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Are native phosphate solubilizing bacteria a relevant alternative to mineral fertilizations for crops? Part I. When rhizobacteria meet plant P requirements.

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Highlights

- P Solubilizing Bacteria were isolated from soils under three different crops
- Most isolated PSB belonged to *Bacillus* and *Pseudomonas* genera
- A *Sanguibacter inulinus* isolate was described as PSB for the first time
- Assessment of PSB abilities of all isolates showed two distinct profiles.
- Plants requirements for P shape PSB structuration

Abstract:

Deployment of Phosphate Solubilizing Bacteria (PSB) can enhance plant P uptake through remobilization of recalcitrant forms of P in soil. In this study, bacterial strains isolated in the rhizospheric soil of rapeseed, winter pea and faba bean, with various P requirements, were screened for their PSB potential through the characterization of several interesting traits linked to P solubilization. In parallel, other Plant Growth Promoting Rhizobacteria (PGPR) traits of interest (indole-3-acetic acid (IAA) production or 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity) were also determined for each isolate. An abundant proportion of PSB strains were successfully emphasized, mainly belonging to *Pseudomonas* and *Bacillus* genera. The present study also reveals for the first time the potential of phosphate solubilization of an isolate belonging to the species *Sanguibacter inulinus*. Interestingly, PSB isolated from rapeseed soil showed higher solubilization efficiency compared to those isolated from faba bean and winter pea rhizosphere. These results highlighted the great influence of plant to shape their associated microbiome in order to fit specific P requirement. Two distinct profiles of PSB isolates could be highlighted, deploying either direct or indirect processes. Plants seemed to preferentially enrich PSB populations presenting complementary characteristics to their own arsenal to favor P nutrition. Valuing native PSB potential, through artificial addition or through the legacy of previous crop for the further culture could represent a promising alternative to sustain plant growth in P-deprived situation.

Keywords: Phosphorus fertilization, Plant nutrition, Plant Growth Promoting Rhizobacteria, Plant-microbial interaction

1. Introduction

Soil phosphorus, even when abundant, is only poorly bioavailable in its orthophosphate form, because 99,9 % is unavailable in other forms (Illmer and Schinner, 1995; Bünemann, 2015). Indeed, the ionized form of P is generally rapidly bound to clay-humic complexes. Up to 80 % of total phosphorus are complexed by sorption or precipitation with mineral matter (principally with calcium) or metals (iron and aluminium oxides and hydroxydes) (Kochian et al., 2004; Menezes-Blackburn et al., 2017), or even through microbial immobilization (Roberts and Johnston, 2015). As a result, crop production has to be sustained by the application of large amounts of phosphate fertilizers. While these inputs are recognized to have deleterious effects on the environment (Grizzetti et al., 2020), they are also rapidly and strongly adsorbed onto soil particles, thus becoming unavailable in soil solution for plant uptake (Helfenstein et al., 2018). Only 10-30% of phosphate mineral fertilization can be really recovered by plants after input (Holford, 1997; Syers et al., 2008). Remobilization from a proportion of the insoluble P pool would be an interesting alternative for reducing the amount of P added to the soil via fertilizer. Some microorganisms can mobilize P complexed with clay particles and metals such as iron and aluminum via several processes. These mechanisms include local soil acidification through direct proton release, linkages to ammonium assimilation, or production of organic acids derived from direct carbon oxidation pathways (Alori et al., 2017; Tian et al., 2021). Another interesting indirect process implicated the production of siderophore production, a common bacterial strategy for iron acquisition. The indirect P solubilization from metal particles represents then a positive externality for plants and microbial nutrition (Ikhajiagbe et al., 2020). Production of HCN, so far rather considered as a biocontrol metabolite in various studies and implied to promote general plant health (Babalola, 2021; Chandra et al., 2007; Kumar et al., 2012a), has been recently proposed to act as a metal chelator and so represents a desirable trait that could play a crucial role in indirect P solubilization (Rijavec and Lapanje, 2016).

Valorization of microorganisms presenting such interesting functional characteristics linked to potentialities of P solubilization, identified as Phosphate Solubilizing Bacteria (PSB), could represent a promising alternative approach to reduce the need of P mineral fertilization, to sustain ecologically responsible intensification of agriculture (Alori et al., 2017; Calvo et al., 2014).

The rhizosphere, hotspot of interaction and exchange between plants and its associated microbiome (Philippot et al., 2013), constitute an environment of great interest to study and better understand the issue of P availability. In the rhizosphere, the abundant microbial communities are highly shaped by plant root exudation (Olanrewaju et al., 2019). To maintain their health, plants attempt to recruit beneficial microorganisms in their rhizosphere, for example in order to fulfill their need in nutrients (Jacoby et al., 2017). So, the plant's rhizosphere could constitute a relevant reservoir to search for beneficial PSM, probably more abundant in this environment.

The aim of this study was (1) to isolate and identify PSB strains from soils sampled from the rhizosphere of three different crops (rapeseed, with high P requirements; faba bean and winter pea, both with medium P requirements), and (2) to assess PSB potential and PGPR traits according to soil origin, plant influence or their environment. We hypothesize that due to their recruitment of adapted beneficial microorganisms from the initial microbial pool present in the soil, plants with high P requirements are promising reservoirs of more efficient PGPR that possess highly interesting P solubilization traits.

2. Materials and methods

2.1 Site description and soil sampling

Samples were collected from two arable soils located in the Pays de Bray in Normandy (France). One plot (49°47'11.364''N, 01°27'5.076''E) was split between two crops: winter pea

(*Pisum sativum*, Balltrap cultivar) and rapeseed (*Brassica napus*, DK Exkio cultivar). The main crops previously cultivated on this plot were wheat for N-1 and flax for N-2. The second plot (49°46'52.716''N, 01°40'49.116''E), 16,5 km away, was planted with faba bean (*Vicia faba*, Nebraska cultivar). The main crops previously cultivated on this plot were wheat for N-1 and rapeseed for N-2. The two plots showed similar textures (Figure S1) and were loamy soils according to the USDA soil classification. Soils were sampled during the flowering stage of the three crops on the 15th of May 2018. For each investigated crop, three independent soil samples were randomly collected using a manual auger from 0 to 15 cm in depth. Each replicate was constituted of a bulk composite of 5 independent and mixed soil samplings. Sampling of soil occurred near the crown of each plant and the samples were named winter pea soil (WPS), rapeseed soil (RS) and faba bean soil (FBS) for soil harvested under the winter pea, rapeseed and faba bean crops, respectively. For the plot with adjacent cultures of rapeseed and winter pea, soil samples assigned to each crop were 10 m distant from each other to avoid at most possible crossed influence of each crop rhizosphere. The soils were then sieved at 2 mm and stored at + 4 °C for bacterial cultures and – 80 °C for molecular biology assays. Physicochemical properties were assayed by standard methods: soil pH was determined using the water-pH method (NF ISO 10390), and soil total and organic carbon were assayed using a Total Organic Carbon (TOC) Analyzer (Shimadzu SSM-5000A/TOC-VCSH Carbon, Shimadzu, Kyoto, Japan). Soil total nitrogen was determined by the Kjeldahl method (Kjeldahl, 1883). Active Calcium Carbonate content was assayed following methods described in standard NF-X31-106 (2002). Phosphorus content was determined using the Olsen method (NF ISO 11263) to estimate directly available P.

2.2 Soil bacteria isolation

Soil bacteria were isolated as follows: 5 g of each sieved soil sample were suspended in 45 mL of NaCl (0.9%) and homogenized by vortexing for 5 min. Serial dilutions of this suspension were then prepared in NaCl (0.9 %). In parallel, initial soil suspensions were also centrifuged for 5 minutes at 1000 rpm and serial dilutions were performed in NaCl (0.9 %). Dilutions of 10^{-3} and 10^{-4} for the non-centrifuged suspension and 10^{-2} and 10^{-3} for the centrifuged suspension were plated on Luria Broth (LB) agar and Reasoner's 2A (R2A) media and incubated for 48 hours at ambient temperature. These multiple procedures were deployed to maximize diversity of culturable strains. About 300 strains from both the LB and R2A media were taken and re-isolated on LB agar medium. To maximize the probability of obtaining different strains, isolates were selected based on their maximal phenotypic differences. Strains were stored in a 15 % glycerol solution at -80°C .

2.3 Screening and characterization of bacterial isolates

2.3.1 PSB abilities

2.3.1.1 Determination of solubilization index

In vitro abilities of phosphate solubilization were determined for all strains by plating on solid Pikovskaya medium (0.5 g.L⁻¹ yeast extract; 10 g.L⁻¹ dextrose; 5 g.L⁻¹ Ca₃(PO₄)₂; 0.5 g.L⁻¹ (NH₄)₂SO₄; 0.2 g.L⁻¹ KCl; 0.1 g.L⁻¹ MgSO₄; 0.1 mg.L⁻¹ FeSO₄; 1.5% agar) (Pikovskaya, 1948) and NBRIP medium (10 g.L⁻¹ dextrose; 2.5 g.L⁻¹ Ca₃(PO₄)₂; 5 g.L⁻¹ MgCl₂; 0.25 g.L⁻¹ MgSO₄; 0.2 g.L⁻¹ KCl; 0.1 g.L⁻¹ (NH₄)₂SO₄; 1.5% agar) as described by (Nautiyal, 1999) and incubated at 30 °C for 4 days. The solubilization index (SI) was calculated as follows:

$$SI = \frac{\text{colony diameter}}{\text{solubilization halo diameter}}$$

Every strain presenting an SI >1 in at least one of the two media was considered as a PSB.

2.3.1.2 *Phytate utilization assay*

The abilities of the bacteria to use phytate, an organic source of P, were tested by plating strains on specific Phytate Screening Medium (PSM medium) (Howson and Davis, 1983) (5 g.L⁻¹ sodium phytate; 10 g.L⁻¹ sucrose; 2 g.L⁻¹ (NH₄)₂SO₄; 3 g.L⁻¹ tryptone; 2 g.L⁻¹ yeast extract; 0.5 g.L⁻¹ KCl; 0.5 g.L⁻¹ MgSO₄; 0.01 g.L⁻¹ MnSO₄; 0.01 g.L⁻¹ FeSO₄; 1.5 % agar). Plates were incubated for 4 days at 30°C and the SI was calculated as previously described.

2.3.1.3. *Siderophore production*

Siderophore production was tested on modified CAS medium as described by (Lakshmanan et al., 2015). Briefly, 60.5 mg of CAS were dissolved in 50 mL of water. Then 270 mg of FeCl₃.6H₂O were dissolved in a 10 mM HCl solution and added to the CAS solution. Then an HDTMA solution was prepared by dissolution of 72.9 mg of HDTMA in 40 mL of water in a beaker under agitation. CAS-Iron (III) solution was then slowly added to the HDTMA, and the mixture was autoclaved. Finally, this sterile solution was added to LB agar medium. Bacteria were cultured for 5 days at 28 °C. Colonies presenting an orange halo were considered as siderophore-producing strains.

2.3.1.4 *Gluconic acid production assay*

Strains were cultured in 50 mL of liquid Pikovskaya medium for 5 days at 30 °C. Bacterial suspensions were then centrifuged for 10 min at 4000 rpm and 4 °C. Supernatant were collected for spectrophotometric assay with a D-Gluconate Assay Kit (Sigma-Aldrich, USA) following the manufacturer's recommendations.

2.3.2 *Other PGPR traits of interest*

2.3.2.1 *IAA production assay*

IAA production was measured with the method described by Kumar et al. (2012a). Strains were pre-cultured in liquid LB medium for 24 h. Then 50 mL of liquid NB medium were supplemented with 0.1 % D-L-tryptophan and inoculated with 500 µL of pre-culture and incubated at 30 °C under orbital agitation (120 rpm) in the dark for 48 h. The quantities of IAA were estimated by colorimetric reaction. Cultures were centrifuged for 10 min at 10,000 rpm and 4 °C. One mL of supernatant was added to 4 mL of Salkowski reagent and the absorbance of the pink coloration was read at 535 nm in a UV/visible spectrophotometer.

2.3.2.2 ACC deaminase activity

ACC deaminase activity was assessed according to the method of Glick et al. (2011). Bacterial strains were precultured overnight on Tryptone Soja Agar. The isolated colonies were then transferred onto nitrogen-free medium (5 g D,L-malic acid; 0.5 g K₂HPO₄; 0.2 g MgSO₄; 0.1 g NaCl; 15 g agar; 4 mL of 1.64 % Fe(III) EDTA; 1 mL of biotin 0.01 % + pyridoxine 0.02 %; 1 L distilled water; adjusted to pH 6.8) with the addition of 120 µL of filter sterilized 0.5 % ACC. Plates were incubated at 30 °C for 4 days. As ACC is the sole nitrogen source contained in this medium, bacterial strains that were able to grow were considered as ACC deaminase producers.

2.3.2.3 HCN production test

Hydrogen cyanide (HCN) production was assessed with the method outlined by Bakker and Schippers (1987). PSB strains were plated on Nutrient Agar. Whatman n°1 filter disks were soaked in 0.5% picric acid prepared in 2% sodium carbonate. The disks were then placed in Petri dish lids and the Petri dishes were sealed and incubated upside down for 4 days at 30 °C. A change in disk color from yellow to brown indicated HCN production by bacterial strains.

2.3.3 PSB identification

PSB strains were cultivated in LB broth for 24 h and then centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet containing bacterial cells was stored at –20 °C. Before analysis, frozen pellets were incubated for a few minutes in a water bath at 60 °C. Amplification of ribosomal 16S DNA was performed by PCR using 1 µL of 63f primer (5'-CAGGCCTAACACATGCAAGTC-3') (Marchesi et al., 1998) and a primer derived from 341f (5'-CTGCTGCCTCCCGTAGG-3') (Muyzer et al., 1993), 2 µL of bacterial culture, and 1X of GoTaq® Green Master Mix (Promega, USA) in a final volume of 50µL. PCR reaction was performed in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, USA). The amplification program was 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. A final extension step was performed for 7 min at 72 °C. PCR product quality was verified by electrophoresis on agarose gels stained with ethidium bromide. Amplicons were then sequenced using the Sanger method by Genoscreen (Lille, France) and aligned using the sequence match tool from the Ribosome Database Project (RDP, Cole et al., 2014).

Sequences obtained by 16S Sanger sequencing were aligned using the ClustalW algorithm implemented in MEGA X. A phylogenetic tree was then constructed following the maximum likelihood method, and bootstrapping (100 replicates) was used to investigate robustness.

2.4 Statistical analyses

All data were analyzed using R 64-bit version 4.0.2. One-way analysis of variance (ANOVA) of the “multcomp” package was performed, followed by a post-hoc LSD Fisher test using the function LSD.test from the R package “agricolae” for comparison between the 3 crops. For proportion differences, chi-square tests were performed on contingency tables using the chisq.test function. P values < 0.05 were considered as significant. Data are expressed as means ± SEM (Standard Error of the Mean). PCA analysis was performed using the package

“Factoshiny” to compare physico chemical properties of native soils and global traits of isolated PSB strains.

3. Results

3.1 *Physico-chemical profiles of native soils*

Both soils were sandy-clay-loam, which is classical for Normandy. All measured physico-chemical properties are summarized in Table 1. FBS was significantly different from WPS and RS (Figure 1). FBS showed lower carbon (C), organic matter, nitrogen (N), calcium carbonate contents and C:N ratio, but higher P availability. The lowest P availability was observed for RS.

3.2 *Screening and evaluation of phosphate solubilization efficiency.*

Phosphate-solubilizing potential and efficiency were screened on both Pikovskaya and NBRIP media. Among 300 culturable bacteria isolated (100 per soil), a total of 155 strains showed a SI > 1 on at least one medium (Pikovskaya or NBRIP), and so were classified here as PSB (Table 2). When considering each medium apart, a total of 117 strains showed solubilization properties (with a SI > 1) on Pikovskaya medium (54 strains from RS, 31 strains from WPS and 32 strains from FBS) and 121 strains on NBRIP medium (46 strains from RS, 29 from WPS, 46 from FBS). Only 79 strains presented a SI >1 when considering both media (33 from RS, 22 from WPS and 24 from FBS). The highest proportion of PSB were observed in RS (66 strains, i.e. 62.9 % of isolated RS bacteria), followed by 51 PSB strains found in FBS, representing 48.3 % of our initial bacterial bank for FBS. The lowest proportion of PSB strains was observed in WPS with 38 strains (42.7 % of isolated WPS bacteria).

SI of PSB screened from RS on Pikovskaya medium (Table 3) reached a significantly higher mean value of $1.61 (\pm 0.04)$ compared to $1.34 (\pm 0.04)$ from WPS and $1.28 (\pm 0.06)$ from FBS. The average SI values obtained on NBRIP medium were higher than those obtained on Pikovskaya medium: $1.77 (\pm 0.11)$ from RS, $1.44 (\pm 0.04)$ from WPS and $1.55 (\pm 0.06)$ from FBS (Table 3). However, no significant differences were found when comparing these SI for PSB isolated from RS, WPS and FBS.

Among the original 155 PSB, 68 presented an ability to solubilize phytate, with 39.4% from RS, 28.9% from WPS and 60.8% from FBS (26, 11 and 31 strains, respectively). The proportion of isolated PSB that degraded phytate was significantly higher from FBS. Conversely, the SI for phytate utilization (Table 3) was highest for PSB from RS, followed by PSB from WPS, and finally PSB from FBS, with values of $2.17 (\pm 0.15)$, $1.90 (\pm 0.12)$ and $1.21 (\pm 0.05)$, respectively.

3.3 Molecular identification of phosphate solubilizing bacteria.

Ribosomal DNA sequencing allowed genus identification for all strains, up to species identification for several of them. *Pseudomonas* was the main genus found in the PSB pool. It represented 42 % of all PSB isolated from RS, and 63 % from both FBS and WPS. The second genera identified was *Bacillus*, representing 41% for RS, 26 % for WPS and 25 % for FBS. *Paenibacillus* were found only in RS and WPS, representing respectively, 3 % and 8 % of isolated PSB. Some other anecdotal PSB genera or species were identified, including *Buttiauxella izardii*, *Microbacterium oleivorans*, *Microbacterium phyllosphaerae*, *Sanguibacter inulinus* and *Plantibacter flavus* from RS. *Erwinia rhapontici* was the only strain found in WPS other than *Pseudomonas*, *Paenibacillus* and *Bacillus*. Other PSB strains in FBS were affiliated to the species *Acinetobacter calcoaceticus*, *Stenotrophomonas rhizophila*, *Erwinia billingiae*, *Pectobacterium wasabiae* and *Serratia sp.*

No specific clusterization related to soil origin was observed according to phylogenetic tree construction for any of the PSB isolates (Figure S2).

3.4 Characterization of functional PGPR traits of interest

Several traits of interest linked with P solubilization were explored. A large proportion of PSB isolates were able to produce siderophores on CAS medium (Table 4), representing 71.0 % of all PSB. This proportion was higher for WPS (84.2 %) than for RS (62.1 %), while no differences were found for FBS (72.4 %) compared to the other soils investigated. The percentages of HCN-producing isolates were the same for each investigated crop soil, representing only a small proportion of all PSB (16.1 % on average). The gluconic acid assay highlighted 41 producing strains from the rapeseed plot (62.1% with a mean production of $139.5 \pm 22.4 \mu\text{g mL}^{-1}$), 23 producing strains from the winter pea plot (60.5% with a mean production of $104.0 \pm 19.6 \mu\text{g mL}^{-1}$) and 36 producing strains from the faba bean plot (70.6% with a mean production of $156.6 \pm 33.0 \mu\text{g mL}^{-1}$) (Table 4). The proportion of PSB strains producing gluconic acid tended to be greater in FBS, and they had higher production abilities. Interestingly, the proportions observed in both WPS and RS were similar.

Concerning IAA, 34.8% of all strains were actually producers. A higher number of IAA-producing strains was observed under FBS (49.0%) compared to RS (24.2%), while no differences were found regarding WPS (34.2%). IAA-producing PSB showed a mean production of $73.3 \text{ ng } \mu\text{L}^{-1}$ (± 9.8), $54.3 \text{ ng } \mu\text{L}^{-1}$ (± 8.4) and $46.9 \text{ ng } \mu\text{L}^{-1}$ (± 5.0) for PSB originating from RS, WPS and FBS, respectively. IAA amounts produced were highest considering PSB strains from RS (from a range of 7.7 to $297.1 \text{ ng } \mu\text{L}^{-1}$), followed by WPS (3.5 to $208.5 \text{ ng } \mu\text{L}^{-1}$) and lowest for FBS (2.6 to $147.6 \text{ ng } \mu\text{L}^{-1}$) (Figure 2). Due to the combination of proportion of IAA-producing PSB and variations in quantities, all PSB strains showed close mean productions of auxin: around $20 \text{ ng } \mu\text{L}^{-1}$ on average. Regarding ACC deaminase activity,

no significant differences were highlighted. About 59.7 % of strains isolated from RS were able to use ACC as an N source, 73.7% for PSB from WPS and 63.0% for PSB from FBS.

Principal Component Analyses (PCA) of these results, plotted according to soil origins, showed a separation in the PSB populations between RS and WPS due to the higher P solubilization values of the RS populations on NBRIP, Pikovskaya and PSM media (Figure 3). The same PCA, plotted according to PSB genera (Figure 4a), highlighted that all PSB genera have similar mean solubilization abilities and PGPR characteristics, except for the genera *Buttiauxella* and *Acinetobacter*. These two genera seemed to show higher SI values on Pikovskaya medium and a higher gluconic acid production. Siderophore-producing PSB showed distinct characteristics from non-producing ones (Figure 4b). Indeed, interestingly, siderophore-producing strains tended to produce more IAA but less gluconic acid and had lower SIs on Pikovskaya medium. Similarly, the presence of ACC deaminase activity seemed to be linked to higher SIs on NBRIP and PSM media (Figure 4c). The SIs on NBRIP medium seemed also to correlate with phytate utilization. Solubilization on Pikovskaya medium seemed to correlate with gluconic acid production (Figure 4d). No distinct profile could be found between HCN-producing PSB and non-producing strains (data not shown).

4. Discussion

4.1 Identification of potential PSB strains from investigated soils

The use of beneficial microorganisms as biofertilizers represents an emerging and promising alternative to mineral fertilizer application (Menezes-Blackburn et al., 2017). Numerous bacterial strains with PSB or PGPR abilities have already been reported in the literature (Alori et al., 2017). In this study, we decided to appoint as PSB all bacterial isolates presenting a SI > 1 on at least one of the tested media (Pikovskaya or NBRIP). The three investigated soils hosted

a great variety of PSB strains, representing a wide range of the overall culturable bacteria. Unsurprisingly, most of the PSB isolates belonged to *Pseudomonas* and *Bacillus* genera, which are among the most represented PSB genera described in the literature (Ghorbanzadeh et al., 2020; Safari et al., 2020; Saxena et al., 2020). The third most abundant genus here was *Paenibacillus* which has also previously been described as an endophytic PGPR mainly isolated from legume nodules and showing P solubilization abilities (Ali et al., 2021). However, surprisingly, this genus was not detected in faba bean rhizosphere. Most of the other genera found in our study also had prior description as potential PSB strains (Araújo et al., 2021; Emami et al., 2020; Mitra et al., 2020; Rodríguez and Fraga, 1999), except for *Sanguibacter*, mainly known as N₂ fixing bacteria with biocontrol properties against nematode infestation (Alishahi et al., 2020; Moussa and Zawam, 2010). To our knowledge, the present study reveals for the first time a *Sanguibacter inulinus* isolate with phosphate solubilization abilities and can thus be described as a PSB.

To characterize the ability of bacterial isolates to solubilize inorganic phosphate, two dedicated media were used: Pikovskaya and NBRIP (Pikovskaya, 1948; Nautiyal, 1999). These two media are classically used for screening of PSM (Suleman et al., 2018; Xiao et al., 2020). NBRIP medium provide a more stringent environment, with lower quantities of C and N sources compared to Pikovskaya medium. Most of our PSB isolates showed positive P solubilization on both medium, despite occasional differences in the SI value range for the same strain. However, some isolates showed a positive solubilization of inorganic P in only one medium. These differences were probably linked to the adaptation of bacterial metabolism to their surrounding environmental conditions, and particularly in response to nutrient ratios (Cui et al., 2018; Manzoni et al., 2017). An accurate evaluation of P solubilization in several

environmental conditions would be a relevant procedure for PSB screening and to better understand and so exploit their potential.

Other direct and indirect mechanisms of P solubilization were explored in parallel. We decided to focus our screening on acidification ability, through the assessment of gluconic acid production as one of the principal organic acids released by PSB (Alori et al., 2017). We also evaluate siderophore production, phytate utilization and HCN production. Beside these specific PSB characteristics, auxin production and ACC deaminase activity, other interesting classical PGPR traits, were also explored according to their possible interest for root growth enhancement, and so potentiality to increase the volume of soil prospected by plants (Ambreetha and Balachandar, 2019; Richardson et al., 2009).

When considering jointly all studied traits, two interesting PSB profiles can be distinguished. Isolates showing better direct solubilization of phosphate also presented high secretion of gluconic acid but lacked siderophore production. So, it could be hypothesized that these isolates would improve P availability in soil rather through direct processes. On the contrary, PSB strains that showed lower direct solubilization correlated with low gluconic acid production were able to produce siderophores and also high levels of auxin, improving P availability in soil rather via indirect processes. PSB strains that are efficient in solubilizing phosphate in more deprived conditions (on NBRIP medium, with lower N and P content) were also able to use phytate and had positive ACC deaminase activity. These strains showed a broad metabolism, with the solubilization of both inorganic and organic P, maybe more adapted to stressful and variable soil conditions. Only little relationship between PSB characteristics and bacterial genus, except in the cases of the *Buttiauxella* and *Acinetobacter* strains, less studied than *Bacillus* or *Pseudomonas*. Indeed, in our study the *Buttiauxella* and *Acinetobacter* strains showed globally higher potential for P solubilization on Pikovskaya medium and high

production of gluconic acid. The other genera studied showed great variability in all studied traits. These results emphasize the importance of exploring total soil microbial diversity, without setting up an *a priori* screening approach.

This screening of bacterial isolates constituted thereafter an opportunity to more deeply explore the *in planta* abilities of some selected strains showing the most promising *in vitro* abilities. This work, subsequently achieved, aimed to determine the possible efficiency of PSB deployment as an alternative of mineral fertilization but also to investigate the factor of success of PSB strains deployment on rapeseed growth according to their rhizospheric origin and their potential metabolic profiles (Amy *et al.*, 2022, “Are native phosphate solubilizing bacteria a relevant alternative to mineral fertilizations for crops? Part II: PSB inoculation enables a halving of P input and improves the microbial community in the rapeseed rhizosphere”).

4.2 The influence of plant type on the beneficial microorganism community

In this work, the presence and diversity of culturable PSB was explored in the rhizosphere of three different crops, each with their own specificities and P requirements. Interestingly, a higher number of PSB isolates were recovered from rapeseed relative to winter pea or faba bean. Because rapeseed is known to have high P requirements compared to legume crops (Bolland *et al.*, 1999; Bolland and Brennan, 2008), this higher PSB abundance and proportion among culturable bacteria was actually expected. Despite that our results could also be influenced by chemical properties of soils from the two distinct plots and cultural practices applied by farmers, our results nevertheless emphasize the substantial influence of the rhizosphere environment on the culturable PSB community, and particularly the specific nutrient preference of each plant species to condition microbiome's shape. Plant influence on the rhizospheric bacterial community is well known (Costa *et al.*, 2006; Gkarmiri *et al.*, 2017). The specific assemblages of the associated microbiome are governed by each plant species'

traits, and more particularly as part of an adaptative strategy to sustain and improve nutrient acquisition (Bakker et al., 2018; Cai et al., 2017; Dastogeer et al., 2020; Fitzpatrick et al., 2018). Some recent studies have assessed plant-microbe interactions and plasticity in terms of the P nutritional status of the plant (Castrillo et al., 2017; Pang et al., 2021). For instance, in the eggplant rhizosphere, Li et al. (2019) demonstrated a correlation between P-requiring developmental stages and PSB abundance and efficiency. In their study, the highest P requirements occurred during flowering and fruit maturation. In the current work, soils were also sampled during flowering in the three crops, which could have maximized the probability of screening efficient and diverse PSB.

A noteworthy trend in the level of phosphate solubilization indexes according to the investigated rhizosphere could be also highlighted. Indeed, in the rhizosphere of rapeseed, where the P requirement was high, the solubilization indexes of isolated PSB were higher than those isolated from the soil under the legumes. PSB found under winter pea and faba bean, with comparable P requirements (Bolland et al., 1999), presented similar P solubilization indexes despite geographical distancing between fields and differences in some soil chemical properties. Enrichment of populations in more efficient PSB by rapeseed supported again the hypothesis of a targeted selection linked to plant nutrient preference. However, the higher proportion of PSB in rapeseed rhizosphere could also be due to the lower content of available P, leading to a higher need of PSB recruitment from the soil microbial pool for the crop, when compared to winter pea and faba bean. Nevertheless, high P requirement of rapeseed, and so nutrient uptake in soil with the help of PSB populations, could also be one of the main reasons explaining the lower observed content of available P in its rhizosphere. Despite enrichment in efficient and diverse PSB could have been constrained by the contrasted chemical properties and particularly P availability observed between the two studied plots, and so interfering with the inherent

nutritional necessity of plants, similarities observed when comparing PSB isolated from legumes remain strikingly astonishing.

A lower diversity in PSB species could be expected in soil under rapeseed (Siebers et al., 2018). Indeed, in the rhizosphere of rapeseed, the rhizodeposition of glucosinolates represented a major constraint, compelling and shaping microbial communities (Bressan et al., 2009). Rumberger and Marschner (2003) showed that these brassicaceous sulphureous metabolites and their products of degradation with bioactive properties are continuously released by living canola roots in the rhizosphere, even if they can be rapidly degraded by some microorganisms (for example, in 90 h for 2-phenylethylisothiocyanate). Nevertheless, in our study, PSB isolates retrieved from rapeseed rhizosphere were affiliated to a larger variety of genera than those from soil harvested under winter pea or faba bean. While different proportions of PSB strains were found in the three investigated rhizospheres, the proportions among the two main genera (*i.e.*, *Pseudomonas* and *Bacillus*) were incredibly similar in the soils under the legumes, despite the geographic distance and differences in measured chemical properties of the soils but were distinct from the rapeseed crop's soil. The abundance of PSB affiliated to *Bacillus* genus was lower under winter pea, compared to rapeseed, which is consistent with the generally overall lower level of *Bacillus* observed in the rhizosphere of legumes (Kumar et al., 2012b). Conversely, a decrease in *Pseudomonas* abundance has been previously observed in rapeseed's rhizosphere (Gkarmiri et al., 2017). However, Gkarmiri et al. (2017) also demonstrated that despite this lower abundance, this bacterial genus seemed to be highly active in the rhizosphere. Nevertheless, it could be interesting to confirm the observed distinction of cultivable PSB diversity between rapeseed and legumes, but also conjunction between winter pea and faba bean, if deployed on the same field.

Because these three crops are known to deploy different physiological and morphological strategies of adaptation to P stress conditions (Honvault et al., 2020), we could hypothesize that each crop would have selected from the microbial pool PSB strains possessing functioning complementary or synergistic with their own P acquisition strategies. Indeed, Honvault et al., (2020) showed that P acquisition strategies for legumes were geared towards direct mechanisms with phosphatase release and decreases in soil pH, while Brassicaceae rather increased fine roots and hair production to explore a higher soil volume and also released carboxylates. In the present work, rapeseed seemed to shape PSB populations towards profiles with direct solubilization mechanisms (high SI), while the PSB from winter pea and faba bean showed an indirect solubilization profile with higher production of siderophores and auxin. However, as our study has only focused on culturable PSB, representing only a small proportion of the total microbiome of the soil (Steen et al., 2019), the hypothesis of this synergy between plants abilities and microbial enrichment of complementary characteristics will require greater exploration.

Even if the chosen *in-situ* approach is undoubtedly influenced by several confounding factors because of the chosen experimental design, our results remain of interest to point out specific plants microbiomes properties in crop rotations and in mixed-cropping. Besides the direct influence of the cultivated crop, the possible influence of the chemical characteristics of soil and particularly P Olsen content could not be excluded. Thus, the lowest observed content of available P in the rhizosphere of rapeseed could also contribute to the higher proportion of PSB, compared to faba bean rhizosphere. Similarly, the entire field history and particularly the legacy of previous crops could also be another factor influencing the microbial assemblage. However, both fields were previously cultivated with wheat, possibly limiting the difference of the initial microbial pool. The observed assemblage of microbial populations could finally be the result

of crop effect combined with all applied farming practices. It would be interesting to deeply explore and disentangle the determinism of each of these factors.

5. Conclusion

In this study, a large proportion and a wide diversity of all isolates showed PSB traits, highlighting their extensive presence in soil. The higher proportion of bacteria isolates, with more efficient direct P solubilization, in the rhizosphere of rapeseed point out the importance of crop's nutritional preference as a factor of modulation shaping their associated microbiome. These crops could further be considered as interesting trap plants, of great potential for future PSB screening. Interesting linkages were highlighted between PGPR traits and P solubilization efficiency, with two distinct isolate profiles of direct or indirect processes. Complementarity between these bacterial traits with those specifically deployed by plants appeared to be an efficient strategy to face nutritional issues in soil. Future work will now focus on the *in-situ* evaluation of bio-inoculants composed of selected PSB isolates on rapeseed growth and P acquisition. In the context of crop rotation, the present work also indicates that plant-driven selection could be considered to fortify soil microbiome at the benefit of subsequent crops, by attracting high-value microbial partners.

Declaration of interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1 : Physicochemical properties of soils sampled from rapeseed, winter pea and faba bean (mean \pm SD). Letters indicate significant differences between the three different soils for each chemical parameter.

	Origin of the sampled soil					
	Rapeseed		Winter pea		Faba bean	
pH	7.40	\pm 0.05 <i>ab</i>	7.90	\pm 0.04 <i>b</i>	7.98	\pm 0.04 <i>a</i>
Total Carbon (g / kg)	19.62	\pm 0.17 <i>b</i>	20.69	\pm 0.44 <i>a</i>	10.64	\pm 0.26 <i>c</i>
Total Organic Carbon (g / kg)	18.74	\pm 0.26 <i>b</i>	19.78	\pm 0.40 <i>a</i>	10.46	\pm 0.28 <i>c</i>
Organic matter (%)	3.37	\pm 0.03 <i>b</i>	3.56	\pm 0.07 <i>a</i>	1.83	\pm 0.04 <i>c</i>
Calcium (ppm)	6.52	\pm 0.17 <i>a</i>	6.56	\pm 0.16 <i>a</i>	3.94	\pm 0.09 <i>b</i>
Total Nitrogen (mg / g)	2.55	\pm 0.03 <i>a</i>	2.43	\pm 0.05 <i>a</i>	1.59	\pm 0.06 <i>b</i>
Olsen Phosphorus (mg / kg)	2.50	\pm 0.10 <i>a</i>	1.18	\pm 1.79 <i>a</i>	22.12	\pm 0.29 <i>b</i>

Table 2 : Number of PSB strain by genus according to their identification by 16S rDNA sequencing in the soil sampled under rapeseed, winter pea and faba bean crops.

	Origin of the sampled soil		
	Rapeseed	Winter pea	Faba bean
<i>Pseudomonas sp</i>	28	24	32
<i>Bacillus mycoides</i>	5	3	3
<i>Bacillus pseudomycoides</i>	7	2	2
<i>Bacillus simplex</i>	11	2	6
<i>Bacillus sp</i>	4	3	2
<i>Paenibacillus</i>	2	3	0
Others	9	1	6
total PSB strains	66	38	51
<i>Pseudomonas</i> proportion	42%	63%	63%
<i>Bacillus</i> proportion	41%	26%	25%
PSB proportion in all isolated bacteria	62.9 %	42.7 %	48.6 %

Table 3 : Phosphate solubilization abilities of PSB strains from rapeseed, winter pea and faba bean soils. The solubilization index (SI) was observed on the two tested media for inorganic P and the phytate solubilization abilities of phytate-solubilizing PSB strains. Letters indicate significant differences between soils for each medium.

		Origin of the sampled soil		
		Rapeseed	Winter pea	Faba bean
SI on NBRIP medium	Mean \pm SE	1.77 \pm 0.11 <i>a</i>	1.44 \pm 0.04 <i>a</i>	1.55 \pm 0.06 <i>a</i>
	Median	1.54	1.46	1.53
	SI max	4.81	2.23	2.69
SI on Pikovskaya medium	Mean \pm SE	1.61 \pm 0.04 <i>a</i>	1.34 \pm 0.04 <i>b</i>	1.39 \pm 0.06 <i>b</i>
	Median	1.53	1.31	1.28
	SI max	3.14	2.64	3.67
SI on phytate medium	Mean \pm SE	2.17 \pm 0.15 <i>a</i>	1.9 \pm 0.12 <i>a</i>	1.21 \pm 0.05 <i>b</i>
	Median	1.29	1.87	1.44
	SI max	4.9	2.62	2.23

Table 4 : Various PGPR traits and solubilization mechanisms of isolated PSB from rapeseed, winter pea and faba bean soil. Data are expressed as a percentage of all isolated PSB and as a proportion. Letters indicate significant differences based on chi-square tests between soils for each tested PGPR trait.

	Origin of the sampled soil					
	Rapeseed		Winter pea		Faba bean	
Siderophore producing strains	62.1 %	<i>b</i>	84.2 %	<i>a</i>	72.4 %	<i>ab</i>
ACC deaminase producing strains	59.7 %	<i>a</i>	73.7 %	<i>a</i>	66.7 %	<i>a</i>
HCN producing strains	13.6 %	<i>a</i>	10.5 %	<i>a</i>	23.5 %	<i>a</i>
IAA producing strains	24.2 %	<i>b</i>	34.2 %	<i>ab</i>	49.0 %	<i>a</i>
Gluconic acid producing strains	62.1 %	<i>a</i>	60.5 %	<i>a</i>	70.6 %	<i>a</i>

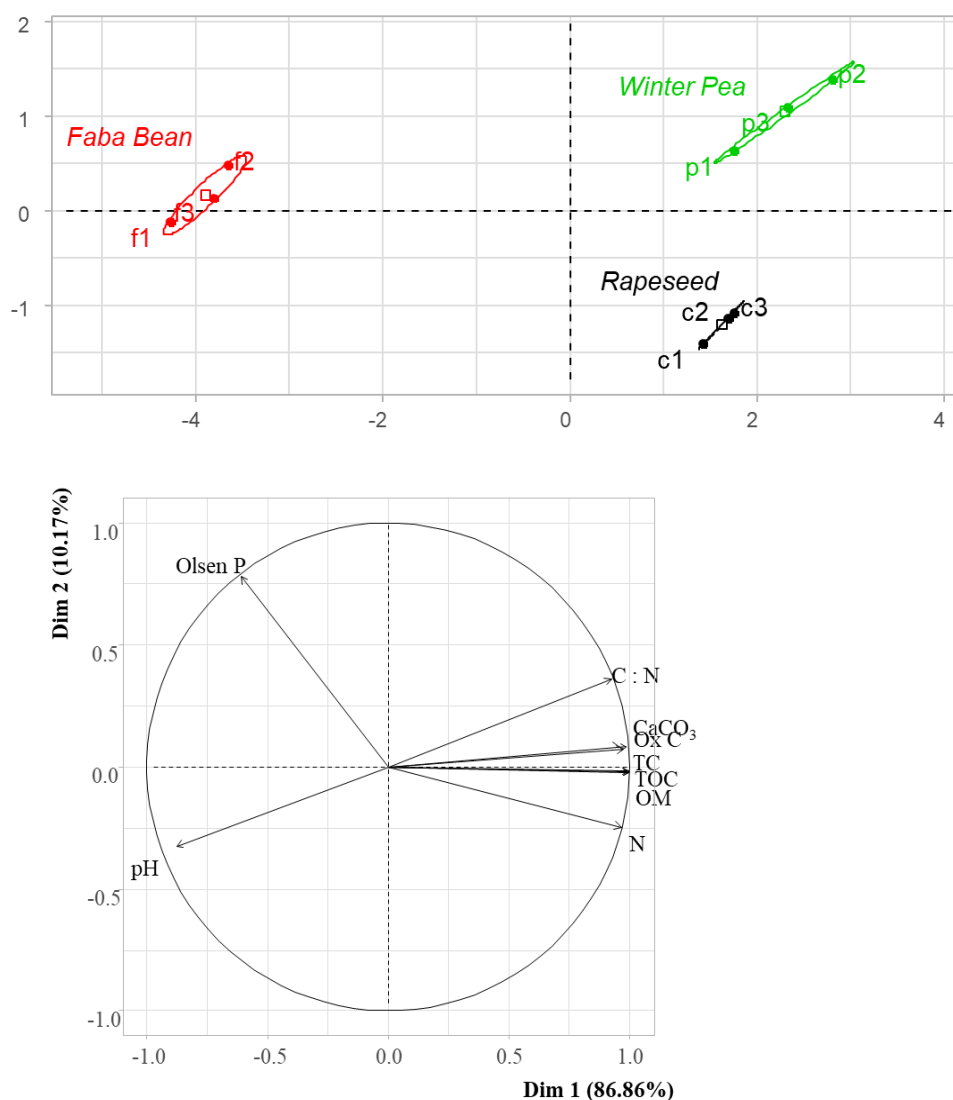


Figure 1: Principal Component Analysis of physicochemical properties of native rhizospheric soils. Letters on individuals topology indicate the soils origin (c : rapeseed (RS) f : faba bean (FBS) and p : winter pea (WPS)). N, nitrogen; OM, organic matter content; CaCO₃, calcium carbonate content; TC, total carbon; TOC, total organic carbon; Ox C, oxidizable carbon; C : N, carbon to nitrogen ratio; Olsen P, directly available phosphorus.

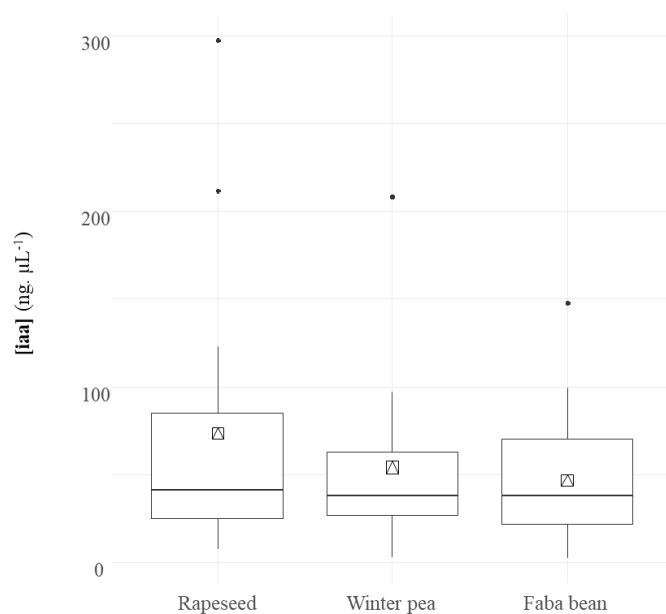


Figure 2: IAA production of auxin-producing PSB isolated from rapeseed (RS), winter pea (WPS) and faba bean soils (FBS). Triangles represent the average IAA production.

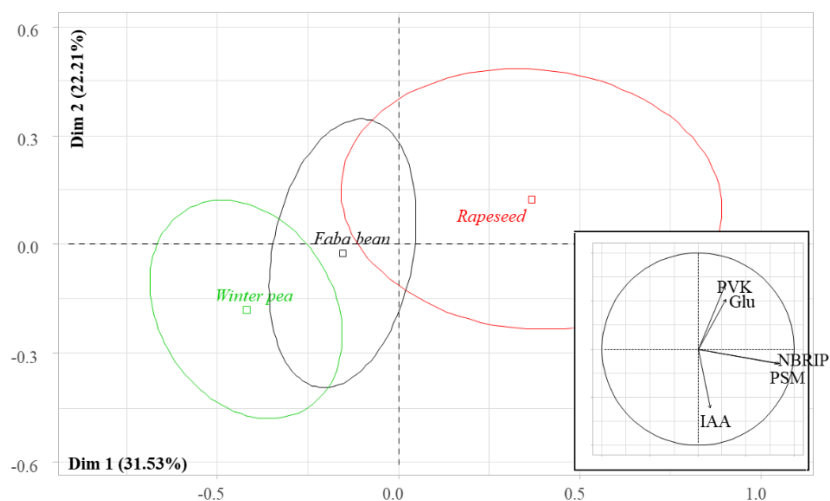


Figure 3: Principal Component Analysis of PSB strains according to their soils of crop origin (rapeseed (RS) faba bean (FBS) and winter pea (WPS)). Correlation circles comprise the solubilization indexes on Pikovskaya (PVK), Phytase Screening (PSM), and NBRIP Media, and the gluconic acid (Glu) and IAA assays.

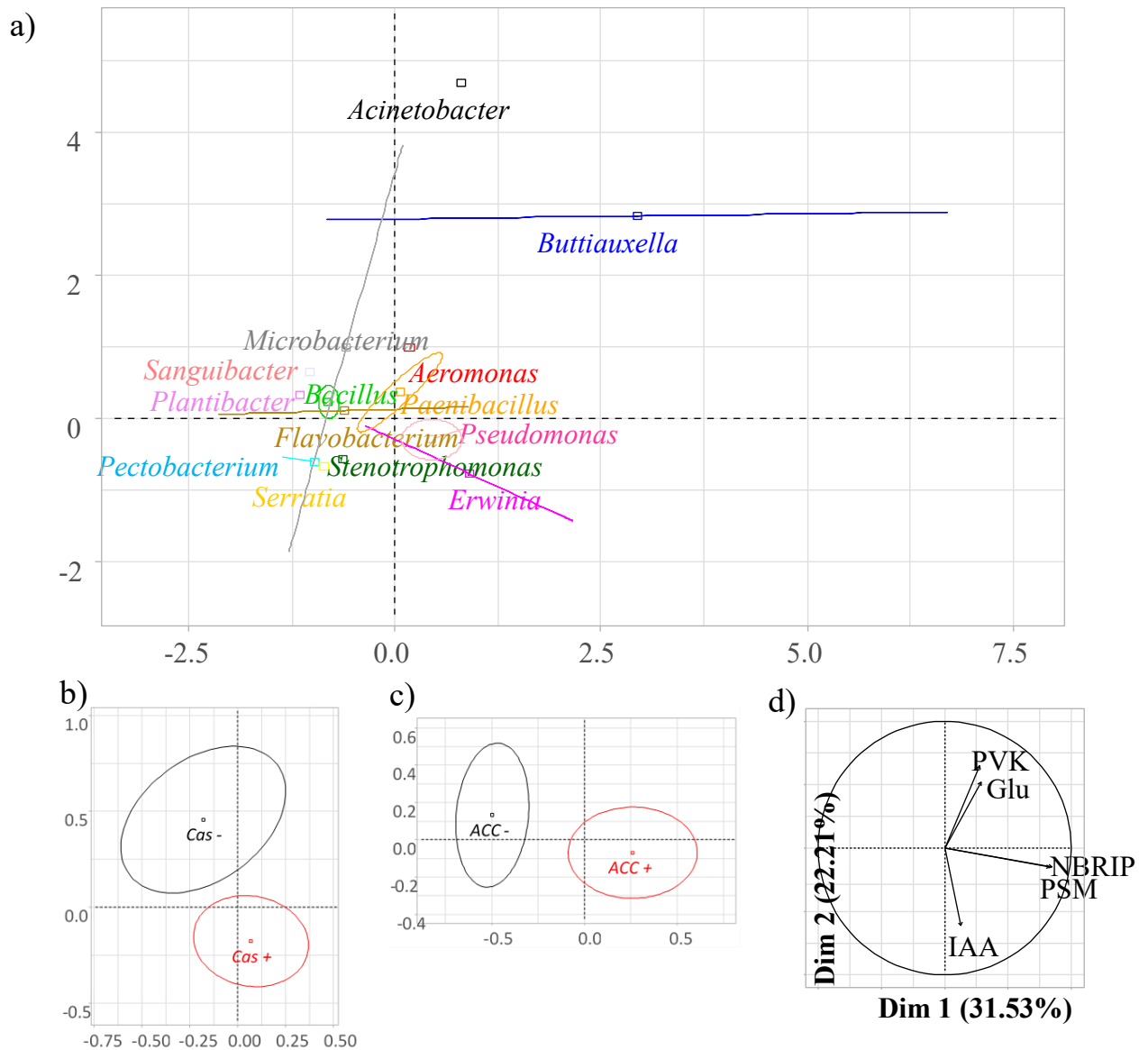


Figure 4: Principal Component Analysis of PSB strains according to their solubilization abilities and PGPR traits. Individuals are plotted according to (a) their genera; (b) abilities to produce siderophores on CAS medium; or (c) to degrade ACC. The correlation circles (d) comprise solubilization Indexes on Pikovskaya medium (PVK), solubilization Indexes on Phytase Screening Medium (PSM), solubilization Indexes on NBRIP medium (NBRIP), Gluconic acid production (Glu) and IndoleAcetic Acid production (IAA).