



RESEARCH ARTICLE - ANTS

Leaf Volatiles from Two *Corydalis* Species Lure a Keystone Seed-dispersing Ant and Enhance Seed Retrieval

Y ZHU, D WANG

School of Life Sciences, Central China Normal University, Key Laboratory for Geographical Process Analysis & Simulation, Wuhan, P.R. China

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Corresponding author

Dong Wang
 School of Life Sciences
 Central China Normal University
 Key Laboratory for Geographical Process
 Analysis & Simulation
 Hubei Province, Wuhan, 430079, China.
 E-Mail: 395351998@qq.com
 dw@mail.ccnu.edu.cn

Abstract

It has been reported that a suit of plant traits can regulate the ant-seed interaction and subsequently affect the seed dispersal. However, the role of plant volatiles in attracting the ants for seed dispersal remains little examined. We used a Y-tube olfactometer to test behavior response of a keystone seed-dispersing ant (*Pristomyrmex pungens* Mayr) to leaves and seeds of five co-occurring myrmecochorous *Corydalis* species (*C. wilfordii* Regel, *C. racemosa* (Thunberg) Persoon, *C. sheareri* S. Moore, *C. balansae* Prain and *C. incisa* (Thunberg) Persoon). Of the five species, only *C. wilfordii* and *C. racemosa* leaves emits heavily volatiles. We also performed seed cafeteria experiments to assess the effect of leaf volatiles from *C. racemosa* on seed retrieval by presenting simultaneously the seeds near the fresh leaf and the leaf immersed by diethyl ether both in the field and lab. The experiment using Y-tube showed that the ants were only significantly attracted by the fresh leaves of two species, *C. wilfordii* and *C. racemosa*. The cafeteria experiments showed that ants spent less time to detect the *C. racemosa* seeds which were near the fresh leaf, and transported these seeds more quickly. This indicated that the leaf volatiles can function as an attractant for the dispersing ants, and ant preference in turn enhance the seed retrieval. The findings reveal that leaf volatiles can play an important but underestimated role in shaping the ant-seed dispersing interactions.

Introduction

Myrmecochory (i.e., seed dispersal by ants) is a common ecological interaction in nature. Ants benefit nutrition from elaiosomes attached to seeds and plants benefit from having their seeds dispersed (Beattie, 1985). The plants that rely on ant dispersal can benefit from interaction in several ways, including directed dispersal, distance dispersal, fire avoidance and seed predator avoidance (Beattie, 1985; Giladi, 2006). Myrmecochorous plants have evolved some features, which referred to dispersal syndromes, to increase the probability and effectiveness of seeds being transported by ants. To date, diverse features of plants such as elaiosome size and chemistry (e.g., Hughes & Westoby, 1992; Gammans et al., 2006), seed morphology (e.g., Nakanishi, 1994), pre-dispersal mode (e.g., Nakanishi, 1994; Giladi, 2006), plant stature (e.g., Leal et al., 2015), the timing of seed release (e.g.,

Oberrath et al., 2002; Boulay et al., 2007; Arandarickert & Fracchia, 2012), and spatial dispersion of seeds (e.g., Gorb and Gorb, 2000), have been reported to influence the association between seed-dispersing ants and myrmecochorous plants.

Many plants emit volatile organic compounds (VOCs) from most parts of their anatomy (Baldwin, 2010). The volatiles, functioning as either a deterrent to defend them against herbivores and pathogens or an attractant to lure pollinators and seed dispersers, are presumed to serve as cues that mediate various interactions with animals (Inui et al., 2003; Raguso, 2008; Unsicker et al., 2009; Baldwin, 2010). Previous studies found that ants which are involved in interactions with plants, such as protective ant-plant interaction (e.g., Brouat et al., 2000; Edwards et al., 2007) and ant-flower interaction (e.g., Wanjiku et al., 2014; Vega et al., 2014), could respond to the VOCs from host plants. However, it is still less known about the role of volatiles in ant-seed



dispersal interactions (Sheridan et al., 1996; Youngsteadt et al., 2008). The few existing results remain inconsistent (Borges, 2015), with ants being attracted by seed odor of Antgarden (AG) plant in Amazonian rainforests (Youngsteadt et al., 2008), but not for the myrmecochorous seeds *Asarum canadense* (Sheridan et al., 1996).

Apart from odor-induced seed retrieval by ants, volatiles from vegetative parts such as leaves can also mediate the interaction between ants and plants (Brouat et al., 2000; Pichersky & Gershenzon, 2002). Until now, whether volatiles from vegetative organs such as leaves can attract the dispersing ants and such the effect of attractiveness it may have on subsequent seed retrieval remains largely unclear. The *Corydalis* (Papaveraceae) is a north-temperate genus with about 465 species, about 357 of which are distributed in China (Zhang et al., 2008). The species of *Corydalis* have elaiosomes attached to seeds to attract ants (Lengyel et al., 2010). Of them, some species can readily emit volatiles from vegetative organs, whereas others do not (Wu et al., 1999; Zhang et al., 2008). We hypothesized that (1) leaf volatiles of *Corydalis* can function as an attractant for keystone seed-dispersing ant, and (2) the resultant attractiveness would influence seed collection.

Methods

Study organisms

In this study, five *Corydalis* species including *C. wilfordii*, *C. racemosa*, *C. shearereri*, *C. balansae* and *C. incisa* were used. All the five species are ant-dispersed plants, and they co-occur in temperate forests at the margins of Mufushan Mountains (31°49'N, 113°55'E), Hubei province, China. Of them, only *C. wilfordii* and *C. racemosa* emit heavily volatiles, especially the leaves (Wu et al., 1999; Zhang et al., 2008). The leaves are still present on the fruiting plants of all five species.

Olfactometer Assay

To test the behavior response of ants to leaves and seeds from the five studied species, we conducted olfactometer assay by using Y-tube olfactometer. Our previous study showed that the *Pristomyrmex pungens* Mayr (Formicidae: Myrmicinae) was a keystone seed disperser of *Corydalis* species in the study site (Zhu & Wang, 2014). The olfactometer was a Y-shaped glass tube with a 2.6cm diameter and each of the three arms 10 cm in length. Air flow of 750 ml/min was generated with an air pump (QC-4S, China) that directed ambient air through a charcoal filter, odor source bottles before entering a Y-shaped Teflon tube that split air flow evenly between the two arms of the olfactometer. Odorant sources were placed into an odor source bottle (the other bottles were left blank), and 50–80 ant workers were induced to the basal arm. The initial choices of the first 30 different ant workers that walked into and proceeded at least 5 cm down the right or left arm

of the olfactometer were recorded. Thirteen replicates were conducted for each treatment. The Y-tube was washed, and the two side arms were exchanged in turn for treatments and control after each replicate. The odor sources tested in the experiments were as follows: (1) Leaf of *Corydalis* spp. vs blank; (2) Seed of *Corydalis* spp. vs blank. The T-test was used to test the response of workers to the odor sources and blank.

Cafeteria experiments

To assess the effect of volatiles from leaf on seed retrieval, we used *C. racemosa* seeds to conduct a cafeteria experiment both in the field and lab. In the field, we assembled 20 quadrats (1 × 1 m) 10 m apart in a 4 × 5 grid at a site which was at least 20 m away from the nearest *C. racemosa* individual. Each quadrat included two seed depots 1 m apart. For each depot, five seeds were randomly placed near two types of leaves which mimic the presence or absence of volatiles in the field: (1) *Fresh leaf*; (2) *Immersed leaf*— leaf was immersed by diethyl ether for 8 h for removal of volatiles, and were then left overnight at room temperature to allow the diethyl ether to evaporate. The experiment was conducted during peak foraging periods (0800–1100 h). Observations lasted 12 min, at which point the number of seeds removed from each depot was recorded. Twenty trials were conducted.

The workers of *P. pungens* could recruit workers to collect seeds of some *Corydalis* species directly from their dehiscing capsules (Fig 1). We collected four colonies of *P. pungens* in the field, and maintained them in the laboratory for bioassays. The colonies were reared in plaster nests (10 × 10 × 0.4 cm) and placed in an area (50 × 37 × 8 cm) with walls coated with Fluon to prevent ants from escaping. Each nest contained approximately 300–400 workers and 30–40 larvae. The laboratory temperature and relative humidity were maintained in a range from 21 to 26°C and approximately 36%, respectively. We supplied ants with water and sucrose water (1M), and twice a week with mealworms.

In the lab, we placed simultaneously five seeds near the two types of leaves (fresh leaf and immersed leaf) on the sites which left ca. 15 cm to the nest entrance in the laboratory (inter-leaf distance ca. 20 cm). Observations lasted 12 min, at which point the number of removed seeds from each leaf was recorded. Eight trials (4 colonies × 2 replicates) were conducted.

Data analysis

SPSS v20 (IBM, Inc.) was used for statistical analyses and the significant difference was set at $p < 0.05$. Data were transformed prior to analysis when necessary. T-test was used to test the response of workers to the odor sources and blank. General linear model (GLM) was used to test the effects of leaf type on the number of removed seeds (ln-transformed) in the lab and nest identity was included in this model as a random factor. T-test was also used to test the effects of leaf type on the number of seed removals in the field.



Fig 1. Workers of *Pristomyrmex pungens* directly collected *Corydalis racemosa* seeds from the dehiscing carpels in the field (A); workers were attracted by capsules of *C. racemosa* in the lab (B)

Results

In olfactometer assay, workers of *P. pungens* significantly preferred the arm containing leaves of *C. wilfordii* ($t = 3.477$, $df = 24$, $p = 0.002$) and *C. racemosa* ($t = 2.657$, $df = 24$, $p = 0.014$) over the empty control arm, while workers showed no obvious preference to leaves of the remaining three species (Fig 2A). Workers did not prefer the arm containing seeds than the empty control arm, regardless of plant identity (Fig 2B).

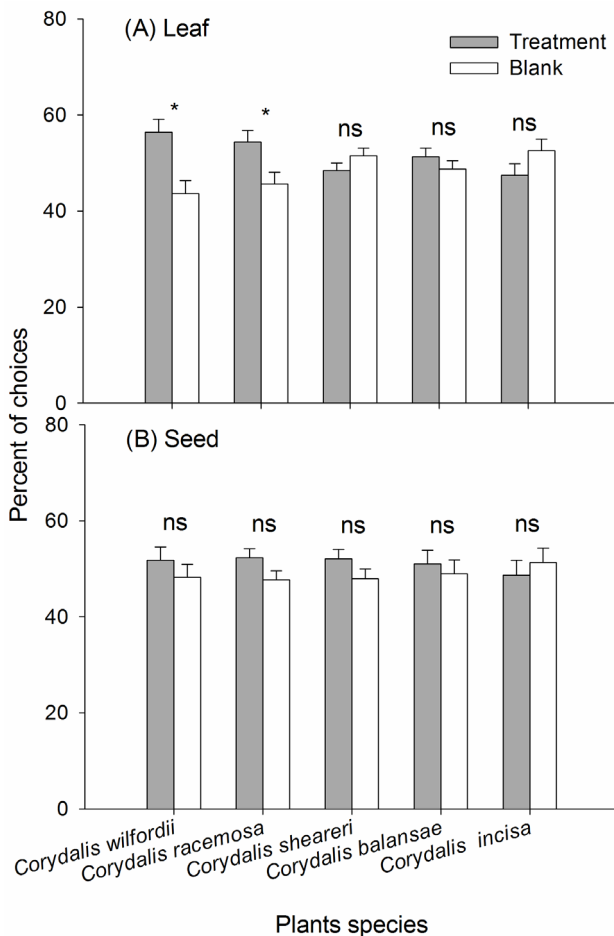


Fig 2. Responses of *P. pungens* (mean \pm SE) in a Y-tube olfactometer when given a choice between plant and clean air. (A) Leaf vs blank, (B) seed vs blank. The asterisk indicates significant difference within a choice test.

In cafeteria experiments, four species of ants were observed to retrieve seeds in the field, with *P. pungens* being responsible for the vast majority of seed removal (73.7%; Table 1). Ants located the seeds which were near the fresh leaf threefold and fourfold earlier than the seeds which were near the immersed leaf in the field and lab, respectively (Fig 3). In the field, the number of seed removals near the fresh leaf was significantly greater than that near the immersed leaf ($t = 3.365$, $df = 23.235$, $p = 0.003$; Fig 3). In the lab, the number of seed

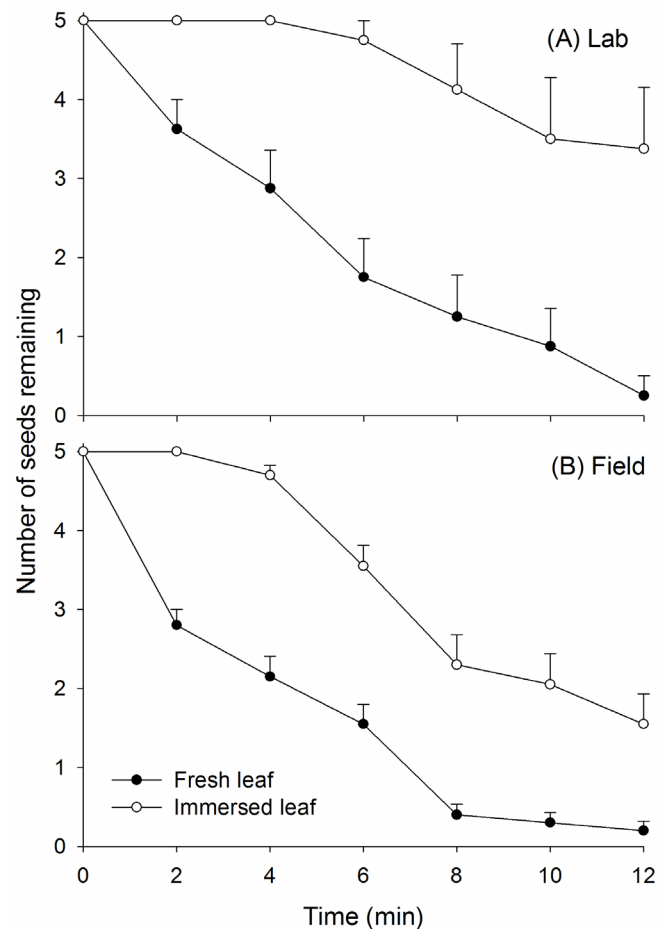


Fig 3. Number of mean seeds remaining (\pm SE) of *C. racemosa* in the field (A) and lab (B).

removals near the fresh leaf was also significantly greater than that near the immersed leaf ($F = 4.572$, $df = 1$, $p = 0.017$), while the nest identity had no effects on seed removals ($F = 1.907$, $df = 3$, $p = 0.305$). Comparing seed-removal dynamics, ants removed nearly all seeds which were near the fresh leaf after 12 min (95% in both the field and lab), whereas they only removed 69.0% and 32.5% of seeds which were near immersed leaf in the field and lab, respectively (Fig 3).

Table 1. Number of removed seeds by each dispersing ant in the field.

Ant species	Fresh leaf	Immersed leaf
<i>Pristomyrmex pungens</i>	74	36
<i>Pheidole nodus</i>	12	7
<i>Paratrechina yerburyi</i>	8	14
<i>Pachycondyla luteipes</i>	1	0

Discussion

This study showed that leaf volatiles from *C. wilfordii* and *C. racemosa* can lure the keystone dispersing ants *Pristomyrmex pungens*, and subsequently promote the seed retrieval. Previous studies have investigated the attractiveness of volatiles to ants in the AG plant and myrmecochorous plants, and some studies found the attractiveness of seed odor to ants (Davidson et al., 1990; Youngsteadt et al., 2008), and others did not (Sheridan et al., 1996; Gammans et al., 2006; Youngsteadt et al., 2010). In the olfactometer assay, we did not observe the behavior response of *P. pungens* to seeds from the five *Corydalis* species, implying that the seeds may not attract dispersing ants by olfactory cues to some extent. The possible reason is that the lower volatility of compounds in the diaspores insufficiently serves as gustatory cues (Borges, 2015). Indeed, Turner et al. (2013) also reported that gas chromatography could not detect volatile chemicals from the diaspores of four typical myrmecochorous plants (i.e., *Asarum canadense*, *Trillium grandiflorum*, *Sanguinaria canadensis* and *T. erectum*).

The plants that rely on ant dispersal can benefit from interactions in several ways (Beattie, 1985; Lengyel et al., 2010). Plants may therefore evolve some dispersal syndromes to enhance seed dispersal effectiveness by ants. For example, oleic acid or its dimer diolein in elaiosome have been found to induce seed carrying behavior (Hughes et al., 1994; Pfeiffer et al., 2010). The time of fruit dehiscence and seed shedding of some ant-dispersed plants at a particular moment greatly enhance the probability of seed being removed by effective seed-dispersing ants (Arandarickert & Fracchia, 2012; Boulay et al., 2007). In the present study, ants can be attracted by volatile compounds released by both *C. wilfordii* and *C. racemosa* plants and ants could use them as chemical cue to locate the plants and, consequently, the diaspores. As a result, the presence of leaf volatiles greatly shortened the ant foraging time and increased the removal rates of seeds.

This implied that the leaf volatiles of some *Corydalis* species may be one important trait promoting the association between plants and ants.

Although the myrmecochorous seeds can be found and then transported by ants because of the attached elaiosome, the results of our study indicated that leaf volatiles of some myrmecochorous *Corydalis* species can function as an attractant for ant dispersers, and such attractiveness in turn enhances the seed retrieval. It suggests that leaf volatiles of some *Corydalis* species may be one of several factors shaping the ant-seed dispersing interactions. Further research is needed to identify the chemical basis of volatiles that trigger ant foraging activity, and to assess to what extent the seed dispersal of *Corydalis* species depend on leaf volatiles.

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