



Plant Archives

Journal homepage: <http://www.plantarchives.org>

DOI Url : <https://doi.org/10.51470/PLANTARCHIVES.2024.v24.no.1.135>

EFFECT OF PLANT VOLATILES ON THE ELECTROPHYSIOLOGICAL AND BEHAVIOURAL RESPONSE OF APHID'S PARASITOID (*DIERETIELLA RAPAE*)

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(Date of Receiving-26-12-2023; Date of Acceptance-04-03-2024)

ABSTRACT

The impact of six herbivore-induced plant volatiles (HIPVs) on the electrophysiology and behavior of the aphid parasitoid, *Dieretiella rapae* was assessed using electroantennogram (EAG) and a Y-tube olfactometer. Among the six compounds tested with EAG, citronellal, cis-3-hexenyl acetate, and citronellol elicited higher EAG responses from the antennae of *D. rapae*. These six compounds were subsequently evaluated with a Y-tube olfactometer to validate the parasitoid's behavioral preferences. Significant differences were observed in the time spent by *D. rapae* on treatment stimuli (the six synthetic compounds) compared to the control (n-hexane). The highest percentage of *D. rapae* was attracted to stimuli of methyl salicylate, (-)- trans-caryophyllene, and cis-3-hexenyl acetate. These results underscore the significance of HIPVs in influencing the behavior of parasitoids.

Keys words : Aphids, *Dieretiella rapae*, Electroantennography, Herbivore-induced plant volatiles, Olfactometry.

Introduction

Aphid parasitoids, initially classified under Aphidiidae, are now commonly regarded as Aphidiinae, a subfamily within the Braconidae family, as proposed by certain authors (O'Donnell, 1989 and Reed *et al.*, 1995). With a documented 400 parasitoid species, their vital role in pest control, notably in Europe and Pakistan has been underscored (Powell, 1982). This study identified eight aphid parasitoids, including *Praon pakistanum* (Kirkland), from eight aphid species, highlighting their significance in the natural enemy guild for effective pest control (Starý, 1987; Hågvar and Hofsvang, 1991). Nonetheless, challenges such as pesticide use and environmental factors may impede their field efficiency (Powell, 1983 and Powell *et al.*, 1983).

The intriguing behavior of female parasitoids in selecting suitable hosts for oviposition has been explored (Mackauer *et al.*, 1996). Host selection is influenced by factors like female fitness and specific attributes

distinguishing hosts from non-hosts (Godfray, 1994 and Mackauer *et al.*, 1996). The success of parasitoids in finding suitable hosts upon emergence is crucial, given potential scenarios of emerging away from suitable aphid populations or in unsuitable environments (Starý, 1988).

Parasitoids utilize various chemical and physical cues during habitat and host location, as well as host examination (Vinson, 1984; Schmidt, 1991 and Vet and Dicke, 1992). Their responses are shaped by genotype, physiological state, and past environmental adaptability (Vet *et al.*, 1990). Non-random searching and learning from different trophic levels are observed during foraging (Vet, 1995). Host selection encompasses discrete steps like habitat location, host location, host acceptance, host suitability, and host regulation (Vinson and Iwantsch, 1980b). This intricate process, guided by chemical and physical parameters, narrow down the search area, leading to host location (Hågvar and Hofsvang, 1991). Understanding parasitoid behavior presents opportunities for manipulating

their actions to enhance aphid control.

Moving on to *Dieretiella rapae* Mac Intosh, a key parasitoid of the cabbage aphid, it is attracted to cruciferous plants through scent and color (Sheehan and Shelton, 1989) or volatile chemicals released due to aphid feeding activity (Reed *et al.*, 1995). Aphids are recognized and accepted through antennae touch. Cornicle secretions serve as a contact kairomone, aiding in host recognition by aphid parasitoids (Battaglia *et al.* 1993). The study's objective was to explore and analyze the volatiles of host plants and prey using electroantennogram (EAG) and these EAG-active compounds were further evaluated in a Y-tube olfactometer to determine their attractancy or repellency. The results obtained from this study could be applied to manipulate the behavior of *D. rapae*, ultimately enhancing the effectiveness in the Brassica ecosystem.

Materials and Methods

Dieretiella rapae : The rearing of parasitic wasp *D. rapae* was initiated by collecting the mummified aphids from brassica fields (Fig. 1). The collected twigs with mummified aphids were kept in separate jar (20×10 cm) and covered with muslin cloth. The temperature of rearing room was 23±1°C and 43±10% RH with a photoperiod of 12:12 (Light: Day). We followed the rearing protocol



Fig. 1 : Experimental crop *B. juncea* (PM-26).

Table 1 : EAG response (mV) of *D. rapae* to OVCs.

S. no.	Compounds	<i>D. rapae</i>
1.	(R)-(+)-limonene	1.10±0.10 ^d
2.	Methyl salicylate	1.06±0.08 ^d
3.	Cis-3-hexenyl acetate	1.42±0.20 ^{ab}
4.	(-)-trans-caryophyllene	1.25±0.07 ^c
5.	Citronellal	1.48±0.12 ^a
6.	Citronellol	1.39±0.12 ^{ab}
7.	Air	0.02±0.001 ^f
8.	Hexane	0.27±0.02 ^c

Mean (±SE) with common letter in column are not significantly different (Fisher's LSD, $P < 0.01$).

for *D. rapae* as described by Souza *et al.* (2017). The cloth was further secured by rubber bands. After 3-5 days, colour of the parasitized aphids changed from brown to golden yellow. The adults emerged from mummified aphids after 8-10 days. These newly emerged adults were collected with help of glass separator and transferred to another jar (20×10 cm). Mustard twigs with sufficient aphids were provided for further multiplication of parasitic wasps. The adults from this was collected into separate vials (2.5×1.2 cm) and provided with 30% honey solution.

Chemicals : Six organic volatile compounds (OVCs) [methyl salicylate (98%), cis-3-hexenyl acetate (99%), (-)-trans-caryophyllene (99%), (R)-(+)-limonene (95%), citronellal (98%), citronellol (98%)] were purchased from Sigma-Aldrich (USA). Five different concentrations of each compound were prepared by serial dilution and these were further used for EAG and olfactometer studies (Table 1).

Electrophysiology : EAG recordings for *D. rapae* were conducted, measuring responses against a varied concentration range of OVCs. The EAG setup (Fig. 2) and protocol employed in this study were previously outlined by Kumar and Paul (2022) for *Bracon bravicornis* (Wesmael), Kumar *et al.* (2017, 2019) for *Dysdercus cingulatus* (Fabricius), Kumar and Paul (2018) for *Pieris brassicae*, Kumar *et al.* (2021) for *Earias vittella*. Newly emerged adult parasitic wasps (2-4 days old) were selected for EAG recording. To facilitate the process, the parasitic wasps were first anesthetized in a refrigerator and subsequently, the heads of the adults were carefully removed using a sharp scalpel. The antennae were then excised and the terminal segments were removed before being positioned between the electrodes (Fig. 2).

For the chemical assay, a 10 µL concentration of each compound was applied onto filter paper strips (1.5×0.5 cm) and allowed to evaporate the solvent for 10 seconds. The filter paper strip with stimuli was then placed inside a Pasteur pipette using forceps. Each session commenced with the application of air as a stimulus, followed by n-hexane (solvent) and subsequently, the specific compound was presented. A minimum of one minute was provided between two stimuli to allow for the recovery of the antenna. The EAG responses obtained from different concentrations were normalized against n-hexane, and the results are presented accordingly (Fig. 2).

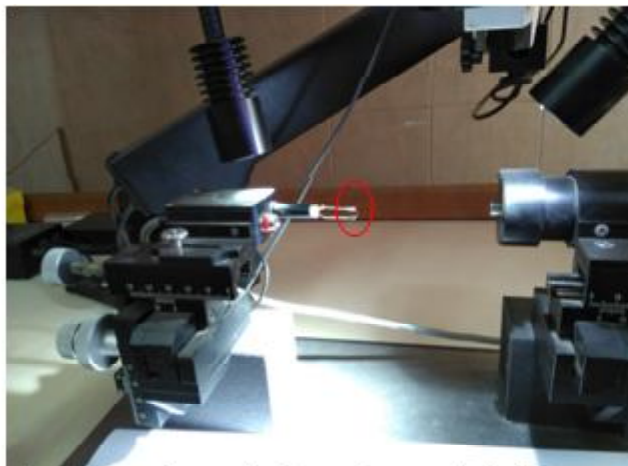
Y-tube Olfactometry : The current investigation utilized a Y-tube glass olfactometer with dimensions of 20×20×20 cm (length) and a diameter of 1.5 cm. The



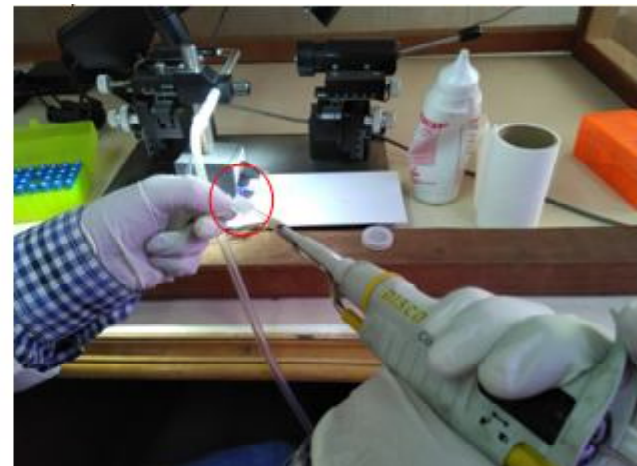
1) Antenna cutting



2) Fixed antenna on electrode



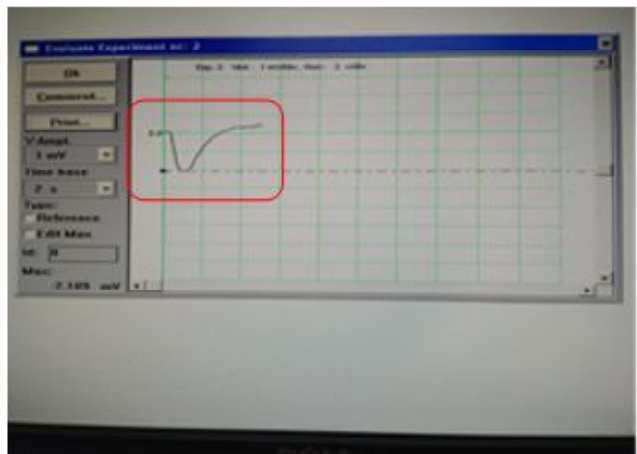
3) Fixing electrode into electrode holder



4) Loading stimuli on filter paper



5) Application of stimuli on antenna



6) EAG response elicited in antenna

Fig. 2 : Steps in electroantennogram analysis.

room temperature was maintained at $26\pm 2^{\circ}\text{C}$, with a relative humidity of $65\pm 5\%$. Illumination was provided by a 40 W fluorescent bulb. A 1% concentration was chosen for the study based on its higher attractiveness to parasitoids, as previously identified by Kumar and Paul (2023). Individual insects were released through the

olfactometer opening, allowing 10 minutes for parasitoids and 5 minutes for beetles to reach the stimuli source. The time spent by individual insects in each olfactometer arm was meticulously recorded.

The recording also included the number of insects attracted to either stimuli source. Each set of ten

individuals constituted one replication, and this process was repeated six times with different individuals. To introduce variability, the location of the stimuli source was alternated after each release. Following each release, the olfactometer setup underwent cleaning with ethanol and was then left to dry in an oven at 200°C.

Statistical analysis : The EAG responses elicited by OVCs from parasitic wasps underwent statistical analysis employing ANOVA (analysis of variance), and subsequent means of treatment were differentiated using Fisher's LSD test ($p < 0.05$). To evaluate the disparity in the time spent between treatment and control, a paired t-test was applied, with significance levels denoted by asterisks (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) through the XLstat software version 2016.

Furthermore, the cumulative responses from both wasps were separately subjected to a chi-square test for goodness of fit, utilizing SAS 4.3. This statistical approach was employed to assess whether the observed responses aligned with the expected distribution.

Results

EAG recording - *D. rapae*

The EAG recordings from the antenna of *D. rapae* wasps exhibited a dose-dependent pattern, with the

maximum EAG response observed at a 1% concentration, as illustrated in Figs. 3 and 4. Notably, among the tested compounds, citronellal elicited the highest EAG response (1.48 ± 0.12 mV), followed by cis-3-hexenyl acetate (1.42 ± 0.20 mV) and citronellal (1.39 ± 0.12 mV), as outlined in Table 1. Importantly, the responses to all OVCs were significantly higher than the control group, as confirmed by Fisher's LSD test ($p < 0.05$).

Olfactometry - *D. rapae*

The Y-tube olfactometer results unveiled significant differences in the time spent by parasitic wasps in each treatment (OVCs) compared to the control (n-hexane) ($t = 4.28$, $df = 5$, $p = 0.007$). Specifically, adult *D. rapae* exhibited increased residence time in the olfactometer arm containing stimuli of (-)-trans-caryophyllene ($t = 4.02$, $df = 5$, $p = 0.01$), cis-3-hexenyl acetate ($t = 6.17$, $df = 5$, $p = 0.001$), and methyl salicylate ($t = 7.90$, $df = 5$, $p = 0.0005$) (Fig. 5). However, no significant difference was observed in residence time between the control and treatments when stimuli were provided from (R)-(+)-limonene ($t = 2.44$, $df = 5$, $p = 0.058$), citronellal ($t = 0.54$, $df = 5$, $p = 0.61$) and citronellol ($t = 2.22$, $df = 5$, $p = 0.076$), as depicted in Fig. 5.

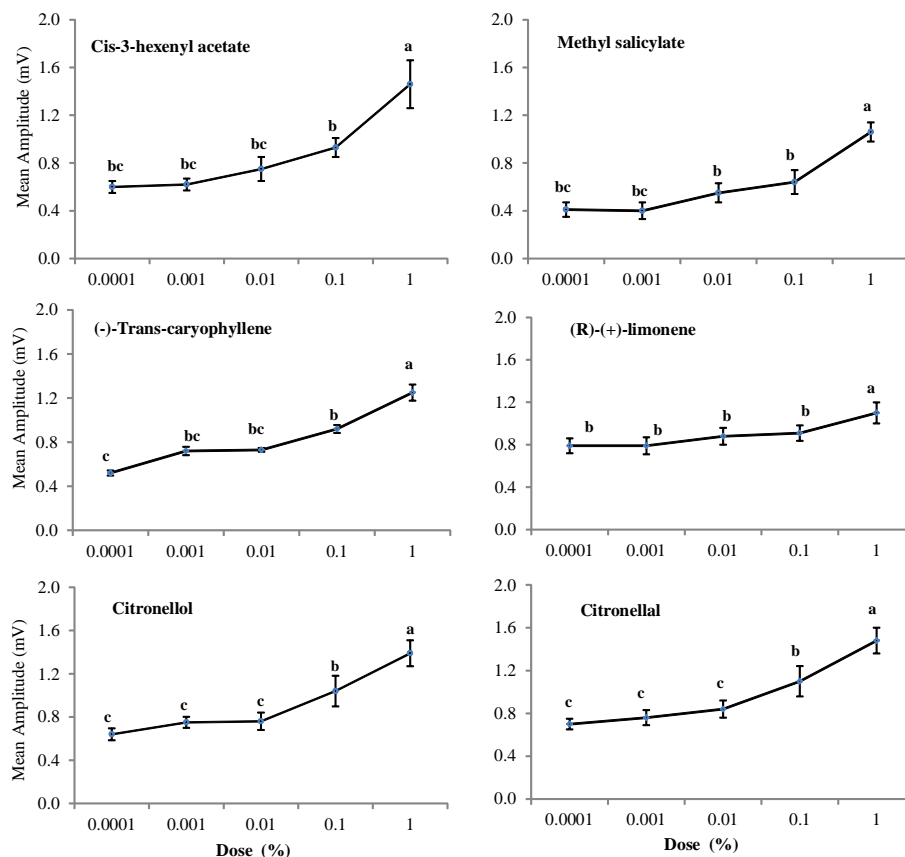


Fig. 3 : EAG responses profile of *D. rapae* to different concentrations.

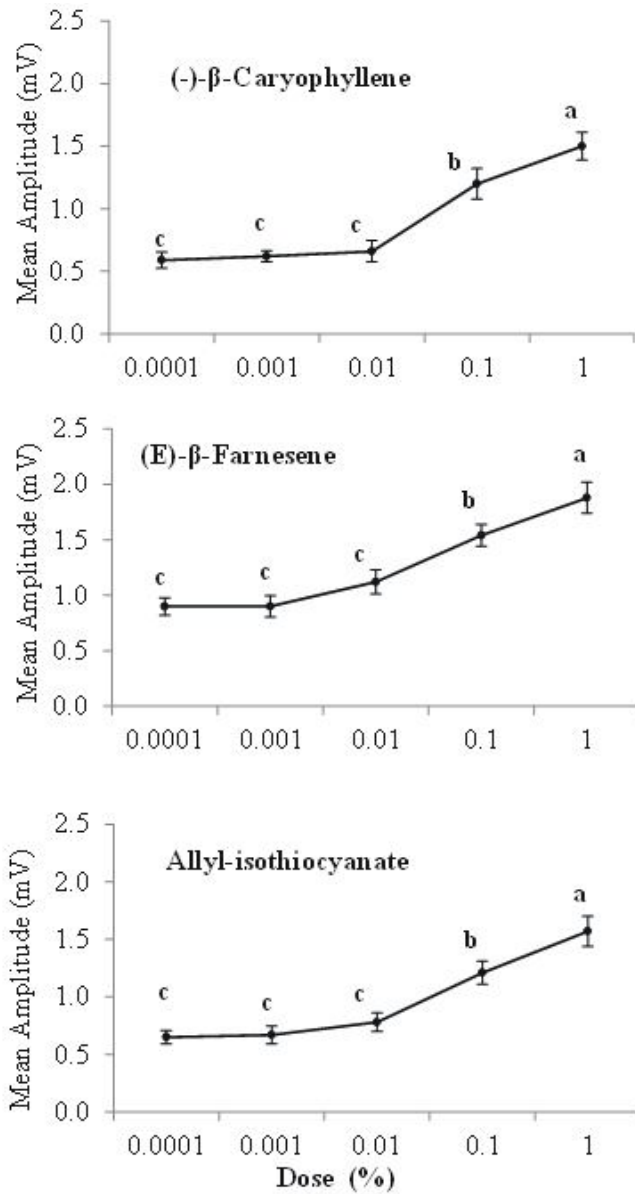


Fig. 4 : Dose-response analysis of selected compounds of *D. rapae*.

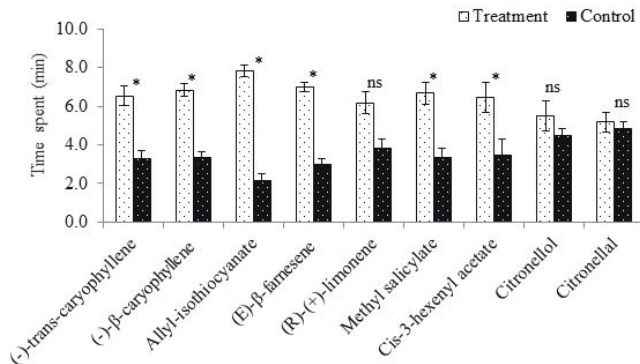


Fig. 5 : Time spent (\pm s.e) for *D. rapae* adult into uninfested OVCs (treatment) and n-hexane (control). An asterisk (*) above the letter indicates a significant difference between male and female.

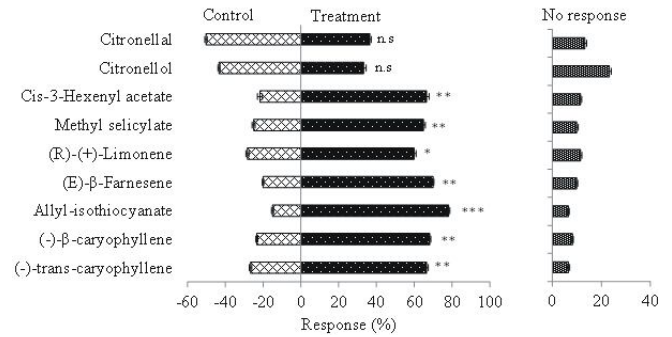


Fig. 6 : Response of *D. rapae* to OVCS in Y-tube olfactometer. [Values are mean \pm SE (n=6). Significant differences at $p < *0.01$ and $**0.001$, respectively in chi-square test for 50:50 distribution in a 2 \times 2 contingency table compared to control stimulus].

Furthermore, the maximum percentage of adult wasps were attracted to (-)-trans-caryophyllene, followed by cis-3-hexenyl acetate and methyl salicylate, with attractancy rates of $66.60 \pm 0.81\%$, $66.60 \pm 1.26\%$ and $65.00 \pm 0.91\%$, respectively as illustrated in Fig. 6.

Discussion

Parasitoids undergo a sequence of behavioral responses to locate and parasitize their hosts, involving five essential steps in host plant selection: host habitat finding, host finding, host recognition, host acceptance and host suitability. Chemical orientation plays a crucial role in each stage, with olfactory cues originating from the plant where herbivorous insects feed, the host itself, associated organisms with the host, or volatile compounds emitted due to the interaction between the host and the plant. Therefore, understanding the chemical cues released from the host plant or host itself is crucial for parasitoids in locating their hosts.

In EAG studies, the parasitic wasp demonstrated higher sensitivity to citronellal, cis-3-hexenyl acetate, and citronellol compared to other compounds. This suggests the presence of specific receptors in the wasps stimulated by citronellal and cis-3-hexenyl acetate. These findings align with Sen *et al.* (2005), who reported higher EAG recordings with citronellal and citronellol than (-)-trans-caryophyllene and (R)-(+)-limonene. Cis-3-hexenyl acetate, known as a herbivore-induced plant volatile that benefits the plant by attracting natural enemies of the host (Mumm and Dicke, 2010), was the second most sensitive compound to *D. rapae* after citronellal.

In contrast to previous studies, our results for (R)-(+)-limonene differed, with a lower EAG response compared to (-)-trans-caryophyllene. These variations may be attributed to the use of different parasitoid species and doses (10 mg/mL). Our findings are consistent with

Sen *et al.* (2005), who reported a strong EAG response in *Trichogramma chilonis* Ishii with (-)-trans-caryophyllene compared to (R)-(+)-limonene.

Methyl salicylate, an aphid-induced volatile attracting several natural enemies (Zhu and Park, 2005), exhibited lower EAG activity in our experiments compared to other compounds. Differences in EAG responses between parasitoids could be due to specific sensilla on antennae responding to this compound. However, in olfactometry, methyl salicylate was equally attractive to *D. rapae* as cis-3-hexenyl acetate and (-)-trans-caryophyllene. This suggests that the parasitic wasp may utilize methyl salicylate in the process of host habitat location, aligning with previous studies showing attraction of parasitoids to methyl salicylate induced by *Pieris rapae* (Linn.) (Snoeren *et al.*, 2010).

Cis-3-hexenyl acetate, another herbivore-induced plant volatile, attracted 61.60% of *D. rapae* adults, consistent with Turlings and Fritzsche (1999), who found *C. marginiventris* attracted to cis-3-hexenyl acetate. Contrary to earlier studies indicating the repellent or deterrent effects of limonene in citrus plants against mealy bugs and scale insects, our olfactometric assays revealed that *D. rapae* showed an attractive response to (R)-(+)-limonene. However, the attractancy to (R)-(+)-limonene was significantly lower than methyl salicylate, cis-3-hexenyl acetate and (-)-trans-caryophyllene.

Interestingly, both citronellal and citronellol, highly sensitive compounds to *D. rapae*, exhibited less alluring activity in Y-tube olfactometer experiments. Adult wasps spent more time in the olfactometer arm containing stimuli of both citronellal and citronellol, suggesting irritation in the antennae, leading the wasps away from these treatments. This effect was reflected in the higher percentage of adult wasps attracted to the control (n-hexane) than to citronellal and citronellol. Similar alluring activity of citronellal and citronellol has been reported in studies on mosquitoes (Park *et al.*, 2001 and Yang *et al.*, 2004). Odalo *et al.* (2005) also investigated the repellent effect of different metabolites of 12 Kenyan plants against *Anopheles gambiae*, with citronellal and citronellol identified as potential repellents.

Conclusion

Our findings indicate that the antennae of *D. rapae* were capable of detecting and recognizing a diverse array of compounds. Notably, citronellal and citronellol exhibited less alluring effects on both wasps, while methyl salicylate, cis-3-hexenyl acetate, (-)-trans-caryophyllene, and (R)-(+)-limonene acted as attractants for both species. These compounds hold the potential to enhance the effectiveness

of parasitoids by manipulating their behavior.

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