Why are flowers ephemeral? Effects of old flower microbes on fruit set in a wild ginger with one-day flowers, *Alpinia japonica* (Zingiberaceae)

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Abstract: Flowers are colonized by and inhabited by diverse microbes. Plants rapidly replace flowers of short lifespan, and old flowers senesce. This may contribute to avoiding adverse effects of the microbes. In this study, we investigate if the flower microbial community on old flowers impedes fruit and seed production in a wild ginger with one-day flowers. We inoculated newly opened flowers with old flower microbes, and monitored the effects on fruit and seed set. We also assessed prokaryotic communities on the flowers using amplicon sequencing. We found five bacterial amplicon sequence variants (ASVs) whose proportions were increased on the inoculated flowers. These ASVs were also found on flower buds and flowers that were bagged by net or paper during anthesis. Fruit set was negatively associated with the proportions of these ASVs, while seed set was not. The results suggest that old flowers harbor microbial communities different from those at anthesis, and that the microbes abundant on old flowers negatively affect plant reproduction. Though the short lifespan of flowers has gotten little attention, it might be an essential defense mechanism to cope with antagonistic microbes that rapidly proliferate on the flowers.

Keywords: flower longevity, anthosphere, pollination, microbiome

INTRODUCTON

Flowers are an elaborated device to exchange pollen among conspecific individuals. Instead of moving around to find a potential mate, the plants disperse and receive pollen relying on different vectors such as insects and wind to achieve sexual reproduction. As a consequence, flowers are also exposed to microbial colonization under natural conditions [1–3]. Flowers have various habitats for microbes. Flowers attract pollinators by secreting nectar, which is rich in sugars and often contains other nutrients, such as amino acids and lipids [4]. The stigma is a germination bed for pollen grains connected to a growth chamber for pollen tubes. It maintains humidity and nutrients necessary for pollen tube growth [5], which would also be beneficial for microbes. Recent studies using high-throughput sequencing have revealed highly diverse microbial communities on flowers of many species in a wide range of habitats [6–8].

Today, flower microbes are increasingly recognized as essential components in the ecology and evolution of plant reproduction (reviewed in [9,10]). Not surprisingly, some flower microbes are known to have negative effects on the survival and reproduction of the host plant. Many pathogens use flowers, especially the stigma and nectary, as a point of entry. Their infection of flowers often results in fruit abortion and systemic infection [11,12]. Other microbes impede various processes of pollination and fertilization. Nectar yeasts are reported to modify pollinator foraging patterns and reduce pollination success [13]. *Acinetobacter* bacteria that commonly inhabit floral nectar exploit pollen nutrition by inducing pollen germination and bursting [14]. Most of these studies examined the effects of an individual microbial species or strains separately rather than that of the whole community.

In general, even newly opened flowers already have microbes at least on some flower tissues. Their abundance increases over time on individual flowers [10]. Flowers have various mechanisms to suppress microbial growth, such as flower volatiles [3,15], reactive oxygen [16] and secondary compounds [17,18]. Besides, an extremely short lifespan of the flowers from one to several days [19], especially in hot habitats [20], is also understood as a compromise to efficiently achieve sexual reproduction while avoiding adverse effects of flower antagonists, including microbes [1,21,22]. Unlike in mammals, flowers, reproductive structures of the plants, are only retained while they are

needed, and are developed *de novo* every season in perennial species [22]. We do not know yet, however, to what extent microbes that are suppressed by the short lifespan of flowers are potentially detrimental to plant fecundity.

In this study, we examine if the flower microbial community on old flowers impedes fruit and seed production in a wild ginger, *Alpinia japonica* (Zingiberaceae), that produces short-lifespan flowers. This perennial herb grows on the humid forest floor of evergreen and deciduous forests in western Japan. It flowers in June, when the climate is hot and humid. Its flowers open in the morning and wilt at sunset within the same day. Therefore, its flowering matches with the most ideal season for microbial growth, while it may avoid the negative effects of microbes by renewing flowers every day. To imitate microbial communities that would appear on the flowers of *A. japonica* if their longevity were longer, we inoculate microbes from old flowers onto newly opened flowers in the morning. Firstly, we characterize the prokaryote communities on *A. japonica* flowers. Secondly, we identify prokaryotes that significantly increased after inoculation by comparing prokaryotic communities between the inoculated flowers and open flower controls. In addition, we examine if these prokaryotes colonize by air or by flower visitors after flowering, or if they had already existed before anthesis. We covered the flowers by net or paper bags to exclude microbial colonization by flower visitors or by both flower visitors and air, respectively. If the old-flower microbes colonize only after anthesis, bagging would reduce the occurrences of these microbes. Lastly, we observe fruit and seed sets of the flowers under the treatments. We expected that the flowers that had more old-flower microbes would show lower fruit and seed set if antagonistic microbes are abundant on old flowers.

MATERIALS AND METHODS

Study species

Alpinia japonica (Zingiberaceae) is a wild ginger distributed in temperate and subtropical regions of eastern Asia. It is a perennial herb 0.5–0.7 m in height occurring mostly in broad-leaved evergreen forests. Inflorescences are compound racemose with 10–60 flowers (37 on average in this population). Flowers are zygomorphic with a prominent labellum 1.1–1.2 cm in length, and white with red stripes at the margin of the labellum (Fig. 1a). Flowering occurs almost synchronously within a population and lasts

for 10–14 days for each plant between late May and June. From one to five flowers open per inflorescence. At anthesis around 0700, the dorsal and lateral petals open, and the labellum inside expands (Fig. 1b). The flowers are known to have volatiles, including 1, 8-cineole, with antimicrobial activities [23,24]. The corolla wilts at sunset within the day. The ovary matures into an ellipsoid, three-loculate fleshy capsule ~2 months after flowering. The species is self-compatible, and pollinated by bees, mainly carpenter bees (*Xylocopa appendiculata*) and bumble bees (*Bombus ardens*) (J. Nuria and S. Sakai, unpublished data).

Study site

Studies were conducted in May to July of 2018, in Seta Park, Otsu, Shiga Prefecture, Japan (34°50'N, 135°50'E). Seta Park has a young secondary deciduous forest where streams are abundant, which provides an optimal habitat for *A. japonica*. The annual mean temperature is 14.9°C, and annual total rainfall is ~1530 mm in Otsu city (Japan Meteorological Agency, https://www.data.jma.go.jp/). Mean daily maximum temperatures in May, June and July 2018 were 24.0°C, 27.3°C and 33.7°C, respectively.

Flower treatments

Alpinia japonica typically presents 2–12 inflorescences per plant at the study site. We selected six plants with at least eight inflorescences with more than 10 flowers on each. Unfortunately, one of them had been damaged, probably by park visitors, before the experiments and sampling. Therefore, we used the remaining five plants (Plant ID = N1, N2, N4, N5, N6). In each target plant, eight inflorescences were tagged and covered with a fine net (Cloth Cabin, Suminoe Teijin Techno, Osaka, Japan) prior to flowering.

We applied one of five treatments to part of the flowers that opened on the inflorescences between 22nd May and 7th June. The flowers under these treatments were used for either identification of microbes on their flowers or for observation of fruit set (Fig. 1c). We could not use the same flower for both, because we should collect the flower on the same day for the former, while we should leave the flower intact for the latter. The five treatments were as follows: 1) open control (OT), in which the mesh bag on the inflorescence was removed during 0700–1700, so that microbes were

127 transmitted to the flower by both air and pollinators; 2) insect exclusion by a net bag
128 (NB), in which the inflorescence was left covered with the net, which allowed air but
129 not insects to get through; 3) paper bagged (PB), in which the mesh bag covering the
130 inflorescence was replaced by a new paper bag (Grape Bag, DAIICHI VINYL, Fukui,
131 Japan) during 0700–1700 to exclude air flow and insect visits; 4) open and microbial
132 inoculation (MI) and 5) open and water sprayed mock (WI) treatments. In MI, the mesh
133 bag on the inflorescence was removed as in OT, and the flower was sprayed with
134 microbial solution. During the spray inoculation, the focal flower was isolated from the
135 rest of the inflorescence by a funnel-shaped plastic sheet to avoid contamination. The
136 microbial suspension for the inoculation was prepared by soaking old flowers in
137 distilled water. Nine old flowers that had opened the previous day from 1–3 individuals
138 were collected from individuals that did not receive the treatments. Microbes were
139 detached by ultrasonic dispersion in 10 ml of distilled water and kept at 4°C until the
140 inoculation (maximum of 36 hours). In WI, the mesh bag covering the inflorescence
141 was removed as in OT, and the flower was sprayed with distilled water as a control for
142 the physical effect of the MI treatment. We did not conduct the treatments on cloudy or
143 rainy days. Flowers that opened on the same day on the same inflorescence were
144 assigned to the same treatments in the case of NB and PB, while OT, MI and WI were
145 occasionally mixed on an inflorescence. This is because we bagged the entire
146 inflorescence rather than a single flower to avoid damaging the flowers. The sample size
147 of each treatment is summarized in Table S1.
148
149 **Identification of microbial communities**
150 To identify microbial communities, we sampled 4–5 flowers with each treatment
151 from each plant. The flowers were cropped at ~1700 of the day of flower opening and
152 separately put into a 5 ml plastic tube after removing the stigma (to use for another
153 study) and petals (Fig. 1b). In addition, five flower buds were collected from each
154 individual to identify microbes that were present inside prior to anthesis with the same
155 procedure. The tube was kept on ice until being brought to the lab within 4 hours after
156 sampling and kept at –20°C until further processing.
157 To detach microbes from the flower surface, we added 3 ml of Phosphate-
158 Buffered Saline solution (PBS buffer, pH 7.4, Nippon Gene Co., Ltd.) to the tube and

159 sonicated it using an Ultrasonic Disruptor Handy Sonic UR-21P (Digital Biology) at
160 Level 5 power for 30 sec. Microbes in the PBS solution were then filtered using a
161 Sterivex Millipore filter (Sterivex-HV 0.22, Merck). Residual PBS on the Sterivex filter
162 was removed using a Vac-Man® Laboratory Vacuum Manifold (Promega).
163 Microbial DNA was extracted with DNeasy Blood & Tissue Kits (QIAGEN)
164 following the manufacturer's instructions. A portion of the 16S small-subunit ribosomal
165 gene was PCR-amplified using the primer pairs, 515F (5'-GTG YCA GCM GCC GCG
166 GTA A-3') and 806R (5'-GGA CTA CNV GGG TWT CTA AT-3') [25] with
167 Nextera DNA index adapters. First PCR was performed in 12 µl reactions, each
168 containing 1 µl of DNA template, 6 µl of KAPA HiFi HotMasterMix (Kapa
169 Biosystems) and 0.7 µl of each primer (5 µM). The first PCR was conducted with a
170 temperature profile of 95°C for 3 min, followed by 35 cycles at 98°C for 20 s, 60°C for
171 15 s, 72°C for 30 s, and a final extension at 72°C for 5 min, followed by storage at 4°C
172 until use. Triplicate PCR products were pooled and purified with Agencourt AMPure
173 XP (Beckman Coulter). P5/P7 Illumina adaptors were then added in a second PCR
174 using fusion primers with 8-mer index sequences for sample identification [26]
175 (forward, 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-[8-mer tag]-TCG
176 TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG -3'; reverse, 5'-CAA GCA
177 GAA GAC GGC ATA CGA GAT-[8-mer tag]-GTC TCG TGG GCT CGG AGA TGT
178 GTA TAA GAG ACA G-3'). This preparation had a total volume of 24 µl with double
179 the amount of each reagent in the first PCR and 10-times diluted first PCR products as
180 DNA template. The temperature profile was 95°C for 3 min, followed by 12 cycles at
181 98°C for 20 s, 68°C for 15 s, 72°C for 15 s, a final extension at 72°C for 5 min, and 4°C
182 until use. Second PCR products were pooled at equal volumes after a
183 purification/equalization process with the same AMPure XP protocol and E-Gel
184 SizeSelect II Agarose Gel kit (Invitrogen). DNA quantitation was performed using a
185 Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific Inc). The pooled library was
186 sequenced on the Illumina Miseq using Reagent Kit V2 (500 cycle Nano Kit).
187 We processed the sequence data following Ushio [27]. The raw MiSeq data were
188 converted into FASTQ files using the bcl2fastq program provided by Illumina
189 (bcl2fastq v2.18). The FASTQ files were then demultiplexed using the command
190 implemented in Claident (http://www.claid ent.org; [28]). Demultiplexed FASTQ files

191 were analysed using the ASV method implemented in the package DADA2 [29] of R
192 [30]. At the quality filtering process, forward and reverse sequences were trimmed at
193 the length of 215 and 160, respectively, using DADA2::filterAndTrim() function.
194 Taxonomic identification was performed for ASVs (Amplicon Sequence
195 Variants; proxy for Operational Taxonomic Units) inferred using DADA2 based on the
196 query-centric auto-k-nearest-neighbour (QCauto) method [28] and subsequent
197 taxonomic assignment with the lowest common ancestor algorithm [31] using the
198 "semiall" database and clidentseq and classigntax commands implemented in claident
199 v0.2.2018.05.29. The reads identified as Bacteria or Archaea were used for further
200 analysis. Blast search was also conducted for the most abundant ASVs for which the
201 above procedure did not provide the identification of the order or lower taxonomic
202 levels. Top matches of the sequences were referred to in order to assign taxonomic
203 identities of the ASVs. Taxonomy, ASV table, and sample information were combined
204 into a single R object for downstream manipulation using the R package phyloseq [32].
205 We also removed ASVs that only occurred in negative control samples. Furthermore,
206 potential contaminant ASVs were identified statistically based on prevalence in negative
207 control samples using the R package decontam [33] based on ASV prevalence and a
208 threshold of 0.5. This resulted in elimination of 79 of 2679 16S ASVs.
209
210 **Fruit set and seed production**
211 The treated flowers for the observation of fruit set were hand-pollinated around
212 1700 using outcrossing pollen to ensure that the fruit set was not limited by pollen.
213 Pollen was taken with a wood toothpick from a cut flower from a pollen donor plant, of
214 which inflorescences had been covered with a mesh bag prior to anthesis to prevent
215 removal of pollen by flower visitors. In total, 251 pollinated flowers were monitored for
216 fruit and seed set (Table S1). Six weeks after the treatment, we surveyed whether the
217 fruits under the treatments were still retained on the inflorescence, and collected the
218 fruits (131 fruits) and counted developing seeds and aborted ovules inside. At that time,
219 the fruits were still green but fully plump.
220
221 **Statistical analysis of microbial community data**

To examine differences in the prokaryotic diversity among the treatments, we evaluated the Shannon diversity index of each sample using the phyloseq::estimate_richness() function. The index did not show a significant association with the total numbers of the reads (the results not shown). The differences among the treatments were tested using a nonparametric Kruskal-Wallis test.

We examined variation in the structure of the prokaryotic communities using the ASVs that were present in 20 or more flower samples (34 ASVs satisfied this criterion). The frequency of each ASV was divided by the number of the total sequences of the 34 ASVs in each sample to convert it to a proportion. The ASVs that accounted for more than 5.0 % in the samples (5 ASVs) on average were further identified by matching with the sequences of the type strains in the NCBI database (https://blast.ncbi.nlm.nih.gov/). The genus of the strain(s) with the highest similarity was assigned.

To compare microbial communities among flowers under the different treatments, we calculated the Bray–Curtis dissimilarity index using the package vegan [34] in R. We performed a permutational multivariate analysis of variance (PERMANOVA) using the vegan::adonis function to examine if flowers of the same individuals nested within under the same treatment have similar microbial communities. Ordinations were plotted with nonmetric multidimensional scaling (NMDS) using the vegan::metaMDS procedure. We then calculated the distances of each flower sample from the centroid of the treatment. Variation in the distances among the treatments were tested using vegan::anova for betadisper object.

To find ASVs that tend to co-occur in the samples, we conducted hierarchical clustering of ASVs based on Bray–Curtis dissimilarity using Ward's minimum variance algorithm using the hclust procedure. The algorithm merges clusters that minimize the increase in the sum of squared distances from the cluster centroid. We chose the number of clusters that maximize the silhouette index [35]. The index was calculated using silhouette in the R package cluster [36]. The proportions of the ASVs in the sample were visualized by a heatmap created using the R package ComplexHeatmap [37]. We identified the cluster that showed the largest difference in the median proportion in the samples under the microbial inoculate (MI) treatment compared with that under the water sprayed (WI) treatment. Differences in the proportions of the cluster between the

MI and WI treatments were also evaluated by the statistic of the Wilcoxon rank sum test.

Statistical analysis of fruit set and seed set data

To assess the factors associated with fruit set and seed production, we used generalized linear mixed models (GLMMs). To evaluate differences in fruit set among the treatments after controlling the difference among the individuals, we modeled fruit set (retained/aborted) with the treatments as a fixed factor and the individuals as a random factor using a binomial error distribution. Then we calculated and plotted estimated marginal means of fruit set for the treatments using the ggpredict() function of the R package ggeffects [38]. Then, to test the association of fruit set with microbial communities, we constructed the second model for fruit set. The model included microbial compositions as a fixed factor and the individuals as a random factor using a binomial error distribution. We identified Cluster 5 to include ASVs that showed the largest increase after the microbial inoculation (see below). Therefore, we used the average proportion of Cluster 5 of each treatment as the variable for the microbial composition. We evaluated the significance of the fixed factor by the Wald test.

We also modeled seed set (retained/aborted) and tested its association with microbial composition. The model included the proportion of Cluster 5 as a fixed factor and the individuals and fruit as nested random factors. We fitted the model with a binominal error distribution. The GLMM analyses for fruit and seed sets were performed using the R package lme4 [39].

RESULTS

Microbial communities

We obtained 804,963 reads of prokaryotic sequences, which were clustered into 2,600 ASVs. Samples without prokaryotic reads were removed from further analysis (four samples). This reduced the number of samples to 142. According to the rarefaction curves (Fig. S1), the sequencing captured most prokaryotic diversity. The median number of reads in the remaining samples was 670. The Shannon diversity index of the samples varied among samples, and the majorities fell between 2.0 and 3.5 (Fig. S2).

285 The diversity index was significantly different among the treatments, and was the
286 lowest in the paper bag (PB) treatment (Kruskal-Wallis χ^2 =18.2, df = 5, p = 0.0054).
287 The 34 ASVs that appeared in 20 or more samples were identified as bacteria,
288 and accounted for 75.7 % of the prokaryotic sequences. NMDS plots of the microbial
289 communities revealed that the flowers under different treatments in each plant
290 individual had different microbial communities, while the same treatment on different
291 plant individuals did not always converge the microbial communities into similar
292 compositions. For example, the OT flowers are plotted on the right of the flower bud
293 samples in the plant N1, while the OT flowers are on the left side of the buds in N5
294 (Fig. S3a). Only MI treatment resulted in the conversion of the prokaryotic
295 communities. PERMANOVA indicated that the communities were significantly
296 different among individuals (F = 5.52, R^2 = 0.106, p < 0.0001) and among the
297 treatments within each individual (F = 2.96, R^2 = 0.355, p < 0.0001). Variation in the
298 microbial communities within a treatment was significantly different among the
299 treatments (ANOVA, df = 5, F = 13.31, p < 0.0001), and it was the lowest in the MI
300 treatment, as suggested from the NMDS plots (Fig. S3b).
301 The cluster analyses grouped the 34 ASVs into 10 clusters (Fig. 2, Table S2).
302 Cluster 5, which accounts for the second highest proportion, showed the largest
303 difference in the proportion between the MI and WI treatments, followed by Cluster 1,
304 the most dominant cluster (Table S2, Fig. S4). The Wilcoxon rank-sum test showed that
305 the difference between the treatments was significant for both clusters (w = 90, p =
306 0.0001 for Cluster 1; and w = 501, p < 0.0001 for Cluster 5), while the difference was
307 not significant in the other clusters.
308 Blast search identified the two most abundant ASVs (T00020 and T00030) in
309 the dataset as *Cutibacterium* (Propionibacteriaceae) and *Ochrobactrum* (Brucellaceae)
310 in Cluster 1 (Table 1, Fig. 2). The third most-abundant ASVs (T00004) showed the
311 highest matches with sequences of *Erwinia* (Erwiniaceae) and the fourth and fifth
312 (T00005 and T0000) did with *Pseudomonas* (Pseudomonodaceae) in
313 Gammaproteobacteria (Table 1). The two *Pseudomonas* ASV sequences were different
314 for only five bases among 253 (2.0 %), so they may belong to the same species. These
315 three ASVs were included in Cluster 5 (Fig. 2).
316

317 **Fruit set and seed production**

318 The probability of fruit set of OT predicted by the first GLMM was 55.7 %, and
319 that of MI was the lowest (34.7 %) (Fig. 3b). Those of the other treatments were
320 between these two (Fig. 3b). On the other hand, the proportion of Cluster 5 showed the
321 opposite patterns; it is the lowest in OT and highest in MI. The GLMM including the
322 proportion of Cluster 5 as the fixed factor indicated that Cluster 5 was significantly
323 associated with low fruit set (GLMM, $z = -2.19$, $p = 0.0288$).

324 Each fruit that remained on the plants under the treatments had 12–24 ovules,
325 55.6 ± 20 % of which had developed. In contrast to fruit set, seed set was not
326 significantly associated with the microbial composition (GLMM, $z = 0.685$, $p > 0.05$).

327

328 **DISCUSSION**

329 **Prokaryotic community on *A. japonica* flowers**

330 *Alpinia* flowers harbored diverse prokaryote communities comparable with those
331 on flowers of other plant species reported so far [6,40]. ASVs that accounted for more
332 than 5% on average were *Cutibacterium* (T00020), *Ochrobactrum* (T00030), *Erwinia*
333 (T0004), and two *Pseudomonas* ASVs (T0005, T0007) (Table 1). The genus
334 *Cutibacterium* (Propionibacteriaceae) has rarely been reported as a dominant taxon in a
335 plant microbiome (but see [41]), and little is known about the role of this genus on
336 plants. On the other hand, *Ochrobactrum* is often found in the rhizosphere of various
337 plant species (e.g., [42]). Some strains of the genus have been reported to positively
338 affect plant survival and growth [43,44]. *Erwinia* and *Pseudomonas* are among the most
339 common constituents of the anthosphere [10]. They include many well-known plant
340 pathogens. *Erwinia amylovora* is one of the best-studied bacterial plant pathogens
341 causing fire blight in apple trees. The pathogen is transmitted among trees by flower
342 visitor insects as well as wind and rain [12]. *Psudomonas syringae* is also a well-studied
343 pathogen with many pathovars, which infect different host plants. *Pseudomonas*
344 *syringae* pv. *actinidiae*, the causal agent of kiwifruit bacterial canker, is vectored by
345 flower visitors, resulting in systemic invasion [45].

346 Though these major taxa were shared among the samples, prokaryotic
347 community compositions were significantly different among these closely related plant
348 individuals. The differences already existed in the buds prior to anthesis. Since flower

349 microbes are often shared by leaves of the same plant [6,7,46], the differences might
350 have originated from vegetative parts of the same individuals. The effects of most
351 treatments were not consistent across the individuals (Fig. S3). Although the paper bag
352 treatment decreased the Shannon diversity index (Fig. S2), we did not find a drastic
353 increase of particular ASVs by the treatment (Fig. 3). Growth of a small number of
354 bacteria species in the bag with high humidity and low UV-irradiance might have
355 reduced the diversity [47]. Prokaryotic diversity on open and pollinator-excluded net-
356 bagged flowers was more or less similar to that on the flower buds (Fig. S2). It has been
357 reported that flower visitors disperse microbes among flowers [2], and often increase
358 microbial diversity [48]. Flower visitors might not have large effects in our system,
359 however, partly due to the relatively low flower visitor activities during this year (N. E.
360 Jimenez and S. Sakai, personal observation).
361
362 **Identification of bacteria that were increased by inoculation**
363 We identified a group of ASVs that showed substantial changes after inoculation
364 of old-flower microbes. The cluster analyses grouped the ASVs into 10 clusters based
365 on the co-occurrence among the samples. The first and second most dominant clusters
366 of the 10 accounted for 41.2 % and 29.3 % of the sequence reads of the samples,
367 respectively, and were significantly different among the treatments. The microbial
368 inoculation treatment drastically increased Cluster 5 (Fig. 4), which contains three of
369 the five most dominant ASVs, *Erwinia* (T00004) and two *Pseudomonas* (T00005,
370 T00007) (Table 1). Microbial communities on old flowers might be dominated by these
371 taxa. The bacteria may have already colonized before anthesis, because these bacteria
372 were also found in flower buds at lower abundances. The proportions of Cluster 5 were
373 also moderately higher in the paper- and net-bagged and water-sprayed flowers than the
374 proportion under the open control treatment (Fig. S3b). Higher humidity due to these
375 treatments might have enhanced growth of these bacteria. In agriculture, it has often
376 been reported that high humidity or precipitation enhance activities of pathogenic
377 *Erwinia* and *Pseudomonas*, and increase damage by diseases [49–51].
378 Though the number of studies that investigate microbial communities on flowers
379 is rapidly increasing, no study has examined microbial communities on old flowers as
380 far as we know. The bacterial families Erwiniaceae and Pseudomonodaceae probably

381 dominating on old flowers of *A. japonicus* are among the most dominant taxa on
382 flowers [10]. Many bacterial species of these families are frequently found both on
383 flowers and inside the plant and/or on leaves [10]. Besides, flowers of unrelated plant
384 species in the same habitats often share common flower bacteria species [7,40].
385 Therefore, we consider that the bacteria of Group 5 may be generalist inhabitants on
386 living plants and senesced plant parts. However, identification of bacteria in most
387 microbial community studies of the anthosphere and phyllosphere are based on short
388 DNA sequences of hundreds of base pairs. To confirm habitat specificity within a plant
389 and adaptation to a specific host, cell lineage tracking using a bacterial strain with a
390 marker gene, or genomic comparisons among flower and leaf isolates from the same
391 and different plant hosts would be necessary [10].
392
393 **Effects of old-flower microbes on fruit and seed production**
394 Do the microbes that become dominant on old flowers negatively affect
395 reproductive success of the plant? We observed the lowest fruit set in the microbial
396 inoculated treatment, which recorded the highest abundance of Cluster 5. Besides, we
397 found significant negative associations between the proportion of the ASVs of Cluster 5
398 and fruit set based on the GLMM analysis, while we did not for seed set. Since *Erwinia*
399 and *Pseudomonas* include plant pathogens that trigger abortion of flowers and fruits
400 [51–53], some of these bacteria might have negatively affected fruit development by
401 infecting a reproductive organ. However, we cannot rule out other potential
402 explanations about the correlation. Fungi, rather than bacteria, that show similar
403 changes among the treatments with Cluster 5 might be the cause of the low fruit set.
404 Alternatively, bacteria and/or fungi dominant on old flowers might have indirectly
405 decreased fruit set by changing the microenvironment of flowers. Some flower microbes
406 are known to inhibit pollen germination or pollen tube growth [14,54], while the effects
407 of microbes on other post-pollination processes are largely understudied [55]. On the
408 other hand, it is unlikely that pollinator behavioral response affected the fruit set as
409 reported in previous studies [56,57], because we hand-pollinated the flowers under the
410 fruit set monitoring.
411
412 **CONCLUSION**

413 Compared with other floral traits, lifespan of flowers has received surprisingly
414 little attention. Some previous studies elegantly explained variation among species
415 based on the balance of the production and maintenance cost and benefit in terms of
416 reproductive outputs [44–46]. However, it has rarely been argued why plants so quickly
417 replace cheap, fragile flowers during the flowering period rather than maintaining
418 expensive but durable flowers [19]. This study is the first to show the potential negative
419 consequence of longer flower lifespan by non-pathogenic (microbes that do not cause
420 apparent symptoms) microbes. It is increasingly recognized that flowers have
421 characteristic microbial communities, but their effects and implications have just started
422 to be explored and our knowledge is still very fragmental [9,10,55,58]. Future studies
423 will elucidate the impacts of microbes on plant reproductive ecology deeply embedded
424 in the evolution of angiosperms [9].
425
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600

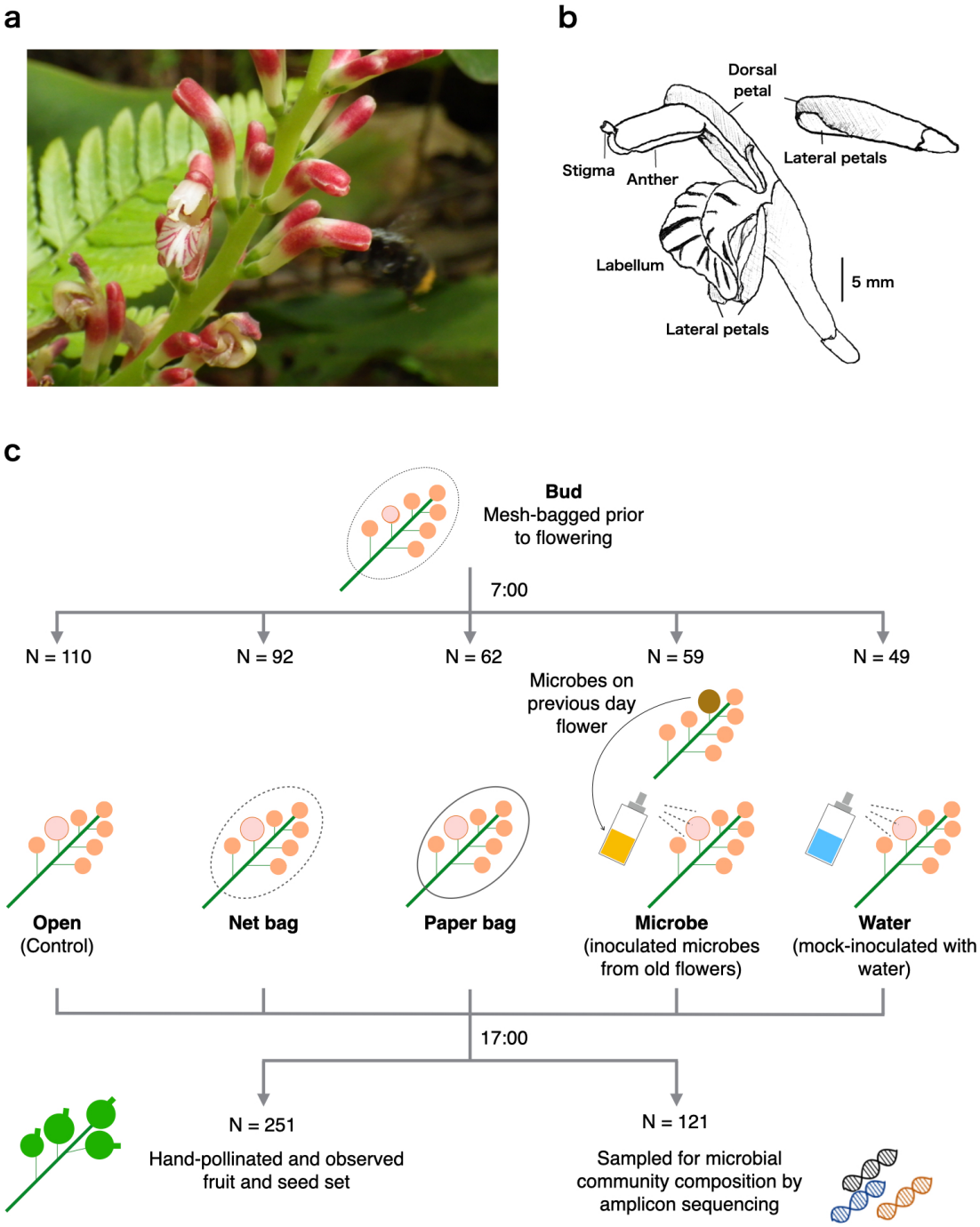
601 Table 1. The results of Blast search of the five dominant ASVs, and their mean percentages in the samples.

602

ASV	Class	Order	Family	Genus	Percentage
T00020	Actinobacteria	Actinomycetales	Propionibacteriaceae	*Cutibacterium*	17.20
T00030	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Ochrobactrum*	12.36
T00004	Gammaproteobacteria	Enterobacterales	Erwiniaceae	*Erwinia*	8.82
T00007	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	8.60
T00005	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	6.13

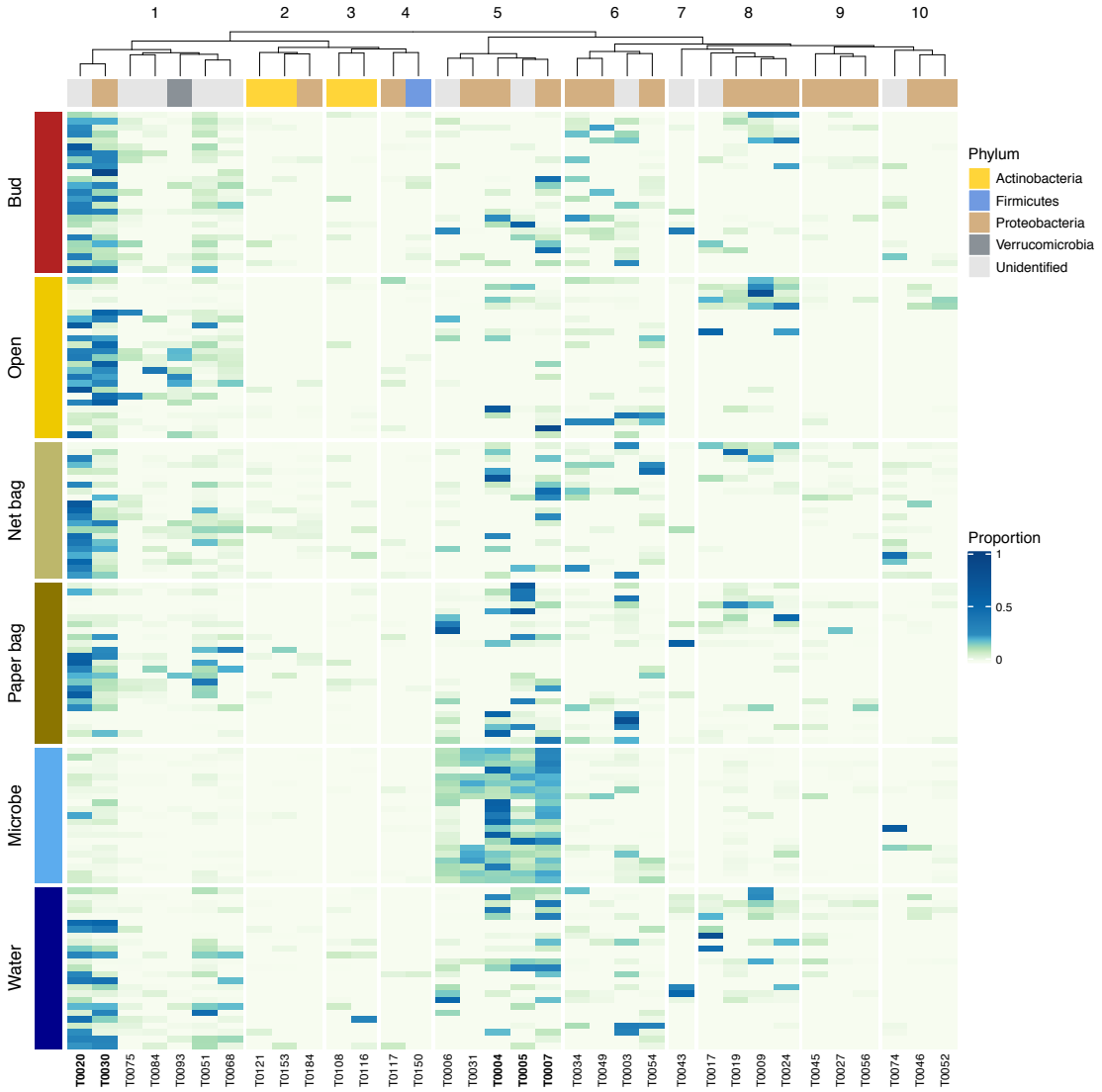
603

604 Figure 1. Experimental design used in this study. a, An open flower and flower buds on
605 an inflorescence of *Alpinia japonica*. b, Floral parts of *Alpinia japonica* in open state
606 (left) and bud state (right). c, Experimental and sampling procedures.

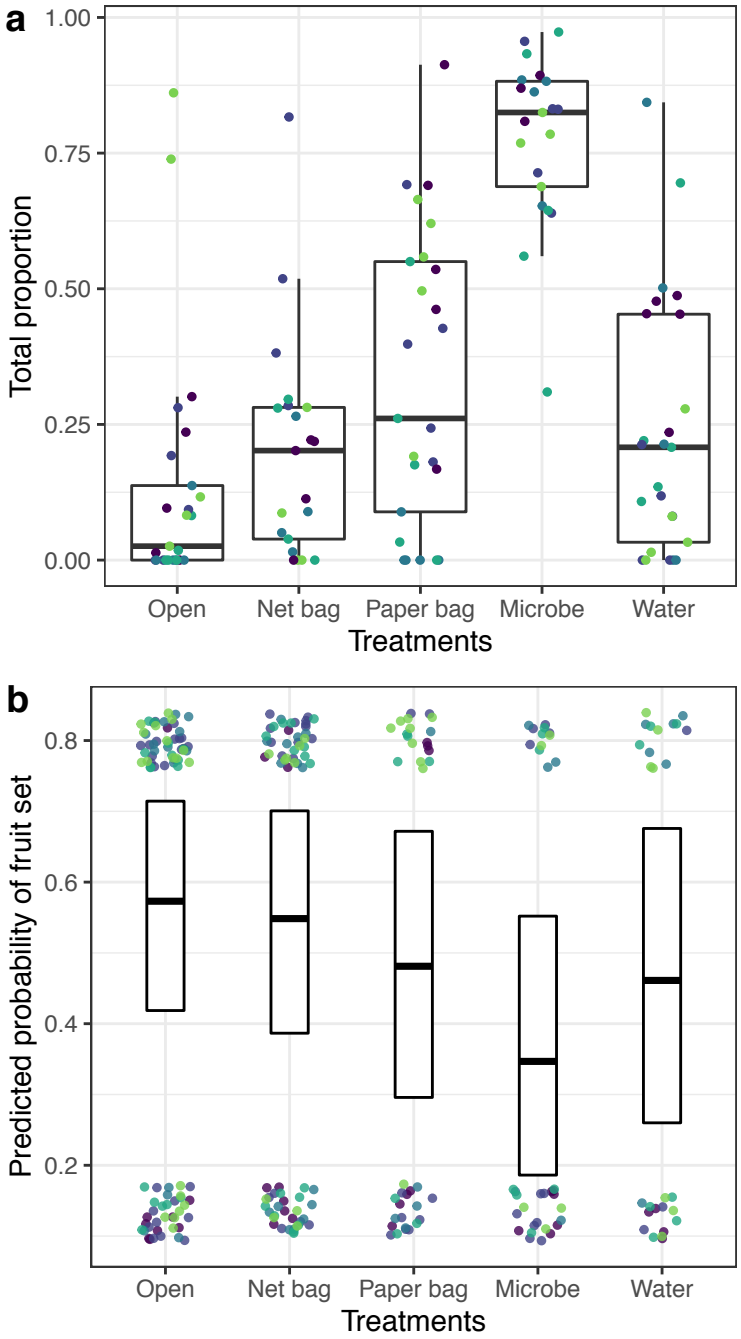


607

Figure 2. Heatmap of the prokaryotic communities of flower buds and flowers under different treatments. Samples were vertically arranged and grouped by the treatments as shown on the right. ASVs were clustered hierarchically based on Bray–Curtis dissimilarity using Ward's minimum variance algorithm. The phylum of each ASV is shown by the color below the tree. The color intensity in each panel shows the proportion in a sample, referring to the color key on the left.



615 Figure 3. Association between the proportion of Cluster 5 and predicted probability of
616 fruit set. (a) Variation in the total proportions of the bacteria among the treatments. (b)
617 The predicted probabilities of fruit set 6 weeks after flowering under the different
618 treatments by the GLMM. The thick lines and the boxes indicate the predicted values
619 and their 95 % confidential intervals. Distribution of flowers that set fruits (top) or were
620 aborted (bottom) are shown by dots. Colors indicate the five individual plants (see the
621 caption of Fig. 2).



622 Table S1. The number of the samples used to monitor fruit set for the five treatments
623 and to analyze microbial communities.

624

	Fruit set and seed production					Microbial community				
Treatments	N1	N2	N4	N5	N6	N1	N2	N4	N5	N6
Bud	-	-	-	-	-	5	5	5	5	5
Open	10	26	14	16	19	5	5	5	5	5
Net bag	9	17	15	17	10	5	4	4	4	4
Paper bag	7	9	5	7	9	5	5	5	5	5
Microbe	6	11	5	7	5	5	5	5	5	5
Water	4	3	6	7	7	3	5	4	5	4

625

626 Table S2. Characteristics of the 10 clusters. The number of ASVs included, the median
627 proportion in the samples of the microbial inoculated (MI) and water sprayed (WI)
628 treatments, and p values of Wilcoxon rank sum tests in the comparisons of the
629 proportions between MI and WI are shown. The asterisks indicate that the proportions
630 are significantly different between the treatments (**, $p < 0.001$; ***, $p < 0.0001$).
631 Cluster 5, which shows significantly higher proportions in MI than in WI, is highlighted
632 with boldface type.

633

Cluster ID	No. ASVs included	Median of proportions		p-value of Wilcoxon rank sum test (MI vs. WI)
		Microbe (MI)	Water (WI)	
Cluster 1	7	0.054	0.326	0.0001**
Cluster 2	3	0.000	0.000	0.4535
Cluster 3	2	0.000	0.000	0.0694
Cluster 4	2	0.000	0.000	0.3206
Cluster 5	**5**	**0.825**	**0.208**	**0.0000*** **
Cluster 6	4	0.027	0.036	0.7899
Cluster 7	1	0.000	0.000	0.1691
Cluster 8	4	0.015	0.075	0.2253
Cluster 9	3	0.000	0.000	0.6284
Cluster 10	3	0.000	0.000	0.2313

634

Figure S1. Rarefaction curves of the samples. The vertical axis shows the number of observed prokaryotic ASVs. The number of sequences per sample is shown on the horizontal axis. The three panels show the same graph with different ranges of the horizontal axis.

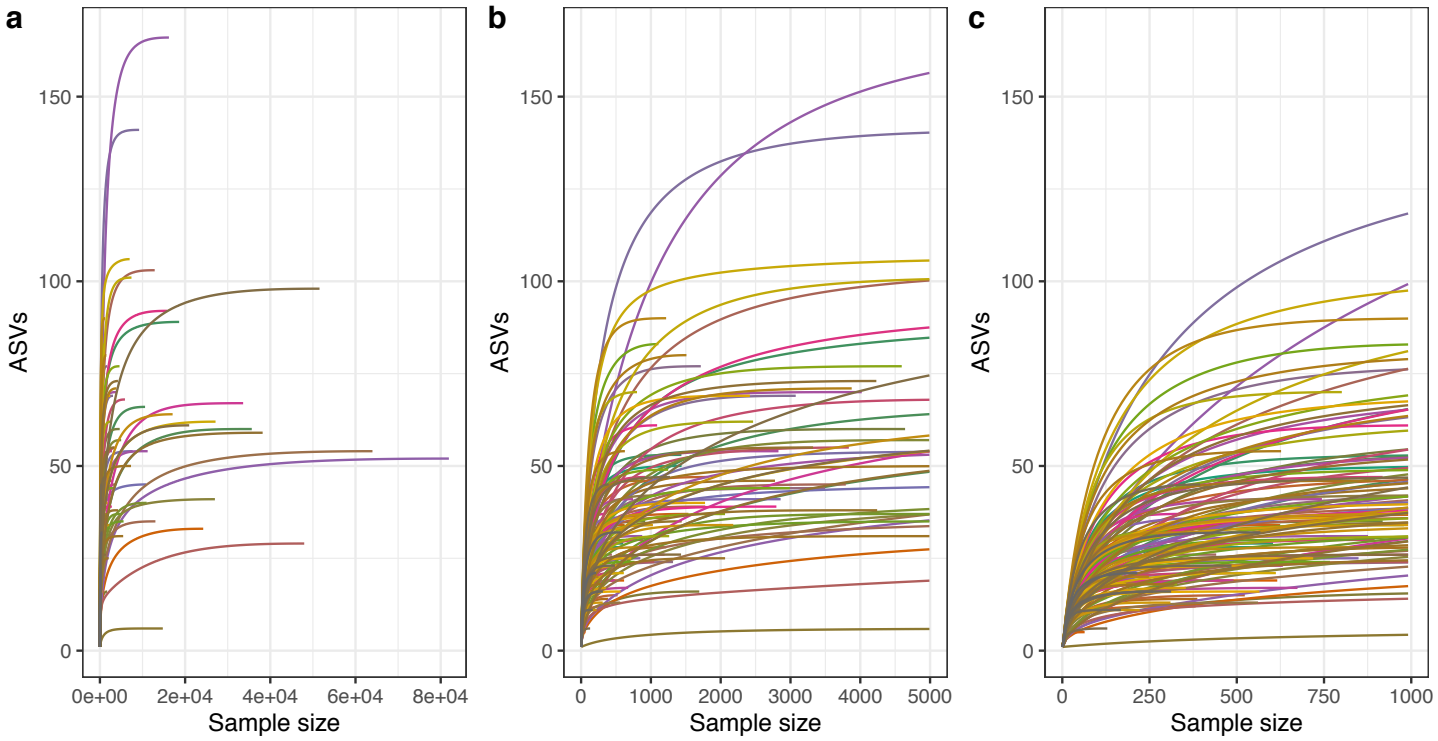


Figure S2. Variation in the prokaryotic diversity among the treatments and samples. The diversity was significantly different among the treatments (Kruskal-Wallis test; p = 0.0054). Colors indicate the five individual plants.

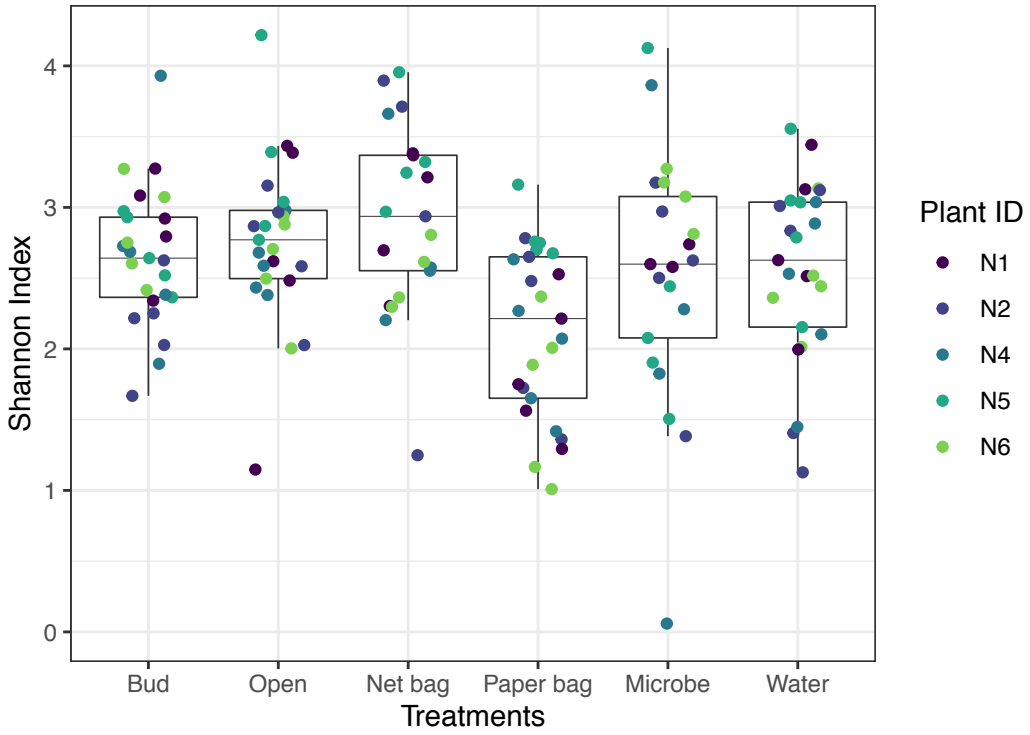
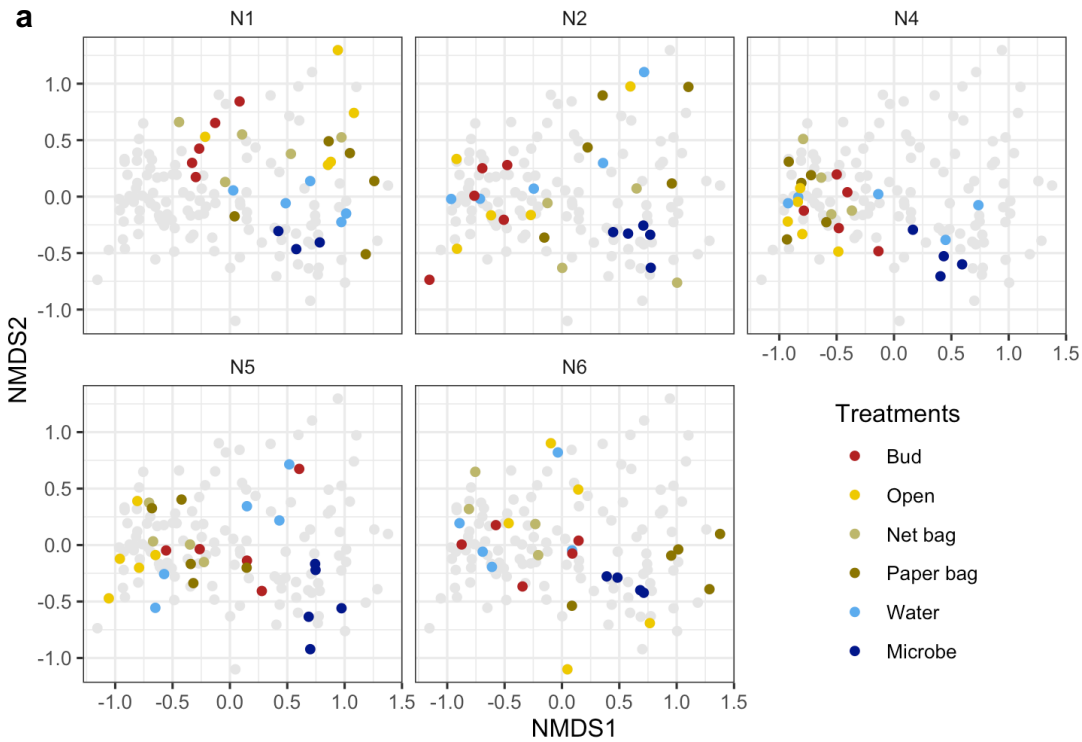


Figure S3. Variation of the prokaryotic communities on the flowers under different treatments. (a) NMDS plots of the microbial communities of flower buds and flowers under different treatments. In the five panels, all microbial samples are plotted with grey points, and samples from each plant are overlayed individually. The five treatments are indicated by different colors. (b) Distribution of the distances from the treatment centroid. The diversity was significantly different among the treatments (Kruskal-Wallis test; p = 0.0026). Colors indicate the five individual plants.



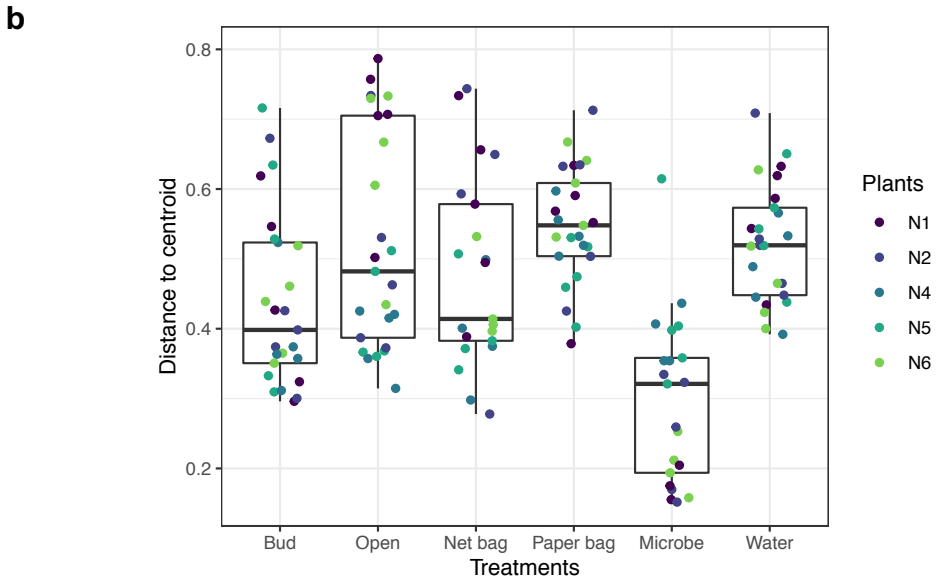
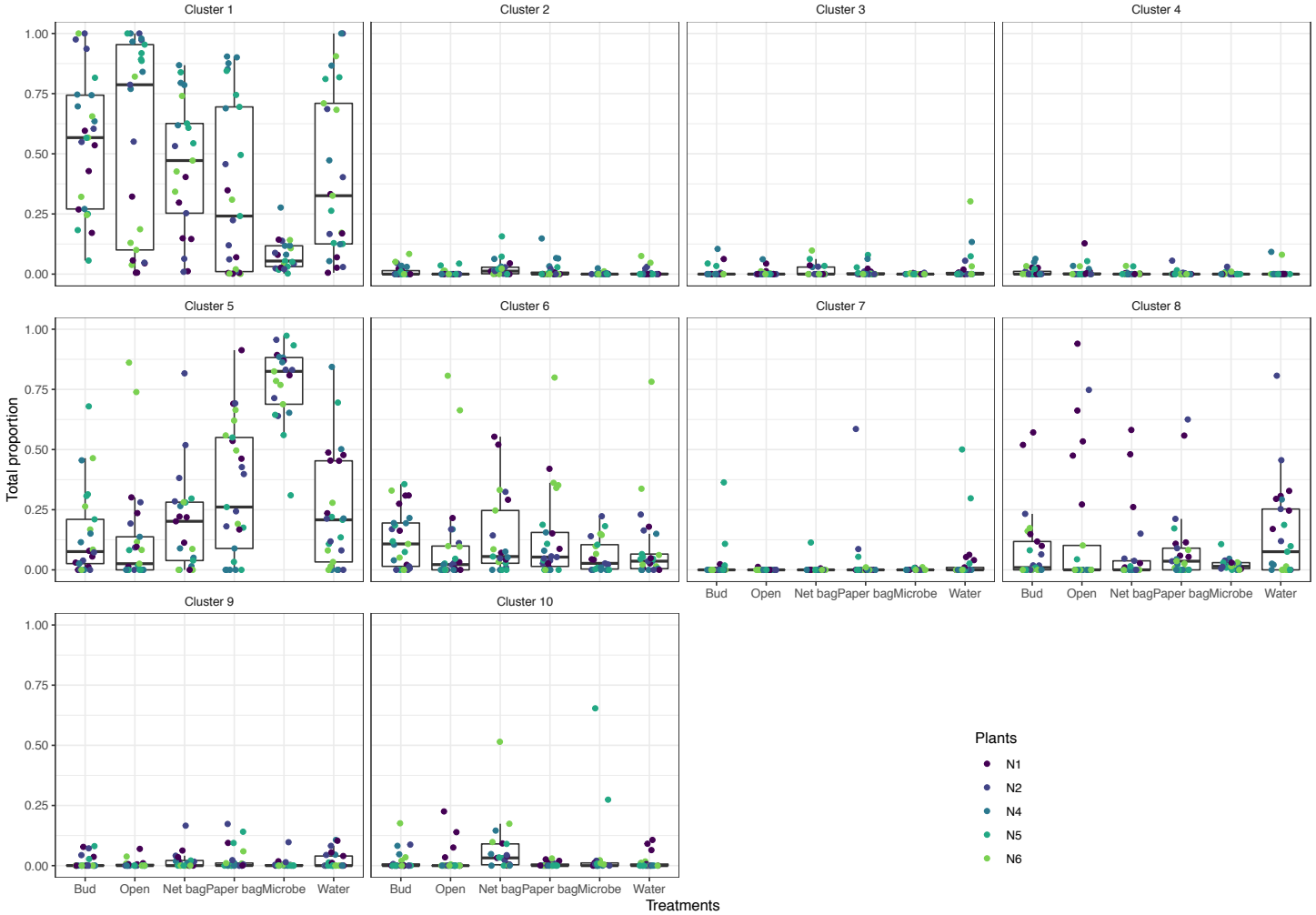



Figure S4. Variation in the total proportions of the 10 ASV clusters among the flower bud and flowers under the five treatments. Colors indicate the five individual plants.