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RESEARCH ARTICLE

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Contrasting effects of nectar yeasts on the reproduction of Mediterranean plant species

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Abstract

Premise: Yeasts are often present in floral nectar and can influence plant fitness directly (independently of pollinators) or indirectly by influencing pollinator visitation and behavior. However, few studies have assessed the effect of nectar yeasts on plant reproductive success or compared effects across different plant species, limiting our understanding of the relative impact of direct vs. indirect effects.

Methods: We inoculated the nectar of six plant species in the field with the cosmopolitan yeast *Metschnikowia reukaufii* to analyze the direct and indirect effects on female reproductive success over 2 years. The pollinator assemblage for each species was recorded during both flowering years.

Results: Direct yeast effects on female fecundity were statistically nonsignificant for all plant species. There were significant indirect, pollinator-mediated effects on fruit production and seed mass for the two species pollinated almost exclusively by bumblebees or hawkmoths, with the direction of the effects differing for the quantity- and quality-related fitness components. There were no consistent effects of the yeast on maternal fecundity for any of the species with diverse pollinator assemblages.

Conclusions: Effects of *M. reukaufii* on plant reproduction ranged from negative to neutral or positive depending on the plant species. The among-species variation in the indirect effects of nectar yeasts on plant pollination could reflect variation in the pollinator community, the specific microbes colonizing the nectar, and the order of microbial infection (priority effects), determining potential species interactions. Elucidating the nature of these multitrophic plant–pollinator–microbe interactions is important to understand complex processes underlying plant pollination.

KEYWORDS

Bombus, female fitness, fruit production, *Macroglossum*, *Metschnikowia reukaufii*, nectar yeasts, pollination, pollinator diversity, priority effects, seed mass

Flowers do not live as isolated, aseptic entities, but are closely associated with microorganisms. The presence of different microbial communities on petals, pistil, pollen, or nectar reflects the ability of the microbes to colonize the distinct microenvironments of ephemeral flowers (Junker et al., 2011; Pozo et al., 2012; Junker and Keller, 2015; Rebolleda-Gómez and Ashman, 2019). Increasing awareness of the collective microbiota living in association with flowers ("anthosphere") is leading to new ideas on floral evolution and providing insight into the complexity of multitrophic relationships involving plants, microbes, and floral visitors (Rebolleda-Gómez et al., 2019; Vannette, 2020; Cullen et al., 2021).

One of the best-studied floral microhabitats for microorganisms is floral nectar, a sugary solution initially sterile, that can be rapidly colonized after anthesis by microorganisms comprising mainly bacteria and fungi that depend predominantly on animal visitors for their dispersal (Álvarez-Pérez et al., 2019; Vannette, 2020). The most widely described nectar–microbial interaction is the one between flowers and *Metschnikowia reukaufii* (Metschnikowiaceae, Saccharomycetales, Ascomycota), a cosmopolitan, specialist yeast species found in floral nectar and on associated pollinators (Brysch-Herzberg, 2004; Pozo et al., 2011; Golonka and Vilgalys, 2013; de Vega et al., 2021). *Metschnikowia reukaufii* is capable of inducing

manifold changes in nectar properties, such as decreasing sugar concentration (de Vega and Herrera, 2012; Vannette and Fukami, 2016), modifying nectar carbohydrate and amino-acid composition (Peay et al., 2012; Vannette and Fukami, 2018; Pozo et al., 2020; Rering et al., 2020), decreasing H_2O_2 concentration and pH (Vannette et al., 2013), warming the flowers (Herrera and Pozo, 2010), and altering floral volatile blends (Rering et al., 2018; Schaeffer et al., 2019; Crowley-Gall et al., 2021).

Recent studies have shown that responses of pollinators to nectar microbes, and particularly to *M. reukaufii*, may vary across insect taxa and depend on the particular behavioral assay employed (olfactory and/or gustatory cues) (reviewed in Crowley-Gall et al., 2021). Reported effects of microbial cues on insects range from eliciting a negative or a nonsignificant influence on foraging preferences (Good et al., 2014; Rering et al., 2018, 2020) to positive effects on feeding and attraction of floral visitors (Herrera et al., 2013; Schaeffer and Irwin, 2014; Schaeffer et al., 2017, 2019; Yang et al., 2019). Thereby, nectar microbes can impact plant fitness directly (independently of floral visitors) or indirectly, by leaving physicochemical footprints on flowers that can influence pollinator foraging preferences and/or the floral microenvironment, which can ultimately affect plant pollination and fertilization success (Herrera and Medrano, 2017; Cullen et al., 2021).

Investigating the intrinsic complexity of species interactions may aid in understanding the overall effects of nectar yeasts on plant reproduction. There are documented priority effects in microbes inhabiting floral nectar, in which the order of the arrival of the microbe species dictates the outcome of species interactions and the diversity of the communities (Peay et al., 2012; Tucker and Fukami, 2014; Mittelbach et al., 2016; Tsuji and Fukami, 2018). Structurally different microbial communities have different outcomes on nectar chemistry (Peay et al., 2012; Vannette et al, 2013; Vannette and Fukami, 2018), which may cascade to impact pollinators and the processes they mediate, ultimately affecting plant reproduction. A further complexity is that different species of pollinators may vary in their response to nectar modifications induced by different microbes (Vannette et al, 2013; Good et al., 2014; Crowley-Gall et al., 2021). In plants interacting with a diverse array of pollinators, nectar microbes may provoke different or even opposite effects on the foraging preferences of different species of pollinators, which could potentially lead to an overall neutral effect for plant reproduction. A different outcome would be expected when plant pollination relies on one or a few pollinator species, as the potential effect of microbes over insects should be more evident in these instances. Overall, nectar microbes would be predicted to indirectly influence plant pollination, but the impacts may depend on the species of the plant, the interacting pollinators, and the microbes involved, which underscores the species-dependency of these tripartite interactions.

We are at the beginning of understanding multitrophic interactions involving plants, nectar microbes, and pollinators. To our knowledge, only four studies have so far tested the effect of nectar microbes, and more specifically of *M. reukaufii*, on the reproductive output of wild plants (Herrera et al., 2013; Vannette et al., 2013; Schaeffer and Irwin, 2014; Yang et al., 2019), and we still know very little about the extent to which total effects of nectar yeasts on plant reproduction are direct versus indirect. These studies have revealed that the nature of the interactions of *M. reukaufii* with plants ranges from mutualistic to detrimental to the plant. However, our knowledge of the effects of microbial communities in plants is taxonomically biased, as they have been tested mainly in closely related plant species (three of four studies involved species of the family Ranunculaceae). More importantly, most of the study systems depended almost exclusively on bumblebees as pollinators (and only one on hummingbirds; Vannette et al., 2013), so the results are heavily biased toward certain pollinator types.

The aim of this study was to assess the degree to which *M. reukaufii* affected the pollination of six Mediterranean plant species in the field during two consecutive years. We focused on the following six plant species due to their importance as pollen and nectar resources during spring for different groups of insect pollinators: *Anchusa calcarea* Boiss. (Boraginaceae), *Asphodelus ramosus* L. (Asphodelaceae), *Echium plantagineum* L. (Boraginaceae), *Lonicera etrusca* Santi (Caprifoliaceae), *Phlomis purpurea* L. (Lamiaceae), and *Teucrium fruticans* L. (Lamiaceae). Using two experimental approaches, we independently evaluated (1) the effects of *M. reukaufii* on pollination success and plant reproduction, independently of the pollinator activity ("direct effects") and (2) whether pollinators discriminate between manipulated flowers, ultimately and indirectly affecting the female reproductive success of flowers ("indirect effects").

MATERIALS AND METHODS

Study area

Six populations of six native Mediterranean plant species were studied over two consecutive years (year 1 and year 2; 2010 and 2011, respectively). Five populations (each for one plant species) were located near Doñana National Park (Huelva Province, southwestern Spain; 37°18′N, 6°25′W, 80–90 m a.s.l.). The other population was located in a small area of Sierra de Cazorla, Segura y las Villas Natural Park (Jaén Province, southeastern Spain; 37°53′N, 2°52′W; 1280 m a.s.l.).

Study species

The main characteristics of the study plant species and sample sizes are summarized in Appendices S1 and S2.

Anchusa calcarea (Boraginaceae) is a short-lived, perennial herb, endemic to western Iberian Peninsula where it occurs on sand dunes. The blue, hermaphroditic,

protandrous flowers last approximately 2–3 days and secrete a daily mean of 0.4 mg sugar per flower (Ortiz et al., 2021). The compatibility system in this species is not well-known, but our preliminary data point to self-incompatibility as in other members of *Anchusa* (Selvi and Bigazzi, 1998). The fruit produces up to four nutlets.

Asphodelus ramosus (Asphodelaceae) is a common, clonal, tuberous Mediterranean perennial herb. It usually produces one flowering scape that bears a paniculate inflorescence composed of tens or hundreds of white flowers. Its daily sugar secretion is 0.96–1.2 mg sugar per flower (Herrera, 1985; Ortiz et al., 2021). The species is self-compatible (Díaz-Lifante, 1990) and anthesis last 1–2 days. The fruit is a loculicidal capsule with up to 6 seeds.

Echium plantagineum (Boraginaceae) is a late-spring, annual, self-compatible species native to the South European Mediterranean region, and currently cosmopolitan. The blue-purple, hermaphroditic, protandrous flowers last 2–3 days and secrete a daily mean of 0.2–0.8 mg sugar per flower (Talavera et al., 1988; Ortiz et al., 2021). The fruit produces up to four nutlets.

Lonicera etrusca (Caprifoliaceae) is a climbing shrub native to the Mediterranean basin. The species is self-compatible and produces fragrant hermaphroditic, protogynous flowers that secrete copious nectar that accumulates at the corolla base, with a mean daily secretion rate of 1.06 mg sugar per flower (Jordano, 1990). The initially white flowers last 1–3 days and turn yellowish with age. The fruit is a reddish berry.

Phlomis purpurea (Lamiaceae) is an evergreen shrub native to North Africa and southern part of the Iberian Peninsula. The species is self-compatible, and the pink, bilabiate, protandrous flowers are arranged in verticillasters and last 2–4 days. Daily sugar secretion is around 1.9–3.6 mg per flower (Talavera et al., 1988; Ortiz et al., 2021). The fruit produces up to four nutlets.

Teucrium fruticans (Lamiaceae) is an evergreen, gynodioecious shrub native to the western Mediterranean. The species is self-compatible, and its pale-mauve-blue, protandrous flowers, lasting 2–3 days, are borne on terminal spikes and secrete a daily mean of 0.5–1.25 mg sugar per flower (Herrera, 1985; Talavera et al., 1988). The fruit produces up to four nutlets.

Pollinator observations

In each population and year, we observed the insect visitors to flowers of the study plant. We observed flowers for visitors for 15 min/h from 10:00 to 16:00 hours each day. During the observation period, we recorded the insect identity to the genus level and to species when possible, verified that the visitor contacted anthers and stigma (pollinators), and noted any visits by nectar robbers. Each population was visited once a week during 4 weeks in the flowering peak of each species (Appendix S3). A total of 12 h of pollinator observations were made for each population and plant species. All observations were done on sunny days.

To test for potential production of fruits in the absence of pollinators (control for automatic self-pollination), 150 flowers for each studied species were covered with a nylon mesh bag before they opened and were kept bagged during the experiment. We recorded the presence/absence of fruits during the fruiting season for each species.

Yeast isolation and characterization

To obtain wild yeast strains for use in the experiments, we isolated *M. reukaufii* from the different species by plating diluted nectar onto YM agar plates (2.0% w/v agar, 1.0% w/v glucose, 0.5% w/v peptone, 0.3% w/v malt extract, 0.3% w/v yeast extract, 0.01% chloramphenicol v/v, pH 6.0) and incubating plates at 22–25°C. Strains were identified by sequencing the D1/D2 domain of the 26 S rRNA gene following the methods of Kurtzman and Robnett (1998) and preserved at −80°C on 10% v/v glycerol and in the Microbank system (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) (see Appendix S3 for detailed methods).

Two of the *M. reukaufii* strains isolated from *T. fruticans* and *A. calcarea* (GenBank numbers MZ562885 and MZ562886, respectively) were freshly streaked on YM agar plates 4 days before the start of the field experiments. The inoculum was prepared by suspending colonies of *M. reukaufii* in sterile distilled water with 20% w/v sucrose for a final concentration of 2×10^4 cells/μL (hereafter, yeast solution). This concentration of yeast cells is commonly found in nectar (Herrera et al., 2009). Control solutions contained 20% w/v sucrose in sterile distilled water but no yeast was added (hereafter, control solution); the concentration is within the range in the study species.

Field experiments

We conducted two experiments to investigate whether inoculating the flowers with the nectar yeast *M. reukaufii* affects plant reproduction, measured as female reproductive success.

Experiment 1. Direct effects of *M. reukaufii* on plant reproduction in hand-pollinated flowers

This experiment was designed to test whether the inoculation of nectar with the yeast *M. reukaufii* had a direct effect on female reproductive success, independently of the pollinator activity ("Direct effects").

Immature flowers or inflorescences were chosen randomly among plants and bagged in nylon mesh (200 μm mesh) before they opened. (On the same plants another set of flowers was bagged for experiment 2, as described below). The remaining flowers on the plant were exposed to

pollinators. Experiments were conducted at the flower level and not at the whole plant level due to the characteristics of the study species, all producing hundreds of flowers per plant. Although the experiments at the flower level may have some limitations (Knight et al., 2006), given the high number of flowers per plant produced by the study species, conducting the experiments at the plant level was unfeasible.

To check that bagged flowers were microbe-free before the inoculations, we plated nectar from bagged flowers on bacterial and yeast media (trypticase soy agar and YM, respectively). Additionally, we used a microscope to observe nectar samples from bagged flowers. None of the plates were colonized by yeasts or bacteria, and we did not observe any yeasts or bacteria in these nectar samples. Therefore, nectar from bagged flowers remained "sterile". During the experiment, we never observed thrips, ants, or other small arthropods inside the bags.

To evaluate any direct effects of yeasts, flowers were tagged at the time of anthesis, and half of them were randomly assigned to a yeast-added treatment (YE, hereafter) and inoculated with 1 μL of yeast solution (see above) using a 0.5–10 μL pipette (Eppendorf, Hamburg, Germany). The rest of the flowers were assigned to a control treatment (CO, hereafter) and were inoculated with 1 μL of control solution consisting of sucrose 20% w/v diluted in sterile distilled water. A different tip was used in each flower to prevent cross-contamination. Immediately after inoculation, flowers were hand-pollinated with a mixture of pollen from 8 to 10 different plants collected 5–25 m from the target plant. We pollinated the stigma of each flower with pollen by brushing the pollen mixture against the stigmas. All flowers were kept bagged until all flowers were withered and corollas were shed. The total sample number for hand pollinations was 280 flowers in *A. calcarea* (35 plants), 154 in *A. ramosus* (20 plants), 256 in *E. plantagineum* (26 plants), 123 in *L. etrusca* (20 plants), 194 in *P. purpurea* (30 plants), and 269 in *T. fruticans* (26 plants).

Flowers from both treatments were left on plants to assess fruit production. Mature fruits were collected to quantify fruit and seed set. For fruits containing seeds, we counted the number of ovules and the number of seeds produced. Seed set was then calculated as the proportion of ovules in each flower that developed into mature seeds. All seeds produced per flower were weighed individually on a digital balance to the nearest 0.01 mg.

Lonicera etrusca produces fleshy fruits. For this species, fruits were oven-dried at 50°C, weighed, and then dissected to remove the seeds, which were then weighed individually. For this species, the number of ovules could not be estimated.

Experiment 2. Indirect effects of *M. reukaufii* on plant reproduction in flowers exposed to pollinators

This experiment was designed to evaluate whether the pollinators discriminated between experimentally inoculated flowers in natural populations, ultimately and indirectly affecting female reproductive success of flowers ("Indirect effects").

Flowers were bagged before opening as in experiment 1 and kept bagged until anthesis to ensure that only flowers with microbe-free nectar would be used for further experiments. As flowers opened, they were tagged and randomly assigned to one of two groups: YE or CO and inoculated, respectively, with either 1 μL of yeast solution or with 1 μL of the control solution (20% w/v sucrose in sterile distilled water). Immediately after the inoculations, all flowers were exposed to natural pollination for their whole lifespan. Consequently, the experiment consisted of a comparison between flowers immediately inoculated with yeasts (YE) and flowers allowed to be potentially colonized by microbes naturally transported by insects (CO).

Densities of yeasts in nectar at the end of the floral lifespan were expected to be significantly higher in yeast-inoculated flowers than in flowers inoculated with control solutions (which could or not be colonized by yeasts), as demonstrated in previous studies (Vannette et al., 2013; Schaeffer and Irwin, 2014). To confirm our expectations, we sampled the floral nectar 3 days after inoculations (the end of floral lifespan) and assessed yeast cell densities as done by Herrera et al. (2009) (see Appendix S4). As expected, cell density in yeast-inoculated flowers was significantly higher by one order of magnitude than the density in the controls ($P < 0.001$). Moreover, while 100% of the yeast-inoculated flowers contained high densities of yeast cells at the end of the floral lifespan, only 48–60% of the control flowers (depending on the species) were colonized by yeasts (Appendix S4). Since flowers of most species lasted 2–3 days (Appendix S1), microbes had approximately the same time to colonize floral nectar in the study species. Species with a significant treatment effect (see results) were not different in terms of flower longevity.

Total sample sizes were 472 flowers for *A. calcarea* (40 plants), 709 for *A. ramosus* (40 plants), 436 for *E. plantagineum* (42 plants), 912 in *L. etrusca* (30 plants), 346 for *P. purpurea* (30 plants), and 475 for *T. fruticans* (30 plants). Flowers from all treatments were left on the plant to assess fruit production. Fruit set, seed set, and seed mass were estimated as in experiment 1.

Statistical analyses

All analyses were conducted using R version 4.0.3 (R Core Team, 2021).

To test for the effects of inoculations on the estimates of female plant reproductive success, we fitted generalized linear mixed models (GLMMs) separately for each plant species. Inoculation treatment (YE or CO), experimental year, and their interaction were the fixed effects considered, and individual plants were included as random intercept terms. The proportion of flowers that set fruits (fruit set), proportion of ovules that set seeds, seed mass, and fruit mass were the response variables. We tested for the effects

of treatment and year on fruit set and seed set by applying a binomial error distribution with a logit link function using the glmer function in the package lme4 v. 1.1.26 (Bates et al., 2015). We added an observation-level random effect (OLRE) (Bolker, 2015) in cases where we detected signs of weak overdispersion. For *L. etrusca*, the effects of treatment and year on the number of seeds (not the proportion of ovules that set seeds as in the other species) were tested by using a truncated Poisson distribution and log-link function with the glmmTMB function in the package glmmTMB v. 1.0.2.1 (Brooks et al., 2017).

Seed mass was analyzed for all plant species using the function lmer in the package lme4. When deviations of normality were found, the bestNormalize function was used in the bestNormalize package 1.8.0 (Peterson and Cavanaugh, 2020) to automatically choose the best transformation to approximate normality.

After model adjustment, in the cases when the interaction term was significant, we calculated marginal post-hoc Tukey tests with the emmeans function in the emmeans package v.1.5.5 (Lenth, 2021) to separately calculate the significance of the factor treatment for each year.

Hand-pollinated flowers received exclusively xenogamous pollen, while insects could deliver autogamous and xenogamous pollen. Fruit set, seed set, and individual seed mass from yeast-inoculated hand-pollinated flowers from experiment 1 were compared with those from flowers pollinated by insects from experiment 2. For that aim, we fitted GLMMs with the same predictors and the same R packages as above, including individual plants as random intercept terms.

All mixed model diagnostics were done using the DHARMa package v. 0.4.3 (Hartig, 2021), which uses a simulation-based approach to create standardized residuals.

RESULTS

Pollinator observations

Four plant species, *A. calcarea*, *A. ramosus*, *E. plantagineum*, and *T. fruticans*, had a diverse pollinator community including several genera of Hymenoptera, Lepidoptera, and Diptera (Table 1, Figure 1). The pollinator community was slightly less diverse in the first year of observations than in the second.

In contrast, *P. purpurea* and *L. etrusca* relied mainly on one insect species each for pollination in the studied populations and years. *Bombus terrestris* was the predominant and almost the only bee species visiting *P. purpurea*, with occasional visits from *Xylocopa cantabrita*. *Lonicera etrusca* was almost exclusively pollinated by the diurnal hawkmoth *Macroglossum stellatarum*, with *X. violacea* and *Bombus* spp. behaving as nectar robbers (Table 1, Figure 1). Because we did not do any nocturnal observations, we could not ascertain whether any strictly nocturnal moth species were involved in the pollination of *L. etrusca*. However, Jordano (1990) showed in the same study area that *M. stellatarum* was the main pollinator for *L. etrusca*.

The six plant species relied on pollinators to set fruits. Spontaneous fruit production by flowers from which the insect pollinators were excluded was very low for the six plant species (*A. calcarea*, 0%; *A. ramosus*, 3.9%; *E. plantagineum*, 3.8%; *L. etrusca*, 0%; *P. purpurea*, 1.8%; *T. fruticans*, 4.8%).

Direct effects of nectar yeasts on female reproductive success

The inoculation of *M. reukaufii* and posterior hand-pollination had no significant direct effect on the measured variables in any of the six plant species when floral visitors were excluded. The number of flowers setting fruits was not affected by yeast inoculation, neither was the seed set or individual seed weight (Table 2; Appendix S5).

Indirect effects of nectar yeasts on female reproductive success

The indirect effect of *M. reukaufii* on female reproductive success varied among plant species exposed to pollinators. For three of the four plant species visited by a diverse pollinator community (*A. calcarea*, *A. ramosus*, and *E. plantagineum*), there was no significant effect of the inoculation treatment on the proportion of flowers setting fruits or on seed set and seed mass in any year (Table 3; Appendix S6). In *T. fruticans*, the effect of yeast inoculation on seed set differed between years (significant treatment × year interaction, Table 3), with an increase in the first year and a decrease in the second. However, in Tukey tests conducted separately by year, the treatment effect was not statistically significant ($Z = 1.920$, $P = 0.220$ for year 1; $Z = -2.031$, $P = 0.177$ for year 2).

Interestingly, for the two species that relied almost on one pollinator, significant differences were observed between YE and CO flowers, but with opposite effects on different fitness components within species, and also on the same fitness component between the two species (Figure 2). In *P. purpurea*, yeast inoculation on average increased fruit set by 83–90% compared to the control treatment, with nearly identical effects in both years (Table 3, Figure 2). Seed set was not affected by the inoculation treatment, but individual seed mass was 7–12% lower in YE flowers than in CO flowers (Table 3, Figure 2).

In *L. etrusca*, the proportion of YE flowers setting fruits was 53–60% lower compared to CO flowers (Table 3, Figure 2). The effect of yeast inoculation on number of seeds produced differed between years (significant treatment × year interaction, Table 3), with an increase in the first year and a decrease in the second. However, in Tukey tests conducted separately by year, the treatment effect was not statistically significant ($t = 1.095$, $P = 0.693$ for year 1;

TABLE 1 Pollinators of the six plant species observed during the 2 years of this study. Numbers in parentheses indicate the number of unidentified pollinator species for each genus

Plant species	Pollinators first year	Pollinators second year
Anchusa calcarea Boiss.	*Amegilla quadrifasciata* (Apidae) *Amegilla* sp. (Apidae) (1) *Anthophora plumipes* (Apidae) *Anthophora dispar* (Apidae) *Anthophora* sp. (Apidae) (1) *Apis mellifera* (Apidae) *Bombus terrestris* (Apidae) *Bombylius* sp. (Bombyliidae) (1) *Xylocopa cantabrita* (Apidae)	*Amegilla quadrifasciata* (Apidae) *Amegilla* sp. (Apidae) (1) *Anthophora plumipes* (Apidae) *Anthophora dispar* (Apidae) *Anthophora* sp. (Apidae) (1) *Apis mellifera* (Apidae) *Bombus terrestris* (Apidae) *Bombylius major* (Bombyliidae) *Bombylius* spp. (Bombyliidae) (2) *Ceratina cucurbitina* (Apidae) *Eucera nigrilabris* (Apidae) *Eucera* spp. (Apidae) (2) *Xylocopa cantabrita* (Apidae) *Zerynthia rumina* (Papilionidae)
Asphodelus ramosus L.	*Anthophora* spp. (Apidae) (2) *Apis mellifera* (Apidae) *Empis tesellata* (Empididae) *Eucera* spp. (Apidae) (2) *Megachile sicula* (Megachilidae) *Xylocopa cantabrita* (Apidae)	*Anthophora dispar* (Apidae) *Anthophora* sp. (Apidae) (1) *Apis mellifera* (Apidae) *Callophris rubi* (Pieridae) *Empis tesellata* (Empididae) *Empis* spp. (Empididae) (2) *Eucera interrupta* (Apidae) *Eucera* sp. (Apidae) (1) *Megachile sicula* (Megachilidae) *Xylocopa cantabrita* (Apidae)
Echium plantagineum L.	*Andrena* spp. (Andrenidae) (2) *Anthophora* spp. (Apidae) (2) *Apis mellifera* (Apidae) *Bombus terrestris* (Apidae) *Bombylius* sp. (Bombyliidae) (1) *Eucera* spp. (Apidae) (2) *Systoechus* sp. (Bombyliidae) (1)	*Andrena* spp. (Andrenidae) (2) *Anthophora* spp. (Apidae) (2) *Apis mellifera* (Apidae) *Bombus terrestris* (Apidae) *Ceratina cucurbitina* (Apidae) *Eucera* spp. (Apidae) (2) *Gonepteryx cleopatra* (Pieridae) *Systoechus* sp. (Bombyliidae) (1)
Lonicera etrusca Santi	*Macroglossum stellatarum* (Sphingidae)	*Macroglossum stellatarum* (Sphingidae)
Phlomis purpurea L.	*Bombus terrestris* (Apidae)	*Bombus terrestris* (Apidae) *Xylocopa cantabrita* (Apidae)
Teucrium fruticans L.	*Anthophora dispar* (Apidae) *Anthophora* spp. (Apidae) (1) *Apis mellifera* (Apidae) *Bombus terrestris* (Apidae) *Eucera* spp. (Apidae) (2)	*Anthophora dispar* (Apidae) *Anthophora* spp. (Apidae) (2) *Apis mellifera* (Apidae) *Bombus terrestris* (Apidae) *Eucera* spp. (Apidae) (2) *Xylocopa cantabrita* (Apidae)

$t = -2.174$, $P = 0.134$ for year 2). Dry mass of fruits was not affected by the inoculation treatment ($\chi^2 = 0.341$, df = 1, $P = 0.559$), or year ($\chi^2 = 1.344$, df = 1, $P = 0.246$), or their interaction ($\chi^2 = 2.261$, df = 1, $P = 0.133$). However, seed mass of YE flowers was on average 21% higher than that of CO flowers (Table 3, Figure 2).

Comparison of hand pollinations with natural pollinations

There were no significant differences in fruit production between natural vs. hand pollination of yeast-inoculated flowers for five of the study species (Figure 2; Appendices S5–S7). Only in *L. etrusca* did hand pollinations lead to higher fruit production compared to insect pollination (Figure 2; Appendices S5 and S7).

In *L. etrusca*, the effect of experiment on seed production depended on year (significant experiment × year interaction, Appendix S7), and analyses conducted separately by year showed that hand pollinations significantly increased seed production compared to natural pollinations only in the second year ($t = -0.069$, $P = 0.999$ in year 1; $t = 4.515$, $P < 0.001$ in year 2; Figure 2 and Appendix S5).

The largest differences between hand and natural pollinations were found for individual seed mass. Hand

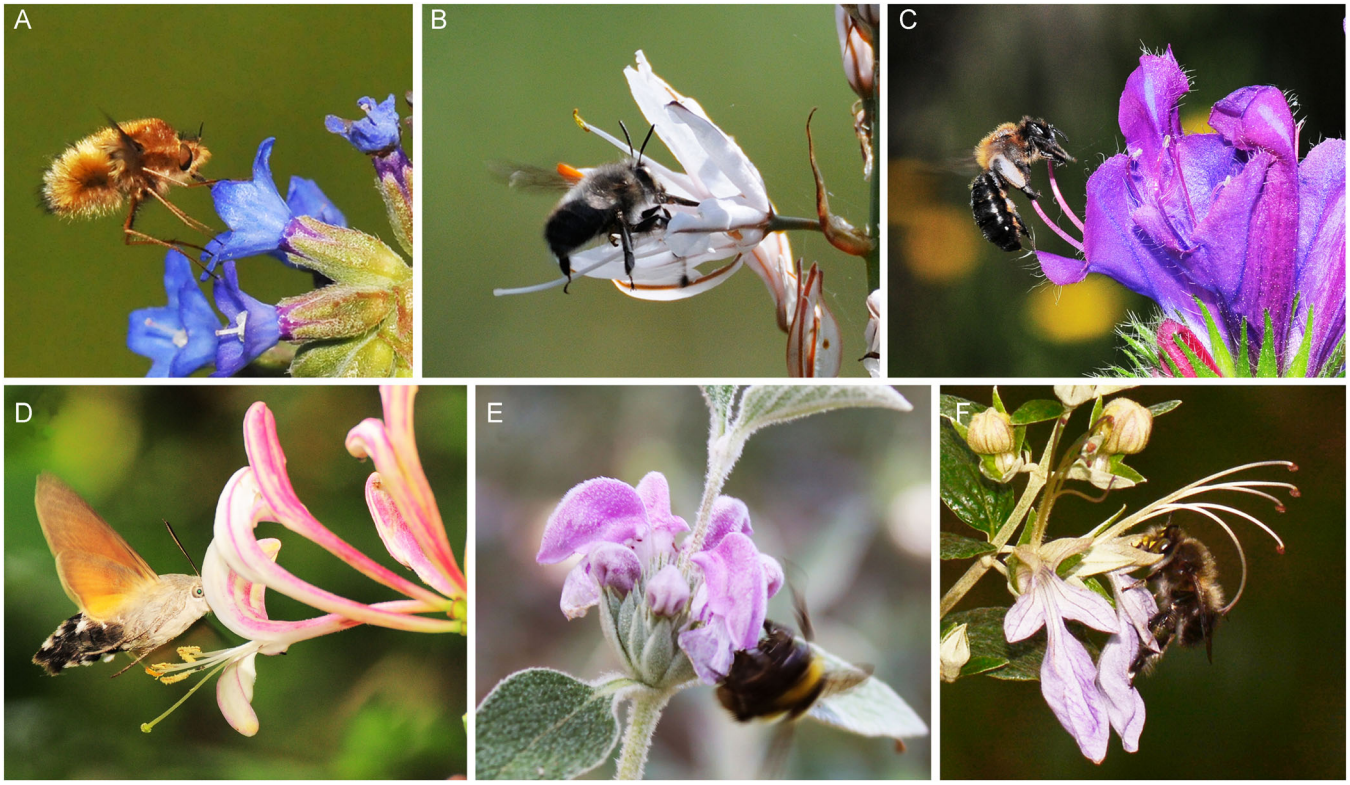

FIGURE 1 Flowers of the six studied plant species that were visited by pollinators. (A) *Anchusa calcarea* and *Bombylius major*; (B) *Asphodelus ramosus* and *Anthophora dispar*; (C) *Echium plantagineum* and *Andrena* sp.; (D) *Lonicera etrusca* and *Macroglossum stellatarum*; (E) *Phlomis purpurea* and *Bombus terrestris*; and (F) *Teucrium fruticans* and *Anthophora dispar*

pollinations led to heavier seeds than natural pollinations in *A. ramosus*, *E. plantagineum*, and *P. purpurea* (Figure 2; Appendices S5–S7). In *A. calcarea* and *T. fruticans*, the effect of experiment on seed mass depended on year (significant experiment × year interaction in Appendix S7). The Tukey tests showed that hand pollinations significantly increased seed production compared to natural pollinations only in the first year in *A. calcarea* ($t = 5.331$, $P < 0.001$, year 1; $t = -2.212$, $P = 0.212$, year 2) and *T. fruticans* ($t = 2.936$, $P = 0.019$, year 1; and $t = 0.829$, $P = 0.841$, year 2) (Appendices S5–S7). In *L. etrusca*, seed mass was similar between hand pollinations and open pollinations in both years (Figure 2; Appendices S5, S7).

DISCUSSION

Microorganisms can affect plant reproduction through both direct and indirect pathways of species' interactions (Afkhami et al., 2020). The present study showed that the inoculation of nectar with the yeast *M. reukaufii* can have different effects on plant reproductive success, including mostly neutral, but also negative or positive effects. Differences in the identity of host plants, pollinators, and species interactions could underlie the variable outcomes observed.

Direct effects of *M. reukaufii* on plant fitness

After hand pollinations of pollinator-excluded flowers, we found no direct effects of *M. reukaufii* on female reproductive success in any of the six species studied. Our findings are consistent with the only previous observations of direct effects on female plant reproduction mediated by *M. reukaufii* on *Delphinium nuttallianum* (Schaeffer and Irwin, 2014). Although our study involved only one species of nectar microorganism, *M. reukaufii* is the dominant yeast species in the study areas (Pozo et al., 2012; Álvarez-Pérez and Herrera, 2013), indicating that that our findings represent realistic outcomes after nectar microbial colonization.

Given the cosmopolitan distribution of *M. reukaufii*, which is the most abundant nectar yeast worldwide (Pozo et al., 2012; Golonka and Vilgalys, 2013; de Vega et al., 2021), it is not trivial to assess whether *M. reukaufii* in general should be considered a pathogen of plants. It is unknown whether the first interactions of a *Metschnikowia* yeast with flowers, thought to have occurred during the Paleocene (approximately 58 Ma; Guzmán et al., 2013), were also commensalistic or whether they evolved from a primitive pathogenic strain of the yeast. Our results derived from the hand pollinations and those of previous studies suggest that *M. reukaufii* is not a plant antagonist, but rather

TABLE 2 Summary of generalized linear mixed models (GLMMs) testing for the direct effects (experiment 1) of inoculation treatment, year, and their interaction on the proportion of flowers that set fruit, seed set, and seed mass of experimental flowers of the six studied plant species pollinated by hand. Numerator degrees of freedom are shown (*df*). Significant effects ($P < 0.05$) are highlighted in bold

Species	Flowers set fruit			Seed set			Seed mass		
	χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*
Species with diverse pollinator community									
Anchusa calcarea									
Treatment	0.004	1	0.949	0.002	1	0.965	0.149	1	0.699
Year	2.690	1	0.101	6.892	1	**0.009**	3.333	1	0.079
Treatment × year	0.025	1	0.874	3.479	1	0.062	1.476	1	0.224
Asphodelus ramosus									
Treatment	0.163	1	0.687	0.222	1	0.637	0.011	1	0.915
Year	0.788	1	0.375	0.176	1	0.675	0.128	1	0.721
Treatment × year	0.014	1	0.906	2.189	1	0.139	0.024	1	0.876
Echium plantagineum									
Treatment	0.076	1	0.783	2.649	1	0.104	1.505	1	0.220
Year	2.050	1	0.152	2.630	1	0.105	0.562	1	0.453
Treatment × year	0.449	1	0.503	0.347	1	0.556	3.756	1	0.056
Teucrium fruticans									
Treatment	0.622	1	0.430	0.277	1	0.599	0.036	1	0.849
Year	0.845	1	0.358	6.296	1	**0.012**	0.007	1	0.932
Treatment × year	3.478	1	0.062	3.679	1	0.055	0.004	1	0.985
Species with limited pollinator community									
Lonicera etrusca									
Treatment	4.045	1	0.065	2.774[a]	1	0.100	0.171	1	0.679
Year	0.183	1	0.669	1.322[a]	1	0.250	0.012	1	0.911
Treatment × year	0.004	1	0.951	2.552[a]	1	0.110	1.899	1	0.168
Phlomis purpurea									
Treatment	0.022	1	0.882	0.304	1	0.581	0.305	1	0.580
Year	0.0001	1	0.994	1.361	1	0.243	0.507	1	0.476
Treatment × year	0.626	1	0.429	0.009	1	0.925	1.772	1	0.183

[a]For *L. etrusca*, the number of seeds per fruit was calculated.

a commensal having no detectable direct fitness costs or benefits for the host plant.

Indirect effects of *M. reukaufii* on plant fitness

The six plant species included in this study strongly depended on pollinators to set fruits, as revealed by their negligible levels of automatic self-pollination. Therefore, any change in the foraging behavior of the pollinators or in the frequency of visits due to the nectar microbes can have different outcomes for the plant, ranging from beneficial or neutral to harmful (Herrera et al., 2013; Vannette et al., 2013; Schaeffer and Irwin, 2014; Yang et al., 2019); and all these possibilities were observed in our study systems.

Early colonization by *M. reukaufii* had significant pollinator-mediated effects on plant reproductive success for *P. purpurea*, which was mainly pollinated by bumblebees, and *L. etrusca*, whose main pollinators were diurnal hawkmoths. For these two species, the sign of pollinator-mediated effects changed depending on whether quantity (proportion of flowers setting fruits) or quality (seed mass as a proxy of allogamous pollination) component of female fitness were considered.

Bumblebees prefer to forage on flowers with concentrated nectar (Cnaani et al., 2006; Kim et al., 2011).

TABLE 3 Summary of generalized linear mixed models testing for the indirect effects (experiment 2) of inoculation treatment, year, and their interaction on the proportions of flowers that set fruit, seed set and seed mas of experimental flowers of the six studied plant species exposed to pollination by insects. Numerator degrees of freedom are shown (df). Significant effects ($P < 0.05$) are highlighted in bold

Species	Flowers set fruit			Seed set			Seed mass		
	χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*
Species with diverse pollinator community									
Anchusa calcarea									
Treatment	0.061	1	0.805	0.139	1	0.709	0.243	1	0.622
Year	21.845	1	**<0.001**	1.047	1	0.306	7.818	1	**0.005**
Treatment × year	0.755	1	0.385	0.472	1	0.492	0.086	1	0.769
Asphodelus ramosus									
Treatment	0.280	1	0.597	2.178	1	0.140	0.682	1	0.408
Year	0.730	1	0.393	0.283	1	0.595	0.268	1	0.605
Treatment × year	2.242	1	0.134	1.469	1	0.225	0.262	1	0.609
Echium plantagineum									
Treatment	2.051	1	0.152	0.922	1	0.337	2.435	1	0.118
Year	3.055	1	0.081	0.433	1	0.511	7.826	1	**0.005**
Treatment × year	0.005	1	0.947	0.057	1	0.810	0.021	1	0.883
Teucrium fruticans									
Treatment	0.289	1	0.591	0.143	1	0.705	1.475	1	0.225
Year	0.437	1	0.509	1.747	1	0.186	4.386	1	**0.036**
Treatment × year	0.712	1	0.399	7.606	1	**0.006**	1.829	1	0.176
Species with limited pollinator community									
Lonicera etrusca									
Treatment	40.076	1	**<0.001**	0.243[a]	1	0.622	19.553	1	**<0.001**
Year	0.081	1	0.776	2.030[a]	1	0.154	0.139	1	0.709
Treatment × year	0.455	1	0.500	5.728[a]	1	**0.017**	0.682	1	0.409
Phlomis purpurea									
Treatment	21.692	1	**<0.001**	0.001	1	0.990	11.453	1	**<0.001**
Year	0.002	1	0.967	0.047	1	0.829	1.418	1	0.234
`Treatment × year	0.028	1	0.867	1.794	1	0.180	0.838	1	0.359

[a]For *L. etrusca*, the number of seeds per fruit was calculated.

Thus, because nectar yeasts render nectar less energetically rewarding (Herrera et al., 2008; de Vega et al., 2009; de Vega and Herrera, 2012), a high yeast incidence is expected cause the bees to leave flowers with diluted nectar. However, early colonization of nectar by *M. reukaufii* appeared to increase bumblebee visitation to flowers of *P. purpurea*, as evidenced by a higher proportion of flowers setting fruits in yeast-inoculated than in control flowers. These findings are supported by previous experiments showing that bumblebees prefer yeast-inoculated flowers over the control flowers in controlled synthetic systems (reviewed by Jacquemyn et al., 2021; Crowley-Gall et al., 2021) and in field experiments (Herrera et al., 2013; Schaeffer and Irwin, 2014; Yang et al., 2019). The proximate mechanisms driving changes in bumblebees' behavior remain unknown. It has been suggested that yeast can constitute a nutritional supplement for insects as a source of vitamins, lipids, and probiotics and can help to reduce the susceptibility of the bumblebee to pathogens (Stefanini, 2018; Pozo et al., 2020). Additionally, fermentation volatiles released by yeasts may act as an honest signal to indicate nectar availability (Raguso, 2004; Schaeffer et al., 2017; Crowley-Gall et al., 2021). Accordingly, the recently proposed dispersal-encounter hypothesis suggests that *M. reukaufii* and bumblebees interact in a

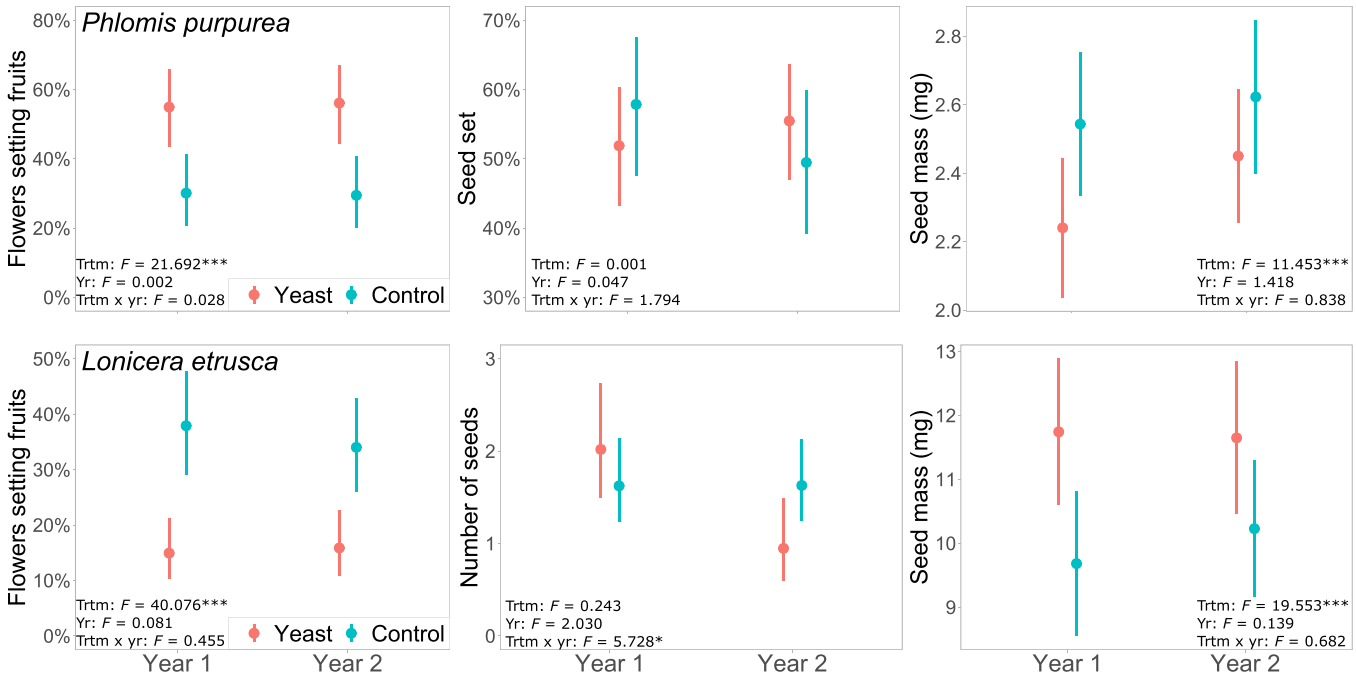

FIGURE 2 Proportion of flowers setting fruits, seed set/number of seeds, and seed mass of open-pollinated flowers in the yeast-inoculated treatment (red) and the control treatment (blue) for the study species *Phlomis purpurea* (above) and *Lonicera etrusca* (below). The results of the GLMM with the factors treatment (Trtm), year (Yr), and the interaction (Trtm × yr) are included in each panel. Asterisks (*) indicate significant differences (*$P < 0.05$; ***$P < 0.001$). The 95% confidence intervals are shown

form of diffuse mutualism, in which yeasts provide sugar as an honest signal and the pollinators in return transport yeasts to new flowers (Madden et al., 2018). These suggestions would partially explain why bumblebees preferred flowers colonized by *M. reukaufii*.

Increased fruit production per se may be a poor descriptor to understand the net effect of nectar yeasts on *P. purpurea*, because offspring quality simultaneously was negatively affected. Seed mass of yeast-inoculated flowers was lower than in control seeds and in hand-pollinated flowers, the latter indicating an effect of supplemental pollination on offspring quality. Lower seed mass of yeast-inoculated flowers has been reported previously for other bumblebee-pollinated plant species (Herrera et al., 2013). The lower seed mass of yeast-inoculated flowers suggests that bumblebees increase geitonogamous pollination, maybe because they spend more time on the flowers (Herrera et al., 2013).

Species-dependent effects were evident in our study, as we found an opposite effect of early colonization of nectar by *M. reukaufii* on female reproductive success in *L. etrusca* versus in *P. purpurea*. The significant lower level of fruit production in yeast-inoculated flowers of *L. etrusca* over control flowers suggests that high densities of *M. reukaufii* led to decreased hawkmoth visitation rates. *Macroglossum stellatarum* can discriminate among flowers that differ in nectar composition and viscosity (Josens and Farina, 1997, 2001), spend more time feeding on more concentrated sugar solutions (Josens and Farina, 1997), and prefer nectar containing sucrose to those with more fructose and glucose (Kelber, 2003). Because *M. reukaufii* at high densities can decrease sugar concentration and change sugar composition by increasing fructose and decreasing sucrose (Herrera et al., 2008; de Vega and Herrera, 2013), it is possible that the metabolic activity of *M. reukaufii* may be driving changes in foraging decisions of the moth. The hawkmoth could prefer microbial-free flowers of *L. etrusca* or even flowers naturally colonized by other microbial species, which may likely cause distinct changes in nectar chemistry.

In contrast, higher yeast densities increased offspring quality in *L. etrusca*. Naturally pollinated flowers inoculated with yeast produced seeds of a similar mass to those produced by hand-pollinated (xenogamous) flowers, but the seeds were significantly heavier than those from control flowers. This result suggests that early colonization of nectar yeasts may cause a decrease in geitonogamy in *L. etrusca*, increasing allogamous pollination. In this way, although early colonization of *M. reukaufii* at high densities may at first seem negative for the plant as the fruit set decreases, the seeds produced are of higher quality. One cannot therefore assume that the net effect of *M. reukaufii* for this species is exclusively positive or negative.

Much of the work to date on understanding indirect plant responses to nectar microorganisms have focused on pairwise plant–pollinator species interactions, in which one species can potentially alter the traits of the other species (Herrera et al., 2013; Yang et al., 2019). Here we also studied microbe–plant species interactions in more complex situations, including plants with diverse pollinator

communities. Our results showed that flowers initially inoculated with yeasts harbored significantly higher microbial densities than those naturally colonized by microbes that were transported by pollinators. It is expected that inoculated flowers would have microbial communities dominated by *M. reukaufii*, while the controls have no microbes or harbor microbial communities dominated by other species. However, the expected differences in nectar chemistry between flowers that received different treatments would not have influenced plant reproduction on most of the species with a high diversity of pollinators. We can hypothesize that the lack of influence on plant reproduction was due to the absence of any microbial-induced effects on the specific community of pollinators for each plant species. However, most of these species were pollinated, among others, by *B. terrestris* that have shown to prefer flowers with high densities of *M. reukaufii* (Herrera et al., 2013; Schaeffer and Irwin, 2014; Yang et al., 2019). Some pollinators could be attracted and others deterred by high densities of *M. reukaufii* or have differ in their preferences for nectar microbial composition, so that the pressures exerted by one species could be erased by those exerted by other species. For the plant species with a diverse pollinator community, yeast inoculation had opposite effects on the seed set of *T. fruticans*, but in the analyses conducted separately by year, the treatment effect was not statistically significant. The tendency for opposite effects in the 2 years could be related to variation in pollination (the pollinator community was slightly less diverse in the first year of observations) or variation in the priority effects and cascade of interactions between species. Field experiments including pollinator observations that rigorously test the preference or aversion of specific insects to control and treated flowers would be required to test the aforementioned hypotheses.

Microbes depend mostly on animal visitors for their dispersal and establishment in nectar (Brysch-Herzberg, 2004; Herrera et al., 2010; de Vega and Herrera, 2012; Schaeffer and Irwin, 2014; Vannette and Fukami, 2017). Once established, nectar microorganisms may modify the plant's floral chemical phenotype and consequently the relationships with their pollinators (Herrera et al., 2008; Peay et al., 2012; Vannette and Fukami, 2018; Pozo et al., 2020; Crowley-Gall et al., 2021). Priority effects, in which the consequences of interactions depend on the order of species arrival is fundamental in determining microbial community assembly (Peay et al., 2012; Tucker and Fukami, 2014; Dhami et al., 2016). In our experiments, early colonization by *M. reukaufii* in the yeast-inoculated flowers exposed to pollinators likely modified the nectar microhabitat rapidly enough to hamper the establishment of late-arriving species transported by pollinators, though this competitive exclusion is certainly not absolute. Therefore, the differences between treatments observed in this study may be driven not only by specific outcomes of *M. reukaufii* on pollinators, but also by *M. reukaufii* interactions with other microbes.

CONCLUSIONS AND PERSPECTIVES

Our study suggests that *M. reukaufii* had no direct effects on plant reproduction, indicating that this nectar yeast should be viewed as a commensal for the study species. Secondly, we have shown that the inoculation with nectar yeasts can affect plant reproduction via pollinator-mediated mechanisms. The variability present in plant–pollinator–microbe interactions observed here demonstrates that we cannot extrapolate the effects of nectar microbes on plant reproduction from one plant species to another.

We are still at the very beginning of understanding the ecological and evolutionary processes underlying the plant–pollinator–nectar microorganism interactions. Pollinators are important selective agents on floral traits, and effects of nectar microbes mediated by pollinators could potentially have an evolutionary impact on plants. How often nectar microbial communities affect patterns of natural selection on plant traits remains an open question.

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AUTHOR CONTRIBUTIONS

C.d.V. and C.M.H. conceived the study and designed the research. C.d.V. and R.G.A. conducted fieldwork. C.d.V. and S.A.P. performed lab work. C.d.V. and R.G.A. performed statistical analyses. C.M.H. and C.d.V contributed reagents and materials. C.d.V. wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Sanger sequences are accessible at NCBI GenBank under accession numbers MZ562885 and MZ562886. Further data associated with this article are available at the Digital.CSIC Repository http://hdl.handle.net/10261/256036.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1. Characteristics of the study plant species.

Appendix S2. Sample size (number of flowers) for each plant species used for the experiments to test for indirect and direct effects of nectar yeasts on female reproductive success.

Appendix S3. Yeast isolation, DNA extraction, and sequencing.

Appendix S4. Comparisons of yeast densities in yeast-inoculated and control flowers exposed to pollinators.

Appendix S5. Proportion of flowers setting fruits, seed set, and seed mass of hand-pollinated flowers in the yeast-inoculated treatment (red) and the control treatment (blue) for the six study species (direct effects). The 95% confidence intervals are shown. The results of the GLMMs are shown in Table 2.

Appendix S6. Proportion of flowers setting fruits, seed set, and seed mass of flowers exposed to pollinators in the yeast-inoculated treatment (red) and control treatment (blue) for the four study species with a diverse pollinator community (indirect effects). The 95% confidence intervals are shown. The results of the GLMMs are shown in Table 3.

Appendix S7. Summary of generalized linear mixed models testing for differences between the fruit set, seed set, and individual seed mass from yeast-inoculated, hand-pollinated flowers in experiment 1 (hand-pollinated flowers) compared with those from flowers pollinated by insects in experiment 2 (flowers exposed to pollinators). Numerator degrees of freedom are shown (df). Significant effects ($P < 0.05$) are highlighted in bold.

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