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Note

Distribution of Pink-Pigmented Facultative Methylotrophs on Leaves of Vegetables

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The distribution of pink-pigmented facultative methylotrophs (PPFMs) on the leaves of various vegetables was studied. All kinds of vegetable leaves tested gave pink-pigmented colonies on agar plates containing methanol as sole carbon source. The numbers of PPFMs on the leaves, colony-forming units (CFU)/g of fresh leaves, differed among the plants, although they were planted and grown at the same farm. Commercial green perilla, *Perilla frutescens viridis* (Makino) Makino, gave the highest counts of PPFMs ($2.0–4.1 \times 10^7$ CFU/g) of all the commercial vegetable leaves tested, amounting to 15% of total microbes on the leaves. The PPFMs isolated from seeds of two varieties of perilla, the red and green varieties, exhibited high sequence similarity as to the 16S rRNA gene to two different *Methylobacterium* species, *M. fujisawaense* DSM5686^T and *M. radiotolerans* JCM2831^T respectively, suggesting that there is specific interaction between perilla and the PPFMs.

Key words: pink-pigmented facultative methylotroph (PPFM); perilla; phyllosphere; *Methylobacterium*; methanol

The plant phyllosphere supports a large and complex microbial community, and bacteria are considered to be the dominant microbial inhabitants of the phyllosphere.[1)] Especially, leaves constitute a very large microbial habitat. The terrestrial leaf surface area that might be colonized by microbes is over 6.4×10^8 km^2, supporting bacterial populations of about 10^{26} cells. As an ecological niche, the plant phyllosphere supports highly abundant *Methylobacterium* species of 10^4–10^7 colony-forming units (CFU) per leaflet.[2)] The bacterial genus *Methylobacterium* is a well-studied example of pink-pigmented facultative methylotrophs (PPFMs) that belong to the *α-proteobacteria* class, and use methanol as sole carbon and energy source. These bacteria are not considered to be passive passengers on plant leaves, but are known to stimulate seed germination and plant development,[2,3)] and to contribute towards the aroma of strawberry.[4)] In the present study, the distribution of these PPFMs on the leaves and seeds of various commercially important vegetables was studied. In addition, we investigated to determine whether these bacteria exhibit any specific interaction with plants.

To test the distribution of PPFMs on vegetable leaves, freshly picked leaves of vegetables (listed in Table 1) planted at a farm (100 m^2) in the suburbs of Kusatsu, Shiga, Japan, were used. Fresh leaves (1 g) were homogenized in ice-cooled sterilized water (100 mL) with Ace Homogenizer (Nihonseiki, Tokyo) at 15,000 rpm for 1 min, and the homogenates were serially diluted and plated onto AMS (buffered ammonium salts solution[5)])-methanol agar medium supplemented with 1% methanol v/v and 10 μg/mL of cycloheximide. After 7–10 d of incubation at 28 °C, pink-pigmented colonies appeared on the plates, and were counted. The numbers of PPFMs (CFU/g of fresh weight) observed on a few vegetable leaves are shown in Table 1. All kinds of vegetable leaves tested exhibited pink-pigmented colonies on agar plates containing methanol as sole carbon source, but the CFU values observed differed between the plants.

Next, the agar impression method[6)] was employed to obtain total profiles of the PPFMs on various vegetable leaves. One cm^2 of disks of leaves was impressed onto AMS-methanol agar containing cycloheximide for 1 min. After removal of the disks, the plates were incubated for 7–10 d at 28 °C. The results are shown in Fig. 1. All kinds of leaves tested, potherb mustard (*Brassica rapa* L. var. *nipposinica*), broccoli (*Brassica oleracea* var. *italica*), crown daisy (*Glebionis coronaria*), rucola (*Eruca vesicaria*), turnip (*Brassica rapa* L. var. *glabra*), qing geng cai (*Brassica rapa* var. *chinensis*), Italian parsley (*Petroselinum neapolitanum*), spinach (*Spinacia oleracea*), Japanese radish (*Raphanus sativus*), Chinese cabbage (*Brassica rapa* L. var. *glabra* Regel), basil (*Ocimum basilicum*), leaf lettuce (*Lactuca sativa* L. var. *crispa*), komatsu-na (*Brassica rapa* var. *pervirides*), and green perilla (*Perilla frutescens viridis*

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Abbreviations: AMS, buffered ammonium salts solution; CFU, colony-forming units; DAPI, 4′,6′-diamidino-2-phenylindole; PPFM, pink-pigmented facultative methylotroph

Table 1. Distribution of PPFMs on Vegetable Leaves of Various Kinds Planted at the Same Farm

Vegetables	Number of PPFMs[a] (CFU/g fresh weight)
Eggplant (*Solanum melongena*)	$(1.5 \pm 0.39) \times 10^7$
Green perilla (*Perilla frutescens viridis* (Makino) Makino)	$(1.3 \pm 0.47) \times 10^7$
Small green pepper (*Capsicum annuum*)	$(1.3 \pm 0.65) \times 10^6$
Pumpkin (*Cucurbita moschata*)	$(1.3 \pm 0.32) \times 10^6$
Bitter melon (*Momordica charantia*)	$(8.2 \pm 0.22) \times 10^5$
Okra (*Abelmoschus esculentus*)	$(6.3 \pm 0.21) \times 10^5$
Tomato (*Solanum lycopersicum*)	$(3.5 \pm 0.21) \times 10^5$

[a]Means ± standard deviations of six replicated measurements are shown.

(Makino) Makino), exhibited pink-pigmented colonies on the plates. Corpe and Rheem reported that epiphytic bacteria are most abundant near the margins of the abaxial surface of leaves.[7] However, in the present study there was little difference in PPFM count values between the adaxial and abaxial sides of the leaves tested (Fig. 1).

Among the tested vegetables, Japanese radish, komatsu-na, and green perilla gave larger numbers of PPFMs (>50 colonies/cm^2) on the plates. The total microbial counts and PPFMs of these three vegetable leaves were determined in order to determine the ratio of distribution of PPFMs to the total microbial count on leaves. PPFMs were counted using the homogenization method, as explained above. The total microbial count of the leaves was measured by the DAPI (4′,6′-diamidino-2-phenylindole)-staining method. Fresh leaves (1 g) were mixed with 25 mL of PBSE buffer (130 mM NaCl, 10 mM phosphate buffer, 1 mM EDTA, pH 7.0) in a 50 mL-plastic tube, and were processed using an ultrasonic cleaning device (UT205S, Sharp, Osaka, Japan) for 15 min, and the aqueous phase was treated with 1% v/v formaldehyde for 30 min. An aliquot of the aqueous phase was filtered with a membrane filter (Isopore™ 0.2 μm GTBP, Millipore, Billerica, MA), the microbes trapped on the filter were stained with 20 μL of DAPI solution (1 μg/mL), and the total number of microbes was counted under a fluorescence-inverted microscope (IX70; Olympus, Tokyo). The highest count of PPFMs was obtained for green perilla leaves (5.0×10^7 CFU/g of fresh leaves), and the ratio of PPFMs to the total microbial count (3.34×10^8 CFU/g of fresh leaves) was 15%. The counts of PPFMs in Japanese radish (1.0×10^6 CFU/g of fresh leaves) and komatsu-na (1.0×10^5 CFU/g of fresh leaves), however, were negligible compared to the total microbial counts (9.7×10^7 CFU/g of fresh leaves and 1.11×10^8 CFU/g of fresh leaves respectively). Trials to reproduce the high PPFM values of the leaves of Japanese radish and komatsu-na were unsuccessful. The reason for this discrepancy is unclear, but might have been any of several factors, including planting location and conditions, growth stage, soil, and surrounding atmosphere.

To determine whether the distribution of PPFMs was dependent on the geographic location of the perilla plants, the PPFMs from commercial green perilla leaves planted at different places were counted. Six samples planted in three prefectures in Japan (Oita, Kochi, and Aichi) were purchased from neighborhood greengrocers and supermarkets, and the PPFMs were counted. Regardless of geographic location, all the leaves exhibited high PPFM values ($2.0–4.1 \times 10^7$ CFU/g of fresh leaves), and the number of PPFMs was independent of planting site. PPFMs were also detected in red perilla (*Perilla frutescens crispa* (Thunb.) Makino) leaves ($10^{5–6}$ CFU/g of fresh leaves).

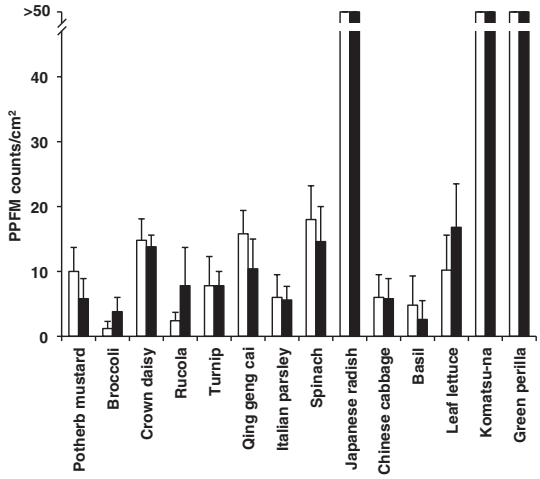

Fig. 1. PPFM Counts for Vegetable Leaves Using the Agar Impression Method.

One cm^2 of disks of leaves was impressed onto AMS-methanol agar containing cycloheximide. After 5–7 d of incubation at 28 °C, pink-pigmented colonies were counted. Hollow bars, PPFM counts on the adaxial side of the leaves; solid bars, PPFM counts on the abaxial ones. Error bars show standard deviations for five replicated measurements. Qing geng cai, spinach, Chinese cabbage, and green perilla were purchased from local supermarkets. The other vegetables were planted at the farm that figures in Table 1.

Although wide distribution and isolation of PPFMs in the phyllosphere has been reported,[5–10] PPFMs of green perilla have not been reported previously. The PPFM counts of perilla were higher than those of other taxonomically closely related species such as *Plectranthus*, belonging to the same family *Lamiaceae* (*Labiatae*). A PPFM count of 3.6–7.5 CFU/cm^2 was detected for two *Plectranthus* species.[11] In comparison, the PPFM level in the leaves of perilla was rather high (>50 CFU/cm^2).

Perilla is widely used as a food in Japan and other Asian countries, and it has medicinal value. There are green and red varieties. The former is a popular potherb, and the latter is used mainly as a coloring ingredient in pickle making. In the Ohara district in the northeastern part of Kyoto, Japan, a characteristic red perilla is used extensively as one of the most important ingredients in shiba-zuke (local pickle) production. We found that the red and green perilla leaves harbored high populations of PPFMs, and investigated to determine whether a specific interaction exists between PPFMs and the two varieties of perilla. We looked into the relationship between the two in terms of PPFM occurrence by 16S rRNA gene sequencing of PPFMs isolated from red perilla seeds (harvested in 2009 by Doi Shiba-Zuke Honpo Co., Kyoto, Japan) and green perilla seeds (Product no. ATY132L15, Takii & Co., Kyoto, Japan). Twenty grains of perilla seeds was suspended in 5 mL of 10 mM PBS (phosphate buffered saline, pH 7.4) in a test tube and shaken for 2 h at 28 °C. The supernatant thus

obtained was streaked on an AMS-methanol agar plate supplemented with cycloheximide. After incubation for 5–7 d at 28 °C, pink colonies were selected and streaked on an AMS-methanol agar plate supplemented with 0.01% of yeast extract (BD Biosciences, Franklin Lakes, NJ) for single-colony isolation. Finally, 12 strains (OR01 to OR12) were isolated from the red perilla seeds and eight strains (TG01 to TG08) were isolated from the green perilla seeds. Sequencing was performed using an automated DNA sequencer (Model 3130; Applied Biosystems, Life Technologies Japan, Tokyo), and partial 16S rRNA gene sequences (1,419 bp) of the PPFM isolates were determined. Nucleotide sequences were deposited in DDBJ under accession nos. AB673234-AB673253. Among the 12 PPFM isolates (OR01 to OR12) from red perilla seeds, the 16S rRNA gene sequence of strain OR09 differed from that of the other 11 strains by one base at position 93. These 11 strains had entirely identical 16S rRNA gene sequences and were found to exhibit highest homology to closest relative *Methylobacterium fujisawaense* DSM5686^T, with one base difference at position 1176. On the other hand, all eight PPFM isolates (TG01 to TG08) from the green perilla seeds had entirely identical 16S rRNA gene sequences, and showed highest sequence homology to another *Methylobacterium* species, *M. radiotolerans* JCM2831^T, with one base difference at position 662. Thus the PPFMs from seeds of the red and green perilla gave different profiles of closest relatives. The 16S rRNA gene sequence similarity between *M. fujisawaense* DSM5686^T and *M. radiotolerans* JCM2831^T is 99.3%.

Literature on the origin of phyllospheric PPFMs abounds, but it is still debatable. Corpe (1985) argues that the paucity of PPFMs in the air makes it unlikely that the atmosphere is a major contributor of the methylotrophs encountered on plant leaves.[6] Holland and Polacco (1992) suggest that leaf-inhabiting PPFMs are probably descendants of seed-borne bacteria rather than bacteria from the air, soil, or other plants.[12] Madahaiyan (2005) has reported that PPFMs are transmitted mostly through seeds.[2] On the other hand, Romanovskaya *et al.* (2001) has reported that leaves were not colonized after seed bacterization or soil application of a PPFM strain, and were colonized only after direct application to the phyllosphere, suggesting that natural leaf colonization occurred *via* transfer of soil particles.[13] There are various reports on the colonization of PPFMs as well. Omer *et al.* (2004) has reported that PPFMs isolated from red clover readily colonized winter wheat leaves and *vice versa* in greenhouse experiments, and that the tested isolates had good potential to colonize the rhizosphere, especially after seed inoculation.[8] According to Knief *et al.* (2010), factors specific to the sites from which the plant species were collected, more than the plant species themselves, have a strong influence on the composition of the phyllospheric *Methylobacterium* community.[14] We found that PPFMs were highly abundant on perilla leaves, regardless of the geographic site from which they were collected. However, our results compared the given species across different geographic locations and did not include interspecies analysis, as did Knief *et al.* (2010).

In this study, we found that red and green perilla harbored a dominant population of PPFMs on their leaves and seeds, and that the closest relatives of PPFMs isolated from red and green perilla seeds differed from each other in terms of 16S rRNA gene sequence showing similarities to two different *Methylobacterium* species. This strongly argues for specific interaction between perilla and PPFMs. Further investigation focusing on the origin and inheritance of the PPFMs on perilla seeds and leaves, and in the surrounding atmosphere and the soil are in progress.

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References

1) Morris CE and Kinkel LL, "Phyllosphere Microbiology," eds. Lindow SE, Hecht-Poinar EI, and Elliot V, APS Press, St. Paul, pp. 365–375 (2002).
2) Madhaiyan M, Poonguzhali S, Lee HS, Hari K, Sundaram SP, and Sa TM, *Biol. Fert. Soils*, **41**, 350–358 (2005).
3) Lidstrom ME and Chistoserdova L, *J. Bacteriol.*, **184**, 1818 (2002).
4) Zabetakis I, *Plant Cell Tissue Organ Cult.*, **50**, 179–183 (1997).
5) Corpe WA and Basile DV, *Dev. Ind. Microbiol.*, **23**, 483–493 (1982).
6) Corpe WA, *J. Microbiol. Methods*, **3**, 215–221 (1985).
7) Corpe WA and Rheem S, *FEMS Microbiol. Ecol.*, **62**, 243–250 (1989).
8) Omer ZS, Tombolini R, and Gerhardson B, *FEMS Microbiol. Ecol.*, **47**, 319–326 (2004).
9) Aken BV, Peres CM, Doty SL, Yoon JM, and Schooner JL, *Int. J. Syst. Evol. Microbiol.*, **54**, 1191–1196 (2004).
10) Kutschera U, Thomas J, and Hornschuh M, *Naturwissenschaften*, **94**, 687–692 (2007).
11) Chanprame S, Todd JJ, and Widholm JM, *Plant Cell Rep.*, **16**, 222–225 (1996).
12) Holland MA and Polacco JC, *Plant Physiol.*, **98**, 942–948 (1992).
13) Romanovskaya VA, Stoyler SM, Malashenko YR, and Dodatko TN, *Microbiology*, **70**, 221–227 (2001).
14) Knief C, Ramette A, Frances L, Alonso-Blanco C, and Vorholt JA, *ISME J.*, **4**, 719–728 (2010).