Characterization of cellular, biochemical and genomic features of the diazotrophic plant growth-promoting bacterium *Azospirillum* sp. UENF-412522, a novel member of the *Azospirillum* genus

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ABSTRACT

Given their remarkable beneficial effects on plant growth, several *Azospirillum* isolates currently integrate the formulations of various commercial inoculants. Our research group isolated a new strain, *Azospirillum* sp. UENF-412522, from passion fruit rhizoplane. This isolate uses carbon sources that are partially distinct from closely-related *Azospirillum* isolates. Scanning electron microscopy analysis and population counts demonstrate the ability of *Azospirillum* sp. UENF-412522 to colonize the surface of passion fruit roots. *In vitro* assays demonstrate the ability of *Azospirillum* sp. UENF-412522 to fix atmospheric nitrogen, to solubilize phosphate and to produce indole-acetic acid. Passion fruit plantlets inoculated with *Azospirillum* sp. UENF-41255 showed increased shoot and root fresh matter, as well as root dry matter, further highlighting its biotechnological potential for agriculture. We sequenced the genome of *Azospirillum* sp. UENF-412522 to investigate the genetic basis of its plant-growth promotion properties. We identified the key *nif* genes for nitrogen fixation, the complete PQQ operon for phosphate solubilization, the *acdS* gene that alleviates ethylene effects on plant growth, and the *napCAB* operon, which produces nitrite under anoxic conditions. We also found several genes conferring resistance to common soil antibiotics, which are critical for *Azospirillum sp.* UENF-412522 survival in the rhizosphere. Finally, we also assessed the *Azospirillum* pangenome and highlighted key genes involved in plant growth promotion. A phylogenetic reconstruction of the genus was also conducted. Our results support *Azospirillum sp.* UENF-412522 as a good candidate for bioinoculant formulations focused on plant growth promotion in sustainable systems.

INTRODUCTION

Modern agriculture production strongly relies on synthetic fertilizers and pesticides to achieve high productivity levels, which are often a matter of environmental and health concerns [1]. The development of bioinoculants has been considered an alternative to reduce the use of such synthetic compounds [2]. These inoculants typically have one or more strains of plant growth-promoting rhizobacteria (PGPR), which compose a heterogeneous group of bacteria that exert mutualistic interactions with plants [3], often playing key roles in the root microbiome [4]. The rhizosphere is the fraction of soil directly influenced by roots exudates [5], which modulate bacterial diversity across the endorhizosphere, rhizoplane and ectorhizhosphere (reviewed by Ahkami et al. [6]). Several *Azospirillum* isolates have been reported as PGPR, typically as soil-borne bacteria that are competent rhizosphere colonizers [7, 8].

The genus *Azospirillum* belongs to the *Rhodospirillaceae* family, which is mainly constituted of aquatic genera. Nevertheless, Azospirilla are mostly soil bacteria that coevolved with vascular plants [9]. Following its original description [10], multiple plant-associated *Azospirillum* species have been shown to perform direct nitrogen fixation [11, 12], phosphate solubilization [13], drought and salt stress alleviation [14, 15], root development promotion [16], among other processes [17]. These desirable properties have led several *Azospirillum* strains to be used as part of commercial soil inoculants [18]. Genes associated with these features have been identified in several publicly available *Azospirillum* genomes, such as *nif* [19], ACC-deaminase (*acds*) [20, 21], *pqq* [22], and indole acetic acid biosynthesis genes (e.g., *iaaH*, *iaaM* and *ipdC*) [23, 24].

Recently, our research group has isolated several plant growth-promoting bacterial strains from vermicompost [25, 26] and from the rhizosphere of tropical fruit trees. Among these strains, *Azospirillum sp.* UENF-412522 was isolated from passion fruit rhizoplane. Here we report *in vitro* and *ex vitro* plant growth promotion capabilities, biochemical tests, microscopy analysis of root colonization, whole-genome sequencing, and comparative genomic analyses to investigate the plant growth promotion properties of this isolate, as well as to uncover genes involved in other ecophysiological processes. Together, our results support the potential of *Azospirillum sp.* UENF-412522 to be used in bioinoculant formulations.

RESULTS AND DISCUSSION

Identification of the isolate and assessment of plant growth promotion properties

The strain UENF-412522 was isolated from passion fruit rhizoplane and formed a pellicle typical of diazotrophic bacteria on N-free semisolid NFb medium. It is a Gram negative, slightly curved rod-shaped bacterium with apparently no polymorphic 1.2 x 0.7 µm cells. In order to characterize the isolated strain, a fragment of the 16S rDNA gene was amplified by polymerase chain reaction (PCR); this sequence was deposited in Genbank under the accession KU836626.1. The 16S rDNA maximum likelihood (ML) phylogenetic reconstruction (Figure S1) has not allowed us to classify the strain at the species level. Nevertheless, it is clear that it belongs to the *Azospirillum* genus, leading us to name it *Azospirillum sp.* UENF-412522.

Given the evidence supporting *Azospirillum* sp. UENF-412522 as a novel *Azospirillum* species, we used an API 50 CH system and found that it grows on 18 out of 49 tested carbon sources: glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, methyl-beta-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, amygdalin, aesculin ferric citrate, D-

melibiose, glycogen, xylitol, D-xylose, D-fucose. On the other hand, this strain was unable to grow on: erythritol, L-xylose, D-adonitol, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, Methyl-alpha-D-mannopyranoside, Methyl-alpha-D-glucopyranoside, N-acetylglucosamine, arbutin, salicin, D-cellobiose, D-maltose, D-lactose (bovine origin), D-saccharose (sucrose), D-trehalose, inulin, D-melezitose, D-raffinose, amidon (starch), gentiobiose, D-turanose, D-tagatose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate. Comparison of carbon utilization patterns of *Azospirillum* sp. UENF-412522 and those of the type strains of *Azospirillum lipoferum* ATCC 29707 [10], *Azospirillum doebereinerae* DSM 13131 [27] and *Azospirillum brasilense* ATCC 29145 showed that four C-sources (i.e. D-ribose, D-mannitol, D-sorbitol and N-acetylglucosamine) could be used to discriminate these isolates (Table S1).

Next, we performed *in vitro* tests that would account for the ability of *Azospirillum* sp. UENF-412522 to promote plant growth. The N_2-fixation was supported by positive growth on N-free semisolid medium and subsequently confirmed by the amplification of *nifH* (Figure 1A). We also confirmed the nitrogenase activity by using the acetylene reduction assay, which showed a rate of 28.3 $\pm$ 5.1 nmol.h^{-1} ethylene (Figure 1B).

Indole-acetic acid (IAA) production was investigated in DYGS medium, with and without L- tryptophan, resulting in IAA concentrations of 51.34 $\pm$ 1.1 µg.mL^{-1} and 37.35 $\pm$ 3.5 µg.mL^{-1}, respectively (Figure 1C), supporting the capacity of *Azospirillum* sp. UENF-412522 to synthesize this important plant hormone.

We also tested whether *Azospirillum* sp. UENF-412522 is able to solubilize Araxá P-rock and calcium phosphate in solid medium. We found that this strain formed a halo with a solubilization index (SI) of 3.33 $\pm$ 0.05 and 1.87 $\pm$ 0.11 for Araxá P-rock and calcium phosphate, respectively (Figure 1D). We confirmed the P solubilization activity using a liquid medium assay, which showed 147.5 $\pm$ 3.9 mg.L^{-1} and 52.2 $\pm$ 8.6 mg.L^{-1} solubilized P from Araxá P-rock and calcium phosphate (Figure 1E), respectively. Conversely, zinc solubilization was not detected, likely because of zinc sensitivity in this strain.

The plant growth-promoting effects of a given bacterial strain often result from several concerted processes. Therefore, we inoculated passion fruit plantlets with *Azospirillum* sp. UENF-412522 and found a clear growth increment in inoculated versus non-inoculated plantlets. After 10 days, inoculated plantlets had a significant increase in height (54.5%), root fresh matter (88.6%), root dry matter (61.4%), shoot fresh matter (13.8%) and root length (40%). No significant difference was found for shoot dry matter (Figure S2).

Under gnotobiotic conditions, inoculated plantlets showed a larger diazotrophic bacteria population associated with rhizosphere, rhizoplane, and roots (Figure S3). For rhizosphere and rhizoplane compartments, the population density was significantly greater than control plantlets (approximately 1.5 $\log_{10}$ bacteria cell per g of soil or root). The bacterial population inside the plantlets showed no difference between inoculated and non-inoculated plantlets, further supporting that *Azospirillum* sp. UENF-412522 preferentially colonizes the rhizosphere/rhizoplane. In parallel, samples from seedlings inoculated with *Azospirillum* sp. UENF-412522 at 1 and 7 days after germination (d.a.g.) were viewed under SEM (Figure 2), confirming the root surface as the preferential colonization site. After radicle emergence, single cells were noted at the bottom region of the radicle (Figure 2A), and bacteria aggregates can be seen in the vicinity of the crack zone between the radicle and the tegument (Figure 2C-D). Interestingly, sparse stomata-like structures were seen at the radicle, which seems to be an

infection point for the bacteria (Figure 2B). Bacteria cells interact with the root surface mainly by apolar attachment and by fibrillar material anchoring the plant cell-wall, establishing an aggregate network (Figure 2E). At seven d.a.g., bacteria successfully colonized the root hair zone (Figure 2F) and the elongation/differentiation zone (Figure 2G) of the main developed root.

Genome assembly and strain identification

The promising *in vitro* and *ex vitro* results prompted us to sequence the *Azospirillum sp.* UENF-412522 genome. We used an Illumina HiSeq 2500 instrument (paired-end mode, 2 × 100 bp reads). Sequencing reads were quality filtered and assembled with SPAdes (see methods for details). The assembly consists of 101 contigs (length ≥ 500 bp), encompassing 7,360,543 bp, with a 67.92% GC content, N50 and L50 of 175,376 bp and 13 respectively. The genome harbors 6,508, 71 and 8 protein-coding, tRNA and rRNA genes, respectively.

Genome relatedness within the *Azospirillum* genus was computed using the average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) of all *Azospirillum* spp. genomes deposited in RefSeq (n = 48). ANI values were used to build a genome-to-genome distance matrix and a neighbor-joining dendrogram (Figure 3A). This analysis clearly shows distinct clusters comprising *A. brasilense* and *A. tiophilum* isolates. In contrast, none of the remaining strains formed a consistent clade. Further, *A. lipoferum* RC and 4B isolates did not cluster together, indicating that they do not belong to the same species. We found greater genetic diversity in the isolates that do not belong to these clusters (Figure 3B). Among those is *Azospirillum* sp. UENF-412522, which does not cluster with any other known strain, suggesting that it represents a novel *Azospirillum* species.

Even though the *Azospirillum* genus has more than 15 described species, genomic data are biased towards *A. brasilense*, which accounts for 37% (19 isolates) of the available genomes (Figure 3). We also noticed that five isolates have similar names and share more than 99.9% and 70% in ANI and dDDH analyses, respectively. We removed these redundant genomes from the downstream analyses and used GCF_001315015, GCF_003119195, GCF_003119115, and GCF_004923295 as representatives of *A. brasilense* Sp7, *A. brasilense* Sp245, *Azospirillum* sp. TSH58 and *Azospirillum* sp. TSH100 respectively. Hence, the dataset used in the next sections comprises 43 publicly available genomes and that of *Azospirillum* sp. UENF-412522 genome.

Pangenome analyses of the *Azospirillum* genus

The pangenome is the complete gene repertoire of a given clade (e.g., a species) [28]. We employed a minimum identity threshold of 50% for gene family identification in *Azospirillum*. The pangenome comprises 42,515 genes, including a core genome (i.e. genes present in all isolates) of only 771 genes, likely because of the high diversity, prevalence of genomic rearrangements, and low synteny of this genus [9]. We clustered the isolates based on their gene presence/absence patterns and confirmed the high genomic heterogeneity across the genus (Figure 4A). Our results also support an open pangenome, as it grows with the addition of new genomes (Figure 4B). Similar results were previously shown in *Alphaproteobacteria* [29], who found 220 core genes across 27 in *Novosphingobium* spp. genomes. The *Azospirillum* core genome reported here is substantially greater than that, which could be at least partially explained by the fact that the vast majority of the isolates studied here were obtained from soil and plant tissues. *Azospirillum* sp. RU37 (GCF_900188305) and *Azospirillum* sp. RU38E (GCF_900188385) share the majority of accessory clusters. The same pattern is observed for

Azospirillum sp. TSH20 (GCF_003115935) and *Azospirillum* sp. TSH7 (GCF_003115945). In both cases, the genomes with unusually high accessory genome similarity also exhibit 100% of ANI and dDDH, indicating that they belong to the same strain.

Next, we analyzed the unique gene complement of *Azospirillum* sp. UENF-412522. Out of the 18,065 unique genes inferred by Roary, 405 belonged to this strain. Among these genes, we identified creatinine amidohydrolase *crnA* (EI613_12750), an enzyme involved in the degradation of creatinine [30], which has been reported as replication-impairing molecule in bacteria [31]. The gene 3-alpha-hydroxysteroid dehydrogenase *hsdA* (EI613_09215), involved in steroid catabolism, is also exclusive to this strain and might assist *Azospirillum* sp. UENF-412522 to use steroids as a carbon source [32]. We also found the two component system *prsDE* (EI613_31600-31605) as exclusive to this strain. PrsDE produces a low molecular weight succinoglycan, which might assist in plant colonization by *Azospirillum sp.* UENF-412522 [33].

Plant growth promotion genes

Plant growth modulation by bacteria is a complex process [34]. Several bacterial genes are often associated with direct beneficial effects, such as those responsible for biological nitrogen fixation (BFN), root growth enhancement, phosphate solubilization, and deviation of the ethylene biosynthesis pathway towards ammonia and α-ketobutyrate. We assessed the presence of genes that are likely involved in direct plant growth promotion across *Azospirillum* strains, with a particular focus in *Azospirillum sp.* UENF-412522. This procedure was guided by a maximum likelihood phylogenetic tree built with the protein sequences encoded by the core-genome, which was combined with a gene presence/absence matrix (Figure 5). Below we discuss these genes in light of the mechanisms by which they promote plant growth.

BNF is a major plant growth promotion mechanism present in several *Azospirillum* strains [8, 35]. This process relies on the nitrogenase enzyme complex, which reduces atmospheric nitrogen (N_2) to ammonium (NH_4). The *nifHDK* genes encode, respectively, a nitrogenase iron (Fe) protein, a nitrogenase molybdenum-iron (MoFe) protein alpha chain, and a nitrogenase MoFe protein beta chain. The FeMo co-factor (FeMoCo) present in the MoFe protein binds N_2, while the Fe protein uses the energy from ATP hydrolysis to drive the reduction of N_2 to NH_4 by FeMoCo [36]. We used the presence of the *nifHDK* genes to predict the nitrogen fixation capacity across *Azospirillum* genomes. All the analyzed genomes have these genes, except for *Azospirillum* sp. RU37A, *Azospirillum* sp. RU38E and *Azospirillum* sp. L-25-5w-1 (Figure 5). *Azospirillum* sp. UENF-412522 possesses the *nifHDK* (EI613_05225-05215) operon and a *nifDK* (EI613_05195-05200) operon, resembling the distribution found in most of the other *Azospirillum* genomes containing *nifHDK*. Some exceptions to this structure are found in *A. brasilense* Sp245, which possesses four *nifDK*, including vanadium nitrogenase variants [37]; *Azospirillum* sp. B506 presents a single *nifK*, and; *Azospirillum* sp. RU37A and *Azospirillum* sp. RU38E have two *nifD*. The presence of more than one *nifHDK* increase nitrogen fixation to the bacteria and associated plants. However, given that BNF is energetically expensive, the selective advantage of such increased activity remains unclear.

Most of the soil phosphate is insoluble and hence unavailable to direct plant absorption [38]. Several PGPRs promote plant growth by releasing organic acids that chelate divalent cations (e.g., Ca^{2+}) in poorly soluble mineral forms (e.g., hydroxyapatite), increasing P availability [39]. Gluconic acid secretion is the best-characterized mechanism of phosphate solubilization, that is performed by pyrroloquinoline quinone (PQQ)-dependent glucose

dehydrogenase (GDH) [40]. We used the presence of the genes encoding the PQQ co-factor as a proxy to understand P solubilization through GDH-PQQ in *Azospirillum* spp. The *pqq* genes found in *Azospirillum* sp. were *pqqABCDE* (EI613_19670-19690). While *Azospirillum* UENF-412522 and *A. halopraeferens* DSM3675 harbor the complete operon, all other strains lack *pqqA* (Figure 5). The *pqq* operon is absent in three strains (*Azospirillum* sp. RU37A, *Azospirillum* sp. RU38E, *Azospirillum* sp. L-25-5w-1). *A. brasilense* FP2 lacks *pqqA* and *pqqD*. Interestingly, phytase genes were only found in the pqq-lacking strains *Azospirillum* sp. RU37A and *Azospirillum* sp. RU38E, indicating that the capacity to mobilize P from organic compounds displaced PQQ-dependent P solubilization in these bacteria.

Although *pqqA* has been regarded as non-essential for PQQ biosynthesis [41], other studies support its role as the backbone for PQQ biogenesis [42]. PqqA binds to PqqD, prior to PqqAD interaction with PqqE [43]. The crucial role of *pqqA* and *pqqB* was also confirmed by knockout mutants of *Rahnella aquatilis* HX2, which showed decreased biocontrol and mineral P solubilization [44]. A direct correlation between *pqqB* and *pqqF* expression and PQQ production has also been shown [45]. Further, the same authors also reported that *Pseudomonas putida* KT2440 *pqqF* is controlled by an independent promoter and terminator, allowing this gene to modulate PQQ levels [45]. The presence of incomplete *pqq* operons in many *Azospirillum* strains allows one to speculate that these isolates have other genes to compensate for the absence of *pqqA*, a hypothesis that warrants experimental validation. Further, upstream of *pqqABCDE*, we found an alcohol dehydrogenase ADH IIB *qbdA* (EI613_19655) and its associated regulator *amgR* (EI613_19660), which are possibly related to acetic acid production mediated by PQQ [46]. This operon also has a a gene encoding a hypothetical protein containing a CXXCW motif, present in several soil bacteria, which might be associated with PQQ catabolism (TIGR03865). Hence, the *pqqABCDE* operon might be co-regulated with *qdbA* in *Azospirillum* sp. UENF-412522.

Ethylene is a plant hormone with a central role in senescence and growth of leaves, flowers, and fruits [47]. Under stress conditions, ethylene can impair plant cell elongation [48]. Ethylene is synthesized by the oxidation of 1-aminocyclopropane-1-carboxylate (ACC) by ACC oxidase. ACC can be broken down into α-ketobutyrate and NH_3 by ACC deaminase [49], an enzyme produced by several PGPR that induce plant growth by lowering ethylene levels under stress conditions. We identified the ACC deaminase gene (*acdS*, EI613_03680) in *Azospirillum* sp. UENF-412522 and other 11 *Azospirillum* sp. strains, supporting that their association with plants can alleviate ethylene-mediated growth inhibition. Importantly, we have not found *acdS* in *A. brasilense* strains.

Considering its gene complement, *Azospirillum sp.* UENF-412522 has a significant potential to promote root growth. Despite the strong experimental evidence supporting IAA biosynthesis, we found none of the classic auxin biosynthesis genes (i.e., *ipdC*, *iaaH* and *iaaM*) [50-52], which prompted us to investigate other IAA biosynthesis pathways [51] in this strain. Interestingly, we found a gene encoding a nitrilase (*nit*, EI613_03300), which is 74% similar that encoded by a *nit* that converts indole-3-acetonitrile (IAN) directly into IAA or into indol-3-acetamide (IAM) in the rhizobacterium *Pseudomonas* sp. UW4 [53, 54]. In the latter pathway, the conversion of IAM into IAA requires the action of IAM-hydrolases, for which we found two candidate genes, EI613_04545 e EI613_07015. These genesare similar to amide hydrolases from *Rhodococcus* sp. (Q53116) [55, 56] and *Agrobacterium fabacearum* (ADY67766.1) [57], respectively. The apparent absence of tryptophan monooxygenase genes, which catalyze the conversion of Trp into IAM, indicates that *Azospirillum* sp. UENF 412522 produces IAA through

an interplay between the IAN and IAM pathways, as reported in *Rhizobium* spp. and *Bradyrhizobium* spp. [53, 58]. Nevertheless, the initial steps in the production of IAN in *Azospirillum* sp. UENF 412522 are yet to be elucidated. In contrast, all *A. brasilense* strains possess the gene *ipdC* (Figure 5), supporting the ability of this species to synthesize IAA by the indole-3-pyruvate pathway, as experimentally demonstrated [59, 60]. Another eight *A. brasilense* strains possess nitrile hydratase (*nthB*) and *iaaH* genes, which contribute to IAA production by the conversion of IAN to IAM, and from IAM to IAA, respectively.

We also investigated *Azospirillum sp.* UENF-412522 genes that might promote root development through the emission of nitrite and nitric oxide. *A. brasilense* is known for secreting nitrite as a product of nitrate respiration [61, 62]. *Azospirillum sp.* UENF-412522 has a nitrate reductase operon *napCAB* (EI613_10540-10550). *napA* encodes a periplasmic nitrate reductase, while *napCB* encodes electron transfer subunits necessary for napA activity. This operon was proposed as an alternative electron acceptor for oxygen in *A. brasilense* sp. 245 periplasm [63] and is possibly responsible for nitrate respiration in *Azospirillum sp.* UENF-412522. The nitrite generated by nitrate respiration can be exported to the environment, absorbed by the plant root, and reduced to nitric oxide. Under acidic pH, nitrite can be reduced to nitric oxide without enzymatic action [64] and absorbed by root cells [65]. The downside of nitrate respiration to plant growth promotion is the reduction of available nitrogen in the system and the production of greenhouse gases. Hence, we hypothesize that *Azospirillum* sp. UENF-412522 nitrogen fixation contributes to nitrate respiration and plant absortion.

Most known *Azospirillum* strains are diazotrophs. N_2-fixation requires a microaerophilic or anoxic environment because nitrogenase metalloclusters are sensitive to oxygen. Previous works in *Azospirillum* proposed that complete oxygen depletion inhibited nitrogenase activity [66], while others suggest otherwise [67]. *Azospirillum sp.* UENF-412522 was isolated from the rhizoplane, where low oxygen conditions are rather common [68]. Given the genomic investigations reported here, we hypothesize that P solubilization, root growth enhancement by nitrate respiration, and N_2 fixation are dependent of an anoxic environment and might be interconnected in the soil-root system (Figure 6). Under low oxygen availability, nitrate is used as final electron acceptor in anaerobic respiration, producing nitrite, which is reduced to nitric oxide under low pH in the periplasm. Such acidic environment can be harnessed by the gluconic acid generated by the PQQ-dependent glucose dehydrogenase. Nitric oxide or nitrite would then be exported to the rhizosphere, stimulating root growth. Finally, gluconic acid is also exported to the rhizosphere, solubilizing phosphate for the root cells uptake.

Antibiotic resistance genes

Azospirillum sp. UENF-412522 has several antibiotic resistance genes, such as those conferring resistance to: fosmidomycin (*fsr*, EI613_04505) [69], bicyclomycin (*bcr*, EI613_29470, EI613_14405) [70], bacitracin (*uppP/BacA*, EI613_27660) [71], tabtoxin (*ttr*, EI613_22745) [72], phenazine (*ehpR*, EI613_22490) [73], and tetracycline (*tetA*, EI613_26440) [74]. We also found multiple drug resistance genes (*mdtABCE*, EI613_19480-19470) [75], *acrAB* (EI613_25875-25880) [76], and *norM* (EI613_15640) [77]. Interestingly, most of these antibiotics, such as bicyclomycin [78], phenazine [79], bacitracin, and tetracycline [80], are commonly produced *Streptomyces*, which is abundant in the soil, where it plays a key role in plant organic matter decomposition [81, 82]. The presence of genes conferring resistance against *Streptomyces*

antimicrobials is likely important to the survival of *Azospirillum sp.* UENF-412522 in the rhizosphere.

CONCLUSION

In the present study we described *Azospirillum sp.* UENF-412522, a new *Azospirillum* plant growth-promoting species. We experimentally demonstrated that this bacterium is able to fix nitrogen, produce IAA, solubilize mineral P, and recognize and colonize seed-root surfaces. This strain is equipped with genes involved in BNF, P solubilization, ethylene catabolism, promotion of root development, and antibiotic resistance in the rhizosphere. Collectively, our results support the biotechnological potential of *Azospirillum sp.* UENF-412522 to be part of bioinoculant formulations for sustainable agriculture. We also propose a system involved in the partitioning of the fixed nitrogen between nitrate respiration and plant absorption, which is likely critical in plant-growth promotion under microaerophilic or anoxic conditions. Finally, this study emphasizes the importance of comprehensive studies using genomic, microscopy, microbiological, and biochemical approaches to characterize novel *Azospirillum* species outside of the *A. brasilense* clade, which could reveal key unlock the genus diversity and open novel possibilities agricultural applications.

METHODS

Bacterial isolation and DNA purification

Passion fruit (*Passiflora edulis* f. *edulis*) root samples were obtained at the Integrated Agroecological Production System (SIPA), Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil (coordinates 22.7635°S 43.6886°W) and taken to the laboratory in a sterilized plastic bag kept in ice. Rhizoplane bacteria isolation was performed by an adapted version of Bramwell et al. (1995)[83]. In summary, root axis segments (10 g fresh weight) were sealed from both tip sides with paraffin, gently washed in tap water, and transferred to a 250 mL glass flask containing 90 mL of saline solution (NaCl, 0.9%). Roots were kept under agitation for 5 min at a rotatory shaker (200 rpm at 30 °C) to remove soil particles. After that, the same sealed root segment was carefully transferred to another 250 mL glass flask containing 90 mL of sterile saline (NaCl, 0.9%) solution plus 10 g of autoclaved sieved sand, where remained under agitation for 10 min at rotatory shaker (150 rpm at 30 °C) to detach bacteria cells from root surfaces (rhizoplane bacteria fraction). After sand decantation, 10 mL of the suspension were transferred to 90 mL of sterile saline solution (10^{-1} dilution), which were used to prepare successive dilutions until 10^{-6}. Three independent 20 µL aliquots of all serial dilutions (10^{-2} to 10^{-6}) were inoculated into glass vials (16 mL volume) containing 5 mL NFb nitrogen-free semisolid medium (composition in g L^{-1}): malic acid, 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$. $2H_2O$, 0.02; micronutrient stock solution ($CuSO_4.5H_2O$, 0.04; $ZnSO_4.7H_2O$, 0.12; H_3BO_3, 1.40; $Na_2MoO_4.2H_2O$, 1.0; $MnSO_4$. H_2O, 1.175. Complete volume to 1,000 mL with distilled water), 2 mL; bromothymol blue (5 g L^{-1} in 0.2 N KOH), 2 mL; FeEDTA (solution 16.4 g L^{-1}), 4 mL; vitamin stock solution (biotin, 10 mg; pyridoxal-HCl, 20 mg in 100 mL distilled water), 1 mL; KOH, 4.5 g; distilled water to bring the final volume to 1,000 mL and adjust pH to 6.5. A quantity of 1.6 g agar L^{-1} was added to prepare the semisolid medium [84].

Inoculated NFb vials were incubated in a growth chamber at 30 °C for 5 to 7 days. The growth of diazotrophic bacteria was characterized by the formation of a subsurface white pellicle. Using a loopful, a sample of the pellicle from the last positive dilution was inoculated in

a fresh NFb N-free semisolid medium and incubated under the same conditions described above. This process was subsequently conducted for three times and the last new pellicle formed was streaked onto DYGS solid medium plates for colony purification. The DYGS composition (g L^{-1}): glucose, 2.0; malic acid 2.0; peptone, 1.5; yeast extract, 2.0; K_2HPO_4, 0,5; $MgSO_4.7H_2O$, 0.5; glutamic acid, 1.5, pH is 6.5. The purified isolate was stored in 10% glycerol at -80 ºC, under the code UENF-412522, as a diazotrophic bacterium associated with the passion fruit rhizoplane.

For DNA extraction, 100 µL of the stored bacteria were transferred to a glass tube containing 5 mL DYGS liquid medium and grown in an orbital shaker at 30 °C and 180 rpm for 48 hours. After that, 20 µL were transferred to the same medium and grown at the same conditions for 24 h. An aliquot of 200 µL was taken, and bacterial genomic DNA was extracted using the QIAamp kit (QIAGEN) following the manufacturer's instructions. DNA was quantified in a 3000 NanoDrop (Thermo Scientific USA). DNA quality was analyzed using a 0.8% agarose gel stained with gel red.

To obtain an initial taxonomic assignment, PCR was conducted with the primers 27F (5`-AGAGTTTGATCMTGGCTCAG 3`) and 1492R (5'-TACGGYTACCTTGTTACGACTT-3`). These reactions were performed using 50 ng of genomic DNA in 25 µL final reaction volume, containing: 2 µL of dNTPs (20 mM each) 2.5 µL of 10 x enzyme buffer, 0.75 µL 50 mM $MgCl_2$, 2.5 µL of each primer (5 mM), 0.3 µL Taq polymerase (5 U/µL). Amplification was performed in a 96-well thermocycler Veriti model (Applied Biosystems), programmed at 9°C for 3 min, 30 cycles of amplification (94°C for 1 min, 55 °C for 30 s, 72 °C for 30 s) at 72 °C for 10 min. The sequencing reactions were performed using the Big Dye Terminator Sequencing Kit-Cycle Sequencing Ready ABI Prism version 3 (Life Technologies, USA), following manufacturer's recommendations. Sequencing was performed on ABI Sequencer model 3130 (Applied Biosystems).

Plant growth promotion traits of UENF-412522 bacterial strain

nifH gene detection

The *nifH* presence were assessed by PCR, with the primers PolF (5'TGCGAYCCSAARGCBGACTC 3`) and PolR (5'ATSGCCATCATYTCRGCCGA 3') [85]. In each reaction, we used 1 µL dNTPs (200 mM each), 5 µL of 10X buffer, 4 µL of 25 mM $MgCl_2$, 0.5 µL of each primer (10 mM), 0.25 µL Taq polymerase (5 U/ µL) and 50 ng DNA, in a final volume of 50 µL. The amplification conditions were denaturation at 94 °C for 5 minutes, then 30 cycles (94 °C for 1 min, 55 ºC for 1 min, 72 °C for 2 minutes) and a final extension step (72 °C for 5 min). PCR products were inspected on 1.5% agarose gels. Bp-100 DNA ladder (Invitrogen) was used as a molecular weight marker.

Acetylene reduction activity assay (ARA)

The *in vitro* nitrogen fixation ability of UENF-412522 was evaluated using the acetylene reduction assay (ARA), as previously described by Baldani [84]. The strain was grown in 16 mL glass vials containing 5 mL of N-free NFB semisolid medium, as described above. These flasks were inoculated with 20 µL of the bacterial inoculum suspended in sterile water, adjusted to an optical density (OD) of 1.0, and incubated at 30 °C for 48 h. After pellicle formation, the vials were closed with a sterilized pierceable rubber stopper of the subseal type. Syringes were used to remove 1 mL of air and to inject 1 mL of acetylene in each vial. The flasks were incubated at 30 °C for 1 h, and 1 mL of the gas phase was analyzed on a gas chromatograph with flame ionization (Perkin Elmer), to determine the ethylene concentration in the sample.

Phosphorus and zinc solubilization

Phosphate solubilization was independently carried out using 1 $g.L^{-1}$ Araxá rock phosphate [$Ca_{10}(PO_4)_6F_2$] and tricalcium phosphate [$Ca_3(PO_4)_2$]. Bacterial cultures grown in DYGS liquid medium adjusted to an optical density of 1.0 were inoculated (20 µL) at the center of the petri dish with agar containing Pikovskaya medium. After this procedure, they were incubated at 30 °C for 7 days. The ability of the isolates in solubilizing P was evaluated by measuring the translucent halo, according to Kumar and Narula formula [86], in which S.I. (solubility index) = halo Diameter (mm)/colony diameter (mm). We conducted three biological replicates of this assay.

We also assessed P solubilization in Pikovskaya liquid medium supplemented with 1$g.L^{-1}$ of the Araxá rock phosphate or $Ca_{10}(PO_4)_6F_2$ was used to quantify the soluble P. Aliquots of 100 µL of bacterial cultures grown in DYGS liquid medium were transferred to 50 mL tubes containing Pikovskaya medium and kept for 7 days under constant agitation at 150 rpm in a rotatory shaker at 30 °C. After this period, cultures were centrifuged at 3200 rpm for 15 minutes, and the supernatant was used for pH determination and quantification of soluble P.

Zinc solubilization was carried out according to Intorne et al [87]. Aliquots of 20 µL of bacterial strains cultivated for 24 h at 30 °C in DYGS liquid medium, adjusted to an optical density of 1.0, were inoculated at the center of the petri dish containing Saravanan medium with agar supplemented with 1$g.L^{-1}$ of ZnO and incubated at 30 °C for 7 days. After this period, the ability to solubilize ZnO was also performed according to Kumar and Narula [86]. We conducted three biological replicates of this assay.

Indole acetic acid (IAA) production

Bacterial isolates were cultured in liquid DYGS medium for 24 h at 30 °C and transferred (25 µL) to test tubes containing 5 mL of the same medium in the presence and absence of L-tryptophan (100 $mg.L^{-1}$) before incubation in the dark for 72 h at 30 °C, shaking at 150 rpm. The cultures were then transferred to 2 mL tubes and centrifuged at 10,000 rpm for 10 min, and the supernatant transferred to a test tube with 2 mL of Salkowski reagent [88]. Tubes were incubated for 30 min in the dark. The production of IAA was evaluated by the presence of pink color in the tubes, and the color intensity was determined with a spectrophotometer at a wavelength of 530 nm. IAA concentrations were measured using a calibration curve.

Biochemical API 50 CH/E test

For the metabolic characterization of bacterial isolates, we used the API 50 CH/E test kit (bioMérieux SA Marcy- l'Etoile/France), which evaluates the ability of the isolates to conduct fermentation of 49 carbohydrates and derivatives. To this end, the isolates were cultured in DYGS medium for 24 h at 150 rpm. After this period, the isolates were suspended in autoclaved water and adjusted to optical density 1.0; 2 mL of this suspension were added to the CHL medium (bioMérieux) and transferred to galleries containing different substrates. Each dome was sealed with a sterile drop of mineral oil and incubated in an environmental chamber at 37 °C. Reads were recorded after 24 h and 48 h after inoculation, and eventual production of organic acids during incubation shifts the pH indicator from red to yellow.

Plant growth promotion assay under greenhouse conditions

A greenhouse assay was carried out to evaluate the effect of UENF-412522 inoculation in plants. To perform it, passion fruit seeds of the cultivar Yellow master FB200 were disinfected in 1% sodium hypochlorite solution for 20 minutes, followed by three rinses in autoclaved distilled water and then transferred to germitest paper moistened with autoclaved water. Then, the seeds were incubated in a growth chamber at 30 °C, for 16 h in the light and 8 h in the dark. After germinating, these seeds were transferred to styrofoam trays containing autoclaved plant substrate under greenhouse conditions. After 30 days of transplanting, seedlings of approximately 6 cm were transferred to a 300 mL plastic bag containing the same autoclaved plant growth substrate. The inoculum of the UENF-412522 strain was prepared in liquid DYGS medium after 40 h of growth in a rotatory shaker at 30 °C and 150 rpm. Bacterial suspensions containing approximately 10^8 cells.mL^{-1} (O.D. = 1.0 at 600 ηm) were inoculated on the region of the plant's neck, and plants were transferred to the greenhouse for 30 days. The assay was set up in a randomized block design, two treatments (inoculated and non-inoculated plantlets) and six replications. The control plants received 1 mL of the liquid DYGS medium containing lysed UENF-412522 cells.

Plant root colonization assay

We evaluated plant-bacteria interaction by determining the population size and root colonization by scanning electron microscopy (SEM). For this, passion fruit seeds of the Yellow cultivar master FB200 were disinfected and placed to germinate as described above. After germination, the seedlings were transferred to a test tube containing autoclaved vermiculite. Then, 1 ml of bacterial suspension containing approximately 10^8 cells mL^{-1} was inoculated on the seedling neck. Seedlings were kept for seven days after inoculation (d.a.i) in a culture room under a temperature of 30ºC (16 h in the light and 8 hours in the dark). For SEM, root samples were collected at 1 and 7 days after the inoculation of the bacterial suspension. Samples for SEM were prepared according to Baldotto et al. [89] and viewed at Zeiss DSEM 962 at 15 Kv voltage in secondary electron detector mode. The bacteria count at rhizosphere, rhizoplane, and roots was performed at seven d.a.i., as described above. The control consisted of seedlings inoculated with the DYGS medium.

Genome sequencing and assembly

The sequencing procedures and basic data processing were performed, as previously described [25]. In summary, paired-end libraries were prepared using the TruSeq Nano DNA LT Library Prep (Illumina) and sequenced on a HiSeq 2500 instrument at the Life Sciences Core Facility (LaCTAD; UNICAMP, Campinas, Brazil). Sequencing reads (2 × 100 bp) had their quality checked with FastQC 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality filtering was performed with Trimmomatic 0.35 [90], and reads with average quality below 30 were discarded. The genome assembly was performed using SPAdes 3.8 [91] and assembly metrics evaluated by QUAST 3.0 [92].

Genome annotation and phylogenetic analysis

The assembled genome was annotated with the NCBI Prokaryotic Genome Annotation Pipeline [93]. The *Azospirillum* sp. UENF-412522 genome was deposited on Genbank under the BioProject PRJNA508400. *Azospirillum* spp. genomes available in RefSeq [94, 95] were downloaded (n = 48, as of June 03 2019) and protein sequences were predicted using PROKKA

1.13.3 [96]. We searched for proteins encoded by genes potentially involved in plant growth promotion using a blastp [97] search on the SwissProt database, with minimum coverage and similarity thresholds of 70% and 60%, respectively. All-against-all average nucleotide identity (ANI) was computed using pyani 0.28 [98]. Pan-genome analysis was performed with Roary 3.12.0, with a minimum identity threshold of 50% [99]. Multiple sequence alignments were performed using Muscle 3.8.30 [100] and phylogenetic reconstructions performed with RAxML 8.2.10 [101].

AUTHOR CONTRIBUTIONS

Conceived the study: FLO, TMV; Funding and resources: FLO, TMV; Data analysis: GLR, FPM, RKG, FP-S, DC-A, IP-O; Performed experiments: PSLG, STS, AFA; Interpretation of the results: GLR, FPM, FLO, TMV; Wrote the manuscript: GLR, FPM, FLO, TMV.

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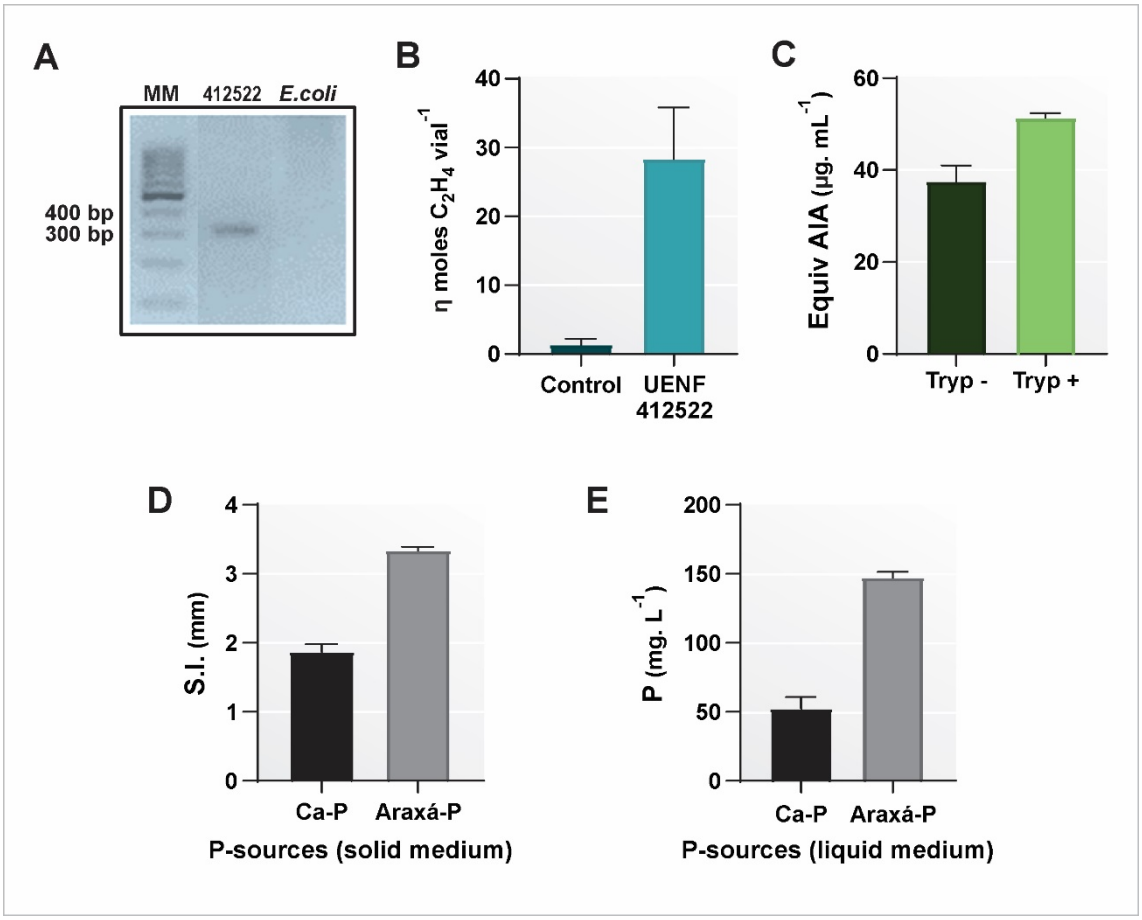
844 **FIGURES**



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846 **Figure 1:** Plant growth promotion *in vitro* traits of *Azospirillum* sp. UENF-412522. (A)
847 Amplification of *nifH* using *E. coli* as negative control; molecular weight marker (MM). (B)
848 Nitrogenase activity by the acetylene reduction assay. (C) Indole acetic acid (IAA) production
849 with and without L- tryptophan. Araxá P-rock and calcium phosphate solubilization in solid (D)
850 and liquid (E) medium.

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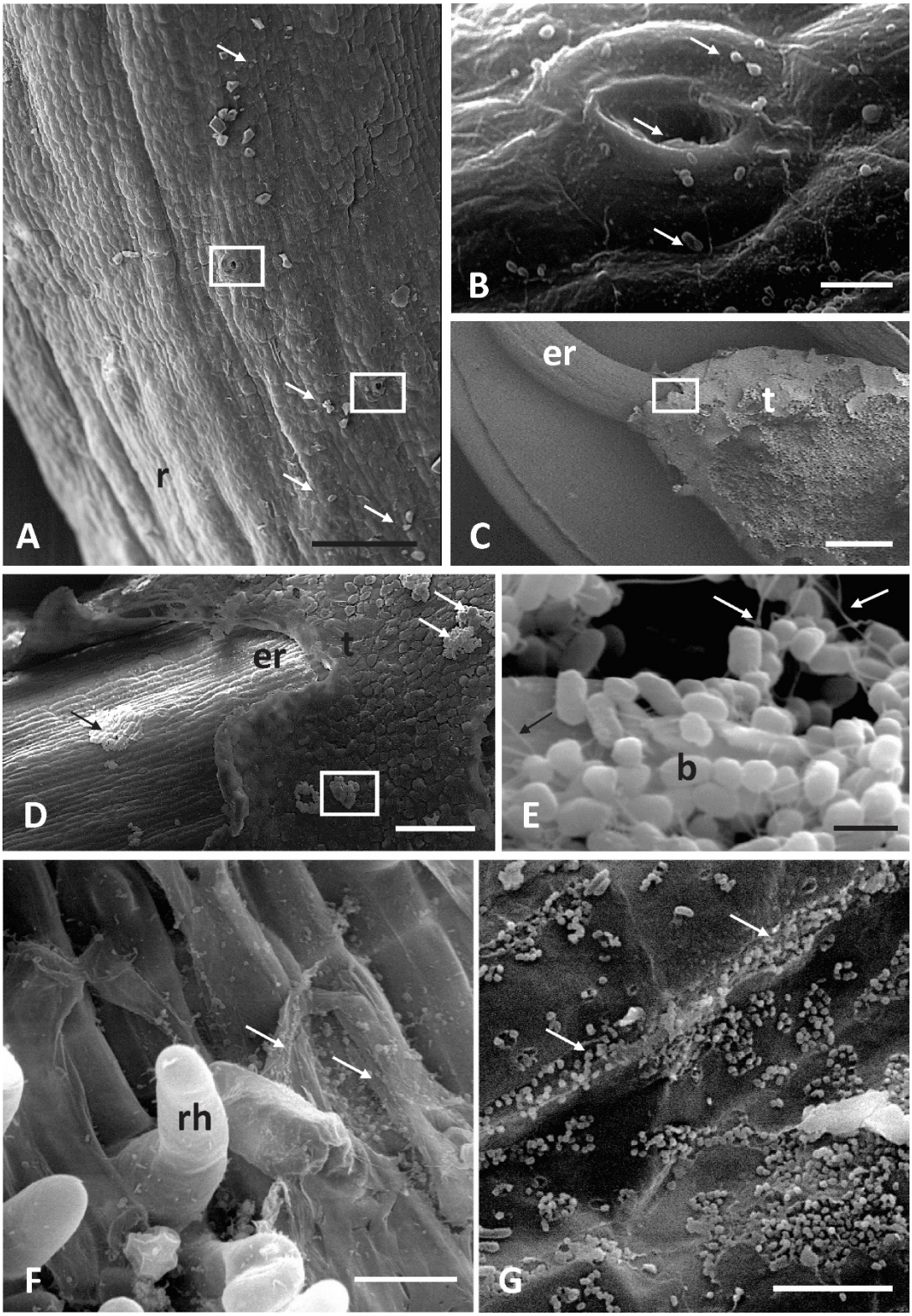

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853 **Figure 2:** Scanning electron microscopy (SEM) of passion fruit (*Passiflora edulis* subsp. *edulis*)
854 seed to seedlings phase inoculated with *Azospirillum* sp. UENF-412522 in an axenic cultivation
855 system at one day (A-E) and seven days after germination (F-G). (A) General view of the emerged
856 radicle surface (r) with single bacteria cells attached (arrows). Note the sparse rudimentary
857 stomata (rectangle magnified in B), bar = 100 µm; (B) Bacteria cells in the vicinity and into the
858 stomata pore (arrows), bar = 5µm; (C) General view of the emerged radicle (er) breaking the
859 seed tegument (t), rectangle magnified in D, bar = 1000 µm; (D) Transition zone between broken

860 tegument (t) and emerged radicle with bacterial aggregates visible in surface tegument (white
861 arrows) and emerged roots (black arrow), rectangle magnified in E, bar = 100 µm; (E) Bacteria
862 aggregates (b) attached by fibrils in the seed surface (black arrow) and linking cell-to-cell (white
863 arrows), bar = 1 µm; (F) Bacteria cells colonizing the root hair (rh) zone as a single-cell by apolar
864 attachment, bar = 20 µm; (G) Aggregated bacteria cells colonizing the elongation-differentiation
865 root zone mainly by apolar attachment and associated to the epidermal cell-wall-junction, bar =
866 10 µm.
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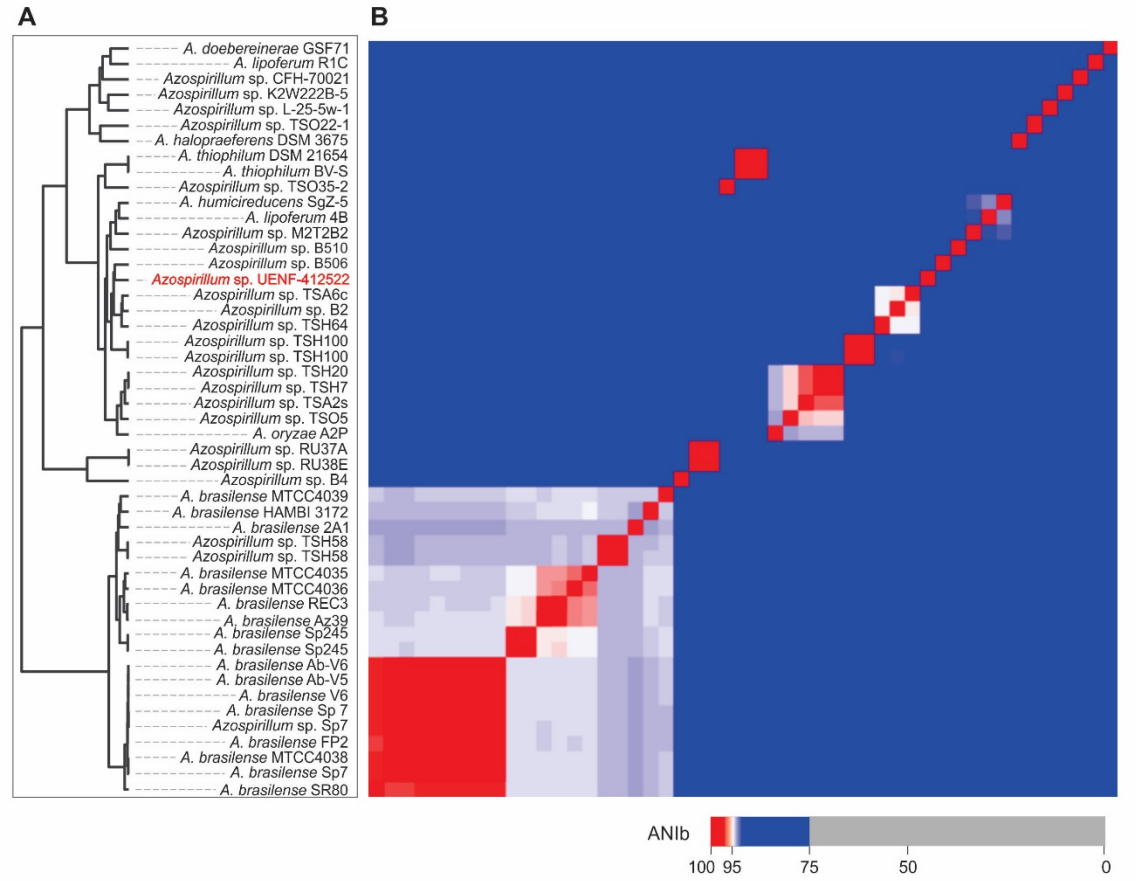

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873 **Figure 3:** Average nucleotide identity (ANI). (A) Neighbor-joining cladogram. (B) Genome-to-
874 genome distance matrix.
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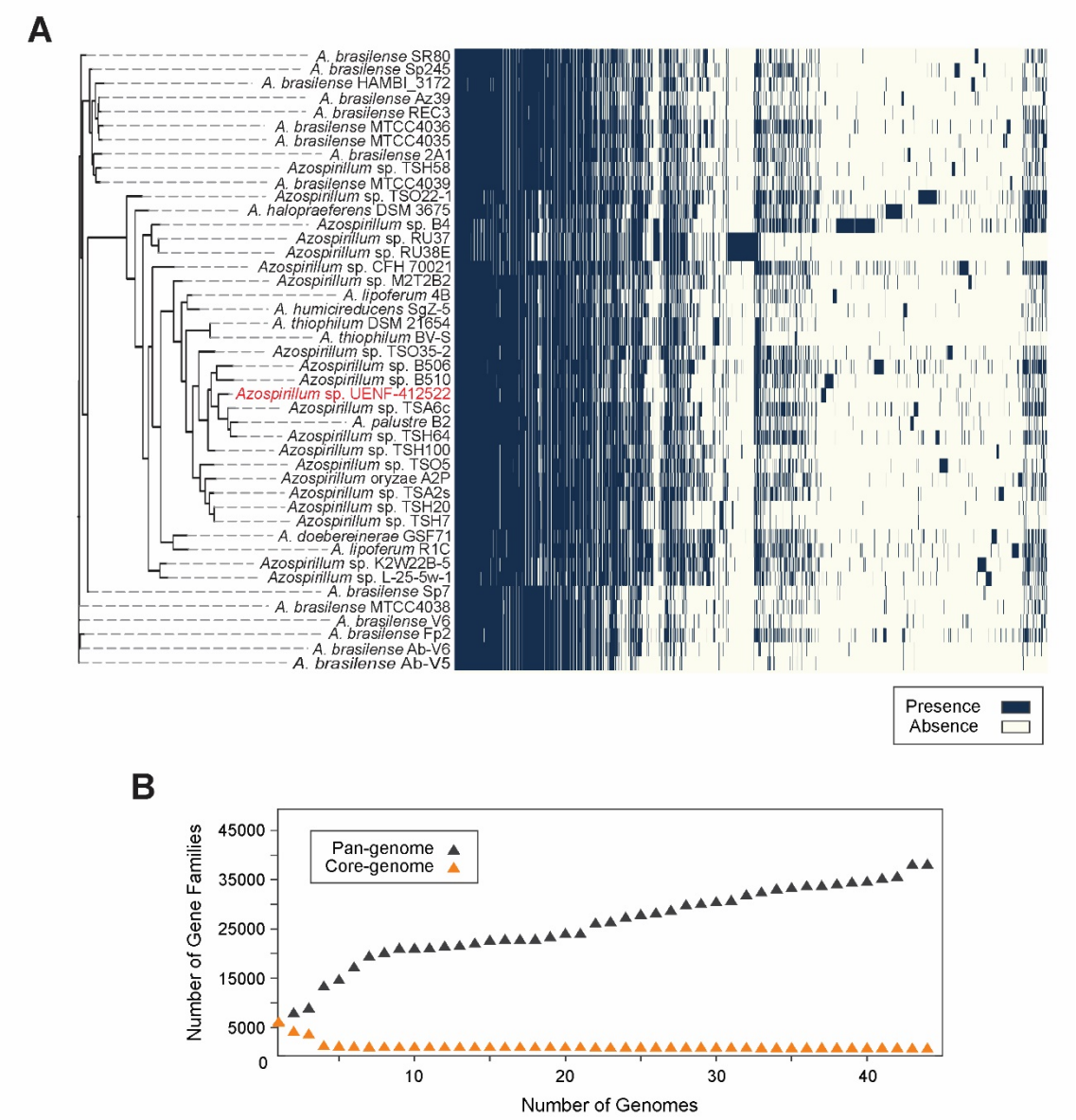

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882 **Figure 4:** *Azospirillum* spp. pan-genome. (A) Gene presence/absence matrix, with gene presence
883 depicted with solid blue boxes. (B) Pan- and core-genome sizes versus new genomes additions
884 to the pan-genome dataset.

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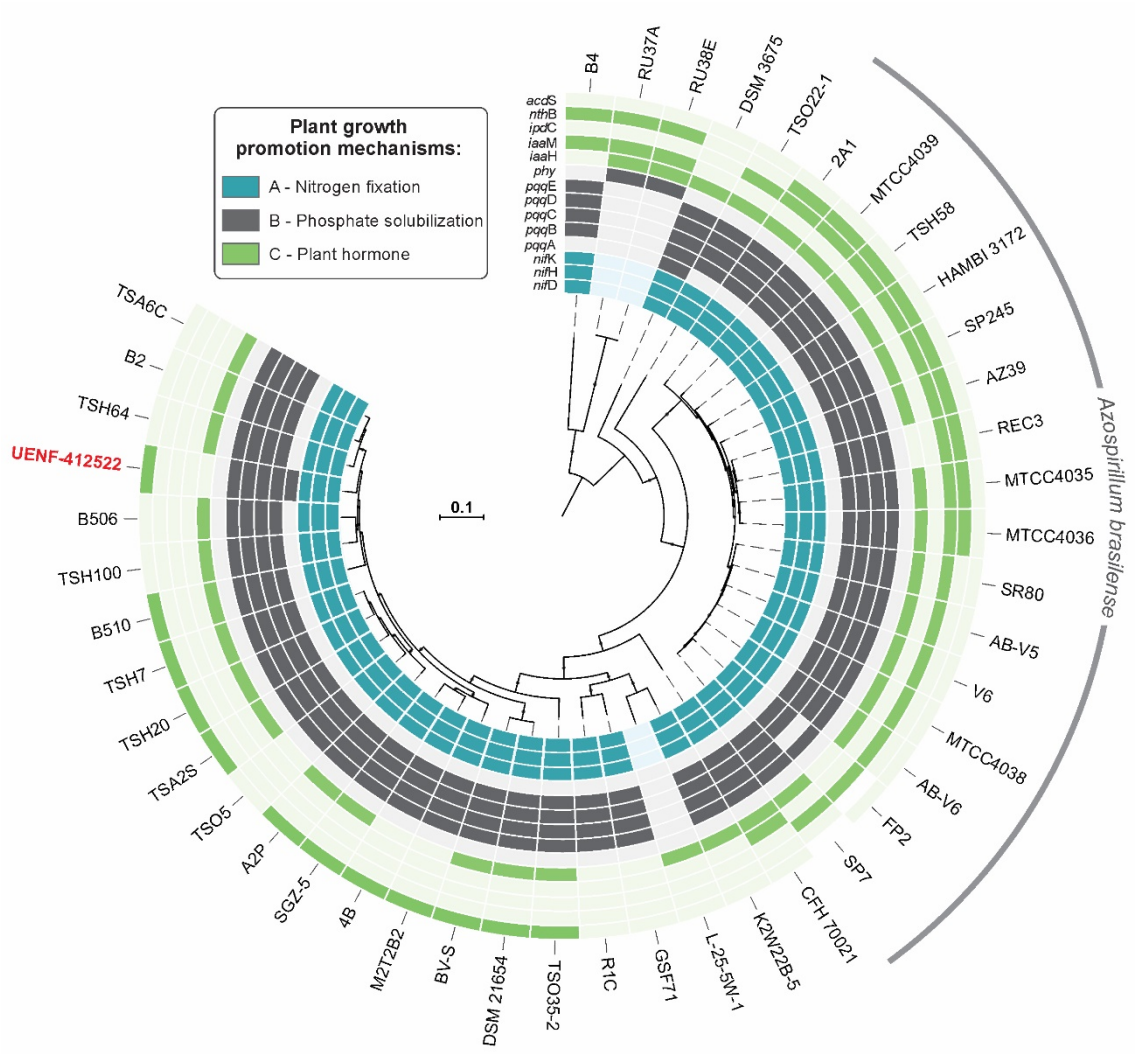

Figure 5: Core maximum likelihood phylogenetic tree. Plant growth-promoting genes were searched, grouped according to the mechanism of growth modulation, and mapped onto the tree.

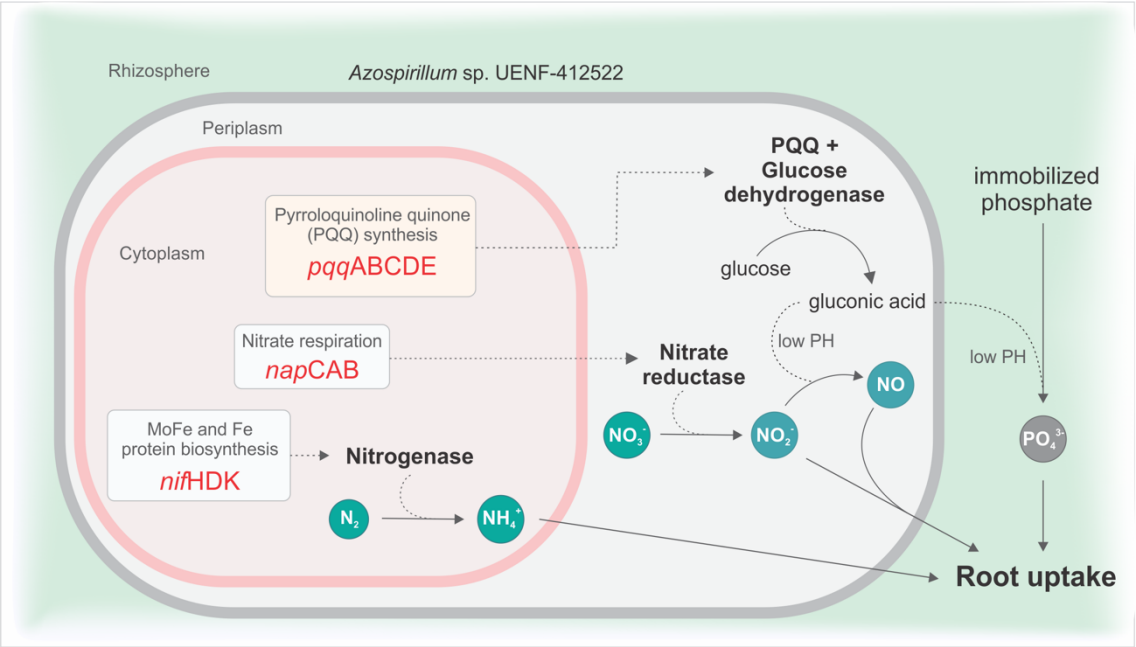

Figure 6: Schematic representation of nitrogen fixation, phosphate solubilization, and nitrite/nitric oxide emission by *Azospirillum sp.* UENF-412522 in the rhizosphere. The identified genes responsible for nitrogen fixation (*nifHDK*), root enhancement (*napCAB*), and phosphate solubilization (*pqqABCDE*) are represented within boxes. Nitrogen fixation occurs in the cytoplasm, while gluconic acid production and nitrate respiration take place at the periplasm. Ammonium generated by the nitrogenase can undergo anaerobic nitrification to nitrite, which can be exported to the periplasm. In the periplasm, glucose dehydrogenase, along with the PQQ co-factor, produces gluconic acid from glucose, lowering the pH. Nitrate is reduced to nitrite by a periplasmatic nitrate reductase napCAB. At low pH, nitrite can be reduced to nitric oxide. Acetic acid, nitrite, and nitric oxide can be exported to the rhizosphere. Acetic acid can reduce the pH in the rhizosphere and solubilize phosphate. The solubilized phosphate, nitrite, and nitric oxide can be captured by root cells.