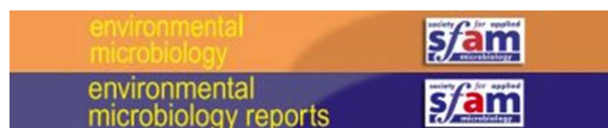

Temperature drives the assembly of endophytic communities' seasonal succession

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1 # Temperature drives the assembly of endophytic communities' seasonal
2 succession

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19

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Originality-Significance Statement

If and how temperature impacts host-associated microbial communities is of critical importance in plant biology in general. So far, a detailed and mechanistic understanding on the temperature- and season-driven changes on plant-inhabiting (endophytic) communities has remained elusive. Recently, some notable attempts have been done in this direction, but these works primarily focus on either field or controlled-environment conditions. Our work innovates, by successfully linking the effects of temperature on host-associated microbial communities in the environment and in controlled conditions, and identifying bacterial co-occurrence networks useful for the development of an interaction model for controlled conditions and field.

Here we discuss two major findings:

- that the dynamics of plant-inhabiting microbes may critically differ from those observed in agricultural soil, and microbial symbiotic communities respond to temperature (and temperature changes) and season in a plant organ-specific manner.
- that plants in controlled environment and in the open field have strong similarities and a few differences, but overall we identified a network of microorganisms with conserved dynamics. These results provide a highly valuable platform able to support the need to generalize results from greenhouse-based experiment to plants in natural, agricultural or wild environments.

How the plant microhabitat influences the response of microbial endophytes to environmental stimuli is a crucial and yet largely uncharted domain of microbial ecology, which we aim at disentangling, starting with this work.

Summary

Endophytic microorganisms asymptomatically colonise plant tissues. Exploring the assembly dynamics of bacterial endophytic communities is essential to understand the functioning of the plant holobiont and to optimise their possible use as biopesticides or plant biostimulants. We studied the variation in endophytic communities in above and below-ground organs in *Vitis vinifera* in the field. To understand the specific effect of temperature, a separate experiment was set up in which grapevine cuttings were grown under controlled conditions at three different temperatures. Our findings reveal the succession of endophytic communities over the year. Endophytic communities of roots and stems differ in terms of composition and dynamic response to temperature. Noticeably, compositional differences during the seasons affected bacterial taxa more in stems than in roots, suggesting that roots offer a more stable and less easily perturbed environment. Correlation abundance networks showed that several taxa (including *Mesorhizobium*, *Burkholderia*, *Ralstonia*, *Bradyrhizobium*, *Dyella* and *Propionibacterium*) are linked in both the field and the greenhouse.

Key Words: grapevine, endophyte, symbiosis, temperature, seasonality

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62 **Introduction**

63 Plants host a wide diversity of microbial inhabitants (Vorholt, 2012). Microorganisms living at least
64 part of their life cycle within plant tissues are termed endophytes (Hardoim, *et al.,* 2015). In
65 contrast with microbes living in abiotic environments, the study of microbial endophytic
66 communities entails not only understanding the interaction between them, but also between such
67 communities and the highly variable and reactive background provided by the living host (Robinson
68 *et al.,* 2010). Plants actively recognise and select the microbial inhabitants in their tissues by
69 modulating gene expression, and consequently the metabolism (Hardoim *et al.,* 2015; Tkacz *et al.,*
70 2015). While endophytes benefit from a substantially protected niche, where nutrition and shelter
71 are provided by the host (Schardl *et al.,* 2004), in the plant they are subject to changes in the
72 host's physiology, which in turn responds to environmental stimuli (Zimmerman & Vitousek, 2012).
73 In animals, symbiotic microbial communities are mostly confined to selected districts connected to
74 the outside environment (i.e. skin, respiratory tract, gut, urogenital tract). Conversely, a variety of
75 plant endophytes colonise all plant organs (Compant *et al.,* 2011). Root-associated communities,
76 including root endophytes, have received considerable attention from science, as they are thought
77 to play a crucial role in plant nutrition and growth, while fewer studies have addressed endophytic
78 communities in the plant canopy. Although knowledge about the composition of plant endophytic
79 communities is increasing rapidly, the forces driving their composition are just starting to be
80 unearthed (Poosakkannu *et al.,* 2015).

81 Perennial deciduous plants in temperate climates experience an annual metabolic cycle that
82 determines (among other things) the loss of leaves and the translocation of nutrients towards the
83 roots in the colder season (Tromp, 1983). These drastic changes are caused by profound
84 metabolic shifts that involve the plant as a whole and are likely to be reflected in the composition of
85 the endophytic communities therein. The weather and, especially temperature, is the main driver of
86 this cycle (Bita & Gerats., 2013). Temperature shifts are also expected to affect the composition of
87 plant-associated microbiota, both directly and through the induced changes in the plant metabolism
88 (Campisano *et al.,* 2014a). In annual plants the soil is the main source of endophytic colonisation,
89 but several vertically-transmitted endophytes are also commonly found (Hodgson *et al.,* 2014;
90 Johnston-Monje *et al.,* 2014). Studies carried out over multiple seasons have so far focussed more
91 on soil and root-associated microorganisms, providing little support for modelling the dynamics of
92 endophytes (Peiffer *et al*., 2013; Shi *et al*., 2015). In perennial woody plants endophyte
93 communities are a combination of pre-existing (resident) and new (pioneer) colonisers. In woody
94 plants vertically-transmitted fungi are less common, probably because of the time span before
95 seed production. The plant's intrinsic features, such as morphology and physiology, may also limit
96 vertical transmission (Saikkonen *et al.,* 2004). In the grapevine we previously hypothesised that
97 vertical transmission of endophytes present in the cuttings may play a bigger role than vertical
98 transmission through seeds (Campisano *et al.,* 2015).

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99 Several attempts have been made to understand the role of climate, including seasonality and
100 temperature variation, in shaping the plant microbiome. Much of the existing information
101 converges, indicating that climate strongly affects fungal assemblages (Coince *et al* 2014; Cordier
102 *et al.,* 2012). On the other hand, bacterial communities correlate better with other, more tissue or
103 compartment-specific variables (Coleman-Derr *et al*., 2016). For example, the variability of
104 bacterial assemblages in Agave plants was linked more to plant compartment than biogeography,
105 suggesting a major role of the plant microenvironment as compared to climate-associated
106 variables (Coleman-Derr *et al*., 2016). However, other relevant factors, such as the influence of
107 soil, cannot be excluded (Bokulich *et al.,* 2014). In addition, climate change can alter the
108 composition and function of endophytes, and with them, the impact on their hosts (Ducklow, 2008;
109 Johnson *et al.,* 2013).
110 To understand the shifts in endophytic bacterial communities over the year, we assessed these
111 communities in the perennial plant *Vitis vinifera L* every three months. By sampling plants grown in
112 the same type of soil along an altitudinal transect, we studied changes in the composition of
113 endophytes inhabiting plants that are exposed to slightly, but consistently different climatic
114 conditions throughout the year (similarly to recent studies on plant-associated fungal assemblages
115 in Cordier *et al.,* 2012; Coince *et al.,* 2014). To evaluate the short term effect of temperature
116 independently of the season, we monitored a set of identical plants kept in greenhouses at three
117 different temperatures (15, 25 and 35°C).
118
119 **Results**
120 The roots of the plants grown in colder conditions were generally more developed than those
121 grown in warmer conditions. This effect was significant in the greenhouse plants, where the root
122 weight of plants growing at 15°C was approximately twice that of plants growing at 25° and 35°C
123 after two months (Tg2). The effect of temperature on the roots of plants growing at different
124 altitudes was less noticeable (Supplementary Figure S1). In these plants the alpha and beta-
125 diversity of their endophytic bacterial communities changed constantly throughout the year (field
126 time-points: Tf0: XX May 2011, Tf1: 4 August 2011; Tf2: 3 November 2011; Tf3: 6 February 2012;
127 Tf4: 7 May 2012) and showed an organ-dependent response to time in the growing season
128 (Figures 1-3).
129
130 *Dominating taxa in the stem and root endosphere*
131 Proteobacteria was the dominant phylum over the whole experimental set (greenhouse and
132 vineyard), with sequence counts ranging from 60% to over 95% of the total. Actinobacteria were
133 also detected, with sequence counts ranging from 3.2% to 32.8% of the total. Bacteroidetes (up to
134 6.1% of sequences) and Firmicutes (up to 6.9% of sequences) were detected at lower levels. The
135 order with the highest average sequence count was Rhizobiales (20.2%), followed by

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136 Pseudomonadales (19.7%), Burkholderiales (15.2%) and Actinomycetales (10.2%). A graphic
137 representation of the OTU table summarised at order level is shown in Figure 1. Sequences
138 assigned to the Burkholderiales order were more common in plant stems (4.6%) than in roots
139 (2.8%, $p = 2.49 \times 10^{-18}$, Wilcoxon rank-sum test, FDR corrected), while the opposite was true for
140 those classified as Rhizobiales, Pseudomonadales and Xanthomoadales ($P = 1.87 \times 10^{-5}$, $P = 1.56$
141 $\times 10^{-6}$ and $p = 9.94 \times 10^{-12}$ respectively) (Supplementary Figure S2).
142

143 *Differentially abundant taxa in the stem and root endosphere*
144 Significant differences ($p < 0.05$, Wilcoxon rank-sum test, FDR corrected) between endophytic taxa
145 found in stems and roots at order and family level are illustrated in Supplementary Figures S2 and
146 S3 respectively. Bacteria from the Nocardioidaceae, Ohtaekwangia, Coxiellaceae,
147 Sinobacteraceae, Methylocystaceae and Hyphomicrobiaceae families were more commonly found
148 in greenhouse plants, while Pseudomonadaceae were more common in field-grown plants. After
149 one and three months in the greenhouse, a shift in the relative abundance of the
150 Conexibacteraceae, Legionellaceae, Coxiellaceae, Sinobacteraceae, Rhizobiales (*incertae sedis*),
151 Hyphomicrobiaceae, Rhodospirillaceae, Haliangiaceae and Aurantimonadaceae bacterial families
152 (in stems), Verrucomicrobiaceae and Ohtaekwangia (in roots and stems), and Sinobacteraceae
153 and Hyphomicrobiaceae (in roots) was observed (Figure 2).
154 Plants grown at different altitudes or different locations did not differ in terms of the composition of
155 their endophytic microbiota (with one exception) and therefore they were considered replicate
156 samples for all statistical purposes (Supplementary Table S4). One exception was noted at Tf2,
157 when root-associated bacterial endophytes were distinct ($p = 0.0184$, PERMANOVA on weighted
158 UniFrac distances).
159 PCoA analysis of the samples' beta diversity using UniFrac distances showed that stem and root
160 bacterial endophytic communities grouped separately. Samples from the field could also be
161 distinguished from those from greenhouses (Figure 3a, Table 1). Clustering in the stem and root
162 was clear in both field-grown plants (henceforth FP, Figures 3b-c) and in greenhouse-grown plants
163 (henceforth GP, Figures 3d-e). Interestingly, samples from FP and GP were separated along the
164 second coordinate (Figure 3a).
165

166 *Temporal changes in endophytic populations inhabiting field plants.*
167 Alpha diversity was generally higher in the endosphere of FP roots as compared to that of FP
168 stems (Welch's t-test $P = 6.8 \times 10^{-13}$). The maximum diversity in FP stems (Figure 4) occurred in
169 summer (Tf1), while it shifted in autumn (Tf2) in FP roots.
170 Analysis of beta-diversity showed that endophytic bacterial communities in stems at Tf1 and Tf2
171 were close together in PCoA plots (Figure 3c), as for the communities at time-points Tf3 and Tf4.
172 Beta-diversity distances from Tf0 samples were higher in stem-associated endophytic communities

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173 than in root-associated ones, both in FP and in GP (Figure 5a). As compared to Tf0, the bacterial
174 communities of roots differed most at Tf2, while they were most similar at Tf1 and Tf4. The cycling
175 trend outlined here (Figure 5a) was reversed when plant stems were considered. Here the
176 distances from TF0 were highest at Tf1 and Tf4 and lowest at Tf2, symmetrically reflecting those
177 observed in plant roots (Figure 5a, Supplementary Table S5).
178
179 To understand the fine shifts in the plant endophytic microbiome, we identified the taxa undergoing
180 significant changes in relative abundance on the basis of time ($p < 0.05$, Wilcoxon rank-sum test,
181 FDR corrected). Many taxa had a maximum relative abundance at Tf1 in plant stems (Figure 2a),
182 including phyla Proteobacteria, Acidobacteria and TM7, and class Sphingobacteria, Flavobacteria,
183 Alpha-, Beta- and Gamma-proteobacteria. In roots (Figure 2b) the relative abundance of
184 sequences in the Actinobacteria phylum peaked at Tf2, while the highest relative abundance of
185 Chlamidiae and Proteobacteria occurred at Tf3.
186
187 In plant stems, the relative abundance of Actinobacteria, Bacteroidetes, Acidobacteria and
188 Firmicutes was highest at Tf1 (Supplementary Figure S4) and lowest at Tf3, while Proteobacteria,
189 which was the most abundant phylum, were least numerous at Tf1. Interestingly, Proteobacteria
190 also (Tg1) decreased readily during greenhouse exposure to fixed temperatures, with the lowest
191 relative abundance recorded in plants exposed to higher temperatures (Supplementary Figure S4).
192 In roots, fewer taxa showed significant differences over the time points (Supplementary Figure S6).
193 In a similar way to the observations in stems, Proteobacteria did not display a unique trend, with
194 Enterobacteriales and Pseudomonadales being the only orders that had the minimum abundance
195 at Tf2 (Supplementary Figure S7).
196
197 *Temporal changes in endophytic populations inhabiting greenhouse plants*.
198 Alpha-diversity indices (Figure 4) were highest in plants growing at high temperature (35°C) and
199 lowest in plants growing at the lowest temperature (15°C). The initial increase in bacterial diversity
200 found at all temperatures in stems was reversed in samples taken at Tg2, when diversity indices
201 were not different from those at Tg0. In roots, alpha diversity increased monotonically between Tg0
202 and Tg2, suggesting a slower response of root endophytic communities to temperature changes.
203
204 PCoA plots (Figure 3d, Table 1) separated Tg0 and Tg1 root samples from Tg2 samples along the
205 main coordinate. A similar separation was apparent in stem samples (Figure 3e). Distances from
206 T0 increased with time in roots, while peaking at Tg1 and decreasing at Tg2 in stems (Figures 3d,e
207 and 5b, Supplementary Table S5).
208

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209 In plant stems, sequences affiliated to Bacteroidetes had their maximum relative abundance during
210 early exposure to fixed temperatures (Tg1), while at the same time Proteobacteria (the most
211 abundant phylum in all samples) had their minimum relative abundance, with the lowest relative
212 abundance recorded in plants exposed to higher temperatures (Supplementary Figure S4).
213
214 In the roots of greenhouse plants (similarly to observations in the field), fewer taxa showed
215 significant differences between time points (Supplementary Figure S6). The relative abundance of
216 Actinobacteria peaked at Tg2, while Verrucomicrobia were most abundant at Tg1. The relative
217 abundance of Proteobacteria was characterised by a sharp decrease over the course of the
218 experiment. Of the Proteobacteria, only Enterobacteriales and Pseudomonadales showed
219 decreasing abundance over time (Supplementary Figure S7).
220
221 *Correlation networks among taxa*.
222 Correlation networks at genus level were detected using pooled data from FP and GP, in order to
223 make the inferred interactions robust. In this representation, taxa were connected if they were
224 either significantly correlated or anti-correlated (Spearman's $|r|>0.7$, Benjamini-Hochberg-Yekutieli
225 FDR corrected $q < 0.05$). Networks in stems and roots were studied separately, as these
226 interactions may be organ-specific. Four sets of correlated taxa were found in plant stems (Figure
227 6a). The most relevant set involved seven genera with a large number of interconnected taxa
228 (*Acidocella*, *Bradyrhizobium*, *Burkholderia*, *Dyella*, *Mesorhizobium*, *Propionibacterium* and
229 *Ralstonia*) and eight less interconnected taxa (*Acinetobacter*, *Anoxybacillus*, *Brevibacillus*,
230 *Cohnella*, *Corynebacterium*, GP2, *Niastella* and *Predibacter*). A second highly connected network
231 included *Aquicella*, *Conexibacter*, *Legionella*, *Hyphomicrobium Pedomicrobium*, *Steroidobacter*
232 and *Mycobacterium*. Three taxa including important plant-associated species (*Massilia*,
233 *Methylobacterium* and *Sphingomonas*) were also connected in a third set. *Aurantimonas* e
234 *Roseomonas* formed the fourth set of correlated taxa in plant stems. Overall the taxa with the
235 highest degree of interaction (measured by the number of links) were *Burkholderia* and
236 *Mesorhizobium*.
237 In roots, four sets of interconnected taxa were observed, but the level of interconnection was
238 generally lower (Figure 6b). One of these sets included the *Actinoplanes*, *Burkholderia*,
239 *Nocardioides*, *Ohtaekwangia* and *Steroidobacter* genera, a second included the *Asticcacaulis*,
240 *Caulobacter*, *Phenylobacterium* and *Pleomorphomonas* genera, while the two remaining sets were
241 both made up of only two genera: *Bradyrhizobium* and *Mesorhizobium*, and *Hyphomicrobium* and
242 *Pedomicrobium*.
243
244 **Discussion**

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245 Although recently more information has been made available on endophytic communities in
246 perennial woody species (Bonito *et al.,* 2014; Lamit *et al.,* 2014), the majority of studies dealing
247 with the composition of microbial communities are based on analysis of perennial and annual
248 herbaceous plants (Garbeva *et al.,* 2001; Long *et al.,* 2010; Sessitsch *et al.,* 2004). As a perennial
249 woody plant, the grapevine offers an interesting model when compared to annual herbaceous
250 plants, as it accumulates stratified endophytic communities during its lifetime (Campisano *et al.,*
251 2015).
252 As several reports have linked typical soil characteristics to wine terroir and the plant microbiome
253 at large (Bokulich *et al.,* 2014; Knight *et al.,* 2015; Taylor *et al.,* 2014; Zarraonaindia *et al.,* 2015),
254 we nullified the effect of this variable by planting the same plants in the same soil in pots, in order
255 to study variables not mediated by the soil. This approach was successful in removing the location-
256 specific effect linked to the type of soil, indeed no statistical differences were present between the
257 samples at the four locations. This approach also allowed us to emphasise other variables
258 affecting the assembly of the plant's endophytic microbiome, such as temperature and season for
259 example, investigating the changes associated with temperature in greater detail.
260 Using this approach we were able to describe the changes in endophytic communities
261 independently of the soil over one year in field conditions. In parallel we observed the changes
262 over a shorter time interval at fixed temperatures. The information from controlled greenhouse
263 experiments provided a valuable source for cross-validating and interpreting temperature-driven
264 community shifts in field plants.
265 The difference between root and stem-associated endophytic communities is not surprising, and it
266 has been reported previously in other model plants (Jin *et al.,* 2014; Mocali *et al.,* 2003; Tian *et al.,*
267 2007). In our experiments, differences were also observed between bacterial communities in field
268 and greenhouse-grown plants. This separation is clearly visible by plotting beta-diversity (Figure 3)
269 and probably originates in the stability of greenhouse conditions and the inherent lack of
270 fluctuations in temperature, wind and humidity. While the specific conditions in the greenhouse and
271 the field favoured the assembly of somewhat different endophytic communities, their microbial
272 communities show ample commonalities (Figure 1). Controlled environmental conditions have
273 previously been used to isolate the impact of variables on soil microbial communities (Corneo *et*
274 *al.,* 2014; Kuffner *et al.,* 2012; Zhang *et al.,* 2013), often showing the limited responsiveness of
275 bacterial assemblies to minor temperature changes. In the field, altitudinal transects have been
276 used to simulate mild to moderate differences in climate (Corneo *et al.,* 2013b; Smith *et al.,* 2002).
277 While in vineyard soils with similar features to those tested here altitudinal transects have been
278 linked to clear-cut shifts in microbial composition (Corneo *et al.,* 2013a), such differences were not
279 found in the grapevine endophytic communities in this study. In addition, we observed that the
280 sampling time during the season was a major driver of diversity in bacterial endophytes. Other
281 researchers (Bevivino *et al.,* 2014) have reported a strong influence of season on the culturable

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282 fraction of bacterial communities in vineyard soils, suggesting that the responsiveness of soil
283 microbial communities to seasonal variations may be too subtle to appear clearly, when
284 confounding factors with a stronger influence are present (Kuffner *et al.,* 2012). Our findings
285 suggest that endophytic communities may be more sensitive to seasonal variations and less
286 sensitive to differences in altitude than those inhabiting the soil. Recent studies have demonstrated
287 that the influence of soil microbial terroir prevails over other factors in shaping the endophytic
288 microbiome (Bokulich *et al.,* 2014; Knight *et al.,* 2015; Zarraonaindia *et al.,* 2015). Our data are in
289 agreement with studies specifically focusing on endophytic bacterial communities in the grapevine
290 (Zarraonaindia *et al.,* 2015). Despite the geographical diversity and although Zarraonaindia and co-
291 workers did not comment on differences connected to different sampling seasons, similarities
292 between our study and their data emerge, including the dominance of Proteobacteria both in the
293 aerial organs and roots of plants. Overall, the community composition at phylum level largely
294 overlaps with previous studies on grapevine endophytes (Campisano *et al.,* 2014a; Zarraonaindia
295 *et al.,* 2015).

296 Our study suggests that the dynamics governing taxonomic composition in soil and the plant
297 endosphere may not overlap. To explain this different response to temperature fluctuations, we
298 hypothesize that the stability of plant physiology may play a major role (as compared to ephemeral
299 soil conditions). Despite higher stability, endophytic communities fluctuated with strong tissue-
300 specific patterns, further supporting the hypothesis that plant physiology is the central
301 environmental constraint for endophytic bacteria. Not only did endophytic communities prove to be
302 more resilient to moderate temperature changes (such as those underlying different altitudinal
303 transects) than soil communities, but the endophytes found in plant roots also responded to
304 temperature-driven changes at a slower pace than those in plant parts above ground. This delayed
305 response to temperature shifts in roots was observed in both field and greenhouse plants. A peak
306 in alpha diversity accompanied both summer warming (for field plants) and the onset of a
307 controlled temperature regime in the greenhouse (Figure 4). In both scenarios, diversity in plant
308 stems increased within a short time interval, but occurred later in roots, over an extended three-
309 month interval.

310 Warming has long been known to positively influence root exudation (Vančura, 1967), and root
311 exudates are reported to play an active role in recruiting microorganisms from the soil to the
312 rhizosphere and then to the endosphere (Broeckling *et al.,* 2008). It is therefore conceivable that
313 the increase in diversity and the alterations in the root-associated bacterial community depend on
314 increased root exudation. On the other hand, an entirely different mechanism may operate in parts
315 of the plant above ground, where a faster response was observed. Besides the reaction to
316 temperature fluctuation, stem-associated endophytic communities may respond more promptly to
317 other stimuli, including those originating from surface-associated microbiota (Copeland *et al.,*

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318 2015). Indeed, phyllosphere microbial communities also follow seasonal variations, regardless of
319 soil communities (Copeland *et al.,* 2015).
320 In both above and below-ground organs, the relative abundance of several taxa followed a
321 seasonal trend (for example for *Sphingomonas*, *Burkholderia*, *Massilia*, *Bacillus* and
322 *Propionibacterium* genera in stems, and *Xanthobacter*, *Nocardia*, *Mycobacterium*, *Pseudomonas*
323 and *Bradyrhizobium* genera in roots). Recently, the notion that below and above-ground plant-
324 associated fungal assemblages are determined by different environmental variables has been
325 proposed, with temperature being relevant only for leaf-associated fungi (Coince *et al.,* 2014).
326 While this may be true for fungi, our data suggest a different effect of temperature on endophytic
327 bacterial communities.
328
329 In greenhouse plants, the alpha and beta-diversity of stem-associated bacterial endophytic
330 communities show a response to temperature perturbation whose extent relates to the temperature
331 value (the higher the temperature, the stronger the response) in the short term (one month), with a
332 subsequent return to a state similar to the initial one after three months (Figure 4b). This behaviour
333 suggests the existence of a mechanism that compensates for the changes in community
334 composition and drives them back towards a stable equilibrium. In root-associated bacterial
335 endophytes this reversion could not be observed, possibly because of the slower response of root
336 endophytic communities over the limited duration of the experiment (three months). Such a
337 mechanism, operating over a time span of between one and three months in stems and three to six
338 months in roots, may keep the plant bacterial endophytic populations in a perpetual dynamic
339 balance, where stability is never achieved because of ever-shifting seasonal changes.
340
341 Despite the diversity in composition (multivariate clustering analysis), correlations among taxa
342 were highly consistent, and indicated a robust network structure, both in the field and in
343 greenhouse conditions. Notably, in stems one principal correlation cluster included bacteria (such
344 as *Burkholderia*, *Mesorhizobium*, *Ralstonia*, *Bradyrhizobium* and *Propionibacterium*) that are well
345 described endophytes present in several land plants (Chaintreuil *et al.,* 2000) and whose presence
346 has been constantly reported in the grapevine (Campisano *et al.,* 2014a; Campisano *et al.,* 2014b;
347 Compant *et al.,* 2008; Zarraonaindia *et al.,* 2015), but also taxa that are only occasionally
348 described as endophytes (such as *Peredibacter* and *Niastella*). In roots, a much smaller set of taxa
349 were linked by correlations. The central roles in networks were played by *Steroidobacter* and
350 *Caulobacter*. OTUs under the genus *Steroidobacter* amounted to up to 20% of the total root-
351 associated endophytic communities and their presence was especially abundant in greenhouse
352 plants. *Caulobacter* was instead present almost exclusively in field plants and at a 10-fold lower
353 concentration than *Steroidobacter*. The presence of *Steroidobacter* as an endophyte has been
354 described in the grapevine (Marasco *et al.,* 2013, Zarraonaindia *et al.,* 2015), where it was found to

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355 be associated with healthy plants (Bulgari *et al.,* 2012). Despite its frequency in other crops as well
356 (Erlacher *et al.,* 2014), the role of these gamma-proteobacteria in plants has still been little studied.
357 These observations underline the strengths and weaknesses of experiments carried out under
358 controlled conditions where only one variable is taken into account, to test hypotheses generated
359 by observations of field-related data. A reductionist approach when studying the influence of
360 environmental or anthropic factors on the composition of plant-associated communities can be
361 satisfied by using plants grown in controlled environments. On the other hand, even excluding the
362 effect of soil, field-grown plants are subject to a much broader spectrum of stimuli, which also
363 interact with each other. For example, temperature fluctuates with the day/night cycle and with the
364 seasons, differing in the soil and air and interacting with other factors such as humidity, rainfall,
365 wind, etc. All these factors and their interaction need to be taken into account when modelling the
366 dynamics of plant endophytes.
367

368 **Experimental Procedures**

369 *Plant material, study site and sampling*.
370 The plants used in this work were one-year-old Chardonnay scions grafted onto Kober 5BB
371 rootstock. They were potted in soil (50% peat and 50% clay/sandy soil) collected in a vineyard in S.
372 Michele all'Adige, Italy and mixed thoroughly just before the start of the experiments. Because
373 much is already known from plant microbiome studies at sites with characteristically diverse soils,
374 we aimed to remove the soil effect entirely from our study, focussing on effects not mediated by
375 soil. For this reason, all the plants were planted in the same soil. Specifically, in field experiments,
376 the potted grapevines were planted in holes dug in vineyard rows, so that the pots would emerge
377 approximately 2 cm from the ground, in order to minimise the effect of soil differences in vineyards
378 on the rhizosphere and root microbiome. The study sites were chosen to represent two transects in
379 the winemaking province of Trentino, Italy. One transect was represented by the sites at San
380 Michele all'Adige (46.192372, 11.134957, ~220 m above sea level, a.s.l.) and Faedo (46.197285,
381 11.171207, ~740 m a.s.l.), the other transect was represented by the sites at Navicello (45.876692,
382 11.020972, ~200 m a.s.l.) and Lenzima (45.869618, 10.983356, ~760 m a.s.l.). The first sampling
383 was performed at the beginning of the experiment at FEM, before plants were moved to the study
384 sites (field time-point Tf0: 4 May 2011) and then every three months (field time-points Tf1: 4
385 August 2011; Tf2: 3 November 2011; Tf3: 6 February 2012; Tf4: 7 May 2012) so that Tf4 occurred
386 one year after Tf0. Four plants were sampled at Tf0 and twelve plants (three per field) were
387 sampled at all successive samplings. Detailed weather data including air and soil temperature,
388 wind speed, humidity, rainfall and solar radiation were recorded by the weather station network of
389 the Fondazione Edmund Mach (FEM) (http://meteo.fmach.it/meteo/). The average soil and air
390 seasonal temperatures at each location during the course of the experiments are available in the
391 supplementary material (Supp. Figure S8).

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392 Greenhouse trials were carried out at FEM, San Michele all'Adige, Italy, between 14 May and 14
393 August 2011. Before the start of experiments, plants were acclimatised in a greenhouse with
394 limited temperature fluctuations for one month. Five plants were sampled at the beginning of the
395 experiment to represent the initial bacterial communities (greenhouse time-point Tg0), then the
396 plants were placed in cells with a fixed air temperature (either 15, 25 or 35°C) and 16:8 h light:dark
397 photoperiod. The soil temperature in the pots was the same as the air temperature, except for a
398 brief fluctuation (approximately 1 hour) after watering (water temperature around 15° C).
399 After one month of incubation in these conditions, five plants per temperature treatment were
400 destructively sampled (greenhouse time-point Tg1). A second sampling of three plants for each
401 temperature treatment (greenhouse time-point Tg2) was carried out after three months.
402 The sampled plants were taken immediately to the lab, quickly washed and air-dried at room
403 temperature. Roots were separated from the stem using clean pruning scissors. Stems were
404 further separated into graft and rootstock. Only the grafted stems were used for further work. After
405 removing all leaves, stems were surface-sterilised and aseptically peeled as previously described
406 Campisano *et al.* (2014a). Roots were washed accurately under a stream of tap water until all the
407 soil was removed. Clean roots were dried and surface-sterilised as described above.
408 After surface sterilisation, as peeling the root epidermis was not possible in small plants, the roots
409 were scrubbed to dislodge surface-attached microorganisms and leave only the endophytic fraction
410 by vortex-mixing with acid-washed glass beads, as previously described (Sessitsch *et al.,* 2002).
411 After surface sterilisation and scrubbing, all plant material was kept in 50 ml tubes at -80°C until
412 processed for DNA isolation. Dried roots were weighed at one timepoint during the course of each
413 experiment: at Tg2 for greenhouse plants, and at Tf1 for field plants.
414
415 *DNA extraction, handling and amplification.*
416 In total 104 and 58 samples were collected from plants grown in field and greenhouse conditions
417 respectively. For DNA extraction, the plant material was homogenized in aseptic conditions by
418 freezing in liquid nitrogen and vigorous shaking in steel jars as described previously (Pancher *et*
419 *al.,* 2012). The finely ground plant material obtained with this procedure was used for extraction
420 using the FastDNA spin kit for soil and a FastPrep-24 mixer (MP Biomedical, USA), according to
421 standard manufacturer protocols. PCR was performed using High Fidelity FastStart DNA
422 polymerase (Roche, USA) and the universal primers 799F (AACMGGATTAGATACCCKG) and
423 reverse primers 1520R (AAGGAGGTGATCCAGCCGCA) with 454 adaptors and a sample-specific
424 barcode on the forward primer, as previously described (Yousaf *et al.,* 2014). The PCR products
425 were separated on 1% agarose gel and gel-purified using Invitrogen PureLink (Invitrogen, USA).
426
427 *Pyrosequencing of the 16S rDNA gene and sequence analysis*

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428 Amplicons were quantified with quantitative PCR, using the Roche 454 titanium Library
429 Quantification Kit (KAPA Biosystems, USA) and pooled in equimolar ratio in the final amplicon
430 library. 454 pyrosequencing was carried out on the Roche GS FLX+ system using the new XL+
431 chemistry dedicated to long reads of up to 800bp, following the manufacturer's recommendations.
432 Pyrosequencing resulted in a total of 2,308,793 16S rDNA reads (1,494,020 in field samples and
433 814,773 in greenhouse samples) and the raw 454 files were demultiplexed using Roche sff file
434 software, available at the European Nucleotide Archive (www.ebi.ac.uk) under the accession study
435 PRJEB11360. Sample accessions and metadata are available in Supplementary Table S1. Reads
436 were pre-processed using the MICCA pipeline (Albanese et al 2015) (version 0.1,
437 http://www.micca.org) Forward and reverse primer trimming and quality filtering were performed
438 using micca-preproc (parameters -f AACMGGATTAGATACCCKG -r
439 AAGGAGGTGATCCAGCCGCA -O 15 -l 250 -q 20), truncating reads shorter than 250 nt. De-novo
440 sequence clustering, chimera filtering and taxonomy assignment were performed with micca-otu-
441 denovo (parameters -s 0.97 -c -d): operational taxonomic units (OTUs) were assigned by
442 clustering the sequences with a threshold of 97% pairwise identity, and their representative
443 sequences were classified using the RDP classifier (Wang *et al* 2007) software version 2.7.
444 Template-guided multiple sequence alignment (MSA) was performed using PyNAST (Caporaso
445 *et., al* 2010) (version 0.1) against the multiple alignment of the Greengenes database (DeSantis *et*
446 *al.,* 2006; release 13_05) filtered at 97% similarity. Finally, a phylogenetic tree was inferred using
447 FastTree (Price *et al.,* 2010) and micca-phylogeny (parameters: -a template --template-min-perc
448 75). Sampling heterogeneity was reduced by rarefaction (4,854 sequences per sample).
449
450 *Data analysis*
451 Statistical analysis and graphic representation of data were performed using the R statistical
452 software suite (R Development Core Team, 2011). Calculations of alpha diversity measures, Bray-
453 Curtis and UniFrac distances and PcoA analysis were performed using the *estimate_richness()*,
454 *distance()*, and *ordinate()* functions, respectively implemented in the R *phyloseq* package
455 (McMurdie & Holmes, 2013). Graphic representation of the data was carried out using the R
456 package *ggplot2* (Wickham, 2009). Correlations were computed using the R package *ccrepe*
457 (https://www.bioconductor.org/packages/release/bioc/html/ccrepe.html), specifically designed to
458 assess the significance in compositional datasets. Cladograms highlighting the most discriminative
459 taxa (LEfSe analysis) were built using the method of Segata *et al* 2011).
460
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466

467 **Author contributions**

468 AC and IP conceived the study; AC, SY and MP carried out the experiments; AC, DA and CD
469 analysed the data; AC, CD, IP and DA wrote the manuscript.
470

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702 **Figure and table legends**

703 Figure 1. Order-level abundance of bacterial endophytes in each treatment (average of….).
704 Samples are coded as follows: for greenhouses (15, 25, 35, temperature of 15, 25, 35°C), plant
705 organ (R, roots; S, stems), sampling time (T1, 1 month;T2, 3 months); for field sampling time (T0,
706 start of the experiment, T1 to T4, every three months), plant organ (R, roots; S, stems), sampling
707 location (L, Lenzima ; F, Faedo; N, Navicello; S, San Michele).

708 Figure 2. Cladograms showing the most discriminative taxa (LEfSe analysis) between time-points
709 in field plants (a and b) and greenhouse plants (c and d). Node colour and shading highlight
710 treatments in which taxa (with names in the legend) had a significantly higher relative abundance
711 (LEfSe Kruskal-Wallis $P<0.01$, Wilcoxon $P<0.01$, logarithmic LDA score > 2). Sample names are
712 defined in the main text.

713 Figure 3. Ordination plots (PCoA on UniFrac distances) showing endophytic communities in: a,
714 roots (filled squares) and stems (circles) of field-grown (green) and greenhouse-grown (red) plants;
715 b, roots of field-grown plants; c, stems of field-grown plants; d, roots of greenhouse-grown plants;
716 e, stems of greenhouse-grown. a, unweighted UniFrac distance; b, c, d, e: weighted UniFrac
717 distance). Colour shades indicate sampling time, shapes indicate greenhouse temperature.
718 Sample legends are defined in the main text.

719 Figure 4. Alpha diversity of endophytic communities in roots (a and c) and stems (b and d)
720 measured as observed OTUs (a and b) and Shannon entropy (c and d).

721 Figure 5. Box plot of weighted UniFrac distances for endophytic communities from time point 0 (Tf0
722 and Tg0) in plant roots and stems of field (a) and greenhouse (b) plants. The upper and lower
723 margin of the boxes represent the first and third quartiles respectively of point distribution. Upper
724 and lower whiskers extend to the highest value within 1.5 * IQR of the box, where IQR is the
725 distance between the first and third quartiles. Asterisks indicate significant differences (Wilcoxon
726 rank-sum test $P<0.05$, FDR corrected).

727 Figure 6. Networks of OTUs in plant stems (a) and roots (b) of grapevine plants. The 8 OTUs with
728 the highest degree of interaction (for each plant organ) are displayed in colour.

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All samples (root + stem) - Group: Type	F	R2	P
uUnifrac ADONIS:	9.5762	0.06	0.0001
wUnifrac ADONIS:	9.0881	0.0571	0.0001
bray-curtis ADONIS:	11.5557	0.0715	0.0001
Root - Group: Time			
uUnifrac ADONIS:	2.317	0.1949	0.0001
wUnifrac ADONIS:	5.8961	0.3812	0.0001
bray-curtis ADONIS:	6.7355	0.413	0.0001
Stem - Group: Time			
uUnifrac ADONIS:	4.3375	0.3056	0.0001
wUnifrac ADONIS:	7.5317	0.4331	0.0001
bray-curtis ADONIS:	7.3125	0.4259	0.0001

Table 1: PERMANOVA multivariate statistical analysis of samples grouped by plant organ and time succession, based on 9999 permutations. Distance metrics: uUnifrac: unweighted UniFrac; wUnifrac: weighted UniFRac; bray-curtis: Bray-Curtis.

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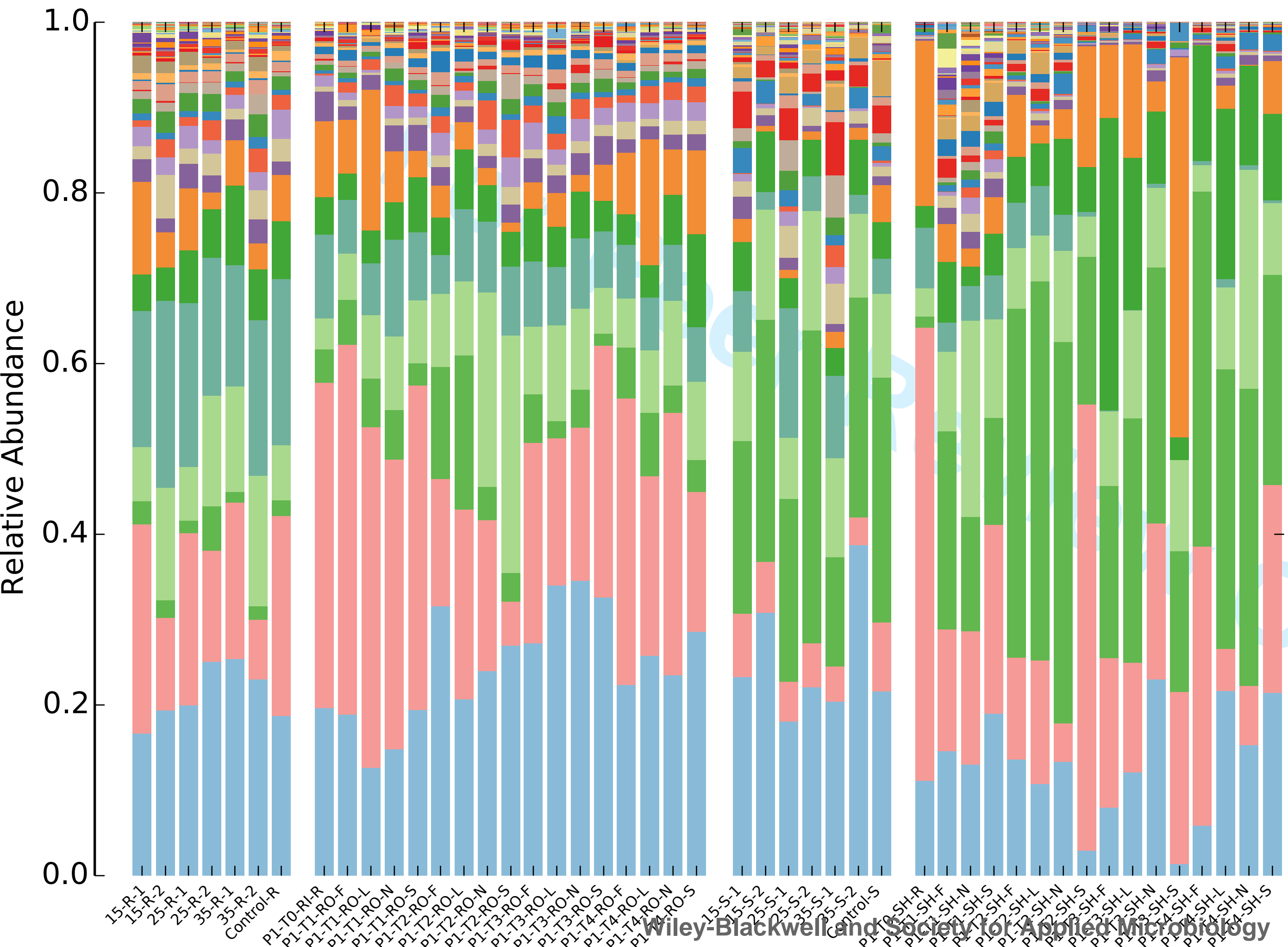

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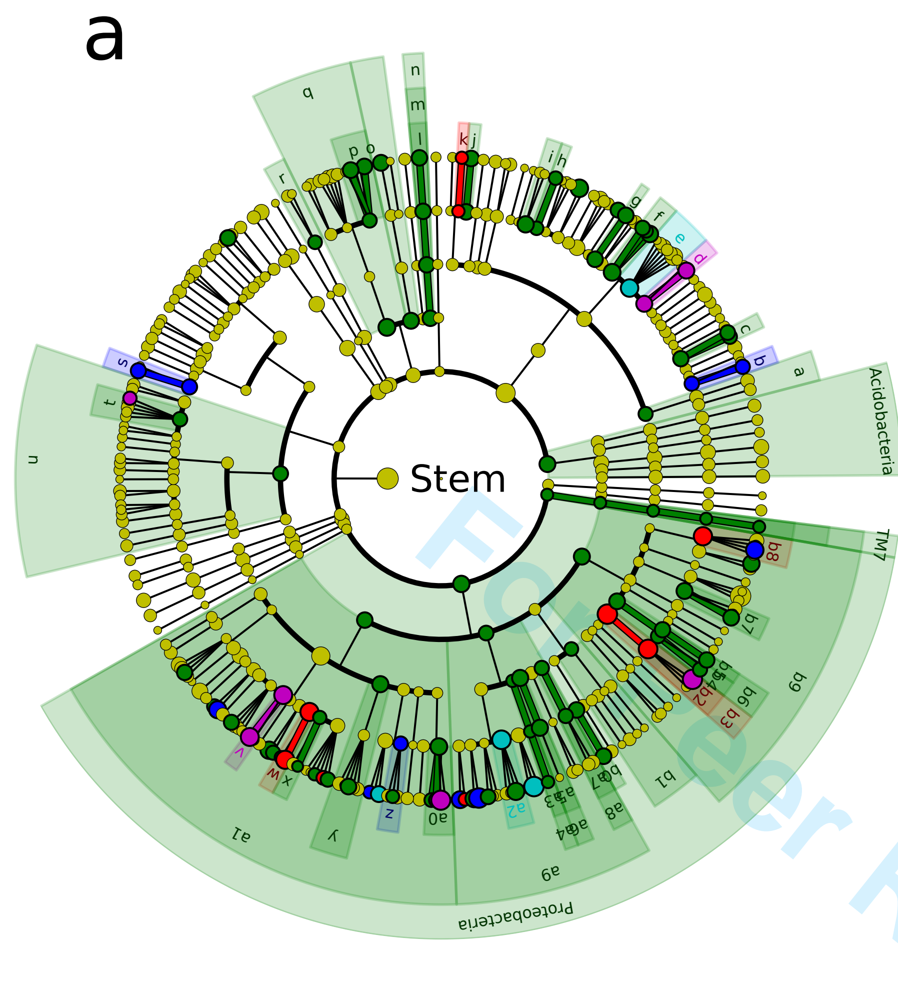
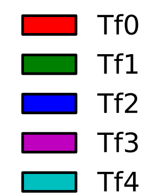
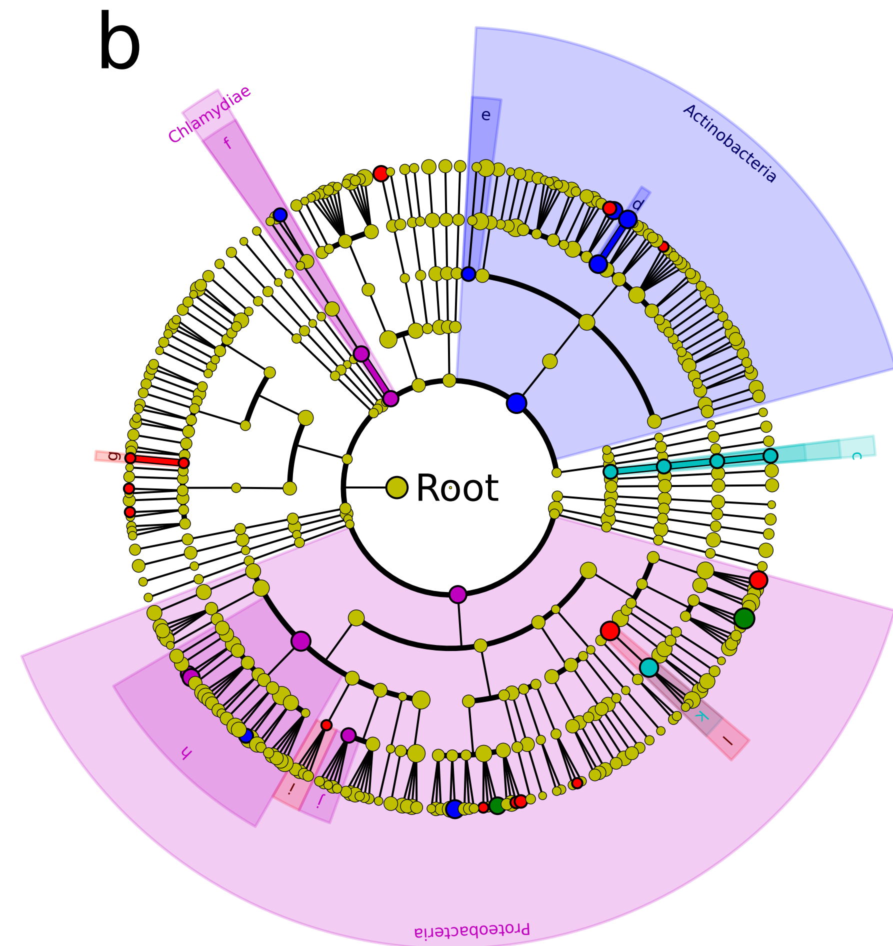
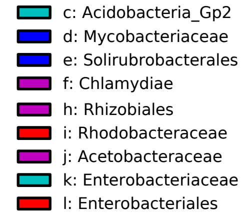
a: Acidimicrobiales
b: Actinomycetaceae
c: Corynebacteriaceae
d: Kineosporiaceae
e: Microbacteriaceae
f: Micrococcaceae
g: Mycobacteriaceae
h: Sanguibacteraceae
i: Streptomycetaceae
j: Conexibacteraceae
k: Patulibacteraceae
l: Ohtaekwangia
m: Ohtaekwangia
n: Bacteroidetes_incertae_sedis
o: Flavobacteria
p: Chitinophagaceae
q: Sphingobacteria
r: Parachlamydiaceae
s: Streptococcaceae
t: Clostridiales_IncertaeSedisXI
u: Clostridia
v: Methylobacteriaceae
w: Rhizobiaceae
x: Rhizobiales_incertae_sedis
y: Rhodobacterales
z: Rhodospirillaceae
a0: Sphingomonadaceae
a1: Alphaproteobacteria
a2: Comamonadaceae
a3: Hydrogenophilaceae
a4: Hydrogenophilales
a5: Methylophilaceae
a6: Methylophilales
a7: Rhodocyclaceae
a8: Rhodocyclales
a9: Betaproteobacteria
b0: Bacteriovoracaceae
b1: Myxococcales
b2: Enterobacteriaceae
b3: Enterobacteriales
b4: Coxiellaceae
b5: Legionellaceae
b6: Legionellales
b7: Moraxellaceae
b8: Xanthomonadaceae
b9: Gammaproteobacteria

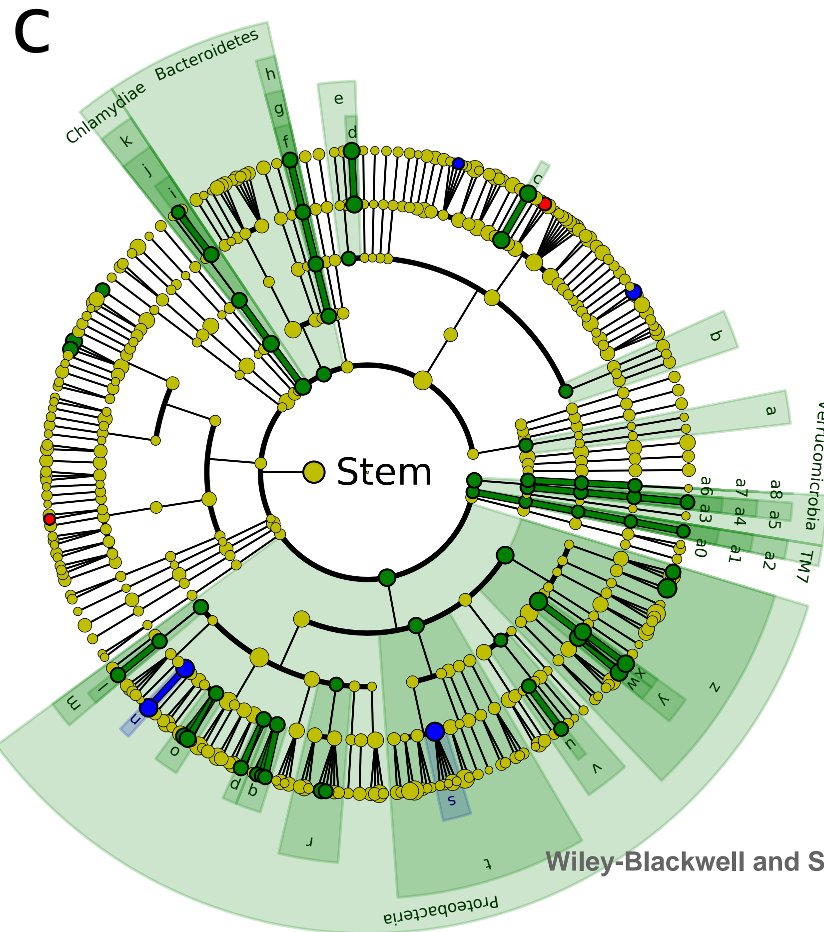


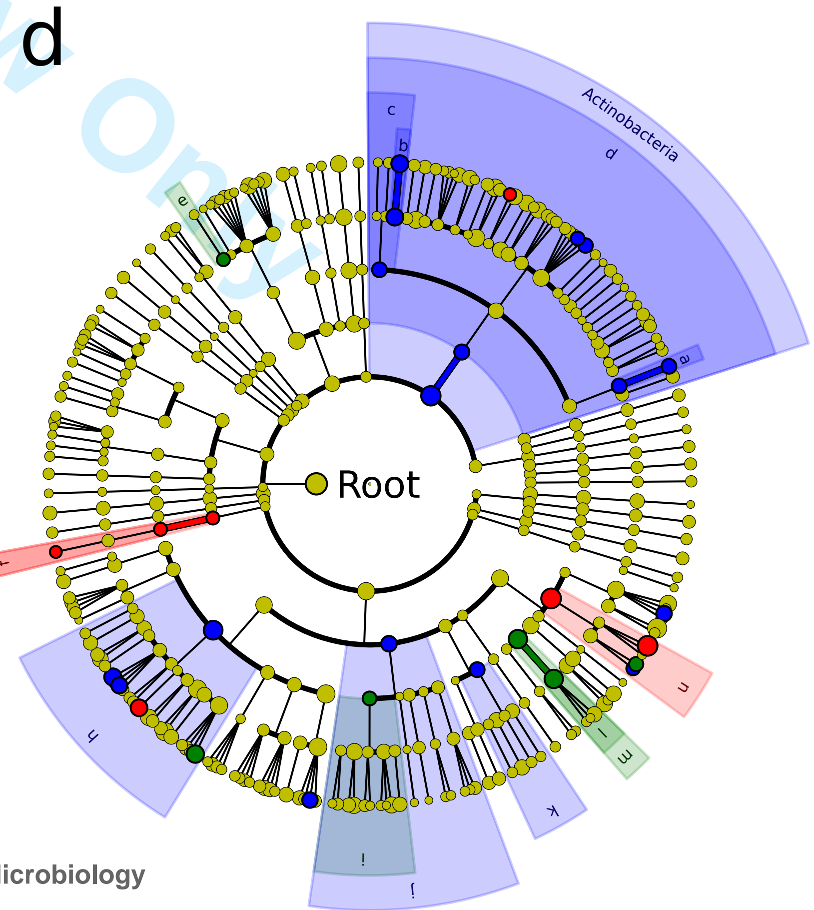


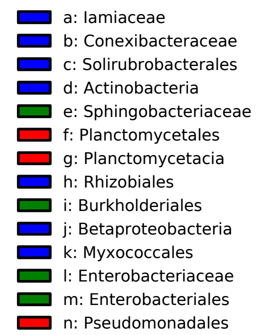




a: Acidobacteria_Gp3
b: Acidimicrobiales
c: Mycobacteriaceae
d: Conexibacteraceae
e: Solirubrobacterales
f: Ohtaekwangia
g: Ohtaekwangia
h: Bacteroidetes_incertae_sedis
i: Parachlamydiaceae
j: Chlamydiales
k: Chlamydiae
l: Rhizomicrobium
m: Alphaproteobacteria_incertae_sedis
n: Aurantimonadaceae
o: Hyphomicrobiaceae
p: Rhizobiales_incertae_sedis
q: Xanthobacteraceae
r: Rhodospirillales
s: Comamonadaceae
t: Betaproteobacteria
u: Haliangiaceae
v: Myxococcales
w: Coxiellaceae
x: Legionellaceae
y: Legionellales
z: Gammaproteobacteria
a0: TM7_genera_incertae_sedis
a1: TM7_genera_incertae_sedis
a2: TM7_genera_incertae_sedis
a3: Subdivision3_genera_incertae_sedis
a4: Subdivision3_genera_incertae_sedis
a5: Subdivision3
a6: Verrucomicrobiaceae
a7: Verrucomicrobiales
a8: Verrucomicrobiae







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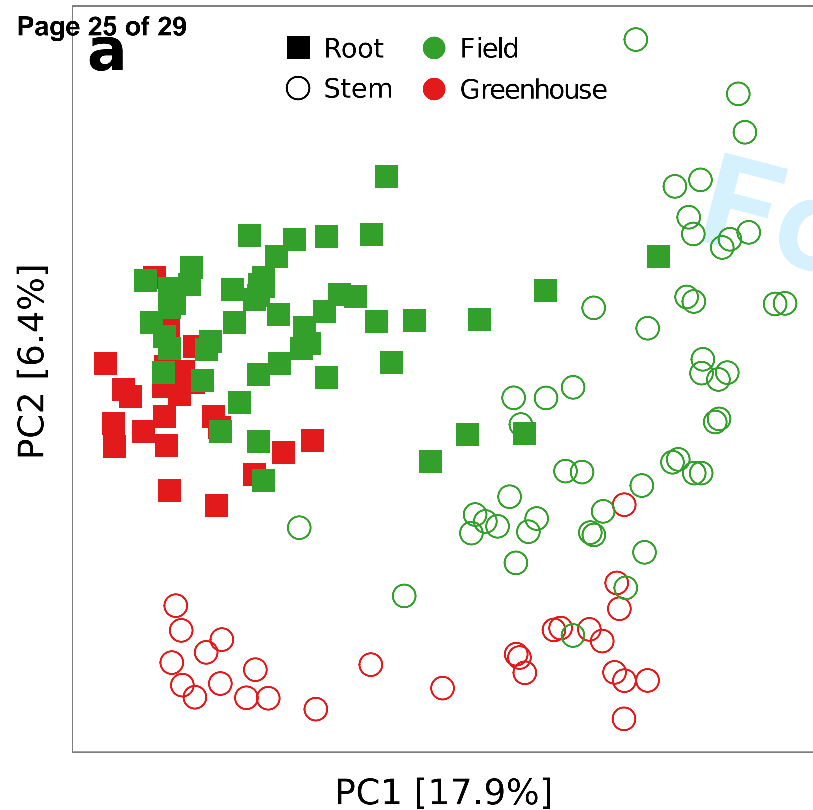

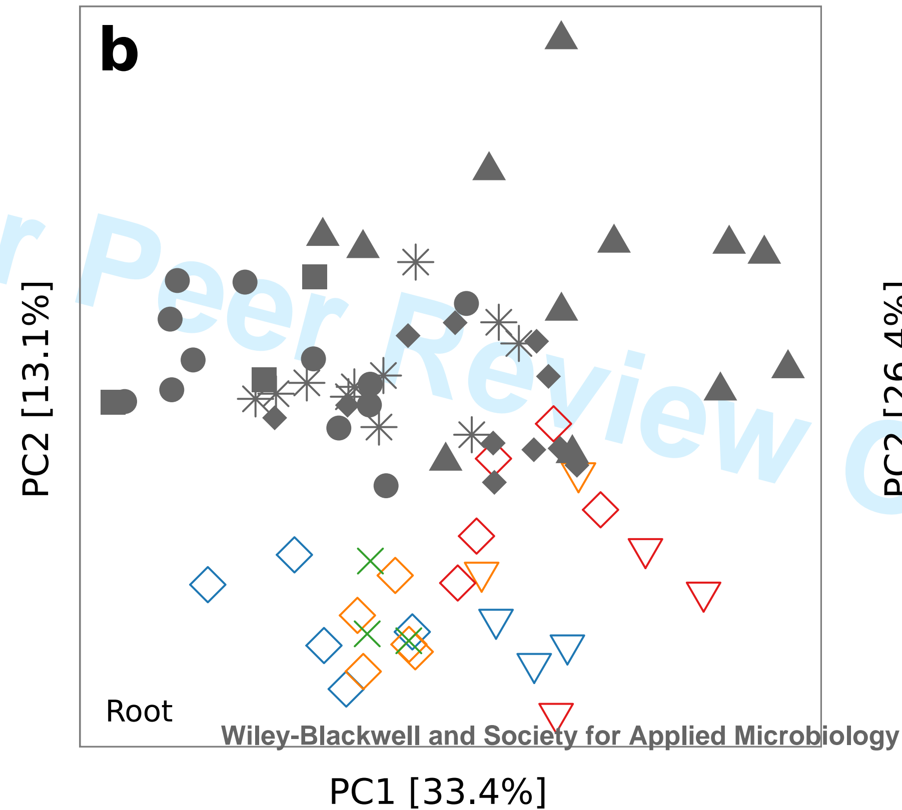

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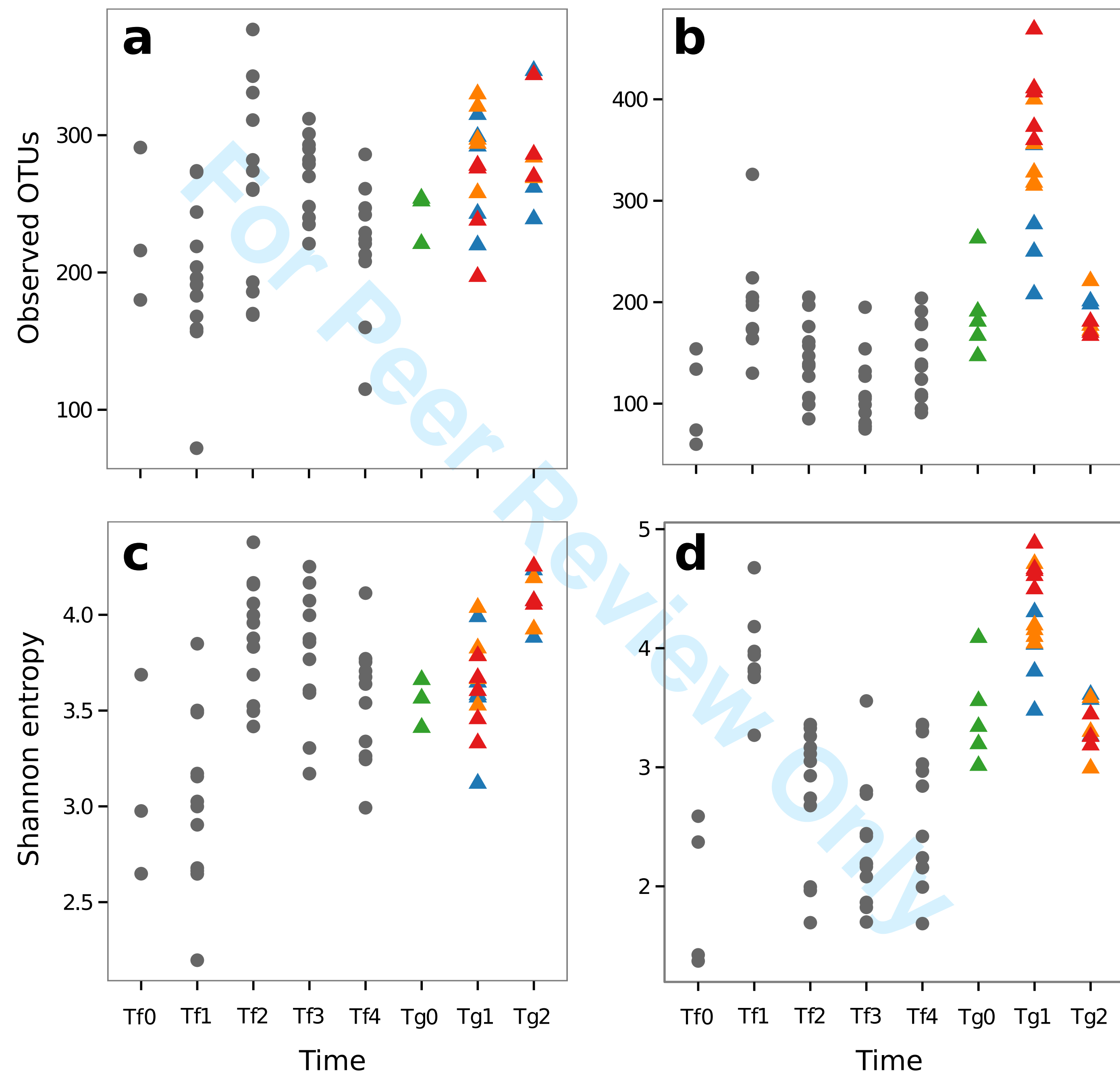

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a

Root

Stem

W. UniFrac distance from Tf0

0.8
0.6
0.4
0.2

Tf1 Tf2 Tf3 Tf4

Time

b

Root

Stem

W. UniFrac distance from Tg0

0.8
0.6
0.4
0.2

Tg1 Tg2

Time

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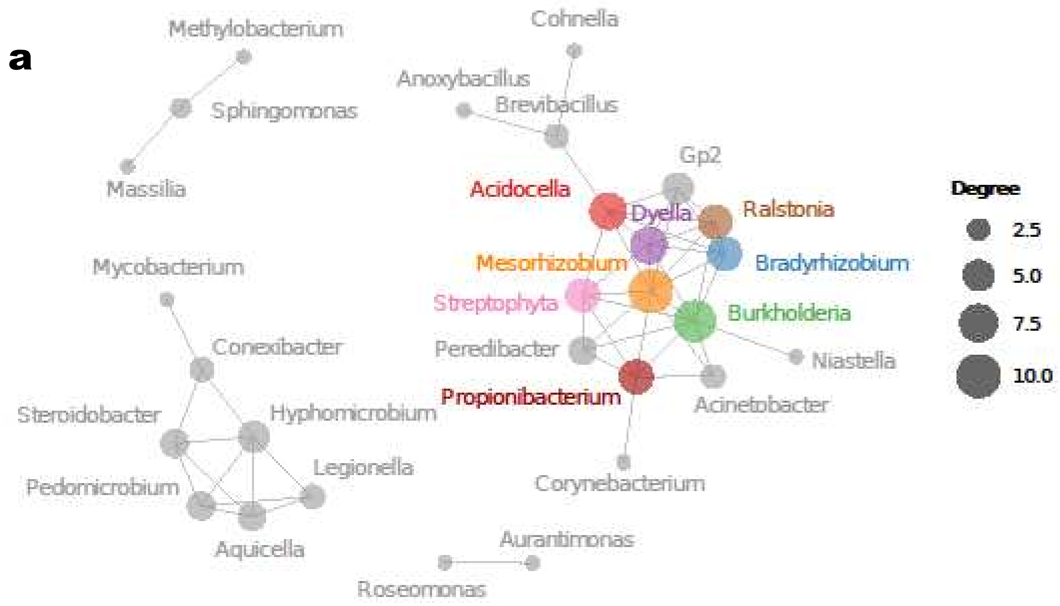
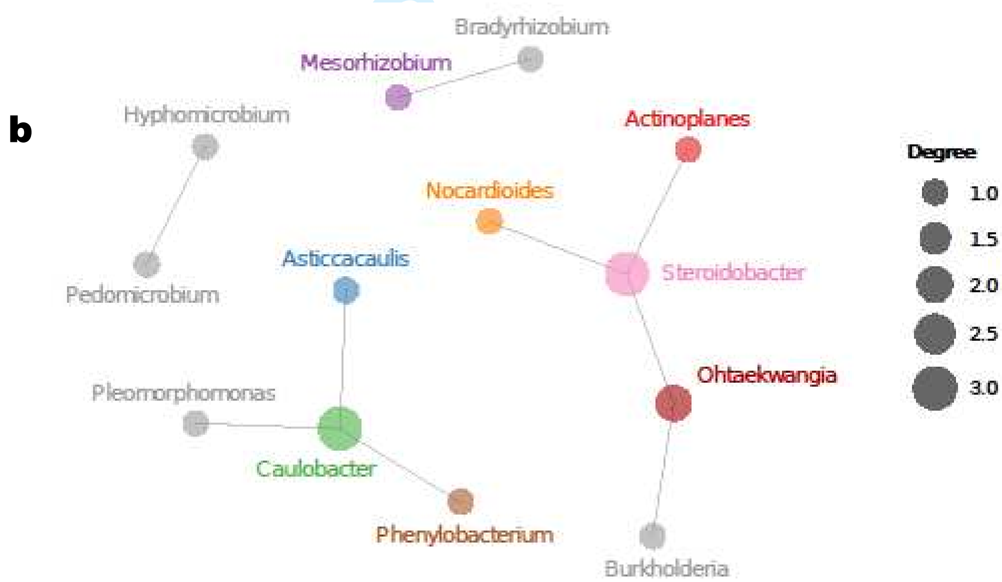

a
Methylobacterium
Sphingomonas
Massilia
Cohnella
Anoxybacillus
Brevibacillus
Gp2
Acidocella
Dyella
Ralstonia
Bradyrhizobium
Mesorhizobium
Streptophyta
Burkholderia
Peredibacter
Niastella
Propionibacterium
Acinetobacter
Corynebacterium
Mycobacterium
Conexibacter
Steroidobacter
Hyphomicrobium
Legionella
Pedomicrobium
Aquicella
Aurantimonas
Roseomonas
Degree
2.5
5.0
7.5
10.0


b
Bradyrhizobium
Mesorhizobium
Hyphomicrobium
Actinoplanes
Nocardioides
Pedomicrobium
Asticcacaulis
Steroidobacter
Ohtaekwangia
Pleomorphomonas
Caulobacter
Phenylobacterium
Burkholderia
Degree
1.0
1.5
2.0
2.5
3.0

All samples (root + stem) - Group: Type	F	R2	P
uUnifrac ADONIS:	9.5762	0.06	0.0001
wUnifrac ADONIS:	9.0881	0.0571	0.0001
bray-curtis ADONIS:	11.5557	0.0715	0.0001
Root - Group: Time			
uUnifrac ADONIS:	2.317	0.1949	0.0001
wUnifrac ADONIS:	5.8961	0.3812	0.0001
bray-curtis ADONIS:	6.7355	0.413	0.0001
Stem - Group: Time			
uUnifrac ADONIS:	4.3375	0.3056	0.0001
wUnifrac ADONIS:	7.5317	0.4331	0.0001
bray-curtis ADONIS:	7.3125	0.4259	0.0001

Table 1: PERMANOVA multivariate statistical analysis, based on 9999 permutations, of samples grouped by plant organ, and time succession. Distance metrics: uUnifrac: unweighted UniFrac; wUnifrac: weighted UniFRac; bray-curtis: Bray-Curtis.

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