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Research

Methods

An optimized hydroponic pipeline for large-scale identification of wheat genotypes with resilient biological nitrification inhibition activity

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Summary

- Several plant species have been reported to inhibit nitrification via their root exudates, the so-called biological nitrification inhibition (BNI). Given the potential of BNI-producing plants to sustainably mitigate N losses in agrosystems, identification of BNI activity in existing germplasms is of paramount importance.
- A hydroponic system was combined with an optimized *Nitrosomonas europaea*-based bioassay to determine the BNI activity of root exudates. The pipeline allows collecting and processing hundreds of root exudates simultaneously. An additional assay was established to assess the potential bactericide effect of the root exudates.
- The pipeline was used to unravel the impact of developmental stage, temperature and osmotic stress on the BNI trait in selected wheat genotypes. Biological nitrification inhibition activity appeared consistently higher in wheat at the pretillering stage as compared to the tillering stage. While low-temperatures did not alter BNI activities in root exudates, osmotic stress appeared to change the BNI activity in a genotype-dependent manner. Further analysis of *Nitrosomonas* culture after pre-exposure to root exudates suggested that BNI activity has no or limited bactericide effects.
- The present pipeline will be instrumental to further investigating the dynamics of BNI activity and to uncover the diversity of the BNI trait in plant species.

Introduction

Nitrogen (N) is the most limiting nutrient for plant growth. While N is abundant in the atmosphere, it is present in the form of inert dinitrogen which requires to be converted into reactive nitrogen (Nr) to become biologically active. The artificial N fixation from the atmosphere with the Haber–Bosch reaction has revolutionized agriculture by providing high levels of Nr for fertilization, leading to increased crop yields and food security world-wide. However, Nr is a pollutant: Volatilization of gases such as ammonia or nitrous oxide (N_2O) or the leaching of N-anions such as nitrate (NO_3^-) cause serious environmental disturbances including climate change and water pollution. A cost–benefit analysis in Europe has estimated that the annual costs related to Nr pollution surpasse the benefits of Nr fertilization (Van Grinsven *et al.*, 2013). Improving nitrogen use efficiency (NUE) of crop plants is an effective approach to reduce N losses from agrosystems. Because of the limited NUE of major global crops such as wheat (Hawkesford & Riche, 2020), it is essential to unlock novel plant traits with potential for reduction in N losses.

One strategy to boost crop NUE is to maintain N in the soil for longer periods. When adequately integrated into the soil, ammonium (NH_4^+), which binds to clay and soil organic matter, is for example less prone to volatilization or lixiviation. However, NH_4^+ pools in the soil can be rapidly diminished by nitrification. Nitrification is the biological process by which nitrifying communities convert NH_4^+ into its oxidized forms, with NO_3^- or N_2O as end products. In nonacidic agricultural soils, nitrification is

mainly driven by ammonia-oxidizing bacteria (AOB; Jia & Conrad, 2009). The field application of synthetic nitrification inhibitors (NI), compounds that transiently block nitrification, has emerged as an efficient strategy to mitigate N losses (Beeckman *et al.*, 2018; Maaz *et al.*, 2021) and to improve at the same time crop NUE (Abalos *et al.*, 2014). Unfortunately, synthetic NI have several limitations: These compounds cannot be used in organic farming; their high costs limit their use in low-input farming systems; their efficiency depends on soil properties and climatic conditions, and thus, multiple applications are often required to ensure a prolonged mitigation of nitrification during the entire crop cycle (Coskun *et al.*, 2017a). Moreover, the use of synthetic NI has been shown to disturb nontargeted soil microbial populations (Corrochano-Monsalve *et al.*, 2021).

Plants interfere with the geological N cycle through different mechanisms, including N absorption, symbiotic N fixation and recruitment of beneficial biota (Hestrin *et al.*, 2019). Interestingly, certain plant species produce compounds limiting nitrification in the soil, a trait known as biological nitrification inhibition (BNI; Subbarao *et al.*, 2009, 2012; Coskun *et al.*, 2017a). The concept of plants limiting nitrification was identified in ecological studies in the 1960s (Munro, 1966), but the study of BNI potential in a diversity of plant species has only emerged in the last decade. The BNI compounds have been first found in plant tissues and root exudates of certain genotypes of the tropical grass brachiaria (Subbarao *et al.*, 2009) and sorghum (Subbarao *et al.*, 2012). Biological nitrification inhibition activities have also been recently characterized in rice (Sun *et al.*, 2016), wheat (O'Sullivan *et al.*, 2016) and maize (Otaka *et al.*, 2021) with some studies indicating intraspecies variation of the BNI trait (Subbarao *et al.*, 2009, 2012; O'Sullivan *et al.*, 2016; Sun *et al.*, 2016). Biological nitrification inhibition can have inhibition activities comparable to synthetic inhibitors and they can contribute to the reduction in N_2O emissions (Coskun *et al.*, 2017b; Yao *et al.*, 2020). The characterization of BNI activities is challenged by several methodological limitations: Microbiological methods to measure BNI are constrained by the difficulty to cultivate several AOB (Meiklejohn, 1950; Hesselsøe & Sørensen, 1999); the contents of free NH_4^+/NO_3 are often masked by plant absorption activities in soil assays so that direct correlation between soil N content and nitrification rate with a plant in the system are challenging to address (Drury *et al.*, 2008); the expression of monooxygenase (*amo*) gene cannot be directly used as a proxy for nitrification activity because gene expression does not necessarily correlate with protein activity; and methods based on isotope discrimination to quantify nitrification rate are accurate but costly and complex to interpret (Granger & Wankel, 2016). Given the aforementioned limitations, *Nitrosomonas*-based bioassays using wild-type or modified strains (Subbarao *et al.*, 2009; Kaur-Bhambra *et al.*, 2022) have become the norm to measure nitrification inhibition. Noticeably, *Nitrosomonas*-based bioassays are only measuring the conversion rate of NH_4^+, and therefore, mechanisms by which nitrification is inhibited are not characterized. Because several synthetic NI have been demonstrated to shape the composition of bacterial communities (Sarr *et al.*, 2020; Wang *et al.*, 2021), there is a need to better understand the modes of action of BNI compounds.

Enhancing screening capacity of crop species with BNI activities could unlock the potential of the BNI trait by facilitating the identification of parental lines and the introgression of the BNI trait in farmer-preferred varieties (Subbarao *et al.*, 2021). Here, we present a unique simple-to-implement pipeline that combines a large and fully randomized hydroponic system for homogenous wheat growth and a high-throughput pipeline for collection of root exudates. The BNI analysis pipeline includes an adapted two-step bioassay enabling the quantification of nitrification inhibition and a characterization of the root exudate effects on *Nitrosomonas europaea*. We demonstrate the reliability of the system to screen for improved BNI capacity in a large number of samples from plants at different developmental stages and exposed to selected environmental conditions.

Materials and Methods

Plant material

Wheat genotypes (Supporting Information Table S1) with known BNI capacity (O'Sullivan *et al.*, 2016) were obtained from the Australian Grains GeneBank (AGG, Horsham, Victoria, Australia) and from the Germplasm Resources Unit Seedstor (UK).

Plant growth conditions

Six seeds per cultivar were sown in 10 × 10 × 10 cm pots filled with calibrated pure quartz sand (N°5; Euroquartz, Hermallesous-Argenteau, Belgium). Water was added at its maximum water-holding capacity. Pots were placed into a dark cold room (4°C) for 2 d. After seed stratification, pots were transferred to a growth chamber where plants were grown for 5–6 d with supplemental lighting (150 µmol m^{-2} s^{-1} photosynthetic active radiation; PAR, 22°C, 16 h : 8 h photoperiod) and low vapour pressure deficit (< 1.5 kPa). When plants homogeneously reached Zadok's stage (Z) Z11, they were moved to the hydroponic growing system.

The hydroponic growing system was established in a glasshouse in Louvain-la-Neuve, Belgium (50°39′57″N, 4°37′1″E). Plants received supplementary lighting (Lumigrow pro 325 W; Lumigrow, Emeryville, CA, USA) when the natural light intensity was lower than 300 µmol m^{-2} s^{-1} PAR. Temperatures were maintained at 22°C : 16°C, day : night with heating and ventilation systems.

Wheat plants were grown in a hydroponic tank filled with a modified Hoagland solution (Table S2) for 16 d (Z15-23) and in two independent experiments, during May 2020 and June 2021. For temperature treatment, the roots were incubated at 8°C during both the NH_4^+ shock treatment and the exudate collection step using a water bath (D77656; Huber, Berching, Germany). For drought stress, roots from plants at stage Z1.4 were placed in dialysis bags (MCO3500 Spectrapor RC membrane; Repligen, Compton, CA, USA) and were grown in a modified Hoagland solution (Table S2) supplemented with PEG6000 (3%) for 5 d in a hydroponic tank. After exposure to PEG

solution, the wheat plants were transferred from the dialysis bags and placed back in the hydroponic tank for a 4 d recovery phase before root exudate collection.

High-throughput hydroponic platform

Hydroponic solution A modified Hoagland solution (Table S2) with N in the form of ammonium nitrate (NH_4NO_3) was used as hydroponic nutritive solution. The nutrient solution was adjusted to pH 7 and buffered with 0.5 mM calcium carbonate ($CaCO_3$). The nutrient solution was replaced every week.

Set-up of the hydroponic system Before transferring the plantlets into the plant holder, roots were carefully cleaned by removing the sand with distilled water. Then, using a long-arm tweezer, each plantlet was placed into a 1.5 ml Eppendorf tube whose bottom part was cut diagonally (Fig. S1). The Eppendorf tube worked as a plant holder to ensure proper stabilization of the plant in the trays, adequate submersion of the root into the nutrient solution and labelling of the plantlet on the tube cap.

Hydroponic system The 120 × 120 × 30 cm tank (Hydrosystem, Quebec, Canada) used for the hydroponic growth had a capacity of 378 l (Fig. 1a,b). The tank was filled with 350 l of hydroponic solution and covered on the sides with aluminium foil to reflect the light and prevent the overheating of the hydroponic solution. The lid of the hydroponic tank consisted of four growing trays and four border trays (Fig. 1a,b). A total of 121 holes with a diameter of 0.5 cm were perforated in each growing tray (Fig. S2A). We kept an empty space of 6-hole space at each corner of the growing trays to avoid interference with the aeration system. Border trays were used to fully cover the open sides of the container. The height of the hydroponic tank (30 cm) helped preventing the roots from reaching the bottom of the tank as they were maintained until stage Z3.1 in the hydroponic tank. The space provided to each plant in the hydroponic system also ensured minimal entanglement between roots.

The aeration was provided by a pump connected to four air diffuser stones of 15 cm long (ProSilent Aeras Micro M; JBL, Neuhofen, Germany) placed at the corners of the tank (Fig. S2A), and one air 10 cm diameter diffuser ball (Air 100 Indoor; Ubbink, Doesburg, The Netherlands) placed in the middle of the tank. Four indoor fountain pumps of 370 l h^{-1} flow (Eli-Indoor 350 I; Ubbink) were fixed at the corners of the tank to allow homogenization of the hydroponic solution.

The dissolved oxygen content was verified twice a week by using an Oximeter (Hanna HI98193, Hanna Instruments, Lingo Tanneries, France). Protocols adapted from Griess (1985) and Patton & Crouch (1977) were followed to ensure that the hydroponic solution was not depleted in NH_4^+ and NO_3^- after solution replenishment. The volume of the hydroponic solution was also constantly monitored to avoid change in electric conductivity. The plants were randomized in the hydroponic tank by changing the position and direction of the growing trays twice a week, which prevents the risk of having entangled roots, a situation that could create root injuries during the subsequent steps.

Exudate collection system

Conditions and solutions for the root exudate collection The collection of wheat root exudates was initiated 3 h after dawn in the same glasshouse where the hydroponic system was placed. In order to minimize the potential effect of the plant circadian clock on the root exudate profile, 120 plants were placed into the root collection system in a period of 90 min. For the root exudate collection, plants were moved to a multistep pipeline (Fig. S2B; Video S1).

Before root exudate collection, wheat roots were immersed for 1 h in a NH_4^+ (1 mM N solution with NH_4Cl and adjusted to pH 7). The so-called NH_4^+ shock treatment helps priming

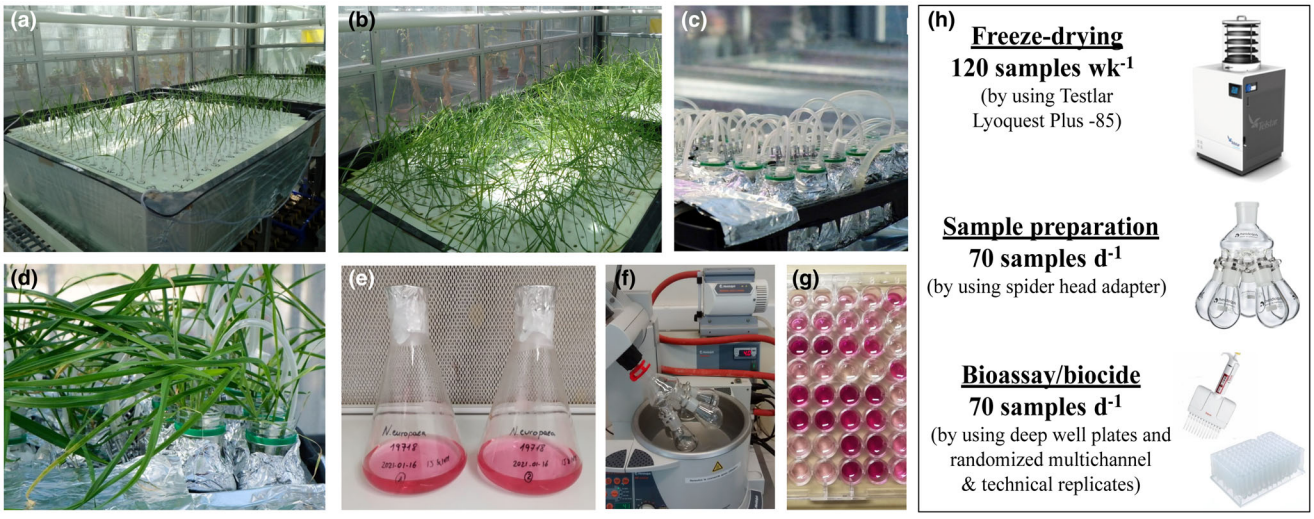

Fig. 1 Overview of the pipeline for identification of biological nitrification inhibition (BNI) capacity in wheat roots. (a, b) Wheat plants growing in the large hydroponic tanks under glasshouse conditions; (c, d) high-throughput root exudate collection system; (e) pure anoxic culture of the wild-type *Nitrosomonas europaea* 19718 in medium 829; (f) concentration process of root exudates with the rotary evaporator; (g) microplate bioassay after Griess staining; (h) timeline for sample processing and analysis.

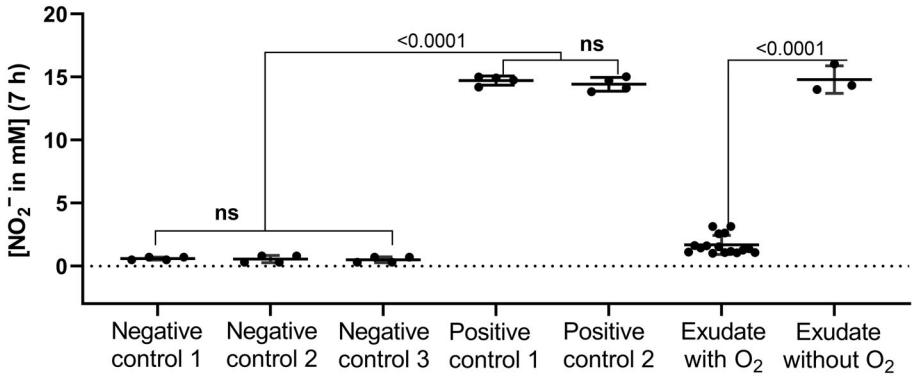

Fig. 2 Nitrite production in the Nitrosomonas-based bioassay showing results for the negative and positive controls as well as for root exudates from wheat line Persia 6 (with and without oxygenation during exudate collection). Negative control 1, absence of NH_4^+ in the bioassay; Negative control 2, presence of synthetic nitrification inhibitor in the bioassay (AT: allylthiourea); Negative control 3, absence of *Nitrosomonas* cells in the bioassay; Positive control 1, HEPES medium; Positive control 2, mock methanol control. Statistics: Welch's ANOVA test and false discovery rate of two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (For bioassay and sample preparation $n = 4$, for Persia 6, $n = 17$, for Persia 6 – O_2 $n = 3$). ns stands for 'nonsignificant'. Error bars represent Standard Deviations (SD).

exudation of BNI compounds as demonstrated in other studies (Subbarao *et al.*, 2007; O'Sullivan *et al.*, 2016). Following the NH_4^+ shock treatment, the root exudates were collected for 22 h. The solutions used for plant growth, priming of exudation and exudate collection (Fig. S2B) were all set at pH 7 in order to avoid any effect triggered by change in pH (Egenolf *et al.*, 2021).

For high-throughput collection of root exudates, batches of 20 plants were moved through the following steps (Fig. 2B):

Step 1: Root cleaning in double distiller water (pH 7, 15 min), equipped with an aeration system;

Step 2: NH_4^+ shock treatment (1 mM N with NH_4Cl, pH 7, 60 min), equipped with an aeration system;

Step 3: Root cleaning in double distiller water (pH 7, 15 min), equipped with an aeration system;

Step 4: Exudate collection in double distilled water (pH 7, 22 h), equipped with an aeration system.

The whole root exudate collection pipeline (Figs 1c,d, S2B) is therefore composed of one tank with the NH_4^+ shock solution (350 l; Hydrosystem) (for step 2), two containers of double distilled water (30 l container; for step 1 and 3) and the exudate collection station (for step 4).

Root exudate collection system The exudate collection station was composed of plastic bottles (polyethylene terephthalate, 250 ml, 38 mm aperture diameter; Mata Pet, Juvasa, Spain) filled with 120 ml of double distilled water (pH 7) after being cleaned with SDS 1%. The plastic bottles were placed into a deep root tray (Nutley, UK) to keep the bottles straight and in dark (see Fig. 1c,d). The aeration in the plastic bottles was provided by a tubing system consisting of a 200 µl tip connected to an air pump using a pipe (diameter 6 mm) placed inside a 15 ml Falcon tube whose bottom part was cut with an angle of 45° (Fig. S1B). This system avoids damaging the root system while maintaining an adequate aeration and homogenization of the trap solution. The system was multiplexed by using 5-headed air manifold diffusers and Y irrigation connectors to reach 121 outlets. The airflow was monitored in each branch with a flow meter (TMR 1 – 011111; Aalborg, Orangeburg, SC, USA). All the used materials were cleaned with SDS 1% and rinsed with double distilled water to avoid degradation of the released compounds. Each plant was individually placed in the plastic bottles (one plant per bottle) with the help of a long-arm tweezer.

After 22 h of exudate collection, the plants were removed from the bottles and the volume of exudate solution was measured with a graduated cylinder before storage at −20°C.

Root exudate sample preparation Root exudates collected in double distilled water were filtered with Acrodisc® Syringe Filters (0.2 µm Supor® Membrane) and further processed in three subsequent concentration extraction steps. A subsample (14 ml) of the collected volume was transferred after filtration to a Pirex glass tube (60 ml, 25 cm long) and frozen in a − 20°C. freezer. The frozen sample was then freeze-dried using a classical freeze dryer (Lyoquest Plus −85 (Testlar, Bensalem, PA, USA)). The described procedure allows lyophilization of 120 samples in 3 working days.

After this step, the obtained dry pellet was resuspended by sonication in 4 ml methanol (34860; Sigma-Aldrich). The methanol resuspension was then evaporated by rotatory evaporation (Fig. 1f) at 35°C and six atmospheres of pressure using a 20 ml flask and a multiplexed spider head (Heidolph Hei-VAP series, Schwabach, Germany). This set-up allows processing batches of five samples at the same time. The obtained residues were subsequently dissolved in 1 ml methanol (34860; Sigma-Aldrich) by sonication and vortexing before storage at −20°C in 1.5 ml conic Eppendorf tubes.

For the *N. europaea* (Winogradsky 1892) inhibition bioassay, the residues dissolved in 1 ml methanol were evaporated using speed vacuum at 30°C and under six atmospheres of pressure. After drying, the samples were dissolved in 2 µl DMSO, and then, 108 µl of HEPES medium (Table S3) was added for a total volume of 110 µl. Full dissolution was obtained after sonication during 10 s and vortexing. Two technical replicates of 50 µl were used for the *N. europaea* inhibition bioassay.

Nitrosomonas europaea inhibition bioassay

***Nitrosomonas europaea* cultivation** The bioassay was established with a wild-type *N. europaea* 19718 strain (ATCC 2021, URL: https://www.atcc.org/products/19718). In our conditions, the Medium 829 (Table S3; Fig. 1e) produced higher cell yield than the ATCC medium 2265 (ATCC 2021, URL: https://www.atcc.org/~/media/a6e62158e40a4799b31d4a5bc81c2323.ashx). *Nitrosomonas europaea* cultures were initiated from a 10% glycerol-concentrated stock. First, a preculture was prepared in 20 ml Medium 829 in a 120 ml Erlenmeyer covered with aluminium foil and shaken during 4 d at 28°C at 180 revolutions per minute (rpm; Eppendorf® New Brunswick™ Innova® 42 Incubator Shaker). During this period, the preculture was maintained at pH 7.8–8.0 by adding sterile 10% $NaHCO_3$ when necessary to avoid the acidification of the medium. After the 4-d incubation, the culture was divided into two 1-l Erlenmeyer flasks containing 200 ml of Medium 829 and cultured for another 7–9 d to an OD_{600} of 0.15. In order to verify the purity of the *N. europaea* cultures, the 7–9-d cultures were plated on tryptic soy broth agar (30 g Tryptic Soy Broth and 15 g agar l^{-1}).

To prepare the *N. europaea* cells for the bioassay, 50 ml of culture was transferred to sterile 50 ml Falcon tubes and centrifuged for 10 min at 6950 ***g***. After that, the pellet was washed three times with N-free HEPES medium and pelleted again by centrifugation. The final pellet was resuspended in N-free HEPES medium to a final OD_{600} of 0.3.

***Nitrosomonas europaea* inhibition bioassay** The quantitative inhibition of the nitrifying activity *of N. europaea* was assessed by measuring $[NO_2^-]$, the end product of ammonia oxidation. The assay was performed for a maximum period of 7 h to avoid *N. europaea* cell division during the assay (Cantera & Stein, 2007), and samples were collected following a time-course sampling at 0, 3, 5 and 7 h after adding the root exudate samples into the *N. europaea* solution.

The bioassay was performed in a 96-deep well plate (BR701350; Brand, Wertheim, Germany), with a total starting volume of 300 µl (3.78 mM $(NH_4^+)_2SO_4$), consisting of the following components added in the indicated order:

200 µl HEPES medium,

50 µl of the root exudate sample

50 µl *N. europaea* cells resuspended in N-free HEPES medium.

The *N. europaea* inhibition bioassay contained the following internal controls:

(**1**) Positive control 1: control HEPES medium: 50 µl of *N. europaea* cells added to 250 µl HEPES medium with DMSO in the same concentration as for the root exudate preparation.

(**2**) Positive control 2: a mock methanol control consisting of methanol (4 ml) subjected to rotatory evaporation step followed by addition of 50 µl of *N. europaea* cells and 250 µl HEPES medium with DMSO in the same concentration as for the root exudate preparation.

(**3**) Negative control 1: bioassay without NH_4^+: 50 µl of *N. europaea* cells added to 250 µl N-free HEPES medium.

(**4**) Negative control 2: bioassay with synthetic inhibitor allylthiourea (AT, 108804; Sigma-Aldrich) at final concentration of 0.27 µM in a solution containing: 50 µl of *N. europaea* cells added to 250 µl HEPES medium with DMSO in the same concentration as for the root exudate preparation.

(**5**) Negative control 3: bioassay without *N. europaea* cells: 300 µl HEPES medium DMSO in the same concentration as for the root exudate preparation.

For every sample (exudate samples and controls), two technical replicates of 50 µl were analysed in the bioassay, each one in a separate 96-well plate. Plates were covered with lids (732-5560; Corning, Glendale, AZ, USA) and parafilm, and placed into the incubator at 28°C and 180 rpm.

A multichannel pipette was used to dispense the HEPES medium and the *N. europaea* cells. Subsamples of 30 µl were collected from each well at 0, 3, 5 and 7 h for quantifying $[NO_2^-]$. The subsamples were then transferred to a 96-well plate in which 2 µl of 2 mM AT solution had been previously added per well in order to stop the nitrification reaction of the collected subsample. The 96-well plates were covered with aluminium foil and, if not directly quantified, stored overnight at −20°C. NO_2^- production in the bioassay was determined by the Griess method (1985) using a microplate reader (Spark 10 K; Tecan, Männedorf, Switzerland). A standard curve using $NaNO_2$ (563218; Sigma-Aldrich) was established with a 0–12 mg NO_2^- l^{-1} range.

To test the long-term linearity rate of NO_2^- production by *N. europaea*, a 24-h incubation assay was carried out with HEPES medium, where $[NO_2^-]$ was determined at 0, 3, 5, 7, 20, 22 and 24 h (Fig. S3).

Root exudate bactericide assay The NO_2^- production capacity of *N. europaea* cells initially pre-incubated with the root exudates was used to assess the bactericide effect of root exudates on *N. europaea*. The root exudate bactericide assay was performed in 1.5 ml Eppendorf tubes using the following steps:

(**1**) Pre-incubation of the *N. europaea* cells with the dissolved root exudates. For this incubation step, 1.5 ml Eppendorf tubes were used to enable easy pelleting of the bacterial cells. SpeedVac-evaporated root exudates were resuspended in 4.6 µl of DMSO (applying sonication (10 s) and vortexing) and then added to 245.4 µl of N-free HEPES medium and 50 µl of *N. europaea* cells in N-free HEPES medium (previously adjusted to OD 0.3). The Eppendorf tubes containing these components (total volume of 300 µl) were shaken at 180 rpm for 3 h at 28°C. This is the pre-incubation step.

(**2**) After pre-incubation, the bacterial cells were recovered by centrifugation using a vertical rotor (centrifuge 2-16Pk rotor 11124; Sigma), at 2710 ***g*** for 10 min. The supernatant, containing the HEPES medium, and the dissolved exudates were carefully removed by pipetting. The bacterial cells were subsequently washed with 1 ml N-free HEPES medium and recovered by pelleting.

(**3**) The bacterial cells were resuspended in 250 µl HEPES medium and 50 µl N-free HEPES (final concentration of

$(NH_4)_2SO_4$ was then 3.78 mM). The Eppendorf tubes were placed into the incubator shaker at 28°C and 180 rpm for 4 h. They were sampled (30 µl) after 0, 1, 2 and 4 h. This is the incubation step.

The following controls were used:

(**1**) Different ratios of alive – dead *N. europaea* cells (100–0%; 75–25%; 50–50%; 0–100%) in the incubation step. Dead bacteria were obtained after treatment with 20 s high-power microwaving and 30 s of sonication.

(**2**) AT: 2.7 µM AT final concentration in the incubation step.

NO_2^- production was determined by the Griess (1985) protocol. In contrast to the *N. europaea* bioassay, we used linear regression to compute the inhibition percentage.

Peat-based experiment

Wheat genotypes of contrasting BNI activity were also grown in peat-based substrate to determine their potential to impact nitrification rate in their rhizosphere. For this, five different wheat genotypes (LG Skyscraper, Persia 6, Persia 6B, Persia 3 and Persia 44) were grown in a peat-based substrate (Agrofino Potground Terrau, Zaaien Semer, Belgium; substrate composed for blond peat, black peat, sand, perlite, fertilizer, NPK 14-16-18 0.6 kg m^{-3}, pH 5.8) in a growth chamber (at 60% WHC and 22°C : 18°C, day : night conditions) and the rhizospheric substrate was collected 22 d after sowing. Net nitrification rate was determined following modified protocols by Hart *et al.* (1994). For this, 1 g of homogenized rhizospheric substrate was incubated for 7 d (60% WHC; 22°C : 18°C, day : night) after addition of ammonium sulphate at a dose of 100 mg $N\text{-}NH_4^+$ kg^{-1} peat substrate. Mineral N was extracted (KCl 2 M, 1 : 3, w/v) at incubation start (t0) and 7 d after (t7) and nitrate content was determined by VCl3-Griess method (sulphanilamide/naphthylamine) as Miranda *et al.* (2001).

Data analysis and statistics

Nitrification inhibition rate (%) exerted by root exudates was computed by comparing their produced NO_2^- in the *N. europaea* bioassay vs the NO_2^- produced by the HEPES medium.

$$\text{Nitrification inhibition (sample)}\% = \left(1 - \frac{(NO_2^- \text{ concentration (7 h) sample})}{(NO_2^- \text{ concentration (7 h) HEPES medium})}\right) \times 100$$

Welch's ANOVA test, Tukey test correcting for false discovery rate of two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli were used to identify differences between the treatments and wheat genotypes. Outliers were discarded using Grubbs' test ($\alpha = 0.05$) in GraphPad Prism 8 (GraphPad, Sand Diego, CA, USA). For the multiple comparison into the elite cultivars and landraces, a Tukey–Kramer (HSD) *post hoc* was used.

Results

Validation of the *N. europaea* inhibition bioassay for high-throughput BNI analysis

After optimization of the Nitrosomonas cell growth conditions, several control samples were run to validate and confirm the accuracy and robustness of the Nitrosomonas-based bioassay. The bioassay did not generate NO_2^- in the absence of NH_4^+ or *Nitrosomonas* cells (Fig. 2, negative controls 1 and 3). Allylthiourea (AT), a standard inhibitor of nitrification (Subbarao *et al.*, 2009), can completely inhibit NO_2^- production in the *N. europaea* inhibition assay at a concentration of 0.27 µM (Fig. 2, negative control 2). Importantly, both the HEPES and MetOH solutions used for collection and preparation of the root exudates did not inhibit NO_2^- production in the bioassay (Fig. 2, positive controls 1 and 2). In order to confirm that the bioassay enables the detection of BNI activities, root exudates from a wheat genotype with demonstrated root-emitted BNI activities (O'Sullivan *et al.*, 2016) were used in the bioassay. Noticeably, the results indicated that oxygenation during the exudate collection was essential to obtain an exudation fraction with BNI activity (Fig. 2). Therefore, oxygenation was implemented in the exudation collection system.

The BNI capacity of a given sample was expressed as the percentage of nitrification inhibition compared with the HEPES control. The percentage of inhibition can be calculated by either using and comparing the slopes of NO_2^- (in mM) regression lines (from time-course activity) or by comparing the nitrite that is obtained after a specific time point (Fig. S4). Given the absence of significative difference between the two methods when comparing the results obtained after 7 h of incubation in the *N. europeae inhibition bioassay* (Fig. S4), values of NO_2^- production after this duration were used to calculate the BNI activity of the root exudate samples. Finally, the exposition to a NH_4^+ shock – while maintaining the pH constant (pH 7) – did not appear to alter the exudation of BNI by roots (Fig. S5).

Confirmation of the BNI trait in selected wheat genotypes with the high-throughput pipeline and a peat-based approach

To test the robustness of our pipeline, three wheat genotypes were selected based on their previously reported and contrasting BNI activity in root exudates (O'Sullivan *et al.*, 2016). Persia 6, Persia 6B and Persia 3 revealed inhibition levels of nitrification ranging from 88% to 6% (Fig. S6). The analysis with the high-throughput also showed that the intragenotype variability for BNI activity was more important for Persia 6B and Persia 3 as compared to Persia 6. The absence of significant differences between the measurements of the same genotypes in two independent experiments confirmed the robustness of the implemented pipeline to measure BNI activity in root exudates (Fig. S6).

Additional wheat genotypes, including elite cultivars and landraces, were characterized using the high-throughput pipeline. The analysis indicated a large variation of BNI activity in the

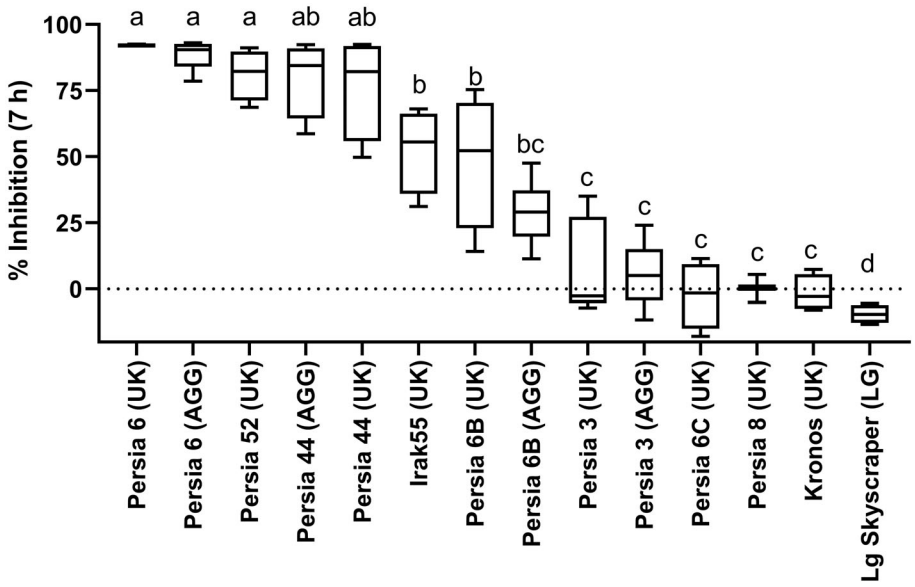

Fig. 3 Biological nitrification inhibition (BNI) activity (inhibition %) in selected wheat genotypes. Box plot with the average and the SD of BNI % is represented. Statistics: letters represent the results of Tukey–Kramer (HSD) *post hoc*. Acronyms in brackets indicate origin. UK, Seedstor; AGG, Australian Grains GeneBank; LG, Limagrain. The horizontal line indicates the level where no BNI activity is measured (0%).

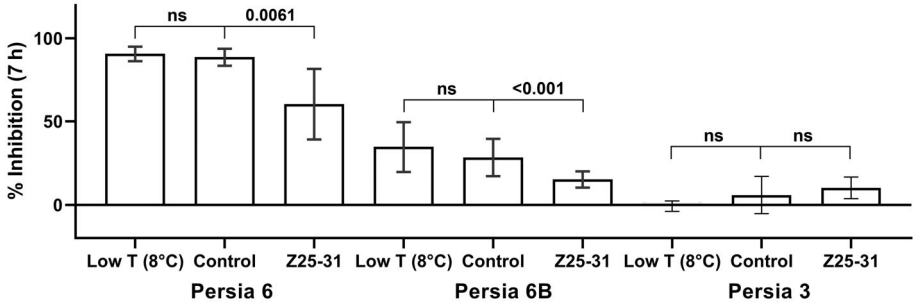

Fig. 4 Biological nitrification inhibition (BNI) activity variation in selected wheat Persia genotypes from AGG. Control treatments correspond to wheat plants at Zadok 15–23 stage and exudation collection operated at 20°C. The average, the SDs and the *q*-values are represented. Statistics: Welch's ANOVA test and false discovery rate of two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (controls $n = 17–18$, treatments $n = 4$). ns, nonsignificant.

selected and tested wheat genotypes. Three genotypes displayed BNI activity in their root exudates superior to 75% inhibition, namely Persia 6, Persia 44 and Persia 52 (Fig. 3). Importantly, the BNI activity also appeared stable in several genotypes (i.e. Persia 6, Persia 44, Persia 6B and Persia 3) sourced from different seed banks (Fig. 3).

Results from the peat-based experiment confirmed the trends observed when plants are not grown in hydroponic system but in substrate-based conditions. Indeed, the quantification of nitrate at the end of the incubation period (Fig. S7) indicates that nitrification rate was significantly reduced in the rhizosphere of BNI active genotypes (Persia 6, Persia 6B and Persia 44).

BNI activity in root exudates was not affected by low temperatures but reduced after drought-rewetting conditions

Because the temperature (20°C) used for the collection of root exudates in the described pipeline is higher than what plants usually experience in soil at early development stages, low-temperature treatment on roots was used to test its potential impact on exudation of BNI compounds. The exudate trap system was set to 8°C and roots from selected wheat genotypes were exposed to the low-temperature treatment during the whole root exudate collection procedure (around 24 h). The results indicated that exposing the roots to 8°C at developmental stage Z15-23 did not significantly alter the BNI activity of root exudates in the selected genotypes (Fig. 4).

In order to assess the potential impact of drought on the exudation of BNI compounds, the roots of the selected wheat genotypes were exposed to a negative osmotic pressure (−1.5 kPa) during 5 d followed by 3 d of rewetting treatment. A trend towards reduced BNI activity of the root exudates after drought-rewetting treatment was observed for all three wheat genotypes albeit the differences were only significant for Persia 6B and Persia 6 (Fig. 5).

BNI activity in root exudates was altered during tillering stage

Studies investigating the BNI activity of wheat root exudates have so far been performed at the early development, on the onset of

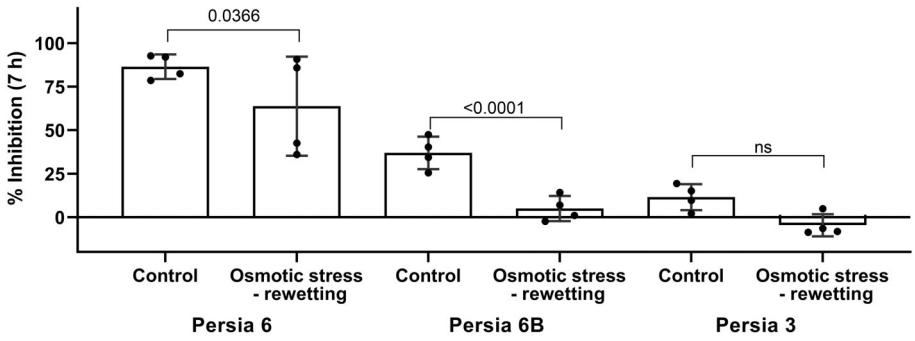

Fig. 5 Biological nitrification inhibition (BNI) activity variation in selected wheat Persia genotypes (AGG) under osmotic stress – rewetting conditions. Individual observations, averages, SDs and the *q*-values are represented. Statistics: Welch's ANOVA test and false discovery rate of two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli ($n = 4$). ns, nonsignificant.

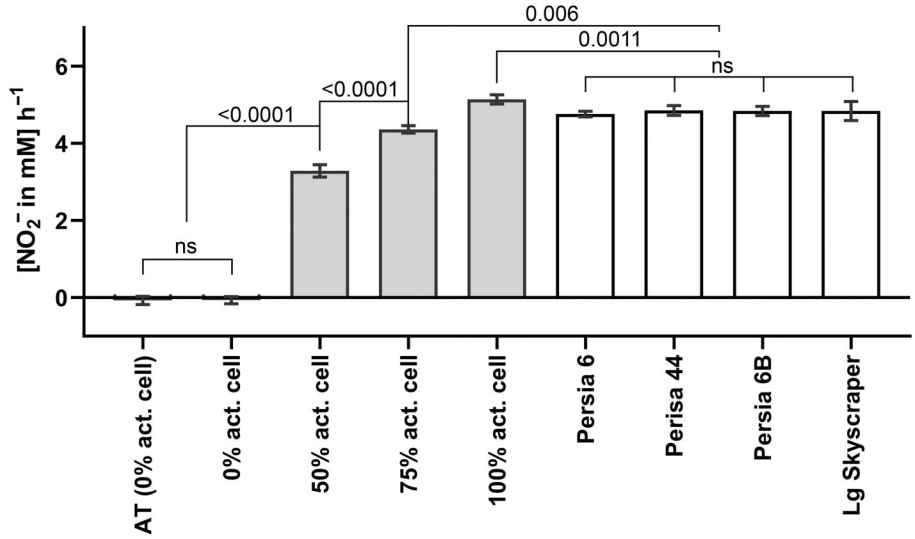

Fig. 6 *Nitrosomonas europaea* viability assay. NO_2^- production activity after pre-incubation (3 h) of the cells with root exudates from wheat Persia genotypes (UK). Values represent the slope ($[NO_2^-]\ h^{-1}$) obtained from a time-course $[NO_2^-]$ accumulation as produced by Nitrosomonas cells. Grey columns represent the control samples with AT (% active cells, act. cells) and an increasing ratio of alive : dead Nitrosomonas cells (% act. cells). SDs and the *q*-values are represented. Statistics: Welch's ANOVA test and false discovery rate of two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli ($n = 3$–6). ns, nonsignificant.

tillering stage (Z 15-23; O'Sullivan *et al.*, 2016). In order to study the expression of the BNI trait during wheat development, root exudates from plants at Z 15-23 and Z 25-31 stages were collected. Despite an increase in root size and biomass (Fig. S8), the analysis of BNI activity in root exudates showed a trend towards a reduction in root exudates from plants at Z 25-31 stage. However, the difference between the Z 15-23 stage and the Z 25-31 stage was only significant for Persia 6 and Persia 6B (Fig. 4). This was confirmed in an independent experiment with the high-BNI activity genotype Persia 6 (data not shown).

Assessing the potential bactericide effect of root exudates on *N. europaea*

In order to further characterize the inhibitory activities of the root exudates, the nitrification rate of *N. europaea* was used as a proxy for survival rate after exposure to the root exudates. The analysis allowed distinguishing inhibiting activities of root exudates on key enzymes involved in NO_2^- metabolism from potential bactericide effect of root exudates on Nitrosomonas cells. A comparison of the slopes of the linear regression of NO_2^- production (incubation phase) indicated that the root exudates had no or limited bactericide effects on *N. europaea* cells (Fig. 6). The same observation was made for all selected wheat genotypes, including those that highly contrast in their BNI activity. Although a small but significant decrease (6%) in the NO_2^- production was observed for the cells exposed to root exudates as compared to same number of cells not exposed to root exudates, the limited difference suggested that most cells remained alive after exposure to the wheat root exudates.

Discussion

The demonstration that plants with improved BNI activity can translate into increased yield (Subbarao *et al.*, 2021) makes BNI a key trait for future wheat breeding strategies. Therefore, there is a need to better characterize the genetic diversity of BNI trait in wheat and to identify novel sources of BNI diversity in yet-to-characterize plant species. The identification of BNI activities in other plant species either mixed-cropped or used as cover and catch crops would further expand the possibilities for a better monitoring of nitrification activities in the field. Efforts for high-throughput screening of BNI activity in plants in field-soil or pot-based conditions have so far been limited because those approaches are time-consuming and tedious (Nuñez *et al.*, 2018; Villegas *et al.*, 2020). Despite the fact that exudate profiles collected in hydroponics might be considered of lower ecological relevance as compared to soil-based methods, the available studies, including ours, investigating Persia genotypes in soil-based experiments suggest that the hydroponics-based pipeline can

provide similar results than in soil conditions (O'Sullivan *et al.*, 2016; Subbarao *et al.*, 2021).

The current pipeline also takes advantage of a bioassay using the wild-type *N. europaea* strain (ATCC 19718), which has been shown to be consistently inhibited by root exudates of several plant species (O'Sullivan *et al.*, 2016, 2017; Sun *et al.*, 2016; Kaur-Bhambra *et al.*, 2022). The optimization of *Nitrosomonas* growth was instrumental to increase the analysis capacity, and the use of a *Nitrosomonas* strain belonging to a biosafety level 1 can also facilitate the implementation of the pipeline in a laboratory environment with limited biosafety requirements. Measuring BNI activities with the *Nitrosomonas*-based bioassay might serve as a good proxy to estimate activities on other nitrifying bacteria. However, future development of the pipeline could also benefit from establishing additional bioassays with other bacterial or archaea strains as only one AOB strain may not represent the full spectrum of inhibition by ammonia-oxidizing microorganisms (Kaur-Bhambra *et al.*, 2022). While the overall BNI activity of the wheat genotypes was in line with previously published data (O'Sullivan *et al.*, 2016), a few discrepancies could also be detected. Differences might be due to variations in the pH value which has been shown to impact the release of BNI compounds in root exudates as it modifies the root transmembrane proton gradient (Egenolf *et al.*, 2021). Differences could also be explained by the bubbling systems which can sometimes lead to root damage and altered release of BNI compounds. Our system that isolates the bubbling in the trap solution was designed to reduce this possible interference (see Fig. S1B). Given the fact that the generation time of *Nitrosomonas* can be as short as 7 h (Cantera & Stein, 2007), the incubation time in the BNI bioassay might impact the measurement of BNI activity. In the present experimental set-up, early measurements in the bioassay also tended to display reduced variations between technical replicates.

The study of BNI production *in planta* and their release under various environmental conditions are important for the optimal use of the BNI trait in various agrosystems. The versatility of the present pipeline can complement other screening methods and offers the possibility to rapidly identify factors altering the stability of the BNI trait. A recent study has demonstrated the impact of drought on the BNI activity of pearl millet using a set of different techniques (Ghatak *et al.*, 2022). The preliminary analysis on wheat root exudates also indicates that the BNI trait is reduced upon an osmotic stress in certain genotypes. The AOB communities are known to be highly resilient to drought-rewetting conditions (Séneca *et al.*, 2020), while those same conditions have also been shown to reduce root exudation (Gargallo-Garriga *et al.*, 2018). Therefore, it is particularly important to investigate the stability of the BNI trait under drought-rewetting conditions for plant genotypes and breeding lines with reported high-BNI activities. The analysis of two development stages also suggests that the BNI trait is altered during development in certain wheat genotypes. Those preliminary results require additional analysis in order to further characterize the modulation of the BNI trait during plant development. Taken together, the different conditions impacting production and /or exudation of BNI compounds could be instrumental to identify genes and/or proteins whose accumulation correlates with the exudation of BNI compounds.

Because root-exudated BNI compounds are organic, they are often considered as environmentally friendly alternatives to their synthetic counterparts. However, some organic compounds limiting nitrification can have broad antimicrobial activity (Lodhi & Killingbeck, 1980; Tahir *et al.*, 2021). Several well-characterized BNIs have also been shown to inhibit bacteria species besides the AOB in the soil (Bending & Lincoln, 2000; Sarr *et al.*, 2020; Schandry *et al.*, 2021; Wang *et al.*, 2021). The deployment and optimal use of the BNI trait in crop plants will undoubtedly require in-depth analysis of their BNI activities on bacterial communities. Measuring the nitrification activities of bacterial cells after their pre-exposure to root exudates in the bioassay offered a simple and cost-effective alternative to flow cytometer-based assays conventionally used to measure bacterial viability. With this approach, the proposed pipeline can provide a preliminary screening of the BNI activity in order to select plant extracts operating by metabolic inhibition of *N. europaea* for further characterization and identification of active compounds.

Conclusion

The proposed pipeline allowed a sensitive, rapid and cost-effective characterization of BNI activity in root exudates. The pipeline could be easily implemented elsewhere as it only requires a controlled glasshouse space, an incubator with shaker, a multiplate reader and the wild-type *N. europaea* strain. Thanks to the implemented optimization, we showed that the proposed method is sensitive enough to detect inhibition rate down to 5%. The versatility of the hydroponic system was demonstrated by studying the impact of different environmental and developmental stages on the BNI trait in selected wheat genotypes. A fast and easy procedure to detect potential bactericidal effect of root exudates on *N. europaea* was also implemented. The pipeline will be instrumental to identify and characterize the BNI trait in cereals as well as in other crop species and should support plant breeders and researchers in introducing a resilient and targeted BNI trait. Including plant species with BNI activity can be particularly interesting for organic agrosystems but also to increase crop NUE for economic and environmental reasons.

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Competing interests

None declared.

Author contributions

All authors contributed to the conceptualization. IJ, IVM and PD contributed to the methodology. IJ, CT and HV contributed to the formal analysis, funding acquisition and visualization. IJ and IVM contributed to the investigation. IJ contributed to the writing – original draft. IJ, CT, IVM, HV and PD contributed to writing – review and editing.

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Data availability

The data that support the findings of this study are openly available in Zenodo at doi: 10.5281/zenodo.7621145.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Details of the plant holder, aeration system and bubbling into the falcon tube.

Fig. S2 Top view of the hydroponic system, and sequence showing the different steps of the exudate collection.

Fig. S3 Production of NO_2^- by *Nitrosomonas europaea* in 24 h ($n = 8$).

Fig. S4 Correlation between inhibition percentages obtained by the single-point 7 h method vs the time-course method (0–3–5–7 h).

Fig. S5 Impact of the 'NH_4^+ shock' at pH 7 on the biological nitrification inhibition activity of selected wheat Persia genotypes from AGG.

Fig. S6 Biological nitrification inhibition capacity of three contrasting wheat Persia's lines (AGG) in two independent experiments.

Fig. S7 Nitrate concentration (mM) increased after 7 d of incubation of rhizospheric substrate collected from five wheat genotypes.

Fig. S8 Representative pictures of wheat plants from three genotypes at two developmental stages.

Table S1 Name and code of the tested wheat genotypes and their corresponding seedbanks.

Table S2 Composition of the modified Hoagland solution.

Table S3 Composition of the Medium 829 and the HEPES medium.

Video S1 Time laps video showing the processing of wheat plants for exudates collection.

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