

1 **Host developmental stage is associated with shifts in the exosphere**
2 **microbiome of urban-farmed Asian green leafy vegetables**

3 Running title: Leafy vegetable microbiome

Shruti Pavagadhi^{a,b}, Aditya Bandla^b, Miko Poh Chin Hong^a, Shivshankar
Umashankar^{a,b}, Yeap Yoon Ting^a, Sanjay Swarup^{a,b,c,d}

4 ^aDepartment of Biological Sciences, National University of Singapore, Singapore

5 ^bSingapore Centre for Environmental Life Sciences Engineering (SCELSE), National
6 University of Singapore, Singapore

7 ^cNUS Environmental and Research Institute, National University of Singapore,
8 Singapore

9 ^dSynthetic Biology for Clinical and Technological Innovation, National University of
10 Singapore, Singapore

11

12 Address correspondence to Sanjay Swarup, dbsss@nus.edu.sg

13 S.P. and A.B. contributed equally to this work

14

15 **Keywords:** Green leafy vegetables, growth stage, plant beneficial functions

16 **Abstract**

17 Green leafy vegetables (GLV's) comprise a significant part of urban South-East Asian
18 (SEA) diets and are intensively farmed in SEA cities, including Singapore. Urban
19 farming practices and urban-adapted vegetable cultivars likely select for specific
20 above- and below-ground microbial communities – microbiomes – that assemble in
21 close proximity to the plant tissues – the exosphere. A healthy exosphere microbiome
22 is important for plant growth and safe human consumption. Using 16S rDNA gene
23 amplicon sequencing compositional analyses, we show here that the exosphere
24 microbiome of two commonly-consumed GLV's – Choy Sum (*Brassica oleracea*
25 *Alboglabra Group*) and Gai Lan (*Brassica chinensis var. parachinensis*) – dominated
26 by *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidia* and *Actinobacteria*. Shifts
27 in exosphere microbiome composition were strongly associated with plant
28 developmental stage. Finally, microbial taxa consistently detected in the exosphere
29 comprise a small subset, which are predicted to harbour plant-beneficial traits.

30

31 **Significance**

32 Among plant crops, GLVs form an integral part of the Asian diet, especially so
33 in Southeast Asia. Some of these GLVs have short life-cycles (~30-45 days), which
34 makes them suitable for urban farms in terms of cost advantage as short cycle crops
35 are preferred in urban farms. From a food-security perspective, GLVs forms an
36 important target food group and efforts are being made to increase its productivity to
37 meet the increasing food demands. Current farming practices often place lot of
38 importance on chemical fertilizers and nutrient inputs to improve the fertility of non-
39 arable urban lands to increase the crop productivity. Furthermore, farms in urban

40 settings are also associated with anthropogenic inputs and eutrophic conditions.
41 These together, contribute to negative environmental externalities questioning the
42 sustainability and eco-sustenance of urban farming. Microbial based management
43 systems can not only resolve these challenging issues, but can also enhance plant
44 growth, nutrient use efficiency and disease tolerance. However, their use as microbial
45 adjuncts to agricultural practices is currently limited in urban environments, which
46 could possibly be due to the restricted knowledge-base on these urban phytobiomes.

47

48 **Observations**

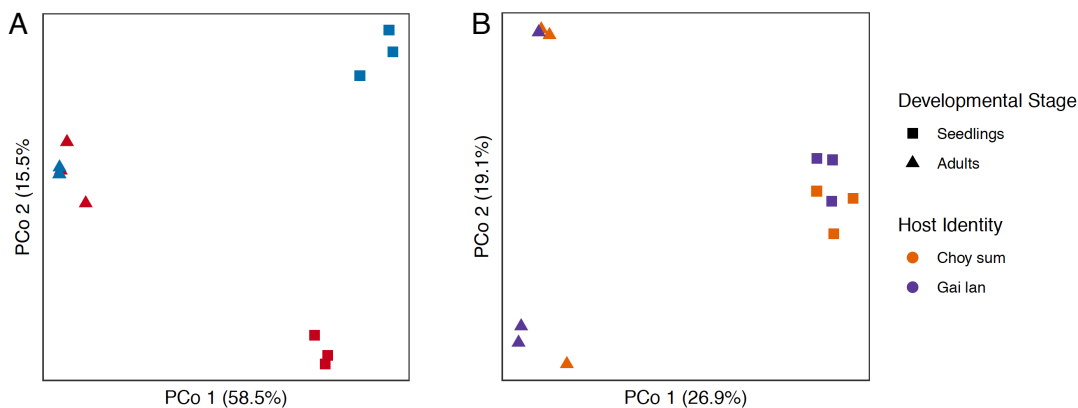
49 **Host developmental stage and host identity shape the exosphere microbiome**

50 Replicated samples (n=3) of two GLV's – Choy Sum (*Brassica oleracea*
51 *Alboglabra Group*) and Gai Lan (*Brassica chinensis var. parachinensis*) – across two
52 major plant developmental stages (seedlings and adults) were randomly collected
53 from the best performing greenhouses in one of Singapore's largest commercial farms
54 involved in green leafy vegetable production. Microbial communities associated with
55 the rhizosphere and phyllosphere – the exosphere, were analysed using 16S rDNA
56 gene amplicon sequencing. A total of 1, 355, 621 (median read count per sample: 25,
57 775; range: 101 – 41425 reads per sample) high-quality reads were obtained, which
58 in turn, mapped to a total of 12, 735 amplicon sequence variants (ASV's). Sample
59 counts were total sum scaled and square-root transformed prior to beta-diversity
60 analysis.

61 Similarity of samples in terms of microbiome composition were visualised using
62 unconstrained principle coordinate analysis (PCoA). Samples separated according to
63 host developmental stage along the first axis for both the rhizosphere and

64 phyllosphere, while separation along the second axis largely corresponded to host
65 identity of adult plants (Figure 1). These patterns were corroborated using
66 Permutational Multivariate Analysis of Variance (PERMANOVA) which showed that
67 host developmental stage accounts for the largest amount of variance in terms of
68 microbiome composition (*SI Appendix, Dataset S1*).

69



70

71 **Figure 1.** Microbiome composition of the plant exosphere is associated with host
72 developmental stage and host identity. (A) Rhizosphere and (B) Phyllosphere

73

74

74 **Microbial taxa in the exosphere are predicted to harbour plant-beneficial traits**

75 Stable and significantly enriched components of the rhizosphere microbiome
76 were identified through differential abundance analyses as well as retaining only those
77 taxa that were detected across all replicates. We found that only a small subset of
78 ASV's were significantly enriched in the rhizosphere of seedling and adult plants (*SI*
79 *Appendix, Dataset S2-S5*). Cumulatively, such ASV's accounted for 15% and 33% of
80 total microbial community in Choy Sum (1753 ASVs; 36 enriched ASVs) and Gai Lan
81 (1638 ASVs; 81 enriched ASVs) seedlings respectively. While for adult plant types, it
82 accounted for 18.6% and 16.1% of the total microbial community in Choy Sum (3802

83 ASVs; 219 enriched ASVs) and Gai Lan adults (3233 ASVs; 119 enriched ASVs)
84 respectively.

85 Stable components of the phyllosphere microbiome were identified as those
86 taxa that were consistently detected across all replicates derived from the respective
87 groups (*SI Appendix, Dataset S6*). Cumulatively, such microbial taxa accounted for
88 39.4 % and 78.4 % of total microbial community in Choy Sum and Gai Lan seedlings
89 respectively. While for adult plant types, they accounted for 48.7 % and 14.8 % of the
90 total microbial community in Choy Sum and Gai Lan adults respectively. Interestingly,
91 most of these prevalent taxa derive from the phyla *Alphaproteobacteria*,
92 *Gammaproteobacteria*, *Deltaproteobacteria*, *Bacteroidia* and *Actinobacteria*.

93 Next, we predicted the genomic repertoire of these taxa using PICRUSt2 (Eddy
94 1998; Langille et al., 2013; Louca and Doebeli, 2018; Barbera et al., 2019, Czech et
95 al., 2019) and then searched for genomic features related to plant beneficial functions
96 (*SI, Appendix, Dataset S7*). Some of these functions pertain to genes involved in
97 nitrogen, sulfur and phosphorus metabolism. These are essential elements for growth,
98 development, and various physiological functions in plants. Furthermore, members
99 from *Brassicaceae* have higher nitrogen, phosphorus and sulfur requirements than
100 other crop species, and therefore, are particularly sensitive to their availability (Walker
101 and Booth, 2003; Albert et al., 2012, Brennan and Bolland, 2009).

102 We found that genes predicted to be involved in ureolysis were the most
103 prevalent across all sample groups i.e. seedling and adult exospheres of both GLV's
104 (*SI, Appendix, Dataset S8*). Although, such genes are involved in the hydrolysis of
105 urea to ammonia, we found ammonia oxidizers to be highly abundant both in the bulk
106 soil and the rhizosphere. We speculate that these taxa may act synergistically to

107 convert urea to plant available nitrates. These nitrates form an integral component of
108 many structural and metabolic compounds in plant cells such as amino acids, proteins,
109 nucleotides, chlorophyll, chromosomes, genes and all enzymes. Nitrogen deficiency
110 in some members from *Brassicaceae* is associated with lowered sensitivity to water
111 stress (Albert et al., 2012)

112 Predicted genomic features associated with phosphate solubilization and
113 sulfate oxidation were also consistently detected in multiple taxa (*SI, Appendix,*
114 *Dataset S8*). Both these nutrients are limited in nature and they are often present in
115 unavailable chemical forms in the soil. Sulfate and phosphate oxidizing microbes
116 convert these to more readily available substrates that can be utilized by the plants.
117 Both, phosphorus and sulfur are essential nutrients required for formation of structural
118 components in plants, including nucleic acids, phospholipids and a variety of
119 secondary metabolites, which play a pivotal role in protecting these plants against
120 abiotic and biotic stressors (Plaxton and Lambers, 2015).

121

122 **Conclusions**

123 These results show that host developmental stage is associated with major shifts in
124 the exosphere microbiome of widely-consumed urban-farmed Asian GLVs. Further,
125 taxa that were consistently detected in the exosphere are predicted to harbour plant-
126 beneficial functions.

127

128 **Supporting Information (SI) Appendix**

129 SI Dataset 1: PERMANOVA results for rhizosphere and phyllosphere microbiome

130 SI Dataset 2: ASVs significantly enriched in the seedling choy sum rhizosphere

- 131 SI Dataset 3: ASVs significantly enriched in the adult choy sum rhizosphere
132 SI Dataset 4: ASVs significantly enriched in the seedling gai lan rhizosphere
133 SI Dataset 5: ASVs significantly enriched in the adult gai lan rhizosphere
134 SI Dataset 6: ASVs consistently detected in all replicates of respective phyllosphere
135 SI Dataset 7: List of plant-beneficial functional genes
136 SI Dataset 8: Frequency of predicted plant beneficial functions in exosphere
137 microbiota

138

139

140 **References**

- 141 Albert, B., Le Cahérec, F., Niogret, M. F., Faes, P., Avice, J. C., Leport, L., &
142 Bouchereau, A. (2012). Nitrogen availability impacts oilseed rape (*Brassica napus* L.)
143 plant water status and proline production efficiency under water-limited
144 conditions. *Planta*, 236(2): 659.
- 145 Barbera, P., Kozlov, A.M., Czech, L., Morel, B., Darriba, D., Flouri, T., Stamatakis,
146 A. (2019). EPA-ng: Massively Parallel Evolutionary Placement of Genetic Sequences,
147 *Systematic Biology*, 68, 365.
- 148 Brennan, R.F., Bolland, M.D.A. (2009). Comparing the nitrogen and phosphorus
149 requirements of canola and wheat for grain yield and quality. *Crop Pasture Sci.* 60:
150 566.
- 151 Czech, L., Barbera, P., Stamatakis, A. (2019). Methods for automatic reference trees
152 and multilevel phylogenetic placement. *Bioinformatics*, 35, 1151.
- 153 Eddy, S.R. (1998). Profile hidden Markov models. *Bioinformatics*, 14, 755.

154 Langille, M. G.I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., a Reyes,
155 J., Clemente, J. C., Burkepille, D. E., Vega Thurber, R. L., Knight, R., Beiko, R. G., and
156 Huttenhower, C (2013). *Nature Biotechnology*, 1-10. 8.

157 Louca, S., Doebeli, M. (2018). Efficient comparative phylogenetics on large
158 trees. *Bioinformatics*, 34, 1053.

159 Plaxton, W.C., and Lambers, H., eds. 2015. *Phosphorus metabolism in plants*. John
160 Wiley & Sons, Ltd., The Atrium, Southern Gate, Chichester, West Sussex, UK.

161 Walker, K.C., Booth, E.J. Sulphur nutrition and oilseed quality. In: Abrol YP, Ahmad
162 A, editors. *Sulphur in Plants*. Dordrecht, The Netherlands: Kluwer Academic
163 Publishers; 2003. pp. 323–339.

164

165 **Acknowledgements**

166 This work was supported by the National Research Foundation, Prime Minister's
167 Office, Singapore under its Competitive Research Programme (NRF-CRP16-2015-
168 04). We also thank the NUS Environmental Research Institute (NERI) and Singapore
169 Centre for Environmental Life Sciences Engineering (SCELSE) for their services and
170 support.

171

172 **Author's contributions**

173 S.P., S.U. and S.S. conceived the study. S.P. and M. P. C. H. were involved in
174 sampling and sample preparation. A.B. was involved in data analysis. A.B. and S.P.
175 were involved in data interpretation and writing the manuscript.

176

177 **Conflict of interests**

178 The authors declare that they have no conflicts of interest.

179

180 **Statement of informed consent, human/animal rights**

181 No conflicts, informed consent, human or animal rights applicable.