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Identifying the Active Microbiome Associated with Roots and Rhizosphere Soil of Oilseed Rape

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ABSTRACT RNA stable isotope probing and high-throughput sequencing were used to characterize the active microbiomes of bacteria and fungi colonizing the roots and rhizosphere soil of oilseed rape to identify taxa assimilating plant-derived carbon following $^{13}CO_2$ labeling. Root- and rhizosphere soil-associated communities of both bacteria and fungi differed from each other, and there were highly significant differences between their DNA- and RNA-based community profiles. *Verrucomicrobia*, *Proteobacteria*, *Planctomycetes*, *Acidobacteria*, *Gemmatimonadetes*, *Actinobacteria*, and *Chloroflexi* were the most active bacterial phyla in the rhizosphere soil. *Bacteroidetes* were more active in roots. The most abundant bacterial genera were well represented in both the ^{13}C- and ^{12}C-RNA fractions, while the fungal taxa were more differentiated. *Streptomyces*, *Rhizobium*, and *Flavobacterium* were dominant in roots, whereas *Rhodoplanes* and *Sphingomonas* (*Kaistobacter*) were dominant in rhizosphere soil. "*Candidatus* Nitrososphaera" was enriched in ^{13}C in rhizosphere soil. *Olpidium* and *Dendryphion* were abundant in the ^{12}C-RNA fraction of roots; *Clonostachys* was abundant in both roots and rhizosphere soil and heavily ^{13}C enriched. *Cryptococcus* was dominant in rhizosphere soil and less abundant, but was ^{13}C enriched in roots. The patterns of colonization and C acquisition revealed in this study assist in identifying microbial taxa that may be superior competitors for plant-derived carbon in the rhizosphere of *Brassica napus*.

IMPORTANCE This microbiome study characterizes the active bacteria and fungi colonizing the roots and rhizosphere soil of *Brassica napus* using high-throughput sequencing and RNA-stable isotope probing. It identifies taxa assimilating plant-derived carbon following $^{13}CO_2$ labeling and compares these with other less active groups not incorporating a plant assimilate. *Brassica napus* is an economically and globally important oilseed crop, cultivated for edible oil, biofuel production, and phytoextraction of heavy metals; however, it is susceptible to several diseases. The identification of the fungal and bacterial species successfully competing for plant-derived carbon, enabling them to colonize the roots and rhizosphere soil of this plant, should enable the identification of microorganisms that can be evaluated in more detailed functional studies and ultimately be used to improve plant health and productivity in sustainable agriculture.

KEYWORDS *Brassica napus*, bacteria, carbon allocation, fungi, high-throughput sequencing, rhizosphere microbiome, root microbiome

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The rhizosphere is an active interface in which plants and microorganisms establish a complex and varied molecular dialogue, involving nutrient transfer as well as specific interactions mediated by the release of signaling molecules from plant roots (1, 2) and resulting in enhanced plant productivity (3). Between 20% and 50% of photoassimilated carbon is transferred to the roots, and half of this is subsequently released

into the soil (4). These exudates affect soil microbial community structure and activity, resulting in the "rhizosphere effect," i.e., elevated numbers of microorganisms (5, 6). These microbes can either help the plant to acquire nutrients from the soil or provide indirect pathogen protection. Therefore, rhizosphere competence implies that plant growth-promoting bacteria are well adapted to utilize carbon resources (7).

Numerous studies performed using the model plant *Arabidopsis thaliana*, a member of the *Brassicaceae* family, have revealed that both soil type and, to a lesser extent, host genotype shape the profiles of root microbiota and that communities associated with the rhizosphere differ significantly from those of the endophytic root compartment (8–10) as well as from those present in the surrounding bulk soil (11). Additionally, the structure of the root microbiomes in *Arabidopsis* spp. and other related species appear to be highly conserved (12) and similar between the monocotyledonous crop barley and the dicotyledonous *Arabidopsis*, despite the existence of some host-specific microbiota (11).

One of the most promising techniques of identifying microorganisms that consume recently fixed plant carbon is stable isotope probing (SIP) (13–16). The technique relies on the incorporation of a stable isotope into nucleic acids from a labeled substrate; thus, microbes that incorporate plant carbon into their biomass become enriched (17). One of the limitations of SIP is the requirement of adding ^{13}C-labeled substrate in large amounts, resulting in an elevated *in situ* availability of carbon and thus a potential divergence between experimental and actual conditions. Long incubation times also potentially lead to nonspecific labeling. One way to minimize such side effects is to apply highly sensitive RNA-SIP and reduce the labeling to a rather short period, depending on the experimental system and the plant species used (14). On the other hand, short incubation times may introduce bias against microorganisms with low growth rates, thus leading to incomplete labeling of the community (18).

Studies tracking metabolically active rhizospheric populations have been published for both bacteria and fungi and in a variety of plant species (19–23). The incorporation of photosynthesized ^{13}C into the biomass of soil microbes occurs rather rapidly ($<$24 h), with maximum incorporation into microbial RNA after 4 to 8 days (24). SIP-based experiments indicate that fungi are important organotrophic organisms in the rhizosphere, receiving considerable amounts of plant-derived carbon (25), and that they can respond rapidly to easily degradable substrates in soil (26, 27).

Oilseed rape is a globally important oil crop cultivated for edible oil, biofuel production, and phytoextraction of heavy metals; however, it is susceptible to numerous diseases (28). The rhizospheric environment of this crop has been studied for its potential to harbor biocontrol bacteria (such as *Serratia proteamaculans*, *S. plymuthica*, *Pseudomonas chlororaphis*, *P. acidovorans*, and *P. putida*) that can protect the plant against fungal pathogens (29, 30). The aims of the present study were to characterize and compare the structure and composition of the root and rhizosphere bacterial and fungal communities of oilseed rape plants and to identify the microbial groups capable of competing for recently fixed carbon and compare these with other less active groups not incorporating a plant assimilate. This was done by labeling the plants with $^{13}CO_2$ and applying RNA-SIP. The experimental approach is summarized in Fig. 1.

RESULTS

^{13}C enrichment in rhizosphere soil. The overall isotopic signatures of δ^{13}C demonstrated that the rhizosphere soil was significantly ($P < 0.05$) enriched in ^{13}C from day 1 postlabeling (see Fig. S1 in the supplemental material). The maximum enrichment was observed at days 3 and 7 postlabeling; but, to focus on the primary consumers of current photosynthates, all further analyses were based on samples from day 3 postlabeling.

Overall structures of bacterial and fungal communities associated with rhizosphere soil and roots. In total, 325,992 bacterial and 350,798 fungal reads were obtained from 454 pyrosequencing of all samples. Following denoising and removal of chimeric sequences, 139,074 bacterial sequences remained. For the fungal data, after

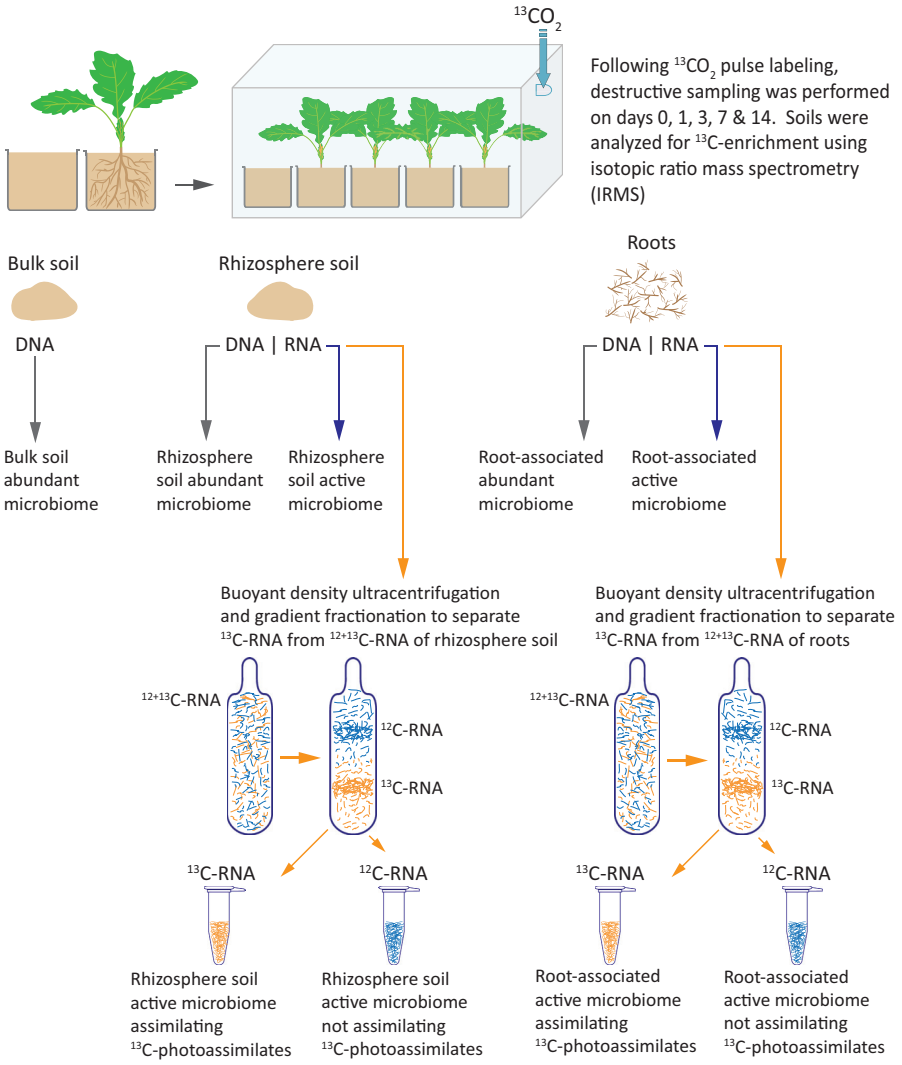

FIG 1 Schematic representation of the experimental approach used for identifying the active microbiome associated with roots and rhizosphere soil of oilseed rape. *Brassica napus* seedlings were grown in pots containing organically managed soil and subjected to $^{13}CO_2$ pulse labeling after 4 weeks growth. Systems were harvested destructively on days 0, 1, 3, 7, and 14, and soils were analyzed for ^{13}C enrichment to determine the stage at which the maximum labeled carbon was allocated to soil through rhizodeposition. Subsequently, the rhizosphere soil and root samples from that time point were used for coextraction of DNA and RNA for analyses of abundant and active bacterial and fungal microbiomes using high-throughput sequencing. ^{12} + ^{13}C-RNA was subjected to density gradient ultracentrifugation to separate ^{13}C-RNA and ^{12}C-RNA fractions that were used to characterize the active bacterial and fungal microbiomes assimilating recent ^{13}C-labeled photoassimilates of plants.

demultiplexing and implementing the quality filtering steps in the split_libraries.py command in QIIME, 123,804 sequences remained.

Nonmetric multidimensional scaling (NMDS) ordinations and nonparametric multivariate analysis of variance (NPMANOVA) for bacteria (Fig. 2A) and for fungi (Fig. 2B) revealed significant differences between DNA-based communities in bulk (plant-free) soil, rhizosphere soil, and roots (Fig. 2A_i and B_i). The active communities colonizing rhizosphere soil and roots (Fig. 2A_{ii} and B_{ii}) were also significantly different. The DNA- and RNA-based communities in rhizosphere soil (Fig. 2A_{iii} and B_{iii}) and in roots (Fig. 2A_{iv} and B_{iv}) also differed from each other significantly. For bacteria, both the ^{13}C-RNA and the ^{12}C-RNA fractions from rhizosphere soil and roots exhibited similar diversity patterns (data not shown). In contrast, for fungi, the ^{13}C-RNA rhizosphere soil and root fractions exhibited unexpectedly greater diversity compared to the respective ^{12}C-RNA

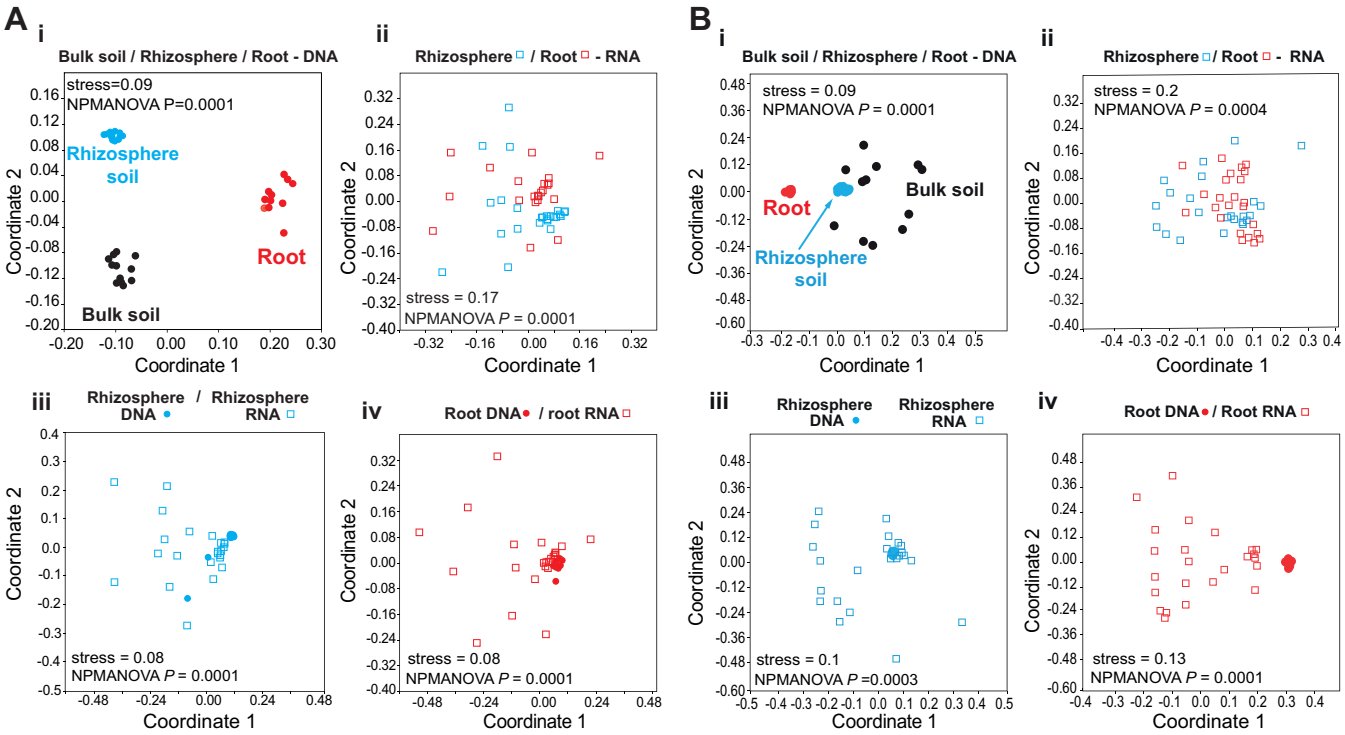

FIG 2 Nonmetric multidimensional scaling ordinations (NMDS) of changes in bacterial (A) and fungal (B) community structures associated with bulk soil DNA, rhizosphere soil DNA, and root DNA (i), rhizosphere soil RNA and root RNA (ii), rhizosphere soil RNA and rhizosphere soil DNA (iii), and root RNA and root DNA (iv).

fractions, probably due to the fact that there was high competition for recently fixed carbon from the plant (data not shown). In the ^{12}C-RNA fractions, some dominant taxa were observed, implying that they do not depend highly on recently fixed C, but most probably live on dead cells or cell walls.

Abundant and active bacteria in the rhizosphere and root compartments. The total numbers of operational taxonomic units (OTUs) are shown in Fig. S2A. In general, similar numbers of OTUs were retrieved for all soil samples (rhizosphere DNA, rhizosphere RNA, and bulk soil DNA). In the roots, the number of OTUs from RNA was almost double that retrieved from DNA; however, there were no differences among the major taxa.

In total, 29 bacterial and two archaeal phyla were identified (Fig. 3A). The relative abundances of *Proteobacteria* were almost equally high in all communities but not in rhizosphere DNA, implying that *Proteobacteria* are proportionally more strongly represented among active bacteria in the rhizosphere than those that were simply present. The relative abundance and activity of *Verrucomicrobia* were much higher in soil samples compared to those in root samples. On the other hand, the relative abundance of *Bacteroidetes* was higher in root samples compared to that in soil samples, and they were proportionally more abundant in the active community in roots. *Actinobacteria* DNA was highly abundant in both the rhizosphere soil and the roots, but the relative activity was much greater in the roots than in the rhizosphere. The abundance of *Actinobacteria* was lowest in the bulk soil DNA-based bacterial community. *Acidobacteria* were more abundant in plant-free (bulk) and rhizosphere soil samples but more infrequent in root samples (<1%). *Chloroflexi* and *Planctomycetes* followed the same trend, being both more abundant and active in rhizosphere soil samples than those associated with roots (Fig. 3A).

Active bacterial communities assimilating plant-derived carbon in the rhizosphere and root compartments. In the rhizosphere RNA-SIP based bacterial community, *Proteobacteria*, *Actinobacteria*, *Planctomycetes*, *Acidobacteria*, *Chloroflexi*, *Verruco-*

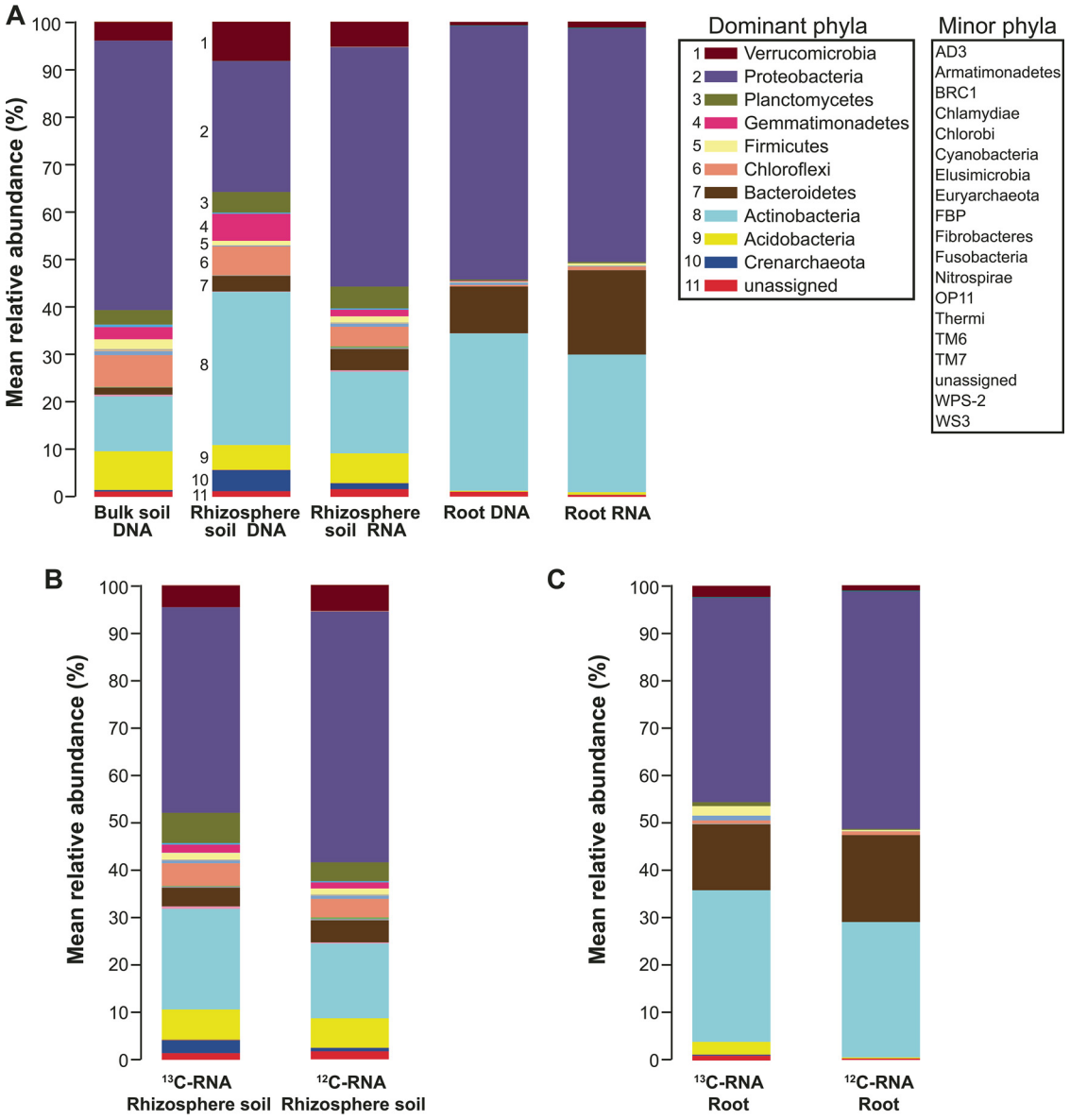

FIG 3 Mean relative abundances of different bacterial phyla in bulk soil DNA, rhizosphere soil DNA and RNA, and root DNA and RNA (A), ^{13}C-RNA- and ^{12}C-RNA in rhizosphere soil (B), and ^{13}C-RNA- and ^{12}C-RNA in roots (C). Taxonomic classification of 16S rRNA gene sequences was performed in QIIME using the Greengenes 16S rRNA reference taxonomy. Colors for dominant phyla that are easily distinguishable are shown in a separate legend, supplemented with a numerical key. Minor phyla are simply listed.

microbia, and *Bacteroidetes* were the most active phyla represented in both ^{13}C-RNA and ^{12}C-RNA fractions, but the abundances of *Planctomycetes* and *Actinobacteria* sequences were higher in the ^{13}C-RNA based community (Fig. 3B). This difference was also true for the archeal phylum *Crenarchaeota* (Fig. 3B). In the root-associated RNA-SIP bacterial community, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the most active phyla, and *Verrucomicrobia*, *Actinobacteria*, and *Acidobacteria* had relatively greater numbers of sequences in the ^{13}C-RNA-based community (Fig. 3C).

At the genus level in all soil samples, the most abundant genera were *Rhodoplanes*, *Kaistobacter*, DA101 ("*Candidatus* Udaeobacter copiosus"), "*Candidatus* Nitrososphaera," *Balneimonas*, and *Luteimonas* (Fig. 4A). In the root-derived abundant and active bacterial communities, the dominant genera identified were *Streptomyces*, *Rhizobium*, *Flavobacterium*, *Devosia*, *Actinoplanes*, and *Agrobacterium* (Fig. 4A). *Rhodoplanes* and *Kaistobacter* were highly active in both the ^{13}C- and the ^{12}C-RNA rhizosphere fractions,

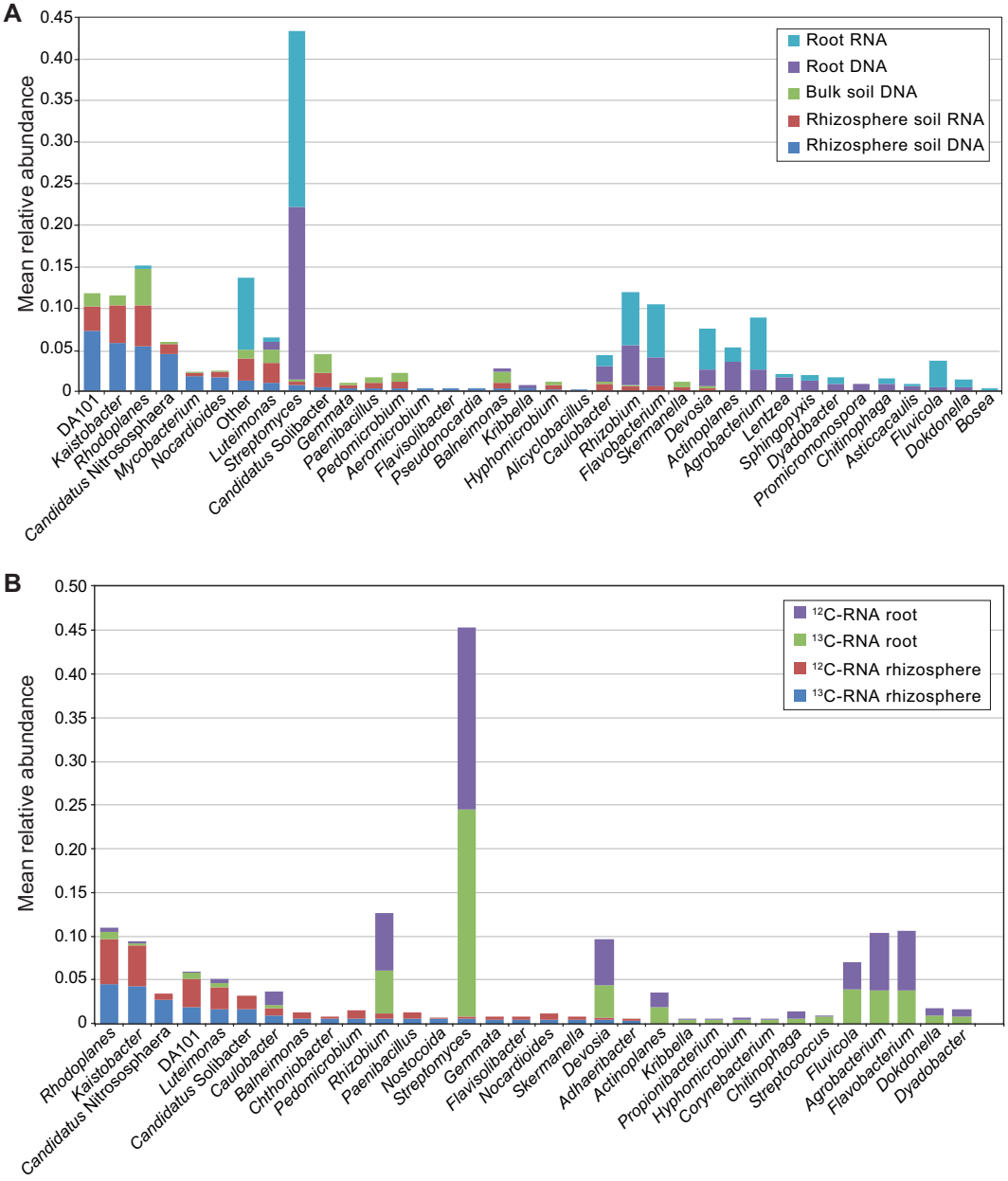

FIG 4 (A) Mean relative abundances of the top 20 most abundant bacterial genera in rhizosphere soil RNA, rhizosphere soil DNA, bulk soil DNA, root RNA, and root DNA. (B) Mean relative abundances of the top 20 bacterial genera found in the ^{13}C-RNA and in the ^{12}C-RNA in the rhizosphere soil and in the root fractions. Taxonomic classification of 16S rRNA gene sequences was performed in QIIME using the Greengenes 16S rRNA reference taxonomy. (Note that more than 20 bars are displayed in each histogram, since the top 20 most abundant genera are not the same in different root/rhizosphere soil DNA/RNA fractions.)

whereas the remaining genera exhibited lower relative activities (Fig. 4B). In the respective root RNA fractions, the highly active genera were *Streptomyces*, *Rhizobium*, *Flavobacterium*, *Agrobacterium*, *Devosia*, and *Fluviicola* (Fig. 4B).

Abundant and active fungi in the rhizosphere and root compartments. The total numbers of OTUs are shown in Fig. S2B. In the soil samples, the number of OTUs retrieved from DNA was double that retrieved from RNA, whereas the opposite trend was observed for the root-derived samples.

In total, five fungal phyla were identified (Fig. 5A). *Basidiomycota* were present in all of the communities but not in root DNA samples, and their relative abundance values imply that they were more strongly represented among active fungi in the rhizosphere,

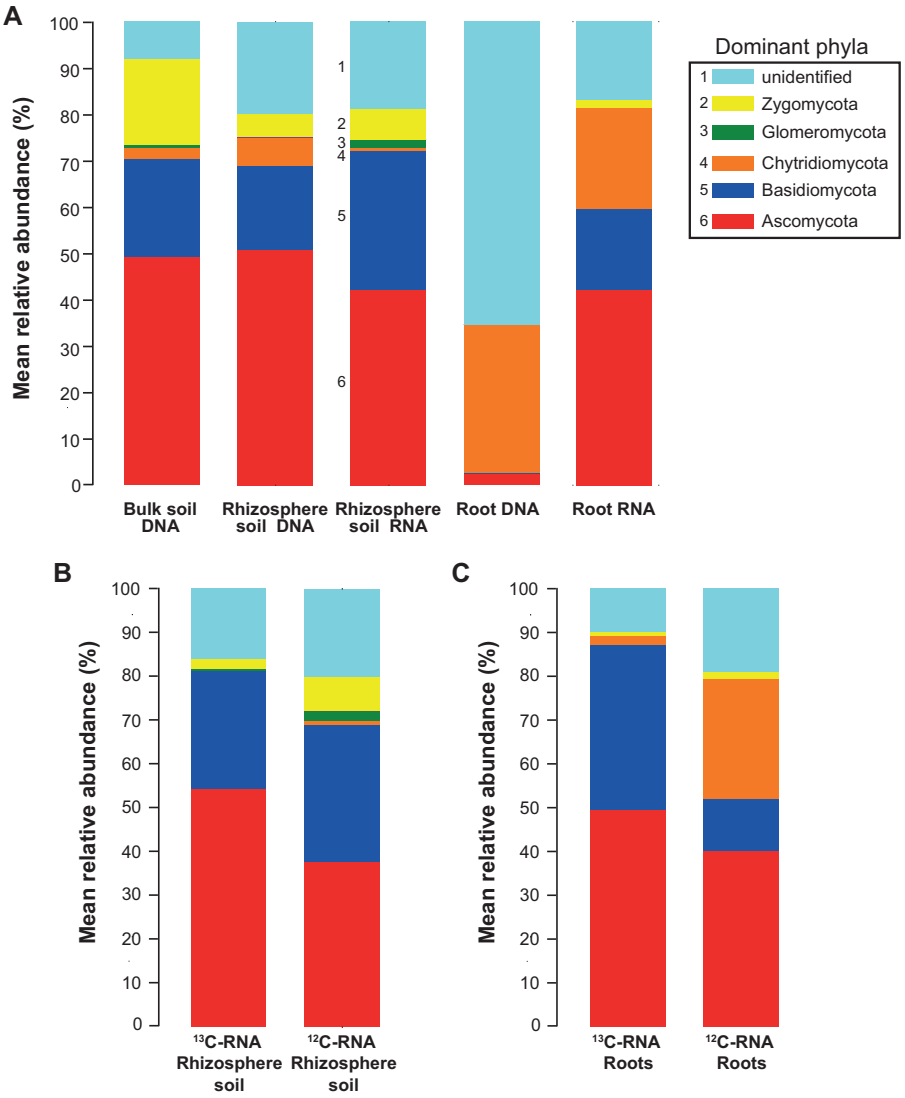

FIG 5 Mean relative abundances of different fungal phyla in bulk soil DNA, rhizosphere soil DNA and RNA, and root DNA and RNA (A), ^{13}C-RNA- and ^{12}C-RNA in rhizosphere soil (B), and ^{13}C-RNA- and ^{12}C-RNA in roots (C). Taxonomic classification of the ITS region was performed in QIIME using the UNITE reference taxonomy.

whereas in the roots, fungi of this phylum were all active. *Ascomycota* were present in all of the communities, with greater relative abundances in all of the soil samples as well as in the root RNA-based community. However, their abundance was very low in the root DNA-based community, suggesting that the *Ascomycota* formed a relatively large proportion of the active fungi in both the rhizosphere soil and the roots, but that they formed a much smaller proportion of the total fungal community that was present in the roots. Fungi of the phylum *Chytridiomycota* exhibited a higher abundance in root DNA, followed by root RNA, rhizosphere DNA, bulk soil DNA, and rhizosphere RNA. *Zygomycota* were mostly abundant in bulk and rhizosphere soil communities, whereas in the roots, they were present only in root RNA samples. *Glomeromycota* were identified only in soil communities with a higher relative abundance in the rhizosphere RNA-based community. However, a large proportion of taxonomic assignments were classified as "unidentified," especially in the root DNA-based community.

At the genus level in all "soil" samples, the most abundant genera were *Cryptococcus* and *Mortierella*, whereas in rhizosphere DNA- and RNA-based communities, *Pseudaleuria*, *Clonostachys*, *Exophiala*, and *Fusarium* were also among the top 20 most abundant/active genera (Fig. 6A). In the roots, *Olpidium* was the most highly abundant

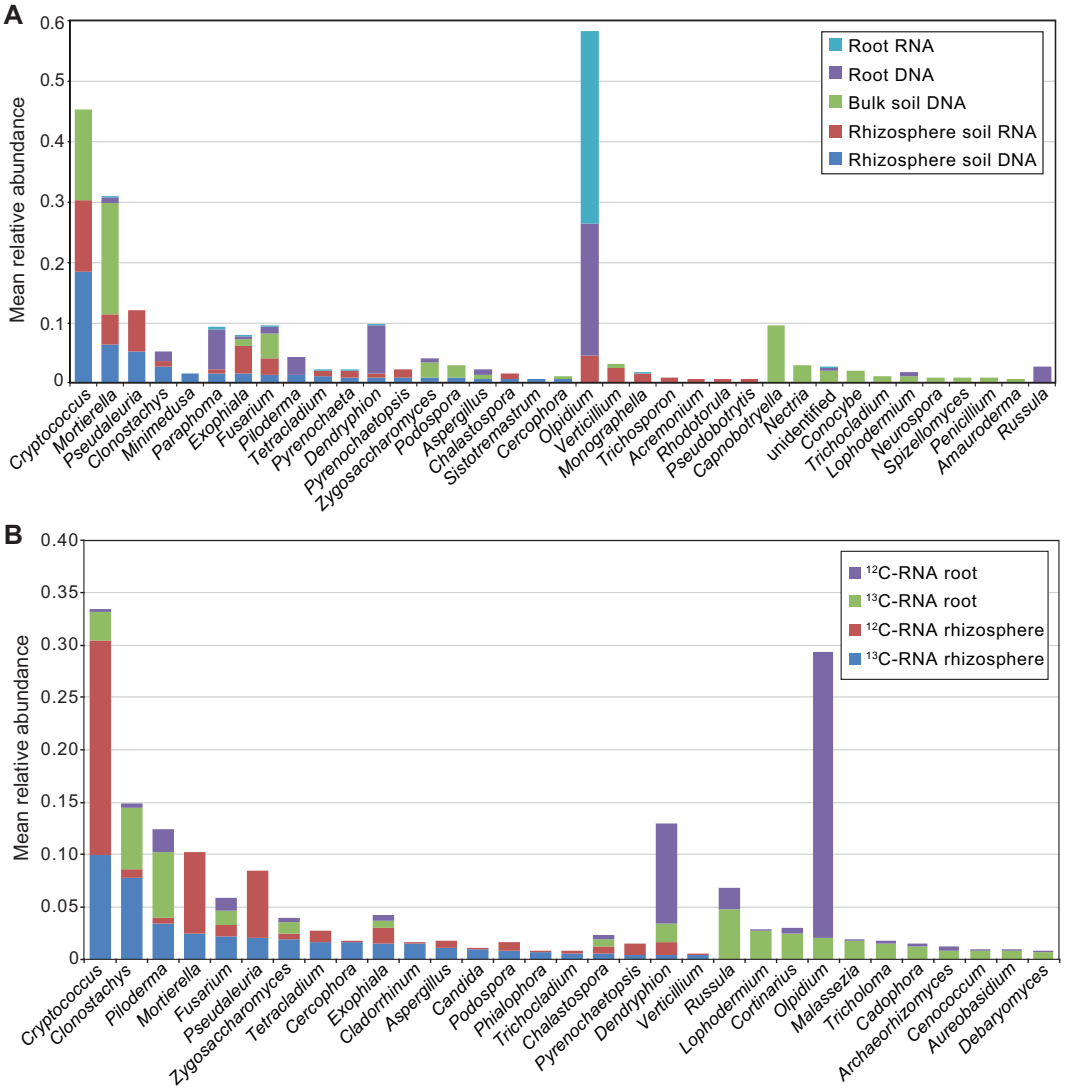

FIG 6 (A) Mean relative abundances of the top 20 most abundant fungal genera in rhizosphere soil RNA, rhizosphere soil DNA, bulk soil DNA, root RNA, and root DNA. (B) Mean relative abundances of the top 20 fungal genera found in the ^{13}C-RNA and ^{12}C-RNA fractions in the rhizosphere soil and in the root fractions. Taxonomic classification of the ITS region was performed in QIIME using the UNITE reference taxonomy. (Note that more than 20 bars are displayed in each histogram, since the top 20 most abundant genera are not the same in different root/rhizosphere soil DNA/RNA fractions.)

and active genus in both the root DNA- and root RNA-based communities and was followed by *Dendryphion* and *Paraphoma*. In the root RNA-derived active community, fungi of the genera *Piloderma*, *Russula*, *Clonostachys*, and *Fusarium* were also in the list of the top 20 most abundant (Fig. 6A).

Active fungal communities assimilating plant-derived carbon in the rhizosphere and root compartments. In the rhizosphere RNA-SIP based fungal community, the highly active OTUs identified in the ^{13}C-RNA fraction belonged to *Basidiomycota* and *Ascomycota* (Fig. 5B). However, the relative abundances of *Zygomycota*, *Glomeromycota*, and *Chytridiomycota* were higher in the ^{12}C-RNA fraction, suggesting that they were probably less reliant on recently fixed plant-derived ^{13}C. Similarly, in the root RNA fractions, *Basidiomycota* and *Ascomycota* became more abundant in the ^{13}C-RNA-based active community, but again, the relative abundances of all other phyla were higher in the ^{12}C-RNA fraction (Fig. 5C).

The rhizosphere ^{13}C- and ^{12}C-RNA-based fungal communities consisted of the genera *Cryptococcus*, *Clonostachys*, *Mortierella*, *Fusarium*, *Pseudaleuria*, and *Tetracladium*

(Fig. 6B). Interestingly, the relative activities of most of them were higher in the ^{12}C-RNA fraction, with the exception of *Clonostachys*, whose activity was much higher in the ^{13}C-RNA-based community. In the root-associated communities, the most active genera were *Olpidium*, *Dendryphion*, *Piloderma*, *Russula*, *Clonostachys*, and *Cryptococcus*. *Olpidium* and *Dendryphion* were more active in the ^{12}C-RNA-based root community (Fig. 6B).

DISCUSSION

We are not aware of any other studies using high-throughput sequencing in conjunction with RNA-SIP to study metabolically active microbial communities associated with the roots or rhizosphere of *Brassica napus*, but interesting differences and similarities exist with older studies. The general predominance of *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* that we observed was expected, since these groups have been identified as common inhabitants in the rhizospheres of potato (31), maize (32), desert, and forest soils, the tundra and grasslands (33), and rice (10). The same phyla were also highly abundant in the rhizosphere soil and roots of *Arabidopsis* spp. (8, 9, 12). Interestingly, *Proteobacteria* and *Actinobacteria* have been suggested to be associated with disease suppression in the rhizosphere of sugar beet plants in a study using DNA metagenomics with PhyloChip (34).

As shown previously, both bacterial and fungal DNA communities originating from bulk soil, rhizosphere soil, and roots were structurally distinct from each other (8–10, 35). DNA- and RNA-based communities of bacteria and fungi were also significantly distinct from each other, both in the rhizosphere soil and the root compartment.

The active bacterial community data in our study suggest that *Streptomyces* were highly active in the root compartment but not in the rhizosphere. Previous studies using denaturing gradient gel electrophoresis (DGGE) and DNA-SIP did not identify *Streptomyces* in either rhizosphere soil or roots of *B. napus* (15). *Streptomyces* were found in the rhizosphere soil of strawberry plants using DGGE, but roots were not examined (36, 37). In our study, the genus *Rhizobium* was abundant in the ^{13}C-RNA fractions of root samples but not in the rhizosphere soil. *Rhizobium* spp. were identified as ^{13}C incorporators in the rhizospheres of rape and wheat, and they were also present in the root DNA-based communities of rape, wheat, and maize (15). *Rhizobium* has also been shown to be abundant in the rhizosphere of the strawberry plant (37). The dominance of *Streptomyces* and *Rhizobium* in the roots of *B. napus* in the present study is supported by earlier studies of *Arabidopsis thaliana* showing higher abundances of *Streptomycetaceae* (8, 9) and *Rhizobiaceae* in the root compartment (8).

Agrobacterium was also abundant in the ^{13}C-RNA root fraction in our study, but it did not incorporate ^{13}C in the rhizosphere and was absent in the root DNA-based community of oilseed rape (15). However, *Agrobacterium rhizogenes* was identified using BOX-PCR fingerprinting of rhizosphere soil in rape and shown to be antagonistic against the vascular pathogen *Verticillium dahliae* (38). DGGE and DNA-SIP in *A. thaliana* revealed that *Agrobacterium* was abundant in the root DNA-based community and also incorporated ^{13}C in the rhizosphere (21). In the present study, abundant ^{13}C-labeled *Flavobacterium* was observed in roots but not in the rhizosphere, a finding supported by earlier studies of the root microbiome of *A. thaliana* demonstrating that bacteria belonging to *Flavobacteriaceae* constitute a significant component of the root microbiome (8, 12). *Devosia* was abundant in the ^{13}C-RNA fraction of the roots but not in the rhizosphere soil. In contrast, these bacteria were abundant in the ^{13}C fraction of the *A. thaliana* rhizosphere but absent in the roots (21). *Actinoplanes* was found exclusively in association with roots in our study and did incorporate ^{13}C. In an earlier study using RNA-SIP (14), the incorporation of ^{13}C by *Actinoplanes* in roots of *Agrostis stolonifera* was demonstrated, but no data were presented concerning the rhizosphere soil. We found abundances of *Rhodoplanes* in both the ^{13}C and ^{12}C fractions of the RNA-based community in rhizosphere soil but not in the root samples; however, these bacteria were abundant in the root DNA-based community of *A. thaliana* (21).

Members of *Streptomyces* are well known for their ability to promote plant growth

and for their biocontrol potential (39–41). They are capable of producing several antimicrobial compounds used in both medicine and agriculture (42), as well as a wide range of volatile organic compounds capable of stimulating plant growth both directly and indirectly (43). Studies have been conducted in nonleguminous crops, including canola, lettuce, and *Arabidopsis*, suggesting the strong potential of *Rhizobium* to colonize roots of nonlegumes effectively, possibly enhancing plant growth (21) via the involvement of plant growth regulators such as indole-3-acetic acid and cytokinin (44). These results suggest that, even in nonlegumes, the presence of appropriate N-fixing bacteria may enable reduced inputs of synthetic nitrogen fertilizers, a practice commonly used in legume crops. A *Flavobacterium* sp. was isolated from the rhizosphere of the bell pepper, and its presence was associated with plant growth promotion and an antagonistic potential against pathogens (45). A study which used ^{15}N-DNA-SIP to investigate soil microorganisms responsible for N fixation identified, among others, bacteria of the genus *Rhodoplanes* as being potential N fixers (46). *Kaistobacter* was abundant in both the ^{13}C- and ^{12}C-RNA fractions of rhizosphere soil in the present study, and members of this genus have been suggested to be involved in the degradation of aromatic compounds (47). "*Candidatus* Nitrososphaera" is an ammonia-oxidizing archaeon, playing a central role in global nitrogen cycling, being highly abundant in all environments, including in soils (48).

We found much higher numbers of *Clonostachys* spp. in the ^{13}C-RNA fraction than in the ^{12}C-RNA fraction of both the rhizosphere soil and root samples, suggesting that this fungus was active in incorporating recently assimilated carbon from *B. napus* in both compartments. In contrast, *Cryptococcus* and *Mortierella* sequences were found primarily in the rhizosphere, and more sequences were associated with the ^{12}C-RNA fraction, suggesting that these fungi primarily assimilated unlabeled carbon, possibly from older structural pools. Abundant *Cryptococcus* and *Mortierella* sequences were found predominantly in the rhizosphere soil of the strawberry plant (35). In our study, *Fusarium* appeared to incorporate recently fixed plant carbon more in the rhizosphere soil than in the roots. High abundances of *Fusarium* spp. in both rhizosphere soil and roots of the strawberry plant have been observed (35). *Fusarium* spp. are common soil fungi that have important roles, not only as plant pathogens but also as saprotrophic competitors against other pathogenic fungi (49).

Clonostachys rosea is a common soil saprophyte and an endophyte in some plants (50). It has been shown to be an effective biocontrol agent against *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Plasmodiophora brassicae*, with mechanisms including mycoparasitism, competition for space and nutrients, antibiosis, and induction of systemic resistance through root colonization (51, 52). The potential for using *Clonostachys* in combination with the biocontrol prodigiosin-producing bacterium *Serratia rubidaea* against the fungal pathogen *Fusarium oxysporum* in tomato plants has recently been demonstrated (53). In natural soils, *Cryptococcus* dominates fungal populations (54, 55), and its predominance could be due to the polysaccharide capsules surrounding it (56) and assisting in nutrient assimilation from soil, resulting in a high competitive ability against other fungi and bacteria (54). *Olpidium* and *Dendryphion* were abundant in the roots of *B. napus* and incorporated ^{13}C in the present study, but substantially higher numbers of sequences were associated with the ^{12}C-RNA fraction. *Olpidium brassicae* is a soilborne obligate parasite that infects plant roots. Its resting spores can remain dormant in the soil for up to 20 years before infecting roots (57). *Dendryphion* is a pathogen and has been shown to be abundant in organically managed potato fields (58). These labeling patterns suggest that either these fungi are slow growing or that they derive carbon from structural pools that were unlabeled.

The ectomycorrhizal fungal genera *Piloderma*, *Russula*, and *Cortinarius* incorporated ^{13}C in the roots of *B. napus* despite the fact that rapeseed is normally considered to be a nonmycorrhizal plant (59). Neither glomeromycotan nor ectomycorrhizal fungal sequences were present in the negative controls or the root or rhizosphere DNA samples, suggesting that the samples are unlikely to have been contaminated. The soil in the present study was taken from a field surrounded by forests on two sides, and it

is likely to have contained ectomycorrhizal spores. It is possible that the activity of ectomycorrhizal fungal spores adhering to *B. napus* roots in our study could have been stimulated by plant-derived carbon, as shown for *Paxillus involutus* (60), explaining the ^{13}C incorporation at an early stage in the present study.

In conclusion, RNA-SIP enabled us to describe the structures and compositions of bacterial and fungal communities associated with the roots and rhizosphere soil of *B. napus* plants and to identify taxa actively assimilating carbon from different plant-derived pools. The higher relative dominance of certain microbial taxa in the roots compared with those in the rhizosphere soil supports the idea of active selection from a more diverse rhizosphere community demonstrated in other plant species. The identification of specific genera such as *Streptomyces*, *Rhizobium*, *Clonostachys*, and *Fusarium* as incorporators of recently fixed C suggests that they may be superior competitors in the *B. napus* rhizosphere and that their potential as inoculants to improve the productivity and health of oilseed crops could be explored. In the 72-h time frame of our SIP analyses, the most abundant taxa were usually well represented in both the ^{13}C- and the ^{12}C-RNA fractions, suggesting that they were active in incorporating both recently fixed carbon and pools of carbon fixed prior to labeling. The spatial and temporal patterns of microbial colonization and acquisition of plant-derived carbon revealed in this study help to identify the microbial genera that can be targeted for more detailed functional studies, including the expression of specific microbial genes involved in plant-microorganism signaling that can be exploited for the sustainable production of oilseed crops such as *B. napus*.

MATERIALS AND METHODS

Greenhouse experiment. The experimental approach is outlined in Fig. 1. The winter *Brassica napus* cultivar "Libraska" was used. Following surface sterilization, seeds were sown on half-strength potato dextrose agar for 10 days to confirm the sterility of the seedlings. Soil was collected from an organically managed field in Ultuna, Sweden (59°49.424′N, 17°39.260′E), in September 2013. The field was plowed prior to collection but was previously planted with *Trifolium pratense*. The soil was homogenized and sieved and transferred to pots (6 cm by 6 cm by 6 cm; 110 g/pot). Two seedlings of uniform sizes were planted in each pot. Twenty replicate pots were used for each sampling occasion, and 5 replicate pots with soil only (bulk soil) were incubated under the same conditions and used as controls. Plants were grown with a 16-h photoperiod at 18 to 20°C and photon flux density of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and an 8-h dark period at 13 to 15°C for 4 weeks. Four days after planting, the seedlings were thinned to one per pot.

$^{13}CO_2$ pulse labeling. Plants were labeled with 99 atoms percent (atom%) $^{13}CO_2$ (Cambridge Isotope Laboratories, Inc., MA, USA) after 4 weeks growth and incubated in a clear Perspex chamber (height by width by length, 30 cm by 48 cm by 98 cm) for a total of 6 h. The total CO_2 concentration was maintained at an average value of 420 ppm during this period by monitoring with an infrared gas analyzer (EGM-4; PP Systems, Hertfordshire, UK) and injecting more labeled gas accordingly. After labeling, the pots were returned to the greenhouse.

Sampling. Three replicate pots were harvested 24 h prior to labeling to monitor the natural abundance of ^{13}C in rhizosphere soil. Following $^{13}CO_2$ labeling, plants were harvested after 24 h, 72 h, 7 days, and 14 days. Since the sizes of the pots were small and root growth was extensive, we considered the whole soil as rhizosphere soil. However, the soil adhering to the roots was collected separately by gently shaking the roots and carefully mixing it with the remaining soil. "Bulk soil" was collected from plant-free pots. Roots were subsequently immersed in water for 10 min and after thorough, but gentle, washing, they were further washed in a 0.1% Triton X-100 solution followed by repeated rinsing with Milli-Q water. Soil and roots were immediately frozen, freeze-dried, and stored at −20°C prior to coextraction of RNA and DNA.

^{13}C enrichment analysis. Freeze-dried rhizosphere soil from different time points was milled to a fine powder and weighed using a microbalance. Five milligrams of soil from each sample was transferred to tin capsules (Elemental Microanalysis, Ltd., Devon, UK) and the $\delta^{13}C$ signatures of these samples were determined with an elemental analyzer (model EuroEA3024; Eurovector, Milan, Italy) coupled online to a continuous flow Isoprime isotope-ratio mass spectrometer (GV Instruments, Manchester, UK). The resulting $\delta^{13}C$ values were expressed in parts per thousand (‰) relative to the international standard of Vienna Pee Dee Belemnite (V-PDB), where $\delta^{13}C = (R_{sample} - R_{standard})/R_{standard} \times 1{,}000$ (‰) and R is the molar ratio of $^{13}C/^{12}C$.

RNA and DNA extraction. Total RNA and DNA were coextracted from 1.0 g of freeze-dried rhizosphere soil and from 50 mg of freeze-dried roots for each sample using the RNA power soil isolation kit (MOBIO Laboratories, CA, USA) and Qiagen plant DNeasy minikit (Qiagen, Germany), respectively (see the supplemental material for further details).

Cesium trifluoroacetate ultracentrifugation. Seven hundred fifty nanograms of RNA from each replicate was fractionated by cesium trifluoroacetate (CsTFA) equilibrium density gradient ultracentrif-

ugation as described before (17) after some modifications. The gradient mixture per sample consisted of 2641.5 μl of a 2.0-g · ml^{-1} CsTFA solution (GE Healthcare, Uppsala, Sweden), 112.5 μl of deionized formamide (Sigma-Aldrich), and 516 μl of PCR-grade sterile H_2O (Sigma-Aldrich). After adding 750 ng RNA to the CsTFA gradient mixture in a 3.3-ml OptiSeal polyallomer centrifuge tube (Beckman Coulter, USA), the tubes were sealed with plugs and spun in a TLN-100 rotor (Beckman Coulter) in an Optima MAX-XP ultracentrifuge (Beckman Coulter) at 140,000 $\times$ *g* for 48 h at 20°C with maximum acceleration and deceleration.

Following the fractionation of gradients using a fraction collector (model 2110; Bio-Rad, CA, USA), the densities of each fraction were calculated by measuring the refractive indices using an automatic benchtop refractometer (model ATR-F Touch; Schmidt + Haensch & Co., Germany). For each gradient, four fractions (with densities of 1.82 to 1.85 g · ml^{-1}) representing ^{13}C-labeled RNA (heavy) and four fractions (with densities of 1.77 to 1.79 g · ml^{-1}) representing ^{12}C-unlabeled RNA (light) were chosen and pooled. RNA fractions were purified by isopropanol precipitation. RNA pellets were air dried in a laminar hood and resuspended in 10 μl of RNase-free sterile water.

Reverse transcription-PCR. Reverse transcription of rhizosphere soil and root RNA (heavy and light fractions) was performed using the iScript reverse transcription Supermix (Bio-Rad, CA, USA) in reactions with final volumes of 20 μl. Prior to cDNA synthesis, the four heavy and light fractions were pooled, resulting in one representative heavy and one light fraction (here called ^{13}C-RNA and ^{12}C-RNA, respectively).

PCR amplification. PCRs were performed using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Germany). The bacterial primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-AACGCACGCTAGGGACTACHVGGGTWTCTAAT-3′) were used to target the variable region V4 (61, 62). The fungal primers fITS7 (5′-GTGARTCATCGAATCTTTG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to target the ITS region (63) (for PCR amplification parameters, see the supplemental material). The primers 806R and ITS4 contained 12-bp and 8-bp barcode sequences, respectively (unique for each sample). The amplification of cDNA samples was performed using 1 μl undiluted cDNA, and for DNA samples, the templates were diluted 10×. Three technical replicates were run for each sample and negative controls were run during all PCRs.

The reactions were run on 1% (wt/vol) agarose gels prestained with Nancy-520 DNA gel stain (Sigma-Aldrich, MO, USA). The triplicate PCR products from each bacterial and fungal amplification were pooled, purified using the Agencourt AMPure kit (Beckman Coulter, USA), and quantified using a Qubit fluorometer (Invitrogen, USA). All bacterial PCR products were pooled in equimolar concentrations and freeze-dried (CoolSafe; ScanLaf A/S, Denmark) for 24 h. The same procedure was followed for fungal PCR products. Pyrosequencing was carried out on 2× one-fourth of a GS FLX titanium Pico titer plate (Macrogen, Seoul, South Korea) according to the manufacturer's recommendations (Roche, Branford, CT, USA).

Microbial community analysis. The sequences obtained were analyzed using QIIME (64) (MacQiime version 1.9.0). Both bacterial and fungal reads were demultiplexed based on the barcode sequences, and forward and reverse reads were combined. Bacterial data were denoised, and sequences from both bacteria and fungi were clustered into OTUs by UCLUST (65) based on 97% similarity (66). For bacteria, the representative sequences for each OTU were aligned using PyNAST (67), and taxonomic classification was done using the Ribosomal Database Project classifier (68) against the Greengenes 16S rRNA database using default parameters. Chimeric OTUs were identified using ChimeraSlayer (69) and removed. Any reads that were identified in the negative PCR controls were eliminated from the final OTU table. For fungi, the UNITE database version 7 (12_11 alpha release) (70) was used as a reference to assign taxonomy against BLAST results (71) using default parameters. Any reads from organisms other than fungi were eliminated from the final OTU table.

Statistical analyses. A multivariate analysis of the OTUs was performed using Paleontological Statistics (PAST version 2-17) (72). Beta diversity community dissimilarity calculations were visualized using nonmetric multidimensional scaling (NMDS) with the Bray-Curtis dissimilarity measure. Nonparametric multivariate analysis of variance (NPMANOVA) was used to estimate the significance of the differences in microbial communities. Venn diagrams were generated using the VENNY online program (http://bioinfogp.cnb.csic.es/tools/venny/).

Accession number(s). The raw sequencing reads were submitted to the NCBI Sequence Read Archive (SRA) under the study number SPR078303, available at http://www.ncbi.nlm.nih.gov/sra/SRP078303.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01938-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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The authors declare no conflict of interest.

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