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RESEARCH

Contrasting Nitrogen Fertilization and *Brassica napus* (Canola) Variety Development Impact Recruitment of the Root-Associated Microbiome

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ABSTRACT

Canola (*Brassica napus*) is an important broadacre crop, produced under high nitrogen (N) fertilizer application. Modern canola varieties are developed under high N rates but the impacts on root-associated microbiomes of different varieties are unknown. We studied eight canola varieties spanning historical Canadian spring canola development at two sites under high and low N fertility and characterized bacterial and fungal microbiomes in the root and rhizosphere using amplicon sequencing. Environmental conditions and the resulting canola varietal responses strongly affected the root-associated bacterial and fungal microbiomes. Microbes regulated by N fertility in each canola variety were mainly Gammaproteobacteria, Bacteroidia, Actinobacteria, Sordariomycetes, Dothideomycetes, and Agaricomycetes classes. Differentially abundant (DA) microbial taxa showed that N more strongly enriched bacteria in the roots and fungi in the rhizosphere. Each variety had its specific pattern of DA amplicon sequence variants (ASVs) responding to soil N availability, and the profile of DA-ASVs in paired canola varieties were also altered by soil N availability, especially bacteria in the rhizosphere. The yield was strongly associated with a subset of microbial taxa, mainly from Proteobacteria, Actinobacteriota, and Ascomycota. These variety-dependent responses to N and links to yield performance make the root-associated microbiome a promising target for improving the agronomic performance of canola by manipulating microorganisms tailored to soil fertility and plant genotype.

Keywords: *Brassica napus*, canola variety, nitrogen fertilization, root-associated microbiome, yield

The plant microbiome plays an essential role in plant nutrient uptake, stress resistance, and productivity, which are the main crop breeding targets (Brown and Caligari 2011; Trivedi et al. 2020). Despite its importance, the plant microbiome is only rarely considered in crop breeding. Although crop varieties are developed through crop breeding based on plant genetic background and field phenotypes, their associated microbiomes are inevitably affected and could influence crop phenotypes and, ultimately, yield. For example, maize historical lines developed over four decades from the 1940s to the 1980s, when the rate of synthetic nitrogen (N) fertilizer application increased from negligible to modern rates, had different associated microbiomes and included shifts in N-cycling functional gene abundances (Favela et al. 2021). In comparison with two *Japonica* rice cultivars, high-yield hybrid rice enriched more rhizosphere microbes with the potential to efficiently mineralize N and phosphorus (P), while mitigating N and potassium (K) losses (Xiong et al. 2021). Therefore, comparing plant microbiomes among crop varieties is fundamental for understanding how plant microbial symbionts contribute to host performance.

Microbes and plants have evolved complex mechanisms of interaction in response to environmental conditions (Mhlongo et al. 2018; Sharma et al. 2020). For example, plant metabolites such as root exudates are a main factor in recruiting and structuring the plant microbiome (Iannucci et al. 2017; Micallef et al. 2009; Singh

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and Mukerji 2006). Changes in plant metabolism under different geographical environments can, in turn, drive changes of plant-associated microbiomes in composition and structure (Iwanycki Ahlstrand et al. 2018; Pang et al. 2021). Together, these plant metabolic responses are expected to contribute to environment-specific adaptation of the root and rhizosphere microbiome. Performance of crop varieties are affected by environmental conditions, known as the genotype–environment interaction (Annicchiarico 2002). Soils at different geographical locations vary in their microbial composition. Because the soil serves as the primary reservoir of microorganisms recruited by plant roots, indigenous communities can also potentially affect plant phenotypes imparted by the root-associated microbiome (Benitez et al. 2021; Howard et al. 2020). Exploring plant microbiomes under different environmental conditions will assist crop breeders to further understand the environment-based variation of crop varietal performance at different locations.

The interaction between roots and soil microorganisms is dynamic and the microbial composition shifts not only with environmental conditions but also with plant growth stage (Li et al. 2022; Taye et al. 2020). For example, the *Arabidopsis* rhizosphere microbial community was different between seedling versus vegetative, bolting, and flowering stages (Chaparro et al. 2014). Rhizosphere microbiomes of cotton were distinct at seedling, budding, and flowering stages (Qiao et al. 2017). Plant nutrient uptake is influenced by plant development stage. For example, N uptake varied from 16 to 185 kg ha^{-1} in the lowland rice (*Oryza sativa L.*) shoot from initiation of tillering to flowering (Fageria 2003). Williams et al. (2021) found higher N uptake by canola at the flowering versus five- to six-leaf vegetative stage and that the relative N uptake of four varieties differed depending on N application rate. Soil nutrient availability affects the assembly of root-associated microbiomes (Ikeda et al. 2014; Yeoh et al. 2016). Considering the close relationship among plant growth stage, nutrient availability, and the root-associated microbiome, the dynamic process of root-microbe assembly during plant growth progression may reflect the response of the plant to nutrient availability (Chen et al. 2019) but may also be stochastic (Bell et al. 2022).

Canola (*Brassica napus*) is an important broadacre cash crop and source of edible and industrial oil (Nath et al. 2016; Raymer 2002). As one of the most widely grown crops in Canada, several hundred canola varieties have been generated to improve yield and oil quality (Brewin and Malla 2013; Government of Canada 2022). Compared with a broadacre cereal such as wheat, canola has a higher demand for N and lower N use efficiency (Brennan and Bolland 2009; Dreccer et al. 2000; Hocking et al. 1997). Considering that modern canola varieties were developed under high N application, it is expected that the root and rhizosphere microbiomes will differ between early and modern, open-pollinated, and hybrid varieties. Given the economic losses and environmental pollution caused by N loss from canola production, it is essential to explore the change of root-associated microbiomes of canola varieties responding to low and high N fertility, which will provide valuable information for engineering the microbiome to increase N use efficiency by canola.

In this study, we compared the fungal and bacterial root-associated microbiomes of eight varieties that spanned historical spring canola development in Canada, grown in the field under high and low N fertility at two different sites in major canola-growing regions of Saskatchewan, Canada. We hypothesized that canola varieties developed over the past 50 years would differ in their recruitment of root-associated microbiomes under high and low N across a variety of environmental conditions (two sites and 2 years).

MATERIALS AND METHODS

Experimental design. Our experiment was established at two field sites, Melfort (52.8185°N, 104.6027°W) and Saskatoon (52.1718°N, 106.5052°W), located on black and dark-brown chernozems, respectively, in Saskatchewan, Canada. The field trials were set up in a randomized complete block design combined with split plot arrangement ($n = 4$). N fertility treatments were assigned to the main plots and canola varieties to the subplots (Supplementary Fig. S1). N fertilizer application (total available N, based on soil test, available N plus fertilizer N added) was targeted to low (60 kg N ha^{-1}) and high (120 kg N ha^{-1}) at Saskatoon and 170 kg N ha^{-1} at Melfort (Smith et al. 2010). We compared eight varieties spanning historical spring canola development: Argentine (released in 1942), Westar (released in 1982), nested association mapping (NAM)-12 (released in 1996), a commercial hybrid (Com Hybrid) (released in 2016), and four unreleased lines: an experimental hybrid (Exp Hybrid) (developed in 2016, where NAM-0 was the pollen donor), NAM-0 (developed in 1999), NAM-17 (developed in 2005), and NAM-72 (developed in 2003). Among the eight canola varieties, NAM-0 is a representation of modern open-pollinated varieties which were released in approximately 2000, prior to hybrid varieties overtaking the canola seed marketplace. Hybrid varieties were generated by crossing two different varieties, which usually grow better and produce higher yield due to the phenomenon of "hybrid vigor". Details for determining soil test available N (NH_4^+-N and NO_3^--N) and fertilizer rates are described in Supplementary Method S1 and Supplementary Table S1. Canola was planted in early June, and samples were collected at early vegetative (approximately five- to six-leaf) and flowering stages in two consecutive years (2019 and 2020). The canola plants were harvested at the maturity stage and yields were determined by the canola breeding crew at Agriculture and Agri-Food Canada.

Sampling methodology. In each plot, three plants from mid-plot rows were randomly selected to avoid edge effects and composited. A trowel was placed vertically adjacent to the canola stem (approximately 2.5 cm in diameter around the stem) to dig out roots and soil to a depth of 10 cm. The root with surrounding soil was placed in a labeled plastic bag. Trowels were cleaned with water and disinfected with 70% ethanol between plots. The samples were kept on ice and transported back to the lab, stored at 4°C, and processed on the following day.

Sample processing. Each sample of roots with soil was processed aseptically on aluminum foil placed on a 70% ethanol disinfected lab bench. Loosely adhering soil was first shaken off the roots; then, the roots with tightly adhering soil were put into a 250-ml Erlenmeyer flask with 100 ml of sterile 0.05 M NaCl solution and shaken at 180 rpm for 15 min (MaxQ 6000; Thermo Scientific, Waltham, MA, U.S.A.). The soil-NaCl solution mixture was filtered into two 50-ml centrifuge tubes through a sterile cotton cloth, which removed plant residues and large sand particles. Tubes were centrifuged (Eppendorf Centrifuge 5910 Ri; Hamburg, Germany) at 5,000 rpm for 15 min at room temperature. The supernatant was decanted, and the rhizosphere soil pellets were transferred into 2.0-ml Eppendorf tubes and stored at −80°C for DNA extraction. The roots were taken out of the flask and rinsed first with tap water, then with sterile deionized water. The washed roots were cut into several pieces using a sterile scalpel blade, placed into 15-ml Falcon tubes, and stored at −80°C for DNA extraction.

DNA extraction and DNA library preparation. The DNA of rhizosphere soil (approximately 0.25 g) and root (approximately 0.1 g) were extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) and DNeasy Plant Pro Kit (Qiagen) according to the manufacturer's protocols. The primers attached with Illumina

adaptors were 342F (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CTA CGG GGG GCA GCA G) and 806R (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACC GGG GTA TCT) (Mori et al. 2014) for amplifying the bacterial 16S ribosomal RNA (rRNA) gene; and internal transcribed spacer (ITS) 1-F_KYO1 (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CTH GGT CAT TTA GAG GAA STA A) and ITS2-R_KYO2 (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTT YRC TRC GTT CTT CAT C) (Toju et al. 2012) for amplifying the fungal ITS. To block the amplification of plant plastid DNA (pPNA) and mitochondrial DNA (mPNA), universal PNA oligos pPNA (5′-GGC TCA ACC CTG GAC AG-3′), and mPNA (5′-GGC AAG TGT TCT TCG GA-3′) (Lundberg et al. 2013), were added into the polymerase chain reaction (PCR) during root 16S rRNA gene amplification. In addition, PCR negative controls and technical duplicates of randomly selected samples were included. The details of the PCR programs and the reaction systems are shown in Supplementary Method S2. The PCR products were checked using electrophoresis in agarose gel (1.2%) and purified with Nucleomag NGS Clean-up and Size Select (D-mark Biosciences, Scarborough, Ontario, Canada) according to the manufacturer's protocol. Then, the purified DNA samples were randomized on three 96-well plates and barcoded using Nextera XT Index Kit V2 (Illumina) through PCR. The PCR program and the reaction system are detailed in Supplementary Method S3. The barcoded PCR products were purified with Nucleomag NGS Clean-up and Size Select and quantified using Qubit 4 (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The purified barcoded amplicons were standardized to 4 nM, and 5 µl of each of the DNA samples were pooled together in a 2-ml Eppendorf tube. The ITS and16S rRNA gene libraries were processed according to the MiSeq System Denature and Dilute Libraries Guide before sequencing on the Illumina MiSeq platform using MiSeq Reagent Kit v2 (500-cycle) for ITS and MiSeq Reagent Kit v3 (600-cycle) for 16S rRNA.

Sequencing data processing. The 16S rRNA gene sequencing data were processed using Qiime2 v. 2020.11.0 (Bolyen et al. 2019). Specifically, the adaptors and the primers of the sequencing reads were trimmed using Cutadapt v. 3.1 (Martin 2011) with default parameters. The trimmed forward and reverse reads were merged and filtered using Qiime2-Deblur flow (Amir et al. 2017; Bolyen et al. 2019). The merged reads after filtering were then denoised using the 'qiime deblur denoise-16S' command, trimming reads at the length of 400 bases, which generated amplicon sequence variants (ASVs), an approach to provide more precise identification of microbes than operating taxonomic units (Callahan et al. 2017). Taxonomy classification of the ASVs was based on the Silva reference dataset v. 138.1 (Bokulich et al. 2020) pretrained with 16S V3/V4 regions using the 'qiime feature-classifier classify-sklearn' command. The files of the ASVs and the taxonomy generated were finally exported in TSV format for downstream analysis.

The adaptors and the primers of the ITS region sequencing reads were removed using Cutadapt with adjusted parameters (-g "max_error_rate = 0.25; min_overlap = 16", -a "max_error_rate = 0.2; min_overlap = 16", -G "max_error_rate = 0.2; min_overlap = 16", -A "max_error_rate = 0.2; min_overlap = 16"); then, the reads were denoised using the 'qiime dada2 denoise-paired' command (Bolyen et al. 2019; Callahan et al. 2016), the forward reads were trimmed at the length of 180 bases and reverse reads at the length of 120 bases, and chimeras were removed. The resulting ASVs were taxonomically classified using a naïve Bayes classifier trained with the UNITE reference dataset (Qiime release v. 8.2). The files of the ASVs and the taxonomy generated were exported in TSV format for downstream analysis. In total, 5,220,600 bacterial and 25,509,045 fungal sequences remained after sequencing data processing. Among the remaining sequences,16,162 unique bacterial and 8,250 unique fungal ASVs were recovered in the 512 root and rhizosphere samples.

Data analysis. The data analysis was handled with R v. 3.5.3 (R Core team 2019) in RStudio (RStudio Team 2019) and visualized using ggplot2 v.3.3.5 (Wickham 2016). Simply, the bacterial ASVs assigned to "chloroplast" and "mitochondria" were removed from the ASV and taxonomy tables. For bacteria and fungi, the sample metadata, ASV, and taxonomy tables were combined using phyloseq v.1.38.0, respectively (McMurdie and Holmes 2013). The PCR negative controls and duplicate samples were removed after testing the quality of sequencing data (Supplementary Results S1). The relative abundances of the bacterial and fungal phyla were analyzed using phyloseq. Samples were rarefied at the threshold of 2,500 and 5,000 reads for bacteria and fungi, respectively, before α-diversity analysis (Shannon index). Shannon index was estimated using the diversity() function in vegan v.2.5.7 (Oksanen et al. 2019). The ASV abundance was applied with Aitchison's log-ratio transformation before principal component analysis (Aitchison 1982). Specifically, rare ASVs with average abundance across all samples < 1 were removed; then, the zero values of ASVs were replaced using zCompositions v.1.4.0 (Palarea-Albaladejo and Martín-Fernández 2015) and the abundance of ASVs was centered log-ratio (CLR) transformed using CoDaSeq v.0.99.6 (Gloor and Reid 2016). Three-way analysis of variance (ANOVA) was applied to determine the significant effect of N treatment, canola variety, and growth stage on the Shannon index based on a linear mixed-effect model. The model was fit with three fixed effects (N fertility, canola variety, and growth stage) and three nested random effects (replication, N treatment, and canola variety) using nlme v.3.1.153 (Pinheiro et al. 2018). To satisfy the assumptions of ANOVA, Shannon index data were transformed using the method suggested by the bestNormalize v.1.8.2 (Peterson 2021) if the residual normality or the homogeneity of variance of the fitting model were not satisfied. Restricted permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) was applied for testing whether sample categories had significantly different microbial community structures using vegan v.2.5.7 (Oksanen et al. 2019) based on the CLR-transformed ASV abundance data. Differential abundance (DA) analyses at the phylum and ASV level were performed using ancombc () function in the ANCOMBC v.1.4.0 (Lin and Peddada 2020) with p_adj_method = holm, zero_cut = 0.9, lib_cut = 1000, max_iter = 100, and $\alpha = 0.05$. The DA-ASV analysis was based on samples grouped by site-years (i.e., Melfort-2019, Saskatoon-2019, Melfort-2020, and Saskatoon-2020). Before DA-ASV analysis, rare ASVs with average abundance over samples in each site-year < 2 were removed. The function of the bacterial community was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States updated (PICRUSt2) (Douglas et al. 2020; Langille et al. 2013). We got Enzyme Commission (E.C.) number abundance of each bacterial ASV after submission of 16S representative DNA sequences using PICRUSt2 v2.4.1. We selected six N-metabolism-related enzymes—that were nitrate reductase, nitrite reductase (NADH), ferredoxin-nitrate reductase, nitric-oxide reductase, nitric-oxide reductase (cyto c), and nitrite reductase (NO-forming)—and extracted the abundances of the six enzymes in the ANCOMBC-identified DA-ASVs. We analyzed the difference between the subset of DA-ASVs and all of the bacterial ASVs in the average copy and prevalence of the six enzymes using Student's *t* test (Student 1908). To identify the bacterial and fungal taxa which were highly associated with canola yield, we applied a greedy algorithm using selbal v. 0.1.0, which outperformed methods commonly used in microbiome analysis such as DESeq2, edgeR, and ANCOM

(Rivera-Pinto et al. 2018). Method selbal can identify the smallest number of taxa with the highest prediction accuracy for our phenotype interest. This algorithm screens the microbial taxa into two groups (i.e., denominator and numerator), which are transformed into a balance using the normalized log ratio of the geometric mean of the abundance value for the two groups of taxa (Rivera-Pinto et al. 2018). The association between canola yield and microbial taxa was indicated by the correlation of canola yield and the balance of the two groups. Prior to selbal analysis, we did microbial taxa filtering. We assumed that the microbial taxa closely associated with yield should popularly cooccur with canola plants (Mamet et al. 2022). We selected microbial taxa presented in ≥75% of samples of each canola variety at each condition (i.e., site, N treatment, growth stage, and compartment [root and rhizosphere]) in two consecutive years, a modified criterion based on Mamet et al. (2022). The microbial taxa identified in root and rhizosphere at two growth stages were annotated in a dendrogram plot using data.tree v. 1.0.0 (Glur 2020) and denextend v.1.16.0 (Galili 2015). Details about the selbal algorithm and how to run the selbal analysis are shown in Supplementary Method S4.

RESULTS

Bacterial and fungal relative abundance and diversity. Bacterial and fungal community composition differed between the canola root and rhizosphere, between site-years, and across early vegetative and flowering stages (Supplementary Fig. S2). Proteobacteria (> 31%) and Actinobacteriota (> 15%) were the dominant bacterial phyla in the root and the rhizosphere across the two experimental sites in both years. Acidobacteriota, Gemmatimonadota, Planctomycetota, Chloroflexi, and Verrucomicrobiota exhibited higher relative abundance in the rhizosphere than in the root (Supplementary Fig. S2a; Supplementary Table S2). The relative abundance of Olpidiomycota in the fungal community was much higher in the root than in the rhizosphere, while Mortierellomycota and Chytridiomycota exhibited higher relative abundance in the rhizosphere than the root (Supplementary Fig. S2b; Supplementary Table S3). Principal component analysis (Supplementary Fig. S2c and d) and PERMANOVA revealed that the rhizosphere versus root explained the most variance in community structure ($F = 292.5$, $P < 0.001$ for bacteria and $F = 614.0$, $P < 0.001$ for fungi), followed by the experimental site ($F = 139.5$, $P < 0.001$ for bacteria and $F = 70.7$, $P < 0.001$ for fungi) and sampling year ($F = 24.9$, $P < 0.001$ for bacteria and $F = 58.0$, $P < 0.001$ for fungi) across site-years.

Growth stage strongly affected the bacterial and the fungal community structures in the root and the rhizosphere across experimental sites in both years (Tables 1 and 2). Variety-driven differences in the bacterial community structure varied across environmental conditions (i.e., site-years) (Table 1); canola variety significantly affected the fungal community structure in the rhizosphere but not in the root for both years at Melfort and Saskatoon (Table 2). N fertilization affected the fungal community structure in the root at Melfort in 2020. However, there were significant two-way interactions between N fertility, canola variety, and growth stage on the bacterial and the fungal community structures in the root and the rhizosphere in some site-years (Tables 1 and 2). The bacterial and the fungal Shannon index in the root and the rhizosphere was altered by canola variety and growth stage in some of the site-years (Table 3). Where Shannon index differences occurred, it was higher in the early vegetative stage than the flowering stage in the rhizosphere but was not consistently higher in the root (Supplementary Fig. S3a and b).

TABLE 1
Effects of nitrogen (N) treatment, *Brassica napus* variety (Var), and growth stage (GS) on the structure of the bacterial communities in the rhizosphere and the roots of the eight *B. napus* varieties grown in Saskatoon and Melfort according to permutational multivariate analysis of variance[a]

	Bacteria											
	Saskatoon						Melfort					
	Root			Rhizosphere			Root			Rhizosphere		
Year, factor	R^2	F	P	R^2	F	P	R^2	F	P	R^2	F	P
2019												
N	0.025	3.211	0.147	0.038	4.667	0.195	0.011	1.538	0.372	0.017	2.171	0.503
Var	0.069	1.246	**0.001**	0.064	1.13	**0.006**	0.057	1.09	**0.004**	0.059	1.108	**0.001**
GS	0.020	2.601	**0.001**	0.018	2.264	**0.002**	0.052	6.922	**0.001**	0.038	4.986	**0.001**
N × Var	0.048	0.871	0.391	0.044	0.785	0.371	0.056	1.062	**0.023**	0.053	0.999	**0.017**
N × GS	0.006	0.746	0.721	0.005	0.591	0.765	0.009	1.219	**0.038**	0.009	1.138	**0.034**
Var × GS	0.039	0.704	0.998	0.029	0.517	1	0.049	0.94	0.581	0.046	0.863	0.798
N × Var × GS	0.038	0.683	1	0.028	0.502	1	0.049	0.938	0.613	0.045	0.839	0.927
2020												
N	0.009	1.165	0.249	0.007	0.98	0.733	0.008	1.091	0.628	0.008	0.993	0.748
Var	0.054	1.049	0.111	0.051	0.994	0.088	0.056	1.079	**0.04**	0.055	1.015	0.138
GS	0.090	12.261	**0.001**	0.103	14.129	**0.001**	0.062	8.266	**0.001**	0.031	4	**0.001**
N × Var	0.050	0.987	0.283	0.051	0.994	0.126	0.052	0.993	0.403	0.053	0.965	0.476
N × GS	0.006	0.87	0.6	0.007	0.931	0.307	0.006	0.87	0.833	0.007	0.908	0.659
Var × GS	0.047	0.917	0.617	0.043	0.845	0.672	0.053	1.012	0.275	0.053	0.975	0.399
N × Var × GS	0.043	0.839	0.932	0.037	0.715	0.993	0.048	0.915	0.942	0.046	0.841	1

[a] Bold indicates P values significant at $P < 0.05$.

Differentially abundant bacteria and fungi under low versus high N. Although N fertilization had a limited effect on the overall α and β diversity of the bacterial and fungal communities, low-N and high-N treatments enriched specific taxa in the root and rhizosphere of the eight canola varieties. The number of bacterial DA-ASVs ($n = 605$) was 2.2 times higher in the root than in the rhizosphere ($n = 270$) (Fig. 1A and B) across the site-years. The opposite trend was observed in the fungal DA-ASVs, where the rhizosphere ($n = 1{,}040$) contained 1.8 times more DA-ASVs than the root ($n = 593$) (Fig. 1C and D).

Argentine and NAM-17 enriched two times more root bacterial DA-ASVs under low N than high N (Fig. 1A) while other varieties had similar DA-ASVs at high versus low N. The root bacterial DA-ASVs under contrasting N fertility were mostly assigned to Gammaproteobacteria (46%), Bacteroidia (23%), and Actinobacteria (18%) (Supplementary Table S4) but the proportions of the three dominant bacterial classes varied distinctly in the eight canola varieties. Argentine, the earliest cultivated canola rapeseed cultivar in Canada, had the lowest proportion of enriched Gammaproteobacteria (36.7%). In the more modern NAM-0 and the Com Hybrid canola variety, Gammaproteobacteria comprised over 50% of the total DA-ASVs (Fig. 1A; Supplementary Table S4).

The profiles of the DA-ASVs at the bacterial class level in the rhizosphere differed from those in the root, even though the Gammaproteobacteria was still a dominant bacterial class. Under high N, Com Hybrid and NAM-0 enriched a higher number of rhizosphere microbial ASVs assigned to Gammaproteobacteria whereas Exp Hybrid and Westar enriched a lower number of microbial ASVs than the other canola varieties (Fig. 1B). Overall, the DA-ASVs identified in the rhizosphere belonged to 23 bacterial classes, over twice as many as in the root across the eight canola varieties (data not shown). This was particularly evident under low N, especially in Argentine and the Com Hybrid, where DA-ASVs were dominated by "others". The proportion of Bacteroidia in the rhizosphere of Argentine (4.1%), NAM-17 (3.2%), and Westar (5.0%) was much lower than that in the root of Argentine (24.5%), NAM-17 (22.7%) and Westar (22.9%) (Fig. 1B; Supplementary Table S4). Within the most dominant class of the DA-ASVs bacterial Gammaproteobacteria, *Pseudomonas* was the dominant genus (30%), containing 36 genera in the root of the eight canola varieties; whereas, in the rhizosphere, *Pseudomonas* and *Serratia* were the dominant genera across the eight canola varieties (Fig. 2A and B).

For the fungal microbiome, the DA-ASVs were mainly assigned to Sordariomycetes (32.3% in the rhizosphere, 38.6% in the root), Dothideomycetes (15.0% in the rhizosphere, 21.8% in the root), and Agaricomycetes (9.8% in the rhizosphere, 11.1% in the root) across the eight canola varieties. In the root, the Exp Hybrid enriched 26.6% and NAM-0 enriched 18.8% more DA-ASVs under high N than low N, and NAM-17 enriched 44.4% more DA-ASVs under low N than high N (Fig. 1C; Supplementary Table S5). NAM-0 and NAM-12 had approximately half less the proportion of Dothideomycetes in the root in comparison with the other canola varieties, whereas they had relatively higher proportions of Agaricomycetes in the root. In the rhizosphere, Argentine, the Com Hybrid, the Exp Hybrid, and NAM-0 enriched 35.2, 24.0, 21.8, and 18.2% more fungal DA-ASVs, respectively, under low N than high N (Fig. 1D; Supplementary Table S5). The Com Hybrid had the lowest proportion of Sordariomycetes in both the root and rhizosphere among the eight canola varieties (Fig. 1C and D; Supplementary Table S5). Unlike bacteria, no dominant genera were present in the profiles of Sordariomycetes in the root or the rhizosphere (Fig. 2C and D).

TABLE 2
Effects of nitrogen (N) treatment, *Brassica napus* variety (Var), and growth stage (GS) on the structure of the fungal communities in the rhizosphere and the roots of the eight *B. napus* varieties grown in Saskatoon and Melfort according to permutational multivariate analysis of variance[a]

	Fungi											
	Saskatoon						Melfort					
	Root			Rhizosphere			Root			Rhizosphere		
Year, factor	R^2	F	P	R^2	F	P	R^2	F	P	R^2	F	P
2019												
N	0.005	0.709	0.493	0.033	4.298	0.101	0.008	1.161	0.526	0.01	1.452	0.609
Var	0.051	1.069	0.282	0.073	1.367	**0.006**	0.048	0.941	0.422	0.087	1.75	**0.001**
GS	0.128	18.538	**0.001**	0.03	3.897	**0.002**	0.083	11.327	**0.001**	0.085	11.936	**0.001**
N × Var	0.051	1.057	0.306	0.039	0.724	0.607	0.057	1.109	0.207	0.04	0.811	0.517
N × GS	0.004	0.546	0.666	0.006	0.724	0.436	0.004	0.532	0.738	0.006	0.795	0.441
Var × GS	0.049	1.024	0.343	0.046	0.868	0.291	0.047	0.914	0.493	0.064	1.278	**0.007**
N × Var × GS	0.051	1.065	0.314	0.039	0.736	0.58	0.05	0.985	0.38	0.026	0.525	0.996
2020												
N	0.006	0.817	0.744	0.01	1.638	0.351	0.009	1.179	**0.002**	0.008	1.106	0.756
Var	0.053	1.035	0.222	0.054	1.25	**0.044**	0.052	1.016	0.352	0.068	1.31	**0.01**
GS	0.095	13.043	**0.001**	0.226	36.356	**0.001**	0.087	11.845	**0.001**	0.049	6.65	**0.001**
N × Var	0.053	1.037	0.229	0.048	1.11	0.1	0.046	0.892	0.592	0.051	0.984	0.321
N × GS	0.004	0.51	0.768	0.005	0.816	0.428	0.015	2.094	0.09	0.006	0.862	0.492
Var × GS	0.049	0.961	0.305	0.036	0.833	0.503	0.04	0.781	0.758	0.054	1.042	0.219
N × Var × GS	0.042	0.828	0.528	0.025	0.576	0.977	0.043	0.832	0.673	0.049	0.933	0.475

[a] Bold indicates P values significant at $P < 0.05$.

In addition, the functional prediction of N metabolism in the bacterial DA-ASVs from both the roots and rhizosphere showed that the average abundance and frequency of nitrate reductase, NADH-nitrite reductase, ferredoxin-nitrate reductase, and nitric-oxide reductase (cytochrome c) were significantly higher in the bacterial DA-ASV community compared with the whole bacterial community (Supplementary Fig. S4a and b). However, the abundance and frequency of nitric-oxide reductase and frequency of nitrite reductase (NO-forming) were not statistically different due to the large differences among the four site-years (Supplementary Fig. S4a and b).

Argentine (1942) was the first rapeseed variety released in Canada, and Westar (1982), NAM-12 (1996), and Com Hybrid (2016) were widely grown commercially for up to 10 years after their respective release dates. We determined the distribution patterns of the bacterial and fungal DA-ASVs in pairwise combinations of the four chronologically released canola varieties as well as the modern open-pollinated NAM-0 in the root and rhizosphere under low and high N (Fig. 3; Supplementary Fig. S5). No consistent bacterial or fungal DA-ASV patterns were observed following chronological comparison of the canola varieties but low N versus high N patterns were distinct between rhizosphere and roots. For example, under low N in the rhizosphere, a diverse assemblage of bacterial DA-ASVs occurred at Saskatoon while Gammaproteobacteria dominated DA-ASVs at Melfort (Fig. 3B and D). In the roots at both low and high N, DA-ASVs were mostly Gammaproteobacteria, Bacteroidia, and Actinobacteria at both Saskatoon and Melfort (Fig. 3A and C). By comparison, fungal DA-ASVs were more similar between low and high N and between sites (Supplementary Fig. S5a to d). Similar to low versus high N comparisons within each variety, Sodiariomycetes, Agaricomycetes, and Dothidiomycetes were commonly found to be differentially abundant between varieties.

Taxa associated with canola yield. The yield of canola varieties varied at different sites ($P < 0.001$), years ($P < 0.001$), and N applications ($P < 0.001$), and the Com Hybrid performed best across all site years (Supplementary Fig. S6a and b). Under each of the low- and high-N conditions, canola yield was strongly associated with a subset of bacterial and fungal communities identified in root and rhizosphere at two growth stages. Over 65% of the bacterial ASVs were assigned to Proteobacteria and Actinobacteriota, and over 68% of the fungal ASVs were assigned to Ascomycota. Only a few ASVs were shared in two or more compartment-stage combinations (i.e., root-early vegetative stage, root-flowering stage, rhizosphere-early vegetative stage, and rhizosphere-flowering stage). Shared ASVs were one bacterial ASV (a *Sphingomonas* sp.) at low N (Fig. 4A), four fungal ASVs (a *Mortierella* sp., an *Exophiala* sp., a *Tetracladium*, and an *Apodus* sp.) at low N (Fig. 4C), and four fungal ASVs (a *Mortierella* sp., an *Alternaria* sp., an *Ophiosphaerella* sp. and a *Gibberella* sp.) at high N (Fig. 4D). For bacteria, three *Sphingomonas* spp. were shown under low and high N (Fig. 4A and B), while three *Streptomyces* spp. were identified at high N (Fig. 4B). For fungi, three *Mortierella* spp. and two *Fusarium* spp. were identified under low and high N. An *Olpidium* sp. was identified at both low and high N (Fig. 4C and D). The number of overlapping bacteria under low and high N was 5, while it was 12 for fungi (Supplementary Table S6). Interestingly, 87 to 95% of yield variation was associated with the identified bacterial community (Supplementary Fig. S7a), while 80 to 94% of yield variation was associated with the identified fungal community (Supplementary Fig. S7b).

TABLE 3
Effects of nitrogen (N) treatment, *Brassica napus* variety (Var), and growth stage (GS) on Shannon index of the bacterial and the fungal communities in the rhizosphere and the roots of the eight *B. napus* varieties grown in Saskatoon and Melfort according to analysis of variance[a]

	Bacteria								Fungi							
	Saskatoon				Melfort				Saskatoon				Melfort			
	Root		Rhizosphere		Root		Rhizosphere		Root		Rhizosphere		Root		Rhizosphere	
Year, factor	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
2019																
N	0.38	0.583	6.63	0.082	0.37	0.586	4.02	0.139	0.13	0.743	<0.01	0.987	0.07	0.808	7.54	0.071
Var	1.02	0.431	0.95	0.481	1.01	0.439	2.25	**0.049**	1.96	0.085	2.35	**0.041**	0.84	0.558	2.95	**0.013**
GS	46.50	**<0.001**	0.03	0.864	0.26	0.615	0.07	0.788	67.01	**<0.001**	3.34	0.074	30.32	**<0.001**	36.39	**<0.001**
N × Var	1.60	0.162	0.38	0.908	0.40	0.896	1.01	0.440	0.87	0.540	0.60	0.749	1.67	0.143	0.79	0.601
N × GS	0.06	0.806	0.66	0.424	0.42	0.520	1.41	0.243	0.35	0.558	0.45	0.503	0.88	0.353	0.69	0.411
Var × GS	1.98	0.079	0.74	0.644	1.07	0.399	1.31	0.270	1.38	0.236	1.59	0.161	1.31	0.266	2.10	0.062
N × Var × GS	0.97	0.462	0.37	0.913	1.73	0.123	1.82	0.108	0.98	0.458	0.84	0.556	1.44	0.210	0.69	0.678
2020																
N	0.62	0.490	0.85	0.424	0.18	0.700	0.12	0.755	0.00	0.975	2.50	0.212	0.16	0.720	2.53	0.210
Var	1.18	0.336	1.78	0.118	0.80	0.590	5.74	**<0.001**	2.06	0.069	0.39	0.905	0.43	0.879	2.74	**0.019**
GS	133.44	**<0.001**	198.30	**<0.001**	<0.01	0.999	26.44	**<0.001**	0.61	0.440	66.98	**<0.001**	38.16	**<0.001**	2.84	0.098
N × Var	0.56	0.783	1.49	0.196	0.61	0.746	1.06	0.404	2.10	0.065	0.13	0.996	1.49	0.197	1.01	0.435
N × GS	1.01	0.319	2.04	0.160	1.15	0.288	0.27	0.605	0.02	0.877	1.68	0.201	2.84	0.098	0.89	0.349
Var × GS	2.14	0.057	2.52	**0.029**	1.13	0.364	5.20	**<0.001**	3.60	**0.003**	0.19	0.985	0.73	0.646	0.90	0.514
N × Var × GS	1.11	0.371	0.92	0.497	0.19	0.986	0.32	0.939	2.07	0.066	1.30	0.270	1.63	0.149	1.01	0.434

[a] Bold indicates *P* values significant at $P < 0.05$.

DISCUSSION

Root-associated microbiomes affected by canola variety and N fertility. The impact of canola variety on the microbial structures was not consistent across the four site-years, indicating that other factors (e.g., soil moisture and temperature) may have a combined effect on shaping the microbial community (Azarbad et al. 2020; Wang et al. 2019). The complex interactions of plant genotype, environmental conditions, and microbiome were recognized in previous studies (Bell et al. 2022; Brown et al. 2020; Morales Moreira et al. 2021; Simonin et al. 2020). The status of N availability significantly affects plant metabolism, which indirectly impacts the root-associated microbiome through plant–soil–microbe communication (Chen et al. 2019; Stitt 1999; Zhao et al. 2021) and directly affects N metabolism in the soil microbiome (Li et al. 2020; Li et al. 2021). Long-term (6 to 50 years) N input has been shown to have a strong influence on the structure and function of soil and root-associated microbial communities (Li et al. 2020; Li et al. 2021; Ren et al. 2020). Moreover, the effect of inorganic fertilizer N input on soil or root-associated microbiomes depends on its application rates (Chen et al. 2019; Zhao et al. 2020). However, in the present study, the contrasting N treatments had a very limited effect on the overall bacterial and fungal community structures in the root and rhizosphere. Canola take up N from two sources, inorganic fertilizer and soil organic matter. A recent greenhouse study showed that over 64% of N uptake in the first 6 weeks of canola growth was from soil organic matter (Carter and Schipanski 2022). It is possible that, here, the signal intensity caused by the two-level inorganic N fertilizer target (low versus high) was not strong enough to significantly affect the overall microbial structure. Rather, N application affected a small subset of the root-associated bacterial and fungal populations through more refined but unmeasured mechanisms.

The patterns of N-affected microbial taxa were different among the eight canola varieties. There were three characteristics of the DA-ASVs of the bacterial and fungal communities under the N treatments: (i) the number of fungal ASVs affected was higher

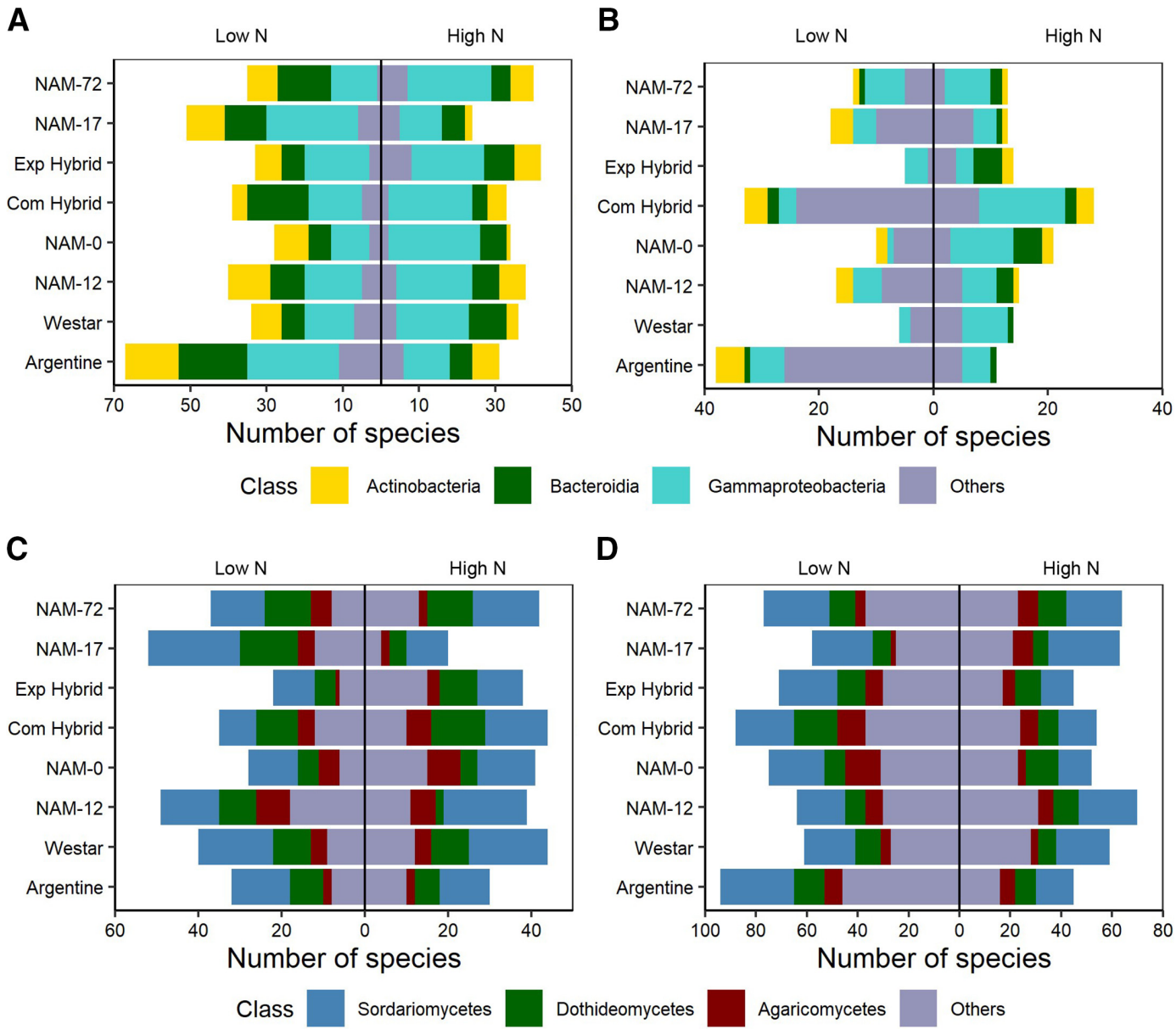

Fig. 1. Number of differentially abundant amplicon sequence variants at class level in the eight canola varieties under low versus high nitrogen (N) addition. **A,** Root bacteria; **B,** rhizosphere bacteria; **C,** root fungi; and **D,** rhizosphere fungi.

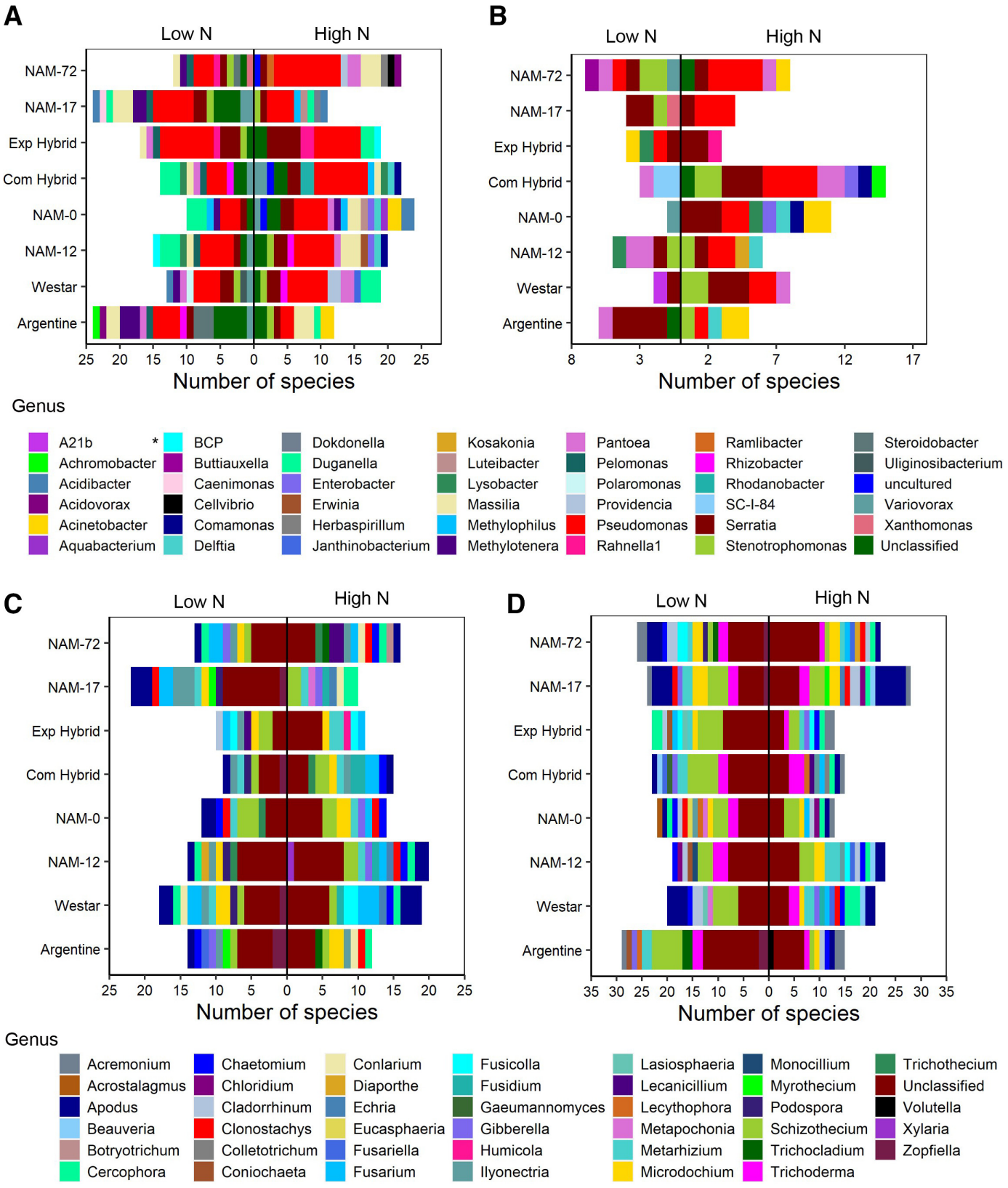

Fig. 2. Number of the differentially abundant amplicon sequence variants assigned to Gammaproteobacteria at genus level in **A,** the root and **B,** the rhizosphere and to Sordariomycetes at genus level in **C,** the root and **D,** the rhizosphere of the eight canola varieties under low versus high nitrogen (N). BCP = *Burkholderia-Caballeronia-Paraburkholderia*.

than bacteria; (ii) the number of the bacterial DA-ASVs was higher in the root than in the rhizosphere, while fungal DA-ASVs were higher in the rhizosphere; and (iii) the DA-ASVs mainly belonged to three classes in both the bacterial (Gammaproteobacteria, Bacteroidia, and Actinobacteria) and fungal (Sordariomycetes, Dothideomycetes, and Agaricomycetes) communities. The relatively higher sensitivity of the fungal community to chemical N fertilizer in comparison with the bacterial community was also reported in long-term fertilized soil and Welsh onion rhizosphere (Pan et al. 2020; Zhao et al. 2020). These differences in the quantity of fungal versus bacterial taxa were at least partially related to their specific abilities to respond to the physiological and biochemical changes of canola plants caused by soil N availability (Chen et al. 2019; Zhao et al. 2021). The other possible reason is that fungi are more sensitive to the soil carbon/nitrogen ratio (C/N) than bacteria (Bahram et al. 2018), and N addition could alter soil C/N in the root region, which affects the fungal community more than the bacterial community.

The opposite distribution patterns of bacterial and fungal DA-ASVs in the root and rhizosphere might be related to their specific functions which assist the plant to adapt to environmental changes (Rodriguez et al. 2009; Sessitsch et al. 2012) and the filtering function of the plant immune system (Hacquard et al. 2017). However, whether the N fertilizer response of root-associated bacterial versus

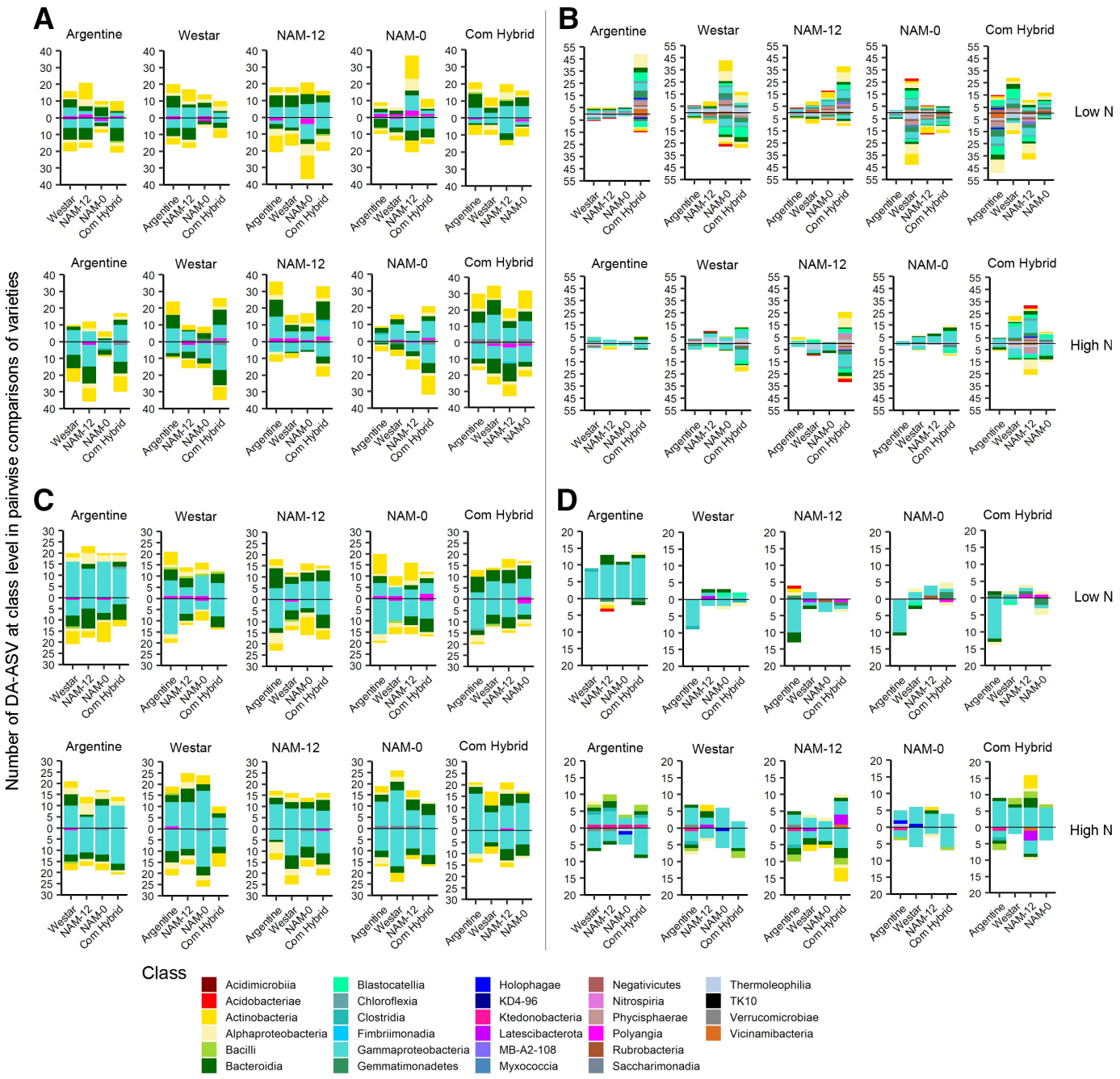

Fig. 3. Number of differentially abundant bacterial amplicon sequence variants at class level in paired comparisons of five canola varieties under low versus high nitrogen (N) in **A,** the root and **B,** the rhizosphere at Saskatoon and in **C,** the root and **D,** the rhizosphere at Melfort. Upper plots were based on low N treatment and lower plots were based on high N treatment. Pairwise varieties shown as one canola variety at the top of each plot versus each of the other four canola varieties along the x-axis.

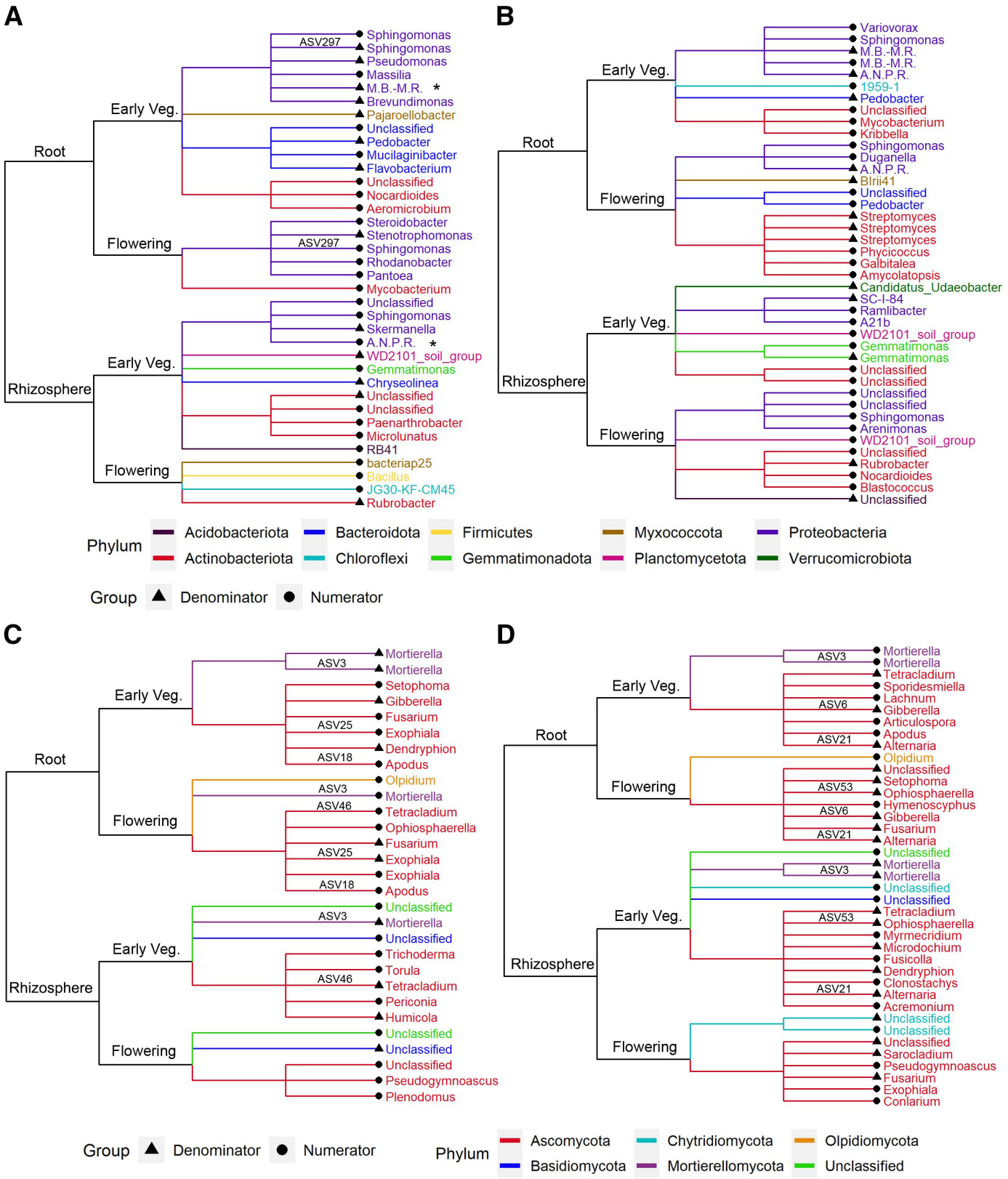

Fig. 4. Bacterial and fungal communities associated with yields of canola varieties at two growth stages in the root and rhizosphere. Bacterial taxa under **A,** low nitrogen (N) fertilization and **B,** high N fertilization and fungal taxa under **C,** low N fertilization and **D,** high N fertilization. Reoccurred amplicon sequence variants (ASVs) were indicated with ASV ID. Early Veg. = early vegetative stage, A.N.P.R. = *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, and M.B.-M.R. = *Methylobacterium-Methylorubrum*.

fungal communities is consistent in other plant species needs further investigation. Gammaproteobacteria, Bacteroidia, and Actinobacteria could play an essential role in assisting canola to respond to soil N availability because PICRUSt2 analysis indicated that the bacterial DA-ASVs contain more average copies and a higher frequency of some N-metabolism genes. *Pseudomonas* was the dominant genus in the differentially abundant Gammaproteobacteria, and it is often reported to promote plant growth and nutrient uptake and mitigate abiotic and biotic stress (Andreolli et al. 2019; Hol et al. 2013; Israr et al. 2016; Nordstedt et al. 2020; Samaddar et al. 2019). A shot-gun metagenomic study reported that Sordariomycetes and Dothideomycetes contained most of the fungal denitrification genes in long-term N-fertilized soil, and Sodariomycetes also holds most of the fungal genes related to N assimilation and dissimilation (Li et al. 2020). This supports the speculation that the N-enriched fungal Sordariomycetes and Dothideomycetes may assist the canola plant to respond to soil N availability. The taxonomic coherence of DA-ASVs within these six classes of bacteria and fungi warrants further investigation into their role in the N response of canola.

The different patterns of DA-ASV profile in paired canola varieties indicate an effect of the canola variety genetic background on the recruitment of microbes. The determinative role of the genetic background on the root-associated microbiome has been reported in many plants such as canola, wheat, maize, and sorghum (Bouffaud et al. 2014; Deng et al. 2021; Kinnunen-Grubb et al. 2020; Taye et al. 2020). Patterns of DA-ASV distribution between canola varieties were also affected by soil N availability, especially bacteria in the rhizosphere, which may reflect the canola varietal difference in utilization of soil N. In addition, the microbial DA-ASV shifts between canola varieties in experimental sites indicated that environmental conditions such as soil physicochemical and biological properties could affect the recruitment of microorganisms by canola varieties (Pii et al. 2016). The specific microbial taxa regulated by plant variety could play an essential role in plant growth and response to environmental challenges; for example, accession-specific differences in root-associated *Pseudomonas* growth enhanced or impaired *Arabidopsis* plant fitness (Haney et al. 2015). *Indica* varieties of rice have better N-use efficiency than *japonica* varieties due to the enrichment of bacterial genera with N metabolism functions (Zhang et al. 2019). Further investigation of which microbial taxa can regulate the N use efficiency of canola varieties is important for future study.

Bacteria and fungi associated with canola yield. A small group of microbial taxa highly associated with canola yield productivity was identified not only at the flowering growth stage but also at early vegetative stage. This indicated that the legacy effect of some microbial taxa at early growth stages might influence canola yield. This finding agrees with Wei et al. (2019), who found that initial rhizosphere microbial composition and functioning predetermined future plant health. Interestingly, some repeatedly identified ASVs under different conditions showed opposite effects on canola yield, indicating that the microbial community network and environmental conditions can influence the function of individual members over time during the plant life cycle. *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was reported as an N-fixing bacteria (Laranjo et al. 2014; Lindström and Mousavi 2020). Our results showed that *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was positively correlated with canola yield at low N but had a negative correlation with canola yield at high N. This was possibly because trade-offs in nutrient N and carbon source between *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and canola were altered under low versus high N fertilization (Sachs et al. 2018). Although the number of yield-associated bacteria identified were higher than the number of fungi under low and high N, the number of overlapped bacteria between low and high N was much lower than that of overlapped fungi, indicating that more diverse bacteria than fungi were associated with canola yield under contrasting N fertilization. Here, we simplified the relationship of microbes and canola yield using their linear correlation; however, the interactions of microbe–microbe, microbe–plant, and microbe–plant–environment are more complicated and might not be linear. Further illustrating how these yield-associated microbes interact with each other and function within the microbiome network under different environmental conditions will contribute to advanced breeding strategies by integrating plant-associated microbiome information.

Conclusion. Based on our findings, Gammaproteobacteria, Bacteroidia, and Actinobacteria bacteria and Sordariomycetes, Dothideomycetes, and Agaricomycetes fungi could be useful targets for more explicit determination of how root-associated microbiomes affect canola N uptake and utilization. High and low N resulted in different DA-ASV profiles both with a single variety and among paired comparisons between varieties. Members of a relatively small but diverse subset of bacteria and fungi were closely associated with canola yield productivity. In the future, the canola varieties' microbial variation responding to soil N availability combined with the information of N use efficiency of canola varieties will identify microbial taxa closely related to canola N uptake and utilization. With this understanding, we can work toward directed breeding strategies that manipulate the root-associated microbiome to optimize the economic and environmental performance of canola.

Data availability. The sequencing data have been deposited in NCBI under the accession number PRJNA765681 (https://www.ncbi.nlm.nih.gov/sra/PRJNA765681).

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