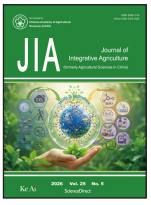
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RESEARCH ARTICLE

Plasticity in within-plant distribution patterns allows aphids to optimize their fitness under contrasting interactions with protective ants and predatory ladybugs

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Highlights

- Phenotypic plasticity is the ability of organisms to adapt to environmental changes. Many aphids have evolved facultative mutualistic relationships with ants, but the colonies must often cope without ants.
- Attendance by red imported fire ants, *Solenopsis invicta*, increased the proportions of cotton aphids (*Aphis gossypii*) distributed on the nutritive parts of cotton seedlings (stem, petioles, and sprouts (SPS)). However, in the absence of tending ants, the aphids on SPS suffered higher predation risk than on leaves. But the aphids can rapidly shift to leaves to reduce the proportions on SPS when the predatory ladybug or its signals were present.
- Aphid distribution patterns have adaptive plasticity which allows aphid colonies to optimize their fitness by responding to the presence of mutualistic ants or predatory threats with flexibility.

Abstract

Phenotypic plasticity is a crucial adaptive strategy that allows organisms to respond to environmental changes. Many aphids have evolved mutualistic relationships with ants, whereby aphids provide honeydew in exchange for protection from natural enemies. Such ant–aphid mutualisms are often facultative and aphid colonies must often cope without ants. We show here that attendance by red imported fire ants, *Solenopsis invicta*, alters the within-plant distribution of cotton aphids (*Aphis gossypii*), resulting in fewer aphids on leaves and more on the stem, petioles, and sprouts (SPS) of cotton seedlings compared to colonies without ant attendance. The nitrogen contents in stems and sprouts were higher than in leaves, which may be a reason for the significantly higher population growth in ant-tended colonies. In contrast, exposure to the signals of a predatory ladybug, *Coccinella septempunctata*, resulted in a remarkably smaller aphid colony size, with lower proportions of aphids distributed on SPS, but a higher proportion on the leaves, compared to those in the predator-free colonies. In addition, ladybug predation risk is considerably higher on SPS than on leaves, and aphids showed rapid positional shifts from stems to leaves upon direct exposure to a ladybug, highlighting their ability to respond swiftly to predator presence. Our findings reveal that adaptive plasticity in aphid distribution patterns enables aphid colonies to optimize their fitness by responding to the presence of mutualistic ants or predatory threats with flexibility.

Keywords: ant–aphid mutualism, natural enemy, phenotypic plasticity, within-plant distribution

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1. Introduction

All organisms have evolved varying degrees of phenotypic plasticity in order to adapt to changes in their environment. This flexibility involves morphological, behavioral, and/or life-history traits that can be adapted in response to changing conditions (DeWitt *et al.* 1998). Safe (e.g., the absence of natural enemies) and unsafe (e.g., the presence of natural enemies) circumstances can lead to distinct changes in survival strategies in various organisms (Agrawal 2001). This is particularly relevant for aphids (Hemiptera: Aphididae). Most aphid species are wingless most of the time and reproduce through parthenogenesis. Yet, when an aphid colony is under attack by natural enemies the aphids may start to produce winged offspring, which is assumed to increase the chance of survival by migration to possibly safer host plants (Weisser *et al.* 1999; Mondor *et al.* 2005). Under safe conditions, aphid colonies usually spend more time feeding and reproducing (Ei-Ziady and Kennedy 1956; Ei-Ziady 1960). Such safe conditions can be provided by mutualistic ants (Hymenoptera: Formicidae).

Various aphid species engaging in facultative mutualism with ants must cope with complex and fast-changing circumstances. These circumstances range from unfavorable conditions (e.g., exposure to natural enemies without ant protection) to favorable situations (e.g., feeding on a suitable host plant and protection by ants). To optimize their fitness, it is critical that facultative myrmecophilous aphids are able to swiftly adapt to these dynamic interactions and balance the benefits and costs in varying situations (Stadler and Dixon 1999). The mechanisms that allow them to respond to their often fast-changing environment, particularly the presence or absence of mutualistic ants and natural enemies, remain poorly understood (Mondor *et al.* 2005, 2008; Keiser and Mondor 2013).

The cotton aphid, *Aphis gossypii*, is a facultative myrmecophile that shows high phenotypic plasticity, including winged morphs (less fecund) and wingless morphs (more fecund) among the parthenogenic generations, and wingless morphs with various body sizes and colors (from yellow to dark green that are correlated with an increase in body size and fecundity) (Wool and Hale 1995; Watt and Hales 1996). Distinct transgenerational changes in phenotype can be adaptively induced by tending ants and predatory ladybugs (Mondor *et al.* 2005, 2008; Styrsky and Eubanks 2010). More winged morphs are produced when the colony is attacked by predatory beetles, which can help the colony survive by escaping to new host plants (Mondor *et al.* 2005). Conversely, aphids produce more green morphs when the colony is tended by ants, contributing to a greater population growth rate (Mondor *et al.* 2008). When tended by the invasive red imported fire ant, *Solenopsis invicta*, cotton aphid colonies exhibit faster population growth (Rice and Eubanks 2013). A recent study found that these changes in the aphid colony are mediated by the perception of the ant's trail pheromone, which leads to reduced dispersal and increased fecundity (Xu *et al.* 2021). The mechanisms and adaptiveness of the specific strategies they employ under different interactions remain to be determined (Mondor *et al.* 2005, 2008).

The performance of aphids is highly dependent on host plant quality (Nevo and Coll 2001; Honěk and Martinková 2002). Importantly, nutritious substances that are essential for aphid development and reproduction, particularly amino acids (Nevo and Coll 2001), are not evenly distributed in a plant (Van Emden and Bashford 1969; Merritt 1996). Yet, aphids do not always feed on the most nutrient-rich plant parts (Banks 1958; Trumble 1982; Jakobs *et al.* 2019), which may be attributed to the higher levels of defense required in these parts (Strauss *et al.* 2004). Aphids may also choose their feeding site based on the risk of exposure to natural enemies, and this risk is expected to be different for aphids with or without ants (Banks 1962; Zhu *et al.* 2006). Ant-tended aphids tend to be concentrated on the young growth parts of host plants for a longer period than untended aphids (Banks 1958). These distinct aphid distribution patterns prompted us to hypothesize that aphids, such as *A. gossypii* which can engage in facultative mutualisms with *S. invicta* on cotton plants (Kaplan and Eubanks 2002), have evolved plasticity in their within-plant distribution patterns that allow them to differentially optimize their fitness depending on the presence of tending ants and predators. Here, we tested this hypothesis with a series of manipulative behavioral and performance assays.

2. Materials and methods

2.1. Insects and plants

Solenopsis invicta colonies were collected on the campus of South China Agricultural University (Guangzhou, Guangdong Province, China), reared in the laboratory (26°C, 70% RH, and 14 h L:10 h D photoperiod), and fed with 10% sugar water and freeze-killed crickets. The cotton aphids used in this study were the clones from an apterous parthenogenetic female originally collected from a cotton field at Langfang Experimental Station, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Hebei Province, China. The aphids were reared on cotton plants (*Gossypium hirsutum* L., cultivar Xinluzao 45 or 72) that were individually grown in 500 cm^3 plastic pots in a climate-controlled incubator (25°C, 70% RH, and 16 h L:8 h D photoperiod). Larvae of the seven-spotted ladybug, *Coccinella septempunctata*, were collected from wheat fields in Shuyang County, Suqian City, Jiangsu Province, China, and reared in the laboratory under ambient temperature (~23°C), and fed with cotton aphids. All manipulative assays were performed using the basic experimental setup shown in Fig. 1. In the assays involving ants, an open transparent plastic container (3.5 L, diameter: 21 cm (open-top) and 17.5 cm (bottom), height: 13 cm) with a hole (1–2 cm diameter) in the bottom center was cut into two halves. The above-ground part of a cotton seedling (~5 weeks old, with two cotyledons and 3–4 fully expanded true leaves) grown in a plastic pot was inserted into the container through a hole in the bottom (2–3 cm above the soil surface in the pot). The two halves of the container were sealed together with a hot melt glue gun. To protect the stem and prevent ants from escaping, the bottom hole was filled with absorbent cotton wool that was tightly wrapped

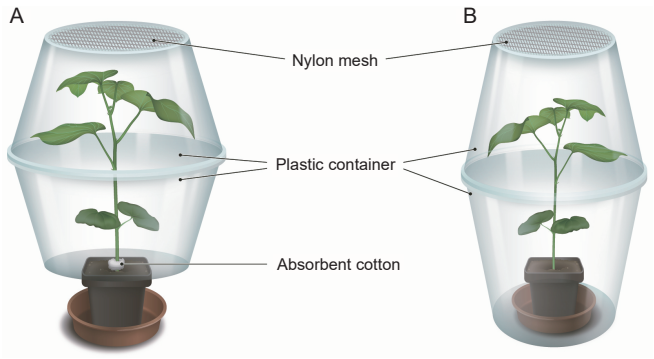


Fig. 1 Schematic diagrams for the experimental setups. A, setup used in investigating the effects of ant attendance or ladybug presence on within-plant distribution patterns of cotton aphids. B, setup used in evaluating the predation risks for the cotton aphids distributed on different parts of a cotton seedling and the within-plant position shifts of the cotton aphids in response to predation.

around the stem, and the inner wall of the container was coated with a polytetrafluoroethylene (PTFE) suspension (Dongguan Mingde Plastic Co., Ltd., Dongguan, China). The seedling was covered with another inverted transparent plastic container of the same size, and its bottom (top in the setup) was cut open and sealed with a piece of nylon mesh for ventilation (Fig. 1-A). In the assays involving ladybugs, a cotton seedling was placed in an open transparent plastic container (3.5 L, diameter: 21 cm (open-top) and 17.5 cm (bottom), height: 13 cm), and covered with another inverted transparent plastic container of the same size (Fig. 1-B).

2.2. Effects of ant attendance on the within-plant distribution of aphids

We first monitored the distributions of cotton aphids on cotton seedlings in the presence or absence of tending *S. invicta*. Fifty apterous cotton aphids (yellow morph, 4th instar) were evenly placed on the upper side of all the leaves of a seedling (cultivar Xinluzao 45) in 16 setups (day 0). After 24 h, the number of aphids was checked and adjusted to 50 per seedling as needed by adding new aphids or removing excess aphids (day 1). Then, the sub-colonies of eight polygynous *S. invicta* colonies, including 1 g of workers (~1,000 individuals), one queen, and 20–30 larvae, were individually introduced into each of the eight systems, while no ants were added to the other eight as the control group (n=8). A plastic Petri dish (6 cm diameter) was used as an artificial ant nest. The inside bottom of the Petri dish was covered with a layer of plaster (~0.5 cm thickness) which was moisturized every other day after the ants were introduced. Black tape was used to cover the Petri dish top to maintain dark conditions inside the nest (Fig. 2-A). The seedlings of the same group were kept together in a climate incubator (25°C, 60–70% RH, and 16 h L:8 h D photoperiod). The numbers of aphids (both alatae and apterae) on the leaves, stem, petioles, and sprouts (new, but not fully expanded leaves) were counted on days 3, 5, 7, 9, 11, 13, 15 and 17. Alate morphs were removed once counted. The seedlings were watered every 2–3 days during the experiment. Water in a 5 mL centrifuge tube sealed with absorbent cotton wool and two dead crickets were supplied to the ants every other day. During the first week, sucrose solution (3% w/v in a 5 mL centrifuge tube sealed with absorbent cotton) was also provided every other day to supplement the insufficient honeydew production by the relatively small number of aphids.

2.3. Effects of ladybug presence on the within-plant distribution of aphids

Because ant-tending was found to cause more aphids to feed on the stems, petioles, and sprouts (SPS), where the aphids are expected to get higher quality phloem, we hypothesized that the risk of predation is one of the reasons that the aphids avoid SPS in the absence of ants. This hypothesis was tested by monitoring the changes in within-plant distribution and population growth of aphid colonies that were exposed to an adult seven-spotted ladybug, *C. septempunctata*. In an aphid-present system as described above, a transparent plastic cup (4.5 cm diameter at bottom×7.5 cm tall×7 cm diameter at top) containing an adult ladybug (1–2 weeks old) was placed next to the middle part of the cotton seedling (cultivar Xinluzao 72; n=10; Fig. 3-A), starting at day 1 after the aphid number had been confirmed. The opening of the cup was covered with a piece of nylon mesh for ventilation, which allowed for the transfer of visual and odorous cues from the ladybug but prevented direct contact with the aphids. Water was supplied in a 0.5 mL centrifuge tube sealed with absorbent cotton wool. The seedlings with (treatment) or without (control) ladybugs were kept in two separate climate-controlled incubators (10 in each, 25°C, 60–70% RH, and 16 h L:8 h D photoperiod). The numbers of all aphids on the leaves, stem, petioles and sprouts of each seedling were counted on days 3, 5, 7, 9, 11, 13, 15, 17 and 19. Every other day, the ladybug was taken out of the cup in the system to allow it to feed on aphids while the aphids were counted and their positions on the seedling were recorded, and it was then put back in the system.

2.4. Predation risks for the cotton aphids distributed on different parts of a cotton seedling

We evaluated whether there were distinct predation risks for the cotton aphids on different parts of a cotton seedling (Fig. 4-A). An adult ladybug (1–2 weeks old) that had been starved for 48 h was released on the inner surface of the upper container in a system containing a cotton seedling (cultivar Xinluzao 72) with 15 yellow apterous cotton aphids (2nd–3rd instar) on each of the six leaves, 10 on the stem, a total of 10 on the petioles of the four true leaves (2, 2, 3, 3, respectively), and 10 on the sprout. This distribution was roughly based on the ratio of approximately 1.5:1:1:1 for the average numbers of aphids on these parts observed on day 15 in the absence of tending ants or a predator (Fig. 2). The lower half of the pot was painted with PTFE suspension, which forced the ladybug to reach the seedling by flight. Each observation session lasted 4 h, beginning from when the ladybug had landed on the seedling. The time spent searching for prey and the numbers of aphids consumed

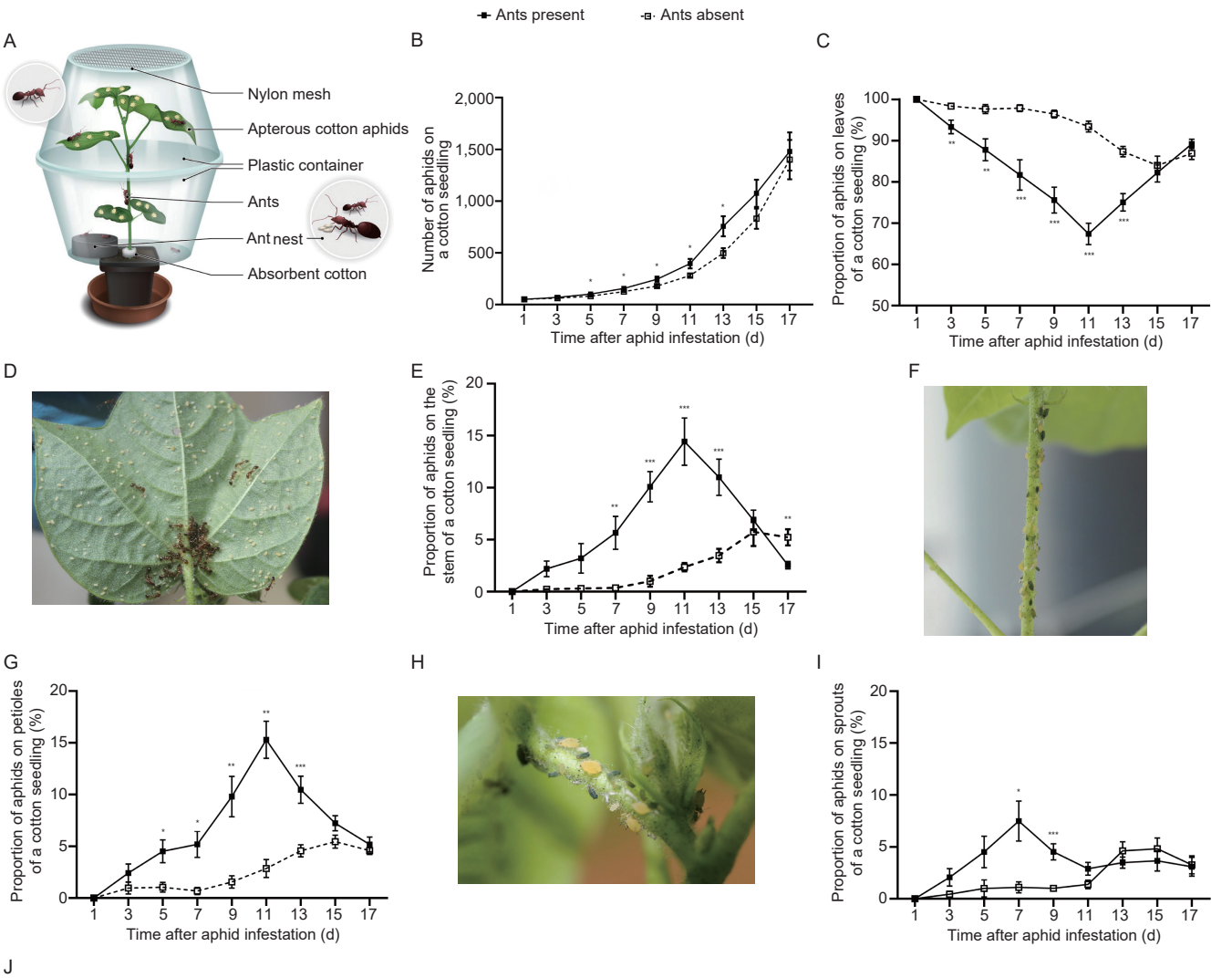


Fig. 2 Effects of *Solenopsis invicta* attendance on the population growth rate and spatial distribution of cotton aphids on cotton seedlings. A, schematic illustration of the experimental setup. B, total number of aphids on each seedling. C, E, G and I, proportions of aphids distributed on the leaves (C), stem (E), petioles (G) and sprout (I) of each seedling. The solid line represents the proportions of aphids in the presence of ants, and the dashed line represents the proportions of aphids in the absence of ants. D, F, H and J, aphids distributed on the leaf (D), stem (F), petiole (H) and sprout (J). Data are shown as mean±SE (n=8). Asterisk(s) indicate statistically significant differences between treatments on the same seedling part on the same day (*, $0.01<P<0.05$, **, $0.001\leq P\leq 0.01$, ***, $P<0.001$).

by the ladybug on leaves and SPS during those 4 h were recorded. An identical system without a ladybug was used to observe the position shifts of aphids between plant parts under enemy-free conditions (control setup). The time that the ladybug spent searching the leaves and SPS of a seedling without aphids (referred to as a blank system) was also recorded. The observations on the aphid-infested seedlings with or without ladybug predation were each replicated 10 times (n=10), and the blank system (aphid-free seedling) observation was replicated 11 times (n=11). All experiments were carried out between 10:00 a.m. and 7:00 p.m., under ambient conditions of 25–27°C, and 30–40% RH.

The predation risks of the aphids were compared on leaves versus SPS by using three indexes: 1) visiting frequency of a ladybug, which was the total times of the ladybug searching for prey either on leaves or on SPS; 2) foraging intensity of a ladybug, which was calculated by dividing the total time spent on leaves or SPS by the total surface area of the corresponding plant parts; and 3) predation rate of the aphids on different parts, calculated as the ratio of the consumed aphids among the total aphids on different plant parts. The percentage of aphids remaining on the leaves or SPS after ladybug foraging (number of remaining aphids/initial number of aphids) was also calculated to evaluate the responses of the aphids on different plant parts to ladybug foraging.

2.5. Within-plant position shifts of cotton aphids in response to predation

Since the foraging intensity of a ladybug and the predation risk for the cotton aphids were both found to be higher on the SPS than on the leaves, the responses of the aphids on leaves and SPS to ladybug presence may be different

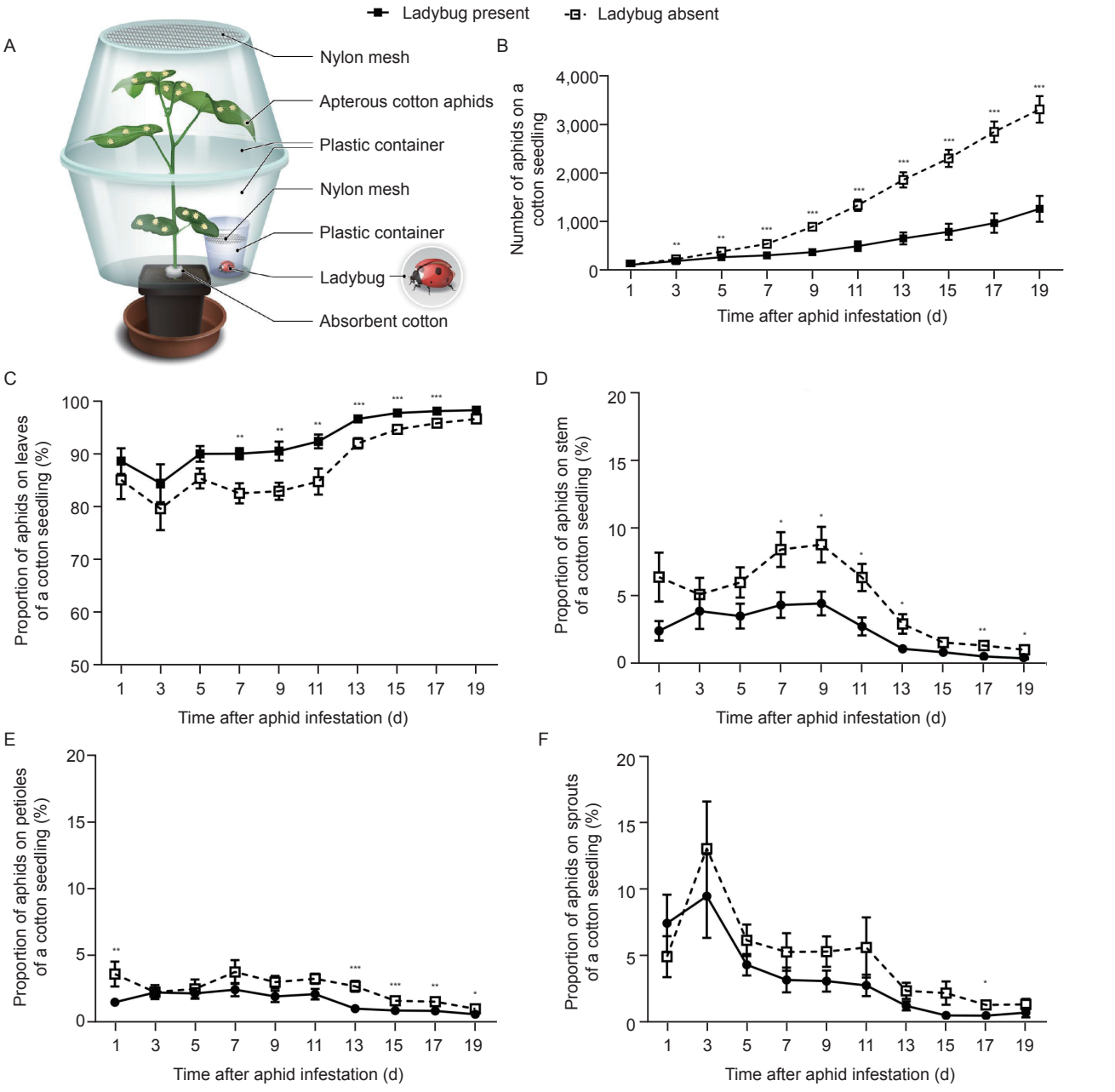


Fig. 3 Effects of predatory ladybug presence on the population growth rate and within-plant distribution of cotton aphids. A, schematic illustration of the experimental setup. B, total number of aphids on each seedling. C–F, proportions of aphids distributed on the leaves (C), stem (D), petioles (E) and sprout (F) of each seedling. The solid line represents the presence of a ladybug, and the dashed line represents the absence of a ladybug. Data are shown as mean±SE (n=10). Asterisk(s) indicate statistically significant differences between treatments on the same seedling part on the same day (*, $0.01<P<0.05$, **, $0.001\leq P\leq 0.01$, ***, $P<0.001$).

(Humphreys *et al.* 2021). To test this, the position shifts of cotton aphids on the leaves or stem of a cotton seedling in response to ladybug presence and predation were observed.

An adult seven-spotted ladybug (1–2 weeks old) that had been starved for 48 h was released in a system as described above, with 30 cotton aphids on the upper part of the stem of a cotton seedling (cultivar Xinluzao 72) (Fig. 5-A). Once the ladybug had landed on the seedling, the observation started, and it lasted for 2 h. During the observation, the numbers of aphids consumed by the ladybug on different plant parts (leaves, stem, petioles and sprouts) were recorded. After 2 h, the numbers of aphids remaining on different plant parts were counted to estimate the number of aphids that had emigrated from the stem and the numbers that had migrated to the other parts. Aphids that were consumed by the ladybug after moving to a new site were also recorded as migrants. The number of aphids that left the seedlings was calculated by subtracting the sum of the remaining aphids plus consumed aphids from the initial aphid number. For the control treatments, no ladybug was released into the same type of setup as described above (Fig. 5-B), and the numbers of aphids on different plant parts were counted at the same time as for the treatments with a predator. Each treatment was replicated 11 times (n=11). The experiments were carried out

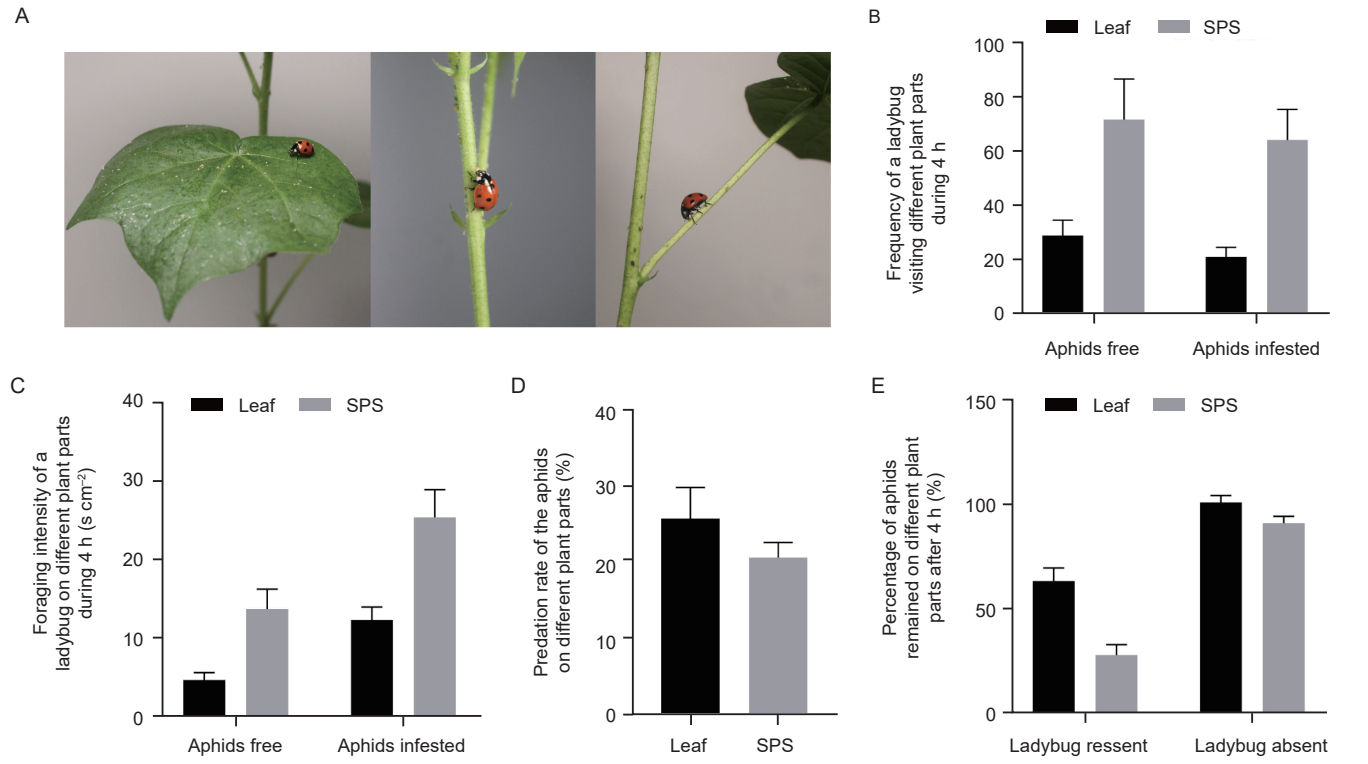


Fig. 4 Predation risks for the cotton aphids distributed on different parts of cotton seedlings. A, ladybug foraging on the leaf (left), stem (middle) and petiole (right) of a cotton seedling. B and C, visitation frequency (B) and foraging intensity (C) of a ladybug on leaves and SPS (stem, petioles and sprout) of an aphid-infested seedling and an aphid-free seedling. D, predation rates of cotton aphids distributed on the leaves and SPS. E, percentages of the aphids remaining on leaves and SPS after a ladybug had foraged on the seedling for 4 h. Data are shown as mean+SE (n=10 for aphids infested; n=11 for aphids free; n=10 for ladybug present and ladybug absent). The black columns represent leaf, and the gray columns represent SPS.

under ambient conditions of (25±2)°C, and 30–40% RH.

Similarly, the position shifts of cotton aphids that were originally on the leaves of a cotton seedling in response to ladybug predation were determined, following the same procedures as above (Fig. 5-D and E), except that 15 aphids were initially placed on the upper side of each of six leaves for a total of 90 aphids per seedling. This experiment was replicated 10 times with or without a predator (n=10).

2.6. Nitrogen contents in different parts of the cotton seedlings

Various studies have shown a positive effect of host plant nitrogen content on the reproduction of cotton aphids (Cisneros and Godfrey 2001; Nevo and Coll 2001; Slosser *et al.* 2004; Chau *et al.* 2005; Hosseini *et al.* 2010; Guo *et al.* 2024). Therefore, we measured the nitrogen contents in different parts of the cotton seedlings used in the present study to test the hypothesis that aphids on SPS ingest more nutritious plant sap than those on leaves. Different parts of cotton seedlings (cultivar Xinluzao 72; ~5 weeks old, with 5–6 fully expanded leaves) were harvested with clean scissors. The separate parts included the mature true leaves (n=5), the upper part of the stem (n=5), petioles (n=5), and the apical sprouts (n=4). The samples were dried at 100°C until constant weight. The dried samples (0.1 g each) were placed in a Kjeldahl flask and 8 mL of concentrated sulfuric acid and potassium sulfate were then added to the flask. Samples were first digested at 280°C on a graphite digestor for 60 min. After cooling, 2 mL of hydrogen peroxide was added into the flask with another digestion at 300°C for 60 min until the solution turned uniformly dark brown. The ammonia in the digested solution was quantified using a Hanon automatic Kjeldahl analyzer (K9860, Hanon Group, China) following the operating instructions. A blank control was prepared using the same reagents by following the above procedure without plant tissues. The nitrogen contents were calculated based on the following equation:

$$N=C\times(V-V_0)\times0.014\times1{,}000\div m$$

where C is the concentration (0.1008 mol L^{-1}) of the standardized hydrochloric acid solution used for titrating the ammonium borate solution; V and V_0 are the volumes of the hydrochloric acid solutions used for titrating the ammonium borate solutions from plant tissue samples and the blank control, respectively; and m is the weight of the test samples.

2.7. Statistical analyses

Generalized linear mixed models (GLMMs, distribution= Gamma, link=log) were used to analyze the effects of the presence of ants or a ladybug, the observation time (as repeated measurements), and their interactions on the total numbers of aphids, the numbers of aphids on leaves, stem, petioles and sprouts, and the proportions of the aphids that were on these plant parts. Two-way ANOVA was used to analyze the effects of plant parts, the presence of aphids, and

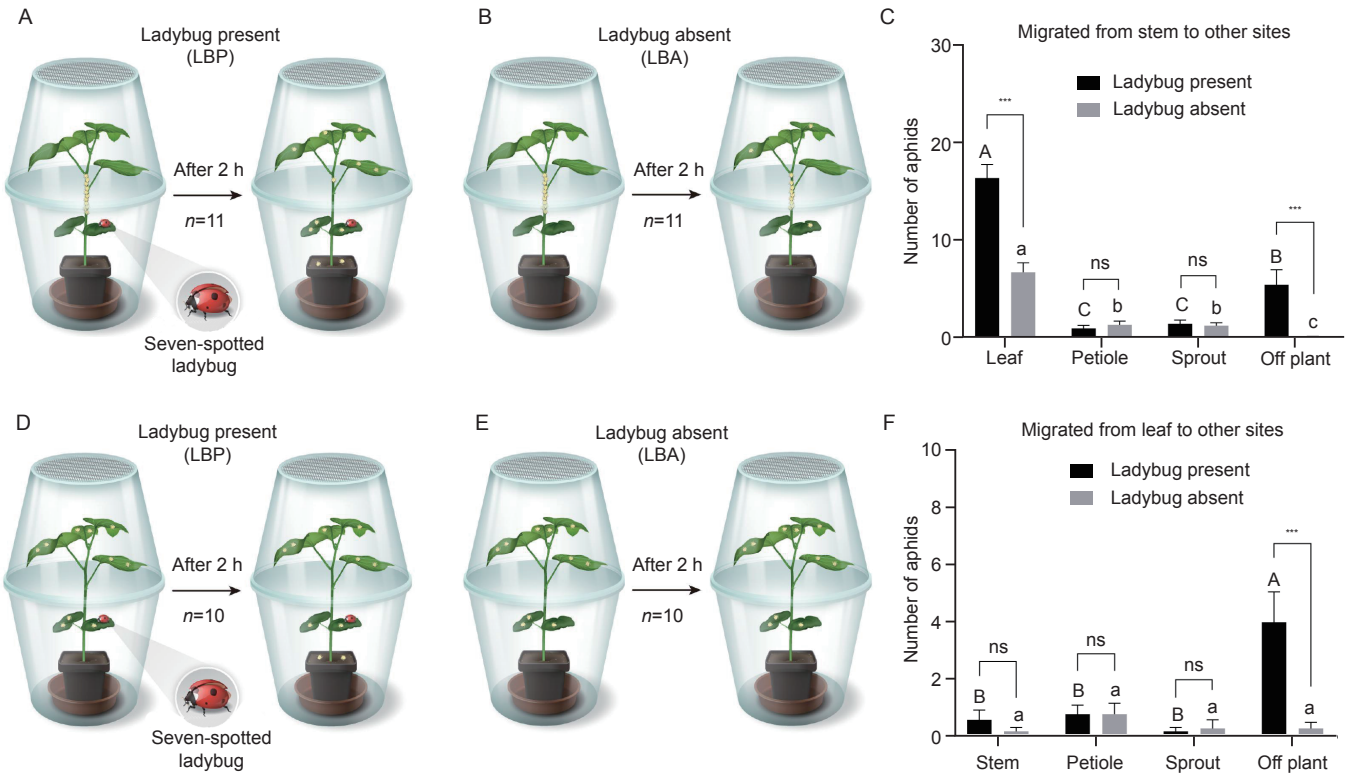


Fig. 5 Position shifts of cotton aphids on a cotton seedling in response to predation. A and B, schematic illustrations of the experimental setups in which the position shifts of the cotton aphids distributed on the stem of a cotton seedling were observed in the presence (A) or absence (B) of a ladybug. C, numbers of emigrated aphids that relocated on the leaves, petioles, and sprout, or left the seedling, after 2 h in the presence (black) or absence (gray) of a ladybug. D and E, schematic illustrations of the experimental setups in which the position shifts of cotton aphids distributed on the leaves of a cotton seedling were observed in the presence (D) or absence (E) of a ladybug. F, numbers of emigrated aphids that relocated themselves on the leaves, petioles, and sprout, or left the seedling, after 2 h in the presence (black) or absence (gray) of a ladybug. Data are shown as mean+SE. The black columns represent ladybug presence, and the gray columns represent ladybug absence. Asterisks indicate statistically significant differences between the ladybug-present treatment and the ladybug-absent control (**, $0.001 \leq P \leq 0.01$, ***, $P<0.001$). Different capital letters above the black columns indicate statistically significant differences between sites in the presence of a ladybug, and the lowercase letters above the gray columns indicate statistically significant differences between sites in the absence of a ladybug.

their interactions on the visitation frequency of the ladybug (log-transformed), the foraging intensity of the ladybug, and to analyze the effects of plant parts, the presence of a ladybug and their interactions on the percentage of the remaining aphids. The predation rates of aphids were compared between leaves and SPS with a paired *t*-test. The effects of plant parts, the presence of a ladybug, and their interactions on the numbers of aphids that migrated to new sites were analyzed using generalized linear models (GLMs) (distribution=Poisson, link=log). Bonferroni adjusted post hoc tests were performed when the interaction effect was significant. The differences of nitrogen contents between different parts of the cotton seedlings were analyzed by one-way ANOVA, followed by Bonferroni adjusted pairwise comparisons. All statistical analyses were performed using SPSS 22.0 (SPSS, Armonk, NY, USA).

3. Results

3.1. Effects of ant attendance on the within-plant distribution of aphids

Ant attendance led to larger aphid population sizes than in untended aphid colonies (GLMM: ants: $F_{1, 112}$ = 25.661, $P<0.001$), with significant differences on days 5, 7, 9, 11 and 13 (Fig. 2-B; Appendix A). The aphids were found to be distributed on leaves, stem, petioles and sprouts of cotton seedlings (Fig. 2-D, F, H and J), but the differences in the proportions of aphids on these parts varied between the tended and untended aphid colonies on different days (GLMM: ants×time: on leaf: $F_{7, 112}$=12.93, $P<0.001$; on stem: $F_{7, 84}$=12.388, $P<0.001$; on petiole: $F_{7, 93}$=5.113, $P<0.001$; on sprout: $F_{7, 89}$=4.77, $P<0.001$). Specifically, the proportions of aphids on leaves were significantly smaller in the tended colonies on days 3, 5, 7, 9, 11 and 13 compared to those in the untended colonies (Fig. 2-C; Appendix B), due to greater proportions of aphids distributed on SPS in these colonies. The differences were statistically significant on days 7, 9, 11 and 13 on stem (Fig. 2-E; Appendix B); on days 5, 7, 9, 11 and 13 on petioles (Fig. 2-G; Appendix B); and on days 7 and 9 on sprouts (Fig. 2-I; Appendix B). Toward the end of the observation period, the differences in the proportions of aphids on these parts between tended and untended colonies disappeared. The proportion of aphids on the stem in untended colonies even became significantly greater than that in tended colonies on day 17 (Fig. 2-E; Appendix B).

This was possibly due to resource depletion, indicated by the fact that tended aphids produced a larger number of alates at the later stage of the experiment (Appendix C). The numbers of aphids distributed on leaves, stem, petioles and sprouts in the tended and untended colonies during the experiment are shown in Appendix D, with the detailed statistical results in Appendix A.

3.2. Effects of predation risk on aphid within-plant distribution

Exposure to a predatory ladybug reduced the aphid population size (GLMM: ladybug: $F_{1, 180}$=164.591, P<0.001), with significant differences between predator-exposed and control colonies beginning three days after the aphids were introduced to the cotton seedlings (Fig. 3-B; Appendix E). The proportions of aphids distributed on different seedling parts in the ladybug-exposed colonies were different from those in the control colonies. In general, the predator-present colonies had a larger proportion of individuals on leaves but smaller proportions on SPS, compared to the predator-free colonies (GLMM: ladybug: on leaf: $F_{1, 180}$=27.24, P<0.001; on stem: $F_{1, 172}$=44.362, P<0.001; on petiole: $F_{1, 175}$=41.382, P<0.001; on sprout: $F_{1, 172}$=12.901, P<0.001). Statistically significant differences were detected on leaves on days 7, 9, 11, 13, 15 and 17 (Fig. 3-C; Appendix F); on stem on days 7, 9, 11, 13, 17 and 19 (Fig. 3-D, Appendix F); on petioles on days 1, 13, 15, 17 and 19 (Fig. 3-E; Appendix F); and on sprouts on day 17 (Fig. 3-F; Appendix F). The numbers of aphids distributed on leaves, stem, petioles and sprouts in ladybug-present and -absent colonies during the experiment are shown in Appendix G, with the detailed statistical results in Appendix E. These results illustrate the opposite effect of predatory ladybug presence on the aphid population growth rate and distribution pattern, compared to ant attendance.

3.3. Predation risks for the cotton aphids distributed on different parts of a cotton seedling

The visitation frequency of the ladybug on SPS was significantly greater than on the leaves of both aphid-infested and aphid-free seedlings (two-way ANOVA: aphid: F=0.336, df=1, P=0.565; plant part: F=30.585, df=1, P<0.001; aphid×plant part: F=0.3, df=1, P=0.587; Fig. 4-B). On both treatment and control seedlings, the ladybug spent much more time searching on leaves than on SPS (two-way ANOVA: plant part: F=54.019, df=1, P<0.001; Appendix H), but the foraging intensity, i.e., time spent searching for prey per unit area, of ladybugs on leaves was significantly lower than that on SPS (two-way ANOVA: plant part: F=22.322, df=1, P<0.001; Fig. 4-C) due to the much larger surface area of leaves compared to SPS (two-way ANOVA: plant part: F=711.958, df=1, P<0.001; Appendix H). These results indicate that ladybugs have a higher probability of encountering cotton aphids feeding on stems, petioles, and sprouts than on leaves.

For the cotton aphids distributed on leaves and SPS, no significant difference was observed in the predation rates, i.e., the ratio of consumed aphids among the total aphids on specific plant parts (Paired t-test: t=1.658, df=9, P=0.132; Fig. 4-D). However, far fewer aphids stayed on SPS compared to leaves on the ladybug-present seedlings, which was not observed on the seedlings without ladybugs (two-way ANOVA: ladybug: F=118.411, df=1, P<0.001; plant part: F=23.949, df=1, P<0.001; ladybug×plant part: F=7.582, df=1, P=0.009; Fig. 4-E). Combined with the results of visitation frequency and foraging intensity of the ladybugs, these findings imply that the more frequent activities of ladybugs on SPS reduced the aphid numbers on these plant parts because of the aphids escaping to other sites. This is mainly due to non-consumptive interactions.

3.4. Within-plant position shifts of cotton aphids in response to predation

Of the 30 cotton aphids originally feeding on the stem, most (over 80%) had escaped to other sites after a ladybug was allowed to forage for 2 h on the seedling, while most of the aphids remained where they were placed on the stem without predation (Fig. 5-A and B). The presence of a ladybug made significantly more aphids move away from the stem to other sites, but this effect depended on the plant part (GLM: ladybug: χ^2=17.407, df=1, P<0.001; plant part: χ^2=215.375, df=3, P<0.001; ladybug×plant part: χ^2=21.975, df=3, P<0.001; Fig. 5-C). The numbers of aphids that migrated to leaves and left the plant, but not to petioles or sprouts, in the ladybug-present treatment were significantly greater than those in the ladybug-absent control (Fig. 5-C; Appendix I). In the presence of a ladybug, leaves were the first choice for the repositioning of the escaping aphids, followed by leaving the plant, while only a few aphids moved to petioles or the sprout (Fig. 5-C; Appendix I). In the absence of a predator, most of the emigrated aphids moved to leaves, followed by petioles and the sprout, and very few left the plant (Fig. 5-C; Appendix I).

Of the aphids that were initially placed on the leaves (15 on each leaf, for a total of 90 on six leaves), only a few (less than 7%) left the original site with the ladybug presence (Fig. 5-D and E), but this was still significantly more than the aphids leaving on the control seedling without a ladybug (GLM: ladybug: χ^2=5.113, df=1, P=0.024; Fig. 5-F). Predator presence and plant part showed a significant interaction effect on the repositioning of the emigrated aphids (GLM: ladybug×plant part: χ^2=13.315, df=3, P=0.004; Fig. 5-F). Compared to the control plants without ladybug predation, only the number of aphids that left the plant was significantly higher in the ladybug-present treatment (Fig. 5-F; Appendix I). Therefore, the first choice of the escaping aphids was to leave the plant in the presence of a ladybug, with a significantly greater number leaving than the number of aphids that moved to the stem, petioles or sprout; but no significant differences were found between the numbers of aphids that moved to these sites in the absence of the predator (Fig. 5-F; Appendix I). Overall, these results confirm that by repositioning themselves to leaves when confronted with a ladybug, cotton aphids effectively reduce their risk of predation.

3.5. Nitrogen contents in different parts of the cotton seedlings

The nitrogen contents in the leaf, stem, petiole and sprout

of the cotton seedlings were measured as a proxy for the nutrient levels in these plant parts. The results revealed significant differences in nitrogen contents among these different parts (one-way ANOVA: F=35.859, df=3, P<0.001; Fig. 6). The sprout and stem contained significantly more nitrogen than leaf and petiole (Bonferroni adjusted pairwise comparisons: P<0.001), but no significant difference was found between sprout and stem (P=0.155), or between leaf and petiole (P=1).

4. Discussion

Researchers have assumed that phenotypic plasticity allows organisms to adjust their survival strategies in order to optimize their fitness under changing circumstances (Agrawal 2001). Here we show that cotton aphids display contrasting phenotypic responses to the presence of mutualistic ants and predation risk. Specifically, when attended by *S. invicta*, aphids changed their within-plant distribution pattern in favor of the stem, petioles and sprouts that contain more nitrogen than leaves. Conversely, exposure to cues from predatory ladybugs, in the absence of ants, resulted in the opposite distribution pattern, in which the aphids avoided plant parts where they were more likely to encounter predators. These results imply that the adjustments of within-plant distribution are adaptive strategies for the facultatively myrmecophilous cotton aphids in response to the opposing biotic interactions.

To attract ants and secure their fidelity, aphids must allocate more resources for producing sufficient ant-preferred honeydew (Stadler and Dixon 2005; Yao 2014). These changes can inflict costs on the aphids, including delayed development or reduced body size and fecundity (Stadler and Dixon 1998; Yao *et al.* 2000; Katayama and Suzuki 2002; Yoo and Holway 2011), which can hinder the evolution of mutualistic relationships (Bristow 1991; Stadler and Dixon 1999). Yet, the adaptive phenotypic changes in one or both of the interacting partners may mitigate the conflicts between

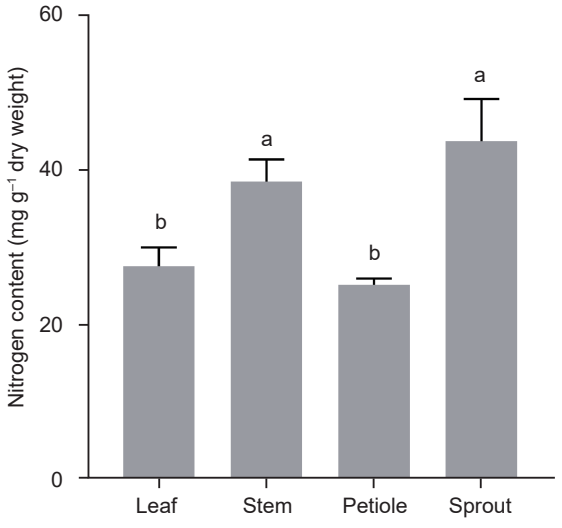


Fig. 6 The nitrogen contents in leaf, stem, petiole and sprout of the cotton seedlings. Data are shown as mean+SE (n=4 or 5). Different letters above the columns indicate statistically significant differences (P<0.05) between parts.

mutualists (Agrawal 2001). More highly fecund green morphs are produced in ant-tended cotton aphid colonies (Mondor *et al.* 2008), which can result in a faster population growth rate (Wool and Hale 1995; Watt and Hales 1996). The larger aphid population may be able to provide more honeydew for the tending ants (Way 1963), thereby stabilizing the mutualism (Leigh 2010).

We found that in the presence of ants, more aphids fed on SPS while the number of aphids on the leaves was not affected. The greater numbers of aphids on SPS contributed to the overall larger population size of tended aphid colonies. Higher nutrient contents can improve development and reproduction, and affect the within-plant distribution of aphids (Banks 1958; Van Emden 1966; Van Emden and Bashford 1969). Nitrogen is a key factor determining the population growth of cotton aphids (Cisneros and Godfrey 2001; Nevo and Coll 2001; Slosser *et al.* 2004; Chau *et al.* 2005; Hosseini *et al.* 2010; Guo *et al.* 2024), and it is usually more concentrated in the growing plant parts, such as the upper part of a stem and sprouting leaves (Van Emden and Bashford 1969; Merritt 1996). We confirmed higher concentrations of nitrogen in these parts compared to the mature leaves of cotton seedlings. Thus, feeding on these plant parts may be a reason for the elevated population growth rate in the ant-tended colonies. Moreover, the more compact aggregations of aphids on SPS may be advantageous to their mutualistic ants, as it concentrates food resources in space (Stadler and Dixon 2005). Previous studies have also shown that dense aphid colonies are more attractive to natural enemies (Schellhorn and Andow 2005; Zhu *et al.* 2006), and more vulnerable to infections with fungal pathogens (Cappuccino 1988). The protection and sanitation services from tending ants may trigger more compact aphid aggregations because of reductions in these risks and better service by the ants.

Natural enemies play an important role in protecting plants from insect herbivores (Pearse *et al.* 2020). Studies have shown that the presence of predators can induce the production of more winged morphs in the offspring of cotton aphids, facilitating their dispersal (Mondor *et al.* 2005). A recent study found that the odors of a predatory ladybug, *Harmonia axyridis*, can disturb the feeding behavior, rather than inducing interplant dispersal, of green peach aphid, *Myzus persicae*, resulting in a decline in the aphid population growth rate (Kansman *et al.* 2023). In this study, the presence of a predatory ladybug, without direct contact, caused the aphids to avoid SPS compared to colonies without a predator. These differential distribution patterns are likely linked to differences in predation risk.

The predation risks for aphids on different plant parts can vary, as is known for the pea aphid, *Acyrthosiphon pisum*. When they feed near petioles, these aphids are at greater risk of predation by coccinellids than individuals further up on a leaf (Keiser *et al.* 2013). Here the aphids on SPS are at greater risk of encountering the seven-spotted ladybug, which may be the main reason that the aphids avoid these parts in the absence of ants. Compared to leaves, SPS have a much smaller surface area, resulting in a much higher prey density on these sites than on leaves. Since the ladybugs tend to spend more time on prey searching in areas with

high prey density (Yasuda and Ishikawa 1999), the aphids that aggregate on SPS may thus be more vulnerable to ladybug predation on these sites. Nevertheless, we observed equivalent predation rates of aphids on the leaves and SPS, possibly due to differential escape responses of the aphids on the different plant parts (Braendle and Weisser 2001). Coccinellid beetles have distinct positive phototaxis and negative geotaxis, preferring to forage on the top parts of a plant (Frazer and McGregor 1994). These innate behavioral features during foraging may be one reason for the increased foraging intensity, but do not explain the high predation rates of aphids on the apical part of cotton seedlings. Here ladybug predation led to a faster and more dramatic reduction in the aphid number on the stem than on leaves. Within 2 h after the introduction of a ladybug, most of the aphids that had been placed on the stem migrated to the leaves or left the seedling, whereas only a few of them migrated when they were placed on leaves. This suggests that the aphids on the stem perceived the higher risk of predation, prompting them to escape to safer sites, which may have contributed to the comparable survival rates between the treatments.

5. Conclusion

Fast-changing environments challenge organisms to quickly adjust their survival strategy. Aphids that have a facultative mutualistic relationship with ants are often confronted with the possibility of losing their protective partners. The selective pressures under such risky conditions must have resulted in efficient phenotypic plasticity to help maximize fitness. Here, we show this to be the case for cotton aphids. This species adaptively responds to contrasting biotic interactions, resulting in subtle but effective changes in the within-plant distribution patterns depending on the presence or absence of mutualistic ants and predators. These abilities can both reduce the costs associated with the mutualism and increase survival probabilities when the mutualism has broken down, resulting in a robust facultative mutualism between *S. invicta* and *A. gossypii*.

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Declarations of competing interest

The authors declare that they have no conflicts of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

All applicable international, national and institutional guidelines for the care and use of animals were followed.

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