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ORIGINAL RESEARCH ARTICLE

# Nectar phenolics drive cross visits between dimorphic flowers by honey bees

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## Summary

Broccoli (*Brassica oleracea*) is widely grown. For hybrid seed production, farmers use a hermaphrodite parent, conventionally called "male", to cross with a male sterile parent, or "female". We fed *Apis mellifera* colonies with 30 % sucrose solution with (P-fed) and without (S-fed) para-hydroxybenzoic acid (a phenolic acid commonly found in nectar), and compared the number of pollen foragers between the P- and S-fed colonies. As compared to the S-fed colonies, the P-fed colonies had higher proportions of foragers visiting the male plants for pollen. Also, they had higher proportions of pollen foragers that switched to visit the female plants. Our study suggests that nectar phenolics may be adaptively suited to augment outcrossing pollination by honey bees.

# Los fenoles contenidos en el néctar llevan a las abejas melíferas a realizar visitas cruzadas entre flores dimórficas

### Resumen

El brócoli (*Brassica* oleracea) es una planta frecuentemente cultivada. Con el fin de producir semillas híbridas, los agricultores utilizan plantas parentales hermafroditas, convencionalmente llamadas "macho", que son cruzadas con un macho estéril, o "hembra". Colonias de *Apis mellifera* fueron alimentadas con solución de sacarosa al 30% con (P-fed) o sin (S-fed) ácido para-hidroxibenzoico (fenol que se encuentra en el néctar), y se comparó el número de pecoreadoras de polen entre las colonias P- y S-fed. Tras dicha comparación, se comprobó que las colonias P-fed contenían una mayor proporción de pecoreadoras que visitaron las plantas masculinas por su polen. Asimismo, éstas mostraron una mayor proporción de pecoreadoras de polen que pasaron a visitar las plantas hembra. Nuestro estudio sugiere que los fenoles del néctar podrían haber contribuido de manera adaptativa al aumento en la polinización cruzada llevada a cabo por las abejas melíferas.

**Keywords:** *Apis mellifera*, nectar, *para*-hydroxybenzoic acid, pollination

## Introduction

In sexually dimorphic plants including dioecious and gynodioecious, female flowers naturally lack pollen and usually produce little nectar (Carlson and Harms, 2006; Delph and Lively, 1992; Willson and Ågren, 1989). Male biased nectar production, relatively common in sexually dimorphic species (Carlson and Harms, 2006), has been shown to attract pollinator visits to males or hermaphrodites, thus potentially increasing pollen export (Bell *et al.,* 1984; Mitchell, 1993). However, few studies have evaluated whether the male biased nectar production promotes pollinators to visit female flowers, thereby potentially increasing the likelihood of pollen receipt by female flowers or raising outcrossing fitness.

Nectars are more than just sugar solutions and commonly contain various secondary compounds such as phenolics (Nicolson and Thornburg, 2007). Studies have shown that foraging bees of *Apis* display reduced sugar response thresholds after the intake of nectar phenolics (Liu *et al.*, 2007; Liu and Liu, 2010). Worker bees with low sugar response thresholds tend to become pollen foragers (Liu *et al.,* 2006; Pankiw and Page, 2000). Therefore, nectar phenolics are expected to encourage foraging bees to visit male or hermaphrodites flowers for pollen. In addition, nectar phenolics can induce increased intake of more dilute nectar by honey bees (Liu *et al.*, 2007; Liu *et al.,* 2004), thus perhaps increasing their seeking other nectar sources and movement between co-flowering individuals. Also, nectar phenolics can augment more foragers to go out of the hive for food collection (Liu *et al.,* 2007). We hypothesize that nectar phenolics may modify honey bee foraging behaviour to increase visits for both male flowers with pollen and female flowers lacking reward to potentially drive outcrossing pollination.

In this study, we examined the effect of nectar phenolics on honey bee visits between the hermaphrodite and female flowers of an artificial breeding system. To achieve this, we fed colonies a pure sugar solution or the same sugar solution with *para*-hydroxybenzoic acid, and compared the proportions of pollen foragers between the two treatments. Also, we tested whether the phenolic acid drove pollen foragers to switch their visits between male and female flowers. For this purpose, we investigated pollen foragers on female flowers, and compared the proportions of pollen foragers visiting female flowers between the two treatments.

# Material and methods

## Artificial breeding system

Broccoli (*Brassica oleracea*) is widely grown throughout the world. In China, farmers use a monoecious parent, conventionally called "hermaphrodite" (Fig. 1a), to cross with a male sterile parent, or "female" (Fig. 1b) to produce hybrid seeds for a cauliflower cultivar (*B. oleracea* var. *botrytis*, Fig. 1), which is usually labelled as '3592' by seed companies. Its hermaphrodites and females typically grow in dense clusters 70-90 cm in height. Both flower synchronously from February until March. The parent lines have hitherto been multiplied by laborious hand pollination for the commercial production of hybrid seeds.

## Plant transplantation

Our study was conducted in an agricultural field in Yuanmo County (101°35′-102°06′ E, 25°23′-26°06′ N), Yunnan Province, China during 2006-2007. Six-week-old female seedlings (ca. 6 - 7 cm tall, derived by tissue culture) and hermaphrodites (derived from seeds) were obtained from the Research Institute of Tropical Eco-agriculture Sciences. A total of 500 plants (250 for each morph) were planted in alternate rows in one *mu* (0.63 ha) in October of each year. Field care including fertilizer, irrigation and pest control was necessary.

## Honey bee colonies and their foraging choices

At the full blossom stage, we evenly split the plot into two parts and separately netted plants with a mesh 3 m in height. Two food-deprived colonies of *Apis mellifera* (with 8,000 worker bees and without brood) were moved into the parts, respectively. In the one part, the colony was fed daily with 200 ml of 30 % (wt/wt) sucrose solution (S-fed). In another part, the colony was daily fed with the same amount of the syrup with 0.08 % 4-hydroxybenzoic acid (P-fed). This phenolic acid is common in floral nectar, and its concentration in natural nectar varies from 0.004 % to 0.05 % (da Silva *et al.,* 2013). The concentration of the phenolic acid used for our study is preferred by honey bees (Liu *et al.*, 2007).

To determine bee foraging choice, we monitored returning foragers at hive entrances on nine days in 2006. To avoid site bias by foraging bees, we used one of two parts for the control colony on 12, 16, 20 and 26 February, and switched it for the treatment colony on 10, 14, 19, 23 and 31 February. The other part was reverse. We performed the similar experiment on seven days in 2007. A given part was first used for the control colony on 19, 23 and 27 February, and then was switched for the treatment colony on 15, 21, 25 and 28 February. Returning foragers to each hive were observed for three times in the morning (1000 - 1200 h) and two times in the afternoon (1400-1500 h). The two periods were the peaks of daily bee foraging activity. Each observation lasted 10 min. Foraging bees were classified

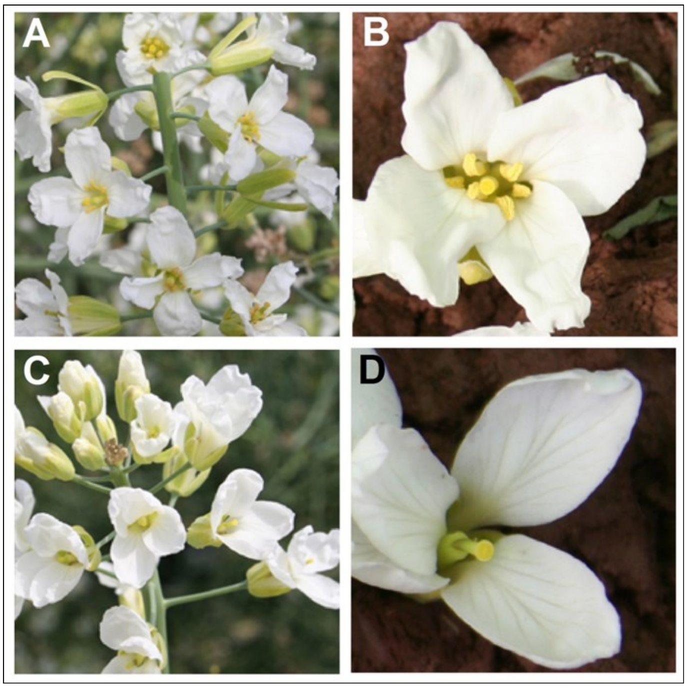

***Fig. 1.*** An artificial breeding system for commercial hybrid seed production of the cauliflower cultivar (*B. oleracea* var. *botrytis*). ***A.*** A branch from the main stem of a hermaphrodite parent, a "male"; ***B.*** A hermaphrodite flower of the male; ***C.*** A branch from the main stem of a male-sterile parent, conventionally called "female"; ***D.*** A male-sterile flower of the female.

as nectar or pollen foragers based on the absence or presence, respectively, of conspicuous yellow pollen loads on the hind legs.

To assess pollen foragers visiting female flowers, we asked observers to walk along plant arrays, and record pollen foragers on female flowers. Each time, observers spent about 15 - 20 min examining all plants in each part. The frequency of data collection was the same as above for bee surveys at the hive entrances, and five observation periods (three in the morning and two in the afternoon) took place at daily peak foraging times.

### Statistical analysis

For the investigation of foragers at each hive entrance, we summed the foragers and pollen foragers in the five observation periods (three in the morning and two in the afternoon) of a day, and calculated the proportion of pollen foragers in a given day for each colony. The proportions of pollen foragers in nine days of 2006 or in seven days of 2007 were compared between the S-fed and P-fed using a chi-square test. We also evaluated the proportions of pollen foragers on females, and compared proportions of female-visiting pollen foragers between the S-fed and P-fed using a chi-square test.

## Results

In the S-fed colonies, most honey bee foragers visited the flowers only for nectar. In 2006, only 30 % of the foragers were found to collect pollen (Fig. 2A). In 2007, the pollen foragers were less than 15 % (Fig. 2B). It was also the case for the P-fed colonies. Nectar foragers were overwhelmingly more than pollen foragers in either 2006 or 2007 (Fig. 2).

We compared the proportions of pollen foragers between the S-fed and P-fed colonies. In 2006, the P-fed colonies had higher (Chi-square test, d.f = 8, $P = 0.046$; Fig. 2A) proportions of pollen foragers than the S-fed ones. Also, the P-fed had significantly (d.f = 8, $P = 0.032$; Fig. 2A) higher proportions of pollen foragers on female flowers than the S-fed. In 2007, as compared to the S-fed colonies, the P-fed ones had significantly (d.f = 6, $P = 0.023$; Fig. 2B) higher proportions of pollen foragers, and also had higher (d.f = 6, $P = 0.016$; Fig. 2B) proportions of pollen foragers on female flowers.

## Discussion

Bees usually show a visitation bias, and visit hermaphrodite flower rather than female flowers. Far from being absolute in preference, bees are often found to move between hermaphrodite and female flowers (Ashman, 2000). Our data show that the phenolic-laced sugar solution could augment bees to visit the hermaphrodite flowers for pollen, and also drove pollen foragers to visit female flowers. This indicates that nectar phenolics exert an additional force which drives forager movement between sexual morphs, thus possibly promoting outcrossing fertilization.

How did nectar phenolics drive bee movement between sexual morphs? A previous study has shown that plant secondary compounds in nectar (alkaloids) can optimize outcrossing through rapid pollinator response via peripheral chemoreceptors (Kessler *et al.*, 2008). It has been also shown that nectar phenolics can modify the sensitivity of bee receptor cells in the peripheral taste system to make them become pollen foragers (Liu and Liu, 2010). As compared to nectar foragers, pollen foragers possess highly variable sugar sensitivity responses and are potentially more flexible in nectar flower choice (Page *et al.*, 2006). Therefore, pollen foragers' flexibility in flower choice and their increased demand for sugar may result in more

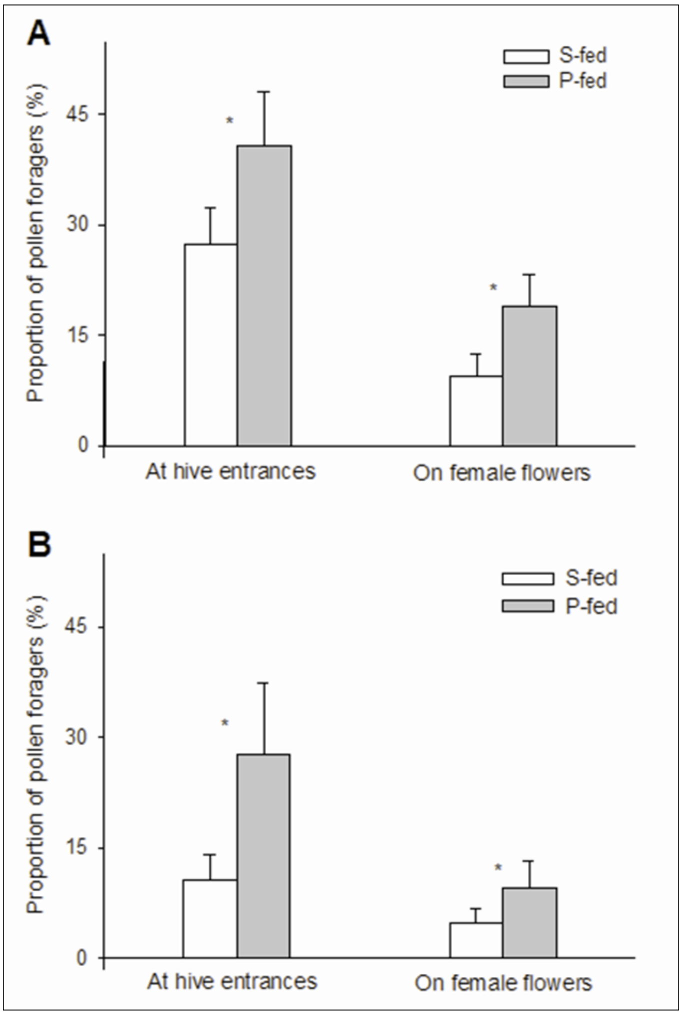

***Fig. 2.*** Comparison of pollen foraging honey bees between from the colonies fed a sugar solution (S-fed) and from those fed a sugar solution with 4-hydroxybenzoic acid (P-fed). As compared to the S-fed colonies, the P-fed colonies had higher proportion of pollen foragers and also had higher proportion of pollen foragers on female flowers either in 2006 ***A.*** or in 2007 ***B.*** Bars denote means ± standard errors. Significances were determined by chi-square tests: $^{*}P < 0.001$.

frequent movement between sexual flowers during foraging bouts.

In tropical forests where dioecious species are common (Bawa, 1980; Bullock, 1985), flower visitors innately prefer a particular colour and scent, and exploit them as cues for floral recognition and selection. Classical arguments state that less rewarding female flowers obtain pollinators by mimicking male or hermaphrodite signals (Ågren, Elmqvist, and Tunlid, 1986; Armstrong, 1997; Bawa, 1980). In fact, however, female flowers are less scented than male flowers (Hemborg and Bond, 2005). We suggest that the lack of female signals may reduce competition between sexual flowers. Our work provides a new insight into the evolution of sexually dimorphic species.

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