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Assessing the efficiency of dimethylpyrazole-based nitrification inhibitors under elevated CO_2 conditions

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ABSTRACT

Nitrification inhibitors (NIs) are useful tools to reduce nitrogen (N) losses derived from fertilization in agriculture. However, it remains unclear whether a future climate scenario with elevated CO_2 could affect NIs efficiency. Thus, the objective of this work was to study whether the increase of atmospheric CO_2 concentration would affect the efficiency of two dimethylpyrazole-based NIs: 3,4-dimethylpyrazol phosphate (DMPP) and 3,4-dimethylpyrazol succinic acid (DMPSA) in a plant-soil microcosm. To do so, *Hordeum vulgare* var. Henley plants were grown in soil fertilized with ammonium sulphate (AS) with or without NIs under controlled environmental conditions at ambient CO_2 (aCO_2) or elevated CO_2 (eCO_2; 700 ppm). In the soil, mineral nitrogen and N_2O emission evolution were monitored together with nitrifying and denitrifying population that were quantified by qPCR. In the plant, biomass, total amino acid content and isotopic discrimination of N and C were measured. Both NIs showed greater efficiency to maintain soil NH_4^+ content under eCO_2 compared to aCO_2, as a consequence of 80% reduction of AOB abundance in eCO_2. Indeed, both inhibitors were able to lessen 53% the N_2O emissions in eCO_2 compared to aCO_2. Regarding the plant, DMPP and DMPSA negatively affected plant biomass at aCO_2 but this effect was restored at eCO_2 due to a better ammonium tolerance associated with an increase in total amino acid content. Overall, DMPP and DMPSA NIs were highly efficient under eCO_2, reducing N_2O emissions and keeping N in the soil stable for longer while maintaining plant biomass production.

1. Introduction

Atmospheric CO_2 concentration will presumably rise to 450 ppm by 2030 and between 750 and 1300 ppm by 2100 (IPPC, 2014), which could produce different effects in the soil–plant system. Among others, Kuzyakov et al. (2019) showed that elevated CO_2 (eCO_2) could modify soil carbon cycling through increasing fluxes of dissolved organic carbon and the turnover of low molecular weight organic acids in the soil. Besides, eCO_2 can also stimulate the productivity of C_3 species (Ainsworth and Long, 2005; Shimono et al., 2019) by increasing photosynthesis (Song et al., 2020), changing plant physiology and metabolism (McGrath and Lobell, 2013; Jauregui et al., 2015) and decreasing plant stomatal conductance improving water use efficiency (Dieleman et al., 2012). This decrease in stomatal conductance has been associated with the root capacity to vary the absorption and assimilation of different nitrogen (N) sources (Torralbo et al., 2019).

Besides the rise in atmospheric CO_2 concentration, agriculture also needs to meet the big challenge to feed the world's growing population, which is expected to evolve from an estimated 7.7 billion people in 2019 to 8.5 billion by 2030 and 10.9 billion people by 2100 (UN, 2019). Among others, N fertilizers are intensively used in order to maximize crop yield. However, a large amount of the N applied is lost to the environment mainly through nitrate (NO_3^-) leaching, ammonia volatilization (NH_3) and the emission of nitrogenous gases such as nitric oxide (NO) and nitrous oxide (N_2O). N_2O is the main greenhouse gas (GHG) generated in agriculture as a consequence of N fertilization, representing 56–70% of anthropogenic N_2O sources (Syakila et al., 2010). N_2O is the single most ozone-depleting molecule (Ravishankara et al., 2009) and it is a GHG with a global warming potential (GWP) 265 times higher than that of CO_2 in a 100 year time horizon (IPCC, 2014). Due to its high GWP, small changes in this gas net flow can contribute significantly to climate change (Robertson, 2004). N_2O is mainly generated by

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microbial nitrification and, especially, denitrification (Li et al., 2016). Nitrification is the sequential aerobic oxidation of ammonium (NH_4^+) to hydroxylamine (NH_2OH), nitrite (NO_2^-) and NO_3^- by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (Arp and Stein, 2003; Könneke et al., 2005). During this process, N_2O is formed by the chemical decomposition of the NH_2OH (Wrage et al., 2001). The NO_3^- formed can be leached due to its low adhesion to soil particles, causing water contamination. Besides, NO_3^- can be also converted to different N-gases (NO, N_2O and N_2) during the denitrification process (Hochstein and Tomlinson, 1988).

Attending to the need of reducing N losses, one efficient strategy is the application of nitrification inhibitors (NI) along with ammonium-based fertilizers. NIs interfere in the nitrification process, slowing down the transformation of NH_4^+ to NO_3^-, thus increasing N-retention on soil particles and reducing leaching. NIs based on dimethylpyrazole (DMP), mostly in the form of 3,4-dimethyl-1H-pyrazol phosphate (DMPP), have been widely used in agriculture since they are able to extend the stability of N in the soil while reducing N_2O emissions and without negatively affecting crop yield (Weiske et al., 2001; Migliorati et al., 2014; Guardia et al., 2017) even in abiotic stress conditions such as salinity (Li et al., 2020). Another DMP-based NI, 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA), has been recently developed to be applied with a wider range of fertilizers compared to DMPP, while maintaining a similar efficiency (Huérfano et al., 2016, 2018). Both DMPP and DMPSA inhibit AOB (Barrena et al., 2017; Torralbo et al., 2017). However, although it is believed that both specifically affect ammonia-monooxygenase enzyme (AMO), there is no evidence demonstrating their mode of action.

Therefore, in the context of climate change, it seems that NIs, including DMPP and DMPSA, may play an increasingly relevant role to try to reduce the impact of agriculture. Yet, since the increment of the CO_2 concentration can modify the N cycle, it is not clear whether NIs efficiency could also be affected. Besides, soil CO_2 concentration is not only affected by atmospheric CO_2 but can also be increased by the use of organic fertilizers, by root and microbial respiration or by certain soil managements such as no-tillage. For this reason, it is necessary to know whether NIs effectiveness would be maintained in future scenarios. Thus, the objective of this experiment was to study if the increase of atmospheric CO_2 concentration would affect DMPP and DMPSA nitrification inhibitors efficiency.

2. Materials and methods

2.1. Soil preparation and experimental design

This experiment was carried out in microcosms in a controlled conditions growth chamber with a daily regimen of 14/10 h day/night cycle with an average day/night temperature of 25/18 °C, a relative day/night humidity of 60/70% and two CO_2 conditions, ambient (aCO_2) or elevated (eCO_2) with a CO_2 concentration of 700 ppm. Soil was collected in June 2019, from a 0–30 cm layer of a Hypercalcic Kastanozem soil (IUSS, 2014) in a wheat field (Table 1) in Arkaute (Basque Country, Spain) (42° 51′ N, 2° 37′ W, 530 m above sea level). Roots and stones were removed and the soil was passed through a 5 mm sieve. In order to increase soil's porosity, it was mixed with sand in proportion of 3:1 soil: sand (v:v). After this, it was air-dried, homogenised and kept at 4° C until the start of the experiment. Thirty-six 5 L pots (20 cm diameter × 16 cm height) were filled with soil and 18 pots were placed in aCO_2 and the remaining 18 in eCO_2. In order to reactivate soil microorganisms, pots were supplied with 14.8 mg of ammonium nitrate (NH_4NO_3) and 2.2 g of glucose (Menéndez et al., 2012; Torralbo et al., 2017) and soil was rehydrated with deionised water up to 60% water filled pore space (WFPS). WFPS was calculated as in Linn and Doran (1984) following the equation:

$$\text{WFPS} = (\text{soil gravimetric water content} \times \text{bulk density}) \times (1 - (\text{bulk density}/\text{particle density}))^{-1}$$

Particle density was assumed to be 2.65 Mg m^{-3} and soil bulk density was determined in the laboratory, resulting in a value of 1.33 Mg m^{-3}.

After 14 days, 3 seedlings of barley (*Hordeum vulgare* var. Henley) were placed in each pot. To do so, seeds were previously germinated on a tray with perlite:vermiculite (1:3) mixture at 20 °C for 6 days. All 36 pots were watered during 15 days after barley sowing to maintain soil WFPS. On the 15th day of watering, 18 pots of each CO_2 concentration were randomly divided into three groups of six pots corresponding to three different fertilizer treatments. The fertilizer treatments were: ammonium sulphate (AS), AS + DMPP (AS+DP) and AS + DMPSA (AS+DS). Nitrogen was applied to soil surface in an equivalent to 180 kg N ha^{-1}, which was achieved by adding 1044 mg of ammonium sulphate in granular form, alone or mixed with nitrification inhibitors at a rate of 0.8% of the applied NH_4^+-N. In order to avoid soil disruption in gaseous measurements three pots per treatment and CO_2 condition were used for gaseous measurements and the resting three for destructive samplings. All of them were watered every two days in order to maintain the WFPS up to 60% during the whole experiment (up to 60 days post-fertilization).

2.2. Plant biomass and metabolite analysis

Biomass production was measured as dry weight (DW). To do so, one plant per pot was dried at 80 °C in a circulation oven for 72 h until a constant DW was reached.

To determine leaf NH_4^+ and total amino acid content, 50 mg of frozen leaf powder was homogenized with 1 mL MilliQ water with a ball miller (Retsch MM 500) at a frequency of 27 s^{-1} for 3 min. Homogenates were incubated at 80 °C for 5 min and centrifuged at maximum speed for 20 min. Supernatants were recovered and stored at −20 °C until use. Free NH_4^+ and total amino acid content was determined in the supernatants as described in Sarasketa et al. (2014).

The N and carbon (C) isotopic composition in leaves was determined by an elemental analyzer (FlashEA1112 ThermoFinnigan) coupled to a mass spectrometer (DELTA^{plus} Finnigan MAT) in the Unidade de Técnicas Instrumentais de Análise, Servizos de Apoio á Investigación

Table 1
Physical and chemical properties of the soil collected in 0 – 30 cm depth layer in Arkaute (42° 51′ N, 2° 37′ W, 530 m above sea level, Alava, Spain) before the addition of sand.

Soil texture			Soil chemical properties								
Sand	Silt	Clay	pH[a]	C:N	N[b]	Organic matter[c]	Carbonate[d]	P[e]	Mg[d]	K[d]	Ca[d]
(%)			(g kg^{-1})					(mg kg^{-1})			
43.4	24.7	31.9	8.0	8.15	1.6	21.2	9.8	59.0	92.4	167	6356

[a] pH (1:2.5 soil:water).
[b] N Kjeldahl digestion (Keeney and Nelson, 1982).
[c] Organic matter (Walkley and Black, 1934).
[d] CaCO3, Mg, K (NH4 AcO, MAPA, 1994).
[e] P (Watanabe and Olsen, 1965).

(SAI), Universidade da Coruña. The values of the isotopic ratio were expressed as $\delta^{15}N$ and $\delta^{13}C$, in parts per thousand (‰) relative to atmospheric N_2 and VPDB (Vienna Pee Dee Belemmite) respectively. The isotope composition values δ (‰) were obtained by the following equation:

$$\delta_{sample}(‰) = \left(\left(R_{sample} - R_{standard}\right)/R_{standard}\right) \times 1000$$

where R_{sample} is the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratio of the plant sample and $R_{standard}$ is the $^{15}N/^{14}N$ ratio of the atmospheric N_2 and the $^{13}C/^{12}C$ratio of VPDB.

2.3. N_2O emission measurement

N_2O soil emission was quantified using the close chamber method (Chadwick et al., 2014). To do so, on sampling days chambers (20 cm diameter × 22 cm height; headspace 6.9 L) were fitted to the pot edge without disturbing the soil and seal was ensured with a rubber band. Chambers height was enough to properly accommodate the plants along the whole experiment. Chambers were removed after measurement. Sampling frequency was 3 times per week post-fertilization along 2 weeks, reducing frequency to 2 times per week in the next 2 weeks and 1 time per week in the following 2 weeks until N_2O emissions were constant. Gas samples were taken just after closing the chambers and after 45 min. 20 mL of gas were taken from each chamber and stored at overpressure in pre-evacuated 12 mL glass vials. The linearity of the fluxes was checked regularly along the experiment taking samples at 0, 15, 30, 45 and 60 min (Chadwick et al., 2014). Samples were analysed in a gas chromatograph (Agilent, 7890A) equipped with an electron capture detector for N_2O detection. A capillary column (IA KRCIAES 6017:240 °C, 30 m × 320 μm) was used, and samples were injected utilizing a headspace auto-sampler (Teledyne Tekmar HT3). Standards of N_2O were analysed as controls. Gas emission rates were calculated as the gas concentration variation during 45 min. Cumulative emissions during the sampling period were estimated using the trapezoidal rule integration (linear interpolation and numerical integration between sampling times) (Levy et al., 2017).

2.4. Geochemical analysis

To measure soil NH_4^+ and NO_3^- content, three soil subsamples were taken from every pot with a hollow sampler (1.5 cm diameter × 7 cm depth) at 0, 5, 10, 30 and 60 days post-fertilization. Holes were refilled with sand and its weight taken into account to recalculate the water needed to maintain the WFPS. Soil subsamples from each pot were homogenized, and then 50 g were mixed with 100 mL 1 M KCl and shaken for one hour at 165 rpm. The soil solution was filtered through Whatman n°1 filter paper (GE Healthcare) to remove particles and, secondly through Sep-Pak Classic C18 Cartridges 125 Å pore size (Waters) to eliminate the organic matter. The filtered solution was used to determine the content of NH_4^+, using the Berthelot method (Patton and Crouch, 1977), and NO_3^-, as described by Cawse (1967).

2.5. Abundance of N-cycle-related microorganisms

Quantitative polymerase chain reaction (qPCR) was used to quantify the abundance of nitrifying and denitrifying genes. Soil DNA from 0, 10, and 30 days post-fertilization was isolated from the same samples used for geochemical determinations. DNA was extracted from 0.25 g of dry soil using the PowerSoil DNA Isolation Kit (Quiagen) including the modifications described in Harter et al. (2014). Extracted DNA concentration and quality were determined spectrophotometrically (NanoDrop® 1000, Thermo Scientific).

16S rRNA gene (for quantification of total bacterial abundance) and functional marker genes involved in nitrification (bacterial *amoA*) and denitrification (*nirK* and *nirS*) were amplified by qPCR using SYBR® Premix Ex TaqTM II (Takara-Bio Inc.) and gene-specific primers (Torralbo et al., 2017) in a StepOne PlusTM Real-Time PCR System. Data analysis was carried out by StepOnePlusTM Software 2.3 (Thermo Scientific). Standard curves were prepared from serial dilutions of linearized plasmids with insertions of the target gene ranging from 10^7 to 10^3 gene copies μL^{-1}. Copy number of target gene per gram of dry soil was calculated according to a modified equation detailed in Behrens et al. (2008):

[(number of target gene copies per reaction × volume of DNA extracted)/(volume of DNA used per reaction × gram of dry soil extracted)]/DNA concentration.

2.6. Statistical analysis

The results obtained in this experiments were analysed with IBM SPSS v. 24.0 statistical software (IBM Corp. Armonk, NY, USA) by two-way (CO_2, C; and fertilizer treatment, T) analysis of variance.

3. Results

3.1. Plant growth

At aCO_2, plant biomass was reduced by 37% in presence of DMPP and DMPSA compared to AS treatment. However, this effect was not observed under eCO_2 and the three fertilizer treatments presented similar plant biomass (Fig. 1A). Regarding CO_2 effect, plant biomass in AS treatments showed no influence of atmospheric CO_2 concentration (Fig. 1A). In contrast, plants grown in presence of NIs at eCO_2 increased their biomass by 44% respect to aCO_2, therefore reaching the same biomass as AS treatment (Fig. 1A). Leaf NH_4^+ content was not affected by CO_2 level nor by fertilizer treatment (Fig. 1B). Amino acid content was always higher in AS+DP and AS+DS respect to plants grown in AS (Fig. 1C). In addition, eCO_2 exerted a positive effect on amino acid accumulation in every fertilizer treatment.

3.2. N and C isotopic composition and accumulation

NIs did not affect plant N content (Supplementary Fig. 1A) at any CO_2 level. However, eCO_2 provoked a decrease in leaf N content regardless of the fertilizer treatment compared to aCO_2 (Supplementary Fig. 1A). The supply of NIs entailed lower values of $\delta^{15}N$ compared to AS, but the N isotopic composition was not affected by the CO_2 level (Fig. 2.A). Regarding C, neither fertilizer treatment nor CO_2 level affected its content (Supplementary Fig. 1B). Nevertheless, although the presence of NIs did not affect plant C isotopic composition in comparison with AS treatment, it was strongly affected by CO_2 concentration with more negative $\delta^{13}C$ values at eCO_2 compared to aCO_2 (Fig. 2.B). The C:N ratio was affected by both fertilizer and CO_2 treatments. Plants grown in AS+DS presented higher values of C:N ratio respect to AS and AS+DP irrespective of the CO_2 level (Supplementary Fig. 1C). Besides, plants from eCO_2 treatments had higher values of C:N ratio than those of aCO_2.

3.3. Soil mineral N

After N fertilization, NH_4^+ content increased in all treatments. As expected, the lowest NH_4^+ content was for AS, and both AS+DP and AS+DS were able to keep high levels of NH_4^+ in the soil for longer (Fig. 3A). On average, AS treatments of both CO_2 concentrations presented similar values of soil NH_4^+ content, while treatments with inhibitors at eCO_2 presented higher NH_4^+ content than at aCO_2 (Fig. 3B). On the other hand, AS treatments presented the greatest NO_3^- content in the soil, notably at eCO_2 (Fig. 3C). AS+DP and AS+DS similarly reduced the apparition of NO_3^- compared to AS with no differences between both inhibitor treatments regardless of the CO_2 level (Fig. 3D). The sum of NH_4^+ and NO_3^- evidences that in eCO_2 soil total N was maintained at a higher level respect to aCO_2 regardless of the fertilizer applied (Fig. 3E).

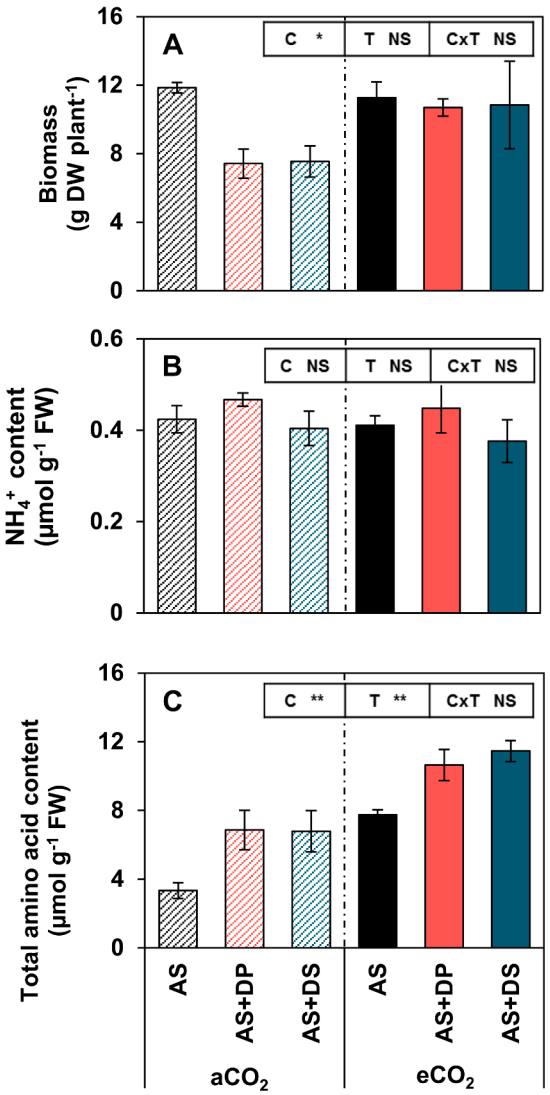

Fig. 1. Shoot biomass of barley (*Hordeum vulgare* var. Henley) plants, (A) leaf ammonium (B) and total amino acid content (c). Pots were fertilized with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). Statistical analysis was made through analysis of variance (two-way ANOVA) showing the effect of CO_2 (C), fertilizer treatment (T) and their interaction (CxT). Significant differences are marked with an asterisk (*) when $p < 0.05$ and double asterisk (**) when $p < 0.01$.

On average, the AS treatment had a significantly higher amount of total N compared to treatments with inhibitors and AS+DS treatment presented more soil total N than AS+DP at both CO_2 conditions (Fig. 3F).

3.4. Nitrous oxide emissions

Daily N_2O emissions ranged from 0.12 to 11.72 g N_2O-N ha^{-1} d^{-1} in aCO_2 and from 0.21 to 7.23 g N_2O-N ha^{-1} d^{-1} in eCO_2 (Fig. 4A). The maximum emissions occurred 9 day post-fertilization (DPF) in AS regardless of CO_2 condition. Unlike AS, treatments with DMPP and DMPSA inhibitors did not show a peak in response to fertilization, as their emissions rates were low and continuous during the experiment. There were no differences between AS+DP and AS+DS in total cumulative N_2O emission and they were greatly reduced respect to AS at both CO_2 concentrations with a reduction of 85% for aCO_2 and 88% for eCO_2 (Fig. 4B). Interestingly, CO_2 affected N_2O emissions in every treatment, with a reduction of 42% for AS of the total accumulated N_2O emissions and a reduction of 53% for both inhibitors treatments in eCO_2 (Fig. 4B).

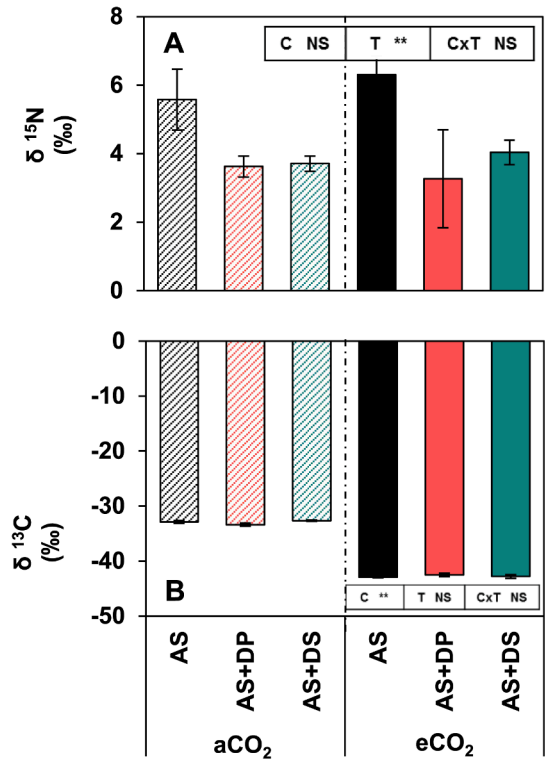

Fig. 2. Leaf δ ^{15}N (A) and δ ^{13}C (B). Pots were fertilized with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). Statistical analysis was made through analysis of variance (two-way ANOVA) showing the effect of CO_2 (C), fertilizer treatment (T) and their interaction (CxT). Significant differences are marked with an asterisk (*) when $p < 0.05$ and double asterisk (**) when $p < 0.01$.

3.5. Abundance of nitrifying and denitrifying bacteria

The total bacterial abundance (measured as *16S rRNA* gene abundance) was affected neither by fertilizer treatment nor CO_2 concentration (Fig. 5A). Nitrification (in terms of bacterial *amoA* gene abundance) was greatly enhanced in AS treatment at both CO_2 concentrations, notably at 10 DPF with respect to pre-fertilization (0DPF) (Fig. 5B). The application of inhibitors was very effective in avoiding the increase of AOB abundance. Indeed, at 10 DPF, in AS+DP and AS+DS at aCO_2 AOB abundance was reduced by 84% and 71% respectively and by 90% and 81% at eCO_2. At 30 DPF, the inhibitors showed a reduction of 70% for DMPP and 60% for DMPSA at aCO_2 and 78% and 66% at eCO_2, respectively. Even though the effect of CO_2 condition over AOB abundance was not significant ($p = 0.082$), it could be appreciated that eCO_2 showed a lower AOB abundance in all fertilizer treatment compared to aCO_2 at 10 DPF.

Denitrification was affected by both the fertilizer treatment and the CO_2 concentration. The abundance of *nirK* at 10 DPF was lower in AS+DP and AS+DS treatments compared to AS, at both CO_2 concentrations. On the contrary, at 30 DPF and aCO_2 *nirK* abundance was higher with in AS+DS while under eCO_2 no differences were observed. Regarding CO_2 effect in AS, at 10 DPF *nirK* abundance was lower in eCO_2 compared to aCO_2 while at 30 DPF there was no effect of CO_2 concentration (Fig. 6A). Remarkably, CO_2 affected *nirK* abundance in presence of any of the inhibitors at both 10 and 30 DPF, with lower abundance values observed at eCO_2. Concerning *nirS*, its abundance at 10 DPF was slightly lower in presence of NIs (Fig. 6B), In contrast, at 30 DPF under aCO_2 AS+DP and AS+DS had higher *nirS* abundance compared to AS treatment. CO_2 concentration did not affect *nirS* abundance at 10 DPF. However, at 30 DPF, its abundance in AS+DP and AS+DS was higher at aCO_2 compared to eCO_2 (Fig. 6B).

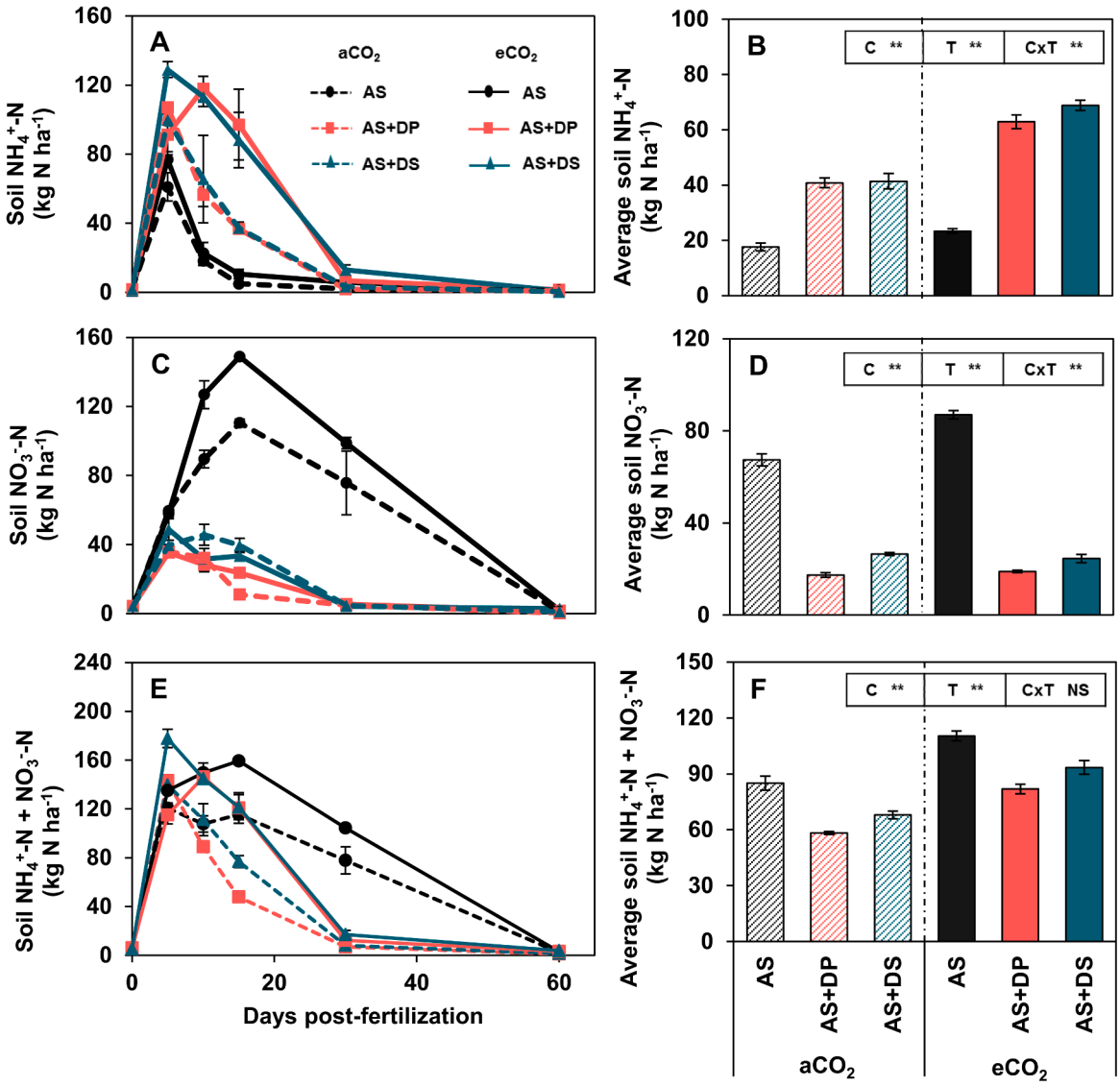

Fig. 3. Evolution during 60 days of experiment and average of soil mineral nitrogen. NH_4^+ (A, B) NO_3^- (C, D) and total nitrogen calculated as the sum of NH_4^+ and NO_3^- (E,F). Pots were fertilized with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). For average soil mineral nitrogen, statistical analysis was made through analysis of variance (two-way ANOVA) showing the effect of CO_2 (C), fertilizer treatment (T) and their interaction (CxT). Significant differences are marked with an asterisk (*) when $p < 0.05$ and double asterisk (**) when $p < 0.01$.

4. Discussion

Plant exposed to elevated CO_2 (eCO_2) commonly display enhanced photosynthesis and increased growth (Ainsworth and Long, 2005). Nevertheless, the effect of eCO_2 depends on the plant species (Wu et al., 2017). Even within the same species, different varieties display different plasticity to eCO_2. For instance, old cultivars tend to show higher C-sink capacity and stronger reactions than modern ones, which may be related to the development of these varieties in a less CO_2-enriched atmosphere (Manderscheid and Weigel, 1997; Ziska, 2008; Clausen et al., 2011). Moreover, plant response to eCO_2 is variable depending on other environmental conditions (Butterly et al., 2016). As an example in barley, adverse conditions such as drought, tend to intensify the responses to eCO_2 through a higher water use efficiency (Wullschleger et al., 2002; Schmid et al., 2016). Nevertheless, plants of this experiment grew in optimal water availability conditions (60% WFPS). In AS treatment, we did not observe an effect of eCO_2 in plant biomass (Fig. 1A). However, even though the effect of eCO_2 was not observed in plant biomass, plants did respond to the increased CO_2 concentration in other terms. For instance, eCO_2 plants showed a higher total amino acid content (Fig. 1C). NH_4^+ nutrition is known to promote the synthesis of amino acids probably to avoid excessive NH_4^+ accumulation in the tissues, which is known to be detrimental for plant performance (de la Peña et al., 2019). This excessive assimilation often entails a limitation in carbon skeletons and thus, an increase in carbon availability has been shown to promote NH_4^+ assimilation (Roosta and Schjoerring, 2008; Setién et al., 2013). Therefore, the extra C available under eCO_2 would be favouring the incorporation of NH_4^+ into amino acids regardless of the supply of nitrification inhibitors (NIs) (Fig. 1C).

The combination of NIs and ammonia-based fertilizers is a widely proven appropriate strategy to maintain NH_4^+ in the soil for longer periods while reducing N losses (Ruser and Schulz, 2015). Although it has been extensively reported that, in general, the use of NIs does not affect negatively the crop yield in field conditions (Huérfano et al., 2015; Guardia et al., 2018a), in our experiment, barley biomass decreased in presence of DMPP and DMPSA under aCO_2 (Fig. 1A). This reduction of biomass could be a result of plants suffering from "ammonium syndrome" due to the higher presence of NH_4^+ in the soil, as a consequence of nitrification inhibition by DMPP and DMPSA (Fig. 3A and B). Ammonium syndrome is characterized by a decreased photosynthesis,

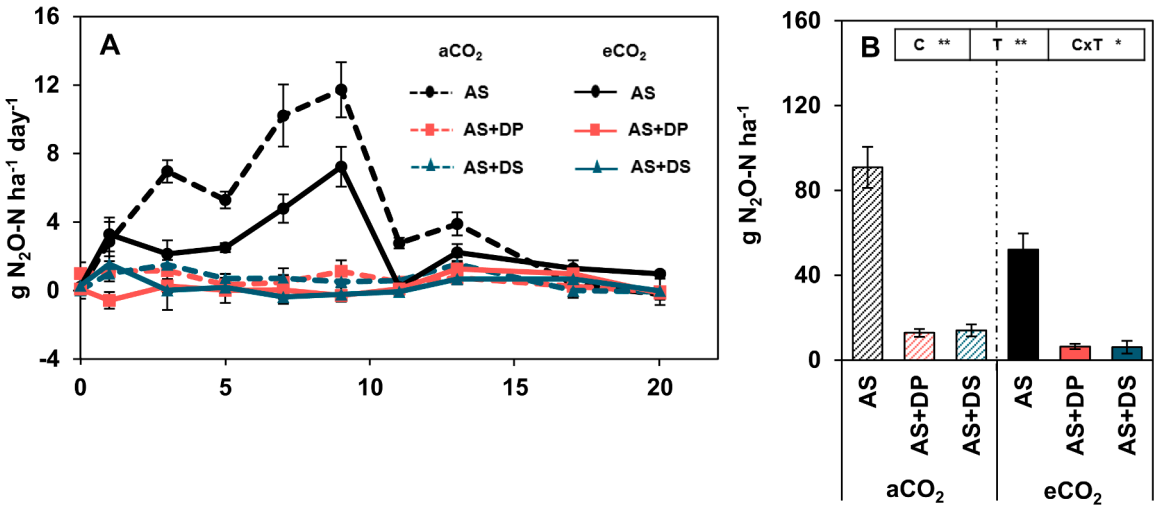

Fig. 4. Daily (A) and cumulative (B) N_2O emission during 20 days of experiment. Pots were fertilized with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+ DS). For cumulative emissions, statistical analysis was made through analysis of variance (two-way ANOVA) showing the effect of CO_2 (C), fertilizer treatment (T) and their interaction (CxT). Significant differences are marked with an asterisk (*) when $p < 0.05$ and double asterisk (**) when $p < 0.01$.

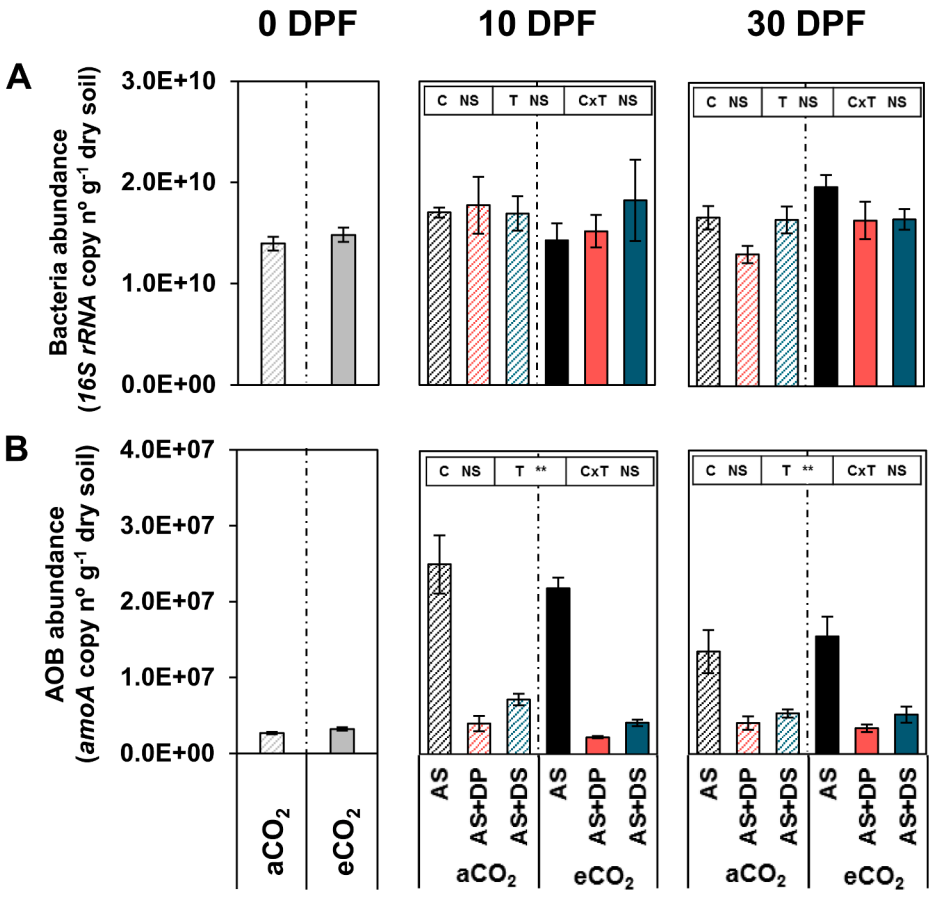

Fig. 5. Bacteria (A) and ammonia oxidizing bacteria (AOB) (B) abundance at 0, 10 and 30 days post-fertilization (DPF) with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). Statistical analysis of 0 DPF was made through *t*-test. Statistical analysis of 10 and 30 DPF were made through analysis of variance (two-way ANOVA) showing the effect of CO_2 (C), fertilizer treatment (T) and their interaction (CxT). Significant differences are marked with an asterisk (*) when $p < 0.05$ and double asterisk (**) when $p < 0.01$.

leaf chlorosis, rhizosphere acidification and other symptoms that contribute to a diminished yield (Britto and Kronzucker, 2002; Liu and von Wirén, 2017). In our experiment, the addition of DMPP and DMPSA was able to delay the oxidation of NH_4^+ and thus, keep it available for longer compared to AS treatment (Fig. 3A and B). Among others, an energy imbalance because of excessive NH_4^+ assimilation has been put forward as one of the causes of ammonium syndrome (Hachiya et al., 2020). Indeed, free amino acid content was higher with NIs supply under both CO_2 conditions with respect to AS (Fig. 1C). However, in line with the carbon shortage hypothesis, the extra supply of C in eCO_2 condition restored barley growth in presence of NIs (Fig. 1A). This has been already reported by other authors such as Setién et al. (2013) in wheat grown with high light, Vega-Mas et al. (2017) in tomato grown under eCO_2 or by Roosta and Schjoerring (2008) with cucumber root

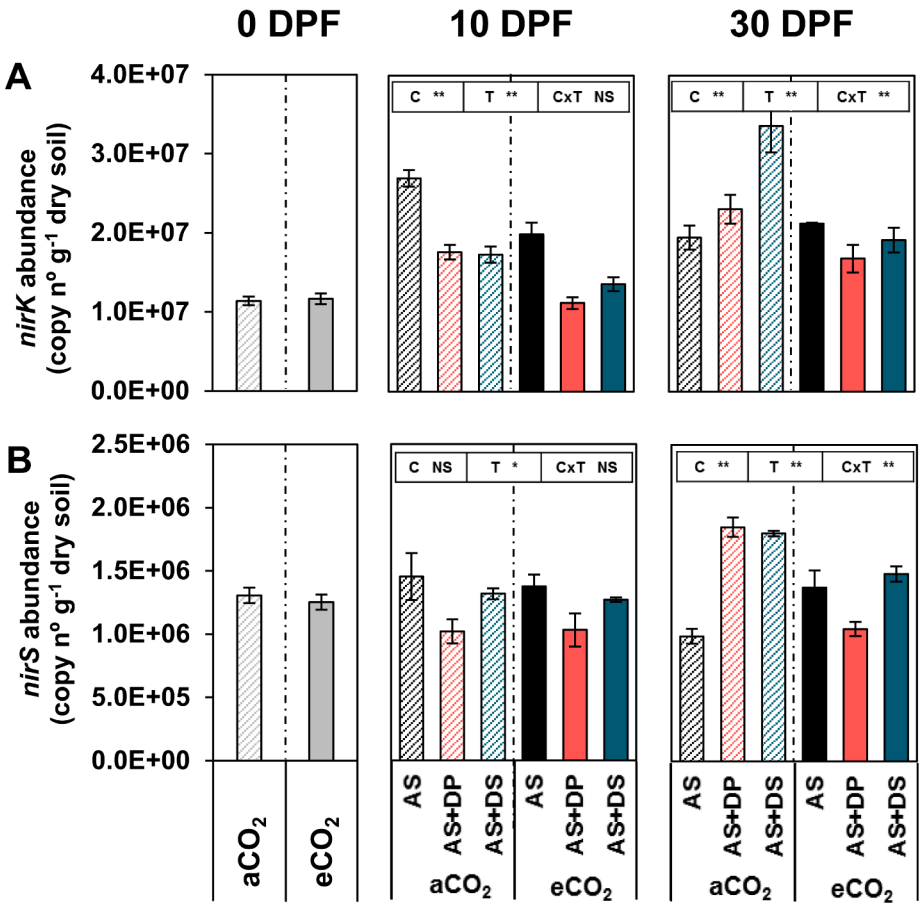

Fig. 6. Abundance of NIR enzyme containing denitrifying bacteria as *nirK* (A) and *nirS* (B) gene copy number g^{-1} dry soil at 0, 10 and 30 days post-fertilization (DPF) with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). Statistical analysis of 0 DPF was made through *t*-test. Statistical analysis of 10 and 30 DPF were made through analysis of variance (two-way ANOVA) showing the effect of CO_2 (C), fertilizer treatment (T) and their interaction (CxT). Significant differences are marked with an asterisk (*) when $p < 0.05$ and double asterisk (**) when $p < 0.01$.

carbon enrichment with carbonates. The absence of differences in leaf NH_4^+ accumulation (Fig. 1B) may be due to the harvest time (60 days post-fertilization) when soil NH_4^+ content was almost undetectable (Fig. 3A) but also because, in general, cereals accumulate NH_4^+ at the root level (de la Peña et al., 2019; Setién et al., 2013). Overall, it seems that even with the higher NH_4^+ content in the soil derived from the use of NIs (Fig. 3A), the great availability of C under eCO_2 relieved ammonium stress symptoms and allowed barley to grow up to the same rates of AS treatment. Future long-term experiments under field conditions using systems such as Free-Air Carbon dioxide Enrichment (FACE) will be useful to validate the results we report in the present short-term experiment.

To further characterize plant performance we determined leaf C and N content together with their isotopic composition. $\delta^{13}C$ is a good tool when studying eCO_2 because of the effect that eCO_2 has on the discrimination of Rubisco. This enzyme discriminates against the heavy isotope of C (^{13}C). Therefore, a greater abundance of atmospheric CO_2 increases rubisco isotopic discrimination, resulting in a lower $^{13}C/^{12}C$ ratio ($\delta^{13}C$) in plant biomass (Farquhar et al., 1982, 1989). This was confirmed because plants at eCO_2 presented a lower $\delta^{13}C$ than plants from aCO_2 in every fertilizer treatment (Fig. 2B). On the other hand, the $\delta^{15}N$ value is indicative of what kind of primary N source plant has had (Werner and Schmidt, 2002) and Ariz et al. (2011) observed that plants fed with NH_4^+ as the sole source of N are depleted of $\delta^{15}N$. In addition, low $\delta^{15}N$ value can be also associated with reduced soil microbial nitrification and thus higher soil ammonium content (Robinson, 2001; Jones and Dalal, 2017). While CO_2 concentration did not affect the discrimination of the heavy isotope of N (^{15}N), DMPP and DMPSA treatments displayed lower $\delta^{15}N$ values compared to AS treatment (Fig. 2A). This indicates that, as observed with soil NH_4^+ content (Fig. 3. A), DMPP and DMPSA made barley plants to be exposed to a preferential ammonium nutrition for a longer period, which is in line with the observed biomass reduction at aCO_2. Regarding leaf C, no differences were found regardless of the concentration of CO_2 or the supply of NIs (Supplementary Fig. 1B). Nevertheless, the effect of eCO_2 provoked a reduction in total leaf N content regardless of the supply of NIs (Supplementary Fig. 1A). Cotrufo et al. (1998) reported that the decrease in N concentration is a fundamental plant response to eCO_2 and indicated several explanations for this effect, such as a preferential N allocation in the root, or the decrease in transpiration rate in plants exposed to eCO_2, which may affect N uptake. Indeed, Shimono and Bunce (2009) observed that after a long-term eCO_2 exposure, N uptake in vegetative stages of rice was reduced in eCO_2 conditions.

As it is also observed in our work (Fig. 3A and B), NIs efficiency to delay NH_4^+ oxidation has been associated with the action that NIs have over AOB (Ruser and Schulz, 2015), which are the main drivers of nitrification in soils receiving high N inputs (Di and Cameron, 2011). As expected, AS+DP and AS+DS were able to reduce the abundance of AOB by more than 70% in aCO_2 and 80% in eCO_2 compared to AS from the 10 days post-fertilization (Fig. 5.B). Interestingly, the efficiency of DMPP and DMPSA to keep NH_4^+ in soil was higher in eCO_2 than in aCO_2 (Fig. 3A and B). This may be due to the observed influence of eCO_2 over AOB abundance. Indeed, although differences between both CO_2 concentrations were not significant ($p = 0.082$), AOB abundance in NIs treatments at eCO_2 was 42% lower compared to aCO_2 (Fig. 5B). This reduced abundance could be responsible for the higher maintenance of NH_4^+ in eCO_2 soils. In agreement with reducing NH_4^+ oxidation, the NIs also diminish NO_3^- apparition in the soil (Guardia et al., 2018b; Corrochano-Monsalve et al., 2020). This was clearly observed in our experiment with the supply of DMPP and DMPSA independently of the CO_2 concentration (Fig. 3C and D). This is especially relevant because in the absence of NIs, eCO_2 promoted higher NO_3^- content in soil than

aCO_2. Altogether, in eCO_2 conditions, NIs increased N-retention in soil due to an increase of soil NH_4^+ content and diminish the amount of NO_3^-. In addition, the reduced availability of NO_3^- in soil due to the inhibition of nitrification by the use of NIs could contribute to decrease the N_2O emissions coming from denitrification, overall reducing N_2O emissions by more than 85% compared to AS at both CO_2 concentrations (Fig. 4A and B). This efficiency to reduce N_2O production is in line with other experiments carried out under controlled conditions, in which higher inhibitions than in field experiments are usually reported (Torralbo et al., 2017; Recio et al., 2018; Corrochano-Monsalve et al., 2021).

Regarding the effect of elevated CO_2 on N_2O emissions, in a *meta*-analysis in uplands with crops such as sorghum, wheat and soybean Dijkstra et al. (2012) reported that elevated atmospheric CO_2 concentration could increase N_2O emissions. According to these authors, this increment could be driven by enhanced denitrification as a consequence of more anoxic conditions in soils due to higher microbial activity and/or improved plant water use efficiency entailing a higher soil water content. Our experiment was performed under controlled water availability and, on the contrary, we observed lower N_2O emissions in every treatment from eCO_2 condition (Fig. 4). This could be a result of a weakened denitrifying pathway at 10 DPF, the moment of maximum N_2O emission peak (Fig. 6), which is in agreement with the higher NO_3^- accumulation in AS at eCO_2 that would not have been consumed by denitrifiers (Fig. 3B). In this sense, Dong et al. (2020) also reported a decrease of *nirK* gene abundance at eCO_2 and suggested that it was due to an increase in the diversity of microorganism competing with denitrifiers. Alternatively, Jin et al. (2019) reported that eCO_2 decreases the exchangeable Cu in the soil. Thus, reduced denitrifiers abundance might be associated with limited Cu availability, a cofactor for the *nirK* encoded nitrite–reductase enzyme (NIR) (Zumft, 1997; Glass and Orphan, 2012). Indeed, the fact that eCO_2 did not affect *nirS* abundance in AS is somehow in agreement with this hypothesis since the hemo-containing NIR enzyme encoded by *nirS* does not need Cu as a cofactor. Future experiments are necessary to enlighten the potential relation between eCO_2, soil Cu availability and denitrification. Regarding NIs effect on denitrifiers, at 10 DPF the use of both DMPP and DMPSA reduced *nirK* abundance at both CO_2 concentrations with respect to AS (Fig. 6A), and, to a lesser extent, the *nirS* abundance (Fig. 6B). This could be a consequence of the observed decrease in NO_3^- formation because of nitrification inhibition (Fig. 3) and/or due to the reduction in AOB abundance that also harbour *nirK*, which has been also reported with the application of other NIs such as dicyandiamide (DCD) (Di et al., 2009). Instead, a potential direct effect of these NIs on denitrifiers cannot be fully discarded.

In conclusion, under an enriched CO_2 atmosphere, both nitrification and denitrification tended to be weakened. NIs showed great efficiency avoiding AOB growth because of fertilization at both CO_2 concentrations. In this manner, NIs exhibited more than 70% and 80% lower AOB abundance than AS in aCO_2 and eCO_2 respectively. Thus, nitrification was even lower in eCO_2 conditions, leading to a higher soil NH_4^+ content and lower NO_3^-. As a consequence of the decreasing in the substrate to denitrify, *nirK* abundance was also reduced with the application of DMPP and DMPSA, which probably also resulted in lower N_2O emissions coming from denitrification. Altogether, N_2O emissions were reduced by half under eCO_2 and the application of NIs caused an 85% reduction of N_2O emissions with respect to AS treatment. Regarding plant performance, plants showed lower biomass because of ammonium stress due to the application of nitrification inhibitors under aCO_2 conditions. Interestingly, this was not observed under eCO_2, probably because the extra carbon availability allowed more efficient NH_4^+ assimilation in agreement with the observed increase in total amino acid content. Overall, the greater efficiency observed for NIs under enriched CO_2 atmosphere highly recommends their use in future climate scenarios in order to reach a sustainable agriculture.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2021.115160.

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