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

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**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 N₂O emissions and maintain soil NH₄⁺ 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 Cu²⁺ cations that the ammonia monooxygenase enzyme (AMO) needs to carry on the first step of NH₄⁺ oxidation. However, the efficiency of DMPP was not altered regardless the Cu²⁺ 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 Cu²⁺ 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*
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\(_2\)O) [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\(_2\)O 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\(_2\)O, are also set free. NO\(_3^-\) leaching causes eutrophication and contamination of groundwater supplies. In addition, N\(_2\)O, 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\(_2\)O-N year\(^{-1}\), which accounts for 19% of total N\(_2\)O global source and 49% of anthropogenic N\(_2\)O emissions [5]. Furthermore, N\(_2\)O 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\(_2\)O 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\(_4\))\(_2\)SO\(_4\) 2.5 g L\(^{-1}\), KH\(_2\)PO\(_4\) 0.5 g L\(^{-1}\), NaHCO\(_3\) 0.5 g L\(^{-1}\), MgSO\(_4\)·7H\(_2\)O 0.1 g L\(^{-1}\), CaCl\(_2\)·2H\(_2\)O 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\(_4\)·4H\(_2\)O 0.045 g L\(^{-1}\), H\(_2\)BO\(_3\) 0.049 g L\(^{-1}\), ZnSO\(_4\)·7H\(_2\)O 0.043 g L\(^{-1}\), (NH\(_4\))\(_2\)MoO\(_4\)·24H\(_2\)O 0.037 g L\(^{-1}\), and CuSO\(_4\)·5H\(_2\)O 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}))) \times 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\(_4\)·7H\(_2\)O and CuSO\(_4\)·5H\(_2\)O. 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.

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\(_4\))\(_2\)SO\(_4\) or NH\(_2\)OH 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\(_4\)NO\(_3\); 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 × bulk density) × (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 (NH₄)₂SO₄ equivalent to 200 kg N ha⁻¹, mixed with DMPP or DMPSA at a rate of 0.8% of the applied NH₄⁺-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 50% during the whole experiment. Soil was destructively sampled for soil NH₄⁺ and NO₃⁻ content measurement and DMP determination on day 8 post-fertilization.

To quantify soil NH₄⁺ and NO₃⁻ 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 n1 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 NH₄⁺ content. The NO₃⁻ 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 NH₄⁺ (a) and NO₂⁻ (b) in the growth medium during 7 days. Bacterial density (OD₆₀₀) 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
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 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.

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

**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)

**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.

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 Cu²⁺ and thus, in general, nitrification is thought to be dependent of Cu²⁺ availability [3, 22, 43]. In this sense, the action of nitrification inhibition of DMP-based NIs has been generally related to their presumed Cu²⁺ 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 Cu²⁺ while for DMPSA the efficiency was 2 molecules of inhibitor per atom of Cu²⁺. The fact that both DMPP and DMPSA were able to bind Cu²⁺ 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 Cu²⁺ chelating capacity. Indeed, if their action would be based on Cu²⁺ chelation many biological processes with Cu²⁺ 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 N₂O reduction, activity performed by the Cu-containing N₂O reductase enzyme encoded by *nosZ* genes [33, 35, 46, 47]. Indeed, the reduction of N₂O to N₂ 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 Cu²⁺. To further investigate this hypothesis, we grew *N. europaea* pure cultures with a higher Cu²⁺ 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 Cu²⁺ chelating capacity, an extra supply of Cu²⁺ would relieve the nitrification

![Figure 5](image-url)
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 \textit{N. europaea} [50]. This dose largely surpasses the theoretical DMP 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 DMP as evidenced by the almost absence of NH_4^+ consumption and NO_3^- production, and the inhibition of \textit{N. europaea} growth (Fig. 5). Consequently, these results together with the inability of DMPSA to inhibit nitrification in pure cultures of \textit{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.

\textbf{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 \textit{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.

\textbf{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 \textit{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 \textit{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.

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**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. CCM: 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.

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**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**

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