Rhizosphere Microbiomes in a Historical Maize/Soybean Rotation System respond to Host Species and Nitrogen Fertilization at Genus and Sub-genus levels

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Abstract

Root associated microbes are key players in plant health, disease resistance, and nitrogen (N) use efficiency. It remains largely unclear how the interplay of biological and environmental factors affects rhizobiome dynamics in agricultural systems. Here, we quantified the composition of rhizosphere and bulk soil microbial communities associated with maize (Zea mays L.) and soybean (Glycine max L.) in a long-term crop rotation study under conventional fertilization and low N regimes. Over two growing seasons, we evaluated the effects of environmental conditions and several treatment factors on the abundance of rhizosphere and soil colonizing microbial taxa. Time of sampling, host plant species and N fertilization had major effects on microbiomes, while no effect of crop rotation was observed. Using variance partitioning as well as 16S sequence information, we further defined a set of 82 microbial genera and sub-genus groups that show distinct responses to treatment factors. We identified taxa that are highly specific to either maize or soybean rhizospheres, as well as taxa that are sensitive to N fertilization in plant rhizospheres and bulk soil. This study provides insights to harness the full potential of soil microbes in maize and soybean agricultural systems through plant breeding and field management.

Introduction

Crop rotations of maize and soybean exploit the symbiotic relationship of legumes with nitrogen (N) fixing bacteria. This rotation system has historically been a widespread practice in the U.S and continues to be employed as a supplement to synthetic N fertilizer (Peterson and Varvel, 1989). Soybean-maize (Jagadamma et al., 2008) and other crop rotations in general (Drinkwater et al., 1998; Peralta et al., 2018) have also shown beneficial effects on crop yield, disease resistance, weed management and soil nutrient conservation. Root-colonizing soil microbes may play a role in N use efficiency (Garnett et al., 2009), plant health (Berendsen et al., 2012) and crop performance (Yadav et al., 2018) in agricultural fields. Furthermore, the capacity of plants to recruit a specific set of beneficial microbes can potentially be employed in plant breeding and genetic engineering to improve disease resistance and yield potential of crop plants while reducing the application of exogenous fertilizer and pesticides (Chaparro et al., 2012; Compant et al., 2010; Haichar et al., 2008; Huang et al., 2014).

Soil and rhizosphere microbial communities have been studied in several major crop species including maize (Peiffer et al., 2013), soybean (Mendes et al., 2014), wheat (Donn et al., 2015) and rice (Edwards et al., 2015), as well as in crop rotation systems, including maize-wheat (Rascovan et al., 2016), wheat-maize-soybean (Gdanetz and Trail, 2017) and more complex systems (Peralta et al., 2018). Similarly, the effects of N-fertilization on microbial communities have been studied in maize (Zhu et al., 2016), wheat (Kavamura et al., 2018), and rice (Ikeda et al., 2014). These studies have shown that crop plant species, N-fertilization, and possibly crop rotation affect rhizosphere microbial community structure. However, it is largely unknown how these factors together shape rhizosphere and soil microbial communities in the context of contemporary farm management practices, and how these factors rank in terms of their impact on the abundance of distinct rhizosphere and soil colonizing microbial taxa. For instance, it has

been unclear whether maize and soybean planted in succession in the same field would adopt similar root microbiomes in response to soil "memory" induced by the previous year's crop (Lapsansky et al., 2016), or if the effect of the host plant would outweigh any crop rotation effects.

Here, we leveraged a long-term experimental field with consistent crop rotations (established 1972) and N fertilizer regimes (established 1983) (Peterson and Varvel, 1989; Varvel, 2000) in a two year replicated experiment. Through 16S sequencing of rhizosphere and bulk soil samples and statistical modeling of individual amplicon sequence variants (ASVs), we aim to rank the impact of agriculturally relevant factors, including environmental conditions (year and month of sampling), biological factors (crop plant species), and agricultural practices (N fertilization and crop rotation) on the abundance of rhizosphere and bulk soil colonizing microbes. We further aim to identify microbial taxa that respond to these diverse treatment factors as consistent units. Among these taxa, we aim to identify the key respondents that are specific to either maize or soybean, and taxa that respond to inorganic N-fertilization or the lack thereof.

Materials & Methods

Experimental design and sample collection

Maize and soybean plots in a historic long-term crop rotation study at the Eastern Nebraska Research Extension Center near Mead, NE (41.167380, -96.418667) were arranged in a randomized complete block design (Peterson and Varvel, 1989). Detailed site, management, yield and long-term weather information can be accessed at the USDA-ARS Agricultural Collaborative Research Outcomes System (AgCROS) website (https://agcros-usdaars.opendata.arcgis.com/). For this study, plants were sampled from two replicate blocks in each of two subsequent years (2017 and 2018). Each replication included four plots planted with continuous maize (M), continuous soybean (S), maize rotated with soybean (MS), and soybean rotated with maize (SM). Each plot contained a subplot with standard N treatment (180 kg/ha annually for maize, 68 kg/ha for soybean) and a subplot with low N conditions (no added N). From each of those subplots (experimental units), two subsamples, each for plant rhizosphere and bulk soil were collected in June, August, and September (7, 14, and 20 weeks after planting). In total, 384 samples were collected (2 years x 3 months x 2 plant species x 2 crop rotations x 2 N treatments x 2 soil compartments x 2 blocks x 2 subsamples = 384), see Fig. 1. This experimental design made it possible to distinguish 5 experimental factors: year of sampling (year 1 or year 2), month of sampling (early, mid and late season), plant species (maize or soybean), crop rotation (continuous vs. rotated), and N treatment (standard N fertilization or low N conditions). All analyses were conducted separately for rhizosphere soil and bulk soil.

16S rRNA sequencing and microbial community analysis

Genomic DNA was extracted from n=192 rhizosphere and n=192 bulk soil samples using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany). Paired-end sequencing of a 300-bp sequence spanning the V4 region of the ribosomal 16 S rRNA was generated using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Overall, sequencing yielded 41.4M raw 16S reads for 384 samples with a median number of 121k reads per sample for rhizosphere and 103k reads per sample for bulk soil samples. ASVs were called using a dada2-based pipeline as described by (Callahan et al., 2016a, 2016b). After a series of quality and abundance filtering steps (see Fig. S1), a final set of 4.3M reads were retained that belong to a curated set of 2,225 unique ASVs derived from both rhizosphere and bulk soil samples. The median read count per sample was 13.1k for rhizosphere and 5.9k for bulk soil samples.

Grouping of ASVs into taxonomic groups

ASVs were initially grouped at the genus level. This is the lowest taxonomic level where groups of operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) can be reliably annotated using short reads of 16S rDNA alone based on the SILVA reference database (Yilmaz et al., 2014). Sub-genus groups were further identified based on taxonomic clustering of each genus' ASVs and associated variance partitioning data. For each of 87 genera, a phylogenetic tree of all ASVs was plotted together with the variance scores. This procedure allowed us to identify a total of 105 genera and sub-genus groups that show distinct and unambiguous responses to treatments. 82 groups that had at least five distinct ASVs were used for subsequent analyses. For each set of ASVs that mapped to a genus in which subgroups were identified, open-reference OTU picking was performed in qiime (Caporaso et al., 2010) to cluster ASVs into OTUs. The number of OTUs generated through this OTU picking procedure

was compared to the number of groups identified through manual identification of genus subgroups (Table S1).

Statistical analysis

Variance partitioning was performed on the ASV table with log transformed relative abundances to estimate the contribution of each treatment factor to changes in microbiome composition in rhizosphere and bulk soil. For each of 2,225 ASVs present in rhizospheres and a subset of 2,014 ASVs present in bulk soil, the fraction of total variance explained by each treatment factor was calculated using R package lme4 (Bates et al., 2015) with the model log(ASV relative abundance) ~ Year + Month + Host species + Crop rotation + Nitrogen + Block + Subsample, where year, month, host species, crop rotation, nitrogen, block, and subsample were all fit as random effects.

Differential abundance of taxonomic groups in response to treatments was calculated with R package DESeq2 (Love et al., 2014): starting from the ASV table with raw sequence counts, ASVs were agglomerated into 82 taxonomic groups identified above, and a +1 pseudocount was added to all table values. Unless stated otherwise, n = 96 samples were used for comparisons, e.g. 96 soybean rhizosphere samples vs. 96 maize rhizosphere samples.

For a detailed description of experimental procedures and data availability view supplementary methods.

Results

Rhizosphere and soil microbiomes in a historical crop rotation system are highly dynamic over time and across niche environments

Because our field experiments are subject to year and seasonal effects (Fig. S2), our first analysis was to assess how rhizosphere and bulk soil microbiomes vary across early, mid and late season sampling time points in two consecutive years. Principal coordinates analysis (Fig2 A) revealed the time of sampling to be the largest source of variation (PCoA axis 1, 34%), followed by soil compartment rhizosphere vs. bulk soil (PCoA axis 2, 20.4%). Time point variation may be attributable to temperature and precipitation patterns. In particular, the last sampling time point in 2018 occurred soon after a major precipitation event associated with drastic changes in microbial community composition (Fig. S2). Rhizosphere and bulk soil microbiomes are more dissimilar in soybean than in maize with clear separation along axis 2 in the PCoA plot. In both soil compartments, we observed higher microbial diversity in 2018 than in 2017 as measured by the Shannon diversity index (Fig2 B). In addition, both bulk soil and rhizosphere microbiomes tended to increase in diversity as the season progresses (Fig2 B).

Environment, host plant, and agricultural practice together shape microbial communities

We fit a mixed linear model for each ASV as a response variable in order to reveal in more detail to what degree microbial communities are influenced by different treatment factors (see materials and methods). Through variance partitioning, we calculated the proportion of total variance attributable to each treatment factor (termed “variance scores”) for rhizosphere (2,225 ASVs) and bulk soil (2,014 ASVs). We tallied the number of ASVs that are responsive to

172 treatment – defined here as any ASVs with a variance score above an arbitrary threshold of 5%
173 – to estimate the relative importance of each treatment factor in shaping microbiome
174 composition (Fig. 3). For rhizosphere data, out of n= 2,225 ASVs, we found 1,115 (50.1%)
175 responsive to year and 835 (37.5%) responsive to month above the 5% threshold. For bulk soil
176 data, out of n= 2,014 ASVs, we found 668 (34.2%) responsive to year and 639 (31.7%)
177 responsive to month. These results agreed with our previous observations (Fig. 2) and
178 suggested environmental factors affect microbiome abundance in the rhizosphere more than in
179 bulk soil.
180
181 Interestingly, microbial communities responded to host plant species to a statistically significant
182 higher degree in the rhizosphere than the bulk soil (Chi-squared test, p-value = 2.2e-16), with
183 variance scores of 618 ASVs in rhizosphere and only 88 ASVs in bulk soil exceeding 5%.
184 Employing a threshold of 10% reveals a similar pattern with 422 ASVs in the rhizosphere and 9
185 ASVs in bulk soil exceeding the threshold (Chi-square p-value = 2.2e-16), and patterns were
186 overall consistent at thresholds of 2.5% or 10% (Fig. S3). For 36 ASVs in the rhizosphere, more
187 than 40% of total variance was explained by host plant species whereas no response was
188 observed in bulk soil. These results are consistent with the idea that rhizosphere ecosystems
189 are home to highly specialised microbes that have co-evolved alongside plant hosts, whereas
190 bulk soil harbors more uniform microbial communities.
191
192 Among factors related to agricultural practice, we found that 5% or more variability was
193 explained by N treatment in 539 rhizosphere ASVs and in 300 bulk soil ASVs (Chi-square p-
194 value = 3.6e-14), with scores exceeding 20% for 71 and 42 ASVs (Chi-square p-value =
195 0.03267), respectively. In contrast, response to crop rotation was negligible in both rhizosphere
196 and bulk soil, suggesting that the previous year's crop has at best a minor effect on microbial

197 community composition in any given year. We detected no noticeable variation due to
198 experimental blocks and subsamples.
199

200 Response to host species and N treatment reveals groups of microbial taxa
201 at sub-genus level

202 As responses to host plant species and N treatment were apparent at the level of individual
203 ASVs, we hypothesized that the responsive ASVs might be clustered into taxonomic groups. In
204 order to address this hypothesis, we binned ASVs into 87 distinct microbial genera based on
205 SILVA taxonomy annotation. However, by plotting all ASVs within each genus against the
206 variance scores in response to plant host species and N treatment, we noticed a high range of
207 values in some cases (Fig. S4), suggesting that there may be distinct groups of ASVs within the
208 same genus that show different responses to treatments.
209 To achieve taxonomic resolution beyond the genus level, we constructed a phylogenetic tree of
210 all ASVs in each genus together with the variance scores in response to host plant species and
211 N treatment in the rhizosphere (see materials and methods). Using this approach, we identified
212 subgroups in 12 genera: *Streptomyces*, *Chitinophaga*, *Flavobacterium*, *Pedobacter*,
213 *Mucilaginibacter*, *Burkholderia*, *Pseudomonas*, *Sphingomonas*, *Sphingobium*, *Mesorhizobium*,
214 *Nitrobacter*, and *RB41* with distinct patterns of variance partitioning (Supplementary file 1). For
215 example, the genus *Burkholderia* (Fig. 4A & 4B), shows two clusters of ASVs (*Burkholderia*_S1,
216 n= 29 ASVs and *Burkholderia*_S2, n= 28 ASVs) that exhibited significantly different variance
217 scores (Wilcoxon rank sum test p-value = 2.2e-16). These clusters are further grouped by
218 phylogeny, which may indicate separate evolutionary lineages. We refer to these groups as sub-
219 genus groups in this study to draw a distinction between groups identified here by 16S
220 phylogeny and variance partitioning, and microbial “species” that are categorized in some 16S

sequence databases other than SILVA based on sequencing information alone. In total, a final set of 82 taxonomic groups (genera and sub-genus groups) was defined that responded to treatments as a unit. These groups spanned 64 genera and 12 classes of prokaryotes and contained between 5 and 102 ASVs, displayed in a phylogenetic tree (Fig. 4C) generated based on 300 bp 16S sequences and rooted using the outgroup *Candidtus_Nitrocosmicus* (*Archaea*). This set of 82 taxa was used for subsequent analyses in this study. Total abundances of each group were estimated by the sum of read counts across all samples (Fig. S5).

To evaluate how our ASV grouping method compares to automated OTU clustering, OTU picking was performed on the sets of ASVs within each of the 12 genera for which we identified sub-genus groups (see materials and methods). The number of sub-genus groups generated by classical OTU picking at a fixed 97% sequence identity threshold was in many cases larger than the number of subgroups identified using our method, which may indicate some redundancy (Table S1). In other cases (including *Burkholderia* in Fig. 4A & 4B), OTU picking failed to identify sub-genus groups altogether, even though variance partitioning data shows a clear distinction in the behavior of groups of ASVs.

Host plants strongly affect rhizosphere microbial communities and have little influence over bulk soil

Fig. 5 shows 26 taxa that are responsive to host plant species in the rhizosphere, using a 5% variance score as a cutoff. The variance scores are reported together with the Log2 Fold Change (Log2FC) differential abundance of ASV counts in n = 96 soybean vs n = 96 maize rhizosphere samples, and ranked by the response to host plant species. In contrast, no taxonomic groups responded to plant species above the 5% threshold in bulk soil, with the exception of *Rudaea* (see Fig. S6 for complete data).

Maize and soybean recruit distinct and highly specialized microbial taxa to rhizospheres

Several rhizosphere-dwelling taxa showed a strong response to host plant species. 9 out of the top 10 taxa responding to host plant species are specific to soybean (Fig. 5). These include *Bradyrhizobium, Rhizobium*, *Nordella*, *Nitrobacter*, *Novosphingobium*, *Phenylobacterium*, *Streptomyces_S1*, *Allostreptomyces*, and *Chitinophaga_S2*. Among the three members of the *Sphingomonadaceae* family, *Novosphingobium* (Log2FC = 5.98, FDR adjusted p-value = 1.44e-105) was highly specific to soybean, whereas *Sphingobium_S1* (Log2FC = -6.88 FDR = 1.67e-93), *Sphingobium_S2* (Log2FC = -6.12, FDR = 1.02e-83) and *Sphingomonas_S1* (Log2FC = -5.03, FDR = 6.97e-32) were specific to maize. *Sphingomonas_S2* (Log2FC = -0.77, FDR = 0.0107) shows no substantial host preference. Similarly, within the *Streptomyces* genus, *Streptomyces_S3* (Log2FC = 4.81, FDR = 7.82e-63) was highly specific to soybean whereas *Streptomyces_S2* (Log2FC = -2.00, FDR = 1.94e-07) showed a preference for maize, and *Streptomyces_S1* (Log2FC = -0.15, FDR = 0.5928) was found in roughly equal proportions in soybean and maize. *Burkholderia_S1* (Log2FC = -5.63, FDR = 2.50e-43) was highly specific to maize whereas *Burkholderia_S2* (Log2FC = 0.01 FDR = 0.9786) appears to have no preference (compare also with Fig. 4B).

Nitrogen treatment affects soil and rhizosphere microbiomes directly and indirectly via host plant effects

Fig. 6 shows microbial taxa that respond to N treatment at a threshold of >5% variance explained. We hypothesized that the N treatment would affect rhizosphere microbiomes of maize and soybean differently, hence differential abundances of microbial taxa were analyzed

separately for n=48 low N vs n=48 std N maize rhizosphere samples and for n=48 low N vs n=48 std N soybean rhizosphere samples(Fig 6A). For comparison, differential abundance of microbes between maize and soybean was shown as before (Fig 6A, rightmost panel). For bulk soil, comparisons of n=96 low N vs n=96 std N samples were made with samples from both maize and soybean fields (Fig 6B). The complete data is shown in (Fig. S7). Overall, more taxa were responsive in rhizosphere samples (n=20) than in bulk soil (n=8) at a threshold of >5% variance explained by N treatment. Notably, several taxa responded to N treatment both in bulk soil and in rhizospheres: *Nitrospira*, *Sphingomonas_S1* & *Sphingomonas_S2*, *Rudaea*, *Nocardioides*, and *UTBCD1* (marked bold in Fig. 6). Among these taxa, *UTBCD1* increased under low N whereas the other groups increased under std N in both bulk soil and rhizospheres. Two subgroups of genus *RB41*, *RB41_S1* and *RB41_S2*, were responsive to N treatment exclusively in bulk soil, whereas *RB41_S3* was responsive in both rhizospheres. *RB41_S1* and *RB41_S2* increased under std N whereas *RB41_S3* was highly increased under low N.

Groups that mainly respond to N treatment in both rhizospheres include *Burkholderia_S1* & *Burkholderia_S2*, *Mucilaginibacter_S1*, *Mesorhizobium_S1*, *Massilia*, *Streptomyces_S2*, *Pseudomonas_S2* & *Pseudomonas_S4*, *RB41_S3*, *Phenylobacterium*, *Sphingobium_S1*, *Gemmatimonas*, *Terrimonas* and *Gaiella*.

These data suggest that N fertilization has a direct effect on the 6 microbial taxa that respond in both rhizosphere and bulk soil environments, as well as an indirect effect on taxa that only respond in rhizospheres, which is likely induced by changes in the host plant rhizosphere.

Maize rhizosphere microbiomes are affected by N-deficiency

Differential abundance of microbial groups tends to be more extreme in maize than in soybean. This was noticed by calculating the means of absolute Log2 Fold Changes (low N ASV counts

vs std N ASV counts) for maize and soybean in rhizospheres (maize mean Log2FC 1.945735 vs soybean mean Log2FC 0.9755595, Welch two sample t-test p-value 1.54e-05) as well as in bulk soil (maize mean Log2FC 1.51321 vs soybean mean Log2FC 0.8643147, p-value 0.001722). The vast majority of taxa responding to N treatment occur in greater numbers in maize than in soybean rhizospheres (Fig 6A, rightmost panel). While responses to N treatments are generally more pronounced in maize rhizospheres than in soybean rhizospheres, the direction of the changes seems to be consistent between host plant species, with a few notable exceptions: *Rudaea* are more abundant under standard N treatment than under low N in maize rhizospheres (and in bulk soil), whereas no response to N treatment was observed in soybean rhizospheres. Similarly, *Pseudomonas_S4* and *Pseudomonas_S2* increase in abundance under low N in maize rhizospheres but not in soybean rhizospheres.

Maize showed a severe N-deficiency phenotype, especially late in the season. This is known to dramatically change root architecture and exudation patterns (Gaudin et al., 2011). In contrast, soybean plants are more tolerant to a wide range of N fertilizer, which is reflected in a more stable root microbiome. Together, these data show that variation in N levels likely has a direct effect on soil microbes as well as an indirect effect through the impact of N levels on plant health and root exudation, which is most apparent in maize.

Discussion

Environmental factors, plant species and N treatments affect rhizosphere microbiota

Through statistical modeling of individual ASVs and variance partitioning we identified three major factors influencing rhizosphere microbial communities: time of sampling, plant species and N treatment. Year-to-year variation due to different weather conditions is common in agricultural experiments, and soil microbial communities are known to be affected by changes in temperature or humidity (Ullah et al., 2019; van der Voort et al., 2016, Fig. S2). Seasonal variation has an additional biological cause as host plant physiology – including root exudation – changes significantly as plants mature (Shi et al., 2015). Apart from environmental factors, the host plant species is the most important factor shaping rhizosphere microbiomes. Genetic distance between plant species (Fitzpatrick et al., 2018) and between genotypes of the same species (Bouffaud et al., 2014) seems to correlate with differences in microbial communities. N fertilization had an effect on both rhizosphere and soil microbial communities, which has been observed before in maize (Zhu et al., 2016). Our data show that both host plant genetics and N fertilization are major factors influencing microbial communities in maize/soybean agricultural systems. It may thus be possible to modify the composition of microbial communities in the field through plant breeding and the mode of fertilizer application, respectively.

Our data do not support a major effect of crop rotation on bulk soil or rhizosphere microbiomes when compared to other environmental and experimental factors. Thus more targeted experiments are required to discern any changes in bulk soil and rhizosphere microbiomes in response to different cropping histories at agricultural field sites.

ASVs and variance data enable unprecedented taxonomic resolution

A common practice in observational microbiome studies is to cluster 16S amplicon sequences into operational taxonomic units (OTUs) in bins of 97% sequence similarity, and conclusions about microbial communities are drawn often at the level of bacterial phyla or classes (Bragina et al., 2015), and rarely at lower taxonomic ranks such as families (Santos-Medellín et al., 2017). However, in a highly competitive environment such as the plant rhizosphere we would expect to find highly specialized groups of microbes that react differently to a variety of treatments and any such effects would not be apparent at higher taxonomic ranks. Moreover, OTUs may not correspond to any established taxonomic rank or experimentally distinguishable group of microbes that can be studied as a unit (Yilmaz et al., 2014).

To circumvent the problems inherent to OTU clustering, we employed variance partitioning on individual amplicon sequence variants (ASVs) and used these data to complement DNA sequence information. This novel approach allowed us to identify biologically relevant taxonomic groups at the genus and sub-genus level. Importantly, we showed that traditional OTU picking would have under- or overestimated the number of sub-genus groups in most cases (Table S1). Most interestingly, the two subgroups of *Burkholderia* identified in this study, which show significantly different responses to host plant species (Fig. 4A & 4B), would have been missed entirely with traditional OTU picking. Thus, we demonstrated that multifactorial experimental designs may be exploited to improve taxonomic resolution in microbiome studies using both 16S sequence information and variance partitioning data. It may be worthwhile to formalize and automate this process using appropriate statistical tools or machine learning approaches, and to re-analyze previously published data sets whenever there are treatment factors involved that could be used to distinguish groups of microbes.

Host plant species are a key predictor of rhizosphere microbial communities

Overall, higher microbial diversity was observed in rhizospheres than in bulk soil, which is consistent with previous studies (Prashar et al., 2014). Also in accordance with previous research (Wang et al., 2017), we observed strong responses to host plant species in both maize and soybean rhizospheres and no response in bulk soil sampled only a few centimeters away from root surfaces. An immediate effect of host plants on bulk soil microbiomes is not expected as root exudate concentrations decline exponentially and reach virtually zero only 7 mm into the soil (Kuzyakov et al., 2003).

The top 6 taxa responding to plant host species are specific to soybean. Unsurprisingly, they include N fixing bacteria such as *Bradyrhizobium*, *Rhizobium* and closely related *Nordella*. These were previously identified as key components of soybean microbiomes (Sugiyama et al., 2014). Nitrobacter is closely related to Bradyrhizobium and involved in Nitrite oxidation (Boon and Laudelout, 1962). *Novosphingobium*, *Phenylobacterium*, *Streptomyces* and *Allostreptomyces* have no known role in the N cycle. One notable observation was that *Novosphingobium* is highly specific to soybean, and *Sphingobium* and *Sphingomonas* are specific to maize, while all three genera are members of the *Sphingomonadaceae* family. This demonstrates once again the need for adequate taxonomic resolution when comparing microbial communities.

Novosphingobium has been found in the rhizosphere of *Arabidopsis* (Lin et al., 2014), maize (Kampfer et al., 2015), lettuce (Schreiter et al., 2014) and rice (Zhang et al., 2016), and to our knowledge it has not previously been reported as a prominent member of soybean rhizospheres. It remains to be confirmed whether *Novosphingobium* can be found in soybean rhizospheres in different geographic locations and in different soybean cultivars. *Sphingomonas* has been isolated previously from maize rhizospheres and proposed as a good candidate for

385 microbial fertilizers due to N-fixation capabilities (Sun et al., 2010). A previous study (Li et al.,
386 2014) has found *Sphingobium* to be significantly enriched in the maize rhizosphere compared to
387 bulk soil, which was consistent with our findings. Sphingobium has also been found in
388 rhizospheres of other grasses such as sorghum (Kochar and Singh, 2016) and common reed
389 (Toyama et al., 2009), as well as in distantly related plants such as pine trees (Lee et al., 2019)
390 and Kumquat (Young et al., 2008). Members of the Sphingobium genus were shown to degrade
391 phenolic compounds such as the biocide pentachlorophenol (Dams et al., 2007) and to
392 solubilize inorganic phosphates (Yongbin Li et al., 2017). Furthermore, An aryloxyalkanoate
393 dioxygenase gene derived from *Sphingobium herbicidivorans* has been successfully expressed
394 in maize to confer resistance to a broad range of herbicides (Wright et al., 2010).
395
396 Together, our results show that the host plant strongly influences microbial communities in the
397 rhizosphere, with minimal effect on bulk soil, and that specific taxonomic groups at the genus
398 and sub-genus level are highly adapted to either host plant. These data are consistent with the
399 idea that maize and soybean rhizospheres are colonized by highly specialized groups of
400 microbes that are likely in a symbiotic relationship with the host plant and may be relevant to
401 plant health and performance.
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404 N treatment affects rhizosphere microbiomes both directly and indirectly via
405 host plant effects

406 The vast majority of taxa responding to N fertilizer are more abundant in maize rhizospheres
407 than in soybean rhizospheres, whereas soybean-specific taxa generally do not respond to N
408 treatments (see Fig. 6A, rightmost panel and Fig. S6). This may be because maize shows a

409 severely stressed phenotype under N-deficiency, especially late in the season, which induces
410 large-scale changes to root architecture, including root hair length and density (Gaudin et al.,
411 2011). N-limited conditions have also been shown to alter plant root exudate profiles (Baudoin
412 et al., 2003; Haase et al., 2007). In contrast, soybean plants are hardly affected if fields are not
413 fertilized. Thus, two factors shape microbial communities in agricultural systems: direct
414 application of N fertilizer to the soil, which should affect both rhizosphere and bulk soil microbes,
415 and changes due to altered root architecture and exudation patterns in response to N
416 deficiency, which should mainly affect rhizosphere microbiomes. In accordance with this, we
417 found more taxa affected by N treatment in rhizospheres than in bulk soil. Microbial taxa directly
418 affected by N treatment are likely the ones that show a response to N treatment in both
419 rhizosphere and bulk soil samples (marked in bold in Fig 6). All other taxa are likely affected
420 indirectly, and reduced abundance under N deficiency may be due to reduced vigor of the host
421 plant rather than due to a simple lack of inorganic N to consume.
422 These findings also support the idea that plant rhizospheres are colonized by highly specialized
423 groups of microbes that are intimately tied to the host.
424
425 Taxa that increase in abundance under standard N fertilization are often capable of directly
426 metabolizing ammonia or nitrate. *Rudaea*, a member of the *Xanthomonadaceae* family, has not
427 been reported in maize or soybean rhizospheres but has been linked to nitrification in
428 wastewater (Dong et al., 2016). Similarly, *Gemmatimonas*, *Nitrospira*, *Mesorhizobium*,
429 *Burkholderia*, *Rudaea*, and *RB41* were shown to be key players in N assimilation (Morrissey et
430 al., 2018). Burkholderia and Sphingomonas decrease in abundance under low N conditions in
431 both maize and soybean rhizospheres, even though many members of the genus have N-fixing
432 capabilities (Caballero-Mellado et al., 2007; Sun et al., 2010). This may indicate that the
433 reduced abundance could also be due to changes in the rhizosphere environment other than a

434 direct lack of N. This reinforces the idea that rhizosphere microbiomes are primarily shaped by
435 host plant effects and to a lesser degree by external treatments such as N fertilization.
436
437 Taxa that increase in abundance under low N conditions in plant rhizospheres may be able to
438 take advantage of reduced plant vigor under N-deficiency. Conversely, we suggest that some
439 microbes may also be actively recruited by plants if they confer a growth or disease resistance
440 benefit under low N stress conditions. The *Pseudomonas* genus contains both opportunistic
441 pathogens and stranis with plant-growth promoting activity (Santoyo et al., 2012) and some
442 groups have previously been observed in maize rhizospheres under low N conditions.
443 *Terrimonas*, *Gaiella* and *Gemmatimonas* have been observed in maize rhizospheres before
444 (Correa-Galeote et al., 2016), although their function is unknown. *UTBCD1* (*Chitinophagaceae*)
445 and *RB41_S3* (*Pyromonadaceae*), both uncultured bacteria, increased the most under low N
446 conditions (Liljeroth et al., 1990). Overall, surprisingly little is known about these taxa that
447 respond positively to N-deficiency in rhizospheres and it remains to be determined whether they
448 are simple opportunists, whether they cause disease, or whether they actively respond to
449 changes in root exudate profiles under low N conditions, and if so, whether they have plant-
450 growth promoting capabilities that could be exploited to improve agricultural production.
451

452 Conclusions

453 In this study, we observed that rhizosphere and bulk soil microbiomes are primarily shaped by
454 seasonal effects due to environmental changes, host plant species, and N treatment, whereas
455 crop rotation of maize and soybean seems to be of minor importance. This suggests that maize
456 and soybean rhizosphere microbiomes can potentially be manipulated through targeted plant
457 breeding and farm management. We defined a set of 82 taxonomic groups at the genus and
458 sub-genus level based on both 16S sequence information and responses to treatment variables.

459 This allowed us to identify biologically meaningful groups of microbes that are relevant in maize
460 and soybean production. We found groups of microbes that are highly adapted to either the
461 maize (e.g. *Sphingobium*) or the soybean host (e.g. *Novosphingobium*), which may be relevant
462 to plant health and performance. Lastly, we showed that N fertilization or the lack thereof has a
463 direct effect on the abundance of several groups of microbes in bulk soil and rhizospheres as
464 well as an indirect effect via reduced host plant vigor that is most apparent in maize. The
465 findings presented in this work enhance our understanding of the key factors that influence
466 rhizobiome compositions in two major crop plants under conventional and N-limited farming
467 practices. Further research in this direction may open avenues to sustainably improve crop
468 performance in agricultural industry.
469

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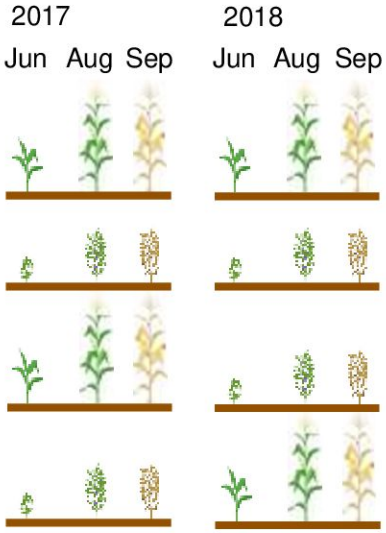
A



M continuous maize

S continuous soybean

MS maize/soybean rotation

SM soybean/maize rotation

C

Experimental factors

2	Years
3	Months
2	Plant species
2	Crop rotations
2	N treatments
2	Soil compartments
2	Blocks
2	Subsamples
384	samples total

B

field layout 2018

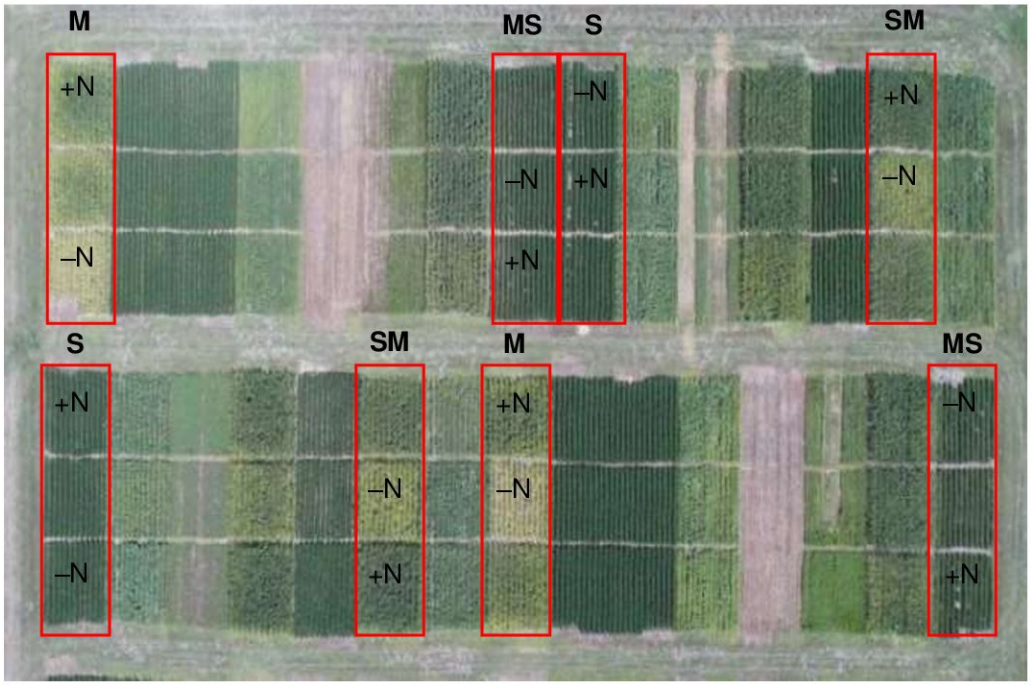


Fig. 2

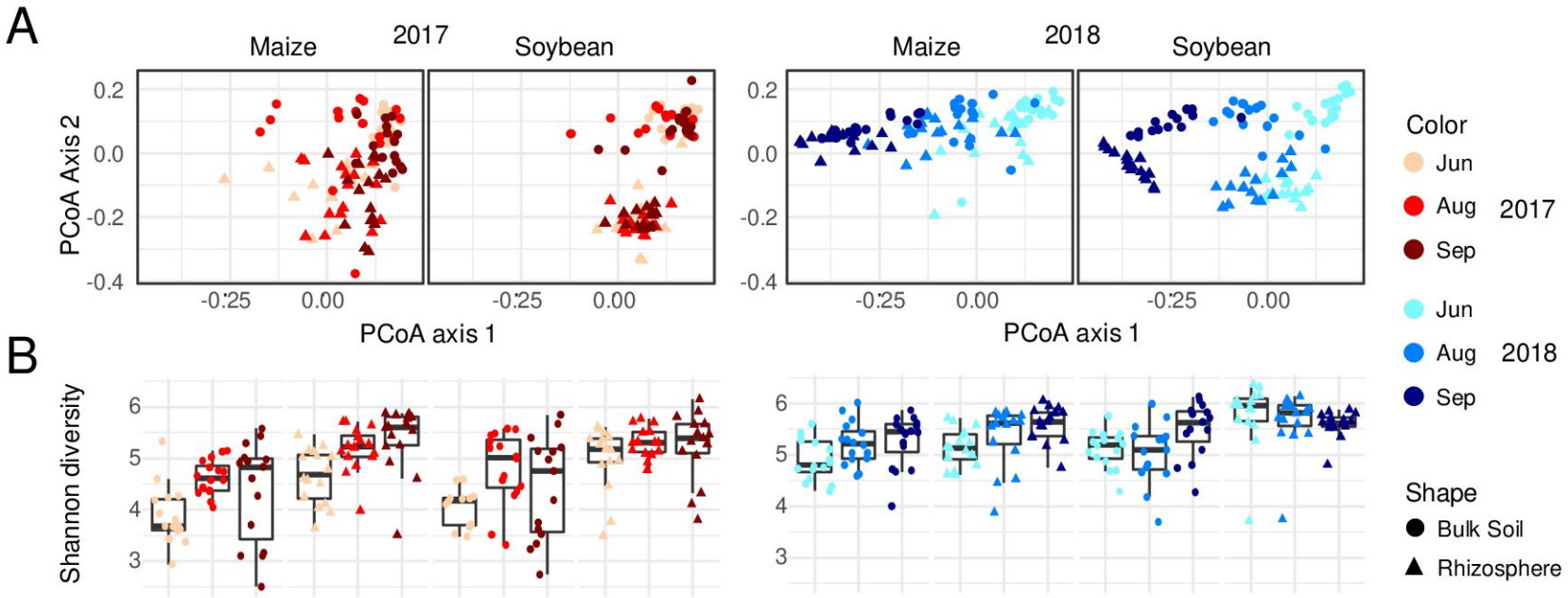

A
Maize
2017
Soybean
Maize
2018
Soybean
PCoA Axis 2
0.2
0.0
-0.2
-0.4
-0.25
0.00
PCoA axis 1
B
Shannon diversity
6
5
4
3
Color
Jun
Aug
Sep
2017
Jun
Aug
Sep
2018
Shape
Bulk Soil
Rhizosphere

Fig. 3

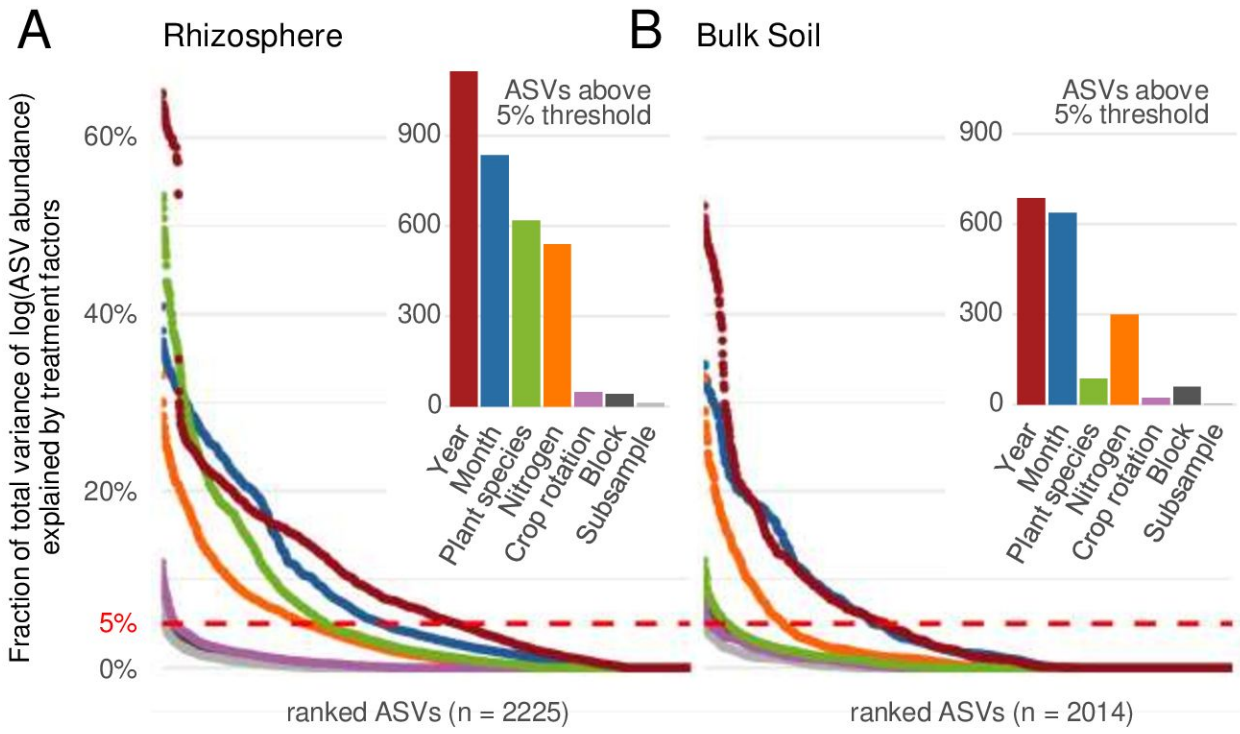

A
Rhizosphere
B
Bulk Soil
Fraction of total variance of log(ASV abundance) explained by treatment factors
60%
40%
20%
5%
0%
ASVs above 5% threshold
900
600
300
0
Year
Month
Plant species
Nitrogen
Crop rotation
Block
Subsample
ranked ASVs (n = 2225)
ranked ASVs (n = 2014)

Fig. 4

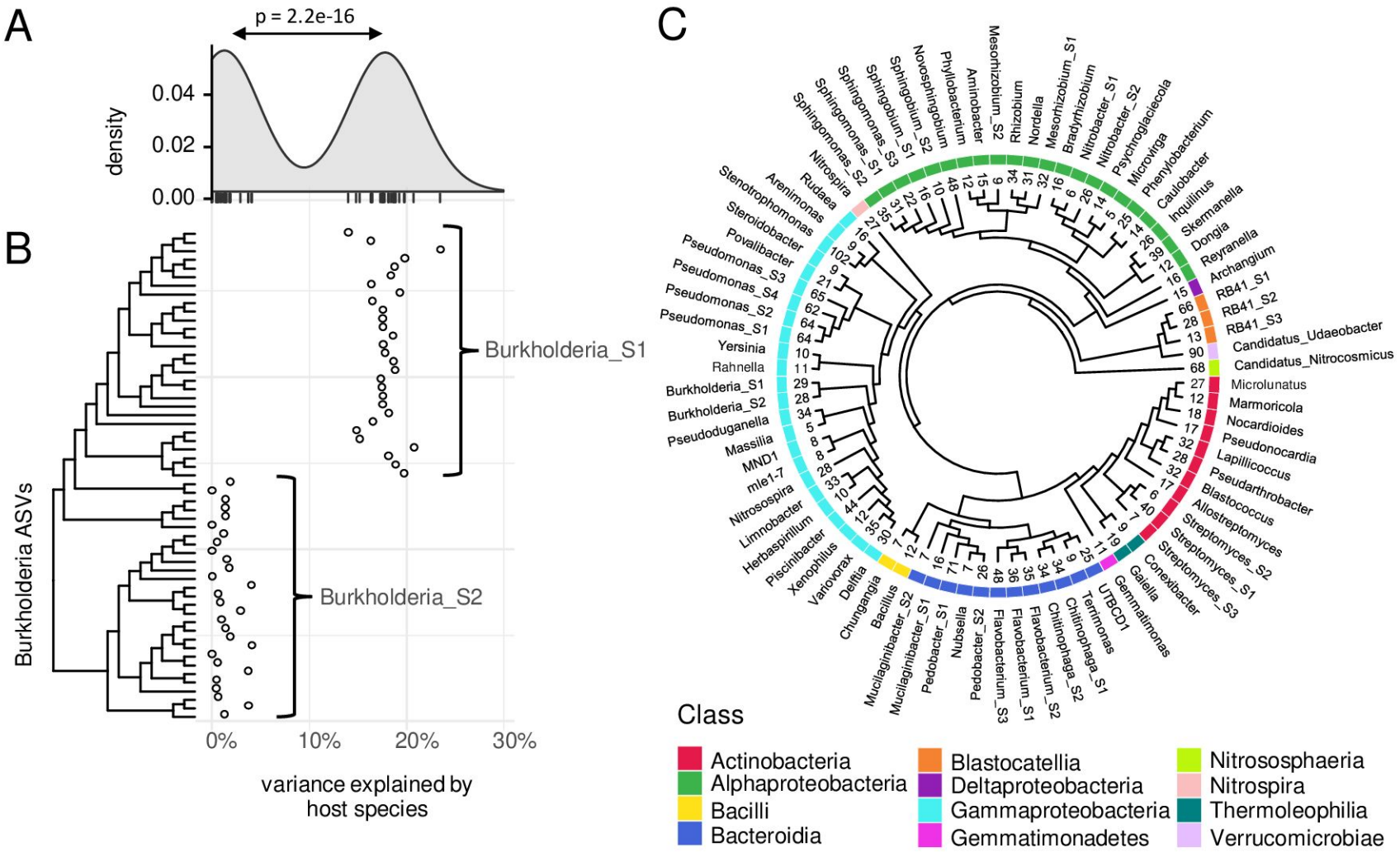

A
p = 2.2e-16
density
0.04
0.02
0.00
B
Burkholderia ASVs
Burkholderia_S1
Burkholderia_S2
0%
10%
20%
30%
variance explained by
host species
C
Mesorhizobium_S2
Rhizobium
Nordella
Mesorhizobium_S1
Bradyrhizobium
Nitrobacter_S1
Nitrobacter_S2
Psychroglaciecola
Microvirga
Phenylobacterium
Caulobacter
Inquilinus
Skermanella
Dongia
Reyranella
Archangium
RB41_S1
RB41_S2
RB41_S3
Candidatus_Udaeobacter
Candidatus_Nitrocosmicus
Microlunatus
Marmoricola
Nocardioides
Pseudonocardia
Lapillicoccus
Pseudarthrobacter
Blastococcus
Allostreptomyces
Streptomyces_S2
Streptomyces_S1
Streptomyces_S3
Conexibacter
Gaiella
Gemmatimonas
UTBCD1
Terrimonas
Chitinophaga_S1
Chitinophaga_S2
Flavobacterium_S1
Flavobacterium_S2
Flavobacterium_S3
Pedobacter_S2
Nubsella
Pedobacter_S1
Mucilaginibacter_S1
Mucilaginibacter_S2
Bacillus
Chungangia
Delftia
Variovorax
Xenophilus
Piscinibacter
Herbaspirillum
Limnobacter
Nitrosospira
mle1-7
MND1
Massilia
Pseudoduganella
Burkholderia_S2
Burkholderia_S1
Rahnella
Yersinia
Pseudomonas_S1
Pseudomonas_S2
Pseudomonas_S4
Pseudomonas_S3
Povalibacter
Steroidobacter
Stenotrophomonas
Arenimonas
Rudaea
Nitrospira
Sphingomonas_S2
Sphingomonas_S3
Sphingomonas_S1
Sphingobium_S1
Novosphingobium
Phyllobacterium
Aminobacter
Class
Actinobacteria
Alphaproteobacteria
Bacilli
Bacteroidia
Blastocatellia
Deltaproteobacteria
Gammaproteobacteria
Gemmatimonadetes
Nitrososphaeria
Nitrospira
Thermoleophilia
Verrucomicrobiae

Fig. 5

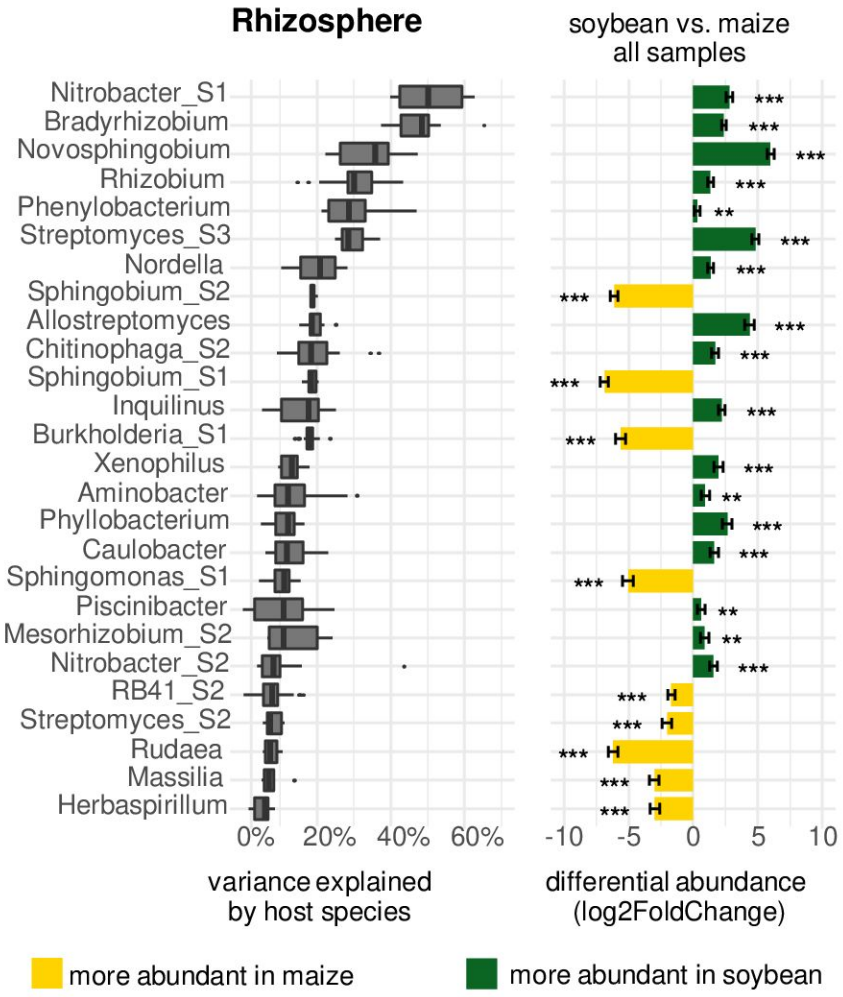

Rhizosphere
soybean vs. maize
all samples
Nitrobacter_S1
Bradyrhizobium
Novosphingobium
Rhizobium
Phenylobacterium
Streptomyces_S3
Nordella
Sphingobium_S2
Allostreptomyces
Chitinophaga_S2
Sphingobium_S1
Inquilinus
Burkholderia_S1
Xenophilus
Aminobacter
Phyllobacterium
Caulobacter
Sphingomonas_S1
Piscinibacter
Mesorhizobium_S2
Nitrobacter_S2
RB41_S2
Streptomyces_S2
Rudaea
Massilia
Herbaspirillum
0%
20%
40%
60%
-10
-5
0
5
10
variance explained
by host species
differential abundance
(log2FoldChange)
more abundant in maize
more abundant in soybean

Fig. 6

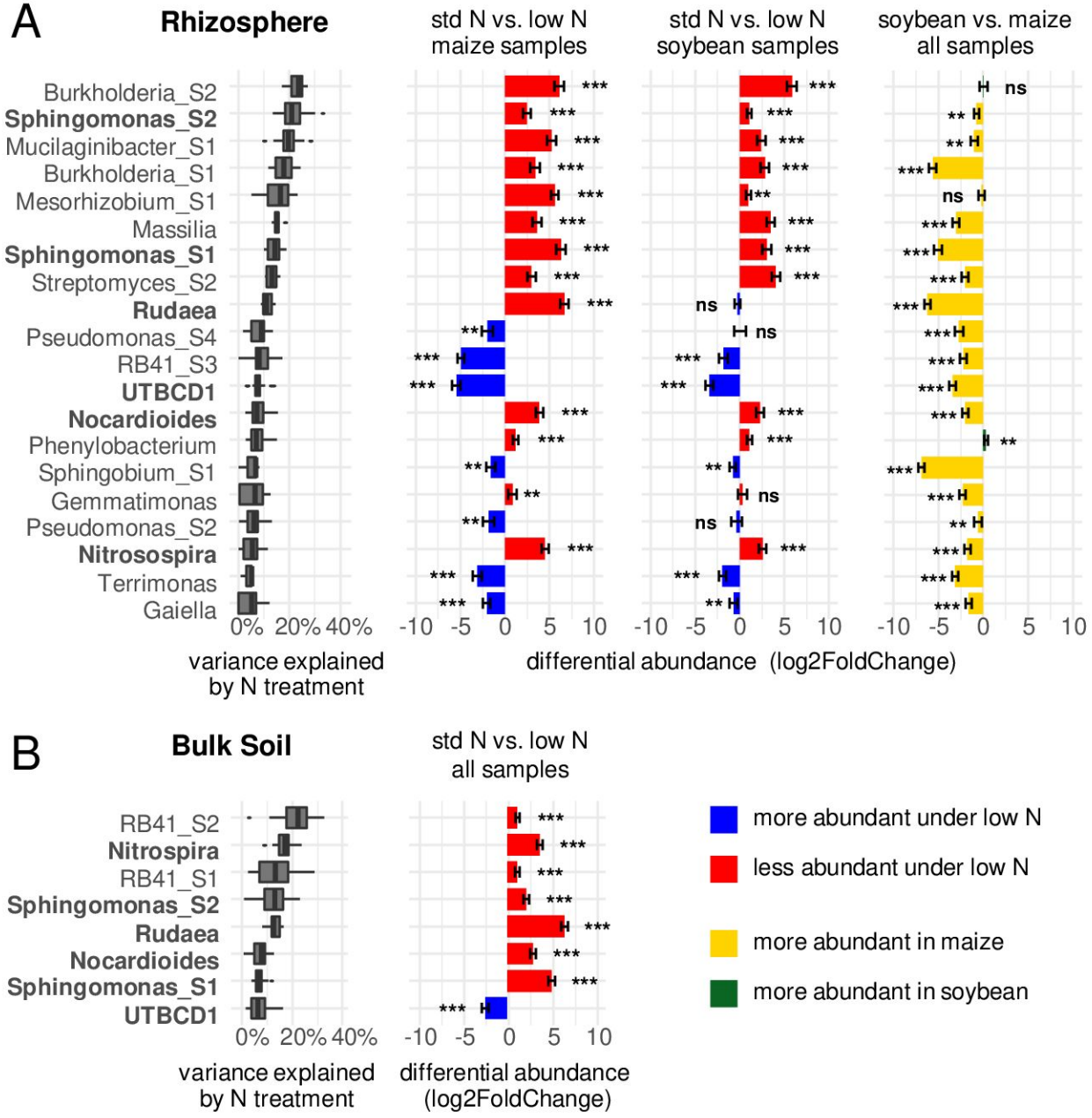

A
Rhizosphere
std N vs. low N
maize samples
std N vs. low N
soybean samples
soybean vs. maize
all samples
Burkholderia_S2
Sphingomonas_S2
Mucilaginibacter_S1
Burkholderia_S1
Mesorhizobium_S1
Massilia
Sphingomonas_S1
Streptomyces_S2
Rudaea
Pseudomonas_S4
RB41_S3
UTBCD1
Nocardioides
Phenylobacterium
Sphingobium_S1
Gemmatimonas
Pseudomonas_S2
Nitrosospira
Terrimonas
Gaiella
0% 20% 40%
-10 -5 0 5 10
variance explained
by N treatment
differential abundance (log2FoldChange)
B
Bulk Soil
std N vs. low N
all samples
RB41_S2
Nitrospira
RB41_S1
Sphingomonas_S2
Rudaea
Nocardioides
Sphingomonas_S1
UTBCD1
0% 20% 40%
-10 -5 0 5 10
variance explained
by N treatment
differential abundance
(log2FoldChange)
more abundant under low N
less abundant under low N
more abundant in maize
more abundant in soybean

Fig. S1

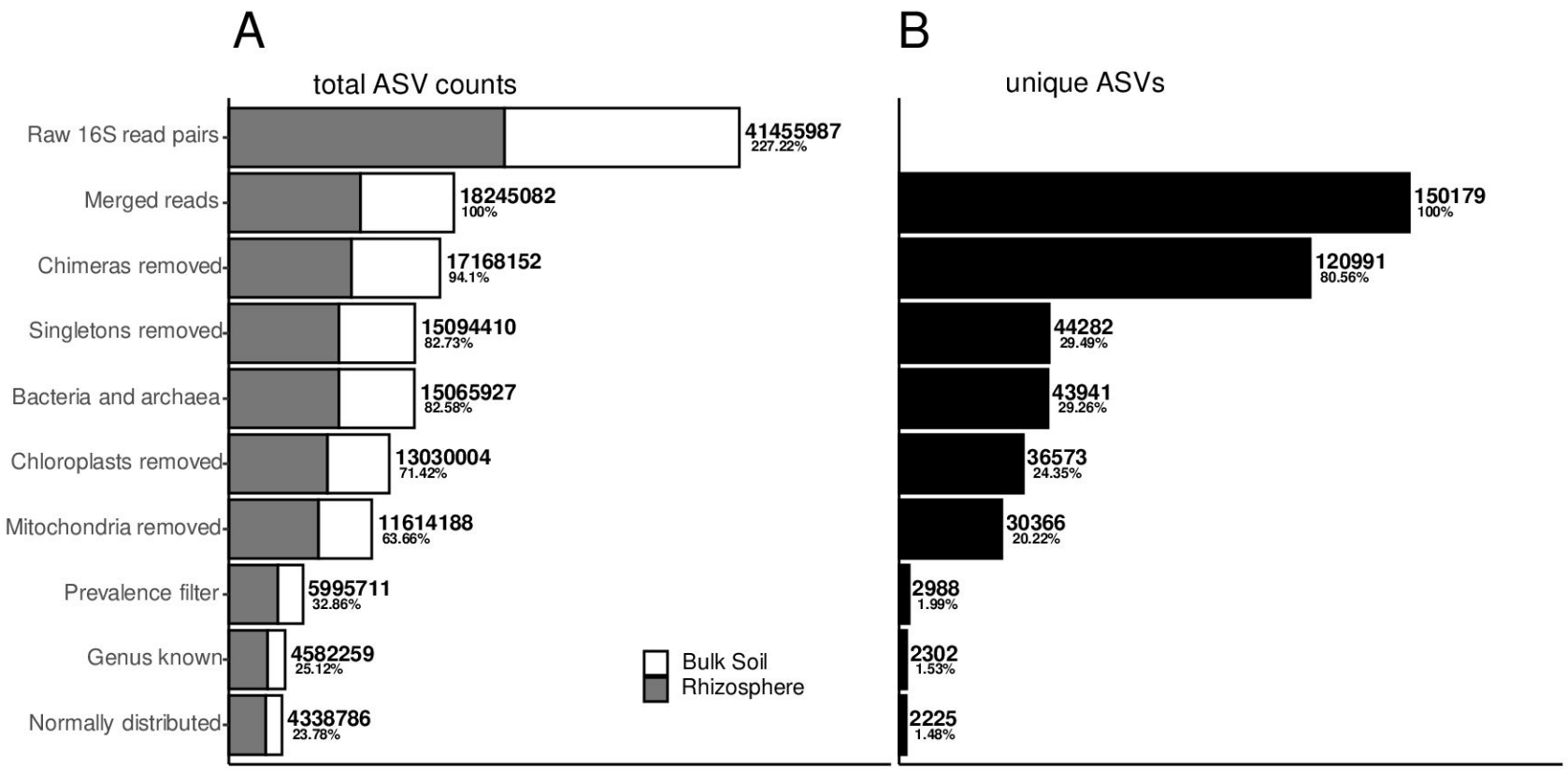

A
total ASV counts
Raw 16S read pairs
41455987
227.22%
Merged reads
18245082
100%
Chimeras removed
17168152
94.1%
Singletons removed
15094410
82.73%
Bacteria and archaea
15065927
82.58%
Chloroplasts removed
13030004
71.42%
Mitochondria removed
11614188
63.66%
Prevalence filter
5995711
32.86%
Genus known
4582259
25.12%
Normally distributed
4338786
23.78%
Bulk Soil
Rhizosphere
B
unique ASVs
150179
100%
120991
80.56%
44282
29.49%
43941
29.26%
36573
24.35%
30366
20.22%
2988
1.99%
2302
1.53%
2225
1.48%

Fig. S2

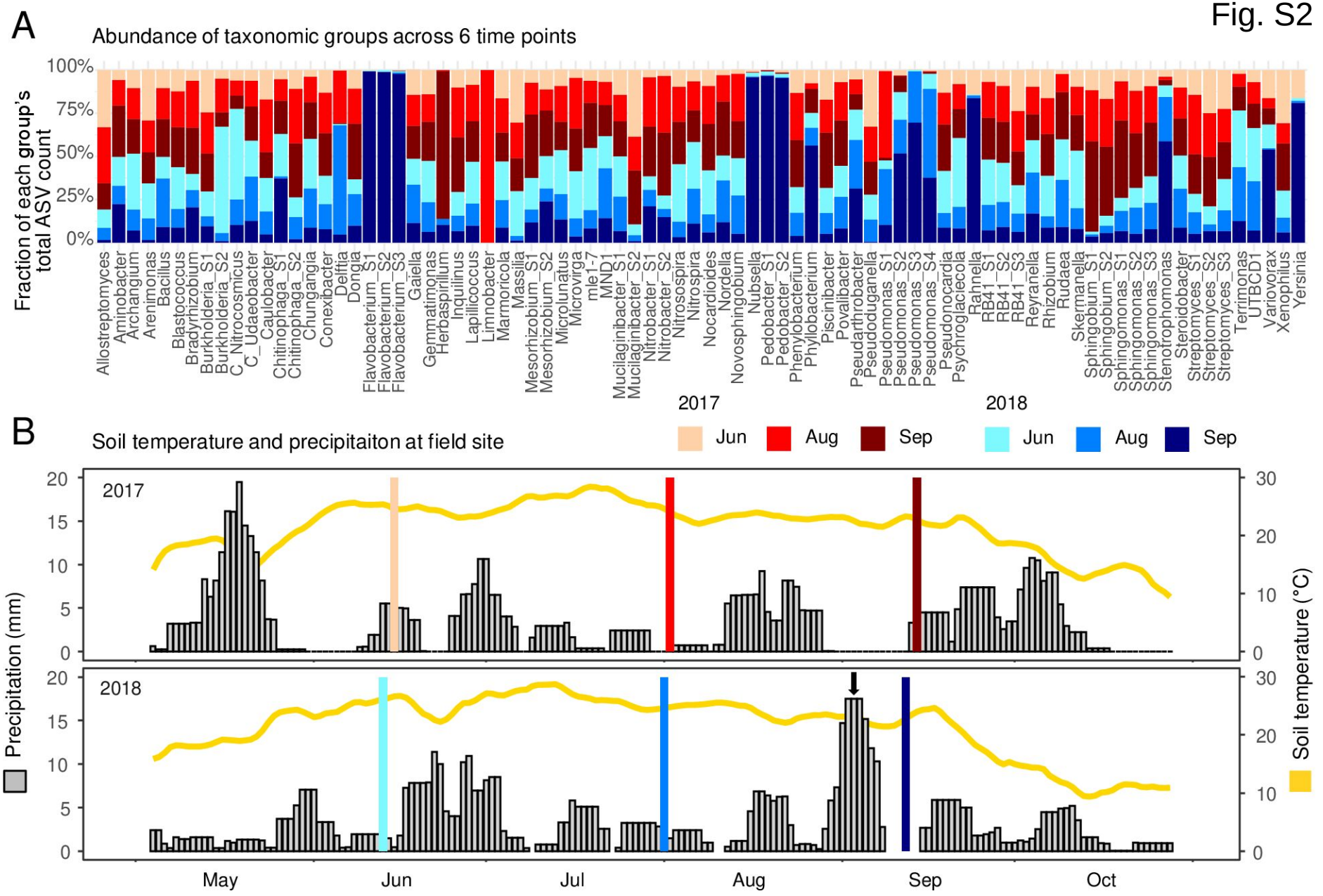

A
Abundance of taxonomic groups across 6 time points
Fraction of each group's total ASV count
100%
75%
50%
25%
0%
Allostreptomyces
Aminobacter
Archangium
Arenimonas
Bacillus
Blastococcus
Bradyrhizobium
Burkholderia_S1
Burkholderia_S2
C_Nitrocosmicus
C_Udaeobacter
Caulobacter
Chitinophaga_S1
Chitinophaga_S2
Chungangia
Conexibacter
Delftia
Dongia
Flavobacterium_S1
Flavobacterium_S2
Flavobacterium_S3
Gaiella
Gemmatimonas
Herbaspirillum
Inquilinus
Lapillicoccus
Limnobacter
Marmoricola
Massilia
Mesorhizobium_S1
Mesorhizobium_S2
Microlunatus
Microvirga
mle1-7
MND1
Mucilaginibacter_S1
Mucilaginibacter_S2
Nitrobacter_S1
Nitrobacter_S2
Nitrosospira
Nitrospira
Nocardioides
Nordella
Novosphingobium
Nubsella
Pedobacter_S1
Pedobacter_S2
Phenylobacterium
Phyllobacterium
Piscinibacter
Povalibacter
Pseudarthrobacter
Pseudoduganella
Pseudomonas_S1
Pseudomonas_S2
Pseudomonas_S3
Pseudomonas_S4
Pseudonocardia
Psychroglaciecola
Rahnella
RB41_S1
RB41_S2
RB41_S3
Reyranella
Rhizobium
Rudaea
Skermanella
Sphingobium_S1
Sphingobium_S2
Sphingomonas_S1
Sphingomonas_S2
Sphingomonas_S3
Stenotrophomonas
Steroidobacter
Streptomyces_S1
Streptomyces_S2
Streptomyces_S3
Terrimonas
UTBCD1
Variovorax
Xenophilus
Yersinia
2017
2018
B
Soil temperature and precipitaiton at field site
Jun
Aug
Sep
Jun
Aug
Sep
2017
2018
Precipitation (mm)
Soil temperature (°C)
0
5
10
15
20
0
10
20
30
May
Jun
Jul
Aug
Sep
Oct

Fig. S3

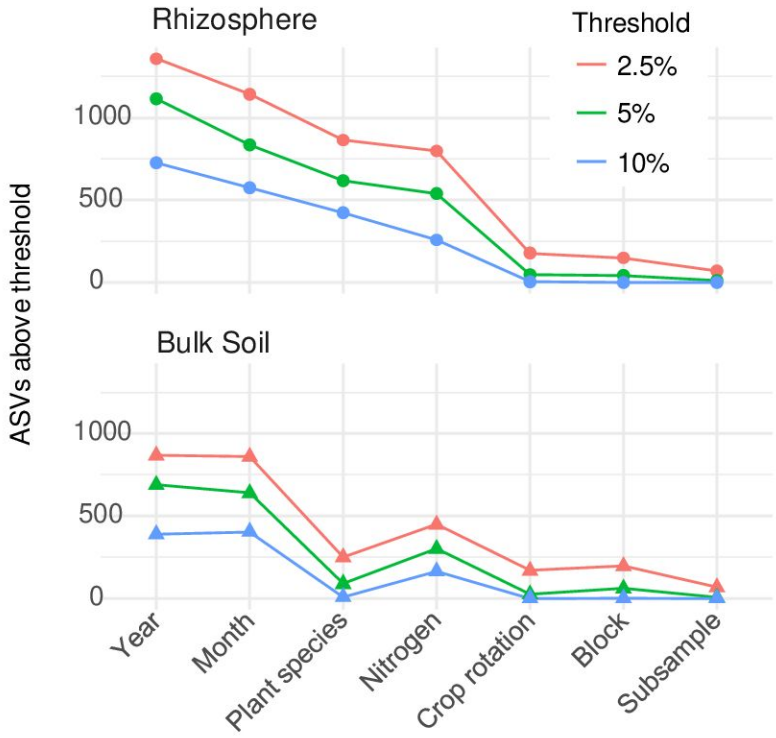

Rhizosphere
Threshold
2.5%
5%
10%
1000
500
0
Bulk Soil
1000
500
0
ASVs above threshold
Year
Month
Plant species
Nitrogen
Crop rotation
Block
Subsample

Fig. S4

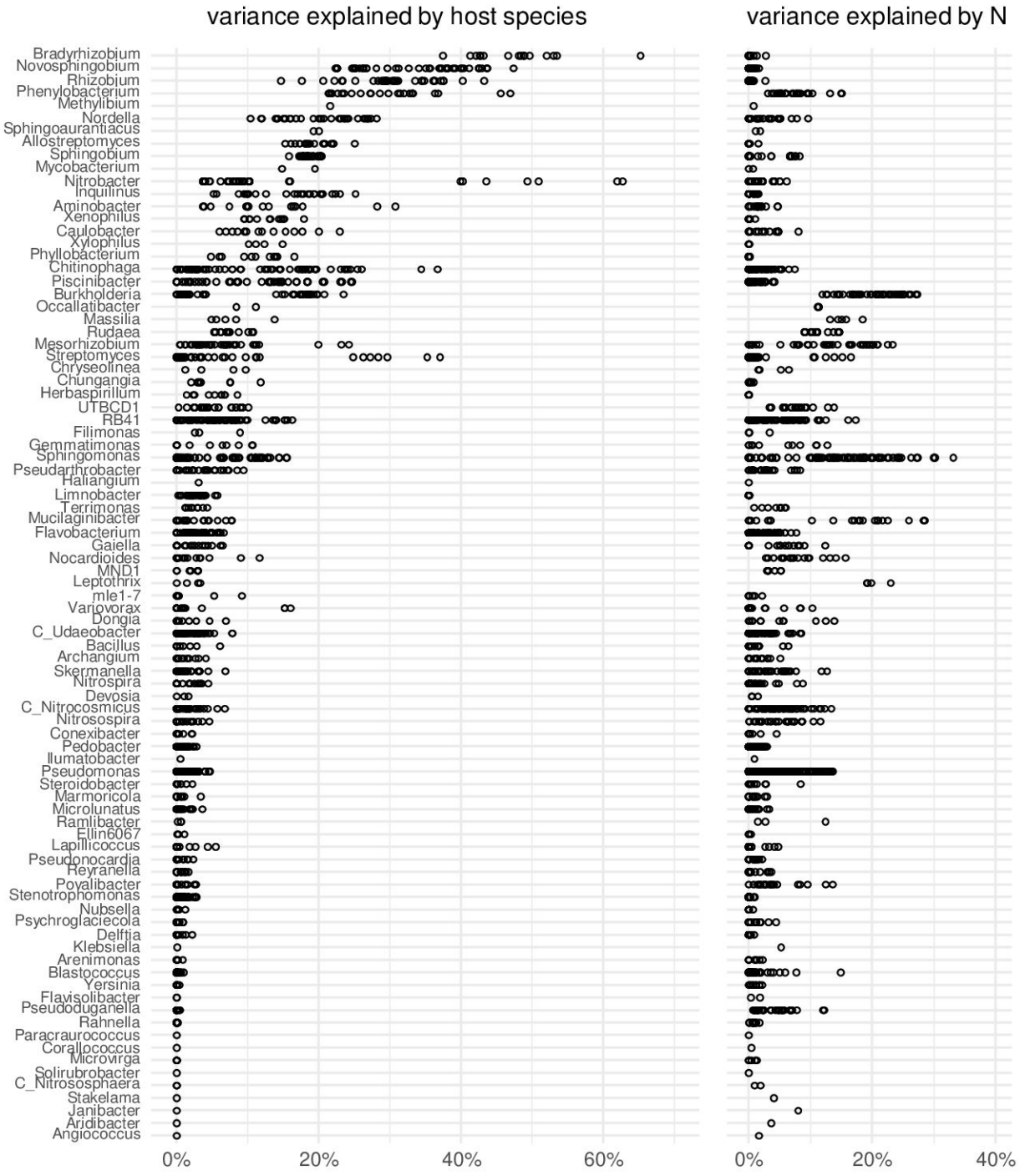

variance explained by host species
variance explained by N
Bradyrhizobium
Novosphingobium
Rhizobium
Phenylobacterium
Methylibium
Nordella
Sphingoaurantiacus
Allostreptomyces
Sphingobium
Mycobacterium
Nitrobacter
Inquilinus
Aminobacter
Xenophilus
Caulobacter
Xylophilus
Phyllobacterium
Chitinophaga
Piscinibacter
Burkholderia
Occallatibacter
Massilia
Rudaea
Mesorhizobium
Streptomyces
Chryseolinea
Chungangia
Herbaspirillum
UTBCD1
RB41
Filimonas
Gemmatimonas
Sphingomonas
Pseudarthrobacter
Haliangium
Limnobacter
Terrimonas
Mucilaginibacter
Flavobacterium
Gaiella
Nocardioides
MND1
Leptothrix
mle1-7
Variovorax
Dongia
C_Udaeobacter
Bacillus
Archangium
Skermanella
Nitrospira
Devosia
C_Nitrocosmicus
Nitrosospira
Conexibacter
Pedobacter
Ilumatobacter
Pseudomonas
Steroidobacter
Marmoricola
Microlunatus
Ramlibacter
Ellin6067
Lapillicoccus
Pseudonocardia
Reyranella
Povalibacter
Stenotrophomonas
Nubsella
Psychroglaciecola
Delftia
Klebsiella
Arenimonas
Blastococcus
Yersinia
Flavisolibacter
Pseudoduganella
Rahnella
Paracraurococcus
Corallococcus
Microvirga
Solirubrobacter
C_Nitrososphaera
Stakelama
Janibacter
Aridibacter
Angiococcus
0%
20%
40%
60%
0%
20%
40%

Fig. S5

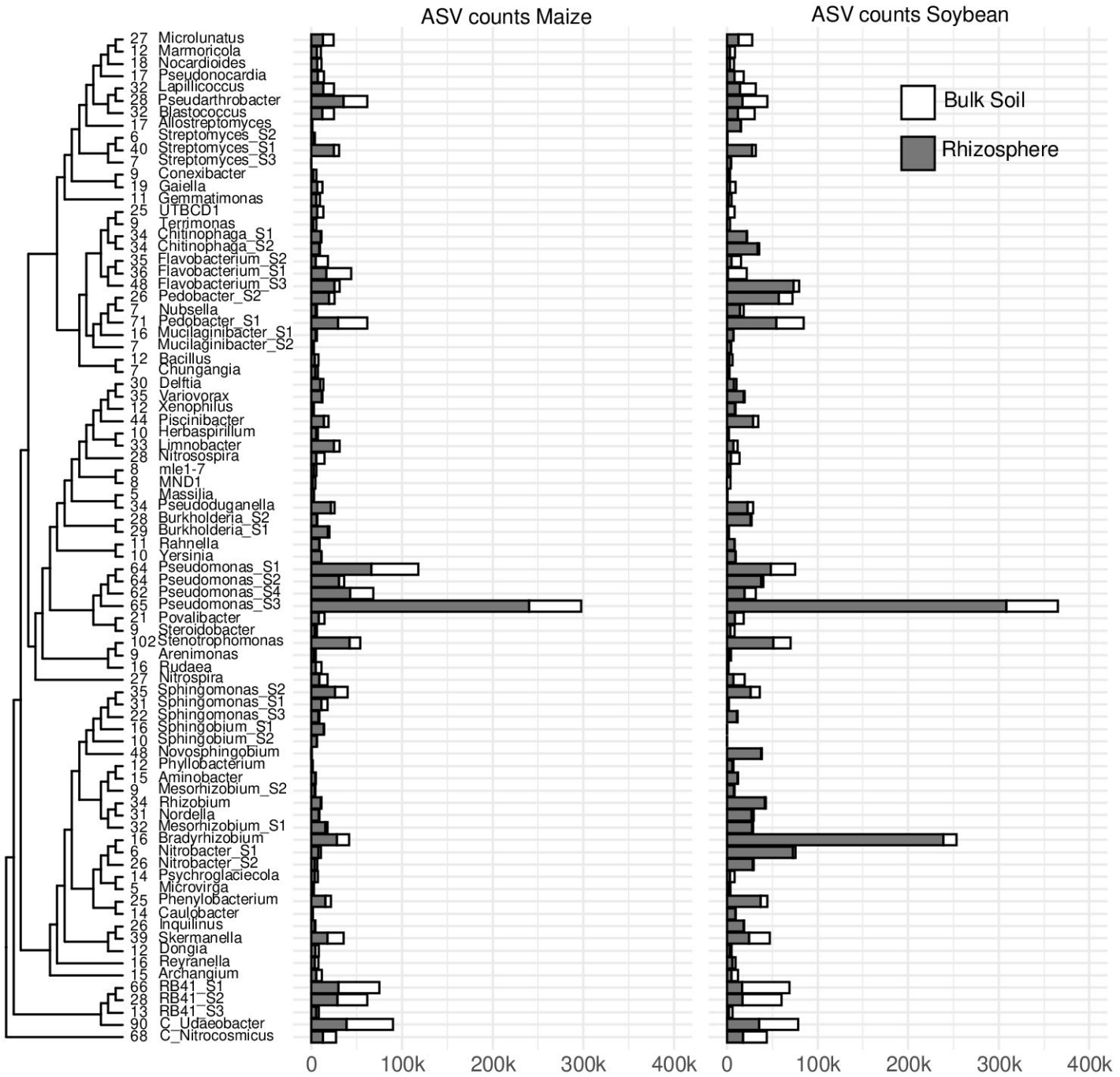

ASV counts Maize
ASV counts Soybean
Bulk Soil
Rhizosphere
27 Microlunatus
12 Marmoricola
18 Nocardioides
17 Pseudonocardia
32 Lapillicoccus
28 Pseudarthrobacter
32 Blastococcus
17 Allostreptomyces
6 Streptomyces_S2
40 Streptomyces_S1
7 Streptomyces_S3
9 Conexibacter
19 Gaiella
11 Gemmatimonas
25 UTBCD1
9 Terrimonas
34 Chitinophaga_S1
34 Chitinophaga_S2
35 Flavobacterium_S2
36 Flavobacterium_S1
48 Flavobacterium_S3
26 Pedobacter_S2
7 Nubsella
71 Pedobacter_S1
16 Mucilaginibacter_S1
7 Mucilaginibacter_S2
12 Bacillus
7 Chungangia
30 Delftia
35 Variovorax
12 Xenophilus
44 Piscinibacter
10 Herbaspirillum
33 Limnobacter
28 Nitrosospira
8 mle1-7
8 MND1
5 Massilia
34 Pseudoduganella
28 Burkholderia_S2
29 Burkholderia_S1
11 Rahnella
10 Yersinia
64 Pseudomonas_S1
64 Pseudomonas_S2
62 Pseudomonas_S4
65 Pseudomonas_S3
21 Povalibacter
9 Steroidobacter
102 Stenotrophomonas
9 Arenimonas
16 Rudaea
27 Nitrospira
35 Sphingomonas_S2
31 Sphingomonas_S1
22 Sphingomonas_S3
16 Sphingobium_S1
10 Sphingobium_S2
48 Novosphingobium
12 Phyllobacterium
15 Aminobacter
9 Mesorhizobium_S2
34 Rhizobium
31 Nordella
32 Mesorhizobium_S1
16 Bradyrhizobium
6 Nitrobacter_S1
26 Nitrobacter_S2
14 Psychroglaciecola
5 Microvirga
25 Phenylobacterium
14 Caulobacter
26 Inquilinus
39 Skermanella
12 Dongia
16 Reyranella
15 Archangium
66 RB41_S1
28 RB41_S2
13 RB41_S3
90 C_Udaeobacter
68 C_Nitrocosmicus
0
100k
200k
300k
400k
0
100k
200k
300k
400k

Fig. S6

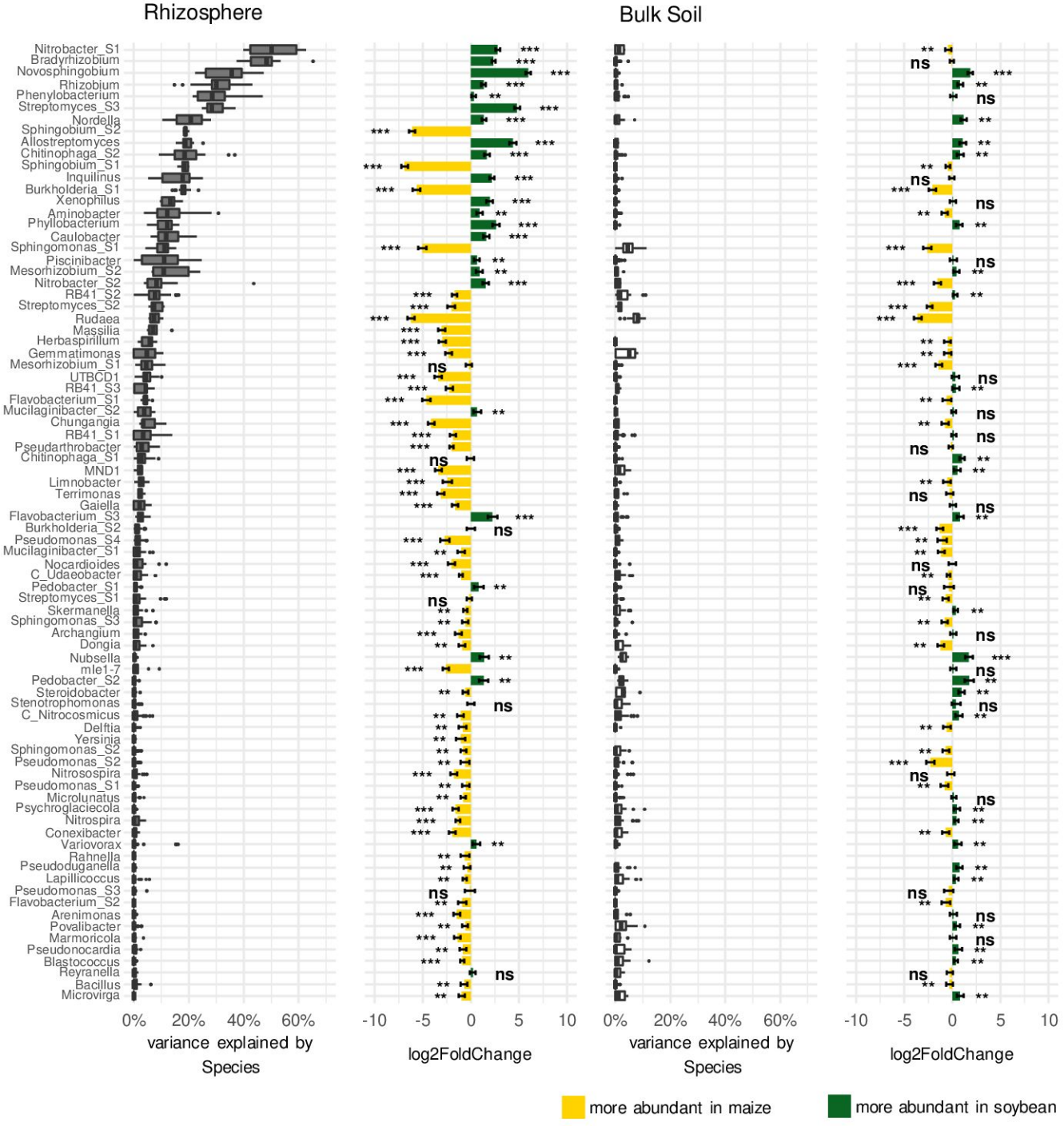

Rhizosphere
Bulk Soil
Nitrobacter_S1
Bradyrhizobium
Novosphingobium
Rhizobium
Phenylobacterium
Streptomyces_S3
Nordella
Sphingobium_S2
Allostreptomyces
Chitinophaga_S2
Sphingobium_S1
Inquilinus
Burkholderia_S1
Xenophilus
Aminobacter
Phyllobacterium
Caulobacter
Sphingomonas_S1
Piscinibacter
Mesorhizobium_S2
Nitrobacter_S2
RB41_S2
Streptomyces_S2
Rudaea
Massilia
Herbaspirillum
Gemmatimonas
Mesorhizobium_S1
UTBCD1
RB41_S3
Flavobacterium_S1
Mucilaginibacter_S2
Chungangia
RB41_S1
Pseudarthrobacter
Chitinophaga_S1
MND1
Limnobacter
Terrimonas
Gaiella
Flavobacterium_S3
Burkholderia_S2
Pseudomonas_S4
Mucilaginibacter_S1
Nocardioides
C_Udaeobacter
Pedobacter_S1
Streptomyces_S1
Skermanella
Sphingomonas_S3
Archangium
Dongia
Nubsella
mle1-7
Pedobacter_S2
Steroidobacter
Stenotrophomonas
C_Nitrocosmicus
Delftia
Yersinia
Sphingomonas_S2
Pseudomonas_S2
Nitrosospira
Pseudomonas_S1
Microlunatus
Psychroglaciecola
Nitrospira
Conexibacter
Variovorax
Rahnella
Pseudoduganella
Lapillicoccus
Pseudomonas_S3
Flavobacterium_S2
Arenimonas
Povalibacter
Marmoricola
Pseudonocardia
Blastococcus
Reyranella
Bacillus
Microvirga
0%
20%
40%
60%
variance explained by Species
-10
-5
0
5
10
log2FoldChange
ns
more abundant in maize
more abundant in soybean

Fig. S7

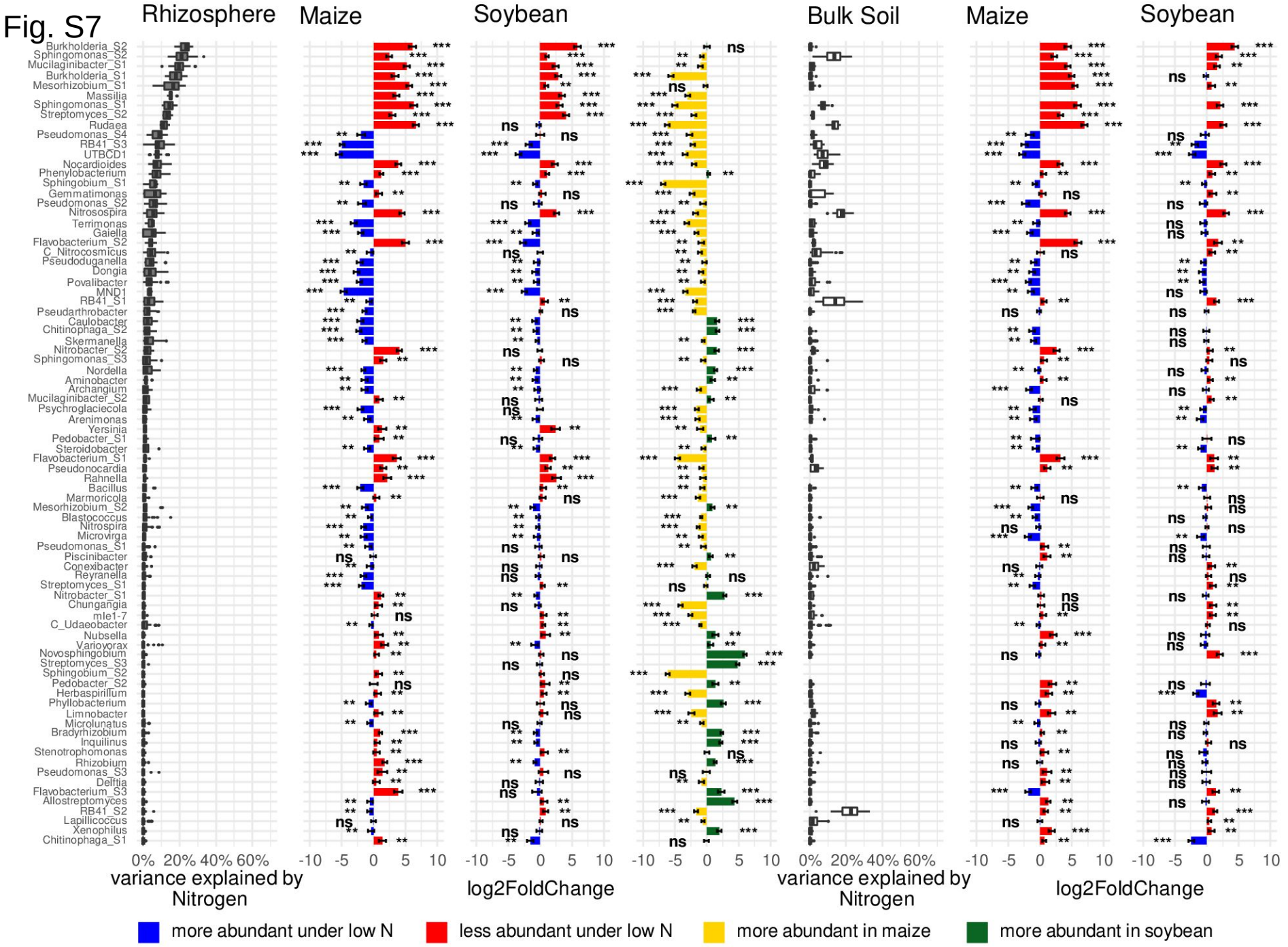

Rhizosphere
Maize
Soybean
Bulk Soil
Maize
Soybean
Burkholderia_S2
Sphingomonas_S2
Mucilaginibacter_S1
Burkholderia_S1
Mesorhizobium_S1
Massilia
Sphingomonas_S1
Streptomyces_S2
Rudaea
Pseudomonas_S4
RB41_S3
UTBCD1
Nocardioides
Phenylobacterium
Sphingobium_S1
Gemmatimonas
Pseudomonas_S2
Nitrosospira
Terrimonas
Gaiella
Flavobacterium_S2
C_Nitrocosmicus
Pseudoduganella
Dongia
Povalibacter
MND1
RB41_S1
Pseudarthrobacter
Caulobacter
Chitinophaga_S2
Skermanella
Nitrobacter_S2
Sphingomonas_S3
Nordella
Aminobacter
Archangium
Mucilaginibacter_S2
Psychroglaciecola
Arenimonas
Yersinia
Pedobacter_S1
Steroidobacter
Flavobacterium_S1
Pseudonocardia
Rahnella
Bacillus
Marmoricola
Mesorhizobium_S2
Blastococcus
Nitrospira
Microvirga
Pseudomonas_S1
Piscinibacter
Conexibacter
Reyranella
Streptomyces_S1
Nitrobacter_S1
Chungangia
mle1-7
C_Udaeobacter
Nubsella
Variovorax
Novosphingobium
Streptomyces_S3
Sphingobium_S2
Pedobacter_S2
Herbaspirillum
Phyllobacterium
Limnobacter
Microlunatus
Bradyrhizobium
Inquilinus
Stenotrophomonas
Rhizobium
Pseudomonas_S3
Delftia
Flavobacterium_S3
Allostreptomyces
RB41_S2
Lapillicoccus
Xenophilus
Chitinophaga_S1
0% 20% 40% 60%
-10 -5 0 5 10
variance explained by Nitrogen
log2FoldChange
more abundant under low N
less abundant under low N
more abundant in maize
more abundant in soybean

Table S1

Genus	subgroups identified by response to treatment	97% OTUs generated from ASVs
Burkholderia	2	1*
Chitinophaga	2	3
Flavobacterium	3	4
Mesorhizobium	2	3
Mucilaginibacter	2	2
Nitrobacter	2	1*
Pedobacter	2	2
Pseudomonas	4	3
RB41	3	7
Sphingobium	2	1*
Sphingomonas	3	4
Streptomyces	3	3