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The diazotrophic bacteria *Azospirillum baldaniorum* and *A. brasilense* improve wheat seedlings' nitrogen budget through ammonia scavenging

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ABSTRACT

Besides N_2 fixation, we consider that other diazotrophic traits can be explored to increase plants' nitrogen (N) budget. Here, we report initial results of the capacity of the diazotrophic plant growth promoting rhizobacteria *Azospirillum baldaniorum* and *A. brasilense* to improve wheat seedlings' N budget through ammonia (NH_3) scavenging. We inoculated wheat seedlings with two *Azospirillum* strains (*A. baldaniorum* Sp245 and *A. brasilense* ARG2) and determined its effect on plant biomass, N content and N isotopic signatures (i.e., $\delta^{15}N$). Furthermore, using bipartite Petri dishes, we grew the *Azospirillum* strains under increasingly alkaline conditions (from pH 7.5 to 10.0), which created a gradient of atmospheric NH_3 concentrations ($[NH_3]$), and we used *Saccharomyces cerevisiae* mutants to explore the involvement of the AMT/MEP/Rh proteins in atmospheric NH_3 scavenging. Wheat seedlings inoculated with *A. baldaniorum* Sp245 and *A. brasilense* ARG2 increased their N content by 65 and 94 % (respectively), and their negative N isotopic signatures (around −10 ‰, which contrasted with positive signatures in control plants) were compatible with NH_3 transport through AMT/MEP/Rh transporters, but not with N_2 fixation. Furthermore, increasing the atmospheric $[NH_3]$ stimulated the growth rate of the *Azospirillum* strains up to 5-fold in relation to ambient atmospheric $[NH_3]$, showing that both *Azospirillum* strains scavenged the atmospheric NH_3 and used it to grow. Our data clearly show that: i) NH_3 scavenging by *A. baldaniorum* Sp245 and *A. brasilense* ARG2 is involved in increasing plant's N budget; and ii) NH_3 transport through AMT/MEP/Rh protein family transporters is involved in microbial NH_3 scavenging. This overlooked microbial trait can be an interesting tool to mitigate atmospheric $[NH_3]$, especially in farming environments.

1. Introduction

Being the prime component of amino acids, proteins, nucleic acids, and chlorophylls (among other key cell components), nitrogen (N) is an essential macronutrient for all life forms. Although N is very abundant in the atmosphere (approximately 80 % of the Earth's atmosphere is N_2), in the molecular form it is inaccessible to most organisms (Bernhard, 2010). Therefore, N is one of the most limiting nutrients in most ecosystems (Agren et al., 2012; Zhang et al., 2023). Once the inert N_2 is converted (by lightnings, N_2 fixation by diazotrophs, or the industrial Haber-Bosch process) into a reactive N form which is ammonia/ammonium (NH_3/NH_4^+), it can be taken up by plants and microbes and cycle along the food webs. Given the critical roles that N plays in all life forms, and the limiting availability at which N naturally occurs in most ecosystems, organisms have evolved high affinity transport systems. In agreement, genes of the high affinity transporter family for NH_3/NH_4^+ (consists of the three major clades AMT, MEP, and Rh, i.e., the AMT/MEP/Rh family) are found in almost all prokaryotic and eukaryotic lineages, including bacteria, fungi, algae, plants, and animals. In microbes and plants, the function of AMT/MEP/Rh proteins is to take up NH_3/NH_4^+ while in animals, it is to excrete NH_3/NH_4^+, which is a by-product toxic to animal cells (McDonald and Ward, 2016). As the affinity for NH_3/NH_4^+ of the AMT/MEP/Rh family transporters is very high [e.g., Km for MEP and AMT in the mM range (Boeckstaens et al., 2008)],

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when N availability is below 0.5–1 mM, NH_3/NH_4^+ transport occurs through these transporters, i.e., the AMT/MEP/Rh family enables its organisms to scavenge the scarce NH_3/NH_4^+.

Besides plants' direct contribution to their N budget, microbes living in the roots and the surrounding soil, such as plant growth promoting rhizobacteria (PGPR), can also contribute to plants' N budget. In particular, the diazotrophic PGPR, which can be symbiotic (e.g., *Rhizobium* sp. which may colonize root nodules in legume plants) or free living (e.g., *Azospirillum brasilense* and *Herbaspirillum seropedicae* which colonize non-legume plants such as cereals) (Ramos et al., 2020). The percentage of plant N derived from free-living diazotrophs can be up to 60 % in sugar cane plants (Boddey et al., 1995), and up to 33 % in the common wetland sedge *Schoenoplectus californicusi* (Rejmánková et al., 2018). So far, it is assumed that the main process through which diazotrophic PGPR contribute to improve plant's N budget is through N_2 fixation. However, besides fixing N_2, diazotrophic bacteria can uptake other N sources, including NH_3/NH_4^+ (Reed et al., 2011). NH_3/NH_4^+, the direct product of N_2 fixation, is well known to inhibit N_2 fixation (Reed et al., 2011), and has been shown to inhibit nitrogenase synthesis at the genetic level through the regulation of *nifA* gene transcription (Dixon and Kahn, 2004). Furthermore, it has been impossible to prove the direct involvement of the nitrogenase in improving plant's N budget through associations with free-living diazotrophic PGPR, which raises the question if these microbes could improve plant N budget through other processes.

Although *Azospirillum*'s capacity to fix N_2 is one of its most documented traits involved in plant growth promotion, many studies have shown that the contribution of N_2 fixation to plants is minimal ranging from 5 to 18 % of the total N of inoculated plants (Cassán et al., 2020). Considering its high energy burden [i.e., 16 adenosine triphosphate (ATP) molecules are needed to break down an N_2 molecule, and another 12 ATP molecules are required for NH_4^+ assimilation and transport (Soumare et al., 2020)], N_2 fixation by *Azospirillum* strains (and by other diazotrophs) only prevails when there are no alternative N sources (Norman and Friesen, 2017) such as NH_3/NH_4^+ (Reed et al., 2011). However, studies on microbial NH_3 scavenging are very scarce. For example, Jones and Rhodesroberts (1980) focused on bacterial NH_3 scavenging but not on its effect on plants, while Hurek et al. (1988) focused on rhizospheric bacterial NH_3 scavenging and its effect on plants, but the bacteria were not diazotrophs. Therefore, the role of NH_3 scavenging by PGPR diazotrophs in contributing to plant's N budget is clearly understudied and possibly an underestimated and overlooked microbial trait.

Here, we tested the capacity of two *Azospirillum* strains to improve plant's N budget through atmospheric NH_3 scavenging. Since *Azospirillum* sp. express high affinity ammonium transport family proteins, namely the AmtB transporter (Moure et al., 2019; Steenhoudt and Vanderleyden, 2000), we hypothesize that if NH_3/NH_4^+ is available, even at low concentrations, NH_3 scavenging will prevail over N_2 fixation, reflecting N_2 fixation's high energy burden (Soumare et al., 2020) and the fact that NH_3/NH_4^+ inhibits N_2 fixation (Reed et al., 2011). NH_3 scavenging by *Azospirillum* strains would therefore be involved in plant growth promotion and improvement of plant's N budget. Finally, we explored the molecular mechanisms behind NH_3 scavenging, namely, the involvement of the AMT/MEP/Rh proteins responsible for the high affinity transport of NH_3/NH_4^+.

2. Material and methods

2.1. Microbial strains, media and culture conditions

The following two *Azospirillum* strains used here belong to Soilvitae's (Lisboa, Portugal - https://soilvitae.com/id/) microbial culture collection: i) *A. baldaniorum* Sp245, former *A. brasilense* Sp245 (Ferreira et al., 2020); and ii) *A. brasilense* ARG2, isolated from a soil in Algeria. For inocula preparation, the *Azospirillum* strains were grown in Nutrient Broth (NB, Biokar Diagnostics) at 28 °C for 20 h with a 120 RPM agitation (Orbital shaker, Optic Ivymen System, Comecta). Calibration curves [i.e., the relation between the optical density at 600 nm (OD600) and the colony-forming units (CFU)] were performed for both strains in the specified growth conditions.

The following four *Saccharomyces cerevisiae* strains used here, including three mutants for the MEP proteins (i.e., MEP1, MEP2 and MEP3), also belong to Soilvitae's microbial culture collection: i) wild-type strain ∑1278b, designated as WT; ii) its isogenic triple mutant (lacks *mep1*, *mep2* and *mep3* genes), designated as ΔMEP; iii) triple mutant complemented with *mep1* genes, designated as MEP1; and iv) triple mutant complemented with *mep2* genes, designated as MEP2. For inocula preparation, *S. cerevisiae* strains were grown in proline liquid medium at 28 °C for 48 h with a 120 RPM agitation. The medium contained 7.6 g L^{-1} of yeast N base (without NH_3 or amino acids), 0.1 g L^{-1} of proline, and 20 g L^{-1} of glucose (Marini et al., 1997). The medium for ΔMEP was further supplied with 12 mg L^{-1} of uracil (Ariz et al., 2018; Marini et al., 1997).

2.2. DNA extraction, PCR, sequencing and phylogenetic analysis of the strain ARG2

The taxonomic status of the strain ARG2 was confirmed through a phylogenetic analysis based on the partial sequence of 16S rRNA gene. The DNA was extracted following the method described by Azevedo et al. (2017), with minor modifications. The bacterial culture was incubated at 60 °C overnight with 0.5 mg mL^{-1} proteinase K (Grisp) in 100 mM Tris-HCl (pH 8.8), and subsequently incubated at 98 °C for 3 min to inactivate proteinase K. The resulting supernatant, containing the extracted DNA, was maintained at 4 °C. The partial 16S rRNA gene was amplified using the forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards et al., 1989) and the reverse primer 1492R (5′-GGTTACCTTGTT ACGACTT-3′) (Stackebrandt and Liesack, 1993). PCR reactions were performed in a Biometra Uno II thermal cycler (Biometra GmbH, Göttingen, Germany) under the following conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s, and final extension at 72 °C for 3 min. The amplified products were sent for Sanger sequencing in a commercial laboratory (StabVida, Portugal) and the newly generated 16S rRNA gene sequences were blasted against GenBank. A maximum likelihood (ML) tree was constructed using MEGA X (v. 10.2.6) and the General Time Reversible (GTR) nucleotide substitution model. The best-scoring ML tree was estimated by conducting a bootstrap analysis of 1,000 replicates.

2.3. Effect of inoculating the Azospirillum strains on wheat seedlings' N budget

We tested the effect of the *Azospirillum* strains in improving wheat seedlings' N budget by applying the following three treatments: i) control (non-inoculated); ii) inoculated with *A. baldaniorum* Sp 245, former *A. brasilense* Sp245 (Ferreira et al., 2020); and iii) inoculated with *A. brasilense* ARG2. Each treatment was replicated in 4 tip boxes, each containing 10 seedlings (Fig. S1).

Before inoculation, the bacterial cells (grown in the conditions described above) were washed to remove all N sources from the culture medium: cells were centrifuged at 3800 ×g for 10 min and the supernatant discarded; cells were resuspended in 10 mL of 0.8 % NaCl solution and centrifuged again under the same conditions. The washing procedure was performed twice. After removing the supernatant from the second wash, 10 mL of 0.8 % NaCl solution were added, and the cells resuspended. For both strains, cell density was adjusted to 10^8 CFU mL^{-1} based on the OD600 (i.e., based on the calibration curves, OD600 of 1, corresponded to 10^8 CFU mL^{-1} approximately).

Wheat (*Triticum aestivum*) seedlings were grown hydroponically. Wheat (variety Preto-amarelo) seeds, donated by Estação de

Melhoramento de Plantas (INIAV, Elvas, Portugal), were surface-sterilized (1.5 min in 70 % ethanol; 1 wash in sterile deionized water, 3 min in sodium hypochlorite; 10 washes in sterile deionized water), soaked for 12 h in sterile deionized water and heat-treated for 10 min at 50 °C. Sterilized seeds were aseptically transferred to Petri dishes containing 1.5 % agar (10 seeds per dish) and kept in a growth chamber (Fitoclima “Walk-In” 5000 EH, Aralab) with a 16-h light/8-h dark photoperiod and temperature of 25/20 °C, relative humidity 70/50 %, for 96 h. Four-day-old seedlings were transferred to tip boxes containing 100 mL of a modified 1/4 diluted Hoagland solution with $(NH_4)_2SO_4$ as the sole N source (which represented the addition of 5.6 mg of N per box, and 0.56 mg of N per seedling), with the leaves emerging from the holes of the rack, and the solution bathing the roots (Fig. S1).

Inoculated wheat seedlings roots received 1 mL of a living *Azospirillum* (*A. baldaniorum* Sp245 or *A. brasilense* ARG2) suspension (with approximately 10^8 cells) (Fonseca et al., 2017), which corresponded to 1.5 ± 0.3 mg of *Azospirillum* biomass, containing between 10 and 15 % of N, so that our *Azospirillum* inoculation corresponded to the addition of 0.15–0.23 mg of N per box, and 15–23 μg of N per seedling. Each box was sealed in a sterilized gas exchange bag (Fig. S1) and maintained in the growth chamber (the same conditions previously described) for 16 days.

Twenty-day old seedlings were harvested, washed with sterile deionized water to remove bacterial cells, and analysed for plant biomass (i.e., plant material was dried to constant mass at 60 °C), N concentration and N stable isotopic signature ($\delta^{15}N$). N concentration and $\delta^{15}N$ were determined at the Stable Isotopes and Instrumental Analysis Facility (SIIAF), Portugal, by continuous flow isotope mass spectrometry (CF-IRMS), on a Sercon Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, joined to a EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion (Dias et al., 2018, 2020). The $\delta^{15}N$ of the $(NH_4)_2SO_4$ used in all experiments was also determined. By combining wheat seedlings biomass and their respective N concentration, we calculated plant N content (i.e., plant’s N budget). To account for the N added in the form of *Azospirillum* (*A. baldaniorum* or *A. brasilense*) suspension at the beginning of the experiment, we divided the maximum estimate (i.e., 0.23 mg N per box) by the number of wheat seedlings per box (i.e., 10) and subtracted this value (i.e., 23 μg of N) from the inoculated plants to obtain the wheat seedling’s N content.

2.4. *A. baldaniorum Sp245 and A. brasilense ARG2 capacity to scavenge atmospheric NH_3*

We tested the capacity of the *Azospirillum* strains to scavenge atmospheric NH_3, using bipartite Petri dishes (Fig. S2). Two milliliters of a 10 mM ammonium sulphate [$(NH_4)_2SO_4$] solution with different pH values were placed in the source compartment. Ammonium (NH_4^+) exists in chemical equilibrium with NH_3. The pK_a for this equilibrium is 9.25 at 25 °C (Ariz et al., 2018), i.e., at pH 9.25 there is approximately as much NH_3 as there is NH_4^+. Therefore, by alkalinizing the pH to 9.5 or higher, this chemical equilibrium favours NH_3, which is volatile. We created an atmospheric NH_3 gradient in the source compartment by adjusting the pH of the $(NH_4)_2SO_4$ solution to the following pH values: 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. The less alkaline solutions hampered NH_3 release into the atmosphere as the chemical equilibrium favoured NH_4^+, while the more alkaline solutions, closer or above the pKa (i.e., 9.25 at 25 °C), favoured NH_3 release into the atmosphere. Besides the atmospheric NH_3 gradient, we used two controls: i) deionized water to control for ambient atmospheric [NH_3]; and ii) sulfuric acid, an NH_3 scavenger, was used as a negative control.

The *Azospirillum* strains were inoculated in the sink compartment (Fig. S2). The bacterial inocula were prepared as previously described, including the washing procedure to remove all N sources from the culture medium. Cell density was determined based on the OD600. When the strains reached an OD600 of 1 (which corresponded to 10^8 CFU mL^{-1}), 125 μL of bacterial inoculum and 12.5 mL of liquid N free medium (NFB) (Melo et al., 2016) were inoculated in the sink compartment. Bacterial strains were grown at 28 °C with a 120 RPM agitation, and their growth (i.e., OD600) was assessed at 0, 7, 14, 17, 32, 56, 63 and 88 h after inoculation. Specific growth rates, designated as growth rates, were calculated using the formula $\mu = (\ln OD600t_2 - \ln OD600t_1) / (t_2 - t_1)$ according to Widdel (2007). Briefly, the OD600 values were ln-transformed and the transformed OD600 at time zero [i.e., ln ($OD600_{t0}$)] was subtracted. The three points that fitted best into the log phase of the culture were chosen and plotted. For our growth conditions, the selected points were: 7, 14 and 17 h. A linear function was calculated using those points, and its slope corresponds to the growth rate.

2.5. *Role of the high affinity ammonium transporters in atmospheric NH_3 scavenging*

Using the same experimental apparatus described for the bacterial strains (Fig. S2), we linked the effect of the atmospheric NH_3 gradient on the growth of the four *S. cerevisiae* strains and on their $\delta^{15}N$, to test the role of the AMT/MEP/Rh proteins in atmospheric NH_3 scavenging. The yeast strains were also grown in bipartite Petri dishes (Fig. S2). Two milliliters of a 10 mM $(NH_4)_2SO_4$ solution pH 9.5 were placed in the source compartment, thus increasing the atmospheric [NH_3]. The yeast strains were inoculated in the sink compartment. Before inoculation, the yeast cells were washed to remove all N sources from the growth medium (the same procedure as described for the bacterial cells). When the strains reached an OD600 of 1, and after washing the cells, 125 μL of yeast inoculum and 12.5 mL of liquid N free medium (NFB). Yeast strains were grown at 28 °C with a 120 RPM agitation (Orbital shaker, Optic ivymen system, Comecta), and its growth (i.e., OD600) was assessed 21, 42, 69 and 90 h after inoculation. At the end of the incubation, the yeast cells were washed as previously described, and the pellets were frozen at −80 °C. Finally, the frozen yeast pellets were freeze-dried, and their $\delta^{15}N$ was determined as previously described for the plant material.

2.6. *Statistics*

The effect of inoculating the *Azospirillum* strains (*A. baldaniorum* Sp245 and *A. brasilense* ARG2) on wheat seedling’s biomass, N content, and $\delta^{15}N$ was tested separately using a one-way analysis of variance with treatment as fixed factor. The effect of increasing the atmospheric [NH_3] (due to increasing the $(NH_4)_2SO_4$ solution’s pH and the controls) on the growth rate of the two *Azospirillum* strains was tested using a two-way analysis of variance with strain and pH as fixed factors. The role of the high affinity ammonium transport family proteins in *S. cerevisiae* strains’ (WT, MEP1, MEP2 and ΔMEP) capacity to grown by scavenging atmospheric NH_3 was tested using a two-way analysis of variance with yeast strain and incubation time as fixed factors. The effect of the presence of the high affinity ammonium transport family proteins in *S. cerevisiae* strains’ (WT, MEP1, MEP2 and ΔMEP) $\delta^{15}N$ was tested using a one-way analysis of variance with strain as fixed factor. Bonferroni post hoc multiple comparisons were used to test for differences ($p < 0.05$). Furthermore, differences between $\delta^{15}N$ values of the N source [i.e., $(NH_4)_2SO_4$] and those determined in the plant material and the yeast cells were analysed by the t-student test ($p < 0.01$). In all cases, preliminary analyses were performed to ensure that there was no violation of the assumptions regarding the tests’ application. SPSS software (version 29.0, IBM, Inc., Chicago, IL, USA) was used for all the analyses.

3. Results

3.1. *Phylogenetic position of the Azospirillum strains*

Amplification of the 16S rRNA gene of the strain ARG2, and an ML analysis generated from 17 nucleotide sequences (including *A. baldaniorum* Sp245), with a total of 1446 positions in the final dataset

(Fig. S3), confirmed the clustering of the strain ARG2 with *A. brasilense* DSM 1690, the type strain of the species.

3.2. *Effect of diazotrophs' atmospheric NH_3 scavenging on plant N budget*

Although the bacterial inoculation had no effect on wheat seedlings' biomass (Fig. 1-a), both *A. baldaniorum* Sp245 and *A. brasilense* ARG2 influenced wheat seedlings' N budget (Fig. 1-b) and their $\delta^{15}N$ signature (Fig. 1-c). Despite the scarce inorganic N in the nutrient solution, inoculation with the *Azospirillum* strains Sp245 and ARG2 improved seedlings' N budget (i.e., seedlings' N content) by 65 and 94 %, respectively. The $\delta^{15}N$ signature of the N sources were the following: i) $\delta^{15}N$ of the $(NH_4)_2SO_4$ solution was −1.0 ± 0.2 ‰; and ii) $\delta^{15}N$ of N_2 is ~0 ‰ (Unkovich, 2013). Wheat seedlings' $\delta^{15}N$ values differed between inoculated seedlings and the control (i.e., −10 ‰ for inoculated seedlings and +3 ‰ for control seedlings), and all differed from the N sources. However, inoculated seedlings' $\delta^{15}N$ values differed much more from the N sources than control ones, showing a great depletion in ^{15}N.

3.3. *Azospirillum strains can scavenge atmospheric NH_3*

The capacity of the *Azospirillum* strains Sp245 and ARG2 to scavenge atmospheric NH_3 (i.e., to grow) was influenced by the pH of the $(NH_4)_2SO_4$ solution (and controls), by the strain and by the interaction between pH and strain (Fig. 2). While the growth rate of both *Azospirillum* strains was very low (~0.01 ln cells h^{-1}) under low atmospheric [NH_3] (i.e., the controls consisting of NH_3 depletion and ambient atmospheric [NH_3]), it increased by up to 5-fold upon increasing the atmospheric [NH_3]. Both *Azospirillum* strains were able to scavenge the atmospheric NH_3 and use it as a N source. However, the *A. brasilense* ARG2 seemed to be more efficient in NH_3 scavenging since it reached higher growth rates than *A. baldaniorum* Sp245. Furthermore, *A. baldaniorum* Sp245 increased its growth rate up to pH 9.0, after which its growth rate dropped, while *A. brasilense* ARG2 increased its growth rate up to pH 9.5, after which it remained high.

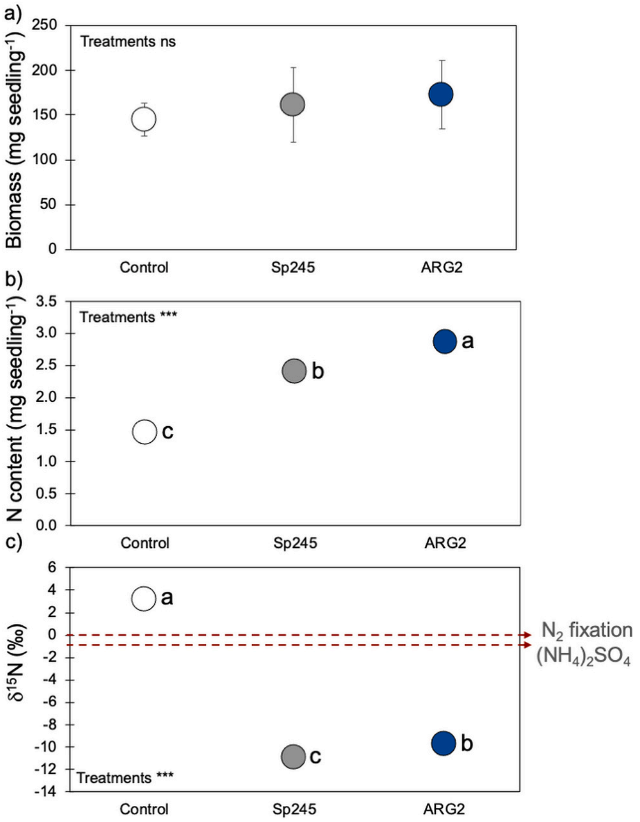

Fig. 1. Effect of inoculating the *Azospirillum* strains Sp245 and ARG2 on wheat seedlings' biomass (a), N content (b), and $\delta^{15}N$ (c). Significant effects are shown: ***0 % level ('ns' means no significant effect). Different letters show significant differences between treatments ($p < 0.05$). Symbols are the mean ± SD ($n = 4$ replicates).

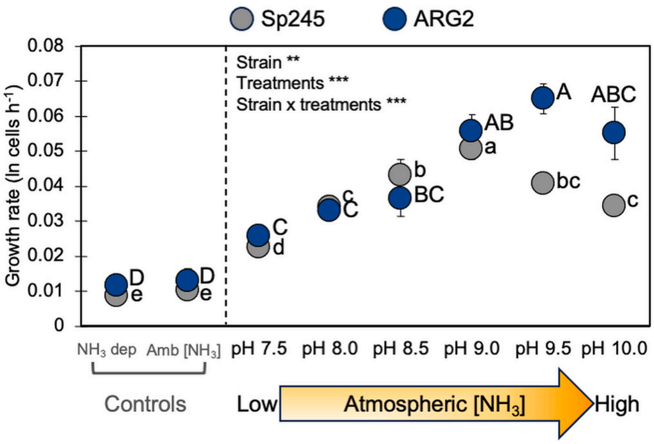

Fig. 2. Effect of increasing the ammonium solution's pH on the growth rate of the *Azospirillum* strains Sp245and ARG2. Increasing the ammonium solution's pH created an atmospheric NH_3 gradient (i.e., the higher the pH, the higher the atmospheric NH_3 concentration). Significant effects are shown: **1 % level; and ***0 % level. Since there was a significant interaction between strain and treatments, different letters show significant differences between treatments ($p < 0.05$) for each strain: lower-case letters for Sp245, and upper-case letters for ARG2. Symbols are the mean ± SD ($n = 3$ replicates).

3.4. *High affinity ammonium transporters are involved in microbial atmospheric NH_3 scavenging*

The capacity of the yeast (*S. cerevisiae*) strains to scavenge atmospheric NH_3 (i.e., to grow) was influenced by the strain, by the incubation time and by the interaction between strain and incubation time. While yeast cells with no Mep protein (i.e., ΔMEP) were unable to grow in NH_3 enriched atmospheres, the yeast mutants with copies of either *mep1* or *mep2* genes (i.e., MEP1 and MEP2) and the wild type (i.e., WT) grew in NH_3 enriched atmospheres (Fig. 3-a). Therefore, the presence of at least one Mep protein enabled atmospheric NH_3 scavenging by *S. cerevisiae*.

Since the ΔMEP did not grow in NH_3 enriched atmospheres, it was not possible to obtain enough biomass to analyse its $\delta^{15}N$ signature. By contrast, the other yeast strains with at least one Mep protein (i.e., WT, MEP1 and MEP2) produced enough biomass to measure their $\delta^{15}N$ signatures. There was no effect of the strains in yeast $\delta^{15}N$ signatures, which were all negative (between −9 and −12 ‰) and all differed from the only N source, which was the $(NH_4)_2SO_4$ solution ($\delta^{15}N = -1.0 \pm 0.2$ ‰) because yeasts cannot fix N_2.

4. Discussion

By growing (i) inoculated wheat seedlings in sterilized bags that allowed for gas exchanges, including of NH_3, and (ii) diazotroph strains in a gradient of increasing atmospheric [NH_3], we showed, for the first time, that NH_3 scavenging by the diazotrophic PGPR *A. baldaniorum* Sp245 and *A. brasilense* ARG2 can be an overlooked microbial trait in improving the host plant's N budget.

4.1. *Effect of diazotrophs' atmospheric NH_3 scavenging on plant N budget*

The wheat seedlings inoculated with both *Azospirillum* strains

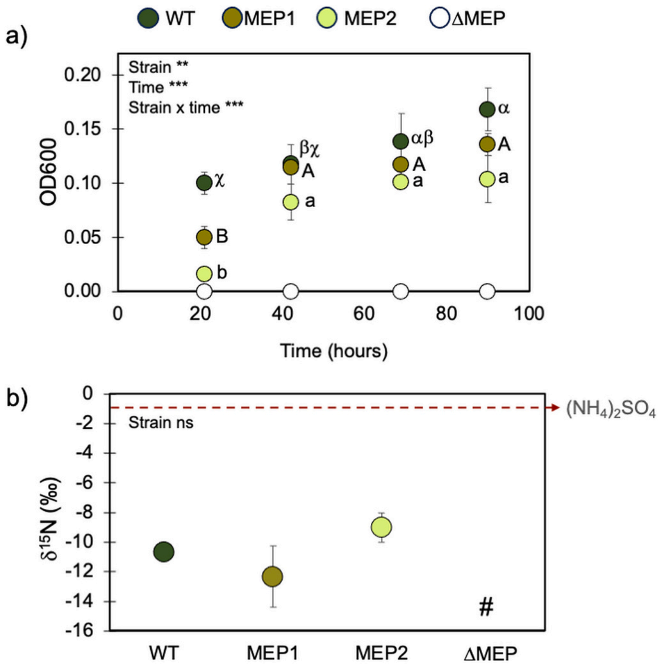

Fig. 3. Effect of the MEP transporters on yeast strains' growth (a) and δ^{15}N (b). # for ΔMEP means no data because the strain did not grow. Significant effects are shown: **1 % level; and ***0 % level ('ns' means no significant effect). Since there was a significant interaction between strain and time, different letters show significant differences ($p < 0.05$) between sampling points for each strain; Greek letters for WT, upper-case letters for MEP1 and lower-case letters for MEP2. Symbols are the mean ± SD ($n = 5$ replicates).

(*A. baldaniorum* Sp245 and *A. brasilense* ARG2) greatly improved their N budget (Fig. 1-b), thus confirming the benefits of the *Azospirillum*-wheat interaction, even in this early phase of the plant's life cycle. When considering the 65–94 % increase on plant's N budget due to *Azospirillum* inoculation in such a short-term experiment, N_2 fixation and N transfer to the host plant seems the most likely mechanism explaining our results. Furthermore, when accounting for all the N sources available for all wheat seedlings (i.e., control and inoculated), we have to consider: i) the inorganic N in the nutrient solution (i.e., 0.56 mg N seedling^{-1}); ii) the N stored in the seed [i.e., considering an average of 50 mg seed^{-1}, and a N concentration of 2 % (De Marco, 1990), each wheat seed represented 1 mg of N]; and iii) the atmospheric NH_3, which we did not measure, but can be taken up by plants (Fangmeier et al., 1994; Tian et al., 2022). The wheat seedlings inoculated with the *Azospirillum* strains had access to an additional N source, which is atmospheric N_2. Indeed, while control wheat seedlings contained approximately all the N they had available (c.a. 1.56 mg seedling^{-1}), the inoculated seedlings contained more N than that provided by the nutrient solution and the seed (>1.56 mg seedling^{-1}). Since the nitrogenase enzyme does not discriminate between N isotopes, the inoculated wheat seedlings' δ^{15}N values we obtained were incompatible with N_2 fixation (Fig. 1-c), thus excluding a major role of N_2 fixation in improving wheat seedlings' N budget. As the wheat seedlings were grown in sterilized bags that allowed for gas exchanges, atmospheric NH_3 needs to be considered as a significant N source. Wheat plants also express NH_3/NH_4^+ transporters from the AMT/MEP/Rh family (Porras-Murillo et al., 2023) that could scavenge NH_3 from the atmosphere and improve plant's N budget. However, Ariz et al. (2011) studied the relationship between the δ^{15}N of the N source and the ^{14}N/^{15}N fractionation in plants and observed that ^{15}N depletion of the plant (i.e., more negative δ^{15}N values) was associated with an increased level of NH_3 uptake at alkaline pH. Therefore, based on the δ^{15}N values of the control seedlings, atmospheric NH_3 does not seem to have been a significant N source in our experiment. Instead, *Azospirillum* expresses the high affinity AmtB transporter (Moure et al., 2019; Steenhoudt and Vanderleyden, 2000), and the *Azospirillum* strains we used were capable of scavenging atmospheric NH_3 (Fig. 2) and use it as a preferential N source when compared to N_2 fixation. Although we were unable to measure the δ^{15}N values of the *Azospirillum* strains growing in the bipartite Petri dishes (Fig. 2) due to very low biomass production, we selected two conditions that promoted the highest bacterial growth and pooled together the biomass obtained in 15 bipartite Petri dishes. The δ^{15}N values of the ARG2 strain growing with pH 8.0 and 9.5 were −36 ‰ and −26 ‰, respectively (data not shown), which together with the δ^{15}N values obtained with the yeast strains scavenging atmospheric NH_3 (Fig. 3-b) all showed a ^{15}N depletion similar to that observed in the inoculated wheat seedlings (Fig. 1-c). Altogether, our data suggest that atmospheric NH_3 scavenging results in ^{15}N depletion in different organisms, and supports that atmospheric NH_3 scavenging by the *A. baldaniorum* Sp245 and *A. brasilense* ARG2 was involved in improving wheat seedlings' N budget (Fig. 1-b). Microbial N scavenging, possibly of NH_3, and N transfer to the host plant, had been previously proposed in the model of oxidative N scavenging proposed by White et al. (2015).

Despite the very short duration of our experiment (i.e., 16 days), the wheat seedlings inoculated with both *Azospirillum* strains tended to accumulate more biomass (Fig. 1-a), which suggests that inoculation with this diazotroph PGPR can also improve wheat's production. Indeed, there are numerous studies showing that inoculation with *Azospirillum* [especially with *A. brasilense* which is tested most often (Cassán et al., 2020)] improves wheat biomass accumulation and grain production under different edapho-climatic conditions [e.g., (Díaz-Zorita and Fernández-Canigia, 2009; Galindo et al., 2022; Kazi et al., 2016; Reynders and Vlassak, 1982)]. Furthermore, Galindo et al. (2022) showed that reducing N fertilization from 100 to 50 kg N ha^{-1} together with *A. brasilense* inoculation resulted in an increase in operating profit of 10.5 %. In view of low economic cost, ease of application, and high probability of a positive response by wheat crops, the authors concluded that inoculation with *A. brasilense* is prone to be a key sustainable management practice to improve wheat production, at least under tropical conditions.

However, it is difficult to provide evidence of the importance of NH_3 scavenging by *Azospirillum* strains in wheat production, and least of all to quantify it. Although NH_3 scavenging seems to be associated with ^{15}N depletion, when plants are grown in the soil there are many N sources (each one with its δ^{15}N value) that need to be considered because plant and grain δ^{15}N values will integrate and reflect all the N sources that were available during wheat cultivation. Controlling the available N sources was the main reason why our experiment was conducted in hidropony. Growing wheat plants until grain production hydroponically and in a sterilized environment poses many challenges.

4.2. *Azospirillum baldaniorum* and *A. brasilense* can scavenge atmospheric NH_3

Diazotrophs such as *A. baldaniorum* Sp245 and *A. brasilense* ARG2 can fix N_2 and uptake other N sources, including NH_3/NH_4^+ (Reed et al., 2011), which is the direct product of N_2 fixation and inhibits nitrogenase synthesis at the genetic level through the regulation of *nifA* gene transcription (Dixon and Kahn, 2004). However, NH_3/NH_4^+ does not inhibit the activity of already synthesized nitrogenase in most organisms, and *A. brasilense* is one of a few diazotrophs that regulate nitrogenase post translationally (Smercina et al., 2019). Therefore, we cannot exclude the possibility that the *Azospirillum* strains used in our study were also fixing N_2. However, the 5-fold increase in their growth rates upon increased atmospheric [NH_3] (Fig. 2), together with the ^{15}N depletion observed when the ARG2 strain was grown with pH 8.0 and 9.5 (data not shown) clearly show that atmospheric NH_3 scavenging is an alternative process capable of supporting bacterial growth. N_2 fixation together with NH_4^+ assimilation and transport require 28 ATP molecules in total (Soumare et al., 2020). Such a high energy burden may explain why the bacterial growth rates were so low under low atmospheric [NH_3]. Since diazotrophs are adapted to very N limited environments, and N_2 fixation is very energy demanding (Soumare et al., 2020), it is only performed by these organisms when there is no other alternative way to acquire N. This means that free-living soil diazotrophs likely rely on soil N pools (including NH_3/NH_4^+) to satisfy their N demand, and only fix N_2 under certain conditions (Norman and Friesen, 2017). Therefore, as hypothesized, by increasing the atmospheric [NH_3], NH_3 scavenging by *A. baldaniorum* Sp245 and *A. brasilense* ARG2 prevailed over N_2 fixation. In agreement, bacterial capacity to scavenge NH_3 as a N source has been previously documented for example for marine bacterial strains growing in N free media (Jones and Rhodesroberts, 1980). Furthermore, rhizospheric *Pseudomonas* sp. strains growing in a N free medium with a $^{15}N_2$ enriched atmosphere were shown to incorporate atmospheric $^{15}NH_3$, but not $^{15}N_2$ (Hurek et al., 1988), thus showing that the bacterial strains were scavenging NH_3 and not fixing N_2.

The fact that both *Azospirillum* strains were able to scavenge the atmospheric NH_3 and use it as a N source shows that this phenomenon is not exclusive to a specific strain. However, the *A. brasilense* ARG2 seemed to be more efficient in NH_3 scavenging and more tolerant to alkaline conditions than the *A. baldaniorum* Sp245 since the former reached higher growth rates and for higher pH values than the latter (Fig. 2). Since the optimum pH for the *A. brasilense* C16 growth is 6.8 (Romero-Perdomo et al., 2015), the alkalinization of the growth medium we induced in our study probably prevented the bacterial strains from taking full advantage of the additional atmospheric NH_3. The fact that the growth rate of *A. brasilense* ARG2 was less affected by the very high pH values than *A. baldaniorum* Sp245 may be related with the former's higher efficiency in atmospheric NH_3 scavenging. Therefore, by scavenging more NH_3 from the atmosphere, *A. brasilense* ARG2 may have prevented the atmospheric NH_3 to deposit in its growth medium, thus slowing down its alkalinization and preventing the negative effects on its growth.

4.3. High affinity ammonium transporters are involved in microbial atmospheric NH_3 scavenging

The fact that the yeast cells with no Mep protein (i.e., ΔMEP) were unable to grow in NH_3 enriched atmospheres, while those with at least one Mep protein (i.e., WT, MEP1 and MEP2) did (Fig. 3-a) shows that besides scavenging NH_3/NH_4^+ from solutions [as previously shown with the same yeast strains growing on N free solid media supplemented with 3 mM of NH_3 (Boeckstaens et al., 2007)], the presence of at least one high affinity ammonium transport protein (i.e., one Mep protein) enables atmospheric NH_3 scavenging. Furthermore, our data extends the relation between ^{15}N impoverishment (Fig. 3-b) and NH_3 uptake through Mep proteins (Ariz et al., 2018) to the uptake of atmospheric NH_3.

Since we had no access to *Azospirillum* sp. mutants for AMT/MEP/Rh proteins, while we had *S. cerevisiae* mutants for those proteins (i.e., MEP1, MEP2 and MEP3), we consider that several reasons justify our use of the *S. cerevisiae* strains to test the role of the AMT/MEP/Rh proteins in atmospheric NH_3 scavenging, namely: i) the high affinity ammonium transport family proteins are expressed in both *S. cerevisiae* (Ariz et al., 2018) and *Azospirillum* strains (Moure et al., 2019; Steenhoudt and Vanderleyden, 2000); ii) AMT/MEP/Rh proteins have the same function in bacteria and yeasts, which is to uptake NH_3/NH_4^+ into the cells; and iii) the *S. cerevisiae* and the bacterial strains used here are the same used by Ariz et al. (Ariz et al., 2018), where the authors show that both bacteria and yeast show an ^{15}N impoverishment when the main source of N acquisition is NH_3/NH_4^+ transported through AMT/MEP/Rh family transporters. In line with this latter argument, it is important to highlight that *Azospirillum*'s slow growth makes it very difficult to produce enough bacterial biomass to measure $\delta^{15}N$. For example, it was necessary to pool together the biomass of 15 bipartite Petri dishes of the strain ARG2 growing with pH values that enabled high growth rates (i.e., pH 8.0 and 9.5) to have enough material for one $\delta^{15}N$ determination. Therefore, our use of a non-diazotrophic microbe (i.e., *S. cerevisiae*) with isogenic mutants for the high affinity ammonium transport family proteins, provided a proof of concept that: i) atmospheric NH_3 can be used as a N source capable of sustaining microbial growth; and ii) $\delta^{15}N$ can be used to signal microbial NH_3 scavenging.

5. Conclusions

The 65–94 % improvement in wheat seedlings' N budget due to *Azospirillum* (*A. baldaniorum* Sp245 and *A. brasilense* ARG2) inoculation could not be explained by N_2 fixation due to incompatibilities with plant $\delta^{15}N$ values. Instead, both *A. baldaniorum* Sp245 and *A. brasilense* ARG2 were able to scavenge atmospheric NH_3 and use it as a N source, and the involvement of AMT/MEP/Rh proteins in NH_3 scavenging was compatible with the ^{15}N depletion we observed in wheat seedlings. Altogether, our data show that under increasing atmospheric [NH_3], NH_3 scavenging by the *Azospirillum* strains Sp245 and ARG2 prevailed over N_2 fixation, and that the scavenged NH_3 was transferred to wheat seedlings, improving their N budget.

The ongoing intensification of agriculture (Martins-Loução et al., 2022; Sutton et al., 2011; Yu et al., 2024) has been increasing atmospheric [NH_3] (Sutton et al., 2011), further aggravating agriculture's ecological footprint and human health problems (Gu et al., 2021). Since atmospheric NH_3 scavenging can reduce the atmospheric [NH_3], this microbial trait can contribute to decrease greenhouse gases emissions and improve air quality. This is especially relevant because: i) reducing atmospheric [NH_3] was highlighted to be a priority for mitigating $PM_{2.5}$ air pollution (Gu et al., 2021); and ii) besides scavenging the scarce NH_3/NH_4^+, AMT/MEP/Rh transporters have been suggested to be responsible for at least a part of cell N supply under abundant NH_3/NH_4^+ in yeast cells (Ariz et al., 2018), which may also occur in other organisms coding for AMT/MEP/Rh transporters, such as plants and bacteria. Although our observations provide evidence for the use of microbial inoculants as relevant tools to mitigate agriculture's footprint, this trait needs to be further studied and confirmed under field conditions.

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CRediT authorship contribution statement

Teresa Dias: Writing – original draft, Validation, Project administration, Investigation, Conceptualization. **Kamran Azmaliyev:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Juliana Melo:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Ana Margarida Santos:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Patrícia Correia:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Cristina Cruz:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105737.

Data availability

Data will be made available on request.

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