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Successive plant growth amplifies genotype-specific assembly of the tomato rhizosphere microbiome

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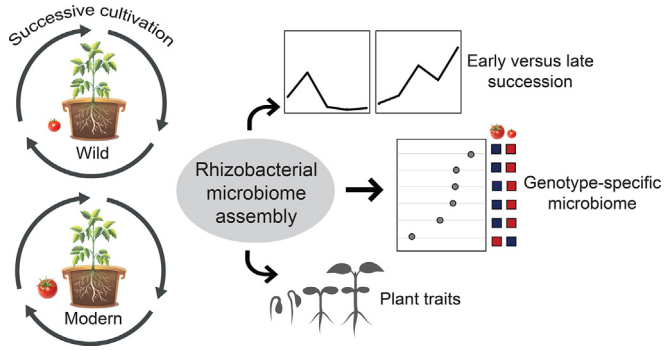
HIGHLIGHTS

- Genotype effects were amplified through successive cultivation.
- Modern and wild tomato microbiomes become more dissimilar over time.
- Early and late successional rhizobacterial taxa were identified.
- Successive cultivation leads to accelerated seedling emergence.

GRAPHICAL ABSTRACT



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ABSTRACT

Plant microbiome assembly is a spatial and dynamic process driven by root exudates and influenced by soil type, plant developmental stage and genotype. Genotype-dependent microbiome assembly has been reported for different crop plant species. Despite the effect of plant genetics on microbiome assembly, the magnitude of host control over its root microbiome is relatively small or, for many plant species, still largely unknown. Here we cultivated modern and wild tomato genotypes for four successive cycles and showed that divergence in microbiome assembly between the two genotypes was significantly amplified over time. Also, we show that the composition of the rhizosphere microbiome of modern and wild plants became more dissimilar from the initial bulk soil and from each other. Co-occurrence analyses further identified amplicon sequence variants (ASVs) associated with early and late successions of the tomato rhizosphere microbiome. Among the members of the Late Successional Rhizosphere microbiome, we observed an enrichment of ASVs belonging to the genera *Acidovorax*, *Massilia* and *Rhizobium* in the wild tomato rhizosphere, whereas the modern tomato rhizosphere was enriched for an ASV belonging to the genus *Pseudomonas*. Collectively, our approach allowed us to study the dynamics of rhizosphere microbiome over successional cultivation as well as to categorize rhizobacterial taxa for their ability to form transient or long-term associations with their host plants.

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1. Introduction

Microbiome assembly is a spatial and dynamic process driven by root exudates and influenced by soil type, plant developmental stage and genotype (Badri and Vivanco, 2009; Chaparro et al., 2013; Chaparro et al., 2014; Cordovez et al., 2019). The effect of plant genotype on microbiome assembly has been reported for crop cultivars and their wild relatives (Bulgarelli et al., 2015; Perez-Jaramillo et al., 2017; Wagner et al., 2016; Zachow et al., 2014), for genotypes of the same plant species (Coleman-Derr et al., 2016; Lundberg et al., 2012; Schlaeppi et al., 2014), and for plants with mutations in specific genes and pathways (Carvalhais et al., 2013; Lebeis et al., 2015; Thiergart et al., 2019). In addition, the genotype effects also vary according to specific root-compartments, as for example, greater selection signal was found in the rhizosphere compared to the endosphere of rice root microbiomes (Edwards et al., 2015). Although it is well recognized that distinct genotype-specific processes (such as root exudation and mucilage formation) mediate microbiome assembly (Micallef et al., 2009a; Miller et al., 2019), the underlying mechanisms by which these processes operate are still poorly understood. Hence, identification of these mechanisms and the plant genes associated with specific microbial taxa assembly is a key step towards the development of new plant breeding strategies that enable the recruitment of beneficial microorganisms and microbial functions that support plant growth and health (Gopal and Gupta, 2016; Wagner et al., 2016).

Comparison of the root and leaf microbiomes of the wild perennial plant *Boechera stricta* showed that plant genotypes differed significantly in richness and composition of the leaf microbiome whereas genetic control of the root microbiome was much weaker (Wagner et al., 2016). Indeed, many studies have shown that the influence of host genetics on rhizosphere microbiome assembly, although significant, is relatively small compared to the impact of other environmental factors, in particular of soil type and pH. These findings have been consistently described for several plant species, including model plant species such as *Arabidopsis thaliana* and *Lotus japonicus* (Lundberg et al., 2012; Micallef et al., 2009b; Thiergart et al., 2019) as well as different crop species (Bulgarelli et al., 2015; Perez-Jaramillo et al., 2017). Moreover, the relative contribution of the plant genotype to microbiome assembly also changes during plant development. For example, significant selection signals on the rhizosphere microbiome of potato and sorghum plants were reported to be detectable only at later plant developmental stages (İnceoğlu et al., 2010; Schlemper et al., 2017).

Investigations into rhizosphere microbiome assembly are generally conducted for a single life cycle of the host plant and a single time point or developmental stage, and thus, little is known about microbial community dynamics in the rhizosphere during plant succession (Chang and Turner, 2019). Several plant-soil feedback studies have demonstrated that plant-microbiome interactions are influenced by successive growth cycles of a given plant species on the same soil (Hendriks et al., 2015; Hu et al., 2018; van der Putten et al., 2013). These feedback mechanisms are driven by successive changes in soil biotic and abiotic factors which in turn affect plant growth, root development and resistance to pests and pathogens below and aboveground (Hannula et al., 2019; Hu et al., 2018). Collectively, these studies highlight the need for a mechanistic understanding of the factors impacting the rhizosphere microbiome assembly within an ecological and evolutionary framework. A recent study employing the passaging of the tomato phyllosphere microbiome over four consecutive cycles elegantly showed the establishment of a stable microbiome (Morella et al., 2020). Moreover, significant effects of the plant genotype on the bacterial community composition in the tomato phyllosphere were found for the first two cycles, but these genotype effects became insignificant at later growth cycles. In the present study, we tested if successive cycling can amplify the genotype selection leading to greater differences in the rhizosphere microbiome of wild and modern tomato species. More specifically, we determined the turnover rate and the taxonomic differences in the rhizosphere microbiomes of the two contrasting tomato genotypes during four consecutive growth cycles. We also highlighted the associations of these changes with plant phenotypic traits, in particular, seedling emergence.

2. Materials and methods

2.1. Soil and plant accessions

The soil was collected from the top 30 cm of a commercial tomato greenhouse in the Netherlands, air-dried, sieved and stored in sealed bags in the dark at room temperature until further use (*i.e.* addition of 25% bulk soil to rhizosphere soils described in Section 2.2 and *in vitro* seedling emergence assays in Section 2.4). Two tomato accessions were used: *Solanum lycopersicum* var. Moneymaker (hereafter: modern) and the wild relative *S. pimpinellifolium* LA1578 (hereafter: wild). These genotypes have been extensively phenotyped for seed and seedling traits as well as for root system architecture (Kazmi et al., 2017; Khan et al., 2012; The 100 Tomato Genome Sequencing et al., 2014).

2.2. Soil experiment

Plants were grown in PVC pots (7 × 7 × 8 cm) containing 310 g of soil with an initial moisture content of 20% (*v*/*w*) in a greenhouse (21 °C/16 °C (−1/+2 °C) day/night; 16 h d^{-1} light, ≥50% RH). Non-sterile seeds were sown approximately 1 cm beneath the soil surface. A total of 24 pots were prepared per tomato accession for the collection of rhizosphere soil and 6 pots containing bulk soil only. For each growth cycle, rhizosphere and bulk soils were sampled 28 days after sowing. The remaining soil from each pot was kept in PVC open boxes in the greenhouse overnight until the start of the next cycle. To compensate for the soil loss due to sampling throughout the different growth cycles, 25% (*w*/w) of bulk soil (Section 2.1) was added at the start of the next growth cycle. During growth cycle 2, 20 ml of nutrient solution (1/2th Hoagland's solution) and during growth cycles 3 and 4, 20 ml of nutrient solution (1/4th Hoagland's solution) was added once a week. Pots containing bulk soil only also received nutrient solution weekly. Root architecture traits (*i.e.* specific root length, specific root area, root density) were assessed by WINRHIZO (Arsenault et al., 1995) and shoot and root dry biomass was determined after drying overnight at 60 °C. Seedling emergence was determined in growth cycles 3 and 4.

2.3. Sampling of rhizosphere soil

Plants were removed from the pots and vigorously shaken to remove the soil loosely adhered to the roots as previously described (Lundberg et al., 2012; Perez-Jaramillo et al., 2017) with modifications. Briefly, roots with tightly adhered soil were placed in a 50 ml tube containing 20 ml of 10 mM $MgSO_4$ buffer. The solution was vortexed at max speed for 1 min, sonicated for 30 s and vortexed again for 30 s. The solution was filtered through two layers of sterile Miracloth and a 50 ml plastic syringe filled with sterile cotton to remove root debris and the filtrate (rhizosphere soil) was collected in a new Falcon tube. Half of this rhizosphere filtrate (10 ml) was added to the remaining soil in each pot and used as rhizosphere inoculum for the consecutive plant growth cycle. The other half of the filtrate was centrifuged at 9500 rpm for 20 min at 4 °C, and the supernatant was discarded. The pellet was re-suspended in 2 ml LifeGuard Soil Preservation Solution (QIAGEN) and stored at −20 °C until further use.

2.4. Analyses of plant phenotypic traits

Shoot and root dry weights were determined after every growth cycle. For the dry weights, root and shoot samples were incubated at 60 °C for 24 h. Root architecture was analyzed with WinRHIZO (Arsenault et al., 1995). Specific root length (cm/g) was calculated by dividing root length by root dry weight; the specific root area (cm^2/g) was calculated by dividing root surface area by root dry weight. Statistically

significant differences between plants grown in cycled and non-cycled soils were tested with Student's *t*-Test.

Seedling emergence was determined in growth cycles 3 and 4 by scoring the day of shoot emergence of each pot containing one seed. Soil from cycle 4 was sterilized by gamma-irradiation and used to determine the microbial contribution to seedling emergence. A total of four technical replicates were used, each consisted of 24-well plates, where 12 wells were filled with sterilized cycled soil and 12 with non-sterilized cycled soil. Each well contained 2 g of soil with an initial moisture content of 20% (*v*/*w*). Non-cycled (bulk) soil was used a control. Plates were kept closed in a greenhouse using the same conditions as the soil experiment (Section 2.2).

2.5. Rhizosphere DNA extractions, 16S amplicon sequencing and bioinformatics

Rhizosphere DNA extractions were carried using DNeasy PowerSoil Kit (QIAGEN, USA). DNA samples were cleaned using the DNeasy PowerClean Pro Cleanup Kit (QIAGEN, USA) and stored at −20 °C until further use. DNA concentrations were measured with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and Qubit 3 fluorometer (Invitrogen, USA). Rhizobacterial community was characterized by sequencing amplicons of the 16S rRNA gene using Illumina MiSeq at BaseClear (Leiden, Netherlands). Primers 341F 5'-CCTACGGG NGGCWGCAG-3' and 785R 5'-GACTACHVGGGTATCTAATCC-3' (V3-V4 region) were used. Demultiplexed paired-end fastq files were processed into quality-filtered reads using error-corrected amplicon sequence variant (ASV) in DADA2 v1.8 (Callahan et al., 2016). Taxonomic assignment was performed using the naïve Bayesian classifier (implemented in DADA2) and the "Silva version 132" database. The ASV table with read counts was filtered based on taxonomy, *i.e.*, ASVs affiliated with "eukaryota", "archaea", "chloroplast", and "mitochondria" were removed. This filtered table was used for further analysis.

2.6. Rhizobacterial community structure and composition analysis

For the alpha diversity, the filtered ASV table was rarefied using Microbiome Analyst (Chong et al., 2020). For the beta diversity, the function calculateEffectiveSamples from the metagenomeSeq R package was applied to the filtered ASV table and features with less than the average number of effective samples in all features were removed (Table S1). The data was further normalized using the Cumulative Sum Scaling (CSS) method, which calculates the scaling normalization factors equal to the sum of counts up to a particular quantile to normalize the read counts (Paulson et al., 2013). A Bray-Curtis dissimilarity matrix was calculated and displayed using Principal Coordinate Analysis (PCoA) in the Vegan package v.2.3-2 (Oksanen et al., 2010) implemented in the Phyloseq package v.1.10 (McMurdie and Holmes, 2013) in R. Although we used a new plant in every cycle, the pots containing the soil were sampled multiple times and replicates were kept the same through all cycles whenever possible. Therefore, we treated the data as a time series experiment and performed repeated measures. Linear mixed models were used to determine the statistical differences in alpha and beta-diversity over time. For that, we used the *lmerTest* package (lmer (Distance~Cycle+(1|Id) and lmer(Distance~Cycle * Genotype+(1|Id)) in R. Pairwise comparisons were determined with *lmeans* and adjusted with Tukey. To determine the overall effect of successive cycling (C1, C2, C3, C4), plant genotype (modern, wild) and soil (bulk, rhizosphere) we performed permutational analysis of variance (PERMANOVA) using the *adonis* function in the Vegan package (adonis(Distance~Soil+Genotype+Cycle)). For determining the effect of the genotype over time, bulk samples were excluded and PERMANOVA was performed (adonis(Distance~enotype+Cycle)).

To better identify ASVs that were progressively enriched through successive growth cycles of each of the two tomato genotypes, a clustering analysis was conducted using the filtered average CSS normalized abundance of each ASV in each treatment and cycle. To select the appropriate correlation cut-off for clustering, a sensitivity analysis was conducted to determine the relationship between the Pearson correlation (0.75, 0.8, 0.85, 0.9, 0.95 and 0.99) and the number of edges in the final network (Fig. S3). Based on this analysis, a cut-off of 0.85 was selected and the Louvain clustering algorithm was implemented in the R package 'igraph' (Csardi and Nepusz, 2005). Clustering was genotype-specific and each cluster was categorized as either bulk, Early Successional Rhizosphere, or Late Successional Rhizosphere. The Early Successional Rhizosphere was defined as those ASVs that increased in the initial cycle, but decreased in the subsequent cycles. The Late Successional Rhizosphere was defined as those ASVs that increased, or maintained high abundance, through each subsequent cycle, whereas the bulk ASVs showed a pattern consistent with enrichment in the bulk and decrease in the rhizosphere and subsequent cycles. When comparing between genotypes, a core and genotypes specific early and late successional rhizosphere communities were identified based on the overlap of ASVs. To further understand the fine-scale network structure, a network was constructed using both genotypes in Cytoscape (Shannon et al., 2003). Only nodes with greater than a median CSS value of 0 were included, and only those ASVs that demonstrated the Bulk, Early Successional Rhizosphere and Late Successional Rhizosphere patterns.

To further identify rhizobacterial ASVs associated with the rhizosphere and genotype effects, linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) was performed using CSS-normalization with MicrobiomeAnalyst (Dhariwal et al., 2017). Differential abundance of ASVs between genotypes at each cycle was tested with DESeq2 in MicrobiomeAnalyst.

3. Results

3.1. Impact of successive cultivation on rhizosphere microbiome assembly

When wild and modern tomato genotypes were grown for four successive cycles of 28 days, alpha-diversity of the rhizosphere bacterial microbiome for both genotypes showed an overall reduction over time until cycle 3 with a 'recovery' in cycle 4 to levels similar to that in cycle 2 (Fig. 1A, B). Using repeated-measures ANOVA, we found that species richness (Observed ASVs) was impacted by the successive cycling ($F_{3,18} = 293.68$, $P < 0.001$) but not by the genotype ($F_{1,6} = 5.27$, $P = 0.061$) whereas species evenness (Shannon index) was affected by both cycling ($F_{3,24} = 82.28$, $P < 0.001$) and genotype ($F_{1,24} = 15.15$, $P < 0.001$). Significant interactions between cycling and genotype were found for both community richness ($F_{3,18} = 11.78$, $P < 0.001$) and evenness ($F_{3,24} = 14.15$, $P < 0.001$). Using Tukey's multiple comparison test, we found a gradual decrease in community richness for each of the genotypes from cycle 1 to cycle 3 ($Observed_{wild}$ ranging from 389 ± 42 to 124 ± 12, $P_{wild} < 0.001$; $Observed_{modern}$ ranging from 469 ± 20 to 81 ± 14, $P_{modern} < 0.001$, Fig. 1A) and evenness ($Shannon_{wild}$ 4.27 ± 0.52 to 2.92 ± 0.30, $P_{wild} < 0.001$; $Shannon_{modern}$ 5.58 ± 0.06 to 2.44 ± 0.40, $P_{modern} < 0.001$; Fig. 1B) from cycle 1 to cycle 3.

To determine the turnover of the rhizosphere bacterial communities during successive growth cycles of the two tomato genotypes, we calculated and visualized beta-diversity based on Bray-Curtis distances and Principal Coordinate Analysis (PCoA) (Fig. 1C). To assess the overall effects of the soil (bulk, rhizosphere, genotype (modern, wild) and successive cycling (C1, C2, C3, C4)), we first performed permutational multivariate analysis of variance (PERMANOVA) on the Bray-Curtis dissimilarity values using all cycled bulk and rhizosphere samples (Fig. S1). We found an overall effect of the soil ($R^2 = 0.204$, $P = 0.001$), genotype ($R^2 = 0.036$, $P = 0.003$) and cycle ($R^2 = 0.379$, $P = 0.001$). To further investigate the overall effect of the cycling on the genotype over time, we performed PERMANOVA using only the rhizosphere samples (Fig. 1C). We found that both the genotype ($R^2 = 0.064$, $P = 0.001$) and the cycle ($R^2 = 0.62$, $P = 0.001$) impact microbiome diversity. To investigate if the rhizosphere effect, *i.e.*, the recruitment and enrichment

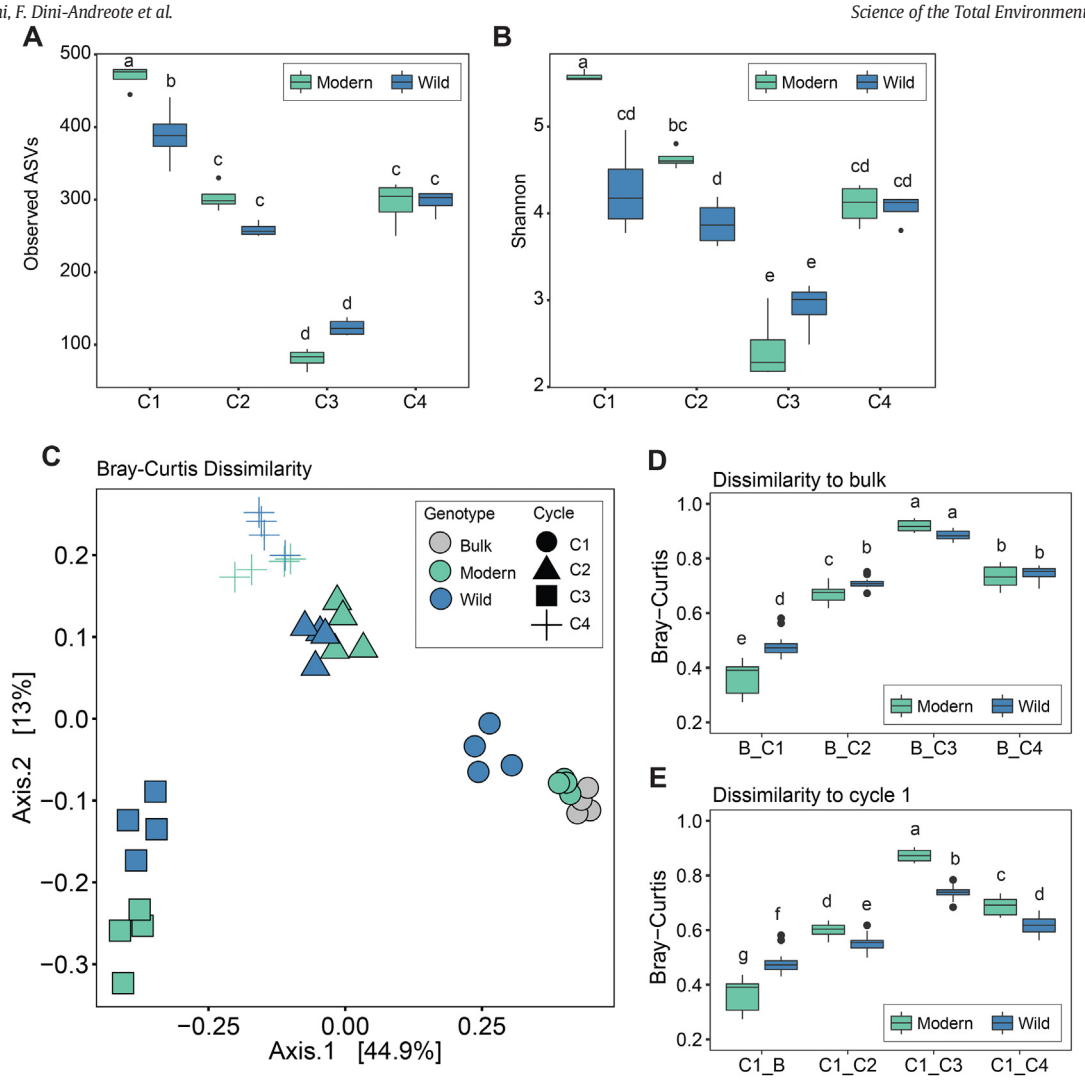

Fig. 1. Rhizobacterial community assembly of modern and wild tomato plants during four successive growth cycles. (A, B) Alpha diversity of 16S rRNA rhizosphere bacterial sequences of modern and wild tomato genotypes at different growth cycles. Observed ASVs were calculated with rarefied counts and Shannon diversity index was calculated with Cumulative Sum Scaling (CSS) normalized counts. (C) Principal Coordinate Analysis (PCoA) of 16S rRNA diversity in the rhizosphere of the two tomato genotypes and the bulk soil (colors) after 1, 2, 3 and 4 cycles (C1, C2, C3,–C4; shapes). (D) Bray-Curtis dissimilarities between bulk and rhizosphere samples in the cycles 1, 2, 3 and 4. (E) Bray-Curtis dissimilarities between cycle 1 and the bulk as well as the consecutive cycles (C1_C2, C1_C3, C1_C4). Dissimilarities were calculated with Cumulative Sum Scaling (CSS) normalized counts. Statistically significant differences are indicated by different letters above the averages (repeated measures ANOVA post-hoc Tukey HSD, $P < 0.05$).

of specific bacterial taxa in the rhizosphere from the bulk soil, was amplified during successive cycling, we compared the Bray-Curtis dissimilarities between bulk from cycle 1 to the rhizosphere soil of each of the consecutive cycles (C1, C2, C3, C4). We observed an increase in the distance between bulk and rhizosphere samples through the growth cycles ('Wild' increasing from 0.48 ± 0.04 to 0.74 ± 0.03, $P < 0.001$; 'Modern' increasing from 0.36 ± 0.05 to 0.73 ± 0.04, $P < 0.001$; Fig. 1D). Through successive cycles, the rhizobacterial communities also became more dissimilar from the original rhizosphere community in the first growth cycle ('Wild' increasing from 0.55 ± 0.03 to 0.61 ± 0.03, $P < 0.001$; 'Modern' increasing from 0.60 ± 0.02 to 0.69 ± 0.03, $P < 0.001$; Fig. 1E). Similar to the alpha-diversity indices, we observed a 'recovery' of the beta-diversity levels in cycle 4 to levels close to that in cycle 2 for both modern and wild genotypes.

Given that the rhizosphere bacterial communities of modern and wild tomato genotypes are significantly different in each cycle (PERMANOVA, $P_{C1} < 0.032$, $P_{C2} = 0.027$, $P_{C3} = 0.031$, $P_{C4} = 0.03114$), we sought to determine whether these differences were amplified by successive cycling. Bray-Curtis dissimilarities between genotypes became higher after cycle 2 (0.47 ± 0.03, Fig. 2A) and cycle 3 (0.43 ± 0.03, Fig. 1F) compared to cycle 1 (0.38 ± 0.03; ANOVA post-hoc Tukey HSD, $P_{C1\_C2} < 0.001$, $P_{C1\_C3} < 0.001$; Fig. 2). Dissimilarities

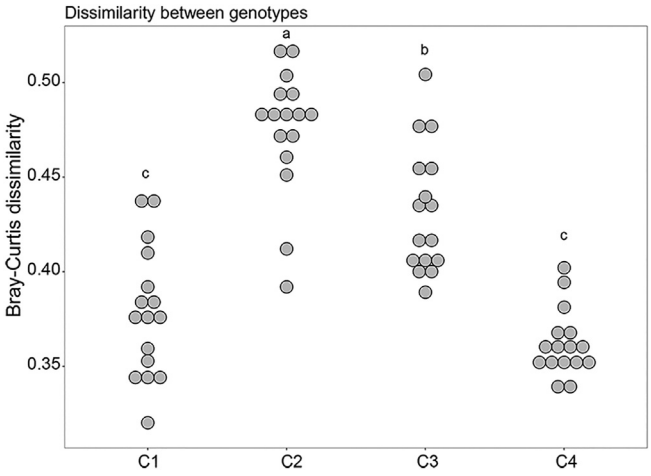

Fig. 2. Temporal turnover of bacterial communities in the rhizosphere of wild and modern tomato plants. (A) Bray-Curtis dissimilarities between wild and modern tomato genotypes over time. C1: cycle1, C2: cycle 2, C3: cycle 3, C4: cycle 4. Bray-Curtis dissimilarities were calculated with Cumulative Sum Scaling (CSS) normalized counts. Statistical differences are indicated by the different letters (repeated measures ANOVA post-hoc Tukey HSD, $P < 0.05$).

between genotypes at cycle 4 were found to be similar to those observed in cycle 1 (0.36 ± 0.02; ANOVA post-hoc Tukey HSD, $P_{C2\_C4} < 0.001$, $P_{C3\_C4} < 0.001$; Fig. 2).

3.2. Dynamic changes in rhizosphere microbiome taxa over time

We used the Louvain clustering algorithm to cluster the 1271 ASVs detected in the bulk soil and rhizospheres of wild and modern tomato genotypes. A total of 10 ASV clusters were identified with 4 clusters from the modern tomato genotype and 6 from the wild tomato genotype. From these, we focused on those clusters which included ASVs that were abundant in the bulk soil compared to the rhizosphere (referred to as bulk ASVs), ASVs which increased in the rhizosphere in the first cycle but subsequently decreased (referred to as Early Successional Rhizosphere ASVs), and ASVs which gradually increased their sum CSS over each cycle (referred to as Late Successional Rhizosphere ASVs). These clusters included a total of 1154 ASVs. For the bulk soil, a total of 458 ASVs were enriched compared to the rhizosphere (Fig. 3A). A total of 320 ASVs was identified as Early Successional Rhizosphere (ESR) ASVs (Fig. 3B) and 376 ASVs as Late Successional Rhizosphere (LSR) ASVs (Fig. 3C). From these, only 32% of ESR ASVs (101 ASVs) were shared by both genotypes, whereas 66% of all LSR ASVs (249 ASVs) were shared by both genotypes. Significantly more 'Modern' ASVs were classified as ESR and LSR than the 'Wild' ASVs (Fig. 3D, Fisher's exact test, $P = 0.016$).

From the network analysis of bulk, ESR and LSR ASVs present in 50% or more of samples, a total of 4775 positive and 1419 negative correlations with an absolute value of 0.85 Pearson correlation or greater were observed. The majority of the positive correlations were between ASVs within a cluster, with 70% of all positive connections found within

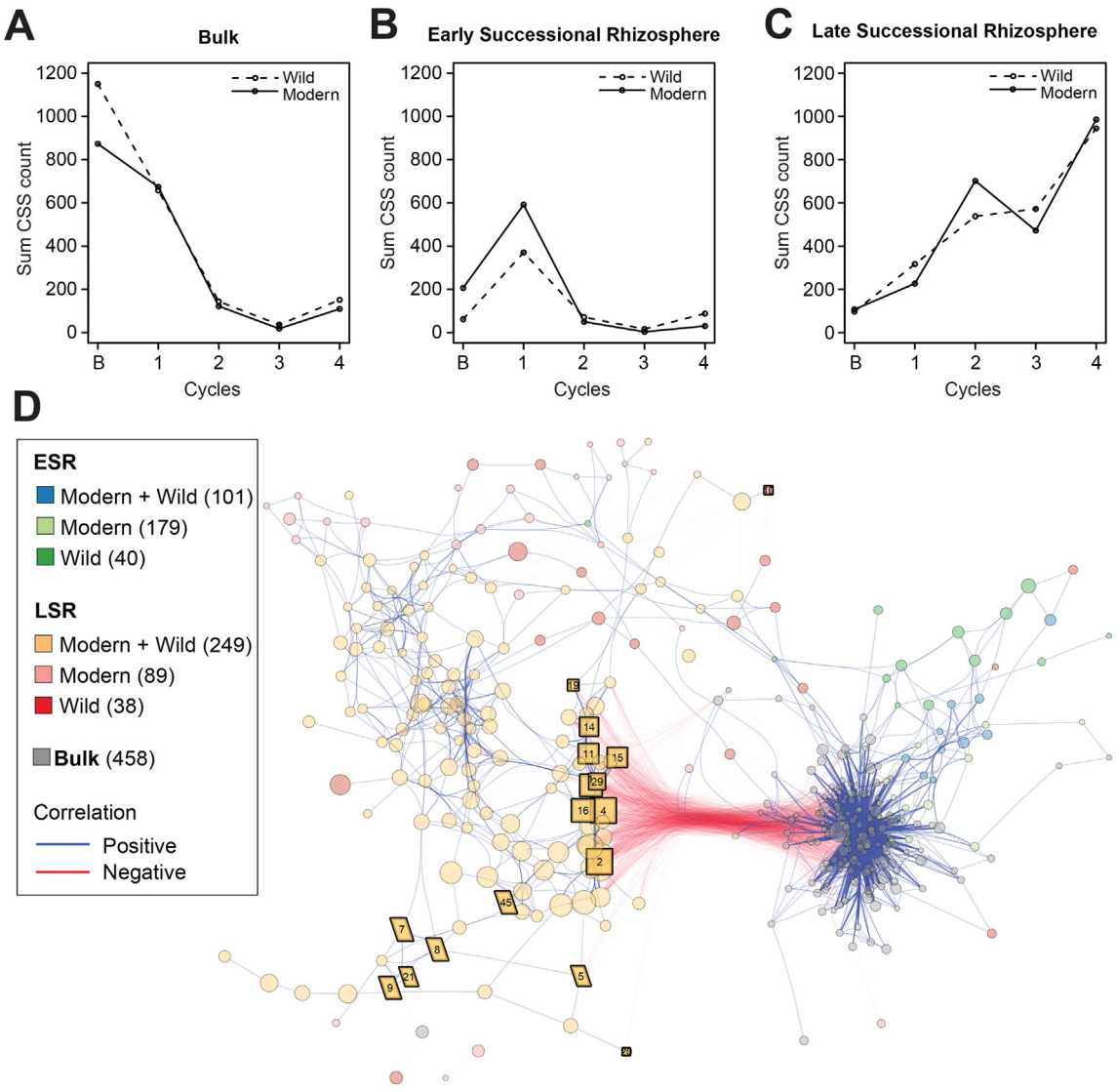

Fig. 3. Dynamic changes in rhizobacterial taxa during successive growth cycles of wild and modern tomato genotypes. Clusters of Amplicon Sequence Variants (ASV) based on Louvain clustering algorithm: (A) Bulk (ASVs enriched in unplanted soils compared to modern and wild rhizosphere), (B) Early Successional Rhizosphere (ESR, ASVs that increased in the first cycle but subsequently decreased), and (C) Late successional rhizosphere (LSR, ASVs enriched over time). (D) Network analysis of co-occurring ASVs (average of Cumulative Sum Scaling normalized counts) based on Pearson's correlation coefficients ($P < 0.01$, $r > 0.85$). The nodes represent ASVs, where the node size is proportional to the average counts for both genotypes in the 4th growth cycle. The edges represent positive (blue) and negative (red) correlations. Different colors denote different clusters identified with the Louvain clustering algorithm. ASVs explaining the rhizosphere effect are represented by the squares and ASVs explaining the rhizosphere and genotype effects by the parallelogram. Total number of ASVs detected for each cluster category is displayed with parentheses. Network was visualized with Cytoscape. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the Bulk ASVs, 13% within the LSR ASVs, and 4% within the ESR ASVs. Positive correlations between ASVs in different clusters were much fewer, the highest fraction coming from connections between ESR and Bulk ASVs (14%), and less than 1% of connections between LSR and bulk, or between LSR and ESR. In contrast, the majority of negative correlations were found between ASVs in different clusters. Specifically, 93% of all negative correlations were between LSR and bulk ASVs, and only 6% between LSR and ESR ASVs (Fig. 3D).

To determine the bacterial taxa that most explain the rhizosphere and the genotype effects, we performed Linear discriminant analysis Effect Size (LEfSe) by coupling statistical test (Kruskal-Wallis) with Linear Discriminant Analysis (LDA) encoding biological consistency and effect relevance (FDR < 0.05, LDA score $\log_{10} > 2$). A total of 16 ASVs across all cycles was found to contribute to the differences between bulk and rhizosphere samples, *i.e.*, explained the amplification of the rhizosphere effect; these ASVs belonged to the genera *Pseudomonas* (ASV_2, ASV_4, ASV_6, ASV_15, ASV_19, ASV_29), *Massilia* (ASV_7, ASV_8, ASV_9), *Pseudoduganella* (ASV_11, ASV_14), *Bacillus* (ASV_10), *Acidovorax* (ASV_5), *Azospirillum* (ASV_20), *Rhizobium* (ASV_16) and *Rubrivivax* (ASV_1) (Fig. 4A). These 16 ASVs associated with the amplification of the rhizosphere effect also accounted for the majority of the negative correlations between bulk and Late Successional Rhizosphere ASVs displayed in the network (Fig. 3D). A total of 7 ASVs across all cycles contributed to the differences between the tomato genotypes, *i.e.*, genotype effect. From these, 6 ASVs were more abundant in the wild genotype through the successive cycling and belonged to the genera *Acidovorax* (ASV_5), *Massilia* (ASV_7, ASV_8, ASV_9, ASV_21) and *Rhizobium* (ASV_45) and one ASV was specifically enriched in the modern genotype and belonged to the *Pseudomonas* genus (ASV_57) (Fig. 4B). A total of 38 ASVs was associated with genotype over time (*i.e.* per genotype per cycle), accounting for 20 genera with the majority of ASVs belonging to the *Pseudomonas* (24%), *Bacillus* (13%) and *Massilia* (11%) (Fig. 5A, B). To further determine which ASVs were significantly different between the modern and the wild genotypes at each cycle, we performed a differential analysis using DESeq2 (FDR adjusted $P < 0.05$). We found a total 33 ASVs differentially enriched at cycle 1, 10 ASVs at cycle 2, 6 ASVs at cycle 3 and 15 ASVs at cycle 4 (Table S2).

3.3. Effects of successive growth cycles on plant phenotypic traits

To determine the association, if any, between microbiome dynamics and plant growth and development over time, we recorded plant biomass, root phenotypic traits (specific root length, specific root area) and seedling emergence. No significant impact of successive cycling was observed on root dry weight, specific root length and specific root area (Table S2). However, in cycled soils the tomato seedlings emerged significantly earlier than those grown in bulk soil (non-cycled soils) (Fig. 6A, B). Modern and wild seedlings were 31% (from 13 to 9 days) and 22% (from 9 to 7 days) faster in reaching at least 70% emergence than seedlings grown in non-cycled soil (Fig. 6A, B).

To investigate the potential effects of organic carbon exuded by the roots accumulated during successive cycling on seed germination and seedling emergence, we determined the carbon content in the cycled soils. No significant differences were observed between the carbon content of non-cycled soil and soil cycled successively with the modern or wild tomato plants (Student's *t*-Test, $P_{modern} = 0.478$, $P_{wild} = 0.217$; Table S4). To test if the earlier seedling emergence has a microbial basis, cycled and non-cycled soils were sterilized by gamma-irradiation. Subsequent *in vitro* bioassays revealed similar emergence time between sterilized and non-sterilized cycled soils for the wild tomato seedlings (Fig. 6C, D). However, modern tomato seedlings were 36% slower (from 7 ± 0.5 to 9.5 ± 0.9 days) in reaching at least 70% emergence in sterilized cycled soils as compared to tomato seedlings grown in non-sterilized cycled soils (Student's *t*-Test, $P < 0.001$; Fig. 6C, D).

4. Discussion

The rhizosphere microbiome is defined by the compositional and functional changes that occur at the interface between the bulk soil (unplanted soil) and the plant roots. These changes occur due to the selection imposed by the plant roots as they modulate carbon, oxygen, pH, and nutrient availability, thereby activating and or suppressing the growth and activity of specific microbial genera (Reinhold-Hurek et al., 2015). Despite increasing recognition of the beneficial effects of the rhizosphere microbiome on plant growth and health, ecological

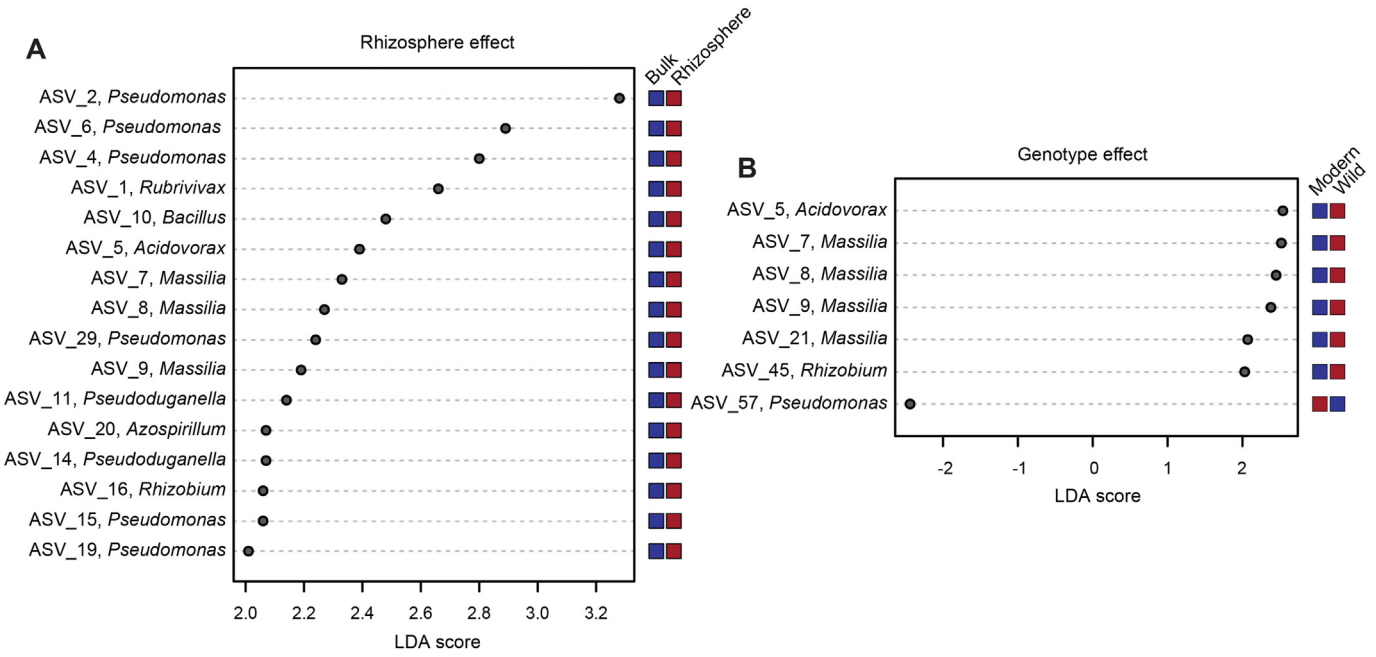

Fig. 4. Taxonomic composition of the rhizobacterial microbiome after successive plant growth. Differential abundance was determined with Linear Discriminant Analysis (LDA) Effect Size (LEfSe) of Cumulative Sum Scaling (CSS) normalized counts. Amplicon Sequence Variants (ASVs) driving the rhizosphere effect (A) and the genotype effect (B). LDA scores are displayed by the grey dots. ASVs in low and high abundances are shown in dark blue and dark red squares on the right, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

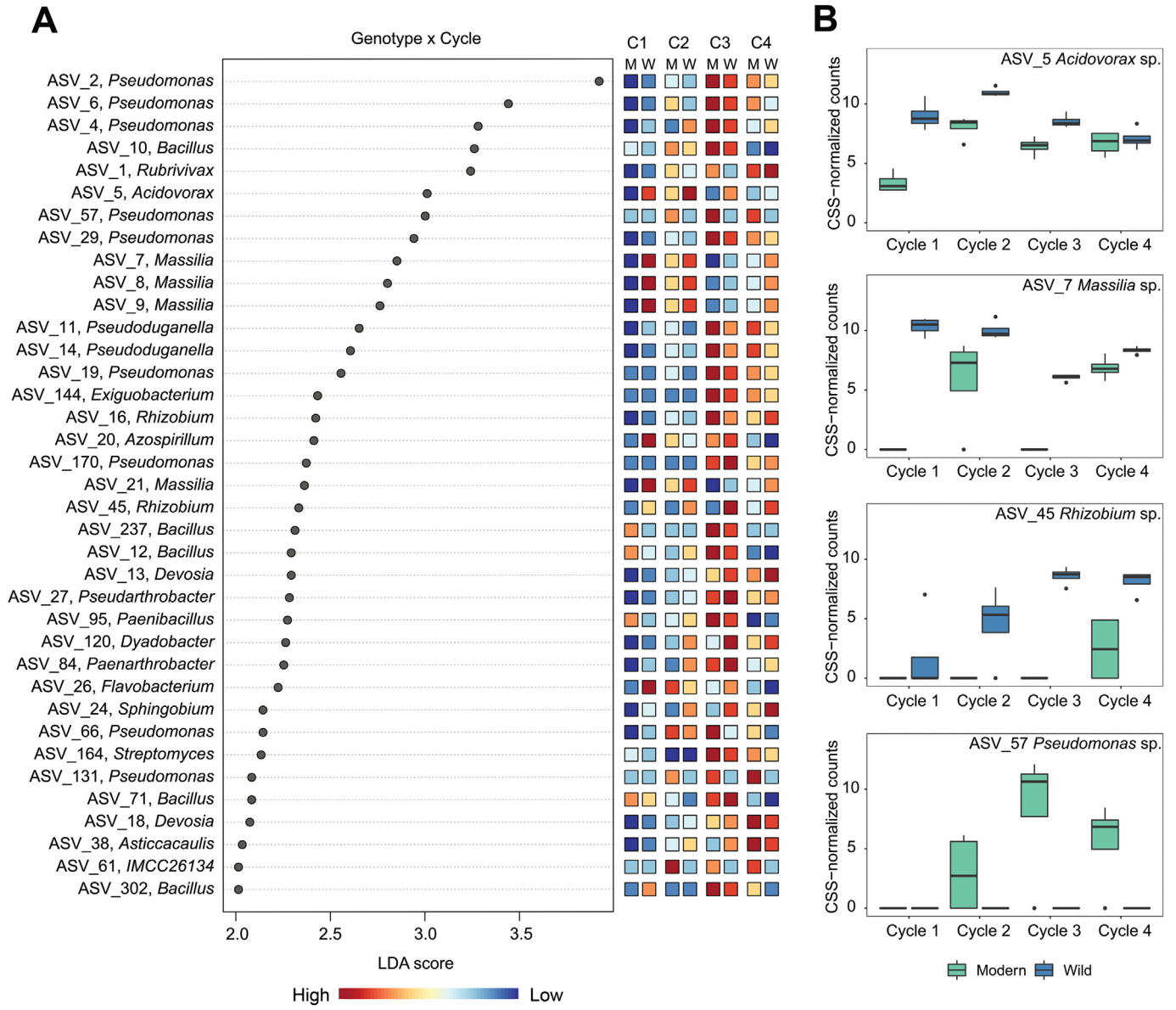

Fig. 5. Effects of the plant genotype on rhizobacterial microbiome composition in the different tomato growth cycles. Differential abundance was determined with Linear Discriminant Analysis (LDA) Effect Size (LEfSe) of Cumulative Sum Scaling (CSS) normalized counts. (A) Amplicon Sequence Variants (ASVs) driving the genotype effect in each growth cycle in the rhizosphere of wild and modern tomato plants. LDA scores are displayed by the grey dots. ASVs in low and high abundances are shown in blue and red squares on the right, respectively. (B) CSS normalized counts of differentially abundant ASVs in the wild and modern rhizosphere per growth cycle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and evolutionary understanding on the mechanisms controlling the microbiome assembly across plant genotypes and developmental stages as well as during successive cultivation is lacking.

Under natural conditions, the same plant species often grow in the same location over subsequent seasons. Thus, while the first generation of plants may recruit a rhizosphere microbiome from the bulk soil, subsequent generations might have home-field advantages as often observed for late successional plant communities (Kardol et al., 2006; Koziol and Bever, 2019). Thus, it plausible that succession within the rhizosphere microbiome over growth cycles plays an important role of plants' life-history. However, little is known about microbial community dynamics in the rhizosphere during succession (Chang and Turner, 2019). Studies investigating the rhizosphere microbiome dynamics during plant development showed that pioneering species assemble from random resource overlap at earlier stages into high-density, functionally complementary climax communities at later stages, providing plant resistance to bacterial pathogens (Hu et al., 2020; Wei et al., 2019). In our study, tomato plants were grown in the same soil for four successive cycles and the rhizosphere samples were collected at the same plant developmental stage (28 days) at each cycle. Our results revealed a gradual amplification of the rhizosphere effect, as both modern and wild rhizosphere microbiomes became more dissimilar from the initial bulk soil over time. We observed that alpha-diversity decreased in both modern and wild rhizosphere, whereas beta-diversity increased over time compared to the first (non-cycled) soil. Although the rhizobacterial community diversities were significantly different from those in the initial cycle, community diversity in cycle 4 was more similar to that in cycle 2. Whether these effects are transient or fixed remains to be investigated by including additional growth cycles. Our initial focus was to investigate the impact of plant microbiome shifts on plant phenotype (*i.e.* seedling emergence/growth) over successive cycling. Since the earlier seedling emergence was already visible at cycle 2 and stabilized in cycles 3 and 4 we decided not to continue cycling in this study.

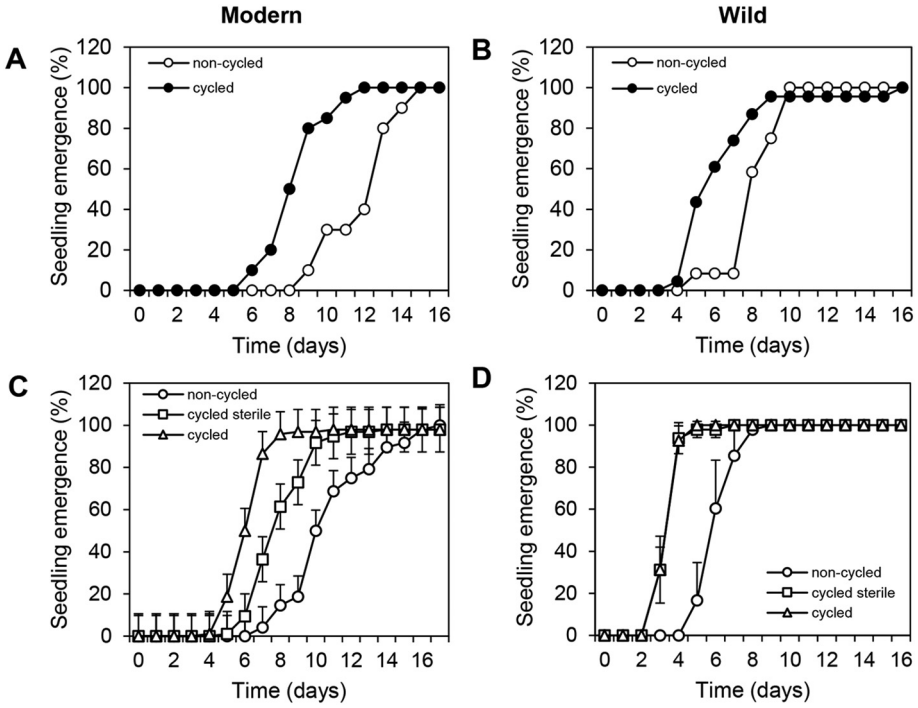

Fig. 6. Impact of successive cultivation on seedling emergence of modern (A) and wild (B) tomato genotypes. Non-cycled samples (white circle) refer to plants grown in bulk soil and cycled samples (black circle) refer to plants growth in soils used for successive cultivation. Modern (C) and wild (D) genotypes sown in sterile cycled soils (square). Non-sterile cycled (triangle) and non-cycled (circle) soils were used as controls.

Lower alpha-diversity in the rhizosphere and over the plant growth cycles suggests that these communities are under increasing selective pressure imposed by the amplification of the genotype effect. In line with these findings, we found that only a small subset of 16 Late Successional Rhizosphere ASVs, representing the genera *Pseudomonas*, *Bacillus*, *Massilia*, *Acidovorax*, *Azospirillum*, *Rhizobium*, *Pseudoduganella* and *Rubrivivax*, was associated with the amplification of the rhizosphere effect and accounted for the majority of the negative correlations with the bulk soil ASVs, suggesting an amplification of competitive interactions between taxa.

We also found that there was not only a significant effect of the tomato genotype, but also that this effect was amplified upon successive cycling, *i.e.* the distances between modern and wild microbiomes through the cycles increased. This effect was driven by seven taxa representing the genera *Massilia*, *Rhizobium*, *Acidovorax* and *Pseudomonas*. Interestingly, these ASVs formed a small connected sub-module and, despite differentiating modern and wild microbiomes, did not exhibit any negative correlations within the Late Successional Rhizosphere. The submodule structure and lack of negative interactions supports the hypothesis of the 'modular microbiome', which postulates that discrete functional communities within the plant microbiome exist (Oyserman et al., 2018). In a recent study, using a successive passage of the tomato phyllosphere microbiome, *i.e.* microbial communities inhabiting the surface and the internal tissue of the leaves, resulted in the assemblage of stable and well-adapted communities (Morella et al., 2020). The authors described that the genotype explained 24% of the variation among samples in the phyllosphere, however this effect was significant only at the first and second passages and decreased over time. Although both the phyllosphere and rhizosphere microbiomes are greatly influenced by the local environmental factors, the main drivers structuring these microbiomes are likely different. Whereas the rhizosphere microbiome is influenced by the pool of taxa existing in the bulk soil and how specific taxa respond to root exudates and mucilage release, the phyllosphere microbiome is mostly influenced by UV exposure and random dispersal events. Therefore, it is reasonable to assume that these different factors will result in distinct genotype effects on the phyllosphere and rhizosphere microbiomes. Furthermore, the different degrees of resistance/susceptibility displayed by different plant genotypes, such as the wild and modern tomato plants used in this study, might impact the abundance of specific members of the rhizobacterial community, including plant pathogens. However, the amplicon data obtained in this study are not discriminative enough identify pathogenic microorganisms within the microbiome. Although, we did not observe any disease symptoms on roots or shoots in any of the two genotypes over the four growth cycles, it will be interesting to investigate whether successive cycling and the concomitant shifts in the microbiome affect tolerance to pathogens or if pathogen infection further amplifies the genotype effect.

Our results also showed that successive cultivation of modern and wild tomato plants led to an acceleration of seedling emergence which was significantly reduced, at least for the modern tomato genotype, by elimination of the microbiome *via* y-irradiation of the cycled soil. It is likely that acceleration of seedling emergence also occurred for the wild tomato genotype but in shorter intervals, *i.e.* hours instead of days, and therefore, was not detected in our daily measurements. Several genera found to be enriched in the rhizosphere of the modern and wild tomato genotypes over time, such as *Pseudomonas* and *Rhizobium*, have been described for their beneficial effects on plant growth and development and are referred to as plant-growth promoting rhizobacteria (PGPR). Furthermore, members of the genus *Massilia* (*Oxalobacteraceae*), here found to be consistently enriched in the rhizosphere of the wild genotype. This genus has been suggested to be successful colonizers in early succession in the rhizosphere, when higher carbon and energy sources are present, but before competition with other rhizosphere microorganisms intensify (Ofek et al., 2012). Successive cycling of *Brassica rapa* also showed an increase in the abundance of *Massilia* spp. in the rhizosphere and correlated with the invasion of the fungal pathogen *Olpidium brassicae* (Tkacz et al., 2015). To what extent the changes in relative abundances (enrichment of specific taxa and decrease in alpha-diversity) described in our study impact seedling

emergence or lead to pathogen susceptibility in our experimental system remains to be investigated.

Increased rhizosphere network connectivity and complexity have been described for bacterial assemblages in the rhizosphere compared to the surrounding (bulk) soil (Shi et al., 2016). Here, we show that clustering of ASVs identified three major patterns: bulk soil, Late Successional Rhizosphere and Early Successional Rhizosphere. Most studies of the rhizosphere microbiome investigate only a single growth cycle making it impossible to distinguish between Early and Late Successional Rhizosphere taxa and assemblages. Our results show that many Early Successional Rhizosphere bacteria have only a transient presence in the rhizosphere over successive cycles. Thus, while these opportunistic rhizosphere microorganisms likely contain traits that make them suitable for colonization of the rhizosphere during early successional stages, competition with other microorganisms over successive cycles ultimately leads to their diminished presence. These findings indicate that while both Early and Late Successional Rhizosphere taxa may be adapted to life in the rhizosphere, Late Successional Rhizosphere taxa likely contain key traits to survive the competition and to accelerate seedling emergence. To identify these traits, it is important that further studies, using metagenome and metatranscriptome comparative analysis, focus on the mechanisms underpinning such interactions.

Microbiome assembly is a dynamic process determined by shifts in the community composition over time in response to environmental changes, plant development, and across growth cycles. Collectively, our study contributes to the understanding of how host genotype shapes rhizosphere microbiome and provides a first step towards understanding the impact of successive cultivation on rhizosphere-driven functions. Understanding of the genetic control by plants over rhizobacterial community composition is of interest to plant breeders as it will determine if the microbiome can evolve in response to the selection imposed by the host plants (Gopal and Gupta, 2016; Wagner et al., 2016). Moreover, categorizing specific taxa within the microbiome based on their ability to establish long-term *versus* transient associations with plants may be the base for developing new hypotheses on plant-microbiome evolutionary interactions. This categorization can also pinpoint bacterial taxa and consortia that are highly compatible to the rhizosphere environment, contributing to the development of new strategies for engineering beneficial rhizosphere microbiomes.

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CRediT authorship contribution statement

VC and JR design the study. VC and CR performed the experiments. VC and CR analyzed the microbiome data with the assistance of FDA and VJC. VC and BO performed the clustering and network analysis. VC drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and materials

The raw sequences were submitted to the European Nucleotide Archive (ENA) under project accession number PRJEB40313.

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