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Rhizobium leguminosarum symbiovar *viciae* strains are natural wheat endophytes that can stimulate root development

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Summary statement

We showed that *Rhizobium leguminosarum* strains that are typical legume symbionts, naturally colonize wheat roots and can stimulate host root development.

Abstract

Although rhizobia that establish a nitrogen-fixing symbiosis with legumes are also known to promote growth in non-legumes, studies on rhizobial associations with wheat roots are scarce. We searched for *Rhizobium leguminosarum* symbiovar *viciae* (*Rlv*) strains naturally competent to endophytically colonize wheat roots. We isolated 20 strains from surface-sterilized wheat roots, and found a low diversity of *Rlv* compared to that observed in the *Rlv* species complex. We tested the ability of a subset of these *Rlv* for wheat root colonization when co-inoculated with other *Rlv*. Only a few strains, including those isolated from wheat roots, and one strain isolated from pea nodules, were efficient in colonizing roots in co-inoculation conditions, while all the strains tested in single strain inoculation conditions were found to colonize the surface and interior of roots. Furthermore, *Rlv* strains isolated from wheat roots were able to stimulate root development and early arbuscular mycorrhizal fungi colonization. These responses were strain and host genotype dependent. Our results suggest that wheat can be an alternative host for *Rlv*; nevertheless, there is a strong competition between *Rlv* strains for wheat root colonization. In addition, we showed that *Rlv* are endophytic wheat root bacteria with potential ability to modify wheat development.

Introduction

Plant roots interact with microorganisms that play key roles in their development, nutrition and protection against pathogens (Ortíz-Castro et al., 2009). Under the influence of root exudates, microbes multiply in the rhizosphere, i.e. the soil portion in proximity to the roots (Haichar et al., 2014; Baetz & Martinoia, 2014; Hu et al., 2018; Huang et al., 2019). Microorganisms inhabiting the rhizosphere are known to play important roles in plant nutrition through various properties such as biological nitrogen fixation, phosphate solubilization or siderophore secretion (de Souza et al., 2015). Among the extremely high diversity of soil/rhizospheric microorganisms, only a small fraction is able to colonize the inner part of the plant root system. Several of these endophytic microbes have been shown to offer important benefits to their hosts displaying Plant Growth-Promoting (PGP) activities or protection against pathogens (Santoyo et al., 2016). Mechanisms including: i) facilitation in acquiring nutrients, ii) interference with plant hormone (auxin, cytokinin or ethylene) homeostasis, iii) pathogen control via antibiosis, have been related to these beneficial activities (Santoyo et al., 2016).

Among endophytic microorganisms, some, such as the Arbuscular Mycorrhizal Fungi (AMF) interacting with most plant species, have the ability to massively colonize plant roots (Bonfante & Genre, 2010). This colonization relies on AMF recognition by plants that is, at least in part, mediated by the perception of fungal LipoChitoOligosaccharide (LCO) signals (Girardin et al., 2019). Non-legume LCO receptors are poorly specific for LCO structural variations (Buendia et al., 2019a; Girardin et al., 2019), which is consistent with poor host-specificity in Arbuscular Mycorrhiza (AM) as almost all AMF species are able to colonize most the terrestrial plants. The LCO perception machinery and the downstream signaling pathway have been recruited in legumes to allow recognition of nitrogen fixing bacteria called rhizobia, which are accommodated in root organs, the nodules (Girardin et al., 2019). Rhizobia also

produce LCOs, called Nod factors, that are recognized by legume hosts (Gough & Cullimore, 2011). Rhizobia are polyphyletic and belong to various bacterial genera. Indeed, the Nod factor synthesis genes (*nod* genes) are frequently located on a plasmid (the symbiotic plasmid), which can be horizontally transferred within or across rhizobial species (Boivin et al., 2020). In contrast with the AM, the rhizobium-legume symbiosis is generally host-specific and bacteria able to nodulate the same host(s) are grouped into ‘symbiovars’. For example, pea, faba bean, vetch and lentil plants are nodulated by *Rhizobium leguminosarum* bacteria of the symbiovar (sv) *viciae* (*Rlv*), while clover is nodulated by *Rhizobium leguminosarum* sv. *trifolii*. Other legumes such as alfalfa and soybean are nodulated by rhizobia from other genera, *Ensifer* (formerly called *Sinorhizobium*) and *Bradyrhizobium* respectively. Nod factor structural variations are associated to this host-specificity (Gough & Cullimore, 2011). In addition, in natural soils, multiple rhizobia of the same symbiovar coexist but display contrasted Competitiveness to Form Nodules (CFN) depending on their host genotype (Boivin et al, 2020; 2021). Together with the host-specificity associated with Nod factor-specificity, CFN contributes in determining partner choices during the rhizobium-legume symbiosis.

Several studies have shown that rhizobia can also interact with non-legumes, including wheat. Wheat core root microbiota have been identified by metabarcoding approaches, and genera that include nodulating rhizobia were frequently found (Simonin et al., 2020; Kavamura et al., 2021). More specifically, *Rlv* were previously isolated from soils used for wheat monoculture (Depret et al., 2004), suggesting that *Rlv* can be maintained in absence of rotation with compatible legumes. Rhizobia belonging to various genera were isolated and/or shown to have PGP activities in rice (Biswas et al., 2000; Chaintreuil et al., 2000; Peng et al., 2002; Yanni & Dazzo, 2010). *R. leguminosarum* sv. *trifolii* (isolated from legumes, rice or wheat) were also shown to colonize and have PGP activities on wheat (Höflich et al., 1995; Webster et al., 1997; Hilali et al., 2001). Although mechanisms involved in AM and rhizobial symbiosis

establishment share similarities, there is little information on the rhizobia-AMF interaction, particularly in wheat. Nevertheless, Raklami and coworkers recently showed that rhizobial inoculation in open field increases wheat colonization by AMF and yield (Raklami et al., 2019). However, tripartite interactions between PGP bacteria, AMF and plant hosts, can have contrasted (synergistic, neutral or antagonist) effects depending on the combinations, including in wheat (Imperiali et al., 2017; Pérez-de-Luque et al., 2017; Vanini et al., 2021).

Here, we performed a functional ecological study on the wheat-*Rlv* interaction. We investigated whether: i) wheat is a natural host for *Rlv* strains, by isolating bacterial strains from wheat grown in open field in the southwest of France, ii) there is partner choice in the wheat-*Rlv* interaction, by co-inoculating strains representative of the known *Rlv* diversity, iii) there are differential abilities among *Rlv* for colonization and stimulation of responses in wheat roots, by measuring *Rlv* epiphytic/endophytic colonization, root development and early AMF colonization.

Material and Methods

Wheat sampling from open field

Wheat plants (*Triticum aestivum ssp. aestivum* and *Triticum turgidum ssp. turgidum*) were collected in the CREABio experimental station de la Hourre in Auch (Southwest France; 43°37'17.7"N 0°34'20.6"E) in March 2014, at the end of the tillering stage. Plants were collected in 2 experimental field plots: LH1 under rotation with pea (*Pisum sativum*) and fertilized with 80 kg/ha of organic fertilizer, and LH7 under rotation with soybean (*Glycine max*) and fertilized with 100 kg/ha of organic fertilizer. Both plots were characterized by a limestone clay soil with a pH of 8.1 and 8.4 for LH7 and LH1, respectively. Specific soil composition for the field plots are reported in Fig. S1. Sixteen wheat varieties, 8 from LH1 and 8 from the LH7, were collected (Table S1). Six plants per variety were sampled and transported in sterilized plastic bags.

Strain isolation and characterization

To isolate *Rhizobium leguminosarum* symbiovar (sv) *viciae* (*Rlv*) strains, 2 methods were employed: a nodule-trapping method and direct root plating. Prior to bacterial isolation, wheat roots of each variety were pooled and surface-sterilized. Roots were first rinsed in sterile water to remove soil, and incubated 10 min in a 1.2 % sodium hypochlorite solution, 10 min in a spiramycine 30 mg/ml solution and 1 min in 70% ethanol, followed by 3 rinses in sterile water. Surface-sterilized roots were mixed in 5 ml of phi liquid medium (10 g/L Bacto Peptone, 1 g/L Casimino acids and 1 g/L yeast extract) and root mixtures were stored in 50% of glycerol at -80°C prior to bacterial isolation. For the nodule-trapping experiment, pea (*Pisum sativum*) and vetch (*Vicia sativa*) plantlets were used as specific hosts. Seeds were surface-sterilized and germinated as described in Method S1. Seedlings were grown in 110 ml glass tubes containing Farhæus agar medium (Catoira et al., 2000). After 10 days, plantlets were inoculated with

wheat root homogenates and incubated for 4 weeks at 22°C. Non-inoculated plants were used as controls. For each of the 16 wheat root homogenates, 3 plants of each legume host were inoculated. After 4 weeks, plants were scored for the presence/absence of nodules (Table S1). Each nodule was collected with a scalpel, homogenized and stored in 50% glycerol at -80°C prior to bacterial isolation. Serial dilutions of nodule homogenates were spread on TY agar medium (16 g/L peptone, 10 g/L yeast extract and 5 g/L NaCl) supplemented with 2 mM CaCl₂. For direct isolation of culturable bacteria, root homogenates were diluted and spread on TY agar medium supplemented with 2 mM CaCl₂ (3 plates / root homogenate). Forty nine colonies were purified and stored in 30% of glycerol/TY medium at -80°C. Bacteria isolated from nodules or by direct plating were tested for presence of the *nodD* gene. We used the Y5 and Y6 primer set developed by Zeze et al., (2001) amplifying an 850bp *nodD* fragment from *Rhizobium leguminosarum* sv. *trifolii* and *Ensifer meliloti*. Strains showing an amplification (18/29 from nodule trapping and 2/49 from direct plating) were then used for *gyrB* amplification by using primers and protocols already described (Martens et al., 2008). Sequences (Table S2) were trimmed and aligned with DAMBE version 6 (Xia, 2017) and neighbor-joining phylogeny in comparison with other *Rhizobium* species (Table S3; sequences retrieved from GenBank <https://www.ncbi.nlm.nih.gov/genbank>), was inferred with MEGA 5 (Tamura et al., 2011) with *p*-distance model. Three strains isolated from wheat roots, FWPou15, FWPou32 and FWPou38 were selected for further analysis and their genomes were sequenced and analyzed as described in Method S3 and Table S4. Genomes are available in GenBank under the accession numbers JACBGR000000000, JACBGQ000000000 and JACBGP000000000. Hierarchical clustering and heatmaps were built using the pheatmap R package (clustering “maximum”, method “wardD.2”). Phenotypic assays were performed on 2 of these 3 strains in comparison with the A34 strain used as control (8401 pRL1JI in Götz et al., 1985; Oono & Denison, 2010).

Metabolic pathways and comparative genomics analyses

A metabolic reconstruction in SBML format (Hucka et al., 2003) was built for each strain with the Carveme software (Machado et al., 2018), a binary matrix containing the presence/absence information for each metabolic reaction in each strain was created from SBML files using ad-hoc Java programs. A correspondence analysis was performed with the `dudi.coa` method of the R `ade4` package (Dray et al., 2007). The correspondence analysis result was then plotted using the `scatterD3` R package. Tryptophan metabolism was analyzed with the `kbase` platform (Arkin et al., 2018) and by using the RAST annotation pipeline (Brettin et al., 2015) and the Model Seed metabolic reconstruction pipeline (Henry et al., 2010). Comparative genomic analysis was performed using `snippy` (<https://github.com/tseemann/snippy>) with default parameters on whole genomes including coding and non-coding sequences. Orthology searches were performed with `OrthoFinder` with default settings (Emms & Kelly, 2019). Functional category assignments and annotations of orthologous groups were made by selecting a representative of each group and annotating with `eggNOG-Mapper` (Huerta-Cepas et al., 2017).

Nodulation tests

Three selected strains FWPou15, FWPou32 and FWPou38 and the A34 strain were tested for their ability to nodulate pea, faba bean, vetch and clover plants. For pea and vetch plants, germ-free seedlings (Method S1) were placed into 110 ml glass tubes filled with attapulgitite and watered with 10 mL Farh eus liquid medium (Method S2) supplemented with 1 mM NH_4NO_3 , and aluminum foil was placed around the bottoms of the tubes. Seedlings were grown for one week before inoculation with 2 ml of 10^8 /ml bacterial suspension of each strain/plant (12 plant replicates / plant species). For faba bean and clover plants, germ-free seedlings were transferred in containers (250 ml of volume) filled with a mix of 50% perlite/50% sand and humidified with 300 ml of sterilized water. Seedlings were inoculated by adding 150 ml of 10^7 /ml bacterial suspension/plant and cultivated in a growth chamber (20°C and 16h/8h light/dark period). Nodules were counted 6 weeks after inoculation. Microscopy analysis was performed on 3

randomly selected nodules for each nodulated plant species (Method S4) to confirm that nodules were colonized by bacteria.

Wheat root colonization in co-inoculation assays

Seven-day old germ-free wheat seedlings (Method S1) of the Energo variety, individually grown in 110 ml glass tubes filled with 70 ml of Farhæus agar medium (Fig. S2; Method S5), were inoculated with FWPou15 strain alone or with bacterial mixtures containing either the FWPou15 or FWPou38 strains plus 22 strains of the *Rlv* core collection representing the known genomic *Rlv* diversity (Table S3; Boivin et al., 2021). Mixtures were made by diluting each strain at a final OD_{600nm} = 0.01 (initial OD_{600nm} are given in Table S5). Each modality consisted in an inoculation of 6 plants; 3 independent replicates (3 temporal blocks) were performed. In total 18 plants were inoculated with each bacterial mixture. Each wheat seedling was co-inoculated with 2 ml of each mixture containing all bacterial strains at the final OD_{600nm} = 0.01 and incubated in a growth chamber at 20°C and a light/dark period of 16h/8h. Roots were collected after 7 days. Half of the roots were surface-sterilized using the following successive treatments: 1 min in pure ethanol, sterilized water, 3 min in 1.2% sodium hypochlorite solution, 3 times in sterilized water, 1 min in pure ethanol and 3 times in sterilized water. The other half was directly analyzed without surface sterilization. For each temporal block, the plant roots of each modality were pooled prior to DNA extraction with the DNeasy Plant Mini Kit following the manufacturer's instructions (Table S5). To characterize the relative abundance of the *Rlv* bacteria in the wheat roots, the 309 bp sequence of the *nodD* coding region was amplified by PCR from the extracted DNAs and the PCR products were sequenced by Illumina MiSeq 2x250 bp technology as described in Method S6. Each strain present in the mixture is characterized by a unique 309 bp *nodD* sequence and was therefore unambiguously identified. Abundance of each strain was estimated by the number of reads corresponding to its unique *nodD* sequence (Method S6, Table S6).

Wheat root and leaf colonization in single strain inoculation assays

To test the colonization level in single-inoculation conditions, experiments were performed as described for co-inoculation assays except that seedlings were inoculated with 2 ml of each bacterial suspension ($OD_{600nm} = 0.08$, see result section for the bacterial strain used). The number of independent replicates (temporal blocks) and the total number of plants analyzed for each strain and conditions are reported in Table S7. Roots and leaves were excised 4, 7, 14 or 21 days post inoculation (dpi) and weighed (fresh weight). Bacterial isolation from roots and leaves was performed by chopping the entire root system and the leaf fragments using a scalpel and by dilution plating on TY plates supplemented with 2 mM $CaCl_2$. To estimate root endophytic growth, wheat roots were surface-sterilized as described for the co-inoculation assay. To determine whether leaf bacteria were endophytic, leaf fragments were plated on the TY medium supplement with 2 mM $CaCl_2$, before leaf chopping. The absence of bacteria on leaf imprints while bacteria were found after leaf chopping corroborated that the inoculated strains were located within the leaves. Plates were incubated 3 days at 24°C before colony counting. The number of Colony-Forming Units (cfu) was normalized by root or leaf fresh weights.

Wheat root colonization by the fluorescent protein-expressing strain

Construction of the strains expressing GFP, mCherry or YFP fluorescent proteins is described in Method S7 and microscopy analysis is detailed in Method S4.

Wheat root development assays

Three-day old germ-free wheat seedlings (Method S1) were placed on 20x20 cm plates containing Farhæus agar medium (Fig. S2, Method S5). Seedlings were grown 4 days prior to inoculation with 1 ml of bacterial suspension ($OD_{600nm} = 0.08$) of each strain (see result section for the bacterial strain used). Control plants were inoculated with 1 ml of sterilized water. The

number of experiments (temporal blocks) and plant replicates are shown in Table S8. Plants were grown at 20°C with a light/dark period of 16h/8h. Roots were removed from the plates 12 dpi, washed with water to remove traces of agar, and imaged with an Epson Expression 10000XL scanner. Total root length was estimated by using WINRhizo software and the number of lateral roots was counted manually on the scanned roots.

Mycorrhiza assays

Three-day old germ-free wheat seedlings (Method S1) were transferred to 50 ml containers filled with attapulgitite supplemented with 20 ml of 1/2 modified Long Ashton liquid medium (containing 7.5 μ M NaH₂PO₄, Method S2) and 200 *Rhizophagus irregularis* DAOM197198 spores (purchased at Agronutrition, Carbonne, France). The substrate was stirred in a beaker after addition of the 200 spores to homogenize spore density. The number of experiments (temporal blocks) and plant replicates are shown in Table S9. After 3 days at 23°C with a 16/8h light/dark regime, 1 ml of each bacterial suspension (OD_{600nm}= 0.08, see result section for the bacterial strain used) was inoculated and plants were further grown for 21 days. Roots were then washed and permeabilized in a 10% KOH solution for 10 min at 95°C and stained with a 5% ink-vinegar solution for 3 min at 95°C. The number of colonization sites was counted on entire root systems using a Leica S6E stereomicroscope. Areas with several neighboring arbuscules were considered as colonization sites.

Statistical analysis

All the statistical analyses performed in the manuscript were carried out in the R environment. Scripts used for the analyses and for the construction of the graphs are detailed in Method S8 and S9. Raw data that were used to run the statistical models are available in: i) Table S10 for the relative abundances of the *R/v* strains in roots during the co-inoculation assays, ii) Table S11 for the numbers of cfu in the root colonization assays, iii) Table S12 for the total root

lengths and lateral root numbers obtained in the root development assays, iv) Table S13 for the number of colonization sites in the mycorrhiza assays.

Results

***Rhizobium leguminosarum* sv. *viciae* (*Rlv*) strains are associated with wheat roots**

In order to determine whether *Rlv* strains naturally interact with wheat, we collected wheat roots from plants under rotation with pea or soybean in open field located in the southwest of France. We surface-sterilized and macerated the roots to extract bacterial endophytes. Several wheat varieties belonging to 2 species (*Triticum aestivum* ssp. *aestivum* and *Triticum turgidum* ssp. *turgidum*, Table S1) were compared. *Rlv* were isolated from these root samples by two approaches. First, pea and vetch seedlings, which are known legume *Rlv* hosts, were inoculated with the root macerates. The nodules formed were individually collected and bacterial clones occupying these nodules were isolated (“nodule trapping”). Second, surface-sterilized macerates from wheat roots were directly plated. Bacteria collected by both methods were PCR screened by using primers that specifically amplify a fragment of the *nodD* gene from *Rlv*, and from a few other rhizobial species (Zeze et al., 2001). We succeeded in isolating 20 strains showing a *nodD* amplicon from 5 of the 8 wheat varieties in rotation with pea, but not from the varieties in rotation with soybean (Table S1). The nodule trapping strategy, from which we obtained 18 of the 20 strains, was more efficient than the bacterial plating strategy. Phylogenetic analysis based on a portion of the chromosomal *gyrB* gene demonstrated that these 20 strains clustered in 2 closely-related clades of the *R. leguminosarum* species complex (Young et al., 2021; Fig. 1a). The first clade includes the *Rlv* strains TOM, FRF1D12, Vaf10 and CCBAU83268 belonging to genospecies gsN (Boivin et al., 2020), and the second clade includes the *Rlv* strain SL16 belonging to the genospecies gsR (Boivin et al., 2020). Three strains (FWPou15, FWPou32 and FWPou38) randomly selected from the most predominant clade, were further characterized. Sequencing a portion of the *nodD* coding region confirmed that they belong to *R. leguminosarum* symbiovar *viciae* (Fig. S3). Phylogeny of the *nodD* gene

suggested that the symbiotic plasmid of these strains is closely related to that of the reference *Rlv* strain 3841 and the control strain A34 (Oono & Denison, 2010). The 3 strains (FWPou15, FWPou32 and FWPou38) were found to nodulate pea, faba bean and common vetch, but not clover (Fig. 1b-1e, Fig. S4-S5), confirming that they belong to symbiovar *viciae*.

Wheat-associated *Rlv* do not display specific genomic or metabolic signatures

Whole genome sequencing was performed on FWPou15, FWPou32 and FWPou38 (Table S4) and genomes of these strains were compared to genomes of *Rlv* isolated from legumes (Boivin et al., 2020). Sequences of the 3 strains shared a ca. 100% Average Nucleotide Identity (ANI). Only one SNP was detected between the strains FWPou15 and FWPou32 or FWPou38. Genomic phylogeny confirmed that the 3 wheat strains clustered with the representatives of genospecies gsN (Fig. 2). Phylogeny on 11 concatenated *nod* genes located on the symbiotic plasmid, showed that the 3 wheat strains cluster with the Nod group B1 that includes the 3841 and FRF1D12 strains isolated from faba bean and pea respectively (Fig. 3; Boivin et al., 2020). The genomes of strains FWPou15, FWPou32 and FWPou38 encode 301 orthologous genes, which are not shared with other *Rlv* representatives. A majority of these genes (200/301) were not assigned to a specific functional category of Clusters of Orthologous Groups (COG). We could not identify functions with an obvious link to symbiotic or endophytic growth among the remaining genes. We further inferred and compared the metabolic potential of the 3 wheat strains and 22 strains isolated from legume nodules, representing the *Rlv* genetic diversity (Table S3, Boivin et al., 2021). We found that all the strains shared most of the metabolic reactions (1,967 reactions, >87%; Fig. S6a) and only 13% of the reactions were specific to a subset of strains. Correspondence Analysis (CA) constructed on the presence/absence of the metabolic reactions, showed a hierarchical clustering similar to that observed for the ANI (Fig. S6b; Fig. 2), in which the wheat isolates grouped with the TOM strain. In conclusion, no

specific genomic and metabolic signatures were detected in the wheat-isolated *Rlv* when compared with *Rlv* isolated from legume nodules.

***Rlv* strains are wheat root endophytes**

We quantified the endophytic and epiphytic wheat root colonization abilities of two strains, FWPou15 and FWPou38, by measuring colony-forming units (cfu) in wheat roots following root surface-sterilization (S) or without root surface-sterilization (NS). Colonization was assessed 4, 7, 14 and 21 days post inoculation (dpi) with single strain inoculations on two *T. aestivum* varieties, Energo and Numeric, in gnotobiotic conditions. The two strains were detected in both NS and S conditions (Fig. S7a), suggesting that they can both colonize roots externally and internally. The data also showed that maximal colonization was reached a few days after inoculation. We thus performed further analyses of epiphytic and endophytic colonization only at 7 dpi. Root endophytic bacteria are also known to spread in plants and colonize leaves. We tested whether FWPou15 and FWPou38 can be detected in wheat leaves. Interestingly, we detected both strains in leaves at 21 dpi (Fig. S7b).

***Rlv* strains colonize wheat roots with different degrees of success in co-inoculation assays**

We then tested whether there are differences among the *Rlv* complex species for wheat root colonization ability. For this, we compared the colonization success of wheat isolates with that of strains isolated from legume nodules in a co-inoculation experiment. We inoculated in gnotobiotic conditions, seedlings of the *T. aestivum* Energo variety with 3 mixtures containing an equal concentration of 23 strains, each mixture consisting of the 22 strains representing the genetic diversity of *Rlv* (Table S3) and either FWPou15 or FWPou38. In each replicate, plantlets were also inoculated with FWPou15 alone, as a positive control to verify the efficacy and the specificity in detecting the *Rlv* strains. We quantified the relative abundance of each strain, 7 days post inoculation (dpi), by the Illumina MiSeq sequencing of a *nodD* gene fragment

(Method S6, Boivin et al., 2021) using DNA extracted after root surface-sterilization (treatment S) or not (NS). In the control plants inoculated with FWPou15 alone, an average of 95% of the total reads corresponded to this bacterium (Table S6), confirming its ability to colonize wheat roots, and suggesting little contamination in the experiment. In the co-inoculated plants, thirteen strains were either not detected (no reads, CZP3H6, GD25 and CCBAU03058, Table S6) or had a relative abundance lower than 1% (BLR195, CCBAU10279, FRP3E11, GB51, GLR2, GLR17, P1NP2K, SL16, TOM, UPM1134; Fig. 4a-b, Table S6), suggesting there are either not able to colonize wheat roots or poor competitors. Alternatively, limitation in their DNA extraction and/or amplification might explain they have not been detected. The other strains, including ten strains isolated from legumes as well as the wheat strains, were detected both in NS and S roots, revealing that epiphytic and endophytic wheat root colonization abilities are widely distributed among the *Rlv* diversity. However, strong differences in the relative abundance of these strains were observed. A generalized mixed-linear model (*glmm*) on the relative abundances of the strains in the 3 mixtures showed a significant effect of the ‘*strain*’ factor, but not of the ‘*sterilization*’ factor, on the colonization success (Table 1). The IAUb11 strain isolated from pea nodules was significantly more abundant than all the other strains in both root compartments (Table S14), with a mean relative abundance of 63% and 45% in NS and S roots, respectively (Fig. 4a-b, Table S6). The wheat strain FWPou38 showed intermediate colonization ability, with a mean relative abundance of 4% in NS roots and 22 % in S roots (Fig. 4a-b, Table S6). Significant differences in their abundance compared to other strains (except IAUb11) were only found in the S roots (Table S14). Interestingly, the strain TOM, belonging to the same *gsN* genospecies as the wheat strains, had very low ability to colonize wheat roots in this assay (read abundance <1%), suggesting that the genospecies does not predict wheat colonization ability.

To confirm the differences between the *Rlv* strains in their abilities to colonize wheat roots when co-inoculated, we constructed an mCherry-tagged version IAUb11 and a YFP-tagged version of FWPou38. Although fluorescence of both strains was detected as free-living bacteria grown on a culture medium or when inoculated separately on Energo roots, almost no FWPou38 was detected on the root surface of Energo when co-inoculated with IAUb11 (Fig. 4c), suggesting that IAUb11 strongly competes with FWPou38.

The colonization success of *Rlv* strains in the mixtures was generally equivalent in S and NS roots, suggesting that epiphytic colonization success is a major determinant of the endophytic colonization success. However, the relative abundance of the strain FWPou38 was higher in S roots than in NS roots, suggesting a stronger endophytic ability of this strain compared to most of the *Rlv* strains isolated from legumes.

***Rlv* strains can colonize wheat roots similarly in single inoculation assays**

We then compared the endophytic and epiphytic wheat root colonization abilities of IAUb11, FWPou38 and FWPou15 in mono inoculation assays using the two wheat varieties, Energo and Numeric. We also included in the analysis the commonly used *Rlv* strain A34 as a control. Colonization was assessed by measuring cfu values of strains isolated from wheat roots 7 dpi following surface-sterilization (treatment S) or not (NS) of root systems. A linear-mixed model (*lmm*) on the cfu /mg of root fresh weight showed a significant effect of the ‘sterilization’, ‘variety’ and ‘strains’ factors as well as the nested ‘sterilization × strain’ and ‘variety × strain’ factors (Table 2). All strains colonized the root surface of the 2 wheat varieties (10^6 to 10^7 cfu/mg, Fig. 5a) and no significant difference was observed between strains and between the wheat varieties in the NS roots (Table S15). By contrast, bacteria differed in their endophytic colonization abilities in the Energo variety. FWPou38 and IAUb11 were found at about 10^4

cfu/mg, while A34 and FWPou15 at about 10^3 cfu/mg, (Fig. 5a, Table S15). No difference in the level of epiphytic and the endophytic colonization were found in Numeric.

To confirm the individual epiphytic and the endophytic abilities of these *Rlv* strains, we constructed fluorescent-tagged versions and observed *in vitro* root colonization of the two wheat varieties by confocal microscopy. We found that all strains massively colonized the wheat root surface (Fig. 5b). A few bacteria were also observed inside the roots, at least around the outer cortical cells, confirming their root endophytic colonization ability.

***Rlv* can stimulate wheat root development**

We investigated the effects of the *Rlv* strains on root architecture. We mono-inoculated A34, FWPou15, FWPou38 or IAUb11 on both Energo and Numeric varieties in gnotobiotic conditions, measured the number of lateral roots and total root lengths at 12 dpi. *Imm* on both variables showed significant effects of the ‘*strain*’ and ‘*variety*’ factors as well as a significant nested ‘*strain* × *variety*’ effect (Table 3). Although for each wheat variety the total root lengths and lateral root numbers were correlated ($R^2 \sim 0.7$, Fig. S8), these traits responded differently to inoculation by the *Rlv* strains (Fig. 6). Inoculation by A34 did not affect root architecture in comparison to non-inoculated plants, in either wheat variety, showing that the impact of *Rlv* on root architecture is not a general trait of all *Rlv*. Concerning lateral root numbers, FWPou38 and IAUb11 induced a specific increase in Energo (on average + 35% each; Fig. 6d, Table S16), while FWPou15 induced a specific increase in Numeric (on average +52%; Fig. 6b, Table S16). Concerning total root lengths, FWPou15 induced a modest but general increase (on average +12 % in Energo and +29% in Numeric; Fig. 6a, Table S16), while FWPou38 induced a weak specific decrease in Numeric (on average -18 %; Fig. 6c, Table S16).

***Rlv* can enhance early colonization of wheat roots by arbuscular mycorrhizal fungi**

We then investigated whether *Rlv* strains have effects on wheat root colonization by AMF. For this, we co-inoculated Energo and Numeric roots with the *Rhizophagus irregularis* isolate DAOM197198 and individual *Rlv* strains (FWPou15, FWPou38, IAUb11 or A34). We measured the number of AMF colonization sites 24 dpi and compared it to wheat plants inoculated only with *R. irregularis*.

lmm showed a significant effect of the ‘*strain*’ factor only on the Energo variety (Table 4). More specifically, when compared to the control, inoculation with the FWPou38 and IAUb11 strains resulted in significantly more fungal colonization sites (on average +55% and +31% respectively; Fig. 7b; Table S17). On the Numeric variety, inoculation with any of the *Rlv* strains did not result in a significant change in the number of fungal colonization sites (Fig. 7c and d; Table S17), reinforcing the influence of the wheat genotype on the response induced by *Rlv*. Taken together, these results suggest that the *Rlv* strains stimulating early AMF colonization in wheat roots are those able to stimulate lateral root formation and/or emergence.

Discussion

Wheat can participate in shaping *Rlv* populations in agronomical soils

Rhizobium leguminosarum sv. *viciae* strains are known to nodulate legume hosts such as pea and faba bean. Here we show that wheat can be an alternative natural host for *Rlv*. This confirmed a previous report describing *Rlv* strains in soils under wheat monoculture (Depret et al., 2004). In our study, we isolated *Rlv* strains from roots of wheat plants cultivated in rotation with pea plants (*Rlv* host), but did not succeed in isolating *Rlv* from wheat plants in rotation with soybean (non-*Rlv* host). This suggests an enrichment of *Rlv* in wheat roots through rotation with a legume host. Although *Rlv* bacteria belong to at least 9 genospecies (Boivin et al, 2021), only members of the genospecies gsN and gsR were identified among the 20 wheat isolated strains. The limited diversity of *Rlv* found in our study might be explained through three non-exclusive hypotheses. Firstly, the sampling we performed may not be saturated. Secondly, we cannot exclude that soils on which wheat have been grown were deprived of other *Rlv* genospecies. However, this is not supported by previous results showing a high *Rlv* diversity in European soils including in the southwest of France (Boivin et al., 2020). Thirdly, the low *Rlv* diversity may be related to a specific partner choice in wheat-*Rlv* endophytic interactions. Supporting this hypothesis, we showed through co-inoculation assays, a strong competition between *Rlv* strains for wheat root colonization. Interestingly, the strain IAUb11, which was the most efficient competitor, belongs to another genospecies (gsE), while other members of the genospecies gsN and R were not efficient competitors suggesting that wheat partner choice is not related to a particular *Rlv* genospecies. Host-specific partner choice also exists in the Fabeae-*Rlv* interaction (Boivin et al., 2021). Interestingly, the pattern of colonization success we found for the *Rlv* strains in wheat roots was different from that observed with the same 22 *Rlv* for the colonization of various legume nodules (Fig. 8; Boivin et al., 2021). This suggests

that *Rlv* strains might carry different competitiveness abilities to colonize legume nodules, wheat roots or other putative hosts. By consequence, multiplicity of plant hosts in fields might participate in maintaining *Rlv* diversity in soils. Field campaigns on a broader geographical scale to investigate the diversity of *Rlv* in wheat roots, legume nodules and in the surrounding soils, should help to validate these hypotheses. Moreover, comparison of the *Rlv* diversity inside roots and in the rhizosphere from field samples would allow to determine whether the low diversity we found is restricted to the endophytic *Rlv* population or whether it is characteristic of the interaction with wheat roots.

Different mechanisms could influence *Rlv* competitiveness for wheat root colonization

The colonization success of a given strain in a natural environment is not only associated with its ability to colonize hosts but also to compete with other microorganisms associated with the same host. Here, we characterized the abilities of 4 strains for epiphytic and endophytic colonization of wheat roots. We did not find any difference for epiphytic growth between the 4 strains tested individually. This suggests that the difference in epiphytic colonization success between *Rlv* strains observed in the co-inoculation assays are likely due to differences in competitiveness rather than in abilities to colonize wheat roots. Competition for nutrient resources is the most common mechanism underlying variations in bacterial root colonization, since strains/species carrying resource-specific pathways are often those able to rapidly grow and dominate niches (Simons et al., 1996; Pieterse et al., 2014; Yang et al., 2019). Other hypotheses to explain different bacterial abilities to compete for root colonization include antagonism *via* the production of secondary metabolites (Weller et al., 2002). However, the minor differences found in this study among the *Rlv* strains in term of metabolic pathways (Fig. S6) did not correlate with the differences observed for wheat root colonization in the co-inoculation assays. Comparative genomics revealed that a cluster of 4 genes annotated as

putative sulfide/taurine transporters was only found in the genomes of the strains FWPou15, FWPou32, FWPou38 and IAUb11 and not in the 21 other strains (gene identifiers in strain IAUb11: RLVI AUB1145.7 - RLVI AUB1145.10). Whether such transporters may be involved in the competitiveness for wheat root colonization of the FWPou38 and IAUb11 strains by facilitating export of sulfate or sulfite (Weinitschke et al., 2007) remains to be investigated.

In the co-inoculation assays performed in this study, the competitiveness pattern for epiphytic root colonization was similar to the pattern found for root endophytic colonization. This indicates that *Rlv* multiplication on the root surface is an important factor contributing to the endophytic success of *Rlv* strains. Nevertheless, mechanisms underlying competitiveness of the *Rlv* strains in colonizing the inner parts of wheat roots might be different from those regulating root epiphytic growth. First, we found differences for endophytic colonization between the strains we tested individually. Secondly, the endophytic/epiphytic colonization ratio was higher for the wheat strain FWPou38 than for the pea strain IAUb11. The ability of strains to escape recognition by hosts and to avoid plant defenses might also account for the ability to multiply inside the roots (Pieterse et al., 2014). A combination of mechanisms might thus modulate the ability of *Rlv* strains to endophytically colonize wheat roots and explain adaptation of some *Rlv* strains to wheat.

Surprisingly, while almost clonal, FWPou15 and FWPou38 strains showed contrasted abilities to colonize and induce responses in wheat roots. Based on short read Illumina genome sequencing, we found only one SNP between the FWPou15 and FWPou38 strains, which is a non-synonymous mutation in a gene encoding a DNA polymerase IV involved in DNA repair. In bacteria in response to stress, DNA polymerase can synthesize error-containing DNA leading to the formation of genetic variants and different phenotypes of the progeny (Foster, 2005). This process, called phenotypic plasticity, also leads to bacterial cells showing different

responses when inoculated in a plant host (i.e. loss of pathogenicity or decrease of epiphytic growth; Bartoli et al., 2015). Further experiments are required to determine whether this mutation, additional SNPs, genome re-arrangements (that could have been missed by Illumina sequencing) or epigenetic modifications are responsible for these phenotypic differences. Whatever the mechanisms behind these phenotypic differences, these variants represent an interesting material to decipher the molecular mechanisms controlling the ability of *Rlv* strains to colonize wheat roots.

Common molecular mechanisms might be involved in the stimulation of wheat root development and AMF colonization

Several bacteria displaying PGP activity, including rhizobia, can enhance wheat interaction with AMF (Germida & Walley, 1996; Russo et al., 2005; Pivato et al., 2009). Soil microorganisms can modulate the auxin-dependent root developmental program leading to lateral root formation in both monocots and dicots (Contreras-Cornejo et al., 2009; Pieterse et al., 2014). Indeed, many PGP bacteria including rhizobia, produce auxins (Camerini et al., 2008; Spaepen & Vanderleyden, 2011; Boivin et al., 2016). The effect of *Rlv* on wheat root development could be attributed to auxin produced by *Rlv* strains. In support of this hypothesis, key enzymes of 3 of the known bacterial auxin synthesis pathways using tryptophan as a substrate, the indole acetamide, the indole pyruvate and the tryptamine pathways (Spaepen & Vanderleyden, 2011), are encoded in the FWPou15, FWPou38 and IAUb11 genomes (Fig. S9). Alternatively, rhizobial LCOs can also affect root development both in legumes and non-legumes through the regulation of plant auxin homeostasis (Herrbach et al., 2017; Buendia et al., 2019a). Stimulation of AMF colonization by *Rlv* can be indirectly favored by the increase in the number of lateral roots, preferential sites for AMF colonization (Gutjahr et al., 2013). Both LCOs and auxin stimulate AMF colonization in legumes and non-legumes plants (Maillet

et al., 2011; Etemadi et al., 2014; Buendia et al., 2019b) and may also directly affect AM. Effects of exogenous application of either auxin or LCO on root development are dependent on their concentrations (Herrbach et al., 2017; Buendia et al., 2019a). Different levels of auxin and/or LCO production could, at least partially, explain the differences between *Rlv* strains on root development and AMF colonization.

Levels of endophytic colonization rather than epiphytic colonization might be critical for *Rlv* ability to induce plant responses

Both co-inoculation and single strain inoculation assays showed that FWPou38 and IAUb11 are efficient wheat endophytic colonizers with $\sim 10^4$ cfu/mg of root tissues on both Energo and Numeric varieties. The bacterial population sizes found in wheat roots for these strains are similar to those previously reported for PGP rhizobia colonizing rice roots (Chaintreuil et al., 2000; Mitra et al., 2016) or other species of PGP bacteria colonizing wheat roots (Dong et al., 2003; Chen et al., 2017; Bernabeu et al., 2018). Both strains can increase the number of lateral roots and stimulate AMF colonization in Energo. This was not observed on the Numeric variety, suggesting that bacterial effects are plant genotype dependent. The effect of wheat genotype on the colonization by PGP rhizobacteria has been described, for example for bacteria of the genus *Pseudomonas* (Valente et al., 2020). Here, we show that beside the colonization level, host responses also vary depending on the plant genotype. Interestingly, FWPou15, for which we found an endophytic colonization of $\sim 10^4$ cfu/mg in Numeric roots, had a stimulation activity on root architecture in this variety, while not on Energo for which we found an endophytic colonization of $\sim 10^3$ cfu/mg. Similarly, the poorest endophytic colonizer in both Energo and Numeric, A34, did not induce any root architecture changes or AMF colonization responses. In this regard, we can hypothesize that *Rlv* strains with the best endophytic colonization ability were able to reach an endophytic population size

sufficient for production of auxins, LCOs or other PGP molecules at concentrations required for stimulating lateral root development and AMF colonization. Further analysis with a larger number of strains should establish whether there is a correlation between endophytic colonization ability and stimulation of plant responses and its genotype-genotype dependency.

In conclusion, our study suggests that *Rlv* competitiveness and level of endophytic colonization are critical for potential PGP activities. These novel concepts will help in understanding and/or in designing microbial consortia based on beneficial bacteria for improving wheat yield/quality in a context of reducing the use of chemical inputs.

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Data accessibility statement

Amplicon sequences are available in Table S2 (Supporting Information). Genomes of *Rlv* are available in GenBank under the accession numbers JACBGR000000000, JACBGQ000000000 and JACBGP000000000. All raw data necessary to confirm our results are available in Supplementary Tables. Scripts and pipelines for data analysis and R figures are available in Supplementary Methods. *Rlv* bacterial collection is available at the LIPME laboratory (INRAE, Toulouse) under request to the corresponding author.

Table 1. Analysis of Deviance on the variation of the strain relative abundance inferred from the total number of reads obtained by Illumina MiSeq sequencing of a *nodD* fragment in the co-inoculation assays. Chisq: value of the type II Wald chi squared, Df: degree of freedoms. Significant results obtained after FDR correction are reported in bold.

	FWPou15 mix			FWPou38 mix		
Factors	Chisq	Df	P value	Chisq	Df	P value
Sterilization	0.08	1	0.777	0.001	1	0.973
Strain	545.25	19	2e-16	605.93	19	2e-16
Sterilization : Strain	27.05	19	0.103	24.83	19	0.166

Table 2. Analysis of Deviance on the variation of bacterial colony-forming units (cfu) / mg of wheat roots obtained after inoculation with the WT A34, FWPou15, FWPou38 or IAUb11 *R/v* strains. Chisq: value of the type II Wald chi squared, Df: degree of freedoms. Significant results obtained after FDR correction are reported in bold.

Energo and Numeric			
Factors	Chisq	Df	P value
Sterilization	1121.64	1	2e-16
Variety	0.005	1	0.003
Strain	53.92	3	0.003
Sterilization : Variety	4.23	1	0.317
Sterilization : Strain	32.78	3	0.035
Variety : Strain	9.86	3	1e-04

Table 3. Analysis of Deviance on the variation of the total root length and number of lateral roots obtained after inoculation with the WT A34, FWPou15, FWPou38 or IAUb11 *R/v* strains. Analysis was performed on two datasets: A34-FWPou15 and IAUb11-FWPou38, as values for controls (plantlets inoculated with water) were different in the two datasets. Chisq: value of the type II Wald chi squared, Df: degree of freedoms. Significant results obtained after FDR correction are reported in bold.

Energ & Numeric							Energ & Numeric						
Total root length			N° lateral roots				Total root length			N° lateral roots			
A34 & FWPou15							IAUb11 & FWPou38						
Factors	Chisq	Df	P value	Chisq	Df	P value	Factors	Chisq	Df	P value	Chisq	Df	P value
Strain	27.89	2	9e-07	24.12	2	6e-06	Strain	1.61	2	0.446	11.87	2	0.003
Variety	23.39	1	1e-06	230.02	1	2e-16	Variety	47.05	1	7e-12	128.79	1	2e-16
Strain : Variety	7.21	2	0.027	21.13	2	3e-05	Strain : Variety	10.22	2	0.006	7.60	2	0.022

Table 4. Analysis of Deviance on the variation of the number of AMF colonization sites after inoculation with the WT A34, FWPou15, FWPou38 or IAUb11 *Rlv* strains. Chisq: value of the type II Wald chi squared; Df: degrees of freedom. Strain refers to wheat roots inoculated with the four *Rlv* strains or sterilized water (control). Significant result is reported in bold corresponding to P-value after FDR correction.

Factor	Ergo			Numeric		
	Chisq	Df	<i>P</i> value	Chisq	Df	<i>P</i> value
Strain	60.73	4	2e-12	3.57	2	0.167

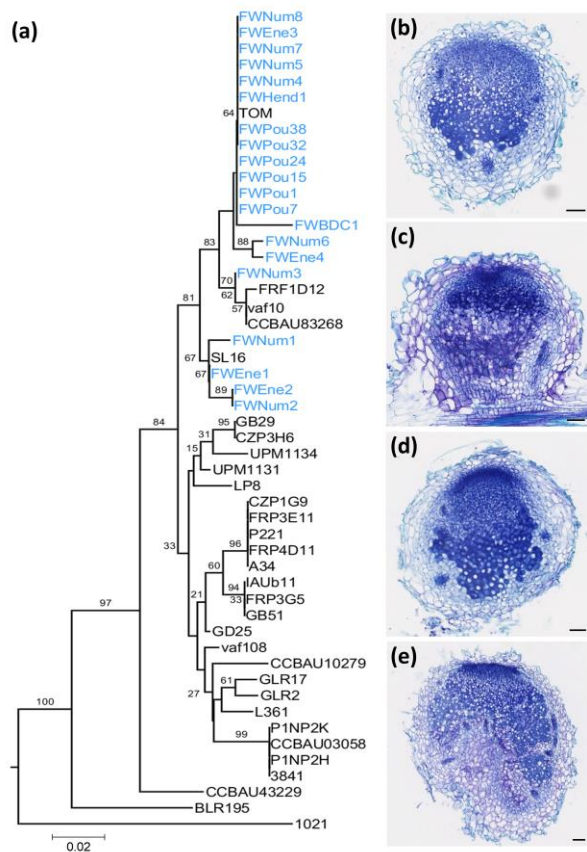


Figure 1. Functional *Rhizobium leguminosarum* sv. *viciae* (*Rlv*) are associated with wheat roots.

(a) Neighbor-Joining tree based on a portion of the chromosomal *gyrB* gene from the 20 wheat-isolated strains (indicated in blue), a set of *Rlv* strains representative of the diversity of the species complex (Boivin et al., 2021), and the strain A34. *Ensifer meliloti* was used as outgroup. The *Rlv* strains isolated from wheat roots cluster with strains from genospecies gsN (TOM, FRF1D12, Vaf10, CCBAU83268) or gsR (SL16). (b-g) Sections of pea nodules colonized by the WT strains, FWPou15 (b), FWPou32 (c), FWPou38 (d) or A34 (e). Scale bars correspond to 100 μm.

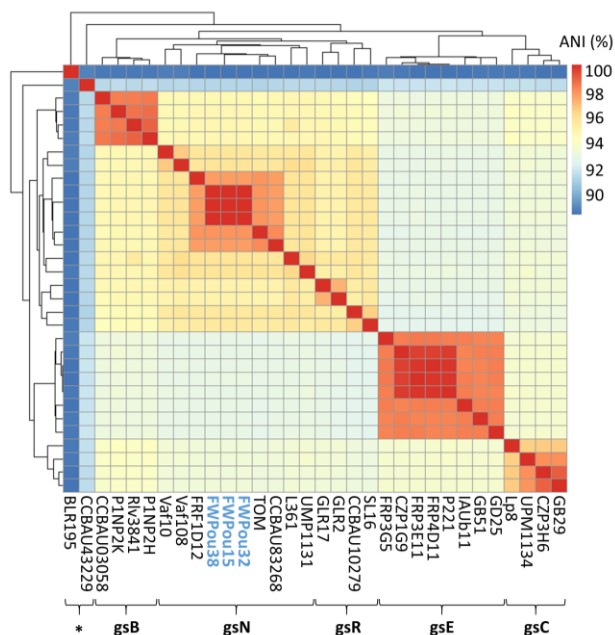


Figure 2. Selected *Rlv* strains for functional analyses belong to genospecies gsN. Hierarchical clustering and heat map based on the Average Nucleotide Identity (ANI) values between each couple of the 3 wheat strains, FWPou15, FWPou32 and FWPou38 (indicated in blue), and a set of *Rlv* strains representative of the diversity of the species complex (Boivin et al., 2021). Genospecies (gs) have been defined using an ANI threshold of 95%. Star indicates phylogenetically distant isolates that do not cluster with any of the genospecies.

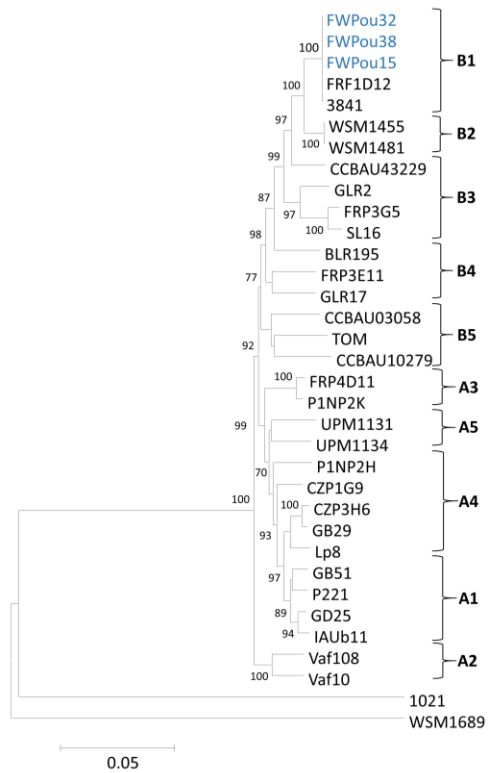


Figure 3. Selected *Rlv* strains for functional analyses belong to the Nod group B1.

Neighbor-Joining tree based on concatenated *nodABCDEFIJLMN* gene sequences of the 3 wheat strains, FWPou15, FWPou32 and FWPou38 (indicated in blue), and *Rlv* strains representative of the Nod groups (Boivin et al., 2021). WSM1689 (*R. leguminosarum* symbiovar *trifolii*) and *E. meliloti* 1021 were used as outgroups.

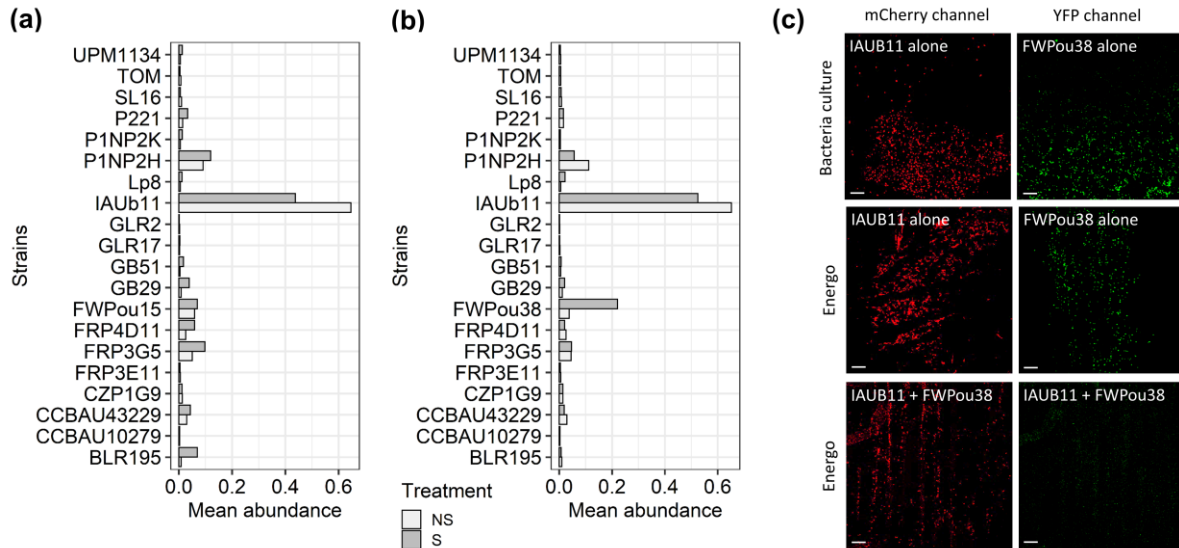


Figure 4. *Rlv* strains colonize wheat roots with varying degrees of success in co-inoculation assays.

(a-c) Least-Squares-Means (lsmeans) of the *Rlv* strain abundance in wheat roots when co-inoculated in an *in vitro* assay. Co-inoculation of 22 *Rlv* strains representing the diversity of *Rlv* (Table S3) together with the WT wheat strains FWPou15 (a) or FWPou38 (b). Each mixture was inoculated in a total of 18 plant root systems of the Energo variety, in 3 independent replicates. Total DNA was extracted from pools of 3 roots systems, with or without surface-sterilization, at 7 dpi, and a *nodD* amplicon was sequenced by MiSeq Illumina. Mean abundance was inferred by normalizing the number of reads corresponding to each strain with the total number of reads obtained in the run. Dark grey bars indicate results obtained from surface-sterilized (S) roots and light grey bars indicate results obtained from non-sterilized (NS) roots. The S and NS treatments allow estimating respectively the relative endophytic and epiphytic abundance of each inoculated strain since endophytic bacteria are in negligible amounts compared to epiphytic bacteria. Three *Rlv* strains GD25, CCBAU03058 and CZP3H6 were not detected by MiSeq sequencing, thus, they are not reported in the barplots. (c) Confocal microscope images of IAUB11 and FWPou38 respectively tagged with mcherry and YFP fluorescent proteins, either grown on culture medium or 7 days post mono-inoculation or co-inoculation on the Energo variety. Scale bars corresponds to 20 μm .

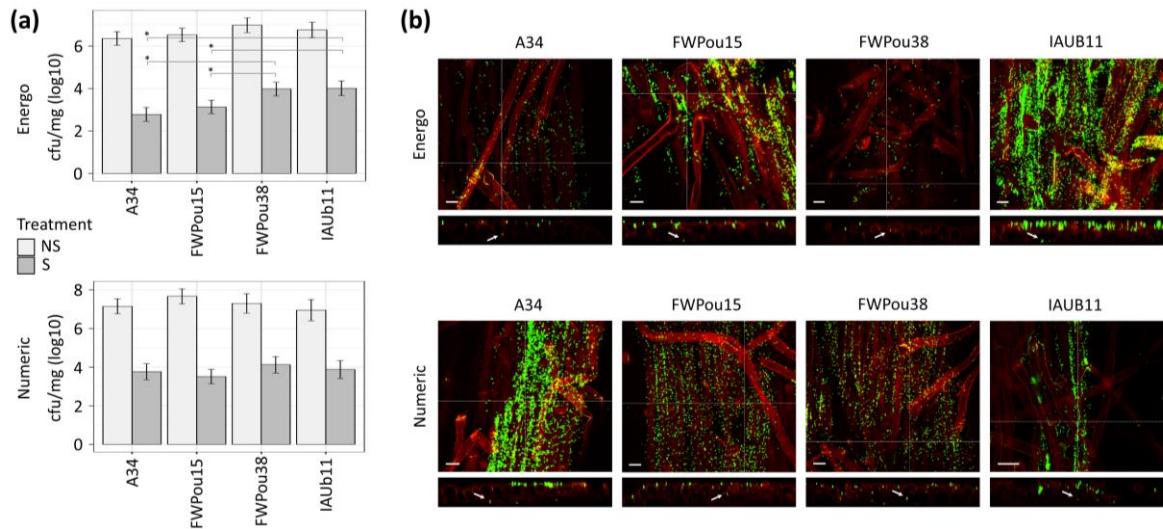


Figure 5. *Rlv* strains can colonize wheat roots in single inoculation assays.

(a) Barplots representing the endophytic (surface-sterilized: S; dark grey bars) and epiphytic (non-sterilized: NS; light grey bars) bacterial abundance in roots. The wheat varieties Energo or Numeric were inoculated with the WT strains reported in the *x*-axis. Least-Squares-Means (lsmeans) and standard deviation of bacterial abundance 7 dpi is expressed as log₁₀ of cfu (colony-forming units)/mg of root fresh weight. Total number of plants analyzed (≥ 6 for NS treatment and 12 for S treatment, in at least 2 independent replicates) are indicated in Table S7. Significant differences were found in the surface-sterilized roots. *P* values (corrected for FDR) ≤ 0.05 are indicated by * (See Table S15). (b) Confocal microscope images of roots of the wheat varieties Energo or Numeric 7 dpi with A34, FWPou15, FWPou38 or IAUB11 strains tagged with fluorescent proteins. For each condition, the upper panel shows a root surface focal plan and the lower panel a transversal section reconstruction. Green corresponds to the bacteria fluorescence and red to cell wall auto-fluorescence. Arrows points endophytic bacteria. Scale bars corresponds to 20 μ m.

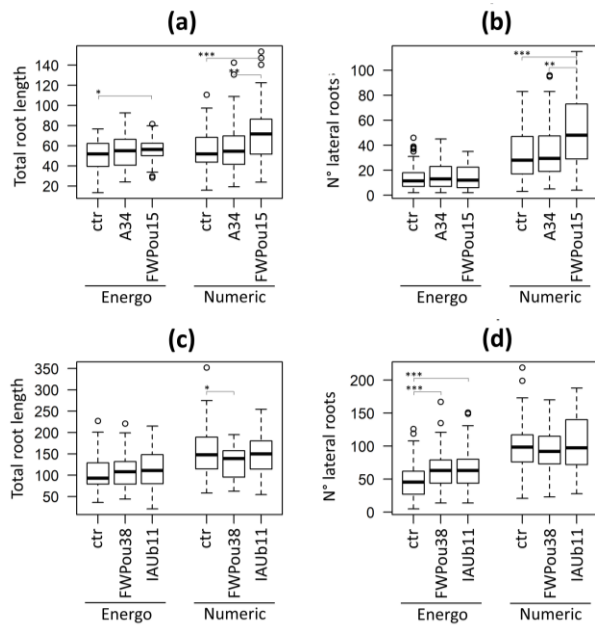


Figure 6. *Rlv* can stimulate wheat root development in a genotype-dependent manner.

Boxplots representing the variation in wheat total root length (a, c; cm) and lateral root number (b, d) 12 dpi in 2 wheat varieties (Energo and Numeric) inoculated with the WT strains, A34 or FWPou15 (a, b), FWPou38 or IAUb11 (c, d). Controls (ctr) are non-inoculated plants. Total numbers of plants analyzed (≥ 30 , in at least 2 independent replicates) are indicated in Table S8. P values (corrected for FDR) ≤ 0.05 are indicated by *, ≤ 0.005 by ** and ≤ 0.0005 by *** (See Table S16).

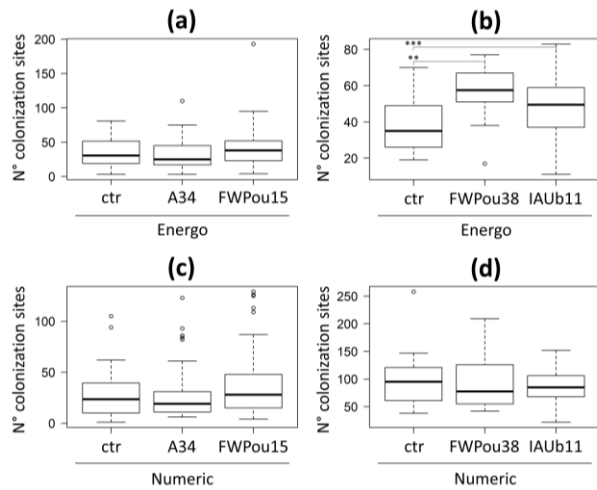


Figure 7. *Rlv* can stimulate wheat root colonization by AMF in a genotype-dependent manner.

(a-c) Boxplots representing the variability in the number of colonization sites by the AMF *R. irregularis* isolate DAOM197198, 24 dpi, when inoculated alone (ctr) or in combination with *Rlv* strains. Two wheat varieties, Energo (a, b) and Numeric (c, d) were co-inoculated with the WT strains, A34 or FWPou15 (a, c), FWPou38 or IAUb11 (b, d). Total numbers of plants analyzed (≥ 31 , in at least 2 independent replicates) are indicated in Table S9. *P* values (corrected for FDR) ≤ 0.005 are indicated by ** and ≤ 0.0005 by *** (See Table S17). Significant differences, not represented on the boxplots, were also found in the Energo variety between A34 vs FWPou38 ($P < .0001$) or IAUb11 ($P = 0.0007$), and FWPou15 vs FWPou38 ($P = 0.0062$).

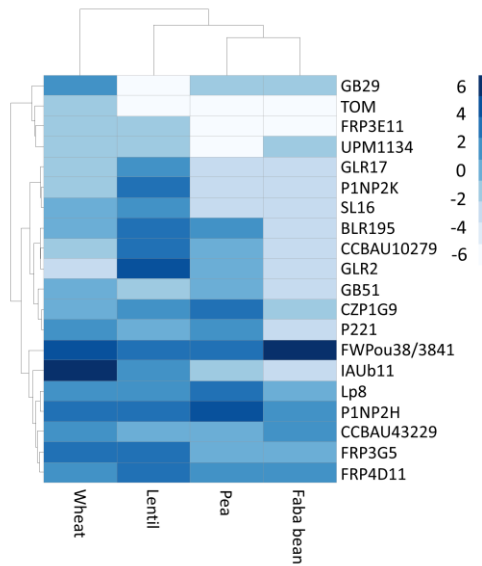


Figure 8. Success of wheat and legume plant colonization in the *Rlv* co-inoculation assays. Hierarchical clustering and heatmap based on the relative abundance values (\log_2) of each *Rlv* found in the wheat roots and legume nodules during co-inoculation assays (Table S6 and Boivin et al., 2021). FWPou38 or 3841 strains were used in the co-inoculation assays in wheat or legumes respectively as they are undistinguishable by Illumina *nodD* sequencing. Wheat cv. ‘Energó’, Lentil cv. ‘Rosana’, Pea cv. ‘Kayanne’, and Fababean cv. ‘Diva’.

Supporting information

