

# Changes in Root Bacterial Communities Associated to Two Different Development Stages of Canola (*Brassica napus* L. var *oleifera*) Evaluated through Next-Generation Sequencing Technology

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**Abstract** Crop production may benefit from plant growth-promoting bacteria. The knowledge on bacterial communities is indispensable in agricultural systems that intend to apply beneficial bacteria to improve plant health and production of crops such as canola. In this work, the diversity of root bacterial communities associated to two different developmental phases of canola (*Brassica napus* L.) plants was assessed through the application of new generation sequencing technology. Total bacterial DNA was extracted from root samples from two different growth states of canola

(rosette and flowering). It could be shown how bacterial communities inside the roots changed with the growing stage of the canola plants. There were differences in the abundance of the genera, family, and even the phyla identified for each sample. While in both root samples Proteobacteria was the most common phylum, at the rosette stage, the most common bacteria belonged to the family Pseudomonadaceae and the genus *Pseudomonas*, and in the flowering stage, the Xanthomonadaceae family and the genus *Xanthomonas* dominated the community. This implies in a switch in the predominant bacteria in the different developmental stages of the plant, suggesting that the plant itself interferes with the associated microbial community.

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## Introduction

Crop production is closely related to the soil and rhizosphere bacteria, which interact with plant roots. These interactions can be beneficial, detrimental, or neutral for the plant, and sometimes the effect of a particular bacterium may vary as a consequence of soil conditions [1]. The knowledge on bacterial community is, therefore, indispensable in agricultural systems that intend to make use of beneficial bacteria to improve crop production. It is well known for a long time that soils represent one of the most complex and difficult environments to study and at the same time are of particular interest as they are considered to harbor the most diverse populations of bacteria of any environment on earth [2]. There is evidence that there might be several thousands of microbial species in one soil sample

and most (99.5–99.9 %) of the soil bacteria observed by microscopy cannot be isolated and cultured in laboratory media. Isolated bacteria may therefore account for only a minor proportion of the total bacterial diversity in soil [3]. Similar difficulty is faced regarding cultivable bacteria present in the rhizosphere or inside plant tissues, which are thought to represent only a small proportion (0.1–10 %) of the total bacteria in these environments [4].

The application of molecular genetic detection and identification methods greatly aids in clarifying the phylogenetic relationships of the microbial community of rhizosphere-associated bacteria. It is generally accepted that only a combination of methods, including classical cultivation techniques and cultivation-independent techniques, allow a comprehensive insight into the bacterial community in environmental habitats [5, 6]. The ribosomal RNA genes of bacteria, especially those for 16S- and 23S-rRNA, are excellent molecular markers for phylogenetic studies due to their functional constancy, their ubiquitous distribution, and elements rising from highly conserved to highly variable regions within the sequence [7–9]. This molecular phylogenetic approach can be used to identify pure isolates and to assess the diversity of complex communities.

Although many of the recent publications have shown the use of next-generation sequencing technologies, using 16S rRNA amplified regions, as a way to get insights into the diversity of certain bacterial communities such as deep sea [10] and human gut [11, 12], not much work has been done on microbial communities in soil or on root-associated bacterial communities. For studying bacterial communities, pyrosequencing of amplified V9 regions of the 16S rRNA genes present in soil DNA or of regions V3 and V6 of the 16S rRNA genes has been successfully demonstrated [12]. However, in both cases, the obtained sequences showed average sizes of 100–200 base pairs (bp), sometimes even shorter (50 bp). With the increased capability of the new generation of sequencing machines to sequence up to 400 bp or more, it is now possible to span most hypervariable regions, multiple adjacent hypervariable regions, or possibly combinations of nonadjacent hypervariable regions through paired-end sequencing strategies [12]. As a consequence, the reads with purpose of molecular ecology studies can be more useful and accurate than the ones generated by classical capillary sequencing [13].

Canola (*B. napus* L.) is a very important oil-producing plant. The oil produced by this plant, besides its benefits to human health, with a large amount of vitamins and omega 3, is also very important for biodiesel production worldwide [14]. Canola is used as a partner in crop rotation systems with other important crops, such as wheat and soybean [15]. However, studies regarding the diversity or the isolation of

the bacterial community that could interact with canola plants are still very preliminary [15–19].

The aim of this work was to estimate the diversity of microorganisms that interact with the roots of canola plants at two different stages of development. By using next-generation sequencing technology, we were able to show that the bacterial community inside the roots changed between these two growth periods, implying in a shift in the dynamics of bacterial populations.

## Methods

### Sampling and Location

Roots of canola (*B. napus* L., variety Hyola 60) were collected in an experimental field from Fundação Estadual de Pesquisa Agropecuária (Fepagro/RS), in the city of Vacaria, south of Brazil (28°30'43" S, 50°56'02" W). The first sample, roots of canola at the rosette stage, was collected in September 14, 2009; the second sample, roots of canola at the flowering stage, was collected in October 15, 2009. For each stage, five independent plants at least 2 m away from each other were taken randomly and bulked to obtain a representative composite sample. Roots were separated from the rest of the canola plants. This material was used for bulk DNA extraction.

### Total Bacterial DNA Extraction

Bacteria that were inside the roots were isolated by surface disinfection performed by washing the roots in running tap water, followed by a 70 % ethanol wash for 1 min, a sodium hypochlorite solution (4 %, v/v) wash for 2 min, and five serial rinses in sterilized distilled water. Immediately after disinfection, the roots were sliced with a sterile scalpel and macerated. A total of 10 g of root segments for each sample were placed in distinct 500-ml Erlenmeyer sterile flasks containing 90 ml of sterile saline solution (0.85 % NaCl). Samples were incubated at 4 °C under agitation (200 rpm) for 3–4 h. DNA from bacterial communities that were inside the roots of canola plants was extracted according to Soares and co-workers [20]. Briefly, bacterial cells were rinsed with TES buffer (10 mM Tris pH 8.0, 25 mM EDTA, and 150 mM NaCl) and suspended in TE buffer (10 mM Tris pH 8.0, 25 mM EDTA). Cell lyses took place in 20 mgml<sup>-1</sup> lysozyme at 37 °C and 4 % sodium dodecyl sulfate. Extractions with phenol/chloroform and precipitation in ethanol were performed. DNA quality and integrity was checked by electrophoresis on 0.8 % agarose ethidium bromide gel. DNA was quantified by spectrophotometer.

### Amplification of the Metagenomic DNA with Primers for the V6 to V8 Region of the 16S rRNA Gene

Amplification was performed by using FastStart High Fidelity PCR system (Roche®). The amount of DNA template was 50 ng per reaction. Primers U968 (AACGCGAAGA ACCTTAC) and L1401 (CGGTGTGTACAAGACCC) [21] flanking a region of about 500 base pairs between nucleotides 968 and 1401 of the *Escherichia coli* 16S rRNA gene, including variable regions V6 to V8 [22], were used. Amplification reactions (25 µl) contained 20 µM of each deoxynucleoside triphosphates, 0.3 µM of each primer U968 and L1401, 2 mM MgCl<sub>2</sub>, and 2 U High Fidelity Taq polymerase (Roche®) in 1X Taq buffer. The amplifications were performed in a PCR Express Temperature Cycling System (Thermo Hybrid) as follows: an initial denaturation step at 94 °C for 5 min followed by 30 cycles at 94 °C for 45 s, 52 °C for 45 s, 72 °C for 45 s, and one cycle at 72 °C for 10 min for final elongation. Whole PCR reaction was purified by gel extraction (Minelute, Qiagen). PCR template for the next reaction was 1 µl of the purified samples and the specific primers were **CCATCTCATCCCTGCGTGTCTCC GACTCAGACGAGTGCGTAAACGCGAAGAACCTTAC** and *CCTATCCCCTGTGTGCCTTGGCAGTCT CAGCGGTGTGTACAAGACCC* for the DNA obtained from bacteria that were inside the roots of canola at the rosette stage; and **CCATCTCATCCCTGCGTGTCTCC GACTCAGACGCTCGACAAACGCGAAGAACCTTAC** and *CCTATCCCCTGTGTGCCTTGGCAGTCT CAGCGGTGTGTACAAGACCC* for the DNA obtained from bacteria that were inside the roots of canola at the flowering stage. The primers are constituted of the Roche adaptors (30 bp, forward is in bold and backward is in italic), Roche multiplex identifiers (10 bp, underlined), and the rDNA specific sequences (17 bp). The underlined bases indicated tags that were later used for the identification of the samples. Amplifications conditions were the same as mentioned above. The PCR probes were purified twice by gel extraction (Minelute, Qiagen).

### Sequencing Procedures

DNA sequencing was performed at CeBiTec (University of Bielefeld, Germany), according to the manufacturers' protocols (Roche/454 Life Sciences, Branford, USA). An aliquot of the DNA preparation served as template DNA for sequencing on the GS FLX Titanium platform. The sequencing library was constructed according to the protocol of the GS Titanium General Library Prep Kit (Roche Applied Science). After titration of the library using the GS Titanium SV emPCR Kit (Roche Applied Science), a full sequencing run was carried out on the GS FLX Titanium

platform. Metagenomic sequence data have been submitted to European Bioinformatics Institute (EMBL-EBI) databank for SRA archive (study accession number is ERP001267).

### Sequencing Data Analysis

Untrimmed raw pyrosequencing reads were consecutively checked for various kinds of quality features according to standardized practice. Filtering of obvious sequencing errors, i.e. (a) reads with ambiguous call bases (N) and (b) incorrect amplification primers, was done using an in-house developed processing pipeline. Inexact matches of the leading and tailing amplification primers to the raw read with up to two mismatches were allowed by this processing step using a fuzzy pattern matching algorithm. Reads not fulfilling these criteria were filtered out; leftover reads were trimmed of the primer sequences.

Operational taxonomic unit (OTU) clustering, species diversity, and species richness estimations were performed using the ESPRIT algorithm [23] with default parameters except for the preprocessing step where only exact matching sequences were considered to form groups of unique sequences.

A rarefaction analysis was employed to assess the coverage of the microbial community by the datasets based on the OTU clustering results. Rarefaction curves were obtained by plotting the sample sizes versus the estimated number of OTUs.

Taxonomic classification of sequencing reads up to the genus level were performed using the RDP naive Bayesian Classifier [24] with an 80 % confidence threshold.

## Results

Canola develops from seeding to rosette, flowering, maturity, postharvest fall stubble, and overwintered stubble stages [18, 25], and it is likely that root-associated bacteria facilitate plant growth during initial stages of plant development, providing the host plants with compounds such as phytohormones that play a critical role in plant growth and development [26, 27]. In this study, the bacterial diversity at the rosette and flowering developmental phases of canola (*B. napus* L.) plants was assessed through the application of new generation sequencing technology. Total bacterial DNA was extracted from root samples of canola grown in Vacaria, south of Brazil. Samples were identified as the following: R1 consisted of DNA extracted from bacteria that were inside the roots of canola plants at the rosette stage and R2 consisted of DNA extracted from bacteria that were inside the roots of canola plants at the flowering stage. These DNA samples were used in PCR reactions to amplify a specific

fragment corresponding to the V6 to V8 region of the 16S rRNA gene using primers with molecular IDs as described in the “Methods” section. Sequencing of the amplicons yielded 64,675 reads for sample R1 and 16,699 reads for sample R2 after the initial filtering step.

Rarefaction curves (Fig. 1), which plot the number of observed OTUs (cluster count) versus the fraction of sample observed to assess coverage, were done for each sample, and all plots approached an asymptote.

In the sample R1, microorganisms that could be identified belonged to several classes, in which Gammaproteobacteria, Flavobacteria, Sphingobacteria, Bacilli, Betaproteobacteria, Actinobacteria, and Alphaproteobacteria were the predominant classes, respectively. The most representative phylum in this sample was Proteobacteria, followed by Bacteroidetes, Firmicutes, and Actinobacteria.

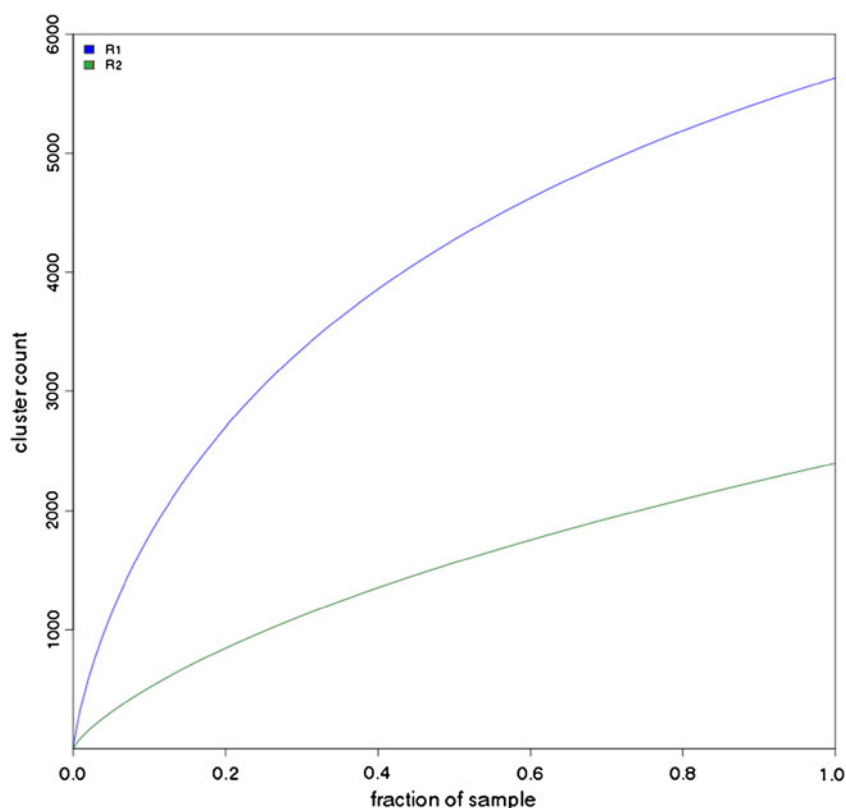
Pseudomonadaceae was the most abundant family in sample R1, followed by Flavobacteriaceae and Enterobacteriaceae families. Xanthomonadaceae, Sphingobacteriaceae, and Paenibacillaceae families were identified as well although less frequently. Other families, like Oxalobacteraceae, Comamonadaceae, Rhizobiaceae, Sanguibacteraceae, and Alcaligenaceae appeared with fewer representatives. Figure 2 shows a phylogenetic tree for the R1 sample up to the family level. About 50 % (51.70) of the sequences of the R1 sample were characterized as belonging to the genus *Pseudomonas*. Other bacterial genera, such as *Chryseobacterium*,

*Sphingobacterium*, *Erwinia*, *Saccharibacillus*, *Stenotrophomonas*, *Azomonas*, *Serratia*, *Riemerella*, and *Xanthomonas*, were also abundant in this sample. In total, 35 bacterial genera were identified in sample R1 (see also Supplementary Material Table S1).

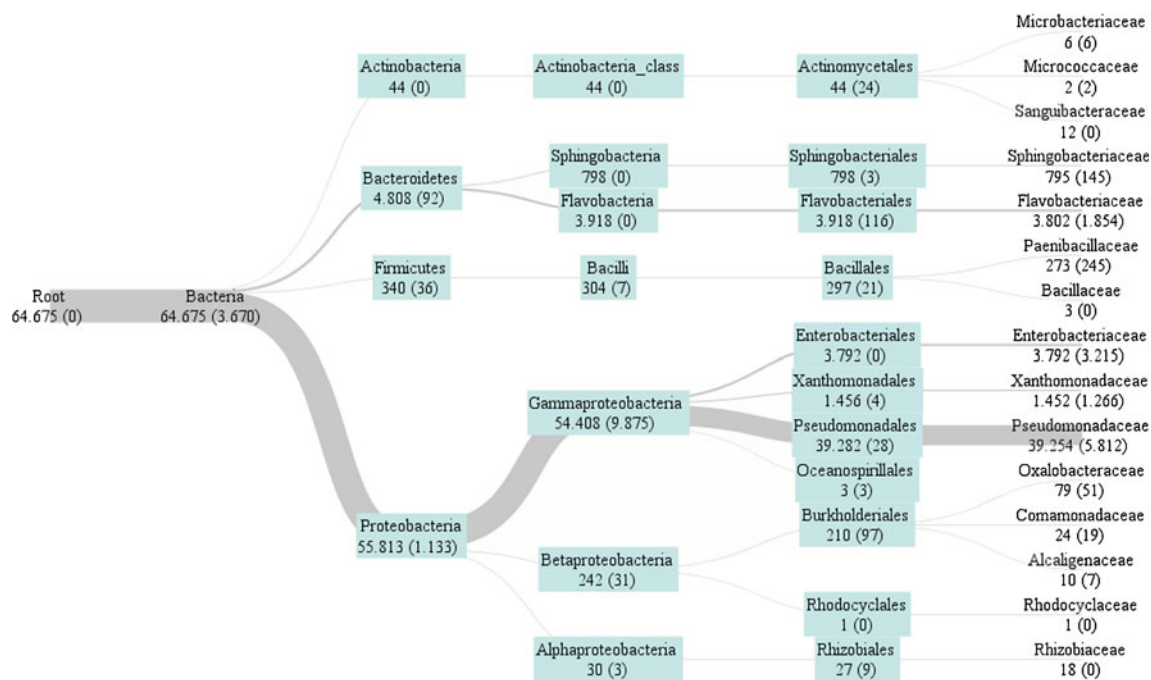
Proteobacteria was also the most representative phylum in sample R2, with almost 70 % (69.21) of the sequences that were characterized belonging to it. The predominating group in this sample was also Gammaproteobacteria, followed by Flavobacteria, Alphaproteobacteria, Betaproteobacteria, Sphingobacteria, Actinobacteria (class), Bacilli, Verrucomicrobiae, and Chlamydiae classes. Sequences belonging to Bacteroidetes, Actinobacteria, Firmicutes, Acidobacteria, Verrucomicrobia, and Chlamydiae phyla were also present.

The most abundant family in sample R2 was Xanthomonadaceae, followed by Flavobacteriaceae, Pseudomonadaceae, Rhizobiaceae, Sphingobacteriaceae, Comamonadaceae, and Enterobacteriaceae. Other families were also identified but with fewer representatives. Figure 3 shows a phylogenetic tree for the R2 sample until the family level. In sample R2, 8.53 % of the identified sequences could be assigned to members of the genus *Xanthomonas*, followed by sequences of members of *Rhizobium*, *Pseudomonas*, *Flavobacterium*, *Stenotrophomonas*, *Chryseobacterium*, *Pedobacter*, *Variovorax*, *Epilithonimonas*, *Sanguibacter*, and *Pigmentiphaga* genera. In total, 57 bacterial genera were identified in sample R2 (see also Supplementary Material Table S1).

**Fig. 1** Rarefaction analysis of the samples. The curves represent the number of observed OTUs (cluster count) plotted against the fraction of sample. *R1*, DNA extracted from bacteria that were inside the roots of canola plants at the rosette stage; *R2*, DNA extracted from bacteria inside the roots of canola plants at flowering stage







**Fig. 2** Taxonomic tree of sample R1 showing the most abundant lineages up to the family level. *Line thickness* indicates the relative abundance of a lineage; the *numbers associated with each node* give

the total number of classified sequences assigned to this taxon; *numbers in parentheses*, the number of sequences that could not be assigned on a more specific level

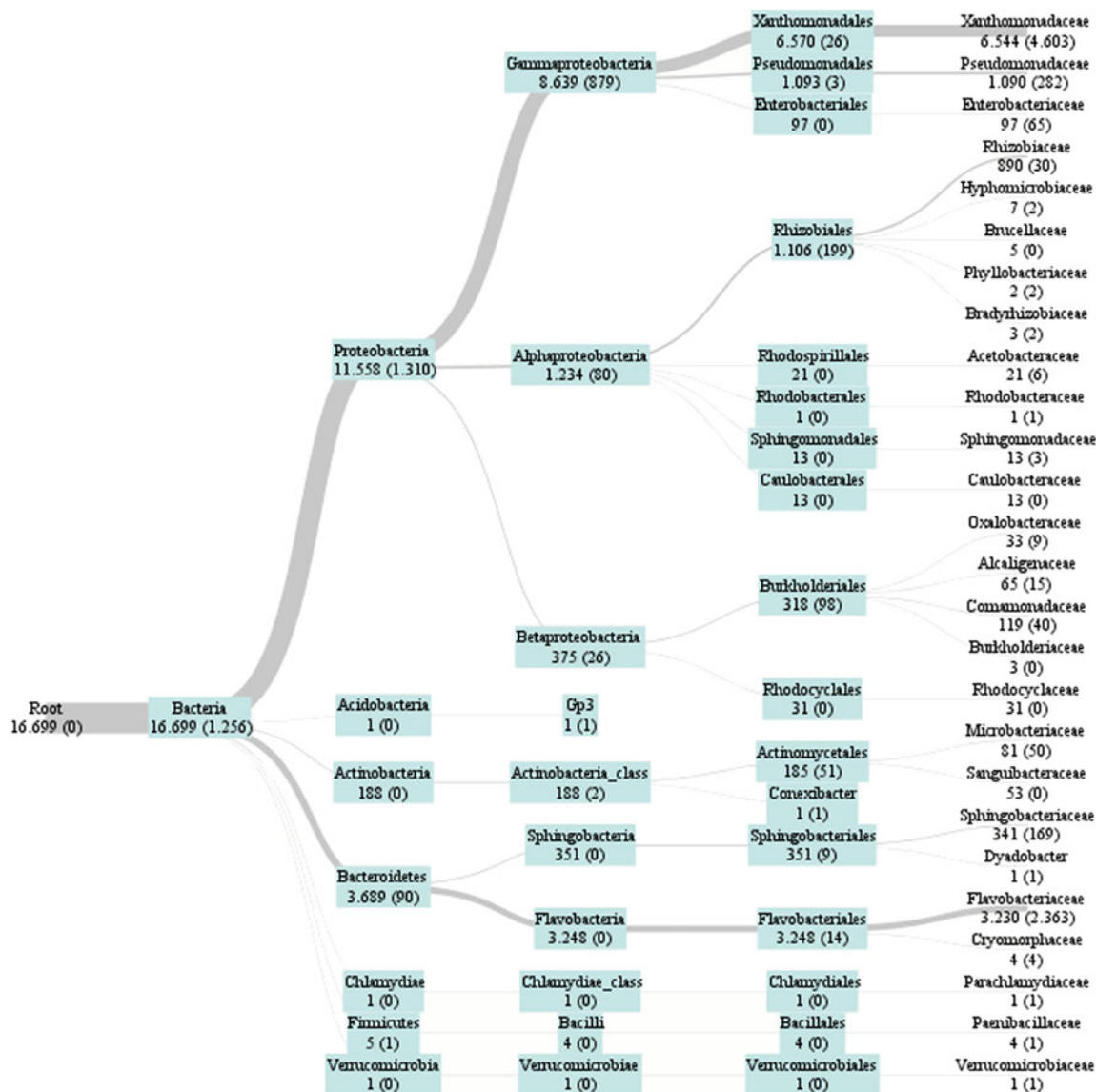
From rosette development stage to flowering stage, we could observe that the bacterial community changed in its composition and abundance. The diversity appeared to increase from rosette to flowering stages. In sample R1, there was a clear predominance of Pseudomonadaceae and Enterobacteriaceae families, whereas in sample R2, Xanthomonadaceae and Flavobacteriaceae families surpass Pseudomonadaceae family, although it was still present. On the other hand, members of the Rhizobacteriaceae family were detected only in the flowering development stage sample (R2). Both of the samples show the presence of members of Sphingobacteriaceae family. Figure 4 summarizes the results concerning to the most abundant families identified for samples R1 and R2.

## Discussion

The occurrence and distribution of microbial communities in the soil and rhizosphere is influenced by many factors like root morphology, the stage of plant growth, root exudates, and the physical and chemical properties of the soil [28]. Whereas for several economically important crops the knowledge concerning the diversity of bacterial communities that associate with those plants is growing fast [28, 29], little information is still available for canola plants. In this work, we made use of the 454 deep sequencing technology to unravel the microbial community associated to two developmental stages of canola (*B. napus* L. v. Hyola 60). This

strategy was chosen since it is the most suitable for ecological diversity studies [2]. Roesch and co-workers [2] performed one of the first works applying the deep sequencing methodology to describe microbial diversity in soil samples. In this work, the authors sequenced the V9 region of the 16S rRNA gene amplified by PCR, and they were able to characterize a great amount of bacteria that were not previously accounted for. This work was also important to increase the estimation of microbial diversity in the soil, showing that this environment is of great importance for microbial ecology. In the present work, the V6 to V8 region of the 16S rRNA gene was chosen for the bacterial identification of the bacterial communities present inside canola roots. This region has also proved to be very useful in the identification of bacteria [21] in most cases until the genus level as could be observed in this work and several others [10–12]. In the future, as the size of the reads increased rapidly, next-generation sequencing technology should result in larger reads. This advance will enable researchers to easily identify bacteria until the species level, making the deep sequencing technology even more suitable for ecological diversity studies.

One of the purposes of our research was to investigate whether the stage of development of the plant plays a role in the diversity of the microbial community associated to it. To achieve this goal, two different plant developmental stages, rosette (R1) and flowering (R2) [18], were used for DNA extraction of the bacterial community present inside canola



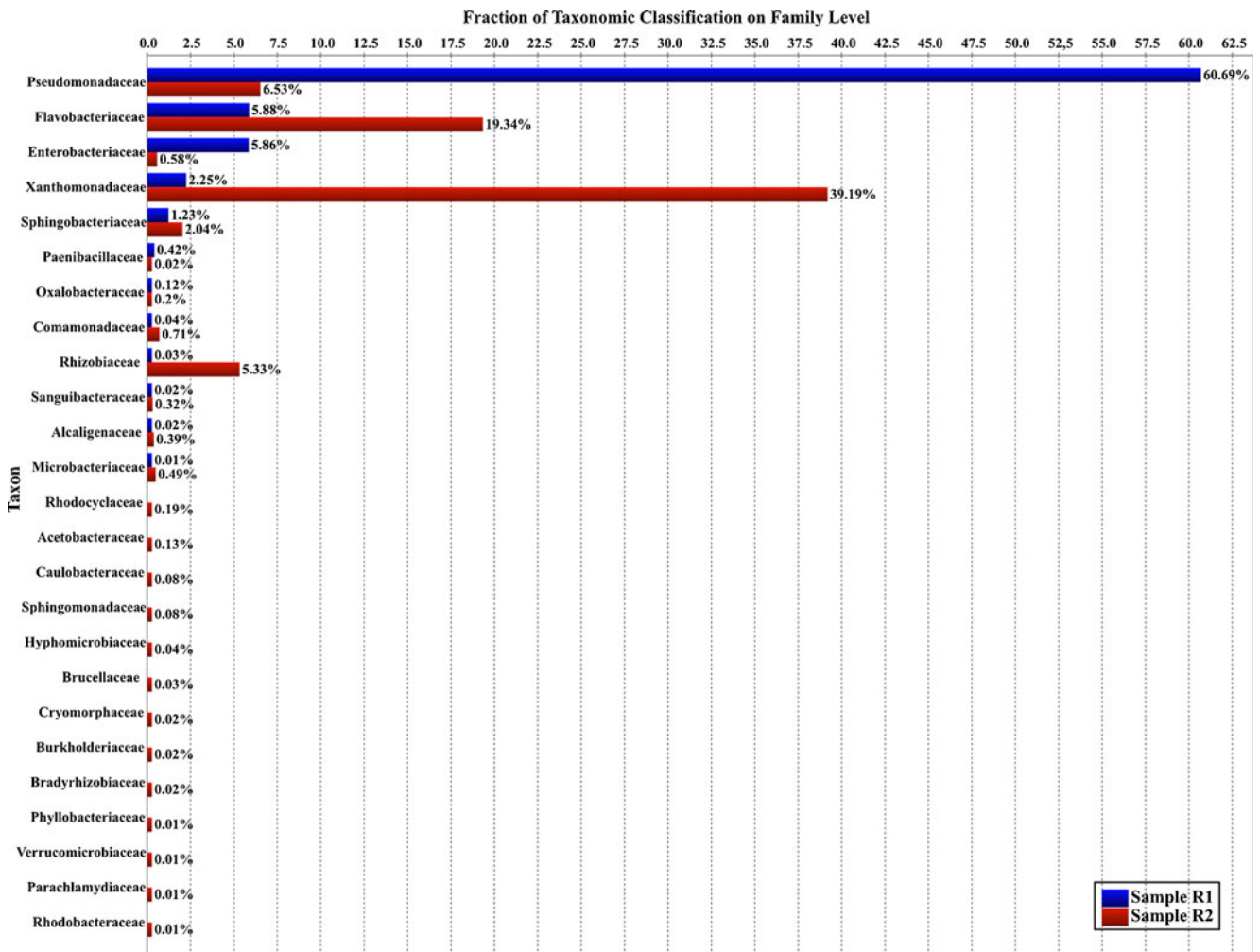
**Fig. 3** Taxonomic tree of sample R2 showing the most abundant lineages up to the family level. *Line thickness* indicates the relative abundance of a lineage; the *numbers associated with each node* give

the total number of classified sequences assigned to this taxon; *numbers in parentheses*, the number of sequences that could not be assigned on a more specific level

roots. These stages of canola plants were chosen since they are critical for the increase of the leaf area and for grain formation, respectively, which are two important periods for canola development and when the bacterial communities that are interacting with its roots must be well established [30, 31]. The results obtained after the deep sequencing analysis showed that the bacterial communities inside canola roots indeed changed with the growing stage of the plants. There was a difference in the abundance of the genera, family, and even the phyla identified for each sample. In both root samples, Proteobacteria was the most common phylum, but sample R1 presented a lower number of representative phyla, four in total, whereas sample R2 presented seven. In this sample (at rosette stage), the most common

bacteria belonged to the genera *Pseudomonas*, and the family Pseudomonadaceae was the most representative family (Figs. 2 and 4). In the flowering stage (R2), the genus *Xanthomonas* was the most common, with the Xanthomonadaceae family the most representative family in this sample (Figs. 3 and 4). Also, the number of bacterial genera identified between the rosette stage (R1=35) and the flowering stage (R2=57) increased. Although root exudation from canola plants has not been assessed, it is likely that the exudation pattern of canola roots changes as plants develop, altering rhizosphere microbial community composition, as reported for maize rhizosphere [32].

Germida and co-workers [15] suggested that plants have a major role in determining the composition of the rhizoplane



**Fig. 4** Comparison of the samples R1 and R2 at the taxonomic rank family. *Numbers* correspond to the fraction of classified reads at the given family. Classifications at fractions below 0.01 % are not shown in this visualization in favor of presentability

and endo-rhizosphere bacterial communities among root-associated bacteria. Several studies on many plant species in different locations, using both culturing and nonculturing (molecular) methods, have also indicated that plant type, genotype, root zone, plant age, and plant community composition are, indeed, more important factors influencing the diversity of microbial communities than, for example, soil abiotic parameters [15, 33–37]. Farina et al. [19] isolated cultivable bacteria associated with soil, rhizosphere, and the roots of canola based on their growth on three selective semi-solid media without nitrogen. Although no dominant group of bacteria was identified in their work, strains belonging to *Agrobacterium*, *Burkholderia*, *Enterobacter*, and *Pseudomonas* genera were the most abundant in all the sampling sites analyzed and, in general, bacteria belonging to the Enterobacteriaceae family were the predominant canola root-associated bacteria. This study also showed that the microbial community structure was influenced by seasonal variation, and in canola rosette stage samples, the diversity of the bacteria

associated with the rhizospheric soil was higher than those associated with the roots of canola. Nevertheless, the results obtained in the present work are in agreement with those of Smalla and co-workers [38] that compared the relative abundance of 16S rDNA targets and found that enrichment of bacterial populations associated with canola was most pronounced when canola was at the flowering stage. Interestingly, both the families (Pseudomonadaceae and Xanthomonadaceae) and genera (*Pseudomonas* and *Xanthomonas*) identified here were found in both developmental stages but at different proportions. This result reinforces the occurrence of a switch in the predominant bacteria in the different developmental stages of the plant, clearly demonstrating that the plant itself interferes with the associated microbial community.

Finally, our results also showed that the percentage of bacteria belonging to the group of bacteria known as plant growth-promoting rhizobacteria is high inside the plant's root samples (R1, 52 %; R2, 59 %). Rhizobacteria are better adapted to colonization of roots than bacteria from non-

rhizospheric soil [39, 40]. Gram-negative bacteria, especially species of *Pseudomonas* genus, are by far the most common rhizobacteria [41], and they have been the most extensive group of bacteria interacting with plants studied, as they are readily isolated from plant tissues, easily handled, and amenable to genetic approaches. The commonly used culture-dependent isolation methods mainly detect *Pseudomonas* and other gram-negative genera [42]. Marilley and Aragno [43] found that the rhizosphere, which is a relatively nutrient-rich niche for bacteria, has a positive selection for the Proteobacteria and reduced the percentage of the Acidobacterium division.

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