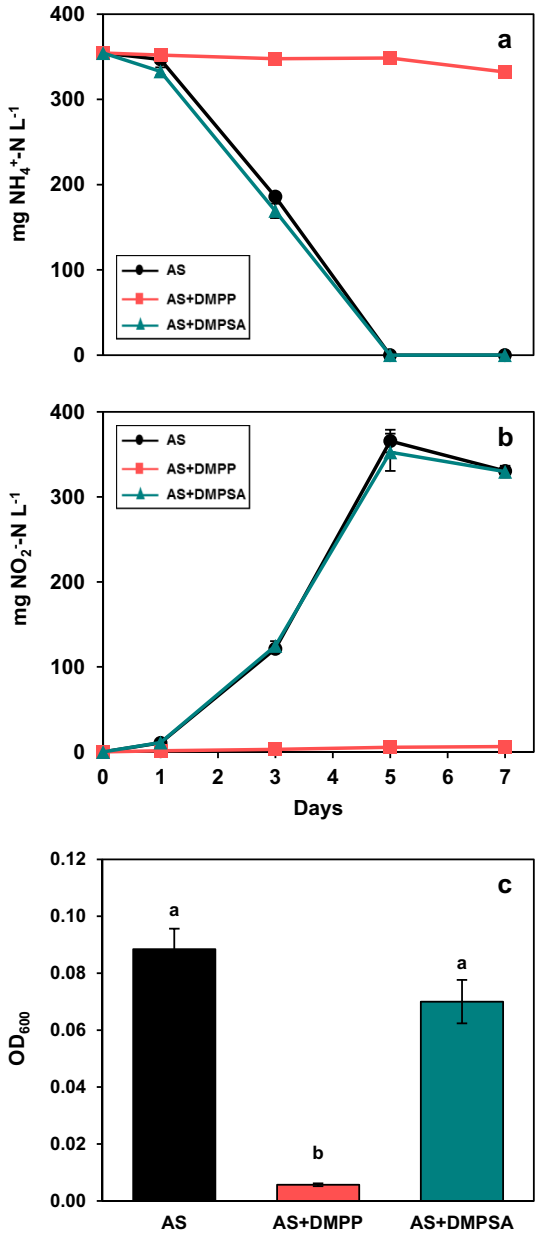


Fig. 1 Effect of DMPP and DMPSA nitrification inhibitors on *Nitrosomonas europaea* pure cultures through monitoring the evolution of NH_4^+ (**a**) and NO_2^- (**b**) in the growth medium during 7 days. Bacterial density (OD_{600}) was determined at the end of the experiment (**c**). For panel C different letters indicate significant differences using one-way ANOVA followed by Duncan's post hoc test ($p < 0.01$; $n = 3$). AS means ammonium sulphate

Statistical analysis

Data were analysed with the SPSS statistical software package (2016, IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, IBM Corp). One-way ANOVA

Table 1 Concentration of DMP and isomers of DMPSA (2,3-DMPSA and 3,4-DMPSA) in *Nitrosomonas europaea* pure cultures grown in presence of DMPP (AS + DMPP) and DMPSA (AS + DMPSA). AS means ammonium sulphate and n.d. not detected

Day	Treatment	mg L^{-1}		
		DMP	2,3-DMPSA	3,4-DMPSA
0	AS + DMPP	5.55 ± 0.16	n.d	n.d
	AS + DMPSA	n.d	0.84 ± 0.01	3.56 ± 0.05
7	AS + DMPP	5.29 ± 0.10	n.d	n.d
	AS + DMPSA	n.d	0.67 ± 0.01	3.45 ± 0.02

was performed with Duncan's multiple range test for separation of means between different treatments. p-value < 0.01 was considered to be statistically significant differences.

Results and discussion

DMPSA is not effective in inhibiting nitrification in *Nitrosomonas europaea* pure cultures

In general, DMPP and DMPSA NIs have shown similar efficiency to inhibit nitrification either in field conditions [19, 20] or microcosm experiments [26, 33]. Surprisingly, when we exposed actively growing *N. europaea* pure cultures to DMPSA, it was innocuous for its growth and nitrification capacity, while, as expected, DMPP completely inhibited *N. europaea* growth (Fig. 1). Briefly, NH_4^+ concentration quickly decreased in AS treatment, indicating that nitrification was taking place (Fig. 1a). On the contrary, the addition of DMPP completely inhibited the growth of the bacteria, so NH_4^+ concentration was maintained during the whole experiment. However, DMPSA had no effect on *N. europaea* performance. Accordingly, *N. europaea* produced a great amount of NO_2^- both in AS and AS + DMPSA treatments; while AS + DMPP showed almost no NO_2^- formation (Fig. 1b). Consequently, AS and AS + DMPSA treatments had a higher OD_{600}, while the presence of DMPP decreased it by 92% (Fig. 1c). To check, whether DMPSA absence of activity was due to its stability, we quantified NIs content in the medium at the end of the growth period and observed both NIs maintained their initial concentration until day 7 (Table 1).

DMPSA is broken into DMP in soils

Although DMPSA is not able to inhibit nitrification in pure cultures of *N. europaea*, its inhibition capacity in soil has been largely proven [19, 20, 22, 34–38]. Therefore, there must be a process that takes place in the soil, but does not occur in nitrifiers' pure culture that allows

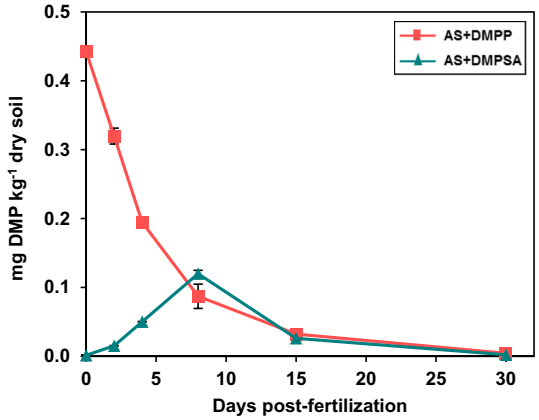


Fig. 2 Evolution of soil DMP content during 30 days of soil incubation with DMPP and DMPSA. AS means ammonium sulphate. Values represent mean ± se ($n = 3$)

DMPSA inhibiting nitrification. Indeed, it has been hypothesized that DMPP and DMPSA act in a similar manner because DMPSA needs to be broken to release DMP (the active constituent of DMPP) in order to be active as an inhibitor [39]. The registration dossier of DMPSA in the European Chemicals Agency (ECHA, EC number 940-877-5) reports that DMPSA is not biodegraded in surface water at least 28 days after its application. However, this dossier also asserts that the degradation of DMPSA into DMP does take place in the soil. Nevertheless, to date, there are no published studies that confirm this aspect. Hence, we monitored DMP apparition in soil incubations with DMPSA, using DMPP as a control (Fig. 2). Immediately after DMPP supply, soil DMP concentration decreased, almost disappearing at 30 days post-fertilization (Fig. 2). This behaviour is in line with the DMPP degradation dynamic recently reported

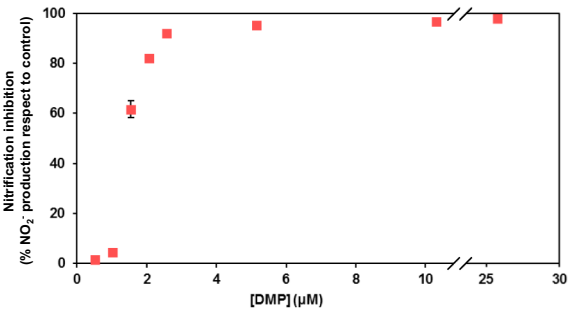


Fig. 3 Inhibition of nitrification in *Nitrosomonas europaea* pure cultures with increasing concentration of DMPP application. Nitrification was determined as NO_2^- production and values are given in % respect to control conditions (in absence of DMPP). Values represent mean ± se ($n = 3$)

by Sidhu et al. [40]. On the contrary, with DMPSA application, DMP apparition was detected 2 days post-fertilization, reaching its maximum 8 days post-fertilization. Interestingly, at day 8, DMP concentration in AS + DMPSA was very similar to AS + DMPP treatment. Afterwards, DMP decreased in a similar way under both conditions. Therefore, this experiment confirms that the covalent bond between the succinic acid and the DMP of DMPSA molecule is broken in the soil since DMP starts appearing shortly after its application. Nonetheless, the maximum DMP concentration in AS + DMPSA treatment is far from the initial DMP concentration of AS + DMPP treatment (4.2 times lower), which eventually might not be sufficient to achieve nitrification inhibition. To check whether this amount of DMP is sufficient to inhibit nitrification, we conducted a DMPP dose–inhibition curve in pure cultures of *N. europaea*. As expected, a decrease in NO_2^- production was observed with an increasing concentration of the nitrification inhibitor DMPP (Fig. 3). In line with the results obtained by O'Sullivan et al. [41] for *N. europaea* and *Nitrosospira multiformis*, we observed that a rate of DMPP of 2.6 µM (10 times lower than the recommended by the manufacturer, 26 µM), was sufficient to achieve an almost complete nitrification inhibition. Based on these results, the DMP concentration found on day 8 post-fertilization of AS + DMPSA treatment (4.2 times lower than the recommended by the manufacturer) (Fig. 2) appears compatible with an efficient nitrification inhibition. Altogether, since DMPSA breakdown did not occur in pure culture (Table 1), these results point out that DMPSA needs to be broken in order to be active as inhibitor; thus, progressively releasing the active molecule DMP to inhibit nitrification.

Soil microorganisms are responsible for DMPSA rupture

The breakdown of DMPSA can unlikely be spontaneous due to the high energy (305 kJ mol^{-1}) required to rupture the covalent C–N bond [42]. Since DMP–succinic acid bond is broken in the soil but not in microbial cultures, it means that DMPSA breakdown must be dependent on (i) soil biological activity, other than exclusive nitrifiers activity, or (ii) soil physical/environmental processes, including UV radiation. To test this hypothesis, we incubated sterile and non-sterile soils with DMPSA. The experiment was performed at a single time point, 8 days after fertilization, time where DMP level was maximum as observed in Fig. 2. As expected, in non-sterile soils the rupture of DMPSA led to DMP apparition in soil (Fig. 4a). In contrast, no DMP could be detected in sterile soil. This result clearly evidences that the breakdown of DMPSA into DMP is mediated by a soil biological process, which does not take place in pure nitrifiers' culture.

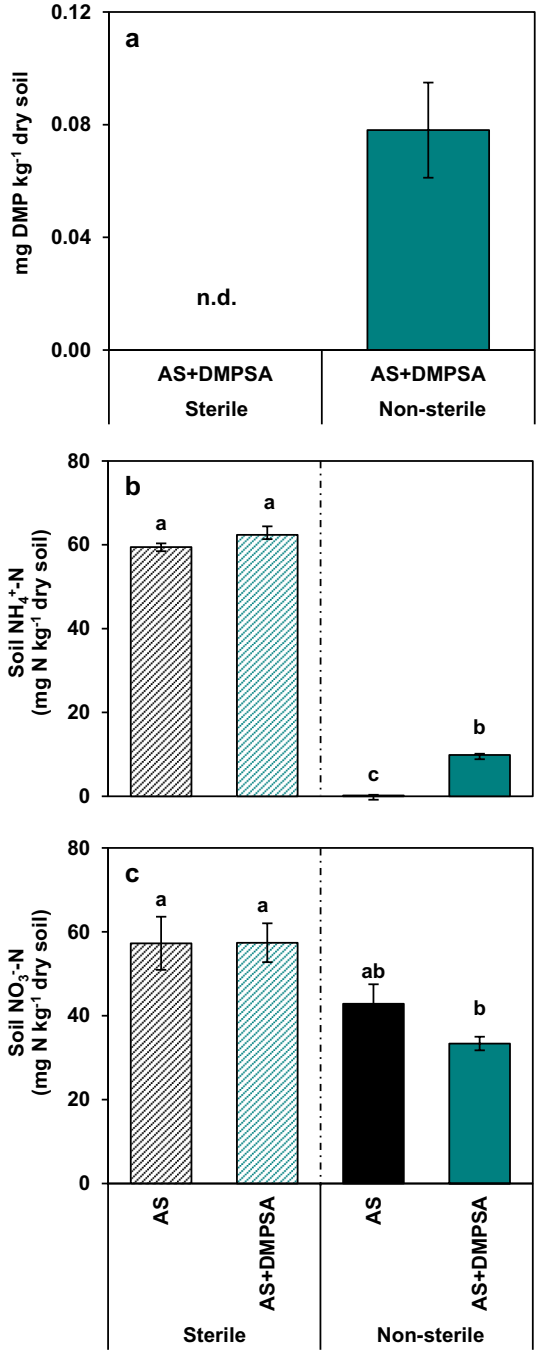


Fig. 4 Soil DMP (**a**), NH_4^+ (**b**) and NO_3^- (**c**) content after 8 days of fertilizer application. Sterile and non-sterile soils were fertilized with ammonium sulphate (AS) and AS + DMPSA. Different letters indicate significant differences using one-way ANOVA followed by Duncan's post hoc test ($p < 0.01$; $n = 4$)

We also determined soil mineral N content. In agreement with DMPSA function, in non-sterile soils the inhibition of nitrification resulted in a higher soil NH_4^+ and lower NO_3^- content respect to AS treatment (Fig. 4b and c). In

sterile soils, as expected considering the absence of biological activity, no differences were observed between AS and AS + DMPSA treatments (Fig. 4b and c). Interestingly, although the DMPSA covalent bond rupture is associated to soil biological activity, the subsequent degradation of the released DMP would take place primarily via chemical processes and not biological. Sidhu et al. [40] suggested that DMP chemical degradation could be initiated by reactive oxygen species (ROS) formed from both biotic and abiotic processes in the soil.

Copper chelation is unlikely the mode of action of DMP-based NIs

The active site of AMO contains Cu^{2+} and thus, in general, nitrification is thought to be dependent of Cu^{2+} availability [3, 22, 43]. In this sense, the action of nitrification inhibition of DMP-based NIs has been generally related to their presumed Cu^{2+} chelating capacity [10]. Corrochano-Monsalve et al. [22] recently confirmed this ability for both DMPP and DMPSA through X-ray crystallography. These authors reported that the chelation efficiency of DMPP is 4 molecules of DMP per atom of Cu^{2+} while for DMPSA the efficiency was 2 molecules of inhibitor per atom of Cu^{2+}. The fact that both DMPP and DMPSA were able to bind Cu^{2+} does not match with the observation that only DMPP was able to inhibit nitrification in the pure cultures of *N. europaea* (Fig. 1). Therefore, we hypothesized that their mode of action is unrelated to their Cu^{2+} chelating capacity. Indeed, if their action would be based on Cu^{2+} chelation many biological processes with Cu^{2+} requirements could be also affected by the use of DMP-based NIs. As example within the N cycle, nitrite reductase encoded by *nirK* [44] is a denitrifiers' copper-containing enzyme. Nonetheless, *nirK* abundance is not affected by the addition of DMP-based NIs [33, 45]. Furthermore, in several works, the application of DMP-based NIs has been shown to induce N_2O reduction, activity performed by the Cu-containing N_2O reductase enzyme encoded by *nosZ* genes [33, 35, 46, 47]. Indeed, the reduction of N_2O to N_2 is not possible without Cu [48, 49]. Altogether, these evidences suggest that DMP-based NIs mode of action may not be related to their ability to chelate Cu^{2+}. To further investigate this hypothesis, we grew *N. europaea* pure cultures with a higher Cu^{2+} concentration in the growing medium. Since DMPSA is not functional in pure cultures (Fig. 1), this test could only be performed with DMPP. If the mode of action of DMPP were based on its Cu^{2+} chelating capacity, an extra supply of Cu^{2+} would relieve the nitrification

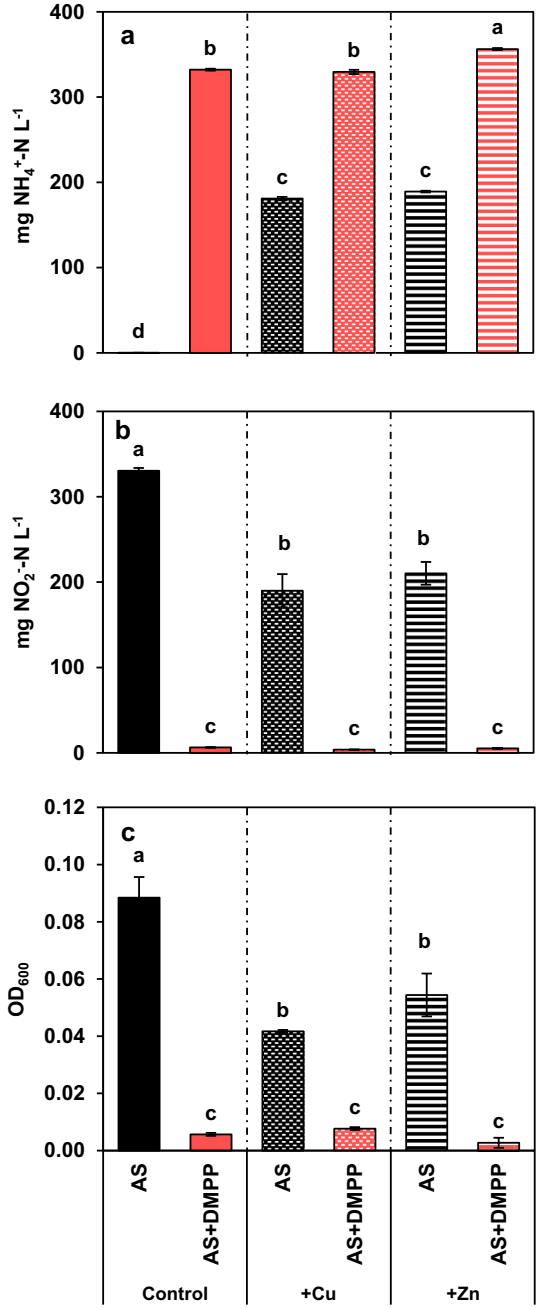


Fig. 5 Effect of increased Cu^{2+} and Zn^{2+} concentration on *Nitrosomonas europaea* growth and nitrification capacity determining NH_4^+ (**a**), NO_2^- (**b**), and bacterial density (OD_{600}) (**c**) in the growth medium after 7 days of treatment application Cu^{2+} and Zn^{2+} concentrations used were 0.01 mg L^{-1} as control dose and 2.50 mg L^{-1} as a higher dose. AS means ammonium sulphate (AS) different letters indicate significant differences using one-way ANOVA followed by Duncan's post hoc test ($p < 0.01$; $n = 3$)

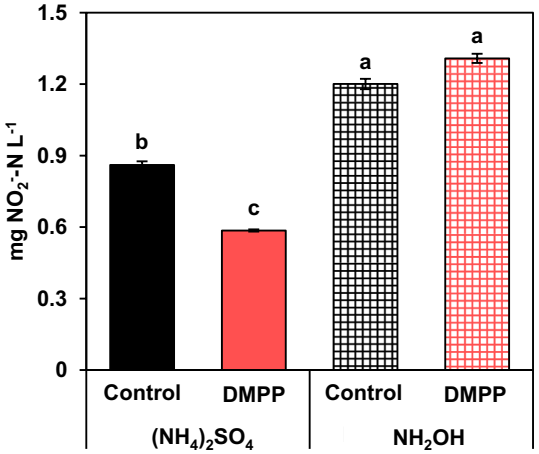


Fig. 6 DMPP capacity to inhibit nitrification in *Nitrosomonas europaea* pure cultures grown with $(NH_4)_2SO_4$ or NH_2OH as nitrogen source by determining NO_2^- in the growth medium after 6 h of N addition. *N. europaea* grew without inhibitor (Control) and in presence of DMPP. Different letters indicate significant differences using one-way ANOVA followed by the Duncan's post hoc test ($p < 0.01$; $n = 3$)

inhibition due to a DMP saturation. We added Cu^{2+} (2.50 mg L^{-1}) in a dose close to its EC_{50} to ensure a concentration was high enough but not completely toxic for *N. europaea* [50]. This dose largely surpasses the theoretical DMPP chelating capacity. In addition, we also tested the growth in presence of a higher Zn^{2+} concentration for two reasons: (i) AMO enzyme also contains Zn^{2+} and it seems to be necessary for its activity [51, 52] and (ii) DMP-based NIs seem also able to chelate Zn^{2+} cations in liquid solutions [22]. In this sense, NH_4^+ oxidation and bacterial growth in + Cu and + Zn mediums was reduced by half compared to the control medium (Fig. 5). Importantly, the higher concentration of Cu^{2+} and Zn^{2+} availability did not alter the inhibitory capacity of DMPP as evidenced by the almost absence of NH_4^+ consumption and NO_2^- production, and the inhibition of *N. europaea* growth (Fig. 5). Consequently, these results together with the inability of DMPSA to inhibit nitrification in pure cultures of *N. europaea* mean that despite DMPP and DMPSA are able to form complexes with Cu^{2+} and Zn^{2+}, their mode of action as NIs seems unrelated to this ability.

The AMO enzyme is the only target of DMP-based NIs

Subbarao et al. [53] already listed 64 synthetic compounds proposed as NIs. This list is continuously growing with new NIs (e.g. [54]) in the attempt of finding molecules with higher efficiency in different soil types, and increased lifetime while minimizing unwanted effects. Most of these NIs act in the first step of nitrification (inhibition of AMO) and their mechanism of inhibition can be divided in three groups: (i) direct binding and interaction with AMO, (ii) removal of co-factors by chelation, and (iii) AMO inactivation through the oxidation of highly reactive substrates [10]. DMPP was assumed to act on the AMO enzyme by chelating the Cu^{2+} co-factor [10, 22]. Nevertheless, to our knowledge, no study truly evaluated whether DMPP inhibits the first step of nitrification. Indeed, since we observed that the mode of action of DMP-based NIs seems not related to their Cu^{2+} chelating capacity, there is the possibility that they might not act on the AMO enzyme. To test this issue, the nitrification inhibitory capacity of DMPP was tested by growing *N. europaea* with hydroxylamine (NH_2OH, substrate of the HAO enzyme) instead of NH_4^+ (substrate of the AMO enzyme). To prevent NH_2OH toxicity, N sources were added in a 1 mg N L^{-1} concentration and the nitrification inhibition was determined just 6 h after N-sources supply [21, 55]. As expected, with NH_4^+, DMPP was efficient to inhibit nitrification as shown by 32% less NO_2^- production compared to control treatment (Fig. 6). On the other hand, DMPP was not able to inhibit nitrification with NH_2OH as the source of N. This evidences that DMPP does not affect the HAO enzyme and confirms its action on the AMO enzyme. Therefore, since DMPP deploys its action on AMO enzyme, we hypothesize that the nitrification inhibition might be due to (i) DMPP attaching directly to the AMO enzyme, modifying its structure by an allosteric-type interaction or (ii) DMPP binds the Cu^{2+} atoms present on AMO active sites inhibiting its ability to catalyse the oxidation of NH_4^+. Nonetheless, more studies will be necessary to confirm whether one of these two or even both hypotheses are correct.

Conclusions

DMP-based NIs are an efficient tool to diminish N losses in agricultural soils. Even though DMPP is one of the most widely used NIs, its mode of action is still unclear and thus, a better understanding of the mode of action of DMP-based NIs and an improvement in their use in the field. Surprisingly, although it is well known that both DMPP and DMPSA are able to delay the oxidation of NH_4^+ in soils, DMPSA did not inhibit nitrification in pure cultures of nitrifying bacteria *N. europaea*. Likewise, we hypothesized that to achieve the inhibition of nitrification in the soil DMPSA needs to be broken in order to release DMP, the active constituent that affects the AMO enzyme. Indeed, we show that DMPSA rupture into DMP takes place in the soil but not in *N. europaea* pure culture. Moreover, we demonstrated with sterile soil that DMPSA breakdown is mediated by a soil biological processes that are yet to be identified. Therefore, since the type of soil and

environmental conditions are known to drive soil microbial diversity, it is key to study DMPSA break and efficiency in different soil types and environmental conditions in order to ensure its effectiveness in agricultural fields. In addition, our results support that the mode of action of DMP-based NIs is not exclusively dependent on their nature as metal chelators.

Abbreviations
AMO: Ammonia monooxygenase enzyme; DMP: Dimethylpyrazole; DMPP: 3,4-Dimethylpyrazole phosphate; DMPSA: 2-(3,4-Dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture; HAO: Hydroxylamine oxidoreductase enzyme; NIs: Nitrification inhibitors.

Acknowledgements
The authors thank Gustavo Garijo from the Public University of Navarre for technical assistance.

Author contributions
ABL: conceptualization, investigation, formal analysis, visualization, writing—original draft. MCM: formal analysis, conceptualization, writing—review and editing. IVM: conceptualization, writing—review and editing. PAT: conceptualization, writing—review and editing, supervision, project administration. CGM: conceptualization, writing—review and editing, supervision, project administration, funding acquisition. DM: conceptualization, writing—review and editing, supervision, project administration, funding acquisition. All authors read and approved the final manuscript.

Funding
This project was funded by the Spanish Government (RTI2018-094623-B-C21 MCIU/AEI/FEDER, UE), by the Basque Government (IT-932-16), and by Eurochem Agro Iberia S.L.U. Dr. Adrián Bozal-Leorri held a grant from the Basque Government (PRE-2020-2-0142).

Availability of data and materials
Raw data used to generate the presented results are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
MCM is an associate editor of Chemical and Biological Technologies in Agriculture. The rest of the authors have no conflicts of interest to declare.

Author details
[1]Institute for Multidisciplinary Research in Applied Biology (IMAB), Public University of Navarre-UPNa, 31006 Pamplona, Spain. [2]Department of Plant Biology and Ecology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Bilbao, Spain. [3]Ikerbasque, Basque Foundation for Science, Bilbao, Spain.

Received: 25 April 2022 Accepted: 17 July 2022
Published online: 16 August 2022

References

1. LeBauer DS, Treseder KK. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecol. 2008;89:371–9.
2. Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Sutton MA. Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. Sci. 2008;320:889–92.
3. Wendeborn S. The chemistry, biology, and modulation of ammonium nitrification in soil. Angewandte Chemie Intern Ed. 2020;59:2182–202.
4. Rockström J, Steffen W, Noone K, Persson Å, Chapin FS III, Lambin E, Foley J. A safe operating space for humanity. Nature. 2009;461:472–5.
5. Coskun D, Britto DT, Shi W, Kronzucker HJ. How plant root exudates shape the nitrogen cycle. Trends Plant Sci. 2017;22:661–73.
6. Syakila A, Kroeze C. The global nitrous oxide budget revisited. Greenh Gas Measur Manag. 2011;1:17–26.
7. IPCC 2014. Climate change 2014 synthesis report summary for policymakers. IUSS working group WRB, 2014. World reference base for soil resources 2014. International soil classification system for naming soils and creating legends for soil maps. World soil resources reports No 106. Rome: FAO
8. Ravishankara AR, Daniel JS, Portmann RW. Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. Sci. 2009;326:123–5.
9. Ábalos D, Jeffery S, Sanz-Cobena A, Guardia G, Vallejo A. Meta-analysis of the effect of urease and nitrification inhibitors on crop productivity and nitrogen use efficiency. Agri Ecosys Environ. 2014;189:136–44.
10. Ruser R, Schulz R. The effect of nitrification inhibitors on the nitrous oxide (N_2O) release from agricultural soils—a review. J Plant Nutr Soil Sci. 2015;178:171–88.
11. Trenkel ME. Slow-and controlled-release and stabilized fertilizers: an option for enhancing nutrient use efficiency in agriculture. Paris: International fertilizer industry association; 2010.
12. Gilsanz C, Báez D, Misselbrook TH, Dhanoa MS, Cárdenas LM. Development of emission factors and efficiency of two nitrification inhibitors DCD and DMPP. Agri Ecosys Environ. 2016;216:1–8.
13. Giltrap DL, Singh J, Saggar S, Zaman M. A preliminary study to model the effects of a nitrification inhibitor on nitrous oxide emissions from urine-amended pasture. Agri Ecosys Environ. 2010;136:310–7.
14. Teske W, Matzel W. Influencing the nitrification-inhibiting effect of dicyandiamide by degradation and translocation in soil. Archiv für Acker-und Pflanzenbau und Bodenkunde. 1988;32:241–6.
15. Weiske A, Benckiser G, Herbert T, Ottow J. Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in comparison to dicyandiamide (DCD) on nitrous oxide emissions, carbon dioxide fluxes and methane oxidation during 3 years of repeated application in field experiments. Biol Fert Soils. 2001;34:109–17.
16. Chen XH, Zhou LX, Zhao YG, Pan SD, Jin MC. Application of nanoring amino-functionalized magnetic polymer dispersive micro-solid-phase extraction and ultra fast liquid chromatography–tandem mass spectrometry in dicyandiamide residue analysis of powdered milk. Talanta. 2014;119:187–92.
17. Zerulla W, Barth T, Dressel J, Erhardt K, von Locquenghien KH, Pasda G, Wissemeier A. 3,4-Dimethylpyrazole phosphate (DMPP)–a new nitrification inhibitor for agriculture and horticulture. Biol Fert Soil. 2001;34:79–84.
18. Rahman N, Henke C, Forrestal PJ. Efficacy of the nitrification inhibitor 3,4-dimethylpyrazol succinic acid (DMPSA) when combined with calcium ammonium nitrate and ammonium sulphate—a soil incubation experiment. Agronomy. 2021;11:1334.
19. Huérfano X, Fuertes-Mendizábal T, Fernández-Diez K, Estavillo JM, González-Murua C, Menéndez S. The new nitrification inhibitor 3,4-dimethylpyrazole succinic (DMPSA) as an alternative to DMPP for reducing N_2O emissions from wheat crops under humid Mediterranean conditions. Eur J Agronomy. 2016;80:78–87.
20. Huérfano X, Estavillo JM, Fuertes-Mendizábal T, Torralbo F, González-Murua C, Menéndez S. DMPSA and DMPP equally reduce N_2O emissions from a maize-ryegrass forage rotation under Atlantic climate conditions. Atmos Environ. 2018;187:255–65.
21. Arp DJ, Stein LY. Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. Crit Rev Biochem Mol Biol. 2003;38:471–95.
22. Corrochano-Monsalve M, González-Murua C, Bozal-Leorri A, Lezama L, Artetxe B. Mechanism of action of nitrification inhibitors based on dimethylpyrazole: a matter of chelation. Sci Tot Environ. 2021;752:141885–96.

23. Patton CJ, Crouch SR. Spectrophotometric and kinetics investigation of the Berthelot reaction for the determination of ammonia. Analytical Chemist. 1977;49:464–9.
24. Snell FD, Snell CT. Colorimetric Methods of Analysis. D. Van Nostrand Co., New York; 1949. p 802–807.
25. IUSS Working Group WRB. World reference base for soil resources 2014. International soil classification system for naming soils and creating legends for soil maps. World soil resources reports no 106. Rome: FAO; 2014.
26. Bozal-Leorri A, González-Murua C, Marino D, Aparicio-Tejo PM, Corrochano-Monsalve M. Assessing the efficiency of dimethylpyrazole-based nitrification inhibitors under elevated CO_2 conditions. Geoderma. 2021;400:115–60.
27. Menéndez S, Barrena I, Setién I, González-Murua C, Estavillo JM. Efficiency of nitrification inhibitor DMPP to reduce nitrous oxide emissions under different temperature and moisture conditions. Soil Biol Biochem. 2012;53:82–9.
28. Linn DM, Doran JW. Effect of water-filled pore space on carbon dioxide and nitrous oxide production in tilled and nontilled soils. Soil Sci Soc Am J. 1984;48:1267–72.
29. Laughlin RJ, Stevens RJ, Müller C, Watson CJ. Evidence that fungi can oxidize NH_4^+ to NO_3^- in a grassland soil. Eur J Soil Sci. 2008;59:285–91.
30. Cawse P. The determination of nitrate in soil solutions by ultraviolet spectrophotometry. Analyst. 1967;92:311–5.
31. Benckiser G, Christ E, Herbert T, Weiske A, Blome J, Hardt M. The nitrification inhibitor 3, 4-dimethylpyrazole-phosphat (DMPP)-quantification and effects on soil metabolism. Plant Soil. 2013;371:257–66.
32. Rodrigues JM, Lasa B, Aparicio-Tejo PM, González-Murua C, Marino D. 3,4-Dimethylpyrazole phosphate and 2-(N-3, 4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture nitrification inhibitors: quantification in plant tissues and toxicity assays. Sci Total Environ. 2018;624:1180–6.
33. Torralbo F, Menéndez S, Barrena I, Estavillo JM, Marino D, González-Murua C. Dimethyl pyrazol-based nitrification inhibitors effect on nitrifying and denitrifying bacteria to mitigate N_2O emission. Sci Reports. 2017;7:13810.
34. Huérfano X, Estavillo JM, Torralbo F, Vega-Mas I, González-Murua C, Fuertes-Mendizábal T. Dimethylpyrazole-based nitrification inhibitors have a dual role in N_2O emissions mitigation in forage systems under Atlantic climate conditions. Sci Total Environ. 2022;807:150670–83.
35. Corrochano-Monsalve M, Huérfano X, Menéndez S, Torralbo F, Fuertes-Mendizábal T, Estavillo JM, González-Murua C. Relationship between tillage management and DMPSA nitrification inhibitor efficiency. Sci Total Environ. 2020;718:134748–61.
36. Corrochano-Monsalve M, Bozal-Leorri A, Sánchez C, González-Murua C, Estavillo JM. Joint application of urease and nitrification inhibitors to diminish gaseous nitrogen losses under different tillage systems. J Clean Prod. 2021;289:125701–14.
37. Recio J, Montoya M, Ginés C, Sanz-Cobena A, Vallejo A, Alvarez JM. Joint mitigation of NH_3 and N_2O emissions by using two synthetic inhibitors in an irrigated cropping soil. Geoderma. 2020;373:114423–32.
38. Montoya M, Vallejo A, Corrochano-Monsalve M, Aguilera E, Sanz-Cobena A, Ginés C, González-Murua C, Álvarez JM, Guardia G. Mitigation of yield-scaled nitrous oxide emissions and global warming potential in an oilseed rape crop through N source management. J Environ Manag. 2021;288:112304–17.
39. Pacholski A, Berger N, Bustamante I, Ruser R, Guardia G, Mannheim T 2016. Effects of the novel nitrification inhibitor DMPSA on yield, mineral N dynamics and N_2O emissions, in: Proceedings of the 2016 International Nitrogen Initiative Conference. Melbourne: "Solutions to improve nitrogen use efficiency for the world"
40. Sidhu PK, Taggert BI, Chen D, Wille U. Degradation of the nitrification inhibitor 3,4-dimethylpyrazole phosphate in soils: indication of chemical pathways. ACS Agric Sci Tech. 2021;1:540–9.
41. O'Sullivan CA, Duncan EG, Whisson K, Treble K, Ward PR, Roper MM. A colourimetric microplate assay for simple, high throughput assessment of synthetic and biological nitrification inhibitors. Plant Soil. 2017;413:275–87.
42. Luo YR. Comprehensive handbook of chemical bond energies. Boca Ratón: CRC Press; 2007.
43. Ensign SA, Hyman MR, Arp DJ. In vitro activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper. J Bacteriol. 1993;175:1971–80.
44. Zumft WG. Cell biology and molecular basis of denitrification. Microbiol Mol Biol Revi. 1997;61:533–616.
45. Duan YF, Kong XW, Schramm A, Labouriau R, Eriksen J, Petersen SO. Microbial N transformations and N_2O emissions after simulated grassland cultivation: effects of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP). Appl Environ Microbiol. 2017;83:e02019-e2116.
46. Fuertes-Mendizábal T, Huérfano X, Vega-Mas I, Torralbo F, Menéndez S, Ippolito JA, Kammann C, Wrage-Möning N, Cayuela ML, Borchard N, Spokas K, Novak J, González-Moro MB, González-Murua C, Estavillo JM. Biochar reduces the efficiency of nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) mitigating N_2O emissions. Sci Report. 2019;9:2346.
47. Castellano-Hinojosa A, Gonzalez-Lopez J, Vallejo A, Bedmar EJ. Effect of urease and nitrification inhibitors on ammonia volatilization and abundance of N-cycling genes in an agricultural soil. J Plant Nutr Soil Sci. 2020;183:99–109.
48. Glass J, Orphan VJ. Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide. Front Microbiol. 2012;3:61–81.
49. Sullivan MJ, Gates AJ, Appia-Ayme C, Rowley G, Richardson DJ. Copper control of bacterial nitrous oxide emission and its impact on vitamin B12-dependent metabolism. Proceed Nat Acad Sci. 2013;110:19926–31.
50. Ore S, Mertens J, Brandt KK, Smolders E. Copper toxicity to bioluminescent *Nitrosomonas europaea* in soil is explained by the free metal ion activity in pore water. Environ Sci Tech. 2010;44:9201–6.
51. Radniecki TS, Ely RL. Zinc chloride inhibition of *Nitrosococcus mobilis*. Biotech Bioeng. 2008;99:1085–95.
52. Chen GC, Tam NF, Ye Y. Does zinc in livestock wastewater reduce nitrous oxide (N_2O) emissions from mangrove soils? Water Res. 2014;65:402–13.
53. Subbarao GV, Ito O, Sahrawat KL, Berry WL, Nakahara K, Ishikawa T, Watanabe T, Suenaga K, Rondon M, Rao IM. Scope and strategies for regulation of nitrification in agricultural systems—challenges and opportunities. Crit Rev Plant Sci. 2006;25:303–35.
54. Taggert BI, Walker C, Chen D, Wille U. Substituted 1,2,3-triazoles: a new class of nitrification inhibitors. Sci Report. 2021;11:14980.
55. Subbarao GV, Ishikawa T, Ito O, Nakahara K, Wang HY, Berry WL. A bioluminescence assay to detect nitrification inhibitors released from plant roots: a case study with *Brachiaria humidicola*. Plant Soil. 2006;288:101–12.

Publisher's Note