Endophytic PGPB Improves Plant Growth and Quality, and Modulates the Bacterial Community of an Intercropping System

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The intercropping of ryegrass and red clover constitutes a sustainable alternative to mitigate the adverse effects of intensive livestock production on grassland degradation by increasing forage yield and quality. The implementation of biofertilization technologies has been widely used to improve soil nutritional properties, and therefore has the potential to ensure the success of this multicrop system. To determine the impact of bioaugmentation on forage growth and quality, as well as the associate change in the rhizosphere bacterial community, we evaluated the inoculation with two plant growth-promoting bacteria (PGPB) under reduced nitrogen usage. Overall, Herbaspirillum sp. AP21 had a larger effect than Azospirillum brasilense D7 on plant growth. Inoculation with Herbaspirillum sp. AP21 together with 50% of the required nitrogen rate increased shoot dry weight, crude protein, and shoot nitrogen content, and decreased the amount of neutral detergent fiber. PGPB inoculation changed the rhizosphere bacterial community structure, which associated with forage growth and quality. We conclude that PGPB inoculation has the potential to improve the growth of the ryegrass-red clover system, decreasing the requirements for nitrogen fertilization.

Keywords: perennial ryegrass, red clover, forage, fertilization, Herbaspirillum, Azospirillum

INTRODUCTION

The growing demand for livestock is progressively resulting in grassland degradation and environmental pollution. Therefore, the pressing challenge is to maintain or increase livestock production, while achieving greater environmental and economical sustainability. This sustainability can be achieved by introducing practices that result in the reduction of soil erosion, nutrient deficiency, weed encroachment, and desertification (Sattari et al., 2016). One of the potential solutions to mitigate the negative environmental impact of intensive livestock production is the implementation of intercropping systems, as the introduction of forage legume crops in grass pasture production systems not only improves soil fertility but also allows the efficient use of water and nutrients, especially under drought conditions (Bi et al., 2019). Grass-legume intercropping systems increase the productivity and persistence of pastures compared to grass monocultures (Elgersma and Soegaard, 2018). Nonetheless, the grass-legume mixture is severely
affected by two environmental factors: drought and soil nutrient availability, especially nitrogen (N) and phosphorus (Yang et al., 2020).

The association of perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.) represents an example of a successful grass—legume intercropping (Hammelehle et al., 2018). Red clover increases pasture yield, as well as N use efficiency by ruminants due to its nutritional composition (Eriksen et al., 2014). In addition, legumes can derive a portion of their N requirements from the atmosphere through symbiotic biological N fixation. This process influences N dynamics in soil that can be extended to grass through a process known as “N transfer” between the legume and the grass, relying on the N exudation rate from the legume and its absorption by the grass (McElroy et al., 2016). The benefits of legume-grass intercropping can be further optimized using efficient plant growth-promoting bacteria (PGPB), which allows reducing the application rates of N fertilizers (Liu and Ludewig, 2020).

The PGPB are a group of microorganisms that improve plant growth by a variety of mechanisms such as symbiotic and associative N fixation, nutrient solubilization and mineralization (e.g., phosphorus, potassium, zinc), synthesis of phytohormones (e.g., indoles, gibberellins, and cytokinins), production of 1-aminoacyclopropane-1-carboxylic acid (ACC) deaminase, synthesis of volatile organic compounds, and induction of osmolyte accumulation, among others (Ramakrishna et al., 2019; Moreno-Galván et al., 2020; Romero-Perdomo et al., 2021). A well-known facultative endophytic bacterial genus with PGPB traits is *Azospirillum* (Cassán et al., 2020). Its inoculation on forage grasses, for instance, has shown to minimize soil degradation risks while improving mass forage production (Boddey et al., 2003). The use of *Azospirillum* allows reducing the amounts of N-fertilizers applied without compromising important yield components, such as dry matter and plant height (Pankievicz et al., 2015; Leite et al., 2019). Another genus of endophytic bacteria capable of asimbiotically fixing N and producing phytohormones is *Herbaspirillum* (Baldani et al., 2014). This genus has demonstrated biofertilizer potential on crops, such as rice, corn, and sugarcane (Afzal et al., 2019). Likewise, *Herbaspirillum* can increase the biomass production of pastures in low-fertility soils without using N fertilization (Marques et al., 2017). Inoculation with *Herbaspirillum* also increased the biomass of *Miscanthus sinensis* and perennial ryegrass plants (Straub et al., 2013; Cortés-Patiño et al., 2021).

Previously, we demonstrated the PGPB potential of two endophytes to promote the growth of both perennial ryegrass and red clover under drought and phosphorus limitation (Cortés-Patiño et al., 2021; Santos-Torres et al., 2021). Here, we further explored the PGPB potential of these two bacterial endophytes to facilitate the growth of an intercropping system conformed by both perennial ryegrass and red clover. We assessed the impact of their inoculation on the growth and quality of the system, and their influence on the diversity of the rhizosphere bacterial community.

### MATERIALS AND METHODS

#### Bacterial Strains and Inoculants

The PGPB strains *Herbaspirillum* sp. AP21 (SAMN15498633) and *Azospirillum brasilense* D7 (SAMN16830199) were provided by the Microorganism Germoplasm Collection of Agrosavia, Colombia. These strains were previously identified by whole genome sequencing and were characterized as facultative endophytes (Cortés-Patiño et al., 2021). For routine experiments, these strains were grown in DYGS culture medium, incubated at 30°C, and agitated at 140 rpm for 48 h (Baldani et al., 2014). For the greenhouse experiment, each bacterial suspension was homogenized at OD<sub>600</sub> = 0.200 and washed twice in a sterile saline solution (8.5 g L<sup>−1</sup> NaCl) to obtain bacterial suspensions of ~10<sup>6</sup> CFU mL<sup>−1</sup>.

#### Evaluation of Plant Growth-Promoting Ability Under Reduced Rates of N Fertilizer

The greenhouse experiments were carried out at the C.I. Tibaitatá of Agrosavia in Mosquera, Cundinamarca, Colombia (4°41'43.6“ N latitude, 74°12'19.9“ W longitude; 2,600 m above sea level). A completely randomized experimental design was used in a factorial arrangement (2 × 4) with ten treatments, three replicates, and three independent experiments. The first factor evaluated was N fertilization (50% and 75%) using urea as the N source. The second factor was PGPB application: without inoculation, *Herbaspirillum* sp. AP21, *Azospirillum brasilense* D7, and *Herbaspirillum* sp. AP21 + *Azospirillum brasilense* D7. Additionally, two fertilization controls without PGPB inoculation were included: 0% N and 100% N, totaling 10 treatments (Supplementary Table 1). Pots with 3 kg of non-sterilized soil were used for the experiment. This soil is classified as Vitrif Haplustands-AMbC and belongs to the Andisol order (Soil Science Division Staff, 2017). Its attributes are the following: pH 5.90, organic matter 53.30 g kg<sup>−1</sup>, effective cation exchange coefficient 11.97 cmol kg<sup>−1</sup>, effective cation exchange coefficient 11.97 cmol kg<sup>−1</sup>, K 1.38 cmol kg<sup>−1</sup>, K 1.38 cmol kg<sup>−1</sup>, Mg 1.65 cmol kg<sup>−1</sup>, K 1.38 cmol kg<sup>−1</sup>, and Na 0.24 cmol kg<sup>−1</sup>. Soil was maintained at field capacity of 80% during the experiment. Perennial ryegrass (*Lolium perenne* L) var. One 50 and red clover (*Trifolium pratense* L) plants were sown in each pot with a planting rate of 60:40 grass-legume (Supplementary Figure 1). Based on the physicochemical characteristics of the soil and the conventional fertilization rates used in the field [urea (150 kg ha<sup>−1</sup>), diammonium phosphate (DAP) (100 kg ha<sup>−1</sup>), and KCl (180 kg ha<sup>−1</sup>)], 5 mL of a solution with the following composition was applied per pot: 31.30 mg L<sup>−1</sup> of urea, 80 mg L<sup>−1</sup> of DAP, and 25 mg L<sup>−1</sup> of KCl. The DAP fertilization was carried out one day before sowing; urea and KCl were applied once the seeds germinated. The bacterial suspensions, or the sterile saline solution for the uninoculated control, were applied directly to the soil surrounding the plant (10 mL pot<sup>−1</sup>). Plant cutting (8 cm high) was performed every 40 days after sowing.

#### Plant Tissue Analysis

The experiment was carried out for 120 days. The intercropping system was inoculated on days 0 and 20 after sowing, and cuts
were performed two times before collecting samples for analysis. Plant tissues were oven-dried at 60 °C for 48 h and shoot dry weight was recorded. The plant material was then ground in a grinder (Wiley Mill, USA) equipped with a mesh wire of a 1 mm opening. The ground material was used to determine the concentration of crude protein, neutral detergent fiber (NDF), and N content as nutritional quality indicators. NDF and crude protein were determined as described by Silva et al. (2009). Nitrogen concentration in shoot tissue (shoot N content) was determined by acid digestion (sulfuric acid) using a semi micro-Kjeldahl distiller Kjeltec™ 8100 (FOSS, Denmark) (Silva et al., 2016).

**High-Throughput Sequencing of the Soil Bacterial Community**

Rhizosphere soil was sampled from each pot, mixed between replicates, and homogenized per treatment and experiment. Rhizosphere was defined as the soil strongly adhered to the root (around 2 mm) that remained attached after vigorous shaking and crumbling. Following harvesting, the sampled soil was mixed and homogenized in a clean plastic bag. A subsample of each treatment per independent experiment (3 replicates) was placed into 10 mL transfer tubes and stored to −80 °C for further analysis. Total DNA extraction from soil was performed using the DNeasy PowerSoil® Kit (Qiagen, Germany) following the manufacturer's instructions. The quality and integrity of the DNA was verified on a 1% agarose gel, and its concentration and purity were quantified utilizing a NanoDrop™ 2000/2000c (ThermoScientific, USA). For construction of the genomic libraries, a first PCR of the V3-V4 region was performed using the primers 16S rRNA-515F and 16S rRNA-806R, modified with pre-adapters (Pacheco-Montealegre et al., 2020). Platinum™ Taq DNA Polymerase (Invitrogen, UK) was used for amplification following the PCR conditions: Buffer (1X), MgCl₂ (1.5 mM), dNTPs (0.2 mM), primers (0.2 mM), and Taq (1U). The PCR program was performed in an Eppendorf® thermocycler (Eppendorf, Germany) as follows: 94 °C for 120 s; denaturation: 94 °C for 45 s; annealing: 50 °C for 60 s, and extension: 72 °C for 90 s for 30 cycles; final extension: 72 °C for 10 min (Pacheco-Montealegre et al., 2020). The PCR products were purified using the Agentcourt® Ampure® XP (Beckman Coulter, USA). The second-round of amplification was performed to include an inline barcode and an Illumina adaptor. For each sample, 2 μL of first-round PCR were used, and the same conditions described above were used for PCR, but only for 12 cycles. The amplicon was again cleaned using Agentcourt® Ampure® XP (Beckman Coulter, USA) (Quail et al., 2009). All samples were mixed into a single batch of V3-V4 region-amplicon pools and quantified using a Qubit Fluorometer (Thermofisher, USA) employing the Quant- iT dsDNA HS Assay Kit (Thermofisher, USA) and sequenced in the Illumina MiSeq platform at the Microbial Genomics Laboratory of the Molecular Genetics and Antimicrobial Resistance Unit of Universidad El Bosque, Bogota, Colombia. The sequencing data were deposited in the NCBI Archive under Bioproject PRJNA627728.

**Data Analyses**

ANOVA (p < 0.05) and Duncan’s test were employed to analyze the plant data and the alfa diversity index. These analyses were performed using IBM SPSS Statistics 22.0.0.0 (IBM, USA) and GraphPad Prism 8 (GraphPad Software, USA).

For the analysis of the rhizosphere bacterial community, reads from 16s rRNA V3-V4 were analyzed using Qiime2 v2019-7 (Bolyen et al., 2019). Quality control and denoise were performed with DADA2 pipeline (Callahan et al., 2016), retaining reads with a length of at least 220 base pairs (forward and reverse reads). Analyses were performed determining the sampling depth as the maximum number of sequences per treatment, obtaining the rarefaction curves (Supplementary Figure 2), and using the median of the number of sequences in the treatments. An optimal number of representative or non-chimeric sequences obtained after cleaning and cutting were determined to normalize the readings for subsequent analyses. In this case, 3,197 operational taxonomic units were obtained. The taxonomic classification was performed using the qiime2 plugin q2-feature-classifier based on a pre-trained naive Bayes classifier and the reference database Greengenes version 13.8 (http://greengenes.lbl.gov).

Alpha and beta diversity analyses were estimated using the R packages: Phylloseq version 1.30 (McMurdie and Holmes, 2013), qiime2R version 0.99.13” (Bisanz, 2019), and dplyr version 1.0.3 (Wickham et al., 2020). For alpha diversity, the Shannon-Weiner index was calculated for observed sequences without rarefying microbial community data, and the interpretation was made according to McMurdie and Holmes (2014). The effect of inoculation and N application on the total structure of the soil bacterial community was assessed by a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) employing the Bray-Curtis distances with 9,999 permutations. A distance-based redundancy analysis (db-RDA) of the Bray-Curtis dissimilarity matrices using the “vegan” package was performed to identify the main plant parameters related to changes in bacterial community structure (Oksanen et al., 2019).

**RESULTS**

**Inoculation With the Endophytes Improves Forage Growth Under Reduced N Rates**

We established a greenhouse experiment on non-sterile soil, using single and mixed microbial inoculations, as well as reduced N fertilization rates, to assess the potential of *Herbaspirillum* sp. AP21 and *Azospirillum brasilense* D7 to improve the growth and quality of the ryegrass—red clover intercropping system. The system was inoculated 0 and 20 days after sowing, and samples were collected after 120 days (Figure 1A). In this experimental setting, nodulation was observed in all treatments. Shoot dry weight ranged between ~32 and ~40 g pot⁻¹ without inoculation, and plant biomass gradually increased with the fertilization rate (Figure 1B). At 50% N and 75% N, for instance, the plant biomass was ~34 g pot⁻¹ and ~37 g pot⁻¹, respectively, validating our experimental setup. We observed that at 50% N, inoculation had a positive effect on shoot biomass. At this
concentration, the largest effect was observed with AP21 and AP21 + D7, followed by D7 and the uninoculated control (Figure 1B). The present data thus suggests that AP21 has a larger potential than D7 to facilitate the growth of the intercropping system. Notably, inoculation with AP21 and AP21 + D7 at 50% N fertilization resulted in comparable plant biomass to the 100% N control. This positive effect, however, largely disappeared when the rate of N fertilization used was 75%.

Inoculation With *Herbaspirillum* sp. AP21 Results in Increased Forage Quality

We next explored the impact of both strains at the different N fertilization doses on forage quality. For this, three different parameters were assessed: crude protein, shoot N content, and neutral detergent fiber (NDF). Inoculation with AP21 led to a dramatic increase in protein content, which phenocopied the 100% N control (Figure 2A). By contrast, the effect of D7 was subtle, and no differences were seen compared with the co-inoculation. The effects due to bacterial inoculation fully disappeared when the N fertilization rate was increased to 75%. Overall, shoot N content and protein content exhibited a similar pattern. As seen before, a positive impact due to inoculation was only seen at 50% N fertilization (Figures 2A,B). The largest increase in shoot N content was also observed when AP21 was used at 50% N fertilization and resembled the N content of the 100% N control (Figure 2B). Finally, we assessed the content of NDF, which is associated with the digestibility of fiber. Smaller values of NDF indicate increased digestibility, and therefore superior forage quality. Noteworthy, the N fertilization rate did not affect NDF without inoculation (Figure 2C). At 50% N fertilization, inoculation with either AP21 or D7 resulted in a significant decrease in NDF; to our surprise, however, the co-inoculation led to opposite results. At 75% N fertilization, AP21 reduced NDF, while inoculation with D7 or AP21 + D7 only showed a slight increase compared to the control treatment.

Inoculation With the Facultative Endophytes Impacts the Rhizosphere Bacterial Community

Next, we sought to determine how inoculation impacted the bacterial diversity of the plant’s rhizosphere by using 16S rRNA metataxonomics. Results showed that the bacterial diversity (alpha-diversity), measured by the Shannon-Weiner diversity index, responded differently between the inoculated and uninoculated treatments. Inoculation slightly decreased the alpha diversity in comparison with the uninoculated treatments at 50% N fertilization rate. Likewise, at the 75% N dose, the treatments inoculated with D7 presented a lower alpha diversity ($p < 0.05$) compared with the uninoculated treatment. Altogether, these results suggest that the application of the PGPB alters the bacterial community associated with the rhizosphere, with an overall decrease in bacterial diversity (Figure 3A).

The distribution of the 20 most abundant orders was studied. In average, the predominant orders found in the treatments were: *Actinomycetales* (28.8%), *Rhizobiales* (13.5%), *Burkholderiales* (12.7%), *Pseudomonadales* (7.7%), *Sphingomonadales* (7.5%), *Gemmatimonadales* (3.5%), *Sphingobacteriales* (2.9%), and *Xanthomonadales* (2.9%) (Figure 3B). At the family level the most predominant families were *Micrococccaeae* (24.5%), *Pseudomonadaceae* (8.3%), *Sphingomonadaceae* (8.0%), *Comamonadaceae* (7.7%), *Chthoniobacteraceae* (6.0%), *Oxalobacteraceae* (5.8%), *Hyphomicrobiaceae* (4.3%) (Figure 3B).
(5.3%), *Bradyrhizobiaceae* (5.1%), and *Rhizobiaceae* (3.1%) (Supplementary Figure 3). To evaluate the impact of the treatments on the structure of the rhizosphere bacterial community, a multivariate statistical analysis (PERMANOVA) was used (Table 1). Interestingly, the inoculation with AP21 and D7, regardless of the N rate, changed the structure of the bacterial community of the rhizosphere (p < 0.05). We did not observe significant changes in the bacterial community due to N fertilization.

**Relationship Between Rhizosphere Bacteriome, and Forage Growth and Quality**

To understand how plant growth and quality correlated with the observed changes in bacterial diversity, we carried out a distance-based redundancy analysis. The db-RDA explained 53.33% of the diversity variation, where db-RDA1 accounted for 28.21% and db-RDA2 explained 25.12% of the total variance. The complete model illustrated the relationship between the variables associated with plant and bacterial diversity (p < 0.05). Besides, the angle in db-RDA included between the arrows pointing at two variables determined the correlation between the parameters: sharp angles defined positive correlations, orthogonal angles defined no correlations, and obtuse angles defined negative correlations (Figure 4; Carvalho-Estrada et al., 2020). Crude protein and shoot N content were the closest correlation parameters, followed by shoot N content, and shoot dry weight. Furthermore, we observed a negative correlation between NDF and crude protein parameters. The treatments inoculated with AP21, D7, and AP21 + D7 fertilized with 50 and 75% N formed a cluster with the positive control treatment fertilized with 100% N. This cluster was positively associated with crude protein, shoot N content, and shoot dry weight increments.

**DISCUSSION**

The implementation of technologies based on the use of PGPB has the potential to improve the environmental and economic sustainability of livestock, via an increase of forage biomass and quality (Chen et al., 2020; Santos-Torres et al., 2021).
In this study, we described the potential of two endophytic strains to promote the growth and improve the quality of an intercropping system conformed by perennial ryegrass and red clover (Figure 5). Our rationale was that inoculation with the facultative endophytes had the potential to improve plant biomass and quality by directly stimulating plant growth or synergistically interacting with the native microbiota.

The effect of microbial inoculation on plant growth was only seen when the N fertilization dose was 50%. Plant nutritional stress thus determines the potential of these microbes to promote growth. Since all plants were nodulated over the course of the experiment with native rhizobia, this implies that the incorporated microorganisms account for the observed differences. Yet, whether these inoculants improve the N fixation process itself or result in a better establishment of the symbiotic interaction legume-rhizobia remains to be determined. The positive effect of rhizosphere microorganisms to support plant growth under suboptimal growth conditions has been noted previously (Rojas-Tapias et al., 2012, 2014; Brisson et al., 2019). The ACC deaminase activity, for instance, has been strongly associated with the capacity of some microbes to facilitate plant growth under stressful conditions. The specific relation between plant nutritional stress and the plant growth potential of these microbes highlights the importance of this technology to improve forage growth and quality in environments that impose stressful conditions.

Inoculation with bacteria reduced the requirement of N and improved both biomass and quality. Inoculation with AP21 at

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**FIGURE 3** | Variations in the Shannon-Weiner diversity index (A) and relative abundance of bacterial orders (B) in the rhizosphere for inoculation with *Herbaspirillum* sp. AP21, *Azospirillum brasilense* D7, and co-inoculation with *Herbaspirillum* sp. AP21 + *Azospirillum brasilense* D7 under different rates of nitrogen fertilization. Different letters indicate significant differences based on Duncan’s test ($p < 0.05$). Each value is the mean of three replicates per treatment.

**TABLE 1** | Effects of PGPB inoculation and N fertilizer rate on the bacterial community structure expressed as R and F values obtained from a one-way PERMANOVA.

|                     | Df | SumsOfSqs | MeanSqs | F.Model | R²     | Pr(>F) |
|---------------------|----|-----------|---------|---------|--------|--------|
| **Inoculation**     |    |           |         |         |        |        |
| Residuals           | 28 | 13.3332   | 0.4762  |         | 0.9623 |        |
| Total               | 29 | 13.8556   |         | 1.000   |        |        |
| **N fertilizer rate**|    |           |         |         |        |        |
| Residuals           | 26 | 12.3984   | 0.4786  | 0.89483 |        |        |
| Total               | 29 | 13.8556   |         | 1.000   |        |        |

*Significance level ($p < 0.05$). N.S., non-significant; Df, degrees of freedom; SumsOfSqs, sum of squares; MeanSqs, mean sum of squares.
50% N, for instance, phenocopied the 100% N chemical control in terms of biomass, total N content, and crude protein. This positive effect of the inoculants is likely to be a direct consequence of microbial N fixation, as both microbes are diazotrophs, or an indirect effect due to their capacity to synthesize phytohormones or produce ACC deaminase activity (Cortés-Patiño et al., 2021). The positive effect of these microbes on plant growth and nutrition has been previously seen in other crops. Inoculation with *Azospirillum* on maize, for instance, has shown to increase grain yield compared with urea fertilization (Martins et al., 2018). In field conditions, *Azospirillum* inoculation increased corn and wheat yield by 27% and 31%, respectively, compared to the non-inoculated control (Hungria et al., 2010). Inoculation of rice plants with *Herbaspirillum* also showed the potential of this microbe to improve N acquisition under phosphorus limitation (Estrada et al., 2013). Likewise, *Herbaspirillum* inoculation with 50% of the N dose in maize crop produced yields comparable to the treatment with 100% N fertilization and increased grain quality (Alves et al., 2014).

NDF values were also affected by inoculation with bacteria. NDF is a quality parameter related to hemicellulose, cellulose, and lignin representing the entire fibrous part of the forage, which associates with increased digestibility (Raffrenato et al., 2017; Hristov et al., 2020). AP21 reduced the levels of NDF and increased the biomass and N content. Co-inoculation, to our surprise, resulted in reduced digestibility, but had a positive effect on growth. At this point, however, it is uncertain how inoculation affects NDF, since co-inoculation of AP21 and D7 resulted in opposite effects. The positive effect of various PGPB on the nutrient and dry matter content of different pastures (e.g., ryegrass, *Brachiaria*, and *Miscanthus*) and legumes (e.g., white clover) has been reported (Zaman et al., 2016; Marques et al., 2017; Akinrinlola et al., 2018; Leite et al., 2019; Liu and Ludewig, 2020).

Facultative endophytes have the potential to modulate the growth and quality of the forage by acting within the inner plant tissue, and by acting on the rhizosphere (Duarte et al., 2020). This effect can be achieved by directly facilitating plant growth and development, or by modulating the microbial structure and diversity of soil (Wang et al., 2021). We observed a decrease in microbial diversity upon inoculation with both microbes. This effect is likely a direct result of the addition of AP21 and D7 as inoculants. This shift indeed correlated with an improvement in the growth and quality of the intercropping system, as seen in other studies (Wang et al., 2018; Zhong et al., 2019; Estrada-Bonilla et al., 2021). The capacity of these microbes to colonize the plant tissue (Cortés-Patiño et al., 2021), potentially ensures that the mutualist relationship between microbes and
Inoculation of the intercropping system with *Herbaspirillum* sp. AP21 brought about changes in the microbial community, which positively correlated with an increase in plant growth and quality. The concomitant use of AP21 and 50% of the fertilization doses phenocopied the complete fertilization control. The use of this technology thus reduces the use of N fertilizers, improving the economical and environmental sustainability of the system.

Collectively, inoculation with *Herbaspirillum* sp. AP21, and *Azospirillum brasilense* D7 at a minor extent, reduced the requirements for N fertilization and improved plant quality, which concomitantly occurred with changes in the rhizosphere bacterial community. Inoculation with AP21 at 50% N fertilization, for instance, phenocopied the complete fertilization control, thereby suggesting that an equivalent to 75 kg of the N-fertilizer ha$^{-1}$ can potentially be substituted in this system by the implementation of this sustainable technology. Interestingly, the microbial inoculants had no effect on plant growth when higher doses of N fertilization were used, suggesting that stressed plants are more responsive to the positive effects of microbial inoculation. Localization and functional studies are therefore needed to understand in detail the mechanisms involved. The implementation of these ecofriendly technologies has thus the potential to reduce the implementation of N fertilizers and can potentially be used as a tool for the restoration of deteriorated agricultural soils.

### DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/?term=SAMN15498633, https://www.ncbi.nlm.nih.gov/bioproject/?term=SAMN16830199, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA627728.

### AUTHOR CONTRIBUTIONS

All authors have made a direct and intellectual contribution to the manuscript. FR-P, JM-L, and SP-D performed the greenhouse experiment, sample collection and processing, and data analysis. EC-R and GE-B carried out the experimental design. SP-D, AS, and DD-D analyzed the metagenomic data. GE-B, DR-T, EC, and FR-P wrote the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.715270/full#supplementary-material
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