Research Article

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Volatiles released by Chinese liquorice roots mediate host location behaviour by neonate *Porphyrophora sophorae* (Hemiptera: Margarodidae)

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Abstract

BACKGROUND: The cochineal scale, *Porphyrophora sophorae* (Hemiptera: Coccoidea, Margarodidae), is one of the most serious arthropod pests of Chinese liquorice, *Glycyrrhiza uralensis* (Fabaceae), an important medicinal herb. The adult females tend to deposit the ovisacs in soil relatively far away from liquorice plants. After hatching, neonates move out of the soil and may use chemical cues to search for new hosts.

RESULTS: We collected and analysed the volatiles from soils with and without liquorice roots, and chromatographic profiles revealed hexanal, β-pinene and hexanol as potential host-finding cues for *P. sphorae*. The attractiveness of these compounds to neonates was studied in the laboratory using four-arm olfactometer bioassays. The larvae showed a clear preference for β-pinene over hexanal and hexanol, as well as all possible combinations of the three compounds. In addition, a field experiment confirmed that β-pinene was significantly more attractive than hexanal and hexanol.

CONCLUSION: Newly eclosed larvae of *P. sphorae* exploit root volatiles as chemical cues to locate their host plant. β-Pinene proved to be the major chemical cue used by *P. sphorae* neonates searching for roots of their host plant.

Supporting information may be found in the online version of this article.

Keywords: *Porphyrophora sophorae*; scale insect; root herbivore; host plant location; β-pinene; attraction

1 INTRODUCTION

In many insect species, adult females are believed to play a major role in determining the host plant on which their offspring will feed, as they choose the host plant on which to lay eggs.[1] However, the chosen oviposition site may be inappropriate or insufficient for the full development of the offspring. For instance, *Pieris virginiensis*, a rare, univoltine butterfly, lays significantly more eggs on the non-host plant *Alliaria petiolata*, a European biennial herb that invades the habitat of *P. virginiensis* in North American forests, than on its native host *Cardamine diphylla*, a most suitable host plant.[2] In such cases, it may be important for neonate larvae to disperse from the oviposition site to a more appropriate host plant via ballooning or crawling.[1] Just like the adults, the larvae may use plant-derived signals to locate these plants.[3]

Subterranean phytophagous insects use chemical cues released from roots to locate host plants. Chemical cues can be primary metabolites, such as CO_2, amino acids and sugars, which are ubiquitous to most plant species, and/or secondary metabolites, which are chemicals produced via secondary chemical pathways and are more host specific.[4–6] With very limited search capacities, neonate larvae must locate the host plant and establish feeding sites on appropriate host tissues within a short time window. Otherwise they will starve to death owing to limited energy reserves.[1] Because of their greatly restricted mobility, the movement of insect larvae through the soil matrix is tedious. It is therefore expected that they make use of long-range chemical signals to assess the availability of host plants from a distance. The effective detection and orientation towards such cues by neonates may also reduce the risk of predation. Similarly to adult

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herbivores,[7,8] neonate larvae are assumed to have an innate ability to differentiate stimuli from suitable host plants and stimuli from non-host plants. Various studies have shown that neonate larvae of lepidopteran species orient towards odours released from their host plants primarily by olfaction.[9–12] Polyphagous root-feeding insects often use common primary metabolites to locate host plant tissues, whereas mono- and oligophagous species tend to use host-specific secondary metabolites.[4,5,13]

The cochineal scale, *Porphyrophora sophorae* (Hemiptera: Coccoidea, Margarodidae), is an important oligophagous pest of Chinese liquorice, *Glycyrrhiza uralensis* (Fabaceae), a flowering plant native to Asia.[14,15] The liquorice root is one of the 50 fundamental herbs used in traditional Chinese medicine, which have been clinically used for over 6000 years.[16]

Larvae of *P. sophorae* feed on roots of wild and cultivated liquorice plants and can cause extensive damage to roots and may even kill plants, especially in poorly managed liquorice field in arid regions of North China. Wingless females and winged males typically emerge from the soil in late August between 8:30 and 11:30 a.m. in order to mate. Immediately after mating, adult females dig 5–10 cm into the soil and then deposit an ovisac containing up to 1000 eggs.[17] The females tend to deposit the ovisacs 10–50 cm away from liquorice plants. Relatively indiscriminate oviposition site selection has been observed in other subterranean insect species, such as black vine weevil, *Otiorhynchus sulcatus* (F.), and grape root borer, *Vitacea polistiformis* (Harris).[12,18] After hatching, neonates move out of the soil and disperse on the soil surface in search of new hosts. The host-searching strategy at ground level facilitates neonate dispersal over a much greater distance with lower energy cost compared with slow movement in the soil matrix. Once a host plant has been located, the larvae burrow downward to depths of 8–25 cm, searching for belowground tissues, and then establish on crown roots. After settling, they complete larval development at the initial feeding site and are unable to relocate until they reach the adult stage. Their highly effective search strategy results in severe infestation by *P. sophorae* larvae. To date, it remains unknown which chemical cues *P. sophorae* neonates use to find their host plant.

The present study aimed (1) to examine the discriminate response of *P. sophorae* neonates to chemical stimuli from soils without and with liquorice roots, (2) to elucidate the chemical identity of potential attractants released from liquorice roots, (3) to evaluate the responses to identified root-produced volatile compounds singly and in various combinations and (4) finally to test the attractiveness of these volatile compounds to neonate larvae in the field. The outcome of this study will increase our understanding of the chemically mediated interactions between *P. sophorae* and its host plant, and may help in the development of strategies for sustainable management of this pest.

2 MATERIALS AND METHODS

2.1 Insects

Newly mated *Porphyrophora sophorae* females were collected in late August from a Chinese liquorice field in Yanchi County, Ningxia Hui Autonomous Region, China. Scouting occurred between 9:00 and 11:00 a.m., when most adult emergence and mating were taking place. Females were placed in containers with soil, where they typically entered into the soil immediately after mating and deposited ovisacs which they kept under their body. Eggs remained undisturbed in the soil and were stored in the laboratory at room temperature. Neonates emerged within 2 months. Newly eclosed and actively crawling larvae were used in bioassays.

2.2 Reagents and test chemicals

HPLC-grade *n*-hexane and dichloromethane (CNW Technologies GmbH, Düsseldorf, Germany) were used for sample preparation. The authentic samples of identified chemicals, hexanal (98%), β-pinene (99%) and hexanol (98%), were all purchased from Sigma-Aldrich, Shanghai. Each compound was diluted with HPLC-grade *n*-hexane to make a 10 $\mu g\,\mu L^{-1}$ solution for behavioural tests. The solutions were kept in a freezer at −20 °C until use.

2.3 Olfactometer bioassays

To determine whether *P. sophorae* neonates use chemical cues from soil actively to select host plants, we used a four-arm olfactometer, which was slightly modified after the four-arm olfactometer described by D'Alessandro and Turlings[19] and Chen *et al.*[20] The olfactometer consisted of a central glass chamber (7 cm i.d., 5 cm length) with four arms (34 mm i.d., 5 cm length), each connected to a glass tube (34 mm i.d., 5 cm length). Each arm was connected via Teflon tubing to a glass vessel that contained the odour source. When eggs started hatching in late September, about 2 kg of three types of soil was collected from a Chinese liquorice field in Yanchi County, Ningxia Hui Autonomous Region, for behavioural bioassays and headspace volatile collections: (1) soil from areas without host plants (bare soil); (2) soil from Chinese liquorice root areas (soil from root areas); (3) soil from Chinese liquorice root areas plus roots (soil containing roots). Soil (ca 500 g) of each type was kept in a glass vessel that served as the odour source. A control glass vessel was left empty. Purified and humidified air entered each odour source vessel at 200 mL min^{-1} via Teflon tubing and carried the volatiles through the connector tube to the olfactometer compartment, and finally was removed by suction via a vacuum pump through the central orifice of the olfactometer at a rate of 1000 mL min^{-1}. The olfactometer was housed in a cardboard box (65 × 65 × 43 cm), with the top left open. The inside surface of the box was covered with white paper, and all edges were strengthened with a wood stake frame. Two fluorescent tubes (25 W) were placed above the box to ensure that each odour chamber received equal illumination.

About 100 neonates were introduced in groups into the central part of the olfactometer chamber with a brush. Neonates that entered an arm of the olfactometer within 30 min were counted as having made a choice for a particular odour source. The larvae that did not enter an arm within this time were considered as 'non-responders'. After each test, the olfactometer was cleaned with acetone and the arms were rotated (90°) to minimise positional effect. Bioassays were replicated 8 times, and all were carried out between 10:00 a.m. and 4:00 p.m.

Bioassay data were determined to be normally distributed and then analysed using one-way analysis of variance (ANOVA) followed by a Tukey–Kramer HSD comparison test ($P < 0.05$) to establish significant differences among the treatments.[21]

2.4 Collection and analyses of headspace volatiles

A quantity of 1 kg of soil of each type was enclosed in a plastic oven bag (Reynolds Oven Bags, 482 by 596 mm; Reynolds Kitchens, Richmond, VA). An adsorbent trap consisting of a borosilicate glass tube (13 cm length, 0.7 cm o.d.) containing Porapak-Q (80–100 mesh, 200 mg; Waters Corp., Milford, MA) was

connected to one side of the bag, and an activated charcoal filter entered the opposite side of the bag. Air was drawn through the bag and adsorbent trap by vacuum (500 mL min^{-1}) for 4 h at room temperature under natural light conditions. After each collection, the adsorbent was rinsed with 1 mL of HPLC-grade dichloromethane, and the eluate was concentrated to 200 µL under a nitrogen stream and stored at −20 °C until use.

Volatiles were analysed with an Agilent 7890A gas chromatograph coupled to a 5975C mass selective detector, with a DB-WAX capillary column (30 m × 0.25 mm i.d., 0.25 µm; Agilent Technologies, Santa Clara, CA). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. Injections (2 µL) were made in splitless mode at an injector temperature of 230 °C. Following injection, the column temperature was maintained at 40 °C for 1 min and then increased to 180 °C at 5 °C min^{-1} and subsequently to 240 °C at 10 °C min^{-1}, with a final 10 min holding time. The total run time was 45 min. The transfer line temperature was kept at 250 °C. Mass spectra were obtained using electron impact (EI, 70 eV). The chemical identities of the dominating peaks that were mainly present in soil containing roots were determined by comparing their mass spectra with those of the NIST 08 library. Spectra and retention times were also compared with those of authentic standards.

2.5 Bioassays with identified chemicals

The responses of *P. sophorae* neonate larvae to individual volatile compounds and to mixtures were examined in the four-arm olfactometer described above. In the first experiment, larval responses to three individual compounds and a solvent control (hexane) were tested. In the second experiment, a three-component mixture was tested against the most active compound, *β*-pinene. In the third to fifth tests, a two-component mixture was tested against *β*-pinene. Each experiment was replicated 8 times on two consecutive days. The individual compounds were tested at 10 µg $µL^{-1}$. Two or three component mixtures were prepared by mixing 10 µg $µL^{-1}$ solutions of individual compounds at appropriate ratios per volume. The ratio of two given compounds was based on the two corresponding peak areas in the GC-MS profile of volatile samples collected from soil with roots.

Each stimulus (or control) was delivered as a 10 µL sample on filter paper strips (1 × 1 cm, Whatman® No. 1). After allowing for solvent evaporation (~15 s), the filter paper strip was inserted into an olfactometer arm. About 100 neonates were tested at a time, which was considered as a replicate. Each bioassay test was replicated 8 times.

2.6 Field evaluation

Synthetic volatile compound (100 mg) dissolved in l mL of hexane was applied on balls of cotton wool in glass vials (15 mL). Each glass vial was left open and was inserted into soil with the opening of the glass bottle slightly lower than the soil surface level. Control vials were loaded with the same volume of hexane. When neonates were observed crawling on the soil surface, trap vials were placed at equal distance (3 m) in the same field. Sets of four traps (three treatments and one control, six replicates each treatment) were randomised and arranged in a complete block design. After 1 week, we counted the number of larvae that had fallen into the different vials. Numbers of neonates trapped with each treatment were analysed by ANOVA (Tukey–HSD test, 5%).

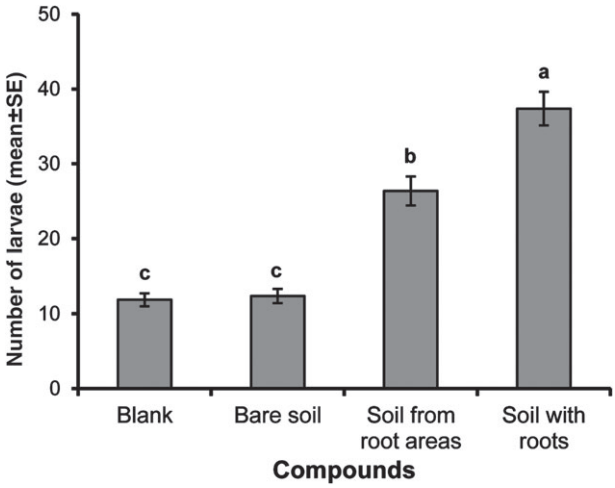

Figure 1. Responses of neonate larvae to odours from different types of soil. The letters above the bars indicate significant differences ($P < 0.05$, Tukey–HSD test).

3 RESULTS AND DISCUSSION

In order to determine whether *P. sophorae* neonates respond to volatiles released from the roots of the host plant, different types of soil were tested for attraction in an olfactometer. Significantly more neonates entered the arm connected to the vessel with soil that contained roots than the arm with odour for soil from the root areas, and the latter was still more attractive than bare soil and the blank control ($F_{3,28} = 57.38$, $P < 0.0001$) (Fig. 1). There was no significant difference in attraction between bare soil and control. This experiment clearly implies that Chinese liquorice roots release behaviourally relevant semiochemicals that may be exploited by cochineal scale neonates to find liquorice roots.

Odours from different types of soil were collected using Porapak-Q and further analysed with GC-MS. Comparison of the chromatographic profiles of volatile collections from bare soil, soil from root areas and soil with roots revealed the consistent presence of three peaks in soil from root areas and soil with roots, but not in bare soil (Fig. 2). Although these three peaks in soil from root areas were small compared with those of the background volatiles, their consistent presence in the collections of root-containing soil strongly suggests that they are root-derived compounds. Peaks 1, 2 and 3 were identified as hexanal, *β*-pinene and hexanol. The identification was confirmed by matching the mass spectrum and retention time of identified compounds with authentic samples of commercial products.

Hexanal and hexanol are ubiquitous green leaf volatiles. They are not commonly found in root emissions, but they are known to be major components of liquorice root (*G. uralensis*).[22] *β*-Pinene has also previously been detected by SPME headspace analysis as a major component of liquorice root (*G. echinata* L.).[23] Both hexanal and *β*-pinene are major components of headspace volatile components of roots, stems, leaves and flowers of *Echinacea* plants of the Compositae family.[24] *β*-Pinene is one of the most abundant monoterpenes emitted into the atmosphere from forest trees,[25,26] such as pine (*Pinus* spp.) and spruce (*Picea* spp.) trees.[27,28] In addition, it is a principal component of the essential oils of various plant species, for instance *Alpinia calcarata* Rosc. (Zingiberaceae).[29] *β*-Pinene has also been found to be a major component of the essential oils from the roots of a number of aromatic plants (supporting information Table S1). The significant amounts of hexanal,

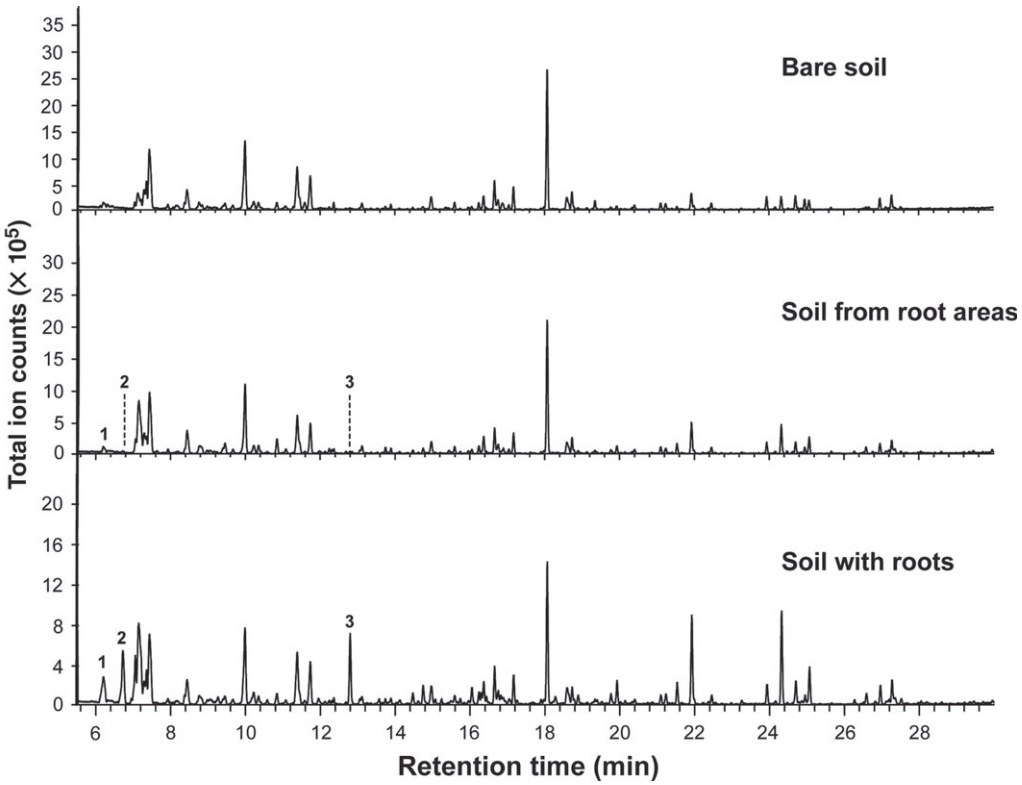

Figure 2. Chromatographic profiles of headspace volatiles released by different types of soil. 1 – hexanal; 2 – β-pinene; 3 – hexanol.

β-pinene and hexanol released from liquorice roots imply that they may play an important role in mediating insect and plant interactions. Hexanal and hexanol have been shown to be attractive to the first-instar larvae of the onion fly, *Hylemya antiqua* Meigen (Diptera: Anthomyiidae),[30] and to the newly hatched larvae of the cabbage maggot, *Delia radicum* (L.) (Diptera, Anthomyiidae).[31] We are not aware of any study showing that β-pinene is attractive to soil-dwelling insects. We first compared the attraction of neonates to the three compounds and solvent only. There was a significant difference in the attractiveness of these various compounds ($F_{3,28} = 43.32$, $P < 0.0001$) (Fig. 3). β-Pinene was significantly more attractive than hexanal and hexanol. The responses to the latter were significantly stronger than to the solvent control.

We then compared the attraction of neonates to different combinations of the three compounds (hexanal, β-pinene, hexanol) and β-pinene alone. The response to the three-component mixture (hexanal + β-pinene + hexanol = 0.22:0.43:0.35) was significantly lower than to β-pinene, but stronger than to both hexane controls ($F_{3,28} = 66.86$, $P < 0.0001$) (Fig. 4A). In general, the two-component mixtures, hexanal + β-pinene, β-pinene + hexanol and hexanal + hexanol, were similarly more attractive than hexane alone, but less attractive than β-pinene alone (Figs 4B to D). Overall, our experiments suggest that, among the three compounds detected in the chemical analyses, hexanal and hexanol are not essential attractants needed by *P. sophorae* neonate to locate its host plant. Neither hexanal nor hexanol increased the attraction to β-pinene.

In the field experiment, glass vials baited with β-pinene caught significantly more larvae than vials baited with hexanal, hexanol or solvent ($F_{3,20} = 27.32$, $P < 0.0001$) (Fig. 5), which confirmed the attraction of *P. sophorae* neonates to β-pinene. Vials baited with hexanal tended to trap more larvae than the unbaited control vials, but the difference was not statistically different, possibly owing

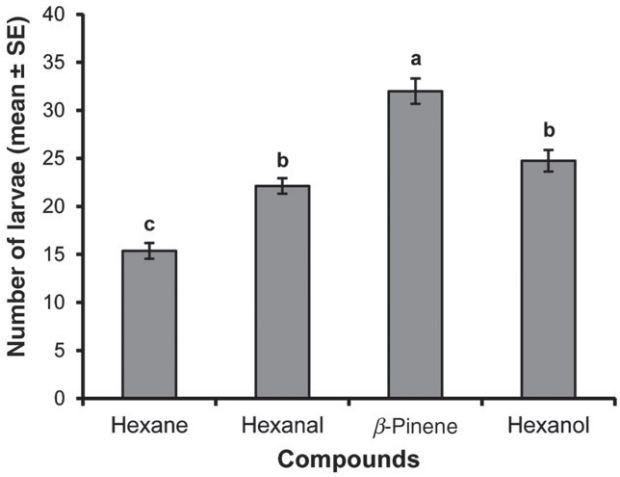

Figure 3. Responses of neonate larvae to the three identified compounds. The letters above the bars indicate significant differences ($P < 0.05$, Tukey–HSD test).

to the relatively small sampling size. The field results match those from the laboratory bioassays and confirm that β-pinene, unlike hexanal or hexanol, may play a crucial role in the attraction of neonates to liquorice root volatiles. Future work should compare trapping effectiveness of different doses of β-pinene in the field, and explore whether a long-lasting and slow-releasing β-pinene dispenser can be used to monitor and perhaps even control populations of *P. sophorae*.[32]

Passive aerial dispersal in many aboveground neonate caterpillars is commonly achieved by silking and ballooning with the wind.[1] Root-feeding larvae usually crawl through the soil and

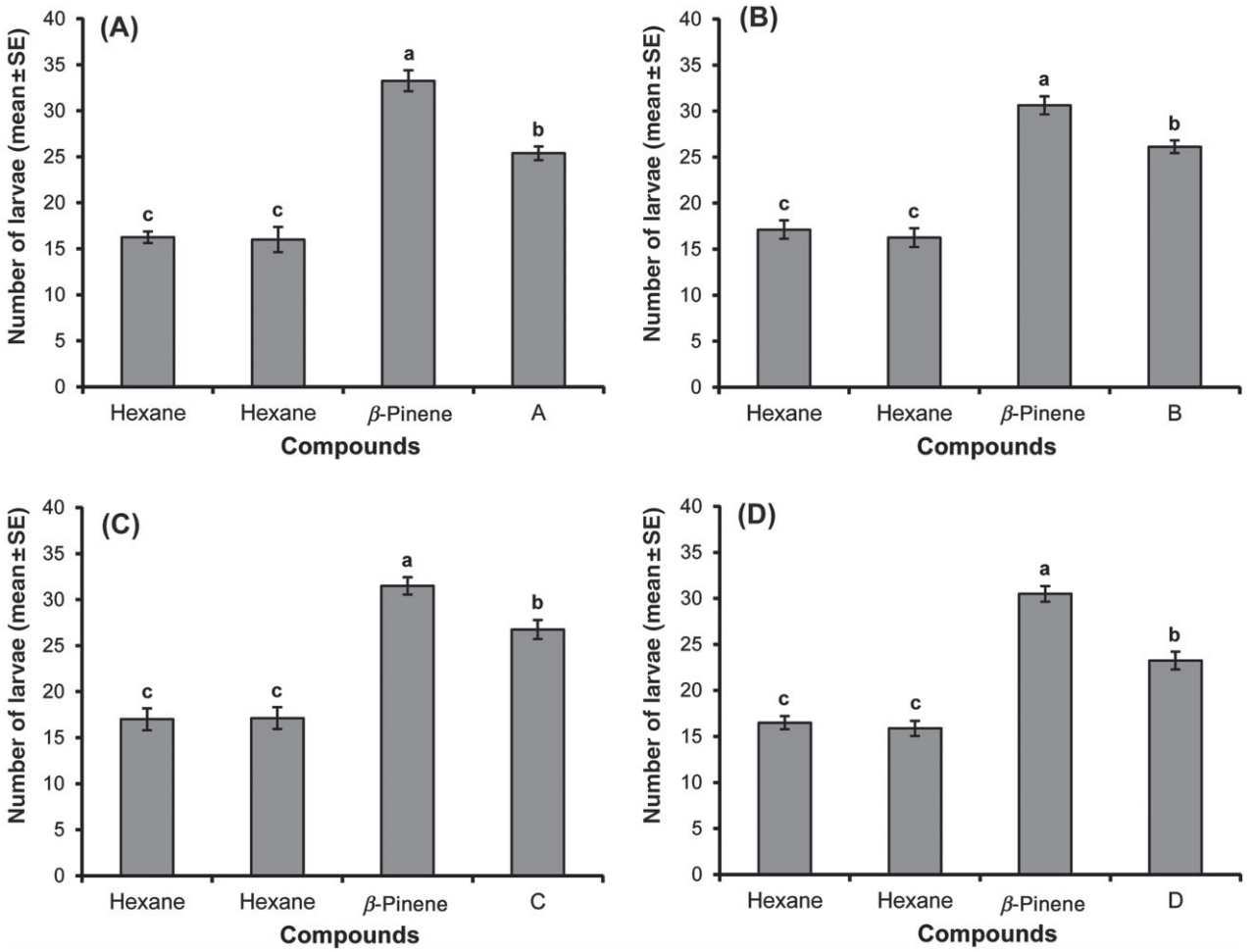

Figure 4. Responses of neonate larvae to different combinations of the three identified compounds. (A) A = hexanal + β-pinene + hexanol 0.22:0.43:0.35; (B) B = hexanal + β-pinene 0.34:0.66; (C) C = β-pinene + hexanol 0.55:0.45; (D) D = hexanal + hexanol 0.39:0.61. The letters above the bars indicate significant differences ($P < 0.05$, Tukey–HSD test).

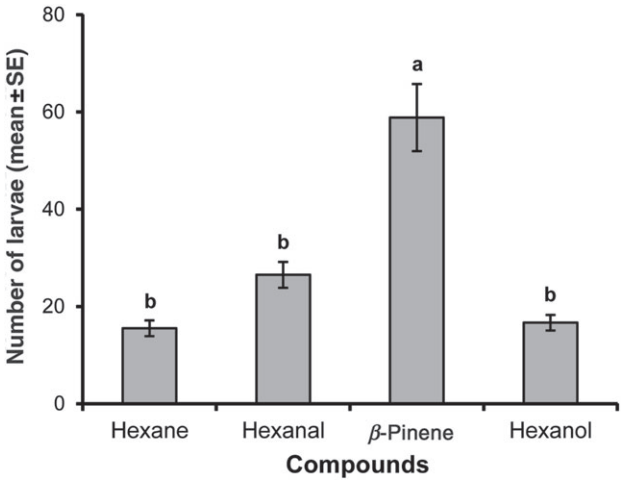

Figure 5. Number of neonate larvae attracted in the field to vials containing hexanal, β-pinene and hexanol. The letters above the bars indicate significant differences ($P < 0.05$, Tukey–HSD test).

exploit chemical cues released from the roots to guide their movement in the soil matrix.[4] Although insects are more likely to use a range of chemicals to locate host plants rather than a single chemical, plant roots compared with leaves release considerably fewer volatile chemicals that can be exploited by belowground organisms.[33] For instance, leaf feeding by caterpillars results in the release of a bouquet of volatiles from maize plants, whereas feeding on maize roots by larvae of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, results in the release of a few sesquiterpenoids, dominated by (*E*)-β-caryophyllene, which is exploited by conspecific larvae for host plant location.[13,34]

The present study demonstrates that *P. sophorae* neonates actively search for host plant tissues at both above- and belowground levels. Aboveground host plant finding can be achieved by tracking volatile chemicals transported rapidly over long distances by wind, whereas belowground host-searching behaviour largely depends on volatile chemicals that slowly diffuse through the soil matrix. Although terpenes are suitable for belowground diffusion, especially in dry soil,[35] horizontal diffusion of β-pinene is very weak compared with its vertical diffusion,[26,28] possibly explaining why *P. sophorae* neonates search for host plant tissues at both above- and belowground levels. The desert-like environment in the dry lands of northern China may favour the observed host location behaviour of *P. sophorae* neonates. *P. sophorae* may serve as a useful model for future research on host searching by subterranean root-feeding insects.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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