Temperature drives the assembly of endophytic communities’ seasonal succession

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<td>Manuscript Type:</td>
<td>EMI - Research article</td>
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<td>Date Submitted by the Author:</td>
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<td>Complete List of Authors:</td>
<td>Campisano, Andrea; Fondazione Edmund Mach, 1. Sustainable Agro-ecosystems and Bioresources Albanese, Davide; Fondazione Edmund Mach, Research and Innovation Centre, Department of Computational Biology Yousaf, Sohail; Quaid-i-Azam University, 3. Department of Environmental Sciences Pancher, Michael; Fondazione Edmund Mach, 1. Sustainable Agro-ecosystems and Bioresources Department Donati, Claudio; Fondazione E. Mach, Computational Biology Pertot, Ilaria; Fondazione Edmund Mach, Research and Innovation Centre, Department of Sustainable Agro-Ecosystems and Bioresources</td>
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<td>Keywords:</td>
<td>grapevine, endophyte, seasonality, symbiosis, temperature</td>
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Wiley-Blackwell and Society for Applied Microbiology
Temperature drives the assembly of endophytic communities’ seasonal succession

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Running title: Endophytes respond to season and temperature

Word Count: 5013 words (Introduction: 811; Materials and Methods: 1147; Results: 1349; Discussion: 1645; Acknowledgements: 61). Figures 1, 2, 3, 4 and 6 should be in colour. Figure 5 in B/W. One Table. Thirteen suppl. material items (8 figures, 5 tables).

Competing interests: the authors declare no competing interests
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 asymptotically 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
Introduction

Plants host a wide diversity of microbial inhabitants (Vorholt, 2012). Microorganisms living at least part of their life cycle within plant tissues are termed endophytes (Hardoim, et al., 2015). In contrast with microbes living in abiotic environments, the study of microbial endophytic communities entails not only understanding the interaction between them, but also between such communities and the highly variable and reactive background provided by the living host (Robinson et al., 2010). Plants actively recognise and select the microbial inhabitants in their tissues by modulating gene expression, and consequently the metabolism (Hardoim et al., 2015; Tkacz et al., 2015). While endophytes benefit from a substantially protected niche, where nutrition and shelter are provided by the host (Schardl et al., 2004), in the plant they are subject to changes in the host’s physiology, which in turn responds to environmental stimuli (Zimmerman & Vitousek, 2012).

In animals, symbiotic microbial communities are mostly confined to selected districts connected to the outside environment (i.e. skin, respiratory tract, gut, urogenital tract). Conversely, a variety of plant endophytes colonise all plant organs (Compant et al., 2011). Root-associated communities, including root endophytes, have received considerable attention from science, as they are thought to play a crucial role in plant nutrition and growth, while fewer studies have addressed endophytic communities in the plant canopy. Although knowledge about the composition of plant endophytic communities is increasing rapidly, the forces driving their composition are just starting to be unearthed (Poosakkannu et al., 2015).

Perennial deciduous plants in temperate climates experience an annual metabolic cycle that determines (among other things) the loss of leaves and the translocation of nutrients towards the roots in the colder season (Tromp, 1983). These drastic changes are caused by profound metabolic shifts that involve the plant as a whole and are likely to be reflected in the composition of the endophytic communities therein. The weather and, especially temperature, is the main driver of this cycle (Bita & Gerats., 2013). Temperature shifts are also expected to affect the composition of plant-associated microbiota, both directly and through the induced changes in the plant metabolism (Campisano et al., 2014a). In annual plants the soil is the main source of endophytic colonisation, but several vertically-transmitted endophytes are also commonly found (Hodgson et al., 2014; Johnston-Monje et al., 2014). Studies carried out over multiple seasons have so far focussed more on soil and root-associated microorganisms, providing little support for modelling the dynamics of endophytes (Peiffer et al., 2013; Shi et al., 2015). In perennial woody plants endophyte communities are a combination of pre-existing (resident) and new (pioneer) colonisers. In woody plants vertically-transmitted fungi are less common, probably because of the time span before seed production. The plant’s intrinsic features, such as morphology and physiology, may also limit vertical transmission (Saikkonen et al., 2004). In the grapevine we previously hypothesised that vertical transmission of endophytes present in the cuttings may play a bigger role than vertical transmission through seeds (Campisano et al., 2015).
Several attempts have been made to understand the role of climate, including seasonality and temperature variation, in shaping the plant microbiome. Much of the existing information converges, indicating that climate strongly affects fungal assemblages (Coince et al. 2014; Cordier et al., 2012). On the other hand, bacterial communities correlate better with other, more tissue or compartment-specific variables (Coleman-Derr et al., 2016). For example, the variability of bacterial assemblages in Agave plants was linked more to plant compartment than biogeography, suggesting a major role of the plant microenvironment as compared to climate-associated variables (Coleman-Derr et al., 2016). However, other relevant factors, such as the influence of soil, cannot be excluded (Bokulich et al., 2014). In addition, climate change can alter the composition and function of endophytes, and with them, the impact on their hosts (Ducklow, 2008; Johnson et al., 2013).

To understand the shifts in endophytic bacterial communities over the year, we assessed these communities in the perennial plant *Vitis vinifera* L every three months. By sampling plants grown in the same type of soil along an altitudinal transect, we studied changes in the composition of endophytes inhabiting plants that are exposed to slightly, but consistently different climatic conditions throughout the year (similarly to recent studies on plant-associated fungal assemblages in Cordier et al., 2012; Coince et al., 2014). To evaluate the short term effect of temperature independently of the season, we monitored a set of identical plants kept in greenhouses at three different temperatures (15, 25 and 35°C).

**Results**

The roots of the plants grown in colder conditions were generally more developed than those grown in warmer conditions. This effect was significant in the greenhouse plants, where the root weight of plants growing at 15°C was approximately twice that of plants growing at 25° and 35°C after two months (Tg2). The effect of temperature on the roots of plants growing at different altitudes was less noticeable (Supplementary Figure S1). In these plants the alpha and beta-diversity of their endophytic bacterial communities changed constantly throughout the year (field time-points: Tf0: XX May 2011, Tf1: 4 August 2011; Tf2: 3 November 2011; Tf3: 6 February 2012; Tf4: 7 May 2012) and showed an organ-dependent response to time in the growing season (Figures 1-3).

**Dominating taxa in the stem and root endosphere**

Proteobacteria was the dominant phylum over the whole experimental set (greenhouse and vineyard), with sequence counts ranging from 60% to over 95% of the total. Actinobacteria were also detected, with sequence counts ranging from 3.2% to 32.8% of the total. Bacteroidetes (up to 6.1% of sequences) and Firmicutes (up to 6.9% of sequences) were detected at lower levels. The order with the highest average sequence count was Rhizobiales (20.2%), followed by
Pseudomonadales (19.7%), Burkholderiales (15.2%) and Actinomycetales (10.2%). A graphic representation of the OTU table summarised at order level is shown in Figure 1. Sequences assigned to the Burkholderiales order were more common in plant stems (4.6%) than in roots (2.8%, \( p = 2.49 \times 10^{-18} \), Wilcoxon rank-sum test, FDR corrected), while the opposite was true for those classified as Rhizobiales, Pseudomonadales and Xanthomoadales (\( P = 1.87 \times 10^{-5} \), \( P = 1.56 \times 10^{-6} \) and \( p = 9.94 \times 10^{-12} \) respectively) (Supplementary Figure S2).

Differentially abundant taxa in the stem and root endosphere

Significant differences (\( p < 0.05 \), Wilcoxon rank-sum test, FDR corrected) between endophytic taxa found in stems and roots at order and family level are illustrated in Supplementary Figures S2 and S3 respectively. Bacteria from the Nocardioidaceae, Ohtaekwangia, Coxiellaceae, Sinobacteraceae, Methylocystaceae and Hyphomicrobiaceae families were more commonly found in greenhouse plants, while Pseudomonadaceae were more common in field-grown plants. After one and three months in the greenhouse, a shift in the relative abundance of the Conexibacteraceae, Legionellaceae, Coxiellaceae, Sinobacteraceae, Rhizobiales (\textit{incertae sedis}), Hyphomicrobiaceae, Rhodospirillaceae, Haliangiaceae and Aurantimonadaceae bacterial families (in stems), Verrucomicrobiaceae and Ohtaekwangia (in roots and stems), and Sinobacteraceae and Hyphomicrobiaceae (in roots) was observed (Figure 2). Plants grown at different altitudes or different locations did not differ in terms of the composition of their endophytic microbiota (with one exception) and therefore they were considered replicate samples for all statistical purposes (Supplementary Table S4). One exception was noted at Tf2, when root-associated bacterial endophytes were distinct (\( p = 0.0184 \), PERMANOVA on weighted UniFrac distances).

PCoA analysis of the samples’ beta diversity using UniFrac distances showed that stem and root bacterial endophytic communities grouped separately. Samples from the field could also be distinguished from those from greenhouses (Figure 3a, Table 1). Clustering in the stem and root was clear in both field-grown plants (henceforth FP, Figures 3b-c) and in greenhouse-grown plants (henceforth GP, Figures 3d-e). Interestingly, samples from FP and GP were separated along the second coordinate (Figure 3a).

Temporal changes in endophytic populations inhabiting field plants.

Alpha diversity was generally higher in the endosphere of FP roots as compared to that of FP stems (Welch’s t-test \( P = 6.8 \times 10^{-13} \)). The maximum diversity in FP stems (Figure 4) occurred in summer (Tf1), while it shifted in autumn (Tf2) in FP roots. Analysis of beta-diversity showed that endophytic bacterial communities in stems at Tf1 and Tf2 were close together in PCoA plots (Figure 3c), as for the communities at time-points Tf3 and Tf4. Beta-diversity distances from Tf0 samples were higher in stem-associated endophytic communities.
than in root-associated ones, both in FP and in GP (Figure 5a). As compared to Tf0, the bacterial communities of roots differed most at Tf2, while they were most similar at Tf1 and Tf4. The cycling trend outlined here (Figure 5a) was reversed when plant stems were considered. Here the distances from TF0 were highest at Tf1 and Tf4 and lowest at Tf2, symmetrically reflecting those observed in plant roots (Figure 5a, Supplementary Table S5).

To understand the fine shifts in the plant endophytic microbiome, we identified the taxa undergoing significant changes in relative abundance on the basis of time (p < 0.05, Wilcoxon rank-sum test, FDR corrected). Many taxa had a maximum relative abundance at Tf1 in plant stems (Figure 2a), including phyla Proteobacteria, Acidobacteria and TM7, and class Sphingobacteria, Flavobacteria, Alpha-, Beta- and Gamma-proteobacteria. In roots (Figure 2b) the relative abundance of sequences in the Actinobacteria phylum peaked at Tf2, while the highest relative abundance of Chlamidiae and Proteobacteria occurred at Tf3.

In plant stems, the relative abundance of Actinobacteria, Bacteroidetes, Acidobacteria and Firmicutes was highest at Tf1 (Supplementary Figure S4) and lowest at Tf3, while Proteobacteria, which was the most abundant phylum, were least numerous at Tf1. Interestingly, Proteobacteria also (Tg1) decreased readily during greenhouse exposure to fixed temperatures, with the lowest relative abundance recorded in plants exposed to higher temperatures (Supplementary Figure S4). In roots, fewer taxa showed significant differences over the time points (Supplementary Figure S6). In a similar way to the observations in stems, Proteobacteria did not display a unique trend, with Enterobacteriales and Pseudomonadales being the only orders that had the minimum abundance at Tf2 (Supplementary Figure S7).

Temporal changes in endophytic populations inhabiting greenhouse plants.

Alpha-diversity indices (Figure 4) were highest in plants growing at high temperature (35°C) and lowest in plants growing at the lowest temperature (15°C). The initial increase in bacterial diversity found at all temperatures in stems was reversed in samples taken at Tg2, when diversity indices were not different from those at Tg0. In roots, alpha diversity increased monotonically between Tg0 and Tg2, suggesting a slower response of root endophytic communities to temperature changes.

PCoA plots (Figure 3d, Table 1) separated Tg0 and Tg1 root samples from Tg2 samples along the main coordinate. A similar separation was apparent in stem samples (Figure 3e). Distances from T0 increased with time in roots, while peaking at Tg1 and decreasing at Tg2 in stems (Figures 3d, e and 5b, Supplementary Table S5).
In plant stems, sequences affiliated to Bacteroidetes had their maximum relative abundance during early exposure to fixed temperatures (Tg1), while at the same time Proteobacteria (the most abundant phylum in all samples) had their minimum relative abundance, with the lowest relative abundance recorded in plants exposed to higher temperatures (Supplementary Figure S4).

In the roots of greenhouse plants (similarly to observations in the field), fewer taxa showed significant differences between time points (Supplementary Figure S6). The relative abundance of Actinobacteria peaked at Tg2, while Verrucomicrobia were most abundant at Tg1. The relative abundance of Proteobacteria was characterised by a sharp decrease over the course of the experiment. Of the Proteobacteria, only Enterobacteriales and Pseudomonadales showed decreasing abundance over time (Supplementary Figure S7).

Correlation networks among taxa.
Correlation networks at genus level were detected using pooled data from FP and GP, in order to make the inferred interactions robust. In this representation, taxa were connected if they were either significantly correlated or anti-correlated (Spearman’s |r|>0.7, Benjamini-Hochberg-Yekutieli FDR corrected q < 0.05). Networks in stems and roots were studied separately, as these interactions may be organ-specific. Four sets of correlated taxa were found in plant stems (Figure 6a). The most relevant set involved seven genera with a large number of interconnected taxa (Acidocella, Bradyrhizobium, Burkholderia, Dyella, Mesorhizobium, Propionibacterium and Ralstonia) and eight less interconnected taxa (Acinetobacter, Anoxybacillus, Brevibacillus, Cohnella, Corynebacterium, GP2, Niastella and Predibacter). A second highly connected network included Aquicella, Conexibacter, Legionella, Hyphomicrobium Pedomicrobium, Steroidobacter and Mycobacterium. Three taxa including important plant-associated species (Massilia, Methyllobacterium and Sphingomonas) were also connected in a third set. Aurantimonas and Roseomonas formed the fourth set of correlated taxa in plant stems. Overall the taxa with the highest degree of interaction (measured by the number of links) were Burkholderia and Mesorhizobium.

In roots, four sets of interconnected taxa were observed, but the level of interconnection was generally lower (Figure 6b). One of these sets included the Actinoplanes, Burkholderia, Nocardioides, Ohtaekwangia and Steroidobacter genera, a second included the Asticcacaulis, Caulobacter, Phenyllobacterium and Pedomorphomonas genera, while the two remaining sets were both made up of only two genera: Bradyrhizobium and Mesorhizobium, and Hyphomicrobium and Pedomicrobium.

Discussion
Although recently more information has been made available on endophytic communities in perennial woody species (Bonito et al., 2014; Lamit et al., 2014), the majority of studies dealing with the composition of microbial communities are based on analysis of perennial and annual herbaceous plants (Garbeva et al., 2001; Long et al., 2010; Sessitsch et al., 2004). As a perennial woody plant, the grapevine offers an interesting model when compared to annual herbaceous plants, as it accumulates stratified endophytic communities during its lifetime (Campisano et al., 2015).

As several reports have linked typical soil characteristics to wine terroir and the plant microbiome at large (Bokulich et al., 2014; Knight et al., 2015; Taylor et al., 2014; Zarraonaindia et al., 2015), we nullified the effect of this variable by planting the same plants in the same soil in pots, in order to study variables not mediated by the soil. This approach was successful in removing the location-specific effect linked to the type of soil, indeed no statistical differences were present between the samples at the four locations. This approach also allowed us to emphasise other variables affecting the assembly of the plant's endophytic microbiome, such as temperature and season for example, investigating the changes associated with temperature in greater detail.

Using this approach we were able to describe the changes in endophytic communities independently of the soil over one year in field conditions. In parallel we observed the changes over a shorter time interval at fixed temperatures. The information from controlled greenhouse experiments provided a valuable source for cross-validating and interpreting temperature-driven community shifts in field plants. The difference between root and stem-associated endophytic communities is not surprising, and it has been reported previously in other model plants (Jin et al., 2014; Mocali et al., 2003; Tian et al., 2007). In our experiments, differences were also observed between bacterial communities in field and greenhouse-grown plants. This separation is clearly visible by plotting beta-diversity (Figure 3) and probably originates in the stability of greenhouse conditions and the inherent lack of fluctuations in temperature, wind and humidity. While the specific conditions in the greenhouse and the field favoured the assembly of somewhat different endophytic communities, their microbial communities show ample commonalities (Figure 1). Controlled environmental conditions have previously been used to isolate the impact of variables on soil microbial communities (Corneo et al., 2014; Kuffner et al., 2012; Zhang et al., 2013), often showing the limited responsiveness of bacterial assemblies to minor temperature changes. In the field, altitudinal transects have been used to simulate mild to moderate differences in climate (Corneo et al., 2013b; Smith et al., 2002). While in vineyard soils with similar features to those tested here altitudinal transects have been linked to clear-cut shifts in microbial composition (Corneo et al., 2013a), such differences were not found in the grapevine endophytic communities in this study. In addition, we observed that the sampling time during the season was a major driver of diversity in bacterial endophytes. Other researchers (Bevivino et al., 2014) have reported a strong influence of season on the culturable
fraction of bacterial communities in vineyard soils, suggesting that the responsiveness of soil microbial communities to seasonal variations may be too subtle to appear clearly, when confounding factors with a stronger influence are present (Kuffner et al., 2012). Our findings suggest that endophytic communities may be more sensitive to seasonal variations and less sensitive to differences in altitude than those inhabiting the soil. Recent studies have demonstrated that the influence of soil microbial terroir prevails over other factors in shaping the endophytic microbiome (Bokulich et al., 2014; Knight et al., 2015; Zarraonaindia et al., 2015). Our data are in agreement with studies specifically focusing on endophytic bacterial communities in the grapevine (Zarraonaindia et al., 2015). Despite the geographical diversity and although Zarraonaindia and co-workers did not comment on differences connected to different sampling seasons, similarities between our study and their data emerge, including the dominance of Proteobacteria both in the aerial organs and roots of plants. Overall, the community composition at phylum level largely overlaps with previous studies on grapevine endophytes (Campisano et al., 2014a; Zarraonaindia et al., 2015).

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

Warming has long been known to positively influence root exudation (Vančura, 1967), and root exudates are reported to play an active role in recruiting microorganisms from the soil to the rhizosphere and then to the endosphere (Broeckling et al., 2008). It is therefore conceivable that the increase in diversity and the alterations in the root-associated bacterial community depend on increased root exudation. On the other hand, an entirely different mechanism may operate in parts of the plant above ground, where a faster response was observed. Besides the reaction to temperature fluctuation, stem-associated endophytic communities may respond more promptly to other stimuli, including those originating from surface-associated microbiota (Copeland et al., 2015).
Indeed, phyllosphere microbial communities also follow seasonal variations, regardless of soil communities (Copeland et al., 2015). In both above and below-ground organs, the relative abundance of several taxa followed a seasonal trend (for example for Sphingomonas, Burkholderia, Massilia, Bacillus and Propionibacterium genera in stems, and Xanthobacter, Nocardia, Mycobacterium, Pseudomonas and Bradyrhizobium genera in roots). Recently, the notion that below and above-ground plant-associated fungal assemblages are determined by different environmental variables has been proposed, with temperature being relevant only for leaf-associated fungi (Coince et al., 2014). While this may be true for fungi, our data suggest a different effect of temperature on endophytic bacterial communities.

In greenhouse plants, the alpha and beta-diversity of stem-associated bacterial endophytic communities show a response to temperature perturbation whose extent relates to the temperature value (the higher the temperature, the stronger the response) in the short term (one month), with a subsequent return to a state similar to the initial one after three months (Figure 4b). This behaviour suggests the existence of a mechanism that compensates for the changes in community composition and drives them back towards a stable equilibrium. In root-associated bacterial endophytes this reversion could not be observed, possibly because of the slower response of root endophytic communities over the limited duration of the experiment (three months). Such a mechanism, operating over a time span of between one and three months in stems and three to six months in roots, may keep the plant bacterial endophytic populations in a perpetual dynamic balance, where stability is never achieved because of ever-shifting seasonal changes.

Despite the diversity in composition (multivariate clustering analysis), correlations among taxa were highly consistent, and indicated a robust network structure, both in the field and in greenhouse conditions. Notably, in stems one principal correlation cluster included bacteria (such as Burkholderia, Mesorhizobium, Ralstonia, Bradyrhizobium and Propionibacterium) that are well described endophytes present in several land plants (Chaintreuil et al., 2000) and whose presence has been constantly reported in the grapevine (Campisano et al., 2014a; Campisano et al., 2014b; Compant et al., 2008; Zarraonaindia et al., 2015), but also taxa that are only occasionally described as endophytes (such as Peredibacter and Niastella). In roots, a much smaller set of taxa were linked by correlations. The central roles in networks were played by Steroidobacter and Caulobacter. OTUs under the genus Steroidobacter amounted to up to 20% of the total root-associated endophytic communities and their presence was especially abundant in greenhouse plants. Caulobacter was instead present almost exclusively in field plants and at a 10-fold lower concentration than Steroidobacter. The presence of Steroidobacter as an endophyte has been described in the grapevine (Marasco et al., 2013, Zarraonaindia et al., 2015), where it was found to
be associated with healthy plants (Bulgari et al., 2012). Despite its frequency in other crops as well (Erlacher et al., 2014), the role of these gamma-proteobacteria in plants has still been little studied. These observations underline the strengths and weaknesses of experiments carried out under controlled conditions where only one variable is taken into account, to test hypotheses generated by observations of field-related data. A reductionist approach when studying the influence of environmental or anthropic factors on the composition of plant-associated communities can be satisfied by using plants grown in controlled environments. On the other hand, even excluding the effect of soil, field-grown plants are subject to a much broader spectrum of stimuli, which also interact with each other. For example, temperature fluctuates with the day/night cycle and with the seasons, differing in the soil and air and interacting with other factors such as humidity, rainfall, wind, etc. All these factors and their interaction need to be taken into account when modelling the dynamics of plant endophytes.

Experimental Procedures

Plant material, study site and sampling.
The plants used in this work were one-year-old Chardonnay scions grafted onto Kober 5BB rootstock. They were potted in soil (50% peat and 50% clay/sandy soil) collected in a vineyard in S. Michele all'Adige, Italy and mixed thoroughly just before the start of the experiments. Because much is already known from plant microbiome studies at sites with characteristically diverse soils, we aimed to remove the soil effect entirely from our study, focussing on effects not mediated by soil. For this reason, all the plants were planted in the same soil. Specifically, in field experiments, the potted grapevines were planted in holes dug in vineyard rows, so that the pots would emerge approximately 2 cm from the ground, in order to minimise the effect of soil differences in vineyards on the rhizosphere and root microbiome. The study sites were chosen to represent two transects in the winemaking province of Trentino, Italy. One transect was represented by the sites at San Michele all’Adige (46.192372, 11.134957, ~220 m above sea level, a.s.l.) and Faedo (46.197285, 11.171207, ~740 m a.s.l.), the other transect was represented by the sites at Navicello (45.876692, 11.020972, ~200 m a.s.l.) and Lenzima (45.869618, 10.983356, ~760 m a.s.l.). The first sampling was performed at the beginning of the experiment at FEM, before plants were moved to the study sites (field time-point Tf0: 4 May 2011) and then every three months (field time-points Tf1: 4 August 2011; Tf2: 3 November 2011; Tf3: 6 February 2012; Tf4: 7 May 2012) so that Tf4 occurred one year after Tf0. Four plants were sampled at Tf0 and twelve plants (three per field) were sampled at all successive samplings. Detailed weather data including air and soil temperature, wind speed, humidity, rainfall and solar radiation were recorded by the weather station network of the Fondazione Edmund Mach (FEM) (http://meteo.fmach.it/meteo/). The average soil and air seasonal temperatures at each location during the course of the experiments are available in the supplementary material (Supp. Figure S8).
Greenhouse trials were carried out at FEM, San Michele all’Adige, Italy, between 14 May and 14 August 2011. Before the start of experiments, plants were acclimatised in a greenhouse with limited temperature fluctuations for one month. Five plants were sampled at the beginning of the experiment to represent the initial bacterial communities (greenhouse time-point Tg0), then the plants were placed in cells with a fixed air temperature (either 15, 25 or 35°C) and 16:8 h light:dark photoperiod. The soil temperature in the pots was the same as the air temperature, except for a brief fluctuation (approximately 1 hour) after watering (water temperature around 15°C). After one month of incubation in these conditions, five plants per temperature treatment were destructively sampled (greenhouse time-point Tg1). A second sampling of three plants for each temperature treatment (greenhouse time-point Tg2) was carried out after three months. The sampled plants were taken immediately to the lab, quickly washed and air-dried at room temperature. Roots were separated from the stem using clean pruning scissors. Stems were further separated into graft and rootstock. Only the grafted stems were used for further work. After removing all leaves, stems were surface-sterilised and aseptically peeled as previously described Campisano et al. (2014a). Roots were washed accurately under a stream of tap water until all the soil was removed. Clean roots were dried and surface-sterilised as described above. After surface sterilisation, as peeling the root epidermis was not possible in small plants, the roots were scrubbed to dislodge surface-attached microorganisms and leave only the endophytic fraction by vortex-mixing with acid-washed glass beads, as previously described (Sessitsch et al., 2002). After surface sterilisation and scrubbing, all plant material was kept in 50 ml tubes at -80°C until processed for DNA isolation. Dried roots were weighed at one timepoint during the course of each experiment: at Tg2 for greenhouse plants, and at Tf1 for field plants.

**DNA extraction, handling and amplification.**

In total 104 and 58 samples were collected from plants grown in field and greenhouse conditions respectively. For DNA extraction, the plant material was homogenized in aseptic conditions by freezing in liquid nitrogen and vigorous shaking in steel jars as described previously (Pancher et al., 2012). The finely ground plant material obtained with this procedure was used for extraction using the FastDNA spin kit for soil and a FastPrep-24 mixer (MP Biomedical, USA), according to standard manufacturer protocols. PCR was performed using High Fidelity FastStart DNA polymerase (Roche, USA) and the universal primers 799F (AACMGATTAGATACCCG) and reverse primers 1520R (AAGGAGGTGATCCAGCCGCA) with 454 adaptors and a sample-specific barcode on the forward primer, as previously described (Yousaf et al., 2014). The PCR products were separated on 1% agarose gel and gel-purified using Invitrogen PureLink (Invitrogen, USA).

**Pyrosequencing of the 16S rDNA gene and sequence analysis**
Amplicons were quantified with quantitative PCR, using the Roche 454 titanium Library Quantification Kit (KAPA Biosystems, USA) and pooled in equimolar ratio in the final amplicon library. 454 pyrosequencing was carried out on the Roche GS FLX+ system using the new XL+ chemistry dedicated to long reads of up to 800bp, following the manufacturer's recommendations. Pyrosequencing resulted in a total of 2,308,793 16S rDNA reads (1,494,020 in field samples and 814,773 in greenhouse samples) and the raw 454 files were demultiplexed using Roche sff file software, available at the European Nucleotide Archive (www.ebi.ac.uk) under the accession study PRJEB11360. Sample accessions and metadata are available in Supplementary Table S1. Reads were pre-processed using the MICCA pipeline (Albanese et al 2015) (version 0.1, http://www.micca.org) Forward and reverse primer trimming and quality filtering were performed using micca-preproc (parameters -f AACMGGATTAGATACCCKG -r AAGGAGGTGATCCAGCCGCA -o 15 -l 250 -q 20), truncating reads shorter than 250 nt. De-novo sequence clustering, chimera filtering and taxonomy assignment were performed with micca-otu-denoovo (parameters -s 0.97 -c -d); operational taxonomic units (OTUs) were assigned by clustering the sequences with a threshold of 97% pairwise identity, and their representative sequences were classified using the RDP classifier (Wang et al 2007) software version 2.7. Template-guided multiple sequence alignment (MSA) was performed using PyNAST (Caporaso et al. 2010) (version 0.1) against the multiple alignment of the Greengenes database (DeSantis et al., 2006; release 13_05) filtered at 97% similarity. Finally, a phylogenetic tree was inferred using FastTree (Price et al., 2010) and micca-phylogeny (parameters: -a template --template-min-perc 75). Sampling heterogeneity was reduced by rarefaction (4,854 sequences per sample).

Data analysis
Statistical analysis and graphic representation of data were performed using the R statistical software suite (R Development Core Team, 2011). Calculations of alpha diversity measures, Bray-Curtis and UniFrac distances and PcoA analysis were performed using the estimate_richness(), distance(), and ordinate() functions, respectively implemented in the R phyloseq package (McMurdie & Holmes, 2013). Graphic representation of the data was carried out using the R package ggplot2 (Wickham, 2009). Correlations were computed using the R package ccrepe (https://www.bioconductor.org/packages/release/bioc/html/ccrepe.html), specifically designed to assess the significance in compositional datasets. Cladograms highlighting the most discriminative taxa (LEfSe analysis) were built using the method of Segata et al. (2011).

Acknowledgements
The authors wish to thank the technical staff of the FEM Sequencing Platform for supporting this work, Lidia Nicola for critically proof-reading this manuscript, Livio Antonielli for ICT support and
the Informative Geographic System (CEM-CTT) for providing climate data. This work was funded by the Autonomous Province of Trento, PAT project - Call 2 Team 2009 - Incoming - Mecagrafic.

**Author contributions**

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

**References**


Figure and table legends

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

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

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

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

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

Figure 6. Networks of OTUs in plant stems (a) and roots (b) of grapevine plants. The 8 OTUs with
the highest degree of interaction (for each plant organ) are displayed in colour.
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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<thead>
<tr>
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<td>0.0001</td>
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<tr>
<td>wUnifrac ADONIS:</td>
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<td>0.0571</td>
<td>0.0001</td>
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<td>0.0715</td>
<td>0.0001</td>
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</tbody>
</table>
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Root Stem

0.2

0.4

0.6

0.8

Tf1 Tf2 Tf3 Tf4 Tg1 Tg2

Time

W. UniFrac distance from Tf0

W. UniFrac distance from Tg0

*a b

Time
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