Acta Societatis Botanicorum Poloniae

DOI: 10.5586/asbp.3626

Publication history
Received: 2018-12-06
Accepted: 2019-05-23
Published: 2019-06-25

Handling editor
Beata Zagórska-Marek, Faculty of Biological Sciences, University of Wrocław, Poland

Authors' contributions
JR, ŁD, and MZ conceived the study; JR collected field samples; JR and PD performed lab analyses and analyzed the data; JR and MZ wrote the first draft of the paper; all authors contributed to the final version

Funding
The metagenomic analysis was financially supported by DSM grant No. 0115000-41 to JR, and sample collection was financed by the National Science Center, Poland, grant No. 2014/15/B/NZ8/00249 to MZ.

Competing interests
No competing interests have been declared.

Citation
Ryniewicz J, Decewicz P, Dziewit L, Zych M. Is bacterial microbiome from the *Polemonium caeruleum* L. (Polemoniaceae) nectar geographically variable? Acta Soc Bot Pol. 2019;88(2):3626. https://doi.org/10.5586/asbp.3626

SHORT COMMUNICATION

Is bacterial microbiome from the *Polemonium caeruleum* L. (Polemoniaceae) nectar geographically variable?

Justyna Ryniewicz[1]*, Przemyslaw Decewicz[2], Lukasz Dziewit[2], Marcin Zych[1]

[1] Botanic Garden, Faculty of Biology, University of Warsaw, Aleje Ujazdowskie 4, 00-478 Warsaw, Poland
[2] Department of Bacterial Genetics, Institute of Microbiology, Faculty of Biology, University of Warsaw, I. Miecznikowa 1, 02-096 Warsaw, Poland

* Corresponding author. Email: j.ryniewicz@biol.uw.edu.pl

Abstract
Floral nectar is one of the key rewards in the mutualistic interactions between plants and pollinators. However, there is a growing amount of evidence that shows that another group of organisms may be involved in the pollination process, namely the microorganisms, which often inhabit floral nectar. However, little is known about the function and taxonomic diversity of microorganisms inhabiting the nectar of plants. Bacterial communities inhabiting nectar of a rare plant species, *Polemonium caeruleum*, in one artificial and two natural populations in Poland were analyzed using a metagenomic approach. *Polemonium caeruleum* is a boreal plant species, and requires appropriate pollinator services for seed production. The reproductive system of this plant may vary between individuals and populations (mixed-mating), as well as with insect visitor assemblages. We considered that nectar-dwelling bacteria of *P. caeruleum* might affect the insects visiting the plant's flowers, which in turn can result in changes in the plant's phenotype and its reproductive system. Bacterial diversity in nectar samples was surveyed using culture-independent 16S rRNA gene amplicon sequencing (MiSeq, Illumina). We found that bacterial communities inhabiting the nectar of *P. caeruleum* differed between populations, although those differences were mostly quantitative. Many of the identified bacterial genera have been found previously in nectar of other plant species, or in the guts of insect visitors, and are described as tolerant of high sugar concentrations and catalase positive (which allows bacteria to survive in the presence of hydrogen peroxide).

Keywords
16S rRNA; flower; operational taxonomy units (OTUs); mutualism; plant–bacteria interactions; plant reproduction; pollination

Introduction

Pollination by animals is one of the fundamental ecological processes that determine the stability of many terrestrial ecosystems. For many years, plant–pollinator interactions were regarded as binary. However, recently, studies have also focused on other groups of organisms that are often involved in pollination and can significantly modify this process, namely bacteria and fungi [1–3].

Angiosperms attract pollinators in various ways to transfer pollen between plants and to achieve reproductive success. One of the most common rewards offered by flowers to floral visitors is nectar, the production of which is costly in terms of energy expenditure. Nectar is essentially a mixture of water and sugars, but it may also contain amino acids, vitamins, secondary metabolites, lipids, and proteins [4]. Many

nectar characteristics, for instance, a high sugar concentration that may generate high osmotic pressure, reactive oxygen molecules, and secondary metabolites, may inhibit the growth of microorganisms. For these reasons, one may expect that only properly adapted microbiota groups are able to colonize floral nectar [4].

Studies of culturable bacterial taxa confirm that the presence of bacterial communities in floral nectar is common but species richness is rather low [3,5]. On the other hand, surveys of unculturable bacterial strains report higher bacterial diversity [6–8]. Both approaches confirm, however, that phylogenetic diversity of nectar-dwelling bacteria is rather restricted [3,5,6]. If bacteria consume nectar, this can influence its chemical profile, and, consequently, plant–pollinator interactions [4], which may result in changes in insect visitation rates [9], and plant reproductive success [2,10].

Currently, little is known about the provenance, taxonomical diversity, and ecological role of microbial communities in floral nectar. A few publications confirm that microorganisms may be transported to nectar by flower visitors [1,7], including herbivores [11] that move between plants and populations. Precipitation and air can be also considered as factors responsible for inoculation of nectar with microorganisms [3].

Bacterial communities inhabiting nectar seem to be species-specific [9,12]; however, little is known about differences between populations of the same species. As nectar composition may vary geographically (e.g., [13]), the composition of nectar-dwelling microbial communities may also change, and even small distances between plant populations can result in significant differences in bacterial microbiomes in nectar [8]. Our understanding of this correlation is, however, quite limited.

For this reason, we chose *Polemonium caeruleum* (also known as Jacob's ladder), as a model plant to study the geographic diversity of nectar bacterial microbiomes. The reproductive biology of this plant is relatively well known [14,15]. It is a boreal, red-listed species, reaching its southern limit range in Poland. Flowers are dichogamous, and produce nectar and pollen, which attract many groups of potential pollinators (mainly Hymenoptera and Diptera), which indicates a generalist pollination system. Plant reproduces only by seeds, and proper pollinator services are necessary for population survival. Nectar is secreted by the rim-like nectary that encircles the base of the ovary, and accumulates in a chamber, where it is protected by a ring of hairs [16]. The nectar is sucrose dominant and proline rich, and nectar production is female-biased [14]. The composition of pollinator assemblages may vary between *P. caeruleum* populations, which probably affects its mating system (Ryniewicz, unpublished data). Therefore, we hypothesized that geographically distant populations would be characterized by different nectar bacterial microbiomes.

Material and methods

We chose three populations of *P. caeruleum* in Poland (Fig. 1A, Tab. 1), separated by the largest possible distance from each other on the N–S line. We isolated inflorescences overnight at the peak of anthesis to prevent insect access. Nectar was collected in sterile plastic sample tubes using sterile tips from flowers in the female stage exposed to insect visitors during the male phase (longevity of a single flower is 7.2 ±1.3 days [14]). We collected nectar from 30–40 randomly selected flowers in each population. Samples were stored in a portable cooler until further lab analysis (which took place at most 12 hours after collection).

Total bacterial DNA was extracted using Mo Bio PowerSoil DNA Isolation Kits, according to the manufacturer's instructions, and stored at −20°C. Using isolated DNA as a matrix, PCR reactions were performed in triplicate (to reduce PCR bias) using primers 16S_V3-F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and 16S_V4-R: 5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3′, to amplify the variable V3 and V4 regions of the 16S rRNA gene.

The electrophoretic analysis was performed for each of the three PCR replicates for the qualitative and quantitative evaluation of the PCR products. Electrophoresis results for one of the populations (KLE) indicated insufficient amount of bacterial DNA in the sample. As such, this sample was replaced with previously collected material

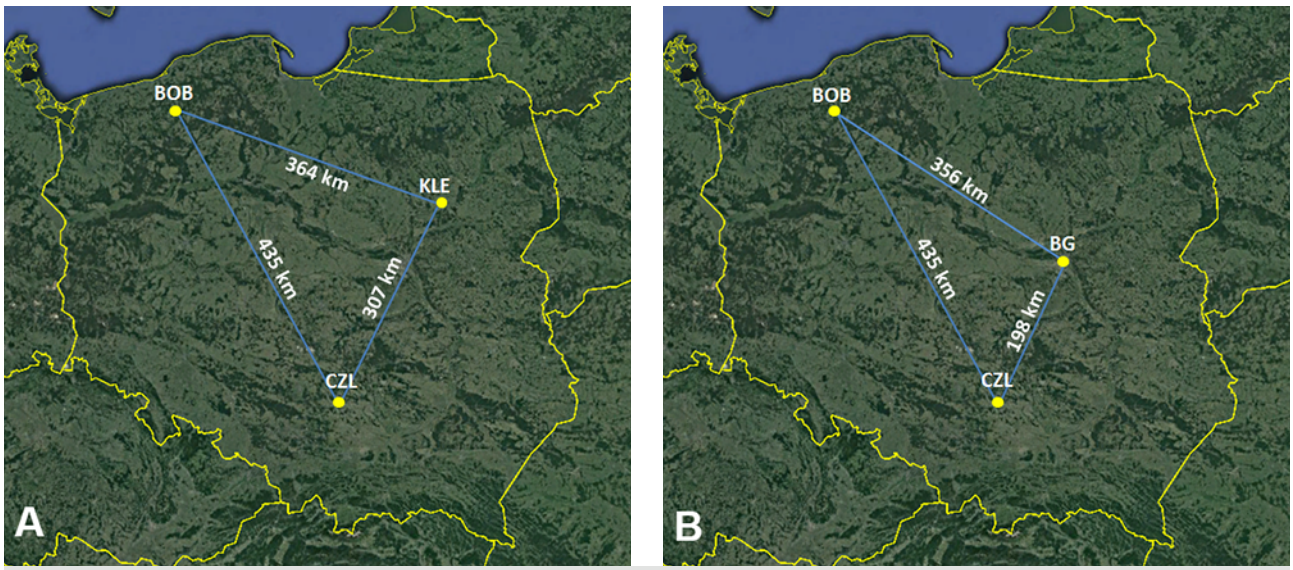

Fig. 1 Geographical distribution of *P. caeruleum* populations originally selected for the studies (**A**) and after replacing one natural population with the planted population in the University of Warsaw Botanical Garden (**B**).

Tab. 1 The features of investigated *P. caeruleum* populations.

	General features			α-Diversity indicators		
Population	No. flowering shoots	Geographical coordinates	Region (voivodeship)	Chao1	Shannon	No. of OTU
BOB	50	53°57′46″ N 16°34′24″ E	West Pomeranian	674	2.69	629
BG	10	52°13′3″ N 21°1′41″ E	Mazovian	755	3.90	684
CZL	500	50°35′45″ N 19°51′46″ E	Świętokrzyskie	528	2.87	385

obtained from plants collected from the University of Warsaw Botanical Garden (site BG; Fig. 1B, Tab. 1).

In the next step, three independent amplicons from each population were combined to one sample used for the DNA sequencing, which was performed using an Illumina MiSeq instrument in paired-end mode using a v3 chemistry kit. The obtained sequence reads were filtered with CUTADAPT ver. 1.9.1 [17] to remove low-quality bases (<Q20) and adapters, and assembled with SEQPREP ver. 1.1 (https://github.com/jstjohn/SeqPrep) with a minimum of 15 bases overlap and a minimum 90% sequence identity in the overlapping region. The resulting assembled reads were subsequently checked for the presence of chimeras with VSEARCH ver. 2.7.1 in both reference- and de novo-based modes [18]. The resulting sequences were then clustered into 97% operational taxonomy units (OTUs) using USEARCH ver. 6.1 [19] in an open-reference scheme with QIIME ver. 1.9.1 scripts. The taxonomy was assigned using RDP classifier ver. 2.2 [20] based on SILVA QIIME release 128 nr 97 reference database [21,22]. After chloroplast and mitochondria sequences were removed from the dataset, on average, 40,000 sequences per sample were included in the biodiversity analysis. Additionally, the values of α-diversity indicators were calculated using Chao1 and Shannon indices.

Results and discussion

The number of OTUs ranged from 385 for the southernmost population (CZL) to 684 for the planted population in the botanical garden (BG). Similar results were observed

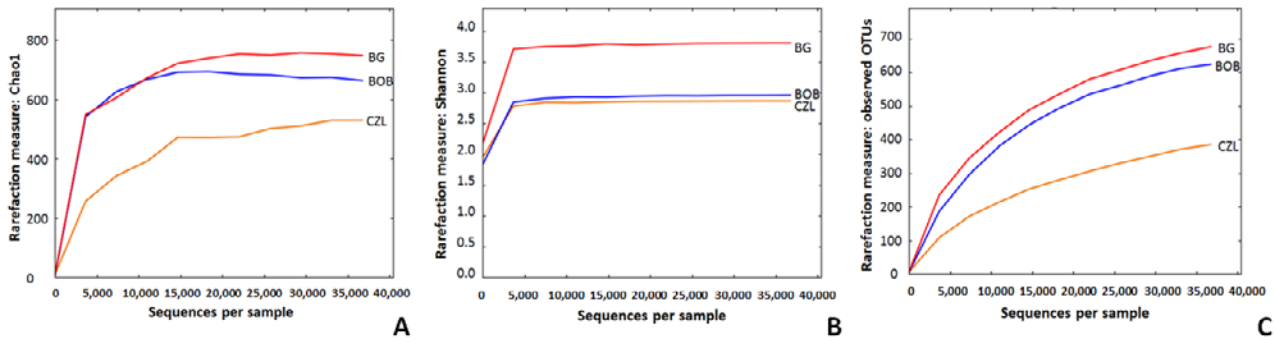

Fig. 2 Rarefaction curves presenting taxonomic richness – Chao1 (**A**) and diversity – Shannon *H′* index (**B**) reaching a plateau above 25,000 and 5,000 sequences, indicating that sequencing was deep enough for establishing those values. Rarefaction curves presenting observed OTUs in nectar samples are not completely saturated, suggesting the sequence identities were not fully surveyed (**C**). Rarefaction-based analyses were applied to assess the relationship between the increase in bacterial α-diversity and the number of randomly sampled sequences. Such analyses generate the expected number of OTUs, Chao1, and Shannon indices in a small collection of *n* samples drawn at random from a large pool of *N* samples.

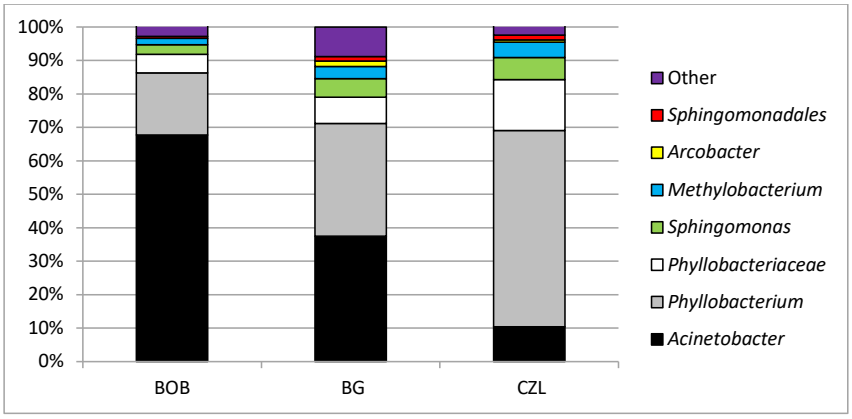

Fig. 3 Percentage of bacterial taxa inhabiting floral nectar of *Polemonium caeruleum* in each of three analyzed populations (see Fig. 1 for locations of populations).

for other indicators of α-diversity (Tab. 1). Contrary to our expectations, there was a negative trend between the number of flowering shoots in the population and the number of observed OTUs, although this may be due to the fact that we sampled a small number of populations, as well as the fact that we replaced one of the natural populations with the population sampled from the botanical garden. The highest diversity in the latter may be a consequence of the highest diversity of coflowering plants, especially generalists, visited by numerous insects [3]. However, the rarefaction curves of OTUs were not completely saturated, suggesting we did not fully survey the sequence identities (Fig. 2C).

The most numerous classes of bacteria inhabiting the nectar of *P. caeruleum* were Alphaproteobacteria (BG: 52%, CZL: 86%) and Gammaproteobacteria (BOB: 68%). The highest percentage of identified bacterial taxa belonged to *Acinetobacter* and *Phyllobacterium*, depending on the population sampled (Fig. 3).

During the analysis, we focused on the most-represented taxa which made up over 1% of sequences in a particular sample. Most of these, e.g., *Methylobacterium*, *Acinetobacter*, *Sphingomonas*, and *Phyllobacterium*, were previously described as colonizing the nectar of other plant species [3,10] or were found in the guts of insect visitors [23,24]. Representatives of *Methylobacterium*, *Sphingomonas*, and *Acinetobacter* were also described as endophytic bacteria [25,26], which can probably occur in nectar. Most of the recorded bacteria genera were previously found on leaf surfaces, flower parts, or roots [11,27,28].

Almost all the bacterial taxa identified in this study are aerobes, exhibiting catalase activity [29]. This makes them resistant to the presence of toxic hydrogen peroxide,

which is often found in nectar. Some of the identified bacteria were already described as tolerating high sugar concentrations, e.g., representatives of the genus *Methylobacterium* (which tolerate sugar concentrations of up to 50%) and the Sphingomondaceae family [3,10].

Some of the recorded bacterial taxa can have a significant effect on the nitrogen and sulfur cycle. *Phyllobacterium*, *Sphingomonas*, *Acinteobacter*, and *Arcobacter* are diazotrophic strains that bind atmospheric nitrogen, and *Arcobacter* can also oxidize sulfur compounds. Many of *Phyllobacterium* isolates are also characterized by antifungal and antimicrobial activities [25,29].

Our analysis shows that taxonomic composition of bacterial communities varies between populations. However, this variation is mainly due to different ratios of bacterial taxa rather than the presence of different bacteria genera in individual populations (Fig. 3). Small differences in taxonomic composition were previously found in studies describing microorganism assemblages in nectar of other plant species [9,10]. The recorded differences may influence nectar composition (content and proportions of sugars, amino acids, pH) and, via the pollinators, may affect the reproductive success of the plant. In order to better understand the connection between plants, pollinators, and microorganisms, it is necessary to extend the survey to other groups of nectar-dwelling microorganisms, especially yeast, which can also have a significant impact on plant-pollinator relationships [1,2].

References

1. Herrera CM, de Vega C, Canto A, Pozo MI. Yeasts in floral nectar: a quantitative survey. Ann Bot. 2009;103:1415–1423. https://doi.org/10.1093/aob/mcp026
2. Vannette RL, Gauthier MP, Fukami T. Nectar bacteria, but not yeast, weaken a plant–pollinator mutualism. Proc R Soc Lond B Biol Sci. 2012;280:e20122601. https://doi.org/10.1098/rspb.2012.2601
3. Jacquemyn H, Lenaerts M, Brys R, Willems K, Honnay O, Lievens B. Among-population variation in microbial community structure in the floral nectar of the bee-pollinated forest herb *Pulmonaria officinalis* L. PLoS One. 2013;8(3):e56917. https://doi.org/10.1371/journal.pone.0056917
4. Roy R, Schmitt AJ, Thomas JB, Carter CJ. Nectar biology: from molecules to ecosystems. Plant Sci. 2017;262:148–164. https://doi.org/10.1016/j.plantsci.2017.04.012
5. Alvarez-Perez S, Herrera CM, de Vega C. Zooming-in on floral nectar: a first exploration of nectar associated bacteria in wild plant communities. FEMS Microbiol Ecol. 2012;80:591–602. https://doi.org/10.1111/j.1574-6941.2012.01329.x
6. Fridman S, Izhaki I, Gerchman Y, Halpern M. Bacterial communities in floral nectar. Environ Microbiol Rep. 2012;4(1):97–104. https://doi.org/10.1111/j.1758-2229.2011.00309.x
7. Aizenberg-Gershtein Y, Izhaki I, Halpern M. Do honeybees shape the bacterial community composition in floral nectar? PLoS One. 2013;8(7):e67556. https://doi.org/10.1371/journal.pone.0067556
8. Junker RR, Keller A. Microhabitat heterogeneity across leaves and flower organs promotes bacterial diversity. FEMS Microbiol Ecol. 2015;91(9):fiv097. https://doi.org/10.1093/femsec/fiv097
9. Junker RR, Romeike T, Keller A, Langen D. Density-dependent negative responses by bumblebees to bacteria isolated from flowers. Apidologie. 2014;45:467–477. https://doi.org/10.1007/s13592-013-0262-1
10. Good AP, Gauthier MP, Vannette RL, Fukami T. Honey bees avoid nectar colonized by three bacterial species, but not by a yeast species, isolated from the bee gut. PLoS One. 2014;9(1):e86494. https://doi.org/10.1371/journal.pone.0086494
11. Samuni-Blank M, Izhaki I, Laviad S, Bar-Massada A, Gerchman Y, Halpern M. The role of abiotic environmental conditions and herbivory in shaping bacterial community composition in floral nectar. PLoS One. 2014;9(6):e99107. https://doi.org/10.1371/journal.pone.0099107

12. Lenaerts M, Pozo MI, Wäckers F, van den Ende W, Jacquemyn H, Lievens B. Impact of microbial communities on floral nectar chemistry: potential implications for biological control of pest insects. Basic Appl Ecol. 2015;17:189–198. https://doi.org/10.1016/j.baae.2015.10.001

13. Zych M, Junker RR, Nepi M, Stpiczyńska M, Stolarska B, Roguz K. Spatiotemporal variation in the pollination systems of a supergeneralist plant: is *Angelica sylvestris* (Apiaceae) locally adapted to its most effective pollinators? Ann Bot. 2019;123(2):415–428. https://doi.org/10.1093/aob/mcy140

14. Zych M, Stpiczyńska M, Roguz K. Reproductive biology of the red list species *Polemonium caeruleum* (Polemoniaceae). Bot J Linn Soc. 2013;173:92–107. https://doi.org/10.1111/boj.12071

15. Ostrowiecka B, Brzosko E, Jermakowicz E, Wróblewska A, Mirski P, Roguz K, et al. Breeding system variability, pollination biology and reproductive success of the rare *Polemonium caeruleum* L. in NE Poland. Acta Agrobot. 2017;70:1709. https://doi.org/10.5586/aa.1709

16. Stpiczyńska M, Kamińska M, Zych M. Nectary structure in dichogamous flowers of *Polemonium caeruleum* L. Acta Biol Crac Ser Bot. 2012;54(2):61–68. https://doi.org/10.2478/v10182-012-0019-6

17. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17(1):10–12. https://doi.org/10.14806/ej.17.1.200

18. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: a versatile open source tool for metagenomics. PeerJ. 2016;4:e2584. https://doi.org/10.7717/peerj.2584

19. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27(16):2194–2200. https://doi.org/10.1093/bioinformatics/btr381

20. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73(16):5261–5267. https://doi.org/10.1128/AEM.00062-07

21. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–D596. https://doi.org/10.1093/nar/gks1219

22. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335–336. https://doi.org/10.1038/nmeth.f.303

23. Mohr KI, Tebbe CC. Diversity and phylotype consistency of bacteria in the guts of three bee species (Apoidea) at an oilseed rape field. Environ Microbiol. 2006;8(2):258–272. https://doi.org/10.1111/j.1462-2920.2005.00893.x

24. Praet J, Parmentier A, Schmid-Hempel R, Meeus I, Smagghe G, Vandamme P. Large-scale cultivation of the bumblebee gut microbiota reveals an underestimated bacterial species diversity capable of pathogen inhibition. Environ Microbiol. 2018;20(1):214–227. https://doi.org/10.1111/1462-2920.13973

25. Pirttilä AM, Frank AC, editors. Endophytes of forest trees. Biology and applications. 2nd ed. Cham: Springer; 2018. (Forestry Sciences; vol 86). https://doi.org/10.1007/978-3-319-89833-9

26. Kandel SL, Joubert PM, Doty SL. Bacterial endophyte colonization and distribution within plants. Microorganisms. 2017;5(4):77. https://doi.org/10.3390/microorganisms5040077

27. Izhaki I, Fridman S, Gerchman Y, Halpern M. Variability of bacterial community composition on leaves between and within plant species. Curr Microbiol. 2013;66:227–235. https://doi.org/10.1007/s00284-012-0261-x

28. Bodenhausen N, Horton MW, Bergelson J. Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. PLoS One. 2013;8(2):e56329. https://doi.org/10.1371/journal.pone.0056329

29. Garrity GM, Brenner DJ, Krieg NR, Staley JT, editors. Bergey's manual of systematic bacteriology. Vol 2. Part B and C. New York, NY: Springer; 2005.