Are microbes growing on flowers evil? 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 and inhabited by diverse microbes. Plants rapidly replace flowers with 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 six 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, suggesting that they had been present in small numbers prior to flowering. 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.

1. INTRODUCTON

Flowers are an elaborate 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 (Burdon et al. 2018; Kaltz and Shykoff 2001; Ushio et al. 2015). 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 (Roy et al. 2017). 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 (Taylor and Hepler 1997), 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 (Gaube et al. 2021; Massoni et al. 2020; Wei and Ashman 2018).

Today, flower microbes are increasingly recognized as essential components in the ecology and evolution of plant reproduction (reviewed in Rebolleda-Gómez et al. 2019; Vannette 2020). Not surprisingly, some flower microbes are known to have strong 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 (Bubán et al. 2003; Vanneste 2000). Other microbes impede various processes of pollination and fertilization. Nectar yeasts are reported to modify pollinator foraging patterns and reduce pollination success (Herrera et al. 2013). *Acinetobacter* bacteria that commonly inhabit floral nectar exploit pollen nutrition by inducing pollen germination and bursting (Christensen et al. 2021). Most studies of these phenomena examined the effects of an individual microbial species or of separate strains 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 (Vannette 2020). Flowers often have characteristics that may suppress microbial growth, such as flower volatiles (Burdon et al. 2018; Huang et al. 2012), reactive oxygen (McInnis et al. 2006) and secondary compounds (Dobson and Bergstrom 2000; Palmer-Young et al.

2019). An extremely short lifespan (from one to several days) of flowers (Primack 1985), especially in hot habitats (Bawa 1990), is also thought to reduce the chance of microbial infection and the proliferation of microbes (Kaltz and Shykoff 2001; Rogers 2006; Shykoff et al. 1996). Unlike the reproductive structures of mammals, flowers, the reproductive structures of the plants, are only retained while they are needed, and are developed *de novo* every season in perennial species (Rogers 2006). We do not know yet, however, to what extent microbes that are suppressed by abscission 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 coincides with the optimal season for microbial growth, while it may avoid the negative effects of microbes by renewing flowers daily. 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 whether these prokaryotes colonize via air or via flower visitors after flowering, or if they had already been present 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 microbe proliferation on flowers is generally to be avoided.

2. MATERIALS AND METHODS

2.1 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 (Asakawa et al. 2017; van Vuuren and Viljoen 2007). 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).

2.2 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.

2.3 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 the 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 transmitted to the flower by both air and pollinators; 2) insect exclusion by a net bag (NB), in which the inflorescence was left covered with the net, which allowed air but not insects to get through; 3) paper bagged (PB), in which the mesh bag covering the inflorescence was replaced by a new paper bag (Grape Bag, DAIICHI VINYL, Fukui, Japan) during 0700–1700 to exclude air flow and insect visits; 4) open and microbial inoculation (MI) and 5) open and water sprayed mock (WI) treatments. In MI, the mesh bag on the inflorescence was removed as in OT, and the flower was sprayed with microbial solution. During the spray inoculation, the focal flower was isolated from the rest of the inflorescence by a funnel-shaped plastic sheet to avoid contamination. The microbial suspension for the inoculation was prepared by soaking old flowers in distilled water. Nine old flowers that had opened the previous day from 1–3 individuals were collected from individuals that did not receive the treatments. Microbes were detached by ultrasonic dispersion in 10 ml of distilled water and kept at 4°C until the inoculation (maximum of 36 hours). Inoculation was conducted on eight different days, and the same inoculant was used for all flowers under MI treatment on the same day. In WI, the mesh bag covering the inflorescence was removed as in OT, and the flower was sprayed with distilled water as a control for the physical effect of the MI treatment. We did not conduct the treatments on cloudy or rainy days. Flowers that opened on the same day on the same inflorescence were assigned to the same treatments in the case of NB and PB, while OT, MI and WI were occasionally mixed on an inflorescence. This is because we bagged the entire inflorescence rather than a single flower to avoid damaging the flowers. The sample size of each treatment is summarized in Table S1.

2.4 Identification of microbial communities

171 To identify microbial communities, we sampled 4–5 flowers with each treatment
172 from each plant. The flowers were cropped at ~1700 of the day of flower opening and
173 separately put into a 5 ml plastic tube after removing the stigma (to use for another
174 study) and petals (Fig. 1b). In addition, five flower buds were collected from each
175 individual to identify microbes that were present inside prior to anthesis with the same
176 procedure. The tube was kept on ice until being brought to the lab within 4 hours after
177 sampling and kept at –20°C until further processing.
178 To detach microbes from the flower surface, we added 3 ml of phosphate-
179 buffered saline solution (PBS buffer, pH 7.4, Nippon Gene Co., Ltd.) to the tube and
180 sonicated it using an Ultrasonic Disruptor Handy Sonic UR-21P (Digital Biology) at
181 Level 5 power for 30 sec. Microbes in the PBS solution were then filtered using a
182 Sterivex Millipore filter (Sterivex-HV 0.22, Merck). Residual PBS on the Sterivex filter
183 was removed using a Vac-Man® Laboratory Vacuum Manifold (Promega).
184 Microbial DNA was extracted with DNeasy Blood & Tissue Kits (QIAGEN)
185 following the manufacturer's instructions. A portion of the 16S small-subunit ribosomal
186 gene was PCR-amplified using the primer pairs 515F (5'-GTG YCA GCM GCC GCG
187 GTA A-3') and 806R (5'-GGA CTA CNV GGG TWT CTA AT-3') (Caporaso et al.
188 2011) with Nextera DNA index adapters. First PCR was performed in 12 µl reactions,
189 each containing 1 µl of DNA template, 6 µl of KAPA HiFi HotMasterMix (Kapa
190 Biosystems) and 0.7 µl of each primer (5 µM). The first PCR was conducted with a
191 temperature profile of 95°C for 3 min, followed by 35 cycles at 98°C for 20 s, 60°C for
192 15 s, 72°C for 30 s, and a final extension at 72°C for 5 min, followed by storage at 4°C
193 until use. Triplicate PCR products were pooled and purified with Agencourt AMPure
194 XP (Beckman Coulter). P5/P7 Illumina adaptors were then added in a second PCR
195 using fusion primers with 8-mer index sequences for sample identification (Hamady et
196 al. 2008) (forward, 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-[8-mer
197 tag]-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG -3'; reverse, 5'-
198 CAA GCA GAA GAC GGC ATA CGA GAT-[8-mer tag]-GTC TCG TGG GCT CGG
199 AGA TGT GTA TAA GAG ACA G-3'). This preparation had a total volume of 24 µl
200 with double the amount of each reagent in the first PCR and 10-times diluted first PCR
201 products as DNA template. The temperature profile was 95°C for 3 min, followed by 12
202 cycles at 98°C for 20 s, 68°C for 15 s, 72°C for 15 s, a final extension at 72°C for 5

203 min, and 4°C until use. Second PCR products were pooled at equal volumes after a
204 purification/equalization process with the same AMPure XP protocol and E-Gel
205 SizeSelect II Agarose Gel kit (Invitrogen). DNA quantitation was performed using a
206 Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific Inc). The pooled library was
207 sequenced on an Illumina Miseq using Reagent Kit V2 (500 cycle Nano Kit).
208 We processed the sequence data following Ushio (Ushio 2019). The raw MiSeq
209 data were converted into FASTQ files using the bcl2fastq program provided by Illumina
210 (bcl2fastq v2.18). The FASTQ files were then demultiplexed using the command
211 implemented in Claident (http://www.claid ent.org; (Tanabe and Toju 2013)).
212 Demultiplexed FASTQ files were analysed using the ASV method implemented in the
213 package DADA2 (Callahan et al. 2016) of R (R Core Team 2016). At the quality
214 filtering process, forward and reverse sequences were trimmed at the length of 215 and
215 160, respectively, using DADA2::filterAndTrim() function.
216 Taxonomic identification was performed for ASVs (Amplicon Sequence
217 Variants; proxy for Operational Taxonomic Units) inferred using DADA2 based on the
218 query-centric auto-k-nearest-neighbour (QCauto) method (Tanabe and Toju 2013) and
219 subsequent taxonomic assignment with the lowest common ancestor algorithm (Huson
220 et al. 2007) using the "merge" database and clidentseq and classigntax commands
221 implemented in claident v0.2.2018.05.29. The reads identified as Bacteria or Archaea
222 were used for further analysis. Taxonomy, ASV table, and sample information were
223 combined into a single R object for downstream manipulation using the R package
224 phyloseq (McMurdie and Holmes 2013). We also removed ASVs that only occurred in
225 negative control samples. Furthermore, potential contaminant ASVs were identified
226 statistically based on prevalence in negative control samples using the R package
227 decontam (Davis et al. 2018) based on ASV prevalence and a threshold of 0.5. This
228 resulted in elimination of 87 of 2915 16S ASVs.
229
230 **2.5 Fruit set and seed production**
231 The treated flowers for the observation of fruit set under all the treatments were
232 hand-pollinated around 1700 using outcrossing pollen to ensure that the fruit set was not
233 limited by pollen. Pollen was taken with a wood toothpick from a cut flower from a
234 pollen donor plant, of which inflorescences had been covered with a mesh bag prior to

anthesis to prevent removal of pollen by flower visitors. In total, 251 pollinated flowers were monitored for fruit and seed set (Table S1). Six weeks after the treatment, we surveyed whether the fruits under the treatments were still retained on the inflorescence, and collected the fruits (131 fruits) and counted developing seeds and aborted ovules inside. At that time, the fruits were still green but fully plump.

2.6 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. Significant differences in the index among treatments were tested using Generalized Linear Mixed Model (GLMM). Prior to the analysis, we confirmed that distributions of the index were not significantly deviated from the normal distribution (Shapiro-Wilk test, $W = 0.983$, $p = 0.0759$). We constructed a GLMM with the treatment as a fixed term and Plant ID as a random factor using the R package lme4 (Bates et al. 2015). Significance of the fixed term was tested by comparing the full model and the model without the fixed term using the anova() function.

We examined variation in the structure of the prokaryotic communities using the ASVs that were present in 20 or more flower samples (36 ASVs satisfied this criterion). The frequency of each ASV was divided by the number of the total sequences of the 36 ASVs in each sample to convert it to a proportion.

To compare microbial communities among flowers under the different treatments, we calculated the Bray–Curtis dissimilarity index using the package vegan (Oksanen et al. 2010) 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 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

267 algorithm using the hclust procedure. The algorithm merges clusters that minimize the
268 increase in the sum of squared distances from the cluster centroid. We chose the number
269 of clusters that maximize the silhouette index (Rousseeuw 1987). The index was
270 calculated using silhouette in the R package cluster (Maechler et al. 2001). The
271 proportions of the ASVs in the sample were visualized by a heatmap created using the R
272 package ComplexHeatmap (Gu et al. 2016). We identified the cluster that showed the
273 largest difference in the median proportion in the samples under the microbial inoculate
274 (MI) treatment compared with that under the water sprayed (WI) treatment. Differences
275 in the proportions of the cluster between the MI and WI treatments were also evaluated
276 by the statistic of the Wilcoxon rank sum test. The identification of the ASVs in the
277 cluster was searched against the Nucleotide database of the National Center for
278 Biotechnology Information (NCBI) with BLAST (Altschul et al. 1990). Top matches of
279 the sequences were referred to in order to assign taxonomic identities (Class, Family,
280 Genus) of the ASVs.
281
282 **2.7 Statistical analysis of fruit set and seed set data**
283 To assess the factors associated with fruit set and seed production, we used
284 generalized linear mixed models (GLMMs). To evaluate differences in fruit set among
285 the treatments after controlling for the difference among the individuals, we modeled
286 fruit set (retained/aborted) with the treatments as a fixed factor and the individuals as a
287 random factor using a binomial error distribution. Then we calculated and plotted
288 estimated marginal means of fruit set for the treatments using the ggpredict() function of
289 the R package ggeffects (Lüdecke 2018). Then, to test the association of fruit set with
290 microbial communities, we constructed the second model for fruit set. The model
291 included microbial compositions as a fixed factor and the individuals as a random factor
292 using a binomial error distribution. We identified Cluster 6 to include ASVs that
293 showed the largest increase after the microbial inoculation (see below). Therefore, we
294 used the average proportion of Cluster 6 of each treatment as the variable for the
295 microbial composition. We evaluated the significance of the fixed factor by the Wald
296 test.
297 We also modeled seed set (retained/aborted) and tested its association with
298 microbial composition. The model included the proportion of Cluster 6 as a fixed factor

299 and the individuals and fruit as nested random factors. We fitted the model with a
300 binominal error distribution. The GLMM analyses for fruit and seed sets were
301 performed using the R package lme4 (Bates et al. 2015).
302
303 **3. RESULTS**
304 **3.1 Microbial communities**
305 We obtained 839,113 reads of prokaryotic sequences, which were clustered into
306 2,828 ASVs. We have removed samples that had < 250 prokaryotic reads (12 samples).
307 This reduced the number of samples to 134. According to the rarefaction curves (Fig.
308 S1), the sequencing captured most prokaryotic diversity in these 134 samples. The
309 median number of reads in the remaining samples was 1354.5. The microbial
310 communities on *Alpinia japonica* flowers were by far dominated by the bacterial
311 phylum Proteobacteria, which accounted for 47.8 % in the samples on average (Fig. 2).
312 At the class level, Gammaproteobaceteria was the most dominant (16.5 %), followed by
313 Alphaproteobacteria (6.1 %) and Betaproteobacteria (5.8 %). The Shannon diversity
314 index of the samples varied among samples, and the majority fell between 1.5 and 3.5
315 (Fig. S2). The diversity index was significantly different among the treatments, and was
316 the lowest in the paper bag (PB) treatment (GLMM, $\chi^2 = 22.23$, $p = 0.0005$).
317 The 36 ASVs that appeared in 20 or more samples were identified as bacteria,
318 and accounted for 75.7 % of the prokaryotic sequences. NMDS plots of the microbial
319 communities revealed that the flowers under different treatments in each plant
320 individual had different microbial communities, while the same treatment on different
321 plant individuals did not always result in microbial communities with similar
322 compositions. For example, the OT flowers are plotted on the right of the flower bud
323 samples in the plant N1, while the OT flowers are on the left side of the buds in N4 and
324 N5 (Fig. S3a). Only MI treatment resulted in the conversion of the prokaryotic
325 communities. PERMANOVA indicated that the communities were significantly
326 different among individuals ($F = 4.89$, $R^2 = 0.100$, $p < 0.0001$) and among the
327 treatments within each individual ($F = 2.85$, $R^2 = 0.366$, $p < 0.0001$). Variation in the
328 microbial communities within a treatment was significantly different among the
329 treatments (ANOVA, $df = 5$, $F = 8.60$, $p < 0.0001$), and it was the lowest in the MI
330 treatment, as suggested from the NMDS plots (Fig. S3b).

The cluster analyses grouped the 36 ASVs into 11 clusters (Fig. 3, Table S2). The first and second most dominant clusters of the 11 include 4 and 6 ASVs and accounted for 33.0 % and 30.6 % of the sequence reads of the 36 ASVs, respectively (Table S2). Cluster 6, which accounted for the second highest proportion, showed the largest difference in the proportion between the MI and WI treatments, followed by Cluster 2, the most dominant cluster (Table S2, Fig. S4). The Wilcoxon rank-sum test showed that the difference between the treatments was the most significant for Cluster 6 ($w = 444$, $p < 0.0001$ for Cluster 6), while Clusters 2 and 5 also showed significant differences ($w = 78.0$, $p = 0.0001$ for Cluster 2 and $w = 127.0$, $p = 0.0071$ for Cluster 5).

The Claident pipeline identified the most abundant 4 ASVs out of the six in Cluster 6 as belonging to *Pseudomonas* (T00005, T00006 and T0007) and *Erwinia* (T0004). The three *Pseudomonas* ASV sequences were different for five to eight bases among 253 (2.0–3.0 %). Blast search further identified the other two ASVs (T00028 and T00031) in the dataset as *Luteibacter* (Rhodanobacteraceae, Gammaproteobacteria) and *Massilia* (Oxalobacteraceae, Betaproteobacteria) (Table 1).

3.2 Fruit set and seed production

The probability of fruit set of OT predicted by the first GLMM with the treatment as the fixed factor was 57.3 %, and that of MI was the lowest (34.7 %) (Fig. 3b). Those of the other treatments were between these two (Fig. 3b). On the other hand, the proportion of Cluster 6 showed the opposite patterns; it was the lowest in OT and highest in MI. The GLMM including the proportion of Cluster 6 as the fixed factor indicated that Cluster 6 was significantly associated with low fruit set (GLMM, $z = -2.21$, $p = 0.0271$).

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

4 DISCUSSION

4.1 Prokaryotic community on *A. japonica* flowers

Alpinia flowers harbored diverse prokaryote communities comparable with those on flowers of other plant species reported so far (Berg et al. 2017; Müller et al. 2016;

363 Qian et al. 2021; Wei and Ashman 2018). They are dominated by the phylum
364 Proteobacteria, most of which belonged to Gammaproteobactera and
365 Alphaproteobacteria. *Erwinia* and *Pseudomonas* of the Gammaproteobactera are among
366 the most common constituents of the anthosphere (Vannette 2020).
367 Though these major taxa were shared among the samples, prokaryotic
368 community compositions were significantly different among these plant individuals.
369 The differences already existed in the buds prior to anthesis. Since flower microbes are
370 often shared by leaves of the same plant (Junker et al. 2011; Massoni et al. 2020; Wei
371 and Ashman 2018), the differences might have originated from vegetative parts of the
372 same individuals. The effects of most treatments were not consistent across the
373 individuals (Fig. S3). Although the paper bag treatment decreased the Shannon diversity
374 index (Fig. S2), we did not find a drastic increase of particular ASVs by this treatment
375 (Fig. 3). Growth of a small number of bacteria species in the bag with high humidity
376 and low UV-irradiance might have reduced the diversity (Hayes et al. 2021).
377 Prokaryotic diversity on open and pollinator-excluded net-bagged flowers was not
378 drastically different from that on the flower buds (Fig. S2). It has been reported that
379 flower visitors disperse microbes among flowers (Ushio et al. 2015), and often increase
380 microbial diversity (Allard et al. 2018). Flower visitors might not have had large effects
381 in our system, however, partly due to the relatively low flower visitor activities during
382 the year of this study (N. E. Jimenez and S. Sakai, personal observation).
383
384 **4.2 Identification of bacteria that were increased by inoculation**
385 We identified a group of ASVs that showed substantial changes after inoculation
386 of old-flower microbes. The cluster analyses grouped the ASVs into 11 clusters based
387 on the co-occurrence among the samples. The microbial inoculation treatment
388 drastically increased Cluster 6 (Figs. 3 and 4), which consists of 6 ASVs. Among the
389 ASVs, four ASVs, one *Erwinia* (T00004) and three *Pseudomonas* (T00005, T00006 and
390 T00007) ASVs accounted for 90 % of the reads of the cluster on average (Table 1).
391 Microbial communities on old flowers might be dominated by these taxa. The bacteria
392 may have already colonized before anthesis, because these bacteria were also found in
393 flower buds at lower abundances as well as in the paper- and net- bagged flowers. The
394 proportions of Cluster 6 were also moderately higher in the bagged and water-sprayed

395 flowers than the proportion under the open control treatment (Fig. S3b). Higher
396 humidity due to these treatments might have enhanced growth of these bacteria. In
397 agriculture, it has often been reported that high humidity or precipitation enhances
398 activities of pathogenic *Erwinia* and *Pseudomonas*, and increases damage by diseases
399 (Billing 1980; Llontop et al. 2020; Pietrarelli et al. 2006).
400 Though the number of studies that investigate microbial communities on flowers
401 is rapidly increasing, no study has examined microbial communities on old flowers as
402 far as we know. Our results suggested that old flowers *A. japonicus* flowers in our field
403 site were dominated by bacteria of *Erwinia* and *Pseudomonas*. Many bacterial species
404 of these genera are frequently found both on flowers and inside the plant and/or on
405 leaves (Vannette 2020). Interestingly, we found all the proteobacterial ASVs in Cluster
406 6 from flowers of an unrelated dioecious tree, *Mallotus japonicus* (Euphorbiaceae). In
407 particular, T0007 was the most abundant and frequent ASV on male flowers of the plant
408 and was present in 72.3 % of male flower samples (Marre et al. 2021 (T0007 is referred
409 to as ASV02P1in this article), and the dataset used for the study (DRA013108 in
410 DDBJ)). Therefore, we consider that the bacteria of Group 6 may be generalist
411 inhabitants on plants, which proliferate on senesced plant parts. It is not uncommon that
412 flowers of unrelated plant species in the same habitats share flower bacteria species
413 (Massoni et al. 2020; Qian et al. 2021). It should be noted, however, that ASVs in this
414 study are distinguished based on relatively short DNA sequences of 253 base pairs, as is
415 often the case in most anthosphere and phylosphere studies. Besides, we have analyzed
416 the anthosphere of only two plant species so far. To confirm the generalist habit of the
417 bacteria, more detailed examination such as sequencing of additional regions and genes,
418 more extensive sampling and culture isolation would be necessary.
419
420 **4.3 Effects of old-flower microbes on fruit and seed production**
421 Do the microbes that become dominant on old flowers negatively affect
422 reproductive success of the plant? We observed the lowest fruit set in the microbial
423 inoculated treatment, which recorded the highest abundance of Cluster 6. Besides, we
424 found significant negative associations between the proportion of the ASVs of Cluster 6
425 and fruit set based on the GLMM analysis, while we did not find such associations for
426 seed set. Since *Erwinia* and *Pseudomonas* include plant pathogens that trigger abortion

of flowers and fruits (Goumas et al. 1999; Llontop et al. 2020; Marre et al. 2022; Young 1988), some of these bacteria might have negatively affected fruit development by infecting a reproductive organ. However, we cannot rule out other potential explanations about the correlation. Fungi, rather than bacteria, that show similar changes among the treatments with Cluster 6 might have been the cause of the low fruit set. Alternatively, bacteria and/or fungi dominant on old flowers might have indirectly decreased fruit set by changing the microenvironment of flowers. Some flower microbes are known to inhibit pollen germination or pollen tube growth (Christensen et al. 2021; Eisikowitch et al. 1990), while the effects of microbes on other post-pollination processes are largely understudied (Cullen et al. 2021). On the other hand, it is unlikely that pollinator behavioral response affected the fruit set as reported in previous studies (Sobhy et al. 2018; Vannette et al. 2013), because we hand-pollinated the flowers under the fruit set monitoring.

5. CONCLUSION

Compared with other floral traits, lifespan of flowers has received surprisingly little attention. Some previous studies elegantly explained variation among species based on the balance of the production and maintenance cost and benefit in terms of reproductive outputs (Ashman 2004). However, it has rarely been argued why plants so quickly replace cheap, fragile flowers during the flowering period rather than maintaining expensive but durable flowers. Short lifespan of the flower may be a defense measure of the plant to escape from negative effects of the microbes that proliferate on flowers during anthesis, though these microbes may not cause apparent symptoms. It is increasingly recognized that flowers have characteristic microbial communities, but the effects and implications of these communities have just started to be explored and our knowledge about them is still very fragmentary (Cullen et al. 2021; Rebolleda-Gómez et al. 2019; Rowe et al. 2020; Vannette 2020). Future studies will elucidate the impacts of microbes on plant reproductive ecology deeply embedded in the evolution of angiosperms (Rebolleda-Gómez et al. 2019).

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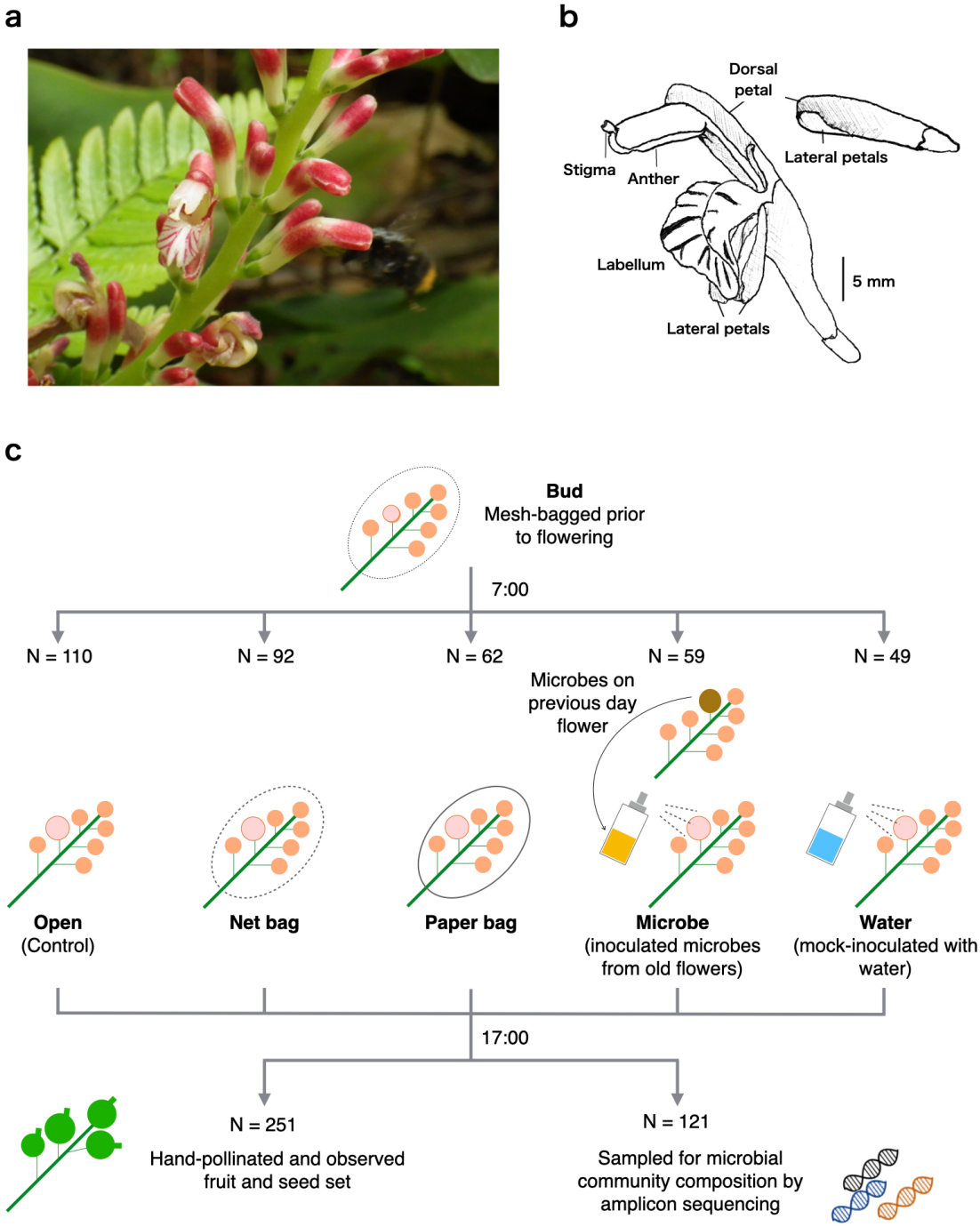
650 TABLE 1. The 6 ASVs in Cluster 6. Taxonomic information was based on Claident (shown by normal face) supplemented by BLAST
651 search (shown by bold face with percent of identical bases). The results of BLAST search of the ASVs in Cluster 6, and their percent of
652 matches.

ASV	Phylum	Class	Family	Genus	Percent identity[1]	Average proportion among samples[2]
T0004	Proteobacteria	Gammaproteobacteria	**Erwiniaceae**	***Erwinia***	100.0	0.086
T0005	Proteobacteria	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*		0.062
T0006	Proteobacteria	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*		0.040
T0007	Proteobacteria	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*		0.086
T0028	Proteobacteria	**Betaproteobacteria**	**Oxalobacteraceae**	***Massilia***	99.2	0.017
T0031	Proteobacteria	Gammaproteobacteria	Rhodanobacteraceae	***Luteibacter***	99.2	0.015

653 Note: 1, The highest percentage of the identical bases that resulted from the BLAST search; it is shown only for the ASVs that were
654 subjected to the BLAST search due to incomplete taxonomic assignments by Claident. 2, Average proportions of the ASV of 36 prevalent
655 ASVs used for the heatmap in Figure 3.

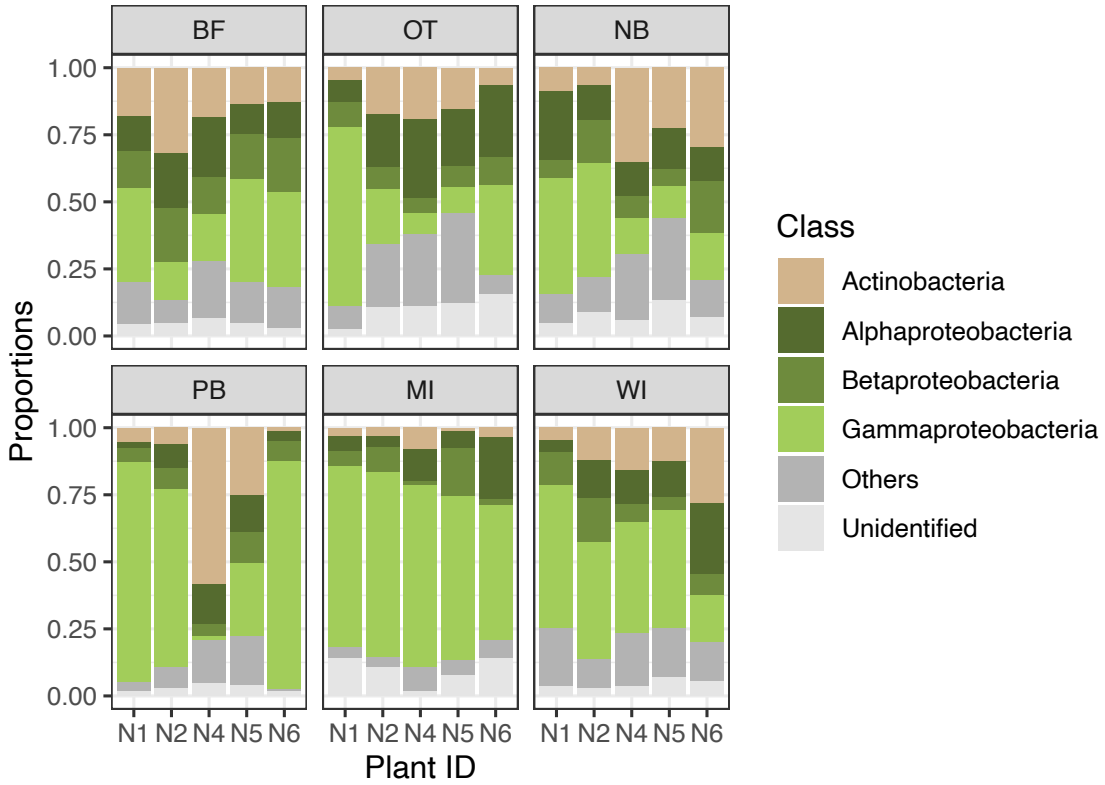
656 FIGURE 1. Experimental design used in this study. a, An open flower and flower buds
657 on an inflorescence of *Alpinia japonica*. b, Floral parts of *Alpinia japonica* in open state
658 (left) and bud state (right). c, Experimental and sampling procedures.
659
660 FIGURE 2. Composition of prokaryotic communities on the flowers of *Alpinia*
661 *japonica*. Proportions in the samples of the same treatment in the same individuals were
662 averaged. See Table S1 for sample sizes.
663
664 FIGURE 3. Heatmap of the prokaryotic communities of flower buds and flowers under
665 different treatments. Samples were vertically arranged and grouped by the treatments as
666 shown on the right. ASVs were clustered hierarchically based on Bray–Curtis
667 dissimilarity using Ward's minimum variance algorithm. The class of each ASV is
668 shown by the color below the tree. The color intensity in each panel shows the
669 proportion in a sample, referring to the color key on the left.
670
671 FIGURE 4. Association between the proportion of Cluster 6 and predicted probability
672 of fruit set. (a) Variation in the total proportions of the bacteria among the treatments.
673 (b) The predicted probabilities of fruit set 6 weeks after flowering under the different
674 treatments by the GLMM with the treatment as the fixed factor. The thick lines and the
675 boxes indicate the predicted values and their 95 % confidential intervals. Distribution of
676 flowers that set fruits (top) or were aborted (bottom) are shown by dots. Colors indicate
677 the five individual plants).

678 FIGURE 1


a
b
Dorsal petal
Lateral petals
Stigma
Anther
Labellum
5 mm
Lateral petals
c
Bud
Mesh-bagged prior to flowering
7:00
N = 110
N = 92
N = 62
N = 59
N = 49
Microbes on previous day flower
Open
(Control)
Net bag
Paper bag
Microbe
(inoculated microbes from old flowers)
Water
(mock-inoculated with water)
17:00
N = 251
Hand-pollinated and observed fruit and seed set
N = 121
Sampled for microbial community composition by amplicon sequencing

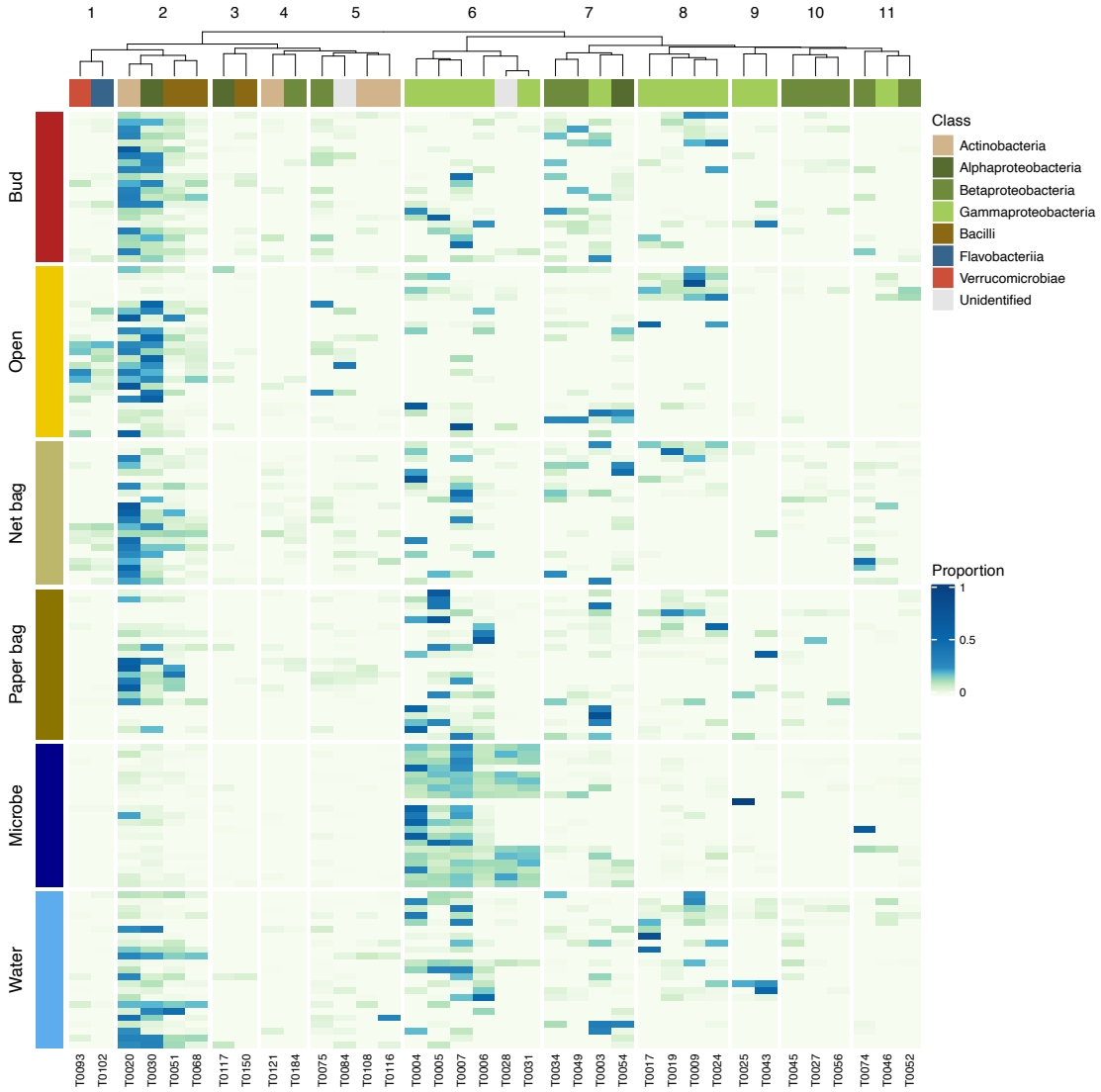
679 FIGURE 2

680

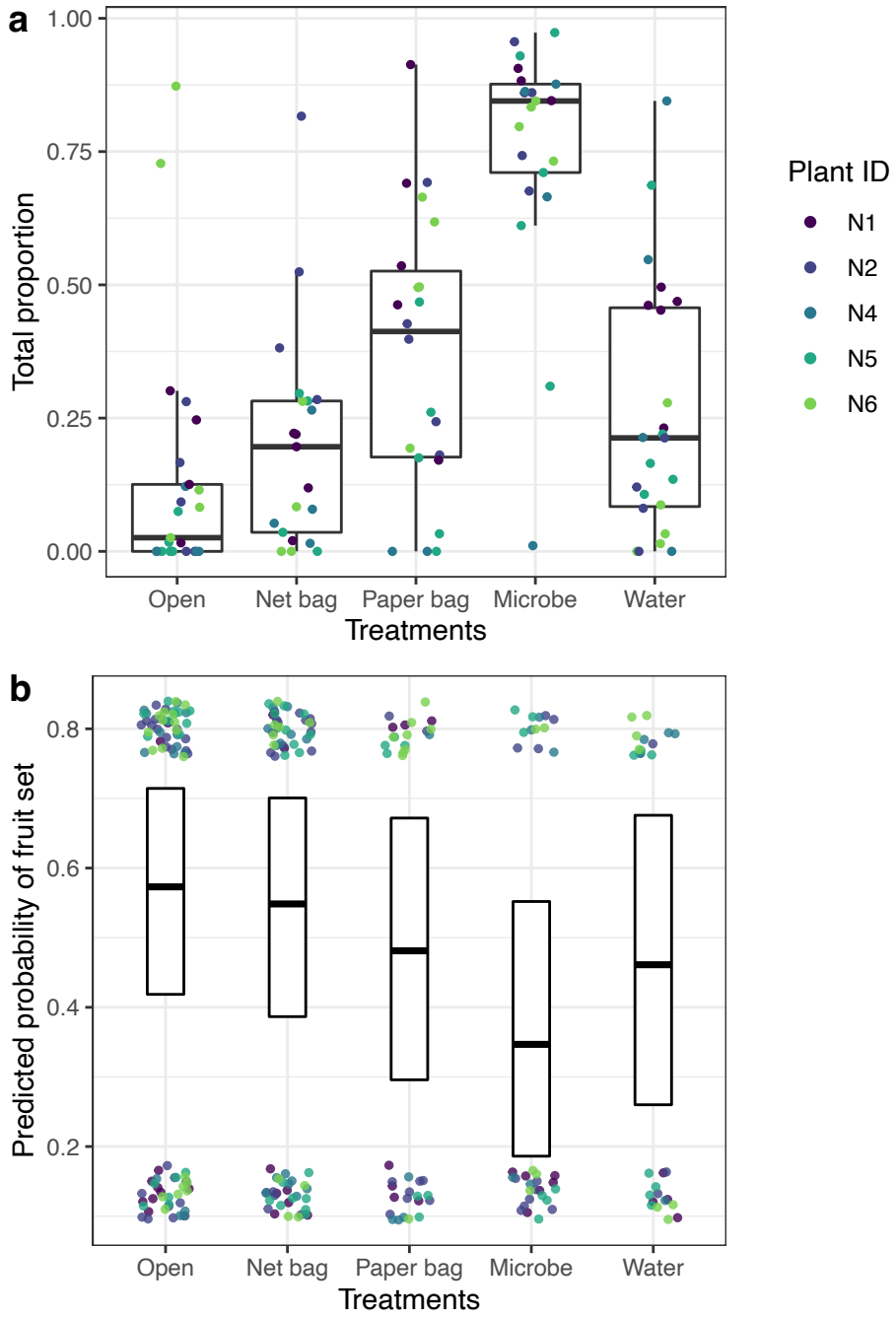


681 FIGURE 3

682



683 FIGURE 4


a
1.00
0.75
0.50
0.25
0.00
Total proportion
Open
Net bag
Paper bag
Microbe
Water
Treatments
Plant ID
N1
N2
N4
N5
N6
b
0.8
0.6
0.4
0.2
Predicted probability of fruit set
Open
Net bag
Paper bag
Microbe
Water
Treatments

684 TABLE S1. The number of the samples used to monitor fruit set for the five treatments
685 and to analyze microbial communities.

686

	Fruit set and seed production					Microbial community				
Treatments	N1	N2	N4	N5	N6	N1	N2	N4	N5	N6
Bud	-	-	-	-	-	5	4	4	5	4
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	2	5	5
Microbe	6	11	5	7	5	3	5	4	5	4
Water	4	3	6	7	7	5	4	4	5	5

687

688 TABLE S2. Characteristics of the 11 clusters. The number of ASVs included, the
689 median proportion in the samples of the microbial inoculated (MI) and water sprayed
690 (WI) treatments, and p values of Wilcoxon rank sum tests in the comparisons of the
691 proportions between MI and WI are shown. The asterisks indicate that the proportions
692 are significantly different between the treatments (*, $p < 0.01$;**, $p < 0.001$; ***, $p <$
693 0.0001). Cluster 6, which shows significantly higher proportions in MI than in WI, is
694 highlighted with boldface type.

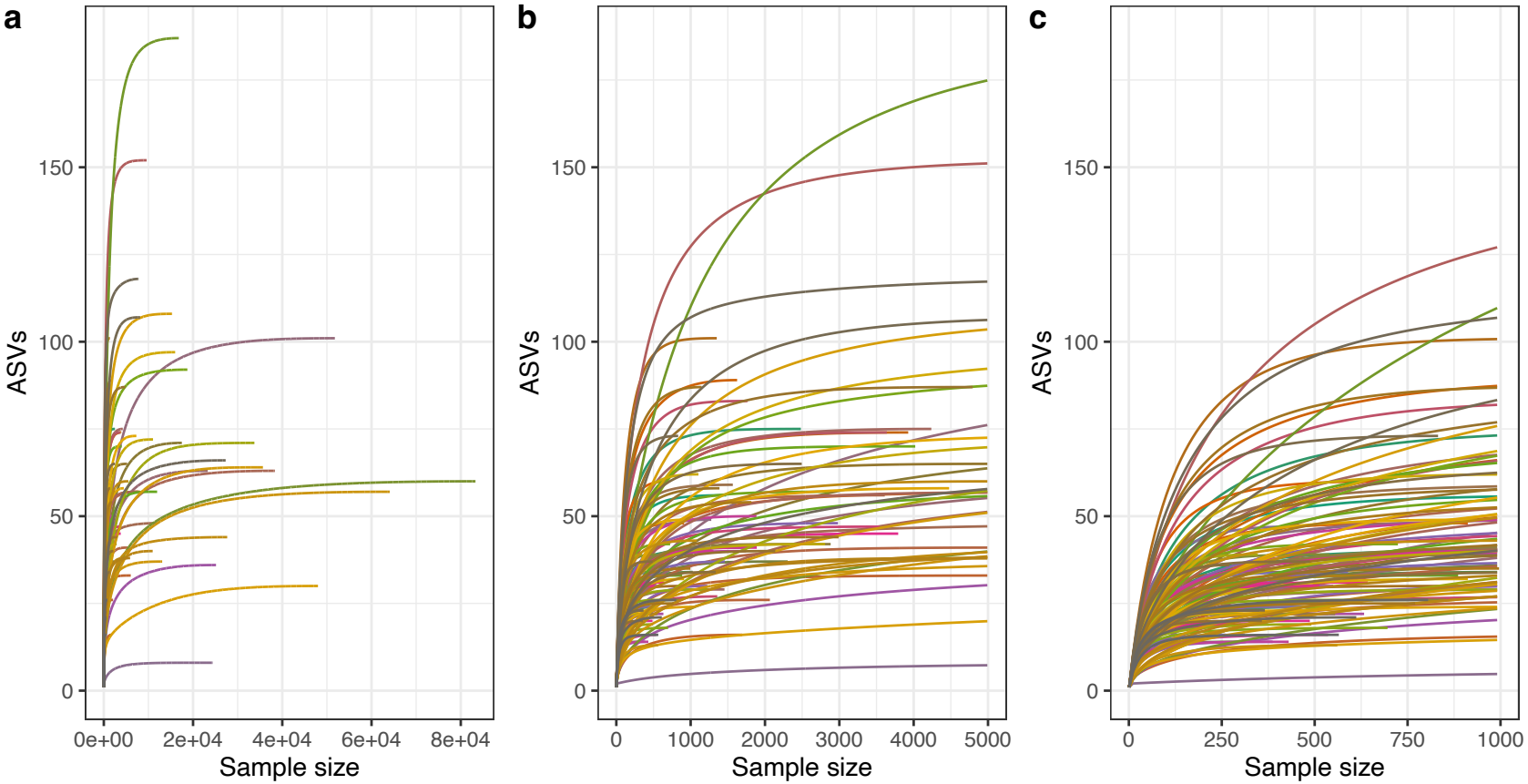
695

Cluster ID	No. ASVs included	Median of proportions		Wilcoxon rank sum test (MI vs. WI)	
		Microbe (MI)	Water (WI)	w	p-values
Cluster 1	2	0.000	0.000	186.0	0.1469
Cluster 2	4	0.038	0.190	78.0	0.0001**
Cluster 3	2	0.000	0.000	267.5	0.4143
Cluster 4	2	0.000	0.000	225.5	0.6198
Cluster 5	4	0.004	0.011	127.0	0.0071*
Cluster 6	**6**	**0.845**	**0.213**	**444.0**	**< 0.0001*** **
Cluster 7	4	0.023	0.036	204.0	0.3826
Cluster 8	4	0.014	0.097	162.5	0.0635
Cluster 9	2	0.000	0.000	224.0	0.6204
Cluster 10	3	0.000	0.000	213.0	0.4531
Cluster 11	3	0.000	0.000	276.0	0.3623

696

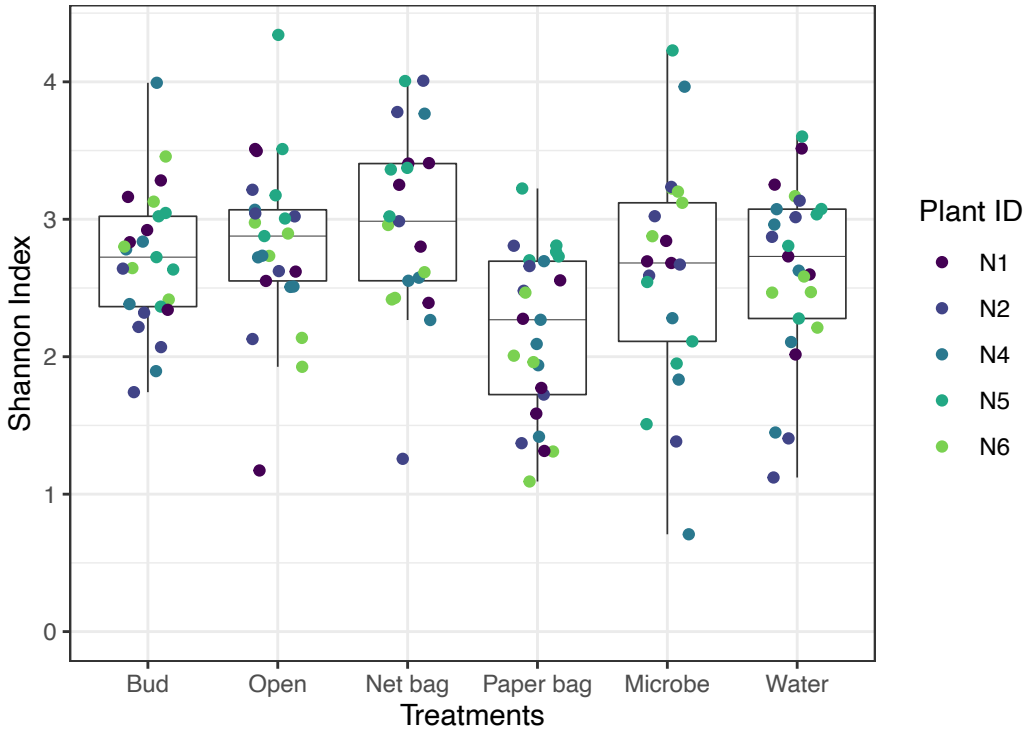
697 FIGURE S1. Rarefaction curves of the samples. The vertical axis shows the number of observed prokaryotic ASVs. The number of
698 sequences per sample is shown on the horizontal axis. The three panels show the same graph with different ranges of the horizontal axis.

699

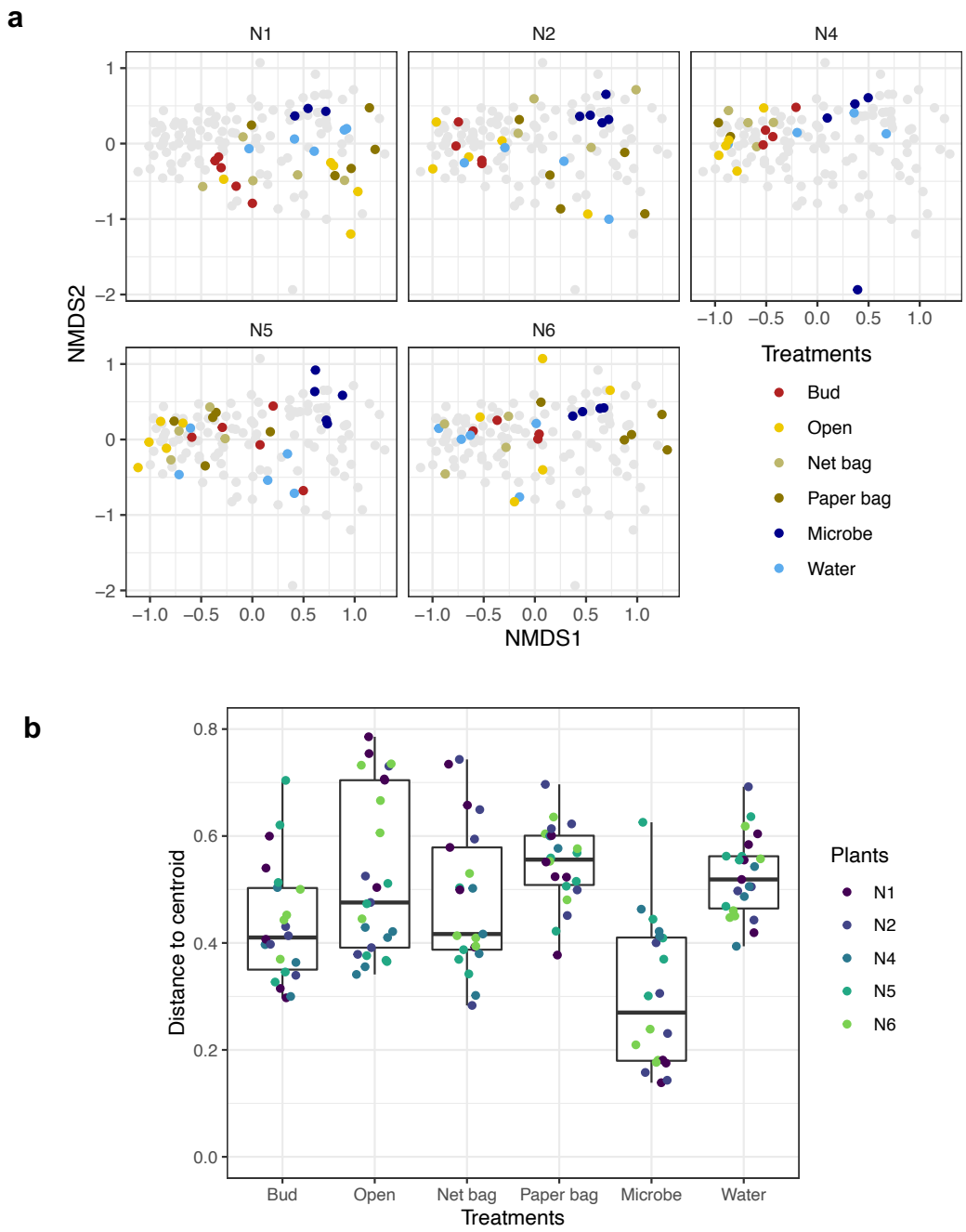
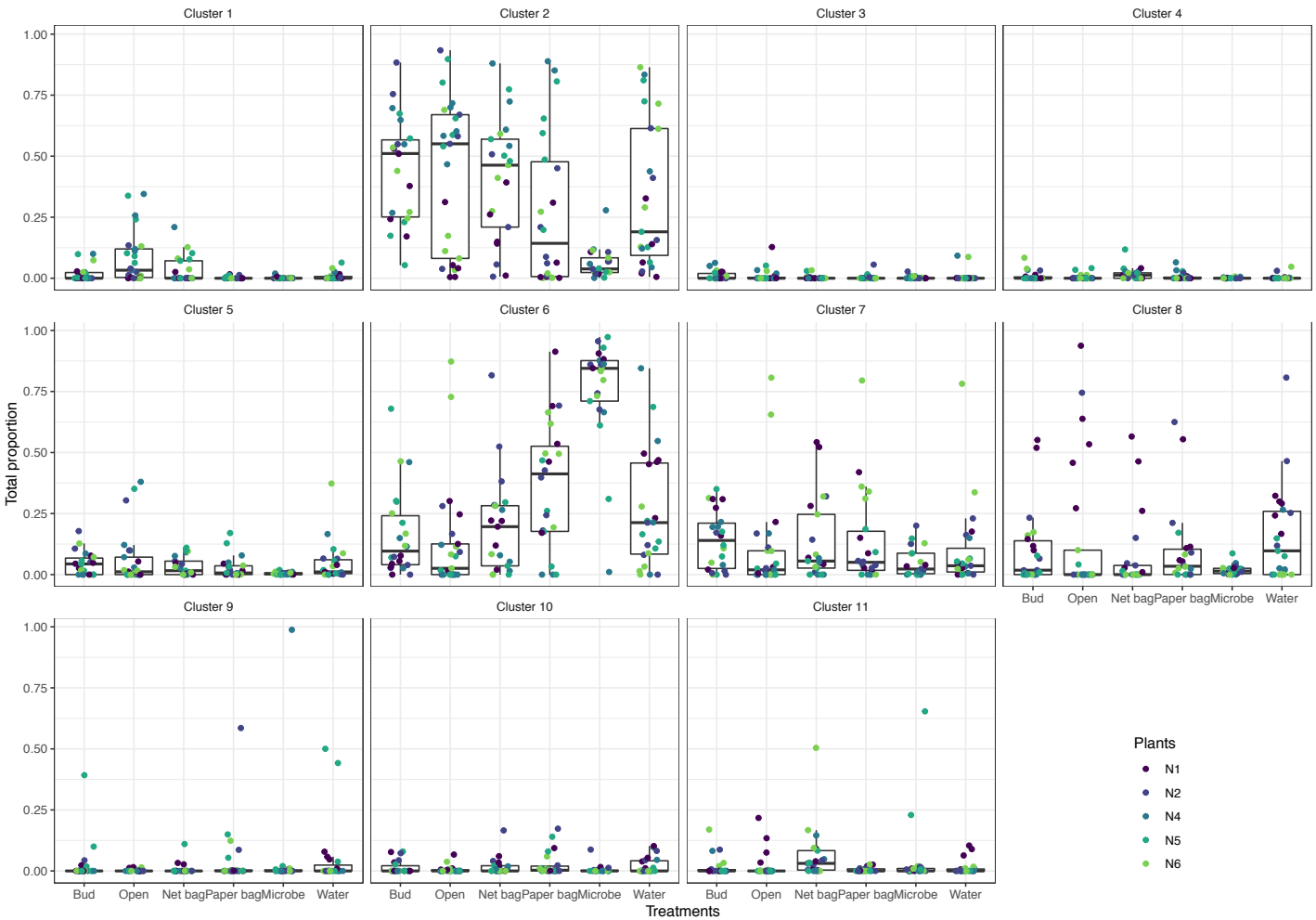


700

701 FIGURE S2. Variation in the prokaryotic diversity among the treatments and samples.
702 The diversity was significantly different among the treatments (GLMM, $\chi^2 = 22.234$, p
703 = 0.0005). Colors indicate the five individual plants.



704 FIGURE S3. Variation of the prokaryotic communities on the flowers under different
705 treatments. (a) NMDS plots of the microbial communities of flower buds and flowers
706 under different treatments. In the five panels, all microbial samples are plotted with grey
707 circles, and samples from each plant are overlayed individually. The five treatments are
708 indicated by different colors. (b) Distribution of the distances from the treatment
709 centroid. Variation in the microbial communities within a treatment was significantly
710 different among the treatments (ANOVA, df = 5, F = 8.60, p < 0.0001). Colors indicate
711 the five individual plants.



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