Temporal variation of endophytic bacterial community structures in relation to the functional foliar traits of sessile oak (*Quercus petraea*)

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2 **functional foliar traits of sessile oak (*Quercus petraea*)**

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ABSTRACT

We studied the relationship between plant functional foliar traits and the endophytic bacterial communities associated in trees, taking the example of sessile oak (*Quercus petraea* (Matt.) Liebl). Forty-five samples of leaves were collected in spring, summer and autumn. Bacterial community diversity was analyzed via Automated Ribosomal Intergenic Spacer Analysis (ARISA). For each sample, the Specific Leaf Area (SLA), level of herbivory, stomatal number, stomatal length, carbon and nitrogen concentration were measured. For statistical analysis, the Canonical Correlation Analysis (CCA) and Non-Parametric Multivariate Analysis of Variance (NPMANOVA) were applied. Significant difference in herbivory, nitrogen and carbon concentration were found between summer and autumn (p value < 0.01). The seasonal differentiation of the bacterial community structure was explained by the first and second axes (29.7 % and 25.3 %, respectively) in the CCA. The most important foliar drivers resulted to be herbivory, nitrogen concentration and stomatal number.

INTRODUCTION

In biological sciences, besides specialization, integrating various scientific disciplines is a current major goal (*Niklas, Owens & Wayne, 2013*). One of the interfaces of biological interaction is between microbiota and plants. While many integrative studies exist, regarding description of bacterial taxa related to host plants and linking bacterial and plant communities across different spatial scales (e.g. symbiosis), temporal scales remain less unexplored. Plant functional traits are increasingly used in ecological research and are a promising avenue to link plant characteristics to environmental factor in interdisciplinary researches (*Cornwell et al. 2008; Wellstein et al. 2011*). In this sense, the leaf environment is characterized by foliar functional traits that are hypothesized to affect the hosted microbiome. In the context of the leaf environment of deciduous trees and inhabiting endophytic bacteria, intra-annual dynamics are of special interest.

Endophytic bacteria are ubiquitous inhabitants that colonize the inner parts of most terrestrial plant species beyond the epidermal cell layers (*Lodewyckx et al. 2015; Santoyo et al. 2016*). Inside the plant, there are diverse ecological niches in which endophytic bacteria can survive and grow, i.e. within cells, in the intercellular space. and in the vascular systems (*Jacobs, Bugbee & Gabrielson, 1985; Bell et al. 1995; Kembel et al. 2014*). Endophytic bacteria are very important to the host plant as they can contribute to the maintenance of its growth and health by,

e.g. promoting nutrient acquisition and defense against pathogens *(Hirano et al. 1982; Afzal, Khan & Sessitsch, 2014)*. This is particularly true for long-lived plant species such as trees and consequently they could affects forest ecosystems *(Tashi-Oshnoei, Harighi & Abdollahzadeh, 2017)*. Endophytic bacteria have often long-term ecological interactions with the host plants including symbiosis, mutualism and commensalism. They can either be obligate or facultative endophytes. Obligate endophytes have a strictly dependent association with the host plant and they are eventually transferred vertically through plant-host generations *(Santoyo et al. 2016)*. Facultative endophytes originate from the surrounding environment and they are often included within epidermal cell layers *(Hardoim, van Overbeek & van Elsas, 2008)*.

Different plant organs have diverse ways of colonization. Roots acquired endophytic bacteria from free-living organisms from the soil *(Bulgarelli et al. 2012; Edwards et al. 2015)*. Leaf endophytic bacteria, especially in case of tall trees, can be acquired from the leaf surface via stomata that represent apertures in the foliar tissue connected to the intercellular space *(Ou et al. 2014; Carrell, Carper & Frank, 2016)*. It has been hypothesized that leaves are a suitable surface for exchange with bacteria inhabiting the atmosphere *(Bowers et al. 2009)*. Microbial communities inhabiting leaves, including endophytic bacteria, appear to be rather specialized, given that they share less than 1% of the bacterial species with soil *(Kim et al. 2012)*.

While numerous publications are focused on leaf epiphytic bacteria *(Hirano et al. 1982; Balint-Kurti et al. 2010; Lopez-Velasco et al. 2011)*, leaf endophytic bacteria remain largely unexplored. Moreover, the possible role of plant functional traits for bacterial community dynamics represents a research gap. In detail, there are a few studies regarding the temporal dynamic and the environmental factors driving the endophytic bacterial communities associated with forest tree species. Previous works revealed that endophytes are subject to leaf age and leaf developmental stage in grapevine and in elm *(Mocali et al. 2003; Bulgari et al. 2014)*. However, the potential drivers affecting the endophytic bacteria composition behind leaf aging are still not well understood, especially in forest plants.

In our study, we aimed to test (i) if there is a temporal gradient associating leaf aging with bacterial turnover and (ii) if foliar plant traits are linked to bacterial community dynamics across time.

MATERIALS AND METHODS

83 **Study site and sampling**

84   The study area is located in the Monticolo nature reserve on the hillslopes of the Mitterberg

85 at 550 m a.s.l. in South Tyrol, Italy. The selected study site is representative of the present oak

86 forest, dominated by sessile oak (*Quercus petraea* (Matt.) Liebl.) and admixed with few specimens

87 of Scots pine (*Pinus sylvestris* L.) in the tree layer as well as of Sweet chestnut (*Castanea sativa*

88 Mill.) and Manna ash (*Fraxinus ornus* L.) in the understory. The forest grows on acidic shallow

89 soil above porphyry bedrock on a west-south-west oriented slope. We selected five individuals of

90 sessile oak within the study site, i.e. a circular plot of 15 m radius (706 m²) representing relatively

91 homogeneous site conditions within the slope. From each tree, we sampled three branches taking

92 eight leaves from the same branch, which were used to assess the endophytic bacteria as well as

93 the foliar functional traits. In detail, we used three leaves for the functional traits measurements

94 and five leaves for the determination of the endophytic bacterial microbial community. We

95 sampled three subsequent seasons in the year 2014, i.e. spring (June 5th), summer (August 25th)

96 and autumn (October 20th). A total of 45 samples was collected.

97   For each season, we determined the Specific Leaf Area (SLA) of sampled leaves following

98 standard protocols (*Pérez-Harguindeguy et al. 2013*). For each leaf, the leaf area was measured at

99 the day of sampling using a scanner (CanoScan Lide, Canon, Cernusco sul Naviglio, Italy).

100 Subsequently, leaves were oven dried at 70°C for 72 hours to obtain their dry weight and the SLA,

101 measured in mm² mg^{-1}, was calculated (*Pérez-Harguindeguy et al. 2013*). Leaf nitrogen (N) and

102 carbon (C) content was determined using an elemental analyzer (Flash 2000 Organic Elemental

103 Analyzer, Thermo Scientific, Milan, Italy).

104   To measure the stomatal characteristics, epidermal impressions of the leaves were

105 examined under an optical microscope connected to a digital microscope camera and the images

106 were analyzed through an image processing software (DeltaPix InSight, DeltaPix, Smorum,

107 Denmark). We used a standard counting area replicated three times on each leaf to determine the

108 stomatal density as number of stomata (STNR) for each standard counting area. On each counting

109 area, the length of the guard cells of stomata (SL) was measured for 15 randomly selected stomata

110 per counting area. Additionally, the percentage of consumed leaf area was estimated to describe

111 leaf-level herbivory. Mean values were calculated per branch, which were used in the statistical

112 analyses.

113

DNA extraction and ARISA

114

115   Leaves used for microbiological analysis were processed within 4 hours as follows: Five
116 leaves for each replicate were disinfected with 70% ethanol twice each for 3 min. Leaves were
117 then washed with sodium hypochlorite (1.5%) and Tween 20 for ten minutes, three rinses in sterile,
118 distilled shaking water. Disinfected leaves were grinded to a fine powder under liquid nitrogen
119 using a sterile mortar and pestle. The disinfected samples were stored at -20°C. Triplicates of the
120 water used in the last rise were used as negative for PCR amplification and plated on a LB and
121 TSA medium to verify the disinfection protocol. Absence of PCR amplification products was
122 observed. Furthermore, absence of bacterial colonies was observed in all the plates after 10 days
123 of incubation at 30°C.

124   DNA was extracted using the Qiagen DNeasy PowerPlant Pro Kit (Qiagen, Milan, Italy)
125 accordingly to the user's manual. Extracted DNA was stored at -80°C. The quality and the size of
126 the soil DNA were checked by electrophoresis on 1.2% agarose gel with a marker (Eurogentec
127 Smart Ladder, Belgio). The absorbance (260 nm) of 2 µl of DNA was used to evaluate the
128 concentration of DNA by NanoVue Spectrophotometer (GE Healthcare, Little Chalfont, UK).

129   PCR was performed using the primers ITSF and ITSReub labeled with 6-FAM according
130 to the chemical and thermal amplification protocol of Cardinale et al. (*2004*). Capillary
131 electrophoresis was done by STAB Vida Lda. (Caparica, Portugal). Data were investigated via
132 Peak Scanner Software 1.0 (Applied Biosystems, Monza, Italy) and the downstream matrix was
133 normalized and analyzed according to Borruso et al. (*2015*).

134

Data analysis

135

136   PAST software (*Hammer, Harper & Ryan, 2001*) was used for the statistical analysis.
137 ANOVA was used to test for differences in the functional leaf traits and the endophytic bacterial
138 richness between seasons. Canonical correspondence analysis (CCA) of the endophytic microbial
139 community structure in dependence of functional leaf traits and season was performed. Non-
140 Parametric Multivariate Analysis of Variance (NPMANOVA) with Bonferroni corrected *p*-value
141 was applied to investigate differences among the endophytic bacterial communities across the
142 three seasons using Bray-Curtis dissimilarity distance.

143

RESULTS

144

Functional leaf traits

Figure 1 shows the functional leaf traits investigated in this study across the three sampling seasons. Leaf nitrogen and carbon concentration was significantly lower in autumn than in spring and summer ($p < 0.05$). The C:N ratio and the level of herbivory were significantly higher in autumn than in spring and summer ($p < 0.05$). Stomatal length, stomatal number and specific leaf area did not show significant differences across the seasons ($p > 0.05$).

Bacterial community structure

An average of 82 ± 15 peaks per sample representing bacterial richness, ranging from 200 bp to 1200 bp, were found. No significant results in terms of number of peaks across the three seasons were found (spring 82 ± 9; summer 86 ± 19 and autumn 79 ± 14; ANOVA p-value: n.s.). NPANOVA showed significant differences between the bacterial community structure of spring and of autumn ($p < 0.001$), while the bacterial community structures of summer did not cluster apart (Table 1).

Canonical correspondence analysis (CCA)

Canonical correspondence analysis (CCA) was used to investigate the effects of functional leaf traits and the level of herbivory on endophytic bacterial communities across the seasons. Differentiation is illustrated by the first and second axes in the CCA (29.7% and 25.3%, respectively) and the leaf features fitted into the CCA. The CCA ordination diagram (Figure 2) revealed first, that community structure variation appeared along season (temporal sequence) and, second, the existence of relationships between plant foliar traits and endofoliar microbiota across the temporal sequence. In detail, the community variation is related mainly to the level of herbivory, leaf nitrogen content and stomatal length, and less to stomatal number, leaf carbon content and specific leaf area (length of vectors in the ordination diagram; Figure 2).

DISCUSSION

We explored the seasonal diversity behavior via fingerprinting ARISA of the of leaf endophytic bacterial communities. ARISA is a corroborate technique used to investigate bacterial structure variations and the correlations with environmental parameters (*Esposito et al. 2013; Borruso, Zerbe & Brusetti et al. 2015; Pioli et al. 2018*) with a comparable robustness as Next Generation

176 Sequencing (*van Dorst et al. 2014*). Although the bacterial community did not show significant

177 differences in alpha diversity across the three seasons, a general trend of the bacterial community

178 structure differentiation mainly between spring and autumn was found (Table 1 and Figure 2).

179 These results support the idea of an intimate association between endophytes and the leaf, seen as

180 a dynamic micro-ecosystem that selects for different specific microbial communities along time.

181 Leaves have been traditionally considered as "short-lived environment", where specialized

182 bacteria can dynamically colonize new niches and leave others according to the leaf continuous

183 modifications over seasons (*Vorholt 2012*). Bulgari et al. (*2014*) hypothesized that the endophytic

184 communities in *Vitis vinifera* should remain stable across the seasons in absence of bacterial plant

185 pathogens such as phytoplasma. However, this is in contrast with our findings and those of other

186 researchers. Influence of the seasonality on endophytic microbial community composition

187 associated with different tree species (i.e. *Acer negundo*, *Ulmus pumila*, and *U. parvifolia*) were

188 also found by Shen & Fulthorpe (*2015*). Moreover, others observed that the bacterial community

189 composition in the phyllosphere was primarily driven by temporal changes and community

190 succession (*Copeland et al. 2015*). In order to shed light on the processes behind community

191 changes, we explored the role of leaf functional traits in bacterial structure dynamics. In fact,

192 previous studies investigated the effect of migration and community succession in the phyllosphere

193 microbiome and suggested that colonization, persistence, and succession of the community may

194 be key-factors driving the phyllosphere microbiome (*Redford & Fierer 2009; Shade, McManus &*

195 *Handelsman, 2013; Maignien et al. 2014; Copeland et al. 2015*). The relevance of environmental

196 conditions such as temperature optima of the bacteria or the changing physiology of tree host

197 species have been discussed for their possible effects (*Jansson & Douglas 2007*). However,

198 previous studies did not look deeper into possible drivers in terms of plant functional

199 characteristics behind the observed dynamical processes of the microbiome.

200   Given that we found a clear endophytic seasonal variation, it is highly interesting to

201 understand better the potential leaf-level drivers behind the compositional variation. In fact, based

202 on our results, we suggest that foliar characteristics related to leaf-level herbivory, nutrient

203 contents and stomatal aperture affect directly the bacterial community composition over time.

204 Indeed, there is evidence that plants regulate to some extent the access of bacteria through stomata

205 (*Gimenez-Ibanez et al. 2017*). In addition, leaf nutrient contents and their changes can influence

206 bacteria. It has been shown that endophytic bacterial taxa able to fix nitrogen occur in oak species

207 (*Tashi-Oshnoei, Harighi & Abdollahzadeh, 2017*) and in *Pinus flexilis* needles (*Moyes et al. 2016*)

208 and, eventually, a drop in leaf nitrogen content could challenge bacterial shifts towards nitrogen-

209 fixing taxa and in contrast to denitrifying bacteria.

210   Our results of diminishing nutrients such as C and N with leaf aging is in line with other

211 studies (*Li et al. 2017*), while other functional traits related to the stomata, such as stomatal length

212 and stomatal density and specific leaf area did not vary significantly along season. Another study

213 on leaf traits showed that even such traits could show seasonal variation (*Römermann et al. 2016*).

214 However, these results are based on different tree species growing in different environments. In

215 comparison, our species *Q. petraea* might have relatively stable stomatal and specific leaf area

216 trait levels based on the relatively rigid leaf. Moreover, herbivory that increases along season due

217 to elongated exposure time, can have direct impact on endophytic bacteria as well as indirect

218 impact influencing other leaf characteristics. For example, the open structures of the leaf, limited

219 to the size and number of stomata in intact leaves, are largely modified by herbivory that exposes

220 further leaf tissue. Due to the increasing rate of changes that can be assumed with aging, we expect

221 that leaf characteristics exert a differential impact during aging on the bacterial community.

222

223 **Conclusions**

224   Based on our findings, we suggest that leaf-level of herbivory, nitrogen content, and size of

225 stomatal aperture are main drivers affecting the endophytic bacterial community composition in

226 oaks growth in alpine forest environments.

227

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231

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366

367 **Figure and Tables captions**

368

369 **Figure 1** Differences of foliar traits (N = nitrogen, C = carbon, C:N = carbon/nitrogen ratio, SLA=
370 specific leaf area, STL = stomatal length, STNR = stomatal number per reference area, HERB =
371 level of herbivory) among three seasons (spring, summer, autumn). Significant differences
372 according to ANOVA followed by post-hoc test are indicated by different lower case letters.
373 Graphics without letters were not significant.

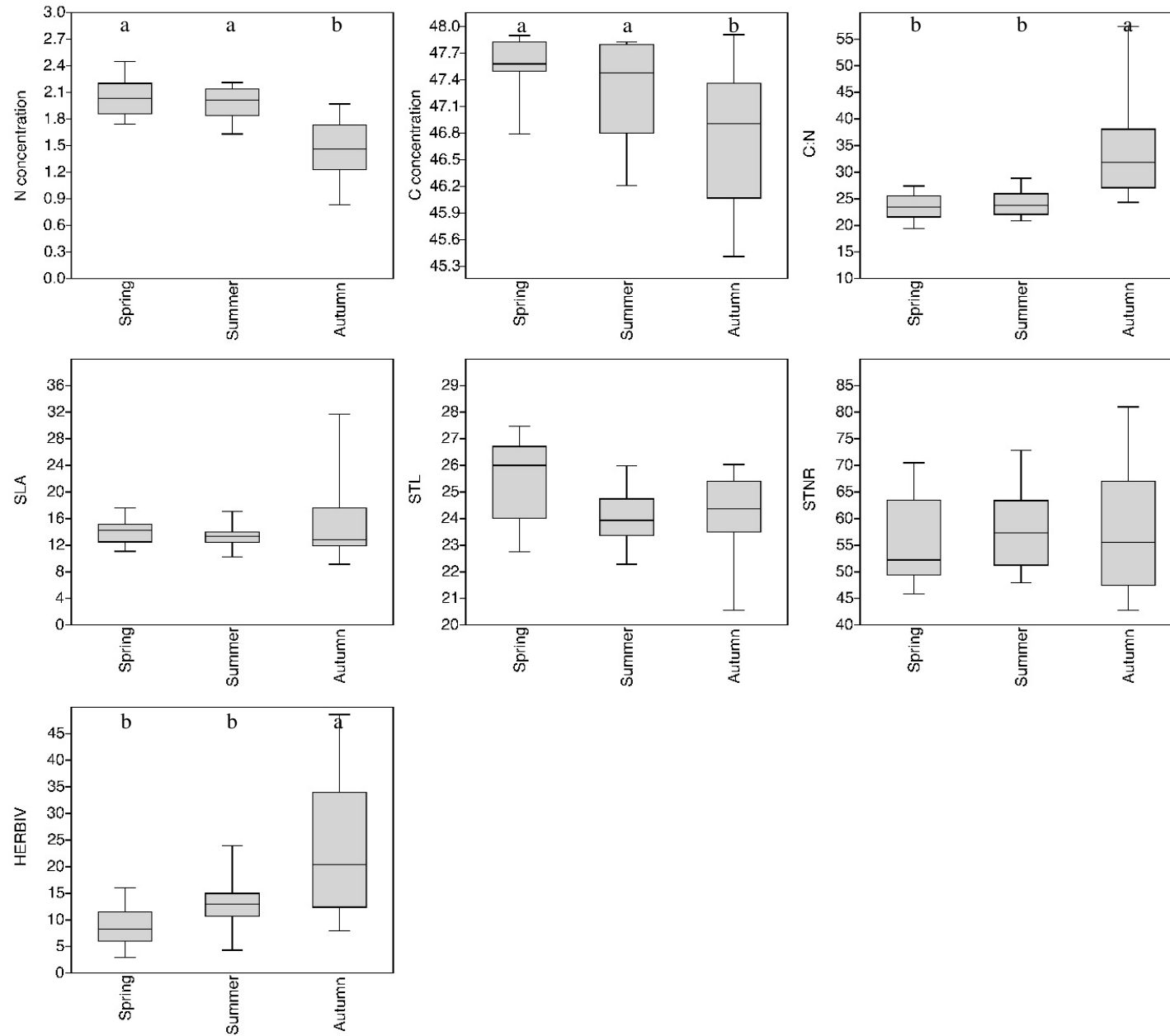
374

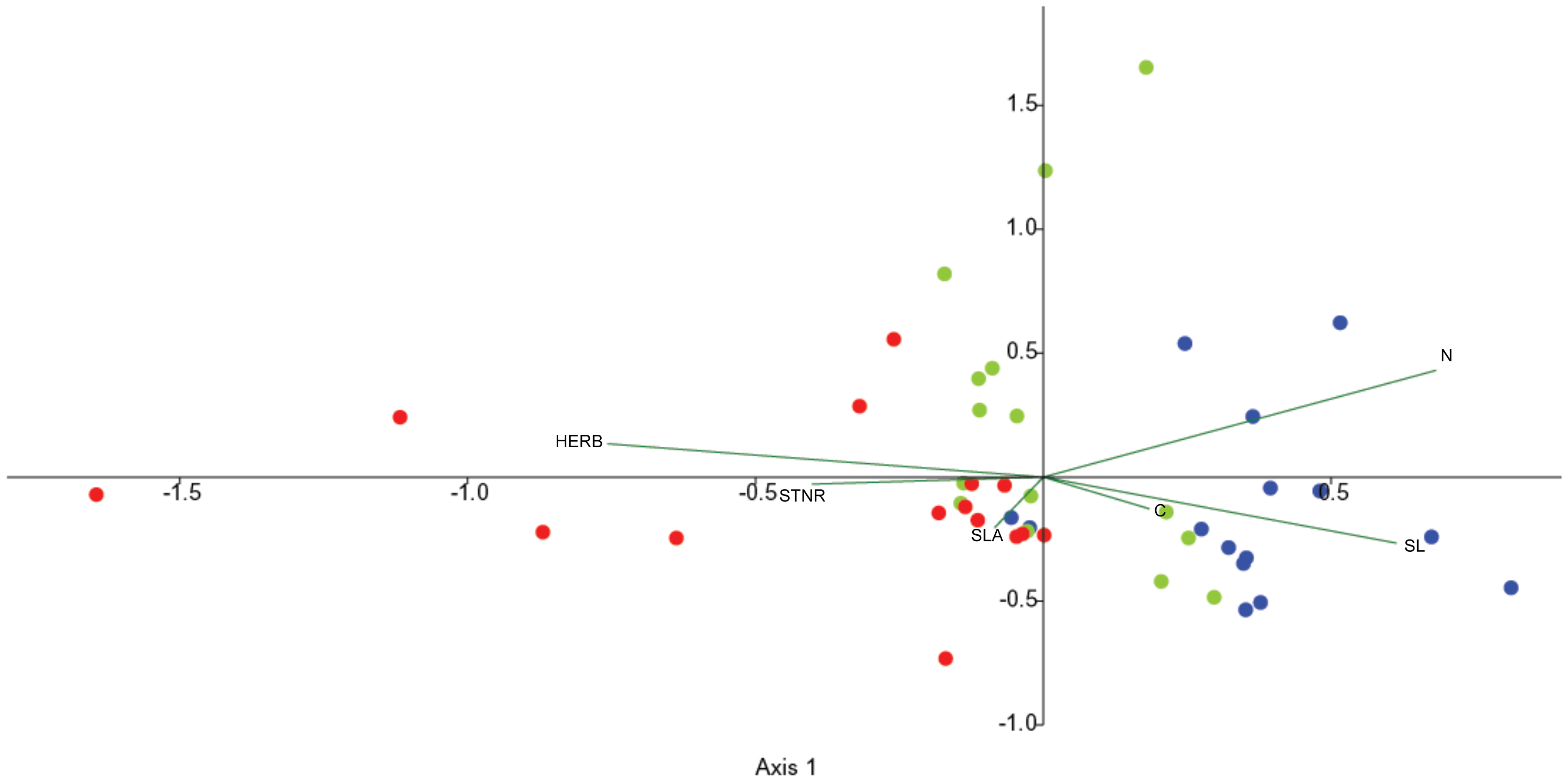
375 **Figure 2** CCA analysis of endophytic communities across a temporal sequence (spring: blue dots;
376 summer: green dots; autumn: red dots) and plant foliar traits. CCA was calculated with the
377 following plant foliar traits: HERB = level of herbivory; STNR = number of stomata; SLA =
378 specific leaf area; SL = length of stomata; N = leaf nitrogen content; C = leaf carbon content.

379

380 **Table 1** *P* value results from Non-Parametric MANOVA (NPMANOVA) with Bonferroni
381 corrected *p* value among endophytic bacterial communities across the three seasons (Bray-Curtis
382 dissimilarity).

Differences of foliar traits among three seasons.

Differences of foliar traits (N = nitrogen, C = carbon, C:N = carbon/nitrogen ratio, SLA= specific leaf area, STL = stomatal length, STNR = stomatal number per reference area, HERB = level of herbivory) among three seasons (spring, summer, autumn). Significant differences according to ANOVA followed by post-hoc test are indicated by different lower case letters. Graphics without letters were not significant.

CCA analysis of endophytic communities across a temporal sequence and plant foliar traits.

CCA analysis of endophytic communities across a temporal sequence (spring: blue dots; summer: green dots; autumn: red dots) and plant foliar traits. CCA was calculated with the following plant foliar traits: HERB = level of herbivory; STNR = number of stomata; SLA = specific leaf area; SL = length of stomata; N = leaf nitrogen content; C = leaf carbon content.

P value results from NPMANOVA

P value results from Non-Parametric MANOVA (NPMANOVA) with Bonferroni corrected *p* value among endophytic bacterial communities across the three seasons (Bray-Curtis dissimilarity).

1   **Table 1** *P* value results from Non-Parametric MANOVA (NPMANOVA) with Bonferroni

2   corrected *p* value among endophytic bacterial communities across the three seasons (Bray-Curtis

3   dissimilarity).

Seasons	Summer	Autumn
Spring	0.0804	0.0009
Summer	/	0.2484