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

Nuria Jiménez Elvira¹, Masayuki Ushio^{2,3}, Shoko Sakai³

¹Centre de Recerca Ecològica i Aplicacions Forestals, 08193 Cerdanyola del Vallès, Barcelona, Spain

²Hakubi Center, Kyoto University, Kyoto University, Kyoto 606-8501, Japan

³Center for Ecological Research, Kyoto University, Hirano 2-509-3, Otsu 520-2113, Japan

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

INTRODUCTION

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

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). 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.

Identification of microbial communities

To identify microbial communities, we sampled 4–5 flowers with each treatment from each plant. The flowers were cropped at ~1700 of the day of flower opening and separately put into a 5 ml plastic tube after removing the stigma (to use for another study) and petals (Fig. 1b). In addition, five flower buds were collected from each individual to identify microbes that were present inside prior to anthesis with the same procedure. The tube was kept on ice until being brought to the lab within 4 hours after sampling and kept at –20°C until further processing.

To detach microbes from the flower surface, we added 3 ml of Phosphate-Buffered Saline solution (PBS buffer, pH 7.4, Nippon Gene Co., Ltd.) to the tube and

sonicated it using an Ultrasonic Disruptor Handy Sonic UR-21P (Digital Biology) at Level 5 power for 30 sec. Microbes in the PBS solution were then filtered using a Sterivex Millipore filter (Sterivex-HV 0.22, Merck). Residual PBS on the Sterivex filter was removed using a Vac-Man® Laboratory Vacuum Manifold (Promega).

Microbial DNA was extracted with DNeasy Blood & Tissue Kits (QIAGEN) following the manufacturer's instructions. A portion of the 16S small-subunit ribosomal gene was PCR-amplified using the primer pairs, 515F (5'-GTG YCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CNV GGG TWT CTA AT-3') [25] with Nextera DNA index adapters. First PCR was performed in 12 µl reactions, each containing 1 µl of DNA template, 6 µl of KAPA HiFi HotMasterMix (Kapa Biosystems) and 0.7 µl of each primer (5 µM). The first PCR was conducted with a temperature profile of 95°C for 3 min, followed by 35 cycles at 98°C for 20 s, 60°C for 15 s, 72°C for 30 s, and a final extension at 72°C for 5 min, followed by storage at 4°C until use. Triplicate PCR products were pooled and purified with Agencourt AMPure XP (Beckman Coulter). P5/P7 Illumina adaptors were then added in a second PCR using fusion primers with 8-mer index sequences for sample identification [26] (forward, 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-[8-mer tag]-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG -3'; reverse, 5'-CAA GCA GAA GAC GGC ATA CGA GAT-[8-mer tag]-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3'). This preparation had a total volume of 24 µl with double the amount of each reagent in the first PCR and 10-times diluted first PCR products as DNA template. The temperature profile was 95°C for 3 min, followed by 12 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 min, and 4°C until use. Second PCR products were pooled at equal volumes after a purification/equalization process with the same AMPure XP protocol and E-Gel SizeSelect II Agarose Gel kit (Invitrogen). DNA quantitation was performed using a Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific Inc). The pooled library was sequenced on the Illumina MiSeq using Reagent Kit V2 (500 cycle Nano Kit).

We processed the sequence data following Ushio [27]. The raw MiSeq data were converted into FASTQ files using the bcl2fastq program provided by Illumina (bcl2fastq v2.18). The FASTQ files were then demultiplexed using the command implemented in Claident (<http://www.claident.org>; [28]). Demultiplexed FASTQ files

were analysed using the ASV method implemented in the package DADA2 [29] of R [30]. At the quality filtering process, forward and reverse sequences were trimmed at the length of 215 and 160, respectively, using DADA2::filterAndTrim() function.

Taxonomic identification was performed for ASVs (Amplicon Sequence Variants; proxy for Operational Taxonomic Units) inferred using DADA2 based on the query-centric auto-k-nearest-neighbour (QCAuto) method [28] and subsequent taxonomic assignment with the lowest common ancestor algorithm [31] using the “semaal” database and clidentseq and classigntax commands implemented in clident v0.2.2018.05.29. The reads identified as Bacteria or Archaea were used for further analysis. Blast search was also conducted for the most abundant ASVs for which the above procedure did not provide the identification of the order or lower taxonomic levels. Top matches of the sequences were referred to in order to assign taxonomic identities of the ASVs. Taxonomy, ASV table, and sample information were combined into a single R object for downstream manipulation using the R package phyloseq [32]. We also removed ASVs that only occurred in negative control samples. Furthermore, potential contaminant ASVs were identified statistically based on prevalence in negative control samples using the R package decontam [33] based on ASV prevalence and a threshold of 0.5. This resulted in elimination of 79 of 2679 16S ASVs.

Fruit set and seed production

The treated flowers for the observation of fruit set were hand-pollinated around 1700 using outcrossing pollen to ensure that the fruit set was not limited by pollen. Pollen was taken with a wood toothpick from a cut flower from a 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.

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 binomial 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).

The diversity index was significantly different among the treatments, and was the lowest in the paper bag (PB) treatment (Kruskal-Wallis $\chi^2 = 18.2$, $df = 5$, $p = 0.0054$).

The 34 ASVs that appeared in 20 or more samples were identified as bacteria, and accounted for 75.7 % of the prokaryotic sequences. NMDS plots of the microbial communities revealed that the flowers under different treatments in each plant individual had different microbial communities, while the same treatment on different plant individuals did not always converge the microbial communities into similar compositions. For example, the OT flowers are plotted on the right of the flower bud samples in the plant N1, while the OT flowers are on the left side of the buds in N5 (Fig. S3a). Only MI treatment resulted in the conversion of the prokaryotic communities. PERMANOVA indicated that the communities were significantly different among individuals ($F = 5.52$, $R^2 = 0.106$, $p < 0.0001$) and among the treatments within each individual ($F = 2.96$, $R^2 = 0.355$, $p < 0.0001$). Variation in the microbial communities within a treatment was significantly different among the treatments (ANOVA, $df = 5$, $F = 13.31$, $p < 0.0001$), and it was the lowest in the MI treatment, as suggested from the NMDS plots (Fig. S3b).

The cluster analyses grouped the 34 ASVs into 10 clusters (Fig. 2, Table S2). Cluster 5, which accounts for the second highest proportion, showed the largest difference in the proportion between the MI and WI treatments, followed by Cluster 1, the most dominant cluster (Table S2, Fig. S4). The Wilcoxon rank-sum test showed that the difference between the treatments was significant for both clusters ($w = 90$, $p = 0.0001$ for Cluster 1; and $w = 501$, $p < 0.0001$ for Cluster 5), while the difference was not significant in the other clusters.

Blast search identified the two most abundant ASVs (T00020 and T00030) in the dataset as *Cutibacterium* (Propionibacteriaceae) and *Ochrobactrum* (Brucellaceae) in Cluster 1 (Table 1, Fig. 2). The third most-abundant ASVs (T00004) showed the highest matches with sequences of *Erwinia* (Erwiniaceae) and the fourth and fifth (T00005 and T00000) did with *Pseudomonas* (Pseudomonadaceae) in Gammaproteobacteria (Table 1). The two *Pseudomonas* ASV sequences were different for only five bases among 253 (2.0 %), so they may belong to the same species. These three ASVs were included in Cluster 5 (Fig. 2).

Fruit set and seed production

The probability of fruit set of OT predicted by the first GLMM was 55.7 %, 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 5 showed the opposite patterns; it is the lowest in OT and highest in MI. The GLMM including the proportion of Cluster 5 as the fixed factor indicated that Cluster 5 was significantly associated with low fruit set (GLMM, $z = -2.19$, $p = 0.0288$).

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, $z = 0.685$, $p > 0.05$).

DISCUSSION

Prokaryotic community on *A. japonica* flowers

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

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

microbes are often shared by leaves of the same plant [6,7,46], the differences might have originated from vegetative parts of the same individuals. The effects of most treatments were not consistent across the individuals (Fig. S3). Although the paper bag treatment decreased the Shannon diversity index (Fig. S2), we did not find a drastic increase of particular ASVs by the treatment (Fig. 3). Growth of a small number of bacteria species in the bag with high humidity and low UV-irradiance might have reduced the diversity [47]. Prokaryotic diversity on open and pollinator-excluded net-bagged flowers was more or less similar to that on the flower buds (Fig. S2). It has been reported that flower visitors disperse microbes among flowers [2], and often increase microbial diversity [48]. Flower visitors might not have large effects in our system, however, partly due to the relatively low flower visitor activities during this year (N. E. Jimenez and S. Sakai, personal observation).

Identification of bacteria that were increased by inoculation

We identified a group of ASVs that showed substantial changes after inoculation of old-flower microbes. The cluster analyses grouped the ASVs into 10 clusters based on the co-occurrence among the samples. The first and second most dominant clusters of the 10 accounted for 41.2 % and 29.3 % of the sequence reads of the samples, respectively, and were significantly different among the treatments. The microbial inoculation treatment drastically increased Cluster 5 (Fig. 4), which contains three of the five most dominant ASVs, *Erwinia* (T00004) and two *Pseudomonas* (T00005, T00007) (Table 1). Microbial communities on old flowers might be dominated by these taxa. The bacteria may have already colonized before anthesis, because these bacteria were also found in flower buds at lower abundances. The proportions of Cluster 5 were also moderately higher in the paper- and net-bagged and water-sprayed flowers than the proportion under the open control treatment (Fig. S3b). Higher humidity due to these treatments might have enhanced growth of these bacteria. In agriculture, it has often been reported that high humidity or precipitation enhance activities of pathogenic *Erwinia* and *Pseudomonas*, and increase damage by diseases [49–51].

Though the number of studies that investigate microbial communities on flowers is rapidly increasing, no study has examined microbial communities on old flowers as far as we know. The bacterial families *Erwiniaceae* and *Pseudomonodaceae* probably

dominating on old flowers of *A. japonicus* are among the most dominant taxa on flowers [10]. Many bacterial species of these families are frequently found both on flowers and inside the plant and/or on leaves [10]. Besides, flowers of unrelated plant species in the same habitats often share common flower bacteria species [7,40]. Therefore, we consider that the bacteria of Group 5 may be generalist inhabitants on living plants and senesced plant parts. However, identification of bacteria in most microbial community studies of the anthosphere and phyllosphere are based on short DNA sequences of hundreds of base pairs. To confirm habitat specificity within a plant and adaptation to a specific host, cell lineage tracking using a bacterial strain with a marker gene, or genomic comparisons among flower and leaf isolates from the same and different plant hosts would be necessary [10].

Effects of old-flower microbes on fruit and seed production

Do the microbes that become dominant on old flowers negatively affect reproductive success of the plant? We observed the lowest fruit set in the microbial inoculated treatment, which recorded the highest abundance of Cluster 5. Besides, we found significant negative associations between the proportion of the ASVs of Cluster 5 and fruit set based on the GLMM analysis, while we did not for seed set. Since *Erwinia* and *Pseudomonas* include plant pathogens that trigger abortion of flowers and fruits [51–53], 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 5 might be 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 [14,54], while the effects of microbes on other post-pollination processes are largely understudied [55]. On the other hand, it is unlikely that pollinator behavioral response affected the fruit set as reported in previous studies [56,57], because we hand-pollinated the flowers under the fruit set monitoring.

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 [44–46]. 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 [19]. This study is the first to show the potential negative consequence of longer flower lifespan by non-pathogenic (microbes that do not cause apparent symptoms) microbes. It is increasingly recognized that flowers have characteristic microbial communities, but their effects and implications have just started to be explored and our knowledge is still very fragmental [9,10,55,58]. Future studies will elucidate the impacts of microbes on plant reproductive ecology deeply embedded in the evolution of angiosperms [9].

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

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

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

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.

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

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

Treatments	Fruit set and seed production					Microbial community				
	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

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

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

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

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639 Figure S2. Variation in the prokaryotic diversity among the treatments and samples. The
 640 diversity was significantly different among the treatments (Kruskal-Wallis test; $p =$
 641 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 overlaid 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.

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