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Rhizobium alamii improves water stress tolerance in a non-legume

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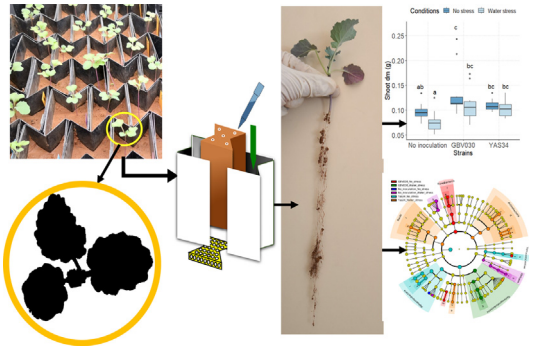
HIGHLIGHTS

- *Rhizobium alamii* strains improved plant growth and tolerance to water stress.
- The impact on the plant growth and root-associated microbiota varied according to the strain.
- *R. alamii* strains modified the microbiota assembly and the soil structure.

GRAPHICAL ABSTRACT



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ABSTRACT

With the increasing demand for alternative solutions to replace or optimize the use of synthetic fertilizers and pesticides, the inoculation of bacteria that can contribute to the growth and health of plants (PGPR) is essential. The properties classically sought in PGPR are the production of phytohormones and other growth-promoting molecules, and more rarely the production of exopolysaccharides. We compared the effect of two strains of exopolysaccharide-producing *Rhizobium alamii* on rapeseed grown in a calcareous silty-clay soil under water stress conditions or not. The effect of factors 'water stress' and 'inoculation' were evaluated on plant growth parameters and the diversity of microbiota associated to root and root-adhering soil compartments. Water stress resulted in a significant decrease in leaf area, shoot biomass and RAS/RT ratio (root-adhering soil/root tissues), as well as overall beta-diversity. Inoculation with *R. alamii* YAS34 and GBV030 under water-stress conditions produced the same shoot dry biomass compared to uninoculated treatment in absence of water stress, and both strains increased shoot biomass under water-stressed conditions (+7% and +15%, respectively). Only *R. alamii* GBV030 significantly increased shoot biomass under unstressed or water-stressed conditions compared to the non-inoculated control (+39% and +15%, respectively). Alpha-diversity of the root-associated microbiota after inoculation with *R. alamii* YAS34 was significantly reduced. Beta-diversity was significantly modified after inoculation with *R. alamii* GBV030 under unstressed conditions. LEfSe analysis identified characteristic bacterial families, *Flavobacteriaceae* and *Comamonadaceae*, in the RT and RAS compartments for the treatment inoculated by *R. alamii* GBV030 under unstressed conditions, as well as *Halomonadaceae* (RT) and several species belonging to *Actinomycetales* (RAS). We showed that *R. alamii* GBV030 had a PGPR effect on rapeseed growth, increasing its tolerance to water stress, probably involving its capacity to produce exopolysaccharides, and other plant growth-promoting (PGP) traits.

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1. Introduction

Field crops are increasingly intensified to meet human food needs and renewable energy resources. However, climate change is forcing us to reorient our strategies and develop a more sustainable and resilient agriculture. Global warming may shorten crop cycles in cold regions, but has serious consequences for agriculture in arid and semi-arid regions (Lotze-Campen, 2011). In these regions, drought is the main factor limiting the productivity of forests (Allen et al., 2010) and field crops (Simelton et al., 2012). Predictions show that crops such as maize, wheat, rice and soybean would be greatly affected if the frequency of drought periods increased (Leng and Hall, 2019). For example, it has been shown that water stress can reduce plant biomass, pod number, oil production and oil quality of canola (Tesfamariam et al., 2010; Moghadam et al., 2011). In addition, water stress affects seed production and produces various losses at different stages of the plant cycle, but plants that suffer water-stressed at early stages are more resistant (Gan et al., 2004). On the other hand, drought induces changes in microbial community composition (Naylor and Coleman-Derr, 2018). Plant health and growth are directly linked to their root-associated microbiota (van der Heijden et al., 2008) as they are able to recruit the microorganisms necessary for their survival (Berendsen et al., 2012). Thus, the search for solutions to reduce the impact of drought on plants has led to focus on understanding the interactions between plants and microorganisms to find solutions to prevent future crop damages caused by climate change (Cavicchioli et al., 2019).

Soil microorganisms are responsible not only for the key steps of the carbon cycle but also for the cycle of mineral elements (N, P and K) that represent the main limiting factors (after water) for plant growth. In addition to their role in the cycling of these mineral elements, the microbiota associated with plant roots, whose source of C and energy is root exudates, also plays a very important role in the transfer of water and these mineral elements to the plant (Guyonnet et al., 2018; Compant et al., 2019). Finally, the microbiota associated with the root and aerial parts of plants was also necessary for their protection against pathogens and predators (Berg, 2009; see Review Olenska et al., 2020).

Among the bacteria that interact directly or indirectly with plants, those that have a beneficial effect on plant nutrition and growth are called "Plant Growth Promoting Rhizobacteria" (PGPR) and are distributed among a very large number of bacterial species. Many mechanisms are involved in the beneficial interaction between PGPRs and the plant, including the production of phytohormones such as auxin (Asghar et al., 2002), abscisic acid (Belimov et al., 2014) and lowering the endogenous ethylene concentration by ACC deaminase activity (Glick et al., 1994; Brunetti et al., 2021). Other bacterial compounds that have been described as beneficial to the plant include volatile organic compounds (VOCs). While they were initially studied for their pathogen resistance inducing properties (Farag et al., 2013), they have been shown to have interesting properties for plant growth by increasing both root number and leaf area (Ruzzi and Aroca, 2015). Another property of PGPR is the ability to protect the plant against abiotic stresses. Stresses such as salt and metal pollution can be mediated either by bacteria capturing the pollutant in their cells (Khanna et al., 2019; Benidire et al., 2020), or by altering plant water use (Ahmad et al., 2013).

It has been shown that inoculation of a single bacterial strain or a combination of bacterial strains can stimulate plant growth and resistance to abiotic stresses (Calvo et al., 2014; Molina-Romero et al., 2017; Rouphael and Colla, 2018; Ullah et al., 2017). This biostimulation of plant growth has been demonstrated in many plants, including chickpea and beans (Hamaoui et al., 2001) and vetch (Benidire et al., 2020) in response to saline stress, as well as in maize (Casanovas et al., 2002) and wheat (Creus et al., 2004) in response to water stress. Among the bacterial genera that have shown a positive effect on plant growth under stress conditions, the most studied are *Brevibacillus*, *Paenibacillus*, *Azospirillum*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Ensifer*, *Serratia* and *Stenotrophomonas* (Berg, 2009; Dimkpa et al., 2009; Ruzzi and Aroca, 2015; Benidire et al., 2020). Several authors have discussed the fact that a bacterial strain has positive and significant effects under controlled conditions, but these effects cannot be demonstrated under field conditions (Compant et al., 2019). Among the properties of selected PGPRs to ensure effective colonization of the root surface and rhizosphere of plants, few authors used the ability of these rhizobacteria to produce exopolysaccharides (EPS) (Amellal et al., 1998; Alami et al., 2000; Bezzate et al., 2000). This phenotypic trait is important for root colonization (Santaella et al., 2008) and for the structuring of root-adhering soil (Amellal et al., 1998; Alami et al., 2000; Bezzate et al., 2000).

Exopolysaccharides are high molecular weight extracellular polymers, generally soluble in water and produced by many rhizobacterial strains (Hebbar et al., 1992). They have a regular chemical structure based on 2 to 8 sugar-repeating units with various physical properties (from thickening to gelling) (Rinaudo, 2004). They are involved in soil structuring, water and nutrient transfer to the roots (Amellal et al., 1998; Alami et al., 2000), and protection against biological or physical stresses (Nwodo et al., 2012; Tecon and Or, 2017). Another property of bacterial EPS is that they help protect the bacteria itself and the plant they colonize from desiccation, predation and toxic compounds. In the case of saline soils, the biofilm formed by EPS reduces the diffusion of salt, thus reducing the adverse effect on plant growth (Hamaoui et al., 2001; Upadhyay et al., 2011; Qurashi and Sabri, 2012; Benidire et al., 2020). Finally, it has been shown that inoculation of EPS-producing bacteria, by modifying soil porosity and thus water conductivity (Rossi et al., 2012), protecting maize (Naseem and Bano, 2014) and sunflower (Alami et al., 2000) from water stress. In the sunflower experiment, inoculation of *Rhizobium alamii* YAS34 increased shoot and root biomass of plantlets, with a modification of rhizospheric soil structure due to an increase in soil macroporosity (Alami et al., 2000). This bacterial strain was also able to colonize rapeseed roots and increased the percentage of water-sable aggregates in the rhizosphere (Santaella et al., 2008). In the present study, we compared the effect of *R. alamii* YAS34 (isolated from the sunflower rhizosphere) with that of *R. alamii* GBV030 (isolated from the rhizosphere of *Arabidopsis thaliana*), which produce the same EPS, on the growth of rapeseed and its root-associated microbiota under water stress (or not).

2. Materials and methods

2.1. Experimental design

Brassica napus (cv. Amazzonite) seeds were sown in a calcareous silty-clay soil taken from the top 20 cm layer of an agricultural site located in Bel Air, near Aix-en-Provence, France (43°33′45.58″ N; 05°28′ 38.78″ E). The soil pH was 8.2 and contained 5.7% sand, 46.7% silt, 47.6% clay, 1.0% $CaCO_3$, 1.8% organic C and 0.18% organic N.

The soil was packed in bottomless 'WM' shaped pots (WM 20-8-5, Thermoflan, Molières-Cavaillac) which were installed in plastic containers to which an anti-mosquito tissue was added at the bottom to prevent soil loss. These pots are made up of two easily detachable interlocked parts and are particularly convenient for root phenotyping and collection of root-adhering soil. In addition, their angular shape prevents the roots from spiraling. Each pot contained 1.5 kg of soil and ten replicates were sown for each treatment with two seeds per pot. After 5 days of germination, only one seedling per pot was kept.

The experiment was conducted according to a completely randomized design in order to test two factors: "soil moisture" (water stress *vs* absence of water stress) and "bacterial inoculation" (inoculation with *R. alamii* YAS34 or GBV030 *vs* no inoculation) corresponding to 6 treatments with 10 replicates for each treatment. Soil moisture was monitored daily using a portable moisture meter (Soil Measurements, DLT/ML3, Ø 40 × L. 158 mm) and soil moisture was adjusted to 9–10% for unstressed conditions and 7% for water stress conditions with tap water.

B. napus seeds were inoculated at sowing and a second time 10 days after sowing with 2 mL of bacterial suspensions of the two bacterial strains, *R. alamii* YAS34 isolated from the sunflower rhizosphere (Alami et al., 2000) and *R. alamii* GBV030 isolated from the *Arabidopsis thaliana* rhizosphere (Berge et al., 2009), at a final concentration of 1.10^8 cfu mL^{-1} for each strain. Both strains were grown in 1:10 diluted TSB (Tryptic Soy Broth, Difco) growth medium for 78 h and the bacterial cells were washed in sterile ultra-pure water after centrifugation. We have shown that the EPS structure of *R. alamii* GBV030 was the same as that of *R. alamii* YAS34 (Villain-Simonnet et al., 2000).

The plantlets were harvested after 8 weeks of incubation under controlled conditions in a growth chamber (Fitotron SGC120, Weisstechnik). Photoperiod was defined as daylight (8 h, 20 °C, 80% humidity) and night (16 h, 16 °C, 80% humidity), with an illumination of 450 $\mu E \cdot cm^{-2}\ s^{-1}$. Plantlets were collected by separating the leaves from the root system. The mass of the fresh shoot was measured as well as the mass of the dry shoot after drying at 85 °C for two days. The root system was manually shaken (30 s) to remove loose soil, and the remaining root-adhering soil (RAS) was separated from the roots by washing in 20 mL sterile ultra-pure water. The mass of fresh root-adhering soil was measured after centrifugation and removal of the supernatant. Root systems were washed with sterile ultra-pure water to remove remaining adherent soil particles and its mass was measured. The samples were frozen and stored at -80 °C for molecular analysis.

2.2. Leave surface phenotyping

After two-week growth, the plantlets were photographed from above with a camera attached to a metal rod, so the photographs were taken at the same distance. The photographs were processed with the ImageJ software using the excess green method, described by Woebbecke et al. (1995), to automatically measure leave surface. A 1 cm^2 cardboard was placed at ground level to adjust the scale in cm/pixel. Each image was separated into a stack of red (R), green (G) and blue (B) channels. The tree channels were merged with the 2*G-R-B formula to create the excess green image. The green of the leaves was then easily selected and used to measure the surface.

2.3. DNA extraction and 16S rDNA metabarcoding

The roots (RT fraction) were rinsed three times in 50 mL Falcon tubes with 30 mL distilled water and stored in 15 mL tubes at −20 °C. They were rinsed again with 6 mL of sterile water, to remove remaining root-adhering soil, wrung out and placed in a sterile mortar. Sterile ultra-pure water (450 μL) was poured into the mortar to grind the root and 450 μL of sterile ultra-pure water was added to recover the grind in a 1.5 mL tube. An additional 400 μL of sterile ultra-pure water was used to recover any DNA that may have remained in the mortar walls. Each tube was stored at −80 °C until the DNA was extracted. Tubes containing RAS fractions were centrifuged for 10 min at 10,000 rpm. After removal of the supernatant, 15 mL of ultra-pure water was added to the pellet and transferred to a 15 mL tube. Each tube was centrifuged again for 10 min at 10,000 rpm. Only the pellet was stored at −80 °C until DNA extraction. Rinsing may have removed some of the DNA, but our aim was to ensure the separation of the different fractions to assess changes in their microbiota.

The FastDNA Spin Kit for Soil was used for DNA extraction from 500 mg of RAS and RT fractions. A slightly modified version of the manufacturer's protocol was used. Each tube was vortexed prior to homogenization in the FastPrep instrument for 60 s. To improve DNA extraction, the highest recommended centrifugation time and addition volume were used, and the incubation time was 5 min at 55 °C before DNA elution. The RT fractions were eluted a second time for 5 min to completely recover the DNA. The extracted sample was directly used for PCR amplification.

The Promega kit and 341F/805R primers were used to amplify the 16S V3-V4 region. For the final 50 μL volume, the following PCR mixtures were used: 10 μL of 5× buffer, 3 μL $MgCl_2$ at 25 mM, 1 μL of dNTP at 10 mM, 2 μL of each 341F/805R primer at 10 μM, 0.25 GoTaq enzyme at 5 U μL^{-1}, 2 μL of extracted DNA and 29.75 μL of ultra-pure water. The PCR protocol started with a 2 min denaturation at 95 °C. The first denaturation step of the cycle at 95 °C for 30 s followed by hybridization at 50 °C starting at 30 s and the extension step at 72 °C for 90 s. This cycle is repeated 34 times before the last extension step at 72 °C for 5 min. Each amplicon was verified by gel migration electrophoresis using the BET method before sending all samples to Biofidal (Lyon, France) for sequencing.

2.4. Sequence analyses

The sequence files were retrieved as demultiplexed sequences from MiSeq pair-end 2 times 300 bp. The QIIME version 2020.2 software was used to process them, first joining the two ends and then filtering them with a quality threshold above 30. The sequences were clipped at nucleotides 45 to 400. Over 75% of the sequences passed the filter for each sample with a minimum of 23,881 sequences. Sequences were then grouped at 97% identity and taxonomy was assigned using the Greengenes 13_8_otus databases. RT and RAS fractions were analyzed separately. Cyanobacteria and mitochondria sequences were removed from the final list of sequences. Depletion was applied for each compartment, RT and RAS, with a depletion of 13,634 and 26,255, respectively. These thresholds corresponded to the minimum number of sequences in a sample for both compartments, as the maximum diversity was achieved. For both compartments, a phylogenetic tree was calculated using the QIIME2 align-to-tree-maft-fastree function. Taxonomic abundance matrices and phylogenetic trees were extracted for statistical analyses.

2.5. Statistical analyses

Alpha-diversity was calculated by taxonomic richness (*i.e.*, the number of taxa, expressed as the number of observed OTUs) and Chao1 index. Shannon and Simpson indices were used then for evenness estimation between samples. All diversity indices were compared among Complines using the *t*-test/ANOVA statistical method. Considering beta-diversity, a Bray-Curtis dissimilarity was used to measure the distance between each pair of samples. This explicit comparison of microbial communities (in-between) based on their composition was tested using a permutational multivariate analysis of variance (PERMANOVA; 999 permutations) and represented by Nonmetric Multi-Dimensional Scaling (NMDS).

LEfSe analysis was performed using LEfSe module for *huttenhower lab galaxy* (Segata et al., 2011). The structure of abundance dataset was modified using R software to fit LEfSe module file format. A threshold 0.05 *p*-value was used for the Kruskal-Wallis and pairwise Wilcoxon tests. The threshold of LDA score was 2. All samples were tested against each other, only the cladograms for RT and RAS were retrieved.

All other statistical tests and graphical representation were performed using R software version 3.5.3. Data processing and graphing were done using *tidyverse* packages (Wickham et al., 2019), correlation between variables were performed using *cor* function. For each pairwise test, *p*-values were adjusted by BH correction. Each univariate analysis was performed using the Kruskal-Wallis test. If a parameter had a significant influence on a variable, a pairwise post-hoc Dunn test was used to determine group significance differences. Homogeneity groups were calculated automatically using the *multcompLetters* function with a *p*-value threshold = 0.05. For multivariate analyses, we performed PCA on plant phenotypic variables using *Factominer* package (Lê et al., 2008). Differences between microbial communities were assessed by calculating the weighted UniFrac distance using the *phyloseq* package as this method can represent microbiota of the samples using OTUs

phylogenetic distance and abundancy (Lozupone and Knight, 2005). The distance matrices were represented in a PCoA using the *ape* package (Paradis and Schliep, 2019) as this is the best way to represent these data (Paliy and Shankar, 2016). Differences in spatial positions in the created 2D space were statistically tested by doing a PERMANOVA test using the *adonis2* function and a beta dispersion test with the *betadisper* function, both from the *vegan* package (Oksanen et al., 2019). When the PERMANOVA showed a significant effect of one factor and beta dispersion showed homogeneity of variance among groups, a pairwise post-hoc PERMANOVA test was performed using the *RVAideMemoire* package (Hervé, 2020).

Sequence data are available in NCBI under BioProject code PRJNA721878.

3. Results

3.1. Effects of water stress and bacterial inoculation on plant growth parameters

Rapeseed plantlets were harvested after 8 weeks of growth in natural soil under controlled conditions. To assess plant growth non-destructively, we estimated leaf surface from photographs processed with the ImageJ software. A significant correlation between leaf surface and fresh and dry shoot biomasses ($p < 0.001$) was found, indicating that the non-destructive measurement of leaf surface is a very good estimator of shoot biomass (Fig. 1).

As expected, only water stress applied to the soil had a significant effect on soil moisture ($p<0.001$) (Table S1). Soil moisture in the water stress treatments was consistent between treatments (no inoculation *vs* YAS34 *vs* GBV030) and was significantly different from their control treatment (no water stress) (Fig. 2). The average decrease (about −20 %) in soil moisture between the 'water stress' treatments compared to 'no water stress' treatments was observed.

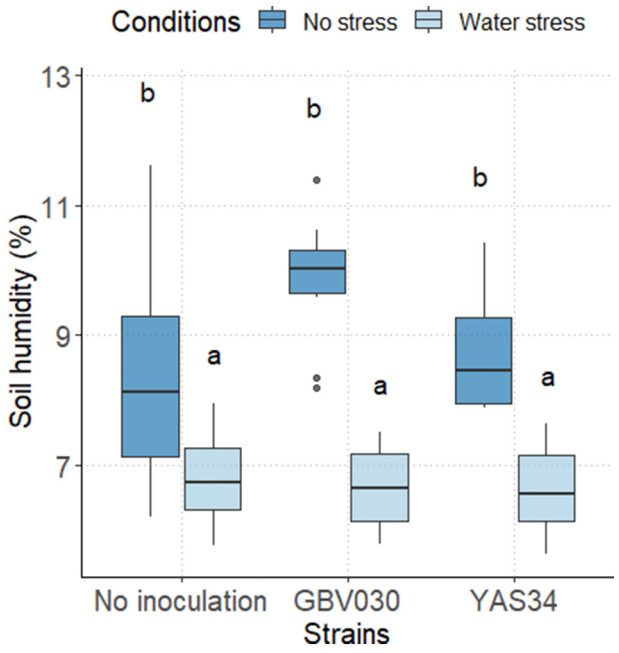


Fig. 2. Soil humidity (%) at the end of the experiment (8 weeks) of rapeseed (*B. napus* L.) grown on a calcareous silty clay soil after inoculation with *R. alamii* GBV030 and YAS34: dark blue boxes correspond to soil without water stress and light blue boxes correspond to soil under water stress. Letters represent homogeneity group based on Kruskal-Wallis *p* values adjusted with BH correction using a 0.05 threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Statistical analysis (Kruskal-Wallis test) of the different parameters measured on the shoots of *B. napus* plantlets (leaf surface, fresh and dry shoot mass) showed a significant effect of the two factors 'water

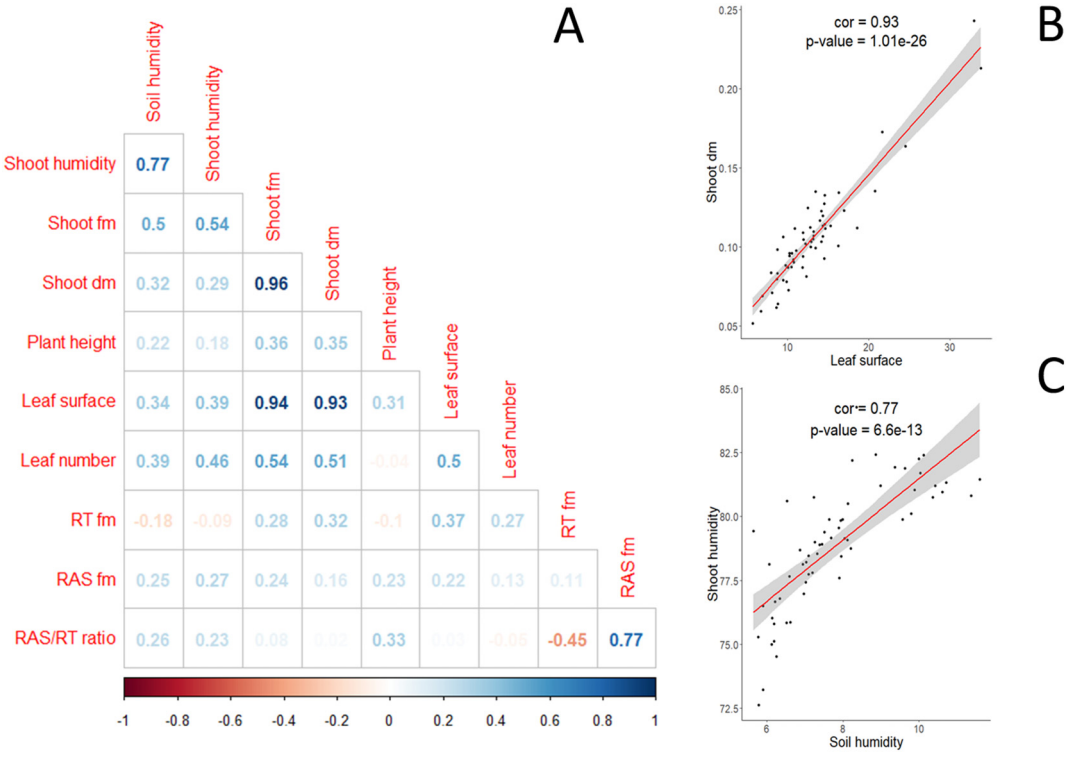


Fig. 1. Correlation between variables measured on rapeseed (*B. napus* L.) grown on a calcareous silty clay soil inoculated with *R. alamii* GBV030 and YAS34 at the end of the experiment (8 weeks). (A) All linear correlations for each paired variable. R coefficient is placed in the intersection of two variables in blue for positive value and red for negative value. (B) Correlation between 'Leaf surface' and 'Shoot dm' (C) Correlation between 'Soil humidity' and 'Shoot humidity': the red line is the fitted linear model based on all values with the standard error in grey area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stress' and 'inoculation' (Table S1). The main significant effect on leaf surface was observed after inoculation of *R. alamii* GBV030 only under water-stressed conditions (Fig. 3A). A significant effect of *R. alamii* GBV030 on dry shoot mass was shown both in water stress (+15%) and under normal water supply conditions (+39%) (Fig. 3B, Table S2). A significant effect on dry shoot mass was observed with *R. alamii* YAS34 only under water-stressed conditions (Fig. 3A–B). Inoculation of both strains (YAS34 and GBV030) under water-stressed conditions produced the same dry shoot mass compared to control/non-inoculated and normal water supply conditions (Fig. 3B), suggesting that the effect of the water stress could be compensated by bacterial inoculation.

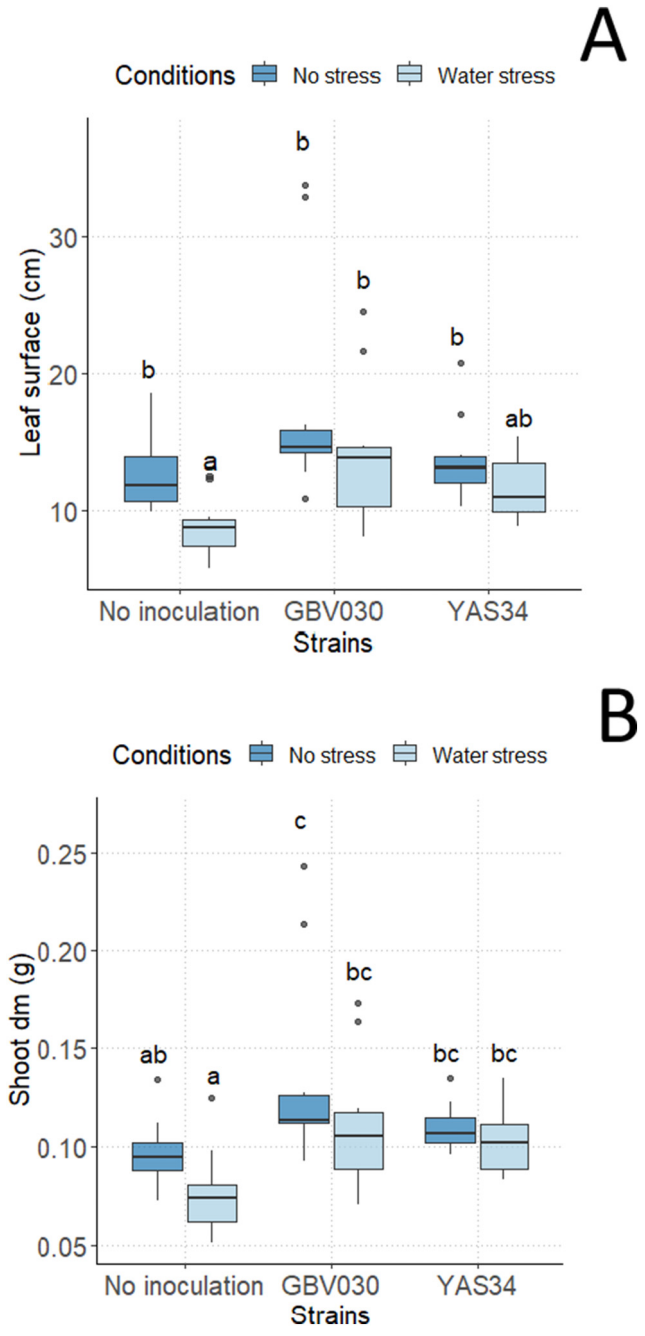


Fig. 3. Leaf surface (A) and shoot dry mass (dm) (B) at the end of the experiment (8 weeks) of rapeseed (*B. napus* L.) grown on a calcareous silty clay soil after inoculation with *R. alamii* GBV030 and YAS34: dark blue boxes correspond to soil without water stress and light blue boxes correspond to soil under water stress. Letters represent homogeneity group based on Kruskal Wallis *p* values adjusted with BH correction using a 0.05 threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For the root system (RT fraction) and the root-adhering soil (RAS fraction), we could not measure their mass of dry matter (dm) because the samples were used for the analysis of the microbiota structure on these two fractions. Therefore, contrary to the classical measurement of the RAS/RT ratio (Amellal et al., 1998; Alami et al., 2000), which is the ratio of the dm of root-adhering soil to the dm of roots, we calculated here the RAS/RT ratio using the mass of fresh matter (fm) of root-adhering soil and of fresh roots No significant effect of 'water stress' or 'inoculation' factors on root biomass (fm) was observed (Table S1). An overall effect of bacterial inoculation on root-adhering soil (RAS fm) was shown ($p<0.05$, Table S1) even though there was no significant effect of either strain compared to their respective control (Fig. 4A), a slight non-significant effect of *R. alamii* GBV030 was observed (Fig. 4A). Finally, the last phenotypic trait measured was the RAS/RT ratio (fm/fm). Only the water stress factor had a significant effect on this ratio (Table S1), and no significant effect of either strain compared to their respective control (Fig. 4B). The RAS/RT ratio was negatively correlated with the root biomass (RT fm) and positively correlated with the amount of root-adhering soil (RAS fm) (Fig. 1). All plant growth and root-adhering soil data are listed in Table S2

3.2. Effects of water stress and bacterial inoculation on plant root-associated microbiota

3.2.1. Alpha-diversity

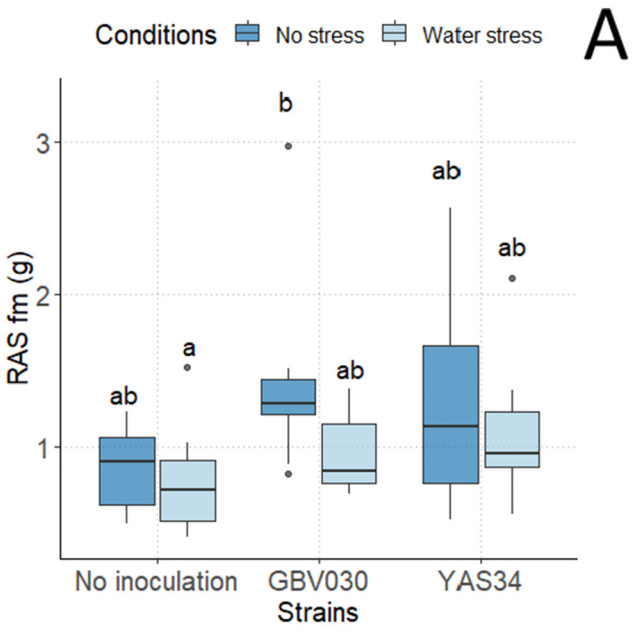
DNA was extracted from root and root-adhering soil (RAS, rhizosphere) samples and the V3-V4 region of 16S rDNA was amplified for metabarcoding analysis.

The alpha-diversity of the root-associated microbiota was highly significantly lower ($p < 0.001$) than that of the root-adhering soil fractions considering richness estimators (OTUs, ACE and Chao1) and evenness estimators (Shannon, Simpson) (Table S3). No significant effect of bacterial inoculation or water stress was observed for the root-adhering soil compartment (Table S4). The same observation was made for root compartment, except for a significant decrease in richness upon inoculation with *R. alamii* YAS34 compared to the control (non-inoculated) or the treatment inoculated with *R. alamii* GBV030 (in the absence of water stress) (Table S3).

3.2.2. Beta-diversity

We analyzed the effect of water stress and bacterial inoculation on the microbiota associated to the roots (RT) and root-adhering soil (RAS) using principal coordinate analysis (PCoA) and weighted UniFrac distance matrices (Fig. 5). For each PCoA, a PERMANOVA and beta scattering were performed to statistically support spatial differences. If a significant difference was found, a PERMANOVA with *fdr* correction was performed. On the root system (RT), both factors (water stress and bacterial inoculation) had a significant effect on root-associated microbiota ($p<0.05$, Fig. 5A). The three stressed treatments (no inoculation, GBV030 and YAS34) are in the center of Fig. 5A, while the 'GBV030/no water stress' treatment is clearly further away from the other treatments. On the contrary, neither water stress nor bacterial inoculation altered the RAS-associated microbiota (Fig. 5B). Homogeneity of dispersion was found for all conditions (beta dispersion, $p = 0.18$), meaning that differences were more likely to be between the barycenter of conditions in the new multidimensional space. The main result was that only inoculation with *R. alamii* GBV030 significantly modified the root-associated microbiota in the absence of water stress, compared to all other treatments (PcoA, PERMANOVA). This effect was not observed with *R. alamii* GBV030 under water-stressed conditions.

At the level of bacterial classes/orders, the RAS-associated microbiota was dominated by *Sphingomonadales*, *Oceanospirillales*, *Actinomycetales*, and a high proportion of undetermined orders (10-12 % of 'other') and did not differ between inoculation treatments or between water supply



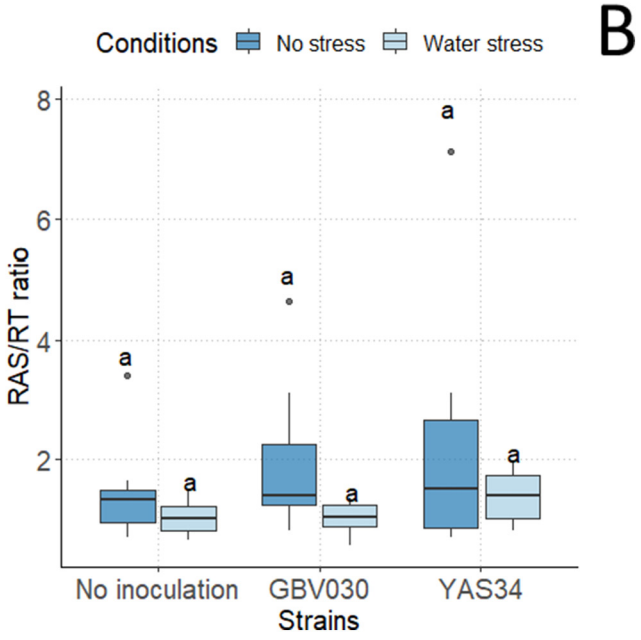
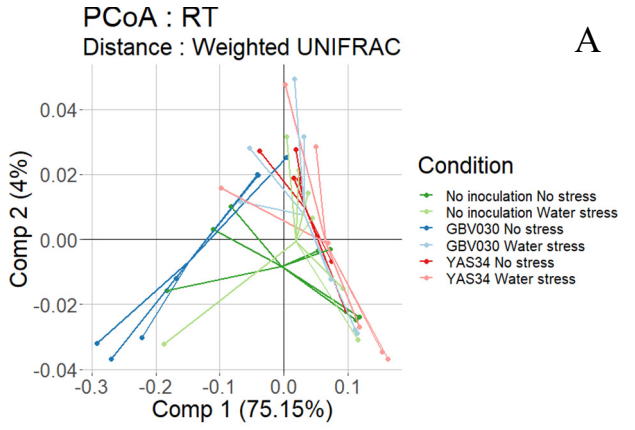
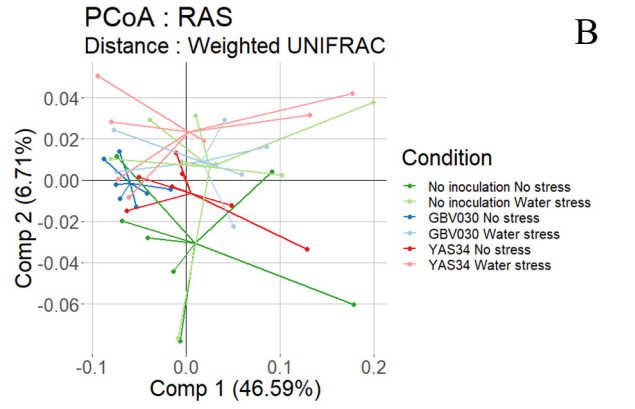


Fig. 4. Root Adhering Soil (RAS) (A) and RAS over Root (RT) ratio (B) at the end of the experiment (8 weeks) of rapeseed (*B. napus* L.) grown on a calcareous silty clay soil after inoculation with *R. alamii* GBV030 and YAS34: dark blue boxes correspond to soil without water stress and light blue boxes correspond to soil under water stress. Letters represent homogeneity group based on Kruskal Wallis *p* values adjusted with BH correction using a 0.05 threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



	Df	SumOfSqs	R2	F	Pr(>F)
Bacterial inoculation	2	0.08488738	0.13994704	3.837766	0.01879812
Water stress	1	0.06389081	0.10533167	5.777019	0.01359864
Interaction	2	0.05964852	0.09833775	2.696715	0.06649335
Residual	36	0.39814120	0.65638354	*NA*	*NA*
Total	41	0.60656791	1.00000000	*NA*	*NA*



	Df	SumOfSqs	R2	F	Pr(>F)
Bacterial inoculation	2	0.01659589	0.04613979	0.9792131	0.3953605
Water stress	1	0.01932877	0.05373772	2.2809241	0.1034897
Interaction	2	0.01869507	0.05197591	1.1030717	0.3333667
Residual	36	0.30506741	0.84814657	*NA*	*NA*
Total	41	0.35968713	1.00000000	*NA*	*NA*

Fig. 5. Comparisons of the microbiota diversity associated with root (RT) compartment (A) and root-adhering soil (RAS) compartment (B) of rapeseed (*B. napus* L.) grown on a calcareous silty clay soil after inoculation with *R. alamii* GBV030 and YAS34 using PCoA analysis. PCoA use a distance matrix to calculate positions of each sample in few dimensions. Only two first axes are shown explaining 53.3% and 79.2% of variability for A and B respectively. We used weighted UniFrac distance matrix, which calculates distances coupling distances between branches in a phylogenetic tree and OTUs abundancies. No inoculation, *R. alamii* GBV030 and *R. alamii* YAS34 are represented in green, blue and red, respectively. Light colours are for water stress conditions and dark colours for absence of stress conditions. Axes represent first two components with their percentage of variance explained. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatments (Fig. 6). The root-associated microbiota was dominated by *Sphingomonadales*, TM7 candidate division and *Actinomycetales* (Fig. 6). The main difference was observed for the treatment inoculated with *R. alamii* GBV030 between water-stressed and unstressed conditions, with a lower proportion of *Rhizobiales* and *Flavobacteriales* under water-stressed conditions (Fig. 6).

These comparisons were also made using LEfSe analysis in the RT and RAS compartments (Fig. 7A–B). The main bacterial groups characterizing the treatments inoculated with *R. alamii* GBV030 were *Flavobacteriaceae* and *Comamonadaceae* in the RT (Fig. 7A) and RAS compartments (Fig. 7B) under unstressed conditions, and *Halomonadaceae* in the RT compartment (Fig. 7A), and several species belonging to *Actinomycetales* in RAS compartment (Fig. 7B) under water-stressed conditions. After inoculation with *R. alamii* YAS34, *Verrucomicrobiaceae* were stimulated on both the RT and RAS compartments, as well as *Rhizobiaceae* in the RT compartment (Fig. 7A), and *Xanthomonadaceae* and *Sphingomonodaceae* in the RAS compartment (Fig. 7B) under unstressed conditions. Under water-stressed conditions, *Actinomycetales* were stimulated in RT compartment (Fig. 7A), and *Sporolactobacillaceae* in both the RT and RAS

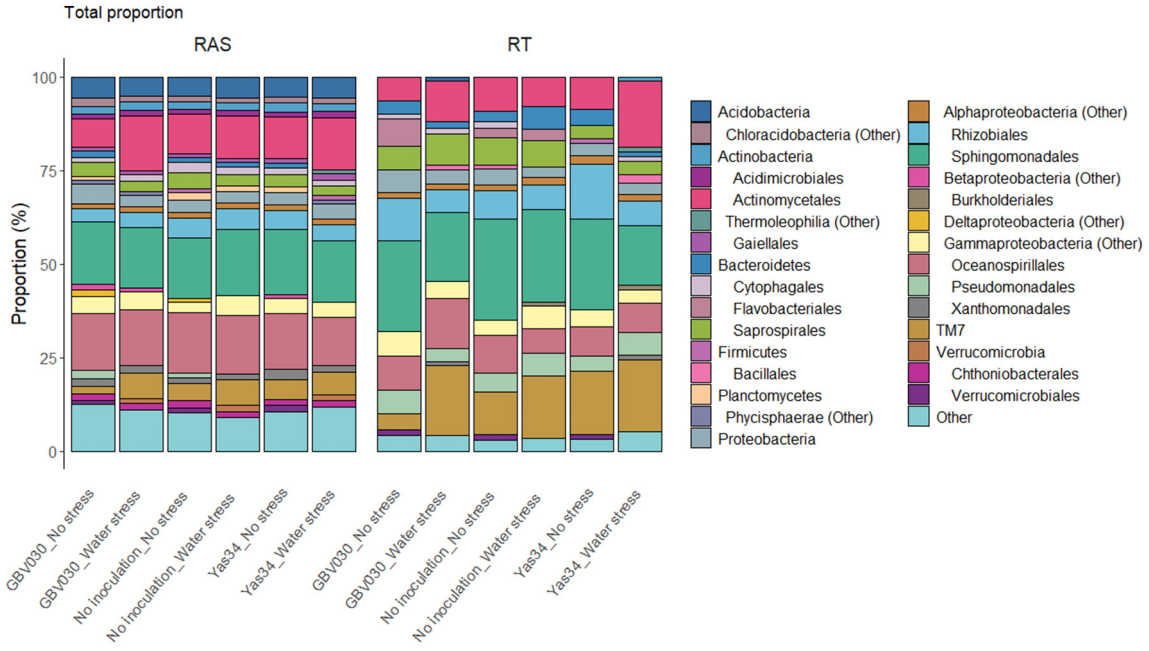


Fig. 6. Analysis of the beta-diversity of the microbiota associated with root (RT) compartment and root-adhering soil (RAS) compartment of rapeseed (*B. napus* L.) grown on a calcareous silty clay soil after inoculation with *R. alamii* GBV030 and YAS34. Relative proportion of orders is shown for each treatment. Identification in the legend separates division from order. Clade followed by "*other*" means that the order was unknown or represented less than 1% of the condition relative proportion. Orders under a clade belong to it. Orders below 1% and/or poorly studied are present in the group "*Other*" at the bottom.

compartments (Fig. 7A–B). There were differences between PCoA analyses and LEfSe analyses. These two methods assess two views of the same microbiota. PCoA measures differences using all OTUs at the same time, whereas LEfSe measures differences in each OTUs separately. Thus, both methods are important and complementary in assessing microbiota comparison.

4. Discussion

4.1. Phytobeneficial effect of inoculation with R. alamii strains

The phytobeneficial effect of *R. alamii* YAS34 has been evidenced on sunflower growth as well as its effect on plant tolerance to water stress (Alami et al., 2000). The main effect of this strain on sunflower growth was attributed to the modification of the rhizosphere soil aggregation involving the production of bacterial EPS. Using a mutant of strain YAS34 impaired in EPS production it was shown that this EPS production was responsible for the positive effect of the wild-type strain YAS34 on water-stable aggregates in the rhizosphere of rapeseed (Santaella et al., 2008). In contrast to the positive effect of *R. alamii* YAS34 on sunflower (Alami et al., 2000), no effect was found on rapeseed growth (Santaella et al., 2008). We confirmed in the present work that YAS34 strain was not effective on rapeseed growth under optimal water supply conditions but, under water stress conditions, this strain was able to compensate for the water stress potentially through its EPS production in the rhizosphere (Fig. 3B). By comparing *R. alamii* YAS34 (isolated from the rhizosphere of sunflower) with *R. alamii* GBV030 (isolated from the rhizosphere of *A. thaliana*, taxonomically close to rapeseed), the objective was to evaluate the level of specificity of bacterial inoculation in using two strains belonging to the same species (*R. alamii*) and producing the same type of EPS, but isolated from two different plant rhizospheres.

In addition to their protection from water stress, rapeseed plantlets inoculated in absence of water stress showed an increase in shoot biomass after inoculation with *R. alamii* GBV030 and YAS34 (+39% and +13%, respectively) (Fig. 3B, Table S2). There was no significant effect of either strain on fresh root biomass and root-adhering soil mass (Fig. 4, Table S2) although we showed an overall significant effect of the inoculation factor on root-adhering soil mass (Table S1). These results confirm those of Saghafi et al (2018) who reported that inoculation of *Sinorhizobium meliloti* and *Rhizobium leguminosarum* strains significantly increased shoot and root biomass after 4 months of growth, and those of Ullah et al. (2017) who evidenced that co-inoculation of *R. leguminosarum* and *Mesorhizobium ciceri* increased grain yield of wheat under water deficit.

In the present work, we evidenced that *R. alamii* GBV030 was also able to compensate for the effect of water stress on rapeseed growth (like strain YAS34) (Fig. 3B) but, in addition, it stimulated plant growth under optimal water supply conditions (unlike strain YAS34), suggesting that properties other than the production of the same type of EPS are involved in this effect of rapeseed growth. These properties can be the synthesis of phytohormones, regulating the hormone balance of their host plant or the production of molecules enhancing the availability of mineral elements essential to plant growth (Lugtenberg and Kamilova, 2009; Olenska et al., 2020). Other *Rhizobium* species than *R. alamii* have been described as PGPRs on different non-legumes of agronomic interest involving various phytobeneficial properties: *Rhizobium leguminosarum*-maize/P solubilization (Chabot et al, 1996), *Rhizobium etli*-maize (Gutiérrez-Zamora and Martínez-Romero, 2001), *R. leguminosarum*-rice/P solubilization/IAA (Yanni et al., 2001; Mishra et al., 2006), *R. leguminosarum*-canola/IAA (Noel et al., 1996), *R. leguminosarum*-tomato and pepper/IAA/siderophores (García-Fraile et al., 2012), *Mesorhizobium japonicum*-barley/P solubilization (Peix et al., 2001). All of these strains of *Rhizobium* (*sensu lato*) are known mainly for being able to nodulate legumes and described more recently to improve the growth of non-legume crops, which contrasts with *R. alamii* described for its PGPR effect on sunflower (Alami et al., 2000) and rapeseed (this work) and, so far, without no legume host identified.

4.2. Effect of inoculation with R. alamii strains on rhizosphere microbiota

In the first part of this work, we showed that the inoculation with *R. alamii* GBV030, and to a lesser extent with *R. alamii* YAS34, increased rapeseed growth and/or tolerance to water stress, probably through the

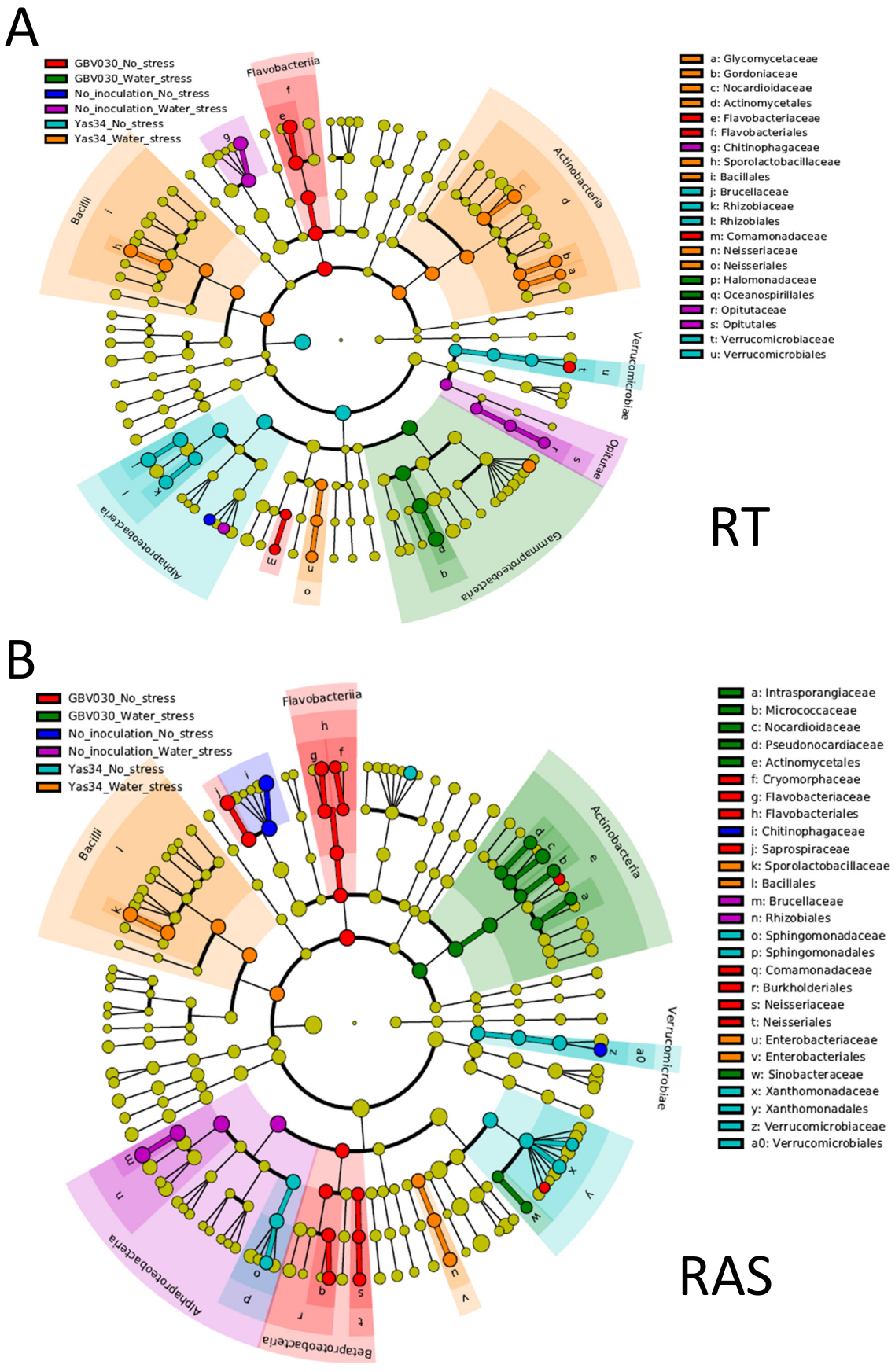


Fig. 7. Analysis of the beta-diversity of the microbiota associated 1037 with root (RT) compartment (A) and root-adhering soil (RAS) compartment (B) of rapeseed (*B. napus* L.) grown on a calcareous silty clay soil after inoculation with *R. alamii* GBV030 and YAS34. LDA Effect Size (LEfSe) represents taxonomic levels that are statistically overrepresented in one condition (plant inoculated or not, water stressed or not). LDA score > 2 was used for both LEfSe algorithms. Only well studied orders were compared.

production of EPS and/or potentially other direct effects on rapeseed growth (*e.g.* phytohormones or VOCs). The second part of this work was devoted to the evaluation of bacterial inoculation on root-associated and rhizosphere microbiota in order to identify which bacterial groups were stimulated, focusing on bacterial family/genera known to have phytobeneficial effects on plant growth that may constitute indirect effects of bacterial inoculation. Evaluation of the effect of bacterial inoculum, especially PGPR *P. fluorescens* strains, on non-target microbial community has ranged from limited and transient effects (Moënne-Loccoz et al., 2001; Bakker et al., 2002) to more significant effects (Walsh et al., 2003; Kozdrój et al., 2004). Surprisingly, the inoculation of *Vicia faba* with *R. leguminosarum* bv. *viciae* altered the composition of bacterial community without increasing faba bean yield (Zhang et al., 2010). More recently the use of Next Generation Sequencing (NGS) technologies has opened the possibility of a more thorough analysis of microbiota diversity using 16S rDNA metabarcoding and a more rigorous method to assess the safety of inoculants on soil microbiota (Martínez-Hidalgo et al., 2019). In a recent work, Jiménez et al. (2020) determined the effect of *P. fluorescens* LBUM677 inoculation over time on native rhizosphere bacterial community associated with three different oilseed crops (including rapeseed). In the rapeseed rhizosphere, using 16S rDNA metabarcoding and LeFSe analysis, they identified only six differentially abundant taxa (including *Paenibacillales*) after inoculation with strain LBUM677 treatment (Jiménez et al., 2020).

In the present work, the beta-diversity of the root-associated (RT) microbiota was significantly modified by water stress and inoculation factors (Fig. 5A). PCoA using weighted UniFrac distance showed that the treatment 'inoculation with *R. alamii* GBV030 in the absence of water stress' was different and clearly further away from the 5 other treatments (Fig. 5A). In contrast, neither water stress nor bacterial inoculation modified the beta-diversity of the RAS-associated microbiota (Fig 5B). In a complementary manner, using univariate LEfSe analysis, we identified several genera overrepresented under different conditions for the RAS and RT compartments (Fig. 6B–C). The differences between multivariate (PCoA using weighted UniFrac distance) and univariate (LEfSe) analysis have long been discussed and several explanations have been suggested by Saccenti et al. (2014). In our case, differences should most likely be due to the high number of uninformative variables used in the weighted UniFrac distance analysis. Thus, the PCoA and PERMANOVA analyses could not reveal differences in beta-diversity, requiring the complementarity of LEfSe calculations. Another explanation for the differences between analyses is the use of different taxonomic levels, as multivariate analysis uses abundance at the species level while the univariate analysis uses the genus level, so the two methods do not overlap perfectly, but should be considered complementary.

Under water stress conditions, inoculation of *R. alamii* strains resulted in an overrepresentation in the RT compartment of *Halomonadaceae* (strain GBV030), and several species belonging to of *Actinomycetales* and *Sporolactobacillaceae* (strain YAS34) (Fig. 7A). In the RAS compartment, under water stress conditions, *Sporolactobacillaceae* (strain YAS34) and several species belonging to *Actinomycetales* were overrepresented (Fig. 7B). *Halomonadaceae* are halophilic or halotolerant Gram-negative (diderm) bacteria (Mapelli et al., 2013) also well adapted to the drought environment. *Actinomycetales* and *Sporolactobacillaceae* are Gram-positive bacteria (monoderm) capable of growing in drought environment (Naylor and Coleman-Derr, 2018). Some genera belonging to *Actinobacteria* can be considered PGPRs (Viaene et al., 2016), and strains of *Halomonas* have also been described as PGPRs that improve water stress tolerance in durum wheat (Albdaiwi et al., 2019).

In the absence of water stress, the main bacterial groups characterizing treatments inoculated with *R. alamii* GBV030 were *Flavobacteriaceae* and *Comamonadaceae* in both the RT (Fig. 7A) and RAS compartments (Fig. 7B). After inoculation with *R. alamii* YAS34, in the absence of water stress, *Verrucomicrobiaceae* were stimulated in the RT and RAS compartments, as well as *Rhizobiaceae* in the RT compartment (Fig. 7A), and *Xanthomonadaceae* and *Sphingomonodaceae* in the RAS compartment (Fig. 7B). Some bacterial strains belonging to *Flavobacteriaceae* have been described for their PGPR potential, such as *Chryseobacterium balustinum* AUR9 eliciting plant protection in *Arabidopsis thaliana* (Ramos Solano et al., 2008), and *Flavobacterium* strains promoting *A. thaliana* growth (Cardoso et al., 2018). On the other hand, the list of bacterial strains belonging to *Comamonadaceae* known for their PGPR potential is very broad with for example *Acidovorax radicis* N35 (Zytynska et al., 2020), *Comamonas acidovorans* RC41 (Erturk et al., 2010), and *Variovorax boronicumulans* CGMCC4969 (Sun et al., 2018). As mentioned previously, several *Rhizobiaceae* strains have phytobeneficial effects on non-legumes (Chabot et al, 1996; Noel et al., 1996; Alami et al., 2000; Gutiérrez-Zamora and Martí´nez-Romero, 2001; Peix et al., 2001; Yanni et al., 2001; Mishra et al., 2006; Mehboob et al, 2009; García-Fraile et al., 2012). Within *Xanthomonadaceae*, several strains of *Stenotrophomonas maltophilia* and *Stenotrophomonas rhizophila* have been characterized (Singh and Jha, 2017; Silambarasan et al., 2020).

In the present work, as in most of the works published to date, the resolution of the diversity is at the family level, making it very difficult to identify species known to have a potentially beneficial effect on plant growth. Within the same genus, and even more so within the same bacterial family, it is possible to find both pathogenic species and PGPR species. At this stage, it is therefore difficult to conclude that the positive effect observed on rapeseed growth following inoculation with *R. alamii* strains is related to an indirect effect on PGPR bacterial populations that benefited from a favorable environment related to the production of EPS or other PGP traits by *R. alamii* strains. However, this hypothesis cannot be ruled out as well as the direct effect of these strains through the production of phytohormones or any other molecule that can stimulate rapeseed growth. Further analysis of the growth-promoting properties of these *R. alamii* strains is in progress using a genomic approach. Finally, the transient character of the modification of the bacterial community structure following inoculation by *R. alamii* remains to be established.

5. Conclusion

The beneficial effect of *R. alamii*, effective on rapeseed growth, allows to consider it as a PGPR acting directly on water stress limitation, through the production of EPS and probably other PGP traits, and also potentially indirectly by modifying the structure of the microbiota on roots and in the rhizosphere (RAS) in favor of species belonging to the families *Flavobacteriaceae* and *Comamonadaceae* which can in turn act as plant growth-promoting rhizobacteria. This indirect effect on the root-associated microbiota is all the more likely as both strains of *R. alamii* produce the same EPS (and probably the same root-adhering-soil structuring effect), while they do not have the same effect on plant growth. The origin of the strains, the rhizosphere of *A. thaliana* for *R. alamii* GBV030 and that of sunflower for *R. alamii* YAS34, and the production of different PGP traits could explain this difference in efficacy on rapeseed and highlight some specificity, apart from nodulation of legumes plants, between a crop plant and a PGPR *Rhizobium* species. The comparison of the available genome of *R. alamii* GBV030 with that of *R. alamii* YAS34 could provide interesting leads.

To verify that the effect on the microbiota is transient, it would be interesting to test the resilience by growing one or two rapeseed cultures on soil that has been inoculated without repeating bacterial inoculation. Such resilience of the soil microbiota would demonstrate the absence of a lasting effect on the non-target microbiota.

CRediT authorship contribution statement

Joris Tulumello: Investigation, Formal analysis, Writing - original draft. **Nicolas Chabert**: Conceptualization, Investigation, Writing-review. **Julie Rodriguez**: Investigation. **Justine Long**: Formal analysis. **Renaud Nalin**: Conceptualization, Writing-review. **Wafa Achouak**:

Conceptualization, Writing-review. **Thierry Heulin**: Conceptualization, Investigation, Writing - review & editing.

Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148895.

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