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Is Endophyte-Plant Co-Denitrification a Source of Nitrous Oxides Emission?—An Experimental Investigation with Soybean

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Abstract: The biological pattern of plants' nitrous oxide (N_2O) generation is not well understood because plant cells cannot form N_2O from nitric oxide (NO) reduction. Hypothetically, we consider that plant-endophytic co-denitrification is potentially capable of making up for the functional loss of NO reduction in the plant cell and is thus the source of plant-derived N_2O. In order to test the above hypothesis, field-cultured soybean seedlings and aseptic-cultured seedlings were used to establish plant holobionts with differentially established endophytes. The N_2O fluxes, copies of the bacterial NO reductase encoding gene, and a diversity of endophytic denitrifying bacteria of these holobionts were observed by gas chromatography, real-time PCR, and 16s rDNA sequencing. The flux of N_2O by the field seedlings was significantly higher than the fluxes of the aseptic seedlings and bulk soil. The N_2O flux of the soybean seedlings was significantly correlated to the abundance of the bacterial NO reductase encoding gene. Eleven genera of denitrifying bacteria were observed in the soybean seedlings, and among them, two genera of aerobic denitrifying bacteria were specifically associated with colonizing plant samples. In this work we have also showed that plant N_2O emission is affected by the abundance of total endophytic denitrifying bacteria. We conclude that plant sourced N_2O is cooperatively generated by the plant-endophyte symbiotic system in which endophytic denitrifying bacteria help plants to emit N_2O by taking over the NO–N_2O reduction process.

Keywords: plant derived N_2O; endophytic bacteria; plant-endophytic co-denitrification

1. Introduction

Nitrous oxide (N_2O), the third most important greenhouse gas, is also an important indicator of biological nitrogen transformations [1]. Because of the uncertainty regarding the sources of N_2O, an imbalance has long existed between estimates of N_2O sources and sinks in the Global Greenhouse Gas Inventory [2,3]. In the agroecosystem, soils are the main source of N_2O, which originates from microbial nitrification and denitrification, and are also regarded as the main N_2O source in inventory calculations of global N_2O emissions. Conversely, plants and mammals are considered uncertain sources of N_2O.

Li and Chen first provided an evidence that soybean plants could generate a considerable amount of N_2O during growth [4,5]. Since then, the N_2O fluxes from 17 species of plants were investigated by Hakata and colleagues, with 16 species of tested plants showing a tiny amount of N_2O emission [6]. However, although plant-derived N_2O emission has been observed for years, plant-sourced N_2O had been always absent from the N_2O emission inventory [1,7].The reason is that the denitrification pathway and the source of N_2O generation in plant cell were still not completely understood [4,8].

In earlier literature, two hypotheses of plant N_2O emission were proposed. One hypothesis is that plants serve as a conduit of soil-sourced N_2O [9], and the second hypothesis is that NO_2^- assimilation by plants is the source of plant N_2O [10]. Since nitrite is toxic to plants, plant NO_2^- reduction probably occurs in the biochemical reaction sequence of "NO_2-NO-N_2O-N_2", which provide a possibility for plants to emit N_2O [11]. Studies have also clearly demonstrated that plants could potentially undertake denitrification to form N_2O from nitrate or nitrite [12,13].

Nevertheless, whether there is a complete denitrification pathway in plants has been a controversial topic [14]. Evidence shows that the NO_2^- level in plant cells is critical for the emission of N_2O from plants [12]. However, a complete NO_2^- reduction pathway in plant has not been recognized and characterized. In plant cells, nitrate could be reduced by the plant's nitrate reductase (nr) and nitrite reductase (nir) to generate NO. Whereas, plant cells do not seem to have the ability to further reduce NO into N_2O. Evidences shows that no single gene encoding NO reductase (nor) was found in any plant genome [15]. Previous results have also found that the fungal nitric oxide reductase gene (P450-*nor*)-transfected tobacco BY-2 cell emits N_2O at a level 59 times higher than compared to the wild-type cell [15]. This finding indicates that plant cell cultures are not capable of generating N_2O, except those under the help of an alien NO reductase.

However, plants are not unicellular organisms. Nowadays, plant holobiont is defined as a symbiont composed of microbial cells and plant cells. Microorganisms abundantly colonize all plant organs and tissues [16]. Thus, the contribution to N_2O emissions by plant-associated microbes should be accounted for plant-based N_2O emissions. In this context, it has been shown that ammonia-oxidizing bacteria (AOB) on grass leaves may be an unrecognized source of N_2O, converting 0.02–0.42% of NH_3 to N_2O [17]. This is the first report that in vitro microbes contribute to plant N_2O emissions. However, no study has been performed to discuss the relationship between plant endophytes and plant N_2O emissions so far.

It is currently postulated that plant tissues could provide denitrifying bacteria with an appropriate in vivo anaerobic environment [18]. There are also earlier pieces of evidence showing that leaves of wheat (*Triticum aestivum* L.) reduce nitrite and release N_2 [14,19], which suggests that plants may have denitrification ability and are thus capable of emitting N_2O as a final product. This prompted us to ask whether endophytic bacteria could help plant cells to establish a NO–N_2O reduction pathway by using their bacterial NO reductase and thus enabling N_2O emission. If this is so, then the existence of the endophytic denitrifying bacteria offers a possible explanation for denitrification in plants.

Based on all the above findings, we hypothesized that endophytic denitrifying microorganisms probably play a critical rule in the N_2O emission of plant holobionts. Specifically, the N_2O flux from plants may be affected by the abundance and composition of the endophytic denitrifying bacteria. To test this hypothesis, field-cultured soybean seedlings and aseptic seedlings were used to perform measurements on the plant holobiont with different endophytic microbiota. The N_2O fluxes of young seedlings were measured and the composition and population of the endophytic denitrifying bacteria were investigated by 16s rDNA metagenomics sequencing. Finally, the abundance of the endophytic bacterial *norB* gene in plant cells was analyzed by using the real-time polymerase chain reaction (PCR). Correlation analysis was performed to test the relationship of the above factors.

2. Experiments

2.1. Plant Cultivation

Field-cultured seedlings were used to investigate the N_2O flux from soybean plants under the intervention of soil microorganisms. Soil samples were collected before seeding from land being sown to serve as background controls. Surface-sterilized seedlings (surface-sterilized seeds were cultured in sterile soil under aseptic conditions) were grown to perform measurements of N_2O flux from soybeans with only endophytic microbiota.

The experimental design is shown in Figure 1 and was performed using the soybean variety "Tie Feng 31", which is widely cultivated in northeast China. Field cultivation was performed at the Institute of Shenyang National Field Observation and Research Station of Shenyang Agroecosystem (41°31' N, 123°24' E). Soybeans were grown in brown earth soil (soil pH: 6.4, organic matter: 16.17 g·kg^{-1}, soil moisture: 45%, and total nitrogen: 0.67 g·kg^{-1}) and the average temperature of the sampled plot was 14–18 °C.
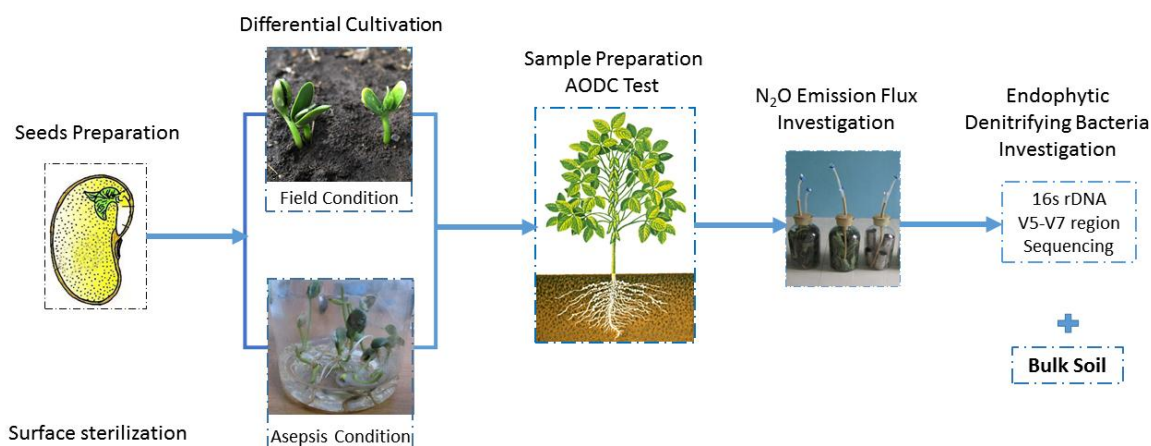
Figure 1. The work flow of this study.

Aseptic seedlings were prepared as follows: First, all seeds were surface-sterilized, and the final wash buffer was tested by the Acridine Orange Direct Count (AODC) method to ensure that no viable microbes were left [20]. Subsequently, the surface-sterilized seeds were aseptically transplanted into a germ-free potted system (diameter × height: 20 × 10 cm) that had been heat-sterilized at 121 °C for 45 min. Seedlings were then aseptically cultured and watered in a germ-free room that received purified air, 16 h of light at 25 °C and 8 h of dark at 20 °C at a relative humidity of 45%.

2.2. Sample Preparation and Determination of Plant Nitrite and N$_2$O Emission

Surface soils (0–10 cm) of the field experiment zone were collected before seedling and marked as "BS" samples. Plant samples were collected on the 20th day after sowing. Field seedlings and the aseptic seedlings were freshly collected synchronously. In the latter, aseptic techniques were strictly followed to prevent the plant from being contaminated. To remove the plant-surface microorganisms, all plant samples were washed and surface sterilized immediately according to the method described by Xiao [21]. The final wash buffer was tested by AODC to confirm all samples were superficially germ-free [20]. Before the gas collection, sterilized plants were aseptically moved into a chamber unit which contained 0.5 kg of sterilized soil [22]. This was to ensure the consistency of the N background for each test while eliminating soil microbes and their interference with N$_2$O emission. The gas samples in the chamber were collected and analyzed for N$_2$O concentration by gas chromatography (GC) on an Agilent 7890D platform [23]. The flux of N$_2$O was measured using the methods previously described [9]. The bacterial counts and the N$_2$O emission rate of the sterilized soil were independently tested by AODC and GC, respectively, to verify sample cleanliness and emission background.

The plant NO$_2^-$-N concentrations in all samples were determined via a spectrophotometric method [24] using an Multiskan FC Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA), while the NO$_3^-$-N contents were analyzed through alkaline distillation.

2.3. Quantitative Real-Time PCR

After the determination N$_2$O Emission, all plant samples were aseptically cut into three different parts: stem, leaf, and root. Subsequently, metagenomics' DNA of all samples (plants and bulk soil) was all extracted using the Power Soil™ DNA Isolation Kit (Qiagen, Valencia, CA, USA).

The copy number of genes encoding bacterial nitric oxide reductase was determined in triplicate for the different samples by applying real-time Q-PCR using a Rotor-Gene 3000 real-time PCR system (Corbett Research Pty Ltd, Mortlake, Australia). Target sequences were amplified using the primer *norB*-2F (5-GAC AAG NNN TAC TGG TGG T-3) and *norB*-6R (5-GAA NCC CCA NAC NCC NGC T-3) [25]. The thermal cycling program was as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s. A total of 40 cycles were run using the annealing temperatures.

Each reaction was performed in 25 mL volume containing 1–10 ng of total DNA, 0.2 mM of each primer, and 12.5 mL of 2 × QuantiNova qPCR SYBR Green Master mix (Qiagen, Duesseldorf, Germany). Product specificity was tested and confirmed using melting curve analysis (65–95 °C, 0.5 °C per read, 2 s hold) and re-visualised on agarose gels, which showed specific product bands at the expected size of 400 ± 10 bps.

The target genes were subsequently connected with the pEASYTM-T5 plasmid vector (Transgene, Shenzhen, China) and then amplified in the *E. coli* host. Extracted plasmid vectors containing the target gene inserts were chosen as the standards for real-time PCR. The concentration of the extracted plasmid was measured using a NanoDrop spectrophotometer (Thermofisher, Waltham, MA, USA). The abundance of genes encoding bacterial nor were calculated from the concentration of extracted plasmid DNA. Ten-fold serial dilutions of a plasmid with a known copy number of the *norB* gene clones were generated to set up the standard curve (from 6.13×10^3 to 6.13×10^9 copies). High amplification efficiencies of 92.5% to 102.4% were obtained, with $R^2 = 0.99$ and slopes from -2.8 to -3.2. The cycle threshold parameter (Ct) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

2.4. Investigation of the Denitrifying Bacteria

Denitrifying bacteria were measured by 16s rDNA metagenomics sequencing. The whole plant was crushed and mixed for DNA extraction. In addition, seedlings were also aseptically cut into 3 parts (i.e., stem, leaf, and root) to investigate the composition of denitrifying bacteria in the different organs. Total DNA of all samples was extracted using the PowerSoil™ DNA Isolation Kit (Qiagen, Valencia, CA, USA).

The hypervariable V5–V7 region of the 16s rRNA gene was chosen as the detection region in order to minimize the contamination from the chloroplast DNA homologue [26]. Target sequences were amplified using the primer 799F (5′-AACMGGATTAGATACCCKG-3′) and 1193r (5′-ACGTCATCCCCACCTTCC-3′) [26]. The thermal cycling program was as follows: 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, with a final extension of 72 °C for 10 min. All libraries were sequenced on an Illumina Miseq 300PE platform at Allwegene Technology Inc. (http://www.allwegene.com/, Beijing, China). High-quality sequences from all samples were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity (OTU97) using the default QIIME pipeline. The representative sequences in each OTU97 were assigned to taxonomic groups using the SILVA classifier with an 80% confidence threshold.

The genera of denitrifying bacteria-related species were selected according to previous studies [27]. Gap analysis and correlation analysis were performed by GraphPad 6 software (GraphPad Software Inc., San Diego, CA, USA), and the "Pheatmap" (v1.0.8) package in R (v3.0.3) software (The University of Auckland, Auckland, New Zealand) was used for heat map visualization. The sequencing data from this study was deposited in the European Nucleotide Archive under the study accession number PRJEB25047.

3. Results

3.1. N₂O Flux of Differently Treated Soybean Seedlings and Bulk Soil

The N_2O flux of the "medium CK" group (chamber units which contained sterilized soil only) was measured first, showing no measurable emission from this group, which indicated that sterile soil did not interfere in the N_2O measurement, as well as in the chamber devices. The N_2O flux of differently treated plants were within a range of 5 to 140 ng N_2O g^{-1} fw h^{-1} (Figure 2). Field seedlings presented the highest flux (111.7 $\pm$ 25 ng N_2O g^{-1} fw h^{-1}), while the flux from aseptic seedlings was 8.56 $\pm$ 2 ng N_2O g^{-1} fw h^{-1}. Statistically, the field seedlings showed significantly higher fluxes than aseptic seedlings ($p = 0.0002$) (Figure 2). Moreover, the amount of N_2O emitted from bulk soil samples was similar to the aseptic seedlings ($p = 0.13$) at 6.81 $\pm$ 2 ng N_2O g^{-1} fw h^{-1} (Figure 2).
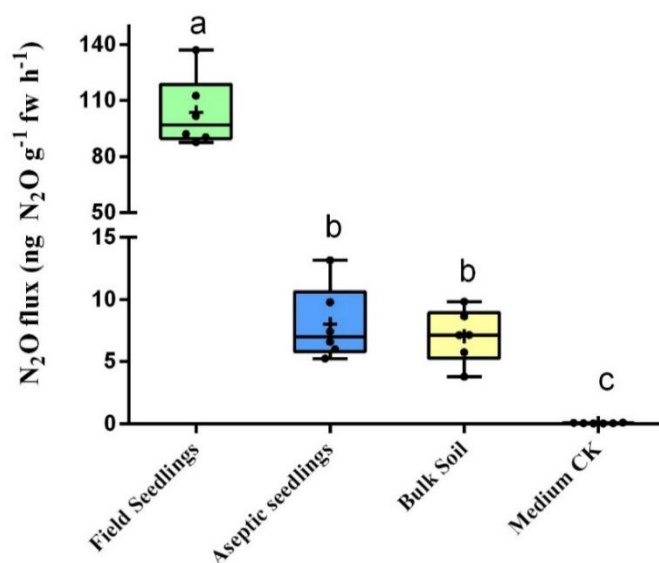

Figure 2. The N_2O flux from differently treated soybean seedlings and bulk soil (45% moisture), means (mean $\pm$ SE, $n = 5$) followed by the same letter are not significantly different at $p < 0.05$.

3.2. NO₂⁻-N, NO₃⁻-N Concentration and Bacterial norB gene Abundance

Figure 3 shows the NO_2^--N, NO_3^--N concentration and the abundance of *norB* in different soybean seedlings, which were measured after the N_2O flux experiment. Insignificant differences were observed in the content of NO_2^- between the field and aseptic seedlings (5.86 and 7.15 µg N g^{-1} fw soybean tissue, respectively). Both types of seedlings have similarities in NO_3^--N concentration ($p = 0.676$), ranging from 552.06 to 502.12 µg N g^{-1} fw soybean tissue. Nevertheless, field seedlings showed significantly higher ($p < 0.01$) abundance of bacterial *norB* (4.2×10^6 copies g^{-1} fw soybean tissue) than in aseptic seedlings (5.8×10^4 copies g^{-1} fw soybean tissue).

To avoid spatial autocorrelation, the correlation analyses were performed in the field seedling group and aseptic seedling group, respectively. Figure 4 illustrates the correlation between the abundance of bacterial *norB* and the fluxes of plant-derived N_2O. Results showed that a positive correlation ($r^2 = 0.81$, $p = 0.002$ in aseptic group, while $r^2 = 0.71$, $p = 0.02$ in field group) was obtained for the N_2O flux and the abundance of bacterial *norB* in both groups.
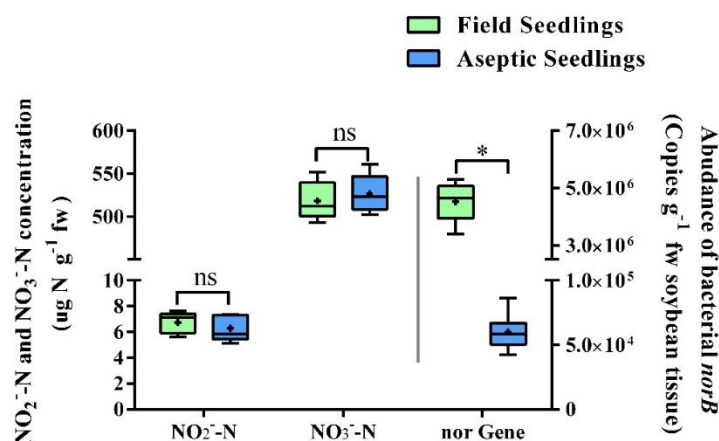
Figure 3. NO$_2$-N, NO$_3{}^-$-N and *norB* gene concentration in field or aseptic soybean seedlings. Error bar indicates the SD. Asterisk indicates high significant differences from the field seedlings only in nor gene abundance (*t*-test, * $p < 0.05$, ns = not significant).
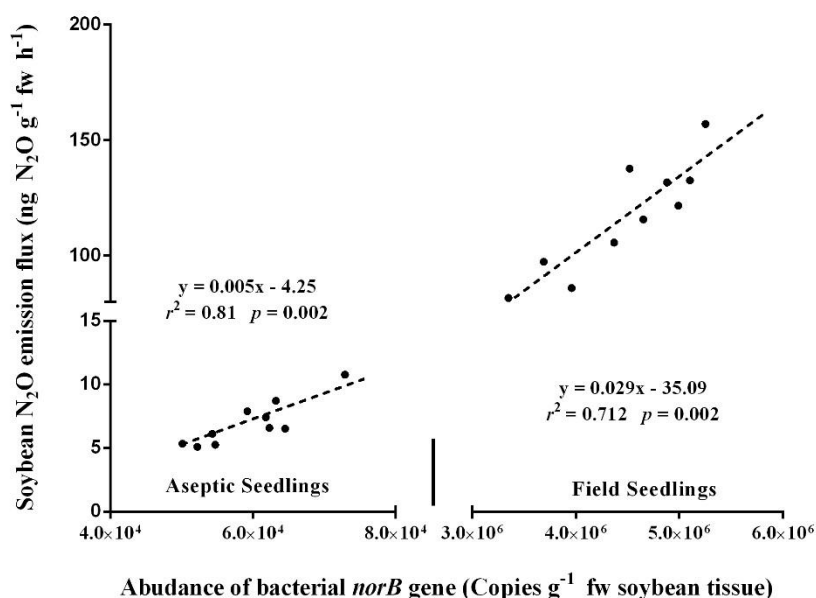

Figure 4. Relationships between plant N$_2$O flux (ng N$_2$O g^{-1} fw h^{-1}) and endophytic nor gene abundance (copies g^{-1} fw soybean tissue), correlation analysis were respectively performed in the field group and aseptic group.

3.3. Composition of the Denitrifying Bacteria and Their Correlation with Plant N$_2$O Emission

Sequencing data shows that field seedlings were enriched the most with denitrifying genera-related OTUs (in total: 4063 ± 1130 OTUs), which is on average 5 times higher than those found in aseptic seedlings (in total: 846 ± 293 OTUs, $p < 0.05$) and the bulk soil (in total: 690 ± 58 OTUs, $p < 0.05$). The denitrifying populations in the field seedlings dominated 14% of the total bacteria, higher than that of the aseptic seedlings (4.1%, $p < 0.05$) and bulk soil (2.2%, $p < 0.05$) (Table S3, Supporting information). Denitrifying bacteria were detected in all organs from the differently treated plants (Figure 5b). A total of 11 genera of denitrification-related bacterial species were found in this study. All genera were plant-associated except for *Leptospirillum*, which was only observed in the bulk soil samples (Table S2, Supporting information). Among these, the genera of *Pseudomonas*, *Arthrobacter*, *Acinetobacter*, *Microbacterium*, *Noviherbaspirillum*, *Leptospirillum*,

Stenotrophomonas, *Sphingobacterium*, and *Bradyrhizobium* are reported as anaerobic denitrifying bacteria. The genera of *Propionibacterium* and *Eubacterium* were previously reported as aerobic denitrifiers. It is worth mentioning that *Propionibacterium*, *Sphingobacterium*, and *Eubacterium* were specifically enriched in both plant samples, which could indicate that these genera are seed-borne denitrifying bacteria (Table S2, Supporting information).
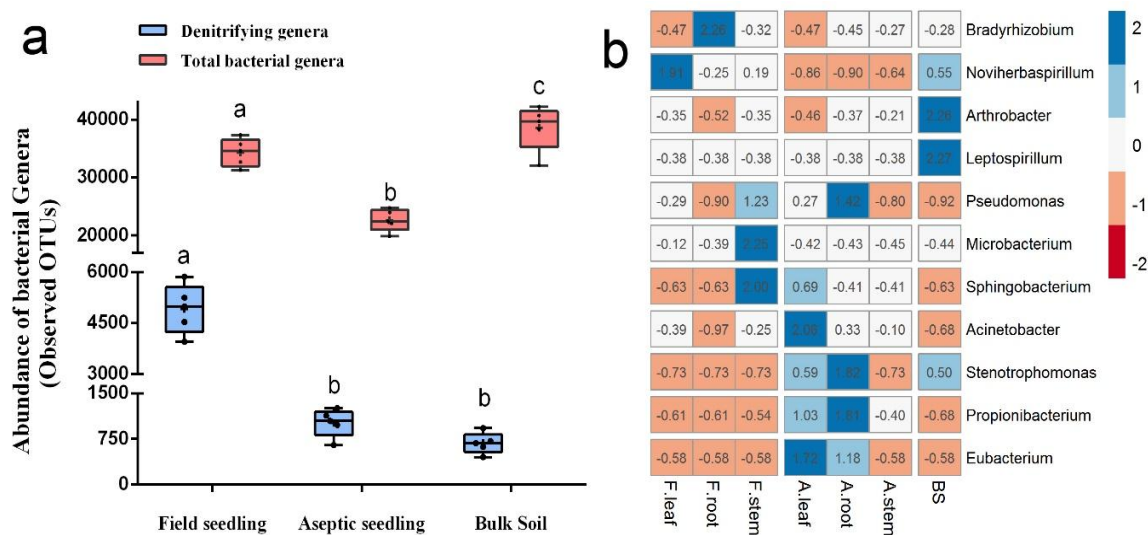
Figure 5. (**a**) Bar plot showing the abundance of total aerobic denitrification related genera and total bacterial genera from a holistic perspective. *t*-test analysis indicates high significant differences obtained from the abundance of denitrifying genera in field seedlings ($p < 0.05$). The same letter on the bars indicate no significant differences between means at ($p < 0.1$). (**b**). Heat map showing the distribution of denitrification related genera in differently cultured soybean organs and bulk soil. The colour of the squares on the left indicates the relative abundance of the genera in each group. Normalized relative abundance (from 2 to −2) was marked on the colour block.

Several denitrifying genera dominated at levels greater than 1% of the bacterial abundance in different organs; these could be considered as "core" denitrifying genera. Among them, *Bradyrhizobium* was dominant by 8.98% in the root and by 0.55% in the stem of field seedlings, while it dominated by 0.71% in the stem of aseptic seedlings (Table S3, Supporting information). *Pseudomonas* dominated by 1.03% of the relative abundance of bacteria in the root fractions of aseptic seedlings and 0.94% in the stems of field seedlings (Table S3, Supporting information). Interestingly, compared to the field seedlings, the aseptic seedlings were significantly enriched with two genera of aerobic denitrifying bacteria: *Eubacterium* and *Propionibacterium* (40 OTUs and 61 OTUs, respectively) ($p = 0.50$ and $p = 0.47$, respectively) (Table S2, Supporting information). These two genera of seed-borne aerobic denitrifying bacteria were not highly abundant in the stem (0.0% and 0.013%, respectively), which is considered an oxygen-containing zone. In contrast, they were more highly distributed in the anaerobic zone, i.e., the roots (0.04% and 0.117%, respectively) and leaves (0.057% and 0.08%, respectively) (Table S3, Supporting information).

4. Discussion

Firstly, in this study, more than a 10-fold variation of N_2O flux was observed between field seedlings and aseptic seedlings. This result suggests that endophytic microbiota may play a critical role in plant N_2O emission. In our work, the N_2O flux that we observed from field seedlings was similar to that observed in previous studies, however, the flux from aseptic seedlings was quite different [4]. Contrary to our observations, Hakata and colleagues previously showed that the highest value for N_2O emission was obtained with aseptic *Hibiscus cannabinus* seedlings (0.45 ± 0.20 ng N_2O/g fw

and week^{-1}) [6]. Hence, the latter study proposes that the activity to convert nitrate nitrogen to N_2O is limited in plants. However, the flux we obtained in this work in aseptic seedlings (8.56 $\pm$ 2 ng N_2O g^{-1} fw h^{-1}) was 100 times higher than what was found in that study [6]. Tracing it to its cause, the different cultivation of plants is what ought to be taken into consideration. While Hakata and colleagues [6] aseptically cultured the plants on the MS medium, we cultured the aseptic seedlings in sterilized soil. The physicochemical properties of the medium will seriously affect the development of plants [28]. This indicates that abiotic factors seriously affected the plant N_2O observation, different culture conditions can lead to different observations. Furthermore, we considered that the N_2O flux of plants is not negligible like what was described in previous studies [6,29]. In fact, the N_2O flux from field soybean seedlings observed in our study was significantly higher than that from the soil from the same field. This suggests that plant-generated N_2O emissions may significantly contribute to the total emissions from the agricultural system.

Nitrite, the substrate of plant N_2O, is closely related to the plant N_2O emission rate [12]. Previous studies of plant N_2O generation suggested that plants may have different N_2O fluxes under different NO_2^- accumulation rates [12,30]. In our study, field seedlings and aseptic seedlings showed similar NO_2^- concentrations. This demonstrates that, under similar abiotic factors, soybean nitrite accumulation was not related to the endophytic microbiota. In other words, nitrite accumulation is mainly accomplished by the plant cell itself and is not affected by endophytic microorganisms. What is more surprising is that under similar nitrite concentrations, field seedlings and aseptic seedlings presented a 10-fold difference in their N_2O fluxes. This is an indication that endophytic microbes may affected plant N_2O emission only by means of contributing to their nitrite conversion activity.

Nevertheless, the nitrite conversion pathway of plant cells is still unrecognized [29]. Specifically, while nitrite can be reduced to NO, plant cells, however, do not seem to have the ability to further reduce NO into N_2O. The plant derived NO reductase and its encoding gene is the key but is lost in plant cells. In this context, Abdel and his colleagues engineered the NO reduction pathway in plant cells by incorporating the fungus-derived P450-*nor* gene, thus enabling N_2O emission [31]. Thus, the purpose of our work is to prove whether there is an exogenous NO reductase in wild plants holobiont, which has similar function to the incorporated P450-*nor* gene.

Results from a Q-PCR assay firmly proved that the bacteria-derived *norB* exists in the plant holobiont. Further, the correlation analysis presented shows that plant N_2O emissions were significantly correlated with the abundance of bacterial *norB* gene. This indicates that although the function of NO reduction in the plant cell is defective, the endophytes filled this functional gap, thus enabling the plant N_2O emission. Besides, our results also suggest that bacterial denitrification quantitively affect plant N_2O generation by taking over the NO-N_2O reduction process. Specifically, plant nitrite accumulation is done by plant cell. The rate of plant nitrite directly affected plant NO concentration, which is a substrate of N_2O production. Subsequently, bacterial NO-N_2O reduction is the final speed limit step of plant N_2O generation. To sum, compared with P450-*nor* gene transfected plant cell cultures, plant holobionts also tackle the N_2O generation pathway with the assistance of endophytic microbes. To the best of our knowledge, this is the first time that a cooperative interaction between the denitrifying bacteria and nitrogen dissimilation in plants has been reported.

Soybean endophytic denitrifying bacteria and its distribution in various organs were determined. Anaerobic and aerobic denitrifying bacterial species were both observed in soybean endophytic microbiota. Similar denitrifying species were observed in field seedlings and aseptic seedlings (Figure 5b, Table S2, Supporting information), which indicates that seed-borne microbiota cover most denitrifying bacterial species and enable to colonize in aseptic-cultured seedlings. In contrast, field seedlings show significantly high abundance of denitrifying bacteria than that in aseptic seedlings (Figure 5a). These observations suggest that soil contact improves the abundance of denitrifying bacteria in all soybean organs. In addition, the genera of denitrifying species (*Propionibacterium*, *Eubacterium and Sphingobacterium*) were specifically observed in plants, and all of those denitrifying species constituted a sizably higher proportion of the plant microbiota than in soil. The reason is

that plants probably provide better anaerobic conditions for endophytic microbes, which ensures the recruitment of more in vivo denitrifying bacteria in plants [32,33]. Thus, we propose that the plant is a more powerful N_2O bioreactor than the soil system. This may explain why plants emit more N_2O than soil samples (Figure 5).

According to the sequencing data, field seedlings were enriched by 5 times higher abundance of denitrifying bacteria than that of aseptic seedlings. These data can be related to the plant N_2O flux data in which field seedlings showed 10 times higher N_2O flux than that of aseptic seedlings (Figure 5). However, at this point, we have not yet definitively proven that sum of total denitrifying bacteria is the only key factor in plant N_2O generation because endophytic microbes may affect plant N_2O emissions from multiple pathways. For instance, the distribution and composition of nitrogen metabolism-related species (e.g., *Bradyrhizobium*) also showed great differences in field seedlings and aseptic seedlings. We found that *Bradyrhizobium* was 100 times more enriched in the roots of field seedlings than that in aseptic seedlings (Figure 5b, Table S2, Supporting information). The biological functions of *Bradyrhizobium* operate in both nitrogen fixation and the denitrification process [33], which are both considered as the source of plant N_2O generation [10,34]. The quantitative variation of *Bradyrhizobium* may significantly affect nitrogen accumulation, including ammonium, nitrate and nitrite accumulation in plants, and successively affect the emission rate of plant N_2O. Therefore, we propose that the contribution of endophytic bacteria to plant-sourced N_2O generation may not be limited to those directly involved in the NO reduction process.

Regrettably, we are not capable of establishing the germ-free soybean seedlings which are more worthy of observation. Tissue culture techniques used to be the way to establish germ-free plants. However, evidence has shown that shoot-tip bacteria are also detected in the plant tissue cultures [35]. Therefore, germ-free seedlings are not established due to this technical limitations. In addition, due to the technical limitations, we were not able to establish a plant-sample queue containing quantitatively different levels of total endophytic bacteria. For these reasons, we are currently incapable of studying the plant N_2O flux in the plant samples with zero endophyte and endophyte gradient. Further, the involvement of endophytic fungi in plant N_2O emission and its potential denitrification activity [36] were not determined in our work. All of this will be addressed in future studies.

5. Conclusions

We measured the N_2O fluxes, endophytic bacteria derived *norB* gene, nitrite concentration and the denitrifying bacterial community of soybean holobionts with differentially established endophytes. Based on the results obtained from this study, we conclude that

(1) Plant-endophytic co-denitrification is capable of making up for the functional loss of NO reduction in plant cell and thus enables plant to generate N_2O. Potentially, plant derived N_2O significantly contributes to the total emissions from the agricultural system.

(2) Endophytic denitrifying bacteria rule the plant N_2O emission by means of contributing to their NO reduction activity. The plant N_2O fluxes significantly correlated to the abundance of their bacteria derived NO reductase encoding gene.

(3) Denitrifying bacteria distribute in all soybean organs, which constituted a sizably higher proportion of the plant microbiota than in soil. Most endophytic denitrifying bacteria are associated with seeds. However, soil contact improves the abundance of denitrifying bacteria in all soybean organs.

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