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RESEARCH ARTICLE

Bacterial populations on the phyllosphere of Mediterranean plants: influence of leaf age and leaf surface

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Abstract **In the present study, we estimated the size of phyllosphere bacterial populations in young and mature leaves from the same plants and also assessed the population abundance on adaxial and abaxial leaf surfaces. We examined eight perennial species naturally occurring in the same area, in Halkidiki (northern Greece). They are *Arbutus unedo, Quercus coccifera, Pistacia lentiscus,* and *Myrtus communis* (evergreen sclerophyllous species), *Lavandula stoechas* and *Cistus incanus* (drought semideciduous species), and *Calamintha nepeta* and *Melissa officinalis* (nonwoody perennial species). Young and mature leaves were examined from the four sclerophyllous evergreen species for their epiphytic bacterial colonization, and it was found that mature leaves were highly populated compared to the younger ones except in *M. communis*. As regards the bacterial colonization of the two leaf surfaces, no differences were found in most species except for the drought semideciduous type where the two leaf surfaces behaved differently.**

Keywords bacterial population, phyllosphere, leaf surface, leaf age, leaf imprints, Mediterranean plants

Introduction

The phyllosphere represents a great habitat for microorganisms in general, and for bacteria in particular. According to an estimate, a total of over 4×10^8 km^2 phyllosphere surface area has supported bacterial population in the region of 1×10^{26} cells (Morris and Kinkel, 2002). In terms of diversity, a study by Lambais et al. (2006) suggested the possible occurrence of 2–13 million phyllosphere bacterial species in 20000 vascular plants in the Brazillian Atlantic forest. Bacterial populations on the phyllosphere vary in size both among and within species, and also over short time scales (Kinkel et al., 1995; Yadav et al., 2004). Furthermore, bacterial populations in the phyllosphere have agricultural and environmental significance as their interactions determine the extent to which human pathogens are able to colonize and survive on plant tissues such as fresh salad, fruit, and vegetable produce (Whipps et al., 2008; Berger et al., 2010).

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Leaf age and position on the plant have been shown to influence the size of the epiphytic microbial population. Inner/younger leaves of lettuce (*Lactuca sativa*), cabbage (*Brassica oleracea* var. *capitata*), whitloof chicory (*Cichorium intybus* var. *foliosum*), broad-leaved endive (*Cichorium endivia* var. *latifolia*) and sugar beet (*Beta vulgaris*) have been reported to carry smaller bacterial population densities than outer/older leaves at a given sampling time during field cultivation (Ercolani, 1976; Geeson, 1979; Van Outryve et al., 1989; Morris and Lucotte, 1993; Thompson et al., 1993; Jacques et al., 1995). Variation of epiphytic bacterial population, as influenced by leaf age and position, were also reported in evergreen trees (Ercolani, 1991; Périssol et al., 1993; de Jager et al., 2001), deciduous trees (Andrews and Kenerley, 1980), and perennial and annual herbaceous plants (Dickinson et al., 1975; Weller and Saettler, 1980; Jones et al., 1985; Thompson et al., 1993). Contrary to the factors "age and position," it appears that there does not exist a pattern as regards the bacterial colonization on the two leaf surfaces. In some instances, there is no difference, while in others it is bigger on the adaxial side, and in others it is bigger on the abaxial side (de Jager et al., 2001; Gau et al., 2002).

This study was designed to investigate how many of the

plant species that we examine follow the general pattern of variability of bacterial colonization of leaves according to their age, and to investigate if there exists any difference in the colonization of the two leaf surfaces.

Materials and methods

Leaf sampling

The study was carried out in Sithonia peninsula (Halkidiki), northern Greece. The climate of the area is Mediterranean, with rather mild and wet winters and hot, dry summers (Yadav et al., 2004a). Eight native and coexisting perennial species, woody and non-woody, were the model plant species. The woody species are the evergreen-sclerophyllous shrubs, *Arbutus unedo* L., *Quercus coccifera* L., *Pistacia lentiscus* L. and *Myrtus communis* L., and the low, drought semi-deciduous shrubs, *Lavandula stoechas* L. and *Cistus incanus* L. They are all common components of the Mediterranean-type ecosystems of the country. The non-woody perennials are *Calamintha nepeta* (L.) Savi and *Melissa officinalis* L.; they occur in less arid microsites of the study site. Sampling of young leaves and, at the same time, of mature leaves for the estimation of bacterial population, was done in April when the four woody species bore young and mature leaves. In other species, sampling of young and mature leaves was practically not feasible. However, to assess bacterial abundance on the adaxial and abaxial leaf surfaces, leaf samples from all eight species were collected. Only healthy leaves were sampled for all purposes.

Estimation of bacterial population

Sampling was done in the morning. After sampling, leaves were placed in sterile plastic bags, transported to the laboratory in an icebox, and analyzed within 24 h. The serial dilution plating method (Lindow et al., 1978) was used to estimate total bacterial population of the phyllosphere. Each sample was weighed and immersed in a 100 mL Erlenmeyer flask with 25 mL sterile phosphate buffer (0.01 M, pH 7.3) supplemented with 0.1% bactopeptone. Flasks were sonicated in an ultrasonic cleaner for 10 min. Portions (100 μL) from the original wash and appropriate dilutions thereof prepared in 0.01 M phosphate buffer (pH 7.3) were plated onto nutrient agar (NAG) medium, supplemented with 2.5% (v/v) glycerol, and amended with 30 $\mu g \cdot mL^{-1}$ natamycin to prevent fungal contamination. Bacterial populations were estimated after incubation at 24°C for 2–5 d. Results are expressed as log (CFU + 1) per gram fresh weight, where CFU corresponds to colony forming units. The reasons for the log (CFU + 1) instead of the common log (CFU) transformation of data were described in detail by Yadav et al. (2004).

To estimate the bacterial population on the adaxial and abaxial leaf surfaces, the leaf-imprint method was followed and imprints were made on NAG media as prepared through the serial dilution plating method. An intact individual leaf was placed onto an agar plate and pressed with the smooth end of a sterile glass rod until a clear imprint of the entire leaf was obtained on the agar surface (Aneja, 2003). Different leaves were used for imprinting the adaxial and abaxial surfaces to avoid disturbances in surface community distribution. After bacterial extraction, each leaf was used to measure its area with a leaf area meter (Ejkelkamp, Agrisearch Equipment, the Netherlands). Plates with leaf imprints were incubated at 24°C for 2–5 d. Bacterial populations were enumerated from the plates and expressed as log (CFU + 1) per square cm.

Statistical analysis

Differences in bacterial population between two leaf ages and two surfaces were determined by Student's *t*-test. The effects of leaf age, leaf surfaces, and their interaction on population abundance were assessed with univariate analysis of variance (two-way ANOVA). All analyses were performed by using SPSS for Windows (11.0.1, SPSS Inc., USA).

Results

Bacterial populations on the phyllosphere of young and mature leaves are presented in Fig. 1. There exist significant differences in bacterial populations between the leaves of two ages. The young leaves support lower size bacterial populations, with the exception of the *M. communis,* where no significant difference is observed.

The results of the 2-way ANOVA showed that the species and leaf age factors had significant effects on the phyllosphere bacterial population of the species studied (Table 1).

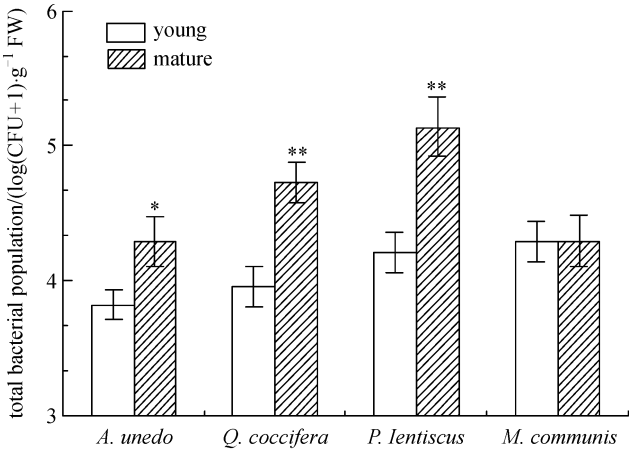

Figure 1 Total bacterial population of young and mature leaves
Note: Columns represent mean bacterial population (n = 16) and error bars represent SE of the means. Significant differences between young and mature leaf populations are indicated by * ($P < 0.05$) or ** ($P < 0.01$).

Moreover, the interactive effect of plant species and leaf age was significant ($P < 0.05$), indicating that the effect of the leaf age on the phyllosphere bacterial population abundance is associated with specific plant species.

Table 1 Results of two-way ANOVA of the total bacterial population of young and mature leaves of four evergreen sclerophyllous species significant *P*-values are indicated in *P* column

source of variation	*SS*	*df*	*F*	*P*
species	6.30	3	4.76	0.004
age	9.39	1	21.26	< 0.001
species × age	3.99	3	3.01	0.033
error	52.99	120	—	—

Imprinting of leaves mostly resulted in non-significant differences in the bacterial population between adaxial and abaxial leaf surfaces (Fig. 2). However, bacterial populations on the two leaf surfaces were significantly different ($P < 0.05$) in the case of the two seasonal dimorphic species. A higher population was found on the adaxial leaf surface of *L. stoechas*, whereas *C. incanus* had the higher population on the abaxial leaf surface.

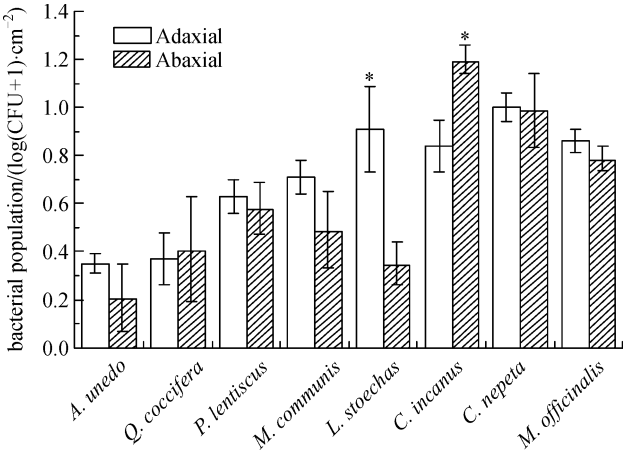

Figure 2 Bacterial population on the adaxial and abaxial leaf surfaces

Note: Vertical bars represent means and the error bars represent SE of the means ($n = 6$). Asterisks indicate significant differences at $P < 0.05$.

Concomitant with this, the two-way ANOVA revealed that leaf surface (adaxial or abaxial) was not a significant factor in bacterial colonization of the phyllosphere (Table 2). Rather, the interaction between plant species and leaf surfaces ($P < 0.05$) indicated that the effect of leaf surface on bacterial colonization was plant species specific.

Discussion

The young leaves of the evergreen-sclerophyllous species maintain smaller sizes of bacterial populations than the mature ones, which is in agreement with earlier reports (Ercolani, 1991; Périssol et al., 1993; Jacques et al., 1995; de Jager et al., 2001). Also, while the species differ with regards to the size of epiphytic bacterial population of their mature leaves, they do not differ for their young leaves. There exist different interpretations for the reasons of this behavior of bacterial abundance. Some attribute this to the time restrictions, since the leaf surface of younger leaves are exposed to colonization only for smaller time periods (Andrews and Kenerley, 1980; Jacques et al., 1995). We also consider this opinion prevailing in the present work in order to interpret our results. Furthermore, despite the differences of species in chemical characteristics of their immature leaves they do not differ in abundance of epiphytic bacteria. However, they differ in bacterial abundance in their mature leaves, indicating corresponding differences in their chemical characteristics.

Table 2 Results of two-way ANOVA of bacterial populations on the adaxial and abaxial leaf surfaces of eight Mediterranean species

source of variation	*SS*	*df*	*F*	*P*
species	5.88	7	10.46	< 0.001
surface	0.15	1	1.88	0.174
species × surface	1.40	7	2.48	0.023
error	6.43	80	—	—

Note: Significant *P*-values are indicated in *P* column.

Other interpretations are related to the low availability of nutrients on the surface since the young leaves are less prone to leaching (Tukey, 1970). Leaf structural features and their content of secondary metabolites are other important attributes affecting the abundance of epiphytic bacterial populations. The population density of epiphytic bacteria is associated with the leaf surface topographic characteristics that vary enormously and evolve with the leaf age (Juniper, 1991). For example, the production and regeneration of wax is very rapid in the young leaves but slows down considerably once a leaf matures completely (Hallam and Juniper, 1971). The wax projections increase the contact angle between the surface and a water drop, leading to reduced wettability, thus rendering the young leaves as a less suitable habitat for epiphytic bacteria. Moreover, the young leaves are not mechanically tough since they are not yet sclerified; they appear to have well equipped defensive system against herbivorous animals (Herms and Mattson, 1992) and microbes, if we consider their high content of phenolic compounds.

In most species, as shown by their imprints, the two leaf surfaces do not differ in their bacterial colonization. Different abundance of bacteria in the two surfaces of leaves is presented by the seasonal dimorphic species without, however, being greater in either of the two leaf surfaces. Similar results have also been reported by other researchers. Mansvelt and Hattingh (1987) and Surico (1993) found

higher numbers of bacteria on the abaxial surface. However, de Jager et al. (2001) did not find significant differences in the colonization of two surfaces of mango leaves, while Gau et al. (2002) observed higher population of *Pseudomonas fluorescens* on the adaxial surface of leaves of *Malus domestica* after inoculation.

In conclusion, plants exhibit higher abundance of bacterial population on the surface of mature leaves as compared to that on the younger leaves, at least in case of woody evergreen sclerophyllous species in this study. However, no difference was found regarding the microbial colonization of the adaxial and abaxial leaf surfaces, except for the two drought semi-deciduous species (*L. stoechas* and *C. incanus*), however showing no pattern.

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