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Article

The Importance of Considering Levels of P and N Fertilization to Promote Beneficial Interaction between Rapeseed and Phosphate-Solubilizing Bacteria

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Abstract: Biointraants constitute a promising opportunity to lower mineral input on rapeseed, characterized by high nutrient requirements. As bio-inoculants, phosphate-solubilizing bacteria (PSB) could increase the amount of available P in a soil solution. However, the deployment of these bio-inoculants in fields is not always successful. Disentangling the factors conditioning their reliability is necessary. Because the activities of microorganisms are particularly subject to nutrient availability, the N fertilization level could represent a key factor for the success of PSB inoculation in the early stages of plant growth. In this study, Pfaba (*Pseudomonas* sp.), a promising plant growth-promoting rhizobacteria (PGPR) strain isolated from soil, was inoculated on rapeseed grown in rhizotrons under two N fertilization levels (N160 or N80) in P labile or P complexed conditions. Pfaba confirmed its PSB potential to solubilize recalcitrant P complexed forms for the benefit of plant growth, but only when the N supply is adequate (N80). In a P complexed environment, Pfaba tended to increase root and shoot biomass (respectively, from 2.17 ± 0.47 g for control modality to 2.88 ± 0.85 g, and from 6.06 ± 1.67 g for control modality to 8.33 ± 1.70 g), increase the P and N contents in roots (respectively, from 0.15 ± 0.09 mg for control modality to 0.70 ± 0.51 mg, and from 37.90 ± 11.09 mg for control modality to 41.34 ± 14.16 mg), and restore root length at a comparable level than plants supplemented with labile P. Conversely, these positive effects were inhibited with lower levels of N fertilization. Our results highlight the importance of nutrient availability to promote beneficial interaction between plants and microorganisms. These findings could also contribute to ensuring the successful deployment of microbial biointraants.

Keywords: biostimulant; plant nutrition; phosphorus; PGPR; rapeseed



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1. Introduction

The fine-tuned exploration of the relationships between plants and microorganisms appears essential to better understanding their involvement in crop productivity and, therefore, agrosystem functioning. In soil, microorganisms perform crucial agronomic and environmental functions, particularly organic matter transformation, thus turning it into available nutrients for plants [1]. Plants construct a real partnership with a rich diversity of microorganisms, especially within their rhizosphere. This partnership is recognized as having a major impact on plant growth and health [2–5]. The collective genome represented by this complex associated microbial community, much larger and diverse than that of the plant, constitutes its second genome and is of great interest in terms of the multiple carried functions [2]. The association of the plant and its overall microbial community is known as a holobiont and represents the great potential of the plant and its second genome (i.e., the hologenome). The rhizosphere is the location of complex interactions between plant and microbial populations but also has strong competition

between microorganisms [6]. This heavily populated environment appears to be a favored reservoir for potentially beneficial microbial agents, constituting the first battle line of plants in soil against abiotic and biotic stresses but also contributing to plant nutrition [7]. Manipulating a native soil microbiome in this habitat to promote plant growth and nutrition represents an interesting, sustainable alternative to chemical inputs [8–10]. The objective is to shape the composition of microbiomes to enhance beneficial interactions [11] and, more specifically, to enrich the soil and associated microbiomes with the taxa of plant growth-promoting rhizobacteria (PGPR) [3,12,13]. Numerous studies have focused on the use of PGPR as bioinoculants to sustain crop development [14–17] because of its numerous well-described beneficial characteristics [18]. Indeed, such PGPR could directly impact plant growth by contributing to the availability of essential nutrients in the soil, improving root absorption, enhancing plant growth through phytohormone production, or indirectly by enhancing plant resistance to abiotic and biotic stresses [19]. Among those PGPR, phosphate-solubilizing bacteria (PSB) constitute a major group of interest in agriculture. These bacteria are known for their ability to turn complexed P into an available form for plants in soil solution. The range of microbial strategies to access recalcitrant P forms is wide. Indeed, P can be solubilized by soil acidification through direct proton release due to NH_4^+ acquisition or secretion of organic acids. Also, P complexed to iron can be solubilized through siderophore production [20,21]. Regarding this latter mechanism, bacteria aim to acquire iron, as free iron is scarce in the environment [22]. Finally, organic P can also represent a variable but non-neglectable proportion of total P in soils (between 20% and 80%) [23]. So, phosphatase and phytase activities can represent desirable traits for an efficient PSB. Independently of phosphate solubilization, other PGPR traits can also be helpful to face plant P starvation, such as enhancement of root growth. Indeed, PSB strains can also possess the capacity to secrete indole-3-acetic acid (IAA), or auxin, a key promoter of plant growth, in their environments, and others are able to degrade ACC [24]. The degradation of this precursor of ethylene [25,26] enables plants to increase root length, thus allowing plants to explore a higher soil volume and maximizing P prospecting [27].

Despite this interesting arsenal to sustain plant nutrition, PSB and, more globally, PGPR inoculation often encounter failures when applied in the field [28]. Indeed, as a prerequisite, microbial inoculants must survive, colonize root surfaces, and express their potential to actually sustain plant health and growth. Dialogue between plants and microorganisms inhabiting the rhizosphere is also primordial to act synergistically as a functional holobiont, i.e., considering the plant host and microorganism as a super-individual [29]. Unraveling the factors of success of PGPR application thus represents a challenge to improving the ecological performance of rapeseed.

Numerous factors could compel microbial inoculants' efficiency in agricultural soil conditions [19,30]. Among abiotic factors, soil temperature has important consequences on microbial biomass development but could also act on their metabolism, particularly gene expression, and so finally could deeply affect their efficacy [31,32]. Schindlbacher et al. [33] showed that warming significantly modulated microbial respiration in soil. Similarly, soil pH could also strongly affect major functions of microbial cells, leading to metabolism modifications such as enzymatic activities [34]. Nadeem et al. [35] showed the great potential of three PGPR strains (*Pseudomonas fluorescens*, *Bacillus megaterium*, and *Variovorax paradoxus*) provided through seed coating to mitigate the alteration of cucumber growth due to salinity stress, particularly shoot length, with nevertheless a substantial variation in observed efficiency. The authors highlighted differences in bacterial competence to colonize rhizospheres as well as a significant modulation of several PGPR trait expressions (IAA production, siderophores production, phosphate solubilization, ACC deaminase activity) according to salinity level. The tested *P. fluorescens*, with the greatest efficiency on cucumber growth, showed a high capacity to maintain in vitro PGPR characteristics and root colonization, whatever the salinity levels, contrary to *Bacillus megaterium* and *Variovorax paradoxus*. Similarly, nutrient availability could be an important factor in ensuring PGPR efficiency for plant growth promotion. Indeed, nutrient depletion could be prejudicial to

rhizosphere colonization by the added PGPR, compelling the efficiency of bio-inoculants in soil. Indeed, nutrient limitation is well known to impact microbial biomass in soil [36]. Aldén et al. [37] showed that nitrogen deprivation constrained microbial growth and activities in soil. Nitrogen availability could also influence the expression of PGPR traits [19]. Martínez et al. [38] showed that the level and nature of the N supply significantly modified PGPR functions, such as the potentiality of P solubilization of the four tested *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Serratia* strains. Nitrogen limitation in soil could be an obstacle to the large-scale deployment of sustainable microbial biosolutions to support crop production by directly affecting the establishment of beneficial plant–microorganism interactions.

Rapeseed is a major oleoproteaginous crop, spread worldwide and characterized by high phosphorus and nitrogen requirements [39,40], leading to the supply of high amounts of fertilizers, often in mineral forms. These inputs are responsible for numerous environmental degradations through leaching or volatilization [41]. Particularly, P is generally quickly complexed to the soils' organomineral particles after application and remains unavailable for plants [42]. The promising potential of promoting beneficial plant–microorganism interactions to sustain rapeseed growth and nutrition while limiting mineral input leads to great interest in expanding sustainable agricultural practices. Previously, Amy et al. [24] showed that rapeseed rhizospheres host an important proportion of PSB strains with a high potential for P solubilization. This specificity of microbial recruitment appears to meet plant nutrient requirements [43]. The benefit of using PSB inoculants to sustain rapeseed growth is gaining more and more attention [44]. Amy et al. [45] described the benefits of applying a PSB strain (*Pseudomonas* sp.), isolated for a faba bean rhizosphere and inoculated at the dose of 1×10^7 cells per shoot in a clay–limestone P-poor soil, to promote rapeseed shoot and root biomasses under a half-reduced P fertilization modality. The authors showed that the inoculation of this *Pseudomonas* strain allowed the maintenance of a P nutrition of rapeseed equivalent to complete fertilization. The added PSB also led to a shift of the associated microbial community in the rhizosphere, with stimulation of *Bradyrhizobium* and *Thiobacillus* populations, with even more potential to facilitate plant nutrition (respectively, nitrogen and sulfur cycles). However, in such experiments with native soil, it could sometimes be difficult to disentangle a direct impact on P solubilization because of several PGPR traits carried by the PSB strain, as well as the observed modifications induced on the associated microbiome.

In this study, part of a PhD work [46], we aimed to evaluate the direct effects of this *Pseudomonas* PSB strain, previously isolated from faba bean rhizosphere, on early stages of rapeseed growth in a rhizotron system, confronted with two levels of N fertilization and two forms of P (P applied in labile or complexed form). The principal objective was to demonstrate the effective promotion of plant nutrition and growth through direct P solubilization. As bacteria can modulate their metabolism to nutrient availability following microbial economics [47], we also evaluate if the N supply could play a role in the expression of desirable traits of inoculated PSB and, therefore, be a determinant for beneficial plant–microorganism interactions.

2. Materials and Methods

2.1. PSB Screening and Application in Plants

In a previous study [24], a PSB screening was performed from soils of three crops: rapeseed, winter pea, and faba bean. Several classical PSB and PGPR traits of interest were determined: solubilization on Pikovskaya and NBRIP media [48,49], phytate utilization, siderophore, HCN and IAA production, and ACC deaminase activity as described by Amy et al. [24]. Three of these PSB strains, showing higher in vitro performances, were also previously tested on rapeseed growth in the first greenhouse experiment [45]. Based on this previous in planta screening, we have selected the Pfaba strain, isolated from soil under the influence of a faba bean culture that belongs to the *Pseudomonas* genus, for further analysis of its effects on root traits and rapeseed growth in axenic conditions. For plant

application, Pfaba was cultured for 24 h at 30 °C in Luria broth. Bacterial concentration was evaluated by optical density measure at 580 nm. The bacterial culture was then centrifuged, and a pellet containing bacterial cells was resuspended in NaCl 0.9% in order to inoculate 1×10^6 bacteria in 1 mL directly around the crown of plants. Control plants received 1 mL of NaCl 0.9%.

2.2. Greenhouse Experiment

Rapeseeds (*Brassica napus* L. cv *Aviso*) were sown directly in rhizotrons (30 × 20 × 1 cm, 1 plant per rhizotron, 5 replicates per modality) filled with autoclaved sand previously sieved at 200 µm and moisturized at 10% with distilled water. Rhizotrons were placed in bins with top apertures, allowing them to remain inclined at 30°. Details of the fertilization modalities are summarized in the experimental design given in Figure 1. The plants received two levels of NH₄NO₃: a high N fertilization (N160: 160 mg of N per plant) or a limited N fertilization (N80: 80 mg of N per plant). The plants also received 56 mg of P in a labile form (LP: KH₂PO₄) or in a complexed form (CP: 10% KH₂PO₄, 2% phytate, 55% Ca₃(PO₄)₂, 23% FePO₄, and 10% AlPO₄). Half of the plants received 1 mL of a suspension of 1×10^6 bacteria mL⁻¹ of the PSB strain Pfaba. The control plants received 1 mL of NaCl 0.9%. Additionally, a total of 3.6 L of modified Hoagland solution, containing neither N nor P, was brought per rhizotrons in bins, allowing percolation of nutrient solution (0.5 mM MgSO₄, 0.2 mM EDTA+ 2 NaFe, 1.25 mM CaCl₂, 1.5 mM KCl, 14 µM H₃BO₃, 5 µM MnSO₄, 0.765 µM ZnSO₄, 0.316 µM CuSO₄, and 0.5 µM (NH₄)₆Mo₇O₂₄, and 0.1 µM CoCl₂). Thirty milliliters of percolated nutritive solution were distributed from bins to the top of rhizotrons in a closed circuit three times per day. The surface of the rhizotrons was covered with a strip of Whatman paper in order to ensure a homogenous horizontal distribution of the solution. Natural light was complemented, if necessary, to maintain photosynthetically active radiation of 400 µmol s⁻¹ m⁻² at the canopy by high-pressure sodium lamps.

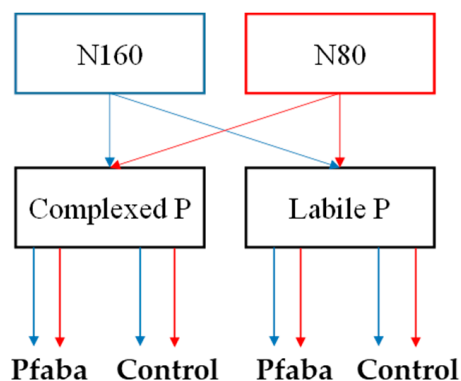


Figure 1. Experimental design of the rhizotrons experiments. Two N fertilization levels were applied: a high (N160: 160 mg per plant) and a lower (N80: 80 mg per plant) N fertilization. For each N fertilization level, P (56 mg per plant) was applied in a complexed or a labile form. Every combination of N and P fertilizations was inoculated with the Pfaba strain or received NaCl 0.9% as the control treatment.

After two months of culture, the plants were harvested, and aerial biomass and roots were separated. Half of the rooting system was conserved in a solution composed of 100 mL 95° ethanol, 50 mL acetic acid, and 850 mL distilled water at +4 °C for further root traits analyses. Leaf area was determined using a Li-Cor3100 area meter (Li-Cor Inc., Lincoln, NE, USA). Aerial parts and roots were dried in an oven at 60 °C, weighed, and ground using the Retsch MM200 (RETSCH, Eragny sur Oise, France) to a fine powder for further elemental analyses.

2.3. Elemental Analyses in Plants

Phosphorus content in shoots and roots was directly measured on powders using X-ray Fluorescence (XRF, S1 Titan, Bruker, Kalkar, Germany). Shoots or root samples were placed in sample cups, and element concentrations were determined with an XRF analyzer. Quantification of P was performed using an external standard calibration curve, and concentrations were expressed in ppm. For the standard calibration curve, linear regression and correlation were assessed by Pearson's test using XLSTAT 2021.3.1 software (Addinsoft, Quebec, QC, Canada) between XRF data and analysis of P concentration given by a high-resolution inductively coupled plasma mass spectrometry (HR ICP-MS, Thermo Scientific, Element 2TM, Bremen, Germany) [50].

2.4. Determination of Root Traits Using WinRhizo™

Previously conserved roots were placed in transparent tanks filled with distilled water and were disentangled before analysis. Tanks were placed in a scanner (Epson Expression 10,000 XL) coupled to the WinRhizo™ version 2007d software (Regent Instruments Inc., Quebec, QC, Canada) to obtain root length, average root diameter, root area, and root length per volume.

2.5. Statistical Analyses

All data were analyzed using R software (4.0.2 version). One-way analysis of variance (ANOVA) of the "multcomp" package was performed for all assay and plant measurements, followed by a posthoc LSD Fisher test using the function LSD.test from the R package "agricolae". Two-way ANOVA tests were performed to assess the effects of PSB application, N fertilization level, P fertilization form, and interaction of these three parameters using the AOV function of the multcomp package. p -values < 0.05 were considered as significant. Principal component analysis (PCA) of plant profiles was obtained using the Factoshiny package. Missing data were imputed through a two-dimensional PCA model.

3. Results

3.1. Characterization of PGPR and PSB Traits of the Strain Pfaba

In vitro, the solubilization abilities of the Pfaba strain (Table S1) showed a solubilization index (SI) of 2.3 in the Pikovskaya medium and 1.4 in the NBRIP medium. Quantification of solubilization performance was $152.1 \mu\text{g P mL}^{-1}$ in liquid Pikovskaya medium, associated with a pH of 6.7 ± 0.1 , and $49.3 \mu\text{g P mL}^{-1}$ in liquid NBRIP medium associated with a wide pH decrease (3.8 ± 0.2). The Pfaba strain was able to produce gluconic acid in both media ($11.0 \mu\text{mol. mL}^{-1}$ in Pikovskaya medium and $8.3 \pm 0.1 \mu\text{mol. mL}^{-1}$ in NBRIP medium). Pfaba was also able to solubilize phytate and showed a SI of 3.5 on PSM medium (Phytate Screening Medium, [24]). Moreover, this strain produced siderophores in CAS medium (Chrome Azurol S, [24]), IAA (on average $18.8 \pm 3.7 \text{ ng } \mu\text{L}^{-1}$), and showed an ACC deaminase activity. In contrast, no HCN production could be highlighted.

3.2. Morphometric Traits of Rapeseed Plants According to N Fertilization, P Forms, and Pfaba Inoculation

After two months, significant differences were found regarding shoot and root dry biomasses (Figure 2). These differences were strongly influenced by N fertilization (Table 1), and despite not being significant from two-way ANOVA tests, they tended to be reinforced by Pfaba inoculation. As expected, shoot dry biomass was lower for N80 (on average $4.29 \pm 1.40 \text{ g}$, Figure 2A) compared to N160 (on average $6.89 \pm 2.01 \text{ g}$). Moreover, Pfaba adjunction combined with N160 showed significantly higher shoot dry biomass (p -value < 0.05) compared to all N80 modalities, regardless of the P forms of P applied (on average, $7.61 \pm 2.02 \text{ g}$). Interestingly, considering the Pfaba addition combined with N160 treatments, shoot dry biomass tended to be higher than without the Pfaba strain, particularly in the case of complexed P forms (Figure 2A). Similarly, root dry biomass was lower for all N80 modalities (on average, $1.47 \pm 0.53 \text{ g}$) compared to N160 modalities

(on average, 2.39 ± 0.64 g), particularly with the Pfaba addition. As previously described, considering the Pfaba application combined with N160 treatments, root dry biomass tended to be higher than without Pfaba, particularly when P was added in a complexed form (2.88 ± 0.85 g, Table S2) (Figure 2B). Leaf areas were lower for N80 modalities compared to N160 (on average, 253.89 cm² and 418.39 cm², respectively, Supplemental Table S2, Figure S1). No difference could be observed for plants inoculated with the Pfaba strain.

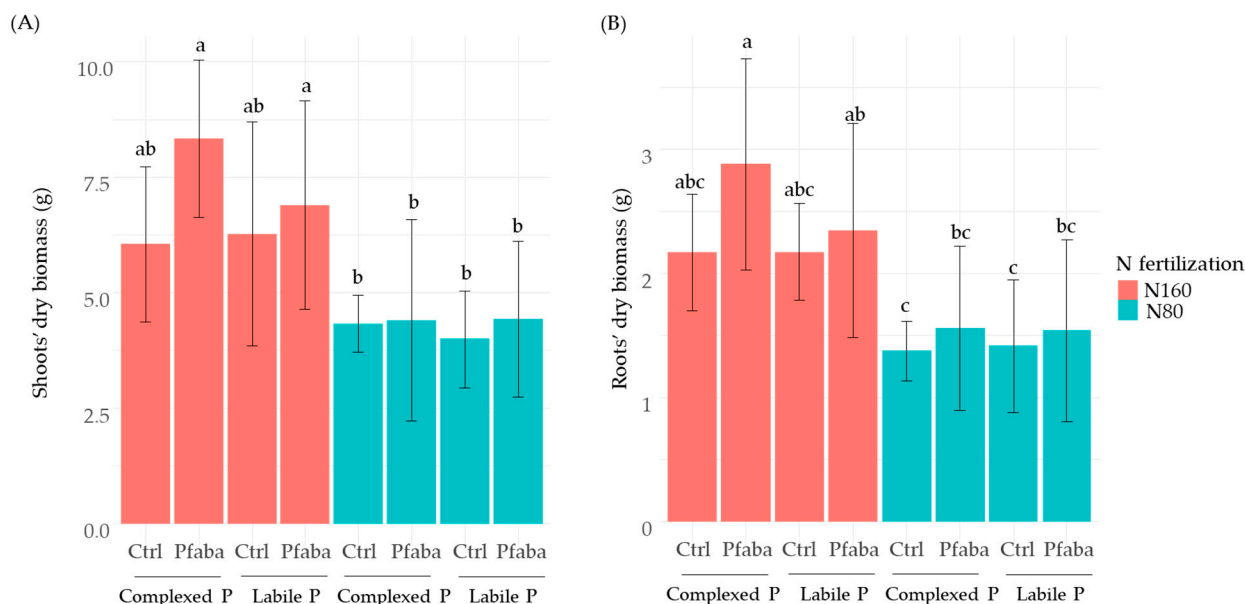


Figure 2. Shoots (A) and roots (B) dry biomasses according to the high (N160, red) or low (N80, blue) N fertilization with P fertilization added in complexed or labile form, without (Ctrl) or with Pfaba inoculation (Pfaba). Letters indicate significant differences obtained by the posthoc LSD Fischer test from the R package “agricolae” (4.0.2 version) (p -value < 0.05).

Table 1. Two-way ANOVA p -values of plant traits according to their N fertilization, P form, and PSB application. ANOVA was performed using the aov function of the multcomp package of R (4.0.2) software.

		PSB	P Form	N Fertilization	P Form × PSB	P × N	N × PSB	N × P × PSB
Shoot	Dry Biomass	0.142	0.507	<0.001	0.579	0.689	0.300	0.383
	P content	0.420	<0.001	0.050	0.583	0.600	1.000	0.929
	P amount	0.100	<0.001	0.231	0.762	0.703	0.166	0.753
	N content	0.367	0.197	0.001	0.260	0.083	0.310	0.613
	N amount	0.412	0.777	<0.001	0.256	0.469	0.685	0.142
	C content	0.641	0.154	0.249	0.576	0.150	0.289	0.503
	C amount	0.185	0.660	<0.001	0.610	0.865	0.405	0.422
	Dry Biomass	0.145	0.531	<0.001	0.465	0.490	0.474	0.557
Roots	P content	0.394	0.001	0.032	0.024	0.254	0.154	0.413
	P amount	0.124	0.082	0.067	0.056	0.661	0.120	0.142
	N content	0.037	0.728	0.966	0.676	0.334	0.204	0.521
	N amount	0.703	0.831	<0.001	0.393	0.891	0.778	0.449
	C content	0.712	0.138	0.661	0.227	0.554	0.750	0.027
	C content	0.135	0.323	<0.001	0.284	0.332	0.446	0.204
	Length	0.043	0.063	0.365	0.104	0.634	0.246	0.546
	Area	0.001	0.171	0.001	0.088	0.407	0.088	0.137
	Diameter	0.052	0.536	0.001	0.556	0.958	0.621	0.254

3.3. P and N Status of Rapeseed Plants according to N Fertilization, P Forms, and the Pfaba Inoculation

P content and amount in shoots were obviously impacted by the form of applied P (Figure 3, Table 1). The P content in shoots was lower when P was applied in a complex form (on average $0.16 \pm 0.15\%$) compared to the labile P form ($0.61 \pm 0.22\%$) (Figure 3A). This effect was also observed even when the PSB strain was applied (Figure 3A). In the same way, the P amount in shoots was lower when P was in complexed forms (on average 0.79 ± 0.49 mg vs. 2.98 ± 0.85 mg for the labile P, Figure 3B). Nevertheless, in N160 fertilization, the addition of Pfaba tended to increase the P amount in shoots slightly in the labile P condition (2.88 ± 0.67 mg for control compared to 3.42 ± 0.54 mg with Pfaba, Table S2) and more widely in complexed P (0.47 ± 0.10 mg for control compared to 1.28 ± 0.71 mg with Pfaba, Table S2). No such tendencies were found for N80. Roots responded differently than shoots in their P content and amounts. As previously described in shoots, the P content was mainly influenced by the P form but also in interaction with PSB application and N fertilization (Table 1). The highest P content in roots was found for the control labile P + N80 ($0.28 \pm 0.06\%$; Figure 4A and Table S2), and the lowest one was found in plants receiving N160, combined with P in complexed form and no PSB ($0.07 \pm 0.04\%$). Whatever the level of N fertilization and the P form, the Pfaba application allowed plants to restore a P content in roots similar to P labile control plants (Figure 4A). Regarding the roots' P amounts, for N160 treatments, higher values were observed when P was supplied in labile form, with an average of 0.52 ± 0.14 mg (Figure 4B) compared with those observed with complexed P with no PSB inoculation (0.15 ± 0.09 mg) (Table S2). Interestingly, when plants were subjected to N160 and complexed P form, Pfaba adjunction increased more than four-fold the P amount in roots compared to control and were able to reach the same values as with labile P (on average, 0.59 mg, Figure 4B).

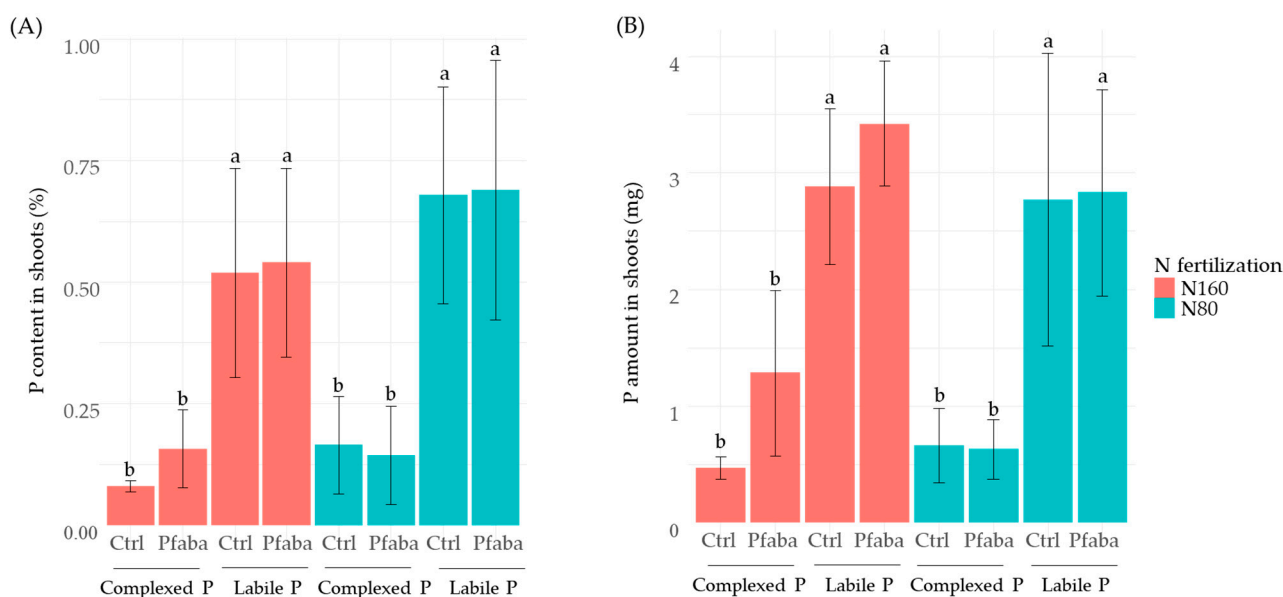


Figure 3. Relative P content (A) and P amount (B) in shoots according to the high (N160, red) or low (N80, blue) N fertilization with P fertilization added in complexed or labile form, without (Ctrl) or with Pfaba inoculation (Pfaba). Letters indicate significant differences obtained by the posthoc LSD Fischer test from the R package “agricolae” (4.0.2 version) (p -value < 0.05).

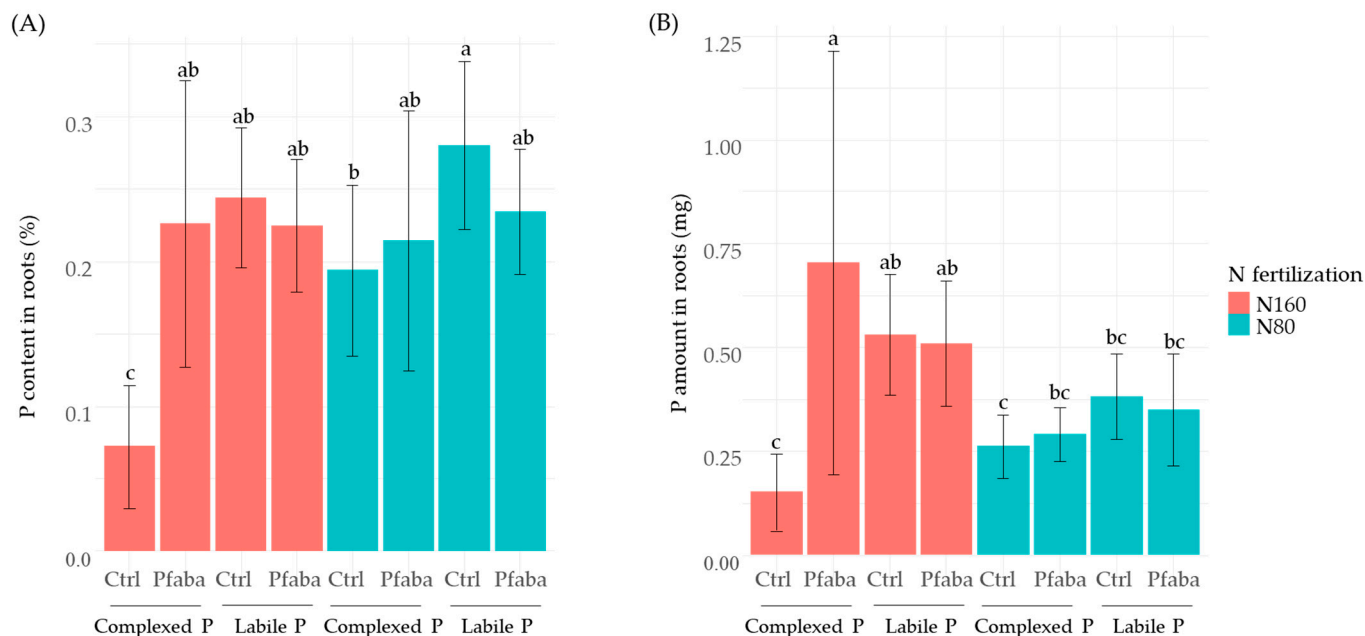


Figure 4. Relative P content (A) and P amount (B) in roots according to the high (N160, red) or low (N80, blue) N fertilization with P fertilization added in complexed or labile form, without (Ctrl) or with Pfaba inoculation (Pfaba). Letters indicate significant differences obtained by the posthoc LSD Fischer test from the R package “agricolae” (4.0.2 version) (p -value < 0.05).

Relative N content in shoots was similar across all N and P fertilizations (around $0.93 \pm 0.09\%$), except in N160–labile P control, which shows the highest value ($1.31 \pm 0.33\%$, Figure 5A, Table S2). As expected, a clear effect of the N fertilization level was found on the N amount in shoots (Table 1). Indeed, the N amount was significantly higher in N160 compared to N80 (73.48 ± 19.80 mg and 36.71 ± 13.14 mg, respectively, Figure 5B). No impact of the Pfaba addition could be noticeably highlighted. On the contrary, the N content in roots (Figure 6A) tended to be slightly influenced by PSB application (Table 1). Indeed, in N160 modalities, Pfaba inoculation led to a decrease of N content compared to control treatments and even in a significant way for labile P (control: $2.06 \pm 0.54\%$ and Pfaba: $1.48 \pm 0.42\%$). When comparing the N amounts in roots, as observed in shoots, the N fertilization effect was predominant (Table 1). The roots’ N amount was significantly higher in N160 compared to N80 (39.78 ± 12.07 mg and 24.59 ± 8.92 mg, respectively, Figure 6B).

3.4. Rapeseed Root Traits according to N Fertilization, P Forms, and the Pfaba Inoculation

Root length was significantly influenced by PSB application (Table 1). In either N160 or N80 fertilization, PSB application led to decreased root lengths, reaching similar values to the P labile control (Figure 7A). In complexed P forms without the Pfaba inoculant, an increase in root length was observed for both N fertilization levels (Figure 7A, Table S3).

Root average diameters varied according to the level of N fertilization (Table 1). Indeed, root diameters decreased when N80 fertilization was applied (0.41 ± 0.05 mm vs. 0.50 ± 0.08 mm for N160, Figure 7B). For N80 treatments, Pfaba inoculation tended to decrease root diameters when P was supplied in a complexed form (0.36 ± 0.02 mm) compared to other N80 treatments with labile P (0.43 mm on average).

The root area was independently influenced by both PSB application and N fertilization (Table 1). N80 fertilization decreased root area (511.00 ± 155.38 cm²) compared to N160 (670.38 ± 112.25 cm², Figure 8). For N80, Pfaba application significantly decreased root surface when P was in its unavailable complexed form (412.12 vs. 749.73 cm² for the control), reaching a similar value as plants grown with labile P (Table S3, Figure 8).

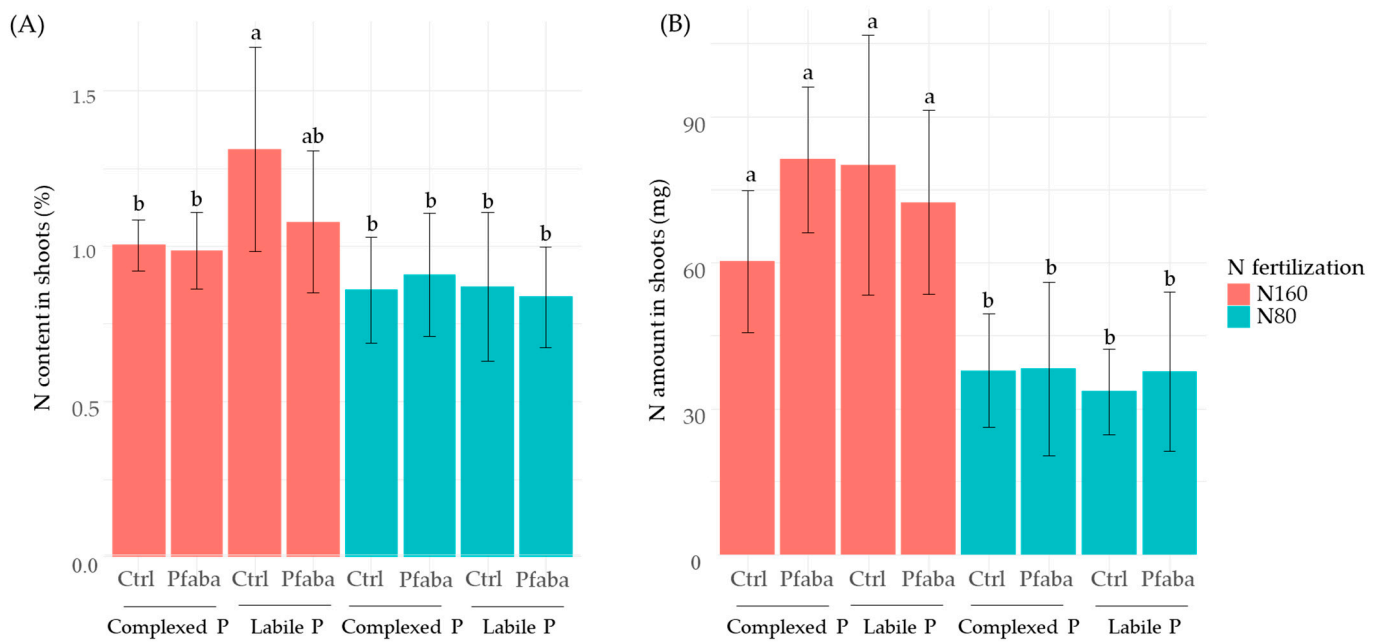


Figure 5. Relative N content (A) and N amount (B) in shoots according to the high (N160, red) or low (N80, blue) N fertilization with P fertilization added in complexed or labile form, without (Ctrl) or with Pfaba inoculation (Pfaba). Letters indicate significant differences obtained by the posthoc LSD Fischer test from the R package “agricolae” (4.0.2 version) (p -value < 0.05).

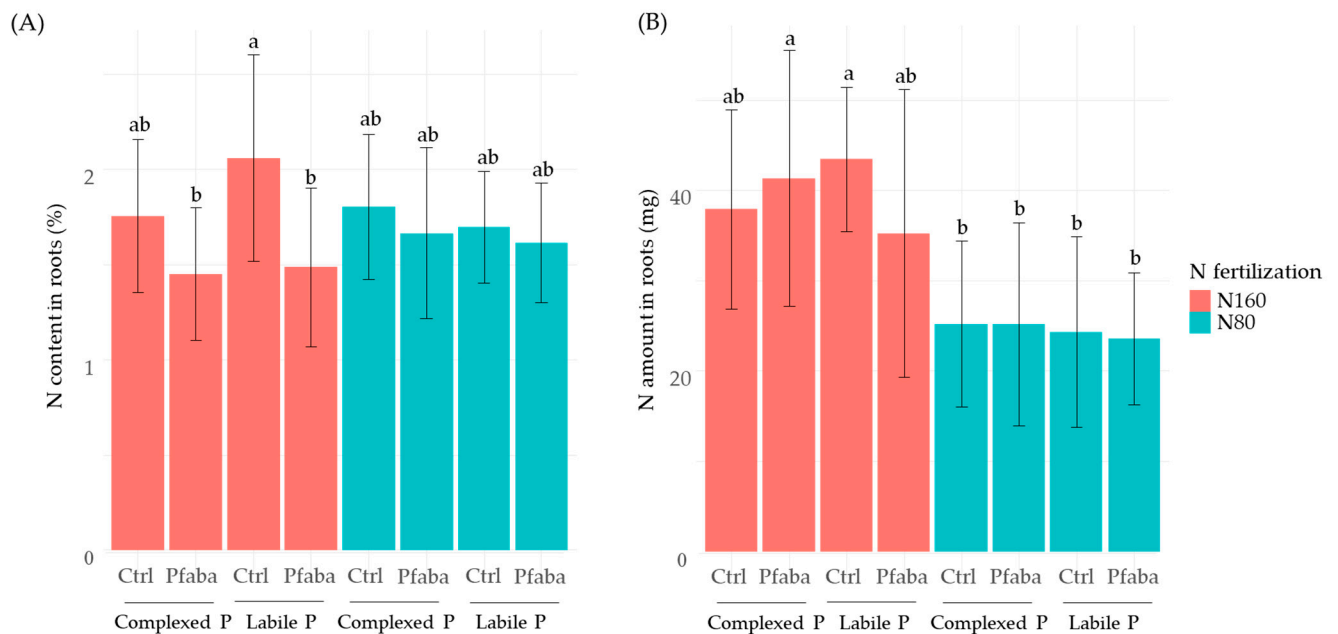


Figure 6. Relative N content (A) and N amount (B) in roots according to the high (N160, red) or low (N80, blue) N fertilization with P fertilization added in complexed or labile form, without (Ctrl) or with Pfaba inoculation (Pfaba). Letters indicate significant differences obtained by the posthoc LSD Fischer test from the R package “agricolae” (4.0.2 version) (p -value < 0.05).

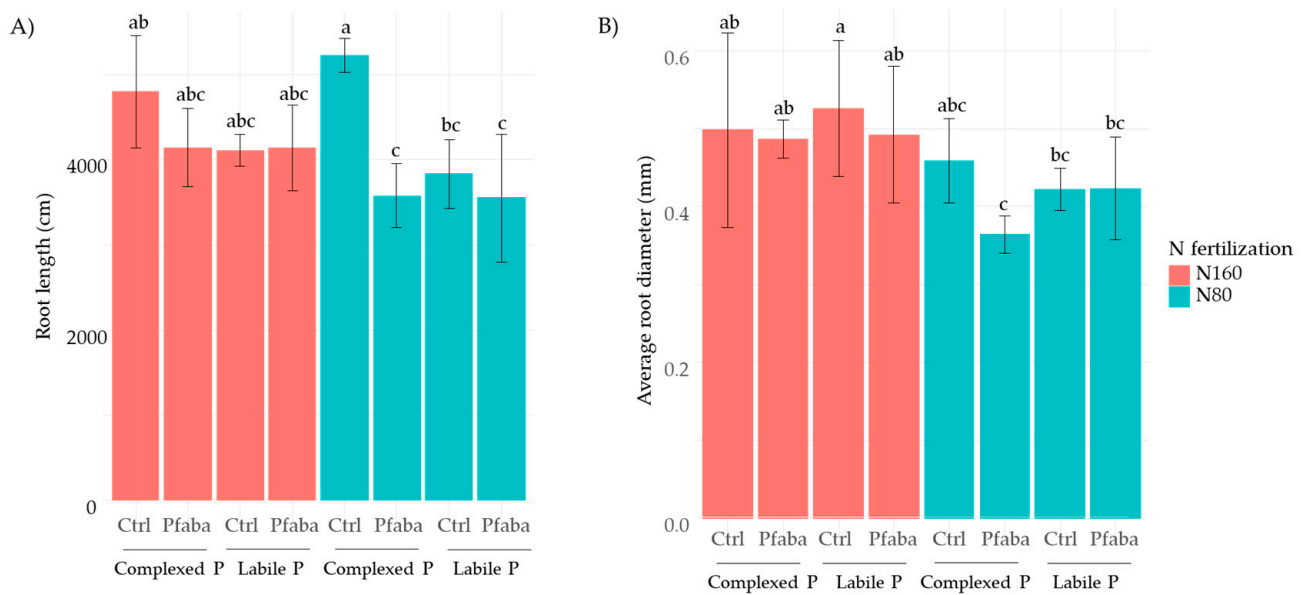


Figure 7. Root length (A) and average root diameter (B) according to the high (N160, red) or low (N80, blue) N fertilization with P fertilization added in complexed or labile form, without (Ctrl) or with Pfaba inoculation (Pfaba). Letters indicate significant differences obtained by the posthoc LSD Fischer test from the R package “agricolae” (4.0.2 version) (p -value < 0.05).

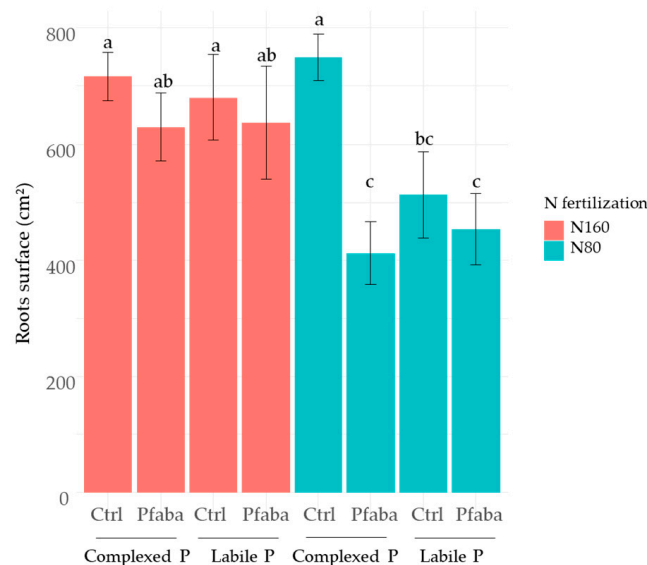


Figure 8. Root surface according to the high (N160, red) or low (N80, blue) N fertilization with P fertilization added in complexed or labile form, without (Ctrl) or with Pfaba inoculation (Pfaba). Letters indicate significant differences obtained by the posthoc LSD Fischer test from the R package “agricolae” (4.0.2 version) (p -value < 0.05).

3.5. General Plant Profiles

PCA of overall plant traits (Figure 9) showed that plants grown in labile P conditions were grouped at the top of individual topology (Figure 9A). As expected, these plants were characterized by high values in P content and amounts in both belowground and aboveground parts. On the contrary, plants grown in complexed P conditions showed low values in these variables and were characterized by higher values in root length and area. No clear separation between control plants and those with Pfaba inoculation could be found. However, interestingly, when P was supplied in complexed forms, plants tended to reach a similar profile to plants grown in labile P conditions, particularly for N160.

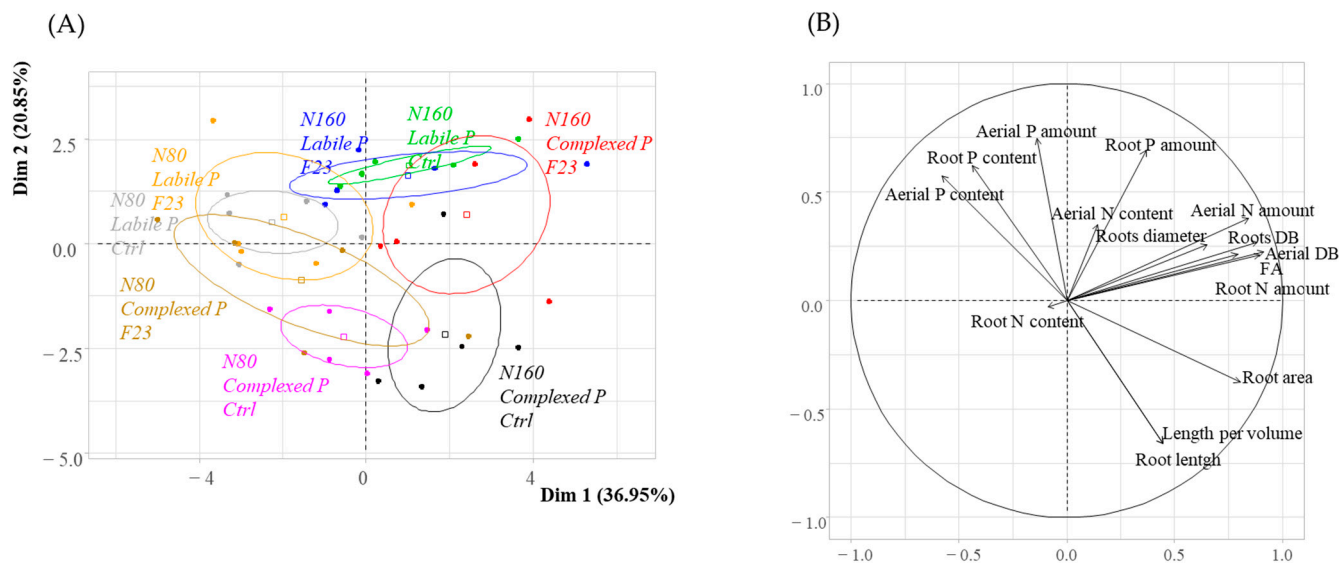


Figure 9. Principal Component Analysis of (A) plant profiles, obtained with two N fertilization levels (N160 or N80) and two P forms (labile or complexed) with PSB application (Pfaba) or not (Ctrl: control). Dimension 1 = 36.95%, dimension 2 = 20.85%. (B) Correlation circle of studied variables. DB: Dry Biomass, FA: Foliar Area.

4. Discussion

In this study, Pfaba inoculation confirmed its PGPR potential on rapeseed growth and, more particularly, its PSB ability by providing plants with access to nonavailable phosphate. Indeed, our data showed that Pfaba is able to synthesize IAA and degrade ACC. These PGPR traits could modify the IAA: ethylene ratio in favor of auxin, sustaining the growth of inoculated plants. Nevertheless, this increase in biomass is explained neither by a higher number of leaves nor by longer or thicker roots.

Evidence of phosphate solubilization was found, considering the higher P content and P amount in roots for Pfaba treatments compared to uninoculated control (Figure 4). Furthermore, for both levels of N fertilization, root length was reduced to the level observed in labile P conditions. Indeed, P starvation is known to modify root traits and particularly root elongation, allowing the plant to prospect a wider soil volume [51]. This phenotype of the elongated root was observed in uninoculated controls when P was supplied in complexed form, but it returned to a phenotype close to plants receiving a labile P source when Pfaba was inoculated (Figure 4).

Regarding N uptake, the benefit of deploying Pfaba appeared to be more tenuous. As N content was quite similar between all modalities, the principal effect to explain the higher N amount in the presence of Pfaba appeared to be a higher shoot biomass. Similarly, root biomass decreased while the N amount remained stable, leading to an apparent decrease in the N content with Pfaba inoculation. But this decrease in N content is probably mainly due to a dilution phenomenon rather than to an adverse effect of the PSB strain or to competition for N uptake. Indirectly, by helping adequate P uptake in a nonavailable P situation, inoculation with Pfaba allowed the N uptake to be maintained in an equilibrium of the ratio N:P [52].

These overall results thus confirm the promising potential of using Pfaba as a PGPR and, more interestingly, as a PSB strain to sustain rapeseed nutrition in low phosphorus conditions through direct processes linked to phosphorus solubilization, as previously hypothesized by Amy et al. [43]. These results are consistent with previous studies concerning the deployment of such microbial bioinoculants on numerous plant species [53–58], confirming the powerful potential of PGPR/PSB utilization. This study also demonstrates that beneficial effects observed on plants are directly due to Pfaba inoculation, as rapeseed

was grown in axenic conditions and independent of associated soil microbial community structure in the soil surrounding the roots.

In addition to the direct effect of the Pfaba strain on P uptake, a possible positive effect linked to classical PGPR traits on inherent P acquisition mechanisms could not be totally excluded. For example, plants are able to exude organic acids to access an insoluble P pool or to increase phosphatase activity [59,60]. Such abilities could be promoted through simple PGPR action.

N Availability as a Key Factor of Success of Plant-PSB Association

In this study, the already well-known effects of N fertilization level were observed on rapeseed growth and N uptake. Indeed, a decrease in N fertilization led to smaller plants and lower N amounts, as frequently reported in previous studies [61–63].

Unexpected results were observed regarding Pfaba's performance in sustaining the growth of rapeseed. Indeed, as positive results were found with N160, a lower level of N fertilization negatively impacted the expression of PGPR/PSB traits. All of the positive effects on plant biomass and N and P uptake observed at N160 were not observed in the N-deprived condition. However, Pfaba inoculation allowed the reduction of root diameter, root length, and root surface. In the same way as P starvation, N limitation leads to an increase in root length, thickness, and volume [64], which was not observed here with Pfaba adjunction or with labile P.

Nevertheless, soil with N availability appeared essential to ensuring the deployment of the PGPR/PSB arsenal of the Pfaba strain and, thus, the pursued interaction with the plant. Indeed, most of PGPR mechanisms rely on amino acid transformation and depend on N availability. More precisely, IAA can be synthesized from tryptophan or its precursors by microorganisms [26]. Tryptophan can be exuded by plants [65] or directly synthesized by *Pseudomonas* [66]. In the same way, pyoverdine, a common siderophore in *Pseudomonas*, is composed of a chromophore derived from 2,3 di-aminodihydroxyquinoline, on which is attached a peptidic functional group containing 6–12 amino acids. In *Pseudomonas*, pyoverdine production is a multistep process comprising more than 11 different proteins [67]. Finally, ACC exuded by plants in the rhizosphere is synthesized from S-adenosyl methionine derived from methionin, a sulfur (S) amino acid [25]. As access to N is more limited in N80, its use by both plants and microorganisms could be preferentially oriented in protein synthesis, plant growth, and maintenance of bacterial multiplication rather than in exudation and expression of PGPR traits. As exudation reflects the nutritional status of plants [68], cooperation and establishment of beneficial interaction between rapeseed and beneficial microorganisms could have been prevented. Moreover, Carvalhais et al. [69] showed a decrease in amino acid exudation in maize in N-deprivation conditions. As amino acids provide an N, C, and S source to microorganisms, additional repression of PGPR/PSB traits could be due to a C allocation to growth or survival mechanisms.

Moreover, N limitation could impede the process of P solubilization. Thus, for example, a N effect has been demonstrated on phytase activities in lichens [70]. Authors have shown a decrease in phytase activity along with the N availability in these complex microorganisms, for which phytate degradation is generally attributed to the fungal part of lichens. We can here hypothesize that this could be similar to bacterial phytase activity. Taken together, these results highlight the primordial role of N on PGPR/PSB efficiency and possibly on the cooperation between plants and bacteria. Indeed, lower N availability could have turned plant–bacteria mutualism into individual behaviors by both protagonists.

Low N fertilization could also have only limited the multiplication of Pfaba in the culture substrate after inoculation. Indeed, N limitation constitutes a substantial factor that could repress bacterial growth in soil [37,71]. Here, the induced low density of the Pfaba population would not have reached a sufficient threshold for which the expression of associated specific PSB and PGPR traits could efficiently sustain plant growth. Because colonization of the rhizosphere by the required beneficial bacterial agent is a critical pre-

requisite, thus conditioning the success of bioinoculation [31], nutrient supply to microbial communities should be taken into account.

More generally, the present results highlighted the necessity at the field scale to consider the effects of fertilization practices for ensuring the success of PGPR/PSB deployment and rhizosphere colonization but also more generally on the perennity of the plants–microorganisms’ dialogue. These observations pointed out the importance of crop production and fertilization practices, not just by considering plant needs alone, but from a more holistic point of view and thus in a holobiont’s functioning [72]. For example, considering that one of the several proposed solubilization mechanisms relies on proton excretion accompanying ammonia respiration of some PGPR, including *Pseudomonas* strains [21], and that rapeseed preferentially absorbs nitrate [73], the use of ammonitrate can be a relevant fertilization practice for the global holobiont.

Further work is now needed to focus on the comprehension of in situ mechanisms that sustain the plants–microorganisms’ cooperation through the exploration of rapeseed exudates by tracing the Pfaba strain and with in situ evaluation of relevant microbial activities, such as siderophore production or determination of phosphatase and phytase activities by zymography [74–76].

5. Conclusions

In this study, the Pfaba strain, a previously tested *Pseudomonas* PSB, was inoculated on rapeseed grown in rhizotrons with contrasted N fertilization levels and P applied under labile or complexed forms. When combined with a higher N fertilization level, Pfaba confirmed its potential as a PGPR and PSB strain by enhancing biomass and root P content, contrary to results observed with a lower level of N fertilization. Whatever the level of N fertilization, in a P complexed situation, Pfaba inoculation allowed a return to a non-P-starved roots phenotype (i.e., to shorten root length, similarly to plants fed with labile P). N limitation seemed to represent an important factor in limiting the competitive issue of resource sharing between plants and microorganisms. These results also suggest that soil N/P balance is a key factor in the establishment of beneficial cooperation between rapeseed and microorganisms, probably through the modulation of the full expression of PGPR/PSB potentialities.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14020334/s1>, Figure S1: Foliar area of plants subjected to high (N160, red) or low (N80, blue) N fertilization, with P fertilization added in complexed or labile form, and without or with Pfaba inoculation; Table S1: In vitro characterization of PGPR traits for the tested PSB isolate; Table S2: Morphometric and elemental NP plants profiles, according to the level of N fertilization (N160 or N80), P added in complexed or labile form and without or with Pfaba inoculation; Table S3: Root traits according to the level of N fertilization (N160 or N80) and P added in complexed or labile form.

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