Universidad del País Vasco
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Nitrification control in the framework of sustainable agriculture: the use of synthetic and biological nitrification inhibitors with ammonium-based fertilizers

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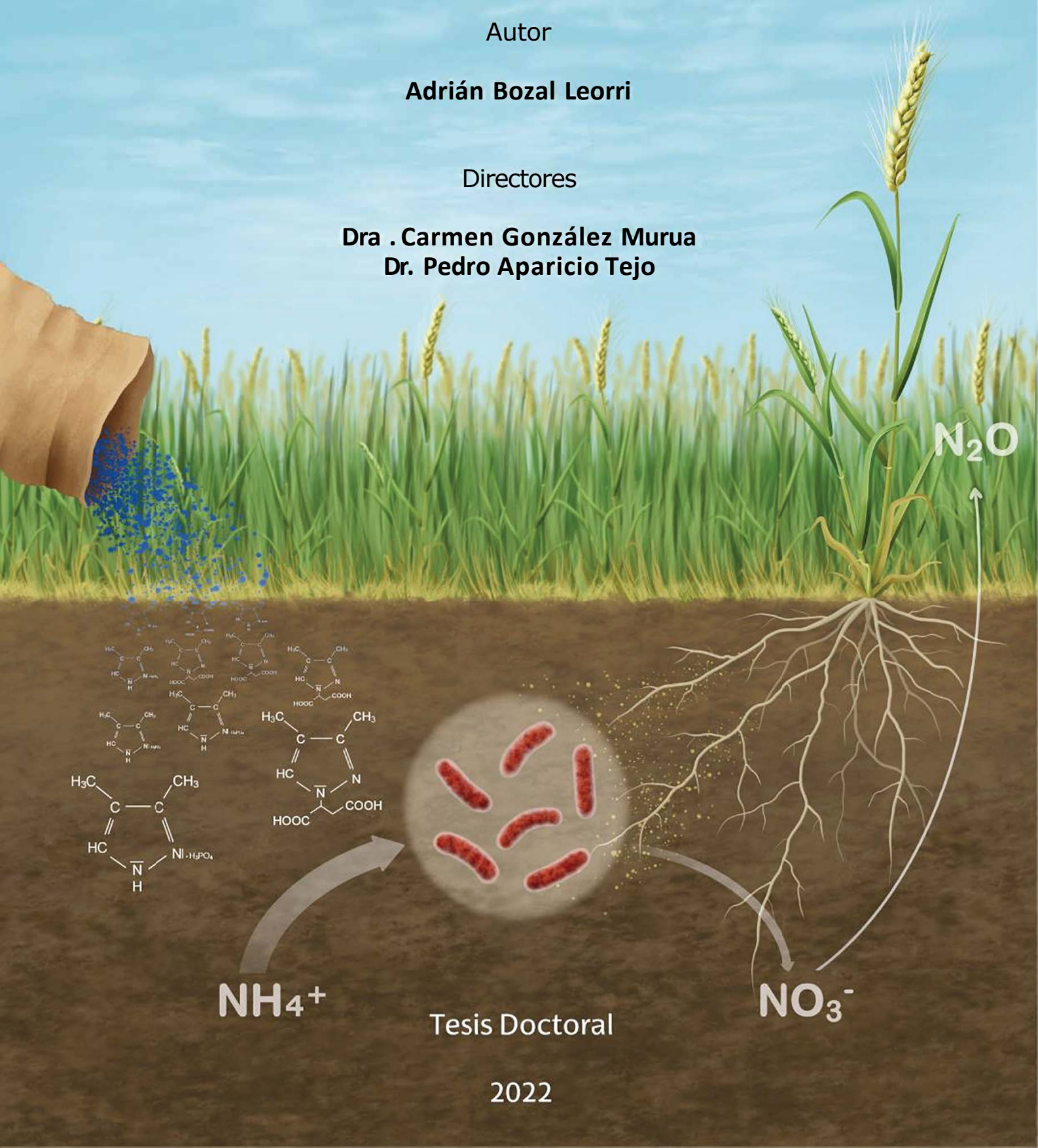


Tesis Doctoral

2022

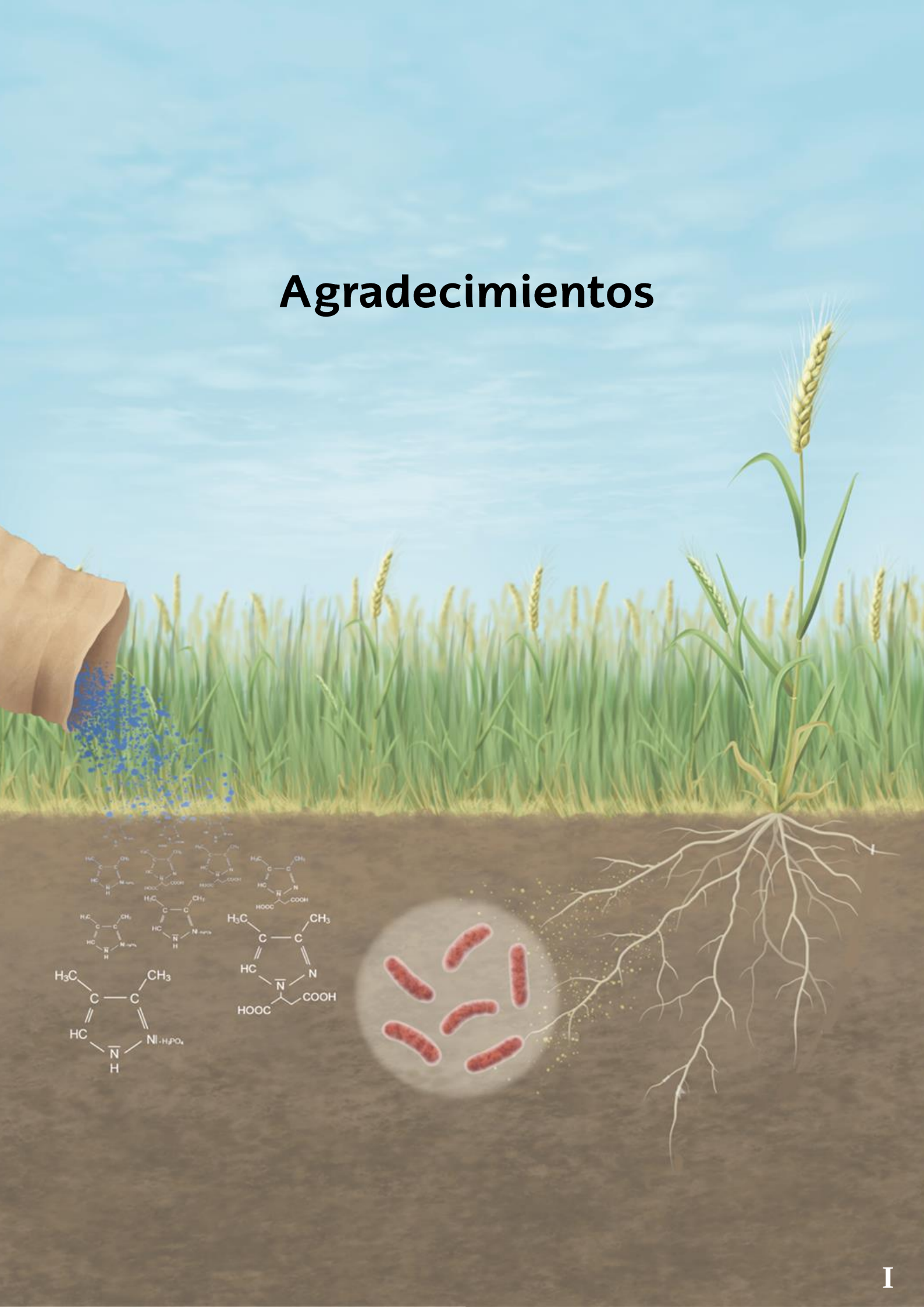
No entres dócilmente en esa buena noche.
La vejez debería delirar y arder cuando se acaba el día.
Rabia, rabia, contra la luz que se esconde.
Dylan Thomas (In Country Sleep, And Other Poems)

No hay nada de noble en ser superior a los demás.
La verdadera nobleza reside en ser mejor que nuestro yo anterior.
Ernest Hemingway

Tomorrow we'll discover
What our God in heaven has in store
One more dawn
One day more!
Claude-Michel Schönberg (Les Misérables)

A mis padres

Agradecimientos



El comienzo de este apartado está dirigido al Adrián del futuro. Si estás leyendo esto en un ejemplar impreso con las firmas del tribunal en él, significa que has sobrevivido a la "aventura" que es hacer un doctorado. ¡Enhorabuena!

Ahora, dejando conversaciones con uno mismo aparte, estoy feliz de poder llegar a escribir esta parte de mi tesis. Aunque no me creáis, es en lo que más he pensado a pesar de que es lo último que estoy escribiendo. Hace ya 10 años que empezó mi etapa universitaria y el destino ha querido que pueda darla por finalizada en una cifra tan redonda. Sí, sé que soy el único que le da importancia a estas cosas, pero me encantan. Han cambiado muchas cosas en todo este tiempo, pero donde más me he visto cambiar y madurar ha sido en los últimos 4 años. Sabía que para meterme en el doctorado había que estar preparado física y mentalmente, pero ¡guau! vaya aventura. He aprendido muchísimo en este capítulo de mi vida, tanto a nivel laboral como personal, y puedo decir que estoy orgulloso del proceso y del resultado. No obstante, siempre me gusta ser realista y sé que este gran trabajo no ha sido solo cosa mía. Soy consciente de la suerte que he tenido y de las personas que han estado a mi lado para que esto fuera posible. Por todo ello, me gustaría agradecer a las entidades, virus y personas que han contribuido a que pudiera realizar mi tesis doctoral.

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No quiero olvidarme del tema del siglo, el COVID19. Gracias a la persona que se comió el murciélago/dejó escapar el virus por la pandemia que ha asolado el planeta a mitad de mi doctorado. Me sirvió para hacer el parón necesario para empezar a analizar los datos de mis experimentos y dar lugar a mi primera publicación.

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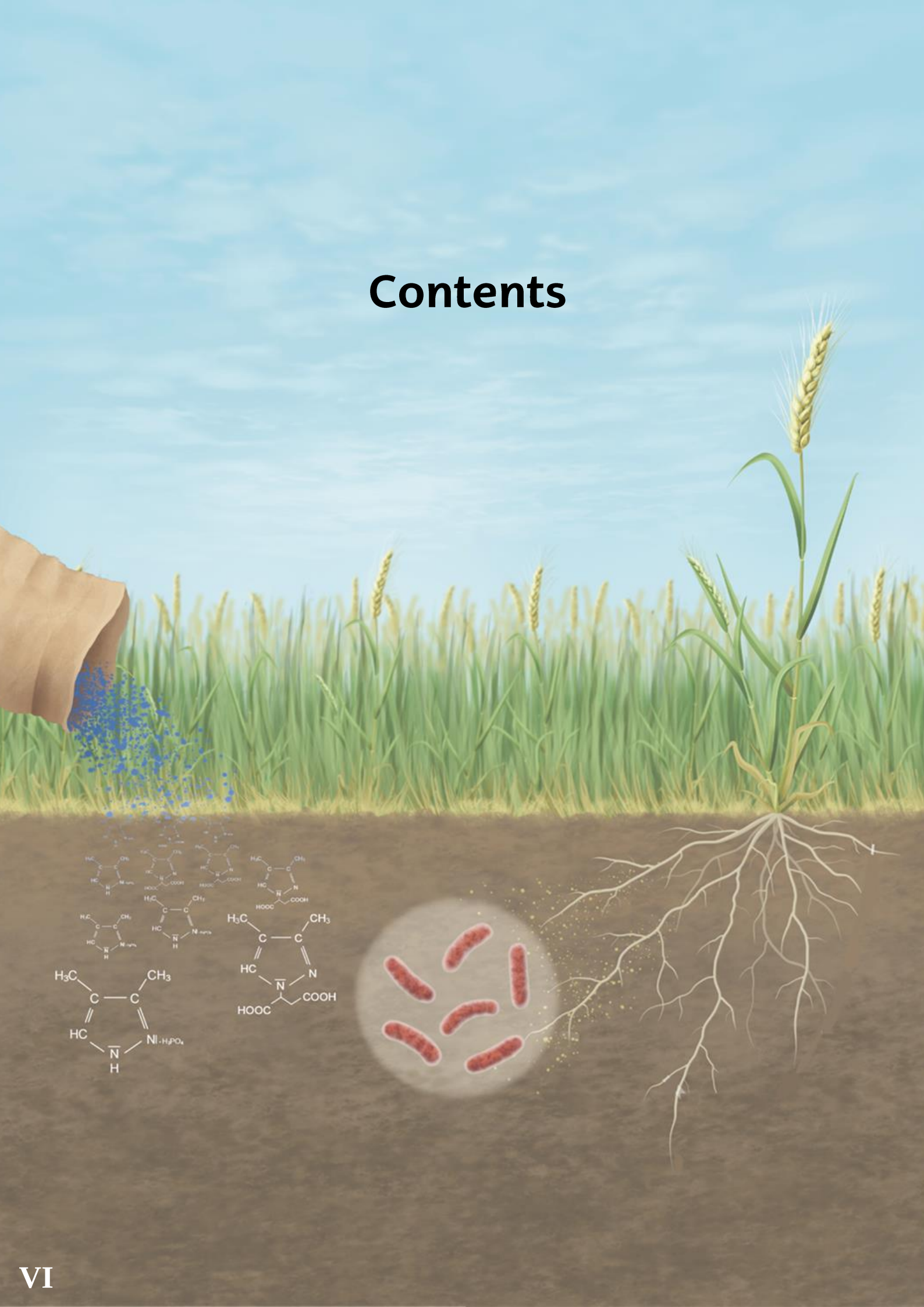
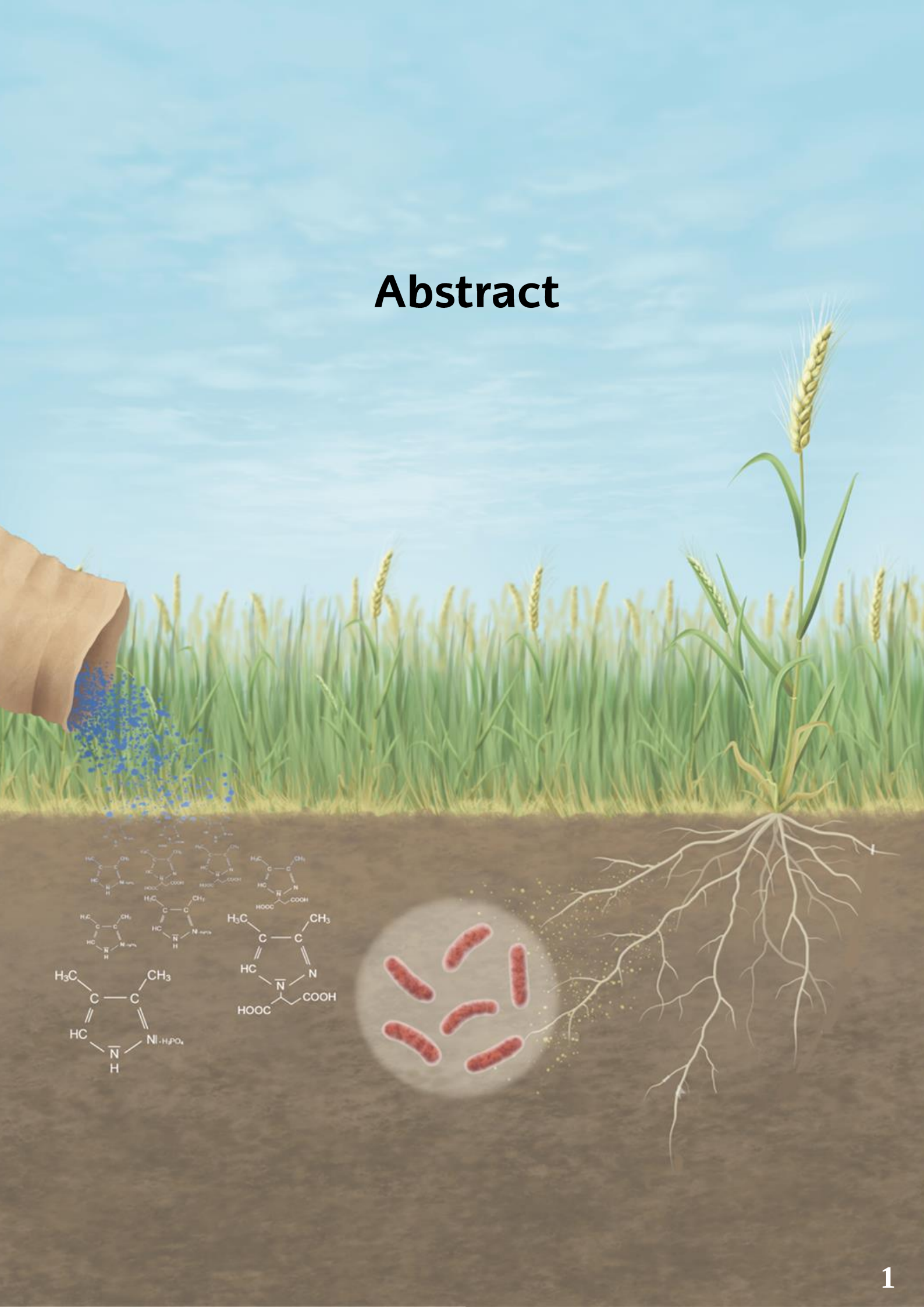
Para terminar, quisiera destinar las últimas líneas de este largo apartado a lo más importante para mí, mi familia. Me gustaría dedicar con cariño esta tesis a mis abuelos, Manuel, Julia, Jesús y Mari. Espero haberos hecho sentir orgullo tanto a los que estáis ahí arriba como a los que estáis aquí conmigo. A la mejor y más "petarda" hermana, Noemí, que a su manera también me ha apoyado mucho durante estos 4 años y comparte todas mis alegrías como si fueran suyas. Que sepas que yo también puedo decir con orgullo que soy tu hermano. A Sandra, mi compañera de vida. Gracias por todo el apoyo que me has dado, toda tu ayuda, por empujarme en mis momentos de bajón, por levantarme la moral, por distraerme con tantas cosas nuevas y por decirme lo que necesito oír en cada momento. A pesar de conocernos a mitad del doctorado, has sido un pilar fundamental para que haya podido llegar hasta aquí. Eres la única persona de este mundo con quien deseo compartir todas las nuevas aventuras que me depara la vida. Te quiero. Finalmente, quiero dedicar esta tesis a las personas más importantes de mi vida, mis padres Manuel y Mariaje. Todo lo que soy hoy, os lo debo a vosotros. Cada esfuerzo y sacrificio que habéis hecho por mí, ha sido un escalón más que me ha facilitado alcanzar el éxito en todo lo que me proponía. Hoy consigo el nivel más alto de educación que puede recibir una persona y yo solo puedo pensar "mirad qué lejos me habéis hecho llegar". Gracias por todo vuestro apoyo y por ser unos padres excepcionales. Sois mi mejor modelo a seguir. Espero poder estar a vuestra altura algún día.

Contents



Abstract

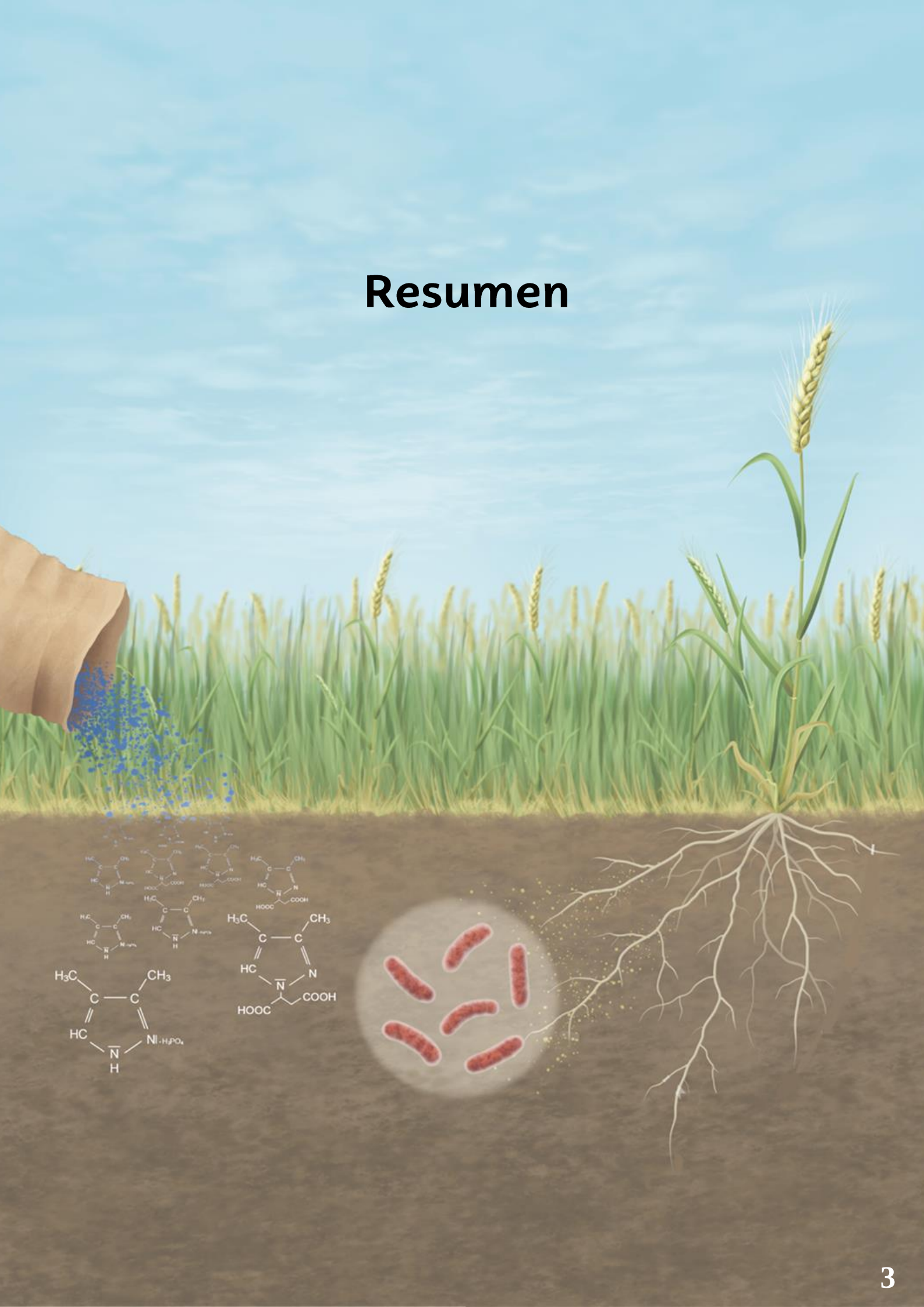


The increasing demand for food production has led to a 10-fold increase in nitrogen fertilizer use since the Green Revolution, turning agricultural soils into high-nitrifying environments that increase nitrogen pollution. Nevertheless, synthetic nitrification inhibitors (SNIs) have been developed to suppress soil-nitrifier activity and decrease nitrogen losses. The SNIs 3,4-dimethylpyrazole phosphate (DMPP) and 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA) can reduce nitrous oxide emissions and maintain soil ammonium for a longer time. However, it remains unclear whether a future climate scenario with increased atmospheric CO_2 concentration could affect their efficiency. Moreover, their mode of action seems to be related to their ability to chelate the Cu^{2+} cations that the ammonia monooxygenase (AMO) enzyme needs, but no more experimental data are available to confirm this. Therefore, a better understanding of the efficiency and mode of action of SNIs is needed to achieve agricultural sustainability.

Although DMPP and DMPSA are efficient tools to minimize nitrogen losses, they are not widely adopted by farmers due to their biologically limited stability and soil mobility. Nonetheless, the allelopathic substances from root exudates from crops such as sorghum, known for their activity as biological nitrification inhibitors (BNIs), have shown as a promising alternative. This makes sorghum a suitable option as a cover crop to be used in sorghum/winter wheat rotation to reduce nitrogen losses. Nevertheless, since BNIs exudation is related to the physiological state and development of the plant, it would also be necessary to determine the effects of abiotic stresses such as drought on the BNIs' release. On the other hand, modern wheat cultivars lack the ability to exude BNIs. Fortunately, the chromosome region controlling BNIs production in *Leymus racemosus*, a wild relative of wheat, was successfully introduced into two elite wheat cultivars, conferring them the ability to inhibit nitrification in acidic soils under ammonium nutrition. This BNI technology presents a worldwide potential use and, therefore, needs to be tested in different soil types and with different nitrogen sources with the aim of accomplishing environmentally friendly agronomic practices.

Overall, this thesis concludes that the control of nitrification allows us to achieve sustainable agriculture by reducing reactive nitrogen losses derived from the use of ammonium-based fertilizers in the environment.

Resumen

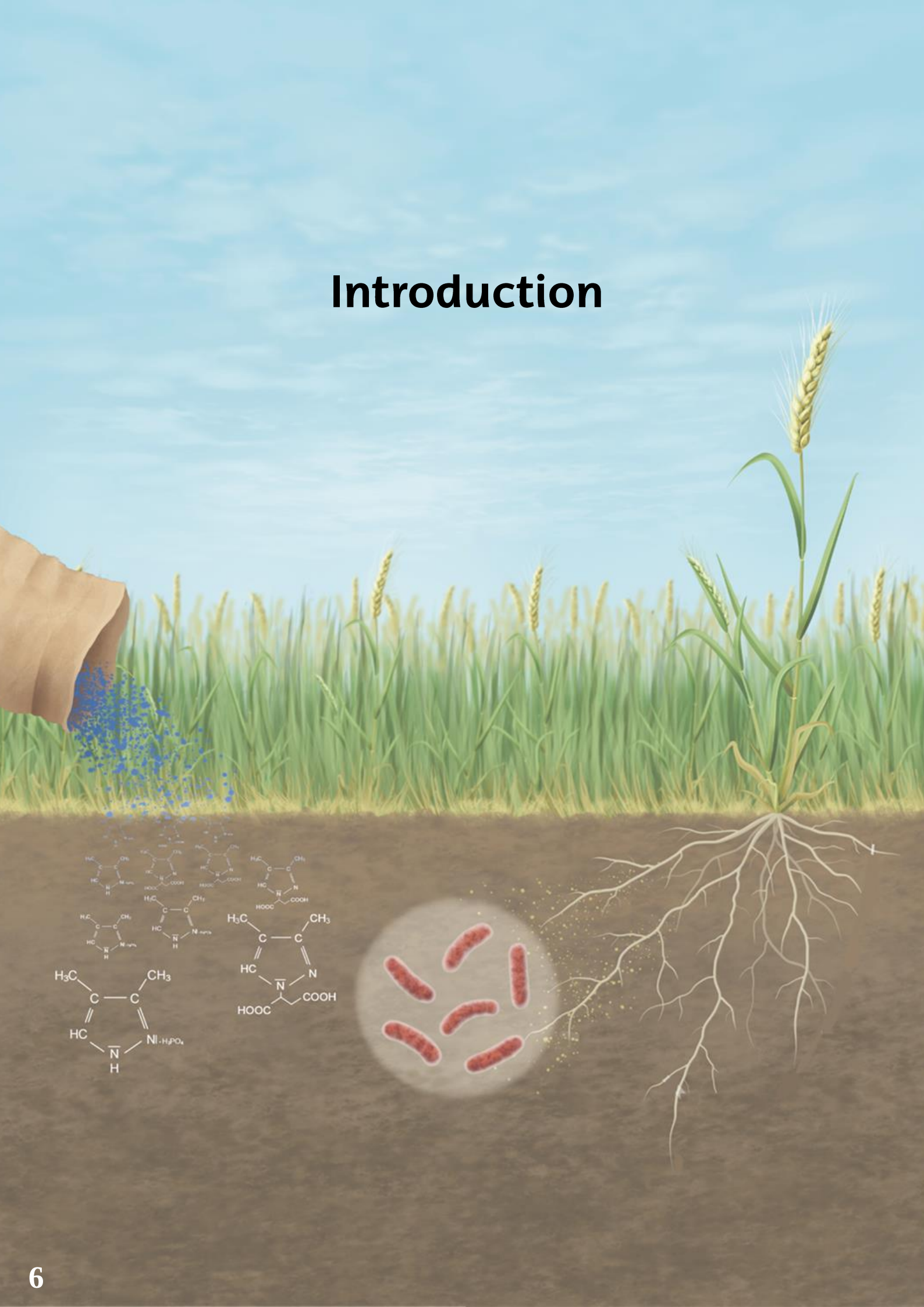


La creciente demanda de producción de alimentos ha llevado a que el uso de fertilizantes nitrogenados se haya incrementado 10 veces desde la Revolución Verde, convirtiendo los suelos agrícolas en ambientes altamente nitrificantes que aumentan la contaminación por nitrógeno, si no se ajustan las dosis a las necesidades del cultivo. Además, para ralentizar la actividad nitrificante del suelo y disminuir las pérdidas de nitrógeno se han desarrollado inhibidores sintéticos de la nitrificación (SNIs). Los SNIs 3,4-dimetilpirazol fosfato (DMPP) y la mezcla isomérica 2-(3,4-dimetil-1H-pirazol-1-il) succinato (DMPSA) pueden mantener el amonio en el suelo durante más tiempo reduciendo las emisiones de óxido nitroso. No obstante, no está claro si las condiciones futuras de cambio climático, como una mayor concentración de CO_2 atmosférico, pueden afectar su eficiencia. Por otro lado, su modo de acción parece estar relacionado con su capacidad para quelar los cationes Cu^{2+} que necesita la enzima amonio monooxigenasa (AMO), pero no hay datos experimentales en cultivos puros de nitrificantes que puedan confirmalo. Por lo tanto, se necesita una mejor comprensión de la eficiencia y del modo de acción de los SNIs para lograr una agricultura sostenible.

A pesar de que el DMPP y el DMPSA son herramientas eficientes para minimizar las pérdidas de nitrógeno, no son ampliamente adoptados por los agricultores debido a su limitada estabilidad biológica y movilidad de suelo. Sin embargo, las sustancias alelopáticas de exudados de raíces de cultivos como el sorgo, conocidas por su actividad como inhibidores biológicos de la nitrificación (BNIs), se han mostrado como una alternativa prometedora. Esto hace que el sorgo sea una opción adecuada para reducir las pérdidas de nitrógeno al ser usado como cultivo de cobertura en la rotación de sorgo/trigo de invierno. No obstante, dado que la exudación de BNIs está relacionada con el estado fisiológico y el desarrollo de la planta, también es necesario determinar los efectos de estreses abióticos como la sequía sobre la liberación de BNIs. Por otro lado, los cultivares de trigo modernos carecen de la capacidad de exudar BNIs. Afortunadamente, la región cromosómica que controla la producción de BNIs en *Leymus racemosus*, un pariente silvestre del trigo, se introdujo con éxito en dos cultivares de trigo de élite, lo que les confirió la capacidad de inhibir la nitrificación en suelos ácidos bajo nutrición amoniacal. Esta tecnología BNI presenta un uso potencial a nivel mundial y, por lo tanto, debe probarse en diferentes tipos de suelo y con diferentes tipos de fertilizante con el objetivo de evaluar la respuesta de estos trigos élite a la nutrición nítrica y amoniacal en el marco de prácticas agronómicas respetuosas con el medio ambiente.

Para finalizar, esta tesis concluye que el control de la nitrificación nos permite avanzar hacia una agricultura sostenible al reducir las pérdidas de nitrógeno reactivo al medio ambiente derivadas del uso de fertilizantes con base amoniacal.

Introduction



1. The challenges that agriculture has to face

Agriculture 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). Fortunately, in the past half-century, world agriculture has succeeded in increasing the production of cereal crops by a factor of three, with only a 30% increase in land-cultivated area during the period generally referred to as The Green Revolution (Wik et al., 2008; Pingali, 2012; Lassaletta et al., 2014a). In addition, the Green Revolution-driven intensification saved new land from conversion to agriculture and allowed for the release of marginal lands out of agricultural production into providing alternative ecosystem services, such as the regeneration of forest cover (Millenium Ecosystem Assessment, 2005). Much of this achievement was caused by the combination of high rates of investment in crop research and infrastructure. Due to the adoption of improved crop varieties, use of pesticides, and increased application of synthetic fertilizers (Tilman et al., 2002; Mueller et al., 2012; Sinclair and Rufty, 2012), the yield of cereal crops such as wheat have experienced a two-fold increase (Fig. 1).

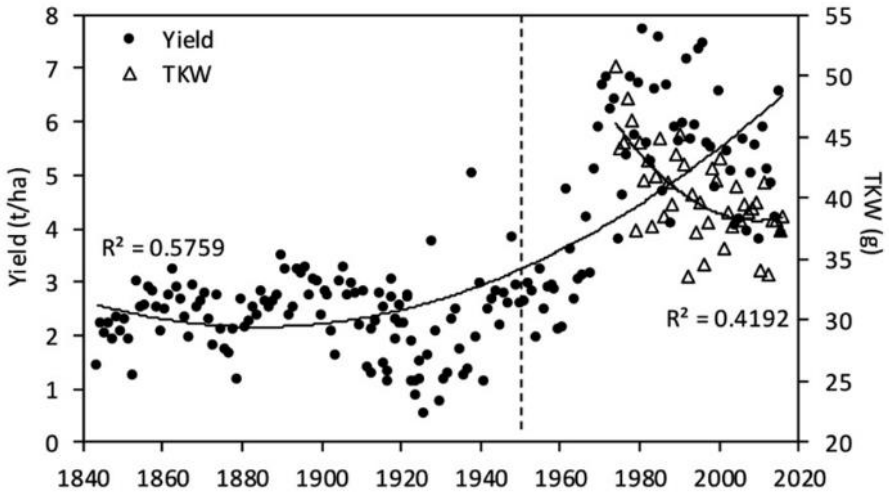


Fig. 1. *Trends in wheat yield and thousand kernel weight (TKW) from 1850 to 2016. Data are means. The dashed line represents the introduction of cultivars from The Green Revolution in 1968 (Mariem et al., 2020).*

Nevertheless, unintended consequences in water use, soil degradation, and chemical runoff have had serious environmental impacts beyond the areas cultivated (Burney et al.,

2010). The environmental consequences were caused by the policy environment that promoted the expansion of cultivation into areas that could not sustain high levels of intensification, and the overuse of inputs such as fertilizers or pesticides (Pingali et al., 2012).

2. Agriculture is the source of N pollution

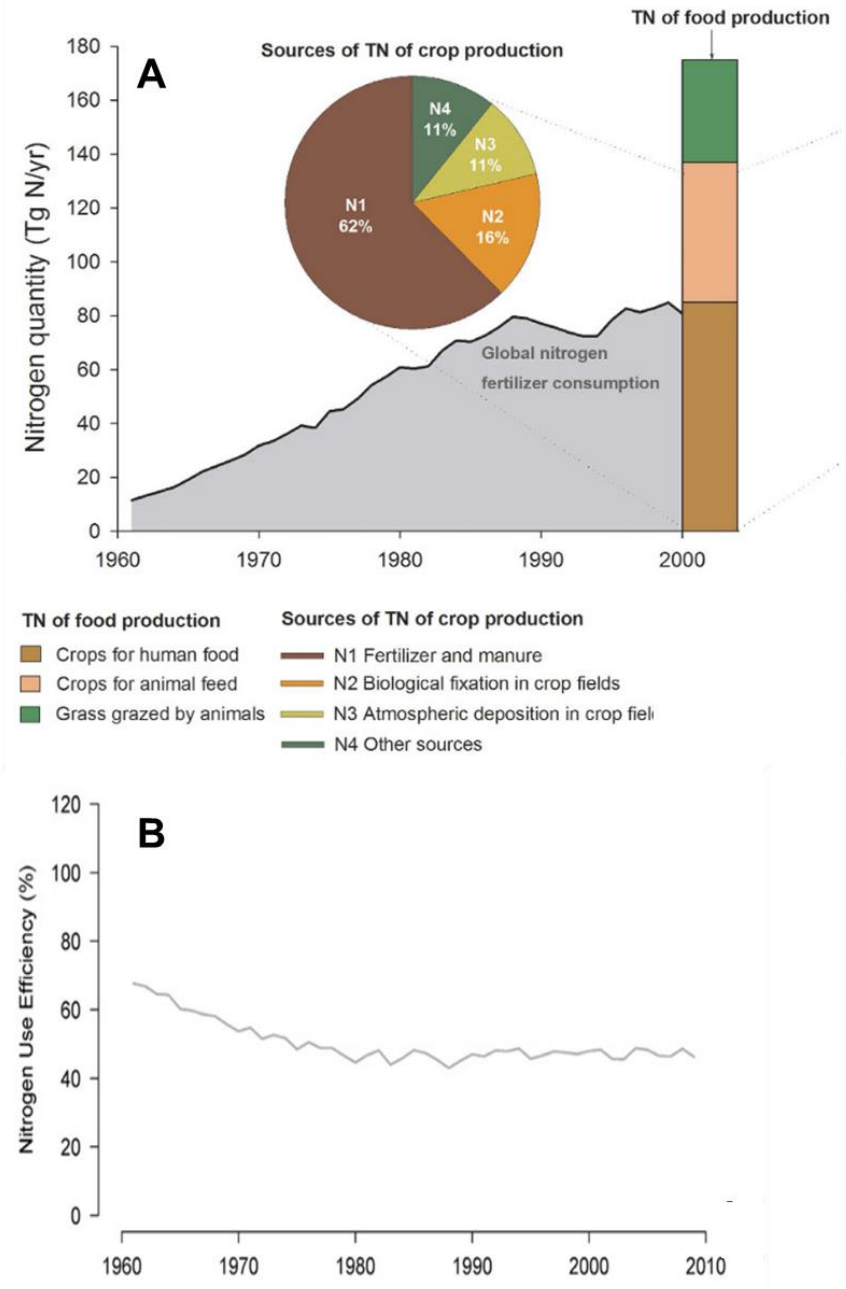


Fig. 2. *Sources of reactive N involved in food production (**A**) (adapted from Liu et al., 2016) and trends in nitrogen use efficiency (NUE) of the global cropping system (**B**) (adapted from Lassaletta et al., 2014b).*

Availability of nitrogen (N) is the major limiting factor for crop growth (LeBauer and Treseder, 2008). Hence, agriculture relies on the intensive use of N fertilizers to maximize crop yields, which uses approximately 2% of the energy consumed in the world for their synthesis (Wendeborn, 2020). The application of N fertilizers has been increasing in the last decades (Fig. 2A) and it is expected to reach 300 Tg N $year^{-1}$ by 2050 (Galloway et al., 2008). However, the entire N applied cannot be taken up by the crop or retained in the soil and, consequently, a great amount is lost as reactive N. Moreover, only 47% of the reactive N currently added globally onto cropland is converted into harvested products, compared to 68% in the early 1960s (Fig. 2B), while synthetic N fertilizer input increased by a factor of 9 over the same period (Lassaletta et al., 2014b). This makes agricultural production to be responsible for the production of more reactive N than all terrestrial natural processes (Rockström et al., 2009). The main pathway for N losses that cause a negative environmental impact is through nitrate (NO_3^-) leaching, ammonia volatilization (NH_3), and emissions of nitrogenous gases such as nitric oxide (NO) and nitrous oxide (N_2O) (Fig. 3).

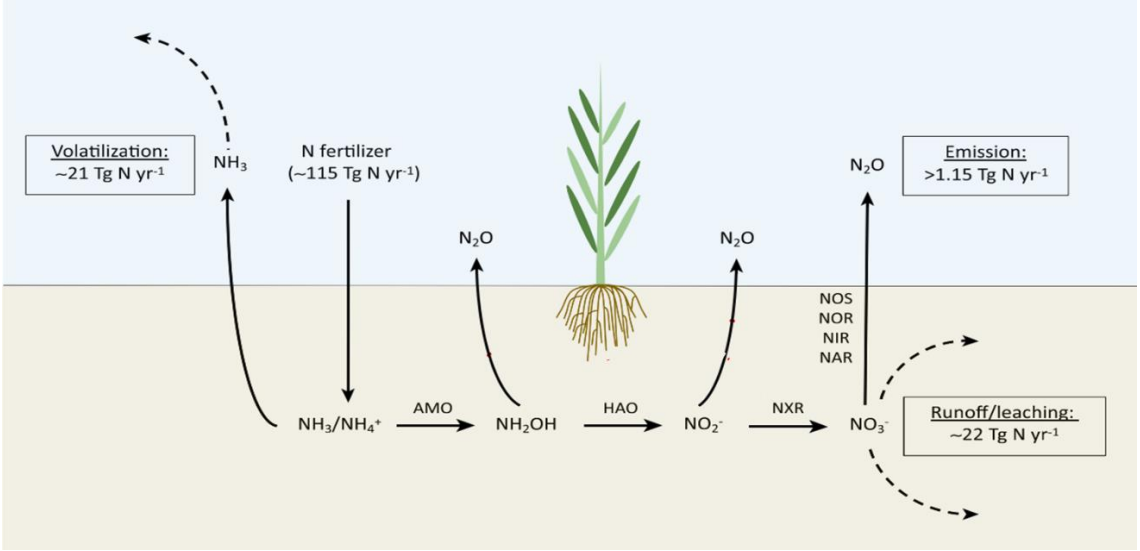


Fig. 3. *Agricultural nitrogen losses with an estimation of 115 Tg N $year^{-1}$ world fertilizer application (adapted from Coskun et al., 2017).*

N_2O is the main greenhouse gas (GHG) generated in upland agriculture derived from the use of N fertilizers (Syakila et al., 2011). Furthermore, it is estimated that agriculture is responsible for the emission of more than 1.15 Tg N_2O-N $year^{-1}$, which accounts for 1% of fertilizer application (Schlesinger, 2009; Reay et al., 2012) and represents around 19%

of total N_2O global source and 49% of anthropogenic N_2O emissions (Fowler et al., 2009). It is the single most ozone-depleting molecule (Ravishankara et al., 2009) with a global warming potential (GWP) between 265 – 298 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 microbial nitrification and, especially, denitrification (Li et al., 2016).

3. Nitrification

Nitrification is the sequential aerobic oxidation of reduced forms of N to NO_3^-, which evolved about 2.5 billion years ago (Berner, 2006). This process represents a key transformation of N in soil-plant systems (Fig. 4) (Stevenson and Cole, 1999).

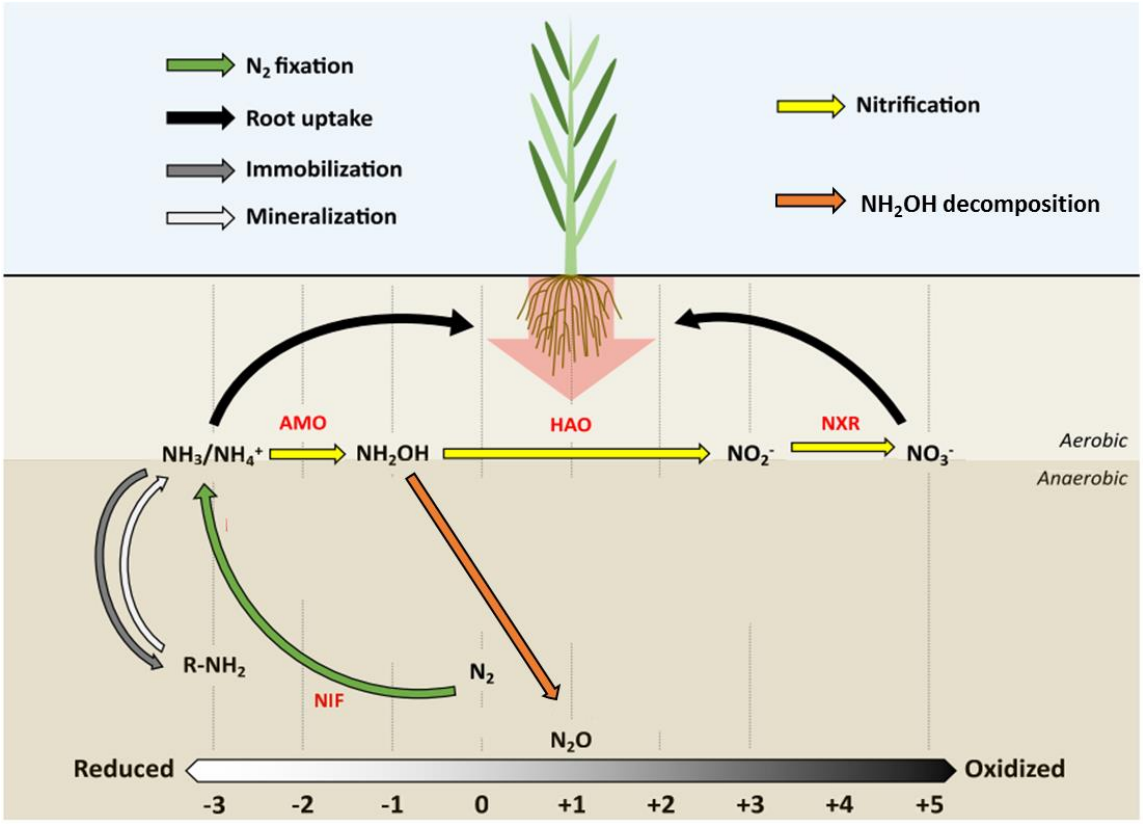


Fig. 4. *A schematic overview of nitrification in soil, pathway of the nitrogen cycle (adapted from Coskun et al., 2017).*

3.1. Ammonia oxidizers

The first step of nitrification is the oxidation of NH_3/NH_4^+ to hydroxylamine (NH_2OH) through the ammonia monooxygenase enzyme (AMO) encoded by the *amoA* gene, and later, NH_2OH is converted to nitrite (NO_2^-) by hydroxylamine oxidoreductase enzyme (HAO) (Arp and Stein, 2003). During this process, N_2O is formed by the chemical decomposition of the NH_2OH (Wrage et al., 2001). The microorganisms that mediate nitrification are two groups of ammonia oxidizers, ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). The AOA are represented by four orders within the phylum Thaumarchaeota: the *Nitrosopumilales, Nitrososphaerales, Nitrosotaleales,* and *Nitrosocaldales* (Könneke et al., 2005; de la Torre et al., 2008; Lehtovirta-Morley et al., 2011; Tourna et al., 2011). On the other hand, AOB communities from the soil are dominated by *Nitrosomonas* and *Nitrosospira* genera within the Betaproteobacteria (Head et al., 1993; Purkhold et al., 2000). The predominance of AOA or AOB carrying nitrification is related to soil pH and NH_4^+ concentration. In acidic soils, AOA are the prevalent group carrying out the nitrification process regardless of NH_4^+ concentration, whereas AOB become relevant as the pH reaches neutral or alkaline values (Nicol et al. 2008). It has been reported that even in neutral-alkaline soils, AOA outnumber the abundance of AOB when soil NH_4^+ content is low, but at the moment that ammonium-based fertilizers are applied, AOA are inhibited and AOB start carrying the nitrification process (Di and Cameron, 2016). As a result, neutral-alkaline soils tend to show an increase in AOB after fertilization (Castellano-Hinojosa et al., 2020). The dependence on soil NH_4^+ content could be related to the AMO differences between AOA and AOB. AMO enzyme can be found in the cytoplasm or bound to the membrane (Gilch et al., 2009). Although its crystal structure has not been already solved, it is known that it is formed by three different subunits (AmoA, AmoB, and AmoC) and contains Cu (6 Cu^{2+} and 3 Cu^{1+}), Fe (4 Fe^{3+}), and Zn (3 Zn^{2+}) (Gilch et al., 2009; 2010). Nevertheless, the AMO enzyme from AOA saturates faster than that of AOB due to a higher NH_4^+ affinity and a lower maximum rate of NH_4^+ oxidation. Therefore, although AOA present greater abundances in agricultural soils (Di and Cameron, 2016), its contribution to nitrification is comparatively lower (Kits et al., 2017). Contrarily, the HAO enzyme is a Fe-dependent oxidoreductase, so it does not need Cu^{2+} or Zn^{2+} (Hendrich et al., 1994).

3.2. Nitrite oxidizers

After the action of AOA and AOB, nitrite-oxidizing bacteria (NOB) oxidize NO_2^- to the final product of nitrification, NO_3^-, through the Fe-Mo nitrite oxidoreductase (NXR) (Lücker et al., 2010). The formed NO_3^- is a negatively charged anion, so it is repelled by negatively charged soil colloids and is thus lost through leaching, causing eutrophication and contamination of groundwater supplies (Fiencke et al., 2005). Among NOB, *Nitrobacter*, *Nitrospira*, *Nitrotoga,* and *Nitrolancea* can be found (Daims et al., 2001; Alawi et al., 2007; Sorokin et al., 2012; Ishii et al., 2017). In addition, van Kessel et al. (2015) discovered that the *Nitrospira* genera is able to perform the complete oxidation of NH_3/NH_4^+ to NO_3^- through the pathway known as comammox. The AMO enzyme of this pathway might present structural differences compared to that of AOB, which increases its affinity for the substrate and it provides an advantage at very low soil NH_4^+ contents (Beeckman et al., 2018).

4. Denitrification

Denitrification is the anaerobic part of the N cycle (Fig. 5) (González-Blanco et al., 2011). It reduces NO_3^- to molecular N (N_2), but several developed intermediates can be set free. The microorganisms that carry out denitrification are widely distributed across the bacterial taxa, including *Pseudomonas*, *Bacillus*, *Thiobacillus,* and *Propionibacterium* (Firestone, 1982). These microorganisms are facultative anaerobes that are able to use NO_3^- in place of oxygen as an electron acceptor in respiration to cope with low-oxygen or anaerobic conditions (Wrage et al., 2001). The intermediates formed are NO_2^-, NO, N_2O, and finally, N_2 through nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR or NOS) enzymes respectively (Hochstein and Tomlinson, 1988). NIR is the key enzyme for an organism to be recognized as denitrifying since it is responsible for the formation of the first gaseous form of N (Zumft, 1997). There are two structurally different, but functionally equivalent NIR enzymes. The first one is encoded by the *nirS* gene and it is a heme-containing nitrite reductase, whereas the second one is encoded by the *nirK* gene and it is a copper-containing nitrite reductase (Zumft, 1997; Glass and Orphan, 2012). In contrast to nitrification, N_2O is a regular intermediate of denitrification, which can be released in high quantities in low-oxygen environments with sufficient NO_3^- (Wrage et al., 2001).

N_2OR enzyme could play an important role in trying to reduce N_2O emissions. This enzyme is encoded by *nosZ* genes and it also requires Cu^{2+} as a cofactor in its catalytic subunit (Pauleta et al., 2013). Recently, a second clade of nitrous oxide reductase has been identified, named *nosZII* (Sanford et al., 2012; Jones et al., 2013) which includes a large fraction of non-denitrifying N_2O reducers, which could act as N_2O sinks without major contribution to N_2O formation (Hallin et al., 2018).

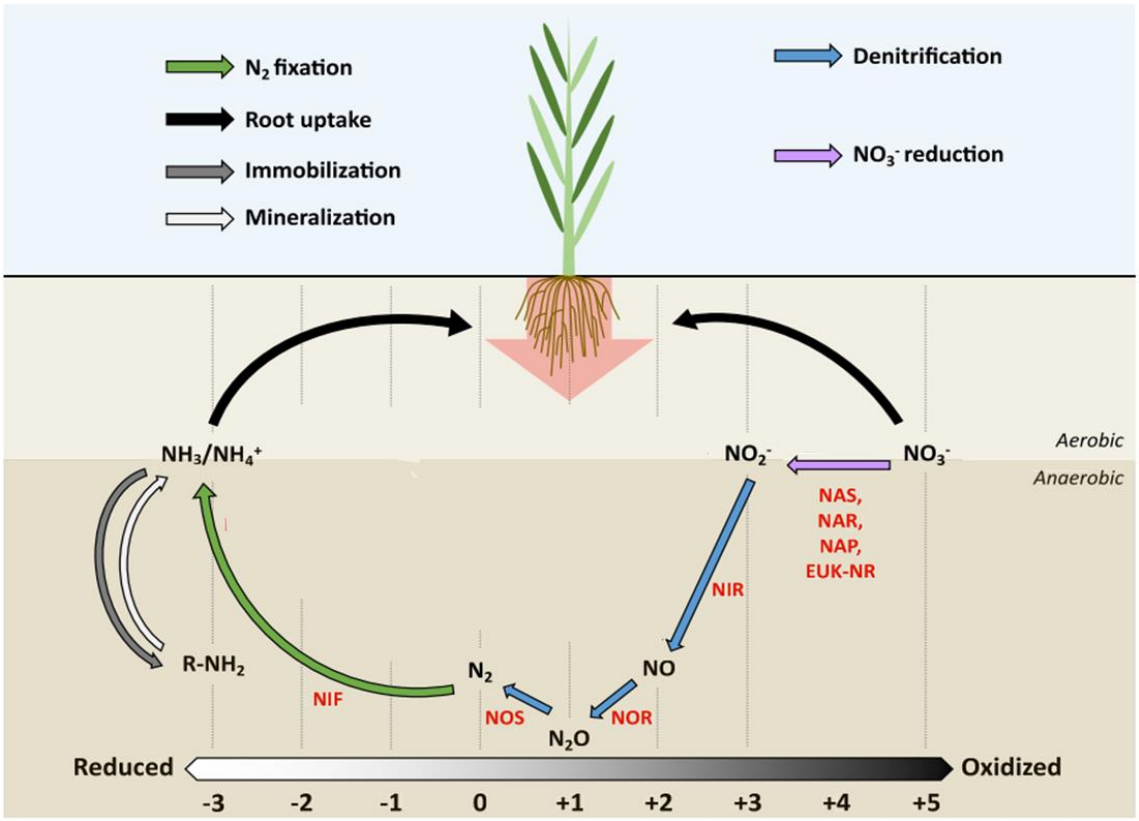


Fig. 5. *A schematic overview of denitrification in soil, pathway of the nitrogen cycle (adapted from Coskun et al., 2017).*

5. Efforts to reduce N pollution

Despite the various problems derived from N losses, environmental N contamination is not always considered as important on a global level as the carbon cycle (Wendeborn, 2020). Therefore, we must guide agricultural systems towards sustainability that allows us to maintain adequate production levels while reducing the amount of reactive N lost to the environment. The practices based on increasing N use efficiency (NUE) are considered good strategies for reducing N-oxide emissions (Sutton et al., 2011). One of

these practices is to optimize N supply and synchronize it with crop demand. To do so, the crop growth, soil type and environmental conditions should be taken into account (Malhi et al., 2001). The use of cover crops instead of fallow during the summer season can also play an important role in improving sustainable agriculture (Schipanski et al., 2014). Cover crops bring multiple environmental benefits such as improved soil fertility, weed control and a reduction in nutrient leaching and soil erosion (Muhammad et al., 2019; Garland et al., 2021). They are a very efficient tool in reducing the amount of leachable nitrate (NO_3^-) in soil (Constantin et al., 2010; Plaza-Bonilla et al., 2015a). In addition, the advantages of cover crops could remain in the following culture. No-tillage could also be a different practice to reach sustainability. No-tillage systems are increasing in Europe because it increases soil C stocks and maintains or increases crop yields (Soane et al., 2012; Plaza-Bonilla et al., 2015b). Moreover, as no-tillage improves water conservation, it decreases the O_2 availability in the soil, which means a reduction in N losses through nitrification (Corrochano-Monsalve et al., 2020a). Another practice is the use of synthetic nitrification inhibitors (SNIs) when applying ammonium-based fertilizers. SNIs have been developed to decrease N losses by suppressing soil-nitrifier activity, maintaining the NH_4^+ content for longer in soil, which reduces the formation of NO_3^- and its subsequent denitrification (Abalos et al., 2014). They are synthetic compounds added to fertilizers, in granular or soluble form, that inhibit the activity of the AMO enzyme (Ruser and Schulz, 2015).

5.1. Synthetic nitrification inhibitors

Currently, the most worldwide used SNIs are nitrapyrin (2-chloro-6-(tri-chloromethyl)-pyridine), dicyandiamide (DCD), and 3,4-dimethylpyrazole phosphate (DMPP) (Fig. 6) (Trenkel, 2010; Gilsanz et al., 2016). Nitrapyrin use reduces NO_3^- leaching and N_2O emissions by 16% and 51% respectively (Wolt, 2004). However, its high volatility makes it necessary to be incorporated into the soil. On the other hand, DCD is a cheaper SNI and its no volatility makes it more suitable to be used as a coating on solid fertilizers (Giltrap et al., 2010). DCD is able to inhibit the growth of AOB and, to a lesser extent, AOA (Ruser and Schulz, 2015). Moreover, a 59% and 57% reduction in NO_3^- leaching and N_2O emissions respectively have been observed after DCD application (Di and Cameron, 2018). Nevertheless, DCD presents a high water solubility, which may cause it to leach out of the action zone, decreasing its efficiency. DMPP has lower volatility than nitrapyrin

and lower mobility than DCD. Furthermore, DMPP shows similar efficiency to DCD with a 10 times lower application rate, which makes it an alternative to DCD due to its reported ecotoxic problems (Zerulla et al., 2001; Weiske et al., 2001; Chen, 2014). The N_2O emissions are reduced by about 33 – 45% with the use of DMPP (Menéndez et al., 2012; Ábalos et al., 2014; Kong et al., 2018).

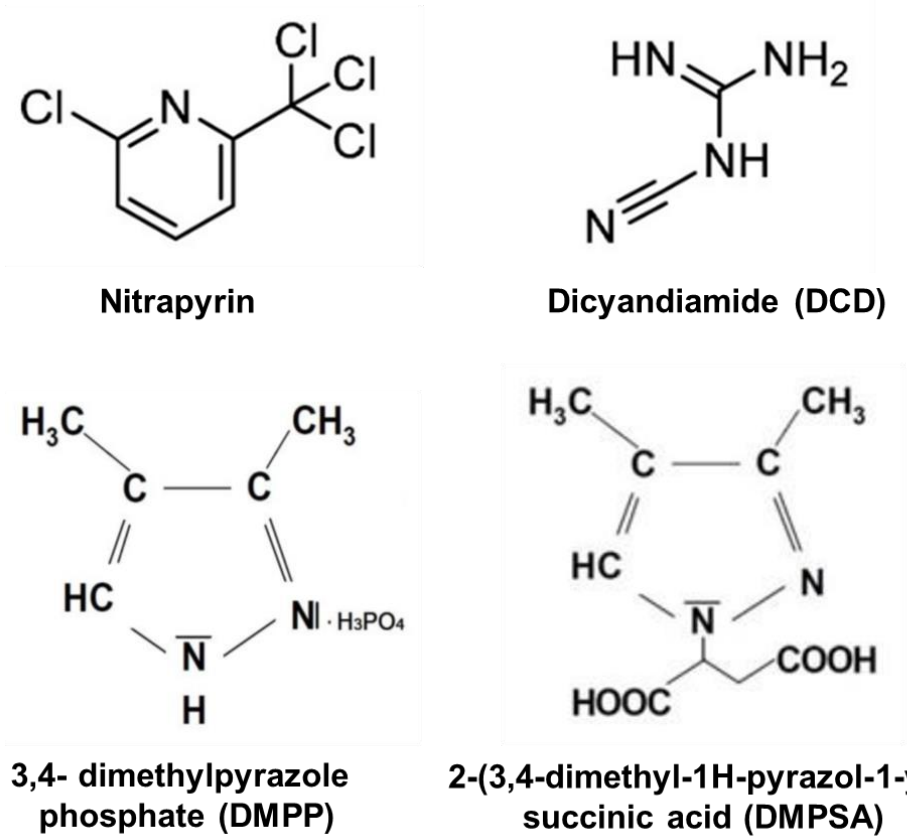


Fig. 6. *Molecular forms of the most used SNIs (adapted from Wendeborn, 2020).*

In addition, another dimethylpyrazole-based SNI, 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA), has been recently developed by Eurochem Agro GmbH (Mannheim, Germany). This new SNI presents a succinic acid covalently bond to the dimethylpyrazole (DMP), instead of a phosphate group as in the case of DMPP, increasing its stability, which allows it to be used with other fertilizers such as calcium ammonium nitrate or diammonium phosphate that cannot be used with DMPP. DMPSA is able to reduce the N_2O emissions and delay NH_4^+ oxidation in field conditions (Guardia et al., 2018a; Corrochano-Monsalve et al., 2020a; Recio et al., 2020; Montoya et al., 2021). Aditionally, both DMPP and DMPSA show a similar efficient at inhibiting nitrification. In field conditions, reduction of around 50% in N_2O emissions are reported

with a higher maintenance of soil NH_4^+ content for longer period (Huérfano et al., 2016; 2018; 2022). In microcosms experiments, the efficiency on reducing N_2O emissions of both SNIs increase up to 90% (Torralbo et al., 2017; Corrochano-Monsalve et al., 2021a). It is suggested that the difference between field and microcosms efficiencies is due to the form that SNIs are applied. DMPP and DMPSA field application is in solid form, while in microcosm experiments they are applied by being previously dissolved in water. Therefore, since DMPP and DMPSA are dissolved in water, a more homogeneous distribution is achieved throughout the soil, which improves their inhibition efficiency.

5.2. Mode of action

To make more efficient use of the compounds, it is desirable to know their mode of action. Nevertheless, the mode of action of the main SNIs remains unknown. Many assumptions have been made that their mode of action is somehow related to their attributed ability to chelate the Cu^{2+} cations that the AMO enzyme needs to carry on the NH_4^+ oxidation. Campbell and Aleem (1965) observed that the nitrification inhibition produced by the use of nitrapyrin could be reversed by the addition of Cu^{2+}. Thus, it was accepted that its mode of action was by chelating Cu^{2+} atoms. Nonetheless, Powell and Prosser (1985) found in pure cultures of *Nitrosomonas europaea* that not only the addition of Cu^{2+} did not relieve the nitrapyrin produced nitrification inhibition, but also enhanced it. Results from these works seem to be contradictory and, lamentably, this aspect has not been further investigated (Wendeborn, 2020). Subbarao et al. (2006a) indicate in their review that DCD is also a Cu^{2+} chelating molecule. These authors also refer to the work of Powell and Prosser (1986) to assert this hypothesis. Nonetheless, this is a misunderstanding since Powell and Prosser (1986) did not study DCD in their work. Therefore, there is no evidence that DCD is able to chelate Cu^{2+} as all the works that claim the chelating capacity of DCD refer to the review of Subbarao et al. (2006a). Ruser and Schulz (2015) report in their review that DMP-based SNIs can chelate Cu^{2+} cations, but this is based on the personal communication of Wissemeier. (researcher from BASF, the company that held the patent of DMPP). Corrochano-Monsalve et al. (2021a) later confirmed that both DMPP and DMPSA are able to chelate Cu^{2+}. These authors also observed that DMPP needs 4 molecules to chelate one atom of Cu^{2+}, whilst DMPSA only needs 2 (Fig. 7). Thus, this makes DMPSA more efficient at chelating Cu^{2+} than DMPP. Nevertheless, although the nitrification inhibition displayed by these compounds seems to be driven by

the interaction between them and Cu^{2+}, no more experimental data are available to confirm that this is the exact mode of action. This knowledge gap is a great opportunity to continue further investigating trying to determine the real mode of action of SNIs.

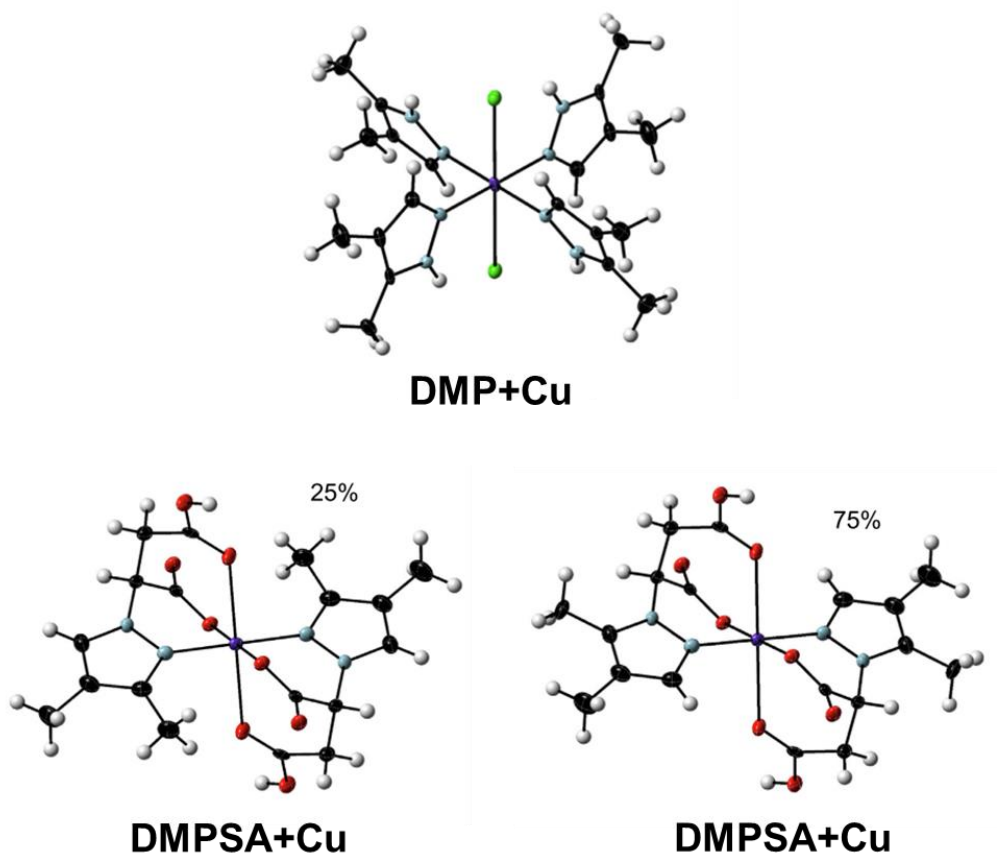


Fig. 7. *ORTEP views of DMP+Cu, and DMPSA+Cu. The two isomers of DMPSA that coexist in the structure of DMPSA+Cu are represented independently. (Color code: Cu, violet; C, black; Cl, green; H, white; N, blue; O, red; S, orange) (adapted from Corrochano-Monsalve et al., 2021a).*

6. Synthetic nitrification inhibitors are affected by soil properties

SNIs efficiency is related to the soil physicochemical composition and, especially, to environmental properties such as temperature and humidity. The application of fertilizer and SNIs one after another might lead to a partial spatial separation of NH_4^+ and the SNI (Azam et al., 2001; Linzmeier et al., 2001). This separation process will presumably depend on the soil texture and it might be faster either the lower the clay content and thus the less adsorption places for cations are available in soil or the lower the amount of clay-

humus-complexes acting as sorption places for organic molecules, and thus retarding the diffusion of SNIs such as DMPP (Ruser and Schulz, 2015). Soil temperature is the most determining factor for the stability and behaviour of SNIs in soils (Irigoyen et al., 2003). The performance of DMPP is suggested to be the best in cold conditions rather in warm ones (Menéndez et al., 2012). The half-life of DCD at a mean soil temperature of 8 ºC is nearly 120 days, but when the temperature is raised at 20 ºC, DCD half-life is reduced to 20 days (Kelliher et al., 2008). Due to the high volatility that nitrapyrin presents, the higher the soil temperature, the more amount that might be volatilized and lost to the atmosphere, reducing its inhibitory effect (Chen et al., 2010). Moreover, SNIs efficiency should also be taken into account in future scenarios such as an increase of soil carbon (C) content due to an increase in atmospheric CO_2. Atmospheric CO_2 concentration will presumably rise to 450 ppm by 2030 and between 750-1300 ppm by 2100 (IPCC, 2014), which could produce different effects in the soil-plant system. Elevated CO_2 can 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 N sources (Torralbo et al., 2019) changing the NH_4^+/NO_3^- balance in soils. Thus, since the increment of the CO_2 concentration can modify the N cycle, it is not clear whether SNIs efficiency could also be affected.

7. Biological nitrification inhibition as a promising alternative

The use of SNIs is not widely adopted by farmers (Subbarao et al., 2006a). They are expensive to apply, their delivery in the field is fraught with many challenges, such as the above-mentioned SNI mobility that could prevent them from acting on the sites of nitrification, and they present a lack of cost-effectiveness (Subbarao et al., 2013a; 2017). Notwithstanding, there is a promising option to alleviate N losses derived from nitrification: the biological nitrification inhibition (BNI) (Fig. 8). The natural ability of some plants to produce and release nitrifier activity in soils is termed BNI (Subbarao et al., 2013a). Most plants release chemical compounds from their roots, which can stimulate or inhibit soil nitrification (Subbarao et al., 2015). Subbarao et al. (2006b) adapted a bioluminescence assay used to monitor nitrification in municipal wastewater

treatments plants to detect whether these released chemical compounds present BNI activity. The assay consists in using a recombinant strain of *Nitrosomonas europaea* that produces bioluminescence due to the expression of the *luxAB* genes (from *Vibrio harveyi*) during nitrification (Iizumi and Nakamura, 1997; Iizumi et al., 1998). Furthermore, this assay allows making a distinction between compounds that affect the AMO or the HAO enzyme. The *luxAB* genes are expressed alongside the genes encoding for HAO because they share the same promoter (Iizumi et al., 1998). *N. europaea* bioluminescence decreases after the addition of biological nitrification inhibitors (BNIs), but if it is recovered after adding NH_2OH, it means that the BNIs only inhibit the AMO enzyme (Subbarao et al., 2006b).

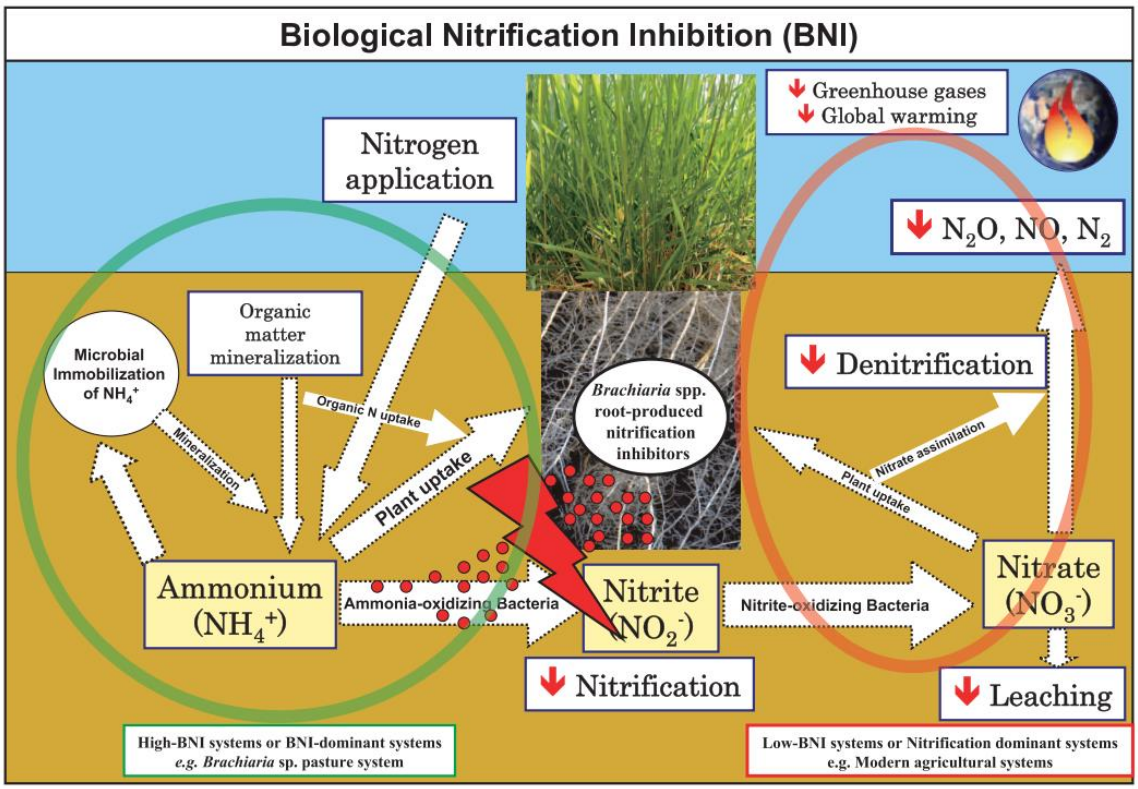


Fig. 8. *Schematic representation where biological nitrification inhibition (BNI) interfaces with the nitrogen cycle. BNI's produced by the root inhibits the process that converts ammonium to nitrate. In ecosystems with large amounts of BNI, such as Brachiaria pastures, the flow of nitrogen from ammonium to nitrate is restricted and ammonium tends to remain or build up in the soil/root system. In systems with little or no BNI, such as modern agricultural systems, nitrification tends to occur at a rapid rate and ammonium is rapidly converted to nitrate that is very susceptible to be lost from the soil/root system (Subbarao et al., 2009a).*

The ability to exude BNIs seems to be related to plants' adaptability to low N environments (Subbarao et al., 2015). *Panicum* spp., a tropical pasture that is adapted to high N environments, present relatively weak BNI activity (Subbarao et al., 2007a). In contrast, *Brachiaria* spp., which is adapted to low N environments, have the highest BNI activity in root systems (Subbarao et al., 2007b). Crude extract of *Brachiaria* BNI exudation inhibited similarly both enzymatic pathways, AMO and HAO (Subbarao et al., 2009b). Through a bioassay-guided fractionation, these authors achieved the isolation of a cyclic diterpene from the crude extract of *Brachiaria* BNI exudation, which they named "brachialactone". Brachialactone also blocked AMO and HAO enzymes activity, but its inhibitory power was lower in HAO compared to AMO (Subbarao et al., 2009b; Egenolf et al., 2020). This indicates that in the crude extract of *Brachiaria* plants might be other BNIs with a different mode of action. Moreover, *Brachiaria* plants not only produce BNIs in their roots but also their leaves. The major BNI compound found in shoot tissues was linolenic acid, which inhibited both AMO and HOA enzymes similar to the crude root exudates (Subbarao et al., 2008). During three years of *Brachiaria* plants growth, high soil NH_4^+ content was determined, while the AOB abundance was very low (Subbarao et al., 2009b). In addition, little effect on the total soil bacterial population was found. Therefore, *Brachiaria* exuded BNIs presents a highly specific inhibitory nature towards AOB. These authors also estimated that *Brachiaria* plants could potentially release an amount of BNIs equivalent to 6.2 – 18 kg nitrapyrin ha^{-1} $year^{-1}$, which is enough to have a significant influence on soil nitrification. Field studies with *Brachiaria* plants indicated a 90% reduction in soil NH_4^+ oxidation and in the N_2O emissions (Subbarao et al., 2009b). Karwat et al. (2017) investigated the residual BNI effect of a long-term *Brachiaria* pasture on subsequent maize (*Zea mays*) crop. The residual BNI effect prevented re-mineralized N from nitrification, which improved maize performance. In this way, the potential use of BNI relies on the residual effect of plants with BNI exudation ability that is noticed in the following crop. Nevertheless, the residual BNI effect may not last enough time to efficiently reduce the N losses coming from nitrification. For example, the residual BNI effect in Karwat et al. (2017) was only significantly evident for less than one year. Hence, another good option would be that the crop itself exude the BNIs.

7.1. *Sorghum bicolor,* the cereal with high BNI capacity

Alike grasses, among field crops, the ability to exude BNIs is also related to low N environments. Sorghum (*Sorghum bicolor*), which is adapted to low N environments, have stronger BNI capacity than crops adapted to high N input environments such as wheat (*Triticum aestivum*) and maize (Subbarao et al., 2007b). Sorghum is a widely cultivated cereal, being the fifth most important after wheat, maize, rice and barley (Dogget, 1988). The heat tolerance and drought resistance that it possesses has made it well adapted to semiarid regions (Smith and Frederiksen, 2000; Hadebe et al., 2017). While Africa and India cultivate sorghum for human food, which accounts for 40% of world sorghum production, countries in North America and Europe use sorghum for biomass production and livestock feed (Dendy, 1995; Rooney and Waniska, 2000). However, sorghum is becoming relevant for industrialized countries because its grain is safe for celiac and gluten-intolerant people (Ciacci et al., 2007). Therefore, it can meet the growing demand for gluten-free foods and beverages from consumers who cannot eat products containing wheat, barley or rye (Taylor et al., 2006). Besides becoming a safe food product, sorghum is also known for the BNI capacity of its root exudates. Sorghum roots release two categories of BNIs i) hydrophilic and ii) hydrophobic. Subbarao et al. (2013b) isolated and characterized 3-(4- hydroxyphenyl) propionate (MHPP) and 5,4′-dihydroxy-7-methoxyflavanone (sakuranetin) within hydrophilic BNIs (Fig. 9). On the other hand, more than 80% of the hydrophobic component of sorghum-exuded BNIs is sorgoleone (Czarnota et al., 2003). Sorgoleone was initially used for its substantial weed-suppressing capability (Alsaadawi et al., 1986), but later its potential as a BNI was discovered. Sorgoleone and sakuranetina efficiently block both the AMO and HAO enzymes, whereas MHPP only inhibits the AMO enzyme in bioassays of recombinant *N. europaea* (Subbarao et al., 2013b). The mode of action of sakuranetina and MHPP remains unknown, but the chemical structure of sorgoleone, which has a hydroquinone head and a fatty acid tail with a terminal double bond, has the potential to disrupt the electron flow between AMO and HAO enzymes and hence the nitrification activity (Rottenberg and Hashimoto, 1986). Nonetheless, although sakuranetina showed inhibitory effect similar to sorgoleone and stronger than MHPP in the bioassays, it has no inhibitory effect on soil nitrification (Subbarao et al., 2013b). This means that not all compounds with BNI activity detected in an in vitro culture bioassay are effective in the soil.

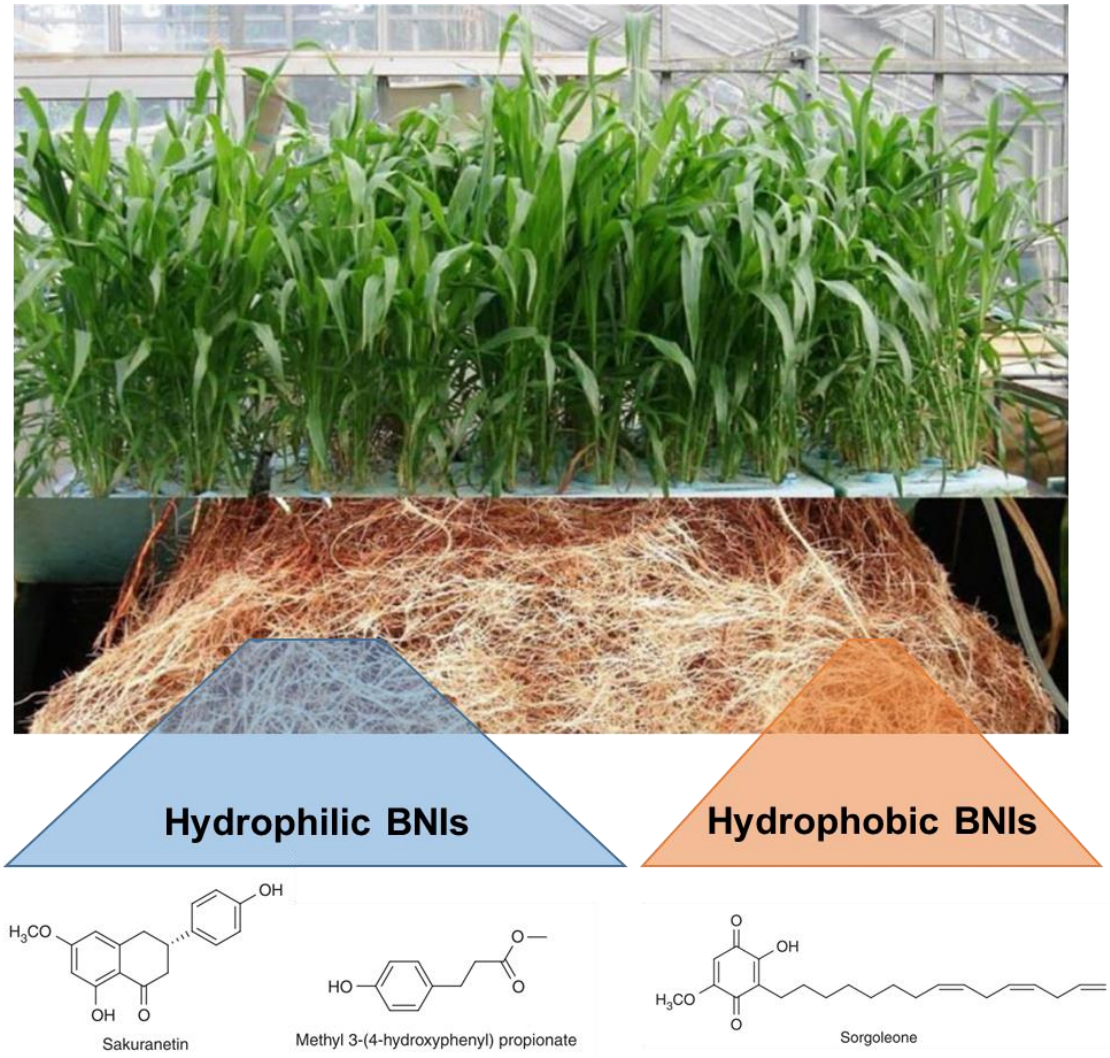


Fig. 9. *Molecular forms of hydrophilic and hydrophobic nitrification inhibitors (BNIs) released from sorghum roots (adapted from Subbarao et al., 2013b).*

Once MHPP and sorgoleone are released from sorghum roots, their nitrification inhibitory action seems to be relatively stable over a pH range of 3.0 to 9.0. This is in contrast to BNIs release from other plants, such as *Brachiaria*, whose molecules are reported to have a total loss of inhibitory function at pH ≥8.0 (Subbarao et al., 2007a). Moreover, the inhibitory effect on soil nitrification of MHPP and sorgoleone appears to be stable in the temperature range of 20 to 30 °C (Subbarao et al., 2013b). Sorghum BNIs are exuded directly into the rhizosphere, which is the main site of nitrification due to the great abundance of AOB and AOA (Nardi et al., 2020). Furthermore, due to the different affinity to water, sorghum BNIs may have complementary roles. The hydrophobic BNIs may remain close to the root systems, as they are strongly absorbed to 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 physiological maturity of the crop (Sarr et al., 2021), which would ensure the presence of nitrification inhibitors during all the stages of crop development. All these could indicate that sorghum is adapted to take NH_4^+ as N source avoiding the effects of the so-called "ammonium toxicity syndrome", which includes leaf chlorosis, ion imbalance, disorder in pH regulation, decrease in net photosynthesis, and changes in metabolite levels including amino acids, organic acids, and carbohydrates (Sarasketa et al., 2014). Anyway, sorghum BNI exudation capacity is highlighted in the framework of sustainable agriculture and its use could drive cultivation systems towards environmentally friendly agronomic practices that allow us to meet global food demand while reducing the loss of reactive N into the environment. Hence, BNI exudation advocates the use of sorghum in crop rotation or as a cover crop. Its BNI ability may reduce nitrifying populations so that in the following crop N pollution is reduced after fertilization due to a decreased nitrification. Sorghum is drought tolerant, so it can develop despite the dry summer climate of areas with humid Mediterranean conditions, and it has a short growth cycle, which provides winter crops enough time to settle. Nevertheless, BNI exudation is related to the physiological state and development of the plant (Sarr et al., 2021), so biotic or abiotic stresses that affect crop growth might modify the rate of BNI exudation or even prevent its release. To date, only Ghatak et al. (2021) studied the effects of abiotic stresses in the BNI root exudation and its composition of pearl millet plants (*Pennisetum glaucum*). These authors found a genotype-dependent enhancement of BNI activity after a defined period of drought stress. Since sorghum could have a potential use as cover crop, it would also be necessary to further investigate the effects of drought stress in its BNI release.

7.2. BNI release mechanisms

Synthesis and release of BNIs is highly regulated by the form of N that it is applied (Subbarao et al., 2007a; Zhu et al., 2012). While plants grown with NH_4^+ as the N source release BNIs from roots, plants grown with NO_3^- do not release them (Zakir et al., 2008; Subbarao et al., 2009b; Zhu et al., 2012). Although high levels of BNIs were detected in

root tissues of plants grown with NH_4^+, they were only released when plants roots were directly exposed to NH_4^+ (Subbarao et al., 2007a; 2007c; 2009a; 2009b). In addition, BNIs release from roots is a localized phenomenon confined to the part of the root system exposed to NH_4^+ and was not extended to the remaining parts of the root system (Zhu et al., 2012). Such localized release of BNIs by roots ensures a relatively high concentration of BNIs in soil microsites where nitrifiers are active, which is often associated with the presence of NH_4^+ (Nardi et al., 2020). Furthermore, the regulatory role of NH_4^+ in the synthesis and release of BNIs suggests a possible adaptive role in protecting NH_4^+ from nitrifiers (Subbarao et al., 2015). It has been hypothesized that the activation and function of the proton pumping activity of root plasma membranes is a functional link between the release of BNI and NH_4^+ uptake and assimilation (Fig. 10) (Zhu et al., 2012). If BNIs are transported through voltage-dependent anion channels, their release will be closely related to the regulation of proton pump ATPase (Subbarao et al., 2015). Therefore, it can be speculated that the release of BNIs through roots cells ATPase is associated with NH_4^+ uptake and assimilation.

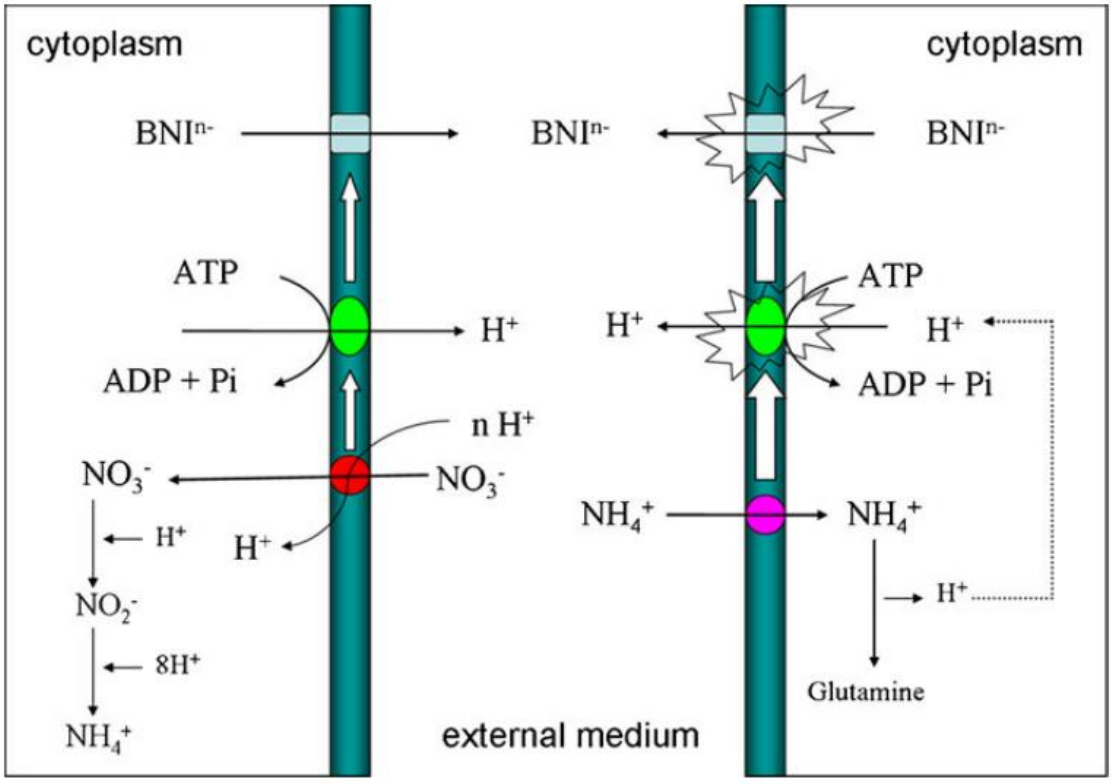


Fig. 10. *Hypothesis on the transport of BNIs, driven by H^+-ATPase, associated with NH_4^+ uptake and assimilation in Sorghum bicolor (Zhu et al., 2012).*

8. Introducing BNI capacity conferring genes into modern wheats cultivars

Wheat is the main crop used for human food and its production is expected to reach 3.8 Mg ha^{-1} by 2050 (Alexandratos and Bruinsma, 2012). This cereal is usually managed under intensive N fertilization (Tilman et al., 2002). Unfortunately, modern wheat cultivars lack detectable BNI capacity in their root systems (Subbarao et al., 2007b) and require application of SNIs or more appropriate N management to reduce N pollution. Several works report a reduction in soil NO_3^- formation and N_2O emissions from wheat crops using SNIs or more appropriate N management without affecting the yield (Matson et al., 1999; Weiske et al., 2001; Migliorati et al., 2014; Huérfano et al., 2015; Dawar et al., 2021). Nevertheless, Subbarao et al. (2007c) reported that *Leymus racemosus*, a wild relative of wheat, possess high-BNI capacity, and the genes responsible for this capacity were located on chromosomes *Lr#n*. With the idea of developing wheat cultivars with BNI capacity, Subbarao et al. (2021) introduced the BNI-controlling chromosome region from *L. racemosus* into several elite high-yielding wheat cultivars (Fig. 11).

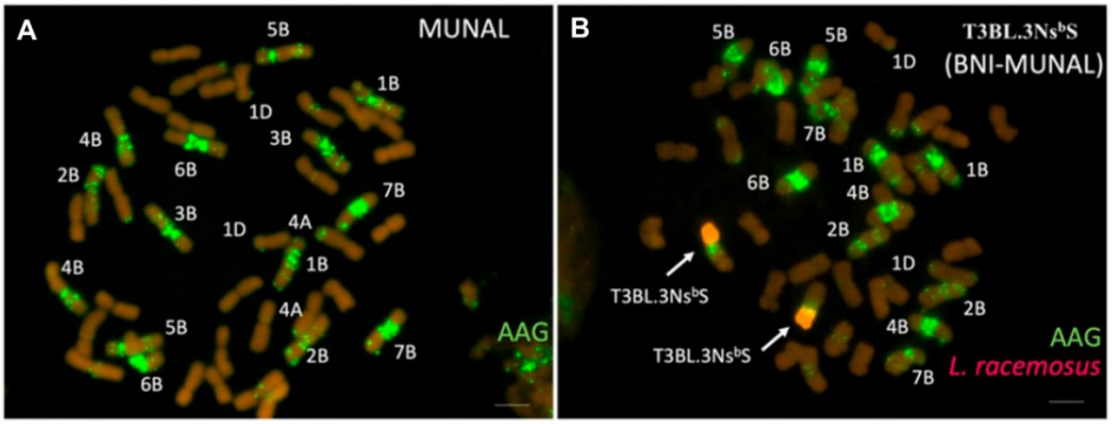
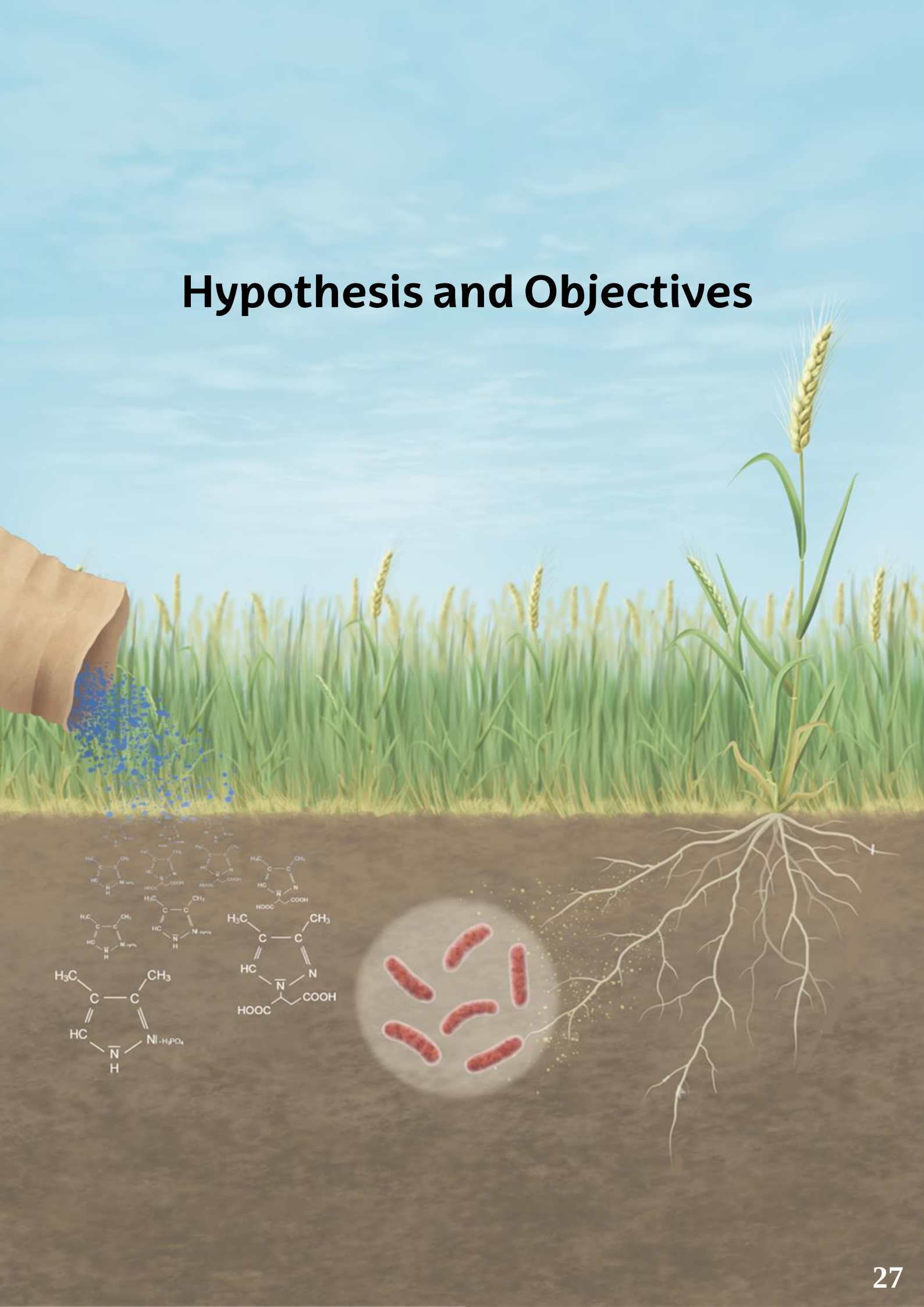


Fig. 11. *Karyotype analysis of BNI isogenic wheat lines, MUNAL-Control (**A**) and MUNAL-BNI (**B**) carrying the BNI-controlling chromosome region from L. racemosus (T3BL.3Ns^{b}S) (Subbarao et al., 2021).*

To do so, they introduced the short arm of *Lr#n* (BNI-trait) into CHINESE SPRING wheat cultivar by backcrossing, achieving a great increment of BNI exudation. Selection for *Lr#n* was done using fluorescence in situ hybridization following Kishii et al. (2004). Later, the BNI-trait was successfully transferred from CHINESE SPRING-BNI into ROELFS and MUNAL wheat cultivar, which present a grain yield potential of > 10 t ha^{-1}, as in Kishii et al. (2004). These authors accomplished near doubling the BNI production

in elite wheat cultivars “ROELFS-BNI” and “MUNAL-BNI” using hydroponically grown plants (Subbarao et al., 2021). Further field studies using isogenic lines of MUNAL (i.e. MUNAL-Control vs MUNAL-BNI) with slightly acidic soils (pH 6.0) indicate significant improvements in grain yields in MUNAL-BNI compared to MUNAL-Control under a wide-ranging N input. In addition, nitrification and N_2O emissions from rhizosphere soils where MUNAL-BNI was grown were significantly lower (about 30%) than in MUNAL-Control (Subbarao et al., 2021). These novel wheat lines mean that a new milestone has been reached for sustainable agriculture since it allows farmers the potential to have highly productive BNI-wheat crops, while reducing N pollution to the environment. The potential use of this new technology is aimed eventually to reach farmers from all over the world. This requires validating the efficiency of BNI-trait in wheat considering different edaphoclimatic conditions, as the type of soil, and different fertilizer types. In the Basque Country, wheat is cultivated in the province of Araba (Alava) under humid Mediterranean climatic conditions, where soils show alkaline pH values. Wheat cultivation in this region presents the handicap of being cultivated in NO_3^--vulnerable zones (Cerro et al., 2014); therefore, it is imperative to look for alternatives aiming to increase N use by wheat crops.

Hypothesis and Objectives



Global food grain production has increased during the last decades due to key elements such as the higher use of chemical fertilizer. However, the nature of these intensive production systems makes agricultural soils to lose nearly 70% of the applied nitrogen to the environment as reactive nitrogen. Therefore, the main objective of this thesis was to study whether the use of synthetic and biological nitrification inhibitors would lead us to reach agricultural sustainability by reducing nitrous oxide emissions and enhancing nitrogen use efficiency when ammonium-based fertilizers are applied.

The principal hypothesis of this thesis was: **the control of nitrification allows us to achieve sustainable agriculture by reducing reactive nitrogen losses derived from the use of ammonium-based fertilizers to the environment.**

To test this hypothesis, six experimental works were conducted, each one focused on trying to test a specific partial hypothesis.

1. Many studies in the literature report that the increment of CO_2 concentration modifies the photosynthetic assimilation and metabolism of plants as well as soil carbon cycle, which could also interfere with the soil nitrogen cycle. Therefore, it is not clear whether dimethylpyrazole-based synthetic nitrification inhibitors' efficiency could also be affected at increased CO_2 conditions. Accordingly:

   Hypothesis 1: **Dimethylpyrazole-based synthetic nitrification inhibitors will remain equally effective under elevated CO_2 conditions.**

   The established objectives to test this partial hypothesis were:

   - To study the effect of ammonium nutrition on the growth of barley plants in a plant-soil microcosm fertilized with ammonium sulphate with or without synthetic nitrification inhibitors at ambient and elevated CO_2 conditions.
   - To compare the evolution of soil mineral nitrogen at ambient and elevated CO_2 conditions.

- To quantify the effects of the use of DMPP and DMPSA on N_2O emissions from nitrifying and denitrifying populations at ambient and elevated CO_2 conditions.

The results are presented in Chapter 1.

2. Although the copper chelating capacity of dimethylpyrazole-based synthetic nitrification inhibitors has been proven, there is no confirmation that their mode of action relies on this ability. Then:

   Hypothesis 2: **The nitrification inhibition mode of action of dimethylpyrazole-based synthetic nitrification inhibitors is based on their copper chelating capacity, leading to an inhibition of AMO activity.**

   The established objectives to test this partial hypothesis were:

   - To evaluate, in pure cultures, the AMO activity and growth of nitrifying bacteria *Nitrosomonas europaea* and the effectiveness of DMPP and DMPSA on inhibiting them.
   - To assess whether the increase of Cu^{2+} concentration in de culture medium reliefs the growth and AMO activity inhibition induced by dimethylpyrazole-based synthetic nitrification inhibitors.

   The results are presented in Chapter 2.

3. Abiotic stresses that affect crop growth might modify the rate of biological nitrification inhibitors exudation since it is related to the physiological state and development of the plant. In plants of pearl millet, an increased exudation of biological nitrification inhibitors has been observed after a defined period of drought stress. Thus:

   Hypothesis 3: **Moderate drought stress will increase the exudation of biological nitrification inhibitors from sorghum to increase the ammonium competition with nitrifying microorganisms.**

The established objectives to test this partial hypothesis were:

- To determine the effects of well-watered and moderate drought conditions on the soil mineral nitrogen content in a sorghum plant-soil microcosm fertilized with potassium nitrate, ammonium sulphate, or ammonium sulphate plus DMPP.
- To study the effects of moderate drought on photosynthesis and growth of sorghum plants.
- To compare the abundance of nitrifying bacteria of soils fertilized with ammonium at well-watered and moderate drought conditions, and the influence of biological and synthetic nitrification inhibitors on the abundance of nitrifying bacteria.

The results are presented in Chapter 3.

4. Synthetic nitrification inhibitors are efficient tools to reduce nitrogen losses. Nonetheless, they are not widely adopted by farmers due to their lack of cost-effectiveness and limited soil stability and mobility. On the other hand, biological nitrification inhibitors seem to be a promising alternative since they are released directly in the place where nitrification is carried out. Accordingly:

   Hypothesis 4: **The biological nitrification inhibition potential makes sorghum a good option as a cover crop in a sorghum/winter wheat rotation since it avoids the nitrogen losses by reducing nitrification in the field. Moreover, the persistence of biological nitrification inhibitors may reduce the need for synthetic nitrification inhibitors.**

   The established objectives to test this partial hypothesis were:

   - To analyse the effects of biological (sorghum crop roots exudates) and synthetic (DMPP) nitrification inhibitors on nitrogen losses through nitrification in a sorghum summer cover crop.

- To evaluate the effects of two different no-till crop rotations (fallow-wheat and sorghum-wheat) on mitigating nitrogen losses through nitrification in a winter wheat crop.
- To determine the effects of sorghum crop precedence on the grain yield of winter wheat crop.
- To compare the potential biological nitrification inhibition activity of sorghum with the use of DMPP in terms of N_2O emissions of a field with winter wheat crop.

The results are presented in Chapter 4.

5. In general, modern cereals lack the ability to exude biological nitrification inhibitors in its root systems. In this way, another step towards enhancing the use of biological nitrification inhibitors is to introduce the genes from wild relatives capable of producing them in elite cereal crops. This was achieved on two elite wheat varieties (ROELFS and MUNAL), getting promising results in acidic soils. However, it is needed validation of their efficiency in different edaphoclimatic conditions and fertilizer types since their potential use is aimed to reach farmers from all over the world. Consequently:

   Hypothesis 5: **ROELFS-BNI and MUNAL-BNI wheat lines are efficient technology to decrease nitrification without affecting their yield. Furthermore, the benefits of harbouring the genes capable of producing biological nitrification inhibitors will avoid the ammonium syndrome under ammonium nutrition.**

   The established objectives to test this partial hypothesis were:

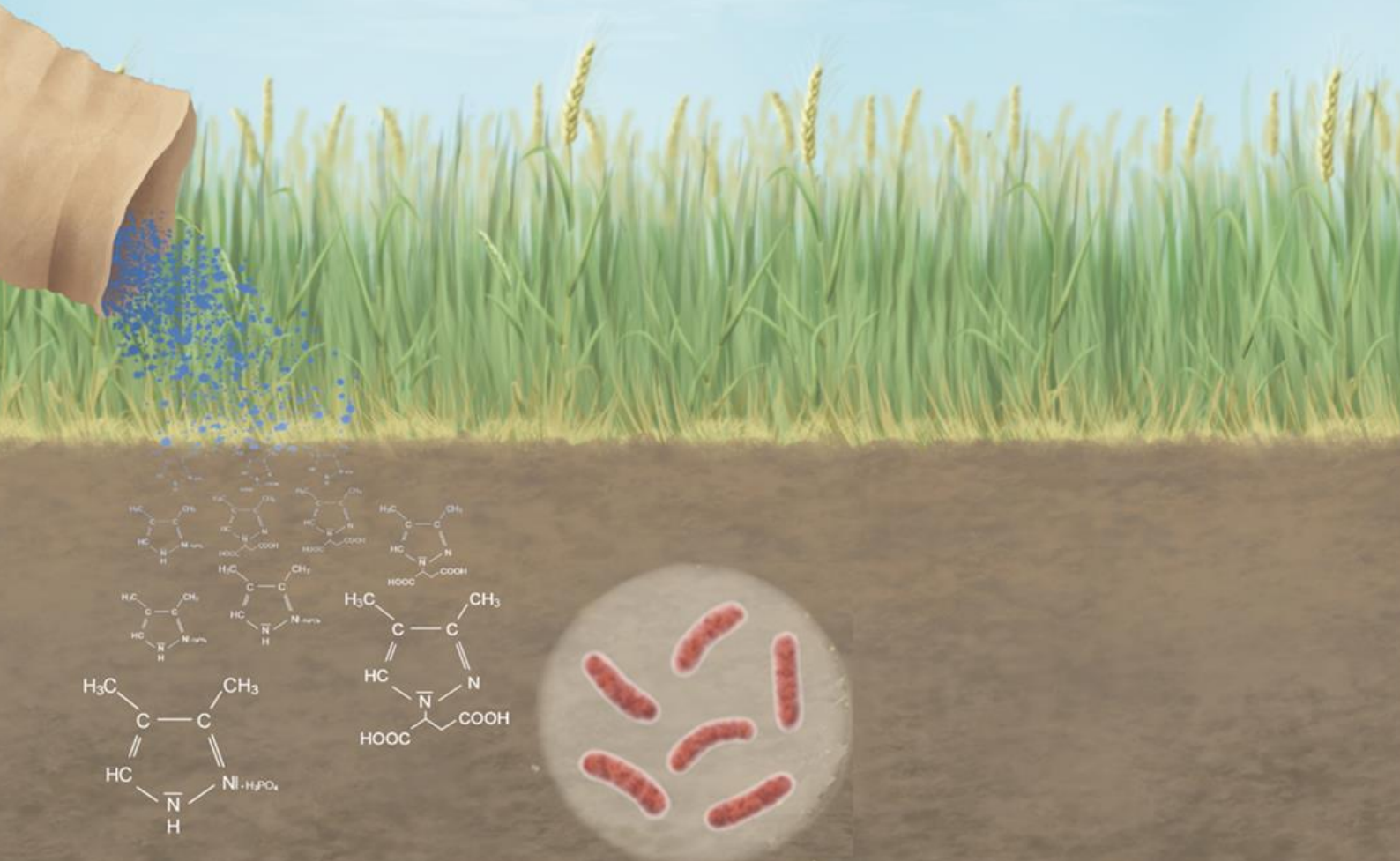
   - To study the nitrogen uptake and plant development of ROELFS-BNI and MUNAL-BNI wheat lines in a plant-soil microcosm fertilized with ammonium sulphate or potassium nitrate.
   - To evaluate the nitrification inhibition potential of ROELFS-BNI and MUNAL-BNI wheat lines in alkaline soil.

- To analyse the effects of the biological nitrification inhibitors released by ROELFS-BNI and MUNAL-BNI wheat lines on denitrifying populations.
- To assess the growth of ROELFS-BNI and MUNAL-BNI wheat lines under ammonium nutrition.

The results are presented in Chapter 5.

Chapter 1

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



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ABSTRACT

Synthetic nitrification inhibitors (SNIs) 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 SNIs 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 SNIs: 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 SNIs 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 SNIs 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 SNIs were highly efficient under eCO_2, reducing N_2O emissions and keeping N in the soil stable for longer while maintaining plant biomass production.

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 (Supplementary 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 x 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:

WFPS = (soil gravimetric water content x bulk density) x (1 - (bulk density / 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}.

Barley plants (Hordeum vulgare) grown in pots with soil in growth chamber

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 hours 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 80ºC for 5 min and centrifuged at maximum speed for 20 min. Supernatants were recovered and stored at -20ºC 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 (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:

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

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

Chambers introduced inside pots for N_2O emissions sampling

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 x 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 minutes (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 x volume of DNA extracted) / (volume of DNA used per reaction x 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.

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

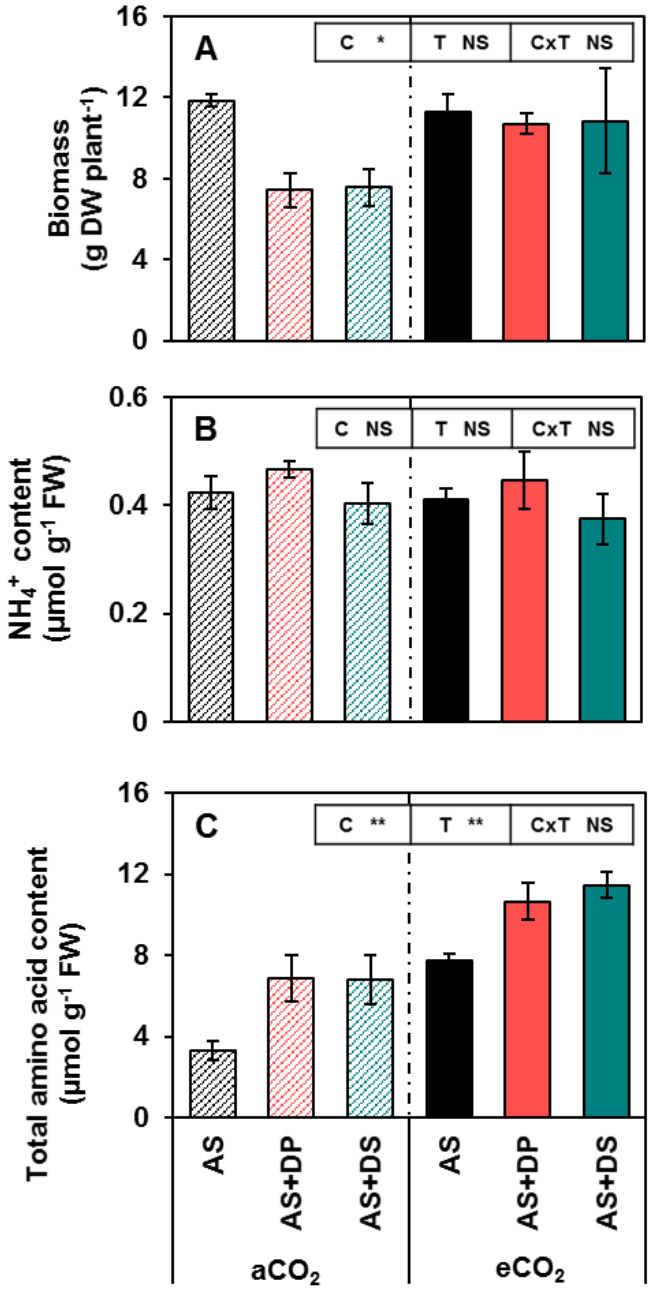


Fig. 1. *Shoot biomass of barley (Hordeum vulgare cv. Henley) plants; (**A**) leaf ammonium (**B**) and total amino acid content (**C**). Pots were fertilized with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). Statistical analysis was made through analysis 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 $p < 0.01$.*

3.2. N and C isotopic composition and accumulation

SNIs did not affect plant N content (Supplementary Fig. 1.A) 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. 1.A). The supply of SNIs 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. 1.B). Nevertheless, although the presence of SNIs 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. 1.C). Besides, plants from eCO_2 treatments had higher values of C:N ratio than those of aCO_2.

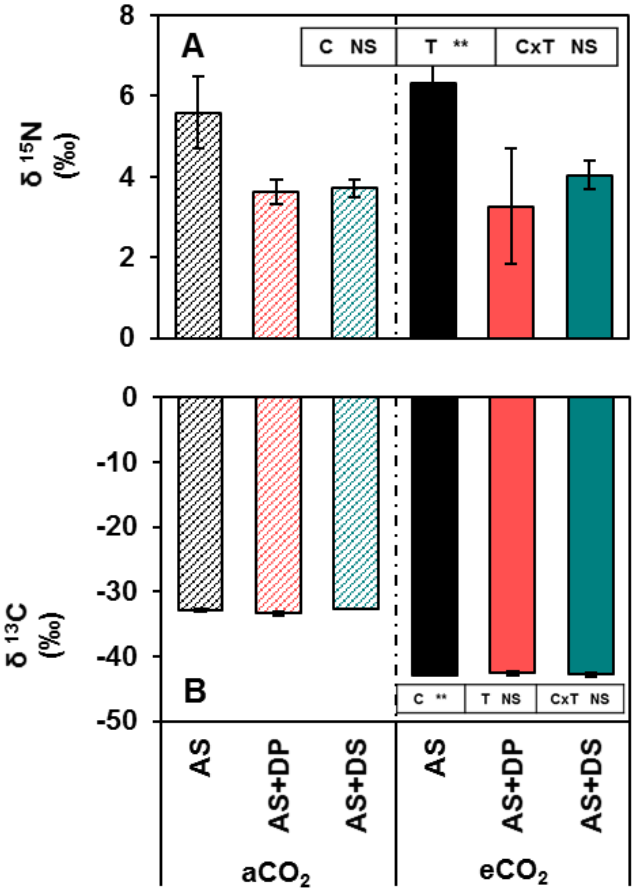


Fig. 2. *Leaf $\delta^{15}N$ (**A**) and $\delta^{13}C$ (**B**). Pots were fertilized with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS).*

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.

3.3. Soil mineral N

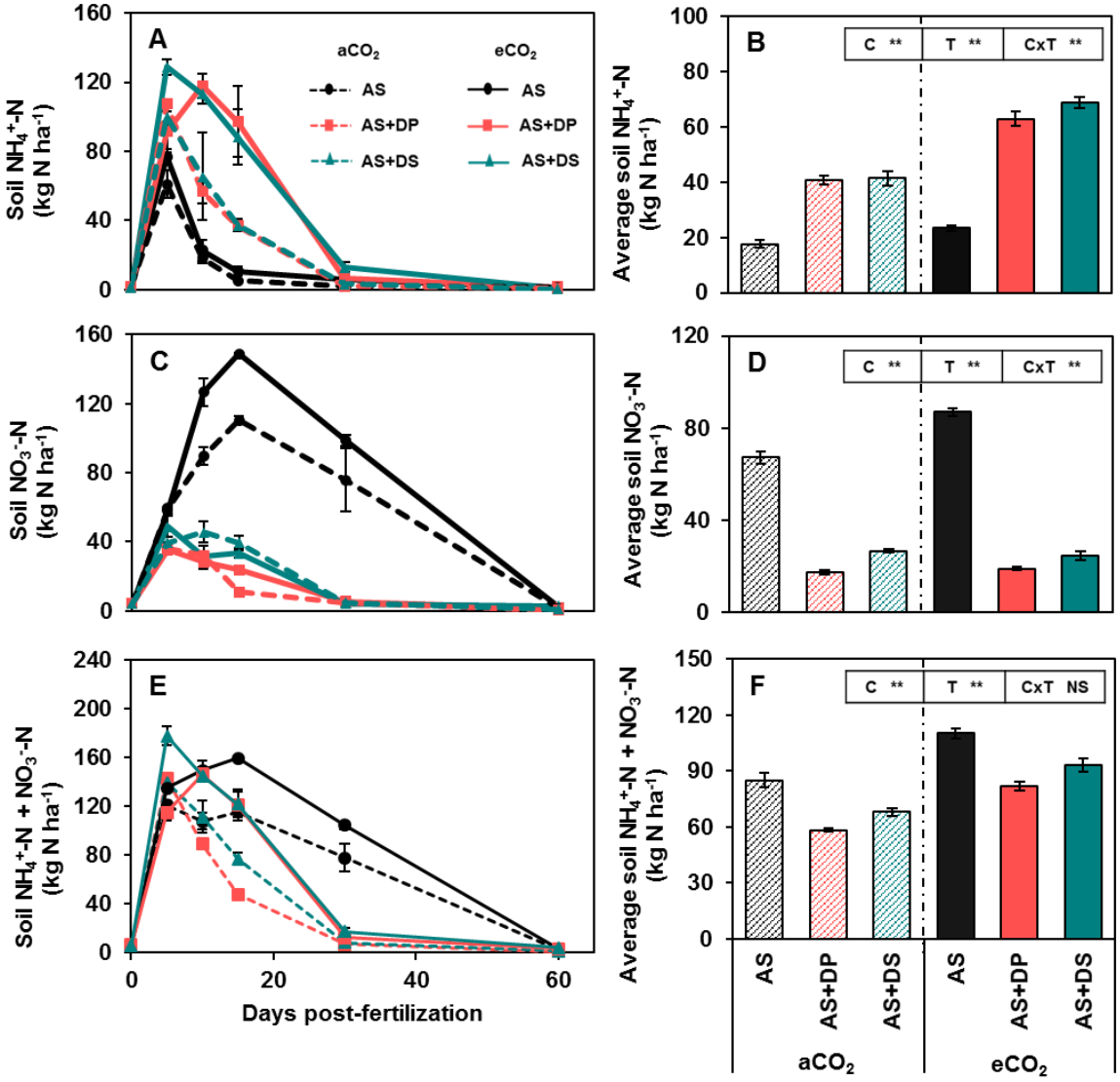


Fig. 3. *Evolution during 60 days of experiment and average of soil mineral nitrogen. NH_4^+ **(A, B)** NO_3^- **(C, D)** and total nitrogen calculated as the sum of NH_4^+ and $NO3^-$ **(E,F)**. Pots were fertilized with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + 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.

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. 3.A). 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. 3.B). On the other hand, AS treatments presented the greatest NO_3^- content in the soil, notably at eCO_2 (Fig. 3.C). 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. 3.D). 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. 3.E). 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 3.F).

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. 4.A). 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. 4.B). 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. 4.B).

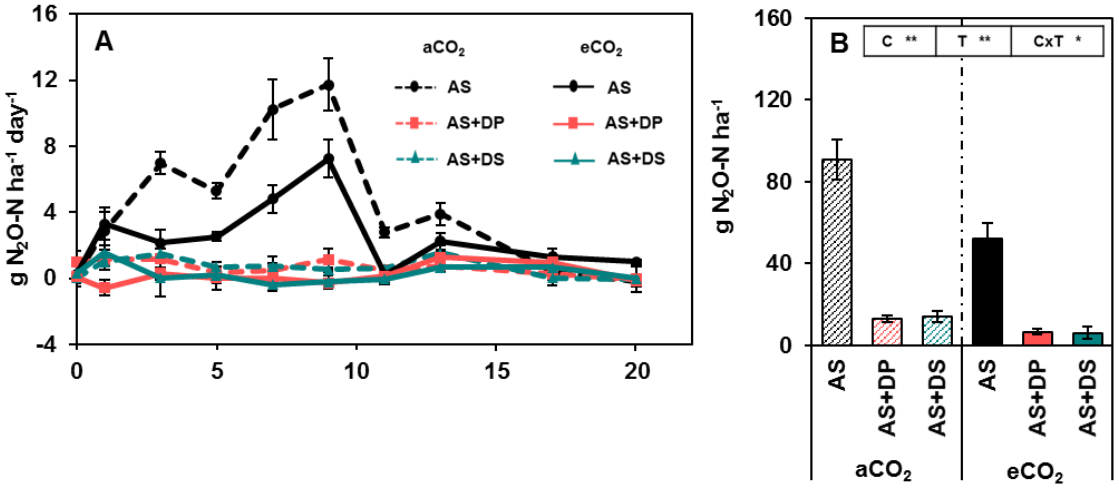


Fig. 4. *Daily (**A**) and cumulative (**B**) N_2O emission during 20 days of experiment. Pots were fertilized with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). For cumulative emissions, statistical analysis was made through analysis of variance (two-way 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 $p < 0.01$.*

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. 5.A). 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. 5.B). 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.

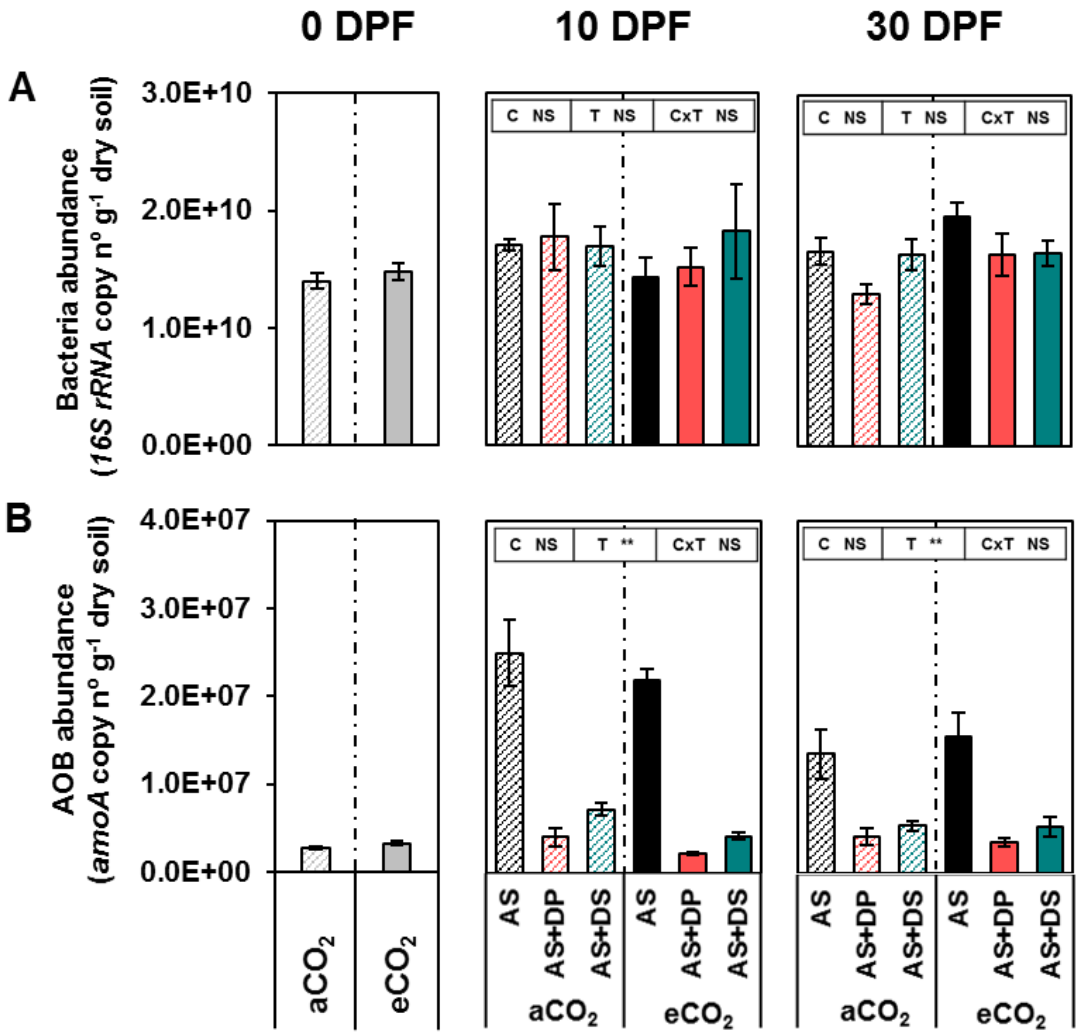


Fig. 5. *Bacteria (**A**) and ammonia oxidizing bacteria (AOB) (**B**) abundance at 0, 10 and 30 days post-fertilization (DPF) with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). Statistical analysis of 0 DPF was made through t-test. Statistical analysis 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.*

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. 6.A). 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 SNIs (Fig.6.B), 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. 6.B).

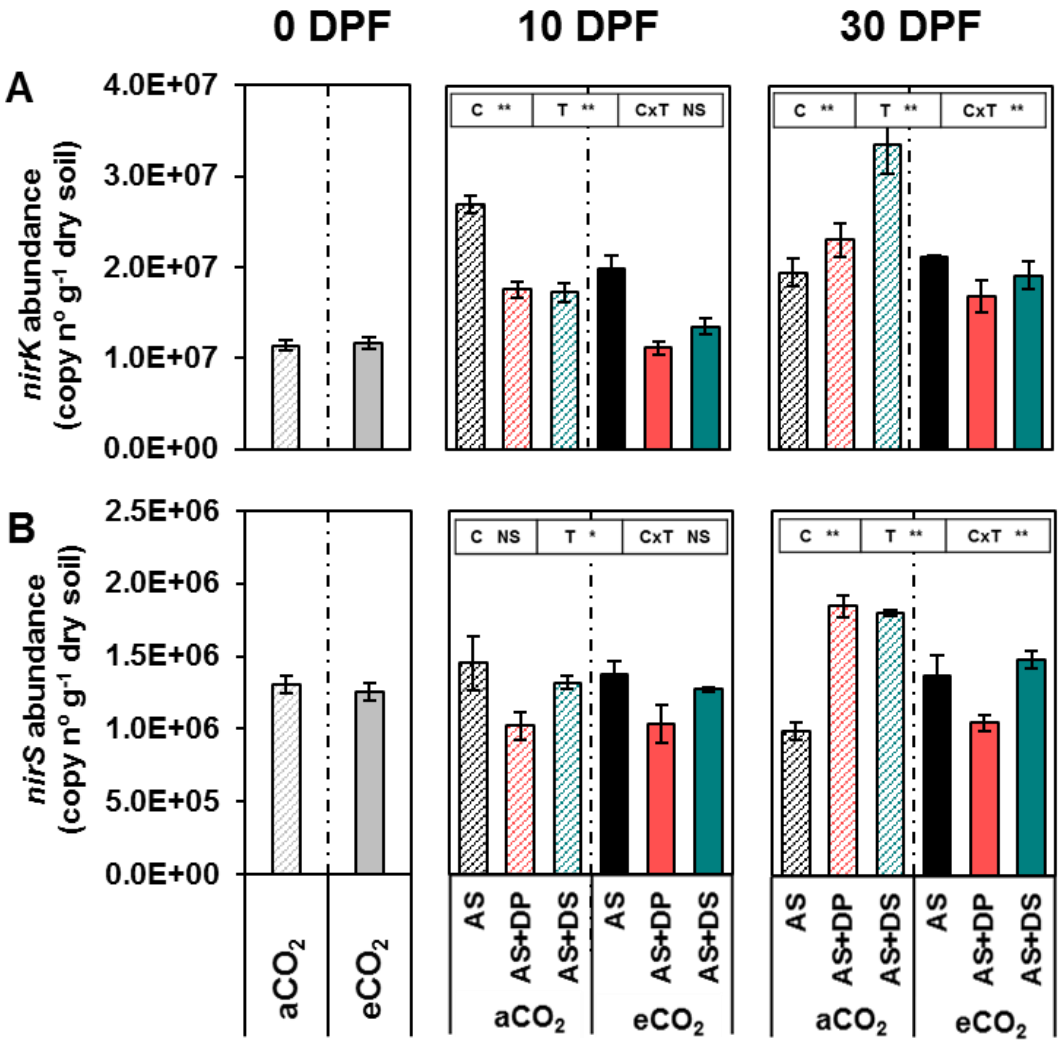


Fig. 6. *Abundance of NIR enzyme containing denitrifying bacteria as nirK (**A**) and nirS (**B**) gene copy number g^{-1} dry soil at 0, 10 and 30 days post-fertilization (DPF) with ammonium sulphate (AS); ammonium sulphate + DMPP (AS+DP) and ammonium sulphate + DMPSA (AS+DS). Statistical analysis of 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

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. 1.A). 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. 1.C). 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 synthetic nitrification inhibitors (SNIs) (Fig. 1.C).

The combination of SNIs 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 SNIs does not affect negatively crop yield in field conditions (Huérfano et al., 2015; Guardia et al., 2018b), in our experiment, barley biomass decreased in presence of DMPP

and DMPSA under aCO_2 (Fig. 1.A). 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. 3.A and 3.B). Ammonium syndrome is characterized by a decreased photosynthesis, 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. 3.A and 3.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 SNIs supply under both CO_2 conditions with respect to AS (Fig. 1.C). However, in line with the carbon shortage hypothesis, the extra supply of C in eCO_2 condition restored barley growth in presence of SNIs (Fig. 1.A). 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 carbon enrichment with carbonates. The absence of differences in leaf NH_4^+ accumulation (Fig. 1.B) may be due to the harvest time (60 days post-fertilization) when soil NH_4^+ content was almost undetectable (Fig. 3.A) 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 SNIs (Fig. 3.A), 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. 2.B). 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. 2.A). 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 SNIs (Supplementary Fig. 1.B). Nevertheless, the effect of eCO_2 provoked a reduction in total leaf N content regardless of the supply of SNIs (Supplementary Fig. 1.A). 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. 3.A and 3.B), SNIs efficiency to delay NH_4^+ oxidation has been associated with the action that SNIs 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. 3.A and 3.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 SNIs 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 SNIs also diminish NO_3^- apparition in the soil (Guardia et al., 2018a; Corrochano-Monsalve et al., 2020a). This was clearly observed in our experiment with the supply of DMPP and DMPSA independently of the CO_2 concentration (Fig. 3.C and 3.D). This is especially relevant because in the absence of SNIs, eCO_2 promoted higher NO_3^- content in soil than aCO_2. Altogether, in eCO_2

conditions, SNIs 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 SNIs 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. 4.A and 4.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., 2021a).

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. 3.B). 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 SNIs 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. 6.A), and, to a lesser extent, the *nirS* abundance (Fig. 6.B). 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 SNIs such as dicyandiamide (DCD) (Di et al., 2009). Instead, a potential direct effect of these SNIs on denitrifiers cannot be fully discarded.

CONCLUSIONS

Under an enriched CO_2 atmosphere, both nitrification and denitrification tended to be weakened. SNIs showed great efficiency avoiding AOB growth because of fertilization at both CO_2 concentrations. In this manner, SNIs 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 SNIs 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 SNIs under enriched CO_2 atmosphere highly recommends their use in future climate scenarios in order to reach a sustainable agriculture.

SUPPLEMENTARY MATERIAL

Supplementary 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**							
						Organic				
Sand	**Silt**	**Clay**	**pH[a]**	**C:N**	**N[b]**	**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

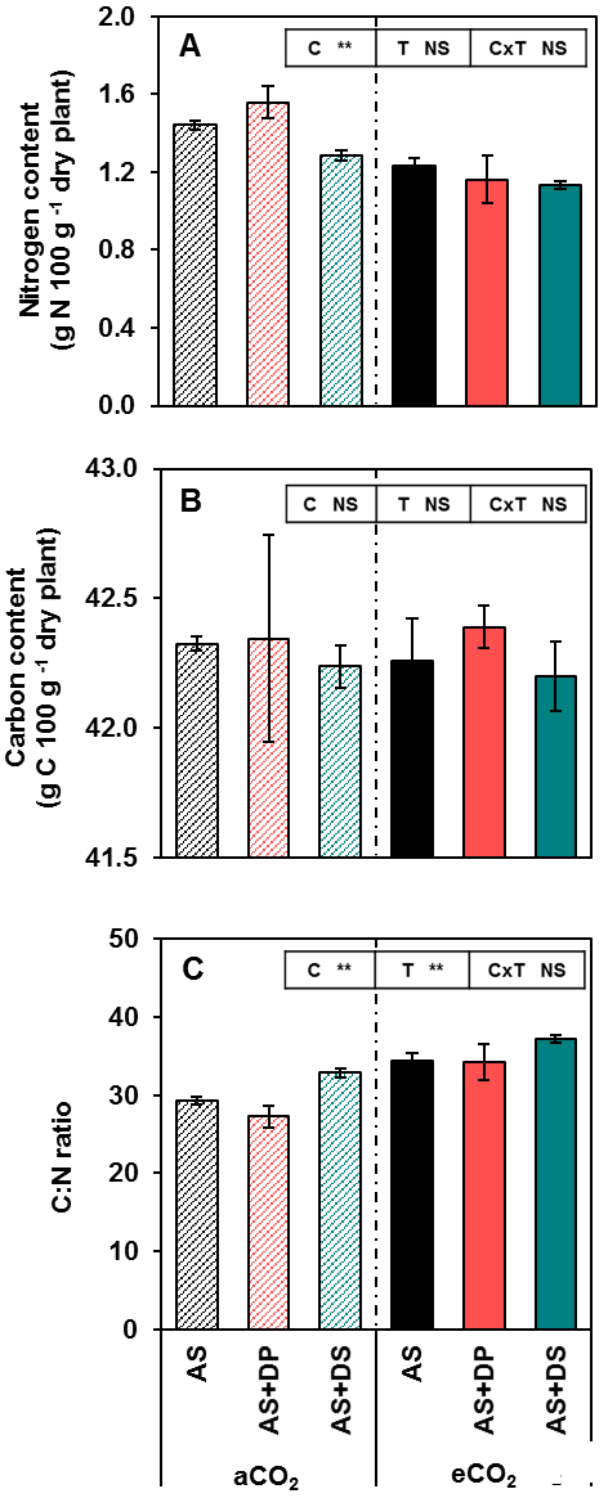
a. pH (1:2.5 soil:water).

b. N Kjeldahl digestion (Keeney and Nelson, 1982).

c. Organic matter (Walkley and Black, 1934).

d. CaCO3 , Mg, K (NH4 AcO, MAPA, 1994).

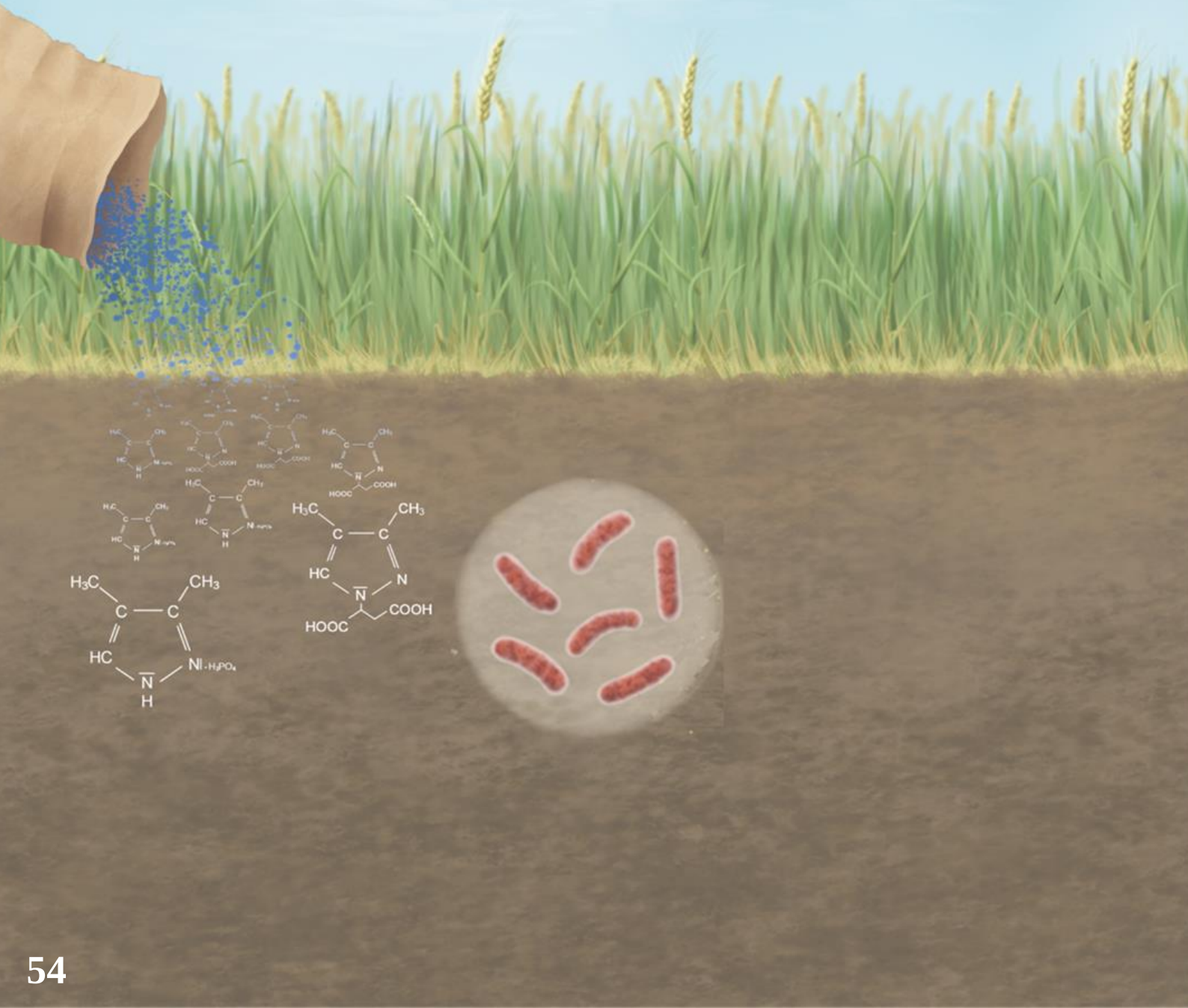
e. P (Watanabe and Olsen, 1965).



Supplemental Fig. 1. *Total leaf nitrogen (**A**) and carbon content (**B**) and C:N ratio (**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 $p < 0.01$.*

Chapter 2

Towards deciphering the mode of action of dimethylpyrazole-based nitrification inhibitors



ABSTRACT

Several changes in agricultural management practices have led to a high-nitrifying soil environment. Nevertheless, synthetic nitrification inhibitors (SNIs) have been developed to suppress soil-nitrifier activity and decrease nitrogen (N) losses. The SNIs 3,4-dimethylpyrazole phosphate (DMPP) and 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA) are able to reduce N_2O emissions and maintain soil NH_4^+ for a longer time. Both dimethylpyrazoles (DMPs) are thought to inhibit nitrification due to their ability to chelate the Cu^{2+} cations that the ammonia monooxygenase enzyme (AMO) needs to carry on the first step of NH_4^+ oxidation. Nevertheless, although the nitrification inhibition seems to be driven by the interaction between DMPs and Cu^{2+}, no more experimental data are available to confirm that this is their exact mode of action. In this work, we aimed for a better understanding of the mode of action of DMP-based SNIs by evaluating the performance of DMPP and DMPSA in pure cultures of nitrifying bacteria *Nitrosomonas europaea* with and without an increase in Cu^{2+} concentration. Additionally, we also studied the behaviour of DMPSA in sterilized and non-sterilized soils. Surprisingly, DMPSA did not inhibit nitrification in pure cultures of *N. europaea*, suggesting that DMPs mode of action may not be related to Cu^{2+} chelation ability. This was later confirmed since the inhibitory capacity of DMPP was not altered regardless of the Cu^{2+} concentration. Moreover, we demonstrated that DMPP exclusively inhibits the AMO enzyme. In the soil, we evidenced that DMPSA needs to be broken into DMP to achieve the inhibition of nitrification. Furthermore, the rupture of DMPSA is through biological processes of soil microorganisms. Therefore, the type of soil and environmental conditions could modify its efficiency.

MATERIALS AND METHODS

2.1. *Nitrosomonas europaea* growth

Pure culture of *Nitrosomonas europaea* ammonia-oxidizing bacteria (strain. ATCC 19718) was supplied by the American Type Culture Collection (Virginia, USA). *N. europaea* was cultivated on growth medium that contained Hepes buffer (pH 8) 11.9 g L^{-1}, $(NH_4)_2SO_4$ 2.5 g L^{-1}, KH_2PO_4 0.5 g L^{-1}, $NaHCO_3$ 0.5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.1 g L^{-1},

$CaCl_2 \cdot 2H_2O$ 0.005 g L^{-1}, NaFe-EDTA 0.004 g L^{-1}, 1 mL L^{-1} of trace elements solution, and 0.5 mL L^{-1} of phenol red solution. The trace elements solution was composed of $MnSO_4 \cdot 4H_2O$ 0.045 g L^{-1}, H_3BO_3 0.049 g L^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.043 g L^{-1}, $(NH_4)_6MO_7O_{24} \cdot 4H_2O$ 0.037 g L^{-1}, and $CuSO_4 \cdot 5H_2O$ 0.050 g L^{-1}. The phenol red solution was prepared by adding 250 mg phenol red to 250 mL distilled water. All the media and solutions were sterilized at 121 ºC for 30 min and immediately cooled to room temperature at a clean bench prior to use. The culture was incubated in an orbital incubator at 28 ºC and 150 rpm. Growth of the culture was monitored through NH_4^+ disappearance, quantified by the Berthelot method (Patton and Crouch, 1977); and NO_2^- apparition, quantified by the Griess reaction (Snell and Snell, 1949). Every two days the medium pH was corrected by adding 10% $NaHCO_3$ until the medium colour was restored.

Nitrosomonas europaea cultures incubated in an orbital incubator

To assess the efficiency of DMPP and DMPSA (hereinafter DMPs) on inhibiting *N. europaea* growth, 100 µL from seven-day-old *N. europaea* culture were added to 20 mL of fresh Hepes medium with or without DMPs. Thus, treatments were i) Control conditions with ammonium sulphate (AS), ii) ammonium sulphate + DMPP (AS+DMPP), and iii) ammonium sulphate + DMPSA (AS+DMPSA). DMPs were

applied in a concentration of 5 mg L^{-1}, representing 0.8% of the present NH_4^+-N, following the same recommendation of manufactures for field application. *N. europaea* was maintained for 7 days. NH_4^+ and NO_2^- evolution was determined on days 0, 1, 3, 5, and 7. At the end of the experiment, the OD_{600} was measured.

For experiments with increased Cu and Zn concentration, 100 µL from seven-day-old *N. europaea* culture were added to 20 mL of three different mediums containing different Cu and Zn concentrations added as $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$. The mediums were i) Hepes medium with 0.01 mg L^{-1} of Cu^{2+} and Zn^{2+} (Standard), ii) Hepes medium with 2.50 mg L^{-1} of Cu^{2+} and 0.01 mg L^{-1} of Zn^{2+} (+Cu), and iii) Hepes medium with 0.01 mg L^{-1} of Cu^{2+} and 2.50 mg L^{-1} of Zn^{2+} (+Zn). Each medium was divided into two different treatments; i) Control conditions with ammonium sulphate (AS), and ii) ammonium sulphate + DMPP (AS+DMPP). DMPP was applied at a rate of 5 mg L^{-1}. NH_4^+ and NO_2^- determinations and OD_{600} measurement were carried out only on day 7.

To evaluate whether DMPP targets AMO or HAO enzyme an actively growing *N.europaea* culture was washed twice with N-free Hepes medium to remove all the residual NH_4^+ and NO_2^-. Then, *N. europaea* was maintained 24 h in N-free Hepes medium to ensure all the possible NO_2^- production from bacteria was released. Later, cultures were washed again twice with N-free Hepes medium. Finally, cultures were resuspended in N-free Hepes medium to which different N sources in a 1 mg N L^{-1} concentration were added; i) $(NH_4)_2SO_4$ ($+NH_4^+$), and ii) NH_2OH ($+NH_2OH$). Each medium was divided into two different treatments; i) Control conditions with ammonium sulphate (AS), and ii) ammonium sulphate + DMPP (AS+DMPP). DMPP was applied at a rate of 5 mg L^{-1}. After 6 h, NO_2^- production was determined.

To address the inhibition capacity of DMPP, 100 µL from seven-day-old *N. europaea* culture were added to 20 mL of fresh Hepes medium holding eight different DMPP rate applications. Rates of DMPP were 0, 0.016, 0.032, 0.048, 0.064, 0.080, 0.160, 0.320 and 0.800 % of the applied NH_4^+-N. 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 uninhibited control (DMPP rate 0):

$$\text{Nitrification inhibition (\%)} = (1 - (\text{rate}_{\text{inhib}}/\text{rate}_{\text{control}})) \times 100$$

2.2. Soil experiments

Soil experiments were carried out in microcosms under controlled greenhouse conditions, with a daily day/night cycle regimen of 25/18 ºC average temperature, and 50/60% relative humidity. Soil was collected in June 2019 from a 0-30 cm layer of a Hypercalcic Kastanozem soil (IUSS, 2014) as in Bozal-Leorri et al. (2021). Before the start of the experiments, soil was supplied with 3.5 mg N kg^{-1} dry soil in the form of ammonium nitrate (NH_4NO_3; equivalent to 10 kg N ha^{-1}) and 500 mg C kg^{-1} dry soil to reactivate soil microorganisms (Menéndez et al., 2012). Later, soil was rehydrated with deionised water up to 50% water-filled pore space (WFPS) calculated as in Linn and Doran (1984):

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

Soil bulk density was determined in the laboratory, with a value of 1.31 Mg m^{-3}, while particle density was assumed 2.65 Mg m^{-3}. Soil was watered every two days to maintain the WFPS at 50% for 14 days.

Pots with soil study the degradation and persistence of DMPP in the greenhouse

To study the degradation and persistence of DMPP and the possible breaking of DMPSA molecule into DMP, six pots (12.5 cm diameter x 7 cm height) were filled with 250 g of dry soil after soil activation. Then, pots were divided into two groups of three pots corresponding to two different treatments; i) AS + DMPP, and ii) AS + DMPSA. 63 mg N kg^{-1} dry soil as $(NH_4)_2SO_4$ was added to soil surface, equivalent to 200 kg N ha^{-1}, mixed with nitrification inhibitor DMPP or DMPSA at a rate of 0.8% of the applied NH_4^+-N. Pots were watered every two 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 x 5 cm depth) on 0, 2, 4, 8, 15, and 30 days post-fertilization.

DMP was extracted from the soil following the protocol described in Benckiser et al. (2013) and it was determined in HPLC as in Rodrigues et al. (2018).

Pots used for assessing DMPSA breakdown in DMP with sterilized and non-sterilized soil

To assess whether DMPSA breakdown in DMP occurs due to abiotic or biotic processes, 72 pots (2.5 cm diameter x 6 cm height) were prepared with 35 g of dry soil and divided into two groups before soil activation, i) sterilized soil, and ii) non-sterilized soil. To sterilize the soil, pots were autoclaved three successive times (121 ºC for 30 min) and later, dried at 80 ºC in a circulation oven for 48 h. Then, soil from all pots was activated, and 3 mg g^{-1} dry soil of cycloheximide was added only to the sterilized soil to further avoid fungal growth (Laughlin et al., 2008). After soil activation, soil from four pots of sterilized and non-sterilized soil was destructively sampled for geochemical determinations. The rest of the pots were divided, within each soil condition, into two groups of 16 pots corresponding to two N treatments. The treatments were i) AS, and ii) AS + DMPSA. 63 mg N kg^{-1} dry soil as $(NH_4)_2SO_4$ was added to soil surface, equivalent to 200 kg N ha^{-1}, alone or mixed with nitrification inhibitor DMPSA at a rate of 0.8% of the applied NH_4^+-N. All of them were watered every two days to maintain the WFPS at to 50% during the 12 days of experiment. Soil was destructively sampled for soil NH_4^+ and NO_3^- content measurement and DMP determination on 0.25, 4, 8, and 12 days post-fertilization.

2.3. Soil determinations

To quantify of soil NH_4^+ and NO_3^- contents, 40 g of fresh soil were mixed with 80 mL 1 M KCl and shaken at 165 rpm for one hour. The soil solution was filtered firstly through Whatman n°1 filter paper (GE Healthcare) and secondly through Sep-Pak Classic C18

Cartridges 125 Å pore size (Waters) to remove particles and organic matter respectively. The Berthelot method (Patton and Crouch, 1977) was followed to quantify the NH_4^+ content. The NO_3^- content was determined according to Cawse (1967).

Extraction of DMP was carried out from 10 g of fresh soil following Benckiser et al. (2013). Briefly, 10 mL of distilled H_2O and K_3PO_4 1 M are added to the samples and shaken upside-down for 2 h at 30 rpm. Later, 0.2 mL $CaCl_2$ 1 M are added followed by another upside-down shake for 1 h at 30 rpm. Subsequently, 1 mL of NaOH is added and shaken again for 30 min at 30 rpm. Finally, samples are shaken again for 1 h at 30 rpm with 15 mL of t-butyl-methyl-ether (MTBE) and, after centrifugation for 5 min at 3000 rpm, DMPP (in the form of DMP) is recovered from the supernatant. DMP was quantified by HPLC (Waters 2690 separation module with a Waters 2487 dual λ absorbance detector) using a 5 µm;25cm×4mmTracer Excel column and a TR-C-160-1 pre-column (Teknokroma) (Rodrigues et al., 2018). HPLC eluent contained 788 mL of phosphate buffer (H_3PO_4 1 mM, KH_2PO_4 5 mM pH 3.3) and 212 mL methanol.

2.4. 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 < 0.001 was considered to be statistically significant differences in pure cultures experiments, while *p*-value < 0.01 was for soil experiments.

RESULTS AND DISCUSSION

3.1. DMPP and DMPSA inhibition is not related to their copper-chelating ability

Crops only use 30 to 50% of the nitrogen (N) applied as fertilizer and the remainder is lost to the environment through NO_3^- leaching, ammonia volatilization, or the emission of greenhouse gasses (GHG) such as nitrous oxide (N_2O) (Wenderborn, 2020). When NH_4^+-based fertilizers are applied, the great majority of these N losses take place after

nitrification transforms soil NH_4^+ into NO_3^-, commonly in less than 10 days (Subbarao et al., 2015). One way to maintain NH_4^+ for longer in the soil and to reduce N pollution, is to slow down nitrification. Synthetic nitrification inhibitors (SNIs) have been proven as an efficient tool to achieve this goal (Subbarao et al., 2006a). The recently developed 2-(3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture (DMPSA) shows the same behaviour as one of the most worldwide utilized SNIs, 3,4-dimethylpyrazole phosphate (DMPP) (Trenkel, 2010), either in field conditions (Huérfano et al., 2016; 2018) or microcosm experiments (Torralbo et al., 2017; Bozal-Leorri et al., 2021). Thus, it was surprising to observe that, in our experiment, DMPSA did not show the same behaviour than DMPP on pure *Nitrosomonas europaea* cultures (Fig. 1). NH_4^+ concentration in the *N. europaea* culture quickly decreased in AS treatment, indicating that nitrification was taking place (Fig. 1A). On the contrary, the addition of DMPP inhibited the growth of the bacteria, so NH_4^+ concentration was maintained during the whole experiment. However, AS+DMPSA presented no signals of inhibition, since NH_4^+ concentration was reduced in a similar way to AS treatment. Accordingly, *N. europaea* produced a great amount of NO_2^- both in AS and AS+DMPSA treatments (Fig. 1B); while AS+DMPP showed almost no NO_2^- formation; another signal of nitrification inhibition by DMPP but not by DMPSA. Consequently, AS and AS+DMPSA treatments had a higher OD_{600}, while the presence of DMPP decreased it by 92% (Fig. 1C). Our results indicate that there was no degradation of any of the DMPs during the experiment; since DMPP and DMPSA maintained their initial concentration in the medium until day 7 (table 1). To date, the mode of action of DMP-based SNIs (hereinafter DMPs) has been barely studied. The majority of the works with DMPs relate their inhibition properties to their attributed Cu^{2+} chelating capacity due to a personal communication of Wiessemeier included in the review of Ruser and Schulz (2015) and later experimentally confirmed in Corrochano-Monsalve et al. (2021a) for both DMPP and DMPSA. In this manner, it is difficult to understand why only DMPP was able to inhibit nitrification in the pure cultures of *N. europaea* (Fig. 1) if the mode of action of both DMPs might be based on their Cu^{2+} chelating capacity. Furthermore, it might be expected that other biological processes with Cu^{2+} requirements within the N cycle could be also affected by the use of DMPs if their mechanism for nitrification inhibition was based on the chelation theory. For example, denitrifiers reduce NO_2^- to nitric oxide (NO) by the copper-containing nitrite reductase encoded by *nirK* (Zumft, 1997). This enzyme can be limited by Cu^{2+} availability. Nonetheless, it has been shown that the *nirK* abundance is not affected by

the addition of DMPs (Duan et al., 2017; Torralbo et al., 2017). Therefore, although both DMPs are capable of chelating Cu^{2+}, our result suggests that their mode of action may not be related to this ability.

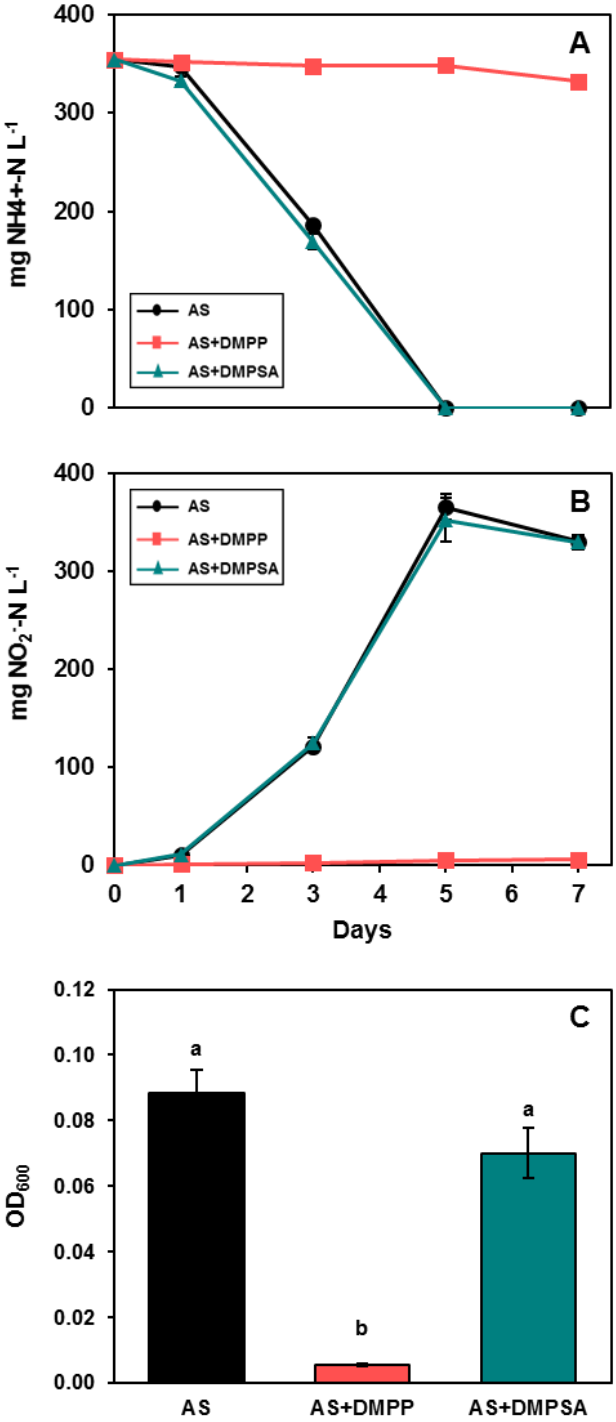


Fig. 1. *Determinations of NH_4^+ (A) and NO_2^- (B) evolution in the Hepes medium during 7 days, and measure of OD_{600} (C) of Nitrosomonas europaea pure cultures at the end of the experiment. N. europaea grew in control conditions with ammonium sulphate (AS);*

ammonium sulphate + DMPP (AS+DMPP), and ammonium sulphate + DMPSA (AS+DMPSA). Different letters indicate significant differences using the Duncan Test ($p < 0.001$; $n = 3$).

To further investigate this hypothesis, we grew *N. europaea* pure cultures with a higher Cu^{2+} concentration. This test was only performed with DMPP since DMPSA was not functional in pure cultures of our experiment. We theorized that, if the mode of action of DMPP were based on the Cu^{2+} chelating capacity, an extra supply of Cu^{2+} would relieve the nitrification inhibition due to a DMPP saturation. We added Cu^{2+} in a similar dose to its EC_{50} to ensure that its concentration was high enough but not completely toxic for *N. europaea* (Ore et al., 2010). In addition, we also tested the growth in presence of a higher Zn^{2+} concentration for two reasons: i) AMO enzyme contains Zn^{2+} and it seems to be necessary for its activity (Radniecki and Ely, 2008; Ruyters et al., 2010; Chen et al., 2014) and ii) DMPs seems to be able to also chelate Zn^{2+} cations in liquid solutions (Corrochano-Monsalve et al., 2021a).

Table 1. *Determination of DMP and both isomers of DMPSA (2,3-DMPSA and 3,4-DMPSA) on days 0 and 7 of growth from Nitrosomonas europaea pure cultures grew in Hepes medium + DMPP (AS+DMPP) and DMPSA (AS+DMPSA).*

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

In AS treatment from the Standard medium, NH_4^+ concentration was almost completely oxidized (Fig. 2A). On the contrary, in +Cu and +Zn mediums, close to EC_{50} Cu^{2+} concentration, *N. europaea* activity of AS treatment was reduced by half, in view of NH_4^+ and NO_2^- concentration in the medium together with bacterial growth (Fig. 2A, B, and C). Importantly, this higher concentration of Cu^{2+} and Zn^{2+} did not alter the inhibitory

capacity of DMPP evidenced by the almost no consumption of NH_4^+, no NO_2^- production, and the absence of *N. europaea* growth in presence of DMPP (Fig. 2).

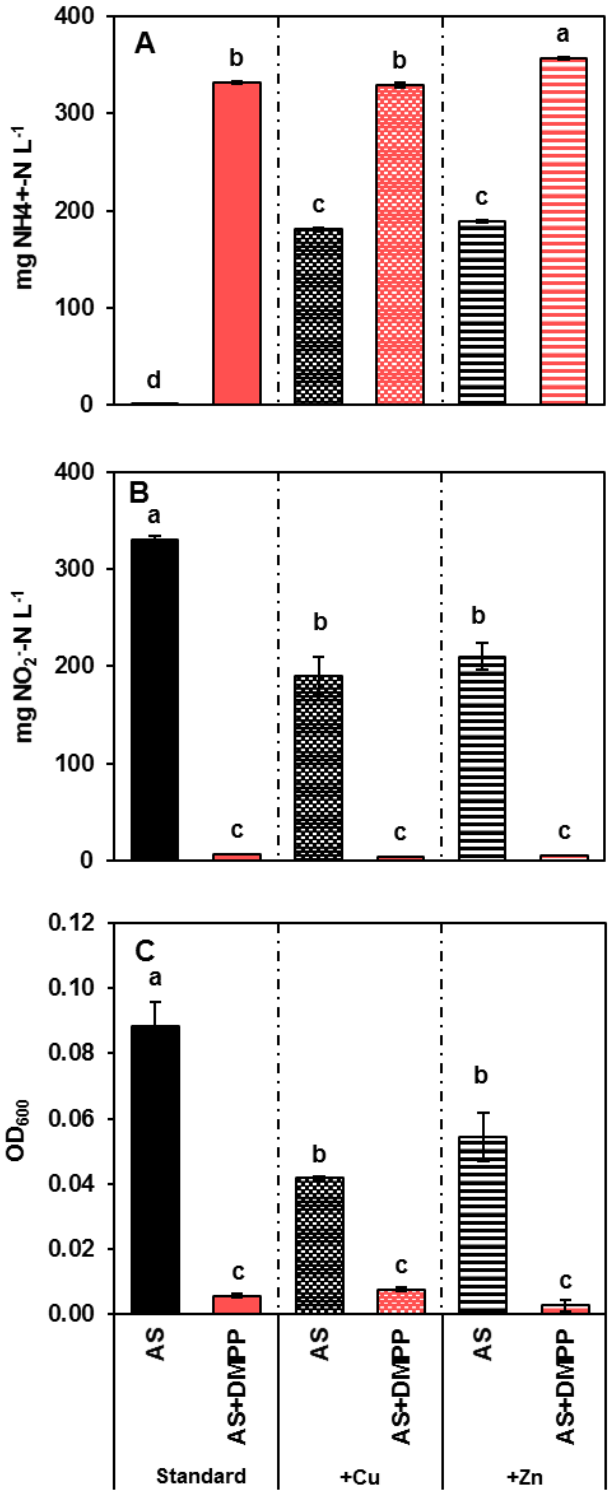


Fig. 2. *Determinations of NH_4^+ (A), NO_2^- (B), and measure of OD_{600} (C) in the Hepes medium with or without an increased concentration of Cu or Zn of Nitrosomonas europaea pure cultures after 7 days of growth. Cu and Zn concentrations increased from 0.01 mg L^{-1} to 2.50 mg L^{-1}. N. europaea grew in control conditions with ammonium*

sulphate (AS) and ammonium sulphate + DMPP (AS+DMPP). Different letters indicate significant differences using the Duncan Test ($p < 0.001$; $n = 3$).

Hu et al. (2003) studied the effect of EDTA chelating agent on nitrification inhibition. These authors observed that EDTA inhibited nitrification but it was relieved when multivalent cations, such as Fe^{3+}, $Ca^{2+,}$ or Mg^{2+} were added to the medium. Furthermore, they showed that the addition of Cu^{2+} partially reduced nitrification inhibition at a 1:1 ratio Cu^{2+}:EDTA. In regards to DMPP, it has been shown that the chelation the chelation efficiency of DMPP is 4 molecules of DMP per Cu^{2+} (Corrochano-Monsalve et al., 2021a). In our experimental conditions, the 20 mL of +Cu medium had 0.83 μmol of Cu (2.50 mg Cu L^{-1}), which means that, according to the findings of Corrochano-Monsalve et al. (2021a), 3.32 μmol of DMPP would be necessary for complete chelation of Cu^{2+}. However, we added 0.56 μmol of DMPP (5 mg DMPP L^{-1}). This means that in case every DMPP molecule was bound to Cu^{2+}, there would still be 83% of the Cu^{2+} available for *N. europaea* to carry out nitrification. However, DMPP in the +Cu medium was just as effective as in the Standard medium. Consequently, these results together with the inability of DMPSA to inhibit *N. europaea* nitrification support that the mode of action of DMPs seems not to be related to their Cu^{2+} and Zn^{2+} chelating capacity. In this way, Corrochano-Monsalve et al. (2021a) could not find convincing reasons to explain the observed induction of the *nosZ* genes (encoding for Cu-containing N_2O reductase enzyme) after DMPs application in several works (Barrena et al., 2017; Fuertes-Mendizabal et al., 2019; Castellano-Hinojosa et al., 2020; Corrochano-Monsalve et al., 2020a) taking into account that the reduction of N_2O to N_2 is not possible without Cu (Glass and Orphan, 2012; Sullivan et al., 2013). Nonetheless, they proposed that the induction of *nosZ* genes might happen because DMPs inhibition of nitrifiers would leave more Cu^{2+} available in the soil that could be used by denitrifiers. Our results support this hypothesis.

3.2. The AMO enzyme is the only target of DMPs

Diverse SNIs have been discovered in the last decades. For instance, Subbarao et al. (2006a) listed 64 synthetic compounds proposed as SNIs. Most of these SNIs act in the first step of nitrification (inhibition of the AMO) and their mechanism of inhibition can be divided into three groups: i) direct binding and interaction with AMO, ii) removal of

co-factors by chelating compounds, and iii) AMO inactivation through the oxidation of highly reactive substrates (Ruser and Schulz, 2015). DMPP was assumed to act on the AMO enzyme by chelating the Cu^{2+} co-factor (Ruser and Schulz, 2015; Corrochano-Monsalve et al., 2021a). However, to our knowledge, no study demonstrated that DMPP inhibits the first step of nitrification. Since we observed that the mode of action of DMPs seems not to be related to their Cu^{2+} chelating capacity, there is the possibility that they might not act on the AMO enzyme. To test this issue, the nitrification inhibitory capacity of DMPP was tested by growing *N. europaea* with hydroxylamine (NH_2OH, substrate of the HAO enzyme) instead of NH_4^+ and compared it to control conditions with 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 nitrification inhibition was determined 6 h after treatment application (Arp and Stein, 2003; Subbarao et al., 2006b). As expected, with NH_4^+ as the source of N, DMPP was efficient to inhibit nitrification as shown by 32% less NO_2^- production compared to AS treatment (Fig. 3). 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 it exclusively inhibits the AMO enzyme.

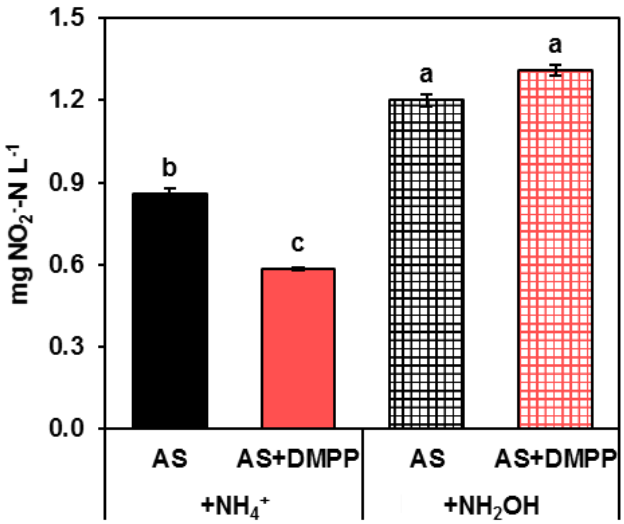


Fig. 3. *Determination of NO_2^- in the Hepes medium with NH_4^+ or NH_2OH as nitrogen source of Nitrosomonas europaea pure cultures after 6 h of the nitrogen addition. N. europaea grew in control conditions with ammonium sulphate (AS) and ammonium sulphate + DMPP (AS+DMPP). Different letters indicate significant differences using the Duncan Test ($p < 0.001$; $n = 3$).*

3.3. DMPSA needs to be broken into DMP to inhibit nitrification

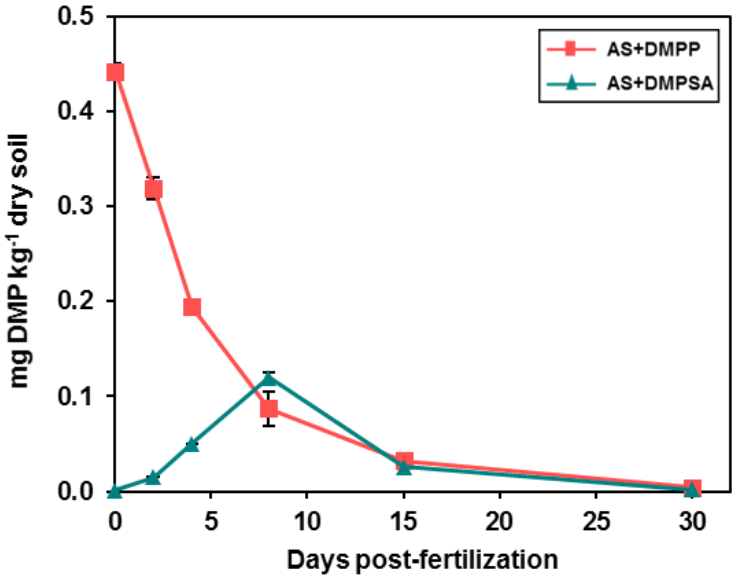


Fig. 4. *Determination of soil DMP content during 30 days of experiment. Pots were fertilized with ammonium sulphate + DMPP (AS+DMPP) and ammonium sulphate + DMPSA (AS+DMPSA).*

Although DMPSA is not able to inhibit nitrification in pure cultures of *N. europaea*, its inhibition capacity in soil has been largely proven (Huérfano et al., 2016; 2018; 2022; Corrochano-Monsalve et al., 2020a; 2021a; 2021b; Recio et al., 2020; Montoya et al., 2021). Therefore, there might be a process taking place in the soil but not occurring in nitrifiers' pure culture. There is another assumption with respect to the mechanism of action of DMPs. Some authors state both DMPP and DMPSA act in a similar manner because it is believed DMPSA needs to be decomposed to dimethylpyrazole (DMP, the active constituent of DMPP) in order to be active as an inhibitor (Pacholski et al., 2016). Indeed, 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, at the same time, this dossier also asserts that degradation of DMPSA into DMP does take place in the soil. Nevertheless, to date, there are no published studies that have confirmed this aspect. Hence, we monitored DMP apparition in soil incubations with DMPSA and we used DMPP as a control. DMP concentration decreased quickly in AS+DMPP treatment, almost disappearing at 30 days post-fertilization (Fig. 4). On the other hand, it seems that DMPSA was broken in the soil

since, from its application, the DMP concentration started to increase after day 2 post-fertilization, reaching its peak on day 8 post-fertilization. At day 8 post-fertilization, DMP concentration in AS+DMPSA was very similar to AS+DMPP treatment. Afterwards, both decreased in a similar way. Thus, it was confirmed that the covalent bond between the succinic acid and the DMP is broken in the soil, in contrast to the registration dossier (EC number 940-877-5) since DMP starts appearing shortly after the 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 may not be sufficient to achieve nitrification inhibition. To answer this question we conducted an experiment in pure cultures of *N. europaea* where we applied different rates of DMPP. As expected, a decrease in NO_2^- production was observed with an increasing rate of application of the nitrification inhibitor DMPP (Fig. 5). Moreover, in line with the results obtained by O'Sullivan et al. (2017) for *N.europaea* and *Nitrosospira multiformis*, we observed that when the rate of DMPP is 10 times lower than the recommended by the manufacturer, it still achieved a high nitrification inhibition (92%), similar to the inhibition at the highest rate (97%). Based on these results, the DMP concentration found on day 8 post-fertilization of AS+DMPSA treatment (4.2 times lower) (Fig. 4) might be enough to efficiently inhibit nitrification. Since DMPSA breakdown did not occur in pure culture (table 1), these results point out that DMPSA needs to be broken into DMP to inhibit nitrification.

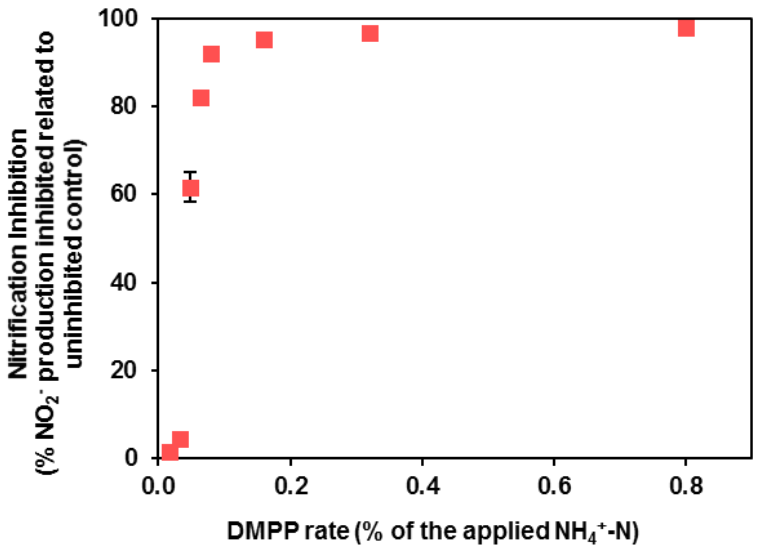
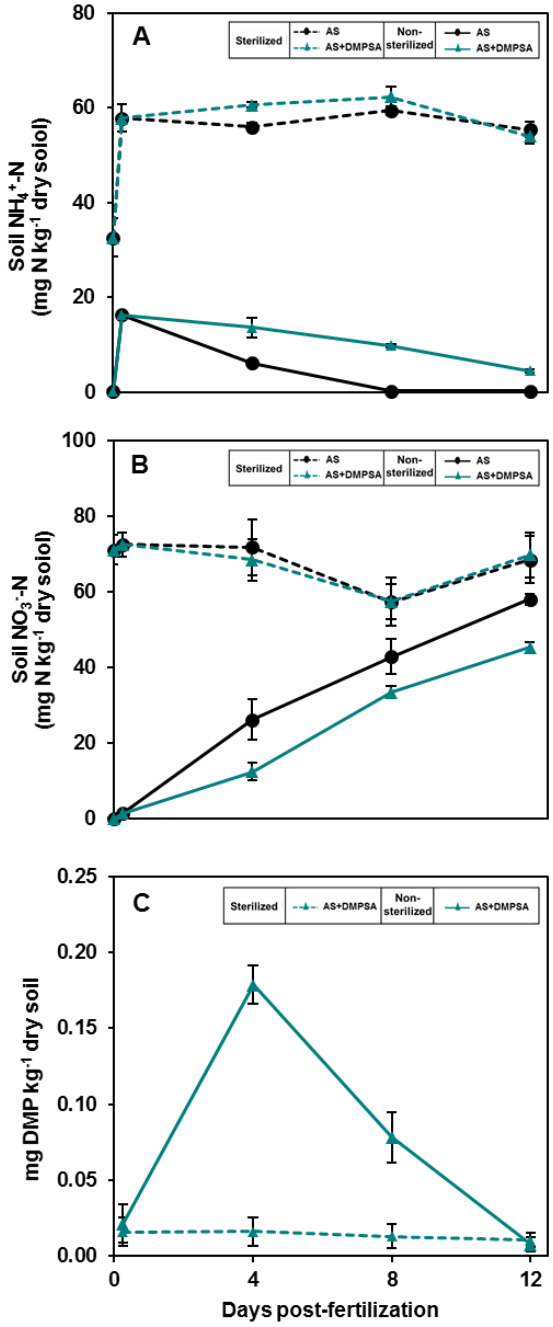


Fig. 5. *Inhibition of nitrification in Nitrosomonas europaea pure cultures with increasing rate of DMPP application related to uninhibited control.*

3.4. Soil microorganisms are responsible for DMPSA rupture

The breakdown of DMPSA was thought to be unlikely spontaneous due to the high energy (305 kJ mol^{-1}) required to rupture the covalent C – N bond (Luo et al., 2007).). Nevertheless, we observed that DMPSA is degraded in the soil but not in microbial cultures, which may indicate i) soil biological activity, other that purely nitrifiers, somehow cooperate in DMPSA break or ii) soil physical/environmental processes, including ultraviolet radiation, could be mediating DMPSA break. To test this hypothesis, we performed an experiment where sterilized and non-sterilized soils were incubated with DMPSA to find out whether the breakdown of DMPSA is through chemical or biological processes. Sterilized soils presented no differences in soil NH_4^+ content between fertilized treatments, maintaining a high concentration during all the experiment (Fig. 6A). On the contrary, soil NH_4^+ content rapidly decreased in AS treatment from non-sterilized soil. Although soil NH_4^+ content of AS+DMPSA treatment from non-sterilized soil was lower than that of sterilized soil, it was able to keep more NH_4^+ in the soil for a longer time compared to AS treatment. Specifically, AS+DMPSA treatment maintained twice and 53-times the amount of soil NH_4^+ on days 4 and 8 post-fertilization respectively. There were no differences in soil NO_3^- content between fertilized treatments from sterilized soil (Fig. 6B). However, AS treatment from control soil showed an increasing soil NO_3^- content during the experiment that reached similar values to those of sterilized soil at day 12 post-fertilization. On the other side, AS+DMPSA treatment reduced the NO_3^- formation 53% on day 4 post-fertilization and 22% on days 8 and 12 post-fertilization compared to AS treatment. It is clear that the prevention of NH_4^+ oxidation to $NO3^-$ in the control soil is a signal of inhibited nitrification by DMPSA (Ekwunife et al., 2021). This inhibition was probably caused by the rupture of DMPSA into DMP since high DMP concentration was observed on day 4 post-fertilization in AS+DMPSA treatment, which was decreasing during the experiment until its disappearance on day 12 post-fertilization (Fig. 6C). No DMP concentration could be noticed in AS+DMPSA treatment from sterilized soil (Fig. 6C). To our knowledge, this is the first confirmation that the breakdown of DMPSA into DMP is through biological processes of soil microorganisms. Furthermore, our results seem to indicate that the microorganisms associated to DMPSA rupture are not *N. europaea*, in agreement with the fact that we did not find DMP in pure cultures that grew with DMPSA. Therefore, the breaking of DMPSA has to be carried out

by another group of microorganisms. Nevertheless, more experiments should be conducted to confirm this hypothesis.



		Days post-fertilization			
		0.25	4	8	12
Sterilized	AS	a	b	a	a
	AS+DMPSA	a	a	a	a
Non-sterilized	AS	b	d	c	b
	AS+DMPSA	b	c	b	b

		Days post-fertilization			
		0.25	4	8	12
Sterilized	AS	a	a	a	a
	AS+DMPSA	a	a	a	a
Non-sterilized	AS	b	b	ab	a
	AS+DMPSA	b	c	b	b

		Days post-fertilization			
		0.25	4	8	12
Sterilized	AS+DMPSA	a	b	b	a
Non-sterilized	AS+DMPSA	a	a	a	a

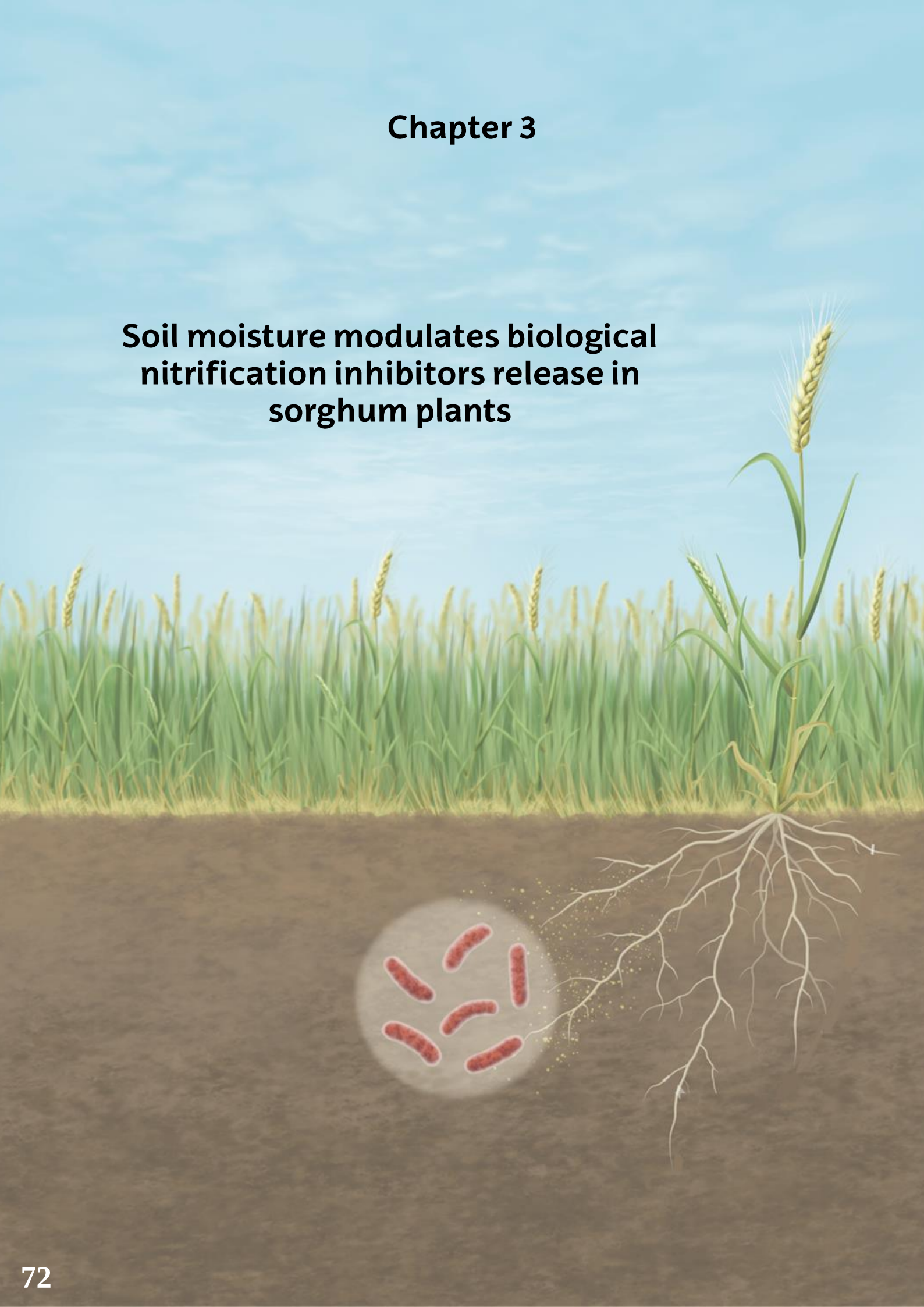
Fig. 6. *Soil mineral nitrogen in the form of NH_4^+ (A) and NO_3^- (B), and determination of soil DMP content (C) during 12 days of experiment. Pots were fertilized with ammonium sulphate (AS) and ammonium sulphate + DMPSA (AS+DMPSA). Different letters in A and B indicate significant differences using the Duncan Test ($p < 0.01$; $n = 4$).*

CONCLUSIONS

DMP-based SNIs are an efficient tool to diminish N losses in agricultural soils. Surprisingly, although it is well known that both DMPP and DMPSA are able to delay the oxidation of NH_4^+ in the soil, DMPSA did not inhibit nitrification in pure cultures of the nitrifying bacteria *N. europaea*. The DMPs inhibition mode of action was thought to be based on the Cu^{2+} chelating capacity, but our results suggests that their mode of action may not be related to this ability. The inhibitory capacity of DMPP was not altered regardless of the Cu and Zn concentrations on the growing medium of *N. europaea*, evidenced by the almost no consumption of NH_4^+, NO_2^- production, and the absence of bacterial growth. Moreover, we also demonstrated that DMPP deploys its action on AMO enzyme, but does not affect the HAO enzyme. On the other hand, we evinced that, to achieve the inhibition of nitrification in the soil, DMPSA needs to be broken to release the active constituent that affects the AMO enzyme, which is DMP. This rupture of DMPSA into DMP take place through biological processes of soil microorganisms, as no DMP concentration could be noticed in the sterilized soil after the application of DMPSA. Therefore, since the breaking of DMPSA is carried out by soil biological activity, the type of soil and environmental conditions might modify its efficiency. To sum up, the experimental evidence that this work provides could lead to a better understanding of the mode of action of DMP-based SNIs and an improvement in their use in the field.

Chapter 3

Soil moisture modulates biological nitrification inhibitors release in sorghum plants



ABSTRACT

Sorghum (*Sorghum bicolor*) is the fifth most cultivated cereal. Since it is adapted to low nitrogen (N) environments, sorghum is able to exude allelochemicals with biological nitrification inhibition (BNI) capacity. These molecules are exuded into the rhizosphere where they delay the oxidation of NH_4^+. Moreover, sorghum might be an option as a 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 or even prevent it. Hence, the objective of this work was to determine the effect of drought stress on the release of BNI from sorghum plants. In field conditions, the effects of sorghum crops over ammonia-oxidizing bacteria (AOB) were monitored for three consecutive years. In a plant-soil microcosm, the soil was divided into two water regimens, Watered (60% WFPS) or Moderate drought (30% WFPS). Soil with or without sorghum plants var. PR88P6 was fertilized with ammonium sulphate (A), ammonium sulphate + DMPP (A+D), or potassium nitrate (K). Soil mineral N was determined together with AOB that were quantified by qPCR. In addition, plant biomass, isotopic discrimination of N and C, and photosynthetic parameters were measured. In the driest year, sorghum was able to reduce the AOB relative abundance by 50% in field conditions. In the plant-soil microcosm, reduced photosynthetic rate, stomatal conductance, and less negative $\delta\ ^{13}C$ values were clear indicators that sorghum plants were suffering from drought stress. In these conditions, AOB abundance of A treatment from Moderate drought regimen was reduced 30% compared to Watered treatment. It seems that under moderate drought conditions, sorghum plants increase their roots exudation and BNI release to increase the N competition with nitrifying microorganisms.

MATERIALS AND METHODS

2.1. Field experiment

This first work was conducted in three different fields from Garinoain, northern Spain (42° 35’ N, 1° 40’ W, 532 m above sea level) during three successive summer seasons 2017, 2018, and 2019. Soil characteristics of the upper horizon of the three lands are

compiled in supplementary table 1. Sorghum (*Sorghum bicolor* var. PR88P68 Pioneer Corteva Agriscience®) was sown at a rate of 15 kg seeds ha^{-1} on May of each year after a previous hairy vetch (*Vicia villosa*) winter cover crop that was terminated with glyphosate and left on the soil surface. The experiment consisted of two randomized blocks and two treatments (5 m x 5 m plots) in each block: 1) fallow (Fallow) and 2) sorghum without fertilization (Sorghum). For fallow plots, sorghum plants were desiccated with a glyphosate-based herbicide one month after sorghum sowing.

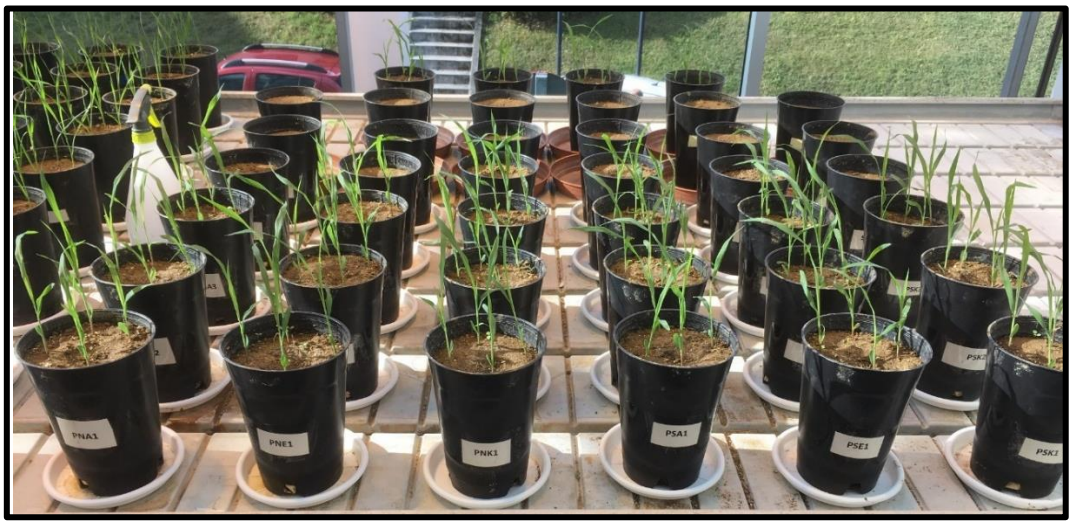
Field with summer fallow (left) and sorghum cover crop (right)

2.2. Greenhouse experiment

This 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 of 25/18° C, a relative day/night humidity of 50/60%. 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, 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 1.35 L pots were filled with soil. In order to reactivate soil microorganisms, pots were supplied with 86 mg of nitrogen 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:

WFPS = (soil gravimetric water content x bulk density) x (1 - (bulk density / 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}.



Pots with soil and with /without sorghum plants in the greenhouse

After 14 days, 4 seedlings of sorghum (*Sorghum bicolor* var. PR88P68 Pioneer Corteva Agriscience®) per pot were placed in eighteen of the thirty-six pots. 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, pots were divided depending on the water regimen. Eighteen pots (nine pots with soil and nine pots with soil and sorghum plants) held "Watered" regimen and the other eighteen the "Moderate drought" regimen. At the same time, pots of each water regimen were randomly divided into three groups of six pots corresponding to three different fertilizer treatments. The fertilizer treatments were: ammonium sulphate (A), ammonium sulphate combined with DMPP (A+D) and potassium nitrate (N). Nitrogen was applied in an equivalent dose to 195 kg N ha^{-1}, which was achieved by adding 1726 mg of potassium nitrate (δ ^{15}N value of 16.5) or 1128 mg 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 treatments application, "Watered" pots increase their WFPS up to 60% while "Moderate drought" pots decrease to 30%. All of them were watered every two days in order to maintain each WFPS for 60 days more.

2.3. Plant biomass and isotopic discrimination

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

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

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.

2.4. Leaf determinations

Gas-exchange measurements were conducted in totally expanded flag leaves using a Li-COR 6400XP portable photosynthesis system (LI-COR Inc.). 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. Maximum quantum of PSII (Fv/Fm) was measured in the centre of the youngest fully developed leaf with a mini-PAM (miniaturized pulse amplitude–modulated photosynthesis yield analyser).

2.5. Geochemical analysis

Measures of soil NH_4^+ and NO_3^- content were made the day after sorghum harvest for field experiment while those of greenhouse experiment were made at 30 and days post-fertilization. Three soil subsamples of 3 cm diameter × 0.3 m depth for field experiment and 1.5 cm diameter × 10 cm depth for the greenhouse experiment were taken. Later, rocks 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 through Whatman n°1 filter papers (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).

2.6. Abundance of nitrifying bacteria

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. 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 (*16S 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 (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 10^2 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 x volume of DNA extracted) / (volume of DNA used per reaction x gram of dry soil extracted)] / DNA concentration.

2.7. 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. The results of leaf gas-exchange measurements, and Fv/Fm determinations were subject to a two-way (water regimen, W; and fertilizer treatment, F) analysis of variance statistical analysis. 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

3.1. Three-year comparison of fallow and sorghum field

Sorghum crop was able to inhibit 50% the *amoA* relative abundance 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 (Fig. 1B). In 2018, summer was moister than the previous one, presenting an accumulated rainfall of 240 L m^{-2}. In turn, the sorghum crop was not able to reduce the growth of nitrifying bacteria, presenting the same *amoA* relative abundance as the soil with fallow. In 2019, the level of accumulated precipitation at the end of the cultivation was average compared to the previous two years, with a value of 200 L m^{-2}. Even though it was not statistically significant, sorghum crop was able to reduce a 16% the *amoA* relative abundance.

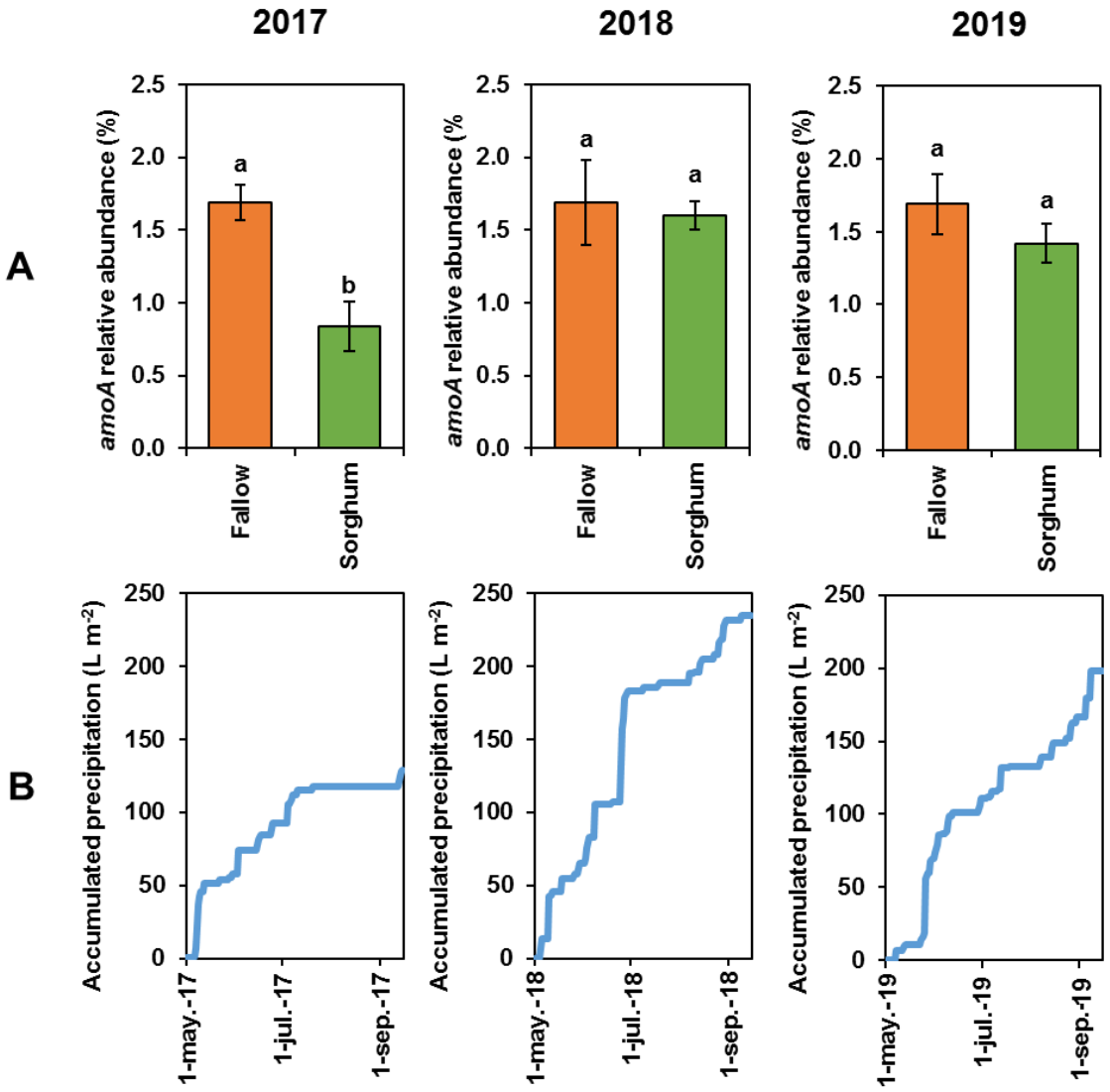


Fig. 1. *Ammonia oxidizing bacteria (AOB) relative abundance (measured as the relative abundance of gene amoA) at the end of sorghum crop (**A**) and accumulated precipitation during sorghum development (**B**). The Mann-Whitney U test was used for the comparison between fallow and sorghum plots. Significant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

On the other hand, the presence of the sorghum crop made a great reduction of soil N content compared to fallow in all three years (Fig. 2). Although there were no differences in soil NH_4^+ content (Fig. 2A) between soil with fallow and soil that held sorghum, the effect was noticeable on soil NO_3^- content. In 2017, the soil that held sorghum reduced 44% soil NO_3^- content compared to soil with fallow (Fig. 2B). In 2018, the soil NO_3^- content from soil with fallow was more than 10 times than that of soil that held sorghum. In 2019, the soil NO_3^- content from soil with fallow was lower compared to the previous

years, but it could be due to a lower soil organic matter (Supplementary table 1). Nevertheless, the soil that held sorghum also presented a reduction of 68% on soil NO_3^- content.

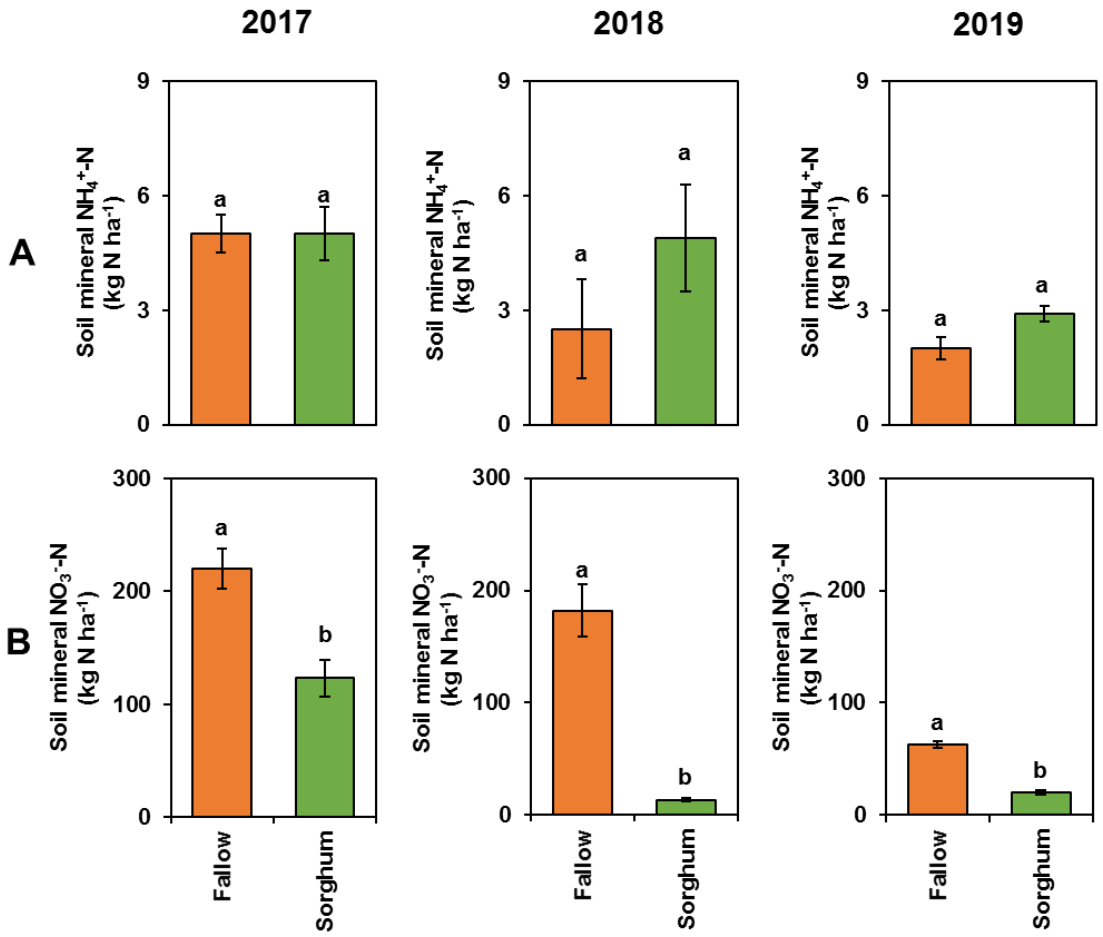


Fig. 2. *Determination of soil mineral nitrogen in the form of NH_4^+ (**A**) and NO_3^- (**B**) at the end of sorghum crop during three years of campaign. The Mann-Whitney U test was used for the comparison between fallow and sorghum plots. Significant differences at p <0.05 are marked with a letter.*

3.2. Effect of moderate drought conditions

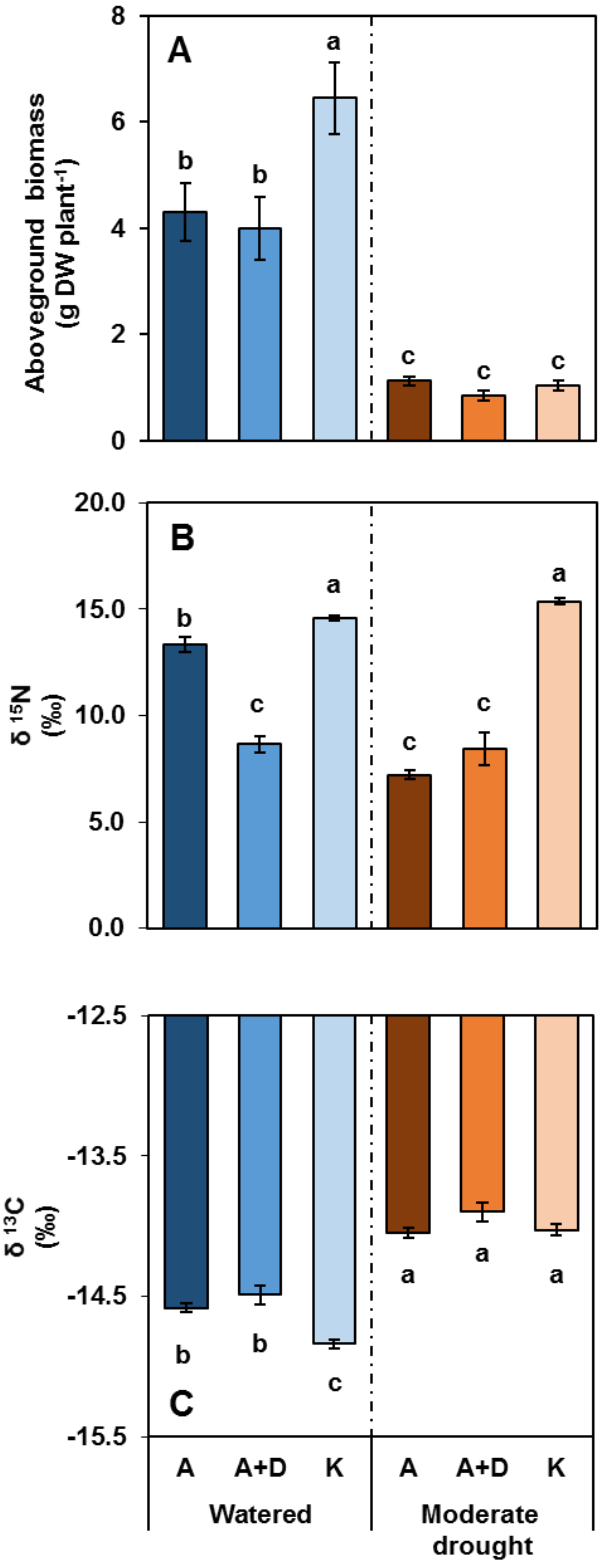


Fig. 3. *Dry aboveground biomass of sorghum (Sorghum bicolor) plants (**A**) and leaf determination of $\delta^{15}N$ (**B**) and $\delta^{13}C$ (**C**). Pots were fertilized with ammonium sulphate (A); ammonium sulphate + DMPP (A+D) and potassium nitrate (K). Significant*

differences between treatments are marked with a lowercase letter (Duncan Test; p <0.05; n = 4).

Regarding sorghum plants photosynthesis, fertilization showed no effect on any of the measured parameters in either Watered regimen or Moderate drought regimen (Fig. 4). Nevertheless, strong differences were found comparing both water regimens. The net photosynthetic rate decreased an average of 47% on treatments from Moderate drought regimen (Fig. 4A). Similarly, the stomatal conductance of treatments from Moderate drought regimen was 48% lower compared to those of Watered regimen (Fig. 4B). On the contrary, the intercellular CO_2 increased in treatments from Moderate drought regimen, being 43% higher compared to Watered regimen (Fig. 4C). Since photosynthesis was decreased in Moderate drought regimen, akin results were found in the maximum quantum of PSII. Although fertilization did not affect Fv/Fm, Moderate drought regimen reduced it by 12% compared to Watered regimen (Fig. 4D).

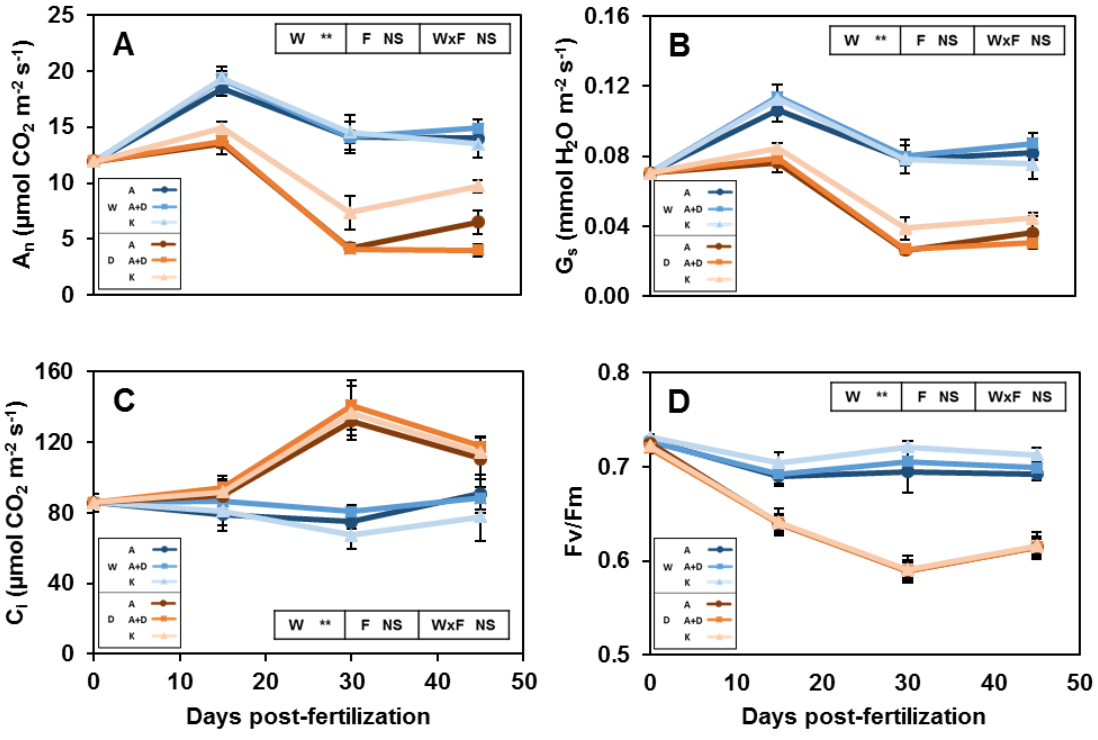


Fig. 4. *Net photosynthetic rate (**A**), stomatal conductance (**B**), intercellular CO_2 mole fraction (**C**) and maximum quantum of PSII (**D**) of sorghum leaves during 45 days post-fertilization. Pots were fertilized with ammonium sulphate (A); ammonium sulphate + DMPP (A+D) and potassium nitrate (K). Statistical analysis was made through analysis*

of variance (two-way ANOVA) showing the effect of water regimen (W), fertilizer treatment (F) and their interaction (WxF). Significant differences are marked with an asterisk (*) when p <0.05 and double asterisk (**) when p <0.01.

In pots of soil without plant, the A treatment could not maintain NH_4^+ in the soil and presented similar soil NH_4^+ content than K treatment, whereas the highest soil NH_4^+ content was found in A+D treatment (Fig. 5A). In the absence of plant, soil NH_4^+ content was not affected by the water regimen. In pots of soil with plant, A+D treatment also showed the highest soil NH_4^+ content. The soil NH_4^+ content of A treatment from Watered regimen was similar to that of K treatment. However, under Moderate drought regimen, A treatment presented more soil NH_4^+ content than K treatment. In addition, water regimen did affect the maintenance of NH_4^+ in the soil when plant is present. A and A+D treatments from Moderate drought regimen kept higher soil NH_4^+ content than Watered regimen.

Regarding soil NO_3^- content, K treatment presented the highest values in pots of soil without plant (Fig. 5B). The addition of the synthetic nitrification inhibitor reduced the apparition 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 is present, K treatment also show the highest soil NO_3^- content. Nevertheless, A and A+D treatments did not present any differences in soil NO_3^- content. In this case, water regimen only affected the soil NO_3^- content in K 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 pots of soil with plant.

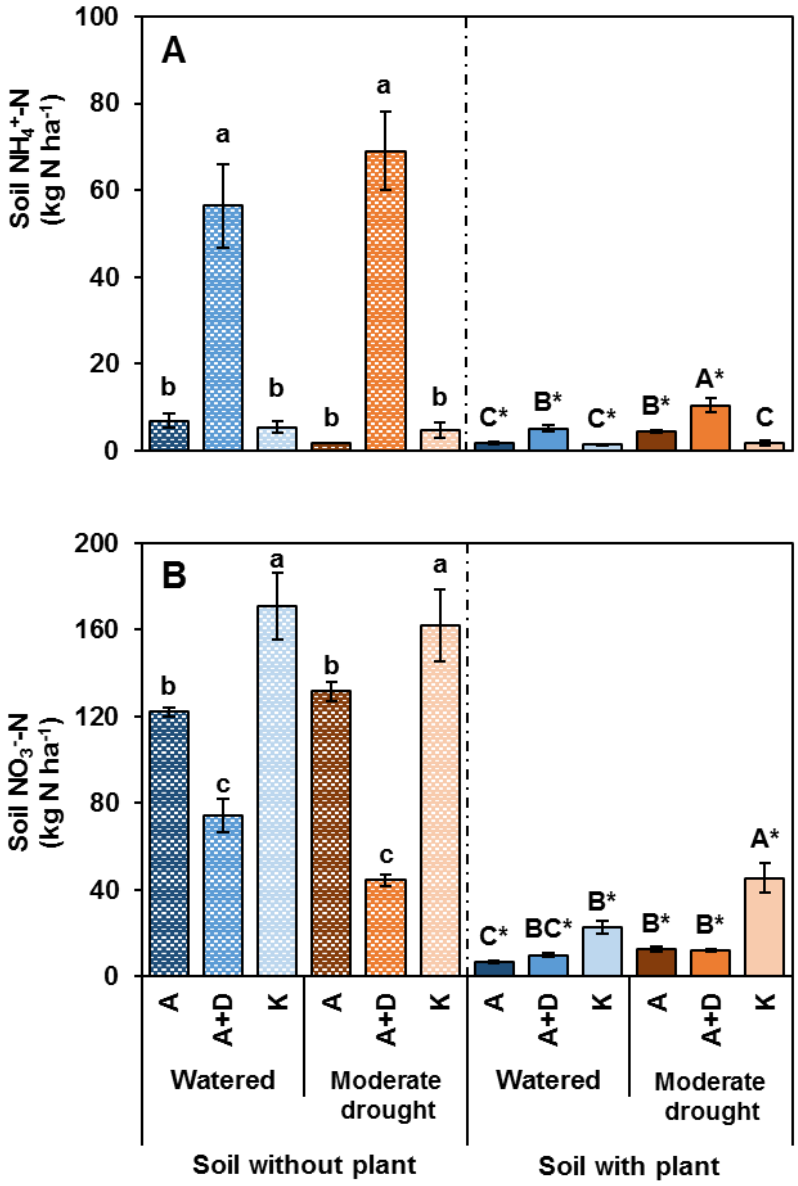


Fig. 5. *Soil mineral nitrogen in form of NH_4^+ (**A**) and NO_3^- (**B**) on pots with soil and pots with soil and plant at 30 days post-fertilization. Pots were fertilized with ammonium sulphate (A); ammonium sulphate + DMPP (A+D) and potassium nitrate (K). Significant differences between treatments 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 ($p < 0.05$; $n = 4$). 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 (*).*

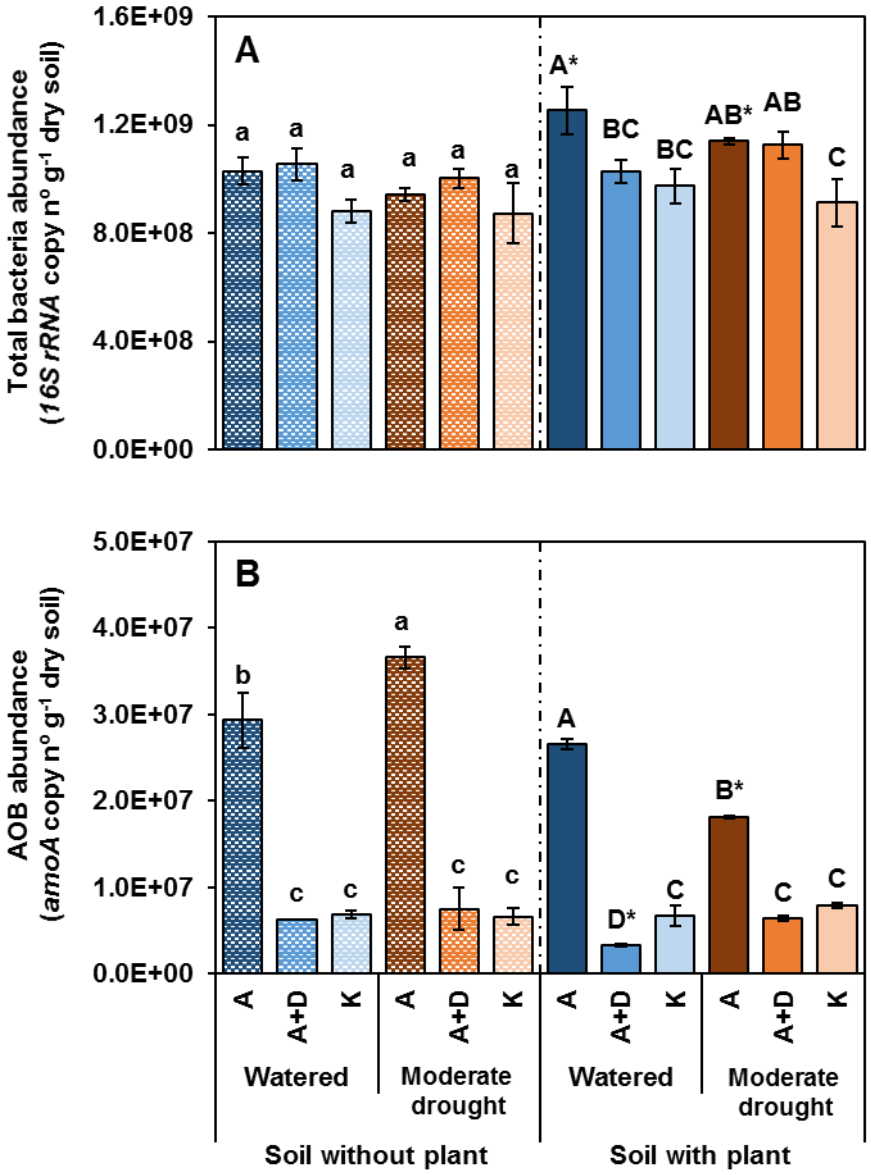


Fig. 6. *Total bacteria (**A**) and ammonia oxidizing bacteria (AOB) (**B**) on pots with soil and pots with soil and plant at 30 days post-fertilization. Pots were fertilized with ammonium sulphate (A); ammonium sulphate + DMPP (A+D) and potassium nitrate (K). Significant differences between treatments 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 ($p < 0.05$; $n = 4$). 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 (*).*

The total bacteria abundance (measured as the abundance of *16S rRNA* gene) was affected neither by the fertilizer treatment nor by the water regimen in the pots of soil without

plant (Fig. 6A). However, it was not so in pots of soil with plant. Under Watered regimen, A treatment showed the highest *16S 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 pots of soil with plants. Nevertheless, A treatment from pots of soil with plant from both water regimens presented higher *16S rRNA* gene abundance than A treatment from pots of soil without plant. Fertilization with NH_4^+ highly increased the *amoA* abundance in pots of soil without plant, especially in Moderate drought regimen (Fig. 6B). However, the application of DMPP diminished the nitrifying microorganisms to levels of K treatment, which means a 77% and 80% reduction of *amoA* abundance compared to A treatment in Watered and Moderate drought regimens respectively. In pots of soil with plant, A treatment had the highest *amoA* abundance, but Moderate drought regimen was 30% lower compared to Watered regimen. A+D treatment decreased nitrifying microorganisms 81% compared to A treatment in Watered regimen, whereas the reduction in Moderate drought regimen was 60%. The presence or the absence of the plant did not affect *amoA* abundance of A treatment from Watered regimen. Nonetheless, the *amoA* abundance of A treatment from Moderate drought regimen decreased by 50% when sorghum plants are present in the soil compared to when they are not.

DISCUSSION

One of the possibilities to achieve suitable use of nitrogen (N) could be a proper crop rotation (Pierce and Rice, 1988). Therefore, it is necessary to determine whether hairy vetch-sorghum rotation in a no-tillage system allows reaching adequate management of N for an optimal settlement of the following wheat crop. The heat tolerance and drought resistance that sorghum presents (Smith and Frederiksen, 2000; Hadebe and Modi, 2017) make it a good option as a catch crop for the summer season after a leguminous crop. The use of this kind of crop absorbs the exceeding soil N, reducing the N 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. 2B). 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 as a biological nitrification inhibitor (BNI). The molecules that sorghum plants exude and reduce nitrification in soil are sorgoleone and 3,4-hydroxyphenyl propionate (MHPP) (Nardi et al., 2013; Sarr et al., 2020). However, field nitrification inhibition only happened the first year (Fig. 1A). The field experiment took place in three close locations with similar physicochemical soil properties (Supplementary table 1). The relative *amoA* abundance from the soil with fallow did not change among the three years. Therefore, soil properties cannot be the reason why nitrification was not reduced in soil with sorghum. BNI exudation is dependent on the physiological state and development of the plant (Sarr et al., 2021). Thus, biotic or abiotic stresses that affect crop growth or nutrient uptake could modify the rate of BNI exudation. In 2017, the period when sorghum grew was very dry (Fig. 1B), but the *amoA* relative abundance of soil with sorghum presented a reduction of 50%. On the contrary, when it was a wetter year like 2018, sorghum was not able to inhibit soil nitrification. Furthermore, although it was not statistically significant, soil nitrification was reduced by 16% in 2019 compared to soil with fallow, when accumulated precipitation was average compared to the previous two years. It seems that BNI activity in sorghum plants may be related to water availability, increasing the BNI capacity when sorghum is under drought stress. Hence, more experiments 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 nitrifying populations.

Several studies 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. We have addressed this question in the present work by applying moderate drought stress to sorghum plants and further investigating the effects on BNI exudation by means of soil nitrification inhibition. Sorghum aboveground biomass was greatly reduced under moderate drought conditions (Fig. 3A) 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 (Fig. 4A). Another way to study photosynthetic performance is to measure chlorophyll fluorescence, which provides information about the state of Photosystem II (PSII) (Petridis et al., 2012). Under drought situations, a reduction of net CO_2 assimilation may provoke an imbalance in the photochemical activity of PSII, leading to an overexcitation and subsequent photoinhibitory damage of the PSII reaction centre (Kaiser, 1987). 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. 4D). The main cause of reduced photosynthesis under drought stress in C4 plants is generally a stomatal closure (Chaves et al., 2011). Tingting et al. (2010) found a close correlation between stomatal conductance and photosynthetic rate in sweet sorghum plants under drought conditions. These authors observed that sorghum plants diminish the photosynthetic rate and stomatal conductance as plant water status decreases. In line with these results, we also found that sorghum plants from Moderate drought regimen presented a 48% reduction in stomatal conductance (Fig. 4B). Furthermore, the carbon (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 increment 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 $\delta\ ^{13}C$ value (Farquhar et al., 1982; 1989). This can be observed in our experiment since sorghum plants from Moderate drought regimen had less negative $\delta\ ^{13}C$ values compared to those of Watered regimen (Fig. 3C). Therefore, our results clearly indicate that sorghum plants were suffering from drought stress.

The inhibition of nitrification withholds the oxidation of NH_4^+ and delays the apparition of NO_3^- in the soil (Clough et al., 2020). For this reason, in the pots where DMPP was applied higher soil NH_4^+ and lower soil NO_3^- content were found (Fig. 5). It can be observed that in pots of soil with plant, A treatment from Moderate drought regimen maintained more soil NH_4^+ content compared to that of Watered regimen, which could

be a signal of inhibited nitrification through BNIs (Subbarao et al., 2017). However, higher soil N content can be noticed in all treatments from Moderate drought regimen (Fig. 5) accompanied by a reduction in dry biomass (Fig. 3A). Therefore, the difference in soil NH_4^+ content may not be fully attributed to nitrification inhibition because plants under drought stress reduce the N uptake showing a lower 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. Changes in $\delta\ ^{15}N$ values indicate the type of N source that the plant had during its development (Werner and Schmidt, 2002). Plants with more ammonium nutrition present lower $\delta\ ^{15}N$ values (Ariz et al., 2011). The $\delta\ ^{15}N$ values of A treatment from Watered regimen were close to those of K treatment (Fig. 3B), which means that NO_3^- was the main source of N during plant growth. Nevertheless, A treatment from Moderate drought regimen showed similar $\delta\ ^{15}N$ values to A+D treatment, indicating that sorghum plants from this treatment had more ammonium nutrition. Thus, this NH_4^+ preference may be a result of a higher soil NH_4^+ availability during plant development due to BNI activity since low leaf $\delta\ ^{15}N$ values are an indicator of BNI activity (Karwat et al., 2018). Furthermore, reduced soil microbial nitrification and, consequently, higher soil NH_4^+ content are associated with low $\delta\ ^{15}N$ values (Jones and Dalal, 2017). The application of the synthetic nitrification inhibitor maintained the *amoA* abundance at the level of K treatment where no NH_4^+ was added (Fig. 6B). These results are in line with other studies where DMPP showed great inhibition of nitrifying population (Bozal-Leorri et al., 2021; 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 pots of soil without plant from Moderate drought regimen (Fig. 6B). 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, when the plant was present, the *amoA* abundance of A treatment from Moderate drought regimen was reduced 30% compared to Watered treatment and 50% compared to A treatment of pots of soil without plant from Moderate drought regimen (Fig. 6B). 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, such as denitrifiers, to increase its abundance (Surey et al., 2020). This matches with the increased total bacterial abundance found in A treatment

from Moderate drought regimen (Fig. 6A). However, nitrifying microorganisms were decreased (Fig. 6B). We suggest that within the increment of root exudations, the exudation of BNI molecules also raised. Therefore, it seems that under drought conditions, sorghum plants increase their roots exudation and BNI release to increase the NH_4^+ competition with nitrifying microorganisms.

CONCLUSIONS

Sorghum crop could be a good option as a catch crop. Sorghum absorbs the exceeding soil N from the previous crop, reducing the soil NO_3^- content between 44% and 92% and, thus, the N losses through NO_3^- leaching. Furthermore, it is able to reduce the *amoA* relative abundance by 50% 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. Moreover, a reduced photosynthetic rate, Fv/Fm, and stomatal conductance and less negative $\delta\ ^{13}C$ values were clear indicators that sorghum plants were suffering from drought stress. In this way, lower $\delta\ ^{15}N$ values were found in A treatment from Moderate drought regimen than those of Watered regimen. This indicates that sorghum plants from this treatment had more ammonium nutrition due to BNI activity. Finally, when the plant was present, the *amoA* abundance of A treatment from Moderate drought regimen was reduced 30% compared to Watered treatment and 50% compared to A treatment of pots of soil without plant from Moderate drought regimen. It seems that under moderate drought conditions, sorghum plants increase their roots exudation and BNI release to increase the NH_4^+ competition with nitrifying microorganisms.

SUPPLEMENTARY MATERIAL

Supplementary table 1 *Physical and chemical properties of the soil collected in 0 – 30 cm depth layer in 2017, 2018 and 2019[1] in Garinoain (42° 35' N, 1° 40' W, 532 m above sea level Navarra, Spain) and in 2019[2] in Arkaute (42° 51' N, 2° 37' W, 530 m above sea level, Alava, Spain).*

	Clasification	**pH[a]**	**C:N**	**Organic matter[b]**	**P[c]**	**K[d]**	**Mg[d]**	**Ca[d]**	**$\delta^{15}N$**
				%	**mg kg^{-1}**				
2017	Pachic Haploxeroll	8.3	10.2	2.5	65.8	209.4	90.1	3150.3	-
2018	Fluventic Haploxerept	8.4	8.6	2.7	7.6	146.5	53.5	3145.7	-
2019[1]	Typic Haploxerept	8.4	9.4	2.0	25.7	181.3	52.3	2358.6	-
2019[2]	Hypercalcic Kastanozem	8.0	8.2	1.6	59.0	167.0	92.4	6356.0	9.0

a. pH (1:2.5 soil:water).

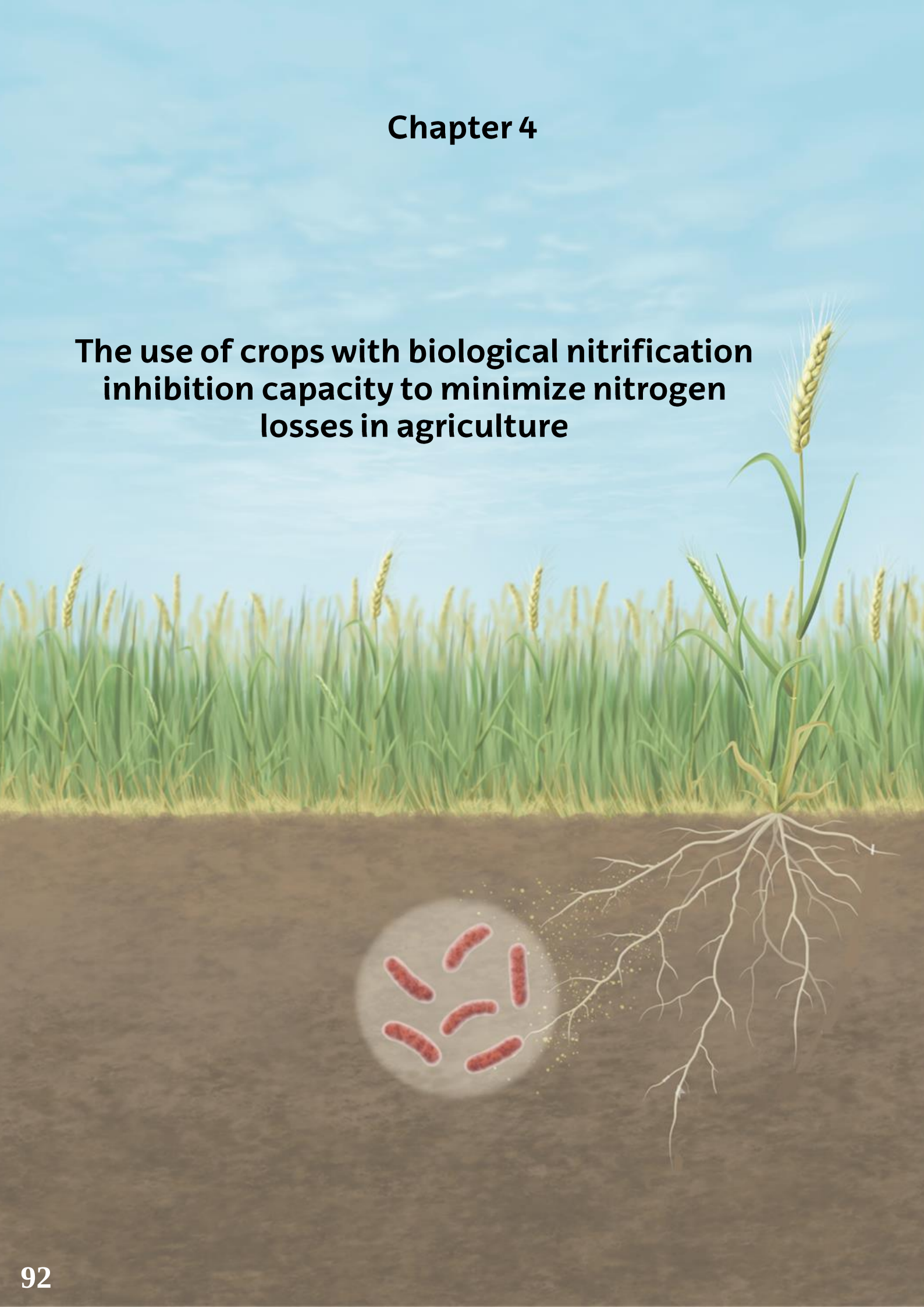
b. Organic matter (Walkley and Black, 1934).

c. P (Watanabe and Olsen, 1965).

d. CaCO3 , Mg, K (NH4 AcO, MAPA, 1994).

Chapter 4

The use of crops with biological nitrification inhibition capacity to minimize nitrogen losses in agriculture



Chapter 4.1

Biological and synthetic approaches to inhibiting nitrification in non-tilled Mediterranean soils

Chemical and Biological Technologies in Agriculture

2021

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ABSTRACT

The increasing demand for food production has led to a 10-fold increase in nitrogen (N) fertilizer use since the Green Revolution. Nowadays, agricultural soils have been turned into high-nitrifying environments that increase N pollution. To decrease N losses, synthetic nitrification inhibitors (SNIs) such as 3,4-dimethylpyrazole phosphate (DMPP) have been developed. However, SNIs are not widely adopted by farmers due to their biologically limited stability and soil mobility. On the other hand, allelopathic substances from root exudates from crops such as sorghum are known for their activity as biological nitrification inhibitors (BNIs). These substances are released directly into the rhizosphere. Nevertheless, BNI exudation could be modified or even supressed if crop development is affected. In this work, we compare the performance of biological (sorghum crop) and synthetic (DMPP) nitrification inhibitors in field conditions. Sorghum crop BNIs and DMPP prevented an increase in the abundance of ammonia-oxidizing bacteria (AOB) without affecting the total bacterial abundance. Both nitrification inhibitors maintained similar soil NH_4^+ content, but at 30 days post-fertilization (DPF), the sorghum BNIs resulted in higher soil NO_3^- content than DMPP. Even so, these inhibitors managed to reduce 64% and 96%, respectively, of the NO_3^--N/NH_4^+-N ratio compared to the control treatment. Similar to soil mineral N, there were no differences in leaf $\delta^{15}N$ values between the two nitrification inhibitors, yet at 30 DPF, $\delta^{15}N$ values from sorghum BNI were more positive than those of DMPP. N_2O emissions from DMPP-treated soil were low throughout the experiment. Nevertheless, while sorghum BNIs also maintained low N_2O emissions, they were associated with a substantial N_2O emission peak at 3 DPF that lasted until 7 DPF. Our results indicate that while sorghum root exudates can reduce nitrification in field soil, even at the same efficiency as DMPP for a certain amount of time, they are not able to prevent the N pollution derived from N fertilization as DMPP does during the entire experiment.

MATERIALS AND METHODS

2.1. Experimental design

This work was conducted in Pamplona, northern Spain (42º 47' N, 1º 37' W, 450 m above sea level), from May to October 2019. Supplementary table 1 describes the soil characteristics of the upper horizon. Daily precipitation and mean temperatures are shown in Supplemental Fig. 1. Sorghum (*Sorghum bicolor* L. var. PR88P68 Pioneer Corteva Agriscience®) was sown under no-tillage at a rate of 15 kg seeds ha^{-1} on 15th May 2019 after a previous hairy vetch (*Vicia villosa* L.) winter cover crop. The vetch was terminated with 1.5 kg ha^{-1} dose of glyphosate on 29th April, rate that is routinely applied in no-till system from this region, and left on the soil surface. This experiment consisted of three randomized N treatments with four replications (5 m x 5 m plots). The N treatments were 1) sorghum without fertilizer (Control); 2) sorghum fertilized with ammonium sulfate (AS) and 3) sorghum fertilized with ammonium sulfate combined with DMPP (AS+D). The fertilizer application rate was 150 kg N ha^{-1} in the form of ammonium sulfate 21%, with the fertilizer hand broadcast on 7th July 2019 in a single application at the beginning of stem elongation (Z30) according to the Zadoks scale (1974). Fertilizer combined with DMPP inhibitor was provided by EuroChem Agro Iberia S.L (ENTEC21). The DMPP rate was 0.8% of the NH_4^+-N applied with the fertilizer. As the purpose of this experiment was only to measure the effects of the sorghum crop on N losses, sorghum was not harvested and it was terminated on 14th October 2019 and left on the soil surface according to usual management practices.

Sorghum bicolor seed and its sow under no-tillage conditions

2.2. Plant analysis

The N isotopic composition in sorghum leaves was determined with an elemental analyser (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. To do so, one sorghum plant per plot was taken randomly at 10, 20, 30, and 60 days post-fertilization and dried at 80 ºC in a circulation oven for 72 hours until a constant dry weight was reached. Later, dry plants were ground with a ball miller (Retsch MM 500) at a frequency of 27 s^{-1} for 2 min. The values of the isotopic ratio of 100 mg of ground material were expressed as $\delta^{15}N$, in parts per thousand (‰) relative to atmospheric N_2. The isotope composition values δ (‰) were obtained with the following equation:

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

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

2.3. Soil analysis

Chambers inserted into the soil for sampling of N_2O emissions

Soil N_2O emissions were measured using the closed chamber method (Chadwick et al., 2014). Gas samples were taken over 60 days post-fertilization at decreasing sampling frequency from three times per week over two weeks to twice per week in the subsequent two weeks and, finally, once per week until the end of measuring time. N_2O samples were measured as detailed in (Corrochano-Monsalve et al., 2021b).

Soil mineral N was determined based on the soil NH_4^+ and NO_3^- contents. Three soil subsamples (3 cm diameter × 0.3 m depth) per plot were taken the day before the treatment application, and later at 10, 20, 30 and 60 days post-fertilization. Then, soil subsamples were homogenized with rocks and roots being removed. The NH_4^+ and NO_3^- contents were determined as described in (Corrochano-Monsalve et al., 2021b). Each day of soil and/or gas measurement two additional soil subsamples (3 cm diameter × 0.3 m depth) were taken randomly from the field to determine soil water content. After removing rocks and roots, they were placed into a circulation oven at 80 ºC for 72 hours until a constant dry weight was reached. Following Linn and Doran (1984), soil water content was described as the percentage of water-filled pore space (WFPS):

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

where 2.65 Mg m^{-3} was used as particle density. The density of the bulk soil was measured at the beginning of the experiment resulting in 1.0 Mg m^{-3}.

Soil samples from mineral N determinations at 20 days post fertilization were used to quantify the abundance of total bacteria (*16s rRNA*), and nitrifying (bacterial *amoA*) and denitrifying (*nirK*) populations. Quantification was done using quantitative polymerase chain reaction (qPCR) in a StepOne PlusTM Real-Time PCR System. Soil DNA isolation and gene amplification were carried out as explained in Bozal-Leorri et al. (2021).

2.4. Statistical analysis

Data obtained in this experiment were statistically analysed with one-way ANOVA followed by Duncan's multiple range tests for separation of means between treatments using SPSS statistical software (IBM Corp. Released 2016. IBM SPSS Statistics for

Windows, Version 24.0. Armonk, NY: IBM Corp). Significant differences are expressed at $p < 0.05$.

RESULTS

Fertilizer treatments did not have any effect on total bacterial abundance (Fig. 1A). Based on the *16S rRNA* gene copy number, bacterial abundance ranged from $1.00 \cdot 10^9$ to $1.10 \cdot 10^9$. Nitrifying bacteria were also not affected by N treatments, having an abundance of between $9.31 \cdot 10^6$ and $1.01 \cdot 10^7$ *amoA* gene copy numbers g^{-1} dry soil (Fig. 1B). Alike AOB, denitrifying microorganisms neither were affected by addition of fertilizer They showed an abundance that varied from $7.31 \cdot 10^5$ to $8.12 \cdot 10^5$ *nirK* gene copy numbers g^{-1} dry soil (Fig. 1C).

After fertilizer application, the soil NH_4^+ content increased in AS and AS+D treatments maintaining higher values during the first 30 days post-fertilization (DPF) (Fig. 2A). At 30 DPF, the soil NH_4^+ content of fertilized treatments decreased to levels that were similar to the control treatment. However, it was observed that the soil NH_4^+ content from the AS and AS+D treatments increased at 60 DPF, which might have been a consequence of mineralization. On the other hand, the AS treatment showed the highest soil NO_3^- content during the 60 days of measurement (Fig. 2B). Although the AS+D treatment showed constant soil NO_3^- content during the first 20 DPF, it was able to diminish its formation to levels of unfertilized treatment until 60 DPF. Control treatment also showed low soil NO_3^- content. Indeed, the highest NO_3^--N/NH_4^+-N ratio throughout the experiment was observed in the control treatment (Fig. 2C). The AS and AS+D treatments had equally low ratios during the first 20 DPF because there were no differences between them in terms of soil NH_4^+ and NO_3^- content. Nevertheless, because the AS treatment did not decrease soil NO_3^- levels at 30 DPF, the NO_3^--N/NH_4^+-N ratio showed a 6-fold increase compared to the AS+D treatment. Still, at 30 DPF both treatments were able to reduce the NO_3^--N/NH_4^+-N ratio in AS and AS+D by 64% and 96%, respectively, compared to the control treatment.

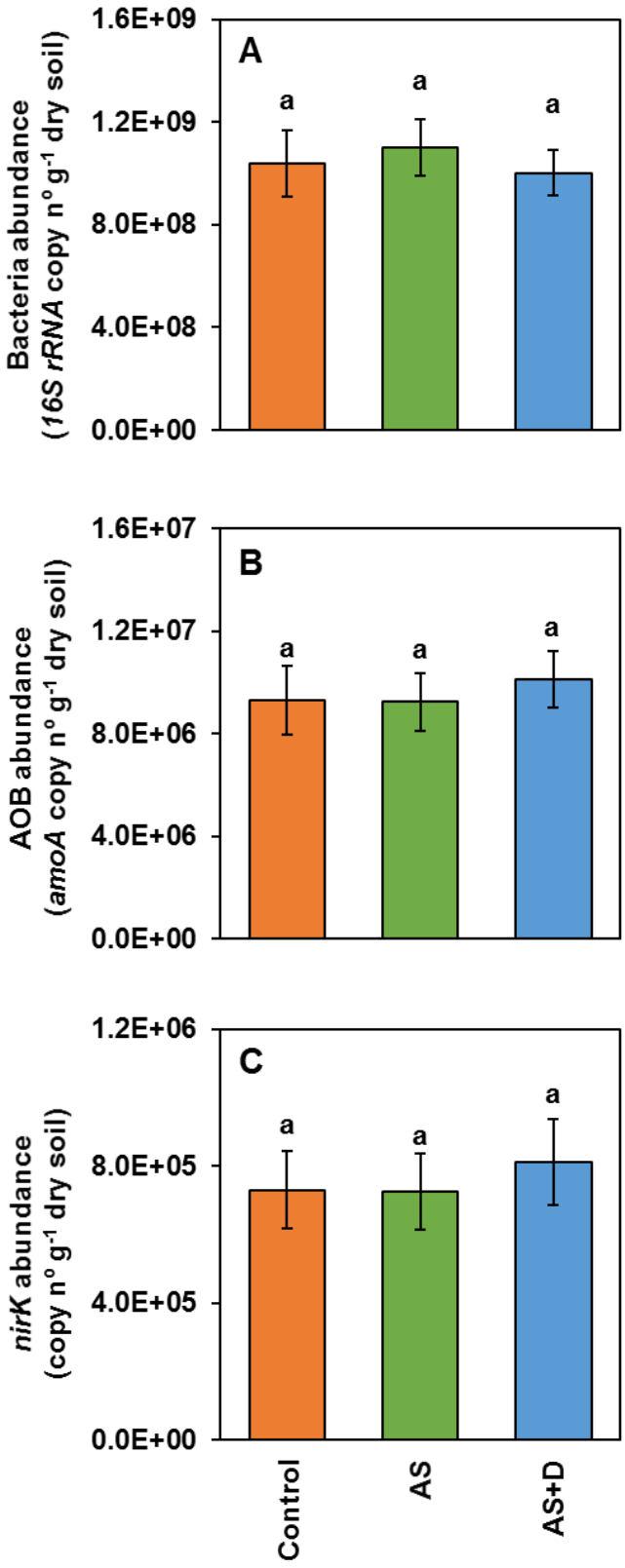
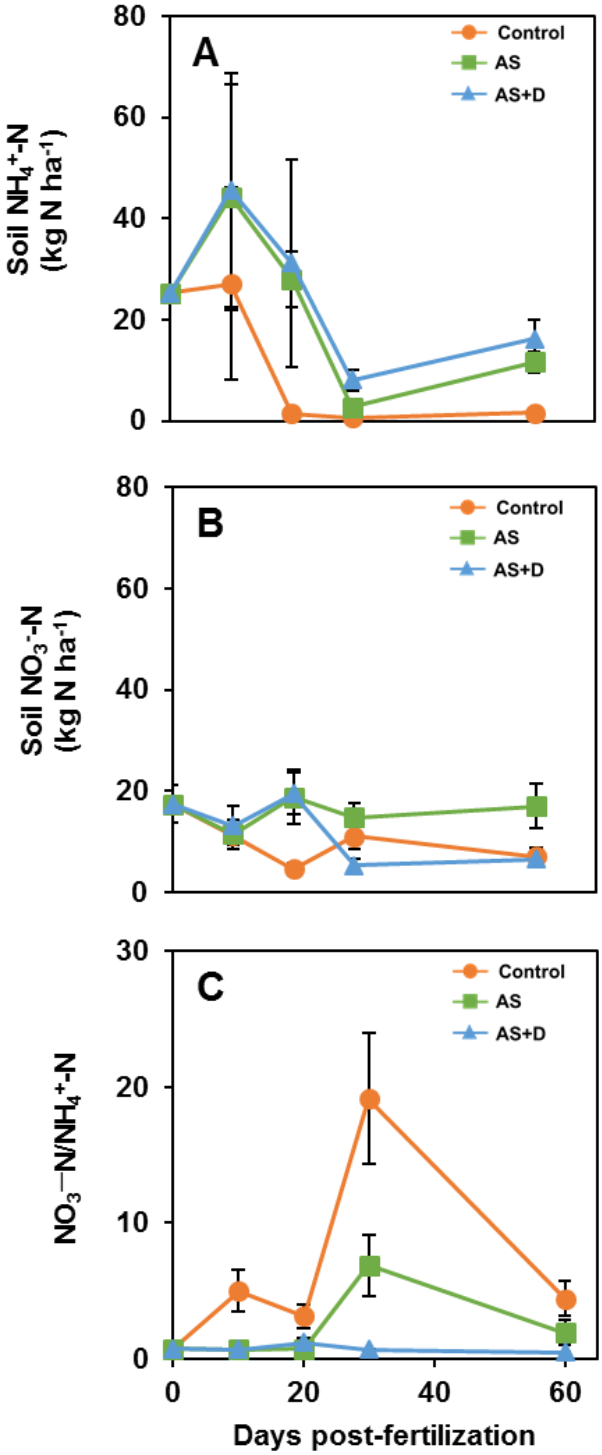


Fig. 1 *Total bacteria (**A**), ammonia oxidizing bacteria (AOB) (**B**) and nirK gene (**C**) abundance at 20 days post-fertilization (DPF). Control = sorghum without fertilization; AS = sorghum fertilized with ammonium sulphate; AS+D = sorghum fertilized with*

ammonium sulphate + DMPP. Different letters indicate significant differences using the Duncan Test ($p < 0.05$; $n = 4$).



	10	20	30	60
Control	a	b	b	b
AS	a	a	b	a
AS+D	a	a	a	a

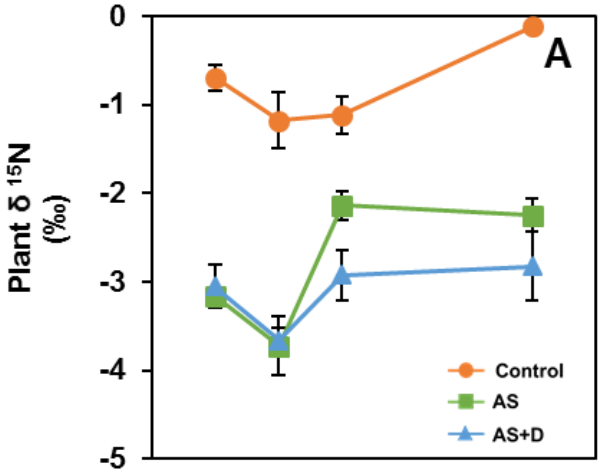
	10	20	30	60
Control	a	b	ab	b
AS	a	a	a	a
AS+D	a	a	b	b

	10	20	30	60
Control	a	a	a	a
AS	b	b	b	ab
AS+D	b	b	c	b

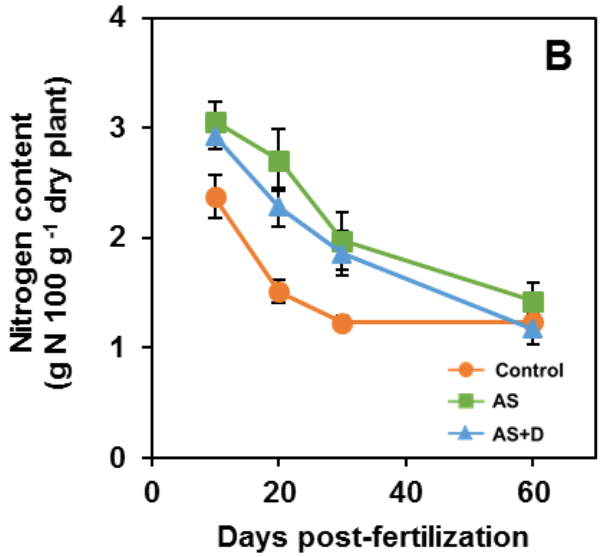
Fig. 2 *Soil mineral nitrogen evolution during 60 days post-fertilization in form of NH_4^+ (**A**), NO_3^- (**B**) and the ratio $NO3^-$-N/$NH4^+$-N (**C**). Control = sorghum without fertilization; AS = sorghum fertilized with ammonium sulphate; AS+D = sorghum fertilized with*

ammonium sulphate + DMPP. Different letters indicate significant differences using the Duncan Test ($p < 0.05$; $n = 4$).

Sorghum leaves from the control treatment showed the least negative $\delta^{15}N$ values (Fig. 3A). The similarity in leaf $\delta^{15}N$ values between the AS and AS+D treatments until 20 DPF indicated no effect from DMPP up to this point. However, at 30 DPF, both fertilizer treatments showed an increase in $\delta^{15}N$ values that was greater in the AS treatment than the AS+D treatment, and the same $\delta^{15}N$ values were maintained until 60 DPF. As expected, the unfertilized treatment possessed the lowest leaf N content (Fig. 3B). Fertilized treatments showed higher N contents that declined throughout the experiment until they reached similar N values to the control treatment at 60 DPF. In this case, there were no differences between the AS and AS+D treatments.



	10	20	30	60
Control	a	a	a	a
AS	b	b	b	b
AS+D	b	b	c	b



	10	20	30	60
Control	b	b	b	a
AS	a	a	a	a
AS+D	a	a	a	a

Fig. 3 *Sorghum leaf determination of δ ^{15}N (**A**) and nitrogen content (**B**). Control = sorghum without fertilization; AS = sorghum fertilized with ammonium sulphate; AS+D = sorghum fertilized with ammonium sulphate + DMPP. Different letters indicate significant differences using the Duncan Test (p <0.05; n = 4).*

The treatment fertilized with AS showed a substantial N_2O emission peak at 3 DPF with an emission of 38.7 g N_2O-N ha^{-1} d^{-1} (Fig. 4A). Nevertheless, the peak was quickly reduced from 7 DPF, with N_2O emissions in the AS treatment maintained between 3.79 and 0.74 g N_2O-N ha^{-1} d^{-1}. In contrast, the N_2O emissions from the control and AS+D treatments were both low throughout the experiment, ranging from 4.23 to 0.67 g N_2O-N ha^{-1} d^{-1} for the control treatment and from 4.01 to 0.31 g N_2O-N ha^{-1} d^{-1} for the AS+D treatment. Although N_2O emissions from AS treatment were reduced to levels similar to the control and AS+D treatments, it had the highest total cumulative N_2O emissions due to the short emission peak (Fig. 4B). There were no differences between control and AS+D treatments in total cumulative N_2O emissions, with reductions of 54% and 59%, respectively, compared to the AS treatment.

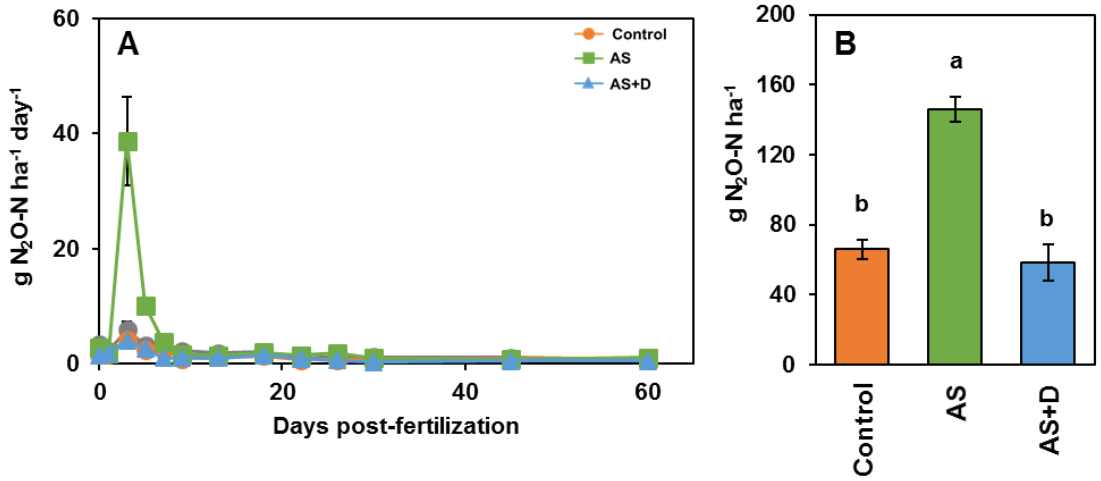


Fig. 4 *Daily (**A**) and cumulative (**B**) N_2O emission during 60 days post-fertilization. Control = sorghum without fertilization; AS = sorghum fertilized with ammonium sulphate; AS+D = sorghum fertilized with ammonium sulphate + DMPP. Different letters indicate significant differences using the Duncan Test (p <0.05; n = 4).*

DISCUSSION

During the last few decades, root exudates from sorghum have been well studied due to the presence of allelopathic substances (Weston et al., 2013). Lately, investigations have focused on their ability to inhibit the nitrification pathway. There are several greenhouse and microcosm studies where molecules such as sorgoleone and MHPP have been characterized and their potential as BNIs investigated (Netzly and Butler, 1986; Zakir et al., 2008; Nardi et al., 2013; Subbarao et al., 2013b). One of the main aspects to consider in the attempt to improve agricultural sustainability is whether these substances could have a negative effect on soil health. Encouragingly, while MHPP molecules reduce AOB abundance, they do not exert a general negative impact on the soil bacterial community, as indicated by maintenance of *16S rRNA* gene abundance in microcosm experiments (Nardi et al., 2013). In the same manner, no effects on total bacterial abundance have been observed in soil from pot-grown sorghum that release different quantities of sorgoleone (Sarr et al., 2020). Here, we corroborate that sorghum plants do not alter total bacterial abundance under field conditions (Fig. 1A). This is a confirmation that both sorghum root exudates and the synthetic nitrification inhibitor (SNI) DMPP do not produce general deleterious effects because DMPP also had no effect on *16S rRNA* abundance, as reported here (Fig. 1A) and in several other studies (Barrena et al., 2017; Luchibia et al., 2020). Nevertheless, previous work with DMP-based nitrification inhibitors and with sorgoleone has shown that there are some shifts in non-target bacterial abundance, even when the total bacterial abundance is not altered, with SNIs associated with decreases in bacterial diversity (Corrochano-Monsalve et al., 2020b) and BNIs associated with changes in bacterial networks (BNIs) (Wang et al., 2021). These studies are still preliminary, so further work should expand these analyses to determine exactly what effects are exerted by these compounds on the soil microbiota.

Fertilizer stimulates root development, changes the soil pH and increases the availability of nutrients for microorganisms and, consequently, the soil bacterial consortia are greatly influenced (Guo et al., 2010; Peng et al., 2017; Zhang et al., 2017). Specifically, the AOB population exhibits a strong response to N fertilizers (Ouyang et al., 2018). Shortly after the fertilizer application, soils tend to show a large increase in *amoA* abundance (Castellano-Hinojosa et al., 2020; Bozal-Leorri et al., 2021). This growth can be avoided

by applying SNIs such as DMPP, and the AOB population size is maintained at the level of unfertilized soils (Fig. 1B) (Duncan et al., 2017; Nair et al., 2021). Furthermore, the objective of BNIs is to suppress soil nitrification by decreasing the ammonia-oxidizing microorganisms' populations (Subbarao et al., 2015). Interestingly, in the absence of DMPP (AS treatment) we also observed no increases in *amoA* abundance in soils. The lack of AOB growth may be associated with the use of glyphosate as an herbicide to terminate winter vetch crop. Several studies have examined the effect of glyphosate on soil microbiology, but the results are highly variable. While some authors described that the use of glyphosate cause negative impacts on microbial community structure (Andréa et al., 2003; Lancaster et al., 2010), others affirmed that glyphosate is able to increase soil microbial biomass and respiration (Wardle and Parkinson, 1990a; 1990b) or, at least, have no significant impact at all (Zabaloy et al., 2012; Rosenbaum et al., 2014). Nevertheless, it has been reported that the application of glyphosate at higher rates than in this experiment (1.5 kg ha^{-1}) had no effect on AOB and AOA abundances (Allegrini et al., 2017; Zabaloy et al., 2017). Moreover, glyphosate is routinely applied in no-till systems from this region. Therefore, it is reasonable that we could conclude that the inhibition of AOB growth was due to the action of BNI molecules present in sorghum root exudates. This is the first field-demonstration that sorghum can avert AOB growth with the same efficiency as SNIs.

Soil mineral N is a useful tool to monitor the activity of AOB based on the oxidation of NH_4^+ to NO_3^-. The use of SNIs such as DMPP maintains soil NH_4^+ for longer periods due to a delay in NH_4^+ oxidation as a consequence of AOB inhibition (Ruser and Schulz, 2015). When ammonium-based fertilizers are applied without nitrification inhibitors, soil NH_4^+ increases substantially followed by a rapid decrease and the appearance of NO_3^- (Corrochano-Monsalve et al., 2020a; Montoya et al., 2021). In our work, AS treatment kept soil NH_4^+ content in parallel with the AS+D treatment (Fig. 2A). This could be a consequence of BNIs released by sorghum, demonstrating the ability to maintain NH_4^+ content at the same level as DMPP, which aligns with the equal AOB populations in both soils (Fig. 1B). Although 20% of total N losses from field-applied N occur through volatilization of ammonia, the great majority of N losses occur after microbial reactions transform NH_4^+ in soils into NO_3^- (Subbarao and Searchinger, 2021). Therefore, it seems that the use of BNI could be a good option to reduce soil N losses due to its ability to withhold NH_4^+ oxidation derived from the inhibition of AOB growth. Nonetheless, the

capacity of BNIs to diminish nitrification seemed to decline over time, as suggested by the daily evolution of soil mineral N (Fig. 2A and B). Although soil NH_4^+ and NO_3^- contents were equivalent between AS- and AS+D-treated soils, differences arose after 20 DPF. The NO_3^--N/NH_4^+-N ratio under AS treatment increased relative to the AS+D treatment (Fig. 2C), which may indicate that the efficiency of BNIs in reducing NH_4^+ oxidation only lasted until 20 DPF. It would be interesting to track AOB abundance over time for longer in further studies to examine the effect of this possible decline in BNI activity on AOB growth. These differences in the NO_3^--N/NH_4^+-N ratio were also associated with an effect on sorghum leaf $\delta^{15}N$ values. The natural variation in the heavy N isotope (^{15}N) has now being used with increasing frequency in physiological studies related to N metabolism (Purnell et al., 2007; Ariz et al., 2011; 2018). For example, N fixation processes, both free and biological, tend to impoverish $\delta^{15}N$ because the value of the atmospheric N_2 is zero. Thus, because the sorghum crop was sown after winter vetch, which is an N-fixing crop, the leaf $\delta^{15}N$ values of the sorghum control treatment were below zero (Fig. 3). In addition, (Ariz et al., 2011) noted that plants grown with NH_4^+ as the sole source of N demonstrated a decrease in $\delta^{15}N$. Therefore, as suggested by Werner and Schmidt (2002), $\delta^{15}N$ can be used as an indicator of the origin of the main N source available to the plant during its development. The lower $\delta^{15}N$ values of the AS and AS+D treatments indicated that the sorghum crops were exposed to a dominating ammonium nutrition for a longer period than the control treatment. Nevertheless, at 30 DPF, the NO_3^--N/NH_4^+-N ratio in soils from the AS treatment showed an increase compared to the AS+D treatment (Fig. 2C), probably due to the aforementioned decline in BNI effectivity. This means that sorghum plants under AS had greater access to NO_3^- than sorghum under the AS+D treatment. As a consequence, the $\delta^{15}N$ values of the AS treatment were less negative than those of AS+D at 30 DPF (Fig. 3A). In addition, $\delta^{15}N$ values of the fertilized treatments did not attain the values of the control without fertilization. This indicates that while BNIs are less efficient than the synthetic inhibitor DMPP, they still promote a certain amount of plant NH_4^+ nutrition.

The main process responsible for N_2O production in soils is denitrification (Davidson et al., 2000). N_2O emissions are related to soil water content (Davidson, 1991), but the humid Mediterranean climate is characterized by a hot and dry summer. The threshold between water-limited and aeration-limited microbial processes is supposed to occur at soil moisture levels of 60% WFPS (Davidson, 1991). Therefore, since the soil WFPS of

the present study did not exceed 25% most of the time (Supplemental fig. 2), it is possible that denitrifiers were not responsible for N_2O emissions. This may have been due to the lack of variation between treatments in the abundance of the *nirK* gene at 20 DPF (Fig. 1C). On the other hand, nitrifying microorganisms can also produce N_2O via nitrifiers' denitrification processes (Wrage et al., 2001). Nevertheless, although nitrifying populations might have been responsible for the emission peak in the AS treatment at 3 DPF (Fig. 4A), at the same time there were no differences in AOB abundance at 20 DPF between AS and AS+D (Fig. 1B). We hypothesized that the presence of the N_2O emission peak at 3 DPF and the inhibition of AOB growth at 20 DPF is related to the amount of BNIs released by the sorghum roots. The release of BNIs is influenced by soil NH_4^+ content, which at higher concentrations has been shown to stimulate greater BNI release in sorghum roots (Subbarao et al., 2012). Therefore, the limited soil NH_4^+ content before the N_2O measurements did not promote the release of enough BNIs to reduce nitrification in the first few days after fertilizer application. Nonetheless, BNI release increased in sorghum after the addition of ammonium-based fertilizer, inhibiting AOB growth at 20 DPF, and this occurred despite a lack of complete inhibition of nitrification during the first 7 DPF. The fact that the N_2O emission peak in the AS treatment was reduced before 7 DPF, while the N_2O emission peaks of fertilized treatments without nitrification inhibitors lasted more than 15 days (Recio et al., 2018; Nair et al., 2021) is in line with this hypothesis. During the rest of the experiment, sorghum exudates were able to maintain low N_2O emissions similar to the AS+D treatment, which showed great efficiency in reducing them, as described in other studies (Huérfano et al., 2015; 2018; Liu et al., 2020). Nonetheless, this indicates that even though BNIs have a similar efficiency to SNIs in reducing N_2O emissions, the delay in BNI release due to the absence of high soil NH_4^+ content does not prevent N_2O emissions in the short-term.

CONCLUSIONS

The use of allelopathic substances from plants to reduce nitrification in the soil is a topic of increasing interest. BNI inhibition could be a nature-based solution to diminish N losses, avoiding reliance on new technologies that are not widely adopted. BNIs from sorghum were able to prevent an increase in *amoA* after N fertilization with the same efficiency as DMPP. Moreover, total bacterial abundance was not affected by either the

presence of sorghum roots exudates or by DMPP. In addition, both BNIs and SNIs maintained similar soil NH_4^+ contents throughout the experiment. However, sorghum root exudates could not prevent the appearance of soil NO_3^- after 20 DPF, which might indicate that the BNI effect decreases in efficiency after a certain amount of time. While DMPP maintained low N_2O emissions throughout the experiment, the AS treatment presented one peak at 3 DPF that lasted until 7 DPF. Since the release of BNIs is related to the soil NH_4^+ concentration, we hypothesize that the limited soil NH_4^+ concentration before the N_2O measurements did not allow release of enough BNIs to avoid this emission peak. Therefore, although sorghum root exudates can reduce nitrification in field soil, even with the same efficiency as DMPP for a certain amount of time, they are not able to prevent the N pollution derived from N fertilization as DMPP does during the entire experiment.

SUPPLEMENTARY MATERIAL

Supplementary table 1 *Physical and chemical properties of the soil collected in 0 – 30 cm depth layer in Pamplona (42° 47' N, 1° 37' W, 450 m above sea level, Navarre, Spain).*

Soil texture			Soil chemical properties								
						Organic					
Sand	Silt	Clay	pH[a]	C:N	N[b]	matter[c]	Carbonate[d]	Mg[d]	K[d]	Ca[d]	P[e]
(%)					(g kg^{-1})			(mg kg^{-1})			
38.6	31.8	29.6	8.3	8.9	1.4	21.5	20.3	53.5	270.0	2735.7	11.5

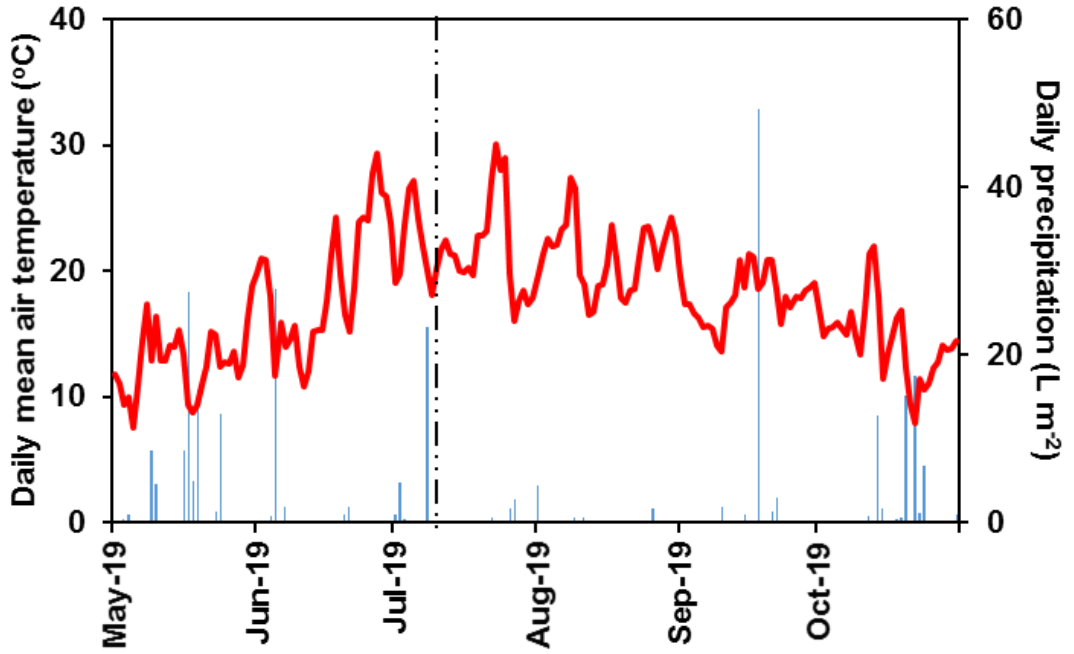
a. pH (1:2.5 soil:water).

b. N Kjeldahl digestion (Keeney and Nelson, 1982).

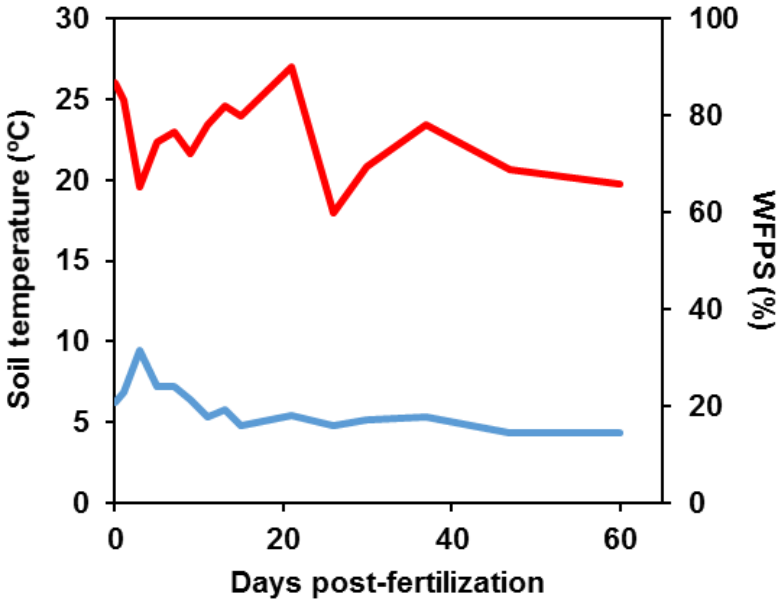
c. Organic matter (Walkley and Black, 1934).

d. CaCO3 , Mg, K (NH4 AcO, MAPA, 1994).

e. P (Watanabe and Olsen, 1965).



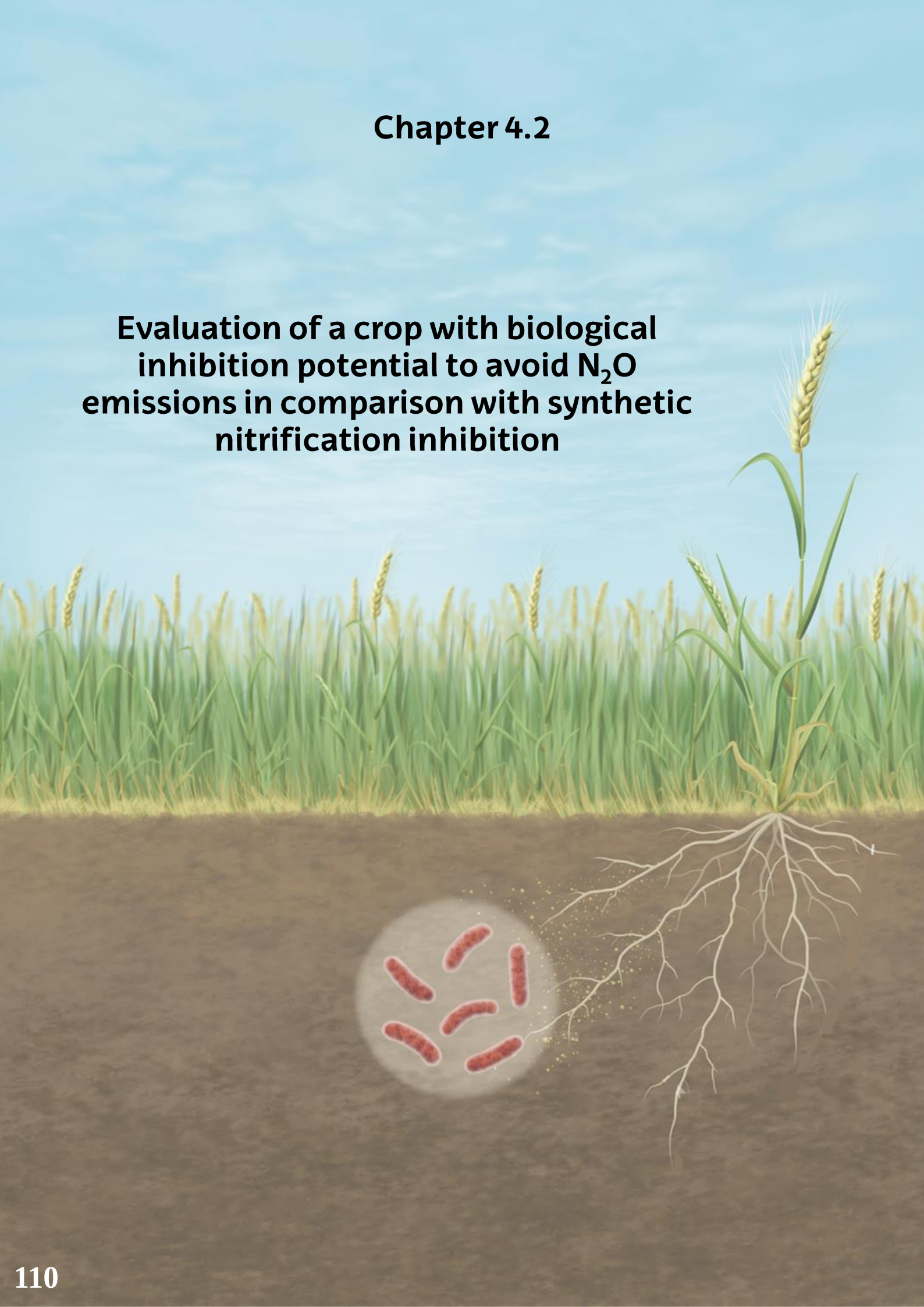
Supplemental fig. 1 *Daily precipitacion (blue bars) and mean air temperature (red line) of summer growing season. Discontinue bar indicates the application of the fertilizer treatments.*



Supplemental fig. 2 *Evolution of soil temperature (0-10 cm) (red line) and soil WFPS (0-30 cm) (blue line) during GHG emissions measurements in sorghum crop.*

Chapter 4.2

Evaluation of a crop with biological inhibition potential to avoid N_2O emissions in comparison with synthetic nitrification inhibition



ABSTRACT

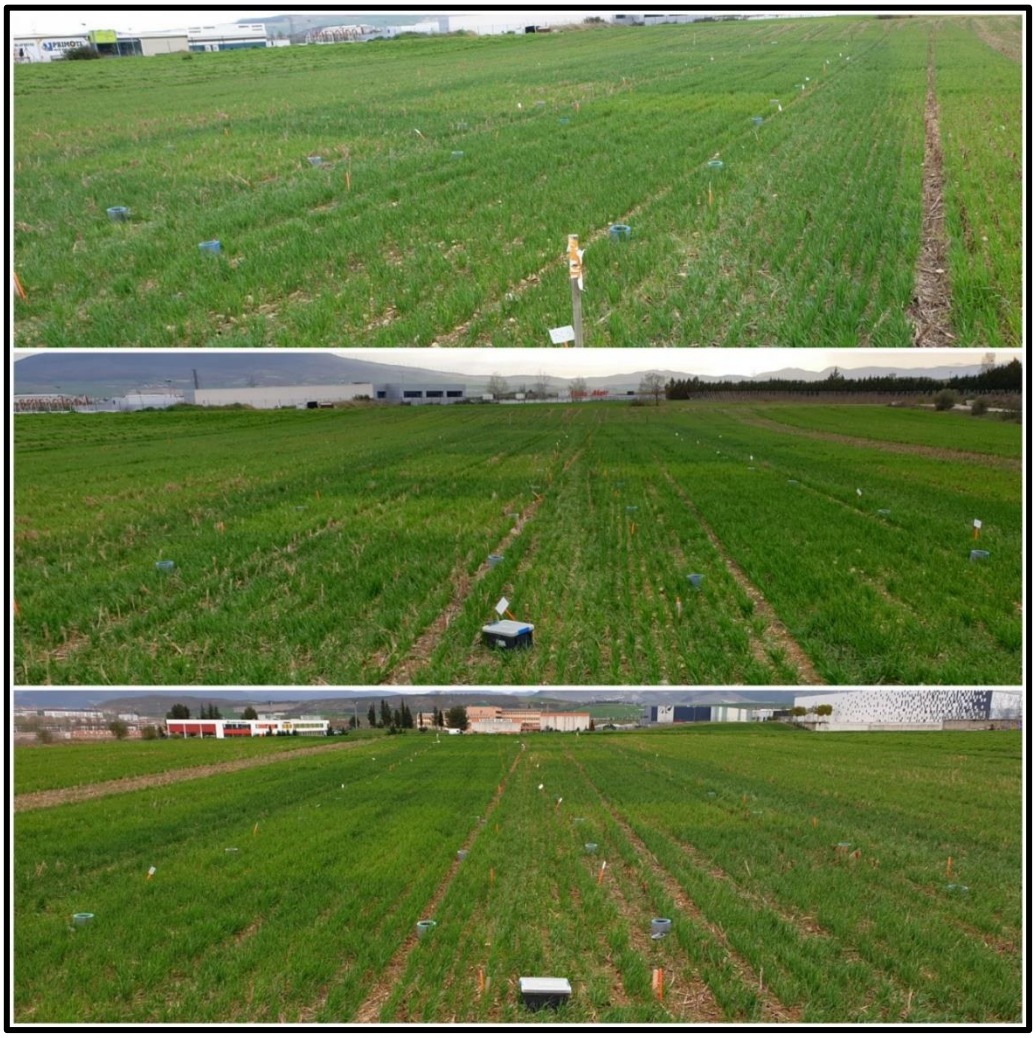
Agriculture has increased the release of reactive nitrogen (N) to the environment due to crops' low N-use efficiency (NUE) after the application of N-fertilisers. Cover crops can be established to reduce soil N pollution and bring multiple environmental benefits. Furthermore, sorghum could be a good candidate as it is drought tolerant and it has the ability to exudate biological nitrification inhibitors (BNI). Otherwise, practices like the use of stabilized fertilisers with nitrification inhibitors such as DMPP (3,4-dimethylpyrazole phosphate) have been adopted. The objective of this work aimed to evaluate the effect of two different no-tilled crop rotations (fallow-wheat and sorghum cover crop-wheat) on N_2O emissions and the grain yield of winter wheat crop. In addition, the suitability of DMPP addition in combination to these alternatives was analyzed. Three fertiliser treatments were applied in each crop rotation: i) control without fertiliser, ii) ammonium sulphate (AS); and iii) AS in combination with DMPP. Soil mineral N and N_2O emission evolution were monitored, and nitrifying (*amoA*) and denitrifying (*nirK, nirS, nosZI* and *nosZII*) bacterial genes were quantified by qPCR. We found no differences in terms of wheat grain yield between rotations. However, the use of sorghum as a cover crop might not be a suitable option to mitigate N_2O emissions in the subsequent crop. Although sorghum–wheat rotation was able to reduce 22% the abundance of *amoA*, it presented an increment of 77% in cumulative N_2O emissions compared to fallow–wheat rotation, which was probably related to a greater abundance of heterotrophic-denitrification genes. This made that the N-yield-scaled N_2O emissions (YSNE) were higher when wheat was grown after sorghum plantation. Therefore, we the application of DMPP would be recommendable not only in fallow-wheat rotations, but especially in sorghum-wheat ones, to avoid N_2O emissions by avoiding the growth of ammonia-oxidizing bacteria and maintaining the N_2O emissions at the levels of unfertilised soils.

MATERIALS AND METHODS

2.1. Experimental design

This work was conducted in Pamplona, northern Spain (42º 47’ N, 1º 37’ W, 450 m above sea level) during the 2019/2020 growing season. The soil characteristics of the upper

horizon before the start of the experiment are given in supplementary table 1, while daily precipitation and mean temperatures are shown in supplementary Fig. 1.



Different perspectives of the winter wheat crop of fallow/wheat or sorghum cover crop/wheat rotation

A bifactorial experimental design (crop rotation and type of wheat fertilisation) was implemented with four random block replications of individual 25 m^2 (5 m x 5 m) plots. The crop rotations were: i) fallow–wheat rotation (fallow–wheat); and ii) sorghum cover crop without N fertilisation–wheat rotation (sorghum–wheat). The soil treatments were arranged in two blocks. In the first block, adventitious plants were desiccated on 20th May 2019 using a glyphosate-based herbicide at a rate of 1.5 L ha^{-1}, dose that is routinely applied in no-till systems from this region. In the second one, sorghum (*Sorghum bicolor* L. var. PR88P68 Pioneer Corteva Agriscience®) was sown under no-till conditions at a rate of 15 kg ha^{-1} on 20th May 2019. The sorghum cover crop was crushed on 14th October

2019 and left on the soil surface. One month before sorghum termination, soil NH_4^+-N content from fallow and sorghum plots were 2.0 and 1.7 kg N ha^{-1} respectively, while the soil NO_3^--N content was 43.7 and 7.0 kg N ha^{-1} for fallow and sorghum plots. Winter wheat (*Triticum aestivum* L. cv. Marcopolo RAGT®) was sown under no-till conditions and over sorghum stover at a rate of 220 kg ha^{-1} on 31st October 2019. Three wheat fertiliser treatments were randomly applied within each crop rotation (fallow–wheat and sorghum–wheat): 1) control without fertilisation (control); 2) fertilised with ammonium sulphate 21% (AS); and 3) fertilised with ammonium sulphate 21% combined with DMPP (AS+DMPP). The fertilisation rate was 90 kg N ha^{-1} applied in a single dose on 28th February 2020 at the beginning of stem elongation (Z30) according to the Zadoks scale (Zadoks et al., 1974). The fertiliser combined with the DMPP nitrification inhibitor was provided by EuroChem Agro Iberia S.L (ENTEC21). The DMPP rate was 0.8% of the NH_4^+-N applied with the fertiliser. The wheat was harvested on 24th July 2020.

2.2. Soil geochemical analysis and water content

Soil NH_4^+ and NO_3^- contents were first determined the day before applying the treatments. Samples were then taken 10, 30 and 60 days post-fertilisation. Three soil subsamples (3 cm diameter × 0.3 m deep) were taken from each plot, rocks and stones were removed and finally they were homogenised. Next, 100 g fresh weight of the homogenised subsamples were mixed with 200 mL of 1 M KCl and shaken for 1 hour at 165 rpm. The soil solution was filtered through Whatman No. 1 filter paper (GE Healthcare) to remove particles and then through Sep-Pak Classic C18 125 Å cartridges (Waters) to eliminate organic matter. The NH_4^+ content of the filtered solution was determined using the Berthelot method (Patton and Crouch 1977) and the NO_3^- content as described by Cawse (1967).

The soil water content was also measured each time the soil and/or GHG were sampled. Two subsamples (3 cm diameter x 0.3 m deep) were taken randomly from the field. Rocks were removed and the soil subsamples were dried at 80 °C in a circulation oven for 72 hours until they reached a constant dry weight. Water content was expressed as the percentage of water-filled pore space (WFPS) as per Linn and Doran (1984), where:

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

Particle density was taken as 2.65 Mg m^{-3}. Bulk density was measured at the beginning of the experiment and was found to be 1.0 Mg m^{-3}.

2.3. Measurement of N_2O emissions

N_2O soil emissions were measured using the closed chamber method (Chadwick et al., 2014). Samples were collected 3 times/week for 2 weeks after wheat fertilisation, then 2/week for the next 2 weeks and 1/week up to day 60. Considering the diurnal variation of emissions (Baggs and Blum, 2004), sampling was performed between 10 a.m. and 1 p.m. To account for soil heterogeneity, four chambers (20 cm diameter x 16 cm high once inserted in the soil) were placed in each plot and two were sampled on alternate days. Linearity was checked and gas samples were taken just after closing the chambers and then 45 minutes later. 20 mL of gas was taken from each chamber and stored at overpressure in pre-evacuated 12 mL glass vials. Samples were analysed in a gas chromatograph (GC) (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 the samples were injected using a headspace autosampler (Teledyne Tekmar HT3). N_2O standards were analysed at the same time as the samples. Gas emission rates were calculated while considering the variation in gas concentration from the beginning to the end of the 45 minutes. 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). Soil temperature (at a depth of 10 cm) was measured before sampling the gas emissions. The air temperature was measured 3 times during the 45 minute gas sampling period to get the average.

2.4. Abundance of N-cycle related microorganisms

Quantitative polymerase chain reaction (qPCR) was used to quantify the abundance of nitrifying and denitrifying genes. Soil DNA was isolated from 0-30 cm soil samples (three subsamples per plot) collected at 10 days post-fertilisation. DNA was extracted from 0.25 g of dry soil using the PowerSoil® DNA Isolation Kit (Qiagen) with the modifications

described in Harter et al. (2014). The concentration and quality of DNA extracts were determined by means of spectrophotometry (NanoDrop® 1000, Thermo Scientific). The *16S rRNA* gene (for quantification of total bacterial abundance) and functional marker genes involved in bacterial nitrification (*amoA*) and denitrification (*nirK, nirS, nosZI* and *nosZII*) were amplified by means of qPCR using SYBR® Premix Ex TaqTM II (Takara-Bio Inc.) and gene-specific primers (Torralbo et al., 2017) in the StepOnePlus™ Real-Time PCR System. Data analysis was carried out with StepOnePlus™ Software 2.3 (Thermo Scientific). Standard curves were prepared from serial dilutions of linearised plasmids with insertions of the target gene ranging from 10^7 to 10^3 gene copies μL^{-1}. The number of copies 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 x volume of DNA extracted) / (volume of DNA used per reaction x gram of dry soil extracted)] / DNA concentration.

2.5. Crop yield parameters

Grain yields were calculated based on a harvested area of 7.5 m^2 (1.5 m x 5 m) per plot and adjusted for a moisture content of 12%. An area of 0.36 m^2 per plot was measured to calculate the number of spikes per m^2 and dry weight of 1,000 grains. The total grain N content was analysed by applying the Kjeldhal procedure (AOAC, 1980) with a Kjeltec Autosampler System 1035 (Tecator) after grinding the grain and passing it through a 1 mm screen. Grain protein content was taken as 5.7 times the total N content (Teller, 1932). The yield-scaled N_2O emissions (YSNE) were expressed as the ratio between the amount of N emitted as N_2O and the aboveground N uptake (van Groenigen et al., 2010). The nitrogen use efficiency (NUE) (kg DM kg^{-1} N) was determined as (kg DM at Nx - kg DM at N0)/kg of applied N where Nx = 90 kg N ha^{-1}, and N0 = no fertiliser application.

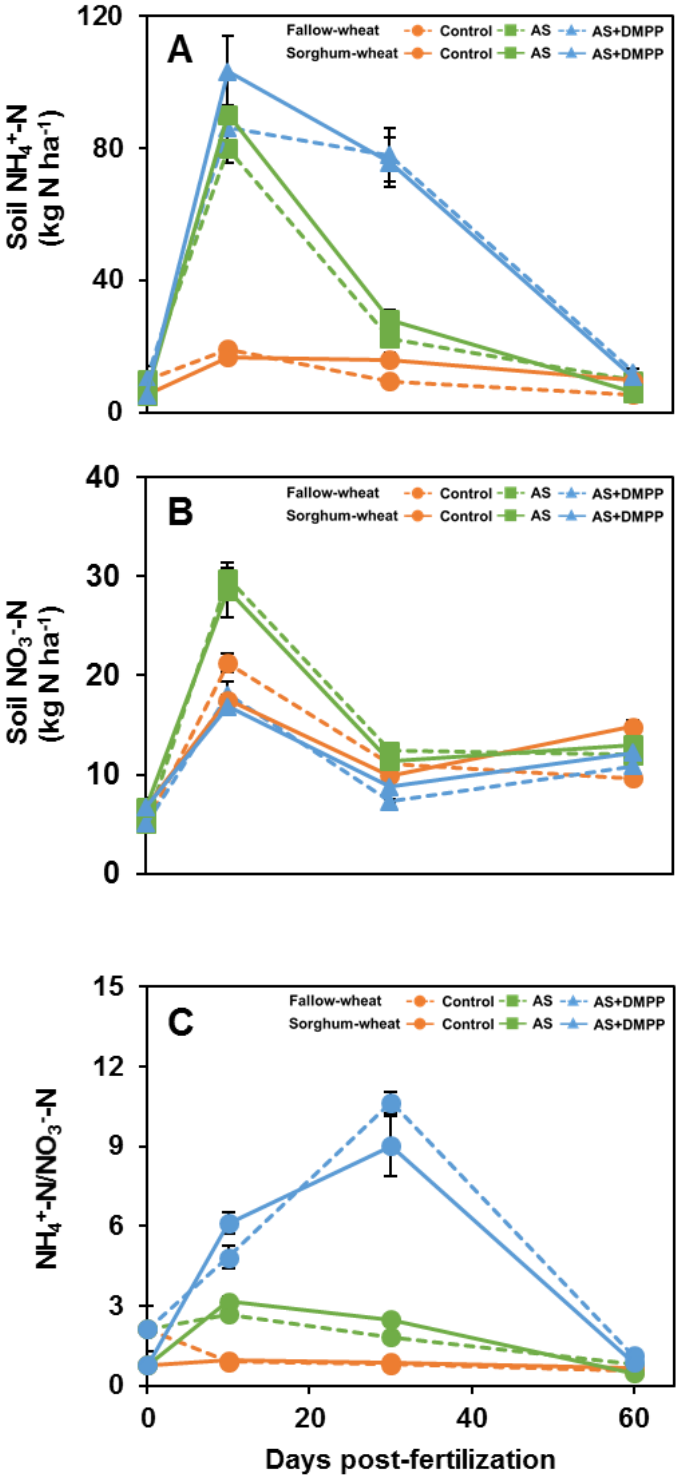
2.6. Statistical analysis

The results from soil mineral nitrogen content determinations were subject to a two-way (crop rotation, R; and fertilizer treatment, F) analysis of variance statistical analysis. The results of N_2O measurements, microbial quantification and grain yield parameters were analysed by one-way ANOVA using Duncan's multiple range test for separation of means

between treatments and the Mann–Whitney U test was used to compare the two treatments with the SPSS statistical software package (2016, IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, IBM Corp). p-values < 0.05 were considered to be statistically significant differences.

RESULTS AND DISCUSSION

Fertilisation had a strong effect on soil mineral N content ($p < 0.01$). Soil from both crop rotations started with a similar soil NH_4^+ content before fertiliser application to the wheat crop (Fig. 1A). After the addition of the N fertiliser, soil NH_4^+ content increased to the same extent in the AS and AS+DMPP treatments for both crop rotations, but there was a significant decrease in the AS treatment at 30 days post-fertilisation (DPF). However, at the same time, the use of DMPP meant the soil retained twice the amount of NH_4^+ compared to the AS treatment. Although the NH_4^+ content for the AS and AS+DMPP treatments dropped to the level of the Control treatment at 60 DPF, DMPP was able to prolong the availability of NH_4^+ at least until 30 DPF. This was in line with other studies that used DMPP as a NI (Huérfano et al., 2015; 2016; Liu et al., 2020). On the contrary, AS+DMPP reduced NO_3^- content to 40% at 10 DPF and 30% at 30 DPF compared to the AS treatment (Fig. 1B). The Control treatment also maintained low soil NO_3^- values throughout the experiment. As NH_4^+ content remained high and NO_3^- content was low due to the delay in NH_4^+ oxidation (Ruser and Schulz, 2015), AS+DMPP presented the highest NH_4^+/NO_3^- ratio of all the fertilised treatments (Fig. 1C). AS treatment showed a higher NH_4^+/NO_3^- ratio than Control treatment but it was only able to maintain 53% and 22% of AS+DMPP ratio at 10 and 30 DPF, respectively. On the other hand, we did not find any differences between the two crop rotations in the maintenance of soil mineral N, in terms of soil NH_4^+ and soil NO_3^- content, during wheat development (Fig. 1A and B). This may indicate that even though cover crops carries benefits for the following culture such as improved soil physicochemical properties (such as water holding capacity, aggregate stability and C stock) (Buyer et al., 2010; Lal, 2015; Poeplau and Don, 2015) or reduced N losses (Kaye and Quemada, 2017), in this case, the use of sorghum as a summer cover crop did not affect the evolution of soil mineral N during the following crop. Nonetheless, the analysis of soil mineral N in the subsequent culture may not be sufficiently sensitive to detect the effects of using sorghum as a cover crop.



	10	30	60
R	*	NS	NS
F	**	**	**
RxF	*	NS	NS

	10	30	60
R	*	NS	NS
F	**	**	NS
RxF	NS	NS	NS

	10	30	60
R	NS	NS	NS
F	**	**	NS
RxF	NS	NS	NS

Fig. 1. *Wheat crop soil mineral nitrogen (0 – 30 cm) evolution during 60 days post-fertilisation in form of NH_4^+ (**A**), NO_3^- (**B**) and the ratio NH_4^+-N/NO_3^-N (**C**). Control = control without fertilization; AS = fertilised with ammonium sulphate; AS+DMPP = fertilised with ammonium sulphate + DMPP. Statistical analysis was made through analysis of variance (two-way ANOVA) showing the effect of crop rotaion (R), fertilizer*

treatment (F) and their interaction (RxF). Significant differences are marked with an asterisk (*) when p <0.05 and double asterisk (**) when p <0.01.

Fertiliser treatment did not affect the total bacterial abundance (measured as *16S rRNA* gene abundance) (Fig. 2A). However, the AS treatment greatly enhanced nitrification (in terms of bacterial *amoA* gene abundance), especially in fallow–wheat rotation (Fig. 2B). Even though adventitious plants were desiccated with glyphosate-based herbicide to create a fallow plot, the great AOB growth in AS treatment in fallow–wheat rotation indicated the lack of deleterious effects of glyphosate on nitrifying microorganisms. This result is in line with previous studies where was demonstrated that higher doses of glyphosate or repeated exposure did not affect nitrifying populations (Allegrini et al. 2017; Zabaloy et al. 2017). The application of the DMPP was very effective at reducing AOB abundance in soils from both crop rotations, even down to the levels of the unfertilised control, with reductions of 56% and 40% compared to AS in fallow–wheat and sorghum–wheat rotations, respectively. Notwithstanding that some studies in microcosms have reached an AOB inhibition of over 85% with the use of DMP-based inhibitors (Torralbo et al., 2017; Corrochano-Monsalve et al., 2021a; Bozal-Leorri et al., 2021), our results are in line with field studies where the AOB inhibition was efficient but at lower percentages (Kleineidam et al., 2011; Duncan et al., 2017). Although the different crop rotations did not affect the soil N content, it did influence soil microbial populations. We observed a significant increase in the total bacterial abundance in soil of sorghum–wheat rotation, as it was 24% and 34% higher compared to fallow–wheat for the control and AS treatments, respectively (Fig. 2A). Furthermore, the type of crop rotation also affected AOB abundance, as the levels for the control and AS treatments for the sorghum–wheat rotation were 35% and 22% lower than the fallow–wheat plots (Fig. 2B). This reduction indicates that, during its development, sorghum might have exuded BNIs that can keep nitrifiers inhibited until the next crop. Dayan et al. (2010) indicated that the inhibitory effect of sorghum could persist for at least 60 days after the harvest was removed. In our case, the BNIs may had a more enduring effect (140 days) because the sorghum was not eliminated from the cultivation soil and the experiment was carried out under no-tillage conditions, which retards BNI degradation (Roth et al., 2000). Thus, leaving the sorghum stover in the soil under no-tillage conditions ensures slower root degradation and the consequent release of exudates with a BNI capacity because such compounds are produced exclusively in the roots (Baerson et al., 2008).

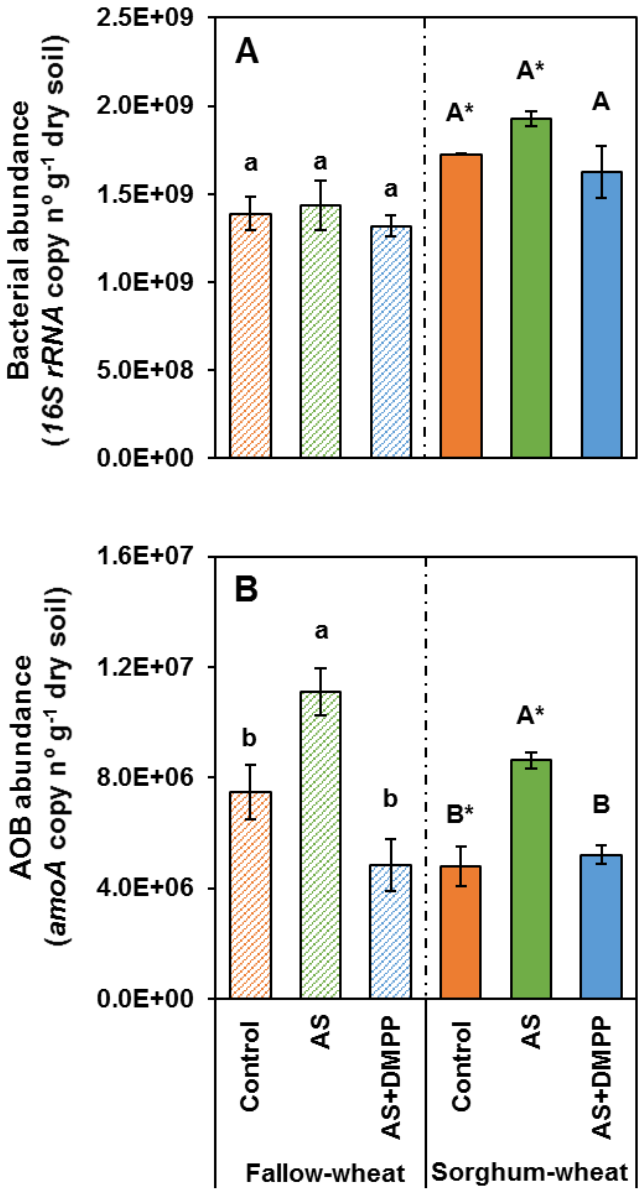


Fig. 2. *Abundance of total bacteria (**A**) and ammonia oxidizing bacteria (AOB) (**B**) at 10 days post-fertilisation (DPF) on soil of wheat crop. Control = control without fertilisation; AS = fertilised with ammonium sulphate; AS+DMPP = fertilised with ammonium sulphate + DMPP. Significant differences between treatments of "Fallow-wheat" rotation are marked with a lowercase letter. Significant differences between treatments of "Sorghum-wheat" rotation are marked with a capital letter. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).*

Daily N_2O emissions ranged from 0.89 to 13.74 g N_2O-N ha^{-1} d^{-1} in fallow–wheat rotation and from 0.38 to 24.58 g N_2O-N ha^{-1} d^{-1} in sorghum–wheat rotation (Fig. 3A). The cumulative N_2O emissions for the AS treatments were the highest of all the fertiliser treatments with 382.9 and 678.3 g N_2O-N ha^{-1} in soil from the fallow–wheat and sorghum–wheat rotations, respectively (Fig. 3B). As is well supported elsewhere (Ruser and Schulz, 2015), DMPP reduced cumulative N_2O emissions to values akin to the Control treatment, corresponding to reductions of 79% and 86% compared to the AS treatment for the fallow–wheat and sorghum–wheat rotations, respectively. Since reduced AOB populations were reduced in the wheat crop, probably because of the BNIs released from sorghum, we also expected a decrease in N_2O emissions (either due to a reduction in N_2O emitted by nitrifiers or a decrease in denitrifying activity because of a delay in the transformation of NH_4^+ into NO_3^-). However, as shown in Fig. 3A, N_2O emissions from the AS treatment of sorghum–wheat rotation were higher than those for the fallow–wheat rotation throughout the entire experiment, resulting in a 77% increase in cumulative N_2O emissions (Fig. 3B). In a meta-analysis of 106 observations, Basche et al. (2014) found that cover crop treatments increased N_2O emissions in the subsequent culture in 60% of cases compared to the land remaining fallow. Han et al. (2017) suggested that different N_2O emission responses are observed because cover crops only tend to decrease N_2O emissions with respect to fallow lands if they are accompanied by a reduction in N fertilisation in the posterior crop. Besides, sorghum stover is an extra C source, and the main mechanism connecting C cycling with N gas emissions is the C availability in the soil that enhances heterotrophic denitrification, which is one of the main processes responsible for N_2O production (Davidson et al., 2000). Furthermore, N_2O emissions are related to soil water content (Davidson, 1991), with a threshold of 60% WFPS between water-limited and aeration-limited microbial processes. In the present work, during N_2O measurements, the soil WFPS remained between 45% and 60% (Supplementary Fig. 2), a range in which denitrifying microorganisms become more relevant for N_2O release. Therefore, we argue that the increment in N_2O emissions from soils in the sorghum–wheat rotation is due to an enhanced heterotrophic denitrification because of a greater C availability, as larger portions of labile C substrates promote denitrification reactions (Surey et al., 2020). This greater abundance of heterotrophic denitrifiers in sorghum–wheat rotations was evidenced by the increased abundance of nitrite reductase (NIR) enzyme containing denitrifying bacteria (Fig. 4A and B).

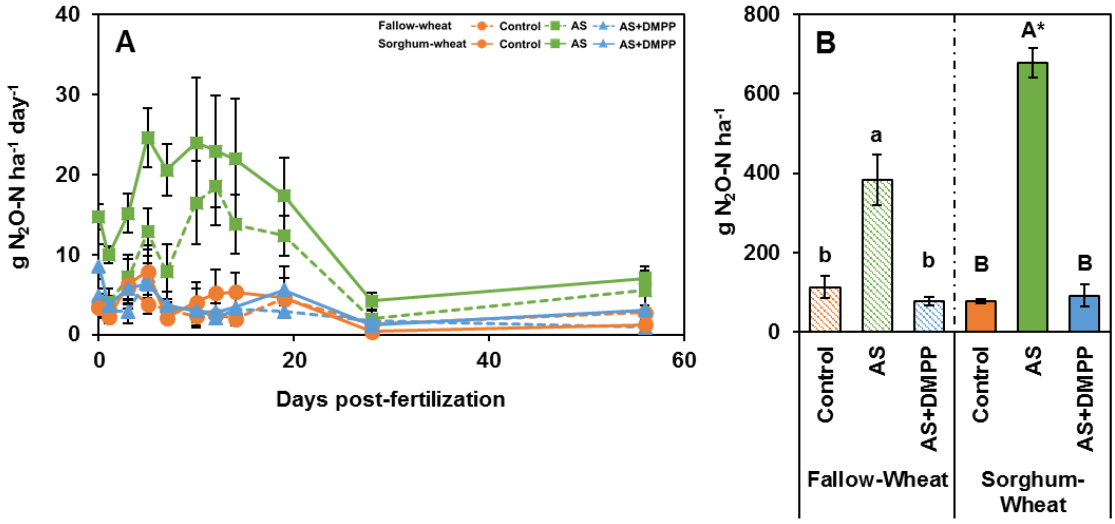


Fig. 3. *Daily (**A**) and cumulative (**B**) N_2O emission during 56 days post-fertilisation on soil of wheat crop. Control = control without fertilisation; AS = fertilised with ammonium sulphate; AS+DMPP = fertilised with ammonium sulphate + DMPP. Significant differences between treatments of "Fallow-wheat" rotation are marked with a lowercase letter. Significant differences between treatments of "Sorghum-wheat" rotation are marked with a capital letter. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).*

The Control and AS treatments from the sorghum–wheat rotation showed a 56% and 73% increase in *nirK* abundance compared to the equivalent treatments on the fallow–wheat rotation (Fig. 4A). On the other hand, the abundance of *nirK* was not affected by any of the treatments (with or without N) on the fallow–wheat rotation, but the AS+DMPP treatment on the sorghum–wheat rotation had a lower *nirK* abundance than the Control and AS treatments. Comparing crop rotations in the case of *nirS* abundance, an increase of 30% was only significant for the AS treatment (Fig. 4B). Similarly to *nirK*, *nirS* abundance was not affected by fertiliser treatment in the fallow–wheat rotation, but the AS+DMPP treatment presented a lower level than the Control and AS treatments for the sorghum–wheat rotation. In this case, regarding complete denitrifiers, neither the crop rotations nor the N treatments affected both *nosZI* and *nosZII* genes abundances (Fig. 4C and D).

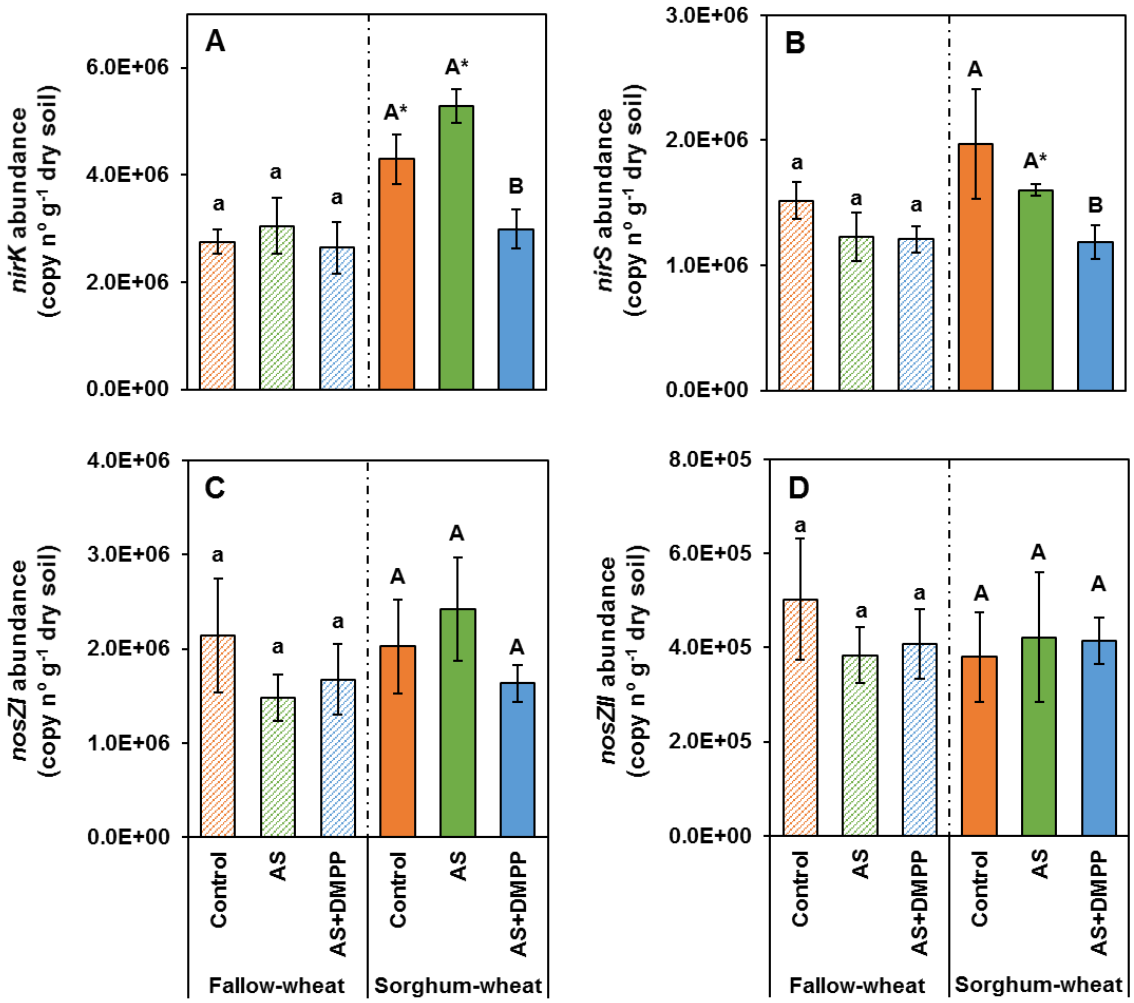


Fig. 4. *Abundance of denitrifying bacteria measured as the abundance of nirK (**A**), nirS (**B**), nosZI (**C**) and nosZII (**D**) genes at 10 days post-fertilisation (DPF) on soil of wheat crop. Control = control without fertilisation; AS = fertilised with ammonium sulphate; AS+DMPP = fertilised with ammonium sulphate + DMPP. Significant differences between treatments of "Fallow-wheat" rotation are marked with a lowercase letter. Significant differences between treatments of "Sorghum-wheat" rotation are marked with a capital letter. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).*

In contrast to the soil mineral N, the changes in the abundances of N-related microorganisms were sensitive enough to detect the effects of using a cover crop. In soil with fallow–wheat rotation, AS treatment presented the highest *amoA*/*nirK* and *amoA*/*nirK*+*nirS* ratios of all three treatments (Table 2). This means that those soils were more balanced towards nitrification since the addition of N-fertilisation increases the

level of nitrification genes (Ouyang et al., 2018). In the same manner, there was a higher ratio between N_2O production in denitrification and N_2O reduction (*nirK*+*nirS*)/(*nosZI*+*nosZII*). On the other hand, the addition of DMPP decreased *amoA* abundance, which is why this treatment presented the lowest *amoA*/*nirK* and *amoA*/*nirK*+*nirS* ratios. Fertiliser treatment did not affect the nitrifying/denitrifying ratios in the sorghum–wheat rotation. Nevertheless, the type of crop rotation influenced these ratios, as there were significant differences between them. The increase of denitrifying microorganisms containing NIR enzyme due to the extra input of C from sorghum stover (Fig. 4A and B), balanced the *amoA*/*nirK* and *amoA*/*nirK*+*nirS* ratios towards denitrification. This produced a 60% reduction in the *amoA*/*nirK* ratio and 55% in the *amoA*/*nirK*+*nirS* ratio for the control treatment on the sorghum–wheat rotation compared to fallow–wheat. Furthermore, the reduction of the AOB population in the AS treatment (Fig. 2B) due to the potential release of BNIs from sorghum roots also contributed to the decrease in *amoA*/*nirK* and *amoA*/*nirK*+*nirS* ratios with a 51% and 54% reduction, respectively, compared to the fallow–wheat rotation. Moreover, although the abundances of *nirK* and *nirS* increased due to the extra C, this did not affect the abundance of *nosZI* and *nosZII* genes. We theorize that, somehow, the use of sorghum as a cover crop favoured a scenario of incomplete denitrification, which increased the emission of N_2O due to the lack of increase of the microorganisms that could reduce it completely to N_2. Consequently, focusing on the capacity to modify the nitrifying/denitrifying ratio of the different crop rotations, we might develop a better understanding of how soil N emissions respond, such as the different N_2O emissions for the AS treatment. However, since the AS+DMPP treatment significantly inhibited AOB growth (Fig. 2B), soil NO_3^- formation diminished compared to the AS treatment and there was no increase in *nirK* and *nirS* genes derived from a greater C availability (Fig. 4A and B), ultimately resulting in similar nitrifying/denitrifying ratios between crop rotations. In addition, AS+DMPP treatment showed the lower (*nirK*+*nirS*)/(*nosZI*+*nosZII*) ratio, resulting in a better balance between N_2O production/N_2O reduction. We therefore suggest the use of synthetic NIs such as DMPP to reduce the pollution derived from the use of sorghum as a cover crop. Moreover, Menéndez et al. (2012) reported that the reduction in N_2O emissions induced by DMPP is conditioned by the magnitude of the losses from the fertiliser without NIs. Thus, DMPP can counteract higher N_2O emissions with greater efficiency, as can be observed in our experiment with a 79% reduction of N_2O emissions with respect to AS in the fallow–wheat rotation versus 86% in the sorghum–wheat rotation (Fig. 3B).

Table 2. *The amoA/nirK, amoA/(nirK+nirS) and (nirK+nirS)/(nosZI+nosZII) ratio on soil of wheat crop. Control = control without fertilisation; AS = fertilised with ammonium sulphate; AS+DMPP = fertilised with ammonium sulphate + DMPP. Significant differences between treatments of "Fallow-wheat" rotation are marked with a lowercase letter. Significant differences between treatments of "Sorghum-wheat" rotation are marked with a capital letter. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).*

		amoA/nirK		***amoA/(nirK+nirS)***		***(nirK+nirS)/ (nosZI+nosZII)***	
Fallow-wheat	**Control**	2.76 ± 0.44	ab	1.77 ± 0.24	ab	1.49 ± 0.25	b
	AS	3.37 ± 0.45	a	2.79 ± 0.61	a	2.27 ± 0.15	a
	AS+DMPP	1.86 ± 0.29	b	1.25 ± 0.19	b	2.36 ± 0.18	a
Sorghum-wheat	**Control**	1.11 ± 0.14	A*	0.78 ± 0.14	A*	2.45 ± 0.14	B*
	AS	1.64 ± 0.09	A*	1.26 ± 0.05	A*	3.00 ± 0.24	A*
	AS+DMPP	1.82 ± 0.32	A	1.29 ± 0.21	A	2.32 ± 0.13	B

Planting winter wheat after a sorghum crop is a common practice, yet it can affect the settlement of wheat seed. Guenzi et al. (1967) demonstrated that water extracts from sorghum residues could inhibit corn and wheat seed germination. This effect is due to the allelopathic substances, such as sorgoleone, that sorghum releases through its roots. Roth et al. (2000) suggested that soil management is the key to counteracting these effects. In soils with conventional tillage management, sorghum exudates are quickly solubilised and degraded. In no-tillage soils by contrast, sorghum remains release their degradation compounds gradually and therefore affect crop yield. Even so, the effects of sorghum residues on wheat seed germination can be mitigated by increasing the seeding rate or delaying the planting of subsequent crops until the residues have decomposed or weathered (Weston et al., 2013). In our experiment, the wheat sowing density was 220 kg ha^{-1}, which seems high enough to palliate the effects of the previous sorghum crop

since the type of crop rotation did not affect the wheat grain yield of fertilised treatments. As expected, wheat grain yield was much higher for the AS and AS+DMPP treatments with 6,616 and 6,133 kg ha^{-1}, respectively, for the fallow–wheat rotation and 6,230 and 6,543 kg ha^{-1} for the sorghum–wheat rotation (Table 3), surpassing the average for that region in 2019, which was 5,000 kg ha^{-1} (MAGRAMA, 2019). Similarly, the use of SNIs did not affect grain yield, which was in line with other works where DMPP maintained the grain yield of rainfed winter cereals compared to fertiliser treatments without inhibitor (Arregui and Quemada, 2008; Huérfano et al., 2016). Furthermore, the number of spikes m^{-2} was also higher in the AS and AS+DMPP treatments in both crop rotations than in the Control treatments. However, even though crop rotations did not influence the grain yield of fertilised treatments, this was not the case for the Control treatment, which presented a decrease in wheat grain yield in the sorghum–wheat rotation (Table 3). This decrease may be due to the competition for nutrients between plants and soil microbes in soils with a low N content. It is assumed that heterotrophic soil microorganisms are stronger competitors for inorganic N than plants (Kaye and Hart, 1997). Moreover, the growth of these microorganisms is C-limited. Therefore, when soil mineral N increased in the Control treatments, such as between 0 and 10 DPF when mineralisation can be observed (Fig. 1A and B), the additional C from sorghum stover led to an increase in the total bacterial abundance compared to the fallow–wheat rotation (Fig. 2A), and therefore to a greater competition against the plants for soil N uptake. In addition, at these stages for wheat plants grown in a sorghum–wheat rotation, the reduction in N uptake was evident as the number of spikes m^{-2} in the control treatment of the sorghum–wheat rotation was lower than those for the fallow–wheat rotation. Despite this, there were no significant differences between fertiliser treatments or between crop rotations on the number of grains per spike and the percentage of grain protein (Table 3).

Table 3. *Wheat grain yield, yield components, grain protein, N yield scaled N_2O emissions (YSNE) and Nitrogen Use Efficiency (NUE). Control = control without fertilisation; AS = fertilised with ammonium sulphate; AS+DMPP = fertilised with ammonium sulphate + DMPP. Significant differences between treatments of "Fallow-wheat" rotation are marked with a lowercase letter. Significant differences between treatments of "Sorghum-wheat" rotation are marked with a capital letter. For both ANOVA, the Duncan Test was used ($p < 0.05$; $n = 4$). The Mann-Whitney U test was used for the comparison between crop rotations within the same fertilization treatment. Significant differences at $p < 0.05$ are marked with an asterisk (*).*

		Grain yield (kg ha $^{-1}$)	Spikes m^{-2}	1000 grains DW (g)	Grains spike^{-1}	Grain protein (%)	YSNE (g N_2O-N kg^{-1} N uptake)	NUE (kg DM kg^{-1} N)
Fallow-wheat	Control	5120 ± 520 b	313 ± 10 b	40.3 ± 1.0 a	39.9 ± 2.8 a	7.1 ± 0.2 a	1.7 ± 0.3 b	
	AS	6616 ± 452 a	452 ± 30 a	40.4 ± 0.5 a	36.9 ± 3.6 a	7.0 ± 0.4 a	4.5 ± 0.6 a	22.0 ± 6.8 a
	AS+DMPP	6133 ± 271 a	395 ± 22 a	39.7 ± 1.1 a	42.0 ± 5.8 a	6.7 ± 0.3 a	1.2 ± 0.2 b	22.2 ± 5.1 a
Sorghum-wheat	Control	3860 ± 103 B*	252 ± 18 B*	38.5 ± 0.4 B	40.0 ± 4.4 A	7.0 ± 0.2 A	1.7 ± 0.1 B	
	AS	6230 ± 362 A	439 ± 21 A	39.9 ± 0.4 A	36.7 ± 4.2 A	7.3 ± 0.2 A	8.7 ± 1.0 A*	26.3 ± 4.0 A
	AS+DMPP	6543 ± 347 A	429 ± 35 A	39.7 ± 0.3 A	40.0 ± 3.6 A	7.0 ± 0.1 A	1.2 ± 0.4 B	29.8 ± 3.7 A

Furthermore, attending to the nitrogen use efficiency (NUE), there were also no differences between fertilisation treatments or crop rotations. Finally, to evaluate the N_2O efficiency of cropping systems and develop strategies for optimal crop productivity, and hence minimise environmental contamination, it may be more informative to express N_2O emissions in relation to crop productivity (YSNE) (van Groenigen et al., 2010; Schwenke and Haigh, 2016). The AS treatment applied to both crop rotations presented an increased YSNE compared to the Control and AS+DMPP treatments, which were equally low. These treatments did not show any differences between the two crop rotations, but fertilising wheat with AS in the sorghum–wheat rotation had a 93% higher YSNE than wheat fertilised with AS in the fallow–wheat rotation. Thus, when sorghum was used as a cover crop, it produced twice the N_2O emissions per kg of N uptake because it increased the gaseous N losses.

CONCLUSIONS

The use of sorghum as a cover crop might not be a suitable option to mitigate the N_2O emissions derived from the N fertiliser application in the subsequent crop. Sorghum–wheat rotation did not present any effect on the maintenance of soil NH_4^+ content during wheat crop development, as the levels were the same as the fallow–wheat rotation. Although the potential release of BNIs from sorghum roots produced a 22% decrease in the growth of AOB in the AS treatment compared to the fallow–wheat rotation, it was not enough to reduce N_2O emissions. We theorize that the 77% increase in cumulative N_2O emissions for the AS treatment applied to the sorghum–wheat rotation was the result of the increase of heterotrophic denitrification due to a higher C availability from sorghum stover, since *nirK* and *nirS* genes were 73% and 30% more abundant compared to the fallow–wheat rotation. Moreover, while the type of crop rotation did not affect wheat grain yields, the higher cumulative N_2O emissions of the AS treatment on the sorghum–wheat rotation produced a 93% increase in the emissions of N_2O per kg of N uptake compared to the fallow–wheat rotation. However, we suggest the use of synthetic NIs such as DMPP to avoid the greater N_2O release derived from the use of sorghum as a cover crop. The application of DMPP maintained AOB growth at the levels of the Control treatment, thereby delaying soil NH_4^+ oxidation. As more soil NH_4^+ content was maintained, soil NO_3^- formation was diminished compared to AS, thus mitigating the

increase of *nirK* and *nirS* genes resulting from the higher C availability in the sorghum–wheat rotation. The cumulative N_2O emissions were also maintained at the levels of the Control treatment. Therefore, as grain yield was not affected by the use of the synthetic NI, the AS+DMPP treatment yielded reductions of 73% and 86% in the emissions of N_2O per kg of N uptake compared to the AS treatment in the fallow–wheat and sorghum–wheat rotations, respectively.

SUPPLEMENTARY MATERIAL

Table 1. *Physical and chemical properties of the soil collected in 0 – 30 cm depth layer in Pamplona (42° 47' N, 1° 37' W, 450 m above sea level, Navarre, Spain).*

Soil texture			Soil chemical properties								
						Organic					
Sand	Silt	Clay	pH[a]	C:N	N[b]	matter[c]	Carbonate[d]	Mg[d]	K[d]	Ca[d]	P[e]
(%)					(g kg^{-1})			(mg kg^{-1})			
38.6	31.8	29.6	8.3	8.9	1.4	21.5	20.3	53.5	270.0	2735.7	11.5

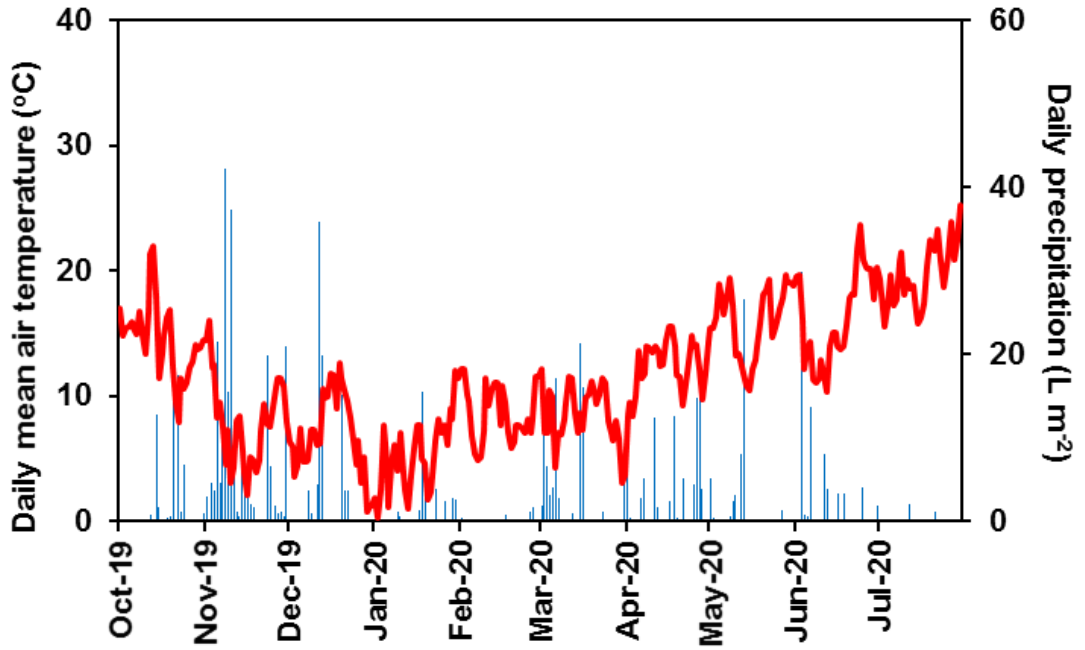
a. pH (1:2.5 soil:water).

b. N Kjeldahl digestion (Keeney and Nelson, 1982).

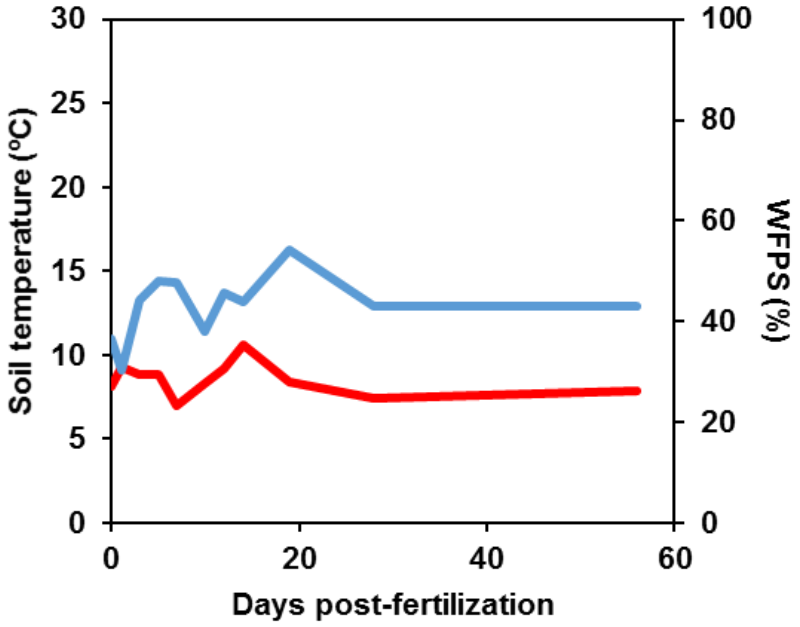
c. Organic matter (Walkley and Black, 1934).

d. CaCO3 , Mg, K (NH4 AcO, MAPA, 1994).

e. P (Watanabe and Olsen, 1965).



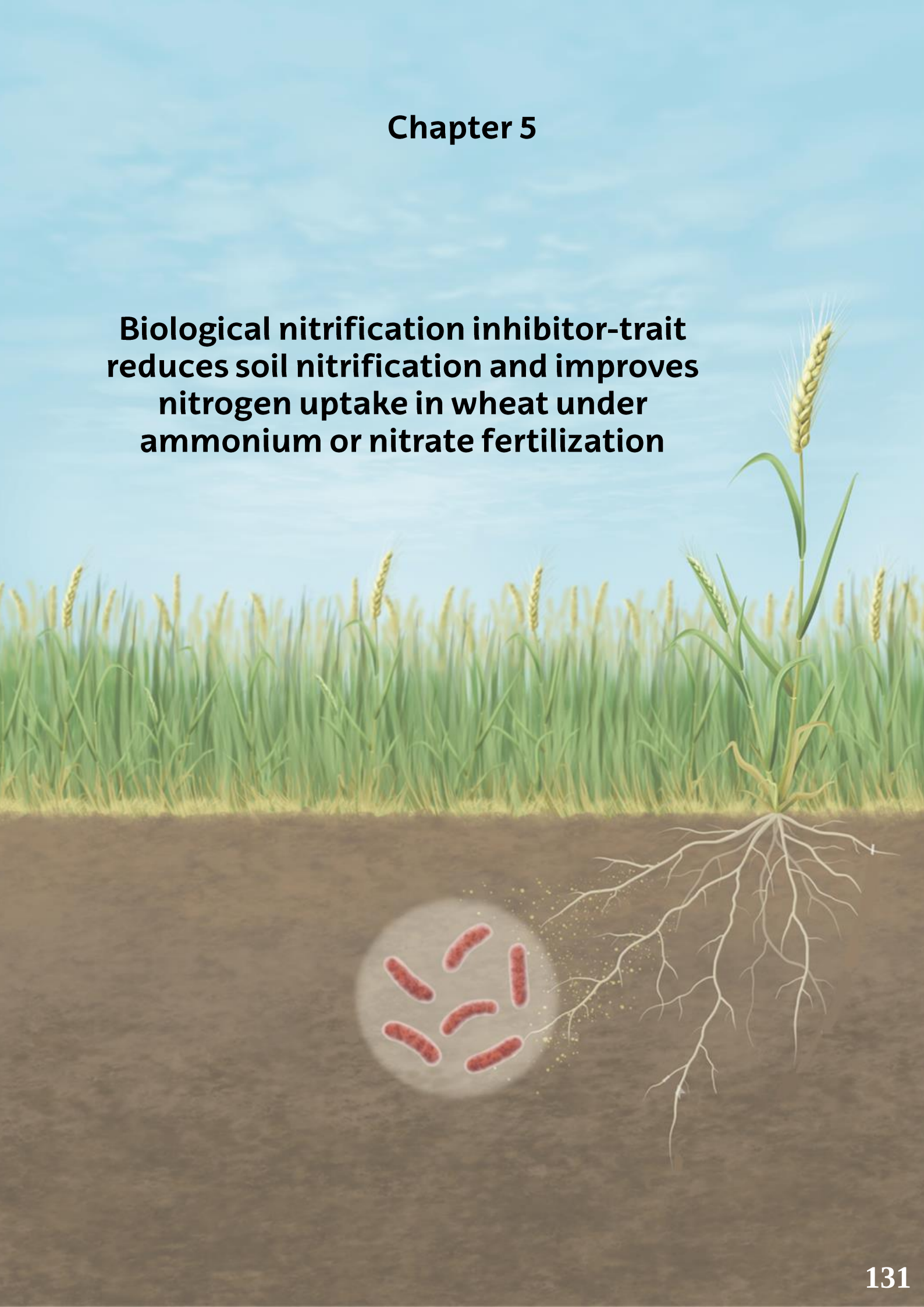
Supplementary fig. 1 *Daily precipitation (blue bars) and mean air temperature (red line) of growing season 2019/2020.*



Supplementary fig. 2 *Evolution of soil temperature (0-10 cm) (red line) and soil WFPS (0-30 cm) (blue line) during N_2O emissions measurements in winter wheat experiment.*

Chapter 5

Biological nitrification inhibitor-trait reduces soil nitrification and improves nitrogen uptake in wheat under ammonium or nitrate fertilization



ABSTRACT

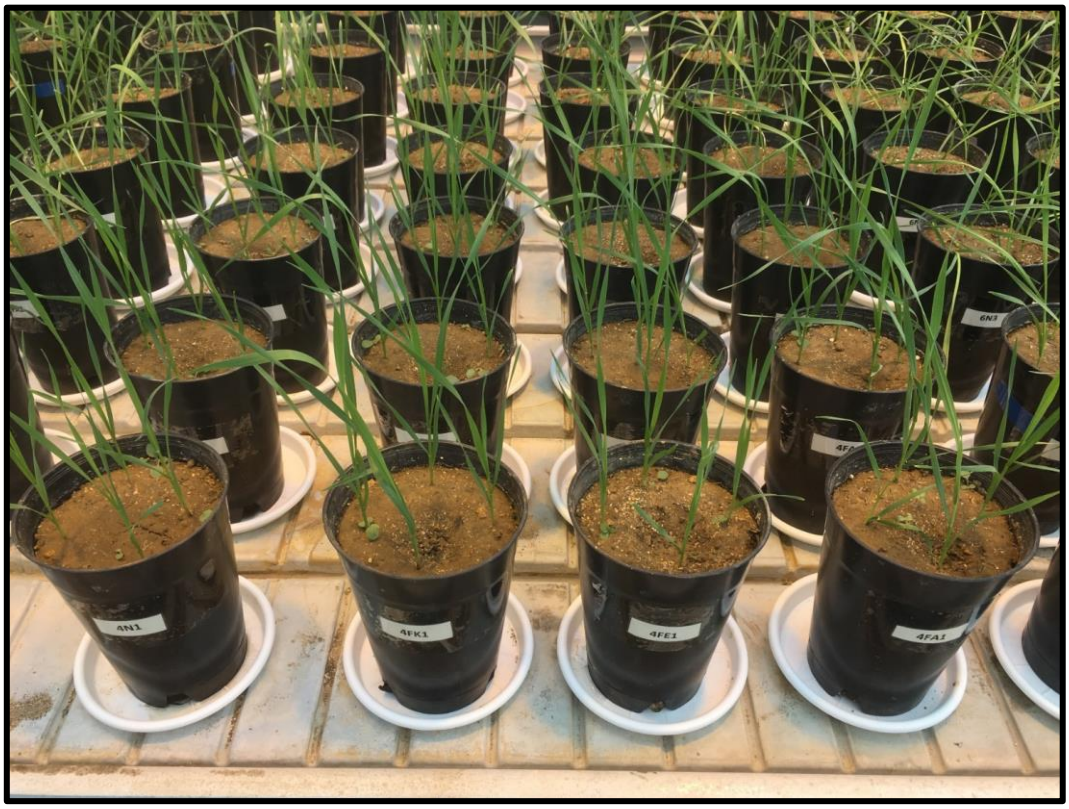
Synthetic nitrification inhibitors (SNI) and biological nitrification inhibitors (BNI) are promising tools to limit nitrogen (N) pollution derived from agriculture. However, SNIs performance varies substantially depending on soil conditions, and modern wheat cultivars lack the ability to exude BNIs. Fortunately, the chromosome region (Lr#n-SA) controlling BNI production in *Leymus racemosus*, a wild relative of wheat, was introduced into two elite wheat cultivars, ROELFS and MUNAL. These two high BNI isogenic lines can inhibit nitrification in acidic soils under ammonium nutrition. We evaluated the BNI trait expression of ROELFS-BNI and MUNAL-BNI under ammonium and nitrate fertilization in alkaline soil. Using BNI isogenic lines could be an efficient technology with potential use worldwide, in support of more sustainable agricultural and environmentally friendly agronomic practices. In the soil, ROELFS-BNI and MUNAL-BNI plants decreased ammonia-oxidizing bacteria (AOB) abundance by 60% and 45% respectively, delaying ammonium oxidation without reducing the total abundance of bacteria or archaea. Moreover, under nitrate fertilization, BNI-isogenic lines reduced soil nitrate content. ROELFS-BNI and MUNAL-BNI plants showed a reduced leaf nitrate reductase (NR) activity and a higher amino acid content compared to BNI-trait lacking lines, indicating that plants preferred ammonium as N source. In addition, the benefits from introduction of BNI-trait into ROELFS and MUNAL wheat cultivars were also noticeable for nitrate fertilization with improved N absorption.

MATERIALS AND METHODS

2.1. Experimental design and plant material

This experiment was carried out in microcosms in a controlled conditions greenhouse with a daily day/night cycle regimen of 14/10 h, average temperature of 25/18 ºC, and relative humidity of 50/60%. Four elite wheat (*Triticum aestivum*) genetic stocks comprising two isogenic lines for BNI-capacity (ROELFS-Control, ROELFS-BNI, MUNAL-Control, MUNAL-BNI) are used in this study (Subbarao et al., 2021). To germinate, 240 seeds per BNI-isogenic line were placed in square Gosselin plates at 5 °C

in darkness for 7 days. Then, seeds were transferred to trays with perlite:vermiculite (1:3) mixture at 20 °C for 4 days.



Growth of ROELFS-BNI and MUNAL-BNI wheat lines in pots in the greenhouse

Soil was collected in June 2019, from a 0–30 cm layer of Hypercalcic Kastanozen soil (IUSS 2014) in a wheat field (Supplementary table 2) in Arkaute (Basque Country, Spain) (42° 51' N, 2° 37' W, 530 m above sea level). Soil was passed through a 5 mm sieve after roots and stones being removed. Soil was mixed with sand in proportion of soil:sand (3:1, v:v) to increase soil porosity and to avoid compaction that would prevent normal root development. Afterward, soil was air-dried, homogenised, and kept at 4° C until the start of the experiment. To reactivate soil microorganisms sixty-four 1.35 L pots (12.5 cm diameter x 17 cm height) were filled with soil, 86 mg of ammonium sulphate ($(NH_4)_2SO_4$) and 1.1 g of glucose (Menéndez et al., 2012) were added to each pot, and soil was rehydrated with deionised water up to 45% water filled pore space (WFPS). WFPS was calculated following the equation described in Linn and Doran (1984):

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

Soil bulk density was determined in the laboratory, resulting in a value of 1.31 Mg m^{-3}, while particle density was assumed at 2.65 Mg m^{-3}. During soil activation, pots were divided into four groups (one per Control and BNI isogenic lines of ROELFS and MUNAL) and three N fertilization treatments with four replications for each isogenic line-N fertilization combination were established. After 14 days of soil activation, 4 seedlings of their corresponding BNI-isogenic line were placed in each pot, and soil was watered for 15 days to maintain the WFPS up to 45%, to maintain nitrifying conditions. On the 15th day, T0 was harvested and N fertilizer treatments were added, as following 1) fertilization with potassium nitrate (KN); 2) fertilization with ammonium sulphate (AS); and 3) fertilization with ammonium sulphate + DMPP (AS+D). N was applied in an equivalent dose to 195 kg N ha^{-1}, which was achieved by adding 1726 mg of potassium nitrate (KNO_3) or 1128 mg of $(NH_4)_2SO_4$, alone or mixed with DMPP (EuroChem Agro Iberia S.L.; ENTEC21); DMPP content represented 0.8% of applied N. To achieve a homogeneous distribution of nitrogen in the soil, fertilizers were dissolved in deionised water and added to the corresponding treatments by pipetting. All the treatments were watered every two days to maintain the WFPS up to 45% during the experiment duration (up to 30 days post-fertilization). After this time, BNI-isogenic lines were harvested for measurements or immediately frozen in liquid N for biochemical and physiological determinations. Soil was sampled in parallel.

2.2. Soil analysis

The abundance of nitrifying and denitrifying genes was quantified through quantitative polymerase chain reaction (qPCR). 0.25 g of dry soil was used to extract the DNA using the PowerSoil DNA Isolation Kit (Quiagen) including the modifications described in Harter et al. (2014). For quantification of total bacterial and archaeal abundance *16 rRNA* gene was used and genes involved in nitrification (bacterial and archaeal *amoA*) and denitrification (*nirK*, *nirS*, *nosZI*, and *nosZII*) were amplified as described in supplementary table 1.

Soil mineral N was determined for soil NH_4^+ and NO_3^- contents. 100 g of fresh soil was mixed with 200 mL 1 M KCl and shaken at 165 rpm for one hour. The soil solution was filtered firstly through Whatman n°1 filter paper (GE Healthcare) and secondly through

Sep-Pak Classic C18 Cartridges 125 Å pore size (Waters) to remove particles and organic matter respectively. The Berthelot method (Patton and Crouch, 1977) was followed to quantify the NH_4^+content. The NO_3^- content was determined according to Cawse (1967).

2.3. Plant determinations and enzymatic activity

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

Leaf NH_4^+, NO_3^-, and total amino acid content were quantified from 50 mg of frozen leaf powder. Plant material was homogenized with 1 mL MilliQ water in a ball miller (Retsch MM 500) for 3 min at a frequency of 27 s^{-1}. Homogenates were incubated at 80 ºC for 5 min and, afterwards, centrifuged at 16,000 *g* for 20 min. Later, supernatants were recovered and stored at -20ºC until metabolite quantification. NH_4^+, NO_3^- and total amino acid content were determined in the supernatants as described in Patton and Crouch (1977), Cataldo et al. (1975), and Yemm et al. (1955), respectively.

Nitrate reductase (NR) activity was determined in leaves by the modified *in vivo* method (Jaworski et al., 1971; Ligero et al., 1987). The NR activity was determined in the flag leaf, which was harvested between Z51 and Z53 stages (Zadoks et al., 1974). Once leaf tissues were removed from the plant, they were immediately sliced in 2 mm-width pieces with a razor blade, 0.2 g FW was placed into test tubes with 10 mL of incubation medium containing 100 mM potassium phosphate buffer, 1 mM EDTA, and 1% (v/v) propanol at a pH of 7.5. Assay tubes were vacuum infiltrated twice at 450 mm Hg for 5 min to get a better accession of the assay solution into the cells. Leaf segments were incubated at 30 ºC in dark for one hour to inhibit nitrite reductase, and afterwards, NO_2^- released into the incubation medium was determined by adding in this order equivalent volumes of 1% sulfanilamide in HCl 1.5 N, and 0.1% Griess reagent (N-(1-naphthyl)-ethylenediamine hydrochloride) (Snell and Snell, 1949). Once the colour was developed and constant (15 min later), the absorbance was determined at 540 nm.

2.4. Field experiment design

A field experiment was established at the Experimental Station Norman Borlaug CENEB at the Yaqui Valley, near Ciudad Obregon, Sonora, Mexico in two growing seasons 2018/2019 and 2019/2020. Soil characteristics were described in Subbarao et al. (2021). The experiment was set up as a split plot design with four replications, the main plot had two levels of nitrogen fertilization, 83 and 250 kg N ha^{-1} with $(NH_4)_2SO_4$, and the subplot were the same four genotypes that were used under controlled conditions (ROELFS-Control, ROELFS-BNI, MUNAL-Control, and MUNAL-BNI). The experiment was also fertilized with 69 kg P_2O_5 ha^{-1} using triple super phosphate and applied pre-plant as a broadcast and then incorporated. The experiment was planted within the optimum planting date, with a seed rate of 250 seed m^{-2}. The experiment received five irrigations through the crop cycle when available soil water reached 50% and all weeds, diseases and insects were controlled. The experimental unit was four beds 75 cm apart and 5 m long. The harvest area was the 2 central beds and the central 3 meters and it was done using a Wintersteiger experimental plot combine.

2.5. Statistical analysis

The data obtained in this experiment were analysed by one-way ANOVA using Duncan's multiple range test for separation of means between N fertilization treatments. The Mann–Whitney U test was used to compare the behaviour of ROELFS-Control and MUNAL-Control plants with ROELFS-BNI and MUNAL-BNI plants within the same N fertilization treatment. Correlation analysis were made with the SPSS statistical software package (2016, IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, IBM Corp). For one-way ANOVA and Mann-Whitney U test p-values < 0.05 were considered to be statistically significant differences, while for correlation analysis they were p-values < 0.01. The field experiment was analysed as a split-plot design using the Least Significant Difference test for mean separation among genotypes. The analysis was done using SAS Institute Inc., statistical software package (V9.4) for Windows.

RESULTS

3.1. Abundance of N-cycle related microorganisms

Fertilization with NO_3^- had no effect on nitrifying bacteria, measured as the abundance of bacterial *amoA* gene (Fig. 1A and B). Under AS treatment, AOB growth was greatly enhanced in soils of ROELFS-Control and MUNAL-Control plants. Nevertheless, ROELFS-BNI and MUNAL-BNI plants were able to reduce the bacteral *amoA* abundance by 60% and 45% respectively compared to their Control BNI-isogenic lines. On the other hand, the presence of DMPP avoided the AOB increase in both BNI-isogenic lines of ROELFS and MUNAL, which was maintained at similar abundance to that of before fertilization (T0). N fertilization also increased the AOA abundance, measured as the abundance of archaeal *amoA* gene, in soils of ROELFS and MUNAL BNI-isogenic lines (Fig. 1C and D). In the case of NH_4^+ fertilization, ROELFS-BNI plants achieved 30% and 18% less AOA abundance in AS and AS+D treatments respectively, while in MUNAL-BNI plants only the AS+D treatment was able to lower by 34% the AOA abundance. Total bacterial abundance (Supplementary fig. 1A and B) and total archaeal abundance (Supplementary fig. 1C and D) remained constant regardless the presence or absence of BNI-trait in ROELFS and MUNAL BNI-isogenic lines, or the kind of N fertilization. Regarding the denitrification pathway, there was almost no effect in any of the different analysed genes (*nirK*, *nirS*, *nosZI*, and *nosZII*) comparing the soils from ROELFS-Control and MUNAL-Control against ROELFS-BNI and MUNAL-BNI (Supplementary fig. 2). The only significant difference was found for MUNAL-BNI lines with nitrate fertilization, which reduced 43% the *nirK* abundance (Supplementary fig. 2B), and 48% the *nosZI* abundance (Supplementary fig. 2F) compared to MUNAL-Control plants.

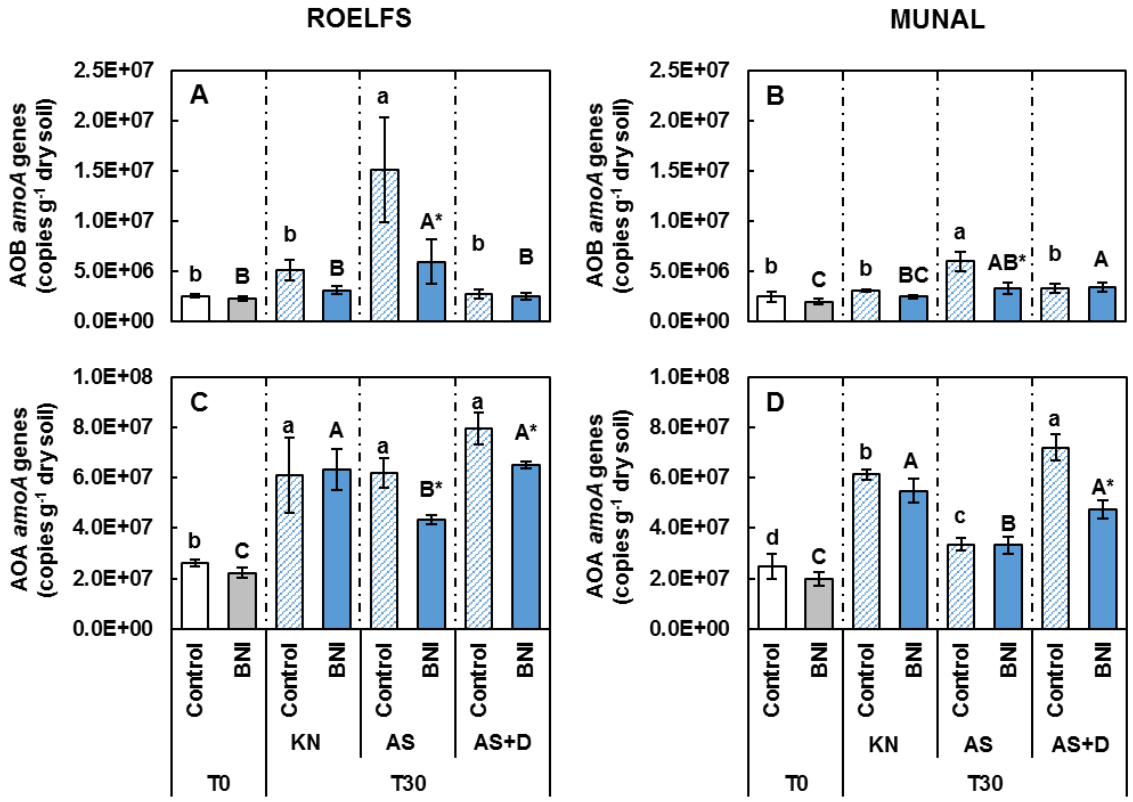


Fig. 1. *Abundance of AOB (**A** and **B**) and AOA (**C** and **D**) of ROELFS (**A** and **C**) and MUNAL (**B** and **D**) BNI-isogenic lines lacking BNI-trait (Control) and with BNI-trait (BNI). Wheat was fertilized with potassium nitrate (KN); ammonium sulphate (AS) and ammonium sulphate + DMPP (AS+D). Significant differences between N treatments of Control isogenic lines are marked with a lowercase letter. Significant differences between N treatments of BNI isogenic lines are marked with a capital letter. The Mann-Whitney U test was used for the comparison between the absence or the presence of BNI-trait within the same fertilization treatment and the significant differences at $p < 0.05$ are marked with an asterisk (*).*

3.2. Soil mineral N

Soil NH_4^+ content of ROELFS-Control and MUNAL-Control plants from AS treatment dropped to values of before fertilization (T0) after 30 days of the experiment (Fig. 2A and B). Fortunately, ROELFS-BNI plants maintained 50% more soil NH_4^+ content than that from ROELFS-Control plants, while MUNAL-BNI plants were able to keep 4 times the soil NH_4^+ content compared to the soil from MUNAL-Control plants. As expected, the addition of DMPP achieved the highest soil NH_4^+ content, which increased around 3 – 26 times compared to AS treatment. However, in presence of SNI, the BNI-trait made

ROELFS and MUNAL behave differently, since soil NH_4^+ content decreased 60% in MUNAL-BNI compared to MUNAL-Control; but, differently, both ROELFS-Control and ROELFS-BNI kept soil NH_4^+ content at maximum.

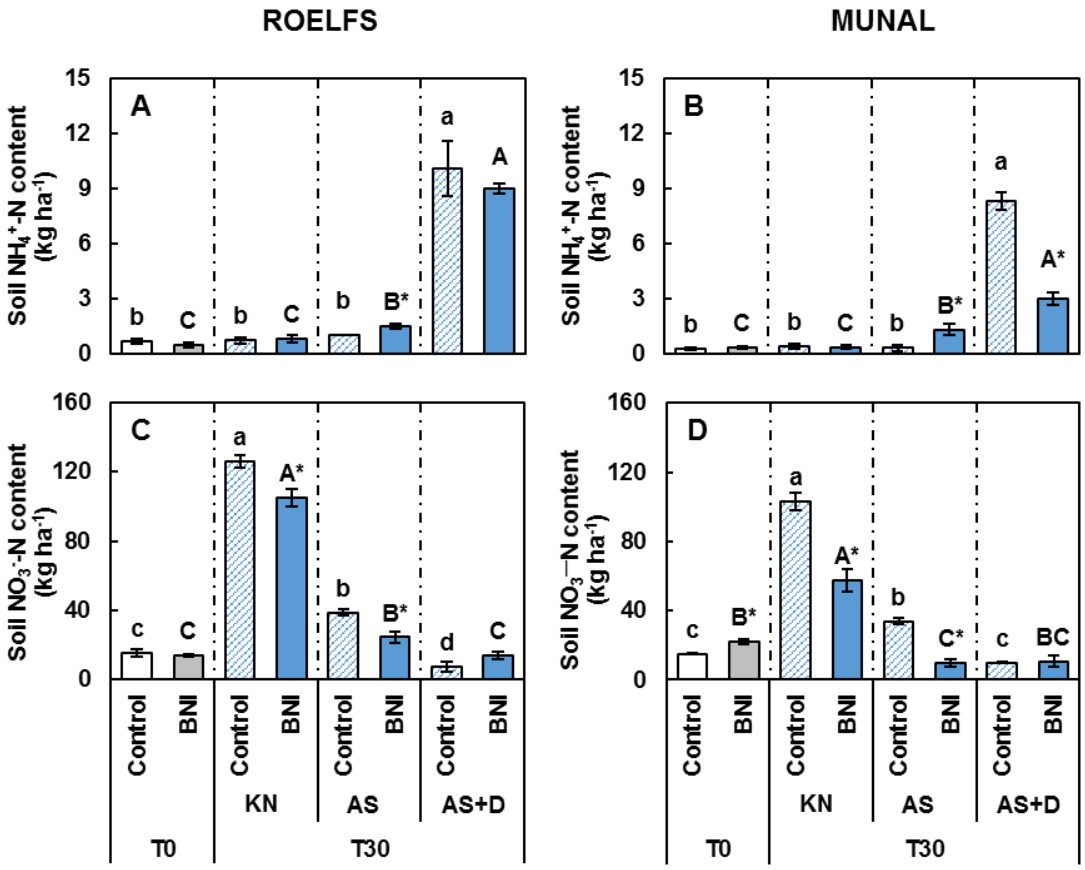


Fig. 2. *Soil mineral nitrogen content such as ammonium (**A** and **B**) and nitrate (**C** and **d**D of ROELFS (**A** and **C**) and MUNAL (**B** and **D**) BNI-isogenic lines lacking BNI-trait (Control) and with BNI-trait (BNI). Wheat was fertilized with potassium nitrate (NK); ammonium sulphate (AS) and ammonium sulphate + DMPP (AS+D). Significant differences between N treatments of Control isogenic lines are marked with a lowercase letter. Significant differences between N treatments of BNI isogenic lines are marked with a capital letter. The Mann-Whitney U test was used for the comparison between the absence or the presence of BNI-trait within the same fertilization treatment and the significant differences at p <0.05 are marked with an asterisk (*).*

Due to fertilization with NO_3^-, the soil of KN treatment from all BNI-isogenic lines of ROELFS and MUNAL showed the highest soil NO_3^- content, followed by AS treatment (Fig. 2C and D). AS+D treatment managed to reduce the apparition of NO_3^- to the level

of soils before fertilization (T0). Comparing soil NO_3^- content, ROELFS-BNI and MUNAL-BNI plants were able to reduce it 16% and 36% respectively in KN treatment. Furthermore, 44% and 74% reduction in soil NO_3^- content was achieved by ROELFS-BNI and MUNAL-BNI plants respectively in AS treatment.

3.3. Plant growth

ROELFS-Control and MUNAL-Control plants reached maximum performance under nitrate fertilization (KN treatment), whereas the lowest growth was found in AS+D treatment (Table 1). The BNI-trait tended to counteract the effect of AS and AS+D fertilization since ROELFS-BNI and MUNAL-BNI plants showed similar biomass regardless of the source of N. Nonetheless, the only difference in plant biomass when comparing Control and BNI isogenic lines was found in AS+D treatment from ROELFS-BNI plants, which showed a 23% increase compared to ROELFS-Control plants. Due to lower growth of ROELFS-Control and MUNAL-Control plants fertilized with NH_4^+ (AS and AS+D treatments) presented the lowest N absorption compared to plants fertilized with NO_3^-. On the contrary, the BNI-trait in MUNAL BNI-isogenic lines made plants to absorb more N regardless the type of fertilization, with increases of 28% for KN, 64% for AS, and 39% for AS+D. Meanwhile, the only treatment that showed differences in absorbed N comparing ROELFS-Control and ROELFS-BNI plants was AS treatment, with a 36% increment.

Table 1. *Biomass (g DW plant^{-1}) and absorbed nitrogen (mg N plant^{-1}) of ROELFS and MUNAL BNI-isogenic lines lacking BNI-trait (Control) and with BNI-trait (BNI). Wheat was fertilized with potassium nitrate (NK); ammonium sulphate (AS) and ammonium sulphate + DMPP (AS+D). Significant differences between N treatments of Control isogenic lines are marked with a lowercase letter. Significant differences between N treatments of BNI isogenic lines are marked with a capital letter. The Mann-Whitney U test was used for the comparison between the absence or the presence of BNI-trait within the same fertilization treatment and the significant differences at $p < 0.05$ are marked with an asterisk (*).*

		ROELFS		MUNAL	
		Biomass (g DW plant^{-1})	**Absorbed nitrogen (mg N plant^{-1})**	**Biomass (g DW plant^{-1})**	**Absorbed nitrogen (mg N plant^{-1})**
KN	**Control**	0.64 ± 0.07 a	20.3 ± 1.5 a	0.63 ± 0.04 a	23.4 ± 0.2 a
	BNI	0.52 ± 0.05 A	15.1 ± 1.2 B	0.64 ± 0.04 A	30.0 ± 0.5 A*
AS	**Control**	0.44 ± 0.03 b	13.3 ± 0.7 b	0.53 ± 0.04 b	19.9 ± 2.3 a
	BNI	0.48 ± 0.05 A	18.1 ± 0.7 A*	0.69 ± 0.11 A	32.6 ± 1.3 A*
AS+D	**Control**	0.40 ± 0.01 b	12.2 ± 0.4 b	0.41 ± 0.03 b	14.1 ± 1.2 b
	BNI	0.49 ± 0.01 A*	12.5 ± 0.2 C	0.56 ± 0.05 A	19.6 ± 1.9 B*

3.4. Leaf NR activity and leaf N content

Fertilization with NO_3^- made leaves of ROELFS-Control and ROELFS-BNI plants duplicate its leaf NO_3^- content after 30 days of fertilization, whereas fertilization with NH_4^+ combined with DMPP reduced leaf NO_3^- content (Fig. 3A and B). In the case of MUNAL BNI-isogenic lines, leaf NO_3^- content was high since the beginning of the experiment, but strongly decreased in AS+D treatment. Moreover, the presence of the BNI-trait in MUNAL caused a contrasting effect in leaf NO_3^- content as it showed a 21% increase in KN treatment, and a 57% decrease in AS+D treatment. As a result of the highest leaf NO_3^- content, MUNAL-Control plants showed higher NR activity than ROELFS-Control plants (Fig. 3C and D). Nevertheless, under NH_4^+ fertilization, ROELFS-BNI and MUNAL-BNI plants diminished 42% and 45% respectively, the leaf NR activity in AS treatment, and 49% and 58% respectively, in AS+D treatment. Regarding the leaf NH_4^+ content, although ROELFS-BNI plants only showed a 25% and 23% decrease in AS and AS+D treatments compared to ROELFS-Control plants, the effect of BNI-trait was more evident in MUNAL since leaf NH_4^+ content was decreased in all treatments, 53%, 49%, and 57% in KN, AS, and AS+D treatments respectively (Fig. 3E and F). Linked to leaf NH_4^+ content, ROELFS-BNI plants had an increase in amino acid content in all fertilized treatments, 39%, 18%, and 19% in KN, AS, and AS+D treatments respectively (Fig. 3G and H). On the other hand, MUNAL-BNI plants were able to increase 39% and 41% the amino acid content in AS and AS+D treatments, but no differences were observed between MUNAL-Control and MUNAL-BNI in KN treatment.

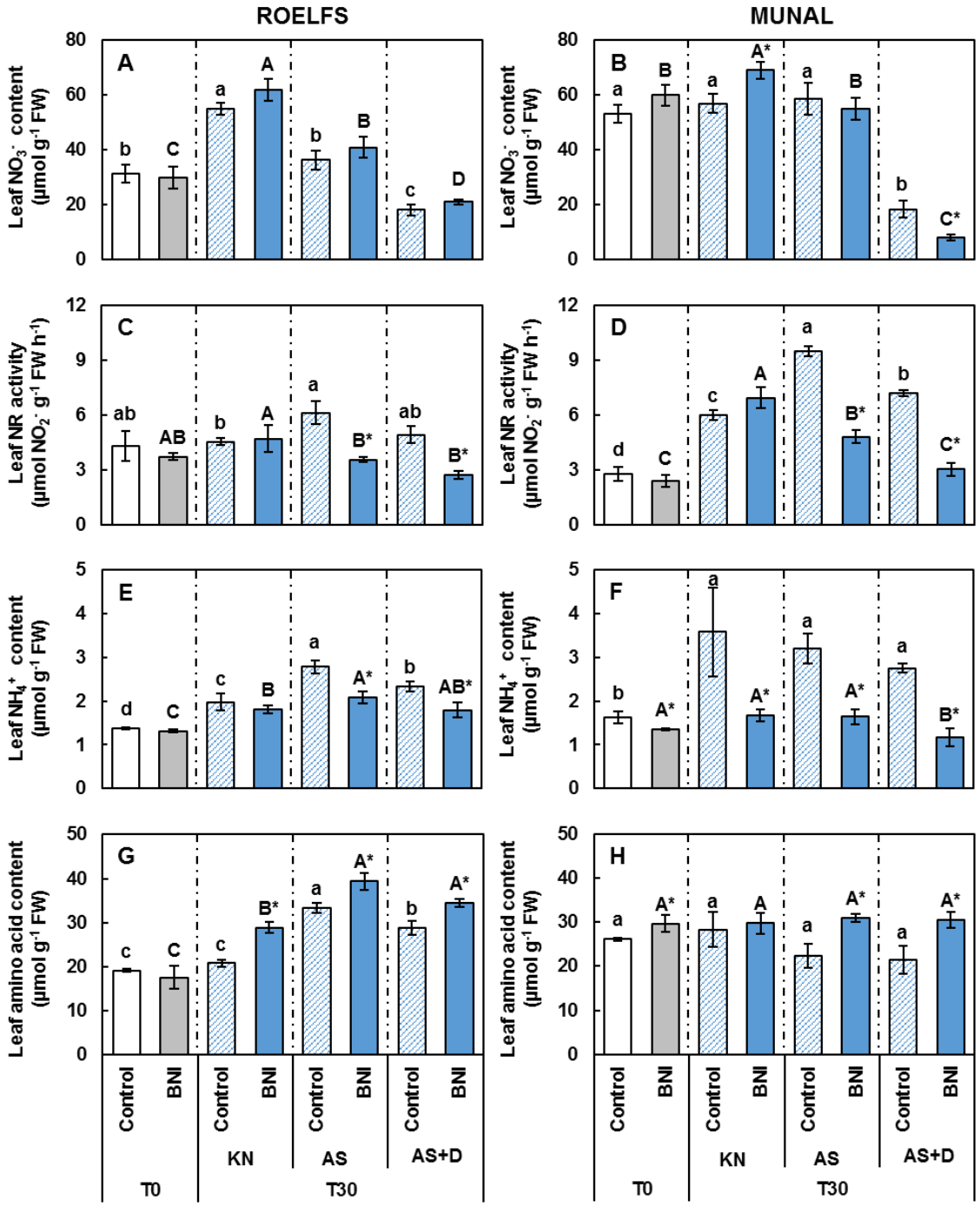


Fig. 3. *Leaf determination of nitrate content (**A** and **B**), nitrate reductase activity (**C** and **D**), ammonium content (**E** and **F**) and amino acid content (**G** and **H**) of ROELFS (**A, C, E** and **G**) and MUNAL (**B, D, F** and **H**) BNI-isogenic lines lacking BNI-trait (Control) and with BNI-trait (BNI). Wheat was fertilized with potassium nitrate (KN); ammonium sulphate (AS) and ammonium sulphate + DMPP (AS+D). Significant differences between N treatments of Control isogenic lines are marked with a lowercase letter. Significant differences between N treatments of BNI isogenic lines are marked with a capital letter. The Mann-Whitney U test was used for the comparison between the absence or the*

presence of BNI-trait within the same fertilization treatment and the significant differences at p <0.05 are marked with an asterisk (*).

3.5. ROELFS and MUNAL BNI-isogenic lines under NH_4^+ fertilization

Table 2. *Gene ratio of bacterial nitrification and denitrification abundancies 30 days after fertilization with ammonium sulphate (AS). The Mann-Whitney U test was used for the comparison between the absence or the presence of BNI-trait and the significant differences at p <0.05 are marked with an asterisk (*).*

	ROELFS		MUNAL	
	Control	**BNI**	**Control**	**BNI**
amoA/nirK	0.38	0.14* (-63%)	0.18	0.11* (-39%)
amoA/nosZI+nosZII	0.95	0.22* (-77%)	0.30	0.14* (-53%)
amoA/(nirK+nirS+ nosZI+nosZII)	0.21	0.08* (-65%)	0.08	0.05* (-40%)
nirK+nirS/nosZI+nosZII	2.27	2.64	1.93	2.06

Under NH_4^+ fertilization, ROELFS-BNI and MUNAL-BNI plants were able to decrease the ratio *amoA*/*nirK* by 63% and 39% respectively compared to Control plants (Table 2). In a similar way, ROELFS-BNI and MUNAL-BNI plants reduced 77% and 53% respectively the *amoA*/*nosZI+nosZII* ratio, whereas the *amoA*/*nirK+nirS+nosZI+nosZII* ratio was diminished by 65% and 40% respectively compared to ROELFS-Control and MUNAL-Control plants. Nevertheless, the presence of the BNI trait in ROELFS and MUNAL wheat did not affect the balance between denitrifiers and complete denitrifiers, since there were no statistical differences in the *nirK+nirS*/*nosZI+nosZII* ratio when comparing Control and BNI plants from ROELFS and MUNAL.

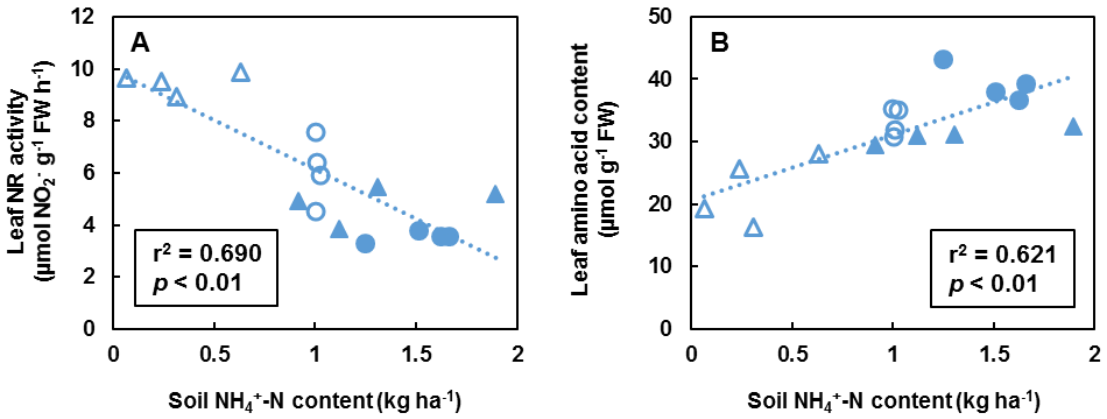


Fig. 4. *Correlation analysis between leaf NR activity (**A**) and leaf amino acid content (**B**) versus soil NH_4^+-N content 30 days after fertilization with ammonium sulphate (AS). Empty circles correspond to ROELFS BNI-isogenic lines lacking BNI-trait (Control), full circles correspond to ROELFS BNI-isogenic lines with BNI-trait (BNI), empty triangles correspond to MUNAL BNI-isogenic lines lacking BNI-trait (Control), full triangles correspond to MUNAL BNI-isogenic lines with BNI-trait (BNI).*

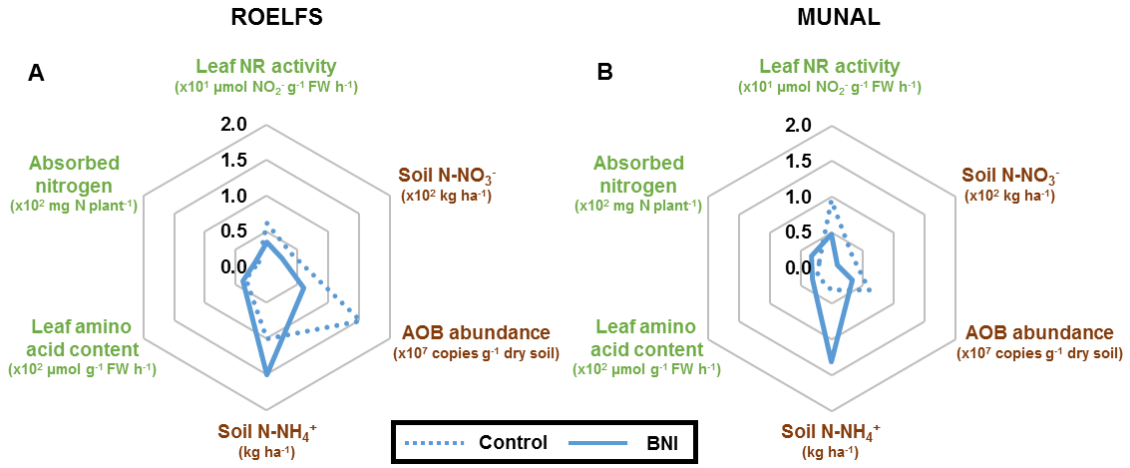


Fig. 5. *Behaviour comparison between Control and BNI plants from ROELFS (**A**) and MUNAL (**B**) BNI-isogenic lines in soil (NH_4^+-N, NO_3^--N and bacteria amoA abundance) and plant (leaf NR activity, absorbed nitrogen, and leaf amino acid content) parameters 30 days after fertilization with ammonium sulphate (AS).*

A negative correlation ($r^2 = p < 0.01$) between soil NH_4^+ content and leaf NR activity was observed for Control and BNI plants from both ROELFS and MUNAL BNI-isogenic lines (Fig. 4A). In the same way, a positive correlation ($r^2 = p < 0.01$) between soil NH_4^+ content and leaf amino acid content was observed for Control and BNI plants from both ROELFS and MUNAL BNI-isogenic lines (Fig. 4B). ROELFS-BNI plants showed

signals of an inhibited nitrification since they maintained greater soil NH_4^+ content due to a reduced AOB population (Fig. 5A). Moreover, ROELFS-BNI plants had a better ammonium tolerance as their N absorption was improved and presented a higher leaf amino acid content. Similarly, MUNAL-BNI plants also presented nitrification inhibition activity since these plants showed greater N absorption, higher leaf amino acid content, and reduced leaf NR activity as a result of a decrease in soil NO_3^- content because of a diminished AOB population (Fig. 5B).

3.6. Crop yield

In field experiment, no statistical differences in grain yield or biomass among the four BNI-isogenic lines were found (Table 3). For the variable harvest index (HI) ROELFS-Control showed a higher value than ROELFS-BNI, but this difference was not large enough to affect the grain yield. In contrast, HI of MUNAL-Control compared to MUNAL-BNI was not different. However, the presence of the BNI-trait affected ROELFS wheat cycle since it took 8 more days to reach anthesis and 6 more days to reach maturity compared to ROELFS-Control plants. On the other hand, MUNAL-BNI plants reached anthesis before MUNAL-Control plants, although both BNI-isogenic lines reached maturity at the same time.

Table 3. *Average wheat grain yield, biomass, harvest index, and days to anthesis and maturity of two levels of fertilization (83 and 250 kg N ha^{-1}) of field experiment in CENEB (Mexico). Least Significant Difference test was used for mean separation among ROELFS and MUNAL BNI-isogenic lines.*

	Grain yield (kg ha^{-1})	**Biomass (kg ha^{-1})**	**Harvest index**	**Days to anthesis**	**Days to maturity**
ROELFS-Control	5025 a	10000 a	0.46 a	87 a	130 a
ROELFS-BNI	4548 a	10063 a	0.41 c	95 b	136 c
MUNAL-Control	4950 a	10139 a	0.44 ab	93 b	132 b
MUNAL-BNI	4532 a	9467 a	0.44 b	89 a	132 b

DISCUSSION

Subbarao et al. (2021) reported the BNI capacity of four elite wheat, ROELFS, MUNAL, NAVOJOA, and QUAIU carrying the T3BL.3Ns^{b}S chromosome from *L. racemosus*. ROELFS-BNI and MUNAL-BNI isogenic lines were the two most successful lines in terms of expressing BNI capacity as both practically doubled the BNI activity released from roots compared to ROELFS-Control and MUNAL-Control plants. These authors conscientiously studied the BNI activity from MUNAL-BNI plants in laboratory soil incubations with ammonium as N source. In this work, we characterized ROELFS-BNI and MUNAL-BNI isogenic lines, being the first demonstration of the BNI-trait expression in microcosms under alkaline pH soil and different N sources. Both BNI-isogenic lines showed obvious indicators of an inhibited nitrification in soils, such as a reduced AOB abundance and higher soil NH_4^+ content (Ruser and Schulz, 2015) and biochemical markers typical of a more NH_4^+ nutrition like decreased leaf NR activity and larger amino acid content (Fig. 5A and B) (Karwat et al., 2019; Bozal-Leorri et al., 2021; Gonzalez-Moro et al., 2021).

The ecological function of biological nitrification inhibitors (BNIs) is to suppress soil nitrification by decreasing the ammonia-oxidizing microorganism populations (Subbarao et al., 2015). BNIs are known to have a stronger inhibitory impact on ammonia-oxidizing archaea (AOA) rather than on ammonia-oxidizing bacteria (AOB) (Shen et al., 2013; Sarr et al., 2020; Kaur-Bhambra et al., 2021). Furthermore, MUNAL-BNI plants are reported to be able to reduce AOA abundance at least by 20% compared to MUNAL-Control plants (Subbarao et al., 2021). However, the type of predominant nitrifying microorganism in soils is dependent on soil properties. In acidic soils, AOA are the predominant group carrying out the nitrification process, whereas AOB are relevant in neutral or alkaline soils (Nicol et al., 2008). This is important because the potential use of these BNI-isogenic lines is worldwide. For example, the soil used in this experiment had a pH value of 8.0 (supplementary table 2); as most agricultural soils that surround Mediterranean Sea are of neutral-alkaline type (Reuter et al., 2008; Fabian et al., 2014). Di and Cameron (2016) reported that in neutral-alkaline soils, AOA outnumber the abundance of AOB when NH_4^+ levels are low, but once ammonium-based fertilizers are applied, AOB outnumber AOA and become major players for carrying the nitrification

process. As a result, soils tend to show an increase in bacterial *amoA* abundance (Castellano-Hinojosa et al., 2020; Bozal-Leorri et al., 2021). The expression of BNI-trait in ROELFS-BNI and MUNAL-BNI plants in AS treatment seems to be effective against AOB in alkaline soils since they were able to reduce 45% – 60% the *amoA* increment after fertilization with NH_4^+ (Fig. 1A and B). In addition, although nitrification is carried out mostly by AOB, AOA are still present in the soil; BNIs exuded by ROELFS-BNI plants decreased 30% the AOA abundance (Fig. 1C). The fact that ROELFS-BNI plants are able to reduce the archaeal *amoA* abundance of AS treatment even in alkaline soils leads us to assume that they would also be able to decrease AOA in acidic soils, where they are the main nitrifiers. These results suggest that ROELFS-BNI and MUNAL-BNI plants could be a very effective in reducing nitrification process in agricultural soils, with pH spanning from acid to alkaline, irrespective of nitrifier population types, i.e. archaea or bacteria. Soil health is an increasingly relevant topic among soil scientists, so, it is necessary to address whether these BNIs could have a negative effect on non-target microorganisms. Here, we prove that the BNIs exuded from ROELFS-BNI and MUNAL-BNI plants do not alter either total bacteria or archaea abundance compared to ROELFS-Control and MUNAL-Control plants regardless of the N fertilization (Supplementary fig. 1). Nevertheless, Wang et al. (2021) have associated changes in bacterial networks with the presence of BNIs from sorghum, even though those molecules did not affect the *16S rRNA* abundance (Nardi et al., 2013; Sarr et al., 2020). In case of SNIs, even though they exert effects on non-target microorganisms, it seems not to be of a sufficient magnitude to have a significant impact on soil health (Corrochano-Monsalve et al., 2021c). Therefore, more experiments should be carried out to verify potential changes in soil microorganism populations due to the release of BNIs from wheat.

The decrease of ammonia-nitrifying microorganisms in soils from ROELFS-BNI and MUNAL-BNI plants is reflected in the soil mineral N. The soil NH_4^+ levels increase considerably after the application of ammonium-based fertilizers. However, when no nitrification inhibitors are added, NH_4^+ is oxidized in the first 30 days after fertilization (Torralbo et al., 2017; Bozal-Leorri et al., 2021). Under these conditions, the efficiency of BNI exudation from ROELFS-BNI and MUNAL-BNI plants was revealed, as they were able to maintain higher NH_4^+ content in soils (Fig. 2A and B). The slowdown in the nitrification process resulted as well in a lower soil NO_3^- content compared to the soil of ROELFS-Control and MUNAL-Control plants (Fig. 2C and D), similar to the behaviour

described for MUNAL-BNI plants in acidic soils (Subbarao et al., 2021). Based on soil NH_4^+ and NO_3^- content, the fertilization with NH_4^+ in AS treatment led to a lower soil N content after 30 days of experiment (Fig. 2), a fact that can be explained by the better N absorption that ROELFS-BNI and MUNAL-BNI plants showed (Table 1). Nevertheless, it seems that MUNAL-BNI plants reduce NH_4^+ oxidation more efficiently than ROELFS-BNI plants because MUNAL-BNI plants kept higher soil NH_4^+ content and lower NO_3^- content (Fig. 2B and D). In hydroponic conditions, MUNAL-BNI plants showed higher BNI activity than ROELFS-BNI plants (Subbarao et al., 2021), which could allow MUNAL-BNI plants to absorb more N than ROELFS-BNI plants when they are grown on soil pots (Table 1). Interestingly, under NO_3^- nutrition BNI-isogenic lines were effective in diminishing soil NO_3^- content, especially MUNAL-BNI (Fig. 2C and D). This may suggest that the expression of the BNI-trait in the plant could allow a higher capacity to absorb N, thus, preventing its loss. The decrease in soil NO_3^- levels due to BNI-trait expression can impact denitrifying populations (Saggar et al., 2013). Nevertheless, there were no differences between soils from ROELFS-BNI and ROELFS-Control plants in the abundances of any of the denitrification genes analysed (*nirK*, *nirS*, *nosZI*, and *nosZII*) after NH_4^+ fertilization (Supplementary fig. 2A, C, E, and G). Even for MUNAL-BNI plants there were no differences in the denitrification genes abundances compared to MUNAL-Control plants (Supplementary fig. 2B, D, F, and H), despite low soil NO_3^- content. To our knowledge, the effect of BNIs from pastures or crop plants on denitrifying populations has not been investigated. On the other hand, Florio et al. (2021) studied the BNI influence of different forest tree species on soil denitrifiers. These authors also suggested that the reduction of NO_3^- formation derived from the action of BNIs over nitrification might affect denitrifiers. Comparable to our results, they discovered that denitrifiers' abundance is not linked to the plant's ability to exude BNIs. Denitrification is a process that depends on several soil environmental variables (Zumft, 1997), such as the level of anaerobiosis, modulated by soil moisture above 60% WFPS (Davidson, 1991). In the present work, the soil moisture was adjusted to a 45% WFPS, which is optimal for nitrification, but not for denitrification. It would be interesting to examine the effects of BNIs on denitrifying populations in soils with anaerobic conditions. Therefore, the lack of response from denitrifiers is the reason why we cannot see any difference in the *nirK+nirS*/*nosZI+nosZII* ratio neither between ROELFS-Control and ROELFS-BNI plants nor between MUNAL-Control and MUNAL-BNI plants (Table 2). Furthermore, the reduction in the nitrification/denitrification ratios is determined by the capacity of

BNI-isogenic lines in reducing the *amoA* abundance. Since ROELFS-BNI plants are more efficient in inhibiting AOB growth (Fig. 1A) than MUNAL-BNI plants (Fig. 1B), the greater reductions of bacterial nitrification/denitrification ratios were achieved by ROELFS-BNI plants. However, *nirK* and *nosZI* abundancies from KN treatment from soils of MUNAL-BNI plants showed a decrease compared to those of MUNAL-Control plants (Supplementary fig. 2B and F). We hypothesise that this reduction of denitrifiers abundance is possibly from reduction in soil NO_3^- content (Fig. 2D), as soil NO_3^- content is the substrate for denitrification, coupled with higher N uptake of MUNAL-BNI plants (Table 1), possibly the reason for lower denitrifiers populations.

Introduction of BNI-trait into MUNAL wheat cultivar improved the grain yield in acidic soils (Subbarao et al., 2021). However, ROELFS-BNI showed lower grain yield compared to ROELFS-Control plants in three different trials in alkaline soils from Obregon (Subbarao et al., 2021). In our work, field experiments in Mexico showed no positive impact from BNI-trait on grain yield for both ROELFS and MUNAL BNI-isogenic lines (Table 3). More studies are needed, however, to understand the implications/impact from BNI-trait on improving nitrogen uptake and improving wheat productivity in different wheat production environments. Introduction of the BNI-trait had a contrasting effect on the number of days to anthesis since it increased in ROELFS but decreased in MUNAL (Table 3). ROELFS had also a crop cycle 6 days later with the BNI-trait compared to the Control, a characteristic that can have an important agronomic relevance since this delay could imply higher risk for the crop. These agronomic characteristics depends on the genetic stock since the crop life cycle was only affected in ROELFS. On the other hand, our results suggest that BNI-trait expression made wheat plants to switch their metabolism more towards ammonium nutrition. BNIs exuded by plants delay soil NH_4^+ oxidation, decreased the formation of soil NO_3^- and its plant uptake. Therefore, the presence of the nitrate reductase (NR) enzyme, which is a substrate-induced enzyme, is reduced in plant tissues (Srivastava, 1980). Karwat et al. (2019) suggested leaf NR activity could be used as BNI-trait indicator for plants grown in greenhouse and field studies. The reduction of leaf NR activity after NH_4^+ fertilization, both in ROELFS-BNI and MUNAL-BNI plants (Fig. 3C and D) indicated the expression of BNI-trait, and its impact on nitrification. Moreover, leaf NR activity levels were similar to SNI treatments. In addition, we found a strong negative linear correlation ($r^2 = p < 0.01$) between leaf NR activity and soil NH_4^+ content under NH_4^+ fertilization for both

ROELFS and MUNAL (Fig. 4A). This response of NR activity to soil NH_4^+ content is interpreted in the sense that more NH_4^+ is maintained in the soil due to the presence of BNIs, and less NO_3^- is absorbed, decreasing the NR activity in leaves. Despite the BNI lines maintained more soil NH_4^+ content (Fig. 2A and B), favouring an ammonium nutrition compared to ROELFS-Control and MUNAL-Control plants, ROELFS-BNI and MUNAL-BNI plants kept less leaf NH_4^+ content (Fig. 3E and F). This lower leaf NH_4^+ content could be explained, in part, because wheat behaves as an "ammonium excluder" (with a root-based mechanism), to cope with excess ammonium, similarly as other gramineae (González-Moro et al., 2021). This ammonium nutrition is associated with enrichment of N-containing compounds (Marino et al., 2016; Coleto et al., 2017). Many cereal species tend to increase their free amino acids content when plants absorb more NH_4^+-N (González-Moro et al., 2021). Thus, the higher amino acid content that ROELFS-BNI and MUNAL-BNI plants presented is indicative of more ammonium nutrition due to higher levels of BNI activity in reducing nitrifier activity. Furthermore, leaf amino acid contents were strongly correlated ($r^2 = p < 0.01$) with soil NH_4^+ content (Fig. 4B). Thus, we suggest that the leaf amino acid content could be used as an indirect marker of plant BNI capacity, since the more nitrification inhibition, the more soil NH_4^+ content, which would result in a higher plant amino acid content.

Improved N-cycling benefits on crop performance, which is shown repeatedly using SNIs (IPCC, 2014). SNIs can increase NUE on average by 7% to 16% and reduce N_2O emission (Kanter and Searchinger, 2018). Unfortunately, several drawbacks limit their acceptance by farmers, as they have high costs without warranting better production (Subbarao et al., 2017). Introduction of the BNI-trait could settle this issue or even reduce the dependence on SNIs. It is evident that the application of SNIs, such as DMPP, inhibits nitrification by decreasing AOB abundance (Bozal-Leorri et al., 2021; Corrochano-Monsalve et al., 2021a). However, their effect on AOA is still inconclusive (Ruser and Schulz, 2015). On the other hand, BNI-trait harbouring wheat could be more effective in decreasing both AOB and AOA and in maintaining soil NH_4^+ for a longer time. In addition, the less drastic inhibition of nitrification would favour the wheat plants to use not only NH_4^+ but also NO_3^-, achieving better growth due to the mixed N source nutrition (Subbarao and Searchinger, 2021). Furthermore, these plants could also improve NUE, even with the addition of a high N fertilizer dose. ROELFS-BNI and MUNAL-BNI plants showed higher N uptake than ROELFS-Control and MUNAL-Control, being higher in treatments

without SNI (Table 1). Subbarao et al. (2021) hypothesize that the increase in N uptake is due to fact that the added BNI-trait also carries genes that improve soil organic matter (SOM) mineralization. Therefore, this kind of wheat variety could displace the use of SNIs since the expression of the BNI-trait would provide the plant a more mixed N nutrition and combine the absorption of mineralized N.

CONCLUSIONS

The introduction of the BNI-trait did not reduce the soil total bacterial or archaeal abundance regardless of the N fertilization treatment. ROELFS-BNI and MUNAL-BNI isogenic lines exhibited inhibited nitrification, decreasing AOB abundance by 60% and 45% respectively in AS treatment. Moreover, ROELFS-BNI also diminished AOA abundance by 30%. The reduction of ammonia-oxidizing microorganisms resulted in maintaining higher soil NH_4^+ and, thus, triggering lower soil NO_3^- content. In this way, ROELFS-BNI and MUNAL-BNI plants showed a reduced leaf NR activity, due to a decreased soil NO_3^- content, and their metabolism shifted to a more ammonium-based nutrition presenting higher amino acid content compared to ROELFS-Control and MUNAL-Control plants. In addition, under NO_3^- fertilization, BNI-isogenic lines reduced soil NO_3^- content. Then, introducing the BNI trait into ROELFS and MUNAL wheat cultivars is not only beneficial under NH_4^+, but also under NO_3^--based fertilization, which is evident from improved N absorption. Therefore, ROELFS-BNI and MUNAL-BNI isogenic lines represent an efficient BNI-technology with potential use worldwide to reach sustainable agriculture based on the use of environmentally friendly agronomic practices.

SUPPLEMENTARY MATERIAL

Supplementary table 1. *Primers pairs and thermal conditions used in real-time qPCR.*

Target group	Primer name	Sequence	Thermal profile	bp lenght	References
Bacterial *16S rRNA*	341F	5'-CCTACGGGAGGCAGCAG-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 60ºC for 30 sec 72ºC for 30 sec 80ºC for 30 sec - x 40 cycles	174	Lopez-Gutiérrez et al., (2004)
	534R	5'-ATTACCGCGGCTGCTGGCA-3'			
Archaeal *16S rRNA*	771F	5´-ACGGTGAGGGATGAAAGCT-3´	95 °C for 2 min – x 1 cycle 95 °C for 15 sec 58 °C for 30 sec 72 °C for 30 sec 80 °C for 30sec – x 40 cycles	226	Ochsenreiter et al., (2003)
	957R	5´ -CGGCGTTGACTCCAATTG-3´			
Bacterial *amoA*	amoA1F	5'-GGGGTTTCTACTGGTGGT-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 54ºC for 60 sec 72ºC for 60 sec - x 40 cycles	491	Rotthauwe et al., (1997)
	amoA2R	5'-CCCTCKGSAAAGCCTTCTTC-3'			
Archaeal *amoA*	ArchamoAF	5´-STAATGGTCTGGCTTAGACG-3´	95 °C for 2 min - x 1 cycle 95 °C for 45 sec 54 °C for 45 sec 72 °C for 45 sec 85 °C for 20 sec - x 40 cycles	635	Francis et al., (2005)
	ArchamoAR	5´-GCGGCCATCCATCTGTATGT-3´			
nirK	NirK 876	5´-ATYGGCGGVCAYGGCGA-3´	95 °C for 2 min – x 1 cycle 95 °C for 15 sec 63 °C for 30 sec (-1 °C /cycle) 72 °C for 30 sec 80 °C for 15 sec – x 6 cycles 95 °C for 15 sec 58 °C for 30 sec 72 °C for 30 sec 80 °C for 30sec – x 40 cycles	165	Henry et al., (2004)
	NirK1040	5´-GCCTCGATCAGRTTRTGGTT-3´			
nirS	cd3aF	5´-GTSAACGTSAAGGARACSGG-3 ´	95 °C for 2 min - x 1 cycle 95 °C for 45 sec 55 °C for 45 sec 72 °C for 45 sec 85 °C for 20 sec - x 40 cycles	410	Michotey et al., (2000)
	R3cd	5´-GASTTCGGRTGSGTCTTGA-3´			Throbäck et al., (2004)
nosZI	nosZ-F	5'-CGCRACGGCAASAAGGTSMSSGT-3'	95ºC for 2 min - x 1 cycle 95ºC for 15 sec 63ºC for 30 sec (-1ºC/cycle), 72ºC for 30 sec 80ºC for 15 sec – x 6 cycles 95ºC for 15 sec 60ºC for 30 sec 72ºC for 30sec 80ºC for 30 sec - x 40 cycles	267	Henry et al., (2006)
	nosZ-R	5'-CAKRTGCAKSGCRTGGCAGAA-3'			
nosZII	nosZ-II-F	5´-CTIGGICCIYTKCAYAC-3´	95 °C for 2 min – x 1 cycle 95 °C for 30 sec, 54 °C for 30 sec 72 °C for 40 sec, 85 °C for 15 sec - x 40 cycles	698	Jones et al., (2013)
	nosZ-II-R	5´-GCIGARCARAAITCBGTRC-3´			

Supplementary table 2. *Physical and chemical properties of the soil collected on 0 – 30 cm depth layer in Arkaute (42° 51' N, 2° 37' W, 530 m above sea level, Alava, Spain). For experiment setup the soil was mixed with sand in soil:sand 3:1 proportion.*

Soil texture			Soil chemical properties							
					Organic					
Sand	Silt	Clay	pH[a]	C:N	N[b]	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

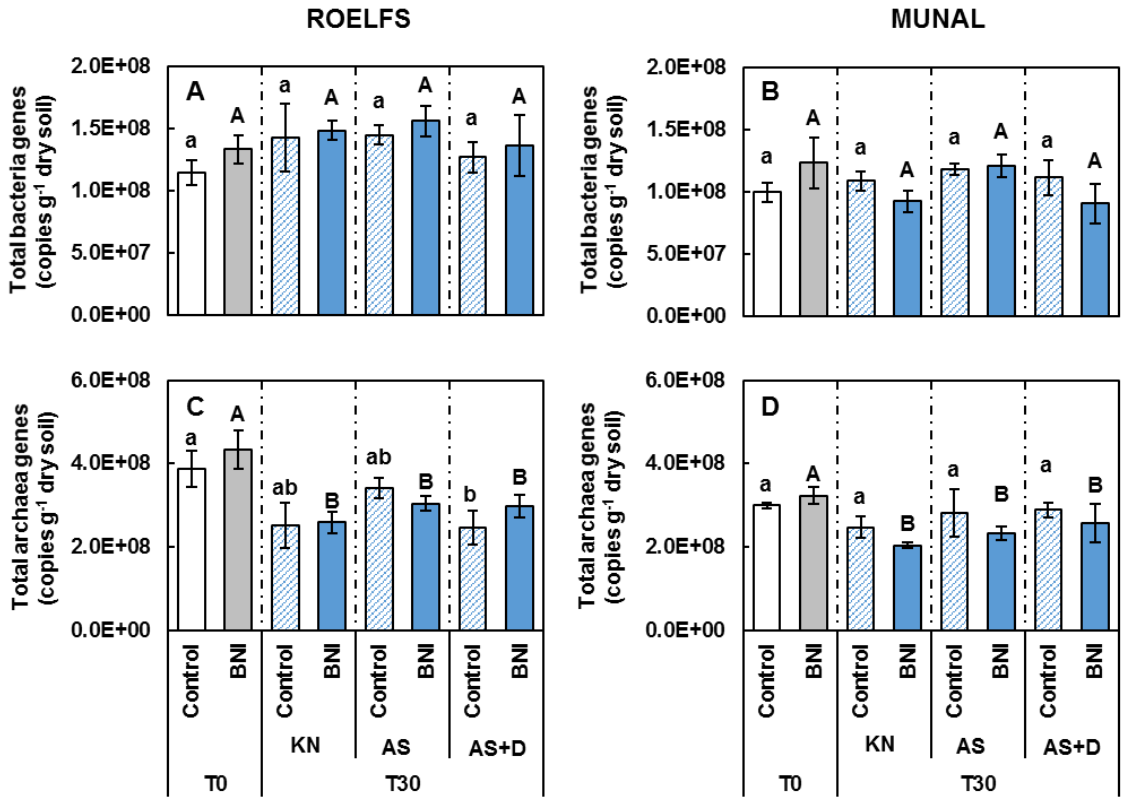
a. pH (1:2.5 soil:water).

b. N Kjeldahl digestion (Keeney and Nelson, 1982).

c. Organic matter (Walkley and Black, 1934).

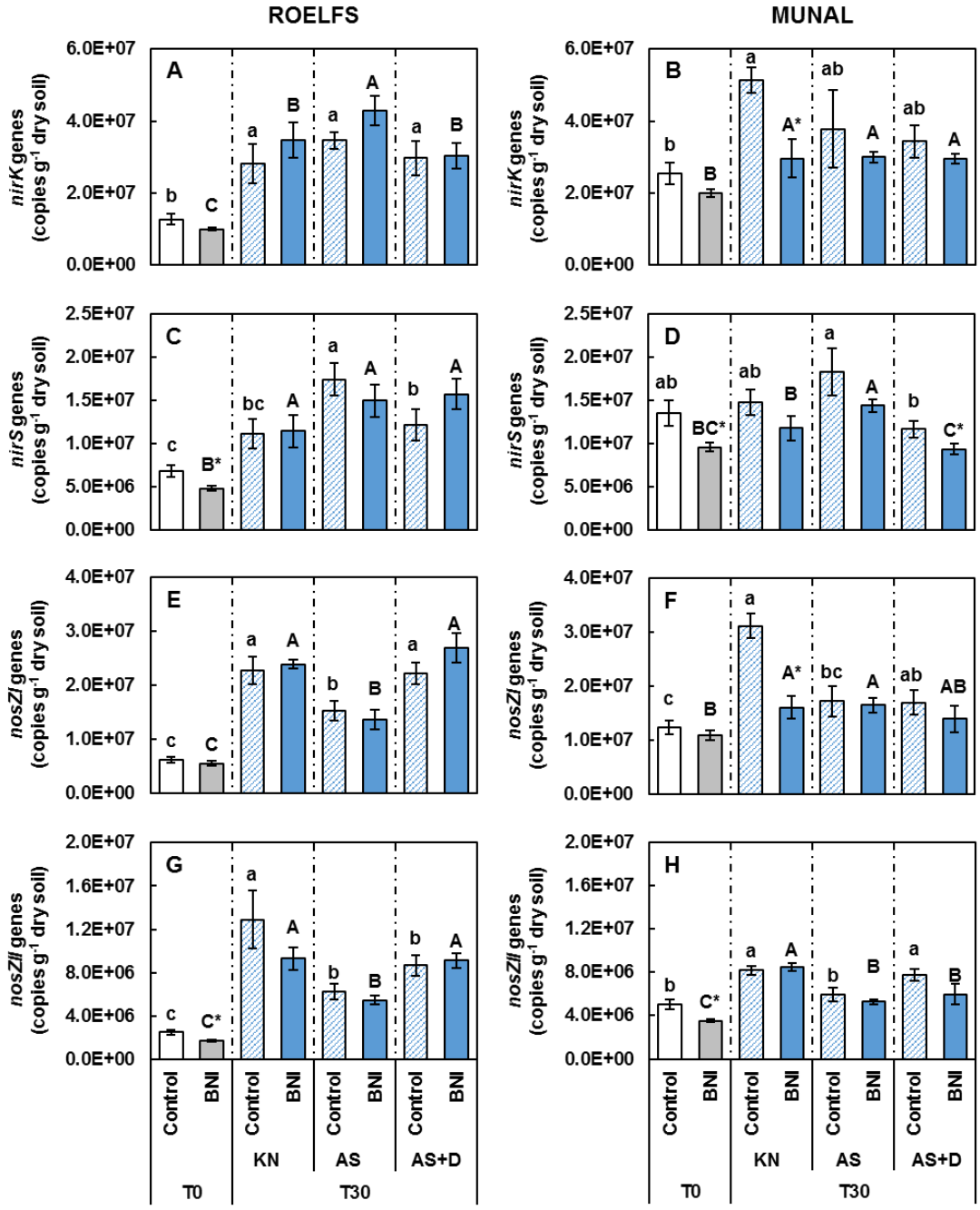
d. CaCO3 , Mg, K (NH4 AcO, MAPA, 1994).

e. P (Watanabe and Olsen, 1965).



Supplementary fig. 1. *Abundance of soil total bacteria (**A** and **B**) and archaea (**C** and **D**) of ROELFS (**A** and **C**) and MUNAL (**B** and **D**) BNI-isogenic lines lacking BNI-trait (Control) and with BNI-trait (BNI). Wheat was fertilized with potassium nitrate (KN); ammonium sulphate (AS) and ammonium sulphate + DMPP (AS+D). Significant*

differences between N treatments of Control isogenic lines are marked with a lowercase letter. Significant differences between N treatments of BNI isogenic lines are marked with a capital letter. The Mann-Whitney U test was used for the comparison between the absence or the presence of BNI-trait within the same fertilization treatment and the significant differences at p <0.05 are marked with an asterisk (*).



Supplementary fig. 2. *Abundance of denitrifying microorganisms expressed as nirK (**A** and **B**), nirS (**C** and **D**), nosZI (**E** and **F**) and nosZII (**G** and **H**) of ROELFS (**A, C, E** and **G**) and MUNAL (**B, D, F** and **H**) BNI-isogenic lines lacking BNI-trait (Control) and with BNI-trait (BNI). Wheat was fertilized with potassium nitrate (KN); ammonium sulphate*

(AS) and ammonium sulphate + DMPP (AS+D). Significant differences between N treatments of Control isogenic lines are marked with a lowercase letter. Significant differences between N treatments of BNI isogenic lines are marked with a capital letter. The Mann-Whitney U test was used for the comparison between the absence or the presence of BNI-trait within the same fertilization treatment and the significant differences at p <0.05 are marked with an asterisk (*).

General Discussion

1. Present and future of synthetic nitrification inhibitors

Synthetic nitrification inhibitors (SNIs) are largely known to be potent agents in increasing fertilizer efficiencies in the field and reducing nitrogen (N) losses (Ruser and Schulz, 2015; Beeckman et al., 2018). The results of this thesis continue to confirm this fact since the SNIs DMPP and DMPSA were able to delay the oxidation of ammonium (NH_4^+) and diminish the nitrous oxide (N_2O) emissions by reducing nitrification in every experiment. However, it is necessary to broaden the focus of analysis and explore whether their mode of action is maintained or modified in future climatic situations, such as the increase in CO_2. The increase of atmospheric CO_2 concentration is expected to alter N_2O-producing soil processes indirectly via changes in plants' N uptake (Kammann et al., 2008). In a meta-analysis, Cheng et al. (2012) found that elevated CO_2 (eCO_2) increased soil nitrate (NO_3^-) content by 26.7%, but decreased soil NH_4^+ content by 7.9%. It seems that the reduction of plant evapotranspiration by the closure of stomata under eCO_2 conditions (Xu et al., 2016) decreases plant NO_3^- uptake. Moreover, Bloom et al. (2014) presented results from a field experiment showing that NO_3^- assimilation was slower under elevated than ambient CO_2 in wheat (*Triticum aestivum*) grown in the free-air CO_2 enrichment experiment. Therefore, under eCO_2 conditions, plant NH_4^+ uptake rather than NO_3^- is enhanced. Nevertheless, in line with the results of Tu et al. (2017), we observed that nitrifying populations are not affected by eCO_2 conditions (Chapter 1, Fig. 5B). Thus, under eCO_2, nitrification would be carried out at the same rate as in present conditions, transforming NH_4^+ into NO_3^- in fewer than 10 days (Subbarao et al., 2015). This would have two potential major implications when applying ammonium-based fertilizers, i) synchronization of plant N needs with fertilizer N inputs will be a more challenging task under future CO_2 scenarios and ii) the increase of soil NO_3^- content together with the increase of soil water content due to the reduction of plant evapotranspiration (van Groenigen et al., 2011) will enhance denitrification activity accelerating the conversion of NO_3^- to N_2O under future CO_2 conditions (Wu et al., 2017). In our experimental conditions, we found a higher soil NO_3^- content in eCO_2 conditions (Chapter 1, Fig. 3C and D), but N_2O emissions were not increased (Chapter 1, Fig. 4) possibly because we controlled the soil water content to avoid differences between both CO_2 concentrations. However, in field conditions with rainfed crop, the soil water content cannot be controlled and it would lead to increase N_2O emissions. In this way, the results of Chapter 1 bring a hopeful solution to mitigate the N losses since, as well as in the present, the use of DMP-

based SNIs will continue to be effective in reducing nitrification in the soil, decreasing the NO_3^- formation and, thus, the N_2O emissions in future conditions.

In addition, the effects of increased CO_2 conditions on the N cycle can also happen before the atmospheric CO_2 concentration changes. 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 the stover of a previous crop in no-till management. Currently, agriculture is also focusing on reducing N emissions in the fallow period, especially when the NUE of the crops is low and, therefore, large amounts of residual N remain in the soil after harvest (Sanchez-Martín et al., 2010). Based on this, the use of cover crops instead of fallow during the summer season has been proposed as an agricultural technique to retain the excess of inorganic N that remains after the harvest of the previous crop (Dinnes et al., 2002). In Chapter 4, sorghum was used as a cover crop in a sorghum-winter wheat crop rotation because i) cereals such as sorghum often decrease the soil N content at early growth stages due to a higher N uptake and decreased drained water, both associated with a faster initial rooting (Thorup-Kristensen et al., 2003) and ii) its biological nitrification inhibition (BNI) activity. In this kind of rotation, the biomass of the cover crop is used as a green manure after harvest to reuse the N retained, which may affect the dynamics of N and C in the soil (Olesen et al., 2007). In this way, the combination of mineral N from fertilizer and C derived from the stover of a previous crop may promote an increase of N_2O emissions in comparison with N fertilizer alone because the increment of C can be used as an energy source for denitrifying microorganisms (Sarkodie-Addo et al., 2003; Abalos et al., 2012). We observed this response in our field experiments. After the fertilizer application of the wheat crop, the abundance of NIR enzyme-containing denitrifying microorganisms incremented in sorghum-wheat crop rotation but not in fallow-wheat (Chapter 4.2, Fig. 4A), leading to an increased cumulative N_2O emission (Chapter 4.2, Fig. 3). Nevertheless, as it was observed in eCO_2 conditions, DMPP is also able to efficiently reduce N losses coming from nitrification regardless of the soil C content. Nitrifying populations and N_2O emissions were equally low in both crop rotations and matches the values of the unfertilized control (Chapter 4.2, Fig. 2A and 3). Therefore, the use of DMP-based SNIs allows farmers to leave the residues of the previous crop without affecting the yield of the next crop due to i) longer maintenance of NH_4^+ in the soil and ii) the mitigation of the impact of the residues on N and C soil dynamics.

The knowledge of the behaviour of DMP-based SNIs in soils with increased C content, either due to an increase in atmospheric CO_2 or due to the residues of previous crops, leads us to make their use more efficient in the soil. These results increase our understanding of how different edaphoclimatic conditions, such as salinity, temperature, soil moisture, and kind of soil affect DMPP and DMPSA (Menéndez et al., 2012; Zhao et al., 2016; Barrena et al., 2017; Torralbo et al., 2017; Guardia et al., 2018a; Li et al., 2020). Research has mainly focused on macroscopic conditions that affect the efficiency of SNIs. Nonetheless, very few works have studied the molecular aspects of SNIs' function. It is also important to understand the chemical and molecular mode of action in order to know how SNIs work. Although there are studies about how different chemical compounds (such as acetic acid, lactic acid, sulfosalicylic acid, or citric acid) affect nitrification (Vandevivere et al., 1998; de Boer and Kowalchuk, 2001; Shi et al., 2015) they are not used as nitrification inhibitors, and all the current studies with the most used SNIs refer to the reviews of Subbarao et al. (2006) and Ruser and Schulz (2015) to explain their mode of action. Recently, Corrochano-Monsalve et al. (2021a) found this knowledge gap and tried to find out the mode of action of DMP-based SNIs. These authors confirmed that DMPP and DMPSA were able to chelate Cu^{2+} (cofactor of the AMO enzyme), which was in line with the personal communication of Wissemeier. In this way, Corrochano-Monsalve et al. (2021a) suggested that the application of DMP-based SNIs counteract the stimulation of AOB communities derived from Cu^{2+} addition to the soil. In order to continue deciphering their mode of action, we performed the experiments presented in Chapter 2. Surprisingly, considering the results we obtained, we cannot maintain the hypothesis that the mode of action of DMP-based SNIs is related to their Cu^{2+} chelation ability. This means that DMPP and DMPSA might form complexes with Cu in the soil but they do not reduce its availability to microorganisms. Therefore, more studies will be necessary to determine the exact mode of action of DMP-based SNIs. Since DMPs inhibit exclusively the AMO enzyme (Chapter 2, Fig. 3) we hypothesize that the nitrification inhibition might be due to i) DMPs attaching directly to the AMO enzyme, modifying its structure by an allosteric-type interaction or ii) DMPs are combined with Cu on active sites of AMO, inhibiting its ability to catalyse the oxidation of NH_4^+.

DMPSA was developed to confer more stability and reduce the high volatility that the use of pyrazole rings presents, bonding a succinic residue instead of the more unstable

phosphate of DMPP. Then, DMPSA can be stable with other fertilizers such as calcium ammonium nitrate or diammonium phosphate (Torralbo et al., 2017). In addition, the DMPSA application present another advantage. It has been observed that the application of N fertilizer decrease the abundance of Cyanobacteria, Armatimonadetes and Fibrobacteres, which are the most important taxa for predicting the multifunctionality of an ecosystem (Chen et al., 2020). In this way, soil functions such as nutrient cycling or the microbial diversity cannot be maintained (Wagg et al., 2014; Bender et al., 2016; Delgado-Baquerizo et al., 2017). On the other hand, the use of DMPSA is able to partially alleviate this negative effect by increasing these taxa abundance in the soil compared to DMPP (Corrochano-Monsalve et al., 2020b; 2021c). Nonetheless, we evidenced in Chapter 2 that DMPSA is not able to inhibit the growth of *Nitrosomonas europaea* (Chapter 2, Fig. 1) and it needs to be broken into DMP through biological processes to achieve the inhibition of nitrification (Chapter 2, Fig. 6). This implies that the type of soil and environmental conditions could modify the rate of DMP liberation from DMPSA, affecting its nitrification inhibition efficiency. However, it has been shown that its efficiency is similar to DMPP in Hypercalcic Kastanozem type of soils (Huerfano et al., 2016; Corrochano-Monsalve et al., 2021a; Bozal-Leorri et al., 2021). Equally important, in view of the potential toxicity of these compounds, we can prove that the changes DMPSA produces in non-target soil microorganisms do not seem to be of a sufficient magnitude to have a significant impact on soil health (Corrochano-Monsalve et al., 2021c). Therefore, both DMPs are equally efficient and the choice for one of them will depend on the type of fertilizer to be used and the physicochemical properties of the soil.

Overall, although counteracting N pollution is a great challenge as it transforms into many chemical forms, DMP-based SNIs will continue to be efficient tools to mitigate the contamination derived from the use of fertilizers. Their use will not only bring environmental benefits but also economic. N pollution, including contributions to climate change and biodiversity loss, costs the European Union between 70 and 320€ billion per year (Sutton et al., 2011). Thus, making policies that encourage farmers to use of SNIs would be necessary to save billions of euros every year.

2. Towards the use of biological nitrification inhibitors

Biological nitrification inhibition (BNI) was discovered 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 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 biological nitrification inhibitors (BNIs) is highlighted in the framework of sustainable agriculture based on the use of environmentally friendly agronomic practices to decrease pollution from the use of fertilizers (Subbarao et al., 2013a; Zhang et al., 2015). On the other hand, non-tillage and cover crops are proposed as good management practices in order to achieve these objectives. Therefore, the use of cover crops capable of producing BNIs represents another promising strategy to control nitrification and, thus, increase the availability of N in the soil for the main crop while reducing N losses from the agrosystem (Karwat et al., 2019; Momesso et al., 2019).

The regulation of the synthesis and release of BNIs 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 BNI by the roots are conditions that have not yet been sufficiently studied. Moreover, the effectiveness of BNI 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 environmental conditions that can modify the production of BNIs by the cover crop is crucial to introduce this quality in agricultural operations such as crop rotation systems. Although droughts 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 negatively affects crop growth and causes a reduction in its yield (Fleury et al., 2010), being the abiotic factor that most limits the productivity of agrosystems. Encouraged by the results of Ghatak et al. (2021) and taking into account that sorghum is drought tolerant and has the ability to produce BNIs (Subbarao et al., 2013b; Hadebe et al., 2017); we thought it was a suitable option to investigate the effects of drought on BNI release. In Chapter 3, we demonstrated that sorghum plants inhibit more efficiently the growth of nitrifying

populations possibly due to an increase in their roots exudation and BNI release. Under drought conditions, plant metabolism and root architecture are modified. Roots are usually the earliest organ to perceive the drought stress and then communicate this to shoots and leaves (del Bianco and Kepinski, 2018). Drought stress usually inhibits shoot growth but stimulates root growth to accelerate the remobilization of photo-assimilates from shoots to roots to cope with drought stress (Yamaguchi et al., 2010). Then, the lack of water during sorghum development will enhance BNI release since drought makes to increase root biomass, and sorghum BNIs are produced exclusively in the roots (Baerson et al., 2008). This makes sorghum a suitable tool to diminish soil nitrification in the hot and dry summer of Mediterranean climate areas, which can be confirmed in Chapters 3 and 4.1. Sorghum maintained a very low abundance of nitrifying bacteria both at the end of the sorghum crop or after fertilizer application (Chapter 3, Fig. 1A and Chapter 4.1, Fig. 1B). In addition, under drought conditions, it has been observed that wheat has fewer roots in the surface soil but also a dense root architecture in the subsoil layer that allows penetration into deep soil to absorb water (Wasson et al., 2012; Lynch and Wojciechowski, 2015; Fang et al., 2017). Therefore, it seems that like sorghum under water deficit, the BNI release from the new ROELFS-BNI and MUNAL-BNI wheat lines could also increase due to higher root biomass. However, more experiments should be carried out to confirm this hypothesis. Anyway, the discovery of the drought effect on the BNI release points to the need for further research on the environmental factors that affect this ability. The successful application and transfer of the use of crops with BNI capacity to productive and sustainable agrosystems will undoubtedly depend on resolving whether BNI release would be maintained in all possible scenarios. Moreover, experiments have to be performed not only focusing on current environmental conditions but also on future ones. In the context of climate change, an elevated atmospheric CO_2 concentration also modifies the structure of roots (Beidler et al., 2015). Furthermore, it maintains more NH_4^+ in the soil (Chapter 1, Fig. 3A and B) facilitating its assimilation over NO_3^- (Vega-Mas et al., 2015; 2017; Rubio-Asensio and Bloom, 2016). Therefore, the use of crops with BNI exudation capacity also appears to be an efficient tool to reduce N losses coming from nitrification in future conditions.

2.1. Comparing biological and synthetic nitrification inhibitors

Using the natural ability of crops to produce BNIs represents a more environmentally friendly alternative to the application of SNIs and, therefore, a sustainable strategy to reduce N losses and improve NUE in crop rotation. Additionally, greater knowledge about the synthesis of BNIs and the environmental conditions that improve its release could lead to a gradual substitution of SNIs by overcoming the drawbacks of manufacturing and application that SNIs present. Nevertheless, if the future of nitrification inhibition will be directed towards the use of BNIs, their effects on soil microorganisms should be further studied in order to avoid the possibility of a negative effect on soil health. The results of this thesis show no deleterious effects in the total abundance of bacteria due to the presence of BNIs (Chapter 4.1, Fig. 1A; Chapter 4.2, Fig. 2A; Chapter 5, supplementary Fig. 1A and B). In the same way, our results are in line with several works that observed no effects of SNIs in the total abundance of bacteria (Barrena et al., 2017; Torralbo et al., 2017; Corrochano-Monsalve et al., 2020a; Luchibia et al., 2020). Therefore, we can assume that BNIs, alike SNIs, could be considered environmentally friendly, although more experiments should be needed to confirm this assumption. However, the fact that BNIs did not affect the total abundance of bacteria does not mean that they do not alter the composition of the microbial community. Wang et al. (2021) suggested that understanding how root exudates modify soil microbiomes might potentially unlock an important tool for enhancing crop sustainability and yield. These authors observed that sorghum roots exudates are able to inhibit the growth and function of a wide range of nitrifying microorganisms but also stimulate the growth of certain groups such as *Methylophilus* and *Nocardia*. Furthermore, Subbarao et al. (2021) hypothesized that the BNI release from ROELFS-BNI and MUNAL-BNI wheat lines could improve soil organic matter (SOM) mineralization. It seems that BNIs not only increase N maintenance in the soil but also modify the bacterial communities that could benefit the plant. Thus, research efforts should be directed towards studying how BNIs shape the bacterial community with the final aim of increasing the productivity of the agroecosystems.

Table 1. *Relative abundance of amoA gene (amoA abundance/16S rRNA abundance). Data from treatments of* ***Chapter 4.1****;* *BNI = AS from sorghum; BNI+SNI = AS+D from sorghum.*

*Data from treatments of **Chapter 4.2**; Control = AS from fallow-wheat rotation; BNI = AS from sorghum-wheat rotation; SNI = AS+DMPP from fallow-wheat rotation; BNI+SNI = AS+DMPP from sorghum-wheat rotation.*
*Data from treatments of **Chapter 5**; Control = ROELFS-Control and MUNAL-Control from treatment AS; BNI = ROELFS-BNI and MUNAL-BNI from treatment AS; SNI = ROELFS-Control and MUNAL-Control from treatment AS+D; BNI+SNI = ROELFS-BNI and MUNAL-BNI from treatment AS+D. Different letters indicate significant differences using the Duncan Test (p <0.05; n = 4).*

	***amoA* relative abundance (%)**			
	Chapter 4.1	**Chapter 4.2**	**Chapter 5 ROELFS**	**Chapter 5 MUNAL**
Control		0.78 ± 0.07 a	10.88 ± 3.11 a	5.17 ± 0.74 a
BNI	0.85 ± 0.11 a	0.44 ± 0.00 b	4.06 ± 1.28 b	2.75 ± 0.25 b
SNI		0.36 ± 0.06 b	2.27 ± 0.37 bc	3.10 ± 0.56 b
BNI+SNI	1.02 ± 0.15 a	0.33 ± 0.05 b	1.92 ± 1.92 c	3.45 ± 0.22 b

The main objective of both SNIs and BNIs is to inhibit the growth of nitrifying communities (Ruser and Schulz, 2015; Subbarao et al., 2017), and it is evident that both nitrification inhibitors are capable of achieving this aim. Nonetheless, if the prospects for a future more environmentally friendly agriculture relies on the use of BNIs, we must ensure that they present similar or even better efficiencies than the inhibition methods used today. In all the chapters of this thesis can be observed that the abundance of nitrifying bacteria experiences a great increase after the application of ammonium-based synthetic fertilizer. In order to reduce this growth, nitrification inhibitors are used. Since SNIs are manufactured products, we have a recommended dose to apply. On the other hand, BNIs are released by plants and this is related to their physiological state and development (Sarr et al., 2020). Then, we cannot control the exudation rate; we can only measure BNIs' effects and compare them with SNIs. However, the BNIs release seems to be enough to match the efficiency displayed by SNIs (Table 1). In Chapter 4.1, BNIs exuded from sorghum roots (BNI) were able to maintain the same *amoA* relative abundance than SNI (Table 1). Moreover, the residual effects of sorghum as a cover crop (BNI) reduced 43% the *amoA* relative abundance compared to AS in the soil of wheat crop (Control) without any differences compared to the SNI treatment (Table 1). This confirms that sorghum is able to control the growth of nitrifying bacteria in a similar way

as SNIs do. In addition, there is also the need to evaluate whether the BNI-trait introduced in ROELFS and MUNAL wheat lines is efficient enough to be able to substitute de use of SNIs. The presence of the BNI-trait in the ROELFS (BNI) wheat line made possible a 63% reduction of *amoA* relative abundance (Table 1). Although there was no difference between the presence of BNIs and the use of SNIs, a greater inhibition was achieved when both nitrification inhibitors were combined (BNI+SNI). MUNAL-BNI was also able to decrease 47% the *amoA* relative abundance without any differences compared to the SNI treatment (Table 1). Therefore, we can conclude that the BNIs are just as efficient as SNIs in inhibiting the growth of nitrifying bacteria. This makes the BNIs a promising alternative to the use of SNIs to reduce N losses and achieve sustainable agriculture.

3. Pathways to achieve sustainable agriculture

The use of cover crops with BNI exudation ability is becoming interesting to the scientific community. This kind of crop could weaken nitrifying populations before the crop of interest is sowed and reduce the N losses in the following culture. Moreover, the results of BNIs on soil nitrification that we obtained in this thesis (Table 1) encourage their use. With this idea, Karwat et al. (2017) studied the residual effects of *Brachiaria humidicola* pasture on N recovery and grain yield of subsequent maize culture. These authors observed that the soil that held the *B. humidicola* pasture presented a decreased nitrification and, thus, higher maintenance of N that was used by the maize crop. In our case, at first, sorghum seemed to be a good candidate because of the results in Chapters 3 and 4.1. Nevertheless, although the residual effects of sorghum as a cover crop were able to decrease nitrification (Chapter 4.2, Fig. 2B), it turned out not to be such a good option. Agrosystems are a complex structure where successive crop rotations take place in order to get high production. This allows the possibility that the effects the previous crop has on the soil may endure into the next one (Garland et al., 2021). Nonetheless, not only the BNI molecules are left in the soil, but also the residues of the crop. In this case, the presence of the sorghum roots meant an extra C source that was used by the denitrification bacteria (Chapter 4.2, Fig. 4A and B), which incremented the N_2O emissions (Chapter 4.2, Fig. 3). Then, although nitrification is reduced in the soil, N is still lost through enhanced denitrification. To our knowledge, the effect of grasses or crop plants' BNIs on denitrifying populations has not been investigated. According to Surey et al. (2020), larger portions of labile C substrates promote denitrification reactions. The

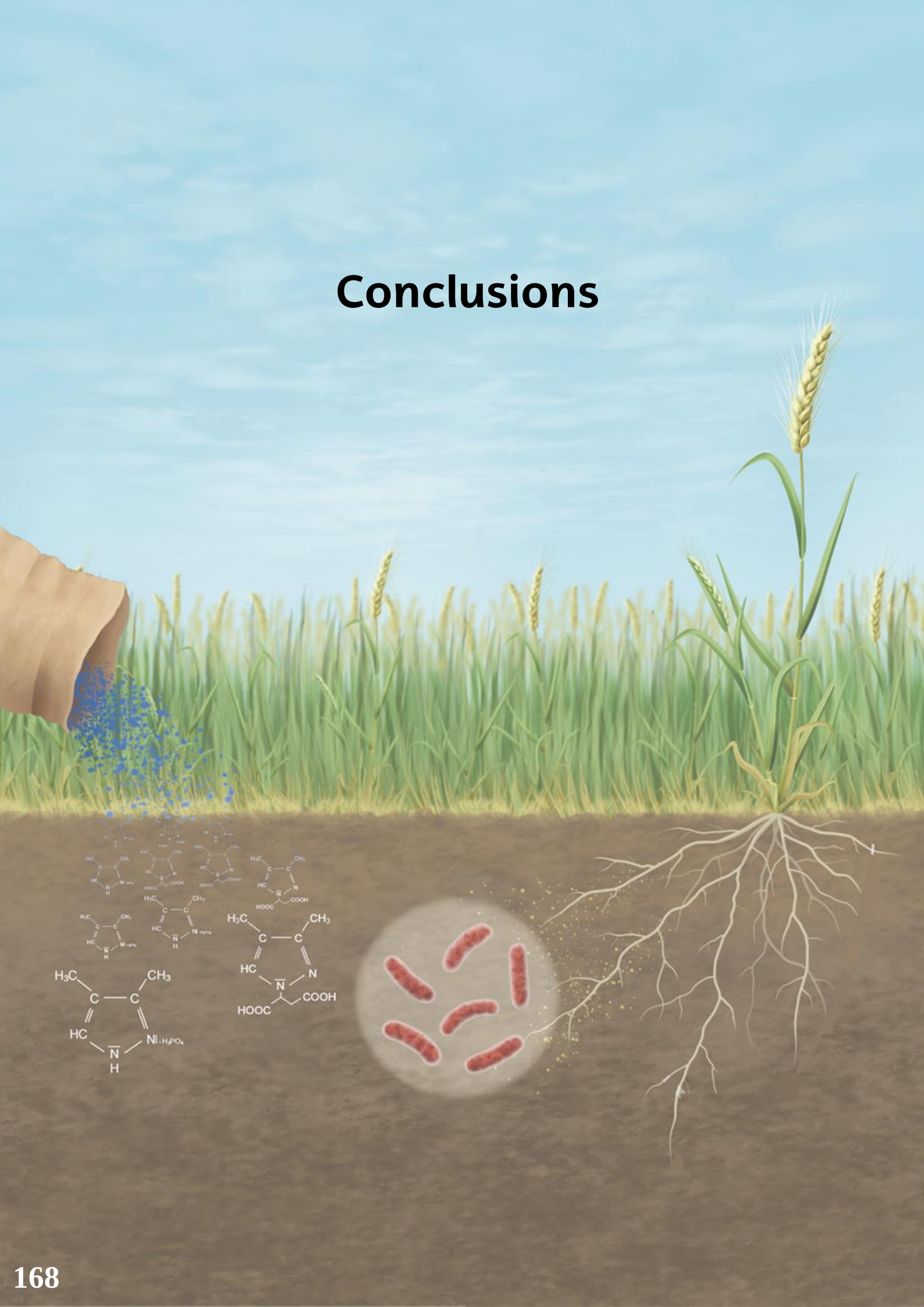
plant BNI activity belongs to the secondary metabolism, and the release of molecules with the capacity to inhibit nitrification implies an extra supply of C to the soil. Since the main mechanism connecting the C cycle with N gas emissions is the availability of C in the soil (Davidson et al., 2000), there could be the possibility that the molecules that inhibit nitrification are, in turn, promoting denitrification, which would cause the loss of N in the form of N_2O emissions. To answer this question we observed the *nirK* relative abundance from our experiments (Table 2). The *nirK* relative abundance from BNI treatments where sorghum, ROELFS-BNI, and MUNAL-BNI were present was not affected compared to Control treatment nor to SNI treatment (Table 2). It only experienced a 40% increase when sorghum was left as a stover to sow the wheat in the sorghum-wheat rotation (Table 2). It seems that the C from BNI molecules does not increase the abundance of denitrifying bacteria, and the increment in *nirK* abundance seen in Chapter 4.2 is exclusively due to C from sorghum residues. Therefore, we have to be careful with the use of cover crops with BNI capacity in crop rotations because we might be inhibiting nitrification, but at the same time, we would be losing N through denitrification.

Table 2. *Relative abundance of nirK gene (nirK abundance/16S rRNA abundance).*
Data from treatments of ***Chapter 4.1****;* *BNI = AS; BNI+SNI = AS+D.*
Data from treatments of ***Chapter 4.2****; Control = AS from fallow-wheat rotation; BNI = AS from sorghum-wheat rotation; SNI = AS+DMPP from fallow-wheat rotation; BNI+SNI = AS+DMPP from sorghum-wheat rotation.*
Data from treatments of ***Chapter 5****; Control = ROELFS-Control and MUNAL-Control from treatment AS; BNI = ROELFS-BNI and MUNAL-BNI from treatment AS; SNI = ROELFS-Control and MUNAL-Control from treatment AS+D; BNI+SNI = ROELFS-BNI and MUNAL-BNI from treatment AS+D. Different letters indicate significant differences using the Duncan Test (p <0.05; n = 4).*

	***nirK* relative abundance (%)**			
	Chapter 4.1	**Chapter 4.2**	**Chapter 5 ROELFS**	**Chapter 5 MUNAL**
Control		0.18 ± 0.03 b	2.39 ± 0.14 a	2.62 ± 0.68 a
BNI	0.08 ± 0.01 a	0.26 ± 0.00 a	2.42 ± 0.08 a	2.50 ± 0.08 a
SNI		0.19 ± 0.02 b	2.40 ± 0.43 a	2.71 ± 0.33 a
BNI+SNI	0.09 ± 0.01 a	0.18 ± 0.03 b	2.31 ± 0.26 a	3.09 ±0.28 a

However, we suggest that the best solution to avoid the threat of enhanced denitrification derived from the extra C of cover crop residues is for the crop itself to exude BNIs. Unfortunately, the plants of the main cereals lack the ability to exude BNIs (Subbarao et al., 2017); while the plants with higher release of BNIs are grasses or cereal crops that do not interest industrialized countries, such as sorghum. Nevertheless, Subbarao et al. (2021) achieved great success by introducing the BNI-trait of *Leymus racemosus* into elite wheat varieties. In this way, a highly productive cereal crop will be inhibiting nitrification by itself, without increasing denitrifying bacteria. Moreover, the use of ROELFS-BNI and MUNAL-BNI wheat lines will be effective in inhibiting nitrification in each crop campaign. In the experiment of Karwat et al. (2017), the residual BNI effect of *B. humidicola* was only effective in the first year. In the second year, they do not find any differences in N_2O emissions between the field with and without *B. humidicola* residues. In addition, the results of Chapter 5 reinforce those obtained by Subbarao et al. (2021) and show that these new BNI wheat lines will be useful and effective under different physicochemical soil conditions and with fertilizer application of different N sources. Wheat, along with rice and barley, is one of the most widely produced crops in the world. Its global production reaches 7.7 Tg per year (Swarbreck et al., 2019). Wheat is a crop that demands high levels of N both to achieve high yields and to increase grain quality (Zorb et al., 2018). This explains why this crop requires practically 20% of the worldwide industrially produced N fertilizers. The ROELFS-BNI and MUNAL-BNI wheat lines can reduce the need for such an amount of N fertilizer. It is estimated that the use of these BNI wheat lines will be able to decrease 15% N fertilizer application, 15.9% the emission of greenhouse gasses (GHG), and improve 16.7% the crop NUE by 2050 (Leon et al., 2021). Therefore, the future of agriculture lies in the introduction of the BNI trait in elite crops of interest. Thanks to Subbarao et al. (2021), a new field of research has been opened and all efforts should be directed to finding BNI traits that can be introduced into crops such as maize (Otaka et al., 2021), barley, and rice.

Conclusions



1. The application of DMPP and DMPSA increases the soil NH_4^+ content, which causes barley plants to show lower biomass because of ammonium stress at ambient CO_2 conditions. However, elevated CO_2 relieves this stress due to the extra carbon availability that allows a more efficient NH_4^+ assimilation.

2. Synthetic nitrification inhibitors are able to inhibit nitrifying microorganisms' growth at both CO_2 concentrations, being more effective in elevated CO_2 conditions. This leads to a decreased substrate to denitrify and, thus, further reduce N_2O emissions.

3. The greater efficiency of both DMPP and DMPSA under elevated CO_2 atmosphere highly recommends their use in future climate scenarios in order to reach a sustainable agriculture.

4. Although DMP-based synthetic nitrification inhibitors are able to delay the oxidation of NH_4^+ in the soil, DMPSA cannot inhibit the growth of nitrifying microorganisms in pure cultures of *Nitrosomonas europaea*. Moreover, the inhibitory capacity of DMPP is not altered regardless of an increase in the concentrations of copper (Cu^{2+}) and zinc (Zn^{2+}) in the medium. Therefore, it seems that the mode of action of DMPs is not related to their Cu^{2+} chelating capacity as it was first suggested.

5. DMPP is highly specific since it exclusively inhibits the AMO enzyme, without any effect on the HAO enzyme of *Nitrosomonas europaea*.

6. To inhibit nitrification, DMPSA has to be broken to release DMP and it is accomplished by biological processes of soil microorganisms. Nevertheless, since the break of DMPSA is carried out by the soil biological activity, the type of soil and environmental conditions could modify its efficiency.

7. Sorghum BNI activity seems to be related to water availability since under drought conditions, sorghum plants increase their roots exudation and BNI release to increase the NH_4^+ competition with nitrifying microorganisms.

8. Sorghum crop is able to avoid an increase in nitrifying microorganisms after nitrogen fertilization with the same efficiency as DMPP, maintaining similar soil NH_4^+ contents throughout the experiment.

9. Sorghum crop is a better alternative to summer fallow in humid Mediterranean conditions since it reduces the exceeding soil nitrogen from the previous crop and their residues weaken nitrifying communities in the following culture.

10. The use of biological nitrification inhibitors is a promising alternative to achieve sustainable agriculture since they reduce the nitrifying microorganisms in the same proportion as synthetic nitrification inhibitors and they do not affect the total abundance of bacteria.

11. Although sorghum residues are able to inhibit the growth of nitrifying microorganisms, the use of sorghum as a cover crop is not a suitable option to mitigate nitrogen pollution in a sorghum/winter wheat crop rotation since the higher C availability from sorghum stover enhances heterotrophic denitrification and, therefore, increases N_2O emissions.

12. To counteract the greater N_2O release derived from the use of sorghum as a cover crop we suggest the application of DMPP since it delays soil NH_4^+ oxidation, diminishing soil NO_3^- formation and, thus, mitigating the increase of heterotrophic denitrification resulting from the higher C availability in the sorghum–wheat rotation.

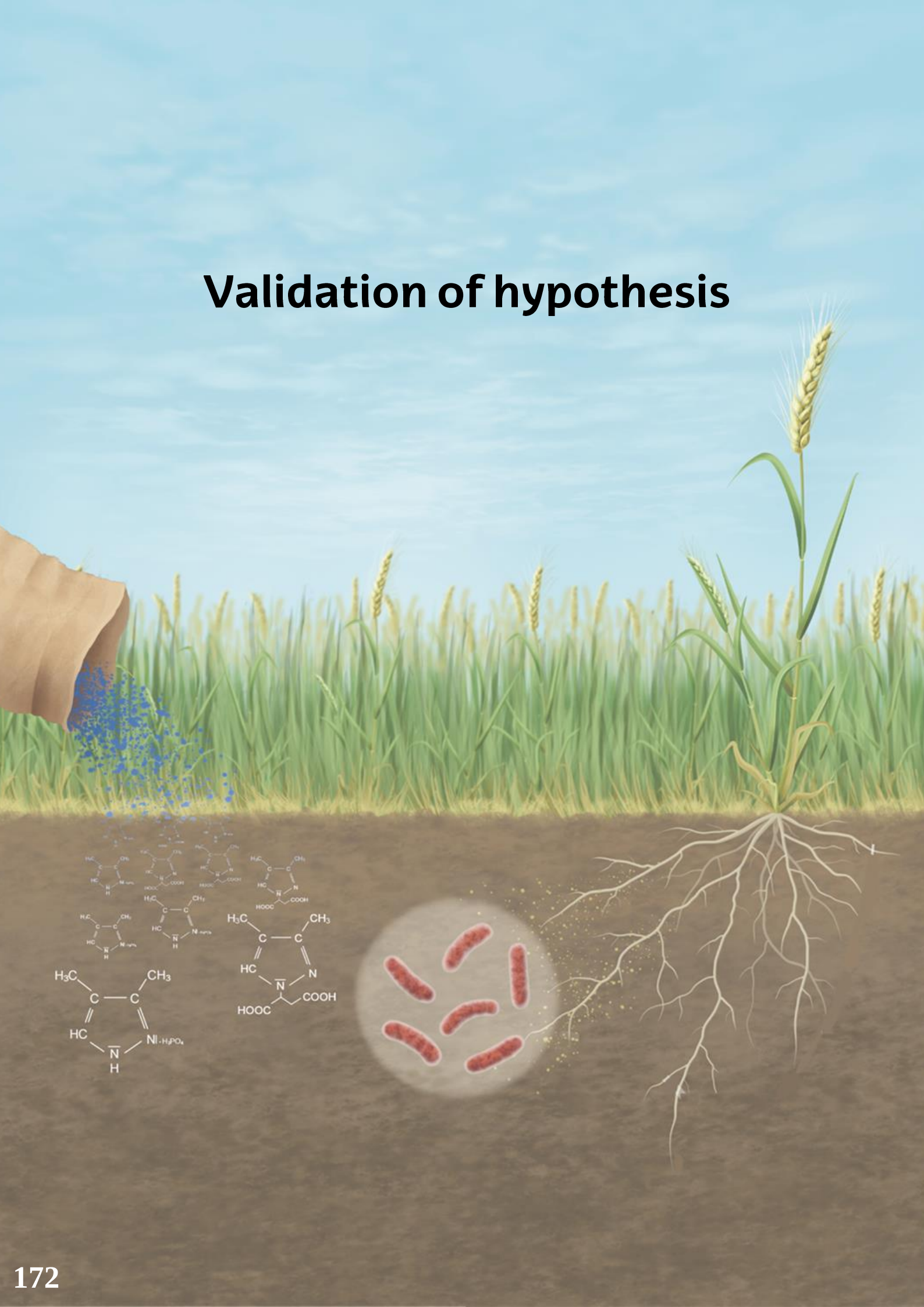
13. The presence of biological nitrification inhibitors does not affect denitrifying microorganisms. Then, the solution to avoid the threat of enhanced denitrification derived from the extra C of cover crop residues is for the crop itself to exude biological nitrification inhibitors.

14. ROELFS-BNI and MUNAL-BNI wheat lines represent an efficient biological nitrification inhibitor technology that is able to reduce the abundance of nitrifying microorganisms, resulting in maintaining higher soil NH_4^+ and, thus, triggering

lower soil NO_3^- content. Additionally, ROELFS-BNI and MUNAL-BNI wheat lines also reduce soil NO_3^- content under NO_3^- fertilization.

15. Due to higher soil NH_4^+ maintenance, ROELFS-BNI and MUNAL-BNI wheat lines are able to shift their metabolism to have more ammonium-based nutrition. Moreover, they can improve the wheat nitrogen-use-efficiency since they enhance nitrogen absorption regardless of the source of nitrogen fertilizer.

Validation of hypothesis



Initial hypothesis 1: Dimethylpyrazole-based synthetic nitrification inhibitors will remain equally effective under elevated CO_2 conditions.

We confirmed that **DMPP and DMPSA inhibited nitrifying microorganisms, and reduced NO_3^- formation, and N_2O emissions at both ambient and elevated CO_2 concentration. Moreover, DMPP and DMPSA showed a higher reduction in *amoA* abundance and N_2O emissions in elevated CO_2.**

Initial hypothesis 2: The nitrification inhibition mode of action of dimethylpyrazole-based synthetic nitrification inhibitors is based on their Cu^{2+} chelating capacity, leading to an inhibition of AMO activity.

Our results proved that **the mode of action of DMPP and DMPSA is not related to their Cu^{2+} chelating capacity. DMPSA was not able to inhibit *Nitrosomonas europaea* growth in pure cultures and DMPP remained efficient even with increased concentrations of Cu^{2+} and Zn^{2+}. In addition, we discovered that DMPSA has to be broken in soils to be effective.**

Initial hypothesis 3: Moderate drought stress will increase the exudation of biological nitrification inhibitors from sorghum to increase the ammonium competition with nitrifying microorganisms.

We corroborated that **sorghum plants decrease considerably the abundance of nitrifying microorganisms due to an increase of their roots exudation and biological nitrification inhibitors' release under moderate drought conditions.**

Initial hypothesis 4: The biological nitrification inhibition potential makes sorghum a good option as a cover crop in a sorghum/winter wheat rotation since it avoids nitrogen losses by reducing nitrification in the field. Moreover, the persistence of biological nitrification inhibitors may reduce the need for synthetic nitrification inhibitors.

It was proved that **the sorghum crop was able of inhibiting the growth of nitrifying microorganisms during its development and in the following wheat crop. However, contrarily to our hypothesis, the use of sorghum as a cover crop cannot reduce the**

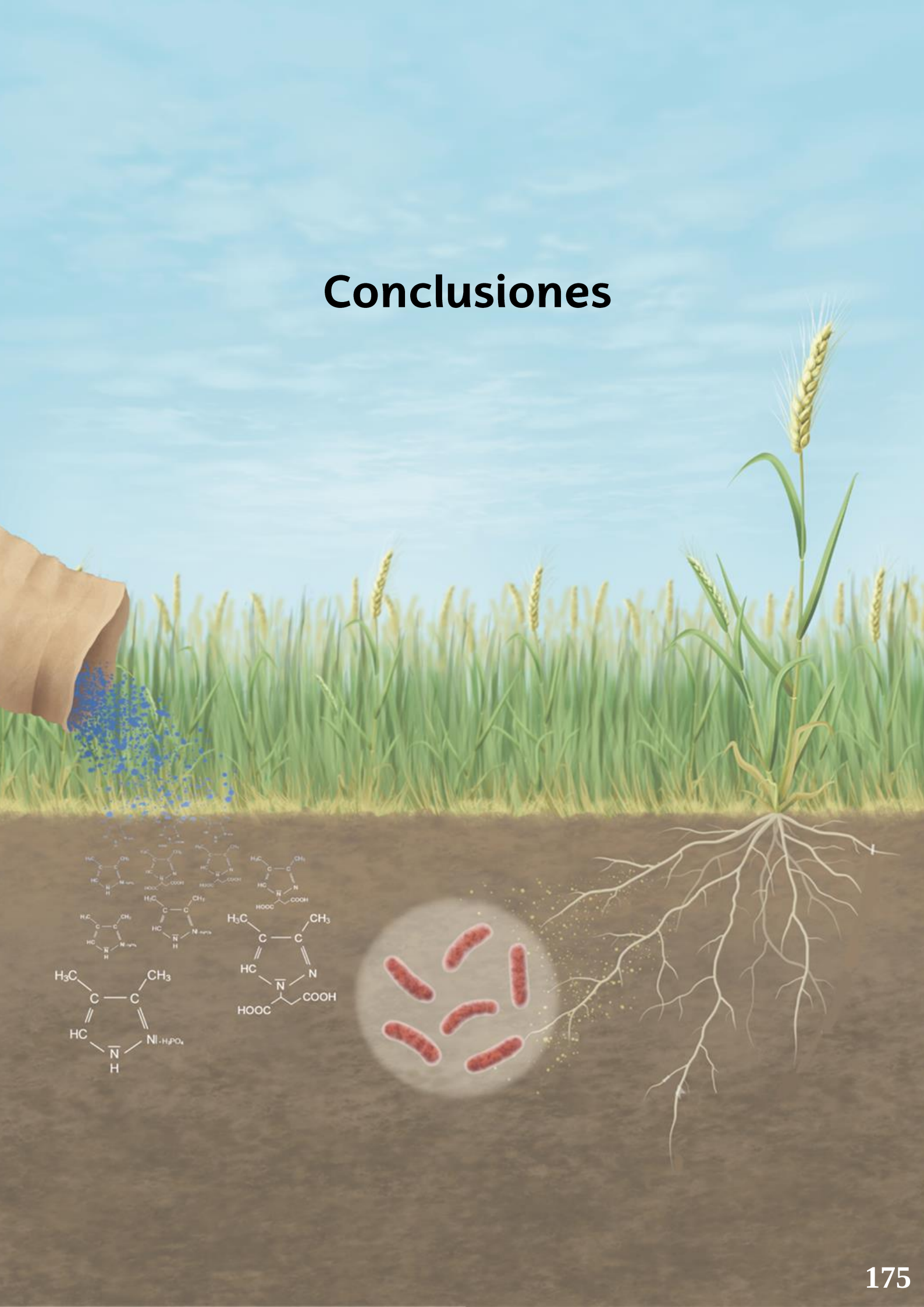
need for synthetic nitrification inhibitors because the extra C availability derived from sorghum stover enhanced heterotrophic denitrification and increased N_2O emissions in the wheat crop, which could only be counteracted with the use of DMPP.

Initial hypothesis 5: ROELFS-BNI and MUNAL-BNI wheat lines are efficient technology to decrease nitrification without affecting their yield. Furthermore, the benefits of harbouring the genes capable of producing biological nitrification inhibitors will avoid the ammonium syndrome under ammonium nutrition.

We demonstrated that **ROELFS-BNI and MUNAL-BNI wheat lines were able to inhibit not only nitrifying bacteria but also nitrifying archaea. They shifted their metabolism to more ammonium-based nutrition without any effect on their biomass. Furthermore, ROELFS-BNI and MUNAL-BNI wheat lines presented an improved nitrogen absorption regardless of the nitrogen source.**

Therefore, all the results of this thesis lead us to confirm our principal hypothesis:
The control of nitrification allows us to achieve sustainable agriculture by reducing reactive nitrogen losses derived from the use of ammonium-based fertilizers in the environment.

Conclusiones



1. La aplicación de DMPP y DMPSA incrementa el contenido de NH_4^+ en el suelo, causando que las plantas de cebada presenten una menor biomasa debido al síndrome de estrés por amonio. Sin embargo, el aumento de la disponibilidad de C en elevado CO_2 alivia dicho estrés permitiendo una asimilación más eficiente de NH_4^+.

2. Los inhibidores sintéticos de la nitrificación son capaces de inhibir el crecimiento de los microorganismos nitrificantes en ambas concentraciones de CO_2. Esto conduce a una disminución del sustrato que puede ser parte de la desnitrificación y, por lo tanto, se reducen aún más las emisiones de N_2O.

3. La mayor eficiencia que presentan tanto DMPP como DMPSA en concentraciones elevadas de CO_2 hace que su uso en escenarios climáticos futuros sea muy recomendable con el objetivo de alcanzar una agricultura sostenible.

4. A pesar de que los inhibidores sintéticos de la nitrificación basados en DMP son capaces de retrasar la oxidación de NH_4^+ en el suelo, el DMPSA no puede inhibir el crecimiento de microorganismos nitrificantes en cultivos puros de *Nitrosomonas europaea*. Además, la capacidad inhibitoria del DMPP no se ve alterada independientemente de un aumento en las concentraciones de cobre (Cu^{2+}) y zinc (Zn^{2+}) en el medio de crecimiento. Por lo tanto, a diferencia de como se ha sugerido inicialmente, parece ser que el modo de acción de los DMPs no está relacionado con su capacidad quelante de Cu^{2+}.

5. El DMPP es altamente específico ya que inhibe exclusivamente la enzima AMO, sin ningún efecto sobre la enzima HAO de *Nitrosomonas europea*.

6. Para inhibir la nitrificación, el DMPSA debe romperse para liberar DMP, lo que se logra mediante procesos biológicos de los microorganismos del suelo. No obstante, dado que la ruptura del DMPSA es realizada por la actividad biológica del suelo, el tipo de suelo y las condiciones ambientales podrían modificar su eficiencia.

7. La actividad BNI en sorgo parece estar relacionada con la disponibilidad de agua ya que, bajo condiciones de sequía, las plantas de sorgo aumentan la liberación de BNIs y los exudados de raíces para aumentar la competencia de NH_4^+ con los microorganismos nitrificantes.

8. El cultivo de sorgo es capaz de evitar un aumento en la abundancia de microorganismos nitrificantes después de la fertilización nitrogenada con la misma eficiencia que el DMPP, manteniendo contenidos similares de NH_4^+ en el suelo durante todo el experimento.

9. El cultivo del sorgo es una mejor alternativa al barbecho durante el verano en lugares de clima tipo Mediterráneo húmedo ya que reduce el exceso de nitrógeno del suelo del cultivo anterior y sus residuos debilitan las poblaciones de nitrificantes en el cultivo siguiente.

10. El uso de inhibidores biológicos de la nitrificación se presenta como una alternativa prometedora para lograr una agricultura sostenible ya que reducen los microorganismos nitrificantes en la misma proporción que los inhibidores sintéticos de la nitrificación, sin ningún efecto sobre la abundancia total de bacterias.

11. Aunque los residuos del cultivo de sorgo pueden inhibir el crecimiento de microorganismos nitrificantes, su uso como cultivo de cobertura no es una opción adecuada para mitigar la contaminación por nitrógeno en una rotación de cultivos de sorgo/trigo de invierno, ya que la mayor disponibilidad de C de los rastrojos de sorgo aumenta la desnitrificación heterótrofa y, por lo tanto, también aumentan las emisiones de N_2O.

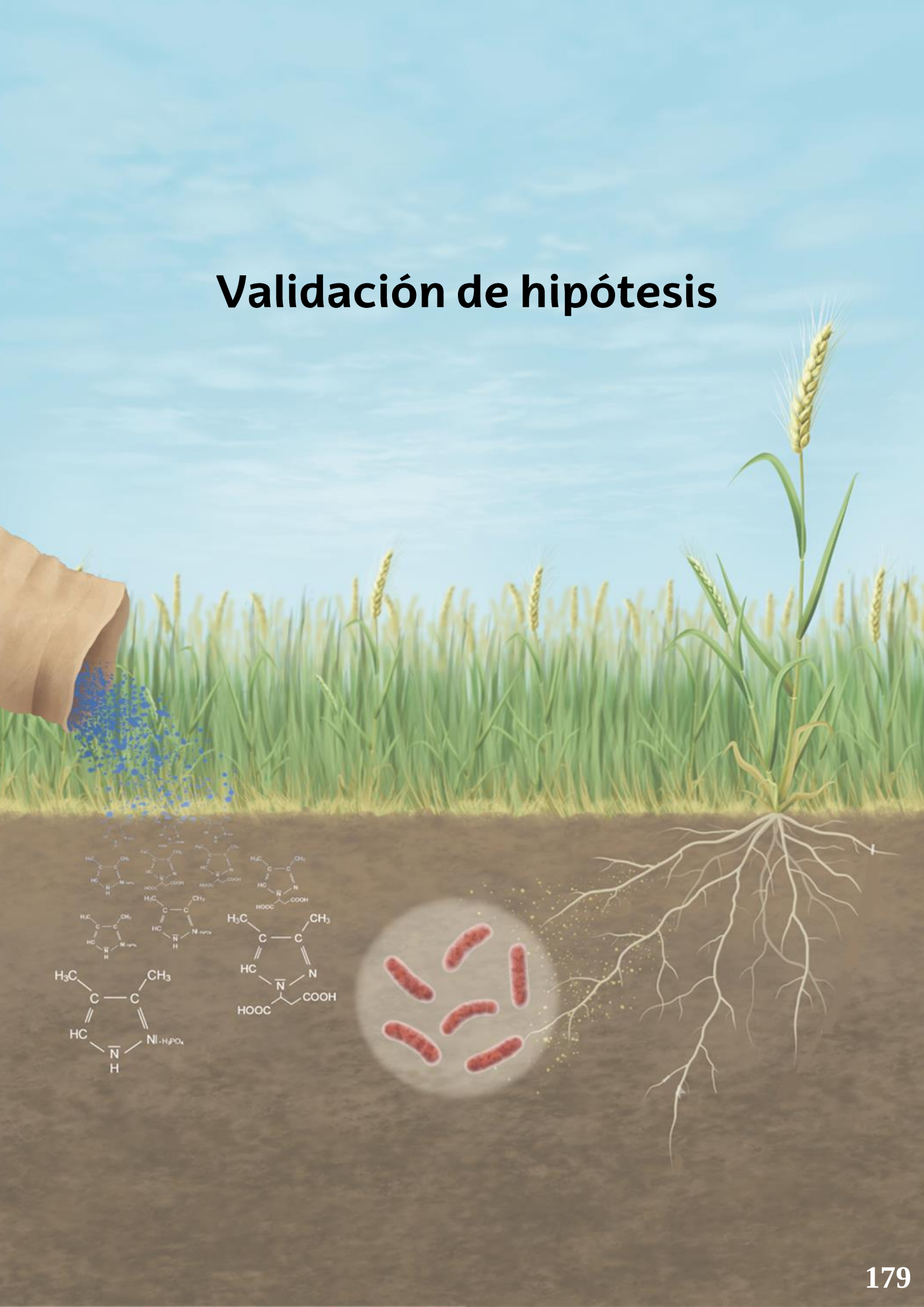
12. Para contrarrestar la mayor liberación de N_2O derivada del uso del sorgo como cultivo de cobertura sugerimos la aplicación de DMPP ya que retrasa la oxidación del NH_4^+ del suelo, disminuyendo la formación de NO_3^- y, por tanto, mitiga el aumento de la desnitrificación heterótrofa resultante de una mayor disponibilidad de C en la rotación sorgo-trigo.

13. La presencia de inhibidores biológicos de la nitrificación no afecta a los microorganismos desnitrificantes. Entonces, la solución para evitar la amenaza de un aumento de la desnitrificación debido a una mayor disponibilidad de C por los residuos del cultivo de cobertura es que sea el propio cultivo quien exude inhibidores biológicos de la nitrificación.

14. Las líneas de trigo ROELFS-BNI y MUNAL-BNI representan una tecnología biológica eficiente que es capaz de reducir la abundancia de microorganismos nitrificantes, manteniendo más altos los niveles de NH_4^+ en el suelo y provocando una reducción en la formación de NO_3^- en el suelo. Además, las líneas de trigo ROELFS-BNI y MUNAL-BNI también reducen el contenido de NO_3^- del suelo bajo la fertilización con NO_3^-.

15. Debido a un mayor mantenimiento de NH_4^+ en el suelo, las líneas de trigo ROELFS-BNI y MUNAL-BNI pueden cambiar su metabolismo para tener una nutrición más amoniacal. Además, pueden mejorar la eficiencia en el uso del nitrógeno del trigo ya que mejoran la absorción de nitrógeno independientemente de cual sea la fuente del mismo.

Validación de hipótesis



Hipótesis inicial 1: Los inhibidores sintéticos de nitrificación basados en dimetilpirazol seguirán siendo igualmente efectivos bajo condiciones de concentración elevada de CO_2.

Hemos confirmado que **el DMPP y el DMPSA inhibieron los microorganismos nitrificantes, reduciendo la formación de NO_3^- y las emisiones de N_2O tanto en concentraciones de CO_2 ambiente como elevada. Además, el DMPP y el DMPSA mostraron una mayor reducción en la abundancia de *amoA* y en las emisiones de N_2O cuando los niveles de CO_2 eran elevados.**

Hipótesis inicial 2: El modo de acción por el que inhiben la nitrificación los inhibidores sintéticos de la nitrificación basados en dimetilpirazol es debido a su capacidad quelante de Cu^{2+}, lo que conduce a una inhibición de la actividad de la enzima AMO.

Nuestros resultados demostraron que **el modo de acción del DMPP y DMPSA no está relacionado con su capacidad quelante de Cu^{2+}. El DMPSA no fue capaz de inhibir el crecimiento de *Nitrosomonas europaea* en cultivos puros y el DMPP siguió siendo eficaz incluso con mayores concentraciones de Cu^{2+} y Zn^{2+}. Además, descubrimos que el DMPSA tiene que romperse en los suelos para que sea eficaz.**

Hipótesis inicial 3: El estrés por sequía moderada aumentará la exudación de los inhibidores biológicos de la nitrificación del sorgo para aumentar la competencia por el amonio con los microorganismos nitrificantes.

Hemos corroborado que **las plantas de sorgo, en condiciones de sequía moderada, disminuyen considerablemente la abundancia de microorganismos nitrificantes debido a un aumento de los exudados de raíces y liberación de inhibidores biológicos de la nitrificación.**

Hipótesis inicial 4: El potencial de la inhibición biológica de la nitrificación que presenta el sorgo hace que este sea una buena opción como cultivo de cobertura en una rotación de sorgo/trigo de invierno, ya que evita las pérdidas de nitrógeno al reducir la nitrificación en el campo. Además, la persistencia de los inhibidores biológicos de la nitrificación puede reducir la necesidad de inhibidores sintéticos de la nitrificación.

Hemos comprobado que **el cultivo de sorgo fue capaz de inhibir el crecimiento de microorganismos nitrificantes durante su desarrollo y el efecto perduró durante el siguiente cultivo de trigo. Sin embargo, contrariamente a nuestra hipótesis inicial, el uso de sorgo como cultivo de cobertura no puede reducir la necesidad de inhibidores sintéticos de nitrificación porque la mayor disponibilidad de C derivada de los residuos de sorgo incrementó la desnitrificación heterótrofa, aumentando las emisiones de N_2O en el cultivo de trigo, lo que solo pudo contrarrestarse con el uso de DMPP.**

Hipótesis inicial 5: Las líneas de trigo ROELFS-BNI y MUNAL-BNI son una tecnología eficiente para disminuir la nitrificación sin afectar el rendimiento del trigo. Además, los beneficios de albergar los genes capaces de producir inhibidores biológicos de la nitrificación evitarán el síndrome de estrés por amonio bajo la nutrición amoniacal.

Hemos demostrado que **las líneas de trigo ROELFS-BNI y MUNAL-BNI fueron capaces de inhibir no solo las bacterias nitrificantes sino también las arqueas nitrificantes. Las líneas de trigo cambiaron su metabolismo para poder tener una nutrición más amoniacal sin ningún efecto sobre su biomasa. Además, las líneas de trigo ROELFS-BNI y MUNAL-BNI presentaron una mejor absorción de nitrógeno independientemente de cuál fuera su fuente.**

Por tanto, todos los resultados de esta tesis nos llevan a confirmar nuestra hipótesis principal:

El control de la nitrificación nos permite conseguir una agricultura sostenible al reducir las pérdidas de nitrógeno reactivo al medio ambiente derivadas del uso de fertilizantes con base amoniacal.

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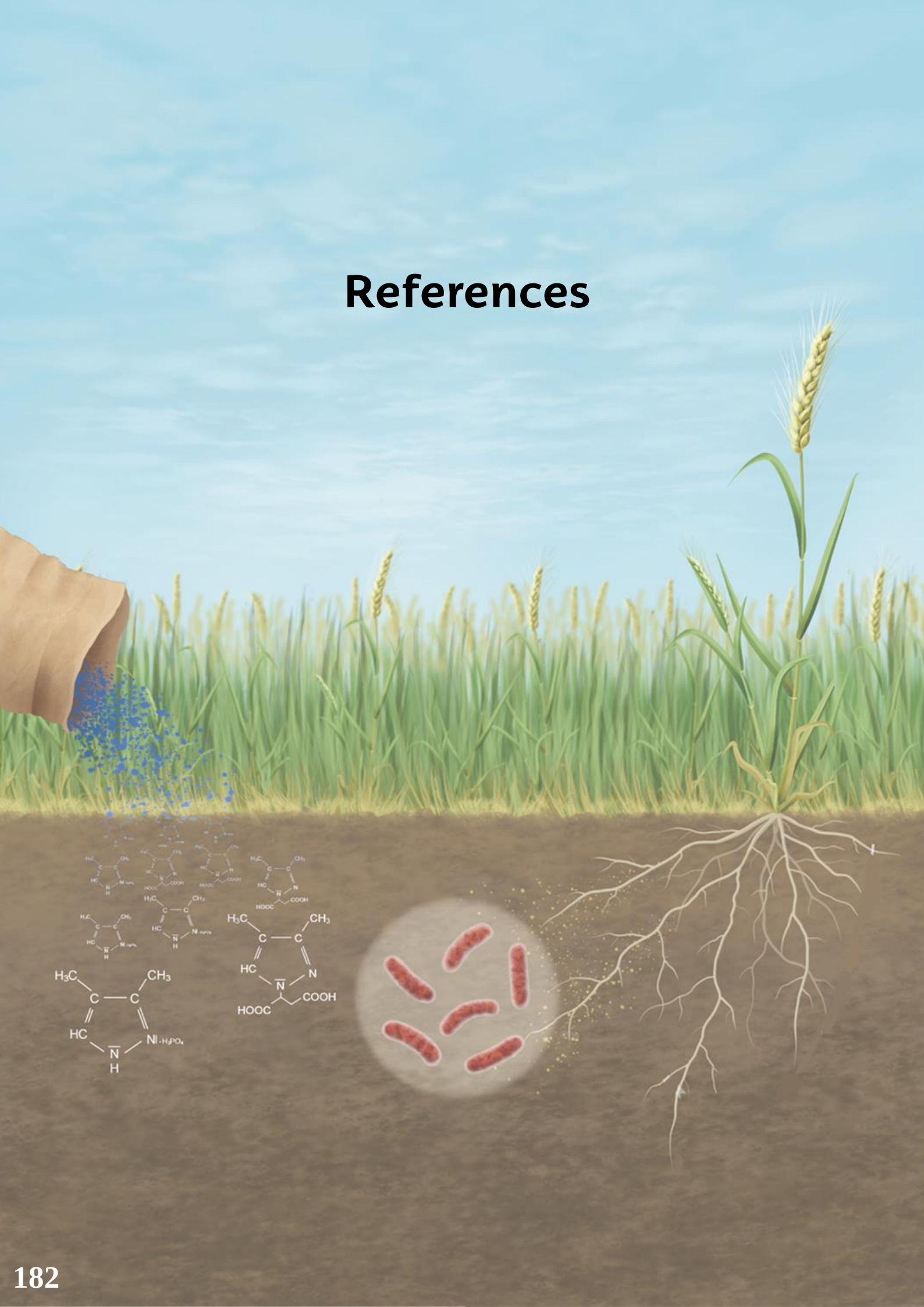

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