RESEARCH ARTICLE

Diversity and multifunctional potential for plant growth promotion in bacteria from soil and the rhizosphere

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Abstract

Beneficial microorganisms play essential roles in soil fertility, plant nutrition, and health. In this study, we examined the potential of a collection of 138 bacterial strains to promote plant growth. The strains were isolated from the rhizosphere of two monocotyledonous and two dicotyledonous plant species and from bare fallow soil, all from the same site. Our interest in this study was to investigate the diversity and the potential for growth promotion in this collection of culturable bacteria. The most common trait was phosphorus (P) solubilization from aluminium phosphate (in 66.7% of the strains), whereas solubilization of P from phytic acid (6.5%) and from iron phosphate (5.8%) was the least common and they were only detected in bacterial strains from faba bean and oilseed rape. All bacterial strains inhibited the growth of *Fusarium graminearum* (from 5.4% to 87.2%). In total, 10 genera were identified among the strains by 16S rRNA sequencing and *Pseudomonas* was the most common in monocotyledonous plants and in bulk soil, while *Stenotrophomonas* was dominant in the rhizosphere of the dicotyledonous plants. Combinations of bacterial strains improved the spectrum of in vitro activity in most cases, however, wheat growth was generally lower. These strains have potential to be used as biofertilizers and/or biocontrol agents and further studies should be pursued to develop them into practical solutions for a more sustainable agricultural production.

KEYWORDS

Fusarium, macronutrients, plant-beneficial bacteria, siderophores, *Triticum*

1 | INTRODUCTION

Plants release exudates that can alter the diversity and abundance of microorganisms in the rhizosphere, which in turn may affect plant development (Jacoby et al., 2017). Some microorganisms can increase the availability of soil nutrients for plants as well as inhibit or kill phytopathogenic microbes thereby increasing plant growth (Mendes et al., 2013; Van Der Heijden et al., 2008). Beneficial microbial populations are essential components of the soil and rhizobacteria are determinants of soil fertility and plant health, enabling plants to overcome both abiotic and biotic stresses (Maheshwari et al., 2013).

Nitrogen (N), phosphorus (P), and potassium (K) are the primary macronutrients for plants with P being the second most applied nutrient in many cropping systems. It is estimated that half of the P applied as fertilizer is not assimilated by plants but rather remains unavailable in recalcitrant forms in soil (Richardson & Simpson, 2011). Phosphorus is involved in many metabolic processes in

plants, and it is also an essential element of several cellular components (Dordas, 2008). Phosphorus is abundant in soil in both organic and inorganic forms and cations such as Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+} can bind phosphate anions and precipitate them, leading to immobilization (Li et al., 2021; Richardson & Simpson, 2011). The fixed P form in alkaline soils is $Ca_3(PO_4)_2$, while aluminium (Al^{3+}) and iron (Fe^{3+}) produce insoluble complexes with the phosphate ion (PO_4^{3-}) in acidic soils, where phosphate mineralisation by plants is low (Alori et al., 2017; Richardson & Simpson, 2011). This is mainly due to the chemical formula of P that is commonly fixed to aluminium (Al) or iron (Fe), which prevent P uptake by plants (Gadagi & Sa, 2002; Reyes et al., 1999). Organic phosphorus forms such as phytic acid may contribute to more than 50% of total P in soil. However, phosphate-mineralizing rhizobacteria can convert organic P in the soil into an inorganic P form that plants can use through for example the activity of phytase enzymes (Richardson & Hadobas, 1997; Richardson & Simpson, 2011; Unno et al., 2005). Furthermore, phosphate solubilizing root associated bacteria are capable of making insoluble inorganic forms of P available for plant uptake through the secretion of organic acids (Richardson & Simpson, 2011). As such, bacterial components of the root microbiome can contribute to P cycling in agricultural systems.

Potassium is the third most important nutrient for plant growth, after N and P. It is directly involved in cell elongation and although it is an abundant element in soil, less than 2% of soil K can be directly absorbed by plants as most of it is fixed in silicate minerals (Zhang & Kong, 2014). A diverse group of microorganisms can release K, solubilizing fixed forms and making them available to plants. Microorganisms solubilize K by secretion of organic acids, acidolysis, chelation, decomposition of organic matter and crop residues, exchange reactions, and complexation (Etesami et al., 2017; Meena et al., 2015; Sarikhani et al., 2018; Sattar et al., 2019). In addition to macronutrients, iron is also very abundant in soils, but its availability to plants is low due to the form it occurs (Jian et al., 2019), and in response, plants release substances into the rhizosphere that promote iron solubility (Tripathi et al., 2018; Zhang et al., 2019). However, under adverse conditions, rhizobacteria benefit plants through siderophore secretion and Fe chelation, which is ultimately made available to the plant upon bacterial cell death (Jian et al., 2019).

Rhizobacteria can enhance plant growth directly by solubilizing and increasing the uptake of nutrients such as N, P and K and indirectly by suppressing phytopathogens through multiple mechanisms. In one of these mechanisms, the bacterial ability to produce siderophores that chelate Fe makes this micronutrient unavailable to plant pathogens. Other mechanisms are the synthesis of antifungal metabolites such as antibiotics and cell wall degrading enzymes, production of volatile organic compounds capable of inhibiting fungal growth, competition with pathogens for nutrients and the capacity to induce plant systemic resistance systems (Maheshwari et al., 2013; Saraf et al., 2011).

Wheat is one of the most important cereals on a global scale and fusarium head blight caused by *Fusarium graminearum* is the most severe floral disease of wheat worldwide. This pathogen reduces yield and contaminates the grains with mycotoxins, mainly nivalenol and deoxynivalenol, which also threaten the health of humans and domestic animals that consume infected crops. Epidemics develop around the fourth or fifth years of wheat cultivation in producing areas such as Europe, United States, Africa, and Brazil (Schumann & D'Arcy, 2010). Several bacteria can inhibit *F. graminearum* and their deployment in agriculture could help to promote plant health and soil fertility.

Most of the fertilizers used in agriculture are obtained from the exploitation of non-renewable mineral sources, frequently at high environmental costs. On the other hand, a diverse group of microorganisms are known to enhance nutrient availability to plants, mainly P, N, K, sulphur (S), calcium (Ca), and zinc (Zn) (Cataldi et al., 2020; Dardanelli et al., 2011; Reid et al., 2021). Nowadays, many of these microorganisms are commercialized as biofertilizers and biofungicides. However, there is a need to increase the availability of products containing these beneficial microbes in the market.

In this study, we aimed at exploring the functional diversity and potential of rhizosphere and bulk soil derived bacteria from different crop hosts in a single soil type to solubilize and mineralise nutrients and antagonize plant pathogens to promote plant growth. The end goal of this study is to employ these bacteria alone or in combinations to develop bioproducts to improve plant growth and contribute to increase the sustainability of agriculture.

2 | MATERIALS AND METHODS

2.1 | Microorganisms used in the study

The bacterial strains were obtained from the rhizosphere of healthy oat (*Avena sativa*), wheat (*Triticum aestivum*), oilseed rape (*Brassica napus*), faba bean (*Vicia faba*), as well as from bare fallow soil. All samples were collected from soil or plants at flowering growth stage grown in the same site, at Furzefield, Rothamsted experimental field, Harpenden, United Kingdom (UK). A

1-g subsample of the recovered rhizosphere soil or bulk soil was diluted in water in 10-fold steps and plated onto 10% tryptic soy broth (TSB) supplemented with 15 g/L of BD Bacto agar™ dehydrated agar (Fisher). Individual colonies were obtained and stored in 25% glycerol at −80°C until required for further studies. A total of 138 bacterial strains were obtained and deposited in the collection of microorganisms maintained at the Molecular Microbial Ecology Group at Rothamsted Research, UK. *Fusarium graminearum* strain 602.10 was obtained from Dr Kevin M. King (Rothamsted Research, UK), grown on potato dextrose agar (PDA) and was preserved at −80°C in 15% glycerol.

2.2 | DNA extraction, PCR, and 16S rRNA gene sequencing

The bacterial strains were subjected to 16S rRNA gene sequencing for taxonomic identification. Bacterial strains were cultured overnight in 1/10 TSB at 28°C and the genomic DNA was extracted using an extraction buffer containing SDS (De Souza et al., 2021). The 16S rRNA gene PCR was carried out with bacterial DNA extracts using the primers FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RD1 (5′- AAGGAGGTGATCCAGCC-3′). PCR reactions were carried out using the DreamTaqTM Green PCR 2x Master Mix (ThermoScientific). Once prepared, the samples were placed in a thermocycler for PCR and subjected to the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 5 min. A single PCR amplicon was generated for each strain with no apparent unspecific amplification in the gel. The purification and sequencing of 16S rRNA gene PCR products with the Sanger method were carried out by Eurofins MWG/Operon (Germany) using a PCR product concentration of 10 ng/µl with the FD1 primer. MegaBlast was used in the searches of the curated rRNA gene database with only type strains of the bacterial species included as subjects in all sequence comparisons.

2.3 | Phosphate solubilization assays (Ca, Al and Fe, NaIHP)

Four different P sources were used and named P1, P2, P3, and P4 as tricalcium, aluminium and iron phosphate, and soluble phytate (NaIHP), respectively. The bacterial strains were grown overnight in TSB 1/10 and 1 µL of each bacterial strain was spotted on agar plates containing the different P sources. A total of 138 bacterial strains were tested and 16 different strains were spotted per Petri dish

(90 mm) containing the different media and the assays were performed in triplicate.

2.3.1 | Tricalcium phosphate (P1)

To detect bacteria that utilize tricalcium phosphate $Ca_3(PO_4)_2$ as a P source, Pikovskaya agar medium (Pikovskaya, 1948) containing per litre, 10 g dextrose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$; 0.2 g NaCl, 0.1 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4.H_2O$, 0.002 g $FeSO_4.7H_2O$, and 15 g agar was used. The plates were incubated at 26°C for 5 days and the strains able to solubilize tricalcium phosphate produced a clear halo around the colony. The solubilization indices were calculated as ((colony diameter + halo diameter) / colony diameter), where 0 indicated no bacterial growth and no solubilization, 1 indicated bacterial growth but no solubilization, and indices higher than 1 indicated bacterial growth and solubilization.

2.3.2 | Aluminium phosphate (P2) and iron phosphate (P3)

To detect bacteria that can utilize aluminium phosphate $(AlPO_4)$ or iron phosphate $(FePO_4)$ as P sources, a modified basal medium was prepared according to Gadagi & Sa, 2002, which contained per litre, 10 g sucrose, 0.1 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.2 g yeast extract; 0.5 g NH_4Cl, 0.1 g $MnSO_4.H_2O$, 2 g $FePO_4$ or 5 g $AlPO_4$, 20 g agar, and 0.025 g bromocresol green. The strains were transferred as described above and plates were incubated at 26°C for 24 h for $AlPO_4$ and for 5 days for $FePO_4$. The ability to solubilize $FePO_4$ was scored as positive or negative, whereas solubilization of $AlPO_4$ was scored as a solubilization index as described above for $Ca_3(PO_4)_2$.

2.3.3 | Soluble phytate (P4)

To detect bacteria that utilize phytic acid or inositol hexaphosphoric acid dodecasodium salt (Na-IHP (Na)) (Sigma Aldrich) as the unique source of P, the solid phytic acid-specific medium was used. This medium was prepared according to Unno et al. (2005), based on modified phytic acid specific broth, which contained per litre, 15 g agar, 10 g Na-IHP, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4·7H_2O$, 7.0 g KCl, 0.1 g $CaCl_2·2H_2O$, 1.0 mL 0.1 M FeNa-EDTA, 1.0 mL of a trace element solution (per litre, 15.0 g $Na_2EDTA·2H_2O$, 0.43 g $ZnSO_4·7H_2O$, 0.24 g $CoCl_2·6H_2O$, 0.99 g $MnCl_2·4H_2O$, 0.22 g $Na_2MoO_4·2H_2O$, 0.19 g $NiCl_2·6H_2O$, 0.08 g $Na_2SeO_3·6H_2O$, and 0.15 g H_3BO_3),

and 0.01 g bromocresol green as a pH indicator. Bacterial strains were distributed on plates in grid format and incubated as described above for tricalcium phosphate. The ability to mineralise phytic acid was scored as negative or positive by the coloration around the colonies, from light to dark green of bromocresol green caused by a change in pH. Negative strains did not show any colour change around the colonies.

2.4 | Casein Hydrolyzation (CH)

Microorganisms with hydrolytic activity can break the peptide bonds that link amino acids in proteins. These bonds and nitrogen are released in the process of protein hydrolysis (Wang et al., 2013). The casein agar medium was used to detect hydrolysing microorganisms (Frazier & Rupp, 1928) and agar supplemented with skimmed milk was used as a casein source. The medium contained per litre, 50.0 g skimmed milk, 5.0 g pancreatic digest of casein, 2.5 g yeast extract, 1.0 g glucose, and 12.5 g agar (Hardy Diagnostics). The growth of bacteria, transfer to plates, and halo scoring for nitrate (NO_3) index calculation were done as described above for $Ca_3(PO_4)_2$. The plates were incubated at 26°C for 24 h for evaluation.

2.5 | Potassium solubilization assay (K)

The 138 strains were tested using the modified Aleksandrow medium to detect bacteria that utilize potassium aluminium silicate as a unique K source. This medium was prepared as described by Zhang and Kong (Zhang & Kong, 2014), using potash feldspar (Bath Potters, UK) as an insoluble potassium source. Aleksandrow's medium contained per litre, 5 g glucose, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCO_3$, 0.005 g $FeCl_3.6H_2O$, 2 g $Ca_3(PO_4)_2$, 2 g washed potash feldspar, 0.5 g $(NH_4)_2SO_4$; 0.2 g NaCl, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4.H_2O$, and 15 g agar. Growth of the inoculum, transfer to plates, incubation at 26°C for 5 days, and halo scoring for solubilization index determination were done as described above for $Ca_3(PO_4)_2$.

2.6 | Siderophore production assay (Sid)

The ability of the bacterial strains to produce siderophores was detected on chrome azurol sulphonate (CAS) agar plates as described by (Louden et al., 2011; Schwyn & Neilands, 1987). Growth of the inoculum, transfer to plates, and halo scoring for siderophore index calculation were done as described above for $Ca_3(PO_4)_2$. The CAS plates were incubated at 26°C for 24 h before evaluation.

2.7 | Antifungal activity

Antagonistic activity of the bacterial strains against *F. graminearum*, abbreviated here as Fg, was verified by using the dual culture technique with three replicates. The bacterial strains were cultured overnight in TSB at 26°C at 180 rpm and 10 μL of each strain was streaked 15 mm from the edge of the 90-mm Petri dish containing a mixture of 25% PDA + 10% TSA and 15 g of agar per litre. After 2 days, a 5-mm mycelial plug of *F. graminearum* was placed in the centre of the Petri dish. Fungal mycelial growth was recorded after 4 days at 26°C and a plug of *F. graminearum* alone in a plate was the control to determine the inhibition percentage.

2.8 | Compatibility among strains and wheat growth promotion

Four strains were selected for the growth promotion studies based on positive outcomes in the multifunctional assays described above. Strain P19(7)a was among the best at solubilizing P from all sources (Ca, Al, Fe, and NaIHP); strain P3(3)a was among the best for siderophore production; P14(9)a was among the best for NO_3 hydrolysation and K solubilization; and P4(20) was among the best for NO_3 hydrolysation. These strains were tested in vitro for their growth compatibility with two-by-two combinations and four replicates were prepared according to Barbosa et al. (2018). Compatibility was tested by streaking the strains vertically and horizontally, crossing each other on 10% TSA medium and incubating at 26°C for 48 h. Growth inhibition indicated incompatibility, whereas overgrowth indicated compatibility.

Strains P19(7)a, P14(9)a, P3(3)a, and P4(20) were tested individually and in all possible combinations of two strains and also all four strains were combined for their capacity to solubilize or mineralise P from different sources, siderophore production, K solubilization, and NO_3 hydrolysation by using the assays described above with four replicates per treatment. For the plate transfers, the OD_{600} value for each strain was adjusted to 0.1 and 1 μL of the suspension was used for individual strains. In combinations of two and four strains, 0.5 μL and 0.25 μL of each strain were used, respectively, to ensure biomass parity regardless of strain mixture complexity.

For plant growth promotion assays, wheat seeds cv. Cadenza were surface-sterilized with 70% ethanol for 10 min and 1.5% sodium hypochlorite for 1 h and rinsed five times with autoclaved distilled water. The bacterial strains were grown overnight, the concentration of the cell suspension was adjusted to $OD_{600} = 0.1$, and the seeds were soaked in individual or combined suspensions

and shaken overnight at 150 rpm. For each treatment (individual or combined bacterial strains), 25 seeds in 4 replicates were incubated 7 days at $20 \pm 2°$ C in germination paper size 25×38 cm (Anchor Paper) and were watered with distilled water once at the first day. Total root length was determined with the WinRhizo™ system, a root-measuring device with a unique overlap correction method (Arsenault et al., 1995).

2.9 | Data analysis

All data were evaluated for normality and homogeneity of variance using Shapiro–Wilk's and Bartlett's tests, respectively. The index data were subjected to the analysis of variance followed by mean comparison with Scott-Knott's test ($P < .05$). Data were transformed when needed and subsequent analysis of variance was carried out. Statistical analyses were conducted using R (R core team, 2013) and the statistical package agricolae (Mendiburu & Yaseen, 2020). A Pearson's correlation analysis was performed between all functional bioassays using the software PAST 4.03 (Hammer et al., 2001).

3 | RESULTS

3.1 | The diversity of culturable bacteria varies according to the isolation source

The electropherograms from the 16S rRNA gene sequences showed two outcomes: 109 strains with clear, single-peak electropherograms with high-quality sequences, whereas 29 strains showed mixed electropherograms, indicative of low-quality sequences. The 109 strains with high quality sequences were identified at the genus level and the 29 strains with low quality sequences could not be identified (Figure S1 and Table S1).

The most abundant genera were *Pseudomonas* (44.2%) and *Stenotrophomonas* (23.2%), which were found in all isolation sources. Eight other genera were also identified (Figure 1). *Pseudomonas* was the most representative genus in strains from the rhizosphere of the grasses (wheat and oat) and bare fallow soil, whereas *Stenotrophomonas* was the most common genus in strains from the rhizosphere of the dicotyledonous species, faba bean, and oilseed rape (Figure 1). However, some bacterial genera appeared to be more specific, for example, *Arthrobacter* was only found
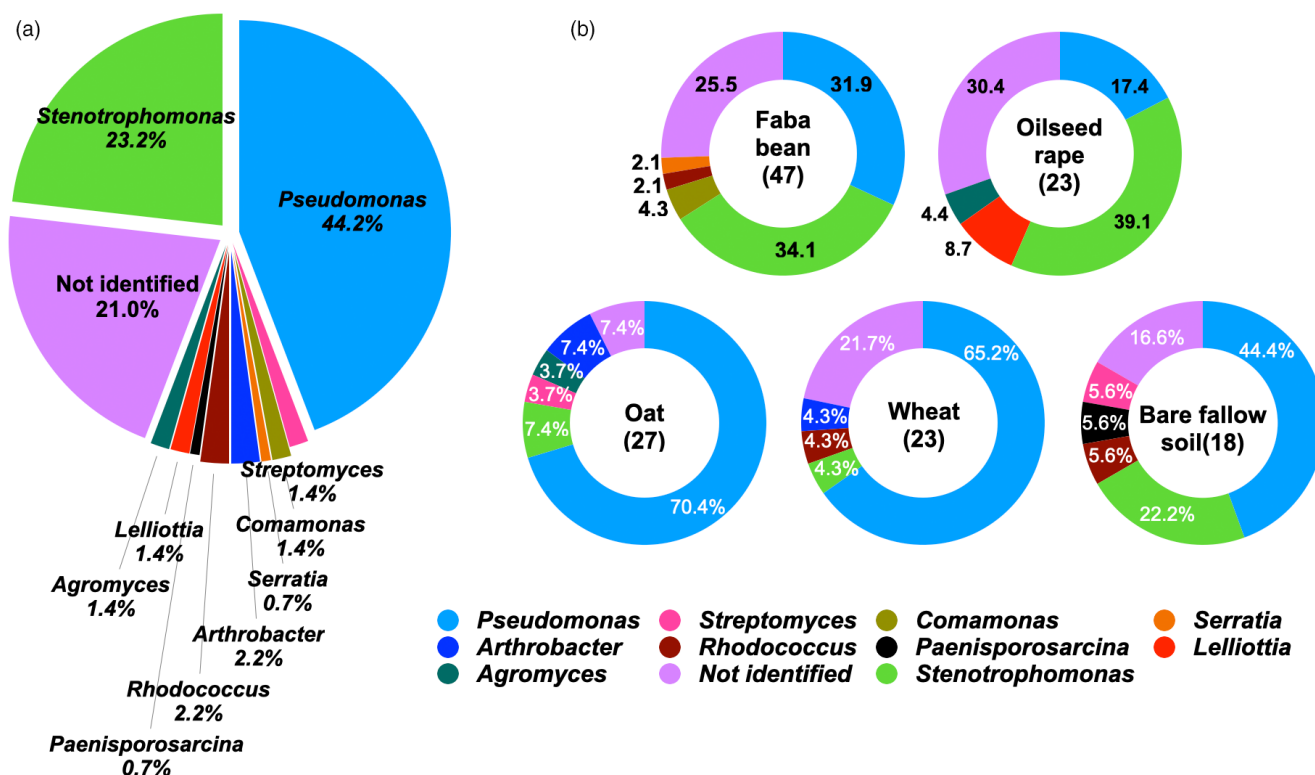

FIGURE 1 Identity at the genus level of the 138 bacterial strains used in this study according to the isolation source. (a) Percentage of each bacterial genus among the 138 studied strains. (b) Distribution of the bacterial genera according to the origin. The numbers between parenthesis represent the number of strains per isolation source. The bacterial strains were identified by sequencing a fragment of the 16S rRNA gene. The identification was done by comparing by Blast searches the sequences obtained in this study with the ones deposited in a curated database containing only type strains of described species. Strains that yielded low quality sequences are labelled as not identified.

in the rhizosphere of wheat and oat, *Comamonas* only in the rhizosphere of faba bean, *Lelliottia* only in the rhizosphere of oilseed rape, and *Paenisporosarcina* was exclusively found in bulk soil (Figure 1).

3.2 | The number of functional groups was higher among strains from dicotyledonous plants

All seven functional groups occurred among strains from faba bean and oilseed rape, whereas only five groups occurred among strains from oat, wheat, and bare fallow soil (Figure 2). The functional group profile in bulk soil most resembled the monocotyledonous grasses (oat and wheat) as opposed to the dicotyledonous plants (faba bean and oilseed rape) (Figure 2). The ability to solubilize P from phytic acid and from iron phosphate was observed only in strains from the dicotyledonous plants (Figure 2).

The most widespread trait was the ability to solubilize P from aluminium phosphate, which was present in 66.7% of the strains studied, followed by K solubilization (61.5%) and P solubilization from tricalcium phosphate (52.2%), while the least common abilities were solubilization of P from phytic acid (6.5%) and from iron phosphate (5.8%) (Figure 2). The capacity to hydrolyse casein and release nitrogen was more common in strains from faba bean (70%) and oilseed rape (78%) than in strains from wheat (8.7%), oat (40%), and soil (45.5%). On the other hand, the ability to synthesize siderophores and to solubilize K was more common in strains from the monocotyledonous plants (70% and 78%) and soil than in strains from the dicotyledonous plants (40 and 48%) (Figure 2).

In addition to the presence or absence of functional ability, bacterial traits evaluated in this study displayed a great quantitative variability among strains. For example, the siderophore production index, which indicates the amount of siderophores secreted, ranged from 0 to 4.58 cm, the aluminium phosphate solubilization index from 0 to 4.38 cm, while nitrogen hydrolysation index ranged from 0 to 2.38 cm (Figure 3; Table S2). Although
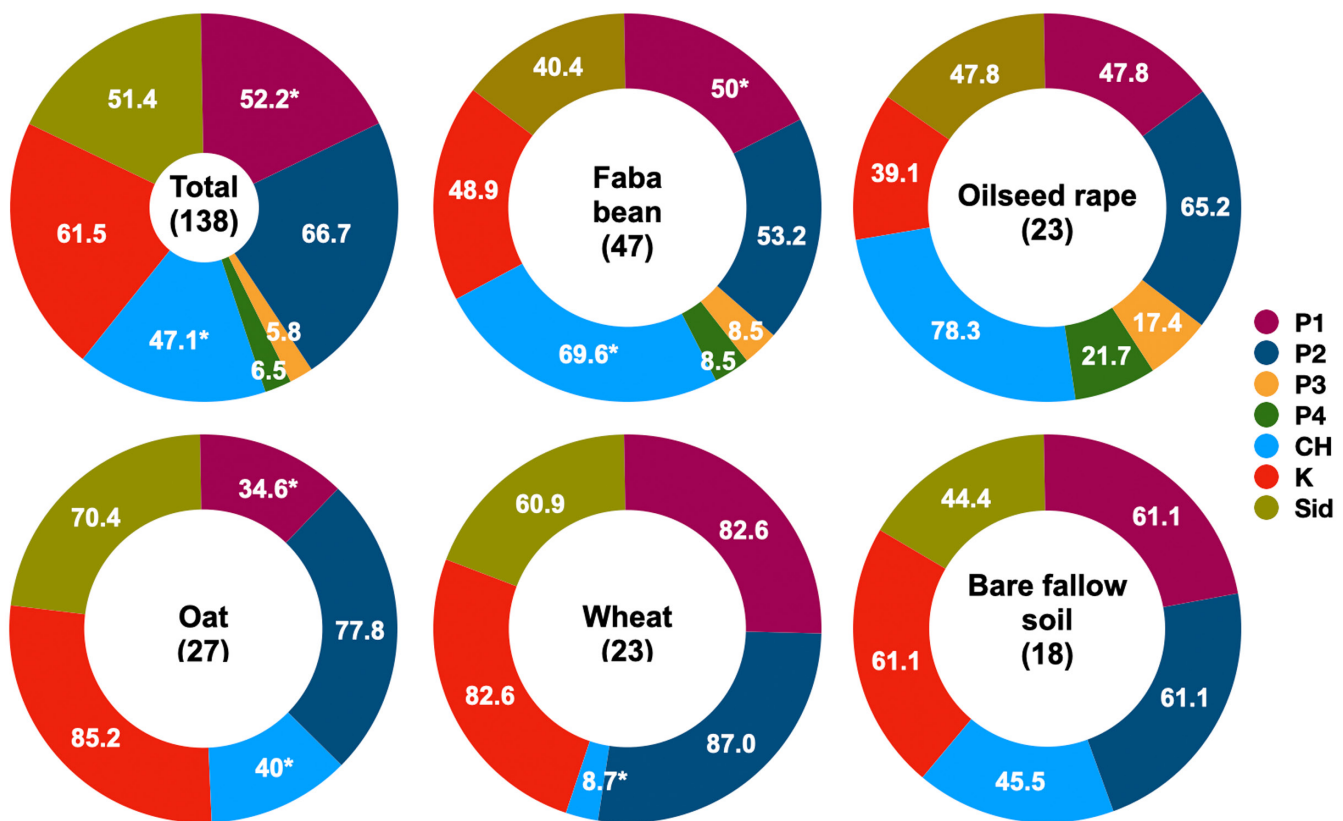

FIGURE 2 Distribution of functional groups of bacterial strains from soil and rhizosphere. The percentages indicate the number of strains that present each respective functional trait. The percentages for each trait do not add up to 100% because most strains possess multiple traits. The numbers between parenthesis represent the total number of strains according to their isolation origin. The functional traits were determined in plate bioassays and scored either as positive or negative for P3 – iron phosphate ($FePO_4$) and P4 – phytic acid (Na-IHP) and as an index for P1 – tricalcium phosphate ($Ca_3(PO_4)_2$), P2 – aluminium phosphate ($AlPO_4$), CH – casein hydrolyzation, K – potassium solubilization and Sid – siderophore production. An asterisk indicates that not all strains were tested.
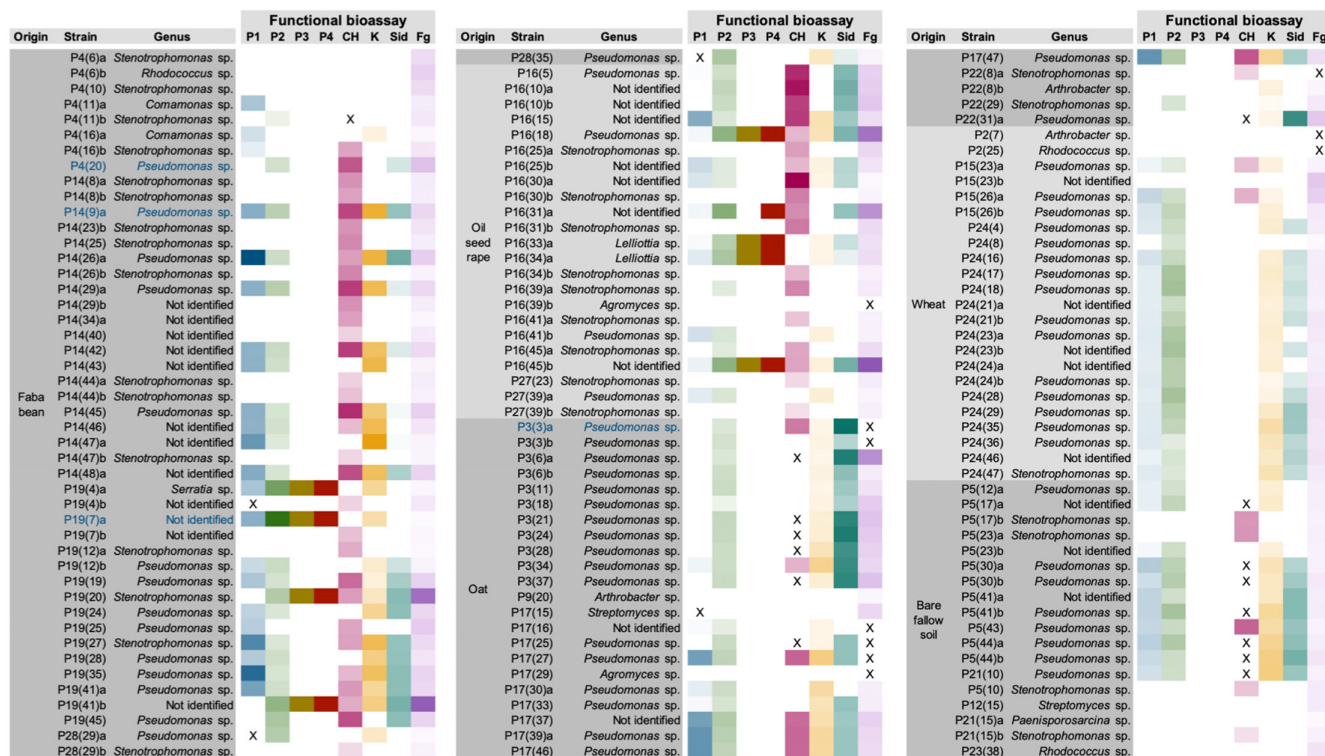
FIGURE 3 Heatmap showing the relative indices for eight different traits in the bacterial strains studied. The shades of grey demarcate the isolation source. The identification of the strains at the genus level was done by 16S sequence analysis. The colours indicate the presence of a respective trait and the strength of the colour indicates the value of the index, whereas the white colour indicates the complete absence of the trait in consideration. The symbol X means that this particular strain not tested. All traits shown were determined in plate assays and scored as positive or negative for P3 (iron phosphate) and P4 (phytic acid) and as an index for P1 (tricalcium phosphate), P2 (aluminium phosphate), CH (casein hydrolyzation), K (potassium) solubilization and Sid (siderophores production). The strains highlighted in blue were selected for the combination studies.

the ability to solubilize P from aluminium phosphate was more common in strains from monocotyledonous plants (77%–88%) against (53%–65%) in dicotyledonous plants; the highest indices of aluminium phosphate solubilization were found among strains from faba bean and oilseed rape (Figures 2 and 3; Table S2). Similarly, K solubilization was more common in oat and wheat (82%–85% of the strains) than in faba bean and oilseed rape (39%–49%), but the highest indices for K solubilization were shown by strains from faba bean (Figure 3; Table S2). Siderophore synthesis was more widespread among strains from monocotyledonous plants; notably, strains from oat exhibited the highest siderophore indices (Figure 3; Table S2).

All bacterial strains had the ability to inhibit the growth of *F. graminearum*, which ranged from 5.4% to 87.2% of inhibition compared to control plates with no inoculant added. However, higher inhibitory activities (>76%) were found only among strains from faba bean and oilseed rape (Figure 3; Table S2). In general, strains from bare fallow soil and wheat had low plant growth promoting potential compared to the other strains in the collection (Figure 3; Table S2).

3.3 | The majority of the strains possess multiple potential growth-promoting traits

A total of 23 unique profiles or functional group profiles were found among the 138 strains studied. These profiles are the individual or combined capabilities shown by the bacterial strains. The only trait common to all bacterial strains was inhibition of *F. graminearum* growth. Most bacterial strains, 125 out of 138, demonstrated more than one of the potential growth-promoting traits evaluated and only two strains possessed all eight traits (Table 1).

The most numerous genera, *Pseudomonas* and *Stenotrophomonas* had at least one representative strain in nine and 13 profiles, respectively (Table 1) and some of the traits were correlated with each other. For example, the majority of the *Stenotrophomonas* strains (22 out of 32) were antagonistic to *F. graminearum* and were also capable of hydrolysing casein, whereas most *Pseudomonas* strains (45 out of 61) shared at least five traits: ability to solubilize P from aluminium and tricalcium phosphate, synthesis of siderophores, K solubilization, and antagonism against *F. graminearum* (Table 1). Strains of *Pseudomonas* were on average more antagonistic against *F. graminearum* than

TABLE 1 Distribution of bacterial strains in unique profiles defined by the eight functional traits evaluated in this study.

P1[a]	P2	P3	P4	CH[a]	K	Sid	Fg[b]	Num.	Genus
○	○	○	○	○	○	○	●	(13)	*Arthrobacter* (2); *Rhodococcus* (2); *Stenotrophomonas* (2); *Rhodococcus* (1); *Paenisporosarcina* (1); *Streptomyces* (2); *Agromyces* (2); Not identified (1)
○	●	○	○	○	○	○	●	(2)	*Pseudomonas* (1); *Stenotrophomonas* (1)
●	○	○	○	○	○	○	●	(1)	*Comamonas* (1)
○	○	○	○	○	●	○	●	(1)	*Arthrobacter* (1)
○	○	○	○	●	○	○	●	(27)	*Stenotrophomonas* (22); Not identified (5)
○	●	○	○	●	○	○	●	(2)	*Stenotrophomonas* (2)
●	○	○	○	●	○	●	●	(2)	*Stenotrophomonas* (1); *Pseudomonas* (1)
○	●	○	○	○	●	●	●	(2)	*Stenotrophomonas* (1); *Pseudomonas* (1)
●	○	○	○	○	●	○	●	(1)	*Comamonas* (1)
○	○	○	○	○	●	●	●	(1)	*Pseudomonas* (1)
○	●	○	○	●	○	●	●	(4)	*Pseudomonas* (2); Not identified (2)
●	●	○	○	○	●	○	●	(11)	*Pseudomonas* (6); Not identified (5)
○	●	○	○	○	●	●	●	(11)	*Pseudomonas* (11)
●	●	○	○	●	○	●	●	(1)	*Pseudomonas* (1)
●	●	○	○	●	●	○	●	(3)	*Pseudomonas* (2); Not identified (1)
○	●	○	○	●	●	●	●	(1)	*Pseudomonas* (1)
●	●	○	○	○	●	●	●	(26)	*Stenotrophomonas* (1); *Pseudomonas* (20); Not identified (5)
●	●	○	●	●	○	●	●	(1)	Not identified (1)
●	●	●	●	○	●	○	●	(2)	*Serratia* (1); Not identified (1)
●	●	○	○	●	●	●	●	(20)	*Pseudomonas* (13); *Stenotrophomonas* (1); Not identified (6)
○	●	●	●	●	●	●	●	(2)	*Lelliotia* (2)
●	●	●	●	○	●	●	●	(2)	*Stenotrophomonas* (1); Not identified (1)
●	●	●	●	●	●	●	●	(2)	*Pseudomonas* (1); Not identified (1)

Note: The bacterial strains were identified by sequencing a fragment of the 16S rRNA gene and the bioassays were done on Petri plates and scored either as positive or negative or as indices. The numbers between parenthesis (Num.) represent the total number of strains that possess the trait under consideration and the number of strains in each genus is shown between parenthesis in each profiles. The traits evaluated were: P1 (tricalcium phosphate), P2 (aluminium phosphate), P3 (iron phosphate), P4 (phytic acid), CH (casein hydrolyzation), K (potassium) and Sid (siderophore production).

[a]For the definition of these profiles, strains not tested for CH and P1 were considered as negative for these traits.

[b]Strains not tested for *F. graminearum* inhibition were considered as positive for this trait because all tested strains showed some level of inhibition.

strains of *Stenotrophomonas* (Table S2). Furthermore, solubilization of K and P from tricalcium phosphate was significantly correlated (R = 0.7; *P* = .0001) as these two traits occurred together in 67 strains (Table 1; Figure S2). The least common traits were solubilization of P from phytic acid and from iron phosphate, which occurred in 8 and 9 strains, respectively. These two traits were highly correlated (R = 0.95; *P* = .0001) and present in strains representative of the following genera: *Pseudomonas* (1), *Serratia* (1), *Lelliottia* (2), and *Stenotrophomonas* (1) and four strains that were not identified (Figure 1; Table 1). Production of siderophores was positively correlated with solubilization of K (R = 0.63; *P* = .0001), where 66 strains had these traits together and 25 strains had either one or the other; it was also correlated with solubilization of P from aluminium

phosphate (R = 0.64; *p* = .0001), with 70 strains sharing this trait and 23 presenting them separately. Similarly, solubilization of P from aluminium phosphate and solubilization of K were positively correlated (R = 0.54; *p* = .0001), with 83 strains showing the traits together and only 12 possessed one but not the other (Table 1; Table S2; Figure S2).

3.4 | Single strains generally promoted more wheat growth than combinations

Four combinations were compatible, while two were incompatible among the four strains selected for this part of the study. Compatibility and incompatibility were verified by the absence or presence, respectively,

of inhibition zones when two strains grow together (Table S3). Strain P19(7)a was the only one compatible with the other three strains. Strain P3(3)a was compatible with P19(7)a and P4(20), but not with P14(9)a. Strain P14(9)a was incompatible with P3(3)a and P4(20). These strains were inoculated on wheat seeds individually or in combinations of two strains as well as one combination of all four strains to test their potential for plant growth. Additionally, the strains were evaluated in in vitro bioassays to verify the interference of the combinations on their ability to solubilize nutrients from different sources and to produce siderophores. It should be noted that P4(20), P14(9)a, and P19(7)a were all isolated from the bean rhizosphere, whereas P3(3)a was isolated from the oat rhizosphere.

In general, the combination of strains increased their spectrum of activity in the in vitro bioassays. For five out of six tested, the presence of the trait in one of the strains led to its addition in the combination. However, the trait solubilization of P from Ca_3PO_4 was suppressed in four of the six combinations with at least one of the strains with this property. Only in one of the combinations (P14(9) a + P19(7)a) an increase in the values of most indices was observed (Figure 4a).
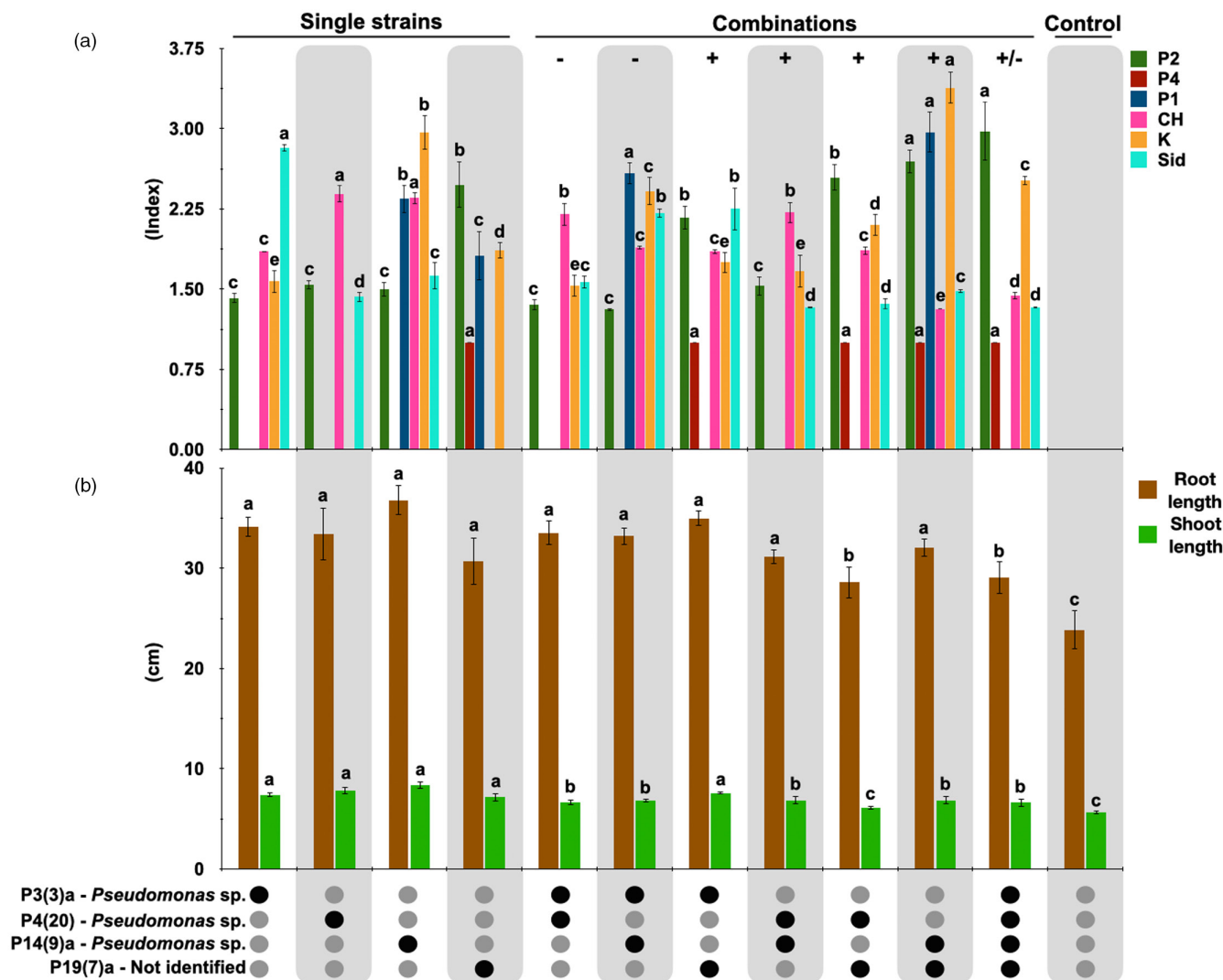

FIGURE 4 In vitro potential grow-promoting traits and wheat growth promotion by four selected bacterial strains and their combinations. (a) Four strains were tested alone or in six two-by-two combinations and one combination containing all four strains for their ability to solubilize P from three sources: P1 – tricalcium phosphate ($Ca_3(PO_4)_2$), P2 – aluminium phosphate ($AlPO_4$), and P4- phytic acid Na-IHP (phytic acid); CH – Casein hydrolysation, K – potassium solubilization, Sid – siderophores production. All bioassays were done in plates and scored as numerical indices according to the size of the halo around the colonies. Solubilization of P from $FePO_4$ was not tested. The signal "+" indicates compatibility, "-" indicates incompatibility and "+/−" indicates the presence of both compatible and incompatible interactions in the combination. (b) Shoot and root length of wheat seedlings 7 days after the treatment. Means were calculated from four replicates of 25 seeds for each treatment. Black circles in the legend indicate that the bacterial strain in consideration was present and grey circles indicate the absence of the strain. The negative control was not treated with any bacterial strain. Columns with the same same letters are not significantly different according Scott-Knott's test at 5% probability.

Root length was significantly longer for wheat plants treated with any bacterial strain applied individually or in combinations when compared to the untreated control. However, in two combinations out of seven, the root length was significantly shorter than in plants treated with individual strains and the other combinations, but it was still longer than the untreated control. Irrespective of the compatibility between strains, combinations in general yielded plants with smaller shoot lengths. The shoot length was significantly shorter in six out of the seven combinations tested when compared with plants treated with individual strains (Figure 4b).

4 | DISCUSSION

The rhizosphere of plants and soils has been shown by many authors to be rich sources for the isolation of culturable plant growth promoting bacteria (Backer et al., 2018). Due to the fact that these beneficial bacteria enhance the fitness of their hosts upon inoculation, they have been intensively exploited in recent times to develop inoculants for agricultural uses (Sharma et al., 2013). Among the benefits brought about by these bacteria, increased growth and defence against pathogens are in the forefront. Growth promotion is mostly induced by beneficial bacteria through the production of phytohormones such as IAA and by the solubilization of recalcitrant nutrients that are otherwise unavailable to plants (Cordero et al., 2018; Sharma et al., 2013; Win et al., 2018). It has recently been shown by multivariate analyses of numerous studies that plants favour associations with hormone-producing bacteria in nutrient-rich soils and with nutrient solubilisers when growing in poor soils (Costa et al., 2014; Reid et al., 2021).

A total of 138 culturable bacterial strains associated with the rhizosphere of healthy faba bean, oilseed rape, oat, wheat plants and bare fallow soil were investigated for their potential to promote plant growth with a variety of bioassays in vitro. These bioassays included the ability to solubilize four different types of P: tricalcium phosphate (P1), aluminium phosphate (P2), iron phosphate (P3), and phytic acid (P4); casein hydrolyzation (CH), potassium (K) solubilization, siderophore (Sid) production and inhibition of *F. graminearum* growth (Fg). Solubilization of nutrients in poor soils has the potential to contribute to modern agriculture by decreasing the use of synthetic and environmentally expensive chemical fertilizers (Backer et al., 2018).

Plants growing in soils with high organic matter contents are less responsive to biofertilizers, i.e., the microbes that assist plants in nutrient acquisition. The best-known effects of biofertilizers are responses from plants growing in nutrient-poor soils (Reid et al., 2021). Several mechanisms are known to be involved in biofertilization by bacteria, including solubilization of nutrients, production of siderophores, and biological fixation of N (Backer et al., 2018). The most exploited microbes in agriculture are the N-fixing bacteria (Toyota & Watanabe, 2013). However, our focus in this study was on nutrient solubilization because after N, P and K are the next most limiting nutrients for plant growth. Furthermore, the root colonization potential of associative nitrogen fixing bacteria is limited when compared to mutualistic associations of rhizobia with legumes. In addition, bacteria that solubilize P and K secrete organic acids that can solubilize recalcitrant forms of these nutrients, that are common in most soils, into forms labile to plants.

Phosphorus is present in soils in two forms: inorganic P (Pi) and organic P (Po), and because only 0.1% of the total P in soils is readily available to plants, chemical fertilizers are applied to cope with this deficiency (Lambers & Plaxton, 2018). However, the phosphate anions in chemical fertilizers are highly reactive and are easily complexed with Ca^{2+}, Fe^{3+}, and Al^{3+}, forming insoluble inorganic salts. Furthermore, organic P is mostly immobilized in organic matter, in the form of salts of phytic acid also known as soil phytate (Alori et al., 2017). Most of the phosphate fertilizers that are not immobilized leach to groundwater and are responsible for water eutrophication that led to poisonous cyanobacterial and algal bloom formation (Schindler et al., 2008). It is estimated that the amount of P accumulated in agricultural soils would be sufficient to sustain maximum crop production for 100 years if it were available (Khan et al., 2007, 2009). Therefore, microorganisms are an eco-friendly and cost-effective approach to solubilize Pi and mineralise Po in soil for the sustainable development of agricultural crops (Sharma et al., 2013). Several mechanisms are employed by bacteria to increase the availability of P to plants: (1) secretion of mineral-dissolving compounds such as organic acids or H^+ ions, protons, hydroxyl anions, and CO_2 or metal chelating agents such as siderophores and HCN; (2) production of extracellular enzymes such as phytase to solubilize Po (biochemical mineralisation); and (3) release of P during substrate degradation (biological P mineralisation) (Alori et al., 2017; Sharma et al., 2013). In this study we considered the first two mechanisms and found that P-solubilizing bacteria are present in soil and in the rhizosphere of different plant species in a manner that depends on the source of P being utilized. While tricalcium phosphate (P1) and aluminium phosphate (P2) solubilisers were present in relatively high numbers in all samples, iron phosphate (P3) and phytate (P4) mineralisers were only found less commonly

in the strains derived from the rhizosphere of dicotyledonous plants. Interestingly, there was a strong positive correlation between the ability of the bacterial strains to solubilize P from iron phosphate and phytate. From the nine strains that possessed these traits, only one was not able to solubilize iron phosphate (Figure 3). These low densities and the co-occurrence of the two traits have been observed previously by other authors (Wang et al., 2021).

Regarding the source of insoluble phosphate used in our screenings, tricalcium phosphate (P1) was used as proxy for inorganic P source in alkaline soils, whereas iron and aluminium phosphate was a proxy for inorganic P in acidic soils and phytate for soils rich in Po (Bashan et al., 2013). Our initial tests for growth promotion were not done in soil and therefore no conclusion can be drawn on the ability of these bacteria to promote growth in soil as several traits besides nutrient solubilization may be involved in the final outcome, such as the capacity to colonize the rhizosphere of a given plant species (Sharma et al., 2013). As such, the ability to solubilize P is not always correlated with plant growth promotion (Collavino et al., 2010).

Potassium is essential for cell turgor and elongation (Sun et al., 2020). Only 1%–2% of the soil K can be directly utilized by plants, whereas 90%–98% is fixed in silicate minerals (Zhang & Kong, 2014). Bacteria and fungi are able to solubilize K by several mechanisms, including secretion of organic acids, chelation, and decomposition of organic matter (Meena et al., 2015; Sattar et al., 2019). In this study, we found that K solubilization was found in more than 60% of the strains, the second most common trait, evidencing the potential for K solubilization in this collection.

The 138 strains identified by 16S rRNA gene sequencing were representative of 10 genera, which varied according to their origin of isolation. Most of these genera were previously reported as plant beneficial in a comprehensive database of plant-associated bacteria (Li et al., 2023). This database may be complemented with the genus *Lelliottia*, which was found in our study to solubilize P and K, and to produce siderophores and antagonize *Fusarium* (Figure 3) and was also reported as a growth promoting genus in another study (Parashar et al., 2023).

The most common genus in dicotyledonous plant rhizospheres was *Stenotrophomonas*, while *Pseudomonas* was the most common in monocotyledonous plants and in soil. The fact that some bacterial genera were more numerous in the rhizosphere of monocotyledonous as opposed to dicotyledonous and vice-versa probably reflects the exudates of these plants that select specific communities with capacity to utilize them more efficiently (Bais et al., 2006). These findings could be important for the design of host specific microbial inoculants. Plants are the key determinants of the composition of microbial communities in the rhizosphere. They determine the diversity, activity and densities of microbes directly through the secretion of root exudates, which vary according to plant species and genotypes and the nutrient status of the soil (Lundberg et al., 2012; Turner et al., 2013). Additionally, plants influence the microbial communities by changing the chemical properties of the soil (Zhou et al., 2017a, 2017b). Previous work has revealed that under Fe deficiency monocots increase their root exudation of phytosiderophores whereas dicots release protons into the soil environment (McNear Jr., 2013). In future studies it will be interesting to discover the differential root exudate chemistry between mono and dicotyledonous plants and how this affects the selection of root associated microbiota.

The number of genera recovered in our study was only 10 because the approach used a single isolation medium (1/10TSA) for subsequent screening for solubilization of nutrients. However, it is well known from NGS and metagenomics data that the total number of bacterial genera in soil and in the rhizosphere is much higher than the number of genera we can cultivate in artificial media (Hirsch et al., 2013). Besides plants, bacteria can also auto-regulate their composition, activity, and diversity by producing quorum sensing molecules that allow them to respond in a coordinated way to external stimuli (Chauhan et al., 2015). Some bacterial groups have a higher degree of hierarchy in soil or in the rhizosphere and are called core species as they regulate broader activities in the bacterial communities (Toju et al., 2018).

It is relatively well established in the scientific literature that dicotyledonous plants, e.g. legumes, exert a stronger effect on the abundance and diversity of microbial communities in the rhizosphere than monocotyledonous plants, e.g. grasses (Chen et al., 2008; Turner et al., 2013; Zhou et al., 2017a, 2017b). In our study, this influence was noticed in a relatively limited collection of culturable bacteria, which may not have a general representativeness, since only a small subset of the bacterial community is culturable (Steen et al., 2019). The range of functional groups in culturable bacteria in the rhizosphere of the dicotyledonous plants (faba bean and oilseed rape) was greater than in the rhizosphere of monocotyledonous plants (oat and wheat) and in soil. Monocotyledonous plants are known to produce root exudates with a higher C:N ratio than dicotyledonous plants, making these exudates more difficult to utilize. On the other hand, dicotyledonous plants secrete more amino acids, sugars, and flavonoids in their root exudates than monocotyledonous plants (Isobe et al., 2001; Wojtaszek & Peretiatkowicz, 1992). These differences could account for the diversity and the number of functional groups we observed in this study. Interestingly,

the functional groups observed among strains from soil were more similar to the ones observed in monocotyledonous plants, which supports the previous observation that dicotyledonous plants exert a stronger influence over their rhizosphere microbiome than monocotyledonous plants, whose community members are more similar to the bulk soil in their PGPR activity profile.

The identification of 29 strains was not possible due to mixed chromatograms that resulted from direct PCR amplifications and sequencing by the Sanger method. These mixed chromatograms probably resulted from the natural heterogeneity in the 16S rRNA gene in genomes of diverse bacterial groups (Silva et al., 2019). Although all bacterial cultures were purified from single colonies and the PCR amplifications have always shown only one visible band in the gels, mixtures of strains cannot be completely ruled out. Variations as high as 9.7% were reported among copies of the 16S rRNA gene in the same bacterial genome (Pei et al., 2010; Sun et al., 2013). This variation is especially high in strains of *Bacillus* (Liu et al., 2015), but also occurs in many other bacterial genera (Silva et al., 2019). It was surprising to us that *Bacillus* spp. were not identified among the strains used in this study. It is possible that at least part of the non-identified strains are *Bacillus* species that could not be identified due to the heterogeneity in the 16S rRNA gene. This explanation is plausible when we consider that *Bacillus* strains can house over 10 copies of the 16S rRNA gene (Mauchline et al., 2010). Indeed, it has been previously shown that 49% of the culturable bacterial strains from cocoa seeds had heterogeneity in their 16S rRNA gene, which greatly complicated their identification (Silva et al., 2019). In our study, approximately 21% of the total number of strains could not be identified at this time, but they may still be identified in the future by methods such as cloning the 16S rRNA gene and sequencing from the vector, or by whole genome sequencing.

The combination of bacterial strains has been shown to increase their effectiveness in agricultural applications (Latha et al., 2009; Roberts et al., 2005; Stockwell et al., 2011; Zangoei et al., 2014). Successful combinations of bacterial strains depend on their compatibility, which may be determined in Petri dish assays (Barbosa et al., 2018; De Boer et al., 2003). The results of this study showed that most combinations, independent of their compatibility, resulted in an increased spectrum of in vitro activity. However, wheat growth was not improved by most combinations. We must view this result with caution though, as the negative treatment was a no inoculant control. As such, it could be the case that the bacterial biomass inoculation could amount to a fertilization effect regardless of PGPR activity. Furthermore, our experiments were done only for the initial phase of wheat growth in

soil-free germination paper, without the addition of recalcitrant nutrient substrates. Future studies should be carried out to verify if the strains and their combinations will improve plant growth in the presence of the relevant recalcitrant nutrients or in soil, where the influence of these strains to solubilize and release of nutrients can be assessed in the context of strain persistence and plant biomass response. In this context, it will also be interesting to test these strains in systems with high and low nutrient status. Other possible explanations for this mutual interference could be nutrient blocking in competitive interactions (Spragge et al., 2023) and self-inhibition (Mazzoleni et al., 2015). Both these hypotheses are warranted future investigations. It should also be noted that we selected the most promising strains for wheat inoculation regardless of the strain source. All selected strains were from either oat or bean rhizospheres, and future wheat *in planta* studies using this collection should also include wheat strains or screen the promising oat and bean strains on the plant species that they were initially isolated from to test the importance of host specificity of inoculants.

To conclude, in this study, we performed the initial stages of a broad characterization of 138 strains with multiple traits that are potentially useful in the development of bacterial-based agricultural products to improve plant growth, with special emphasis on nutrient solubilization.

5 | CONCLUSIONS

In this study, we observed that dicotyledonous plants held a higher number of functional groups of culturable bacteria than monocotyledonous plants. While strains of the genus *Stenotrophomonas* were predominant in the rhizosphere of dicotyledonous plants, *Pseudomonas* was more numerous in soil and monocotyledonous plants. The combination of bacterial strains as inoculants in the wheat rhizosphere increased their spectrum of nutrient solubilization, but did not influence the initial growth of the plants.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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