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Short-range allelochemicals from a plant–herbivore association: a singular case of oviposition-induced synomone for an egg parasitoid

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SUMMARY

Oviposition-induced plant synomones are semiochemical cues used by egg parasitoids during host selection, and are therefore considered important elements of plant defence. In this paper we show that, in the tritrophic system *Brassica oleracea–Murgantia histrionica–Trissolcus brochymenae*, the latter responded in a closed arena and in a static olfactometer to induced chemicals that are perceived from a very short range and after parasitoid contact with the leaf surface opposite the treated surface. An additive or synergistic effect due to (1) egg deposition, (2) feeding punctures and (3) chemical footprints of *M. histrionica* was observed. When all three phases were present, the parasitoid reacted to the induced synomone locally on the treated leaf area, at a close distance to the treated area, and on the leaf above the treated one, showing that there is also a systemic effect. When plants with host footprints combined with feeding punctures or with oviposition were tested, responses were obtained both locally and at a close distance, whereas in the remaining assays only local responses were observed. Induction time was less than 24 h, whereas signal duration was apparently related to the suitability of the host eggs, as parasitoids did not respond to plants carrying old or hatched eggs. These oviposition-induced short-range plant synomones might have an important role in the host location process after parasitoid landing on the plant, in different combinations with the host kairomones involved in the system studied here.

Key words: *Murgantia histrionica*, Pentatomidae, *Trissolcus brochymenae*, Scelionidae, egg parasitoid, induced synomone, induced defense, oviposition.

INTRODUCTION

The interactions between plants, herbivores and carnivores are the subject of intriguing and extensive studies that involve different disciplines, from entomology to plant physiology, as well as the use of different techniques (Vinson, 1985; Turlings et al., 1990; Vet and Dicke, 1992; Dicke and Hilker, 2003; Dicke et al., 2003; Fatouros et al., 2008). One of the main causes of such large interest is that virtually all plants are attacked by herbivores and most of these are insects, which embody the large majority of all existing animals. In turn, practically all herbivorous insects are attacked by one or more carnivores (entomophages), namely predators and parasitoids, which results in an amazing variety of mechanisms that characterize these tritrophic interactions (Price, 1997).

Particularly interesting are the mechanisms involved in host location by parasitoids. In combination with physical cues, parasitoids generally exploit plant synomones and/or host kairomones, which act both from a distance and from a short range (reviewed by Fatouros et al., 2008; Hilker and Meiners, 2010). Specifically, plant synomones play a crucial role, especially when they are emitted as a result of herbivore attack, thus acting as an indirect plant defence mechanism against herbivores (Karban and Baldwin, 1997; Dicke and Hilker, 2003).

The induction of plant synomones is known for a wide array of tritrophic systems, as a consequence of feeding by insects or mites (reviewed by Felton and Tumlinson, 2008; Mithöfer and Boland, 2008). However, for several systems it has been found that the emission of plant synomones can be induced by insect oviposition, and that these synomones will attract specific egg parasitoids (Meiners and Hilker, 1997; Meiners and Hilker, 2000; Hilker and Meiners, 2002; Hilker et al., 2002a; Colazza et al., 2004a; Colazza et al., 2004b; Fatouros et al., 2005; Fatouros et al., 2007; Fatouros et al., 2008; Fatouros et al., 2009). From an evolutionary point of view, both symbionts will take advantage of this 'early alert' (*sensu* Hilker and Meiners, 2006) mechanism, as the egg parasitoids would use such highly detectable and reliable volatiles induced in plants soon after herbivore eggs are laid, whereas the plants would increase their fitness by recruiting natural enemies of the herbivore eggs before significant damage has occurred, i.e. before the herbivore eggs have hatched. This discovery is considered to be of particular interest also for application purposes, because egg parasitoids would prevent plant loss.

Most oviposition-induced synomones known so far are perceived by the parasitoids as olfactory stimuli (volatile synomones) and are exploited by species of Eulophidae, Scelionidae and Mymaridae in a few systems based both on arboreal, perennial plants and on herbaceous, annual plants (Meiners and Hilker, 2000; Hilker and Meiners, 2002; Hilker et al., 2002a; Colazza et al., 2004a; Colazza et al., 2004b; Manrique et al., 2005). The mechanisms underlying synomone emission are associated with the presence of plant damage, either during oviposition or as a consequence of unrelated feeding behaviour on the host plant, in combination with elicitors contained in the reproductive system secretion and/or in the saliva of the herbivore (Hilker et al., 2002a; Hilker et al., 2002b; Rodriguez-Saona et al., 2002; Manrique et al., 2005; Colazza et al., 2004a; Colazza et al., 2004b).

The egg parasitoids might also respond to short-range induced synomones that are perceived after they have alighted on the plant. Oviposition by *Pieris brassicae* L. (Lepidoptera: Pieridae) on Brussels sprouts (*Brassica oleracea* var. gemmifera) and on *Arabidopsis thaliana* induces changes in the chemistry of the leaf

surface, both locally and systemically, that arrest the egg parasitoids *Trichogramma brassicae* Bezdenko and *T. evanescens* Westwood (Hymenoptera: Trichogrammatidae) (Fatouros et al., 2005; Fatouros et al., 2007). Similarly, Brussels sprout plants induced by singly laid eggs of *P. rapae* L. (Lepidoptera: Pieridae) arrest *T. brassicae* wasps (Fatouros et al., 2009). The elicitor of this defensive response was found to be benzyl cyanide, an anti-aphrodisiac pheromone transferred from the male to the female of *P. brassicae* during mating; mated females release this compound during oviposition together with the secretion of the accessory reproductive glands (Fatouros et al., 2005; Fatouros et al., 2008; Fatouros et al., 2009; Blenn et al., 2009). The chemical changes on the plant surface are quantitative rather than qualitative (Blenn et al., 2009).

In this paper, by investigating the tritrophic system *Brassica oleracea* L.–*Murgantia histrionica* Hahn (Heteroptera: Pentatomidae)–*Trissolcus brochymenae* Ashmead (Hymenoptera: Scelionidae), we focus on the parasitoid's behaviour after it alighted on the plant and its responses to short-range allelochemicals originating from such plant–herbivore association. *Murgantia histrionica* mainly attacks Cruciferae and Capparidaceae and is considered to be a pest of cabbage and other brassicaceous crops in North America (McPherson, 1982; Schaefer and Panizzi, 2000). *Trissolcus brochymenae* is an egg parasitoid reported to be associated with at least 11 pentatomid species including *M. histrionica*. This tritrophic system is characterized by the possible contemporary presence on plant leaves of chemical traces (footprints) left by the herbivore (Conti et al., 2003), feeding damage and egg deposition. These features might act singly or in combination to induce chemical changes in the plant (induced synomone) that could be perceived by the parasitoid.

MATERIALS AND METHODS

Insects

Murgantia histrionica was originally collected from cabbage in the Beltsville area, MD, USA in 2000. Adults of *T. brochymenae* were obtained from *M. histrionica* eggs laid on *Isomeris arborea* Nutt. (Capparidaceae) in San Diego, CA, USA in 2000. Both insects were maintained in quarantine conditions in the entomology laboratories of the University of Perugia, Perugia, Italy.

The colony of *M. histrionica* was reared in a controlled condition chamber (25±1°C, 60±5% relative humidity (RH), 15 h:9 h light:dark), inside clear plastic food containers (300×195×25 mm high) with 5 cm diameter mesh-covered holes. Separate containers were used for nymphs and adults. All stages were fed vegetative and reproductive parts of cabbage and broccoli (*Brassica oleracea* L.). Food was changed every 2–3 days, and water was provided weekly through soaked cotton wool. The colony of *T. brochymenae* was reared on eggs of *M. histrionica* that were glued on strips of paper, and therefore they had no experience with plants during emergence. Adult wasps were kept in 85 ml glass tubes and fed small drops of Safavi (Safavi, 1968) diet, under controlled conditions in an incubator (25±1°C, 80±5% RH, 15 h:9 h light:dark). After emergence, male and female parasitoids were kept together for mating.

Two- to 5-day-old female wasps were individually isolated in small vials (25 mm×10 mm diameter), which contained a drop of the Safavi (Safavi, 1968) diet, 16–17 h before the bioassays, and allowed to acclimatize in the bioassay room for at least 30 min before the experiments started. Only naïve female parasitoids were used for the experiments, i.e. females that had never oviposited in the host and had no experience with the host beyond that which occurred during development and eclosion (Vet et al., 2003).

Plant cultures

Seeds of *Brassica oleracea* var. sabauda (cv. Salto, kindly provided by Royal Sluis Brand, Seminis, Parma, Italy) were individually planted in pots filled with peat and, after ~7 days, were transplanted into plastic pots filled with agriperlite and vermiculite mixture. Plants were kept in the greenhouse under controlled conditions (25±1°C, 50–60% RH, 12 h:12 h light:dark), watered daily and fertilized with a water solution of Flory 9 Hydro (Planta Regenstauf, distributed by Agrimport SPA, Bolzano, Italy), sequestrene and urea (1 liter solution: 1 g Flory 9, 0.04 g sequestrene and 0.1 g urea). All experiments were carried out using 4- to 5-week-old plants.

Treatments

The plants used in the bioassays were subjected to the following treatments: (1) *M. histrionica* feeding punctures, which are inevitably combined with chemical traces (footprints) left by the bugs on the substrate; (2) a combination of feeding punctures, a deposited egg mass (oviposition) and footprints; (3) oviposition, combined with footprints as above; (4) an egg mass artificially applied on a leaf, therefore without footprints; (5) fresh ovarian eggs artificially applied on a leaf to form an egg mass, also excluding the presence of footprints; (6) *M. histrionica* footprints; (7) *M. histrionica* inhibited to contact the leaf surface directly because of a filter paper disk placed between the bug and the leaf, thus preventing leaf contamination with bug footprints, but allowing possible contamination with bug volatiles.

Treatments 1–3 and 6 were carried out in order to verify the role of feeding punctures, oviposition, chemical footprints and the different combinations in synomone induction. Treatment no. 4 was conducted to tentatively identify the source of a possible synomone elicitor, whereas treatment no. 5 was carried out with the same purpose, as well as to verify the effect of oviposition by excluding host footprints. Finally, treatment no. 7 was carried out with the aim of evaluating whether volatiles from *M. histrionica* females, which are known to have kairomonal activity for *T. brochymenae* (Conti et al., 2003), might be adsorbed by the leaf surface and then perceived by the parasitoid.

For treatments 1–3, 6 and 7, a mated *M. histrionica* female was placed for 24 h on the lower surface of a leaf of the central plant portion, inside a small cage, made from two Petri dishes (35 mm diameter, 10 mm height), with the bottoms substituted with a fine nylon mesh and each rim of the opposite side covered with a small foam rubber ring, which was kept tightened to the leaf with the help of a clip. These plant–bug complexes were kept in a controlled environment cabinet (25°C, 80±5% RH, 15 h:9 h light:dark) for the total duration of the treatment.

In particular, to prevent bug feeding and to obtain plants with oviposition or with chemical traces (treatments 3, 6 and 7), gravid females with excised stylets were used. For stylet excision, females were previously anaesthetized inside a glass tube with CO_2 for 4–5 s in order to immobilize their labium. Afterwards the stylets were drawn from the labium with an entomological pin (no. 000), to amputate half their length using precision micro-scissors under a stereomicroscope (Zeiss Stemi SV8) with optical fibre illumination (Intralux 5000). The treated females were then placed inside a plastic dish (12 cm diameter) for 24 h allowing them to recover, and subsequently they were used to infest cabbage plants as described.

To obtain plants with an artificially applied egg mass (treatments 4 and 5), deposited egg masses were collected from the rearing containers of adult *M. histrionica* and were applied onto the leaf surface using suitable pincers and kept in place for 24 h. In the case of plants with ovarian eggs, eggs were collected from the oviduct

after dissection of gravid *M. histrionica* females. After careful cleaning in a saline solution, they were applied, together with the secretion produced by the follicular cells, onto the leaf surface using a very tiny brush, to form an artificially applied egg mass. They were kept in place for 24 h under controlled conditions, as described above.

As a consequence of the treatment procedures, the chemical footprints left on the lower leaf surface by *M. histrionica* females were present in all treatments except for treatments 4, 5 and 7.

Behavioural assays and data analysis

The variously treated plants were assayed with the female parasitoids following different procedures, with the aim of evaluating several aspects of induction, as described in the following paragraphs.

(1) To define the timing of possible synomone induction in plants. For this aim, behavioural assays were conducted at different time intervals elapsing after the end of the treatments (0 h, 24 h, 48 h and >96 h). This should be considered as the minimum time elapsing after insect activity (oviposition, feeding, walking) on the leaf, which might have occurred anytime during the 24 h duration of the treatment. However, because carrying out the same experiment at all planned intervals was very time consuming, the number of intervals considered was reduced when appropriate, depending on the type of treatment and on the results obtained in the course of the bioassays. In detail, if local response was not significant at a given time interval, this interval was not considered for bioassays on leaf portions close to the treated area and on a different leaf (i.e. local, close distance and systemic effects; see below); similarly, if the local response was significant but the response at a close distance was not, then the systemic effect was not tested.

(2) To assess possible induction in the leaf portion that had been directly affected by bug activity, i.e. that leaf surface portion on which the host had walked, fed and/or oviposited (local induction) and in a portion at a close distance to the attacked area on the same leaf (close distance induction). The latter was achieved by testing an untreated leaf area that was 8.0–10.0 mm away from the treated area.

(3) To verify possible systemic effects of the induction, i.e. synomone emission from leaves that had not been treated. These leaves were collected one node above or below the treated leaf (systemic induction).

(4) To evaluate possible short-range perception of the synomones by the female parasitoid, and therefore a possible short-range volatility of the synomones. Bioassays were carried out on leaves with a combination of feeding punctures and a deposited egg mass using a static olfactometer, described below.

(5) To evaluate whether it is possible to exclude contamination of plants by volatile compounds emitted by *M. histrionica* during the experiments, which perhaps might be adsorbed by the leaf epicuticular waxes and then perceived by the parasitoids. Therefore, in a specifically designed treatment, a filter paper disk was placed between the leaf area and the *M. histrionica* female to prevent any direct contact between plant and bug.

All bioassays were conducted using leaf squares (200 mm^2) cut using a razor blade from leaf portions that had been directly exposed to the bug or from portions of the same leaf that were close (about 8–10 mm) to the treated area. In addition, bioassays were also conducted on leaves excised from nodes above or below those affected by damage and/or oviposition. Each leaf square was used for about five wasps. The number of replicates for each bioassay is reported in the figures.

In all experiments, only the upper leaf surface (i.e. the surface opposite that affected by *M. histrionica*, as explained above) was tested in order to avoid the presence of perceivable host kairomones on the plant. Moreover, to avoid possible cues originating directly from deposited or artificially applied egg masses, these were removed from the leaf surface before the bioassays. The responses of *T. brochymenae* females to short-range cues from treated plants (treatment) and untreated, healthy plants (control) were evaluated in a closed arena. The arena was assembled from a plexiglass plate with a circular hole in the middle (25 mm diameter and 5 mm height), sandwiched between two glass plates (bottom and cover). A sheet of filter paper was placed between the bottom plate and the Plexiglas® arena, whereas two leaf portions (treatment and control) were placed on the filter paper, at a 5 mm distance from each other, and the whole system was centered on the arena floor.

The static olfactometer (i.e. without air flow) used to evaluate whether the parasitoid is able to perceive the stimuli as short-range volatile cues was similar to the closed arena previously described, the only difference being a fine mesh (200 US mesh, SaatiTech, Somers, NY, USA) placed between the leaf surface and the observation chamber, to prevent direct contact between the parasitoid and the leaf surface.

All experiments were carried out from 09.00 h–13.00 h, in an isolated room at 25±1°C and 50±10% RH with the arena illuminated by two 12 V halogen lamps. During the bioassays, a single female was gently released in the middle of the arena and observations lasted for the total duration of 5 min. Parasitoid behaviour was recorded using a zoom-equipped (125–175 mm) video camera (JVC KY-M280) connected to a video capture device (Pinnacle Dazzle DVC 100) to allow digitization of the images (25 frames s^{-1}). The behavioural data were collected using software for behavioural observations (The Observer Video-Pro version 4, Noldus Information Technology, Wageningen, The Netherlands).

The time spent by the female wasp searching on the treated leaf, on the control leaf or on the arena surface was scored and the percentages of residence time on either the treatment or the control related to total time on the leaf surface were calculated. The residence time properly describes the wasp searching behaviour, which is characterized by returning several times to the treated area followed each time by an examination of the surface around the treated area. When doing a comparative bioassay, the different treatments were tested on several days and they were alternated on the same day after having tested a group of wasps for each arena.

Before the analysis, Box–Cox transformation was used to reduce data heteroscedasticity and then the data were analyzed with the Student's *t*-test for dependent samples [Statistica 6.0, Statsoft, 2001, Vigonza (PD), Italy] (Zar, 1999).

RESULTS

Parasitoid response to cues from plants with feeding damage, a deposited egg mass and footprints

The female *T. brochymenae* showed a higher residence time on cabbage leaves with *M. histrionica* feeding damage, a deposited egg mass and footprints than on leaf portions excised from healthy plants (control). This response was significant at time intervals from 0 to 48 h after the treatment (0 h: t=9.228, d.f.=28, P<0.001; 24 h: t=3.192, d.f.=25, P=0.004; 48 h: t=4.629, d.f.=31, P<0.001), whereas differences were not significant after 96 h (t=1.735, d.f.=40, P=0.090), when the host eggs had hatched. A very similar response was scored when leaf areas that were close to the treated portion were tested (0 h: t=10.215, d.f.=27, P<0.001; 24 h: t=3.398, d.f.=23, P=0.002; 48 h: t=5.498, d.f.=30, P<0.001; >96 h: t=1.400, d.f.=35, P=0.170). When leaves that had been excised from the node above the treated leaves were bioassayed, the parasitoid spent a longer

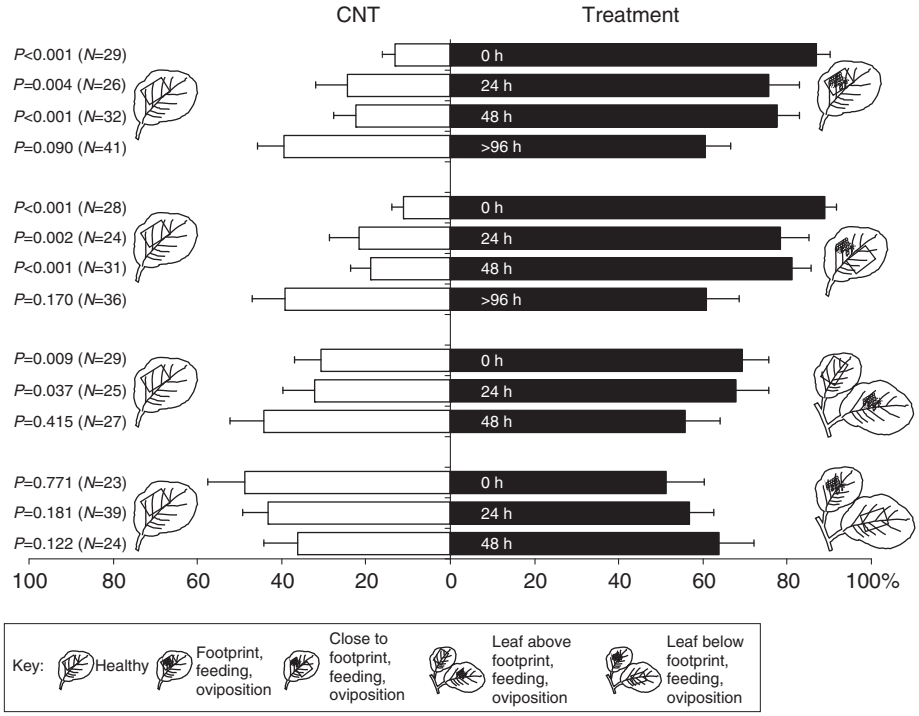

Fig. 1. Response (% residence time, s) of *Trissolcus brochymenae* females to a cabbage leaf portion with feeding damage, a deposited egg mass and chemical footprints by *Murgantia histrionica*, to a portion close to the treated area, and to leaves above or below the treated leaf (Treatment) *vs* response to healthy leaves (CNT), at increasing time intervals after the treatment. Bars indicate means ± s.e.m. (Student's *t*-tests for dependent samples).

time on such leaves at 0 h and 24 h after the treatment (0 h: t=2.814, d.f.=28, P=0.009; 24 h: t=2.203, d.f.=24, P=0.037), but not after 48 h (t=0.829, d.f.=26, P=0.415). By contrast, the parasitoid did not show any significant response towards leaves that were collected from the node below the treated leaf (0 h: t=0.295, d.f.=22, P=0.771; 24 h: t=1.364, d.f.=28, P=0.181; 48 h: t=1.606, d.f.=23, P=0.122) (Fig. 1).

Parasitoid response to cues from plants with feeding damage and footprints

The parasitoid females also reacted to cabbage leaves with harlequin bug feeding punctures and footprints, but without eggs, showing a higher residence time on treated leaves than on control leaves. In this case, the parasitoid response was significant at all tested time intervals from the treatment, including at 96 h, although the difference decreased slightly at this time (0 h: t=7.360, d.f.=24, P<0.001; 24 h: t=6.338, d.f.=69, P<0.001; 48 h: t=7.025, d.f.=30, P<0.001; >96 h: t=2.944, d.f.=33, P=0.006). When leaf areas that were close to the feeding damage areas were bioassayed, the residence time of the parasitoid on such areas was significantly higher than that on the control, but only when 48 h had elapsed from the treatment (t=5.288, d.f.=20, P<0.001); such differences were not significant at 0 h and 24 h (0 h: t=0.608, d.f.=27, P=0.548; 24 h: t=0.846, d.f.=26, P=0.405). When the leaf located immediately above the damaged leaf was tested with a female parasitoid, no significant response was elicited (t=1.836, d.f.=23, P=0.079) (Fig. 2).

Parasitoid discrimination between cues from plants with feeding damage, a deposited egg mass and footprints *vs* feeding damage plus footprints

To evaluate whether the combination of feeding punctures, a deposited egg mass and footprints elicits a different response by the parasitoid to that from plants with only feeding damage and

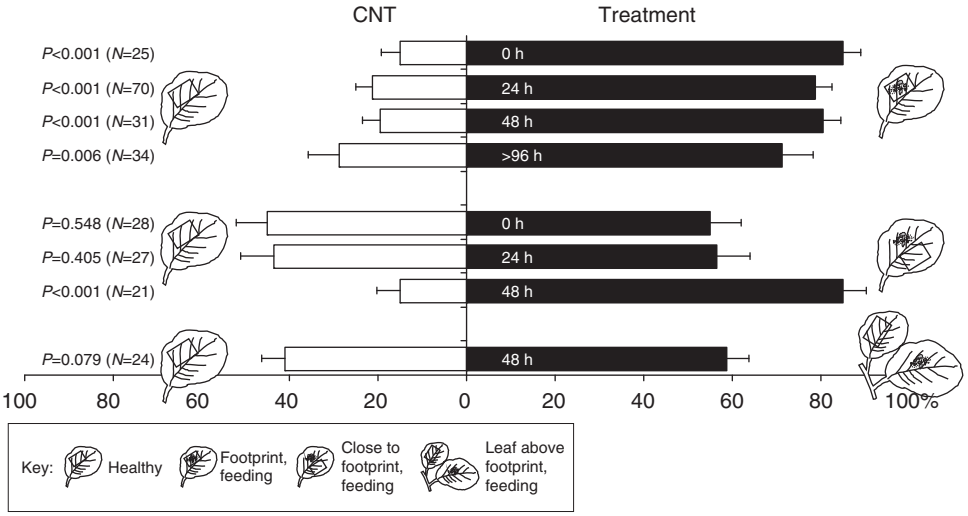

Fig. 2. Response (% residence time, s) of *T. brochymenae* females to a cabbage leaf portion with feeding damage and footprints by *M. histrionica*, to a portion close to the treated portion, and to leaves above the treated leaf (Treatment) *vs* response to healthy leaves (CNT), at increasing time intervals after the treatment. Bars indicate means ± s.e.m. (Student's *t*-tests for dependent samples).

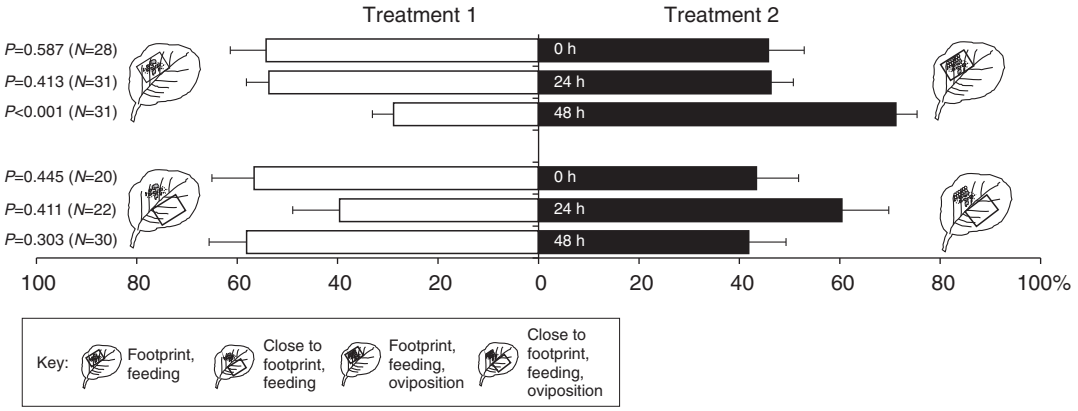

Fig. 3. Response (% residence time, s) of *T. brochymenae* females to a cabbage leaf portion with, or close to, feeding damage, an egg mass and footprints by *M. histrionica* (Treatment 1) *vs* a cabbage leaf portion with, or close to, feeding damage plus footprints (Treatment 2) at increasing time intervals after the treatments. Bars indicate means ± s.e.m. (Student's *t*-tests for dependent samples).

footprints, both types of treated leaves, from different plants, were compared in the closed arena. Differences were not significant at 0 and 24 h after the end of the treatments (0 h: t=–0.459; d.f.=27; P=0.587; 24 h: t=–0.831, d.f.=30, P=0.413), whereas after 48 h (t=–4.894, d.f.=30, P<0.001) the parasitoid residence time was significantly higher on the cabbage leaves that carried also an egg mass than on leaves that did not. However, when the same treatments were compared on leaf areas close to the treated areas, no significant differences in the parasitoid residence time were scored (0 h: t=–0.780, d.f.=19, P=0.445; 24 h: t=0.838, d.f.=21, P=0.411; 48 h: t=–1.049, d.f.=29, P=0.303) (Fig. 3).

Parasitoid response to cues from plants with a deposited egg mass and footprints

The residence time of the parasitoid on leaves with a deposited egg mass and footprints but without feeding damage (female bugs with excised stylets) was significantly greater than that on healthy leaves at 0 h to 48 h from the treatment (0 h: t=4.089, d.f.=35, P<0.001; 48 h: t=2.149, d.f.=52, P=0.036), but not after 96 h, when all eggs had hatched (t=1.431, d.f.=25, P=0.165). The parasitoid reacted similarly to short-range cues that were close to the oviposition area, showing a significant increase of residence time compared with that on the control at 0 to 48 h (0 h: t=3.398, d.f.=26, P=0.002; 48 h: t=2.520, d.f.=43, P=0.016). When a leaf from the node above the treated leaf was tested, no significant response was scored just after the treatment (0 h: t=1.352, d.f.=21, P=0.191) (Fig. 4).

Parasitoid response to cues from plants with artificially applied host eggs

The parasitoid significantly reacted to leaves treated with ovarian eggs compared with the control leaves 24 h after the treatment (t=2.157, d.f.=21, P=0.043), whereas the response was marginally not significant at 0 h (borderline; t=1.896, d.f.=35, P=0.066) and not significant at 48 h (t=0.397, d.f.=34, P=0.694). The parasitoid did not react to the leaf surface close to the treated portion, regardless of the time elapsed after treatment (0 h: t=–0.723, d.f.=36, P=0.474; 24 h: t=–0.992, d.f.=19, P=0.334; 48 h: t=–0.152, d.f.=35, P=0.880). In the case of egg masses previously deposited in the rearing cage and then artificially applied onto the cabbage leaves, no response was observed from *T. brochymenae* females either 24 h or 48 h after the treatment (24 h: t=–0.233, d.f.=25, P=0.818; 48 h: t=1.087, d.f.=22, P=0.289) (Fig. 5).

Parasitoid response to cues from plants contaminated with chemical traces (footprints and possible volatiles adsorbed by epicuticular waxes) of host female

Cabbage leaves contaminated with chemical footprints left by mated females of *M. histrionica* elicited a significant response by *T. brochymenae* at all time intervals (0 to 48 h) tested (0 h: t=4.824, d.f.=18, P<0.001; 24 h: t=6.178, d.f.=21, P<0.001; 48 h: t=3.493, d.f.=20, P=0.002). By contrast, areas close to the host-contaminated areas did not elicit any significant reaction by *T. brochymenae* at any of the intervals considered (0 h: t=1.369, d.f.=19, P=0.187; 24 h: t=–0.354, d.f.=18, P=0.728; 48 h: t=1.343, d.f.=19, P=0.195). In the case of treatments conducted with a filter paper disk placed between the bug and the leaf, no response from the parasitoid was observed (t=–0.526, d.f.=19, P=0.605) (Fig. 6).

Parasitoid response to short-range volatile cues from plants with feeding damage, a deposited egg mass and footprints

Trissolcus brochymenae reacted in the static olfactometer to short-range volatiles from cabbage leaves with feeding damage, oviposition and footprints by *M. histrionica*; the response was

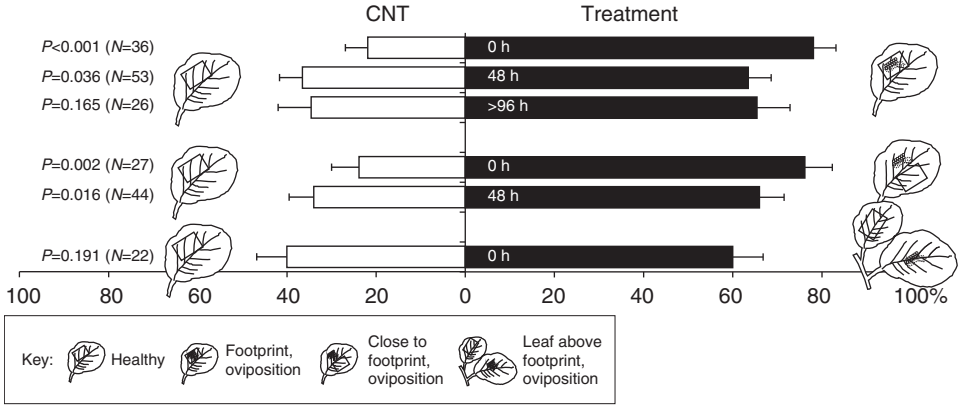

Fig. 4. Response (% residence time, s) of *T. brochymenae* females to a cabbage leaf portion with an egg mass and footprints by *M. histrionica*, to a leaf portion close to the treated area, and to a leaf above the treated leaf (Treatment) *vs* response to healthy leaves (CNT), at different time intervals after the treatment. Bars indicate means ± s.e.m. (Student's *t*-tests for dependent samples).

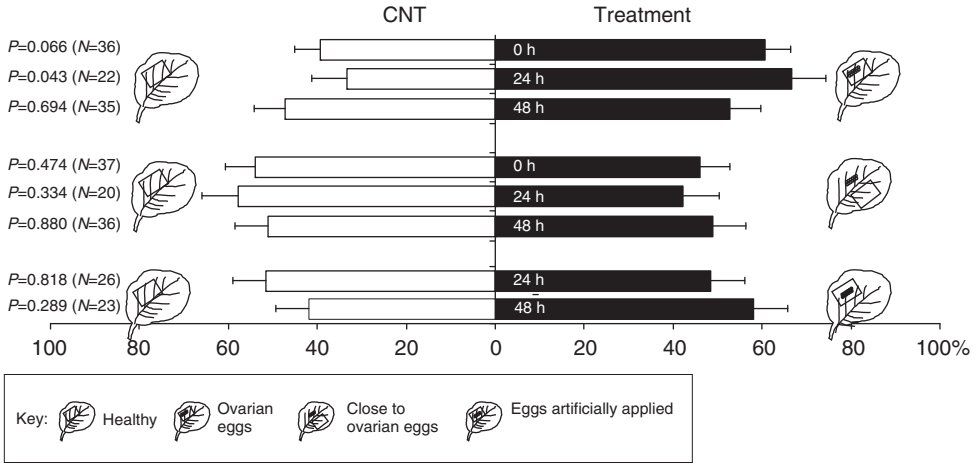

Fig. 5. Response (% residence time, s) of *T. brochymenae* females to a cabbage leaf portion with artificially applied ovarian eggs, to a leaf portion close to the treated area, and to a leaf portion on which an egg mass had been artificially applied (Treatment) *vs* response to healthy leaves (CNT), at increasing time intervals after the treatment. Bars indicate means ± s.e.m. (Student's *t*-tests for dependent samples).

significant at both 0 h and 48 h after treatment (0 h: t=2.277, d.f.=34, P=0.029; 48 h: t=2.614, d.f.=23, P=0.016) (Fig. 7).

DISCUSSION

Through laboratory experiments carried out using specific arenas for evaluating parasitoid behavioural responses to short-range chemicals, it was shown that females of *T. brochymenae* increased their residence time on cabbage plants attacked by *M. histrionica*. When walking in the arena and encountering a host-treated leaf, the parasitoid behaviour was different from the behaviour observed when it encountered the short-range kairomone from the chemical footprints of its host (Conti et al., 2003; Salerno et al., 2009), as no arrestment was observed. By contrast, the parasitoid continued walking on the leaf and, although it seemed to decrease its linear speed, the antennal clubs, rather than showing an intense examination of the substrate, were kept quite vertical and quickly touched the substrate only with their tips. Therefore, the allelochemicals eliciting such behaviour seemed to be perceived, at least partially, from a very short-range through olfactory rather than gustatory sensilla, as also suggested by the experiment conducted in the static olfactometer.

A major question is whether the parasitoid is responding to a host-induced plant synomone, to host kairomones, or to both of them. In all of the experiments reported here, with the aim of avoiding possible effects of kairomonal cues from the host (Conti et al., 2003; Salerno et al., 2009), only the lower leaf side was exposed to the host for the treatments, while the upper side was bioassayed. Translaminar movements of host-derived cues from deposited eggs or the host footprints from the lower to the upper leaf surface, across the epicuticular layers, appear highly improbable. In addition, deposited egg masses were always removed before the experiments. However, the fact still remains that the effect of the feeding punctures by *M. histrionica* reaches the opposite side of the leaf, with visible leaf damage (Velikova et al., 2010). Therefore this aspect needs to be taken into consideration when discussing the results of our study. Concerning volatiles from the host, considering that *T. brochymenae* never responded to leaves that had been exposed to gravid host females that had no direct physical contact with the leaves (use of filter paper disk), possible absorption by the leaf epicuticular waxes of volatile kairomones emitted by the different host stages (Conti et al., 2003), as previously observed in other systems (Noldus et al., 1991; Müller and Riederer, 2005), can be excluded. In the present experiments it was shown also that the parasitoid responded to surface chemicals of leaves located above the treated leaf, indicating that these systemic chemical cues must be plant synomones, rather than host kairomones, especially because of the high detection rate shown by the parasitoid females.

To date, plant defence mechanisms that are in part similar to those shown here have been reported for the following model: cabbage and *Arabidopsis–Pieris* spp.–*Trichogramma* spp. (Fatouros et al., 2005; Fatouros et al., 2007; Fatouros et al., 2008; Fatouros et al., 2009; Blenn et al., 2009). However, in this tritrophic system only

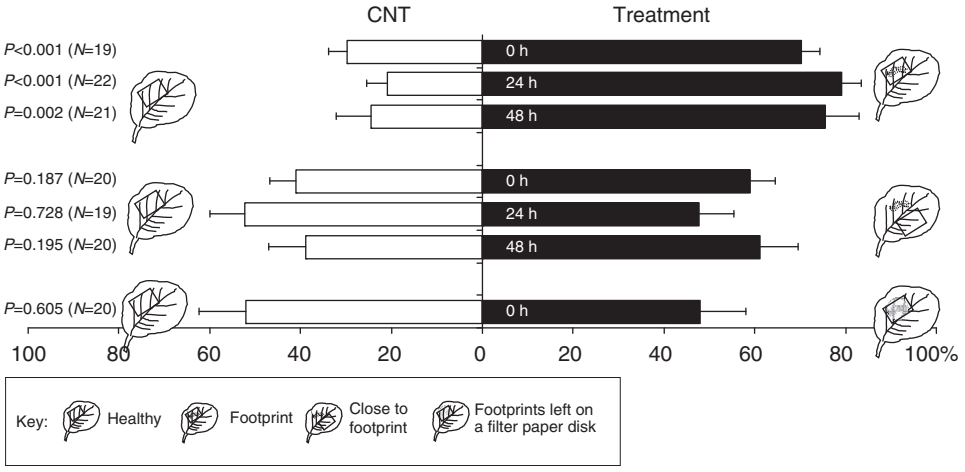

Fig. 6. Response (% residence time, s) of *T. brochymenae* females to a cabbage leaf portion with chemical footprints of *M. histrionica*, to a leaf portion close to such treated area, and to a leaf portion on which a filter paper disk prevented the bug from reaching the leaf surface with the legs or other body parts (Treatment), *vs* response to healthy leaves (CNT), at increasing time intervals after the treatment. Bars indicate means ± s.e.m. (Student's *t*-tests for dependent samples).

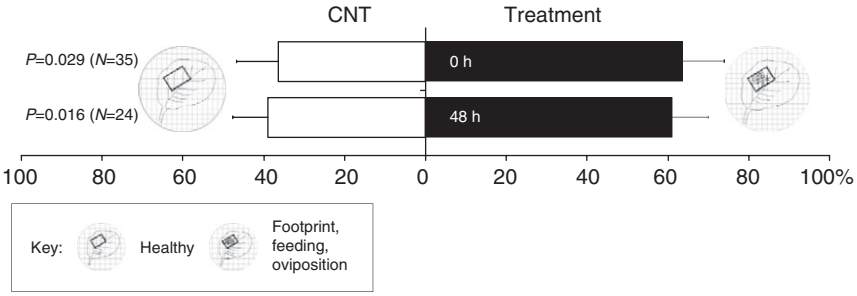

Fig. 7. Response (% residence time, s) of *T. brochymenae* females in a static olfactometer to short-range volatiles from a cabbage leaf portion with feeding damage, a deposited egg mass and footprints by *M. histrionica* (Treatment) *vs* response to healthy leaves (CNT), at increasing time intervals after the treatment. Bars indicate means ± s.e.m. (Student's *t*-tests for dependent samples).

experienced parasitoid females were responsive to the treatments, indicating that parasitoid behaviour depends on associative learning. On the contrary, in our system the responding females were naïve, i.e. they had no host experience beyond that which occurred during development within and eclosion from the host (Vet et al., 2003), which suggests that there is an innate, genetically programmed mechanism.

In detail, females of *T. brochymenae* responded both locally and in the vicinity of the treated portion of the cabbage leaves with feeding damage, a deposited egg mass and footprints. Such response was always significant during the time interval from 0 to 48 h from the end of the treatment, but faded after 96 h. This is consistent with the fact that after 96 h host eggs hatched, and therefore they were no longer suitable for parasitization (E.C. and G.S., personal observation).

Trissolcus brochymenae also responded to leaves that were above a leaf with *M. histrionica* feeding punctures, oviposition and footprints, whereas they did not react to leaves that were below the treated leaf. These results indicate that there is a systemic effect of the induction both in the leaf and in the plant, and that in the plant this effect is acropetous. Whether such systemic effect is the consequence of internal or external signalling within the plant (Heil and Silva Bueno, 2007) is unknown, and needs further investigation for this and other systems. Indeed, almost all of the oviposition-induced synomones known so far show a systemic distribution in the plant (Colazza et al., 2004a; Fatouros et al., 2005; Fatouros et al., 2008; Hilker et al., 2002a; Hilker and Meiners, 2006). This is important for both long-range and short-range synomones. In the first case, by maximising the releasing surface and because of its biomass, the plant is expected to emit high amounts of volatile synomones, making it easily detectable by the parasitoids. In the second case, by changing the chemistry of a large surface area, the plant would inform the parasitoid of the presence of the host eggs independent of the alighting site. The systemic synomone emission by the plant faded quicker than the local emission from the treated leaf portion and in the vicinity from the same leaf, as the parasitoids stopped responding 48 h after the end of the treatment. Although the reasons for such a difference between local and systemic effects have not been elucidated yet, it appears that the plant stimulates host searching in the parasitoid only when the herbivore's eggs are still fresh and suitable for successful parasitization. Therefore, synomone distribution in time seems to be finely tuned with parasitoid behaviour and biology, as has also been observed for a similar tritrophic system, bean–*N. viridula*–*T. basalis*, when volatile synomones were used as cues by the scelionid egg parasitoid (Colazza et al., 2004a), as well as for the cabbage–*P. brassicae*–*T. maidis* system (Fatouros et al., 2005).

The systemic induction is quite fast, i.e. 0–24 h, if it is considered that the plant is exposed to *M. histrionica* for 24 h and the insect activity on it might have started from the beginning. In other systems, the time needed for signal activation ranged from a few hours to a few days in the case of volatile synomones (Colazza et al., 2004a; Hilker and Meiners, 2002), and about 72 h in the case of the synomone perceived upon contact with the leaf, in the cabbage–*Pieris*–*Trichogramma* system (Fatouros et al., 2005; Fatouros et al., 2009).

Considering the results of the experiment with plants exhibiting only feeding punctures and footprints, the parasitoid responded locally at all time intervals, even when more than 96 h had elapsed from the treatment, whereas in the vicinity of the treated area, it responded only after 48 h, and no systemic effect was scored. This suggests that the early parasitoid response to leaf portions directly damaged by host feeding might depend on the host saliva or on its effects on the plant's chemistry, such as primers of metabolic processes and/or compounds derived from tissue hydrolysis, that are initially present only at the damaged site, but that after 48 h are also present in the vicinity of the damaged site on the same leaf. Indeed, feeding by *M. histrionica* on treated cabbage leaves causes changes in the photosynthetic gas exchange and the chlorophyll fluorescence parameters, for at least 72 h, with a substantial decrease in photosynthesis (Velikova et al., 2010).

Interestingly, when cabbage leaves carrying *M. histrionica* feeding punctures, oviposition and footprints were compared with leaves exhibiting only feeding damage and footprints, after 48 h, *T. brochymenae* was locally more responsive to the former treatment, which suggests that there might be a synergistic, or additive effect of feeding and oviposition, as has previously been observed in the bean–*N. viridula*–*T. basalis* system (Colazza et al., 2004a; Colazza et al., 2004b). Indeed, Velikova et al. (Velikova et al., 2010) found that the combined presence of feeding punctures and an egg mass of *M. histrionica* causes a greater alteration of photosynthesis in cabbage plants than does each factor separately, which could support our hypothesis. In the behavioural assays, if *M. histrionica* was only allowed to oviposit on the cabbage leaf, but not to feed, the parasitoid showed an early response (0–48 h), both locally and in the vicinity of the treated area, but not systemically on the plant, which again suggests that a combination of more factors is necessary for systemic induction. However, because of the difficulty in obtaining oviposition by *M. histrionica* females with excised stylets, it was not possible to carry out additional tests to evaluate the systemic effect after 48 h. Although the presence of such effect appears unlikely, it cannot be stated here with certainty that it does not exist.

Trissolcus brochymenae also reacted to cabbage leaf portions on which ovarian eggs of *M. histrionica* had been artificially applied and then removed at the end of the treatment. However, the response was significant only locally at 24 h and was marginally not significant at 0 h, probably because of inevitable differences between natural oviposition and the artificial application of eggs. In addition, before applying the ovarian eggs to the plant surface, they were rinsed in a saline solution to remove the haemolymph and therefore probably contained a lower amount of follicular secretion than freshly deposited eggs. This seems to be confirmed

by the fact that leaf areas close to artificially applied ovarian eggs did not show any short-range induction of synomones. However, because artificially applied egg masses did not elicit parasitoid response, it appears clear that the emission of chemical cues, at least locally, is associated with the interaction between the leaf and the freshly laid eggs covered by the female follicular secretion (Bin et al., 1993; Conti et al., 2003), as was also observed in the case of volatile synomones induced by *N. viridula* on bean plants (Colazza et al., 2004a), as well as for different herbivores (reviewed by Hilker and Meiners, 2010).

In any case, because natural oviposition was not effective in eliciting a systemic response in *T. brochymenae*, it is evident that the combined presence of oviposition and feeding damage is necessary for synomone induction, as was also observed in *N. viridula* (Colazza et al., 2004a). In the case of eggs, whether the chemical(s) involved originates from the egg itself or from the follicular secretion is unknown. Tooker and De Moraes (Tooker and De Moraes, 2005) indicated the presence of jasmonic acid in the eggs of several Lepidoptera, which might possibly provide an explanation for the oviposition-induced plant resistance. However the possible presence of this compound in the eggs of the herbivores considered here is unknown.

When a gravid female bug with excised stylets was confined to the lower side of a leaf and did not oviposit, the only contamination of the plant substrate was that of the chemical traces, or footprints (Conti et al., 2003; Salerno et al., 2009). *Trissolcus brochymenae* spent a significantly longer residence time on the upper surface of such leaf portions than on leaves from untreated plants, at 0–48h after treatment, but did not respond to portions from the vicinity of the treated ones. This would suggest a local plant response to the touch of the herbivore, as has already been indicated for other systems (reviewed by Hilker and Meiners, 2010). The knowledge that cabbage plants might respond promptly and for an extended period to the touch of *M. histrionica*, and that this elicits locally a behavioural reaction in *T. brochymenae*, suggests that the chemical footprints left by the herbivore on the plant substrate not only act as a source of kairomone for the parasitoid, but might also be part of the factors inducing synomone emission in the plant. A combination of chemicals from the herbivore eggs, saliva and footprints, then, would act synergistically as elicitors of indirect plant defence if the hypotheses above are confirmed. Because such factors act in different phases, i.e. during walking, feeding and egg laying, the dynamic of the attack and the duration of the effects might be important in obtaining elicitation, as has been suggested for other systems (reviewed by Hilker and Meiners, 2010).

The parasitoid behavioural responses discussed here occurred after it alighted on the plant substrate. However, when its direct contact with the plant substrate was prevented by using a very fine mesh net, *T. brochymenae* was also able to perceive locally induced volatiles. Therefore, it appears that such short-range allelochemicals might be perceived as olfactory stimuli, although only from a very short distance. This aspect is quite interesting because in previous papers evidence has been reported for different quantitative/qualitative modifications in the volatile profile of cabbage plants as a consequence of feeding and/or oviposition by *M. histrionica* (Conti et al., 2008; Velikova et al., 2010). This information might support the hypothesis that cabbage induced volatiles are partially adsorbed by the leaf epicuticular waxes and then slowly released. Alternatively, it can be hypothesised that different compounds, possibly exudating from the leaves, are used as a short-range synomone by *T. brochymenae*. In both cases, the epicuticular waxes of cabbage plants could have an important role in adsorbing and releasing such induced synomone (Müller and Riederer, 2005), and such a role is currently under investigation.

The following question arises at this point: what is the ecological function of an oviposition-induced short-range synomone in the host selection process of *T. brochymenae*? In the olfactometer, *T. brochymenae* is attracted to volatile compounds from different instars of the host, including the eggs, but shows a preference for mated host females that have not yet oviposited (Conti et al., 2003; Salerno et al., 2009). These cues are therefore used to localize the host community (Conti et al., 2003). In the successive step, just after landing on the plant, the presence of a short-range synomone, induced by host oviposition, would provide the parasitoid with reliable information on the presence of host eggs on the plant, suitable for parasitization. However, because such synomone is probably distributed on the whole plant, it would not provide information on where the eggs have been laid. Therefore, in the last steps of host location, the parasitoid would exploit chemical footprints left on the substrate by gravid females and other host instars, and finally a short-range kairomone from the host eggs in combination with visual cues (Conti et al., 2003). We can then conclude that the short-range synomone studied here appears to have an important role in the host location process after the parasitoid alights on the host plant, acting as a component of the hierarchic sequences of volatile (long and short range) and contact cues involved in this cabbage–herbivore–parasitoid system. Further research is in progress or is projected in order to evaluate the role of epicuticular waxes in synomone perception by the parasitoid, and to identify the chemical compounds acting as synomones, as well as the elicitors, their sources, and the molecular features underlying synomone emission.

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