Colonization of axenic beet by rhizosphere bacteria takes place in discreet phases regardless of bioinculation with next generation bioinoculant

Marcin Gołębiewski[1,2*], Marcin Sikora[2], Justyna Mazur[2], Sonia Szymańska[3], Jarosław Tyburski[1], Katarzyna Hrynkiewicz[3], Werner Ulrich[4]

1 – Department of Plant Physiology and Biotechnology, Nicolaus Copernicus University in Torun, Lwowska 1, 87-100 Torun, Poland

2 – Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University in Torun, Wilenska 4, 87-100 Torun, Poland

3 – Department of Microbiology, Nicolaus Copernicus University in Torun, Lwowska 1, 87-100 Torun, Poland

4 – Department of Ecology and Biogeography, Nicolaus Copernicus University in Torun, Lwowska 1, 87-100 Torun, Poland

* - corresponding author,

Keywords: endophytic bacterial community, 16S rRNA sequencing, bioinoculant, community assembly, *Beta vulgaris*, rhizosphere

Abstract

Background: Bioinoculation improves nutrient access and increases plant resistance to pathogens, thus facilitating higher crop yields under environmental stress. Next generation inoculants (NGIs) consisting of microbial communities instead of strains may be of broader use than standard ones, as different plants might recruit specific organisms from a large pool. Inoculation influences rhizosphere and endophytic community structure modifying community assembly process, which is driven by selection due to the host and by microbe-microbe interactions. Plant colonization by microbes is an instance of succession, with its distinct phases differing in community structure and diversity. This process needs to be studied to determine the optimal timing for bioinoculation and studying its effects. We wanted to learn if, and if so, when stable bacterial communities form during

axenic beet plants colonization by bacteria from soil, either inoculated with NGI or non-inoculated and if this process depends on beet genotype and soil.

Results: Here we show that regardless of bioinoculation, soil type and plant genotype bacteria colonize axenic beets rhizosphere and tissues in two phases differing in bacterial load, degree of nestedness, community structure and diversity and aligned with taproot development. Both rhizosphere and endosphere communities remain stable after five weeks of growth in soil. Alpha-diversity was higher and bacterial load was lower in late samples than in early ones. Communities were more similar at the level of predicted functional potential than at the level of ASVs. Time, soil type and genotype determined community structure but not alpha-diversity, bacterial load or nestedness both in rhizosphere and in endosphere. Inoculation slightly but significantly changed community structure both in rhizosphere as well as in roots and leaves. *Pseudomonadota* and *Bacillota* of low abundance coming from the NGI were found to be recruited by beets.

Conclusions: Axenic beets colonization runs through phases similar to those in other instances of microbial succession and random organisms are recruited. Transition from the early to late phase involves culling of a majority of bacterial cells from plant tissues, apparently on random basis. Five weeks of growth in soil enable formation of stable bacterial communities both in rhizosphere and endosphere. Inoculation only slightly influences bacterial communities and the influence seems to be mostly indirect, probably due to microbe-microbe interactions.

Background

Bioinoculation, defined as the introduction of beneficial endophytic microbes into plant tissues, effectively improves nutrient access as well as increases resistance to pathogens, and, consequently, facilitates higher crop yields in plants, particularly grown under environmental stress [1–3]. The successful construction of a bioinoculant (sometimes termed biofertilizer) requires solving three problems: i) finding appropriate microorganism(s), ii) devising formulation and iii) finding the most effective way of its application. Regarding the first problem, next generation bioinoculants – microbial communities, and not single strains – are a possible solution promising broader applicability/ensuring possibility of application to various plant genotypes (strains, varieties, etc.) in different soils, as each of the genotypes may recruit specific microbes according to their needs. For example, bioinoculation with synthetic microbial community was shown to restore growth under stress conditions in *Arabidopsis thaliana* [4]. We found previously that beet genotype

influences both endophytic and rhizosphere bacterial communities, moreover, lyophilized beet roots can be a source of viable microorganisms for inoculation [5]. As to the problem of devising formulation, various strategies based on liquids, slurries or solids are used, reviewed e.g. in [6], and the bioinculants are applied as seed coats [7], mixed with soil [8] or as foliar sprays [9].

From an ecological point of view, inoculation influences the endophytic microbial community structure, probably modifying the community assembly process (e.g. [10]). Research on the dynamics of plant microbial colonization has shown that the process is rapid [11,12] and driven by host plant interaction-driven selection [13,14] and probably also by microbe-microbe interactions [15]. Accordingly, the host genotype is one of the key factors influencing endophytic community structure [16], and consequently different varieties of the same species could respond differently to a given bioinoculant. The soil proved to be the main source of endophytes, but in some cases seeds seem to be more important [17], while the phyllosphere might be an additional source [18]. Thus, the soil might also contribute to the success or failure of a bioinoculant.

Endophytic communities evolve over time [19–21], and respond to changing host developmental stages [22,23] and environmental conditions [24]. In fact in the case of axenic plants, plant colonization might be regarded as an instance of primary microbial succession. Microbial primary succession was showed to be similar to plant succession in different environments and run through similar phases, e.g. [25]. However, plant colonization is different from other succession instances, as plant adds another layer to environment disturbances and to some extent plant development governs this process .

Many ecological sets of communities were found to be nested, that is species poorer communities are proper subsets of more species rich communities. Nestedness analysis is therefore a common tool to disentangle richness and structural effects on changes in community composition [26].

The tracing of whole plant lifespan is often impractical, particularly for large and/or perennial species, thus application and assessment of bioinoculants require determination of time when they would make sense. Therefore, questions arise of whether and, if so, when endophytic communities become compositionally stable and when to examine inoculant influence on the host plant. For practical reasons, it is convenient to analyze the earliest stages, such as seedlings, but it might not be the best strategy if colonization needs more time. It seems also interesting to learn how a bioinoculant influences rhizosphere and endophytic community and if the influence depends on soil and plant genotype, as such data might be useful in engineering novel bioinoculants.

Beet is an important crop and exceptional plant – one of the few whose undomesticated ancestor still grows in the wild. The ancestor, sea beet, is genetically very similar to domesticated beets, but strongly differs in ecology [27]. Beets are cultivated for their high sucrose content (sugar

beet), as a root (red beet) or leafy (chard) vegetable or as fodder. According to recent FAO data, about 30% of the world sucrose production comes from sugar beet [28]. Beet taproot forms early in plant development and its growth can be divided into three phases differing physiologically and biochemically: pre-storage, transition/secondary growth and sucrose accumulation [29], and presumably also in quantity and quality of root exudates.

The goals of our study were threefold: i) to find how much time is needed for establishing stable endophytic communities in beet plants – as analyzed growth period comprised all three phases of root development, we hypothesized that it would happen within six weeks after planting in soil in all genotypes, ii) to assess if nestedness varies over time and in different genotypes – we thought that , iii) to check to what extent and how inoculation with sea beet root community influences bacterial communities in rhizosphere soils and bacteria-free sugar beet and sea beet plants. To answer these questions we analyzed bacterial communities via 16S rRNA gene fragment sequencing at five timepoints in rhizosphere and roots as well as leaves endosphere of three beet genotypes cultivated in two soils of contrasting edaphic properties either inoculated with next generation bioinoculant or non-inoculated.

Methods

Experimental design

We grew three beet varieties in two soils, each variety (genotype) × soil variant was either inoculated with native microbiome of *B. vulgaris* ssp. Maritima growing in the wild or non-inoculated. We analyzed soils, roots and leaves at five timepoints: T0 – four weeks after planting in soil (prior to inoculation), T1 – twenty-nine days after planting (one day post-inoculation), T2 – thirty-five (7 days post-inoculation), T3 –fifty-six (28 days post-inoculation) and T4 – eighty six (58 days post inoculation). A single plant was considered to be a technical replicate, twenty-five plants were grown in one pot, which was treated as a biological replicate. Five biological replicates per experimental variant were used. At each sampling step we removed five plants from each pot. The design is depicted schematically in Fig. 1.

Soils

We used two soils from commercial sugar beet plantations. Their physicochemical characteristics are given in Table SR1. Soils were pasteurized (autoclaved twice over a week for 1 h at 121ºC) to reduce influence of their native microbiomes on plants.

Plants

We used two varieties of sugar beet (*Beta vulgaris* ssp. *vulgaris* cv. 'Bravo' and cv. 'Casino') as well as sea beet (*Beta vulgaris* ssp. *maritima*). The plant material was generated either from commercial seeds (sugar beet cultivars, KHBC, Kutno, Poland) or from seeds obtained from the collection of the National Germplasm Resources Laboratory (Beltsville, MD). The seeds (in the case of commercial ones, coating was removed) were surface sterilized. We assessed the sterilization efficiency via plating, as describe in details in the Supplementary Methods SM1. After germination, the seedlings were dissected and micropropagated in presence of cefotaxime and vancomycin. Resulting plantlets were subjected to three round of micropropagation on an antibiotics-supplemented medium. Next we rooted and acclimated the explants to *ex vitro* conditions (for details see Supplementary Method SM2) and planted them in respective soils.

Inoculant preparation

Sea beet plants growing along the northern Adriatic coast of Croation were collected in August 2017. As the collection took place on non-protected areas of public grounds and the collected plants were used solely for research purposes, no specific permission had to be obtained in accordance with the Nagoya protocol and EU, Croatian as well as Polish law. Collected plants were immediately refrigerated in styrofoam boxes filled with cooling pads frozen at -80ºC. Upon transfer to the laboratory, roots were separated from above-ground parts and the former were surface sterilized, homogenized with Warring blender, mixed with trehalose (1 mg/g) and lyophilized as previously described [5].

Culturable bacteria density assessment and determination of inoculant microbiome salinity resistance

We assessed bacterial density on Luria-Bertani (LB) agar (BD Difco, Poland) supplemented with 100 µg/ml nystatin (Sigma Aldrich, Poland) to prevent fungal growth. Plates were incubated for 7 days at 26ºC.

Salt tolerance was estimated according to previously published methodology [5] using Biolog Micro Station plate reader (Biolog, CA).

Plant growth conditions

Plants were grown in an artificially-lit growth chamber, with ventilation outlets secured with HEPA filters. The chamber was sterilize with UV radiation using flow lamps (ULTRAVIOL, Poland). Temperature was maintained at 20ºC and the photoperiod was 16 h/8 h day/night throughout the experiment. LED lighting panels generating white light were set to obtain 100 µmol * m^{-2} * s^{-1} PAR intensity. Plants were watered according to their needs with sterile deionized water.

Inoculation

We inoculated plants with 100 mg of bioinoculant, which was mixed with surface soil immediately adjacent to the plants (~ 1 cm radius). On the day of inoculation the plants were watered twice, prior to and after inoculation, each time with half the usual amount of water.

Sampling

We sampled soil, roots and leaves with sterile tools. The soil samples were immediately snap-frozen in liquid nitrogen, roots were surface sterilized before freezing, while leaves were not sterilized. Details of the surface sterilization procedure can be found in the Supplementary Methods SM3.

DNA extraction

DNA was isolated from all types of samples using combination of bead beating and flocculation coupled with silica columns purification. The detailed protocols can be found in the Supplementary Methods SM4.

Libraries preparation and sequencing

We prepared and sequenced 16S rRNA gene fragments libraries and sequenced them on MiSeq (Illumina, CA) at CMIT NCU as described earlier [30] with modifications allowing a decrease of host rRNA amplification (details in the Supplementary Methods SM5).

qPCR

We performed real-time PCR on a LightCycler 480 machine (Roche, Switzerland) using the LightCycler 480 SYBR I Master kit (Roche, Switzerland) and Roche consumables. Reactions were carried out in 10 µl volume using 1 ng of template DNA and 5 pmol of each primer per reaction. Primers sequences and cycling conditions can be found in the Supplementary Methods (Table S1 methods in SM6). Each reaction was performed in four technical replicates. We used purified amplicons to generate standard curves. The C_t vlaues determined with the second derivative algorithm of the LightCycler software were exported into csv files and analyzed in R.

Bioinformatics analyzes

We denoised and merged the sequencing reads with DADA2 [31] and used amplicon sequence variants (ASVs) in downstream analyses. The sequences were classified with the assignTaxonomy function of DADA2 using SILVA v.132 [32] as a reference. A Relaxed Neighbor-Joining tree was constructed using clearcut [33], based on an alignment computed with Mothur v.1.44.3 [34] with SILVA v.132 as a reference as described earlier [5]. Alpha-diversity was assessed as Shannon's H', evenness as Shannon's E, and species richness as observed number of ASVs. For analysis of beta-diversity generalized UniFrac distance matrices were computed based on the tree and ASV table using the GUniFrac Package [35]. To make the alpha and beta-diversity indices comparable accross samples of different coverage, we subsampled the ASV table 100 times to 900 sequences per sample and averaged values rounded to the nearest integer were used in downstream analyses [36]. Primers specific for particular ASVs were designed using the DECIPHER package [37]. Bacterial communities metabolic potential was reconstructed using PICRUSt2 [38] using default parameters. The relevant R and Mothur scripts can be found in the Supplementary Methods SM7.

Statistics

We carried out the comparison of sample means with standard R functions or robust ANOVA implemented in the Rfit package. The NODF metric [39] was used to assess the degree of nestedness, the analysis was carried out using the NODF software run under Windows 7. We used

vegan [40] for ordinations and testing of grouping significance. Pairwise PERMANOVA tests were performed using pairwiseAdonis [41]. sPLS-DA analysis was performed with mixOmics package [42] using leave-one-out cross-validation, and differentially abundant ASVs as well as PICRUSt2-predicted traits were identified with ALDEx2 [43] and DESeq2 [44], while signature ASVs were identified with biosigner [45]. The code allowing performing the computations can be found in the Supplementary Methods SM7.

Results

Beet plants generated by micropropagation of seedlings emerging from surface-sterilized seeds are nearly axenic

Beet seeds turned out to be virtually devoid of bacteria, regardless of genotype. Surface sterilization caused a decrease of bacterial 16S rRNA gene counts below the detection limit (Fig. SR2A). Seedlings emerging from sterilized seeds and propagated once on Murashige and Skoog medium supplemented with cefotaxime and vancomycin (see Supplementary Method SM2) proved to be axenic (Fig. SR2B). Therefore, further passages were carried out on an antibiotic-free medium, as antibiotics impaired plantlets growth.

Communities in soils, roots and leaves differ significantly

Bacterial communities in soils roots and leaves of axenic beet plants grown in pasteurized soils differed significantly in terms of structure (Fig. 2A, 9.57% of variance explained, PERMANOVA $F = 69.45$, $p = 0.001$, permutational test for homogeneity of multidimensional variance (betadisper) $F = 68.89$, $p < 0.001$), alpha-diversity, where diversity and richness of ASVs followed a pattern of soils > roots > leaves (Shannon's H': robust ANOVA $F = 583.60$, $p < 0.001$; Shannon's E: $F = 39.97$, $p < 0.001$; richness: $F = 583.60$, $p < 0.001$) and bacterial load, which was lower in leaves than in roots (Wilcoxon test $W = 259\ 411$, $p < 0.001$) (Fig. 2C). Globally, ASV80, classified as *Achromobacter*, and ASV23, classified as *Chryseobacterium*, were found to be characteristic for plant tissues, while ASV3, ASV21 and ASV27 (*Pseudoxanthomonas*, *Sphingopyxis* and *Pedobacter*, respectively) were characteristic for soils. ASV7 (*Cellvibrio*) was typical for roots, and ASV13 (*Sphigobacterium*) for leaves. Generally, differentially abundant ASVs affiliated with *Gammaproteobacteria* were characteristic for roots and soils, while *Bacteroidia*-afiliated ones were typical for leaves (Fig. 2CD, Table SR3). Core microbiome in the three compartments was limited

to a few ASVs (8 in soils, 3 in roots and 1 in leaves, Table 1), mainly members of *Alpha*- and *Gammaproteobacteria* as well as *Bacteroidia*.

The differences, albeit smaller, were also visible at the level of PICRUSt2-predicted metabolic capabilities, which also grouped according to material (Fig. 3A, 6.70% variance explained, significance of a dbRDA model permutational test: $F = 42.16$, $p = 0.001$; betadisper: $F = 26.21$, $p < 0.001$) and alpha-diversity of functions followed the pattern observed for ASVs (Fig. 3B, Shannon's H': robust ANOVA $F = 104.44$, $p < 0.001$; Shannon's E: $F = 173.20$, $p < 0.001$; richness: $F = 851.62$, $p < 0.001$). Functions related to completition between microorganisms (antibiotic resistance and biosynthesis, quorum sensing) appeared to be characteristic for soils and roots, while carbohydrate metabolism ones were predicted to be more frequent in genomes of soil and leaves-dwelling bacteria (Fig. 3C; Table SR4).

As material explained far greater fraction of variance than any other variable (Table SR5), to make the influence of other variables visible, further analyses were carried out on data divided into soils, roots and leaves sets.

First weeks of axenic beets growth in soil can be divided into two stages differing in community structure, diversity, bacterial load, predicted metabolic capabilities and nestedness

Timepoint was the second most important grouping variable, regardless of material (5.82% of variance explained in whole data, PERMANOVA $F = 27.65$, $p < 0.001$; betadisper $F = 46.29$, $p < 0.001$). Three 'early' timepoints (T0, T1 and T2) clustered together and were significantly different from the 'late' ones (T3 and T4) according to permutational testing of a dbRDA model (Fig. 4ACE leaves: $F = 62.88$, $p < 0.001$; roots: $F = 13.47$, $p < 0.001$; soils: $F = 84.96$, $p < 0.001$). Percent of variance explained by this grouping was 15.64% in leaves, 3.05% in roots and 9.67% in soils. Henceforth, the belonging of a sample to the 'early' or 'late' cluster will be called its 'status'.

The difference between the early and late clusters was observed regardless of material, soil and genotype (Figs. SR4,5,6) and was also visible in alpha diversity measures, which were higher, and, in the case of plant tissue samples, in bacterial load, which was lower in late samples (Fig. 4BDF). The former effect was most pronounced in leaves, and least conspicuous in soils, while the drop of the number of bacterial 16S rRNA gene sequences was greater in roots than in leaves. Different organisms were characteristic for (i.e. significantly more abundant in) early and late samples in soils, roots and leaves. The organisms characteristic for soils and roots were rare ones (Fig. SR8ACE; SupplementaryResultsF1).

Traits characteristic for genomes of organisms thriving in late and early samples differed in soils, roots and leaves (Fig. 5ACE; SupplementaryResultsF2). Early soils harbored organisms whose genomes were enriched in genes involved in diverse functional arrays, among which methane metabolism, protein and nucleotide rescue from glyoxal glycation, as well as heavy metals resistance were most prominent. On the other hand, metabolism of aromatic compounds was characteristic for genomes of organisms dwelling in late soil samples (Fig. SR9F). In roots biofilm formation, exopolysaccharide synthesis and regulation of aminoacids pool was characteristic for early samples, and metabolism of aromatic compounds was characteristic for late ones (Fig. SR9D). Toxin/antitoxin systems were characteristic for leaves in general, and polysaccharide (chitin, pectin) utilization was characteristic for early leaf samples, while aromatic compounds metabolism was typical for late ones (Fig. SR9B). Functional diversity was significantly higher in late samples coming from leaves and soils, but not in roots (Fig. 5BDF).

The degree of nestedness was very low, essentially did not deviate from expected derived from a null model (SupplementaryFile3) and decreased with time. The difference between early and late samples was significant for all combinations of soil and genotype but S1C (Fig. 6; Table SR6).

Genotype influences both soil and endophytic bacterial communities regardless of status

Bacterial communities and sets of imputed KO functions clustered according to genotype regardless of material, soil as well as status and the clustering was significant according to permutational tests of a dbRDA model (Figs. SR13, SR16, SR19 and SR22, SR25, SR28 for ASVs and KO functions, respectively). Genotype influence was strongest in soils and stronger at the level of ASVs than of KO functions.

Alpha-diversity varied depending on material, soil and status at the level of ASVs. Bacterial load differed onl in early root samples, where it was highest in genotype M (Figs. SR14, SR17 and SR20). In general, in soil S1 diversity was highest in genotype B samples, regardless of material, in soil S2, the same was true for genotype M. The functional diversity pattern was essentially the same (Figs. SR23, SR26 and SR29). In all materials and regardless of status ASVs classified as *Gammaproteobacteria* were characteristic for sea beet (genotype M), while *Alphaproteobacteria* and *Bacteroidia* were typical for cultivated beets (genotypes B and C) (Figs. SR15, SR18 and SR21). Sets of KO functions characteristic for particular genotypes also depended on soil and status, and the number of differentially represented functions was highest in soils and lowest in leaves. Moreover, endophytic bacterial communities were functionally more diverse in early samples than in late ones.

Regardless of soil and status the degree of nestedness was higher in sea beet than in sugar beet varieties, however in late samples grown in soil S1 this difference was not significant (Fig. SR31).

Inoculation with lyophilized wild beet roots influences bacterial communities in soils and plants

Inoculant characterization

Reads affiliated with *Pseudomonadota* (formerly *Proteobacteria*), *Bacteroidota* (formerly *Bacteroidetes*) and *Bacillota* (formerly *Firmicutes*) were found in libraries prepared from DNA isolated from inoculant samples. The most abundant genera were *Pseudoxanthomonas* and *Brevundimonas* (>5% each), while *Pedobacter, Devosia, Caulobacter, Flavobacterium, Rhizobium, Sphingobacterium, Pseudomonas, Cellvibrio, Thermomonas,* and *Dyadobacter* were less frequent (~2-5%; Fig. 6A). Rare genera (<2% abundance) comprised 55% of reads. Diversity, measured as Shannon's H', was 4.40 ± 0.75, evenness 0.93 ± 0.003, and 144 ± 125 ASVs were observed in the inoculant samples (rarefied data, n = 3), while 437 were found in the non-rarefied dataset. Culturable bacterial density was $4.0 \pm 0.09 \times 10^5$cfu/g (n = 6), while bacterial 16S rRNA counts were $2.0 \pm 0.5 \times 10^4$ copies/ng of DNA, which translated into $1.4 \pm 0.587 \times 10^8$ copies/g of inoculant (n = 8). The bacteria were able to grow in presence of up to 900 mM NaCl (Fig. 7B).

Community structure in inoculated and non-inoculated samples differs significantly and the differing ASVs depend on compartment, soil and genotype

The overall effect of inoculation on bacterial communities was small, but significant in each of the studied compartments (permutational tests of dbRDA models: soils - F = 5.983, p = 0.001, roots = F = 1.589, p = 0.017, leaves = F = 3.779, p = 0.001; betadisper: soils – F = 10.985, p < 0.001, roots – F = 0.469, p = 0.494, leaves – F = 18.024, p < 0.001) and greater in soils than in roots or leaves (0.48, 0.17 and 0.09 percent of explained variance, respectively, Fig. 8ACE). Mean d05 generalized UniFrac distances between inoculated samples was 0.3810 ± 0.0547, and between non-inoculated ones it was 0.3699 ± 0.0554 and the difference was significant (Wilcoxon test, W = 3.3482e+10, p < 0.001). Further analyses showed that inoculation significantly impacted bacterial communities in all experimental variants in soils, regardless of status, but only in certain variant in the case of roots and soils (Figs. SR32-34, Table SR7). At the level of metabolic potential there was no significant differences between inoculated and non-inoculated plant samples, however in soils the effect of

inoculation was significant (permutational tests of dbRDA models: soils – F = 9.691, p < 0.001, roots – F = 1.705, p = 0.114, leaves – F = 1.390, p = 0.227; betadisper: soils – F = 0.345, p = 0.557, roots – F = 0.003, p = 0.958, leaves – F = 0.337, p = 0.562; Fig 9ACE). Inoculation influenced only early samples of genotypes C and M and late ones in genotype B in soils (Figs. SR36-38 and Table SR7).

Generally, inoculation did not influence alpha diversity neither at the ASVs nor at the metabolic potential level and the same applied to bacterial load in plants with exception of early leaf samples (Fig. 8BDE, 9BDE, SR35 and SR39). Only soil S2 samples of genotype C, and early leaf samples of genotype B grown in S1 differed at the level of ASVs (Fig. SR35A-C). Bacterial load in plant samples did not differ in inoculated and non-inoculated ones, regardless of experimental variant (Fig. SR35D). There were no differences in nestedness, apart from late samples of genotype C grown in S1 (Fig. SR35E).

Sets of ASVs and functions characteristic for inoculated and non-inoculated samples were different early and late samples as well as in each experimental variant (Figs. SR40 and SR42-47, SR41 and SR48-53, respectively as well as SupplementaryResultsF1 and SupplementaryResultsF2). Globally, 29 ASVs were identified with biosigner as signature for either inoculated or non-inoculated samples. The ASVs were classified mainly as *Alpha-* and *Gammaproteobacteria* as well as *Flavobacteria* and *Chitinophaga* (Table SR8). Characteristic ASVs could be found mainly in soils, in case of plant samples it was possible only in certain variants. The organisms that differentiated inoculated late samples from non-inoculated ones were different in each combination of material, soil and genotype. The influence of inoculation was most visible in soil samples (greatest number of ASVs differentiating inoculated and non-inoculated samples), while in roots and leaves there were only single ASVs in certain variants. Only fifteen ASVs (Table SR9) present in inoculant were simultaneously present in inoculated samples and absent from non-inoculated ones. They belonged mainly to *Proteobacteria* and *Firmicutes*. Differences in functional potential comprised diverse functions, and genes involved in antibiotics biosynthesis and resistance were frequently found to be higher represented in inoculated samples than in non-inoculated ones.

Discussion

How much time is needed to establish stable endophytic communities in axenic plants of different genotypes grown in various soils?

Changes in endophytic communities over time due to developmental stage and seasonality were demonstrated in various plants, both perennial [21] and annual [22,46] ones, and might be considered succession, but we are not aware of a plant study reporting such microbial succession in axenic, *in vitro*-propagated plants. In the case of plant colonization by microbes, the succession should be modulated by plant development, as conditions in plant interior depend on developmental stage, which was demonstrated e.g. for beet roots [29] and could be influenced by host genotype.

Indeed, both soil type and genotype influenced both rhizosphere and endophytic community structure, but not alpha diversity. We expected that sea beet, as a wild plant, would recruit more diverse bacterial communities than sugar beet cultivars, both at the level of taxonomy and functions, as wild plants need to cope with a broader spectrum of environmental conditions. However, t here was no clear trend in alpha-diversity, suggesting that under conditions used here there was no need to recruit more diverse communities with a greater potential and revealing the greater plasticity of the wild beet would require more adverse conditions, such as draught, salinity or infertile soil. A relatively small genotype effect was also found in other studies, e.g. on cotton rhizosphere [47] or in willow rhizosphere and root communities [48].

In spite of differences in community composition caused by soil and beet genotype, identical pattern was found in each experimental variant: samples collected until 35th day after planting in soil (early ones) were similar in all measured parameters and the same applied to the samples collected after day 35 (late ones). Such situation seems to be common in microbial succession (e.g. [25]) and is similar to the classical plant succession model of Cowles [49]. Interestingly, rhizosphere communities evolution over time followed the pattern described above. On one hand, this similarity might be interpreted as a result of rhizosphere community being driven by plant developmental stage, possibly via changes in root exudation (reviewed e.g. in [50]). On the other hand it was found that time is a stronger driver of rhizosphere bacterial community structure than the stage of plant development [51].

The phases observed here seem to be in line with beet taproot growth phases, our early phase corresponding to transition/secondary growth onset and the late one to the beginning of sucrose

storage phase [29]. Therefore, we think that under stable environmental conditions the late phase could last at least until flowering (in the case of a perennial – sea beet) or overwintering (in the case of a bi-annual – sugar beet), which are next major events in beet life. In this sense, the beet endophytic and rhizosphere communities become stable three weeks after planting the axenic plants in soil.

Does nestedness differ over time and in various genotypes?

The fact that alpha-diversity followed a pattern of soils > roots > leaves, prompted us to perform nestedness analyses on soil, roots and leaves triplets, which allowed to speculate on possible community assembly mechanisms . The NODF index was consistently low in all variants, and in most cases did not deviate from that expected by chance which, together with little overlap between soil, root and leaf communities, points at random colonization being the prevailing community assembly mechanism . This low level of nested might be explained, at least in part, by relatively low coverage in our samples – organisms which were actually not 'selected out' might have escaped detection and disturb the nested pattern. Therefore, a greater sequencing depth would be needed to improve nestedness analysis. Nevertheless, significant difference in the degree of nestedness between the early and late phase and sea beet and sugar beet varieties suggests that stochastic/deterministic processes ratio might also differ.

As nestedness might be caused both by random (e.g. random sampling (colonization) or incidental death) and deterministic (environmental filtering and selection) extinction processes, it is hard to infer mechanisms behind it based on nestedness alone . In this study we observed a lowered nestedness and concomitant drop in bacterial load in late samples, which suggested that observed higher nestedness in early ones could be caused by ordered extinction. This in turn could be due to higher selection, which then remained stable in late samples.

How does inoculation with wild sea beet root community influence bacterial communities in axenic beets?

Inoculation resulted in stable communities after one week (three weeks of growth in soil), However, as mentioned earlier, there may be other phases of microbiome evolution in line with plant development. Inoculation influenced beet-associated bacterial communities only slightly, but significantly. As we used lyophilized roots as an inoculant, its effect may stem not only from bacteria being introduced to soil but also from organic matter (particularly nitrogen compounds and carbohydrates). Therefore, we expected that inoculant influence would be greater in less N and OM-

rich soil (S1). As, conversely, the influence was smaller in S1 than in S2, we conclude that it was mainly caused by organisms added to soil. As we expected the influence of inoculation to be small, to make it more conspicuous we grew (nearly) axenic plants in pasteurized soils. The presence of a handful of ASVs coming from inoculant in inoculated plant samples and their absence from non-inoculated ones suggested that organisms thriving in the inoculant entered the plants. Their rarity suggests that microbe-microbe interactions and/or fertilization effect are responsible for majority of differences in bacterial community structure observed between inoculated and non-inoculated samples. However, it is possible that they were not detected in non-inoculated samples due to their low abundance - as always, it is impossible to prove that an entity is not present in a given sample. The greatest inoculation influence in late soil and early plant samples suggests that selective pressure in soils is weaker than in plants and/or organisms not being able to enter plants start growing during week 8 of growth in soil.

We expected that inoculation would cause homogenization of communities (i.e. would make mean distance between inoculated communities smaller than between non-inoculated ones) but this did not prove to be true. It seems that the inoculant increases the number of organisms that may be recruited by plants, causing decrease of similarity. Notably, inoculation did not change time- and genotype-driven beta-diversity patterns, which agreed with low values of variance explained by this variable. This fact suggests that the right choice of inoculation time may be crucial for successful application of biofertilizers, at least in beet.

Conclusions

Regardless of soil and genotype, colonization of axenic beet plants takes place in two at least two phases, up to 35th day of growth in soil and past that day until the end of experiment, akin to microbial primary succession in other environments. Bacterial succession in rhizosphere soil follows the same pattern as succession of endophytic communities. Bacterial communities are compositionally stable and bacterial load and nestedness is much lower in the late phase than in the early one. Colonization is largely random – similar strains (ASVs) encoding similar functions are recruited from a pool of functionally redundant soil bacteria. Regardless of soil and genotype, inoculation influences bacterial communities slightly, but significantly and rare organisms are recruited by plants. The inoculation acts mostly indirectly, probably via microbe-microbe interactions. As the early-late phase shift involves purging most cells from bacterial community, early application of bioinoculant seems to be the right choice.

Acknowledgments

We are grateful to Prof. Wiesław Nowak and Dr. Łukasz Pepłowski for making available the computational resources.

List of abbreviations

NGI – next generation inoculant

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated during this study are available in NCBI's SRA repository under BioProjects no. **PRJNA615328** (soil communities), **PRJNA713813** (root communities), **PRJNA713992** (leaf communities) and **PRJNA847007** (inoculant and some leaf and root communities). qPCR and colony counts data are available on figshare (10.6084/m9.figshare.20652645 and 10.6084/m9.figshare.20652915). R scripts allowing generation of all figures and tables are part of Supplementary Methods SM7. Materials used in this work, excluding samples used in their entirety, are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

The study was supported by the National Science Centre Poland, project no. 2016/21/B/NZ9/00840 to MG. The funder had no role in study design, data interpretation or writing the manuscript.

Authors' contributions

MG concieved the study, obtained financing, designed experiments, participated in laboratory work, analyzed data, supervised the study and drafted the manuscript, MS performed laboratory work, participated in writing, JM performed laboratory work, SS performed laboratory work, JT participated in designing experiments, performed laboratory work, participated in writing, KH participated in designing experiments, participated in writing, WU analyzed data and participated in writing. All authors read and approved the final manuscript.

References

1. Compant S, Cambon MC, Vacher C, Mitter B, Samad A, Sessitsch A. The plant endosphere world – bacterial life within plants. Environmental Microbiology. 2021;23:1812–29.

2. Trivedi P, Leach JE, Tringe SG, Sa T, Singh BK. Plant–microbiome interactions: from community assembly to plant health. Nature Reviews Microbiology. Nature Publishing Group; 2020;18:607–21.

3. Santos MS, Nogueira MA, Hungria M. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. AMB Express. 2019;9:205.

4. Hou S, Thiergart T, Vannier N, Mesny F, Ziegler J, Pickel B, et al. A microbiota–root–shoot circuit favours *Arabidopsis* growth over defence under suboptimal light. Nat Plants. Nature Publishing Group; 2021;7:1078–92.

5. Szymańska S, Sikora M, Hrynkiewicz K, Tyburski J, Tretyn A, Gołębiewski M. Choosing source of microorganisms and processing technology for next generation beet bioinoculant. Sci Rep. Nature Publishing Group; 2021;11:2829.

6. Berninger T, González López Ó, Bejarano A, Preininger C, Sessitsch A. Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. Microbial Biotechnology. 2018;11:277–301.

7. Rocha I, Ma Y, Souza-Alonso P, Vosátka M, Freitas H, Oliveira RS. Seed Coating: A Tool for Delivering Beneficial Microbes to Agricultural Crops. Frontiers in Plant Science [Internet]. 2019 [cited 2023 Jan 13];10. Available from: https://www.frontiersin.org/articles/10.3389/fpls.2019.01357

8. O'Callaghan M, Ballard RA, Wright D. Soil microbial inoculants for sustainable agriculture: Limitations and opportunities. Soil Use and Management. 2022;38:1340–69.

9. Preininger C, Sauer U, Bejarano A, Berninger T. Concepts and applications of foliar spray for microbial inoculants. Appl Microbiol Biotechnol. 2018;102:7265–82.

10. Lau JA, Lennon JT. Rapid responses of soil microorganisms improve plant fitness in novel environments. Proc Natl Acad Sci USA. 2012;109:14058–62.

11. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, et al. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature. 2012;488:91–5.

12. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, et al. Structure, variation, and assembly of the root-associated microbiomes of rice. Proceedings of the National Academy of Sciences. Proceedings of the National Academy of Sciences; 2015;112:E911–20.

13. Khare E, Mishra J, Arora NK. Multifaceted Interactions Between Endophytes and Plant: Developments and Prospects. Frontiers in Microbiology [Internet]. 2018 [cited 2023 Jan 16];9. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02732

14. Xiong C, Zhu Y-G, Wang J-T, Singh B, Han L-L, Shen J-P, et al. Host selection shapes crop microbiome assembly and network complexity. New Phytologist. 2021;229:1091–104.

15. Youseif SH, Abd El-Megeed FH, Abdelaal AS, Ageez A, Martínez-Romero E. Plant–microbe–microbe interactions influence the faba bean nodule colonization by diverse endophytic bacteria. FEMS Microbiology Ecology. 2021;97:fiab138.

16. Clouse KM, Wagner MR. Plant Genetics as a Tool for Manipulating Crop Microbiomes: Opportunities and Challenges. Front Bioeng Biotechnol. 2021;9:567548.

17. Hereira-Pacheco SE, Navarro-Noya YE, Dendooven L. The root endophytic bacterial community of Ricinus communis L. resembles the seeds community more than the rhizosphere bacteria independent of soil water content. Sci Rep. Nature Publishing Group; 2021;11:2173.

18. Bodenhausen N, Horton MW, Bergelson J. Bacterial Communities Associated with the Leaves and the Roots of Arabidopsis thaliana. PLOS ONE. Public Library of Science; 2013;8:e56329.

19. Robinson RJ, Fraaije BA, Clark IM, Jackson RW, Hirsch PR, Mauchline TH. Endophytic bacterial community composition in wheat (Triticum aestivum) is determined by plant tissue type, developmental stage and soil nutrient availability. Plant Soil. 2016;405:381–96.

20. Carper DL, Carrell AA, Kueppers LM, Frank AC. Bacterial endophyte communities in Pinus flexilis are structured by host age, tissue type, and environmental factors. Plant Soil. 2018;428:335–52.

21. Wagner MR, Lundberg DS, del Rio TG, Tringe SG, Dangl JL, Mitchell-Olds T. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nature Communications [Internet]. 2016 [cited 2019 Feb 11];7. Available from: http://www.nature.com/articles/ncomms12151

22. Xiong C, Singh BK, He J-Z, Han Y-L, Li P-P, Wan L-H, et al. Plant developmental stage drives the differentiation in ecological role of the maize microbiome. Microbiome. 2021;9:171.

23. Edwards JA, Santos-Medellín CM, Liechty ZS, Nguyen B, Lurie E, Eason S, et al. Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. PLOS Biology. Public Library of Science; 2018;16:e2003862.

24. Zimmerman NB, Vitousek PM. Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. Proceedings of the National Academy of Sciences. Proceedings of the National Academy of Sciences; 2012;109:13022–7.

25. Ortiz-Álvarez R, Fierer N, de los Ríos A, Casamayor EO, Barberán A. Consistent changes in the taxonomic structure and functional attributes of bacterial communities during primary succession. ISME J. 2018;12:1658–67.

26. Ulrich W, Almeida-Neto M, Gotelli NJ. A consumer’s guide to nestedness analysis. Oikos. 2009;118:3–17.

27. Dohm JC, Minoche AE, Holtgräwe D, Capella-Gutiérrez S, Zakrzewski F, Tafer H, et al. The genome of the recently domesticated crop plant sugar beet (Beta vulgaris). Nature. 2014;505:546–9.

28. OECD, Nations F and AO of the U. Sugar [Internet]. Paris: OECD; 2021 Jul. Available from: https://www.oecd-ilibrary.org/agriculture-and-food/oecd-fao-agricultural-outlook-2021-2030_969526b0-en

29. Jammer A, Albacete A, Schulz B, Koch W, Weltmeier F, van der Graaff E, et al. Early-stage sugar beet taproot development is characterized by three distinct physiological phases. Plant Direct. 2020;4:e00221.

30. Thiem D, Gołębiewski M, Hulisz P, Piernik A, Hrynkiewicz K. How Does Salinity Shape Bacterial and Fungal Microbiomes of Alnus glutinosa Roots? Frontiers in Microbiology [Internet]. 2018 [cited 2019 Feb 11];9. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2018.00651/full

31. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. Nature Publishing Group; 2016;13:581–3.

32. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research. 2013;41:D590–6.

33. Sheneman L, Evans J, Foster JA. Clearcut: a fast implementation of relaxed neighbor joining. Bioinformatics. 2006;22:2823–4.

34. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75:7537–41.

35. Chen J, Bittinger K, Charlson ES, Hoffmann C, Lewis J, Wu GD, et al. Associating microbiome composition with environmental covariates using generalized UniFrac distances. Bioinformatics. 2012;28:2106–13.

36. Gołębiewski M, Tarasek A, Sikora M, Deja-Sikora E, Tretyn A, Niklińska M. Rapid Microbial Community Changes During Initial Stages of Pine Litter Decomposition. Microbial Ecology. 2019;77:56–75.

37. Wright ES, Yilmaz LS, Ram S, Gasser JM, Harrington GW, Noguera DR. Exploiting extension bias in polymerase chain reaction to improve primer specificity in ensembles of nearly identical DNA templates. Environmental Microbiology. 2014;16:1354–65.

38. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, et al. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol. Nature Publishing Group; 2020;38:685–8.

39. Almeida-Neto M, Ulrich W. A straightforward computational approach for measuring nestedness using quantitative matrices. Environmental Modelling & Software. 2011;26:173–8.

40. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community Ecology Package [Internet]. 2020. Available from: https://CRAN.R-project.org/package=vegan

41. Arbizu PM. pairwiseAdonis [Internet]. 2022 [cited 2022 Mar 10]. Available from: https://github.com/pmartinezarbizu/pairwiseAdonis

42. Rohart F, Gautier B, Singh A, Cao K-AL. mixOmics: An R package for ‘omics feature selection and multiple data integration. PLOS Computational Biology. Public Library of Science; 2017;13:e1005752.

43. Fernandes AD, Macklaim JM, Linn TG, Reid G, Gloor GB. ANOVA-Like Differential Expression (ALDEx) Analysis for Mixed Population RNA-Seq. PLOS ONE. Public Library of Science; 2013;8:e67019.

44. Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. Microbiome. 2014;2:15.

45. Rinaudo P, Boudah S, Junot C, Thévenot EA. biosigner: A New Method for the Discovery of Significant Molecular Signatures from Omics Data. Frontiers in Molecular Biosciences [Internet]. 2016 [cited 2022 Oct 25];3. Available from: https://www.frontiersin.org/articles/10.3389/fmolb.2016.00026

46. Thiergart T, Durán P, Ellis T, Vannier N, Garrido-Oter R, Kemen E, et al. Root microbiota assembly and adaptive differentiation among European Arabidopsis populations. Nat Ecol Evol. Nature Publishing Group; 2020;4:122–31.

47. Yang C, Yue H, Ma Z, Feng Z, Feng H, Zhao L, et al. Influence of plant genotype and soil on the cotton rhizosphere microbiome. Frontiers in Microbiology [Internet]. 2022 [cited 2022 Dec 13];13. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1021064

48. Koczorski P, Furtado BU, Go\lębiewski M, Hulisz P, Baum C, Weih M, et al. The Effects of Host Plant Genotype and Environmental Conditions on Fungal Community Composition and

Phosphorus Solubilization in Willow Short Rotation Coppice. Frontiers in Plant Science [Internet]. Frontiers Media SA; 2021;12. Available from: https://doi.org/10.3389%2Ffpls.2021.647709

49. Cowles HC. The Ecological Relations of the Vegetation on the Sand Dunes of Lake Michigan. Part I.-Geographical Relations of the Dune Floras. Botanical Gazette. The University of Chicago Press; 1899;27:95–117.

50. Sasse J, Martinoia E, Northen T. Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? Trends in Plant Science. 2018;23:25–41.

51. Dibner RR, Weaver AM, Brock MT, Custer GF, Morrison HG, Maignien L, et al. Time outweighs the effect of host developmental stage on microbial community composition. FEMS Microbiology Ecology. 2021;97:fiab102.

Tables

Table 1. Core microbiomes of soil, roots and leaf samples. Prevalence cutoff of 0.1% and detection of 90% were used.

ASV	Detection[1]	Prevalence[2]	Taxonomy
		Soil	
ASV3	98.84	7.04	*Pseudoxanthomonas*
ASV5	98.20	2.77	*Delftia*
ASV6	87.90	2.91	*Brevundimonas*
ASV11	92.92	1.37	*Caulobacter*
ASV14	99.87	1.80	*Devosia*
ASV27	93.05	1.18	*Pedobacter*
ASV38	90.86	0.57	*Hydrogenophaga*
ASV44	94.72	0.58	*Reyranella*
		Root	
ASV4	96.26	7.26	*Flavobacterium*
ASV5	97.01	5.30	*Delftia*
ASV14	91.29	1.18	*Devosia*
		Leaf	
ASV4	97.77	6.80	*Flavobacterium*

1 – percent of the number of samples in which a given ASV was present, 2 – mean abundance of a given ASV in a given compartment

Table 2. Late and early core microbiomes. Prevalence cutoff of 0.1% and detection of 90% were used.

ASV	Detection[1]	Prevalence[2]	Taxonomy
		Soil early	
ASV3	97.94	4.22	*Pseudoxanthomonas*
ASV4	96.11	3.74	*Flavobacterium*
ASV5	96.80	3.14	*Delftia*
ASV6	90.16	3.77	*Brevundimonas*
ASV14	99.77	1.58	*Devosia*
ASV44	91.76	0.31	*Reyranella*
		Soil late	
ASV3	100	10.66	*Pseudoxanthomonas*
ASV5	100	2.28	*Delftia*
ASV10	93.53	1.83	*Pseudomonas*
ASV11	98.24	1.27	*Caulobacter*
ASV14	100	2.09	*Devosia*
ASV21	90.88	2.37	*Sphingopyxis*
ASV24	95.88	1.59	*Pseudohangiellaceae, BIyi10*
ASV27	98.53	2.05	*Pedobacter*
ASV33	99.12	1.54	*Micropepsaceae*
ASV38	94.71	0.78	*Hydrogenophaga*
ASV44	98.53	0.93	*Reyranella*
ASV54	92.35	0.73	*Shinella*
ASV58	96.47	0.37	*Sphingobacteriaceae*
ASV59	96.76	0.68	*Bosea*
ASV74	92.65	0.66	*Gemmatirosa*

		Root early	
ASV4	98.50	7.92	*Flavobacterium*
ASV5	98.80	5.75	*Delftia*
ASV14	94.31	1.21	*Devosia*
		Root late	
ASV4	91.18	4.03	*Flavobacterium*
		Leaf early	
ASV4	97.30	8.72	*Flavobacterium*
ASV23	93.69	8.24	*Chryseobacterium*
		Leaf late	
ASV3	96.74	3.63	*Pseudoxanthomonas*
ASV4	98.91	2.19	*Flavobacterium*
ASV6	92.39	2.45	*Brevundimonas*
ASV11	91.30	1.70	*Caulobacter*
ASV13	94.57	2.31	*Sphingobacterium*
ASV15	97.83	4.18	*Flavobacterium*
ASV17	96.74	2.43	*Thermomonas*

1 – percent of the number of samples in a given set in which a given ASV was present, 2 – mean abundance of a given ASV in a given set of samples

Table 3. Inoculated and non-inoculated samples core microbiomes. Prevalence cutoff of 0.1% and detection of 90% were used.

ASV	Detection[1]	Prevalence[2]	Taxonomy
		Soil early inoculated	
ASV5	96.80	5.81	*Delftia*
ASV6	90.16	6.97	*Brevundimonas*
ASV14	99.77	2.92	*Devosia*
ASV44	91.76	0.57	*Reyranella*
		Soil early non-inoculated	
ASV3	97.94	9.18	*Pseudoxanthomonas*
ASV4	96.11	8.14	*Flavobacterium*
		Soil late inoculated	
ASV5	100	4.44	*Delftia*
ASV10	93.53	3.57	*Pseudomonas*
ASV11	98.24	2.47	*Caulobacter*
ASV14	100	4.07	*Devosia*
ASV21	90.88	4.60	*Sphingopyxis*
ASV44	98.53	1.81	*Reyranella*
ASV54	92.35	1.43	*Shinella*
ASV58	96.47	0.73	*Sphingobacteriaceae*
ASV59	96.76	1.32	*Bosea*
ASV74	92.65	1.28	*Gemmatirosa*
		Soil late non-inoculated	
ASV3	100	21.95	*Pseudoxanthomonas*

ASV24	95.88	3.27	*Pseudohallangiaceae, BIyi10*
ASV27	98.53	4.22	*Pedobacter*
ASV33	99.12	3.17	*Micropepsaceae*
ASV38	94.71	1.60	*Hydrogenophaga*
		Root early inoculated	
ASV5	98.80	10.48	*Delftia*
ASV14	94.31	2.20	*Devosia*
		Root early non-inoculated	
ASV4	98.50	17.56	*Flavobacterium*
		Root late inoculated	
		No core ASVs	
		Root late non-inoculated	
ASV4	91.18	7.85	*Flavobacterium*
		Leaf early inoculated	
		No core ASVs	
		Leaf early non-inoculated	
ASV4	97.30	16.95	*Flavobacterium*
ASV23	93.69	16.03	*Chryseobacterium*
		Leaf late inoculated	
ASV6	92.39	4.63	*Brevundimonas*
ASV11	91.30	3.21	*Caulobacter*
ASV13	94.57	4.37	*Sphingobacterium*
ASV15	97.83	7.90	*Flavobacterium*
		Leaf late non-inoculated	
ASV3	96.74	7.71	*Pseudoxanthomonas*
ASV4	98.91	4.64	*Flavobacterium*
ASV17	96.74	5.17	*Thermomonas*

1 – percent of the number of samples in which a given ASV was present, 2 – mean abundance of a given ASV in a given compartment

Fig. 1. Experimental design. Single experimental variant is shown, there were two soils S1 and S2 and three genotypes (B – *Beta vulgaris* ssp. *vulgaris* cv 'Bravo'; C - *B. vulgaris* ssp. *vulgaris* cv. 'Cassino', M – *B. vulgaris* ssp. *maritima*), either inoculated with lyophilized roots of sea beets growing in the wild (I) or non-inoculated control (C). Each timepoint (soil × genotype × inoculation) consisted of five biological replicates (BRs - pots), and there were five technical replicates (TRs - plants) in each BR. Leaves, roots and rhizosphere soil were collected from each TR. Leaves and soils were directly snap-frozen in liquid nitrogen, roots were surface-sterilized prior to freezing.

Fig 2. Communities in soils, roots and leaves differ in terms of structure, alpha-diversity and bacterial load. L – leaves, R – roots, S – soils, I – inoculant (lyophilized wild-growing sea beet roots). A) NMDS analysis of d05 generalized UniFrac distance matrix, black – inoculant (lyophilized wild sea beet roots), blue – soils, green – roots, red – leaves; shape shows timepoint, circles – two weeks after planting in soil (T0), squares – fifteen days (T1), diamonds – three weeks (T2), up pointing triangles – four weeks (T3), down pointing triangles – six weeks (T4). B) Alpha-diversity of ASVs. Boxplots of Shannon's diversity index (H') - upper left, Shannon's evenness – upper right, richness (observed number of ASVs) – lower left, number of bacterial 16S rRNA gene sequences per ng of DNA – lower right. C) Ternary plot of ASVs displaying differential abundance in soils, roots and leaves. Mean ASV's abundance is shown as circle size, color denotes classification at the class level; Alphaproteobacteria – red, Bacilli green, Bacteroidia – blue, Gammaproteobacteria - purple. D) Taxonomic structure of soils (S), roots (R), and leaves (L) communities.

Fig. 3. Sets of functions predicted to be encoded in genomes of organisms thriving in soils, roots and leaves and diversity of these sets differ. A) dbRDA of Morisita-Horn distance matrix derived from functions sets imputed by PICRUSt2, black – inoculant (lyophilized wild sea beet roots), blue – soils, green – roots, red – leaves; shape shows timepoint, circles – two weeks after planting in soil (T0), squares – fifteen days (T1), diamonds – three weeks (T2), up pointing triangles – four weeks (T3), down pointing triangles – six weeks (T4). B) Alpha-diversity of functions. Boxplots of Shannon's diversity index (H') - upper left, Shannon's evenness – upper right, richness (observed number of functions) – lower left. C) Ternary plot of pathways displaying differential abundance in soils, roots and leaves, scale is arbitrary. Color shows to which broad category a given pathway belongs; antibiotic resistance and biosynthesis – red, carbohydrates metabolism – yellow-green, heavy metals resistance – green, quorum sensing – blue, secretion systems - purple.

Fig. 4. Bacterial communities in T0, T1 and T2 ('early') samples form a cluster distinct from the one formed by T3 and T4 ('late') ones. AB – leaves, CD – roots, EF – soils. ACE – dbRDA analysis of d05 generalized UniFrac distance matrix (timepoint was a covariate), T0 – circles, T1 – squares, T2 – diamonds, T3 – up pointing triangles, T4 – down pointing triangles. 'Early' cluster – black fill, 'late' cluster – red fill. P-values of a permutational test for dbRDA model (ANOVA) and multidimensional homogeneity of variance (betadisper) as well as percent variance explained by 'early' – 'late' status (Var. expl.) are given on the plots. BDF – bacterial load (lower right), richness (lower left), Shannon's E (upper right) and Shannon's H (upper left). 'Early' cluster – black frame, 'late' cluster – red frame. Significance of late – early difference (Wilcoxon test) is shown over the plots: *** - $p < 0.001$, ** - $p < 0.01$, * - $p < 0.05$, ns – not significant.

Fig. 5. Sets of functions predicted to be encoded by genomes of organisms thriving in T0, T1 and T2 ('early') samples form a cluster distinct from the one formed by T3 and T4 ('late') ones. AB –

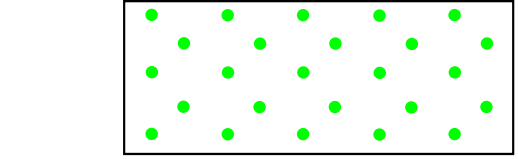
leaves, CD – roots, EF – soils. ACE – dbRDA analysis of Morisita-Horn distance matrix (timepoint was a covariate), T0 – circles, T1 – squares, T2 – diamonds, T3 – up pointing triangles, T4 – down pointing triangles. ‘Early’ cluster – black fill, ‘late’ cluster – red fill. P-values of a permutational test for dbRDA model (ANOVA) and multidimensional homogeneity of variance (betadisper) as well as percent variance explained by ‘early’ – ‘late’ status (Var. expl.) are given on the plots. BDF – richness (lower left), Shannon’s E (upper right) and Shannon’s H (upper left). ‘Early’ cluster – black frame, ‘late’ cluster – red frame. Significance of late – early difference (Wilcoxon test) is shown over the plots: *** - $p < 0.001$, ** - $p < 0.01$, * - $p < 0.05$, ns – not significant.

Fig. 6. Nestedness is higher in early samples than in late ones. Weighted NODF index means and standard deviation are plotted for soil – roots – leaves triplets. ‘Late’ timepoints are framed in red, ‘early’ ones in black. Significance of late – early difference (Wilcoxon test) is shown over the plots: *** - $p < 0.001$, ** - $p < 0.01$, * - $p < 0.05$, ns – not significant.

Fig. 7. Inoculant characterization. A – taxonomic structure of bacterial community. B – bacteria from inoculant are able to grow in presence of up to 900 mM NaCl; I – inoculant, NC – non-inoculated control.

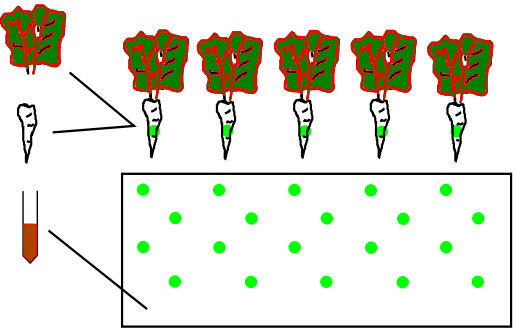
Fig. 8. Bacterial communities from inoculated and non-inoculated samples form clusters regardless of material, but inoculation does not influence alpha-diversity and bacterial load. AB – leaves, CD – roots, EF – soils. ACE - dbRDA analysis of d05 generalized UniFrac distance matrices, inoculated samples – black fill, non-inoculated samples – red fill. P-values of permutational tests for dbRDA models (ANOVA) and multidimensional homogeneity of variance (betadisper) as well as percent variance explained by inoculation (Var. expl.) are given on the plots. BDF – Shannon’s H (upper left), Shannon’s E (upper right), richness (lower left) and bacterial load (lower right). Bacterial load was not measured in soils. Significance of inoculated-non-inoculated difference (Wilcoxon test) is shown over the plots: *** - $p < 0.001$, ** - $p < 0.01$, * - $p < 0.05$, ns – not significant.

Fig. 9. Sets of functions predicted to be encoded by genomes of organisms thriving in inoculated and non-inoculated samples form clusters in soils, but inoculation does not influence alpha-diversity. AB – leaves, CD – roots, EF – soils. ACE - dbRDA analysis of Morisita-Horn distance matrices, inoculated samples – black fill, non-inoculated samples – red fill. P-values of permutational tests for dbRDA models (ANOVA) and multidimensional homogeneity of variance (betadisper) as well as percent variance explained by inoculation (Var. expl.) are given on the plots. BDF – Shannon’s H (upper left), Shannon’s E (upper right) and richness (lower left). Significance of inoculated-non-inoculated difference (Wilcoxon test) is shown over the plots: *** - $p < 0.001$, ** - $p < 0.01$, * - $p < 0.05$, ns – not significant.



0 Planting in soil

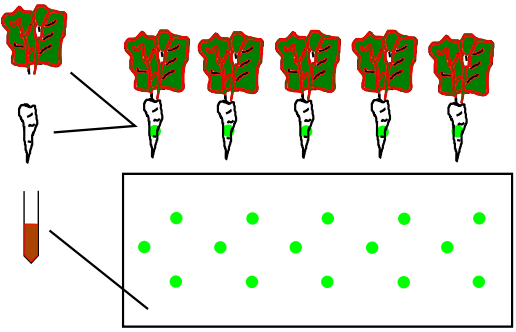
28 days



28 T0 - prior to inoculation

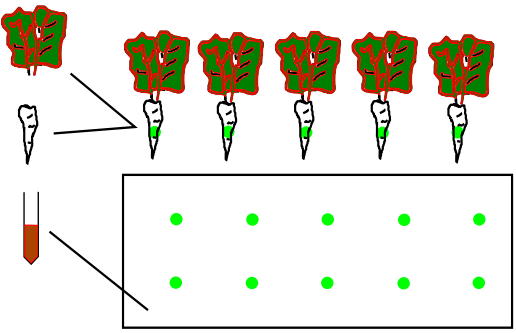
← inoculation

1 day



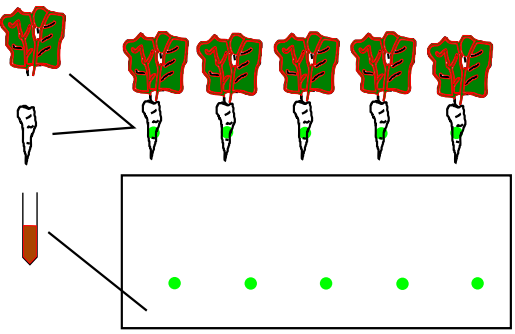
29 T1 - 1 day post-inoculation

6 days



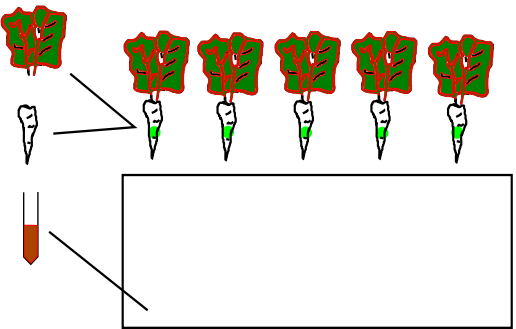
35 T2 - 7 days post-inoculation

21 days

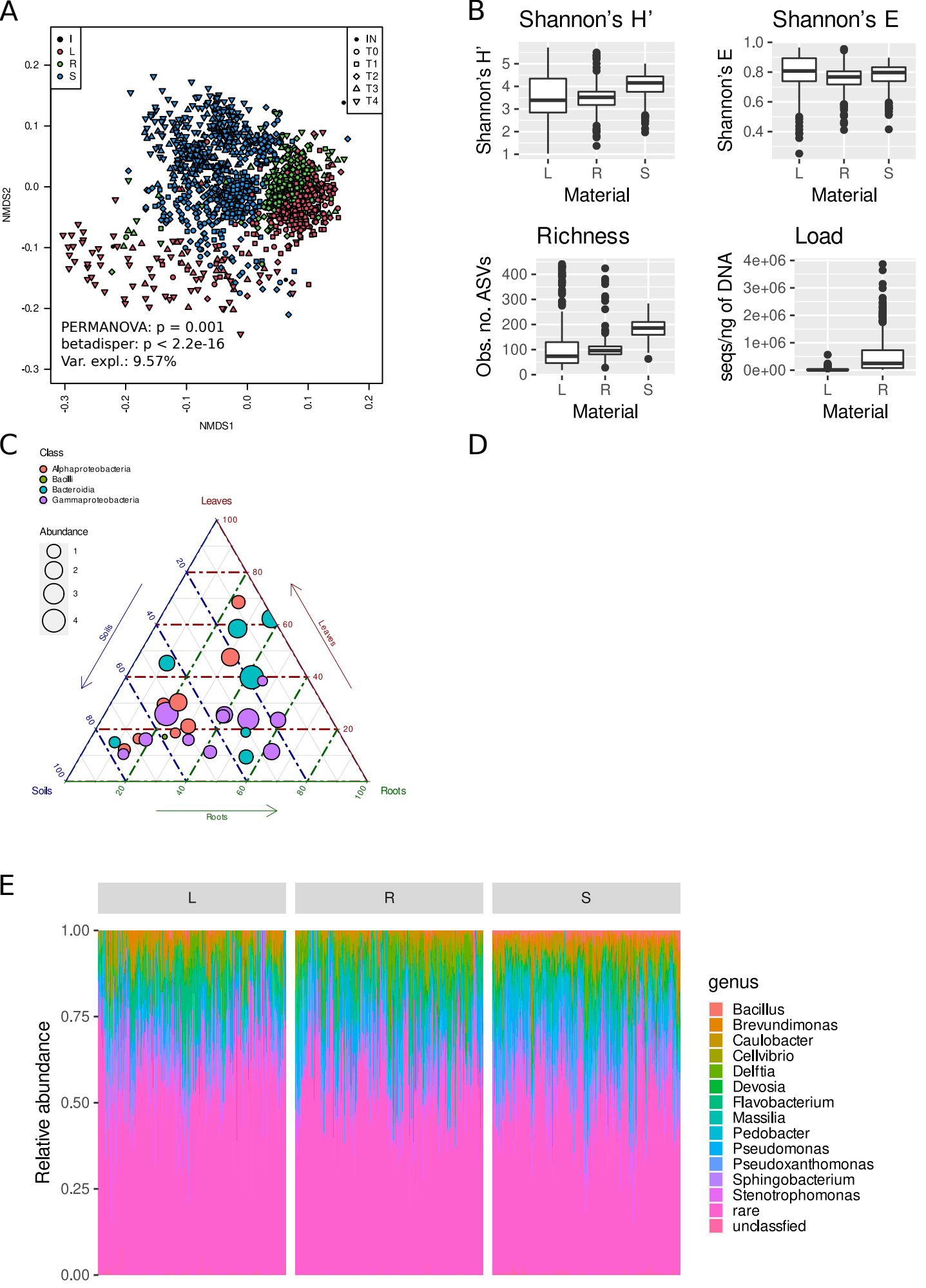


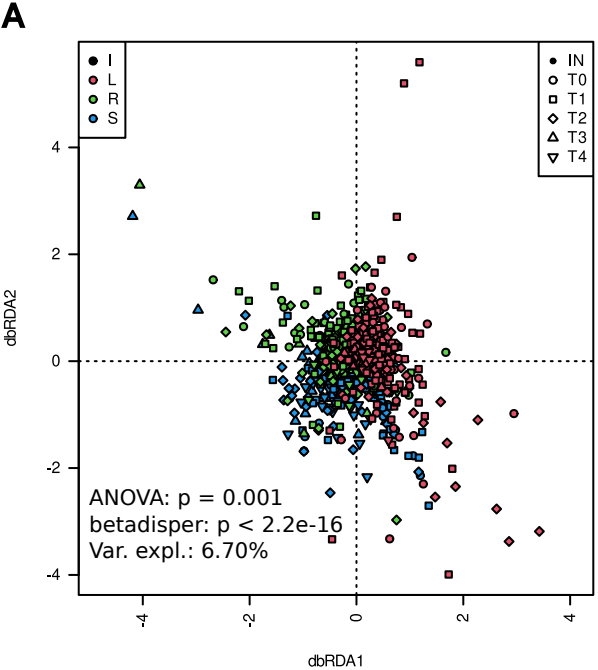
56 T3 - 28 days post-inoculation

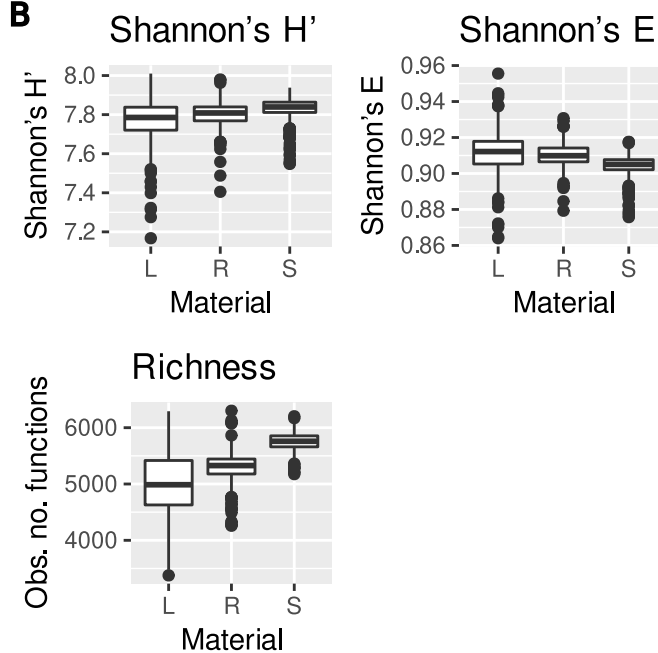
30 days

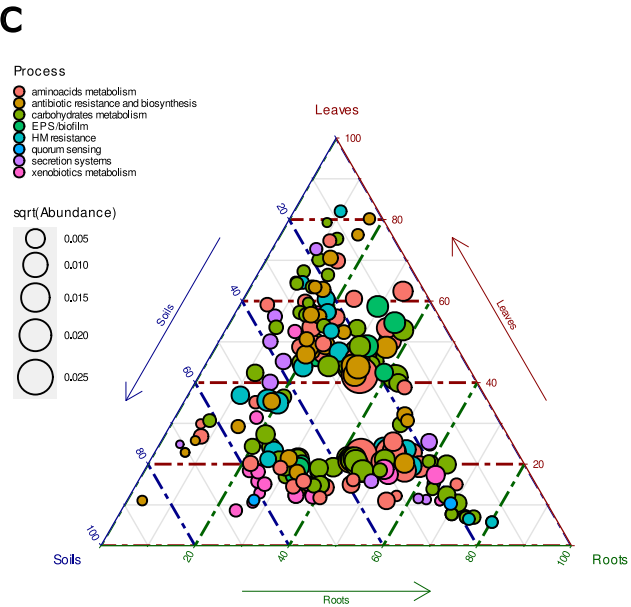


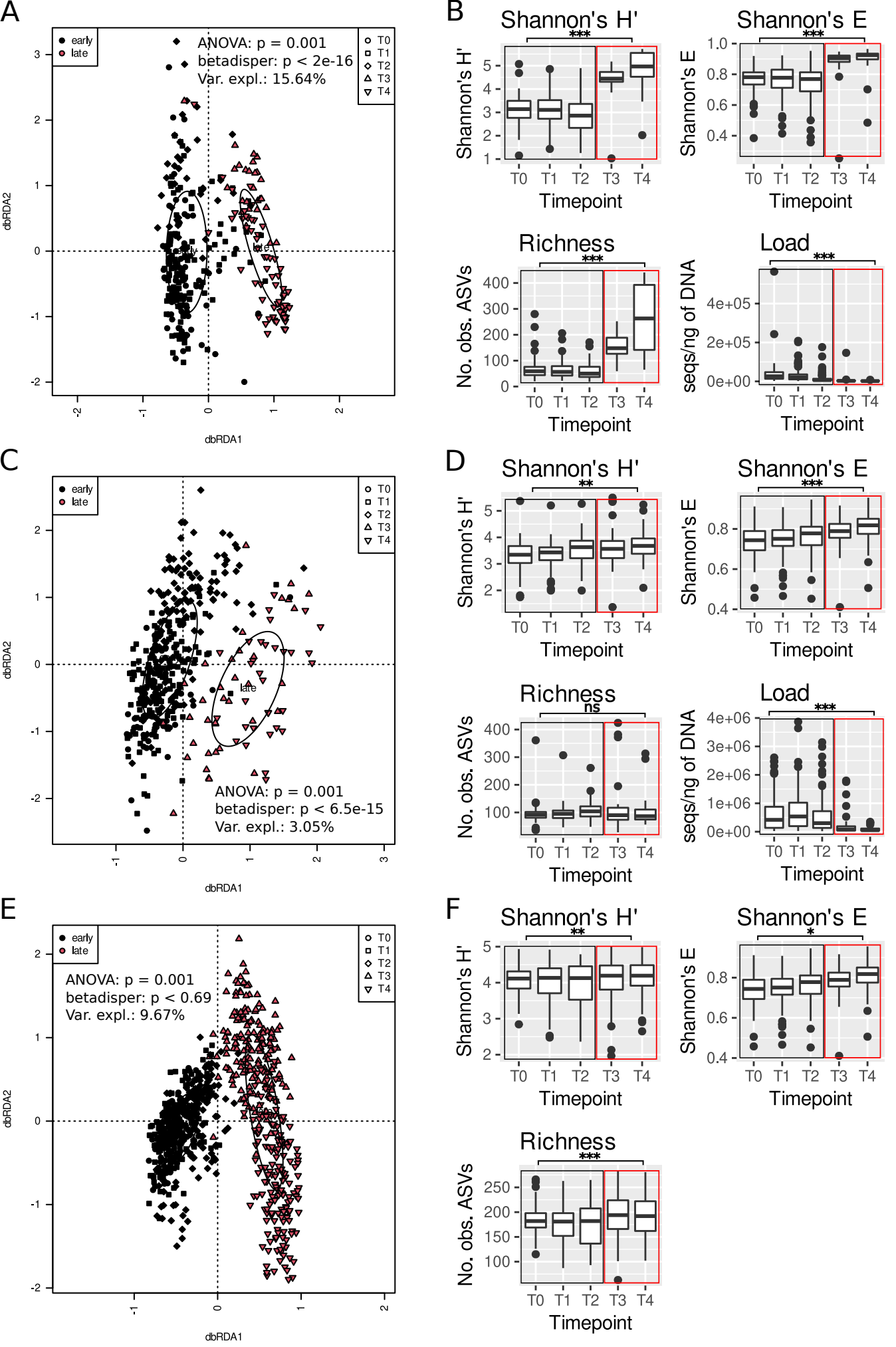
86 T4 - 58 days post-inoculation

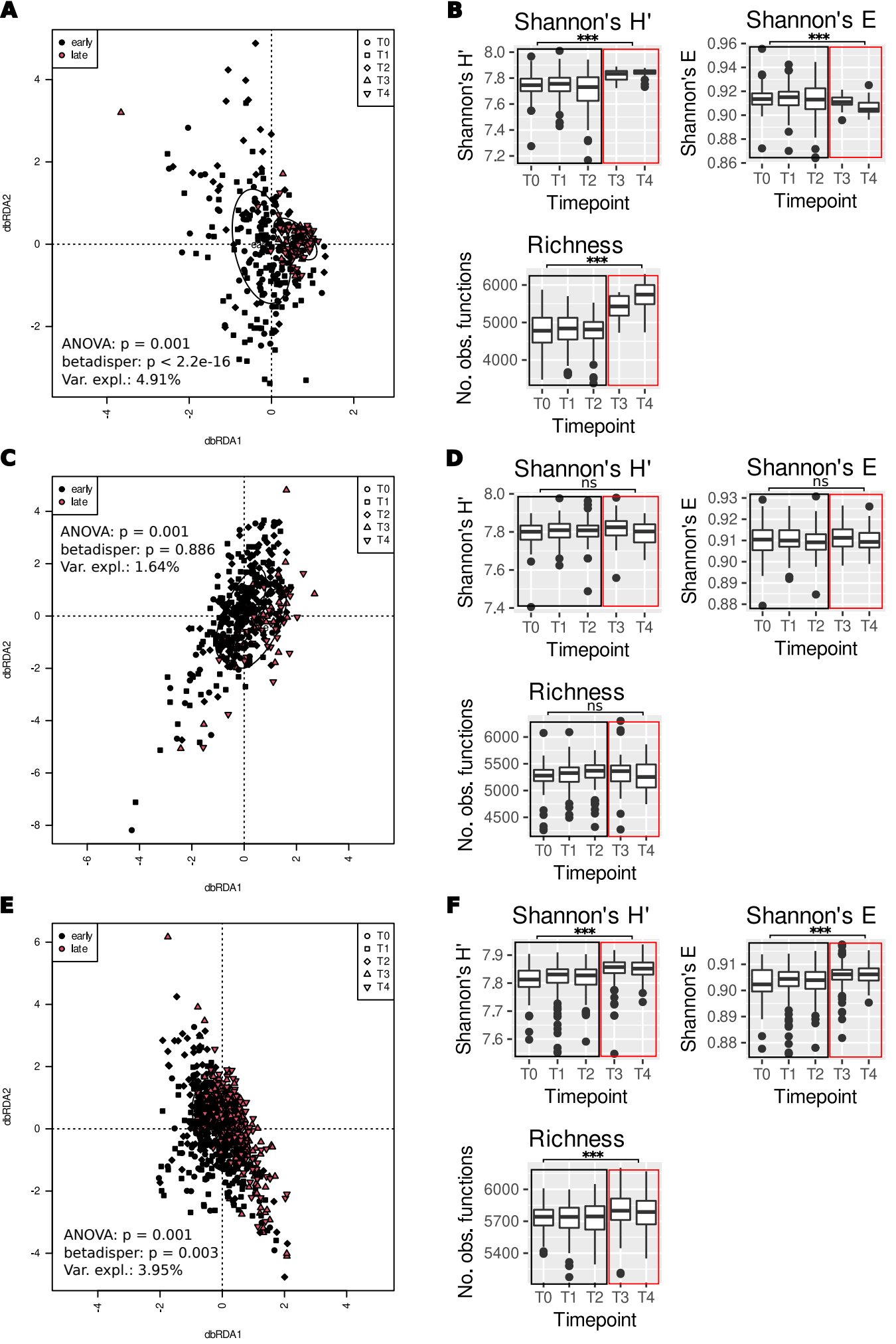

A
I
L
R
S
IN
T0
T1
T2
T3
T4
PERMANOVA: p = 0.001
betadisper: p < 2.2e-16
Var. expl.: 9.57%
NMDS1
NMDS2
B
Shannon's H'
Shannon's E
Richness
Load
Obs. no. ASVs
seqs/ng of DNA
Material
C
Class
Alphaproteobacteria
Bacilli
Bacteroidia
Gammaproteobacteria
Abundance
Leaves
Soils
Roots
D
E
L
R
S
Relative abundance
genus
Bacillus
Brevundimonas
Caulobacter
Cellvibrio
Delftia
Devosia
Flavobacterium
Massilia
Pedobacter
Pseudomonas
Pseudoxanthomonas
Sphingobacterium
Stenotrophomonas
rare
unclassfied

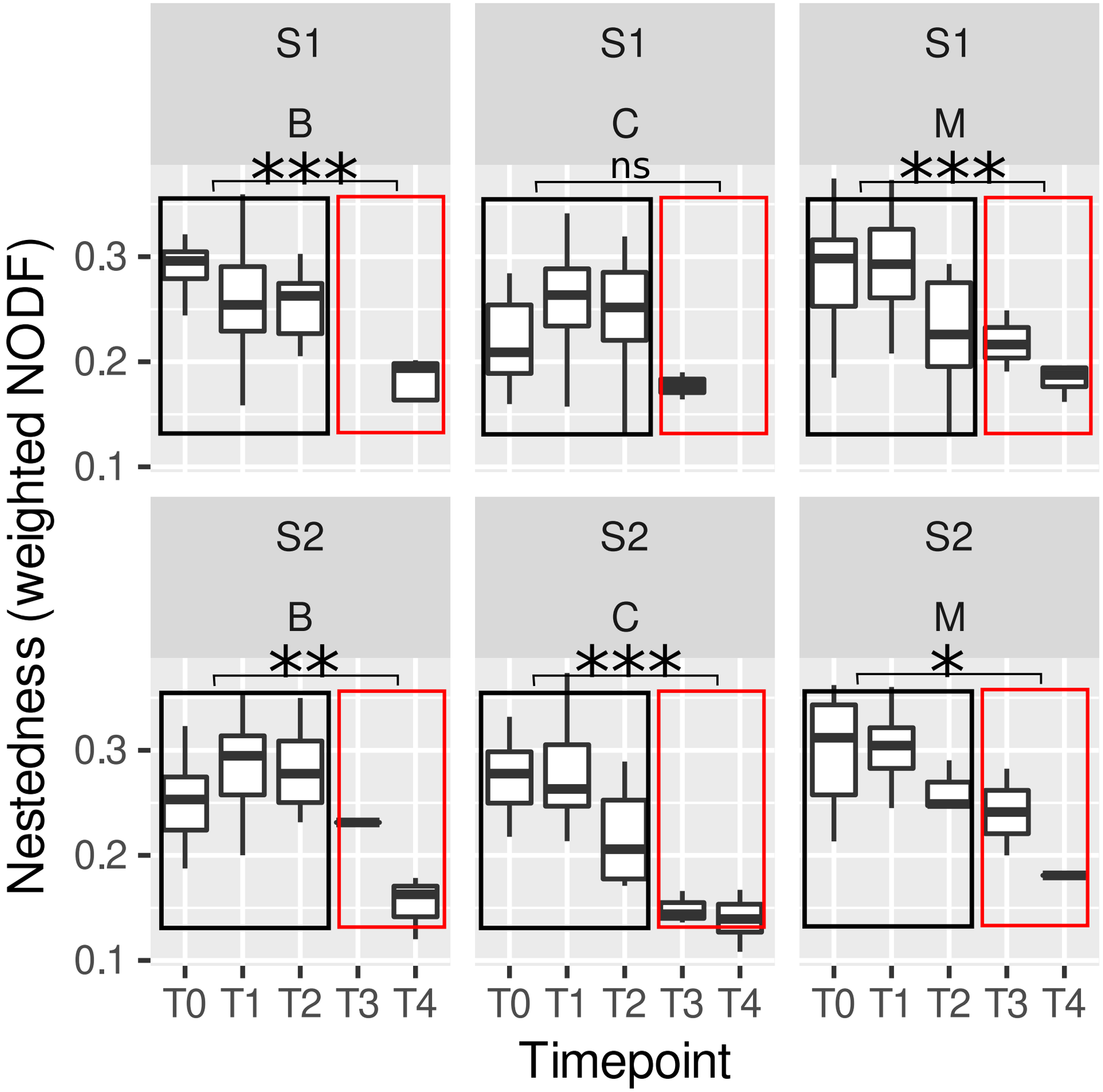

A
I
L
R
S
IN
T0
T1
T2
T3
T4
ANOVA: p = 0.001
betadisper: p < 2.2e-16
Var. expl.: 6.70%
dbRDA1
dbRDA2

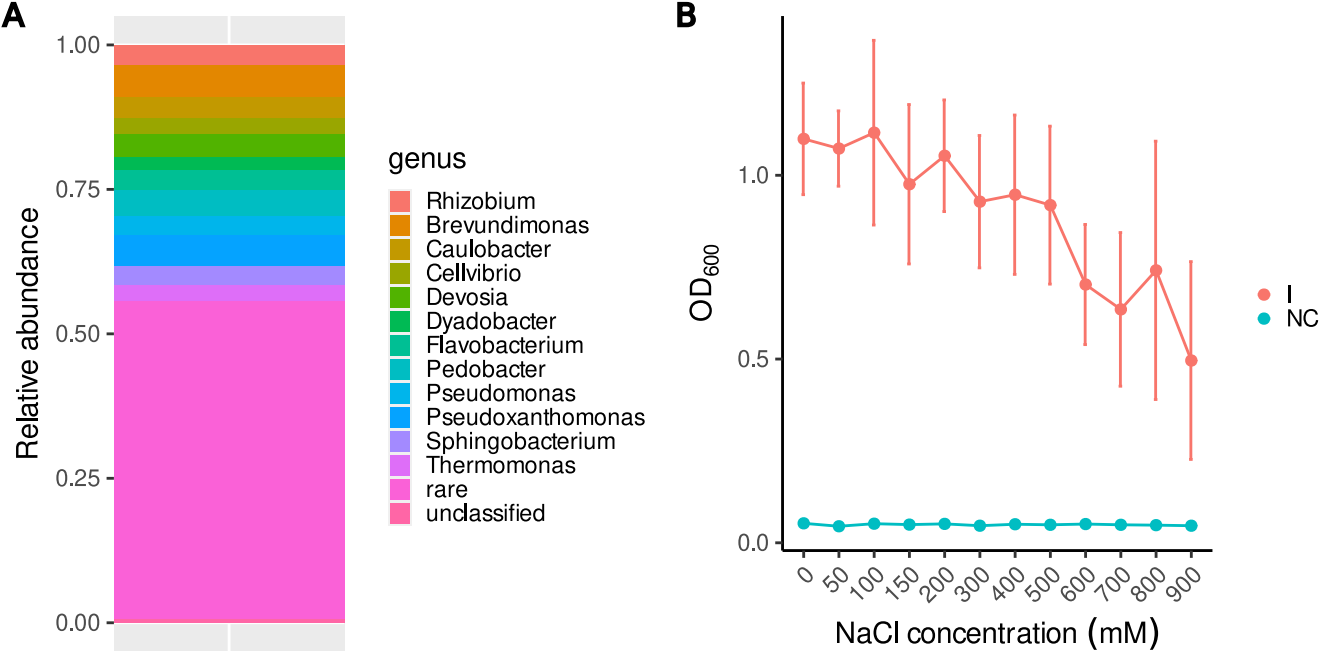

B
Shannon's H'
Shannon's E
Richness
Shannon's H'
Shannon's E
Obs. no. functions
Material
L
R
S

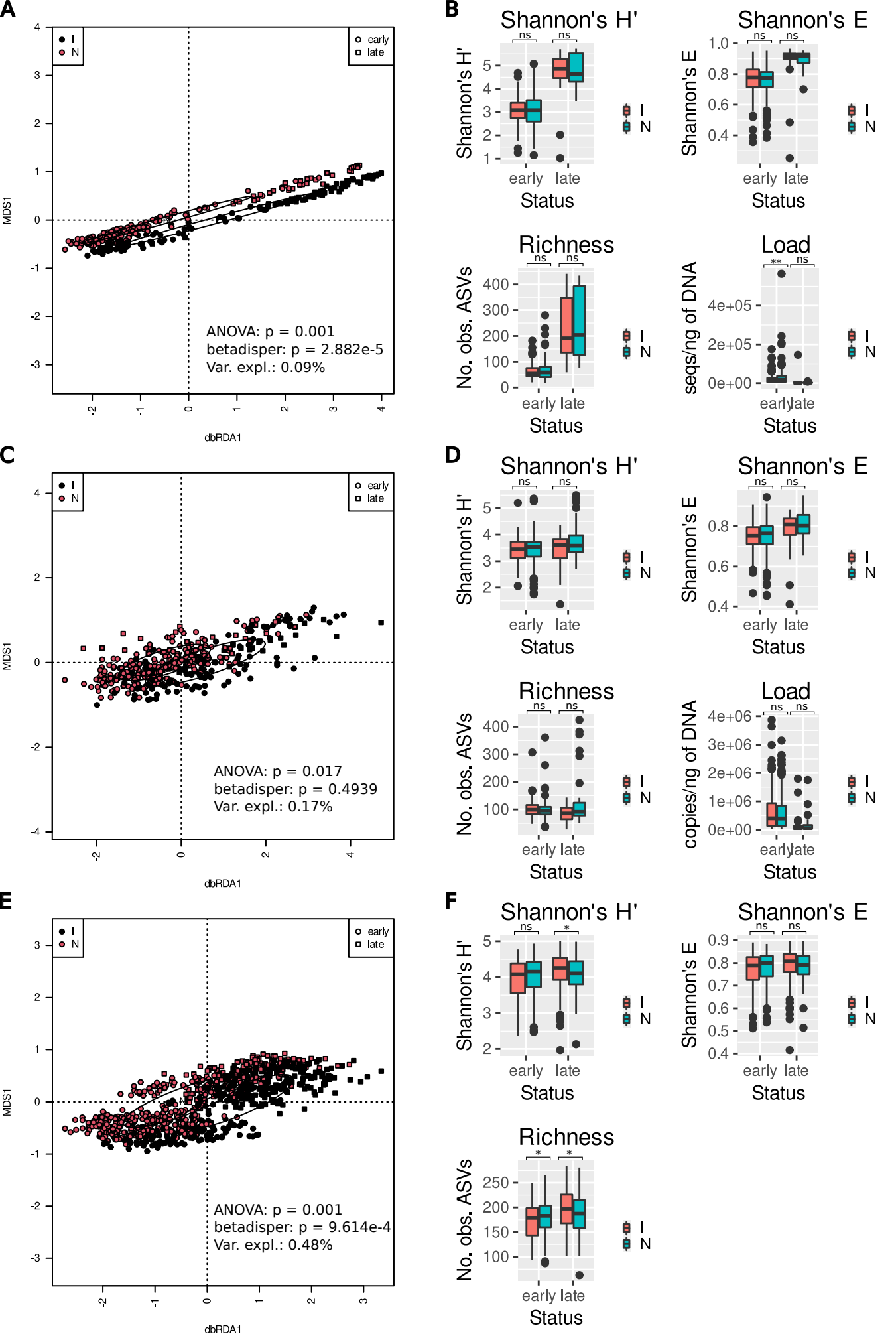

C
Process
aminoacids metabolism
antibiotic resistance and biosynthesis
carbohydrates metabolism
EPS/biofilm
HM resistance
quorum sensing
secretion systems
xenobiotics metabolism
sqrt(Abundance)
0.005
0.010
0.015
0.020
0.025
Leaves
Soils
Roots

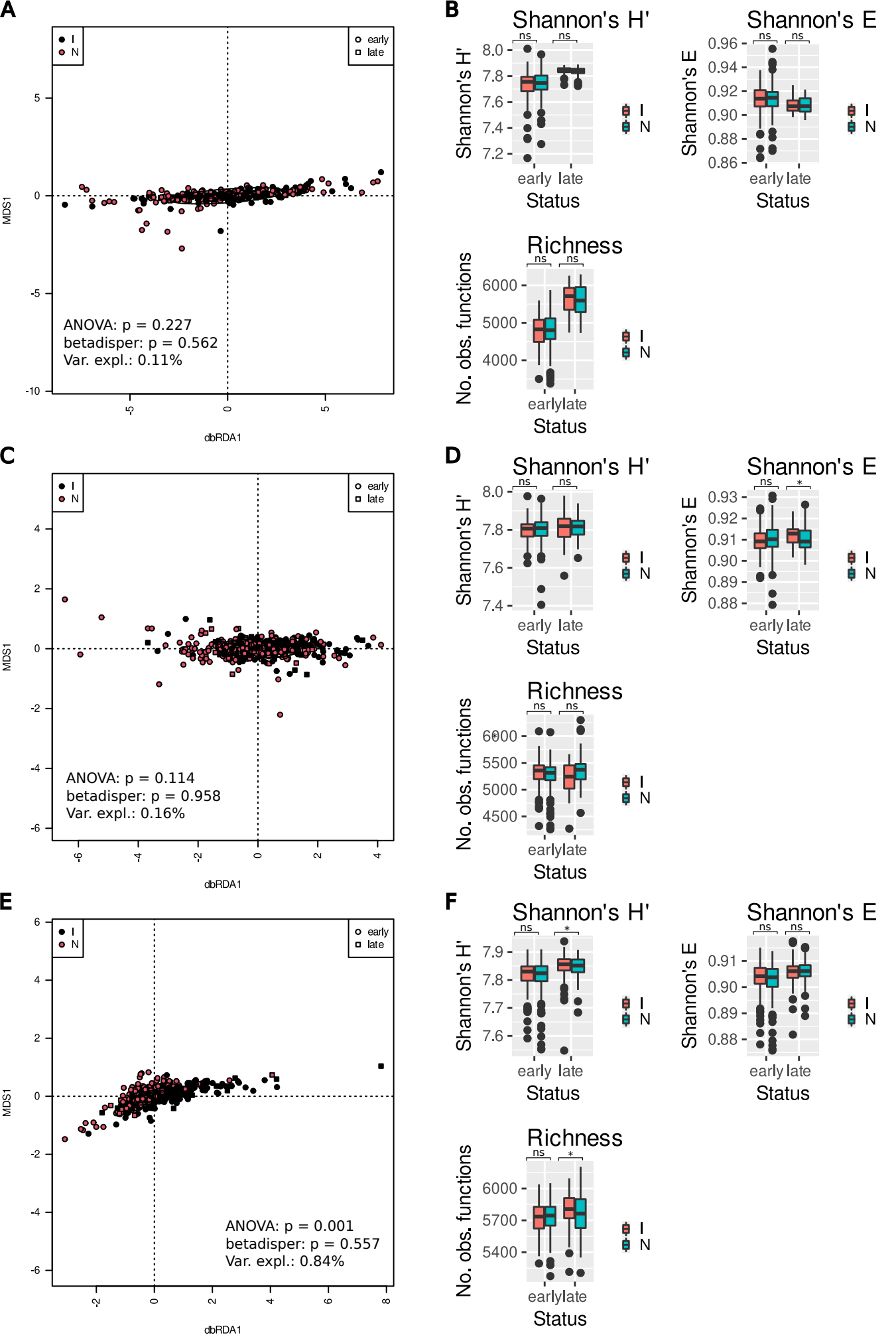

A
early
late
ANOVA: p = 0.001
betadisper: p < 2e-16
Var. expl.: 15.64%
T0
T1
T2
T3
T4
dbRDA1
dbRDA2
B
Shannon's H'
Shannon's E
Richness
Load
Timepoint
No. obs. ASVs
seqs/ng of DNA
C
ANOVA: p = 0.001
betadisper: p < 6.5e-15
Var. expl.: 3.05%
D
ns
E
ANOVA: p = 0.001
betadisper: p < 0.69
Var. expl.: 9.67%
F


A
early
late
T0
T1
T2
T3
T4
ANOVA: p = 0.001
betadisper: p < 2.2e-16
Var. expl.: 4.91%
dbRDA1
dbRDA2
B
Shannon's H'
Shannon's E
Richness
No. obs. functions
Timepoint
***
C
ANOVA: p = 0.001
betadisper: p = 0.886
Var. expl.: 1.64%
D
ns
E
ANOVA: p = 0.001
betadisper: p = 0.003
Var. expl.: 3.95%
F


S1
B
***
S1
C
ns
S1
M
***
S2
B
**
S2
C
***
S2
M
*
0.3
0.2
0.1
Nestedness (weighted NODF)
T0 T1 T2 T3 T4
Timepoint


A
1.00
0.75
0.50
0.25
0.00
Relative abundance
genus
Rhizobium
Brevundimonas
Caulobacter
Cellvibrio
Devosia
Dyadobacter
Flavobacterium
Pedobacter
Pseudomonas
Pseudoxanthomonas
Sphingobacterium
Thermomonas
rare
unclassified
B
1.0
0.5
0.0
OD600
0
50
100
150
200
300
400
500
600
700
800
900
NaCl concentration (mM)
I
NC


A
I
N
early
late
ANOVA: p = 0.001
betadisper: p = 2.882e-5
Var. expl.: 0.09%
MDS1
dbRDA1
B
Shannon's H'
Shannon's E
Richness
Load
No. obs. ASVs
seqs/ng of DNA
Status
early
late
ns
**
C
ANOVA: p = 0.017
betadisper: p = 0.4939
Var. expl.: 0.17%
D
copies/ng of DNA
E
ANOVA: p = 0.001
betadisper: p = 9.614e-4
Var. expl.: 0.48%
F
*


A
I
N
early
late
ANOVA: p = 0.227
betadisper: p = 0.562
Var. expl.: 0.11%
MDS1
dbRDA1
B
Shannon's H'
Shannon's E
Richness
No. obs. functions
Status
early
late
ns
C
ANOVA: p = 0.114
betadisper: p = 0.958
Var. expl.: 0.16%
D
*
E
ANOVA: p = 0.001
betadisper: p = 0.557
Var. expl.: 0.84%
F