Does the generalist parasitoid *Trichogramma brassicae* use an oviposition-induced synomone of *Brassica oleracea* to locate its host *Pieris brassicae*?

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Abstract

By already responding towards egg deposition, plants can defend themselves against future herbivory by insect larvae. One of these responses can be the release of volatiles, thereby attracting parasitoids that can eliminate herbivore eggs. The enemy of herbivore eggs can also be arrested by induced alterations in leaf surface chemicals (i.e. contact cues). If egg deposition of *Pieris brassicae* on Brussels sprouts (*Brassica oleracea*) can attract or arrest female *Trichogramma brassicae* egg parasitoids via the release of volatiles and/or contact cues is tested. Static olfactometer bioassays showed that volatiles from egg infested Brussels sprouts plants did not attract the egg parasitoid. Herbivore induced contact cues were examined by comparing residence time of *Trichogramma brassicae* in setups with a control versus both combinations of kairomones, synomones and arresting substances alone. *Trichogramma brassicae* showed a marked preference for leaves onto which host egg were deposited over a clean leaf, even when the butterfly eggs themselves are removed (p<0.01). To exclude the effect of wing scales and other butterfly deposits, residence time on leaf squares with fresh and 3d old *Pieris brassicae* deposits were tested. Although fresh deposits did arrest *Trichogramma brassicae* (p<0.05), deposits near eggs highly suitable for parasitisation (i.e. 3d old) were no more attractive to the parasitoid than a clean leaf square. Arrestment of *Trichogramma brassicae* by a 3d old clean leaf square from a plant with egg masses, as our tests revealed on local scale, can be attributed to a change of leaf surface chemicals in combination with kairomones such as oviduct secretions (p<0.05). When isolated from another, the effect of either of both factors was not strong enough to arrest the egg parasitoid in its host searching behaviour. An effect of systemic induction could not be detected.

By arresting *Trichogramma brassicae* in the vicinity of host eggs from its long range search, production of locally oviposition-induced changes of leaf surface chemicals can prevent hatching of larvae, thereby ultimately reducing herbivory on the plant.

Abbreviations and Symbols

α	= level of significance
et al.	= and others (in case of more than two authors per article)
c.	= contact bioassay
h	= hour (60 minutes)
L.	= Linnaeus
L:D	= ratio of hours with light versus darkness
n	= sample size
NR	= non-responder
o.	= olfactometer bioassay
p	= probability value
P.	= *Pieris*
rh	= relative humidity (%)
SEM	= standard error of the mean
t	= temperature (°C)
T.	= *Trichogramma*
var.	= botanical variety

*	= *p-value* ≤ 0.05
**	= *p-value* ≤ 0.01
***	= *p-value* ≤ 0.001

Definitions

Allelochemical = an infochemical that mediates an interaction between two individuals that belong to different but associated species (Vinson, 1984, Dicke and Sabelis, 1988), often from different trophic levels (Nordlund *et al.*, 1988; Noldus, 1989a)

Associative learning = responses to stimuli can be newly acquired (i.e. experienced) or enhanced by linking them to a reinforcing stimulus (Vet and Dicke, 1992), thereby redirecting and broadening the response(s) temporally without influencing the genetic make-up of the parasitoid population (Vinson, 1998). The result is a flexible system that allows the animal to respond to changing environments

Contact cue = either emanated from the host, or exposed by the host plant by physically altering its leaf surface. Important in host finding and the succeeding steps of host selection behaviour, by stimulating searching behaviour at close range in the female parasitoid. Alternative to '**Volatile emission**'

Direct defense = induction of the plant's defense mechanism in general by herbivore feeding or oviposition, restricted to the tissue onto which the infestation occurred and the immediately adjacent tissue. In case of herbivore frass, it can result in the production of toxic, digestibility reducing and/or deterrent plant chemicals. In case of egg deposition and/or pathogen infection oviposition deterrents, the formation of neoplasms or a hypersensitive response can occur (Dicke and Sabelis, 1988; Dicke, 1999b; Hilker and Meiners, 2002; Hilker *et al.*, 2002b). Alternative to '**Indirect defense**'

Elicitor = a physical or chemical stimulus of an animal that triggers a direct and/or indirect defense response in a plant

Experience = the behaviour or preference of an animal is affected, redirected or broadened temporally after encountering a new stimulus. See also '**Associative learning**'

Host community location = see '**Host habitat location**'

Host habitat location = the oriented locomotory response of female parasitoids towards olfactory and/or visual cues generally released by organisms associated with the host; host plants included (Vinson, 1998; Steidle and van Loon, 2002). These cues are considered highly detectable but of lower reliability since the presence of the host is not guaranteed (Vet and Dicke, 1992; Vet *et al.*, 1995; Vinson, 1998; Steidle and van Loon, 2002). According to Nordlund (1994), it is the arbitrary first step from the continuum of host selection behaviour. The next step can be host finding: see '**Host finding**'

Host finding = this involves the response of female parasitoids to short range volatiles, pheromones, secretions and waste products, generally released by the host itself (free from Vinson, 1998). These visual, mechanosensory, olfactory and/or gustatory cues are considered most reliable but limited in use by lower detectability, especially at larger distances, since the host is under strong selective pressure not to emit any of these infochemicals (Vet and Dicke, 1992; Vet *et al.*, 1995; Vinson, 1998; Steidle and van Loon, 2002). Following to host finding (arbitrary second step from the continuum of host selection behaviour (Nordlund, 1994)), host recognition, host acceptance and finally oviposition can occur (Vinson, 1998)

Host searching = see '**Host habitat location**'

Indirect defense = induction of the plant's defense mechanism by in general herbivore damage or oviposition, which can result in the systemic production of specific plant volatiles that can promote the effectiveness of natural enemies of the herbivore (Dicke and Sabelis, 1988; Dicke, 1999b). In the indirect defense of plants, at least parts of the plant has to be 'designed' to have no unfavourable features for the predator, like glandular hairs or sequestered toxins (Dicke and Sabelis, 1988). Alternative to '**Direct defense**'

Induced defense = this plant's reaction is activated in response to natural enemy presence, damage and/or even from volatiles emitted by neighbouring plants (Dicke, 1999b). The defensive plant's response can either be '**Direct**' or '**Indirect**'

Infochemical = a chemical that, in the natural context, conveys information in an interaction between two individuals, evoking in the receiver a behavioural or physiological response that is adaptive to either one of the interactans or to both (Dicke and Sabelis, 1988). Chemical information can either originate from 1- the herbivore (or its products), 2- from its food, 3- from (micro-)organisms associated with herbivore presence, 4- from interactions between these sources (Vet and Dicke, 1992)

Infochemical detour = when the herbivore stage of interest is badly detectable to its natural enemy or cues are not reliable, information from other, more conspicuous stages of the herbivore is resorted to, provided that these stage do provide reliable and/or detectable information to the predator or parasitoid (Vet and Dicke, 1992; Vet *et al.*, 1995; Romeis *et al.*, 1997; Steidle and van Loon, 2002)

Kairomone = an allelochemical that is pertinent to the biology of an organism (organism A) and that, when it contacts an individual of another species (organism B) evokes in the receiver a behavioural or physiological response that is adaptively favourable to organism B (= receiver) but not to organism A (= emitter) (Dicke and Sabelis, 1988; Nordlund *et al.*, 1988). Chemicals originating from plants that stimulate host/prey selection behaviour of entomophagous insects should also be considered as kairomones if they are released <u>only</u> as a result of the activities of the herbivore or are active <u>only</u> in combination with materials of the herbivore (Nordlund *et al.*, 1988)

Klinokinese = when an animal gets closer to the source of a stimulus, the number of curves in the walking path is increased, leading to intensified and extended searching in host locations and thereby host finding (Noldus, 1989a)

Learning = see '**Associative learning**'

Local response = the infested plant tissue and the immediately adjacent region is changed in response to herbivory or egg deposition

Long-range cue = emanated from the host, its food, shelter, or associated organisms. Important in host habitat location and host finding (Vinson, 1984). Alternative to '**Short-range cue**'

Micro-habitat location = see '**Host habitat location**'

Oviposition deterrent = marking substance induced by egg deposition. Next to the intraspecific use by deterring conspecifics, also following visits of the same species to the same spot is avoided; thereby evenly distribute eggs over available resources (Blaakmeer *et al.,* 1994a).

Parasitoid = parasitoids have the following characteristics: 1- the developing individual destroys (eventually) its host, 2- only immatures are parasitic whereas the adults are free-living, 3- they are homoecious, i.e. they do not switch during the development of host species (Vinson, 1984) and 4- they can be separated according to host preference: either free-living (in general larvae) or sessile (eggs) hosts

Pheromone = an infochemical, that is pertinent to the biology of an organism, that mediates an interaction between organism of the same species whereby the benefit is to the origin-related organism ((+,-) pheromone), or to the receiver ((-,+) pheromone), or to both ((+,+) pheromone) (Dicke and Sabelis, 1988)

Phoresy = passive form of distribution, for instance being transported as egg parasitoid by attaching to a adult host to freshly laid eggs

p-value = in statistics, the *p-value* is a norm for the probability that the result described is founded on coincidence. When the *p-value* is sufficiently low, this is indicative for an effect of the treatment

Semiochemical = *(σημειου^{Greek} = sign, signal)* a chemical involved in the chemical interaction between organisms (Noldus, 1989b; Vet and Dicke, 1992). When semiochemicals convey information, they are termed infochemicals, see '**Infochemicals**'

Short-range cue = usually emanated directly from the host. Important in host finding and the succeeding steps of host selection behaviour by stimulating searching behaviour in the female parasitoid, thus resulting in a search of the immediate surrounding area (Vinson, 1984). Alternative to '**Long-range cue**'. See also '**Contact cue**'

Synomone = an allelochemical that is pertinent to the biology of an organism (organism A) and that, when it contacts an individual of another species (organism B) evokes in the receiver a behavioural or physiological response that is adaptively favourable to both organism A (= emitter) and organism B (= receiver) (Dicke and Sabelis, 1988; Nordlund *et al.*, 1988)

Systemic response = the response is larger than at the <u>infested</u> plant tissue alone, which increases the chance of interception of the emitted volatiles by downwind predators or parasitoids (Dicke, 1999b)

Tritrophic interaction = tritrophic interaction(s) occurs when an event at one trophic level (in)directly affects two other trophic levels. The first trophic level are the primary producers (plants in general), second trophic level is made up of the organisms which feed on the primary producers (called primary consumers or herbivores), the upper or third trophic levels is occupied by predators which feed on the primary consumers (i.e. carnivores)

Volatile emission = due to herbivore induction of the plant's defense systems, in response volatile emission can be elicited (Dicke, 1999b; Paré and Tumlinson, 1999). In each plant-herbivore complex, volatile signals used by foraging parasitoids can originate from the host, but also from the plant or from an interaction between the two (Cortesero *et al.*, 1997). See also '**Pheromone**'. Alternative to '**Contact cue**'

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1. Introduction

1.1 Herbivore or egg induced plant's defense

1.1.1 Synomones

When a certain herbivorous species is feeding on a plant, in reaction the plant can systemically produce so-called infochemicals (Dicke and Sabelis, 1988) in order to attract or arrest the natural antagonist of the herbivore. Infochemicals[i] are the plant's <u>active</u> response to (future) herbivore damage. The infested plant can respond by producing mixtures of volatiles which can be highly specific for both plant as herbivore species (Dicke, 1999b; Paré and Tumlinson, 1999). These volatiles are not released, or only in trace amounts, by intact or mechanical damaged plants (Turlings *et al.*, 1990; Dicke and van Loon, 2000). Induced emission of blends of plant volatiles through herbivore feeding, thereby attracting their antagonists is demonstrated in a number of systems, for instance with spider mites (Dicke and Sabelis, 1988; Dicke *et al.*, 1999), caterpillars (Turlings *et al.* 1990) or bugs (Weißbecker *et al.* 1999; van Loon *et al.* 2000) as herbivores. While the predatory bug, as member of the tri-trophic interaction van Loon *et al.* (2000) described, did respond towards volatiles of damaged plants, the prey in isolation of the plant did not elicit any response of the carnivore. While plants can lose their attractiveness to antagonists shortly after the damage is mechanically inflicted, attraction in feeding damaged plants can be long-lasting (Weißbecker *et al.*, 2000). Even oviposition (Hilker and Meiners, 2002; Meiners and Hilker, 1997) can induce a long-lasting release of infochemicals.

Herbivores are under strong evolutionary pressure **not** to emit any cues that allow predators or parasitoids[ii] to detect them, and if they produce odours at all, it will be in minute quantities (Vet and Dicke, 1992). Plants, having in general a much larger biomass than herbivorous insects, emit large quantities of volatiles on a longer range (Dicke, 1999a, Dicke, 1999b). If detectable and herbivore-specific, plant volatiles can be reliable host finding cues due to their high turnover rate (Turlings *et al.*, 1990), allowing oriented locomotion of predators and parasitoids to their prey (Vet and Dicke, 1992; Steidle and van Loon, 2002) especially if no long distance cues more directly connected to the host are available (Noldus, 1988; Lewis *et al.*, 1982). Thus, volatiles emitted by infested plants make the herbivore more apparent for predators (Paré and Tumlinson, 1999; Dicke, 2000), especially when uninfested neighbouring plants are able to activate their defenses in response to infochemicals of (conspecific) infested plants (Dicke *et al.*, 2003). While herbivores sometimes have developed resistance against direct plant defense mechanisms (for instance digestion inhibiting chemicals), it is difficult to adapt as herbivore to indirect plant defense mechanisms. Attraction/arrestment of the natural antagonists of the herbivore via the induced release of synomones like plant volatiles can thus result in an additional advantage to the plant, thereby maintaining selection pressure on this trait (Turlings *et al.*, 1990). The benefits for plants luring egg parasitoids are also evident: parasitisation does result in a reduction in the number of viable eggs, and thus, of the avoidance of future feeding damage (Hilker *et al.*, 2002b). Therefore, mutualistic interactions between plants and herbivores' antagonists are expected to be common.

Recently, investigators have been able to demonstrate the plant's response induced by egg deposition in three separate systems (Meiners and Hilker, 1997; Meiners *et al.,* 2000; Colazza et al., 2004; Hilker *et al.,* 2002b; Hilker and Meiners, 2002). In these systems (respectively the elm, the pine and the bean system), an eulophid wasp or a scelionid wasp uses oviposition induced plant volatiles to find its host eggs (for more detailed overview: see Table 1.1). Thus, the plant can adequately react before the actual damage, the feeding of the larvae, arises. Please note three

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[i] When infochemicals or volatiles are mentioned, always <u>herbivore</u> or <u>oviposition</u> induced <u>plant</u> chemicals are meant.
[ii] When written parasitoids, please read adult <u>female</u> parasitoids.

things in the enumeration: 1- the plants are all perennial (except for the bean system)[i], 2- the third trophic level is always represented by a parasitoid and not a predator and 3- the parasitoids are, except for the scelionid wasp in the bean system, all specialists[ii].

In the bean-system, as tested by Colazza *et al.* (2004), the parasitoid uses synomones from the plant to locate host eggs. This reaction of the plant seems to be elicited without any damage prior to oviposition to the plant, while in the elm-system the grooving of the leaf surface is part of a necessary step to induce the plant's synomone production (Meiners and Hilker, 2000). Eggs are laid into the grooves, attached by oviduct secretions. An elicitor inducing elm leaves to produce volatiles that attract egg parasitoids is located in this oviduct secretion, but is only activated when the leaf surface is slightly damaged (Meiners and Hilker, 2000). Beside host accessory gland secretions present on and around lepidopteran eggs (Noldus, 1988; Meiners and Hilker, 2000), substances in the oral secretions of herbivores can also serve as elicitor (Paré and Tumlinson, 1999) causing arrestment and induce host recognition by the parasitoid.

Table 1.1 Overview of three separate systems demonstrating that egg-parasitoids are attracted to the plant's induced response by host egg deposition (adapted from Hilker et al., 2002b).

	Elm system	**Pine system**	**Bean system**
Described in	Meiners and Hilker, 1997; Meiners and Hilker, 2000; Meiners *et al.*, 2000	Hilker *et al.*, 2002a; Mumm *et al.* 2003; Mumm *et al.* 2004	Colazza *et al.,* 2004
Plant	*Ulmus minor* (*Ulmaceae*), field elm	*Pinus sylvestris* (*Pinaceae*), Scotch pine	*Vicia faba*, (*Fabaceae*) broad bean and *Phaseolus vulgaris*, (*Fabaceae*) French bean
Herbivore	*Xanthogaleruca luteola* Müller (*Coleoptra: Chrysomelidae*), elm leaf beetle	*Diprion pini* L. (*Hymenoptera: Diprionidae*), pine sawfly	*Nezara viridula* L. (*Heteroptera: Pentatomidae*), southern green stink bug
Parasitoid	*Oomyzus gallerucae* Fonscolombe (*Hymenoptera: Eulophidae*)	*Chrysonotomyia ruforum* Krausse (*Hymenoptera: Eulophidae*)	*Trissolcus basalis* Wollaston (*Hymenoptera: Scelionidae*)
Induction of synomones by oviposition	+	+	+
Local induction	+	+	+
Systemic induction	+	+	+
Elicitor	leaf-scratching <u>and</u> oviduct secretion	oviduct secretion	ovarian glue (?)

As feeding-induced volatiles, oviposition-induced synomones can also be highly specific (Mumm *et al.*, 2004). Only eggs of *Xanthogaleruca luteola* deposited onto *Ulmus <u>minor</u>* leaves induce the release of volatiles and a subsequent behavioural response in the specialist parasitoid *Oomyzus gallerucae* (Meiners *et al.,* 2000). Specificity assures a high reliability of host location cues for the egg parasitoid (Hilker and Meiners, 2002). *Trichogramma brassicae* is seen as a generalist parasitoid, so the use of more general cues is perhaps more likely.

Volatiles emitted by infested plants can on the other hand be 'illegitimately' exploited by other (species of) herbivores (Meiners and Hilker, 2000; Hilker and Meiners, 2002; Horiuchi *et al.,* 2003 and references therein). Whether an infested plant is attractive and/or arresting or conversely repelling to an herbivore can depend on the rate of infestation. Avoidance of infested leaves can be

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[i] Especially annual plants should benefit from the release of oviposition-induced synomones (Hilker *et al.*, 2002b). Due to their short lifetime and lower biomass compared to perennials, feeding damage in an herbaceous plant can be much more costly in terms of fitness than in a perennial plant (Hilker *et al.*, 2002a; Hilker *et al.*, 2002b).
[ii] Learning to respond to infochemicals was found more frequently in a generalist than in specialists (Vet *et al.*, 1995; Steidle and van Loon, 2002)

advantageous to the herbivore, since it would avoid competition over food resources and also avoid sites that are attractive to predators/ parasitoids (Hilker and Meiners, 2002). Volatiles from slightly infested leaves may on the other hand be a signal for the herbivore, indicting available food resources and a patch where predators are not (yet) present (Horiuchi *et al.,* 2003), but conspecifics are. The latter can as well be used in the mate finding process (Hilker and Meiners, 2002). The nature of infochemicals can also differ over time: Romeis *et al.* (1997) determined the influence of the developmental stage of the plant on the emitted cue to a certain *Trichogramma* species. It was indeed arrested by volatiles from the reproductive pigeonpea plant, the stage preferred for oviposition by the parasitoid, while the vegetative stage was actually repelling the parasitoid.

1.1.2 Kairomones
Random searching for hosts by larval parasitoids is highly unlikely, because of the small size of the host and the location at the undersides of the leaves (Paré and Tumlinson, 1999). In case of egg parasitism, host finding is even more complicated because the hosts are sessile and also eggs release far fewer cues than free living hosts (Vinson, 1998). Thus, egg parasitoids face an even greater challenge in locating their hosts and may have to rely on infochemicals from the host adult instead of the host itself (= infochemical detour), since those cues are much easier to detect although mostly only on short range. However, these cues are less reliable since they are **not** derived directly from the host. Long range cues (mostly volatiles) are discussed in the previous paragraph. More or less succeeding volatiles in the process of host finding, kairomones from the host or the host adult can act on a short range. In other words, the parasitoid is attracted by volatiles to an area in which a host can be found (i.e. host habitat location; Vinson, 1984). Now short range cues, in general non-volatile herbivore products (Lewis *et al.*, 1979; Gardner and van Lenteren, 1986), arrest the egg parasitoid in the vicinity of host eggs (i.e. host location). Such an arrestment response, like inhibition of flight or klinokinese, leads to intensified and extended searching (Noldus, 1989a and references therein).

1.1.3 Ovipositing experience
Learning (i.e. experience) is especially adaptive to a generalist parasitoid, i.e. with multiple hosts species and/or when a certain host is foraging on different plant species (Kaiser *et al.*, 1989b; Paré and Tumlinson, 1999; Steidle and van Loon, 2002). Less host specific but on the other hand very detectable cues like plant cues released after (recent) herbivore infestion can still be used by generalist species, while a specialist is more likely to use innate host specific cues, like host frass volatiles (Cortesero *et al.*, 1997).
Due to the earlier ovatiposing experience, experienced parasitoids 1- have a shorter latency to start searching for hosts than naive, inexperienced wasps (Noldus and van Lenteren, 1983; Noldus and van Lenteren, 1985a; Hérard *et al.*, 1988; Vinson, 1998), 2- exhibit more directed flights to a host-plant complex than naive females (Vinson, 1998), 3- have an exponentially decreased duration of successive ovipositing behaviour (Wajnberg, 1989) and 4- have a higher affinity for the host (Kaiser *et al.*, 1989a). While learning can occur very rapidly, the effects are not lasting in the absence of continued experience (Vet and Dicke 1992; Turlings *et al.*, 1993).

1.2 Model system
This research is designed to investigate tritrophic interactions between the annual cabbage plant *Brassica oleracea* Linnaeus var. *gemmifera* var. Cyrus (Brussels sprout), the herbivore *Pieris brassicae* Linneus [Lepidoptera, Pieridae] (Large Cabbage White) and the generalist parasitoid *Trichogramma brassicae* Bezdenko [Hymenoptera, Trichogrammatidae]. *Pieris* spp. are known pest organisms of Brassicaceae in Holland (Noldus and van Lenteren, 1985b). *Trichogramma* spp. are generalist[i] egg-parasitoids of several lepidopteran species. Because of the immense use of *Trichogramma* spp. in

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[i] *Trichogramma* spp. are considered to be polyphagous in host choice (Kaiser *et al.*, 1989b; Boo and Yang, 2000).

biological control (Noldus, 1989a; Noldus, 1989b; Nordlund, 1994) host searching behaviour of *Trichogramma brassicae* or *Trichogramma* spp. in general have already been intensively investigated.

To determine host finding cues for *Trichogramma brassicae*, all kinds of kairomones from host adults are researched. *Trichogramma brassicae* females show an increased upwind locomotion in an olfactometer towards volatile substances from host eggs or egg extracts over the control (Frenoy *et al.*, 1992; Renou *et al.*, 1992). Cabbage leaves treated with extracts of eggs of *Pieris brassicae* are explored for a longer time by *Trichogramma brassicae* females than control leaves (Noldus and van Lenteren, 1985b). Also compounds present on the surface of host eggs may play a role in host searching and location by females of *Trichogramma brassicae,* acting by short range attraction and/or by contact to these chemicals (Boo and Yang, 2000 and references therein).

Noldus and van Lenteren (1983) and also Gardner and van Lenteren (1986) demonstrated that female *Trichogrammas* search significantly longer for hosts on cabbage leaves treated with wing scales of *Pieris brassicae* over untreated leaves. Lewis *et al.* (1975) and Vinson (1984) found that *Trichogramma brassicae* is stimulated to search for it host by tricosane, which is the most active component of host wing scales (Jones *et al.*, 1973).

Noldus and van Lenteren (1983, 1985a) also described attraction of female *Trichogramma brassicae* to volatile effluvial substance(s) released only by virgin females of *Pieris brassicae* or *Mammestra brassicae*. The same effect was found by Frenoy *et al.* (1992) for *Ostrinia nubilaris,* which eggs can also be of host of *Trichogramma brassicae.*

Frenoy *et al.* (1992), Noldus *et al.* (1991b) and Nordlund (1994) stated the role of sex pheromones of female moths as an arresting kairomone to *Trichogramma brassicae*. Sex pheromones can be indicative to the parasitoid of reproducing adults and therefore an indirect cue for the presence of eggs in the area (Noldus *et al.*, 1991a; Nordlund, 1994; Vinson, 1998; Boo and Yang, 2000). Prolonged searching in areas where pheromones are perceived (i.e. mating sites) can also increase the probability that the wasp contacts other cues with a closer spatial relation with oviposition sites (Noldus *et al.*, 1991a).

Noldus and van Lenteren (1983) found that an oviposition deterrent to *Pieris brassicae* acts as a contact 'kairomone' leading to the arrestment of *Trichogramma brassicae*. Remarkable about these oviposition deterrents is that they are produced systemically by the cabbage plant in reaction to egg deposition and not by the butterfly or the eggs itself (Blaakmeer *et al.,* 1994b). The term contact 'kairomonal' effects used by Noldus and van Lenteren (1983) is therefore not correct, and should be replaced by contact 'synomonal' effects. If oviposition deterrents are indeed used as synomones, a tri-trophic interaction even without any prior injury inflicted by the (potential) herbivore insect to the plant is documented.

1.3 Central questions
1.3.1 General question
Can *Trichogramma brassicae* use synomones released by *Brassica oleraceae*, induced by the egg deposition of *Pieris brassicae,* as host finding cues?

1.3.2 Subquestions
a) Does egg deposition of *Pieris brassicae* induce the release of synomones in *Brassica oleraceae*, which are detectable for *Trichogramma brassicae*?

b) Is this synomone volatile or does the parasitoid have to make contact with the plant to notice induced alterations in the surface of the leaf?

c) Is the reaction of the plant, induced by the egg deposition of *Pieris brassicae,* local or systemic?

d) To what extent does the synomone alter the host searching behaviour of respectively 1- a naive parasitoid or 2- a parasitoid with oviposition experience? In other words, is the parasitoid able to learn in this regard?

If the answer to sub questions a) is "no", then sub questions b) to d) cannot be answered.

1.4 Hypothesis

Based on the literature on *Trichogramma brassicae* and kairomones or synomones and previous work done by the Berlin-group (Fatouros, *unpublished*) on the model for tritrophic interactions between *Brassica oleracea, Pieris brassicae* and *Trichogramma brassicae* (i.e. sprout system)*,* we assume that in the sprout system egg-induced synomones are likely to be non-volatile and locally released, i.e. this parasitoid uses short-range cues to locate its host or the host's (micro)habitat by contact with the induced leaf, which can be optimised by former oviposition experience.

2. Materials and Methods

To test our hypothesis, several contact bioassays were conducted. To be able to determine if the parasitoid uses olfactory cues, olfactometer bioassays were done as well. The effect of oviposition experience of the parasitoid was tested as well.

2.1 Insects and plants

➲ *Brassica oleracea* L. var. *gemmifera* var. Cyrus was reared in a greenhouse (18 ± 2°C; 70% rh; L16:D8), to avoid modulated volatile emission due to reduced light from shorter day length (Paré and Tumlinson, 1999). For the experiments and the rearing of *Pieris brassicae* 8 to 12 weeks old plants were used (Figure 2.1). The plants were placed during daytime into a cage (80*100*80 cm^3) with more than 100 adults of *Pieris brassicae* for about 8 hours (or 24h for experiment c.3 and c.5) to allow deposition of any kind like eggs, wing scales, *et cetera*. Afterwards the plants were stored in another greenhouse (18 ± 2°C, 70% rh, L16:D8) until the age of both induced plant and butterfly eggs is appropriate. Control plants were

Figure 2.1 Cabbage plant (seen from the top)

never in contact with *Pieris brassicae* or any insects feeding and/or egg deposition in general, but further reared and kept under the same abiotic conditions as treated plants but apart from the latter. Since variation in the size of the leaves could account for differences in experimental results (Noldus and van Lenteren, 1983), always two leaves of corresponding size were used. To equal the control and treated leaf as possible, also the position in regard to the topmost leaves was always the same. Only turgid leaves were used. When leaf squares were used, corresponding sections were cut from the control and the treated leaf, so size and structure equaled as much as possible.

➲ *Pieris brassicae* L. adults (Figure 2.2) were mated and oviposited under restricted conditions on cabbage plants (23 ± 5°C; 50-70% rh; L16:D8). The eggs were used as substrate for *Trichogramma brassicae* egg deposition (Figure 2.3). The age of the butterfly eggs varied between 2 and 3 days, when used for the experiments and parasitoid rearing. Older eggs may be rejected by the parasitoid. Furthermore butterfly's faeces, deposits and/or wing scales were used in order to determine the nature of host finding cue(s) for the female *T. brassicae*.

Fig. 2.2 Female Pieris brassicae

Figure 2.3 Pieris brassicae eggs [©photo: Fatouros]

➲ *Trichogramma brassicae* Bezdenko (isogenetic strain Y175) wasps were reared in a climate chamber (25 ± 2°C; 50-70% rh; L16:D8) and supplied with honey. To increase host searching efficiency, the parasitoids were reared in *Pieris brassicae* host eggs for more than ten generations (Figure 2.4). Naive but mated *Trichogramma brassicae* females have never had any oviposition experience, whereas experienced wasps had the opportunity to ovipose for 18h on 2-3 days old *Pieris brassicae* eggs deposited on Brussels sprouts leaves. To prevent waning, experience was always given prior to the experiment. For the experiments, 2-4 days old wasps were used. This age is chosen because wasps younger than 48h are not strongly motivated to react to host seeking cues (Noldus and van Lenteren, 1983) and also to avoid the strong phototropism shown by younger individuals, which could modify the behavioural response (Gardnier-Geoffroy *et al.*, 1996). All females were used only once, because of practical reasons and also to avoid negative experience (habituation) and waning of the experience.
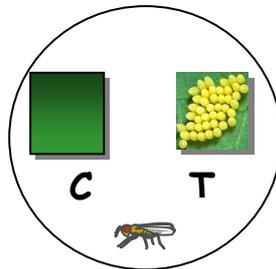

Figure 2.4 T. brassicae female oviposing on P. brassicae eggs [©photo: Fatouros]

2.2 Contact bioassays

In a small Petri dish (4 cm in diameter) made of glass, a single parasitoid was observed for 300 seconds. The animal was simultaneously offered a treated and a control leaf square ($2.0*2.0$ cm^2) upside-down[i] in a two-choice situation on filter paper (Figure 2.5). Plant material was changed and total set-up rotated 180° after each series of three observations. The wasp was released in the centre of the Petri dish. The Petri dish was illuminated from above (Euromex coldlight illuminator EK-I). The total duration time of 300 seconds is divided into the part the animal was spending on respectively 1- the control leaf square (C), 2- the treated leaf square (T) or 3- the area besides both the squares (white area in Figure 2.5). The latter was scored as 'no choice'. If the animal did not respond to any choice during the given time, it was recorded as a 'non-responder'. Non-responders were not included in the analysis. The number of visits and also the duration of time spent on either square were recorded by using the program The Observer® (Wageningen, the Netherlands).

Figure 2.5 Schematic drawing of the two test areas (C and T: respectively control and treated leaf squares) in a Petri dish contact bioassay. The parasitoid represents the starting point from where the insect is released.

Experiment c.1 ("eggs") To determine whether female parasitoids were arrested by *Pieris brassicae* infested plants a) naive or b) 18 hours experienced *Trichogramma brassicae* on either a control leaf square cut from a clean plant, or a test leaf square with a 44±4h old egg deposition (i.e. an egg clutch of about 50 eggs of *Pieris brassicae*) and deposits of the butterfly are tested (Figure 2.6). The plant was for 8h during day time in the butterfly cage.
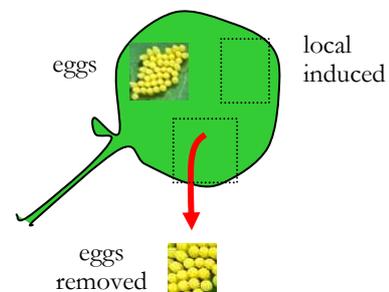
Experiment c.2 ("eggs removed") To further determine the nature of the host finding cue(s), residence time was determined of experienced *T. brassicae* on: 1- a control leaf square, or 2- a leaf square where eggs (72 h old) had been removed with a brush prior to the experiment (Figure 2.6). The plant was for 8h during day time in the butterfly cage.

Figure 2.6 Schematic drawing of different treatments. Not shown are the butterfly deposits on the treated leaf.

Experiment c.3 ("deposits") A follow-up experiment was conducted to exclude the influence of butterfly deposits like wing scales. Covered completely with gauze (mesh size: $1*1$ mm^2), the treated plant was placed for 8 hours in a cage with over 100 adult *Pieris brassicae* butterflies to allow deposition of scales *et cetera*. The gauze prevented butterfly' oviposition on the plant. The control plant was also covered in gauze while the treated plant was placed in the cage. The gauze was removed afterwards and leaf squares from both plants were tested a) immediately or b) 72 hours after exposure. Only experienced wasps were used in these series of experiments.

Experiment c.4 ("local induced with deposits") To determine if plants react locally towards egg deposition, residence time of experienced *Trichogramma brassicae* on 1- a control leaf square, or 2- a clean leaf square proximate to an egg deposition, were tested. The section was about 0.5 cm away from an egg clutch of about 50 eggs of *Pieris brassicae* (Figure 2.6). *Pieris brassicae* egg deposition on the test plant was allowed for 8 hours a) 48 hours or b) 72h prior to the experiment.
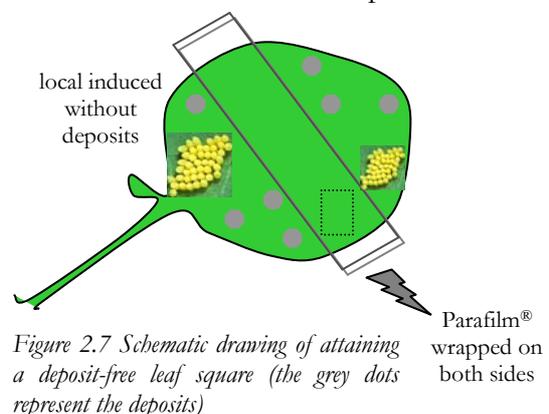
Figure 2.7 Schematic drawing of attaining a deposit-free leaf square (the grey dots represent the deposits)

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[i] Because eggs of *Pieris brassicae* are predominantly laid in batches on the underside of leaves (David and Gardiner, 1962), *Trichogramma* spp. are likely to spend relatively more time at this side of the leaves (Noldus *et al.*, 1991b).

Experiment c.5 ("local induced without deposits") Here, residence time of experienced *Trichogramma brassicae* on a local induced leaf onto butterfly eggs were deposited three days before, was again determined. This time the leaf squares were taken from a section where <u>no deposition of any kind</u> was allowed. The section was as close as possible, respectively a) 1-10 cm and b) 1-5 cm, to an egg clutch of as many eggs of *Pieris brassicae* as possible (10-50 eggs). Sections of the leaves of both control and treated plants were covered with Parafilm® for a) 8 hours or b) 24 hours, i.e. respectively 64-72h and 48-72h prior to the experiment (Figure 2.7).

Experiment c.6 ("systemic induced") To find out if plants can react systemically three days after egg deposition of *Pieris brassicae*, residence time of experienced *Trichogramma brassicae* was determined on 1- a control leaf, or 2- a leaf of an egg-carrying <u>plant</u> without egg deposition. Selected leaves from the egg-carrying plant had been covered with gauze (mesh size: 1.5*1.5 mm^2) to prevent egg deposition for 8 hours (64-72h prior to the experiment). Leaves from the control plant were equally covered for the period when the treated plant was placed in the cage with butterflies. Only leaves that had been covered were tested.

2.3 Olfactometer bioassays

For the olfactory bioassays, a static 2-chamber olfactometer was used (Figure 2.8). The test area (1 cm high, 9 cm in diameter) consisted of a removable walking arena of thin gauze (mesh size: 0.5*0.5 mm^2), on top of a cylinder made of acrylic glass (18 cm high, 12 cm in diameter) which was divided by a vertical plate into two chambers. No airflow was generated. The walking arena area was covered by a glass Petri dish. The wasp was released in the middle of the two sections on the walking arena and observed for 300 seconds. The entire cylinder was randomly rotated after each observation and was illuminated (Euromex coldlight illuminator EK-I) from above. The duration time was recorded using stopwatches.

Experiment o.1 ("eggs versus air") To determine whether females of *Trichogramma brassicae* were arrested by volatiles from egg-carrying leaves an experienced female *Trichogramma brassicae* were tested in a static olfactometer. One halve was filled with clean <u>air</u>, the other contained a leaf with eggs and deposits of *Pieris brassicae*. All deposits were three days old (i.e. deposited 64-72h prior to the experiment) and presented on short range (within 5 mm away from the walking arena).

Experiment o.2 ("long and short range") The arrestment of an experienced female of *Trichogramma brassicae* towards an egg-carrying leaf against a clean leaf was determined for a) short-range (< 5 mm) and b) long-range (5-10 cm) plant volatiles. The treated leaves had eggs and deposits of *Pieris brassicae* with a maximum of 72h old (in Figure 2.8).

Figure 2.8 The static 2-chamber olfactometer with two odour fields. Please notice that the 'no-choice' option is excluded. The star represents the starting point at which the wasp is released.

2.4 Statistics

The data set is analysed with the Observer® (Wageningen, the Netherlands) and the Statistical Package for Social Sciences (SPSS® 10.0 for Windows). Both contact bioassays as the olfactometer assays are analysed with the non-parametric Wilcoxon's matched pairs signed rank test (α=0.050). The ratio responding parasitoids versus non-responders was tested by χ^2- analysis with continuity (i.e. Yates) correction.
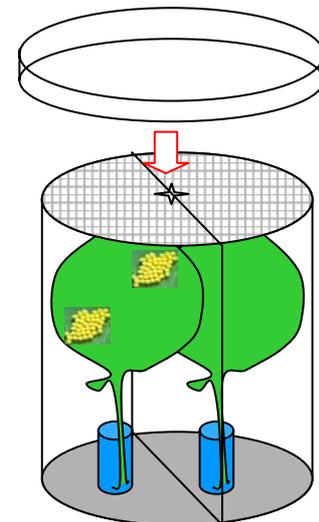
3. Results

3.1 Contact bioassays

3.1.1 Leaf squares with eggs of Pieris brassicae (c.1)

The possible preference of the *Trichogramma brassicae* for a leaf square with an egg clutch of *Pieris brassicae* was tested against a leaf square taken from a clean plant serving as the control. Females of *Trichogramma brassicae* were spending most of the observed residence time in the 'no choice' area (on average over 40% over all experiments). The remaining time of wasps was statistically tested and compared within each treatment. It appeared that parasitoids with no oviposition experience did not discriminate between the two leaf squares (p=0.98, Wilcoxon matched pairs test; c.1 a), Table 3.1). Subsequently parasitoids that were given the opportunity to oviposit for 18 hours in *Pieris brassicae* eggs were tested. The experienced parasitoid did show a significant preference for leaves with egg depositions (p=0.04, Wilcoxon matched pairs test; c.1 b), Table 3.1). When compared with a 2x2 contingency table the response towards a leaf square with eggs is much higher for experienced wasps (2 non-responders out of 23 samples) over the response of naive wasps (14 NR; n= 30) (p<0.005; χ^2-test with continuity correction).

3.1.2 Leaf squares with removed eggs of Pieris brassicae (c.2)

To reveal whether the eggs itself are attractive or if plant cues are involved, we tested leaves from which the *Pieris* eggs (72h old) were removed with a brush, prior to the experiment. Experienced wasps spend significantly more time on leaf squares were eggs had been removed than on clean leaf squares (p=0.01, Wilcoxon matched pairs test; c.2, Table 3.1).

Table 3.1 *Results of contact bioassays with either naive or experienced females of T. brassicae. The codes refer to the experimental setup (see also §2.2 Contact Bioassays); n= sample size; NR = number of non-responders; * = p-value ≤ 0.05 and ** = p-value ≤ 0.01. The no choice option is left out.*

"treatment" plant	code exp.	experience [h]	n	NR	res. time on clean leaf; mean ± SEM (sec)	res. time on treated leaf; mean ± SEM (sec)	p-value (Wilcoxon)
eggs	c.1 a)	0	30	16	38.4 ± 17.49 (51%)	36.4 ± 13.65 (49%)	0.98
eggs	c.1 b)	18	23	2	26.0 ± 13.44 (20%)	106.2 ± 25.85 (80%)	0.04*
eggs removed	c.2	18	35	2	42.1 ± 11.76 (22%)	128.2 ± 20.00 (78%)	0.01**
deposits (fresh)	c.3 a)	18	54	1	74.2 ± 12.78 (36%)	130.1 ± 15.47 (64%)	0.03*
deposits (72h)	c.3 b)	18	64	1	91.4 ± 12.30 (45%)	110.2 ± 12.94 (55%)	0.40
local induction (48h)	c. 4 a)	18	18	1	129.2 ± 30.52 (57%)	97.4 ± 28.88 (43%)	0.65
local induction (72h)	c.4 b)	18	91	12	70.1 ± 10.64 (39%)	111.5 ± 12.06 (61%)	0.03*
local ind. (for 8h) without deposits	c.5 a)	18	47	3	103.5 ± 16.25 (50%)	104.5 ± 15.74 (50%)	0.95
local ind. (for 24h) without deposits	c.5 b)	18	71	3	89.6 ± 10.63 (44%)	116.1 ± 13.78 (56%)	0.20
systemic induction	c.6	18	57	4	88.1 ± 13.47 (46%)	105.1 ± 14.48 (54%)	0.60

3.1.3 Leaves with deposits of Pieris brassicae versus control leaves (c.3)

To rule out any effect of host cues from adult butterflies, fresh butterfly deposits (defined broadly) were tested against a clean leaf. It appeared that residence time on a leaf with fresh deposits was higher than on the control leaf (p=0.03, Wilcoxon matched pairs test; c.3a), Table 3.1). Also 72h old butterfly deposits were tested. The results from that experiment revealed that experienced parasitoids show a random distribution over the two test areas (p=0.40, Wilcoxon matched pairs test; c.3b), Table 3.1).

3.1.4 Local induced leaf squares (c.4 and c.5)

In order to find out whether an oviposition of *Pieris brassicae* eggs induces a local response of plant synomones, possible preference of *Trichogramma brassicae* for egg free squares of a leaf with egg clutches of *Pieris brassicae* was tested against a leaf square of a clean plant. The results showed a prolonged residence time of T. brassicae on the test leaf squares 72h after egg deposition (p=0.03, Wilcoxon matched pairs test; c.4b), Table 3.1), but a random distribution over test and control squares after 48h of egg deposition (p=0.65, Wilcoxon matched pairs test; c.4b), Table 3.1). Without deposits of *Pieris brassicae*, no significant differences in residence time on the either the control or the other leaf square were found, not even when the egg density on the plant was increased (respectively p=0.95 and p=0.20, Wilcoxon matched pairs test; c.5a) and c.5b), Table 3.1). In case of the latest, only a slight trend was found.

3.1.5 Systemic induced leaves versus control leaves (c.5)

The next step was to examine whether the plant can react systemically to 72h old eggs. In the setup with possible systemically induced leaf squares (and deposits) compared with clean leaf squares, the parasitoids showed a random distribution (p=0.60, Wilcoxon matched pairs test; c.6, Table 3.1).

3.2 Olfactometer bioassays

3.2.1 Short range volatiles from leaves with eggs of Pieris brassicae versus clean air (o.1)

To determine possible olfactory components in host finding cues derived from the plant for *Trichogramma brassicae*, residence time of an experienced parasitoid in a static olfactometer with different odour fields was determined. When provided to *Trichogramma brassicae* on short range, no significant differences were found in residence time in either the clean air field versus the odour field from a leaf with eggs and other deposits of *Pieris brassicae* (p=0.33, Wilcoxon matched pairs test; o.1, Table 3.2).

3.2.2 Volatiles from leaves with eggs of Pieris brassicae versus control leaves (o.2)

An experienced parasitoid did **not** discriminate in terms of residence time between odours of a leaf with host eggs or a clean leaf on short range nor on long range (respectively p=0.80 and p=0.51, Wilcoxon matched pairs test; o.2a) and o.2b), Table 3.2).

Table 3.2 *Results of olfactory bioassays with 18h experienced parasitoids of Trichogramma brassicae, in order to reveal the nature of host finding cue(s) of these parasitoids. The codes refer to the experimental setup (see also §2.3 Olfactometer bioassays s), n= sample size and NR = number of non-responders*

"treatment" plant	code exp.	experience	n	NR	res. time on clean leaf; mean ± SEM (sec)	res. time on treated leaf; mean ± SEM (sec)	p-value (Wilcoxon)
eggs versus clean air (short range)	o.1	18h	48	1	165.8 ± 23.93 **(55%)**	134.2 ± 19.37 **(45%)**	0.33
eggs versus clean leaf (short range)	o.2 a)	18h	51	3	154.0 ± 21.57 **(51%)**	146.0 ± 20.44 **(49%)**	0.80
eggs versus clean leaf (long range)	o.2 b)	18h	23	2	161.7 ± 33.72 **(54%)**	138.3 ± 28.83 **(46%)**	0.51

4. Discussion

Our results suggest that egg deposition of *Pieris brassicae* can induce the production of locally released plant synomones. This study shows that these oviposition-induced synomones can arrest the generalist egg parasitoid *Trichogramma brassicae* to its hosts, when according to Fatouros *et al.* (2005, *in press*) most suitable for parasitization [i.e. three day old eggs under conditions used]. Tests revealed that only fresh butterfly deposits were arresting to the wasps, whereas 3d old kairomones did not. Neither could cues from clean leaf squares from egg-carrying plants, i.e. no deposition of <u>any</u> kind allowed on these specific sections, arrest the wasps. Since both factors alone could not evoke a response on itself, a co-effect of both eggs and deposits is suggested. *Trichogramma brassicae* did not respond to volatiles from leaves with egg depositions of *Pieris brassicae*.

In accordance with the findings of Noldus and van Lenteren (1985b) *Trichogramma brassicae* females had a longer time residence time on cabbage leaves infested with eggs of *Pieris brassicae* than on control leaves, although only when the wasps had prior oviposition experience. For parasitoids Vet and Dicke (1992) reported that their behaviour can change in a repeatable way through experience, like a (temporally) increased responsiveness of the wasp to host associated chemicals (Paré and Tumlinson, 1999). This form of learning can occur very rapidly, even after a single oviposition (Vet and Dicke, 1992). As our results confirm, oviposition experience did enhance host searching behaviour.

Chemical compounds present on the surface of host eggs or in the oviduct secretion may play a role in the host searching of *Trichogramma* wasps (Boo and Yang, 2000), but by only testing leaf squares 1 cm or more away from the nearest egg clutch possible effects of (diffusion of) egg substances or oviduct secretion were excluded. However, other factors could also have caused the arrestment of *Trichogramma brassicae*. In accordance with the results of Noldus and van Lenteren (1983), Vinson (1984) and Gardner and van Lenteren (1986) fresh deposits like host wing scales on egg-free cabbage leaves were demonstrated to make female *Trichogramma brassicae* search significantly longer for hosts. However, contact with three days old deposits in a similar setup did not result in arrestment. Arrestment of the parasitoid by kairomones derived from an earlier developmental stage of the adult of the host contributing to host finding, is an example of infochemical detour (Vet and Dicke, 1992).

Gardnier-Geoffroy *et al.* (1996) stated the stimulating effect of a possible chemical trail left after sweeping the surface of the leaf by a host species' extruded abdominal tip (*Ostrinia nubilalis*) to host location at short range of *Trichogramma brassicae*. These 'trail-substances' are pertinent not (absorbed) pheromones and also deposits like wing scales do not appear to constitute to the suggested trail (Gardnier-Geoffroy *et al.*, 1996). *Pieris brassicae,* unlike this moth species, was never observed sweeping the leaf surface during egg deposition. Apart from this different oviposition behaviour, one can question if such a trail would last for days since wing scales and other butterfly deposits could only arrest the parasitoid when fresh… Visual cues on the other hand can be used by the parasitoid for as long as there are eggs on the leaf. However, these cues cannot explain the arrestment of *Trichogramma brassicae* by leaf squares from which the eggs are removed. To explain the attractiveness of those leaves, we assume that the leaf is emitting arresting synomones. Noldus and van Lenteren (1983, 1985b) demonstrated an arresting effects at short range to *Trichogramma brassicae* of an oviposition deterrent supposedly placed by females of *Pieris brassicae*. Blaakmeer *et al.* (1994b) stated that these chemicals deterring conspecific butterflies and arresting egg parasitoids were not derived from butterfly' eggs or adult host' kairomones but from the infested plant, since extracts from cabbage leaves from which *Pieris* eggs were removed were still deterring to the butterfly for oviposition. Oviposition-induced plant synomones have been described since then in three other tri-trophic systems, respectively the

elm, pine and bean system (Table 4.1). The sprout system is added to the table to complete the overview.

Table 4.1 *Comparison of the sprout system with the three systems where oviposition-induced plant volatiles had been demonstrated before (adapted from Hilker et al., 2002b).*

	Elm system	Pine system	Bean system	Sprout system
Described in	Meiners and Hilker, 1997; Meiners and Hilker, 2000; Meiners *et al.*, 2000	Hilker *et al.*, 2002a, Mumm *et al.*, 2003; Mumm *et al.* 2004	Colazza *et al.*, 2004	Fatouros *et al.*, 2005 *in press*
Plant	*Ulmus minor* (*Ulmaceae)*, field elm	*Pinus sylvestris* (*Pinaceae)*, Scotch pine	*Vicia faba*, (*Fabaceae)* broad bean and *Phaseolus vulgaris*, (*Fabaceae)* French bean	*Brassica oleracea* (*Brassiceae)*, Brussels sprout
Herbivore	*Xanthogaleruca luteola* Müller (*Coleoptra: Chrysomelidae)*, elm leaf beetle	*Diprion pini* Linnaeus (*Hymenoptera: Diprionidae)*, pine sawfly	*Nezara viridula* L. (*Heteroptera: Pentatomidae)*, southern green stink bug	*Pieris brassicae* Linnaeus (*Lepidoptera, Pieridae)*, great cabbage white
Parasitoid	*Oomyzus gallerucae* Fonscolombe (*Hymenoptera: Eulophidae)*	*Chrysonotomyia ruforum* Krausse (*Hymenoptera: Eulophidae)*	*Trissolcus basalis* Wollaston (*Hymenoptera: Scelionidae)*	*Trichogramma brassicae* Bezdenko (*Hymenoptera, Chalcidoidea)*
Induction of synomones by oviposition	volatiles	volatiles	volatiles	contact cues
Local induction	+	+	+	+
Systemic induction	+	+	+	-

While the pine and elm-system concern a perennial tree, the bean and sprout system review an annual crop; respectively the broad or the French bean (Colazza *et al.*, 2004) and Brussels sprouts. Feeding damage in an herbaceous plant can be much more costly in terms of fitness than in a perennial plant, due to the short lifetime and also because of a much lower biomass of annual plants compared to perennials (Hilker *et al.* 2002a; Hilker *et al.* 2002b). Parasitizing herbivorous eggs, thereby inhibiting feeding damage of hatching larvae, can very well be beneficial to the plant (Hilker and Meiners, 2002). A strong response of the annual plant to oviposition is therefore to be expected, but was only demonstrated weakly in the sprout system. An explanation can be that in contrast to the three other systems, the adults in the second trophic level of the sprout system are not feeding from (as in the bean system: Colazza *et al.*, 2004) or wounding (as in the elm system: Meiners and Hilker, 1997; and pine system: Hilker *et al.* 2002a) the plant prior to oviposition. An adult female of *Pieris brassicae* is a nectar feeder and just briefly drums on a leaf with its fore legs and tips with the abdomen to test if a leaf is suitable for oviposition (David and Gardiner, 1962). Oviduct secretion alone, cementing eggs of *Pieris brassicae* on the leaf, is a candidate to elicit the production of plant synomones in the sprout system. The cautious way of *Pieris brassicae* females laying their eggs on the plant can evoke a local plant response as demonstrated, most likely by changing the chemical composition of the leaf surface. A systemic induction extending to egg-free leaves of the plant could not been demonstrated here. This does not imply that Brussels sprouts plants are unable to react systemically. On the contrary, Mattiacci *et al.* (2001a; 2001b) found a systemically-induced response of *Brassica oleracea* against larval feeding of *Pieris brassicae*. Not only was the development of larvae fed on systemically induced leaves longer than those fed on non-induced leaves of *Brassica oleracea* (= direct defense), also herbivore antagonists were attracted to damaged plants (= indirect defense). The age of the leaf

was the most important factor in the expression of the systemic response (Mattiacci *et al.* 2001a). Leaves used in this series of experiments were assigned to as 'middle' or 'old' by Mattiacci *et al.* (2001a); i.e. groups in which systemic induction was detectable after being damaged. Younger leaves would be less exposed to oviposition and herbivory than older ones and therefore less likely to attract parasitoids.

Not only age of the plant or its leaves, but also time window might be very important since synomones can be more variable over time than kairomones (Dicke, 2000). Both fluctuations of the plant's response during the day are recorded (Dicke, 1999b; Paré and Tumlinson, 1999) as distinct seasonal trends of volatile release in cabbage plants over the year (Blaakmeer *et al.*, 1994c), declining drastically in autumn. However time of day seem to have no effect on the behaviour of *Trichogramma* in response to host semiochemicals, at least not in the flight cage and also the wind tunnel experiments conducted by Noldus *et al.* (1991a). By rearing Brussels sprouts under controlled conditions, it was tried to minimize any fluctuations of the plant's response.
Meiners and Hilker (2000) stated that a three day (72h) time lapse was necessary for systemic induction in the elm system. This study showed that three days after egg deposition the parasitoid was arrested by cues from local induced leaf squares while not under the same conditions after a two day time lapse. Apparently two days were too short for the indirect local defense of Brussels sprouts plants to become operational. Perhaps a systemic response in the sprout system needed even more time to start up. Immediate action was not necessary, since the threat of herbivory was only apparent after hatching of the eggs. Under standardized conditions used, Fatouros (2005, *in press*) demonstrated that three days old *Pieris* eggs were most suitable for parasitizing to *Trichogramma brassicae*. In the field, the time window in which *Trichogramma brassicae* can parasitize *Pieris brassicae* eggs is most likely prolonged since conditions like temperature (David and Gardiner, 1962) are suboptimal for larval development. A plant's response to lure *Trichogramma brassicae* to eggs over three days old is therefore still possible. Even when infested with herbivores, *Brassica oleracea* needs at least three days of higher levels of continuous larval feeding to invoke a systemic response and then only if additional damage is inflicted on uninfested cabbage leaves (Mattiacci *et al.* 2001b).

Besides timing, the value of infochemicals also depends on the contexual variation (Dicke, 2000). From a leaf square in a Petri dish in absence of other stimuli for comparison, responses of the parasitoid to stimuli recorded might represent a response to 'something' versus 'nothing' rather than a host directed response (Noldus *et al.*, 1990). Perhaps this is why both deposits alone (3d old) and local induced leaf squares without deposits are not arresting *Trichogramma brassicae*, while the combination does. Maybe field studies can give us a more detailed insight into host searching behaviour of *Trichogramma brassicae* by the use of oviposition-induced synomones of *Brassica oleracea*.

Unlike the pine and elm-system, the bean and sprout system include a generalist parasitoid, respectively *Trissolcus basalis* and *Trichogramma brassicae*. Since oviposition-induced synomones can be highly specific for both plant as herbivore species (Meiners *et al.,* 2000), it is more likely for a specialist to use these specific plant cues. We demonstrated however, in accordance with Colazza *et al.* (2004), that synomones can also be used as host finding cues by polyphagous species, which *Trichogramma* spp. are known to be. Perhaps this was due to the manner of rearing since the parasitoids were reared for several generations in *Pieris brassicae* eggs on *Brassica oleracea* leaves. This could have resulted in a preference of this specific host (Vinson, 1984; Kaiser *et al.*, 1989b; Cortesero and Monge, 1994; Vinson, 1998), or could also have enhanced the insect's response to host plant's odours (Turlings *et al.*, 1993; Cortesero and Monge, 1994; van Emden *et al.*, 1996; Douloumpaka and van Emden, 2003) although our olfactometer assays revealed that *Trichogramma brassicae* did not respond to plant volatiles. The latter can be due to the minute size of an adult female (0.5 mm). Upwind flight in open air for *Trichogramma* spp. is therefore

practically impossible, so net dispersal is likely to be drifting passively downwind (Noldus, 1989b; Noldus *et al.*, 1991a; Nordlund, 1994; Vet *et al.*, 1995) or by walking within the host habitat (Hilker *et al.*, 2002a). Since volatiles disperse downwind, orientation to the upwind stimulus source by these minute insects over any significant distance (i.e. attraction by long range cues) is thus unlikely (Nordlund, 1994; Romeis *et al.*, 1997). Arrestment or repellence of *Trichogramma* by non-volatile contact cues on a short range is more likely. Noldus *et al.* (1991a) concluded that *Trichogramma* spp. were definitely arrested by short-range cues in areas where it perceives a stimulus, while leaves with host eggs or host deposits did not cause long-range attraction in *Trichogramma brassicae* (Noldus and van Lenteren, 1983), neither were host eggs themselves known to (Noldus *et al.*, 1991a). Although from the same plant as an egg-infested leaf, an egg-free leaf might be too far away from the egg-carrying leaf for *Trichogramma brassicae*, making systemic released infochemicals non-reliable host-finding cues.

Within the elm system, Meiners and Hilker (1997) could not detect a preference of the egg parasitoid *Oomyzus gallerucae* for non-volatile plant cues. The pine and the bean system were not tested for contact cues (yet).

Survival and reproductivity of parasitoids depend mainly on their ability to locate hosts. From the above findings, one can argue that egg parasitoids may use oviposition-induced plant synomones in the process of host finding. An arrestment of *Trichogramma brassicae* to a local response of Brussels sprouts induced by host egg deposition was shown. A plant's systemic response did not seem to serve as host finding contact cue. Further work on molecular and chemical basis is needed to identify specific elicitor and to fully understand mechanisms and genetics of induced indirect defense in Brussels sprouts under varying circumstances with respect to behavioural and also ecological aspects as emphasized by Hilker and Dicke (2003). Also time window of the plant's response under different circumstances needs to be evaluated. Since *Trichogramma* spp. are gregarious (Schmidt and Smith, 1985), results from this research and follow-up studies can be of practical use for the mass-rearing of herbivore antagonists with increased host searching efficiency in biological pest control.

Epilogue

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Annexes

Annex I Dataset

All unanalyzed data were copied to enclosed floppy disk. Original files were recorded in the program The Observer® (Wageningen, the Netherlands). A hardware key is needed to access these files. For those who do not have such a key, all files were exported to MS Excel for Windows®. Additionally, the Excel files were sorted per series of experiments (olfactometer or contact bioassays) and original comments included.