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RESEARCH ARTICLE

Soil moisture modulates biological nitrification inhibitors release in sorghum plants

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Abstract

Background and aims Sorghum (*Sorghum bicolor*) is able to exude allelochemicals with biological nitrification inhibition (BNI) capacity. Therefore, sorghum might be an option as cover crop since its BNI ability may reduce N pollution in the following crop due to a decreased nitrification. However, BNI exudation is related to the physiological state and development of the plant, so abiotic stresses such as drought might modify the rate of BNI exudation. Hence, the objective was to determine the effect of drought stress on sorghum plants' BNI release.

Methods The residual effects of sorghum crops over ammonia-oxidizing bacteria (AOB) were monitored in a 3-year field experiment. In a controlled-conditions experiment, sorghum plants were grown under Watered (60% WFPS) or Moderate drought (30% WFPS) conditions, and fertilized with ammonium sulphate (A), ammonium sulphate + DMPP (A+D), or potassium nitrate (KNO_3^-). Soil mineral N was determined, and AOB populations were quantified. Additionally, plant biomass, isotopic discrimination of N and C, and photosynthetic parameters were measured in sorghum plants.

Results In the driest year, sorghum was able to reduce the AOB relative abundance by 50% at field conditions. In the plant-soil microcosm, drought stress reduced leaf photosynthetic parameters, which had an impact on plant biomass. Under these conditions, sorghum plants exposed to Moderate drought reduced the AOB abundance of A treatment by 25% compared to Watered treatment.

Conclusion The release of BNI by sorghum under limited water conditions might ensure high soil NH_4^+-N pool for crop uptake due to a reduction of nitrifying microorganisms.

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Keywords Ammonium · Cover crops · Drought stress · Nitrate · Soil mineral nitrogen

Abbreviations

AOB	Ammonia-oxidizing bacteria
BNI	Biological nitrification inhibition
BNIs	Biological nitrification inhibitors
DMPP	3,4-dimethylpyrazole phosphate
SNIs	Synthetic nitrification inhibitors
WFPS	Water filled pore space

Introduction

The availability of nitrogen (N) is the major limiting nutrient for crop growth (LeBauer and Treseder 2008). Although agriculture relies on the intensive use of N fertilizers to maximize crop yields, a great amount is lost as reactive N since crops cannot take the entire N applied and it cannot be retained by soils (Lassaletta et al. 2014). The main pathways for N losses that cause a negative environmental impact are through nitrate (NO_3^-) leaching, ammonia (NH_3) volatilization, and emissions of nitrogenous gases such as nitric oxide (NO) and nitrous oxide (N_2O) (Coskun et al. 2017). Nitrous oxide, one of the main greenhouse gasses (GHG) generated in upland agriculture (Syakila and Kroeze 2011) with a global warming potential (GWP) between 265 and 298 times higher than that of CO_2 in a 100-year time horizon (IPCC 2014), is mainly generated by microbial nitrification and denitrification processes (Li et al. 2016). There are several approaches to reduce N losses derived from fertilization, e.g., the use of synthetic nitrification inhibitors (SNIs), such as 3,4-dimethylpyrazole phosphate (DMPP), when applying ammonium-based fertilizers (Huérfano et al. 2015, 2018). Unfortunately, the use of SNIs is not widely adopted by farmers due to having some disadvantages such as additional product and field application costs and low cost-effectiveness for farmers (Subbarao et al. 2006, 2013a, 2017). Notwithstanding these drawbacks, the use of crops with the capability of producing biological nitrification inhibitions (BNIs) has become in a promising option to alleviate N losses derived from nitrification.

The biological nitrification inhibition (BNI) was firstly described in 1966 in *Hyparrhenia filipendula*, but it was not termed as BNI until 2003 when Ishikawa et al. (2003) tried to describe the capacity of *Brachiaria humidicola* to inhibit the ammonium (NH_4^+) oxidation to NO_3^-. Moreover, the opportunity to exploit this strategy in agricultural systems to minimize the problem of N losses has gone unnoticed until recently (Subbarao and Searchinger 2021). This ability to produce BNIs is highlighted in the framework of sustainable agriculture based on the use of environmentally friendly agronomic practices to decrease pollution derived from the use of fertilizers (Subbarao et al. 2013a; Zhang et al. 2015). Therefore, the use of cover crops capable of producing BNIs represents another promising strategy to control nitrification and, thus, to increase the availability of N in the soil for the next crop while reducing N losses from the agrosystem (Karwat et al. 2019; Momesso et al. 2019). The ability to exude BNIs seems to be related to plants' adaptability to low N environments (Subbarao et al. 2015). Regarding field crops, unlike crops adapted to high N input environments such as wheat (*Triticum aestivum*) and maize (*Zea mays*), the strongest BNI capacity is found in sorghum (*Sorghum bicolor*) since it is adapted to low N environments (Subbarao et al. 2007). Sorghum roots release two categories of BNIs, hydrophilic and hydrophobic, which may have complementary roles. The hydrophobic BNIs may remain close to the root systems, as they are strongly absorbed by soil mineral or organic particles, which may further increase their persistence (Dayan et al. 2010; Subbarao et al. 2013b). In contrast, hydrophilic BNIs are more likely to move out of the rhizosphere, which may enhance their capacity to suppress nitrification in bulk soil (Nardi et al. 2013; Subbarao et al. 2013b). In addition, BNIs from sorghum can be released until close to the physiological maturity of the crop (Sarr et al. 2021), which would ensure the availability of N during all the stages of crop development.

Increasing efforts are taken to identify and characterize BNI molecules, but the regulation of their synthesis and release is still barely understood. In general, BNI activity depends on the presence and direct contact of NH_4^+ in the root environment (Subbarao et al. 2017; Afzal et al. 2020). Nevertheless, the influence of soil physicochemical factors like texture, composition, and pH on the release of BNIs by roots has not yet been sufficiently studied. Moreover, the effectiveness of BNIs in a climate change scenario such as the increase of environmental

temperature or water scarcity during plant growth remains to be evaluated. Therefore, the study of the impact of environmental conditions on the production of BNIs by crops is crucial to introduce this quality in agricultural operations such as crop rotation systems (Bozal-Leorri et al. 2023). Although drought periods happen naturally, it is estimated that their frequency and severity will increase in most of the world's crop-producing regions as a result of climate change (Hochholdinger 2016; Daryanto et al. 2017). Drought stress is the abiotic factor that most limits the productivity of agrosystems, reducing significantly crop growth and yield (Fleury et al. 2010). Since BNI release is related to the physiological state and development of the plant (Sarr et al. 2021), stresses that affect crop growth might also limit or modify the rate of BNI exudation. To date, only Ghatak et al. (2021) have studied the effects of abiotic stresses on the BNI root exudation and its composition in pearl millet plants (*Pennisetum glaucum*). Indeed, these authors found a genotype-dependent enhancement of BNI activity after a defined period of drought stress.

The sorghum ability to produce BNIs (Subbarao et al. 2013b), and, more importantly, the fact of being a drought-tolerant crop (Hadebe et al. 2017) lead us to hypothesize the BNI release in sorghum could be stimulated by soil water scarcity, which will benefit the following crop since sorghum has been postulated as a potential catch crop or cover crop (Bozal-Leorri et al. 2021a, 2023). Therefore, the aim of this work was to determine the impact of drought stress on BNI activity of sorghum plants, and the effects of exuded BNIs on soil mineral N content and nitrifying microorganisms.

Materials and methods

Field experiment

The field experiment was conducted in three different fields from Garinoain, Northern Spain (42° 35' N, 1° 40' W and 532 m above sea level) during three successive summer seasons 2017, 2018, and 2019. Soil characteristics of the upper horizon of the three fields are compiled in Supplementary Tables 1, and daily air mean temperature and accumulated precipitation of the three summer seasons are presented in Supplementary Fig. 1. Sorghum (*Sorghum bicolor* var. PR88P68 Pioneer Corteva Agriscience) was sown in no-till conditions at a rate of 15 kg seeds ha^{-1} in May of each year after the previous hairy vetch (*Vicia villosa Roth*) winter cover crop that was halted with glyphosate and left on the soil surface. The experiment consisted of two randomized blocks with three replications and two treatments (5 m x 5 m plots) in each block: (1) uncultivated soil after the termination of the precedent crop (Fallow) and (2) sorghum without fertilizer application (Sorghum). For fallow plots, sorghum plants were desiccated with RoundUp (a glyphosate-based herbicide; 36% w/v. Monsanto) one month after sorghum sowing, according to manufacture recommendations in no-till systems from this region, 1.5 L ha^{-1}. The use of glyphosate was chosen because application at higher rates than in this experiment has been reported to have no effect on nitrifying bacteria (Allegrini et al. 2017; Zabaloy et al. 2017), and so it was observed in previous works from our group (Bozal-Leorri et al. 2021a, 2023).

Greenhouse experiment

The second experiment was carried out in microcosms in a greenhouse under a daily regimen of 14/10 h day/night cycle with an average day/night temperature and relative humidity of 25/18°C and 50/60%, respectively. The soil was collected in June 2019, from a 0–30 cm layer of clay loam soil in a wheat field (Supplementary Table 1) in Arkaute (Alava, Spain; 42° 51' N, 2° 37' W and 530 m above sea level). Roots and stones were removed and the soil was passed through a 5 mm sieve. Soil was mixed with sand in 3:1 soil:sand (v:v) proportion to increase soil porosity. After this, it was air-dried, homogenised and kept at 4 °C until the start of the experiment. In order to reactivate soil microorganisms, pots were supplied with 86 mg of N in the form of ammonium sulphate ($(NH_4)_2SO_4$), an equivalent dose to 15 kg N ha^{-1}, and soil was rehydrated with deionised water up to 50% water filled pore space (WFPS). WFPS was calculated as in Linn and Doran (1984) following the equation:

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

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 1.31 Mg m^{-3}.

A trifactorial experimental design (presence/absence of sorghum plant, water regimen, and type of fertilization) with three replications was implemented in thirty-six 1.35 L pots. After soil activation, eighteen microcosms were planted with 4 seedlings of sorghum (*Sorghum bicolor* var. PR88P68 Pioneer Corteva Agriscience) per microcosm and the other eighteen microcosms were kept with only soil. Sorghum seeds were previously germinated on a tray with perlite:vermiculite (1:3) mixture at 20 °C for 6 days. All 36 microcosms were watered for 15 days after sorghum transplanting to maintain soil WFPS. On the 15th day of watering, two groups of 9 microcosms were established within each group of microcosms with soil and with soil and plant. One group held "Watered" regimen and the other group held the "Moderate drought" regimen. At the same time, each group was randomly divided into 3 groups of three microcosms corresponding to three different fertilizer treatments. The fertilizer treatments were: ammonium sulphate (A), ammonium sulphate combined with DMPP (A+D), and potassium nitrate (KNO_3^-). Nitrogen was applied in an equivalent dose to 195 kg N ha^{-1}, which was achieved by adding 1.726 g of potassium nitrate (δ ^{15} N value of 16.5) or 1.128 g of ammonium sulphate, alone or mixed with DMPP (δ ^{15} N value of -1.2 and −0.9 respectively) (EuroChem Agro Iberia S.L.); DMPP content represented 0.8% of the applied NH_4^+-N. In order to achieve a homogeneous distribution of nitrogen in the soil, fertilizers were dissolved in deionised water, ready to be added to the corresponding treatments by pipetting. After treatment application, "Watered" pots increased their WFPS up to 60%. On the other hand, "Moderate drought" pots decreased to 30% of WFPS by stopping the watering until they reached the calculated weight for that moisture level. All of them were watered every two days in order to maintain each WFPS for another 60 days.

Plant biomass and isotopic discrimination

Above plant biomass production in the microcosm experiment was measured at 60 days post-fertilization as dry weight (DW) from sorghum plants at vegetative stage 4 (Vanderlip 1993). To do so, one sorghum plant per microcosm was dried at 80 °C in a circulation oven for 72 h until a constant DW was reached.

For a deeper understanding of plant N source acquisition and drought effect, N and C isotopic composition in leaves of sorghum 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 (SAI), Universidade da Coruña. The absorption of different sources of N by the plant changes the δ ^{15} N values (Werner and Schmidt 2002). On the other hand, the C isotopic signature has been widely used as indicator of plant's water use efficiency and drought stress (Eggels et al. 2021). The values of the isotopic ratio were expressed as δ^{15}N and δ^{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:

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

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

Leaf determinations

Leaf gas-exchange parameters were conducted in the totally expanded upper leaf using a Li-COR 6400XP portable photosynthesis system (LI-COR Inc., Lincoln, Nebraska, USA). The rate of CO_2 assimilation (A_N), stomatal conductance (g_s), and intercellular CO_2 (Ci) parameters were determined under light-saturated conditions with a photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1} at 25 °C and with a CO_2 concentration of 400 ppm. The maximum quantum of PSII (Fv/Fm) was measured under light-adapted conditions in the centre of the youngest fully developed leaf with a mini-PAM (miniaturized pulse amplitude–modulated photosynthesis yield analyser).

Geochemical analysis

Soil NH_4^+ and NO_3^- contents were determined the day after sorghum harvest for field experiment while those of greenhouse experiment were made 30 days post-fertilization. Three soil subsamples of 3 cm diameter × 0.3 m depth for the field experiment and 1.5 cm diameter × 10 cm depth for the

microcosms experiment were taken. Plant debris and stones from soil subsamples were removed and finally, they were homogenized. Then 100 g were mixed with 200 mL 1 M KCl and shaken for one hour at 165 rpm. The soil solution was filtered twice through Whatman n°1 filter papers (GE Healthcare) and Sep-Pak Classic C18 Cartridges 125 Å pore size (Waters) to remove big soil particles and organic matter, respectively. 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).

Abundance of nitrifying bacteria in soil

Quantitative polymerase chain reaction (qPCR) was used to quantify the abundance of nitrifying genes. Soil DNA was isolated from the same samples used for geochemical determinations (at the end of the sorghum crop for the field experiment, and 30 days post-fertilization for greenhouse experiment). 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). Quantification of total bacteria abundance (*16 S rRNA*) and functional marker genes involved in nitrification (bacterial *amoA*) were amplified by qPCR using SYBR® Premix Ex TaqTM II (Takara-Bio Inc.) and gene-specific primers in a StepOne PlusTM Real-Time PCR System (Torralbo et al. 2017). 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 10^2 gene copies μL^{-1}. The *amoA* relative abundance was calculated following this equation:

(*amoA* absolute abundance/16*S rRNA* absolute abundance) × 100

where the absolute abundance of the *16 S rRNA* and *amoA* genes (copy number of target gene per gram of dry soil) were 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.

Statistical analysis

Data was analysed with IBM SPSS v. 24.0 statistical software (IBM Corp. Armonk, NY, USA). Comparisons of two soil conditions (fallow and sorghum crop) from the field experiment were made using the Mann-Whitney U test. Leaf gas-exchange and Fv/Fm parameters were subject to a two-way (water regimen, W; and fertilizer treatment, F) analysis of variance. The results of soil mineral N, microbial quantification, aboveground biomass, and leaf $\delta^{15}N$ and $\delta^{13}C$ were analysed by one-way ANOVA using Duncan's multiple range test for separation of means between treatments. The Mann–Whitney U test was used to compare the effect of the absence or presence of sorghum plant within the same treatment. p-values < 0.05 were considered to be statistically significant differences.

Results

Three-year comparison of fallow and sorghum field

Cumulative precipitations during the early development of the sorghum crop (May to July) were similar (Supplementary Fig. 1), but the rain events from July to the end of the crop made a great difference between the three years. The *amoA* relative abundance was reduced by 50% in the soil with sorghum crop compared to soil with fallow in 2017 (Fig. 1a). The accumulated precipitation at the end of the crop was 120 L m^{-2} since it was a dry summer compared to years 2018 and 2019, with 240 and 200 L m^{-2}, respectively (Fig. 1a). In turn, the growth of nitrifying bacteria during these two years was not statistically affected by the presence of sorghum in relation to fallow soil. However, even though it was not statistically significant, the *amoA* relative abundance in the sorghum crop was reduced a 16% regarding the fallow in the year 2019. In addition, the presence of the sorghum crop reduced significantly soil N content compared to fallow in all three years (Fig. 1b). Although no differences were observed in soil NH_4^+ content between soil with fallow and soil that held sorghum (Fig. 1b), the effect of sorghum presence did reduce soil

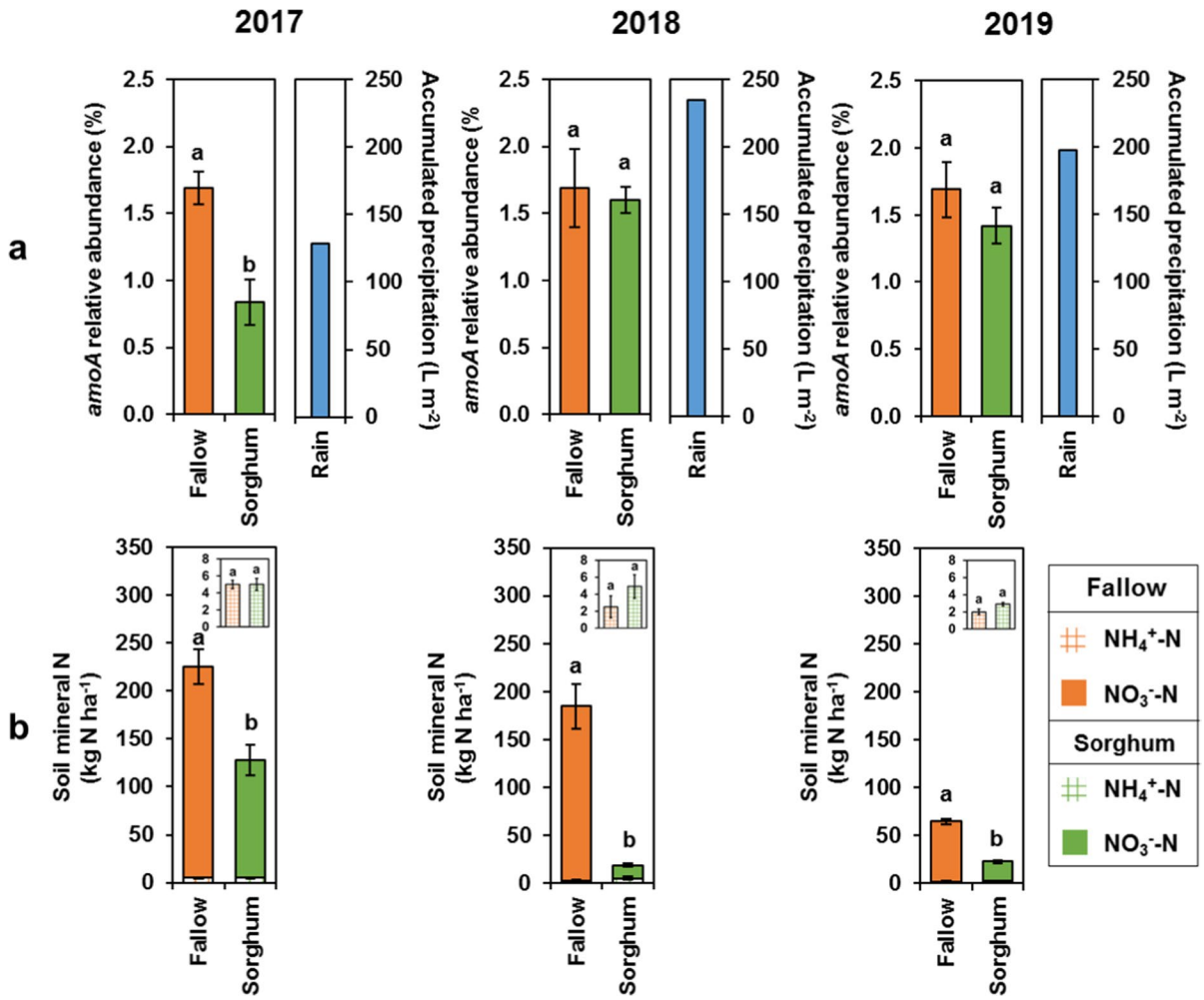


Fig. 1 Ammonia-oxidizing bacteria (AOB) relative abundance (measured as the relative abundance of gene *amoA*) at the end of sorghum crop and accumulated precipitation during sorghum development (**a**) and soil mineral nitrogen at the end of sorghum crop (**b**) during three years of campaign. The Mann-Whitney U test was used for the comparison between fallow and sorghum plots. Significant differences at $p < 0.05$ are marked with a letter

NO_3^- content by more than 40%, 90% and 68% in soil that held sorghum compared to fallow soil (Fig. 1b) in years 2017, 2018 and 2019, respectively. The lower soil NO_3^- content from the soil with fallow detected in year 2019 compared to the previous years could be due to a lower soil organic matter (Supplementary Table 1).

Effect of moderate drought conditions

Sorghum plants reached the highest aboveground biomass under KNO_3^- treatment from Watered regimen, while A and A+D treatment presented similar aboveground biomass (Fig. 2a). Fertilizer treatment had no effect on aboveground biomass under Moderate drought regimen. Nevertheless, the water regimens affected the sorghum plants growth since plants under Watered regimen presented 2–5 times the aboveground biomass than those of Moderate Drought regimen. The application of fertilizers, as well as the water regimens, affected the leaf δ^{15} N values. Sorghum plants fertilized with NO_3^- presented the highest δ^{15} N, whereas A+D treatment had the lowest, regardless of the water availability (Fig. 2b). However, A treatment showed lower

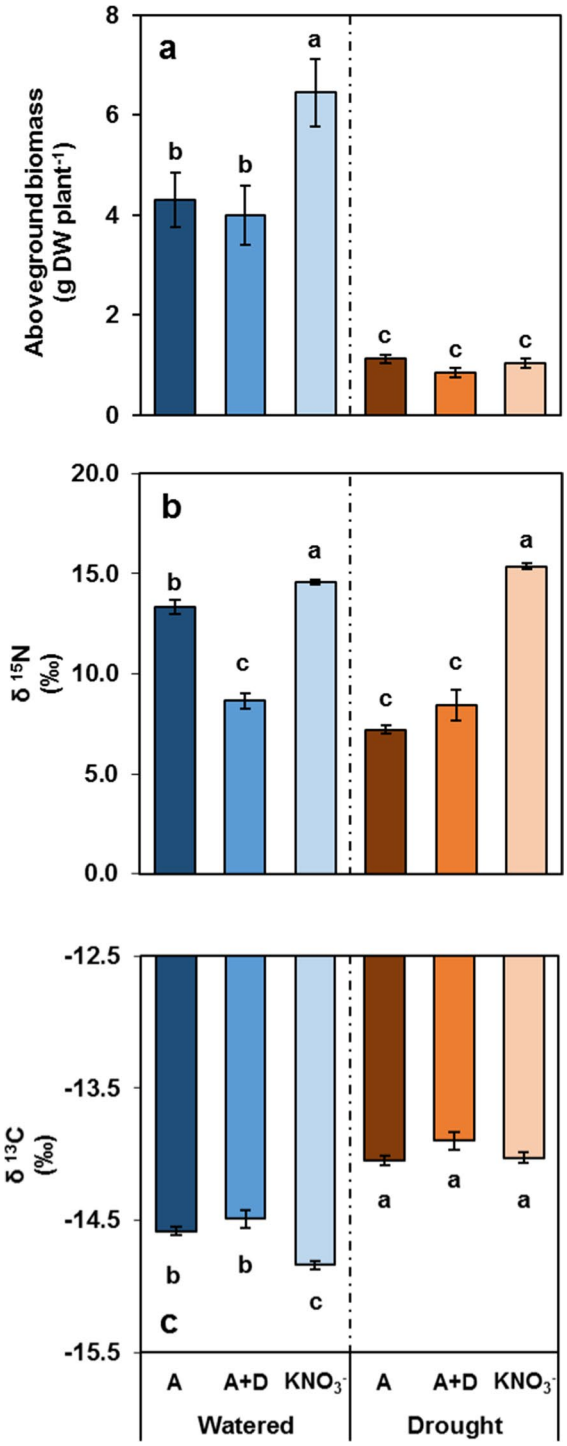
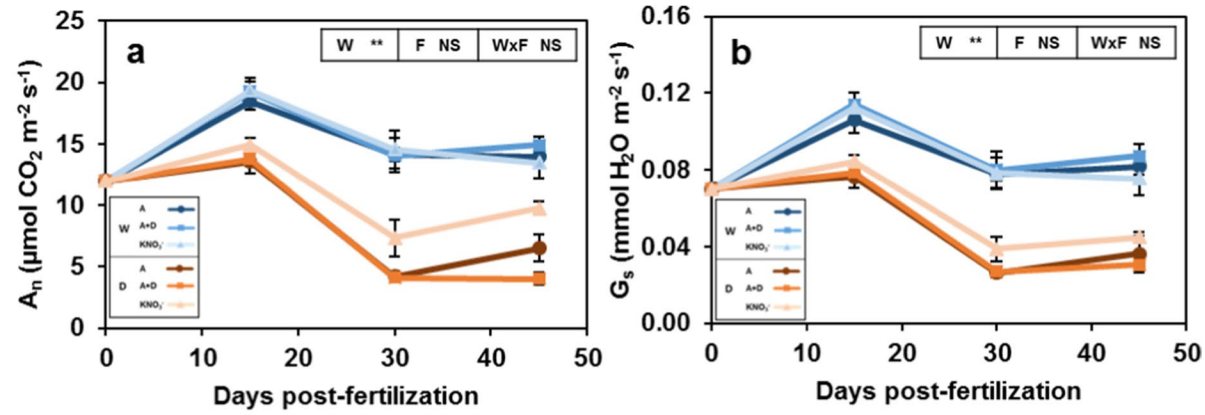


Fig. 2 Dry aboveground biomass of sorghum (*Sorghum bicolor*) plants (**a**) and leaf determination of $\delta^{15}N$ (**b**) and $\delta^{13}C$ (**c**). Pots were fertilized with ammonium sulphate (A); ammonium sulphate + DMPP (A+D) and potassium nitrate (KNO_3^-). Significant differences between treatments are marked with a lowercase letter (Duncan Test; $p < 0.05$; $n = 3$)

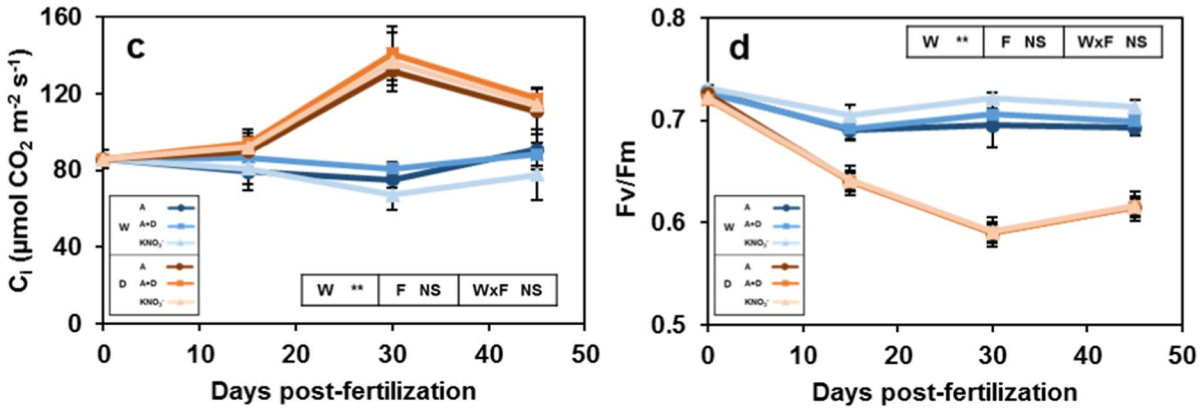
leaf δ^{15} N values in Moderate drought regimen than in Watered regimen. Under Watered regimen, KNO_3^- treatment showed the most negative leaf δ^{13} C values, whilst A and A+D treatment had similar values (Fig. 2c). No effect of fertilization was found on leaf δ^{13} C values of Moderate Drought regimen, but this regimen presented less negative leaf δ^{13} C values than Watered regimen. Fertilizer application had not a very strong effect on leaf gas exchange parameters in either Watered regimen or Moderate drought regimen (Fig. 3). Nevertheless, strong differences were found comparing both watered regimens. The net photosynthetic rate decreased by an average of 47% on treatments from Moderate drought regimen in respect to Watered regimen (Fig. 3a). Similarly, the stomatal conductance of treatments from Moderate drought regimen was 48% lower compared to those of Watered regimen (Fig. 3b). On the contrary, the intercellular CO_2 increased in treatments from Moderate drought regimen, being 43% higher compared to Watered regimen (Fig. 3c). Since photosynthesis was decreased in Moderate drought regimen, akin results were found in the maximum quantum of PSII. Although fertilizer application did not affect Fv/Fm, Moderate drought regimen reduced it by 12% compared to Watered regimen (Fig. 3d).

In microcosms of soil without plant, the NH_4^+ added in A treatment was not kept in the soil and presented similar soil NH_4^+ content than KNO_3^- treatment, whereas the greatest soil NH_4^+ content was found in A+D treatment (Fig. 4a). In the absence of plant, soil NH_4^+ content was not affected by the water regimen. In microcosms of soil with plant, A+D treatment also showed the highest soil NH_4^+ content, and soil NH_4^+ content of A treatment from Watered regimen was similar to that of KNO_3^- treatment. However, under Moderate drought regimen, A treatment presented greater soil NH_4^+ content than KNO_3^- treatment. In addition, A and A+D treatments from Moderate drought regimen kept higher soil NH_4^+ content than Watered regimen when plants were present. Regarding soil NO_3^- content, KNO_3^- treatment presented the highest values in pots of soil without plant (Fig. 4b). The addition of the synthetic nitrification inhibitor reduced the generation of NO_3^-, showing the lowest soil NO_3^- content. As with the soil NH_4^+ content, the water regimen did not affect the soil NO_3^- content when the plant is absent. On the other hand, when the plant was present, KNO_3^- treatment also showed the highest soil



	0 DPF		15 DPF	30 DPF	45 DPF
Watered	a	A	a	a	a
		A+D	a	a	a
		KNO_3^-	a	a	a
Drought	a	A	b	c	d
		A+D	b	c	c
		KNO_3^-	b	b	b

	0 DPF		15 DPF	30 DPF	45 DPF
Watered	a	A	a	a	a
		A+D	a	a	a
		KNO_3^-	a	a	a
Drought	a	A	b	b	b
		A+D	b	b	b
		KNO_3^-	b	b	b



	0 DPF		15 DPF	30 DPF	45 DPF
Watered	a	A	a	b	ab
		A+D	a	b	b
		KNO_3^-	a	b	b
Drought	a	A	a	a	ab
		A+D	a	a	a
		KNO_3^-	a	a	a

	0 DPF		15 DPF	30 DPF	45 DPF
Watered	a	A	a	a	a
		A+D	a	a	a
		KNO_3^-	a	a	a
Drought	a	A	b	b	b
		A+D	b	b	b
		KNO_3^-	b	b	b

Fig. 3 Net photosynthetic rate (**a**), stomatal conductance (**b**), intercellular CO_2 mole fraction (**c**) and maximum quantum of PSII (**d**) of sorghum leaves during 45 days post-fertilization. Pots were fertilized with ammonium sulphate (A); ammonium sulphate + DMPP (A+D) and potassium nitrate (KNO_3^-). Statistical analysis was made through the Mann-Whitney U test ($p < 0.05$; $n = 3$) for the comparison between watered and Moderate drought regimens of each parameter at 0 day post-fertilization (DPF); ANOVA ($p < 0.05$; $n = 3$) for the comparison of all treatments from both water regimens at 15, 30, and 45 DPF; and to analyse the effect of water regimen (W), fertilizer treatment (F) and their interaction (WxF) was made through analysis of variance (two-way ANOVA; significant differences are marked with an asterisk [*] when $p < 0.05$ and double asterisk [**] when $p < 0.01$)

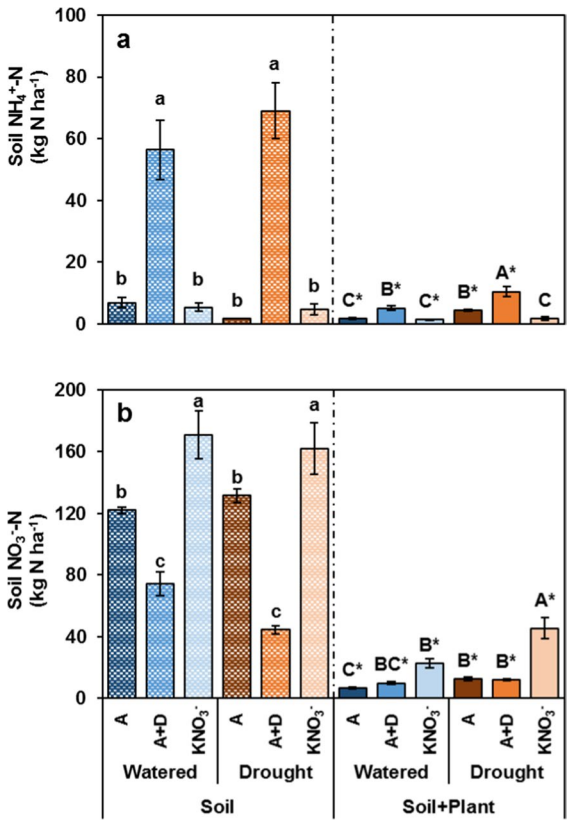


Fig. 4 Soil mineral nitrogen in form of NH_4^+ (**a**) and NO_3^- (**b**) on pots with soil and pots with soil and plant at 30 days post-fertilization. Pots were fertilized with ammonium sulphate (A); ammonium sulphate + DMPP (A+D) and potassium nitrate (KNO_3^-). Significant differences between treatments from pots with soil are marked with a lowercase letter. Significant differences between treatments from pots with soil and plant are marked with a capital letter. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 3$). The Mann-Whitney U test was used for the comparison between the absence or presence of plant within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*) only in the "Soil + Plant" treatment

NO_3^- content. Nevertheless, A and A+D treatments did not present any differences in soil NO_3^- content. In this case, the water regimen affected the soil NO_3^- content in A and KNO_3^- treatment, where Moderate drought regimen showed higher values than those of Watered regimen. The presence of the plant greatly modified the soil mineral N since the lowest values of soil NH_4^+ content and soil NO_3^- content were found in the microcosm of soil with plant.

The total bacteria abundance (measured as the abundance of *16 S rRNA* gene) was affected neither by the fertilizer treatment nor by the water regimen in the microcosms of soil without plant (Supplementary Fig. 2a). However, the presence of plants altered the total bacteria abundance. Under Watered regimen, A treatment showed the highest *16 S rRNA* gene abundance; while in Moderate drought regimen, A and A+D treatments were the ones with higher values. Water regimen also had no effect on the total bacteria abundance in microcosms of soil with plants. Nevertheless, A treatment from microcosms of soil with plant from both water regimens presented higher *16 S rRNA* gene abundance than A treatment from microcosms of soil without plant. Fertilization with NH_4^+ highly increased the *amoA* gene abundance in both microcosms of soil without plant and soil with plant (Supplementary Fig. 2b). However, the observed changes in the total abundance of bacteria could mask the real power of sorghum inhibition; thus, the relative abundance of *amoA* gene is presented (Fig. 5). The application of DMPP in the microcosms of soil diminished the *amoA* relative abundance up to levels of KNO_3^- treatment, which means a 78% and 81% reduction compared to A treatment in Watered and Moderate drought regimens, respectively. In microcosms of soil with plant, A treatment had the highest *amoA* relative abundance, but its abundance was 25% lower under Moderate drought compared to Watered regimen. A+D treatment decreased nitrifying-gene abundance by 85% compared to A treatment in Watered regimen, whereas the reduction in Moderate drought regimen was 64%. The presence of sorghum plant affected *amoA* relative abundance of A treatment from both water regimens. While the *amoA* relative abundance of A treatment was reduced by 25% in watered regimen when sorghum plants were present in the soil compared to when they were not, it was also decreased by 59% in Moderate drought regimen.

Discussion

One of the possibilities to achieve suitable use of nitrogen (N) could be a proper crop rotation (Macdonald et al. 2005; Moreau et al. 2012). The heat tolerance and drought resistance that sorghum presents (Smith and Frederiksen 2000; Hadebe et al. 2017) make it a good option as a catch crop for the summer season in a crop rotation. The use of this kind of crop absorbs the exceeding soil N from precedent crop, reducing the N

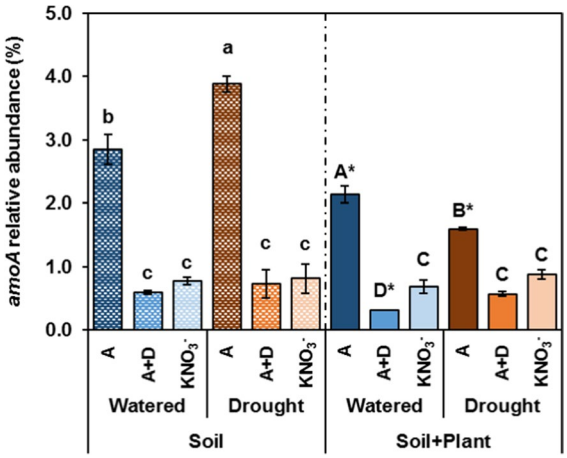


Fig. 5 Ammonia oxidizing bacteria (AOB) relative abundance (measured as the relative abundance of gene *amoA*) at 30 days post-fertilization. Pots were fertilized with ammonium sulphate (A); ammonium sulphate + DMPP (A+D) and potassium nitrate (KNO_3^-). Significant differences between treatments from pots with soil are marked with a lowercase letter. Significant differences between treatments from pots with soil and plant are marked with a capital letter. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 3$). The Mann-Whitney U test was used for the comparison between the absence or presence of plant within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*) only in the "Soil + Plant" treatment

losses through NO_3^- leaching (Askegaard et al. 2005; Berntsen et al. 2006). This is in line with our results since in the three years of experiment, soil NO_3^- content from soil that held sorghum presented a decrease between 44% and 92% compared to soil with fallow (Fig. 1b). In this way, the use of sorghum reduces the amount of NO_3^- that can be leached during heavy rain events from the autumn-winter period (Gabriel et al. 2012), which happens before the following wheat crop develops a high N uptake capacity. Moreover, the substrate for denitrifying bacteria that usually produces greenhouse gases, such as N_2O (Smith et al. 2008), is also reduced. On the other hand, sorghum is also an interesting crop due to its allelopathy. Although firstly was used as a weed control alternative (Purvis et al. 1985), now it is studied for its potential BNI role. The inhibition mediated by BNIs is time dependant, but also highly conditioned by the environment, and hence the nitrification inhibition under field conditions was only detected during the first year of our experiment (Fig. 1a). The field experiment took place in three close locations with similar physicochemical soil properties (Supplementary Table 1) as it can be noticed by the lack of effect over the relative *amoA* abundance from the soil with fallow among the three years. Therefore, soil properties cannot be the reason why nitrification was not reduced in soil with sorghum. In addition, BNI exudation is dependent on the physiological state and development of the plant (Sarr et al. 2021), but also on the soil water status and temperature. Thus, both biotic and abiotic stresses that affect crop growth or nutrient uptake might modify the rate of BNI exudation. The growing period of the year 2017 was drier compared to the years 2018 and 2019 (Fig. 1a), ensuring an aerobic soil condition that favour nitrification. However, the *amoA* relative gene abundance of soil that held sorghum presented a reduction of 50% compared to fallow soil. On the contrary, the years 2018 and 2019 were characterized by higher soil moisture and reduced soil air pores that might reduce nitrification processes (Menéndez et al. 2012; Barrena et al. 2017; Torralbo et al. 2017). Although sorghum was not able to significantly inhibit soil nitrification under these conditions, soil nitrification was reduced by 16% in the year 2019 compared to soil with fallow, when accumulated precipitation was average compared to the previous two years. Therefore, BNI activity in sorghum plants may be related to water availability, increasing the BNI capacity when sorghum is under drought stress. Hence, more experiments at different times during the sorghum and the following crop periods should be carried out to confirm this hypothesis, since sorghum could be a good candidate as a catch crop by reducing soil NO_3^- content and affecting the nitrifying populations for the following crop.

Several studies have addressed the effects of drought stress on sorghum plants (Saini and Westgate 1999; Hadebe et al. 2017; Prasad et al. 2021). However, there is not much knowledge of how stress affects the regulation of metabolites present in root exudates, such as BNIs. To our knowledge, only Ghatak et al. (2021) studied the BNI root exudation and its composition of pearl millet plants (*Pennisetum glaucum* L.) under different drought stresses. These authors found a genotype-dependent enhancement of BNI activity after a defined period of drought stress. In climate change scenarios where aridity is increasing in several areas of the globe (Greve et al. 2019), it can be of importance to investigate the effects of drought stress on the BNI exudation capacity from other types of plants. Because of the environmental conditions seemed to condition BNI release, sorghum plants were

exposed to different water stress under controlled conditions, which allowed us to investigate in more detail the effects on potential BNI exudation by means of soil nitrification inhibition. As expected, sorghum aboveground biomass was greatly reduced under moderate drought conditions (Fig. 2a) indicating that dry matter accumulation, which is the result of photosynthesis and nutrient uptake from the soil, was seriously affected (Hasan et al. 2017). Regardless of N fertilization, sorghum plants from Moderate drought regimen showed a 47% reduction in net photosynthesis as a consequence of a low stomata conductance. During the early stages of drought stress (10 days post-fertilization), stomata regulated the amount of water transpired while maintained similar net photosynthetic rate, g_s and Ci that before the imposition of the water deprivation. However, both net photosynthetic rate and g_s decreased but Ci gradually increased as long as drought stress progressed, which may indicate other factors rather than stomatal closure might be the main responsible of the reduction in photosynthetic rate. Similar results have been observed in naked oat (Zhang et al. 2022). In addition, the reduction of net CO_2 assimilation, but the increase of Ci might indicate an imbalance in the photochemical activity of PSII, leading to an overexcitation and subsequent photoinhibitory damage of the PSII reaction centre (Kaiser 1987; Meng et al. 2016). This coincides with our results where Moderate drought stress made 12% lower the sorghum leaves Fv/Fm from all fertilized treatments compared to those of Watered regimen (Fig. 3d). Although non-stomatal processes such us damages in chloroplast might have an impact on decreasing photosynthetic rates under drought stress, the main cause of reduced photosynthesis under drought stress in C4 plants is generally a stomatal closure (Chaves et al. 2011), which present a close correlation between stomatal conductance and photosynthetic rate in sweet sorghum plants under drought conditions (Tingting et al. 2010). In line with these results, we also found that sorghum plants from Moderate drought regimen presented a 48% reduction in stomatal conductance (Fig. 3b). Furthermore, the C isotopic signature has been frequently used as an indicator for water use and drought stress of plants (Eggels et al. 2021). Under water deficit conditions, plants do not discriminate against the uptake of the heavier C isotope (^{13}C) due to closing stomata, leading to an enrichment of ^{13}C in biomass that increases the δ^{13}C value (Farquhar et al. 1982, 1989). Our results from the microcosm experiment are in line with that since sorghum plants from Moderate drought regimen showed higher δ^{13}C values compared to those of Watered regimen (Fig. 2c).

The inhibition of nitrification withholds the oxidation of NH_4^+ into NO_3^- in the soil (Clough et al. 2020). For this reason, microcosms of soil with plant from Moderate drought presented higher soil NH_4^+ contents (Fig. 4a) and lower *amoA* relative abundance (Fig. 5) than that of Watered regimen, which implies a possible signal of inhibited nitrification through BNIs (Subbarao et al. 2017). However, higher soil NH_4^+ and NO_3^- content can be noticed in all treatments from Moderate drought regimen compared to Watered regimen (Fig. 4) accompanied by a reduction in dry biomass (Fig. 2a). Therefore, the difference in soil NH_4^+ content may not be fully attributed to nitrification inhibition since plants under drought stress might reduce water and nutrient uptake that show low plant N content (Ogbaga et al. 2016; Sun et al. 2020). To further characterize plant N acquisition N isotopic composition from sorghum leaves was determined. Since the plant δ^{15}N value tend to be similar to that of the acquired N source, changes in δ^{15}N values could indicate the type of N source that the plant had during sorghum plants from this treatment its development (Werner and Schmidt 2002). Plants under ammonium nutrition often present low δ^{15}N values (Ariz et al. 2011). It is known that drought produce changes in the soil pH (Wang et al. 2021) and this, in turn, modifies the NH_3/NH_4^+ balance, which could affect the plant δ^{15}N. However, Ariz et al. (2018) described a depletion of δ^{15}N values when NH_4^+ was the N source in a wide range of external pH, indicating its independence of NH_3 formation in solution. Then, any change in NH_3/NH_4^+ balance should not affect the plant to present low values of leaf δ^{15}N when NH_4^+ is the source of N. Nonetheless, the δ^{15}N values observed in A treatment from Watered regimen, which were not so distant from those of KNO_3^- treatment (Fig. 2b), could suggest that the main source of N while plants were growing was the NO_3^- formed during nitrification since this process enriches the soil δ^{15}N (Delwiche and Stein 1970; Herman and Rundel 1989; Jones and Dalal 2017). Nevertheless, A treatment from Moderate drought regimen showed similar δ^{15}N values to A+D treatment, indicating that sorghum plants from this treatment may had ammonium nutrition rather than nitric nutrition. This preference for NH_4^+ uptake could be a result of higher BNI activity and a reduction of nitrifiers (Fig. 5). In this sense, low leaf δ^{15}N values are also reported as an indicator of BNI activity and reduced soil microbial nitrification (Jones and Dalal 2017; Karwat

et al. 2018). The application of the synthetic nitrification inhibitor maintained the *amoA* relative abundance at the level of KNO_3^- treatment where no NH_4^+ was added (Fig. 5). These results are in line with other studies where DMPP showed great inhibition of nitrifying population (Barrena et al. 2017; Torralbo et al. 2017; Bozal-Leorri et al. 2021b; Corrochano-Monsalve et al. 2021a). On the other hand, nitrifiers from the A treatment experienced a huge increase after the application of NH_4^+-based fertilizer, especially in microcosms of soil without plant from Moderate drought regimen (Fig. 5). The low WFPS from Moderate drought regimen was a more aerobic environment where nitrifiers could carry on the nitrification process (Arp and Stein 2003). Nevertheless, the *amoA* relative abundance of A treatment from Moderate drought regimen was reduced by 25% compared to Watered treatment when sorghum plants were present and by 59% compared to A treatment of pots of soil without plant from Moderate drought regimen (Fig. 5). It is generally accepted that the uptake of nutrients by crop plants is reduced in dry-soil conditions (Ogbaga et al. 2016), but plants may also increase the production of root exudates in order to increase chelation and uptake of nutrients (Henry et al. 2007). The increase of root exudates is an extra C supply that can be used by heterotrophic microorganisms, to increase its abundance (Surey et al. 2020). This matches with the increased total bacterial abundance found in A treatment from Moderate drought regimen (Supplementary Fig. 2a). However, nitrifying microorganisms were decreased (Fig. 5). Based on our results, we suggest that the exudation of BNI molecules is also raised within the increment of root exudations, indicating that sorghum has the potential to increase BNI release under limited water conditions to ensure high soil NH_4^+-N pool for crop uptake due to a reduction of nitrifying microorganisms.

Conclusion

Sorghum crop could be a good option as a catch crop to reduce potential N losses through NO_3^- leaching because the presence of sorghum reduced the soil NO_3^- content between 44% and 92% compared to fallow soils. Furthermore, the presence of sorghum reduced the *amoA* relative gene abundance by 50% presumably due to its BNI exudation ability. However, BNI activity in sorghum plants may be related to water availability since they only reduced the *amoA* relative abundance during the driest year. Under moderate drought conditions, sorghum plants presented a great reduction of aboveground biomass together with reduced photosynthetic rate, stomatal conductance, Fv/Fm, and less negative δ^{13} C values. In this way, lower δ^{15} N values detected in A treatment from Moderate drought regimen than those of Watered regimen indicated that sorghum plants from this treatment had more ammonium nutrition. Finally, sorghum plants had the potential to reduce *amoA* gene relative abundance by 25% under Moderate drought regimen compared to Watered treatment and by 59% regarding microcosms without plants. Moderate drought conditions under controlled conditions confirmed the observed reduction of *amoA* relative abundance in the year 2017, which presumably might have happened through the increase of root exudates like BNIs caused by lower water availability.

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Author contributions ABL conducted the experiments and was the main contributor in the data processing and interpretation, also writing the manuscript. LMA prepared the experimental field site, sowed the sorghum crop and participated in formal analysis, writing, reviewing and editing. FT participated in the field experiment and in formal analysis, writing, reviewing and editing. MBGM reviewed the discussion section after journal's revision. PAT and CGM supervised all phases of data analysis and interpretation and reviewed the entire manuscript. All authors read and approved the final manuscript.

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Data availability Raw data used to generate the presented results are available from the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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