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Predators and the accessibility of herbivore refuges in plants

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Predators and the accessibility of herbivore refuges in plants

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This thesis is affectionately dedicated to my loving parents
for their love, care and sacrifices

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chapter 1

General introduction and outline

Indirect plant defense against herbivores

Herbivory by arthropods induces an array of defense mechanisms in plants (Karban and Baldwin, 1997; Agrawal *et al.*, 1999). These mechanisms of plants are either constitutive or inducible. Constitutive defenses, for example impenetrable leaf cuticles or sticky and toxic glandular trichomes, are present on plants independent of herbivore attack. Defenses may also be induced by herbivory, either at the wound-site alone (local plant response) or plant-wide (systemic response). These responses include measures to seal off the wound (cell wall lignification or production of gum, callose, resin) and production of secondary metabolites (enzymes, such as protease inhibitors, polyphenol oxidases, chitinases and glucanases, and other organic compounds, such as terpenoids, alkaloids and phenolics).

The induced secondary compounds may directly deter or hinder the herbivore (direct plant defense). Alternatively, they may promote the effectiveness of the natural enemies of the herbivore (indirect plant defense). Examples are volatile chemicals that inform natural enemies on the presence of herbivores, their prey. During the past two decades much information has surfaced to explain that herbivore-induced plant volatiles (HIPV) are used by parasitoids and predators to find their prey (Dicke and Sabelis, 1988; Dicke *et al.*, 1990; Turlings *et al.*, 1990; Dicke and Vet, 1999; Kessler and Baldwin, 2002; Sabelis *et al.*, 2006). Herbivore-induced plant volatiles differ from those produced by artificially wounded plants (Bouwmeester *et al.*, 1999; Lou *et al.*, 2005; Chapter 5). There is substantial evidence on the active role of plants in releasing HIPV: (1) HIPV compounds

occur in plants (Dicke *et al.*, 1990; Turlings *et al.*, 1990; Scutareanu *et al.*, 1997), (2) biosynthetic pathways of HIPV compounds occur in plants (Paré *et al.*, 1999; Bouwmeester *et al.*, 1999), (3) HIPV are systemically induced in the plants (Turlings and Tumlinson, 1992; Dicke, 1994; Guerrieri *et al.*, 1999), (4) herbivores themselves and their products (faeces, silk) are not attractive to their natural enemies (Sabelis *et al.*, 1984), (5) application of jasmonic acid to wild-type plants and defense-signaling mutants induce the production of HIPV identical to those volatiles induced by herbivores (Ozawa *et al.*, 2000; Gols *et al.*, 2003; Ament *et al.*, 2004), (6) genes expressed in plants due to herbivory are similar to those induced by jasmonates (Kant *et al.*, 2004; Ament *et al.*, 2004), (7) elicitors for HIPV production are proven to be present in insect saliva (Turlings *et al.*, 1993; Alborn *et al.*, 1997; Paré *et al.*, 1998).

Apart from chemical alarms, plants may also promote the effectiveness of natural enemies by providing alternative food (nectar, pollen) and refuges (domatia) that favour the herbivore's enemies more than the herbivores themselves (Walter, 1996; Van Rijn *et al.*, 2002). The reason why plants profit is that herbivores would increase the risk of encountering natural enemies frequenting locally available refuges or sites with alternative food. However, many species of herbivores inhabit or even induce plant structures that are concealed and relatively hard to reach by natural enemies. For example, root-feeding and gall-inducing arthropods are less exposed to natural enemies, than above-ground and vagrant herbivores. Little is known of the ways in which plants defend themselves against herbivores living in refuges on or near plants. This thesis is the first study to systematically investigate how plants can make refuge-seeking herbivores above- and below- ground more vulnerable to their natural enemies.

Part one

Above-ground, refuge-seeking herbivores in a tritrophic context

To reduce the risk of being eaten by their enemies, some animals display morphological and behavioral adaptations such as changes

in their own morphology (Van Buskirk and McCollum, 2000), changing foraging site or foraging time in the presence of predators (Edwards, 1983; Festa-Bianchet, 1988; Larsson *et al.*, 1997; Huhta *et al.*, 1999; Magalhães *et al.*, 2002; Onzo *et al.*, 2003). Others change their reproductive strategy as a response to the presence of predators (Fontaine and Martin, 2006) or move into a refuge that makes them inaccessible to predators (Eubanks *et al.*, 1997; Oku *et al.*, 2003). Many arthropods are known to build shelters from the plant tissues they inhabit. Some have evolved to live in naturally existing concealed plant parts that are not modified by the inhabitants, while others induce plants to produce galls that provide them with refuge from unfavourable biotic and abiotic factors. Thus, even when plants release 'alarm calls', herbivores in a plant gall run less risk of being eaten by their natural enemies.

What countermeasures would plants take when their indirect defenses against herbivores evidently failed due to concealment in plant parts where the herbivores find a hide-out from predators? One way of combating refuge-seeking herbivores is to create chemical or mechanical barriers, but this comes at a cost to the plant. A cheaper alternative would be that plants change their morphology so as to expose their herbivores to the natural enemies. In that case, it would pay the plants to attract predators by releasing HIPV.

To study the latter plant defense strategy, eriophyoid mites were selected as the focal group of herbivores that seek refuge in plants. They are the smallest arthropods in the world and this is the key to their ecological success (Sabelis and Bruin, 1996). Minute size and the absence of hind legs restrict their capacity for ambulatory movements (Sabelis and Bruin, 1996). In one way, this is advantageous because it decreases the contact rate with infective spores of acaropathogenic fungi. In the other way, smaller size and slower movements than the predatory mites, mostly phytoseiids, decrease their ability to escape from predation (Sabelis and Bruin, 1996). Nevertheless, they persist under natural conditions and this raises the question how they persist despite their vulnerability to

predators. Hypotheses explaining their persistence have in common that eriophyoid mites hide away from their predators in spatial or temporal refuges (Sabelis, 1996; Sabelis and Bruin, 1996). In fact, eriophyoids show a range of morphological characters that make them ideal refuge seekers on plants (Lindquist and Oldfield, 1996). They have a long, vermiform body with a small cross-sectional diameter (40-100μm, Sabelis and Bruin, 1996) that allow them to reach places inaccessible to predatory mites that are much bigger (Sabelis, 1996; Thistlewood *et al.*, 1996). The absence of posterior two pairs of legs has also been suggested as an adaptation to live in confined microhabitats (Lindquist and Oldfield, 1996). They either live in concealed plant structures such as in leaf sheaths, within buds and between bulb scales or induce the host plant to produce growth abnormalities such as galls, erinea and leaf edge curls inside which they can feed on and reproduce without being attacked by the predators (Sabelis and Bruin, 1996).

Yet, the refuge hypothesis is not watertight: eriophyoids are extremely vulnerable to predation and phytoseiids can suppress eriophyoid populations to very low levels (Duso and de Lillo, 1996; Conijn *et al.*, 1996; Lesna *et al.*, 2004). Sabelis (1996) challenges the refuge hypothesis of eriophyoids with several arguments. First, the difference in the size of eriophyoids and that of the immature stages of phytoseiids may be less dramatic and, hence, the mobile immature stages may penetrate into narrow spaces in plants where eriophyoids feed. Second, phytoseiids may be able to attack eriophyoids in galls either when the aperture of the gall has become large enough for them to enter. Third, the degree of protection to the eriophyoids from the erinea may vary with age and variety of the host plant. Fourth, during the development of the gall, which could take from many hours to a few days, gall founders are vulnerable to predation. Finally, gall and bud mites have to leave the refuge at some point during their life cycle to find a new host. During their migration, they become exposed to predators and to adverse climatic conditions. Whether the refuge hypothesis stands up to scrutiny in the face of all these five arguments, remains to be seen.

Several predatory mites belonging to Stigmaeidae, Tydeidae and especilly Phytoseiidae have shown significant impact on eriophyoid mites (Sabelis, 1996; Thistlewood *et al.*, 1996; De Moraes and Zacarias, 2002). There are several ways in which predators may attack herbivores in refuges. One way is by pulling apart the plant structure in which the prey is in refuge. However, so far we do not know any morphological adaptation of predatory mites that will help them to open and enter the concealed plant parts. The alternative for the predatory mites is to have morphological and behavioural adaptations that help them to enter the plant structures in the same way as the eriophyoids. Some species of predatory mites are known to stretch their legs, thereby bringing their soma close to the plant surface and enabling them to reach concealed places on the plant (I. Lesna, personal observation). Certain predatory mites, such as *Neoseiulus baraki* Athias-Henriot and *N. paspalivorus* DeLeon (Acari: Phytoseiidae) show morphological features which qualify them to be successful natural enemies of herbivores in concealed plants parts. They have flat and elongated idiosoma which enable them to reach the meristematic tissue under the perianth of the coconut where their prey usually feeds on (De Moraes *et al.*, 2004). Thus, it is likely that species of predatory mites differ in their ability to get access to the concealed places in plants, but whether there are at least some species sufficiently equipped to suppress populations of these eriophyoid mites, is an open question that is to be answered by the experiments presented in this thesis. To this end, I studied the coconut mite, *Aceria guerreronis* Keifer (Acari: Eriophyidae), that feeds on the meristematic tissue under perianth of fruits of the coconut palm (*Cocos nucifera* L.) and I tested the ability of the predatory mite, *N. baraki*, to get access to the space under the perianth and how this access depends on the response of the coconut palm to infestation by coconut mites.

Study system: Coconut, coconut mite and predatory mites

The coconut mite is one of the most serious pests of coconut palms. It was first reported in Mexico in 1965 (Keifer, 1965) and

since then it has been found in many coconut growing countries in America and Africa. In Asia, it was first reported as a pest in Sri Lanka (Fernando *et al.*, 2002) and India (Sathiamma *et al.*, 1998). The loss of copra (endosperm of the coconut) has been estimated to be 10% in Benin (Mariau and Julia, 1970), 16% in Ivory Coast (Julia and Mariau, 1979), 30% in Mexico (Hernandez Roque, 1977) and 11-28% in St Lucia (Moore, 1986). In Sri Lanka, the loss in annual national coconut production was recently estimated to be 2-3% (Peiris, Fernando and Wickramananda, unpublished data).

Aceria guerreronis lives under the perianth (collectively, the tepals that are often referred to as bracts) of the fruit of the coconut (commonly termed as 'nut') causing cosmetic damage to the nut. The perianth of the nut protects the meristematic zone of the female flower and the developing nut thereafter. In unfertilized flowers and younger nuts (*i.e.* 1-2 months after the fertilization), the perianth is firmly attached to the surface of the nut, leaving only a narrow space. The edge of the perianth is so closely appressed to the fruit that it leaves even less space for any mite to enter. However, the coconut mite is able to creep through to enter under the perianth (Howard and Abreu-Rodriguez, 1991). In general, the highest population of coconut mites is found in nuts that are 3-7 months old (Moore and Alexander, 1987; Fernando *et al.*, 2003) and the population declines thereafter. As the nut expands in size, surface of the nut becomes necrotic and suberized which leads eventually to distorted nuts.

In Sri Lanka, several short-term recommendations have been made to manage the pest (Fernando *et al*, 2002; Chandrasiri and Fernando, 2004). But considering the nature of the plant (tall and bearing nuts throughout the year), nature of the pest (small, living in a hidden habitat, with a high propensity to multiply), the nature of the growers (marginal income owners), plus the negative impact on the environment, biological control is preferred over chemical control as a more sustainable, long-term strategy to contain and suppress the pest. Microbial control using *Hirsutella thompsonii* Fisher has shown some promising results, but long-term field

evaluation trials are yet to be done (L.C.P. Fernando, Coconut Research Institute of Sri Lanka, personal communication). Several predatory mites have been reported worldwide (Moraes and Zacarias, 2002) including Sri Lanka (Moraes *et al.*, 2004). So far, their effect on the population of coconut mites has not been extensively studied. In Sri Lanka, *N. baraki* is by far, the most abundant predatory mite associated with the coconut mite (Moraes *et al.*, 2004). This species was previously described as *N.* aff. *paspalivorus* (*e.g.* Fernando *et al.*, 2003) but was later confirmed as *N. baraki* by Moraes *et al.* (2004). Previous field experiments revealed similar trends in abundance of *N. baraki* and *A. guerreronis* within the palm: the pest population reached its peak in 5 month old nuts whereas the predator showed a peak one month later (Fernando *et al.*, 2003), a delay typical for prey-predator interactions. Over time, *N. baraki* populations in the field tend to increase (Fernando and Aratchige, 2003, 2006). Moreover, *N. baraki* shows morphological features that may enable access to the perianth, such as a flat and elongated idiosoma (Moraes *et al.*, 2004). All these features make *N. baraki* a potential candidate for biological control of coconut mite.

Studies on the prey-predator interactions of this system are still in their infancy. The most striking feature of this system is that the prey is in a partial refuge under the perianth and therefore the predator has problems to reach its prey. Apart from the flat body, *N. baraki* is not known to show any morphological or behavioral adaptations that make it capable of reaching the prey. If coconut mites under perianth would be safeguarded from their predators, their populations are expected to grow exponentially and develop into a pest, thereby causing considerable damage to the nut. I hypothesize, however, that coconut palms are not passively suffering from coconut mite attack, but take countermeasures in response to this herbivore by making the fruit-perianth connection less tight, thereby providing more space under the perianth and causing the herbivores to be more exposed to their predators.

Chapter 2 – Plant morphological changes induced by herbivory: Do mite-infested coconuts allow predatory mites to move under the perianth?

In this chapter I discuss how the morphology of the perianth on the fruit of the coconut is changed in response to herbivory by coconut mites. The distance between the perianth and the surface ('perianth-fruit distance') of infested and uninfested nuts in three different cultivars, extensively grown in Sri Lanka, was measured and these measurements were compared to the body size of the coconut mite and its predator. It was found that the mean of the highest perianth-fruit distances at the perianth edge of uninfested nuts is in the range of 40-68 µm. This distance allows coconut mites to enter under the perianth because they are worm-like and 36-52 µm wide (Keifer, 1965). Moreover, 79% of the uninfested nuts had a distance of less than 100 µm suggesting that phytoseiid mites with a width of *ca.* 3 times that of the coconut mite are unable to reach under the perianth in most of the uninfested nuts. Interestingly, when the nuts are infested, the perianth-fruit distance is increased to such an extent that even the predatory mites can enter under the perianth.

The perianth-fruit distance may increase at the expense of plant performance. For example, the meristematic tissue under the perianth may run more risk to desiccate and become more exposed to larger herbivores. Moreover, the impact of predators might become less pronounced if hyperpredators move under the perianth too. However, by limiting the distance to a certain level so that only predatory mites can move under the perianth, nuts may optimize their herbivore-induced indirect defenses. If so, this leaves room for the hypothesis that plants may profit from a change in morphology that causes refuge-seeking herbivores to be more exposed to their predators. Whether these mite-induced changes in plant morphology indeed promote predator access and go together with increased attraction of predatory mites to the mite-induced plant was studied in Chapter 3.

Chapter 3 – How herbivorous mite-induced changes in coconuts affect the searching behaviour of predatory mites

In this chapter, first I test whether infested coconuts attract the predatory mite, *N. baraki*. The study was conducted in a set-up where predatory mites were allowed to move inside a T-tube olfactometer with the arms of the tube connected to containers with the odour sources. Predatory mites showed a preference for the odours emanating from infested nuts by the coconut mite when the alternative odours were from uninfested nuts. However, the preference was bordering significance.

Next, I conducted a release-recapture experiment where predatory mites were given an option to choose among the nuts in a more complex environment where infested and uninfested nuts were arranged alternately in a square-shaped arena. In this set-up, predatory mites were unable to distinguish between the infested and uninfested nuts. These results revealed that predatory mites cannot differentiate between infested and uninfested nuts in a complex environment where odour plumes are not neatly separated, a situation that is likely to occur when infested and uninfested nuts are on the same bunch.

However, quite interestingly, *ca.* 50% of the predatory mites on infested nuts was found under the perianth, whereas on uninfested nuts most predators were wandering on the nut outside of the perianth. Probably, the increased perianth-fruit distance induced by coconut mite attack (Chapter 2) allowed more predatory mites to move under the perianth of infested nuts.

In another experiment, I manipulated the coconuts so as to increase the perianth-fruit distance without induction by coconut mites. As expected, more predatory mites were observed under the perianth of the manipulated nuts when the alternative was an intact nut. The fraction of predatory mites under the perianth of manipulated nuts was close to 50% and hence similar to that on infested nuts.

In conclusion, a role of herbivore-induced plant volatiles in attracting predatory mites cannot be excluded and herbivore-induced changes in the perianth-fruit distance in coconuts determine predator access to the space under the perianth.

Part two

Below-ground, refuge-seeking herbivores in a tritrophic context

Plants may also respond to below-ground herbivores so as to increase their exposure to natural enemies. On the one hand, below-ground herbivores may be protected from predators, larger than themselves. On the other hand, the plant may betray the presence of below-ground herbivores to predators by releasing herbivore-induced volatiles and somehow by promoting (below-ground and above-ground) predator access to the herbivore's feeding sites below-ground.

I studied a tritrophic interaction that occurs in a below-ground plant part. It involved the predatory mite *Neoseiulus cucumeris* Oudemans (Acari: Phytoseiidae), the herbivore, *Aceria tulipae* Keifer (Acari: Eriophyidae) – also called dry bulb mite – and tulip bulbs, *Tulipa* sp. Unique features of this tritrophic system are that the predatory mites live in the litter layer and on plants (Moraes *et al.*, 1986; I. Lesna, personal observation) and that the herbivorous mite feeds on the outer bulb scale as well as on the inner bulb scales where it is less exposed to the much bigger predatory mites. It has been shown that tulip bulbs infested by dry bulb mites increase the distance between the inner bulb scales, thereby allowing predatory mites to enter the bulb (Lesna *et al.*, 2004). This herbivore-induced response in the tulip bulbs promotes elimination of dry bulb mites by predatory mites, but whether the tulip bulbs also attract predatory mites has not yet been assessed. This is the topic of the second part of this thesis.

Despite mounting evidence for the role of HIPV in attracting natural enemies to above-ground plant parts attacked by herbivorous mites and insects, there is only limited evidence for a role of HIPV in below-ground plant parts. Roots of a coniferous plant, *Thuja occidentalis*, release chemical signals upon damage by larvae of the weevil, *Otiorhynchus sulcatus* Fabricius (Coleoptera: Curculionidae), and these signals are found to be attractive to the entomophagous nematode, *Heterorhabditis megidis* (Van Tol *et al.*, 2001). In another study, an endoparasitoid, *Trybliographa rapae* Westwood (Hymenoptera:

Figitidae) was found to be attracted to roots of turnip plants infested by larvae of the cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae) (Neveu *et al.*, 2002). While the former two tritrophic systems have not been explored for the identity of HIPV, this was done in yet another tritrophic system, consisting of maize, *Zea mays* L., western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), and the entomophagous nematode *Heterorhabditis megidis* (Rassman *et al.*, 2005). However, none of these has been studied for herbivore-induced plant responses that give the natural enemies better access to their victims. Using the tritrophic system involving tulips, dry bulb mites and predatory mites, I investigated whether and how the induced morphological change promoting predator access to dry bulb mites inside the bulb was coordinated with the induced production of volatiles in tulip bulbs damaged by dry bulb mites.

Study system: Tulip bulbs, dry bulb mites and predatory mites

Aceria tulipae, commonly known as the dry bulb mite or the wheat curl mite, is the most important eriophyoid mite attacking bulbous crops (Conijn *et al.*, 1996). They have been reported as pests of crops belonging to the other plant families such as Graminae (Styer and Nault, 1996; Frost and Ridland, 1996). Their impact on flower bulbs and grasses is not limited to herbivory as they act as vectors of plant viruses too, *e.g.* wheat streak mosaic virus, wheat spot mosaic virus, onion mosaic virus, onion mite-borne latent virus, and shallot mite-borne latent virus (Oldfield and Proeseler, 1996; Conijn *et al.*, 1996).

In tulip bulbs, *A. tulipae* feeds on the fleshy outer scale, mostly in the hairy area in the basal plate and near the tip of the bulb where it can access the interior of the bulb through its 'nose', a small opening at the top of the bulb. The symptoms start as red-to-purple or cream-to-yellow spots on the scales. These spots enlarge to ultimately cover the whole outer scale of the bulb. Finally, the outer and inner scales dry out, thus giving way to degradation of tissues. Under severe infestation, many thousands of

mites (up to 50,000) can be found on the tulip bulb. Heavily infested bulbs eventually dry out (Conijn *et al.*, 1996).

Conijn *et al.* (1996) suggest using predatory mites for the control of *A. tulipae* in bulbs. Lesna (unpublished data) found *Lasioseius bispinosus*, *L. fimetorum*, *N. cucumeris*, and *N. barkeri* to be associated with *A. tulipae* infesting tulip bulbs in storage rooms. Two predator species, *N. cucumeris* and *N. barkeri*, have proven to be effective in controlling populations of *A. tulipae* in small scale laboratory experiments (Lesna, unpublished data). The former species was selected because of its higher tolerance of low humidity conditions prevailing in storage rooms and it was proven to be a successful biocontrol agent, albeit dependent on the exact way in which the tulip bulbs are stored (Lesna *et al.*, 2004). For the experiments described below, I therefore used *N. cucumeris*.

Chapter 4 – Below-ground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites

In Chapter 4 I investigated whether rust mite-infested tulip bulbs produce volatile plant signals that are attractive to the predatory mite, *N. cucumeris*. I found that hungry female predatory mites preferred odours released from bulbs infested by dry bulb mites over those from intact, uninfested bulbs or artificially wounded, uninfested bulbs (Aratchige *et al.*, 2004; Chapter 4).

The predation risk of *A. tulipae* varies depending on where it feeds on the tulip bulb. When it feeds on the outer scale of the tulip bulbs, like any other free-living herbivore, it is extremely vulnerable to be attacked by natural enemies. But when they feed inside the bulbs, they run less risk of being eaten by predatory mites since they are usually larger in size and more mobile. In the absence of predatory mites, rust mites grow exponentially inside the tulip bulbs, ultimately causing them to desiccate. The tulip bulb responds by changing its morphology so as to make dry bulb mites more exposed to predatory mites. This response was inferred from a series of biocontrol experiments (Lesna *et al.*, 2004). In closed carton boxes in the laboratory, predatory mites effectively controlled the rust mite pop-

ulations in the tulip bulbs, whereas, in open trays and chicken mesh boxes, predatory mites were unable to reduce the rust mite populations (Lesna *et al.*, 2004). It was suggested that ethylene influences the changes in bulb morphology, thereby providing the predatory mites with better access to rust mites feeding inside the tulip bulbs. In subsequent experiments, they found that ethylene triggers the same morphological changes as that are induced by herbivory in tulip bulbs and that effective biocontrol was only achieved when the bulbs received one-day ethylene exposure, not when they received exposure to methyl-1-cylcopropene (MCP), an inhibitor of ethylene perception (Lesna, Conijn and Sabelis, unpublished data). Thus, ethylene mediates accessibility of the bulb's interior to predatory mites. The question remaining was whether ethylene changes plant chemistry, in addition to the plant morphology, and thereby increases the attractiveness of the plants to the natural enemies of the herbivores. This hypothesis on ethylene-mediated coordination of the induced morphological and chemical SOS response was tested in Chapter 5.

Role of ethylene in plant defense chemistry

Before moving on to the results in Chapter 5, it is relevant to provide a general picture of what is known about the role of ethylene in plant defense and other processes in the plant. Ethylene is a plant hormone with a very simple chemical structure. It plays a role in many physiological and developmental processes throughout the life span of plants, including seed germination, breaking seed and bud dormancy, fruit ripening, cell expansion, cell differentiation, flowering, senescence and abscission (Taiz and Zeiger, 1998), as well as in the response to abiotic and biotic stress (Bleecker and Kende, 2000). The production of ethylene in plants is induced when plants are exposed to adverse environmental conditions such as floods, chills, and during temperature or drought stress (Taiz and Zeiger, 1998) and mechanical wounding (Hyodo and Nishino, 1981). Several pathogens (Hoffman *et al.*, 1999) as well as non-pathogenic bacteria (Hase *et al.*, 2003) are known to induce the production of ethylene in various plants. The role of ethylene in

pathogenesis-related defense responses has been studied extensively during the last few decades. For example, it can induce the production of phytoalexins (Fan *et al.*, 2000), pathogenesis-related proteins (Rodrigo *et al.*, 1993; Tornero *et al.*, 1997) and it reduces the susceptibility of plants to pathogens too (Díaz *et al.*, 2002). Evidence for the role of pathogenesis-related defense responses has been provided, using ethylene-insensitive tobacco lines (Knoester *et al.*, 1998) and soybean mutants (Hoffman *et al.*, 1999). Depending on the plant-pathogen combination, ethylene can also be involved in increasing disease susceptibility and symptom expression (Hoffman *et al.*, 1999; Bleecker and Kende, 2000).

Although the role of ethylene in pathogenesis-related defense responses has been studied extensively, this does not apply to herbivore-induced defense responses. It is commonly thought that jasmonic acid (JA) is essential for anti-herbivore defenses, such as the production of proteinase inhibitors and terpenoids present in blends of HIPV (Ament *et al.*, 2004; Kessler *et al.*, 2004). Ethylene, however, is now known to modify JA-regulated wound and anti-herbivore responses. (Kahl *et al.*, 2000; Stotz *et al.*, 2000; Walling, 2000; Horiuchi *et al.*, 2001; Schmelz *et al.*, 2003a).

Herbivore-damage stimulates ethylene emission in several crops (Shain and Hillis, 1972; Kendall and Bjostad, 1990; Kahl *et al.*, 2000; Schmelz *et al.*, 2003a,b). It is not merely a component in the volatile blend from herbivore-infested plants, but it plays a role in both direct and indirect defense responses against herbivores. In indirect plant defense, ethylene greatly promotes JA-regulated volatile emission elicited by volicitin, a compound which arises from the interaction between damaged leaf cells and oral secretions of beet armyworms (Schmelz *et al.*, 2003a). Moreover, exogenous application of JA together with 1-aminocyclopropane-1-carboxilic acid (ACC), an intermediate compound in the conversion of methionine to ethylene, has been shown to greatly promote Lima beans to produce at least three JA- induced volatiles, namely (*E*)- and (*Z*)-β-ocimene and (*Z*)-3-hexenyl acetate (Horiuchi *et al.*, 2001) and to attract more females of the predatory mite, *Phytoseiulus per-*

similis Athias-Henriot (Acari: Phytoseiidae), when compared to Lima bean plants treated with JA alone.

Antagonistic effects of JA and ethylene have also been reported in plants. In tobacco, herbivore-induced ethylene inhibited jasmonate-induced nicotine production (Kahl *et al.*, 2000; Winz and Baldwin, 2001). In other words, ethylene appears to mediate switching from direct defense to putative indirect defense in response to attack by an adapted herbivore (Kahl *et al.*, 2000).

I conclude that exposure of undamaged plants to ethylene alone is not known to have an impact on the production of herbivore-induced plant defense volatiles (*e.g.* Horiuchi *et al.*, 2001). In Chapter 5, I investigated whether ethylene alone could trigger HIPV production in addition to changes in bulb morphology, two responses that together represent an indirect defense syndrome.

Chapter 5 – Ethylene induces tulip bulbs to attract predatory mites

In this Chapter I discuss the results of a series of experiments where intact uninfested tulip bulbs were exposed to ethylene alone. I tested the preference of the predatory mite *N. cucumeris* when the alternative consisted of odour from infested bulbs or uninfested bulbs previously exposed to either an ethylene inhibitor (MCP) or to ambient air. Using a Y-tube olfactometer I found that odour from bulbs previously exposed to ethylene was more attractive than that from bulbs previously exposed to ambient air or MCP, and equally attractive to odour from bulbs infested by dry bulb mites. Then the volatiles from the bulbs of each treatment was analysed, using TOF gas chromatography and mass spectrometry (GC-TOF-MS). This enabled me to correlate differences in behavioural responses of predatory mites to those of the volatile blends. It was found that bulbs infested by dry bulb mites emit the same volatiles (benzoates and intermediate amounts of toxic tuliposides), as those previously exposed to ethylene, but volatiles that are different from uninfested bulbs, artificially wounded bulbs, MCP-pre-exposed bulbs and ambient-air-pre-exposed bulbs (no benzoates and sometimes very high amounts of tuliposides under artificial wounding).

In summary, I showed that infestation by coconut mites causes coconut palms to increase the distance between the perianth and coconut to an extent that is just sufficient to provide access of predatory mites to the space under the perianth (Chapter 2). This response in the coconuts is possibly associated with the release of HIPV, but this effect was bordering significance (Chapter 3). To investigate the coordination between the herbivore-induced morphological response and the release of HIPV in more detail, I studied another tritrophic system involving predatory mites, dry bulb mites and the below-ground parts (bulbs) of tulips. Here, ethylene induces microscopic changes in the distance between bulb scales to an extent that is just sufficient for predatory mites to enter the inside of the bulb and to control dry bulb mites that would otherwise be in the enemy-free space. I showed that predatory mites are attracted to bulbs infested by dry bulb mites (Chapter 4) and to bulbs previously exposed to ethylene alone (Chapter 5). Moreover, I demonstrated that ethylene alone can induce the tulip bulb to emit volatiles that correspond to those released from bulbs infested by dry bulb mites (Chapter 5).

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PART 1

Above-ground refuge-seeking herbivores in a tritrophic context

c h a p t e r 2

Plant morphological changes induced by herbivory: Do mite-infested coconuts allow predatory mites to move under the perianth?

N.S. Aratchige, M.W. Sabelis and I. Lesna

Being minute in size, eriophyoid mites can reach places that are small enough to be inaccessible to their predators. The coconut mite, *Aceria guerreronis*, is a typical example; it finds partial refuge under the perianth of the coconut fruit. However, some predators can move under the perianth of the coconuts and attack the coconut mite. In Sri Lanka, the phytoseiid mite *Neoseiulus baraki* is the most common predatory mite that is found in association with the coconut mite. This predatory mite is *ca.* 3 times larger than the coconut mite. Nevertheless, taking this predator's flat body and elongated idiosoma into account, it is – relative to many other phytoseiid mites – better able to reach the narrow space under the perianth of infested nuts. On uninfested nuts, however, they are hardly ever observed under the perianth. Prompted by earlier work on the accessibility of tulip bulbs to another eriophyoid mite and its predators, we hypothesized that the nuts change their morphology in response to damage by eriophyoid mites and as a result allow predatory mites to enter under the perianth of infested nuts. This was tested in an experiment where we measured the distance between the perianth and the coconut fruit surface in 3 cultivars (Ordinary Tall, Dwarf Green and Dwarf x Tall hybrid) that are cultivated extensively in Sri Lanka. It was found that the perianth-fruit distance in uninfested nuts was significantly different between cultivars: the cultivar 'Dwarf Green' with its smaller and more elongated nuts had a larger perianth-fruit distance. In the uninfested nuts this distance was large enough for the coconut mite to creep under the perianth, yet too small for its predator *N. baraki*. However, when the nuts were infested by coconut mites, the perianth-fruit distance increased to such an extent that also the predatory mites could move under the perianth.

To reduce the predation risk, some organisms show behavioural or morphological changes that are induced by their predators (Wiackowski and Staronska, 1999; Buskirk and McCollum, 2000; Oku *et al.*, 2003). Some have adapted to find refuge in such a way that predators cannot reach them. Eriophyoid mites have a worm-like body with a very small cross-section diameter (40-100 μm) that allows them to reach concealed plant parts or to live in self-induced, small plant galls where they find protection from biotic and abiotic stresses (Sabelis and Bruin, 1996). The coconut mite, *Aceria guerreronis* Keifer (Acari: Eriophyidae), is adapted to find partial refuge under the perianth of the fruit of the coconut palm (commonly called 'nut' even though this term is inappropriate from a botanical perspective). Here, it feeds on the meristematic tissue of the fruit. Among the predatory mites that have been reported to be associated with the coconut mite in Sri Lanka, *Neoseiulus baraki* Athias-Henriot (Acari: Phytoseiidae) is the most frequently found species (Fernando *et al.*, 2003; Moraes *et al.*, 2004). This species was previously referred to as *N. paspalivorus* (Fernando *et al.*, 2003) but was later confirmed as *N. baraki* (Moraes *et al.*, 2004). It can creep under the perianth due to its flat and elongated idiosoma (Moraes *et al.*, 2004). Although it was not observed with *N. baraki*, some predatory mites have shown behavioural adaptations to creep through narrow spaces. For example, *Hypoaspis aculeifer* Canestrini (Acari: Laelapidae) can stretch its legs and make its body as flat as possible against the surface to creep in between the scales of lily bulbs (I. Lesna, personal observation).

When the coconut mites are outside the perianth they are exposed and vulnerable to predators, but under the perianth of the coconut they face less risk of being eaten. In the absence of natural enemies, coconut mite populations may grow exponentially and the development of the nut will be impaired. Therefore, it can be expected that the coconuts will somehow defend themselves directly against the coconut mites and/or indirectly by promoting the efficiency of predators against these herbivores.

In a different tritrophic system, Lesna, Conijn and Sabelis (2004) found that in response to attack by herbivores concealed

within plant structures, plants change their morphology to provide better access to the natural enemies of their herbivores. These authors have found that when tulip bulbs are attacked from within by the eriophyoid mite *A. tulipae* Keifer (Acari: Eriophyidae), bulbs increase the distance between scales to such an extent that predatory mites can enter the interior of the bulbs. Therefore, in the present study, we hypothesized that, when infested by coconut mites under the perianth, the nuts change the perianth morphology which in turn will give predatory mites better access to the coconut mites.

The perianth functions as a protective cover to the female flower and the tender meristematic zone of the growing nut. In young nuts (*i.e.* 1-2 months after fertilization) the perianth is tightly appressed to the surface of the nut (Howard and Abreu-Rodriguez, 1991), but, as the nut grows, the distance between the perianth and the surface of the nut ('perianth-fruit distance') is increased providing more room for the coconut mite to infest the nut. Tightness of the perianth (Howard and Abreu-Rodriguez, 1991), bract arrangement (Moore, 1986) and shape (Mariau, 1986) of the nut have been shown to affect the susceptibility of nuts to the coconut mites.

Coconut mites usually do not infest the meristematic zone of unfertilized coconut flowers (Mariau and Julia, 1970; Hall and Espinosa-Becerril, 1981; Moore and Alexander, 1987). After fertilization, nuts of all stages are susceptible to mite attack but in general, peak populations occur in 3- to 7-months-old nuts (Moore and Alexander, 1987; Ramaraju *et al.*, 2002; Fernando *et al.*, 2003). To measure the perianth-fruit distance, we used 4-month-old nuts of three cultivars, commonly grown in Sri Lanka. Cultivar 'Dwarf green' (DG) usually has small, elongated nuts, whereas cultivars 'Ordinary Tall' (TT) and 'Dwarf x Tall' hybrid (DT) have larger and more round-shaped nuts. The perianth-fruit distance in infested and uninfested nuts was compared among the three cultivars. Finally, this distance in uninfested and infested nuts was compared with the size of *N. baraki* to make inferences on accessibility of the space under the perianth.

In this chapter, we analyze statistical relations between plant cultivar, plant state (infested, uninfested), perianth-fruit distance and coconut mite density. This analysis has prompted mechanistic studies to reveal how the morphological changes of the nut affect the searching-behaviour of predatory mites, the results of which will be shown in sequel to this chapter (Chapter 3).

Materials and Methods

Perianth-fruit distance measurement

Four-month-old nuts (*i.e.* four months after fertilization) were collected from palms of the three cultivars TT, DG and DT. After bringing the nuts to the laboratory they were split into two halves to remove nut water. This made it easier to dissect the nut into four longitudinal sections across the perianth (Figure 1). Dissected nuts with disturbed perianth structure and loosened fibres at the nut surface were discarded from the measurements.

After splitting nuts into four sections, perianth-fruit distance was measured at two different places on each section (Figure 2). The first measurement (L-1) was made at the rim of the perianth where it touches the nut surface (Figure 2). The edge of each bract of the perianth has two different positions: (1) the edge that directly touches the surface of the nut, (2) the edge that overlaps (or is overlapped by) another bract. Measurements were not taken at the latter position, as it was difficult to dissect the nuts along this position of the bract without disturbing the structure of the perianth. The second measurement (L-2) was taken 1 cm away from position L-1, higher up along the surface of the nut. These two perianth-fruit distances were measured in infested and uninfested nuts.

Mite Census

After measuring the distance between the surface of the nut and the perianth, tepals of each nut were removed to enable counting the number of mites under the perianth. Total number of nymphs and adults of *N. baraki* on the inner perianth as well as the surface of the nut was counted under a stereomicroscope. Number of *A.*

guerreronis was estimated by counting the total number of mites from six randomly selected circular patches (three on the perianth and three on the surface) of 1 cm diameter.

Size of the predatory mites

Predatory mites were kept in a petri dish on wet cotton wool at 3°C for *ca.* 3 hours and then the highest somal height of each mite was measured under the stereomicroscope. During the time in which the measurements were made, the petri dishes were kept on ice to reduce the activity of the mites. Female deutonymphs just before their last moult and adult females (1-day- or more-than-5-days-old since their last moult) were used.

Statistical analysis

The perianth-fruit distance at the four different measurements sites per nut appeared to exhibit much variation. The height of adult female *N. baraki* was larger than the mean value of these four

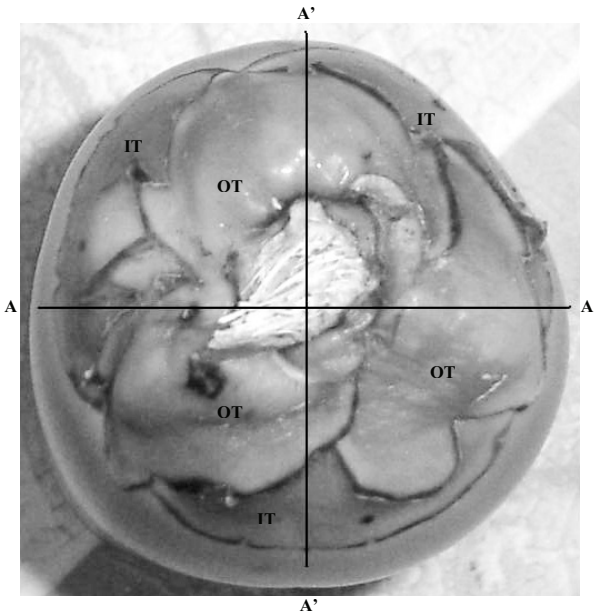

Figure 1 Bract arrangement of the perianth on the nut. Longitudinal sections were taken along A-A and A'-A' lines (see the text for description). OT-Outer tepals of the perianth, IT-Inner tepals of the perianth

measurements in a considerable number of coconuts, yet smaller than the highest perianth-fruit distance measured on the same coconuts. We hypothesized that the predatory mites can find the entrance to the interior of the perianth if there are places with a sufficiently large distance between fruit and perianth. Thus, the mean value of the perianth-fruit distance is less informative if it concerns perianth accessibility to the predatory mite. Therefore, it was assumed that the highest distance observed from data obtained from four places in each nut as the most relevant variable to be taken into account in the data analysis. Generalized Linear Models (GLM) were used to test differences in the perianth-fruit distance between main factors (category of nuts *i.e.* infested and uninfested nuts and cultivar) and to assess the interactions between the main factors. The difference in the density of coconut mites and predatory mites among cultivars were analyzed using one-way ANOVA for log-transformed data. All analyses were carried out using Minitab®, Version 11.

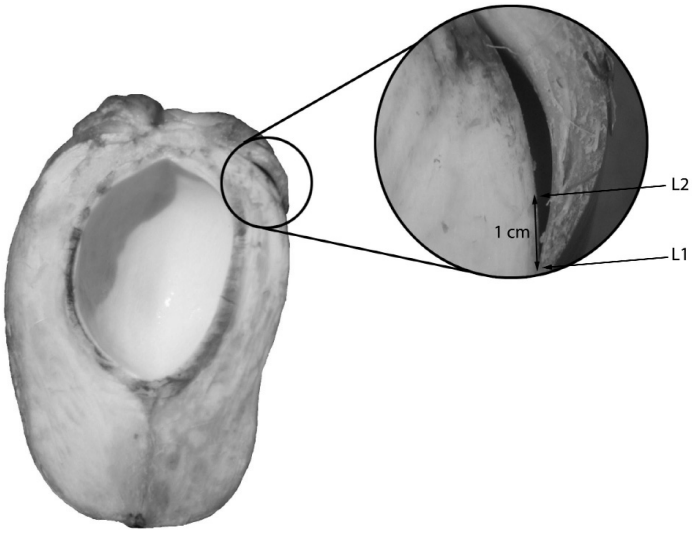

Figure 2 Longitudinal section of a nut showing positions L-1 (Edge of the bract touching the perianth) and L-2 (1 cm above L-1 along the surface of the nut)

Results

The mean of the highest perianth-fruit distances at L-1 and L-2 in infested and uninfested nuts in 3 cultivars are shown in Figure 3. In uninfested nuts these were 41, 68 and 40 µm at L-1 and 39, 78 and 45 µm at L-2 in TT, DG and DT respectively, whereas in infested nuts these were 80, 75 and 99 µm at L-1 and 84, 107 and 94 at L-2 in TT, DG and DT respectively. Thus, irrespective of the cultivar, the perianth-fruit distance at L-1 and L-2 was significantly higher in infested nuts than in uninfested nuts (Table 1). No significant difference was observed in the perianth-fruit distance among cultivars at L-1. However, the perianth-fruit distance was significantly affected by the cultivar at L-2 (Table 1). The interaction between the category of the nut (infested and uninfested) and the cultivar was significant at L-1, not at L-2 (Table 1).

The frequency distribution of nuts over different distance classes showed different patterns in infested and uninfested nuts (Figure 4). At L-1, 79% of the uninfested nuts had a highest perianth-fruit distance less than 100 µm whereas 68% of the infested nuts had a highest perianth-fruit distance exceeding 100 µm (Figure 4). The frequency distribution of perianth-fruit distance at L-2 followed the same pattern as that at L-1 (data not shown). In conclusion, the distance between perianth and surface of the nuts increased dramatically when infested by *A. guerreronis*.

The mean height of the female deutonymphs of *N. baraki* was estimated to be *ca.* 95 µm (n=12). In adult female predatory mites, the mean heights were *ca.* 100 µm in mites 1-day-old after their last moult (n=10) and *ca.*110 µm in mites older than 5 days after their last moult (n=10).

The number of *A. guerreronis* varied from 0 to 6491 with means of 1178, 1302 and 1093 in six circular patches of 1 cm diameter in TT, DG and DT respectively (Figure 5). Their density (per six circular patches of 1 cm diameter) was not significantly different among the infested cultivars (P=0.322 for log-transformed data). The number of *N. baraki* ranged from 0 to 55 with means of 9, 13 and 8 mites per nut in TT, DG and DT, respectively (Figure

5). There was a significant difference in the density of *N. baraki* among infested cultivars (P=0.024 for log transformed data): cultivar DG had a significantly higher density of predatory mites than the other two cultivars.

Discussion

We found that the perianth-fruit distance was different among cultivars TT, DG and DT of the coconut palm. Except at L-1 in infested nuts, among the three cultivars used in our study, cultivar DG had the highest mean distance. In general DG bears smaller and more elongated nuts than the other two cultivars. As observed by Mariau (1977), varieties with smaller nuts are more susceptible to coconut mites than varieties with larger nuts. This may well be because the perianth of the smaller nuts is less firmly attached to the nut, giving mites better access to the space under the perianth. In our study the highest mean number of coconut mites was also found on DG. However, the density of coconut mites did not significantly differ among cultivars. Therefore the results of our study

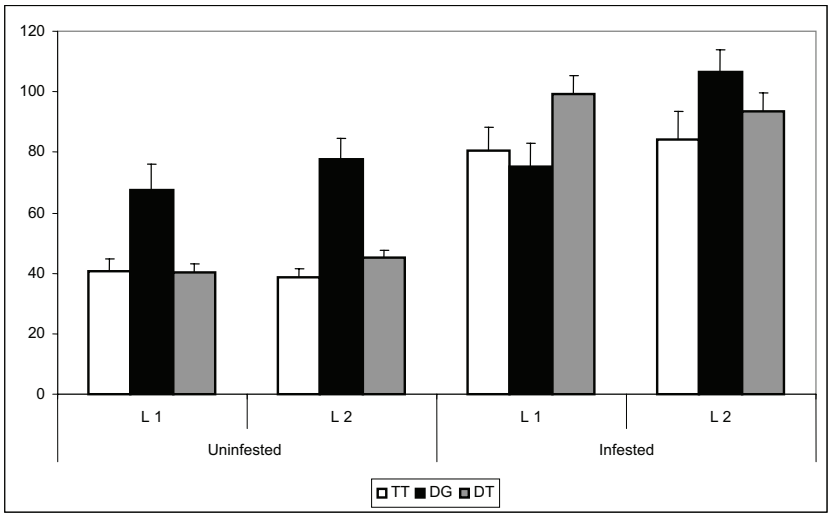

Figure 3 Mean (±SE) of the highest distance at L 1 and L 2 in TT, DG and DT

Table 1 Analysis of variance of distance between the perianth and the surface of the nut

Sources of Variance	*df*	MS	F	P
At L-1				
Category*	1	254670	33.88	<0.001
Cultivar	2	5908	0.79	0.457
Category* x Cultivar	2	40287	5.36	<0.01
Residual	265	7516		
At L-2				
Category*	1	582235	49.27	<0.001
Cultivar	2	56586	6.87	0.001
Category* x Cultivar	2	11831	1.44	0.239
Residual	265	8232		

*Infested and uninfested nuts

did not firmly support the idea that the nuts with larger distance between perianth and the surface of the nut (in other words nuts with loosely attached perianth to the nut) are more susceptible to the coconut mite. It should be noted that the mean highest distance

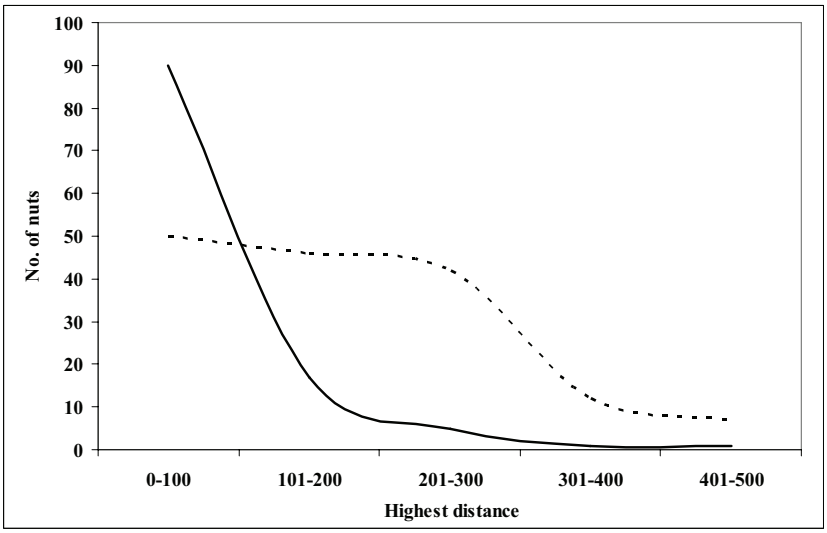

Figure 4 Distribution of the nuts in the highest distance classes in infested (dashed line) and uninfested nuts (solid line)

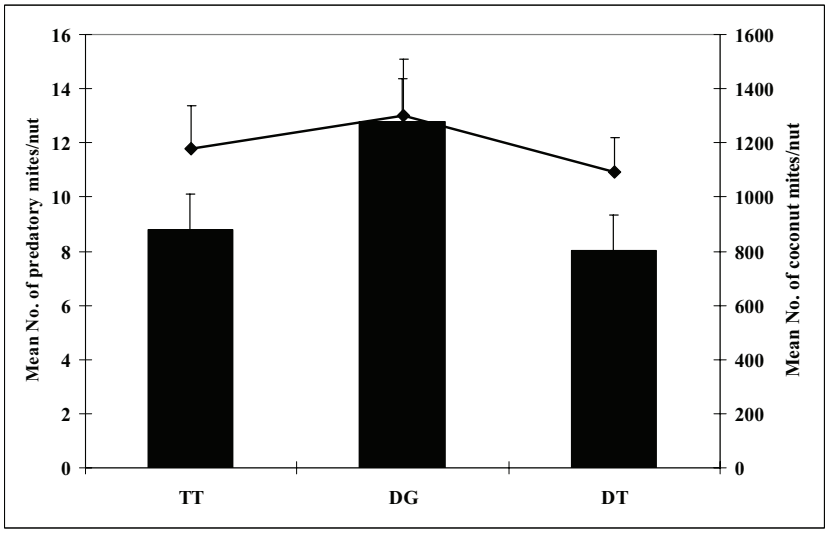

Figure 5 Density of coconut mites (lines) and predatory mites (bars) on cultivars TT, DG and DT

between the perianth and the surface of the uninfested nuts exceeded 40 µm in all three cultivars, which is larger than the cross-diameter of adult female coconut mites (36-52 µm; Keifer, 1965). Hence, the perianth-fruit distance in uninfested nuts of any of the three cultivars is large enough for coconut mites to enter the space under the perianth of the nuts. We conclude that the three cultivars in our study are equally susceptible to coconut mites at least in terms how accessible the sub-perianth space on the nut is to the coconut mites.

The largest perianth-fruit distance of the uninfested nuts was less than 100 µm in 79% of the nuts we observed in our study. However, the average highest somal cross-diameter per adult female *N. baraki* was equal to *ca.* 110 µm. Therefore it is clear that the perianth-fruit distance of most uninfested nuts is not large enough for the females of *N. baraki* to enter the space under the perianth. Thus one can expect that, in this coconut system, the pest organism is well protected from predatory mites. Interestingly, we found that the distance in coconut-mite-infested nuts was signifi-

cantly higher than that in the uninfested nuts. Sixty eight percent of the infested nuts we used in our study had a perianth-fruit distance above 100 µm at L-1 which is large enough for *N. baraki* to creep under the perianth. Thus, when infested by coconut mites, the coconuts increase the distance between the perianth and the surface of the nut to such an extent that the predators can reach their otherwise-concealed prey. Our results are consistent with observations by Lesna, Conijn and Sabelis (2004) on tulip bulbs. When infested by the rust mite, *A. tulipae*, tulip bulbs become attractive to the predatory mite, *N. cucumeris* Oudemans (Acari: Phytoseiidae) (Aratchige *et al.*, 2004, Chapter 4). Having small cross-sectional area, rust mites can easily move into the spaces in between bulbs scales where they are in refuge because the so called 'nose' of the bulbs is too small for predators to move in. However, in response to damage by rust mites, bulbs start to produce ethylene which triggers the bulbs to widen the space in the nose just enough to allow the predatory mites to enter (Lesna, Conijn and Sabelis, 2004). However, the mechanism underlying the morphological changes in the perianth of coconuts in response to coconut mite attack is yet to be elucidated.

For herbivore-induced increase of the perianth-fruit distance to be a successful plant defense strategy, the benefits should exceed the costs. Clearly when the perianth-fruit distance is increased, coconuts are more vulnerable to herbivores that are somewhat larger than eriophyoid mites. This may cause the coconut to invest in the defense against larger herbivores. However, this investment may be offset by allowing a generalist predatory mite like *N. baraki* who would feed on the juvenile stages of other larger herbivores as well.

Herbivore-induced increase in perianth-fruit distance may not only provide the enemies of eriophyoid mites with better access to the space under the perianth, but also the enemies of their enemies. Given that most hyperpredators or intraguild predators are larger (as is the case for most heteropteran predators, ladybeetles and spiders), one may ask whether predatory mites, such as

N. baraki, are safeguarded from their predators under the perianth of an coconut-mite-infested nut. This may be at least partially true, because infested coconuts have a perianth-fruit distance rarely exceeding 300 µm, which is only three times the size of *N. baraki* and clearly smaller than several species of hyper- or intraguild-predators such as *Lasioseius* sp. We therefore hypothesize that the maximum perianth-fruit distance created by the coconut palm represents a compromise between benefits in terms of protection by predatory mites and costs in terms of desiccation and impact of enemies of the predatory mites.

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c h a p t e r 3

How herbivorous mite-induced changes in coconuts affect the searching behaviour of predatory mites

N.S. Aratchige, M.W. Sabelis and I. Lesna

Despite a wealth of studies on how herbivores seek refuge from predation on plants, little is known to what extent predators can overcome the barriers preventing entrance to the refuge. In this study we investigated how plants promote attraction and access of predators to the refuge of their herbivores. The eriophyoid mite, *Aceria guerreronis*, feeds under the perianth of coconuts, which may induce its host plant to produce odours. Experiments with a T-tube olfactometer showed – albeit bordering significance – that female predators of *Neoseiulus baraki* discriminate between an air current carrying odours from nuts infested by coconut mites and an air current with odours from uninfested nuts. Whether the odours are produced by the herbivore, by the plant induced by purely mechanical damage resulting from herbivory or by the plant induced by physiological interaction with the herbivore, remains to be elucidated. Using a release-recapture set-up under still air conditions, it was found that predatory mites did not discriminate between infested nuts over uninfested nuts and between uninfested, perianth-manipulated nuts over uninfested, intact nuts within the period of 1.5 hours. This indicates equal attractiveness of infested, intact-uninfested and manipulated-uninfested nuts.

Of the predatory mites recaptured on the coconuts, a much larger, per-nut fraction was found under the perianth of infested nuts than under the perianth of uninfested nuts. When the perianth structure was manipulated to increase the distance between the perianth and the surface of the nut, the per-nut fraction of predatory mites under the perianth of manipulated nuts was larger than that under the perianth of intact, uninfested nuts. There was no difference between the per-nut fraction of predatory mites under the perianth of infested nuts and that under the perianth of uninfested, manipulated nuts. Evidently, a change in the structure of the perianth, either due to herbivory or due to mechanical manipulation, stimulates

predatory mites on the nut to move under the perianth. The most parsimonious explanation for these results is that the mechanical effect of herbivory induces increased accessibility of the space under the perianth and possibly also increased attractiveness of the meristematic area under perianth. However, a role for plant volatiles induced by physiological interaction between herbivore and plant cannot be excluded.

As a rule, herbivorous arthropods are exposed to a suite of predator species, most of them larger than their prey (Sabelis, 1992). To reduce the risk of being eaten, the herbivores may gain by seeking refuge, *e.g.* in constitutive structures on the host plant or in plant structures induced by the herbivore (*e.g.* galls). However, plants may take countermeasures by avoiding to create such structures, by preventing them from being induced by the herbivore, by making them uninhabitable or – of central interest in this chapter – by making them accessible to the natural enemies of herbivores. Many studies have demonstrated how herbivore-induced morphological changes in plants benefit the inducer (*e.g.* Fukui, 2001; Fournier *et al.*, 2003), but there are virtually no studies testing whether there is a benefit to the plant. As argued by Lesna *et al.* (2004), a possible advantage to the plant may arise when the change in plant morphology causes otherwise predator-free space to become accessible to predators. In this chapter, we study the behaviour of predatory mites in response to herbivore-induced and artificial changes in the structure of the coconut perianth, a plant structure that otherwise encloses refuge space for the eriophyoid mite, *Aceria guerreronis* Keifer (Acari: Eriophyidae).

Members of the family Eriophyidae have characteristics that are ideally correlated with living in confined microhabitats such as galls or erineal growths (Lindquist and Oldfield, 1996). Their vermiform shape and most minute size among the arthropods (Sabelis and Bruin, 1996) enable them to dwell in places, which are generally inaccessible to their predators, mostly phytoseiid mites that are usually six times bigger than eriophyoid mites. The coconut mite (*A. guerreronis*) has a vagrant life style, but it also lives well protected within plant structures, such as under the perianth of the fruit of the coconut

palm (further referred to as 'nut'). Coconut mites outside the perianth are exposed to predators that are usually more mobile than their prey. However, under the perianth, coconut mites cannot be reached by many of these predators since they are larger than their prey. Therefore, in this tritrophic system, the plant would benefit by causing its herbivores to be more exposed to their enemies. The overall objective of the study reported here was to determine the countermeasures taken by the nut to promote the entry of predators into the refuge used by the coconut mite. The specific objectives were to determine the role of herbivore-induced plant volatiles (HIPV) in guiding predators to the site of infestation and changes in the perianth structure of infested nuts that would allow predators better access to the place where the coconut mites cause the damage.

Aceria guerreronis feeds on the meristematic tissue under the perianth of the nut causing considerable damage to the crop (Moore and Alexander, 1987). Several predators have been reported to be associated with the coconut mite from many coconut growing countries in the world (Moraes and Zacarias, 2002). However, in Sri Lanka only a few species of predators have been reported from samples taken under the perianth. *Neoseiulus baraki* Athias-Henriot (Acari: Phytoseiidae) – a species previously described as *N.* aff. *paspalivorus* (Fernando *et al.*, 2003) but later confirmed as *N. baraki* – is by far the most abundant predatory mite (Moraes *et al.*, 2004). Adult females of this species have a flat and elongated idiosoma that enables them to creep under the perianth (Moraes *et al.*, 2004). Previous experiments in the field have revealed that they have a strong temporal relationship with the density of coconut mites (Fernando *et al.*, 2003). However, their prey location behaviour is unexplored and this information is important in understanding the potential impact of this predator on coconut mites. In this prey-predator system, the perianth plays a crucial role, as the prey hides under the perianth of the nut. Therefore, it is important to investigate whether the infested nuts are induced to become attractive to the predatory mite *N. baraki* and whether they alter their perianth morphology so as to promote access of this predator to the coconut mites under the perianth.

In this study, we first investigated whether *A. guerreronis*-induced odours from nuts elicit a response of *N. baraki* in a T-tube olfactometer. Then we studied the prey location behaviour of this predatory mite in a more complex environment where predators were released at the centre of a square with nuts at its corners. This set-up served to test whether prey-related odours from infested nuts increase the attraction of predators in a more complex environment than in a T-tube olfactometer. To eliminate the effect of light on the response of predators to the nuts, a release-recapture experiment was conducted in a closed setup. The distribution of *N. baraki* under and outside the perianth of the infested and uninfested nuts was assessed in the release-recapture experiments, to study whether nuts allow predators into the place where the prey herbivores find their refuge. In a previous study, it was observed that the distance between the perianth and the surface of the nut is increased in infested nuts (Chapter 2). How these morphological changes influence the predatory mite, *N. baraki*, to reach the space under the perianth is investigated here by assessing the effect of artificial and herbivore-induced modification of the perianth structure.

Materials and Methods

Predatory mites

Neoseiulus baraki, collected from infested nuts in the field, was reared on black plastic sheets placed on plastic foam pads saturated with water in a plastic box. Wet tissue papers along the periphery of the plastic sheet prevented the escape of the predators and provided them with drinking water. Three such boxes were maintained in a tray with water and covered with another tray to provide high humidity and shade. Cultures were maintained in an incubator at *ca.* 25°C. Coconut mites were provided as food on every second day.

Odour sources

Young (*i.e.* 3 months after fertilization) nuts of the cultivar Tall x Tall (TxT) were used as the odour sources. Nuts that were 3

months old were specifically selected because they usually contain a substantial number of coconut mites, and no (yet sometimes a few) predatory mites (Fernando *et al.*, 2003).

Olfactometer experiments

Response of predatory mites to volatiles released by nuts was studied in an olfactometer which consisted of two glass tubes (each 12 cm long) connected by a plastic tube in the form of a T (Figure 1). A glass capillary tube, which enabled the predatory mites to walk on it, was held inside the glass tubes and served to 'railroad' the predatory mites. The base of the plastic tube was connected to a vacuum pump, causing air to move through the glass tubes to the base of the T-shaped plastic tube. Two identical glass jars that contained odour sources were connected to two small plastic containers via plastic tubes. The air that entered the glass jars was filtered through charcoal. The plastic containers had wet filter paper on

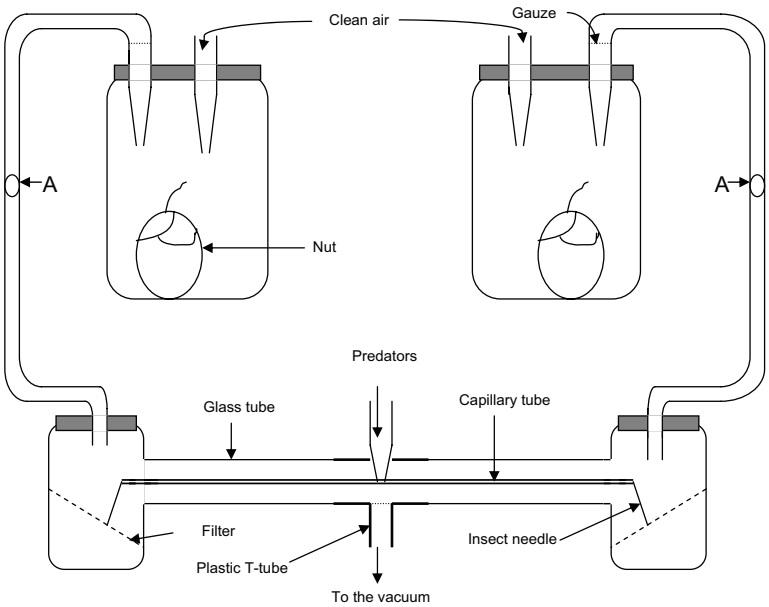

Figure 1 Set-up of the T-tube olfactometer. A: Holes where hot-wire anemometers were inserted into plastic tubes (during the experiments holes were covered with parafilm)

cotton wool at the bottom and they were connected to the glass tubes. At the end of each glass tube, an insect pin (bent at *ca.* 90°) was connected to the capillary tubes. The insect pin was used to hold the capillary tube in position as well as to direct the predatory mites from the capillary tube to the center of the wet filter paper. The air speed inside the olfactometer was approximately 0.5 m/s as measured by a hot-wire anemometer inserted in-between the plastic containers and glass jars (see Figure 1). Predatory mites were starved in a pipette tip for 4 hours before start of the experiment. When they were introduced by connecting the pipette tip to the center of the T-shaped plastic tube that connected the two glass tubes, they started to move on the capillary tube towards the end of a glass tube and entered the wet filter paper in the plastic container via the insect pin. Thirty minutes after the introduction of the predatory mites into the set up, the plastic containers were disconnected from the glass tubes and the number of mites on the wet filter paper in each plastic container was counted. One infested nut in one glass jar and one uninfested nut in the other glass jar served as the odour sources in each replicated experiment. In total, three replicate experiments (on three consecutive days) were carried out, each with 30 predatory mites. Different sets of predatory mites and odour sources were used in each replicate experiment. At the start of each replicate experiment, odour sources were interchanged between the left and right arm of the T-tube. In this way, the overall response of the predatory mites towards the odours is less affected by unforeseen directionality in the environment outside the T-tube.

Release-recapture experiments

Three-months old nuts were arranged at the four corners of an arena consisting of 30 x 30 cm black plastic sheet placed on plastic foam. The arena was positioned in a plastic tray and wet tissue paper strips that were in contact with the water surrounding the arena were laid along the periphery of the plastic sheets to prevent the predators from escaping. Predatory mites, starved for 6 hours prior

to the experiments, were introduced in a Petri dish (ø 3.5 cm) at the centre of the arena. Nuts were destructively sampled 1.5 hours after the introduction of predatory mites. The number of predatory mites on each nut, under and outside the perianth was counted. Presence of predators on the nuts prior to the experiments would lead to overestimation of the response and should therefore be avoided. This could be solved by marking the released, adult female predators, but this appeared far too laborious. A practically feasible solution to this problem was to inspect the nuts destructively after the experiment and to discard replicate experiments from further analysis in case they contained nuts with any juvenile stages or males of predatory mites. In addition, replicate experiments with less than 100 coconut mites per nut were excluded from the analysis in all tests. To exclude the possibility that the introduced predatory mites move under the perianth of the nuts to avoid the light, the tray with the arena was covered with another tray to create darkness inside. The number of mites on the nuts was counted after a fixed time since the introduction of the mite but not by continuous observation because this would require a light source.

Following the above procedure, three experiments were conducted with different odour sources: (1) Infested nuts versus uninfested nuts, (2) Uninfested, manipulated nuts versus intact, uninfested nuts, and (3) Manipulated nuts versus infested nuts. Nuts were called 'manipulated' when the distance between the perianth and the surface of the nut was increased by carefully inserting three insect pins at three different positions in between the perianth and the surface of each nut. Care was taken not to damage the meristematic tissue under the perianth while inserting the pins.

Each experiment was replicated four times with a different set of nuts and group of mites on different days. Sixty females that aged 3-7 days since their last moult were used in each replicate experiment. In each replicate experiment the two types of nuts were arranged alternately at the four corners of the arena. The position of different types of nuts used in these experiments was interchanged in each replicate experiment to prevent effects of any

unforeseen asymmetry in the set-up. For example, infested nuts were positioned on top-left and bottom-right positions in the first two replicate experiments and on top-right and bottom-left positions in the subsequent replicate experiments. All experiments were conducted in a room at *ca.* 20°C.

Statistical analysis

For the T-tube olfactometer experiments, data were analyzed using a replicated goodness-of-fit test (G-statistics) against the null hypothesis of 1:1 (Sokal and Rohlf, 1997). To test whether predatory mites can distinguish between different odour sources in release-recapture experiments, first the data was subjected to a replicated goodness-of-fit test (G-statistics) against the null hypothesis of 1:1 distribution for the total number of mites (under the perianth + outside the perianth) that were recaptured on nuts. Then a chi-square analysis was used to test the distribution of predatory mites under the perianth of nuts that received different treatments. In addition, a replicated G-test was carried out to test whether the predatory mites distribute themselves randomly over the nut. Under this null hypothesis we expect 30% of the predators under the perianth, since this structure covers *ca.* 30% of the nut surface. Rejection of this null hypothesis indicates a preference to reside under or outside the perianth. The test was carried out on the predators recaptured on the two equally treated nuts together in each replicate experiment.

Results

In the olfactometer experiments, 63% of predatory mites out of the total recovered were collected on the wet filter paper in the plastic container connected to the glass jar with infested nuts (Table 1). None of the replicate experiments was significantly different from a 1:1 ratio. Heterogeneity was not observed among the replicate experiments which allowed for pooling of the data. The pooled results showed a deviation that was bordering significance (Table 1).

Table 1 Results of T-tube olfactometer tests and replicated goodness-of-fit tests for the responses of *N. baraki* to infested (+) and uninfested (-) nuts; *n*=number of predators to (+), (-) or none (0); *N*=*n*(+)+*n*(-)+*n*(0)

Olfactometer tests				
	n(+)	*n*(-)	*n*(0)	*N*
Replicate 1	13	7	10	30
Replicate 2	11	5	14	30
Replicate 3	11	9	10	30
Total	35	21	34	90
Replicated goodness-of-fit tests				
Source	df	G-statistics	Critical value	
Replicate 1	1	1.82	0.18 (ns)	
Replicate 2	1	2.30	0.13 (ns)	
Replicate 3	1	0.20	0.65 (ns)	
Pooled	1	3.54	0.06	
Heterogeneity	2	0.77	0.68 (ns)	
Total	3	5.03	0.17 (ns)	

In the release-recapture experiments 69% of mites were recaptured on nuts when they were offered infested and uninfested nuts as the odour source (Table 2). The number of mites recaptured on infested and uninfested nuts was not significantly different from a 1:1 ratio; on average 53% of mites were recaptured on infested nuts (Table 2). Of those recaptured on infested nuts, 52% was recaptured under the perianth (Figure 2) whereas, of those recaptured on uninfested nuts only 3% of predatory mites were collected under the perianth (Figure 2). Chi-square analysis of the data showed that the fraction of mites recovered under the perianth of infested nuts is significantly different from that of uninfested nuts (χ^2=50.288, df=1, P<0.001). A replicated G-test was conducted to assess whether the predatory mites are distributed randomly over the surface of the nut. This showed that the fraction of predators under the perianth of infested nuts is higher than the 0.3 fraction expected under the null hypothesis (Table 5).

Table 2 Results of release-recapture tests and replicated goodness-of-fit tests for the responses of *N. baraki* to infested (+) and uninfested (-) nuts; *n*=number of predatory mites* to (+), (-) or none (0); *N*=*n*(+)+*n*(-)+*n*(0)

Olfactometer tests				
	n(+)	*n*(-)	*n*(0)	*N*
Replicate 1	19	21	20	60
Replicate 2	23	16	21	60
Replicate 3	25	19	16	60
Replicate 4	21	23	16	60
Total	88	79	73	240
Replicated goodness-of-fit tests				
Source	df	G-statistics	Critical value	
Replicate 1	1	0.10	0.75 (ns)	
Replicate 2	1	1.26	0.26 (ns)	
Replicate 3	1	0.82	0.36 (ns)	
Replicate 4	1	0.09	0.76 (ns)	
Pooled	1	0.49	0.49 (ns)	
Heterogeneity	3	1.79	0.62 (ns)	
Total	4	2.27	0.69 (ns)	

*Total number of predatory mites under and outside the perianth of the nut

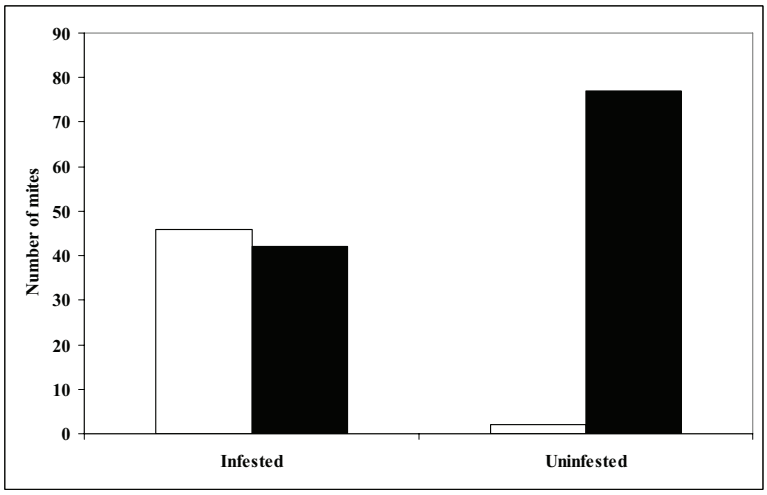

Figure 2 Distribution of recaptured mites under the perianth (open bars) or on the nut surface (solid bars) of infested and intact, uninfested nuts in release-recapture experiments

Table 3 Results of release-recapture tests and replicated goodness-of-fit tests for the responses of *N. baraki* to uninfested, manipulated (+) and uninfested, intact (-) nuts; *n*=number of predatory mites* to (+), (-) or none (0); $N=n(+)+n(-)+n(0)$

Olfactometer tests				
	n(+)	*n*(-)	*n*(0)	*N*
Replicate 1	17	14	29	60
Replicate 2	22	18	20	60
Replicate 3	20	16	24	60
Replicate 4	16	9	35	60
Total	75	57	108	240
Replicated goodness-of-fit tests				
Source	df	G-statistics	Critical value	
Replicate 1	1	0.29	0.59 (ns)	
Replicate 2	1	0.40	0.53 (ns)	
Replicate 3	1	0.46	0.50 (ns)	
Replicate 4	1	1.99	0.16 (ns)	
Pooled	1	2.46	0.12 (ns)	
Heterogeneity	3	0.66	0.88 (ns)	
Total	4	3.12	0.54 (ns)	

*Total number of predatory mites under and outside the perianth of the nut

When uninfested nuts with manipulated perianths and uninfested intact nuts were used in the release-recapture experiments, 55% of the released predatory mites were recaptured on the nuts (Table 3). Fifty seven percent of recaptured predatory mites were collected on manipulated nuts, but this did not differ from a 1:1 ratio (Table 3). Of the recaptured mites on manipulated nuts 48% were collected under the perianth while it was only 2% in intact nuts (Figure 3). According to the results of the chi-square analysis, there is a significant difference between the fraction of mites collected under the perianth of manipulated nuts and that of uninfested intact nuts ($\chi^2=26.3$, df=1, $P<0.001$). Results of the replicated G-test for the distribution of predatory mites over the surface of the nut showed that the fraction of predators under the perianth is significantly higher than the 0.3 fraction expected under the null hypothe-

Table 4 Results of release-recapture tests and replicated goodness-of-fit tests for the responses of *N. baraki* to uninfested, manipulated (+) and infested (-) nuts; *n*=number of predatory mites* to (+), (-) or none (0); $N=n(+)+n(-)+n(0)$

Olfactometer tests				
	n(+)	*n*(-)	*n*(0)	*N*
Replicate 1	18	18	24	60
Replicate 2	20	21	19	60
Replicate 3	18	14	28	60
Replicate 4	12	25	23	60
Total	68	78	94	240
Replicated goodness-of-fit tests				
Source	df	G-statistics	Critical value	
Replicate 1	1	0.0	0.10 (ns)	
Replicate 2	1	0.02	0.88 (ns)	
Replicate 3	1	0.50	0.48 (ns)	
Replicate 4	1	4.67	0.03	
Pooled	1	0.69	0.41 (ns)	
Heterogeneity	3	4.51	0.21 (ns)	
Total	4	5.19	0.27 (ns)	

*Total number of predatory mites under and outside the perianth of the nut

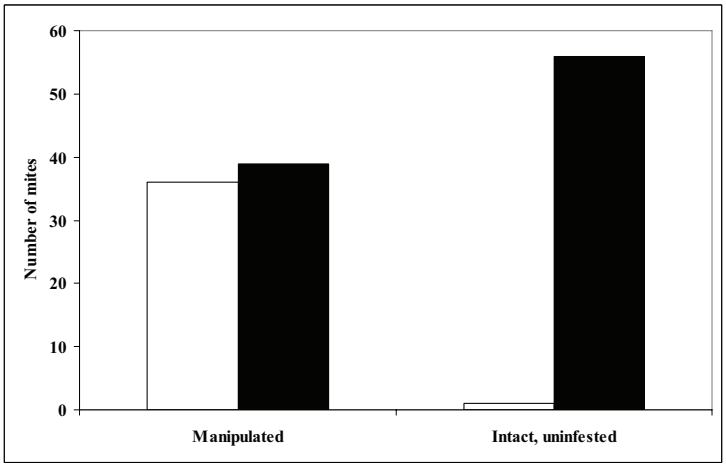

Figure 3 Distribution of recaptured mites under the perianth (open bars) or on the nut surface (solid bars) of manipulated nuts and intact, uninfested nuts

Table 5 Results of the per-nut distribution of predatory mites and replicated goodness-of-fit tests for the distribution of predatory mites in under the perianth (+) and outside the perianth (-) of infested, uninfested-intact nuts and uninfested-manipulated nuts; *n*=number of predatory mites to (+) or (-); *N*=*n*(+)+*n*(-)

	Olfactometer tests			*Replicated goodness-of-fit test*		
	n(+)	*n*(-)	*N*	df	G-statistics	Critical value
Infested nuts						
Replicate 1	14	5	19	1	15.1	0.0001
Replicate 2	11	12	23	1	3.0	0.08
Replicate 3	11	14	25	1	1.98	0.16
Replicate 4	10	11	21	1	2.68	0.10
Pooled				1	18.07	<0.0001
Heterogeneity				3	4.71	0.19
Total	46	42	88	4	22.8	0.0001
Uninfested-intact nuts						
Replicate 1	0	21	21	1	-	-
Replicate 2	0	16	16	1	-	-
Replicate 3	2	17	19	1	4.15	0.04
Replicate 4	0	23	23	1	-	-
Pooled				1	41.2	<0.0001
Heterogeneity				3	-	-
Total	2	77	79	4	-	-
Uninfested-manipulated nuts						
Replicate 1	9	8	17	1	3.71	0.05
Replicate 2	9	13	22	1	1.02	0.31
Replicate 3	12	8	20	1	7.46	0.006
Replicate 4	6	10	16	1	0.31	0.58
Pooled				1	10.0	0.001
Heterogeneity				3	2.49	0.48
Total	36	39	75	4	12.5	0.01

sis (Table 5). Thus, the predators tend to aggregate under the perianth of uninfested-manipulated nuts. However, results of the replicated G-test on the mites distributed on uninfested-intact nuts reveal that the predatory mites are distributed outside the perianth more than under the perianth of uninfested-intact nuts (Table 5).

When infested nuts and manipulated nuts were offered as the odour sources in release-recapture experiments, in total 60% of the released mites were recaptured, out of which 46% was from manipulated nuts which was not significantly different from 1:1 (Table 4). Of recaptured mites on manipulated nuts 56% were collected under the perianth while 51% of recaptured mites on infested nuts were collected under the perianth (Figure 4). Chi-square analysis of these data showed that fraction of mites recaptured under the perianth of manipulated nuts is not significantly different from that of infested nuts (χ^2=0.428, df=1, P=0.5).

Discussion

The results of this study indicate that females of the predatory mite, *N. baraki,* prefer odours from nuts infested by *A. guerreronis* when odours from uninfested nuts were the alternative. The alternative interpretation that they avoid uninfested nuts and therefore end up moving to odours from infested nuts cannot be rejected based on the experiments presented here, but seems less likely given the predator's interest to find prey.

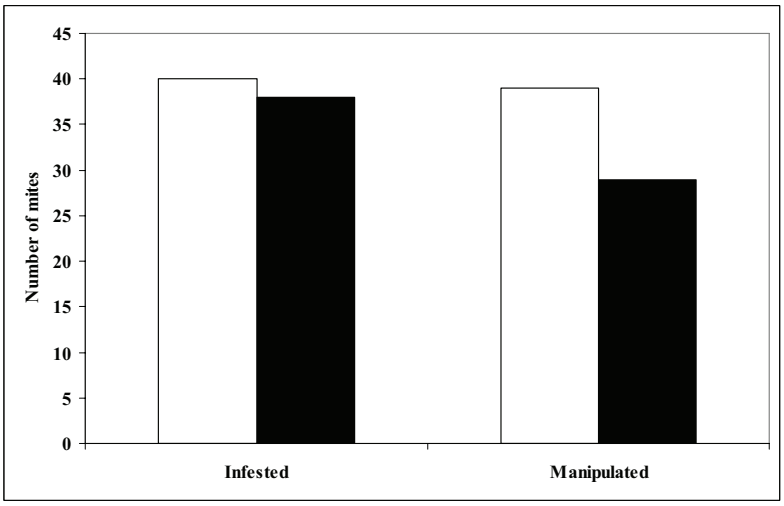

Figure 4 Distribution of recaptured mites under the perianth (open bars) or on the nut surface (solid bars) of infested and uninfested, manipulated nuts

Our olfactometer setup differed from that of Sabelis and van de Baan (1983) in that groups of predatory mites were released at the base tube and collected on wet tissue paper. This greatly facilitated assessment of the choices made by the sluggish and whitish *N. baraki* mites in our study. However, it could be argued that in this setup, the behaviour of one individual mite is influenced by other individuals. If this were the case, it is hard to understand why most mites move towards the odour from infested nuts in all replicate experiments: any of the firstly tested predatory mites that would accidentally make another first choice would then trigger others to follow. Therefore, we believe that the behaviour of individual mites used in this study is independent from that of the others and that they are attracted to the odours from infested nuts.

Based on the data presented in this Chapter it is not possible to determine whether the attractive odours originate from the herbivore alone, the host plant alone or due to mechanical and/or physiological interaction between the herbivore and the host plant. This would have required testing the response to artificially wounded nuts in the T-tube olfactometer. Unfortunately, such a treatment is not feasible. Coconut mites feed on the meristematic tissue under the perianth of the nut and it is not possible to simulate this damage at the site where the mites cause damage to the nut without removing the perianth. However, removal of the perianth itself causes damage to the nut. Hence, one can never be sure whether the odours result from removing the perianth or from the artificial damage inflicted under the treatment. Therefore, we did not conduct experiments to elucidate the origin of odours using artificially wounded nuts.

In contrast to the results from our T-tube experiments, infested nuts were not attractive to the predatory mites in release-recapture experiments with four equidistant coconuts under still air conditions; predatory mites were homogeneously distributed over infested and uninfested nuts. In the natural situation in the coconut palm tree, nuts are borne close together at the base of the long peduncle of the leaves and this configuration resembles that in the

release-recapture experiment. Hence, wind-borne mites land more likely on leaves than directly on nuts. Once they are on leaves, the volatiles may play a key role in informing the predatory mites whether to leave the palm and become wind-borne again or to move towards the cluster of nuts that are either infested or uninfested. This situation is reflected in our release-recapture experiments and may explain why predatory mites do not distinguish between infested and uninfested nuts within clusters, whereas they do discriminate – as in the T-tube – at a larger spatial scale within the palm tree.

The most striking observation in the release-recapture experiments is the per-nut distribution of predatory mites under the perianth of infested and uninfested nuts. The number of predatory mites per unit area was higher under the perianth than outside the perianth in infested and artificially manipulated nuts. It could be hypothesized that the predatory mites move under the perianth in search of food or shelter. From our experiments search for shade is excluded as a possibility because the experiments were conducted under dark conditions. Out of the total number of mites recaptured on the infested nuts, 52% were recaptured under the perianth of infested nuts, whereas only 3% of the mites recovered from uninfested nuts had moved under the perianth. This prompted us to hypothesize that the coconut, when infested, somehow allowed the predatory mites to move under the perianth. In a separate study it was observed that the distance between the perianth and the surface of the nut is increased in response to infestation by coconut mites (Chapter 2), thereby giving the predatory mites better access to the space underneath the perianth and promoting the clean up of herbivorous mites that would otherwise be enemy-free. When this was experimentally simulated by inserting insect needles in between the perianth and the surface of the nut, more predatory mites were recaptured under the perianth of manipulated nuts than under the perianth of uninfested intact nuts. This observation was corroborated in another experiment showing that coconut mites were more or less equally abundant under manipulated and infest-

ed perianths. We conclude that the space under the perianth plays a major role as a refuge, but that the infested plant modifies exposure of herbivores under the perianth to their enemies by altering the structure of the perianth and perhaps also the attractiveness of the space under the perianth.

Studies on tulip bulbs provide a good example of the active role of plants changing their morphology so as to expose herbivorous mites to their predators (Lesna *et al.*, 2004). Dry bulb mites (*Aceria tulipae* Keifer, Acari: Eriophyidae) find refuge in between the scales of tulip bulbs because they are very tiny in size and can move inside the bulb via the so called 'nose' of the bulb. In response to dry bulb mite infestation, bulbs produce ethylene which increases the distance between bulb scales in the nose, thereby allowing predatory mites to move in. For coconuts, however, the causes of changes in the perianth structure upon the infestation by coconut mites are not yet clear. We suspect, however, that ethylene is among the candidate plant hormones regulating herbivore-induced plant morphological changes.

Based on the results of this experiment it is hard to conclude whether the increase in distance between the perianth and the surface of the nut is brought about directly by the coconut mite (for its own benefit) or whether it is a response shown by plants (for their benefit) as a response to herbivore damage. As measured at the rim of the perianth that touches the nut surface, the average distance between the perianth and the surface of the uninfested nuts of the cultivar TxT was 41 μm (Chapter 2), which is just enough for the coconut mites to creep under the perianth, yet too small for *N. baraki*. Therefore, it is not reasonable to expect the coconut mite to increase the distance between the perianth and the surface of the nut because this would make them more exposed to the predators. The plant seems therefore a more likely candidate for modifying its perianth structure in response to herbivory. It remains to be elucidated whether this effect results from purely mechanical (hence unspecific) damage by the herbivore or from physiological (possibly more specific) interaction between the her-

bivore and the plant (*i.e.* the meristematic tissue under the perianth). Moreover, we do not yet know whether the plant's response to herbivory are local or systemic (*i.e.* extending to other nuts on the same tree). Much of the same questions can be posed with respect to the mechanical and/or physiological factors that elicit the plant's attractiveness to predatory mites.

We hypothesize that plants that harbour herbivores in refuges coordinate different indirect defense mechanisms, namely by releasing volatile signals to attract predators and by establishing morphological changes that increase accessibility to the plant-based refuges. Increased distance under the perianth may allow larger herbivores to enter the space under the perianth and the meristematic region of the nut to desiccate. However, by allowing the predators to enter under the perianth of infested nuts where the herbivores do the damage (and by not allowing them under the perianth of uninfested nuts), coconut palms may be promoting the impact of natural enemies on their herbivores, thus increasing the overall vigor of the plant. Desiccation of the meristematic region may have been minimized by limiting the increased distance between the perianth and the surface of the nut just enough to accommodate access of the predatory mites under the perianth (Chapter 2).

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PART 2

Below-ground refuge-seeking herbivores in a tritrophic context

c h a p t e r 4

Below-ground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites

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Although odour-mediated interactions among plants, spider mites and predatory mites have been extensively studied above-ground, below-ground studies are in their infancy. In this paper, we investigate whether feeding by rust mites (*Aceria tulipae*) cause tulip bulbs to produce odours that attract predatory mites (*Neoseiulus cucumeris*). Since our aim was to demonstrate such odour and not their relevance under soil conditions, the experiments were carried out using a classic Y-tube olfactometer in which the predators moved on a Y-shaped wire in open air. We found that food deprived female predators can discriminate between odours from infested bulbs and odours from uninfested bulbs or artificially wounded bulbs. No significant difference in attractiveness to predators was found between clean bulbs and bulbs either wounded 30 minutes or 3 hours before the experiment. These results indicate that it may not be simply the wounding of the bulbs, but rather the feeding by rust mites which causes the bulbs to release odours that attract *N. cucumeris*. Since bulbs are below-ground plant structures, the olfactometer results demonstrate the potential for odour-mediated interactions in the soil. However, their importance in the actual soil medium remains to be demonstrated.

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Plants defend themselves in two ways against herbivore attack (Price *et al.*, 1980): (1) directly, for example, by producing secondary plant metabolites such as toxins, repellents and antifeedents, and (2) indirectly, for example, by providing food and shelter for members of the third trophic level, such as predators and parasites. It is now well established that indirect plant defense can

also be mediated by the release of herbivore-induced plant volatiles (HIPV) and that these alarm chemicals influence the foraging behaviour of carnivorous arthropods at the third trophic level (Sabelis and van de Baan, 1983; Dicke and Sabelis, 1988; Dicke *et al.*, 1990; Turlings *et al.*, 1991; Drukker *et al.*, 1995; Shimoda *et al.*, 1997; Dicke and Vet, 1999; Sabelis *et al.*, 1999; Kessler and Baldwin, 2001). First proof that the volatiles are indeed of plant origin came from experiments showing that they are produced not only in infested leaves but are also systemically induced in uninfested leaves (Turlings and Tumlinson, 1992; Dicke *et al.*, 1993). The elicitor for HIPV production is now proven to be in the saliva (Turlings *et al.*, 1993; Paré *et al.*, 1998).

Evidence for HIPV as part of indirect defense comes largely from studies on above-ground plant parts. The possibility of below-ground plant parts emitting chemical signals upon damage by herbivores has largely been ignored. Recent evidence showing role of HIPV for below-ground plant predator interactions comes from a study on thuja roots, root weevil and entomophagous nematodes (van Tol *et al.*, 2001) and another one on turnip roots, cabbage fly larvae and an endoparasitoid (Neveu *et al.*, 2002). Apart from roots, there are other below-ground plant parts and one may wonder whether they are also active in releasing HIPV after herbivore attack. We studied the interaction between tulip bulbs (*Tulipa* sp.), a herbivorous rust mite (the so called dry bulb mite, *Aceria tulipae* Keifer, Acari: Eriophyidae) and a plant- and litter-inhabiting predatory mite *Neoseiulus cucumeris* Oudemans (Acari: Phytoseiidae) (Conijn *et al.*, 1996). This system has several features that make it quite distinct from the other below-ground systems studied so far. First, predatory mites are relatively more mobile than entomophagous nematodes and unlike the adult endoparasitoids they live in the upper part of soil, especially the litter layer (Moraes *et al.*, 1986; Lesna, personal observation). Second, the herbivorous rust mite has a minute size and a worm-like body, which allows them to live hidden between bulbs scales thus making them less exposed to natural enemies (Sabelis, 1996; Sabelis and Bruin, 1996). But, if the

rust mites are feeding on the outer scale of the bulb, they are just as exposed to natural enemies, as other root feeding arthropods are. Compared to the soil systems studied earlier, these differences in mobility (of predators versus herbivore) and accessibility (to sites occupied by herbivore) may give rise to plant-predator interactions that operate at a different spatial scale. In this article, we do not focus on the effective scale, but rather on the ability of predatory mites to respond to chemical signals that tulip bulbs produce in response to herbivore attack.

Materials and Methods

Rearing of predatory mites

A base culture of *N. cucumeris* for further rearing was obtained from Mitox Consultants BV (Amsterdam, the Netherlands). This culture was reared on black, plastic tiles on foam pads saturated with water in a plastic tray. Wet tissue papers along the periphery of the black plastic tile served to prevent the predators to escape and to provide them with drinking water. Tanglefoot® applied along the periphery of the tissue papers acted as a second-line barrier to escape. Both the filter paper and the tangle foot also helped preventing invasions of other arthropods. Cultures were kept in a climate room at *ca.* 25°C and 60% RH. A mixture of pollen from broad bean, walnut and apple was used to feed the predatory mites.

Tulip bulbs

Uninfested tulip bulbs *cv.* 'Yokohama', stored for 5-7 months at the Bulb Research Centre (Lisse, the Netherlands), were kept separately at 5°C and 60% RH. Next, they were brought to the laboratory where they were kept for 1-2 weeks at 17°C and 70% RH, until they were used in the experiments. Infested bulbs were obtained from the uninfested stock by placing clean bulbs next to infested ones in a tray for 3 weeks at 20°C and 60% RH. Subsequently, they were brought to the laboratory where they were kept for 1-2 weeks at 17°C and 17% RH until use in the experiments.

Olfactometry

To study the response of adult female predators to an odour, a Y-tube olfactometer was used consisting of a glass tube in the form of a Y with a white Y-shaped metal wire in the middle to railroad the mites (Sabelis and van de Baan, 1983). The base of the Y-tube was connected to an air pump that produced a unidirectional air-flow from the arms to the base of the Y-tube. The arms were connected via a plastic tube to two identical plastic boxes containing one odour sources each. The boxes had an inlet and outlet covered with gauze. The air speed inside the arms of the olfactometer was equal to 0.5±0.03m/s as measured by a hot wire anemometer inserted in-between the air outlet of the box and the arms of the olfactometer. When the air flow in the both arms was equal, the odour plumes formed two neatly separated fields in the base of the Y-tube, with the interface coinciding with the metal wire (Sabelis and van de Baan, 1983). After disconnecting the air pump from the base of the Y-tube, an adult female predator starved for 3 hours before start of the experiment was introduced onto the metal wire. When the pump was connected, the predatory mite usually started walking upwind to the junction of the wire, where it was given 5 minutes to decide to move into either of the two arms. If no decision was made, it was removed and a new female was introduced. After a series of five mites, the odour sources were interchanged to correct for any unforeseen asymmetry in the experimental setup. Each experiment was replicated 4-6 times (4-6 consecutive dates) with 20-25 individuals per replicate. Fresh odour sources were used in each replicate. The age of the test mites varied between 2 and 10 days after their last moult.

Following the procedure described above, three experiments were carried out that differed in the odour sources offered to the predators in the two boxes: (1) eight infested bulbs versus eight uninfested bulbs (2) eight artificially wounded, uninfested bulbs versus eight uninfested bulbs (3) eight infested bulbs versus eight artificially wounded, uninfested bulbs. Artificial wounding was achieved by 100 needle punctures through the outer scale of the

bulbs, taking place either 30 minutes (as in experiment 2) or 3 hours (as in experiment 2 and 3) preceding the experiment. To make sure that the infested bulbs were exclusively damaged by rust mite, the interior of the bulbs had to be inspected (destructively) for the presence of other arthropod species after the olfactometer experiment. It appeared that some bulbs were infested with *Tyrophagus putrescentia*e Schrank (Acari: Acaridae), an astigmatic mite frequently found in stored products and bulbs. The replicates that had bulbs contained with *T. putrescentiae* or less than 30 rust mites per bulb, were excluded from further analysis.

Statistical analysis

To enable testing for heterogeneity among replicates in each experiment, we first applied a replicated goodness-of-fit test (G-statistics) against a 1:1 null hypothesis (Sokal and Rohlf, 1997). Differences between treatments were tested in pair-wise comparisons among pooled results, using 2x2 contingency tables and G-test of independence. Females that did not make a choice within 5 minutes were not included in this two-step analysis. However, they were used separately in a 2x3 test of independence to see whether the fraction of non-responders is independent of the treatment.

Results

When tulip bulbs infested by rust mites were offered in one arm of the olfactometer (Table 1), 61% out of the total 111 predatory mites moved into the arm connected to the box with infested bulbs. Where as the pooled results showed this to be a significant deviation from 1:1 ratio, only two out of six replicate experiments were significantly different. However, the replicate experiments did not show significant heterogeneity, which can therefore not explain the significant deviation of the overall response from the ratio expected under the null hypothesis (Table 1). We interpret this result as either a preference for odour emanating from rust-mites infested bulbs or avoidance of odour from uninfested bulbs.

Table 1 Results of olfactometer tests and replicated goodness-of-fit tests for the responses of *N. cucumeris* to infested (+) and uninfested (-) bulbs; *n*=number of predators to (+), (-) or none (0); $N=n(+)+n(-)+n(0)$

Olfactometer tests				
Bulb treatment	*n*(+)	*n*(-)	*n*(0)	*N*
Infested vs. uninfested				
	13	7	0	20
	14	5	1	20
	14	5	1	20
	9	8	3	20
	11	7	2	20
	12	6	2	20
Total	73	38	9	120
Replicated goodness-of-fit tests				
Source	df	G-statistics	Critical value	
Replicate 1	1	1.82	0.18 (ns)	
Replicate 2	1	4.44	0.04	
Replicate 3	1	4.44	0.04	
Replicate 4	1	0.06	0.81 (ns)	
Replicate 5	1	0.90	0.34 (ns)	
Replicate 6	1	2.04	0.15 (ns)	
Pooled	1	11.23	0.0008	
Heterogeneity	5	2.47	0.78 (ns)	
Total	6	13.70	0.03	

Predators were not attracted to odour from artificially wounded, uninfested bulbs when offered against odour from intact, uninfested bulbs (Table 2). This result did not depend on whether the bulbs were wounded 3 hours or 0.5 hours before the olfactometer experiment. Neither the replicate experiments nor the pooled data were significantly deviating from 1:1 ratio and they did not show heterogeneity among replicates (Table 2).

When one of the arms of the olfactometer was connected to a box with infested bulbs and the other to one with artificially wounded, uninfested bulbs, 64% of the total number of predatory mites moved into the arm with odour from infested bulbs (Table 3). Whereas only one out of five replicate experiments was signifi-

Table 2 Results of olfactometer tests and replicated goodness-of-fit-tests for the responses of *N. cucumeris* to odours from artificially wounded (0.5h or 3h before experiment), uninfested bulbs and intact uninfested bulbs; *n*=number of predators to (+), (-) or none (0); *N*=*n*(+)+*n*(-)+*n*(0)

Olfactometer tests				
Bulb treatment	*n*(+)	*n*(-)	*n*(0)	*N*
Wounded (0.5h) before experiment vs. clean				
	4	10	7	21
	13	6	1	20
	10	10	0	20
	8	10	2	20
	11	8	1	20
Total	46	44	11	101
Wounded (3h) before experiment vs. clean				
	5	9	6	20
	7	7	6	20
	8	10	2	20
	7	12	1	20
Total	27	38	15	80

Replicated goodness-of-fit tests	*Wounded 0.5h before experiment*			*Wounded 3h before experiment*		
Source	df	G-stat	Critical value	df	G-stat	Critical value
Replicate 1	1	2.66	0.10 (ns)	1	1.16	0.28 (ns)
Replicate 2	1	2.64	0.10 (ns)	1	0.29	0.59 (ns)
Replicate 3	1	0.20	0.65 (ns)	1	0.22	0.64 (ns)
Replicate 4	1	0.22	0.64 (ns)	1	1.33	0.25 (ns)
Replicate 5	1	0.48	0.49 (ns)	-	-	-
Pooled	1	0.04	0.84 (ns)	1	1.87	0.17 (ns)
Heterogeneity	4	5.95	0.20 (ns)	3	0.84	0.84 (ns)
Total	5	5.99	0.31 (ns)	4	2.71	0.61 (ns)

cantly different from a 1:1 ratio, the pooled results showed a significant deviation, which cannot be attributed to heterogeneity among replicate experiments (Table 3). Contingency table analysis applied to the pooled results per experiment showed: (1) the time of

Table 3 Results of olfactometer tests and replicated goodness-of-fit tests for the responses of *N. cucumeris* to infested (+) and artificially wounded (3h before experiment), uninfested (-) bulbs; *n*=number of predators to (+), (-) or none (0); $N=n(+)+n(-)+n(0)$

Olfactometer tests				
Bulb treatment	*n*(+)	*n*(-)	*n*(0)	*N*
Infested vs. wounded				
	12	6	2	20
	15	5	0	20
	13	7	0	20
	15	9	1	25
	12	8	0	20
Total	67	35	3	105
Replicated goodness-of-fit tests				
Source	df	G-statistics	Critical value	
Replicate 1	1	2.04	0.15 (ns)	
Replicate 2	1	5.23	0.02	
Replicate 3	1	1.83	0.18 (ns)	
Replicate 4	1	1.52	0.22 (ns)	
Replicate 5	1	0.81	0.37 (ns)	
Pooled	1	10.21	0.001	
Heterogeneity	4	1.21	0.88 (ns)	
Total	5	11.42	0.04	

wounding preceding the experiment (3 vs. 0.5 hours) did not affect the response, (2) the response to odours from infested versus clean bulbs is independent from the response to odours from wounded (3 hours), uninfested versus intact, uninfested bulbs, and (3) response to odours from infested versus clean bulbs is independent from response to odours from infested versus wounded (3 hours) bulbs. Tests against wounded (0.5 hours) bulbs gave similar results, as expected from (1). Therefore, it can be concluded that food-deprived females of *N. cucumeris* discriminate between volatiles emitted from infested and wounded or intact, uninfested tulip bulbs. Apparently, wounded and clean bulbs emit odours that

are different from those emanating from tulip bulbs infested by rust mites. Response can be expressed not only in the fraction of + responders out of all responders (+ and -), but also in the fraction non-responders (0) out of all predatory mites tested in the olfactometer (+, – and 0). Recall that odours from the two arms come together in the base tube of the olfactometer and thus also at the release point, where the predatory mites may decide to move upwind or downwind or to stay put. A 2x3 test of independence using G-test showed that the fraction non-responders within 5 minutes experimental time is not independent of the treatments. Scrutiny of the precise data shows that this percentage non-responders is lowest (*ca.* 3%) when choice is between infested bulbs and wounded bulbs followed by the experiment where the choice was between infested bulbs and uninfested bulbs (7.5%) and finally by the experiments where the odour sources were artificially wounded bulbs (0.5 or 3 hours before experiment) and clean bulbs (11 and 19% respectively). Thus, the fraction non-responders decreases with the extent to which the experiment yielded responses to one of the odour sources offered.

Discussion

We show that females of the predatory mite, *N. cucumeris* move to volatiles from tulip bulbs infested by *A. tulipae*, when volatiles from either intact, uninfested bulbs or artificially wounded, uninfested bulbs were the alternative. This results can be in principle emerge from attraction to the former odour source, avoidance of the latter or both. We have two arguments why movement towards rust-mite infested bulbs is more likely to result from attraction. The first argument is based on the consideration that, if the two alternative odours induce avoidance in the predatory mite, then it is quite unlikely that they do so to an equal extent. Thus, the fact that the predatory mites exhibited 1:1 '+ versus –' responses when odours from intact bulbs were offered against those from either of two types of wounded bulbs, is more readily explained as absence of avoidance (rather than equal avoidance). The second argument

comes from the observation that the fraction of non-responding predatory mites (among +, – and 0) increases when the fraction of + responders (among + and -) approaches 0.5. This trend is an important observation, because absence of a response, as well as a positive response to one odour source can be interpreted as two alternative ways to express avoidance of the alternative odour source. Thus, the low fraction non-responders in the case of response deviating from 1:1 indicates absence of avoidance. We therefore conclude that the positive response to odour from rust mite-infested tulip bulbs result from attraction, rather than avoidance of the alternative odour source.

We show that volatiles from tulip bulbs infested by *A. tulipae* are attractive to the predatory mite, *N. cucumeris*, but the volatiles from artificially wounded, uninfested bulbs are not. Also, there is no difference in attractiveness of odours from clean bulbs and bulbs wounded either 0.5 or 3 hours before the experiment. One interpretation is that artificial wounding does not mimck wounding by rust mites in terms of duration and mechanism, and triggers the release of the same volatiles in the plant, yet in different amounts. This, however, would not explain why artificially wounded bulbs are not even a bit more attractive than intact bulbs. This suggests an active response of the plant in producing predator attractants upon herbivore attack, but to proof this will require more in-depth studies to identify the attractants, to elucidate their biosynthesis in the plant and to unravel the signal transduction that acts in the plant after herbivore feeding and leads to the release of volatile chemicals from the plant.

It is not the first time that an eriophyoid mite has been shown to generate such a response in a plant. Apple leaves have been shown to become attractive to the predatory mite *Typhlodromus pyri* Scheuten after being infested by the rust mite, *Aculus schlechtendali* Nalepa (Dicke and Groeneveld, 1986; Dicke *et al.*, 1988). However, the active role of the plant in this plant-herbivore interaction has so far not been proven. This is partly because the apple rust mites are relatively hard to rear in the laboratory and therefore it is more difficult to

ensure pure infestation of the host plant by one (and no more than one) species of herbivore alone. Because *A. tulipae* is relatively easy to rear on stored tulip bulbs and by post-experimental checks for other herbivore species (especially astigmatic mites) are feasible, our findings are a breakthrough on our way to prove an active physiological response of plants to feeding by eriophyoid mites.

Another novelty of our experimental results is that they relate to an interaction between below-ground parts of a plant. However, one may still wonder how the olfactory responses of predatory mites to HIPV from bulbs are manifested in the soil. Clearly, our olfactometer experiments were done in open air, just like those on the endoparasitoids of cabbage root flies (Neveu *et al.*, 2002). One may expect HIPV first to move through soil, and then to enter the laminar air layer above the soil from where it diffuses into open air. A parasitic wasp is likely to experience HIPV under the latter condition, where as predatory mites moving on top of the litter layer will experience the former. This in fact justifies why both the study on the predatory mites and those on the parasitic wasps were carried out in open air. However, the results of these studies do not explain the whole process of searching because the prey is to be found below the soil surface. How do predatory mites and parasitic wasps actually reach the below ground sites where the herbivores feed on the plant? One can imagine that either the soil medium is open enough for the predatory mites to move to deeper layers or that there is interspace between the plant (especially that larger plant structures, such as main roots and bulbs) and the soil. If present, these are the accessible spaces in the soil and provide the routes along which HIPV will travel to the open air. However, the question remains how accessibility of the soil depends on soil structure and the size of the natural enemy. The only olfactometer study, in which these factors have been taken into account, was published by van Tol *et al.* (2001). They filled a compartmentalized type of olfactometer with silver sand and assessed displacement of entomophagous nematodes by filtering them out from sand in each compartment.

If the prey is a herbivore that externally feeds on the plant, then it is also directly vulnerable to any predator arriving at the infested plant site. However, for predatory mites searching for *A. tulipae*, the searching process does not stop there, because part of the prey population resides inside the bulb. Tulip bulbs have an opening *ca.* 5 cm below the soil surface and it may well be that HIPV come out to the open air through this opening (the so called 'nose'of the bulb). Alternatively, herbivore damage in below-ground parts may systemically induce the production of volatiles in above ground parts, as suggested from work on another type of indirect plant defense (Wäckers and Bezemer, 2003). Once they reach the plant, predators can move down the stem and reach the opening at the tip of the tulip bulb. However, relative to their prey, predatory mites are at least six times larger in diameter and they do not have a body shape suited to enter narrow space inside the bulb (Sabelis, 1996). This contrasts with the rust mite *A. tulipae*, which has a worm-like body and a length of only *ca.* 200-300 µm (Sabelis and Bruin, 1996). Thus the rust mites can more easily move through the narrow bulb opening to spaces in between the bulb scales. If the predatory mites are unable to do the same, then the rust mites 'eat the bulbs from inside' (*i.e.*, outer cells from bulbs scales are emptied and scales dry out). It appears that, in response to attack by *A. tulipae*, tulip bulbs increase the distance between bulb scales in such a way that predatory mites can enter the bulb and clean up herbivorous mites (Lesna, Conijn and Sabelis, unpublished data). How such morphological changes in bulb structure are brought about, has not yet been assessed, but we speculate that this is triggered by ethylene, a gaseous plant hormone, which is known to be released upon damaging the tulip bulbs and to affect bulb structure. It would be interesting to study whether ethylene not only exerts morphological effects on the bulbs, but also directly represents the attractant or exhibits cross-talk with signal transducing systems in the plant, involved in triggering the production of HIPV (*e.g.* jasmonic acid and salicylic acid or their methylated forms). Future studies should have reveal whether the bulbs

responds to attack by *A. tulipae* by a cascade of processes involving a morphological change that increases predator access to the interior of the bulb, as well as a change in secondary plant chemistry involving the release of bulb volatiles attracting predators.

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c h a p t e r 5

Ethylene induces tulip bulbs to attract predatory mites

N.S. Aratchige, I. Lesna, C.G.M. Conijn, M.R. Kant and M.W. Sabelis

Ethylene is known to mediate various morphological and physiological changes in plants and to establish cross-talk with the jasmonic acid signaling pathway that is implicated in the production of secondary plant compounds involved in direct and indirect plant defense. We studied a system of tulip bulbs (*Tulipa* spp.), tiny herbivorous mites (the dry bulb mite, *Aceria tulipae*) and predatory mites (the phytoseiid mite, *Neoseiulus cucumeris*). In tulip bulbs, the scales in the 'nose' of the bulb are tightly packed. This disables most arthropods (including the predatory mites) to access the inner bulb, but does not prevent access to dry bulb mites, which are among the smallest arthropods on earth. However, dry bulb mite herbivory induces ethylene-mediated processes that increase the distance between bulb scales to an extent that is just enough to allow the predatory mites to enter the bulb's inside and exterminate the dry bulb mites. It is also known that herbivory induces tulip bulbs to produce volatile chemicals that act as attractants for predatory mites. In this study, we investigated whether ethylene alone elicits attraction of predatory mites and/ or whether it triggers the emission of predator attractants in tulip bulbs. Ethylene alone had no effect in attracting predatory mites in Y-tube olfactometer experiments. Hence, we continued testing predatory mites in the Y-tube for their response to odours from tulip bulbs that had previously been exposed (1) to ethylene, (2) to ambient air or (3) to infestation by dry bulb mites. We found that bulbs exposed to ethylene emit predator attractants, especially on the 5th day after bulb exposure. Moreover, bulbs pretreated with 1-methylcyclopropene (1-MCP: an ethylene-perception inhibitor) prior to the ethylene treatment, did not attract predatory mites in Y-tube experiments. Odours from infested bulbs and bulbs exposed to ethylene were equally attractive. To assess which bulb volatiles might explain the increased attraction of preda-

tory mites to induced bulbs, we collected and identified the volatiles from the headspace of bulbs that were simultaneously assessed for their relative attractiveness in Y-tube experiments. Using GC-TOF-MS (Gas Chromatography – Time-of-Flight Mass Spectrometry), 22 volatile chemicals were identified from tulip bulbs. The attractiveness of tulip bulbs appeared to be related to the presence of three tuliposides (dihydro-3-methyl-2(3H)-furanone, dihydro-3-methylene-2(3H)-furanone isomer 1 and dihydro-3-methylene-2(3H)-furanone isomer 2) and two benzoates (with similarity to butylbenzoate and hexylbenzoate). When tuliposides and benzoates were not emitted (intact, uninfested bulbs, bulbs exposed to 1-MCP) bulbs were not attractive to the predatory mites in the olfactometer. Furthermore, predatory mites did not prefer bulbs that released volatile tuliposides in high concentrations but not the benzoates (artificially wounded bulbs). When benzoates were present, as well as tuliposides in intermediate concentrations (bulbs exposed to ethylene and infested bulbs), predatory mites were attracted. However, when – in addition to benzoates – tuliposides were present at high concentrations, bulbs were not attractive to the predatory mites (bulbs exposed to ambient air). None of the bulbs in the various treatments emitted the benzoates without the tuliposides. Thus, predator attraction is either due to the benzoates alone or due to a combination of benzoates with moderate concentrations of tuliposides.

When attacked by herbivores, plants can defend themselves by attracting the natural enemies of the herbivores (Price *et al.*, 1980). One way is by emitting specific volatile plant-signals that guide predators and parasitoids to their prey (Dicke and Sabelis 1988; Sabelis *et al.*, 1999, 2006).

Ethylene is one of the volatiles that are emitted when plants are infested by herbivores (Kendall and Bjostad, 1990; Kahl *et al.*, 2000; Schmelz *et al.*, 2003a,b). It is a plant-hormone involved in plant defenses as it was found to regulate – via cross-talk with the jasmonic acid signaling pathway – the production of herbivore-induced plant volatiles (Kahl *et al.*, 2000; Schmelz *et al.*, 2003a; Horiuchi *et al.*, 2001; Ruther and Kleiner, 2005) as well as plant-volatiles-mediated interactions between the plant and the natural enemies of the herbivores. (Horiuchi *et al.*, 2001). Ethylene alone

has also been shown to be involved in the oxidative responses of barley plants (Argandoña *et al.*, 2001). Yet another example of the role of ethylene in plant defense related responses stems from work by Lesna *et al.* (2004). They showed that the predatory mite, *Neoseiulus cucumeris* Oudemans (Acari: Phytoseiidae), is able to control dry bulb mites (DBM), *Aceria tulipae* Keifer (Acari: Eriophyidae), on tulip bulbs in closed carton boxes, but they were unable to control the pest in chicken mesh boxes and open trays. When the bulbs in open trays were repeatedly exposed to ethylene, *N. cucumeris* gained control over DBM, whereas no control was achieved when the bulbs were repeatedly exposed to 1-MCP or to ambient air. This result was confirmed in an independent series of replicated experiments in which the population dynamics of DBM and predatory mites were quantified and the simultaneous changes in bulb morphology were assessed on bulbs that were repeatedly exposed for 24 hours to ethylene, to 1-MCP or to ambient air (Lesna, Conijn and Sabelis, in prep.).

Ethylene is known to be emitted by DBM-infested tulip bulbs (Henk Gude, Laboratory for Plant and Environment, Lisse, the Netherlands, formerly Bulb Research Centre, personal communication). DBM feed on the outer scales of tulip bulbs initially in the hairy area around the basal plate and near the tip of the bulb. They enter the inner whorls of scales through the tip of the bulb and, here, they run less risk of being eaten by predatory mites because these are much bigger than their prey. However, upon infestation the bulb appears to 'open up': the distance between bulb scales increases to such an extent that also the predatory mites can enter the bulbs even though they are at least 6 times bigger than the prey mite. It appeared that upon exposure to ethylene, the distance between bulbs scales increases in a similar way as in infested bulbs suggesting a central role of ethylene in bulb defenses (Lesna, Conijn and Sabelis, in prep.). The fact that ethylene can change tulip bulb morphology such that it facilitates indirect plant defenses, prompted us to test if it simultaneously changes the bulbs' defense chemistry.

Using a Y-tube olfactometer (Sabelis and van de Baan, 1983) we investigated whether bulbs exposed to ethylene emit volatile signals that act as attractants to the predatory mites. In our experiments we used ethylene alone, infested bulbs and bulbs exposed either to ethylene or to ambient air or pretreated with 1-MCP. Then, volatiles from the same bulbs that were used in the olfactometer studies were collected and analyzed using GC-TOF-MS. This enabled us to correlate the behavioural responses of the predatory mites to the changes in the plant's secondary chemistry.

Materials and methods

Rearing of predatory mites

The predatory mite, *N. cucumeris*, obtained from Mitox Consultants BV (Amsterdam, the Netherlands), was reared on black plastic tiles on a plastic foam pad saturated with water in a plastic tray. Wet tissue paper strips along the periphery of the arena prevented them from escaping and provided them with water. Tanglefoot was applied along the periphery of the tissue papers as a second-line insect glue barrier to predatory mites. Both water and Tanglefoot barrier also prevented invasion of other arthropods into the culture. *Typha* pollen was used to feed the predatory mites. Cultures were maintained in a climate room at *ca.* 25°C and 60% RH.

Olfactometry

A Y-tube olfactometer, consisting of a Y-shaped glass tube with a white Y-shaped iron wire in the middle of the tube to provide a 'rail-road' to the mites, was used to study the response of predatory mites to the odours (Sabelis and van de Baan, 1983). The base of the Y-tube was connected to an air pump that produced a unidirectional airflow from the arms towards the base of the Y-tube. The arms were connected via plastic tubes to identical plastic boxes that contained one odour source each. The inlet and the outlet of the plastic boxes were covered with gauze. The airspeed inside the arm of the Y-tube was adjusted using a hotwire anemometer inserted in-between the air outlet of the box and the arms of the Y-tube.

At airspeed of 0.5±0.05m/s in each arm, the odours form two neatly separated plumes in the base of the Y-tube, with the interface coinciding with the metal wire (Sabelis and van de Baan, 1983).

Adult predatory mites of 2-6 days after their last moult were starved for 3 hours prior to each experiment. After disconnecting the air pump from the tube, they were introduced, one at a time, to the metal wire at the base of the Y-tube. After the pump was reconnected, the mites usually started to walk upwind to the junction of the wire where they choose to move into one of the arms. Each female was observed until it had reached the end of the arm or for a maximum of five minutes and was subsequently removed to introduce the next female to the set up. After each series of five mites, the odour sources connected to either of the two arms were interchanged to correct for any unforeseen asymmetry in the experimental set up. Each mite was tested only once and each experiment was repeated four times with different sets of bulbs and a fresh group of female predatory mites. Depending on the availability of mites, 15-30 females were used in each replicate.

Following this procedure, five distinct olfactory choice experiments with different combinations of odour sources were carried out:

(a) Ethylene versus Control

0.07 g of Ethephon (2-chloroethyl phosphonic acid) was dissolved in 100 ml of 0.01 M MES buffer [2-(N-Morpholino) ethane sulfonic acid]. At pH 6 there is a steady emission of ethylene from this solution for at least 96 hours (Domir and Foy, 1978) and 5 ml of ethephon solution gave away 32 nl ethylene per minute. Therefore, 5 ml of the MES/ethephon solution (pH 6) was used as the odour source in one arm, while 5 ml of MES buffer solution (pH 6) was used as the alternative odour source.

(b) Bulbs exposed to ethylene versus bulbs exposed to ambient air

Uninfested, tulip bulbs *cv.* 'Yokohama' were exposed to 10 ppm of ethylene or to ambient air for 24 hours in closed containers (1 m^3). After the exposure to ethylene and ambient air, bulbs were ventilat-

ed to remove any traces of ethylene in the bulbs and they were used in the olfactometer for six consecutive days (a new set of bulbs was used on each day). Eight bulbs from each treatment were used in each replicate experiment.

(c) Ambient air versus bulbs exposed to ethylene

To check if the predatory mites show a preference to bulbs exposed to ethylene or if they avoid bulbs exposed to ambient air, each of the two odour sources should be tested against ambient air in separate olfactometer experiments. However, only the experiment with bulbs exposed to ethylene against ambient air (without bulbs) was conducted. Bulbs were exposed to 10 ppm of ethylene as in experiment *(b)*. Experiments were conducted for six consecutive days with a new set of bulbs on each day.

(d) Bulbs exposed to ethylene versus bulbs pretreated with 1-MCP and then exposed to ethylene

To avoid the effect of ethylene on tulip bulbs we first exposed bulbs to 1-MCP for 24 hours and then to 10 ppm of ethylene as in experiment *(b)*. As the bulbs were in the closed container for 48 hours in total, we exposed another set of bulbs to ambient air for 24 hours first and then to 10 ppm of ethylene for another 24 hours. The experiment was conducted on 4-, 5- and 6-day after exposure (from hereafter 'day/s after exposure' will be referred to as 'DAE') with a new set of bulbs on each day.

(e) Bulbs exposed to ethylene versus infested bulbs

Bulbs were exposed to 10 ppm of ethylene as in experiment *(b)*. Uninfested tulip bulbs were stored at 5°C and 60% RH for 1-2 months at the Bulb Research Centre, Lisse, the Netherlands. Then they were brought to the laboratory where they were kept at 17°C and 70% RH for 1 week before they were infested by placing them next to the infested bulbs. Infested bulbs were maintained at 17°C and 60% RH in a climate box. Bulbs that were infested for 42-49 days were tested against bulbs that were exposed to ethylene (3-, 4- and 5-DAE).

Collection of plant volatiles

After each replicate experiment in the olfactometer, tulip bulbs were transferred to 40-litre desiccators that were closed with a glass lid provided with an air-inlet and an air-outlet (Figure 1). Carbon-filtered air entered the desiccator through the air-inlet at approximately 300 ml/min. The air outlet was connected to one end of a 5 mm glass tube which contained 300 mg of Tenax TA to collect organic volatiles from the air that passes through (as described in Kant *et al.*, 2004). The other end of the glass tube was connected to a vacuum pump. All tubes and connections were of Teflon or glass and were sealed with Teflon tape. Glass sampling tubes containing Tenax were covered with aluminium foil to avoid degradation of trapped-volatiles by light. Volatiles were collected from this set-up during 24 hours in a climate room at *ca.* 25°C and 60% RH. After collecting the volatiles, the fresh weight of bulbs was measured. After sampling, the tubes were eluted with 2 ml pentane:diethylether (4:1) into amber vials and stored at 4°C until used for further analysis.

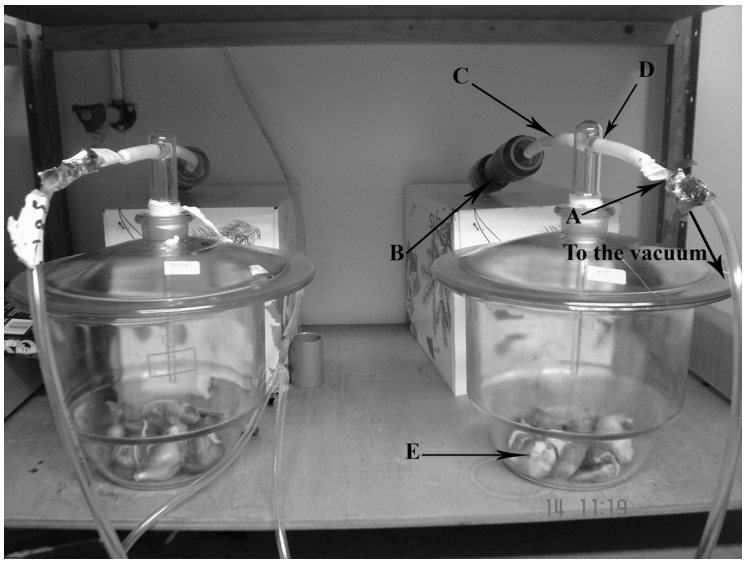

Figure 1 Volatile collection apparatus (A-Tenax TA tubes, B-Carbon filter, C-Air inlet, D-Air outlet, E-Tulip bulbs)

Analysis of plant volatiles

Of the volatiles trapped in pentane:diethylether, 1 µl was injected into an Optic (ATAS, GL International) injection port at 50°C, which was heated to 275°C at 4°C per second. The split flow was 0 ml per minute for 2 minutes and then 25 ml per minute until the end of the run. Compounds were separated on a capillary DB-5 column (10 m x 180 micrometer, film thickness 0.18 micrometer: Hewlett Packard) at 40°C for 3 minutes and then at 30°C per minute to 280°C with He (37kPa) as the carrier gas. The column flow was 3 ml per minute for 2 minutes and 1.5 ml per minute thereafter. Mass spectra of eluting compounds were collected on a Time-of-Flight-MS (Leco, Pegasus III) with a 120 seconds solvent delay. Compounds were identified on the basis of their retention index (Kovats Index, IK) using Adams (2001) and Haagen-Smit Laboratory (1997) and cross comparing the mass spectra with those in the National Institute of Standards and Technology (NIST) library.

After collecting volatiles, uninfested bulbs and bulbs exposed to ambient air, ethylene or 1-MCP were destructively inspected to ascertain that bulbs were not infested by DBM or *Tyrophagus putrescentiae* Schrank (Acari: Acaridae), an astigmatic mite often found in stored products and bulb. Results of the replicate experiments in which bulbs were infested by DBM or *T. putrescentiae* were discarded from further statistical analysis. Number of DBM on infested bulbs was estimated by counting the number of mites on 1 cm^2 on top, mid and bottom of outer scale and the total number of mites inside the bulb. In all replicate experiments total number of DBM was estimated to be more than 687 mites (more than 86 mites per bulb). When destructively inspected after each replicate experiment, none of the infested bulbs was infested by *T. putrescentiae*.

Statistical analysis

In all olfactometer experiments, data were analyzed using replicated G-tests against 1:1 null hypothesis (Sokal and Rohlf, 1997). The amounts of each plant volatile recorded in the GC-TOF-MS spec-

tra for the various treatments were subject to a one-way ANOVA using Minitab®, Version 11.

Results

Olfactory responses

None of the treatment combinations of different odour sources used in the olfactometer led to a significant heterogeneity among replicate experiments. Hence, conclusions can be drawn from analyzing the pooled results.

When ethylene was offered as the odour source in one arm of the olfactometer, 54% of 85 mites who made a choice, moved to the arm connected to the box with ethephon in MES buffer as a source of the ethylene (Figure 2). Neither replicate experiments nor pooled data were significantly deviating from a 50:50 distribution expected under the null hypothesis (Table 1). Hence, it can be concluded that ethylene alone is not attractive to *N. cucumeris*. In this experiment, 15% of the predatory mites tested did not make a choice within 5 minutes.

When the predators were offered a choice between bulbs previously exposed to 10 ppm of ethylene and bulbs exposed to ambient air, they were not attracted to odour from bulbs exposed to ethylene on 1st, 2nd and 3rd DAE (Figure 3). However, from the 4th day onwards, predatory mites started to show a significant preference for bulbs that had been exposed to ethylene. On the 4th day, 64% of 86 predatory mites who made a choice were attracted to odour from bulbs exposed to ethylene (Figure 3). The pooled results showed a significant deviation from 50:50 distribution expected under the null hypothesis (Table 2). Among replicate experiments, however, only one experiment yielded a response that was significantly different from 50:50 distribution when tested separately (Figure 3). The same accounts for the 5th day, where 65% of 98 predatory mites preferred odour from bulbs exposed to ethylene (Figure 3). In this case, pooled data differed significantly from 50:50 distribution (Table 2) and two of the replicate experiments yielded significant deviations from the null hypopthesis when tested sepa-

Table 1 Results of olfactometer tests and replicated goodness-of-fit tests for the responses of *N. cucumeris* to ethylene (+) and control (-); *n*=number of predators to (+), (-) or none(0); *N*=*n*(+)+*n*(-)+*n*(0)

Olfactometer tests				
Treatment	*n*(+)	*n*(-)	*n*(0)	*N*
Ethylene vs. control				
	12	13	5	30
	12	11	7	30
	10	8	2	20
	12	7	1	20
Total	46	39	15	100
Replicated goodness-of-fit tests				
Source	df	G-statistics	Critical value	
Replicate 1	1	0.04	0.84 (ns)	
Replicate 2	1	0.04	0.84 (ns)	
Replicate 3	1	0.22	0.64 (ns)	
Replicate 4	1	1.33	0.25 (ns)	
Pooled	1	0.58	0.45 (ns)	
Heterogeneity	3	1.06	0.79 (ns)	
Total	4	1.64	0.80 (ns)	

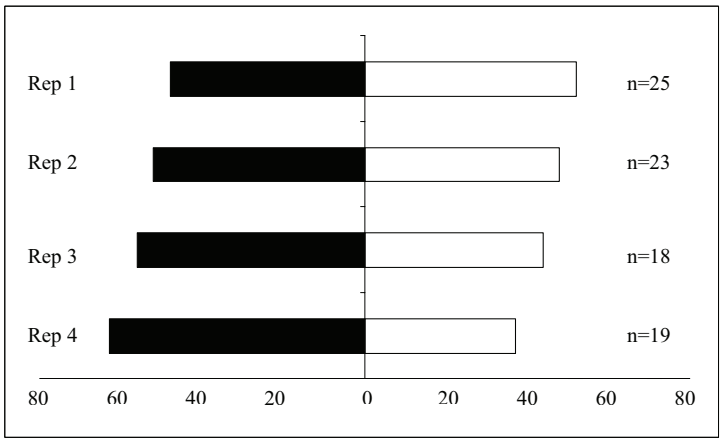

Figure 2 Response of *N. cucumeris* to ethylene from 0.07g ethephon in 100ml MES buffer in four replicate experiments in the Y-tube olfactometer (solid bars-% predatory mites choosing arm containing box with ethephon, open bars-% predatory mites choosing arm containing box with MES buffer solution)

Table 2 Results of the replicated goodness-of-fit tests for the responses of *N. cucumeris* to bulbs exposed to ethylene and bulbs exposed to ambient air

Days after exposure	*Heterogeneity* G-stat.	Critical value	*Pooled* G-stat.	Critical value	*Total* G-stat.	Critical value
1	1.89	0.59 (ns)	2.93	0.09 (ns)	4.82	0.31 (ns)
2	1.31	0.73 (ns)	0.78	0.38 (ns)	2.1	0.72 (ns)
3	5.34	0.15 (ns)	0.43	0.51 (ns)	5.8	0.22 (ns)
4	5.98	0.11 (ns)	6.79	0.009	12.8	0.01
5	2.32	0.51 (ns)	9.33	0.002	11.7	0.02
6	0.36	0.95 (ns)	3.62	0.07 (ns)	3.97	0.41 (ns)

rately (Figure 3). On the 6th DAE, 56% of 90 predatory mites preferred odour from bulbs exposed to ethylene (Figure 3). The pooled data did not differ from 50:50 distribution (Table 2). None of the replicate experiments deviated from 50:50 distribution when tested separately (Figure 3). The increased preference of predatory mites to bulbs exposed to ethylene was also reflected in the fraction of non-

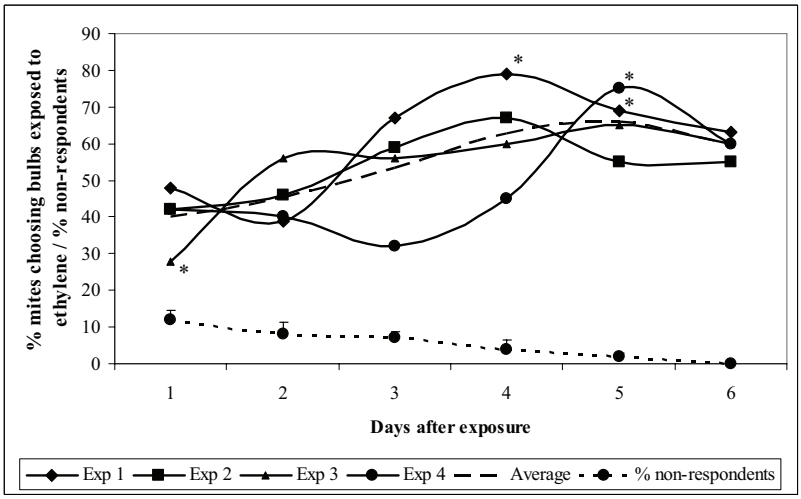

Figure 3 Response of *N. cucumeris* to odour from bulbs exposed to ethylene (10ppm) (alternative odour source-bulbs exposed to ambient air) in the Y-tube olfactometer. Replicates marked with * are significantly deviating from 50:50 distribution

responding predatory mites. There was a decreasing trend in the fraction of non-respondents when the attractiveness of the bulbs exposed to ethylene was increased over time (r^2=0.695, slope= -0.374, P=0.012; Figure 3). The behaviour of predatory mites moving more towards the odours emanating from bulbs exposed to ethylene could be interpreted either as an attraction to odours emanating from bulbs exposed to ethylene or as an avoidance of odour from bulbs exposed to ambient air. To ascertain whether this represents attraction towards bulbs exposed to ethylene or avoidance of bulbs exposed to ambient air, we tested predatory mites for their response to bulbs exposed to ethylene with ambient air (no bulbs) in the other arm of the olfactometer. This resulted in 51, 54, 44 and 51% of the mites that moved to the arm connected to bulbs exposed to ethylene on the 1st, 2nd, 3rd and 4th DAE respectively (Figure 4). Neither the data from the replicate experiments (Figure 4), nor the pooled data tested significantly different from the ratio expected under the null hypothesis (Table 3). On the 5th DAE, 63% of 79 mites who made a choice within 5 minutes chose for the odour from bulbs exposed to ethylene (Figure 4). Although the pooled data showed a significant deviation from the 50:50 distribution expected under the null hypothesis (Table 3), only one out of four replicate experiments showed a significant deviation from 50:50 distribution (Figure 4). On day 6, 61% of 76 mites who made a choice, preferred odours from the bulbs exposed to ethylene (Figure 4). Pooled data did not deviate from 50:50 distribution (Table 3) and none of the replicate experiments was significantly different (Figure 4) when tested separately. The above experiment also yielded non-respondents and these were subject to a separate analysis. Fraction of non-respondents showed a decreasing trend with the increase in the preference of predatory mites to the bulbs exposed to ethylene (r^2=0.347, slope=-0.309, P=0.065; Figure 4). Unlike in the experiment where predatory mites were offered odours from bulbs exposed to ethylene and bulbs exposed to ambient air, the fraction of mites who moved to the arm connected to the box with bulbs exposed to ethylene, showed an irregular pattern in this experiment (Figure 4).

Table 3 Results of the replicated goodness-of-fit tests for the responses of *N. cucumeris* to ambient air and bulbs exposed to ethylene

Days after exposure	*Heterogeneity* G-stat.	Critical value	*Pooled* G-stat.	Critical value	*Total* G-stat.	Critical value
1	3.30	0.35 (ns)	0.05	0.82 (ns)	3.35	0.50 (ns)
2	5.89	0.12 (ns)	0.49	0.49 (ns)	6.37	0.17 (ns)
3	0.19	0.98 (ns)	0.94	0.33 (ns)	1.13	0.89 (ns)
4	1.66	0.64 (ns)	0.05	0.82 (ns)	1.72	0.79 (ns)
5	1.67	0.64 (ns)	5.64	0.02	7.32	0.12 (ns)
6	3.30	0.35 (ns)	3.40	0.07 (ns)	6.70	0.15 (ns)

Based on the results from experiments where mites were given a choice either between odour from bulbs exposed to ethylene and bulbs exposed to ambient air or between bulbs exposed to ethylene and ambient air, we conclude that when tulip bulbs are exposed to ethylene at 10 ppm for 24 hours, they start to emit odours that are attractive to the predatory mites four days after the exposure and this attractiveness lasts for at least one more day.

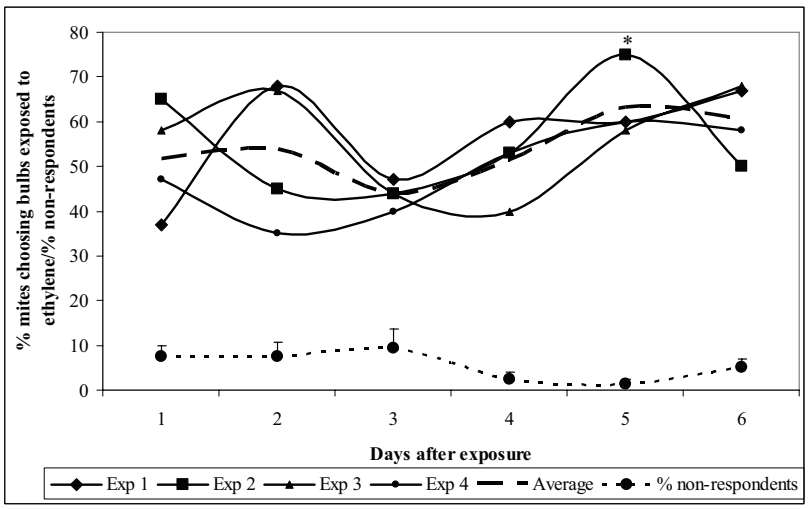

Figure 4 Response of *N. cucumeris* to bulbs exposed to ethylene (alternative odour source-ambient air) in the Y-tube olfactometer. Replicates marked with * are significantly deviating from 50:50 distribution

Table 4 Results of the replicated goodness-of-fit tests for the responses of *N. cucumeris* to bulbs exposed to ambient air and then to ethylene and bulbs exposed to 1-MCP and then to ethylene

Days after exposure	*Heterogeneity* G-stat.	Critical value	*Pooled* G-stat.	Critical value	*Total* G-stat.	Critical value
4	4.11	0.25 (ns)	0.12	0.73 (ns)	4.23	0.38 (ns)
5	0.80	0.85 (ns)	1.54	0.22 (ns)	2.34	0.67 (ns)
6	0.33	0.99 (ns)	3.78	0.05	4.11	0.53 (ns)

To further test the involvement of ethylene in increased bulb-attractiveness, we pretreated tulip bulbs with the ethylene-perception inhibitor 1-MCP after which they were exposed to 10 ppm of ethylene for 24 hours. Bulbs pretreated with 1-MCP prior to ethylene were tested in the olfactometer against the bulbs pre-treated with ambient air prior to ethylene. From previous experiments we concluded that tulip bulbs exposed to ethylene become attractive not before 4 DAE (Figures 3 and 4). Hence, in the 1-MCP-experiment, we tested bulbs only on the 4th, 5th and 6th DAE. As expected, odours from bulbs that were treated with

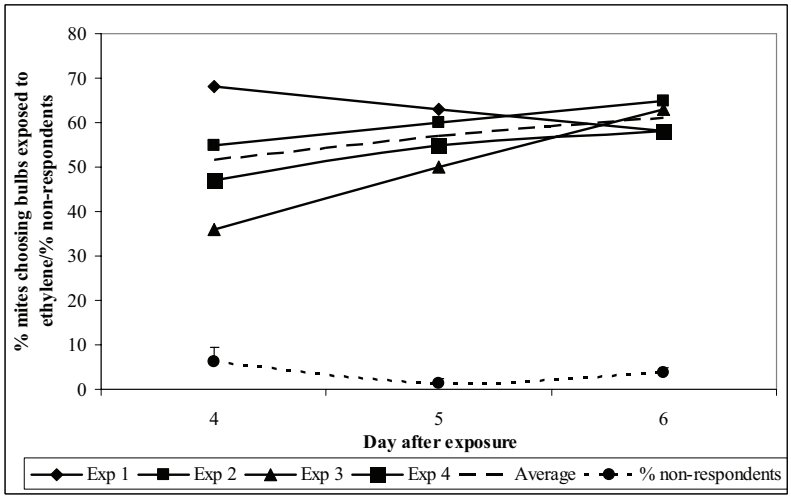

Figure 5 Response of *N. cucumeris* to bulbs exposed to ambient air first and then to ethylene (alternative odour source-bulbs exposed to 1-MCP first and then to ethylene) in the Y-tube olfactometer

ambient air followed by ethylene were more attractive to the predatory mites than the odour from bulbs that had been pretreated with 1-MCP prior to ethylene (Figure 5). Of the mites who made a choice, 52% and 57% preferred odour from bulbs exposed to ambient air and then to ethylene on respectively the 4th and 5th DAE. This was not a significant deviation from the 50:50 distribution of mites in the two odour sources (Table 4). However, after 6 days the trend became significant since 62% of predatory mites chose for the bulbs pre-exposed to ambient air over those pre-exposed to 1-MCP. None of the replicate experiments yielded data deviating from a 50:50 distribution (Figure 5). However, the pooled data showed a significant deviation from 50:50 distribution expected under the null hypothesis (Table 4). Thus these results confirm that exposure of bulbs to ethylene increases their attractiveness to predatory mites and that ethylene perception is required for this change. Also in these experiments, the fraction of non-respondents showed a decreasing trend when the mites started to show a preference to odour from bulbs exposed to ambient air and then to ethylene. However, in this experiment decrease in the fraction of non-respondents was not correlated with increase in the attractiveness of the bulbs to the predators (r^2=0.03, slope=-0.35, P=0.49, Figure 5).

Neoseiulus cucumeris can distinguish between odour from infested bulbs and uninfested bulbs (Aratchige *et al.*, 2004, Chapter 4). Given that this predator is attracted to odour from bulbs exposed to ethylene, we investigated whether it discriminates between the odour from bulbs exposed to ethylene and from infested bulbs. Since we previously showed that the tulip bulbs become attractive only 4 DAE and later (Figures 2 and 3), we tested the attractiveness of infested bulbs over bulbs exposed to ethylene on the 3rd, 4th and 5th DAE. On none of these days predatory mites discriminated between the odour from infested bulbs and from bulbs exposed to ethylene (Figure 6, Table 5). None of the replicate experiments using bulbs 3, 4 or 5 DAE tested significantly different from a 50:50 distribution (Figure 6). Therefore we con-

Table 5 Results of the replicated goodness-of-fit tests for the responses of *N. cucumeris* to bulbs exposed to ethylene and infested bulbs

Days after exposure	*Heterogeneity* G-stat.	Critical value	*Pooled* G-stat.	Critical value	*Total* G-stat.	Critical value
3	6.45	0.08 (ns)	0.12	0.73 (ns)	6.76	0.15 (ns)
4	4.06	0.26 (ns)	1.85	0.17 (ns)	5.86	0.21 (ns)
5	1.71	0.40 (ns)	2.86	0.09 (ns)	4.57	0.37 (ns)

clude that, odour from bulbs exposed to ethylene (on the 3rd, 4th or 5th DAE) are equally attractive to odour from bulbs infested by DBM. When bulbs exposed to ethylene from some randomly selected replicate experiments were tested against uninfested bulbs, predatory mites showed attraction towards the bulbs exposed to ethylene, but this response was bordering significance (replicated G-test: P=0.06 for pooled data, P=0.98 for heterogeneity).

Volatile analysis

A large variation in quantity and quality of volatiles collected from the tulip bulbs in our experiments was observed between and with-

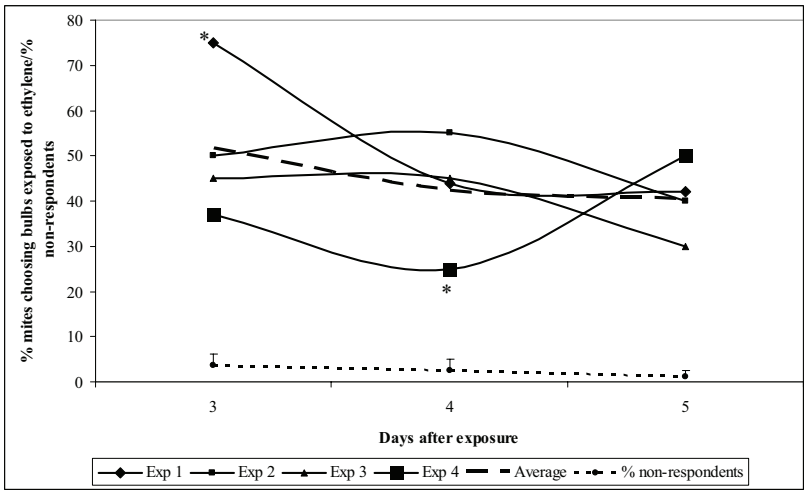

Figure 6 Response of *N. cucumeris* to bulbs exposed to ethylene (alternative odour source-infested bulbs) in the Y-tube olfactometer. Replicates marked with * are significantly deviating from 50:50 distribution

in treatments. Some volatiles were identified from only a small number of samples. Only volatile compounds identified in at least 50% of each set of replicate samples were included in the analysis. Based on this criterion, 22 volatiles remained, nine of which were present exclusively in DBM-infested bulbs which were attractive to the predatory mites (Table 6). These nine volatiles were not present in bulbs exposed to ethylene which were equally attractive to predatory mites as the DBM-infested bulbs. Assuming attraction was due to shared volatiles in these two odour blends, we infer that tuliposides (dihydro-3-methyl-2(3H)-furanone, dihydro-3-methylene-2(3H)-furanone isomer 1 and dihydro-3-methylene-2(3H)-furanone isomer 2) and benzoates trigger attraction in predatory mites. Therefore, we focus on these volatiles in interpreting our data (Figure 7).

Intact, uninfested bulbs emitted decanal and a farnesyl acetone (Figure 7a, Table 6), but no tuliposides nor benzoates. When the bulbs were artificially wounded, one tuliposide (dihydro-3-methylene-2(3H)-furanone isomer 1) was emitted in very large amounts (Figure 7b, Table 6) in all the replicates. The odour blend also contained two other tuliposides (dihydro-3-methyl-2(3H)-furanone, 1,2,4-trimethyl benzene, dihydro-3-methylene-2(3H)-furanone isomer 2), plus decanal and farnesyl acetone. However, none of the benzoates were emitted (Figure 7b). Since artificially wounded bulbs were not attractive to the predatory mites (Aratchige *et al.*, 2004, Chapter 4), high amounts of tuliposides in the absence of benzoates not likely elicit positive responses in foraging predatory mites.

DBM-infested bulbs emitted 16 volatiles (Figure 7c, Table 6), including one tuliposide (dihydro-3-methyl-2(3H)-furanone) and the two putative benzoates (benzoate 1 and benzoate 2) (Figure 7c, Table 6). Other compounds in DBM-infested-bulb odour-blend were nine benzene derivatives, three terpenoids and an aldehyde (Table 6). Out of the volatiles emitted by infested bulbs, propylbenzene, cumene, 1,3-methylethyl benzene, 1,2-methyl-i-propylbenzene, limonene, 1,3-methyl-n-propyl benzene, 1,2-diethyle ben-

Table 6 Volatiles and their relative quantities from tulip bulbs that received different treatments (IK= Kovats Index value derived from the retention times and an alkane series (using a DB5 column as described in Kant *et al.*, 2004); Intact=Intact; uninfested bulbs; Art=Artificially wounded bulbs; Infested=Bulbs infested by *A. tulipae*, >300 mites/bulb; AA= Bulbs exposed to ambient air; Et=Bulbs exposed to ethylene;1-MCP=Bulbs pre-exposed to 1-MCP and then to ethylene)

Volatile		*Bulb treatment*					
	IK	Intact	Art	Infested	AA	Et	1-MCP
Dihydro-3-methyl-2(3H)-Furanone	895		++	+	++	+	
Propylbenzene	912			+			
Cumene	931			+			
1,3-methylethyl benzene	954			++			
Dihydro-3-methylene-2(3H)-Furanone isomer 1	961		++++		+++	++	
1,3,5-trimethyl benzene	962			++			+
1,2,4-trimethyl benzene	983		+	+		+	
Dihydro-3-methylene-2(3H)-Furanone-isomer 2	984		+		+	+	
1,4-methyl i-propyl benzene	1010					+	
1,2-methyl-i-propyl-benzene	1027			+			
Limonene	1029			+			
1,3-methyl-n-propyl benzene	1042			+			
1,2-diethyl benzene	1051			++			
1,2-dimethyl-4-ethyl benzene	1075			+++			
1,3-dimethyl-2-ethyl benzene	1081			+			

Table 6 Continued

Volatile		*Bulb treatment*					
	IK	Intact	Art	Infested	AA	Et	1-MCP
1-(2-butoxyethoxy) ethanol	1197				+		
Decanal	1202	+	+	+	+	+	
Cis-Geranylacetone	1436				+		
Dodecylacetate	1605				+		
Farnesyl acetone	1843	+	+	+	+		
Benzoate 1 (similar to butyl benzoate)	2179			+	+	+	
Benzoate 2 (similar to hexyl benzoate)	2255			+	+	+	
*Attractiveness to mites**		*neg*	*neg*	*pos*	*neg*	*pos*	*neg*

+: <1 arbitrary unit, ++: 1-3 arbitrary units, +++: 3-6 arbitrary units, ++++: >6 arbitrary units (arbitrary units are the total peak areas of selected ion counts derived from the deconvoluted Analytical Ion Chromatogram from GC-TOF-MS). * *neg*: not attractive, *pos*: attractive.

zene, 1,3-dimethyl-2-ethyl benzene and 1,2-dimethyl-4-ethyl benzene were unique to the DBM-infested bulbs (Figure 7c, Table 6).

Predatory mites started to show a preference to bulbs exposed to ethylene from the 4th DAE. Moreover, volatile analysis for 1-3

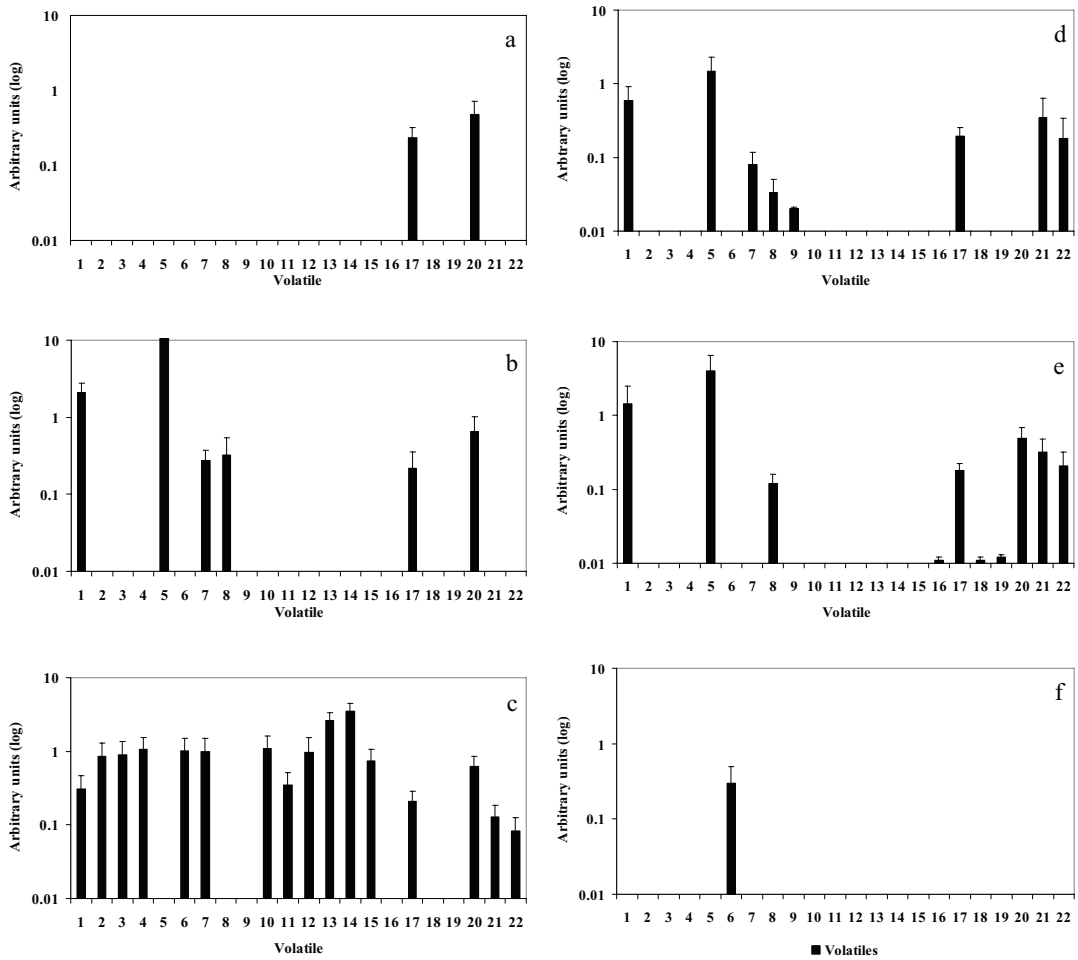

Figure 7 Volatiles emitted by from uninfested (a), artificially wounded (b), infested bulbs (c), and bulbs exposed to ethylene (d), to ambient air (e) or to 1-MCP (f). Bars are corresponding to the amounts of volatiles + SE and numbers on the x axis are corresponding to the volatiles listed in Table 6. Arbitrary units are the total peak areas of selected ion counts derived from the deconvoluted Analytical Ion Chromatogram from GC-TOF-MS

DAE gave erratic results. Therefore, it made sense to seek for potential predatory-mite attracting volatiles in the blends of bulbs 4 DAE. Exposure of tulip bulbs to ethylene at 10 ppm induced the production of at least eight major volatiles (Figure 7d, Table 6). All three tuliposides and the two benzoates were present in the odour emanating from bulbs exposed to ethylene. (Figure 7d, Table 6).

Bulbs exposed to ambient air also released dihydro-3-methyl-2(3H)-furanone and dihydro-3-methylene-2(3H)-furanone isomer 1 in high concentrations. High concentration of tuliposides might thus have contributed to the lower attractiveness of bulbs exposed to ambient air. In addition to tuliposides, the two benzoates, 1-(2-butoxyethoxy) ethanol, decanal, Cis-Geranyl acetone, dodecylacetate and the farnesyl acetone were also present in the headspace of bulbs exposed to ambient air (Figure 7e, Table 6).

Tulip bulbs pre-exposed to 1-MCP emitted neither tuliposides nor benzoates. Only 1,3,5-trimethyl benzene was emitted (Figure 7f, Table 6).

Discussion

Here, we demonstrated that exposure to ethylene stimulates the attractiveness of tulip bulbs to the predatory mite, *N. cucumeris*. In a previous study, bulbs infested by DBM were shown to be more attractive than intact or artificially wounded uninfested bulbs (Aratchige *et al.*, 2004, Chapter 4). In the present study, we found that DBM-infested bulbs and bulbs previously exposed to ethylene are equally attractive to the predatory mites. Predatory mites were not attracted if the bulbs were treated with an ethylene inhibitor (1-MCP) prior to ethylene exposure. Moreover, they did not respond to pure ethylene as such. This suggests that ethylene plays a key role in triggering bulbs to produce volatiles that act as predator attractants.

Hence, we analysed the volatiles released from bulbs infested by DBM and by uninfested bulbs that were either untreated or received different pretreatments (artificial wounding, exposure to ambient air, ethylene or 1-MCP+ethylene). To analyze volatiles, we

used the tulip bulbs that were tested in the Y-tube olfactometer studies. We consider this to be a novel approach in which, by using the bulbs from the olfactometer tests for GC-TOF-MS analysis, we were able to find a positive correlation between the behavioural responses of predatory mites from a given replicate experiment and the volatiles released by the bulbs in that same replicate experiment. Whenever positive responses of the predatory mites were observed, the blends emanating from the bulbs contained pronounced peaks of three tuliposides and two benzoates, as was the case for bulbs, heavily infested by dry bulb mites, and uninfested bulbs exposed to ethylene. In contrast, no response of the predatory mites was observed whenever both tuliposides and benzoates were absent (as was the case in uninfested bulbs and bulbs exposed first to 1-MCP and then to ethylene). Moreover, predatory mites also did not respond whenever benzoates were absent and tuliposides peaked, as was the case for artificially wounded bulbs. From these observations we concluded that a mixture of tuliposides and benzoates is essential to make the odour attractive to predatory mites.

However, in one treatment we recorded no response of the predatory mites whereas benzoates as well as tuliposides were present. This was the case where tulip bulbs were previously exposed to ambient air. Scrutiny of the GC-TOF-MS data showed that the amount of tuliposides in this treatment was much higher than in all other treatments that gave rise to positive responses of the predatory mites. Why do predatory mites refrain from being attracted when benzoates are present and the amount of tuliposides is so high? The chemical properties of tuliposides may hold the answer. Tuliposides are the precursors of tulipalines that have fungitoxic and bacteriotoxic properties and may therefore function as a direct plant defense against opportunistic pathogens colonizing wounded plant tissue (van Rossum, 1998). Artificial wounding and even the slightest stress to the tulip bulbs (including keeping bulbs at low oxygen and high carbon dioxide concentration inside closed containers) induce a copious amount of tuliposides (Henk Gude, per-

sonal communication). Tuliposides can be toxic to plants as well as to arthropods and this may be the reason why the response of predatory mites wanes when tuliposides are present in too high concentrations.

The putative negative effect of tuliposides on the response of the predatory mites is further supported by the association between the ratio of tuliposides to the sum of tuliposides and benzoates, and the response (fraction (+)) in the olfactometer experiments (Figure 8). Given the negative effect of tuliposides on the response of predatory mites to mixtures of tuliposides and benzoates, either

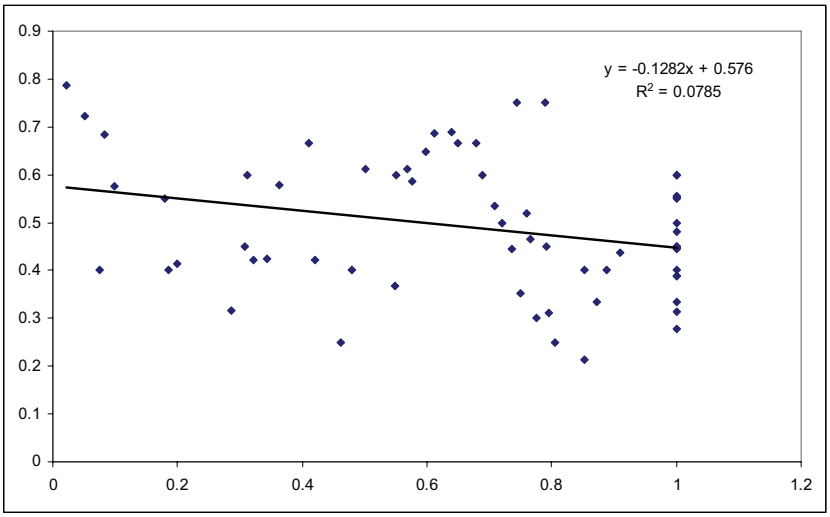

Figure 8 Relationship between the response of predatory mites (Y-axis) and the ratio of tuliposides to the sum of tuliposides and benzoates (X-axis; each compound expressed in GC-TOF-MS arbitrary units, excluding cases where both tuliposides and benzoates had zero values), based on 70 replicate experiments that pertained to 4, 5 and 6 DAE to ethylene or ambient air (thus excluding <4 DAE), infested bulbs, uninfested bulbs and uninfested-artificially wounded bulbs. Linear regression: Y=-0.1282 X + 0.576 with R^2=0.0785. According to GLM analysis (Crawley, 2002), the slope of the linear relation between predator response an fraction tuliposides was significantly different from zero with P=0.00428. Extending the model with the sum of tuliposides and benzoates and the interaction between that variable and the fraction tuliposides gave no significant improvement.

the benzoates alone function as attractants or the combination of benzoates and not too high amounts of tuliposides are required for attraction. Unfortunately, none of the treatments yielded a peak of benzoates without the presence of tuliposides, so that we cannot discriminate between these two possibilities. Future olfactometer tests to analyze the role of benzoates and tuliposides in generating predator attraction await elucidation of the exact chemical structure of these compounds.

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Summary

During their life time, plants become victims of attacks by several pathogens and herbivores. The intriguing defense responses of plants that are induced by such biotic agents have been the topic of much research conducted during the last few decades. Herbivore damage, the external biotic factor that is under consideration in this thesis, necessitates multiple defense strategies in plants out of which two will be addressed here.

Which countermeasures would a plant can take when its interior direct defense against refuge-seeking or refuge-inducing herbivores fails or when the predatory mites, part of the plant's battery of indirect defenses, cannot get access to the herbivores' feeding sites? Answering this question is the overall objective of the studies reported in this thesis. I hypothesized that the plants change their morphology in response to damage by herbivores and in turn, these morphological changes act so as to promote access of the natural enemies to the herbivores in their refuge. In the first part of this thesis, I studied a system consisting of the fruit of the coconut palm (*Cocos nucifera* L.), the coconut mite (*Aceria guerreronis* Keifer, Acari: Eriophyidae) and the predatory mite (*Neoseiulus baraki* Athias-Henriot, Acari: Phytoseiidae). For this tritrophic system, I showed that in response to herbivore damage, the distance between the perianth and the surface of the coconut fruit is increased and that these changes suffice to give predatory mites access to the space under the perianth (Chapter 2). When this phenomenon was simulated in the laboratory by artificially increasing the perianth-fruit distance, it was observed that the predatory mites enter the space under the perianth of the coconut fruit even without the presence of coconut mites, their cues or herbivore-induced plant cues (Chapter 3). It is still not clear how the morphological changes are initiated in the nuts. Probably the changes in the plant chem-

istry after the herbivore damage contribute to the changes in the morphological changes. As shown by Lesna *et al.* (2004), tulip bulbs infested by the dry bulb mite, *A. tulipae* Keifer, release the plant hormone ethylene and this in turn triggers morphological changes in the bulb. Possibly, this mechanism is also responsible for what happens to coconuts after infestation by coconut mites, but this remains to be investigated. Whether the infested coconuts also release herbivore-induced volatile (HIPV), that attract predatory mites, was tested in olfactometer experiments, but the results were bordering significance.

In the second part of this thesis, I used the tritrophic system involving tulip bulbs to test whether the morphological response is associated with the release of herbivore-induced plant volatiles (HIPV) and how these two responses are coordinated. I hypothesized that ethylene not only mediates the morphological response to herbivory, but also the production of HIPV. It was found that when damaged by dry bulb mites, tulip bulbs emit volatile chemicals that are attractive to the predatory mite, *N. cucumeris* Oudemans (Chapter 4). While it is known that ethylene induces morphological changes in tulip bulbs, I show in addition that bulbs infested by dry bulb mites, as well as uninfested bulbs previously exposed to ethylene alone, are induced to release HIPV and thereby attract the predatory mites. Gas Chromatography-TOF-Mass Spectrometry analysis of the volatiles from tulip bulbs showed that the predatory mites are attracted to the tulip bulbs when tuliposides are not too high and benzoates are produced. Since tuliposides are known to be toxic to fungi and arthropods, I suggested that the presence of benzoates overrules the effect of tuliposides.

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Samenvatting

Tijdens hun leven vallen planten ten prooi aan aanvallen door diverse pathogenen en herbivoren. De intrigerende verdedigingsreacties van planten die geïnduceerd worden door dergelijke belagers vormden het onderwerp van veel onderzoek over de laatste decennia. Schade door herbivoren, de factor waarop dit proefschrift betrekking heeft, brengt meervoudige defensiestrategiëen in stelling in planten en twee daarvan komen hier aan bod.

Welke tegenmaatregelen zou een plant kunnen nemen wanneer zijn interne directe verdediging tegen beschuttingzoekende of gal-inducerende herbivoren tekortschiet of wanneer roofmijten, onderdeel van de externe indirecte verdediging van een plant, geen toegang hebben tot de plaatsen waar herbivoren zich voeden en de schade aanrichten? Deze vraag staat centraal bij de diverse studies in dit proefschrift. Ik stelde de hypothese op dat planten hun morfologie veranderen in reactie op schade door herbivoren, en dat deze veranderingen natuurlijke vijanden van de herbivoren in staat stellen door te dringen in de schuilplaats van hun prooien.

In het eerste deel van dit proefschrift bestudeerde ik een systeem dat bestaat uit kokosnoten van de kokospalm (*Cocos nucifera* L.), de zeer kleine (0.1 mm) kokosnootmijt (*Aceria guerreronis* Keifer) (Acari: Eriophyidae) en de vijfmaal grotere roofmijt *Neoseiulus baraki* Athias-Henriot (Acari: Phytoseiidae). Voor dit tritrofe systeem toonde ik aan dat in reactie op herbivoorschade de afstand tussen de perianth (bloembekleedsel) en het oppervlak van de kokosnoot vergroot wordt, waardoor de roofmijten onder de perianth kunnen kruipen (hoofdstuk 2). Wanneer in het laboratorium de afstand tussen perianth en noot kunstmatig werd vergroot, kropen roofmijten inderdaad onder de perianth, ook in afwezigheid van prooimijten, fysieke sporen van prooimijten of door prooimijten geïnduceerde plantenstoffen (hoofdstuk 3). Het is nog onduidelijk hoe de morfologische veranderingen van de

kokosnoot totstandkomen. Waarschijnlijk zijn chemische veranderingen in de noot, in reactie op vraat door de kokosnootmijten, hieraan debet. Lesna en collega's (2004) toonden aan dat tulpenbollen die waren aangetast door bollenmijten (*Aceria tulipae* Keifer; Eriophyidae) het plantenhormoon ethyleen afgeven, wat vervolgens een verandering veroorzaakt in de vorm van de bollen. Mogelijk is eenzelfde mechanisme verantwoordelijk voor de verandering van kokosnoten na vraat door kokosnootmijten, maar dit moet nog worden onderzocht. Of de aangevreten kokosnoten ook vluchtige signaalstoffen afgeven waarmee roofmijten kunnen worden aangetrokken werd getoetst in het laboratorium met behulp van een zogenaamde reukmeteropstelling – de resultaten waren bemoedigend, maar net niet statistisch significant.

In het tweede deel van dit proefschrift heb ik in een tritroof systeem gebaseerd op tulpenbollen onderzocht of de morfologische verandering na vraat samenhangt met het afgeven van vluchtige signaalstoffen door de plant, en hoe beide reacties worden gecoördineerd. Ik stelde de hypothese op dat ethyleen niet alleen de morfologische reactie op vraat medieert, maar ook de afgifte van de geurstoffen. Het bleek dat bij aantasting door bollenmijten, de tulpenbollen geurstoffen afgeven die aantrekkelijk zijn voor de roofmijt *Neoseiulus cucumeris* Oudemans (Phytoseiidae) (hoofdstuk 4). In aanvulling op het bekende effect van ethyleen op tulpenbollen (vormverandering), liet ik zien dat door bollenmijten aangevreten bollen, evenals onaangetaste bollen die tevoren blootstonden aan ethyleen, geurstoffen gaan afgeven en aantrekkelijk worden voor roofmijten. Gaschromatografishe en massaspectrometrische analyse van de geurstoffen toonde aan dat de tulpenbollen aantrekkelijk waren voor roofmijten wanneer er benzoaten werden afgegeven en niet te grote hoeveelheden tuliposiden (hoofdstuk 5). Aangezien van tuliposiden bekend is dat ze giftig zijn voor schimmels en geleedpotigen, heb ik geopperd dat de aanwezigheid van benzoaten het negatieve effect van tuliposiden overschaduwt.

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About the author

Nayanie was born to Kingsley and Chandra Fernando on 18th October, 1970 in Bandarawela, Sri Lanka. She started her primary education in 1977 at Dharmashoka Primary School in Bandarawela. Later she moved to Gothami Girls High School in Colombo, where she completed her high school education in 1990. In 1993 she entered the University of Peradeniya, Sri Lanka and graduated in 1997, specializing in Plant Pathology and Microbiology. Soon after her University education she joined the Coconut Research Institute of Sri Lanka as a Research Officer attached to the Crop Protection Division. With the outbreak of coconut mite in late 1998, she was appointed to work on the same. Later she was sent for training at the International Institute of Tropical Agriculture in Benin where she met Dr Maurice Sabelis who later offered her a PhD position in his Department. In 2002, Nayanie started her studies under the guidance of Dr Maurice Sabelis and Dr Iza Lesna at the Population Biology Department of the University of Amsterdam, the Netherlands. The thesis you read today is the product of her research conducted during the last four years. She returned to Sri Lanka in 2005 to continue her work at the Coconut Research Institute as a Research Officer and presently engaged in research on biological control of coconut mite using predators. Nayanie is married to Deepal and blessed with a son, Ravindu.

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