Reproductive isolation and host plant specialization in European corn borer pheromone strains

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REPRODUCTIVE ISOLATION AND HOST PLANT SPECIALIZATION IN EUROPEAN CORN BORER PHEROMONE STRAINS

Isolement reproducteur et spécialisation à la plante-hôte chez les phérotypes de Pyrale du maïs (*Ostrinia nubilalis*)

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Acknowledgements

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This thesis is based on the following papers, which are referred to in the text by their Roman numerals

I   Leppik, E.; Frérot, B. (2011). The chemical landscape of maize field for host-seeking moth. Agricultural and Food Chemistry, *submitted*.

II Leppik, E.; Tammaru, T.; Frérot, B. (2011). Diel patterns of volatile organic compounds released by maize plants: The chemical environment of the *Ostrinia nubilalis* moth. Phytochemistry, *submitted*.

III Leppik, E.; Frérot, B. (2011). Volatile organic compounds and host-plant specialization in European corn borer E and Z pheromone races. Chemoecology, *submitted*.

IV Leppik, E.; Frérot, B. (2011). Assortative mating between European corn borer pheromone races- the role of male hair-pencil in the reproductive isolation. Journal of Chemical Ecology, *submitted*.

Introduction

Study objectives

The present PhD study investigates the aspect of chemical ecology in the European corn borer (ECB, *Ostrinia nubilalis* Hübner, Lepidoptera: Crambidae): pherotypes reproductive isolation and host plant specialisation.

All the experiences were designed to have ecologically relevant information about the chemical signals released or perceived by ECB moth during the reproduction period. Most of the work was conducted close to natural conditions and taking into account all the behavioural traits of ECB.

-First we investigated what can be the chemical landscape of maize field for host-seeking ECB moth. The whole volatile blends that make up the olfactory environment of maize field biotope was identified according to the ECB oviposition behaviour (Paper I).

-Secondly taking into account the diel periodicity of ECB behaviour we studied the diel changes of volatile profiles released from maize plant and from maize field (Paper II).

-Thirdly, we studied the process of host fidelity within the ECB host plants. The volatiles released from different host plants at the time of ECB oviposition flight were identified and the oviposition behaviour was studied in wind tunnel to evidence the host plant preferences of ovipositing females (Paper III).

-Fourthly, we address the question on assortative mating in ECB pherotypes and we investigated the chemical signals involved in the reproductive isolation in ECB pherotypes. We focused on the male pheromone produced by hairpencils and on courtship behaviour (Paper IV).

Insect-plant relationship

Plants and insects make up about half of all known species of multi-cellular organisms. They have co-evolved over one hundred million years and provide a passionate subject of study for every branch of biology from genetics to plant physiology and from behaviour to ecology. Over the years the plant-insect relationship researches have lead to deeper understanding of plant chemistry, insect physiology and ecology and behaviour.

Plants in general are not solely a source of food to herbivorous insects, but they provide also shelter, place to aggregate for mating and oviposit. The evolution of insects is closely related to the evolution of their host plants. The most studied plant-insect interaction is mutualistlic relationship of flower and pollinisator and the antagonistic interactions between crops and herbivorous insects. Host plants volatiles are involved in many levels in the life cycle of phytophagous insects. Females, to locate a suitable plant where to lay their eggs, use the volatile cues from host-plants, assuming that the chosen plant provides food for the progeny. Host plant quality influences the larvae fitness and moths reproductive performance and therefore the female choice of oviposition site has a crucial place in the life cycle of an insect.

Depending on their host-range, phytophagous insects are generally arbitrary divided into three categories: monophagous, oligophagous and polyphagous. Insects that eat only one or few closely related plant species are called monophagous. Those, which eat a limited range of foods, are referred to as oligophagous. For example, the insect species that seem to exercise little choice and accept many plants from different plant families are classified as polyphagous. The monophagous and oligophagous insect taxons are referred as specialist whereas polyphagous are denominated generalist. Even though a species is found to be an extreme generalist over its geographical range, larvae from restricted populations may be very specialized in their diet (Pashley, 1986) and in closer examination found to be divided into host races or sibling species (Drès and Mallet, 2002). For example the Larch budmoth (Zeiraphera diniana Guenée, Lepidoptera: Tortricidae) has distinct host races which show a high degree of fidelity to their specific host species (Emelianov et al., 2001), as it is the case as well for the Juniper hairstreak butterfly (Mitoura gryneus Hübner, Lepidoptera: Lycaenidae) (Downey and Nice, 2011) and for the Apple maggot

(Rhagoletis pomonella Walsh, Diptera: Tephritidae) whose larvae are host-specific and mate selection is directly coupled to host plant recognition (Prokopy and Boller, 1971; Moericke et al., 1975).

During my thesis project I studied the chemical signals implicated in the interactions between European corn borer (ECB) moths and its host plants. The ECB species is an interesting and special study subject for many aspects. First, the species express female sex pheromone polymorphism and secondly the species is composed of host plant populations. The ECB population that use different female sex pheromone are reproductively isolated and specialized on different host plants. The reason behind the sympatric speciation of the pherotypes and host plant role in reproductive isolation of the ECB populations is a subject that continues to fascinate scientists throughout the world.

Chapter 1: Study subjects

European corn borer

European corn borer	
Kingdom:	Animalia
Class:	Arthropoda
Order:	Lepidoptera
Family:	Crambidae
Genus:	*Ostrinia*
Species:	*O. nubilalis*
Binominal name	
Ostrinia nubilalis (Hübner, 1796)	

Table 1. Taxonomy of European corn borer.

Figure 1. Different development stages of ECB. Mating pair (A), hatching eggs (B), 5th instar larvae (C), pupae and larvae (D), larvae damaged maize plant (E). (Photos: E. Leppik)

The European corn borer (ECB) is a brownish microlepidoptera, belonging to the grass moth family (Crambidae) (Table 1) and known as a major pest of sweet corn. Male and female ECB moths are similar in appearance. Moths are about 2 cm long, their wings have alternating yellow and brown wavy lines across each wing, although the males have a darker pattern and are smaller in size (Figure 1a).

The ECB distribution range is throughout the northern hemisphere (Figure 2). ECB is native in Europe, North Africa and in Asia (Guennelon, 1972). It was unintentionally introduced into North America from Hungary and Italy in shipments of broomcorn between 1909 and 1914 (Vinal, 1917; Caffrey and Worthley, 1927). ECB is absent in Asia where another species of *Ostrinia* genus is present. In Asia the Asian corn borer (ACB) (*Ostrinia furnacalis* Guenée, Lepidoptera: Crambidae*)* occupies the ecological niche of ECB (Ishikawa et al., 1999).

Figure 2. Estimated distribution of ECB. The species has been observed in most Eurasia as well as North America and North Africa. The Eastern limit of ECB distribution may be underestimated since there taxonomic status of several members *Ostrinia* genus is debated (Frolov et al., 2007).

In Europe this moth exhibits one to two flight periods per year, the first on starting at the end of June or the beginning of July, when adult moths emerge from the overwintering pupae (Figure 3). The flight period takes two or three weeks during which ECB females attract males by releasing a sex pheromone from the fourth hour after sunset about three hours. After mating, females lay eggs in clusters of 30-40 eggs underside of the leaf of a host plant (Vaillant and Hawlitzky, 1990).
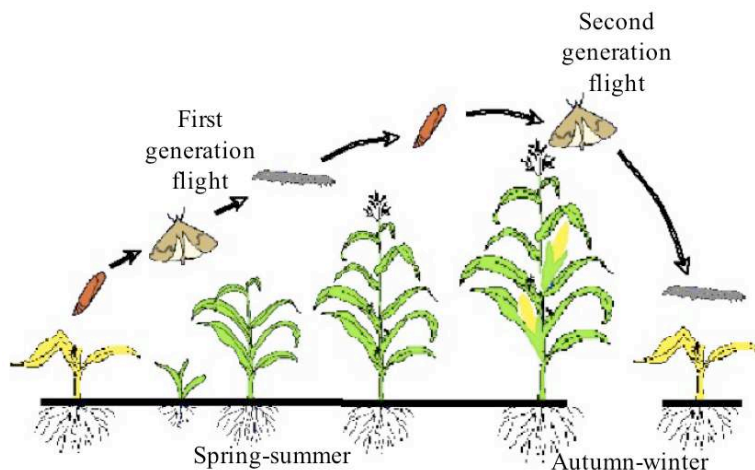
Figure 3. ECB life-cycle on maize. (Source: Pelozuelo 2004)

Egg masses are cream-colored white when first laid and become translucent as time goes on. As larvae mature in the egg, the black colour of the head capsule is visible through the egg (Figure 1b). Eggs hatch in approximately in one week time. The neonates immediately feed on different external parts of the host plant. Soon after,

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young larvae bore into the host plant stalk or stem where it destroys phloem and xylem (Figure 1e). The larvae create large cavities within the stalk or stem where it passes through four to five larval stages. On a maize plant, the behaviour of the young larvae depends on the growth stage of the plant. The larvae may feed on the leaves, kernel, or on the stalk of the maize plant. As larva matures to the 3rd instar, it bores into the stalk where they develop and pupate. The 5th instar larvae are about 20 cm long and have two distinct brown spots (tubercles) on each abdominal segment (Figure 1c). The larvae of the last instar pupate or enter diapause to overwinter during late summer or early autumn. Diapausing larvae are sheltered in host plant stem or in crop residues where they pupate and emerge as moths. Pupae are brown, about 2 cm long, and have a round head region and abdomen area tipped with a small hook (Figure 1d). The emergence of the moth pupae is about ten days after pupation.

According to the climate, the life cycle of ECB is uni-, bi-, and multivoltine. In France the ECB population in the north are univoltine and bivoltines in the south (Figure 4). Both genetic and environmental factors determine voltinism phenotypes, resulting in north-south clines that correspond to barrier latitudes that limit univoltine ecotypes to a northern distribution (Showers, 1979; Showers, 1993).
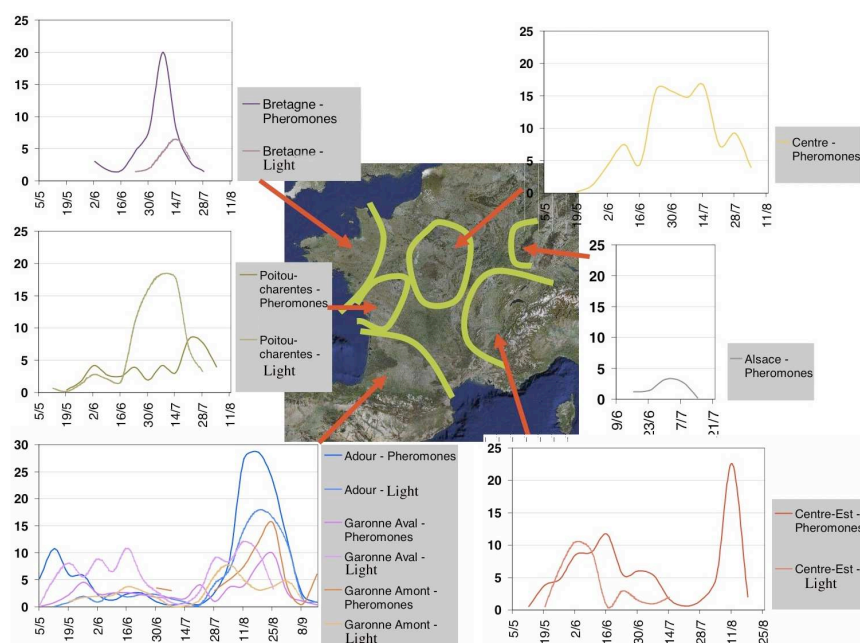
Figure 4. Weekly captures of ECB in France (2009). (Source : Arvalis)

The ECB moths are nocturnal behaving. During the daytime they hide in field edges and grassy areas. The congregations areas or so called "action sites" contain dense

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vegetation and are very humid creating optimal conditions for moths to mate, rest and drink water (Showers et al., 1976). In the middle of the night females start to emit sexual pheromone to attract males. The night after mating, gravid females fly out shortly after sunset and lay eggs on their host plant. Females mate several times. The lifetime of a moth is about a week, during witch they do not need to eat, all the efforts go to ensure the procreation.

Chemical communication in European corn borer

To communicate with other members of their species, as well as with other organisms, insect use semiochemicals. When the interaction is intraspecific, the chemical messages are called pheromones. The term pheromone is defined as "substances secreted to the outside of an individual and received by a second individual of the same species in which they induce a specific reaction, for example, a definite behaviour or developmental process" (Karlson and Luscher, 1959). The sex pheromones are typically volatile chemicals produced by either male or female members of a species, whose release and detection by the partner are essential prerequisites to successful courtship and mating. In most moth species, it is the sexually mature females which attract at long-range conspecific males (Ando, 2004). A little bit less than four hundred molecules have been documented as sex pheromone for moth species with close to 3000 different combinations (Byer, 2006).

Table 2. ECB female sex pheromone composition.

Compound	Chemical structure	E-race	Z-race	Reference
14:Ac		Present	Present	(Klun and Junk 1977; Kochansky and others 1975)
E11-14:Ac		96-99%	3%	(Cardé and others 1978; Klun and others 1973)
Z11-14:Ac		1-4%	97%	(Klun and others 1973)
Z11-16:Ac		Present	Present	(Peña and others 1988)

The chemical communication *via* pheromones has been extensively studied in ECB. The first time the ECB sex pheromone was isolated from 10 000 female moths (Klun, 1968). The sex pheromone that elicited male response was identified as (Z)-11-tetradecenyl acetate (Z11-14:Ac) (Klun and Brindley, 1969). With extensive field

captures (Klun et al., 1973) and supplementary gland extraction (Kochansky et al., 1978) it was shown that an E isomer of Z11-14:Ac is required for efficient male attraction. The ECB was the first discovery of intraspecific sex pheromone polymorphism. The ECB females were found to produce either 97:3 Z/E11-14:Ac or 1:99 to 4:96 ratio of Z/E11-14:Ac (Kochansky et al., 1975; Glover et al., 1987; Peña et al., 1988) and divide into two behavioural strains. Additionally small amounts of (Z)-11-hexadecenyl acetate (Z11-16:Ac) were identified in extracts from E phenotype females, without noticeable behavioural effects in the field tests (Peña et al., 1988; Pelozuelo et al., 2004) (Table 2).

The E and Z pherotypes are found throughout Europe either sympatrically or allopatrically (Buechi et al., 1982; Anglade et al., 1984). In North America, North Africa and China, the most prevalent pherotype is the Z (Klun and cooperators, 1975; Anglade et al., 1984; Klun and Huettel, 1988), whereas E pherotype has a rather restricted distribution in North America, limited essentially to the sites of introductions.

Taxonomy of European corn borer pherotypes

The *Ostrinia* genus is composed of 20 species, that all have a similar morphology and ecology and several of them are important agricultural pests. Frolov (2007), who has studied extensively the *Ostrinia* genus distribution in Eurasia, proposes that the morphologically indistinguishable E and Z pherotypes of ECB are sibling species *sensu* Drès and Mallet (2002). Based on host plant type, as the primary character delineating taxas and on tibia morphology, the ECB E pherotype is considered to be the Adzuki bean borer (*Ostrinia*

Box 1. Definitions of host race, sibling species and Darwinian species.	
Host races	**Sibling species**
Host race is a population of a species that is partially reproductively isolated from other conspecific populations as a direct consequence of adaptation to a specific host (Diehl and Bush, 1984).	Sibling species are species that are very similar in appearance, behaviour and other characteristics while they are reproductively isolated and incapable of producing fertile hybrids (Drès and Mallet, 2002).
Darwinian species	
A group of organisms that shares an ancestor a lineage that maintains its integrity with respect to other lineages through both time and space. At some point in the progress of such a group, some members may diverge from the main population and evolve into a subspecies, a process that eventually will lead to the formation of a new full species if isolation (geographical or ecological) is maintained.	

scapulalis Walker, *sensu nov.*) and the ECB Z pherotype *Ostrinia nubilalis (sensu nov.)*. The reconsideration of *Ostrinia* genus taxonomy has not received unanimity by the scientific community and thus E pherotype of ECB species is in some publications considered as *Ostrinia scapulalis* and in others as a pheromone strain of *Ostrinia nubilalis*. We consider that the criteria based on what the ECB pherotypes are regarded as sibling species, are not justified in view of the morphology and genetical divergence of pherotypes. The pherotypes are reproductively isolated due to the polymorphism of female sex pheromone, but the hybrids obtained under laboratory conditions are fertile and viable. The hybrid incompatibility between host associated populations is an indicator that host races are in reality sibling species (Bush, 1992), which is not the case for ECB pherotypes. No term is more difficult to define than "species" and the species concept changes with the biological model. We consider, based on Darwinian species concept (Box 1) that ECB E and Z pherotypes belong to the same species. Throughout this report the E and Z pherotype are considered as pheromone and host races of the same *Ostrinia nubilalis* species that are in process of sympatric speciation.

We investigated the morphological trait that according to Frolov *et al.,* (2007) differentiate the two *Ostrinia* species: the male mid-tibia morphology. We measured the lengths and the areas of coxa, trochanter, femur, tibia and tarsus of E and Z pherotype individuals from four different populations. The resulting data and comparative analysis are not treated and shown in this report, but will be subject of research paper. The main conclusion from morphological analyses of ECB mid-tibia was that no difference in term of size or shape was detected between the ECB strains we worked on. The results obtained reinforce our standpoint that ECB pherotypes are part of the same species.

European corn borer in agriculture

Plant-insect relationship has a crucial importance in the crop protection. Most of the plant-insect relationships in natural ecosystems are formed over millions of years of co-evolution. Introduced crops are confronted to local insect species against which their resistance is unadapted. The majority of crop plants are cultivated over wide areas as monocultures and serve as new ecological niche for insects with abundant food (Schoonhoven et al., 2005) and lower load of insect predators and parasitoids, than natural ecosystems (Ode, 2006). Using these advantages, pests cause annual crop loss of about 10% despite the use of insecticides (Schoonhoven et al., 2005).

Most of the pests are native and for example in Europe only 20% of pest are introduced accidentally (Pimentel, 1991). The introduced pests are often rather successful in the absence of their natural predators and competitors like for example the moth *Paysandisia archon* Houlbert (Lepidoptera: Castniidae), which was accidentally introduced in Europe in the mid 1990. *P*. *archon* is spreading rapidly in Southern of Europe damaging native and exotic palm trees. Another pest introduced unintentionally to Europe and doing considerable damage to maize is the Western corn rootworm (*Diabrotica virgifera* LeConte, Coleoptera: Chrysomelidae). It was introduced from Northern America to Serbia (Yugoslavia) in the 1990s and has spread throughout Europe causing serious yield losses in maize crop (Hemerik et al., 2004).

Pest species are usually oligophagous or polyphagous. Originally polyphagous pest may evolve to monophagy when food resources are not limited and relatively

predictable (Bernays and Chapman, 1994). Deterrent chemicals and physical defences affect the infestation rate of particular plant species. These characters are often altered in crop by the breeding process and eventually may favour feeding by some insect pests.

To fight against the pests, insecticides are regularly used in excessive amounts resulting high selection pressure that leads to resistance. Additionally, insecticides are generally not species specific and their being extremely toxic may have harmful effect on beneficial insects and on other non-target organism. The integrated pest management (IPM) is defined by as a decision-based process involving coordinated use of multiple tactics for optimizing the control of all classes of pests (insects, pathogens, weeds, vertebrates) in an ecologically and economically sound manner (Koul et al., 2004). In IPM strategy, the utilization of insecticides is limited or replaced by other control methods. The application of IPM requires knowledge on the biology of the pest species, with comprehension of the life cycle, the behaviour and preferences. It is possible to develop specific methods to control pest or use an insecticide at a right moment.

Control methods for European corn borer

ECB has long been recognized as economically important pest causing important yield losses in the maize crop in the Northern Hemisphere. The resulting damage from larva activity favours also the development of different fungi like *Fusarium moniliforme* and *Gibberella zeae*, which leads finally to an additional loss of maize production and quality.

Recently, control methods other than chemical insecticides were considered more and more as insecticide residues are undesirable and pests tend to develope resistance against insecticides. Furthermore, the legislation and regulation of pesticide use in many countries have undergone a major change and many commonly used molecules are withdrawn from use. In European Union, up to 50% of all current pesticides might have to be withdrawn (Blackwell and Freedman, 2011).

Genetic control

Products based on toxins from the soil microorganism *Bacillus thuringiensis* (Bt) have been developed for controlling a variety of insect pest. Bt toxins are largely used microbial insecticides and several strains are highly toxic to certain insect pests, including ECB larva. The insecticidal activity of Bt was first discovered in 1911 by German biologist Ernst Berliner. The ubiquitous Bt bacterium produces crystal proteins with insecticidal activity during sporulation. This trait is used in major crops plants (maize, cotton, potato, rice) that have been engineered through recombinant DNA to carry genes responsible for producing these crystal proteins and providing plant resistance to insect pests. In the United States the first genetically modified Bt maize was commercialized in 1996. In 2001 more than one quarter of all maize cultivated in the United States was Bt-inclusive (Shelton et al., 2002).

Since then, the use of transgenic plants expressing insecticidal proteins from the bacterium has reduced the employ of foliar insecticides. Currently, Bt is the only "microbial insecticide" in widespread use. However, the use of Bt maize in large scale poses the risk of resistance development and threatens parasitoids of ECB. Bt bacterium resistance in ECB is presently managed by implementing a high-dose refuge strategy (Alstad and Andow, 1995). The high-dose refuge strategy consists in cultivating non-toxic conventional crops in the proximity to transgenic crops that produce Bt toxins and utilization of high toxin doses (Vacher et al., 2004). However, recent studies suggests that, although ECB populations are found on different plants of the refuge areas, the ECB populations on non-maize plants constitute separate subpopulations and, therefore, cannot necessarily be viewed as alternative refuges areas (Bourguet et al., 2000). Thus knowing the process of plant specialisation and reproductive isolation, only refuge area of non-Bt maize can prevent emergence of Bt resistance.

Chemical Control

The use of chemicals to kill or to repel insect pests is the oldest pest control method. Greeks used sulphur against pests 3000 years ago and Chinese arsenic compounds

against garden pests before 900 A.D (Pfadt and Brown, 1985). Until the Second World War only "inorganic" and "botanical" insecticides were used.

With the discovery of DDT in 1939, a golden age of chemical pesticides started and the synthetic organic insecticide industry took off. Effective new molecules were available and all of the risks and dangers to human health and the environment were not yet known.

The chemical control of ECB populations consist of spraying toxic molecules on large areas on the crop. Mostly, these are synthetic pyrethroids that require a physical contact or ingestion by the pest larva, to be efficient. The chemical control has to be preventative and is only effective during the short period between egg hatch and feeding by young larva. Once the larva enters in the maize stem, the insecticides pulverized on the surface of the plant have no effect on it. Furthermore the use of insecticide has to be reasonable as ECB have the potential to develop resistance to insecticides rapidly. Resistance occurs particularly when insecticides are used repeatedly and at high concentrations. In addition, frequently utilized insecticides (such as oxadiazine, pyrethroid and organophosphates) have adverse effects on non-target insects including natural enemies and pollinators.

Biological Control

Biological control is the regulation of pest populations by natural enemies like parasites, predators and pathogens (Gillott, 2005). Several biological control methods are actually used for ECB. One method consists of releasing tiny parasitic wasps *Trichogramma brassicae*, Bezdenko (Hymenoptera: Trichogrammatidae). The female wasp searches out ECB eggs and lay eggs within them. One ECB egg will contain a single parasitoid larva. When the eggs hatch, the larva consumes the egg contents and then pupates within the empty eggshell of the host. This method prevents the feeding of larva and makes insecticide pulverization on plants needless. Timing is critical for success. It is crucial to know periods when ECB females oviposition flight occurs and release parasitoids only when fresh egg masses are found on the crop. Larvae, pupae and adults of ECB are not affected in any way by the parasitoid wasp. Cardboard capsules containing developing *T. brassicae* are manually hanged in the maize field. The capsules protect the parasitoids from predators and weather extremes until the

adults emerge and exit through small holes in the capsules. Released *T. brassicae* are at different developmental stages so that adults emerge from the capsules over extended period of time. The wasps need to be released each year, as they are not capable to overwinter in large numbers under European conditions. This method is commercialized and largely used by farmers in Europe.

Two types of pulverized biological insecticides are used to control ECB populations. One is based on a ubiquitous soil fungus *Beauveria bassiana* and causes the white muscadine disease in insects. The second biological insecticide is based on the use of spores and crystalline insecticidal proteins produced by soil-dwelling bacterium Bt. Bt based insecticides are applied as liquid sprays on crops, where it must be ingested to be effective (Madigan et al., 2005; Lemaux, 2008).

Cultural Control
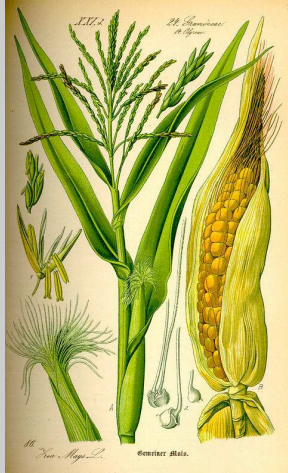
Cultural control is a long-established method of modifying the habitat to reduce the prevalence of unwanted pests (Gillott, 2005). The aim is to reduce rather than eradicate pest populations and it is typically used in conjunction with other control methods. For ECB population control it is recommended to cut stems close to the ground, pulverize crop residues and crop rotation to prevent build-up of pest populations. The crop residues pulverization destroys the diapausing 5t[h] instars ECB larva and thus decrease the number of emerging adults and number of eggs laid in the new crop.

In this context, our research is aiming to understand how insects colonize the cultivated maize plants and provide new tracks to develop new methods to monitor ECB populations. Some of the possibilities are to consider volatiles released by plants as selection criteria and by targeting certain volatiles by selection or by genetic engineering of the chemical signal released by the maize.

European corn borer host plants

Table 3. The taxonomy of the three most common ECB host plants: maize, mugwort and hop. (Images from Thomé 1885)

Kingdom	Plantae		
Order	Poales	Asterales	Rosales
Family	Poaceae	Asteraceae	Cannabaceae
Genus	*Zea*	*Artemis*	*Humulus*
Species	*Z. mays* L.	*A. vulgaris* L.	*H. lupulus* L.
Common names	Maize, corn, sweet corn	Mugwort, sagewort, felon herb, naughty man	Hop, lupulin, pliny the elder

Maize

The maize (*Zea mays* L.) is part of the family Poaceae, plants of this family are usually called grasses, the family has some 9000-10 000 or more species of grasses (Table 3). Maize is a vigorous and tall-growing (1-4 m) annual grass that reproduces exclusively by seed (Figure 5). Maize forms a seasonal root system. Leaves are large and have a distichous leaf arrangement. Each leaf consists of a sheath surrounding the stalk and an expanded blade connected to the sheath by the blade joint, or collar. The leaf number, size and orientation vary considerably between maize races. Maize is a summer-growing crop requiring warm daytime temperatures and has a C4 photosynthetic pathway, which allows a continued response to increasing radiation up to full sunlight coupled with low levels of photorespiration.

Maize is a cereal first cultivated by indigenous peoples in Western Mexico and Mesoamerica in prehistoric times (around 5000 BC). Currently, there are five species included in the genus *Zea*. *Z. mays* ssp. *mays* is the only cultivated species; the other

species and subspecies are wild grasses, referred to as teosintes. Maize is believed to be derived from the wild grass teosinte (*Zea mays* spp. *parviglumis*) (Galinat et al., 1988). In the late 15th and early 16th centuries maize was introduced to Europe and to other countries through trade (Brandolini, 1970) and spread across the world due to its popularity and ability to grow in diverse climates, particularly in temperate zones.

Figure 5. Young maize plants and maize field. (Photo: E. Leppik)

Maize is predominantly cross-fertilizing plant with a wide genetic base and is very adaptive to environmental changes. It has diverse morphological and physiological traits resulting from a wide genetic variability. Maize has a long history of domestication as the breeders have selected it for desirable traits for more than 5000 years, and the professional breeders for approximately 150 years (Matsuoka et al., 2002). The breeding has lead to a wide variety of primitive races, local varieties and cultivars. Breeding has improved or changed traits such as plant height, ear number, yield, maturity, nutrient content, kernel properties, and disease and pest resistance. Increased production per unit area is the primary objective in most maize breeding programs. Genetic transformation protocols for maize have been developed and improved over the last 20 years. The main focus in the production of genetically modified (GM) plants has been on resistance to pests *i.e.*, ECB and tolerance to herbicides. Resistance to diverse pests has been achieved by using *cry*-genes from *Bacillus thuringiensis* (Bt) and tolerance to the herbicides glufosinate ammonium and glyphosate by using the *pat*-gene from *Streptomyces viridochromogenes* and the *cp4epsps*-gene from *Agrobacterium* spp., respectively. Bt maize is genetically altered to express the bacterial Bt toxin, which is poisonous to ECB larva. The Bt toxin brings about the formation of pores in the ECB larval digestive tract and let the

naturally occurring enteric bacteria, such as *E. coli* and *Enterobacter*, to enter the hemocoel, where they reproduce and cause septicemia (Broderick et al., 2006). The ECB, one of the main pests Bt is intended to target, has been shown to be capable of developing resistance to the Bt protein (Ostlie et al., 1997).

The maize releases certain number of volatile organic compounds (VOCs) from leaves, roots and from kernel. The volatile blends change according to the physiological state, genetic, diurnal cycle and other factors influencing the plant. Overall, about 40 VOCs have been identified from maize headspace samples. Maize releases a complex VOCs blend of generally occurring monoterpenes (MT), sesquiterpenes (SQT) and some green leaf volatiles (GLV) into atmosphere. The maize VOCs are ubiquitous, found through out the plant taxa that lack qualitative taxonomic specificity.

Mugwort

The mugwort (*Artemisia vulgaris* L.) is part of the family *Asteraceae* or *Compositae* (Table 3) which the largest family of vascular plants, and the family has more than 22,750 species. Mugwort is a high (1–2 m) herbaceous perennial plant with a woody root. The leaves are 5–20 cm long, dark green, feather-like, with white hairs on the underside (Figure 6). Mugwort small (5 mm) flowers are wind-pollinated.

It is native to temperate Europe, Asia, northern Africa and Alaska and non-native in North America, where it is considered as an invasive weed. It is a very common plant growing on uncultivated areas, often forming dense monospecific stands, along roadsides, urban lots and waste places. Mugwort has aromatic leaves and bitter taste from terpenoids and SQT lactones, which is believed to exists as an adaptation to discourage herbivory. Nevertheless a great number of species of Lepidoptera feed on the leaves and flowers.

Figure 6. Mugwort plants. (Photo: E. Leppik)

Mugwort leave chemicals are mainly studied for its medicinal and allelopathic properties. Several mugwort MT inhibit other plants species seedling root and shoot growth with specific cytotoxic effects (Peñuelas et al., 1996). The mugwort chemical composition is generally obtained from crushed plants or by steam distillation (essential oils). Mugwort leafs contain essential oils (cineole, thujone), flavonoids, triterpenes, and coumarin derivatives. The composition of the essentials oils and the concentration of the main constituents are greatly influences by the geographic location of the plant. More than 80 compounds have been isolated from the foliage of mugwort populations around the world, mainly MT (Milhau et al., 1997).

Hop

The hop (*Humulus lupulus* L.) is part of the family *Cannabaceae*, which also includes the genera *Cannabis*, and *Celtis* (Table 3). The hop is a vigorous climbing perennial herbaceous plant (Figure 7). It is a bine and has a stout stem with stiff hairs to aid in climbing. Early in spring it sends up new shoots and dies back to the cold-hardy rhizome in autumn. Hop shoots grow very rapidly and at the peak of growth can grow 20 to 50 cm per week and individual bines typically grow between 2 to 15 m depending on what is available to climb on. The leaves are opposite, with a 7 to 12 cm leafstalk and a heart-shaped, fan-lobed blades are 12 to 25 cm long and wide; the edges are coarsely toothed. Hop male and female flowers develop on separate plants. The female flower clusters, often called "cones", are known as hops. It is native to

Eurasia and was first domesticated in central Europe (Behre, 1999) and is currently naturalized throughout the northern temperate regions of the world as well as some temperate regions in Australia, South Africa, and South America.

Figure 7. Hop plant. (Photo: E. Leppik)

For the need of beer industry over 100 hop cultivars have been breed with different flavours.

The difference between hop cultivars comes from various amounts of bitter acids and volatile oils they contain (Neve, 1991). More than 400 chemicals have been detected from hops essential oils, and only about 200 of them have been identified. The main compound found in hop essential oils is myrcene that gives hop an unpleasant and harsh odour. The major SQT of hop essential oils are α-humulene, β-caryophyllene, farnesene, selinene and bergamotene (Chadwick et al., 2006).

Chapter 2: Volatile organic compounds

Volatile organic compounds in plants

A walk through a pine forest or a meadow on a summer evening provides convincing evidence that plants release substantial quantities of volatiles from vegetative as well as floral organs. Plants have a tendency to perfume the atmosphere around them; they release a great number of volatile organic compounds (VOCs) that include terpenes, terpenoids, alcohols, aldehydes, ethers, esters and carboxylic acids (Figure 8). The term VOCs (Box 2) includes organic

atmospheric trace gases other than carbon dioxide (CO_2) and monoxide (CO) and has many different meanings, depending on the frame in which the term VOCs is used and there is no clear and widely supported definition. Now, more than thousand VOCs were reported to be emitted from plants, although a comprehensive list is available only for floral scent (Knudsen et al., 2006). Even though a bewildering array of VOCs were identifies in plants, a great number of plant families share ubiquitous VOCs that are present in more than half of seed plant families (Table 4).

Table 4. VOCs occuring in more than half of the families of seed plants.

VOCs	%	Molecule
Limonene	71%	
Ocimene	71%	
Myrcene	70%	
Linalool	70%	
α-pinene	67%	
Benzaldehyde	64%	
β-pinene	59%	
Caryophyllene	52%	

All data from Knudsen et al.,(2006).

In plants, VOCs can be formed as by-products of plant processes and are emitted to air due to their volatility with no specific role for every VOC released (Peñuelas and Llusià, 2004). The evolution of plant secondary compounds, *i.e.,* VOCs, is often considered to be closely related with defence against herbivores and other parasites, but recently it was proposed that plant chemical defence could be primarily aimed at abiotic stresses, such as photodamage and climate changes (Close and McArthur, 2002; Peñuelas and Llusià,

2003). VOCs may also serve as hormones and parts of the cell membrane (Degenhardt and Lincoln, 2006; Sharkey et al., 2008). The hypothesis that plants started to produce volatiles to attract enemies of herbivores is questionable because it would necessitate a simultaneous change in the plant (to synthesize) and in predators and parasitoids of the plant (to respond) to these VOCs (Janssen et al., 2002).
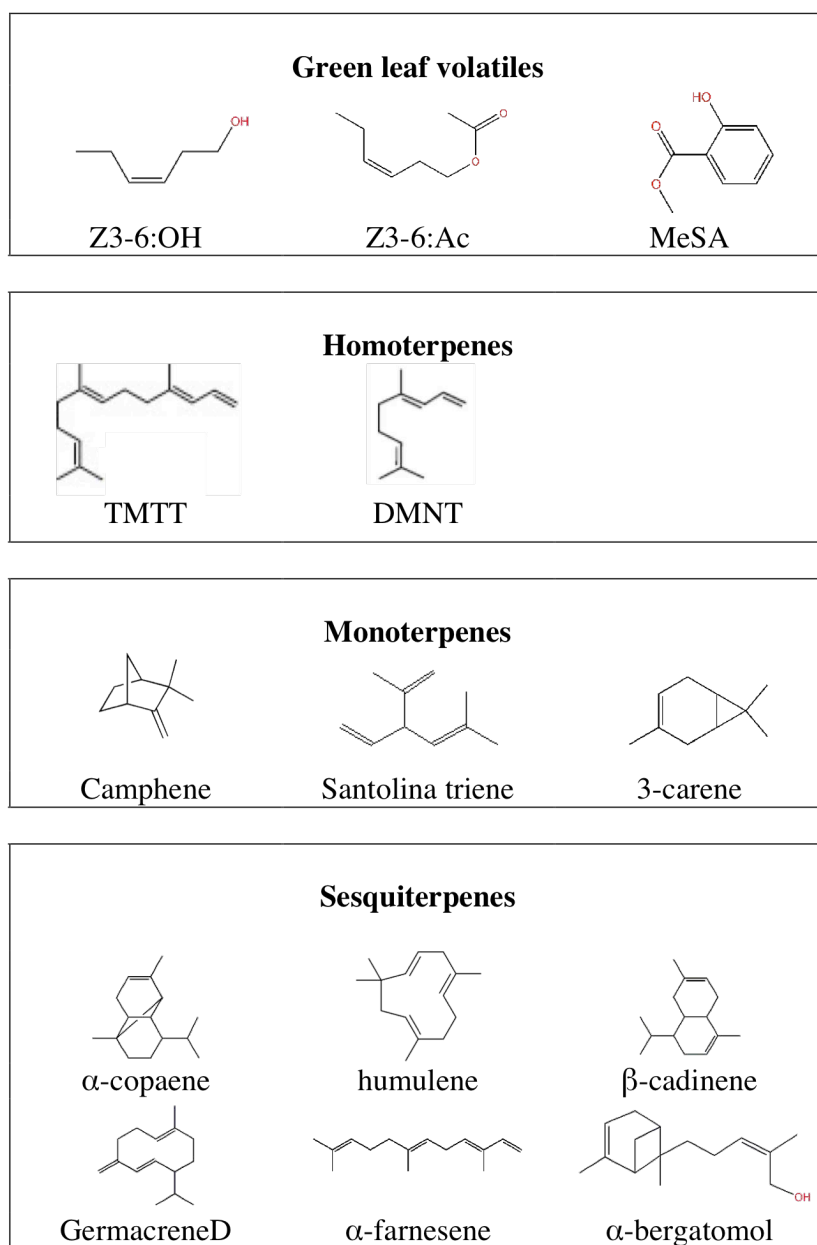
Figure 8. Examples of chemical structures of some plant VOCs.

Nevertheless, natural selection has worked to take advantage of VOCs volatility and some VOCs have become signals for plant protection and communication. In some plants, VOCs are stored in specialized organs such as resin ducts or glandular

trichomes and, when emitted, act as repellents against pathogens or herbivores or are directly toxic to them. VOCs can act as wound sealers or as relatively non-polar solvents for higher molecular weight defensive compounds that would otherwise not go into solution (Pichersky and Gershenzon, 2002). However, VOCs that are not accumulated in specialized organs, and which are commonly light dependent, *i.e.,* their emission depends on instantaneous photosynthetic processes providing energy and carbon for the biosynthesis, have unknown roles. Light-dependent VOC emissions are often the most abundant VOC emissions from ecosystems. The vast majority of the VOCs emitted in a light-dependent manner are either MTs, five-carbon alcohols or methylbutenol. Interestingly, no plant taxa produces more than one kind of light dependant VOC. The exact function and adaptive value of these light-dependant VOCs is still unclear. Since the three types of light-dependent VOCs share great deal of similarities in their chemical structure, biosynthetic pathway and physiological regulation, it has been suggested that these compounds might serve similar or identical functions in the plants that produce them.

Table 5. Impact of selected abiotic stresses on emitted amounts of VOCs in maize.

VOCs	Drought [a]	High temperature [a]	Low light intensity [a]	Air humidity [a]	Nutrient deficiency[a]	Nitrogen deficiency [b]
Green leaf volatile (GLV) (C_6)						
Z3-6:Ac	↔	↑	↑	↔	↓	↔
Z3-6:OH, MeSA	nd	nd	nd	nd	nd	nd
Homoterpenes (HT) (C_{11} and C_{16})						
DMNT	↑	↔	↔	↔	↓	nd
TMTT	↔	↑	↓	↔	↓	↔
Monoterpenes (MT) (C_{10})						
Myrcene	↔	↔	↔	↔	↔	↔
Linalool	↓	↔	↔	↔	↔	↔
Sesquiterpenes (SQT) (C_{15})						
β-caryophyllene	↔	↑	↔	↔	↓	↑
α-bergatomene	↔	↔	↔	↔	↔	↑
β-farnesene	↔	↔	↔	↔	↔	↑
α-farnesene	↔	↔	↑	↔	↓	↔
Trans-nerolidol	↔	↑	↓	↔	↓	↔

Carbon content of VOCs is indicated in parentheses after the chemical class. ↔ = no effect, ↑ = significant increase ↓ = significant decrease, nd = not detected. [a] Gouinguene and Turlings (2002), [b] Schmelz and others (2003).

The abiotic factors cause changes in emission of VOCs relative ratios, showing that climatic conditions and nutrient availability are important factors in determining the intensity and variability in the release of plant volatiles (Table 5).

Green leaf volatiles

The smell of freshly mown lawn is one of the scents that can be easily recognized by man. The intense grassy-green odour is released by plant when it is crushed or suffers otherwise tissue damage. The volatiles that compose the characteristic fresh smell are called green leaf volatiles (GLV). GLV are C_6 saturated or monounsaturated aldehydes, alcohols, and their esters produced from linolenic and linoleic acids *via* the lipoxygenase (LOX) pathway. Most of the GLVs are widespread volatiles in the plant kingdom (Knudsen et al., 2006). Intact plants emit low levels of GLVs while they can account more than half of the emissions from damaged plant parts (Whitman and Eller, 1990). Plants start to form GLVs in great quantities immediately after disruption of their tissue and after suffering abiotic or biotic stresses (Fall et al., 1999). Some GLVs *e.g.*, cis-3-hexenyl acetate (Z3-6:Ac) are released even from young intact leaves of herbivore attacked plants, suggesting that the LOX pathway can be activated in intact leaves (Dudareva et al., 2004).

GLVs, especially (E)-2-hexenal, (E)-3-hexenal and (Z)-3-hexenal, show antibacterial and fungicidal activities (Nakamura and Hatanaka, 2002; Almeras et al., 2003), bacteria and fungi on the newly cut surface are presumably killed by leaf alcohol. Since GLVs are toxic to several phytopathogenes, it has been suggested that one physiological functions of GLVs is to protect plants from infection (Blée, 2002). Recently, studies investing the physiological significance of GLVs in plants have evidenced that GLVs are also important for signalling within and between plants and for allowing plants and other organisms surrounding them to recognize or compete with each other (Matsui, 2006). Some of the GLV elicit the syntheses of jasmonic acid (an important plant hormone implicated in plant acquired resistance against herbivores) and the production of *novel* HT and SQT in intact receiver plants as well as the behavioural response of arthropod parasitoids and predators (Reddy and Guerrero, 2000) which make a distinction between infested and uninfested plants, and thus locate hosts or prey (Agrawal, 2000; De Boer et al., 2008). For example lima bean (*Phaseolus lunatus*, Fabaceae) plants and apple trees *(Malus domestica,*

Rosaceae*)* produce GLV that attract a carnivorous mite (*Phytoseiulus persimilis,* Mesostigmata) when injured by two-spotted spider mite (*Tetranychus urticae,* Trombidiformes) (Takabayashi and Dicke, 1996; Sabelis et al., 2007). Maize and cotton plants release also GLV when damaged that attract hymenopterous parasitoids that attack larva of several species of Lepidoptera (Tumlinson et al., 1993). Most of the herbivorous insects perceive GLVs at the peripheral level and their responses differ from species to species (Bruce et al., 2005). In some insects, GLVs have a synergistic effect on the aggregation and on the action of sex pheromones (Reddy and Guerrero, 2004).

Terpenes

Terpenes and terpenoids constitute a large and diverse class of VOCs produced by a wide variety of plants and more rarely by insects. They are the major components of resin, and of oils and extracts produced from resin. Terpenes are derived biosynthetically from units of isoprene (C_5H_8) and are some of the most structurally diverse natural products. The isoprene units may be linked together forming linear chains (α-farnesene, α-ocimene) or they may form cycles (α-humulene, limonene). Terpenes are technically only hydrocarbons and the oxygen-containing compounds such as alcohols, aldehydes or ketones are terpenoids.

The biological precursors to isoprene are isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). There are two biosynthetic pathways to terpenes: the mevalonate (MVA) pathway in the cytoplasm and the methylerythritol (MEP) pathway in plastids. In both pathways the IPP is isomerized to DMAPP by the enzyme isopentenyl pyrophosphate isomerase (Dudareva et al., 2005). As the isoprene units are linked together, the resulting terpenes are classified sequentially by size as hemiterpenes, monoterpenes (MT), sesquiterpenes (SQT), diterpenes, sesterterpenes, triterpenes, tetraterpenes, and polyterpenes (Table 6). The prefix in the name indicates the number of isoprene units linked together. The terpenes composed from more than three isoprene units are no more considered as VOCs as they are rather heavy and not volatiles. Conventional pathway allocation had suggested that C_{10} precursors of MT are mainly synthesized within plastids by the MEP pathway, whereas C_{15} precursors of SQT are produced *via* the MVA pathway

and the two pathways are strictly independent (Dudareva et al., 2004). However, there is emerging evidence that a small amount of cross-talk between the two pathways occur, implying that the pathways are not completely autonomous (Dudareva et al., 2005). The structural diversity of terpenes is due to the diversity of terpene synthases (TPSs). TPSs are often multiproduct enzymes, and thus even a single TPS can contribute significantly to the plasticity of blends, especially blends produced in response to herbivory (Köllner et al., 2004).

Table 6. Examples of different classes of terpenes that are considered as VOCs.

Chemical class	C_5 units	Formula	Mw	Synthesis	Example
Hemiterpenes	single	C_5H_8	68	MEP and MVA pathways in plastids and cytosol	Isoprene, isovaleric acid
Monoterpenes (MT)	two	$C_{10}H_{16}$	136	Mainly in plastids by the MEP pathway	Limonene, β-myrcene
Sesquiterpenes (SQT)	three	$C_{15}H_{24}$	204	Mainly in cytosol by the MVA pathway	β-caryophyllene, α-copaene
Homoterpenes (HT)		$C_{16}H_{26}$ $C_{11}H_{18}$	218 150	Oxidative degradation of nerolidol	TMNT DMNT

Monoterpenes

Highly volatile MT molecules ($C_{10}H_{16}$) are made of two isoprene units (Table 6). Usually MT are strong smelling, barely water-soluble, and found in plants as well as in animals and microorganisms. They are known to constitute the main fraction of "terpenic oils" or "essential oils" that are produced and stored in plant secretory organs. The MT comprises acyclic (ocimene, myrcene), and monocyclic (limonene), bicyclic (α-pinene), and some rare tricyclic structures (tricyclene) (Figure 8). Oxygenated monoterpenes and their derivates are often summarized as monoterpenoids (linalool, geraniol, camphor).

MT emissions by plants are generally considered light-independent, because most plants that synthesize MT store them in special organs such as resin ducts or glandular trichomes (Monson et al., 1995). In MT storing species their emission is mainly temperature-dependent, *i.e.,* it increases exponentially with temperature (Kesselmeier and Staudt, 1999). MT non-storing plant species synthesize and release them in a light

and temperature-dependent way. However, some of the MT, *e.g.*, oxygenated compounds such as linalool, are *de novo* synthesized after herbivore damage and released systemically from the whole plant.

Sesquiterpenes

Sesquiterpenes (SQT, $C_{15}H_{24}$) are made from three isoprene units and approximately 5000 SQT have been reported. They are typical flower fragrances (Knudsen et al., 1993), but considerable amounts of SQT are also emitted from the herbivore-damaged foliage of plants. For example MT form nearly 100% of the total VOCs emissions from intact cabbage plants (*Brassica oleracea ssp capitata*, Brassicaceae), whereas in herbivore damaged plants, total MT emission increases but the relative proportion drops below 60% because of novel induced compounds such as HT and SQT (Vuorinen et al., 2004b). Among the most typical inducible SQT emitted by herbivore damaged plants are β-caryophyllene and α-farnesene. Compared with other VOCs, SQT are highly reactive with atmospheric O_3 and are often completely oxidized in the atmosphere before their detection for analysis.

Homoterpenes

The irregular acyclic homoterpenes (HT), 4,8-dimethyl-1,3,7- nonatriene (DMNT) and 4,8,12-trimethyl- 1,3,7,11-tridecatetraene (TMNT) are the most typical compounds related to herbivore feeding, to O_3-damage and compose the so-called "white floral image" of night-scented flowers (*e.g.,* Orchidaceae, Magnoliaceae, Liliaceae) (Holopainen, 2004; Vuorinen et al., 2004a). They are emitted by a wide variety of plant species, but are not generally detected as VOCs from undamaged and mechanically damaged foliage. Therefore, the HT emission by plants is considered as specific response to herbivore attack and as a reliable indicator of herbivore damage for other organisms. Together with other VOCs they act as airborne "alarm calls", attracting carnivorous arthropods to the injured plant, leading to a reduction in the population of the attacking herbivore. The biosynthesis of DMNT proceeds *via* the conversion of SQT alcohol, trans-nerolidol from farnesyl diphosphate, the universal precursor of sesquiterpenes (Degenhardt and Gershenzon, 2000).

Inducible volatile organic compounds

Healthy plants, undamaged by herbivores, generally store and release small amounts of volatile chemicals, such as MT, SQT, and aromatics, in specialized storage sites in the leaves until needed (Paré and Tumlinson, 1997). Insect feeding inevitably leads to mechanical damage of pre-existing internal or external secretory structures in which the volatiles are synthesized and stored and triggers the synthesis and emission of VOCs, which are referred as inducible volatile organic compounds (IVOCs) or herbivore-induced plant volatiles (HIPVs). In contrast to the constitutive VOCs, the IVOCs are synthesized *de novo* only when needed and consequently they are more efficient in terms of carbon usage and do not reduce plant fitness (Kessler and Baldwin, 2002; Dicke and Baldwin, 2010). Plants synthesize a bewildering array of IVOCs, including terpenoids, fatty acid derivatives, alkenes, alkanes, benzenoids and alcohols (Dudareva et al., 2004). The

Box 3. Herbivore induced VOCs

Herbivore induced VOCs include an array of terpenes, C_6 alcohols, esters, and aldehydes (GLV) that are the result of an increased release of constitutively emitted volatiles as well as volatiles synthesized de novo. The constitutive volatiles that are regularly released from healthy plants become inducible volatiles when they are produced in larger quantities or in different ratios in response to attack.

dominating IVOCs released into the air at the site of injure or systematically from undamaged parts of affected plants tend to be terpenes and GLV (Figure 9) (Van Den Boom et al., 2004; Heil and Ton, 2008). Among the most commonly induced VOCs are the oxygenated MT linalool, the SQTs β-caryophyllene and α-farnesene, and the HTs DMNT and TMTT (Holopainen, 2004). The IVOCs blends often contain hundreds of VOCs, some of which are not produced by undamaged or mechanically wounded plants and others of which are specifically synthetized *de novo* in response to herbivore attack. The GLV are released immediately whereas the terpenes are synthetized after the damage and released after the initial injury occurred. The activation of genes behind the synthesis of IVOCs (Van Poecke et al., 2001) and their systemic emission (Paré and Tumlinson, 1999) support the hypothesis that they are not emitted passively. The VOCs blends that are released in response to herbivore feeding are different to those induced by artificial damage done by mechanical means (Turlings et al., 1995). The most extensively studied aspect of IVOCs is their induction by mechanical damage and their implication in recruiting natural enemies of herbivores.
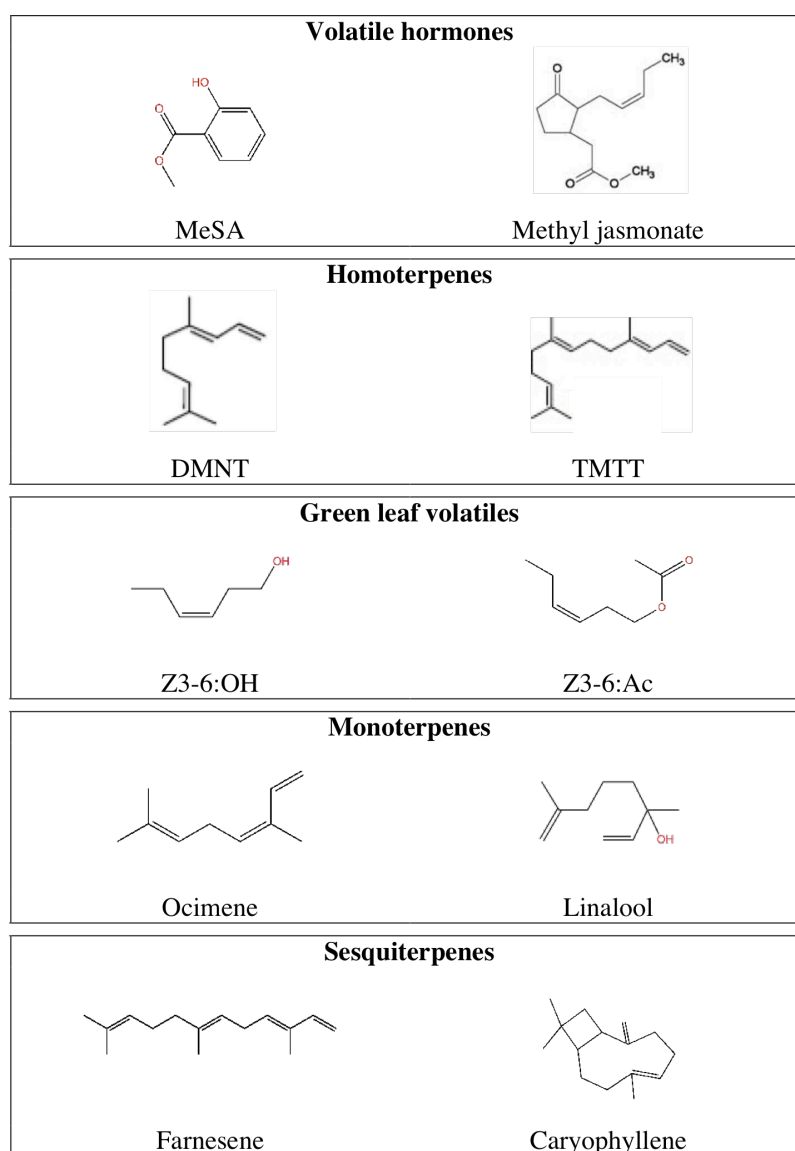
Figure 9. Examples of chemical structures of some inducible plant volatiles.

It has been found that the IVOCs act as an indirect plant defence- "a cry for help"- to attract parasites and predators of the attacking herbivore and to fend off conspecific herbivores (Figure 10) (De Moraes et al., 2001; Mumm and Hilker, 2006; Heil and Ton, 2008). Numerous behavioural studies show that carnivorous arthropods discriminate herbivore-induced plant volatiles when they are searching for their herbivorous hosts or prey (Turlings et al., 1995). In some few cases the composition of the IVOCs blend to which the carnivores respond, has been identified. The IVOCs blends are commonly composed of 20 to over 200 compounds and are chemical diverse, which makes it extremely difficult to establish the composition of behaviourally active blend (Obonyo et al., 2008).

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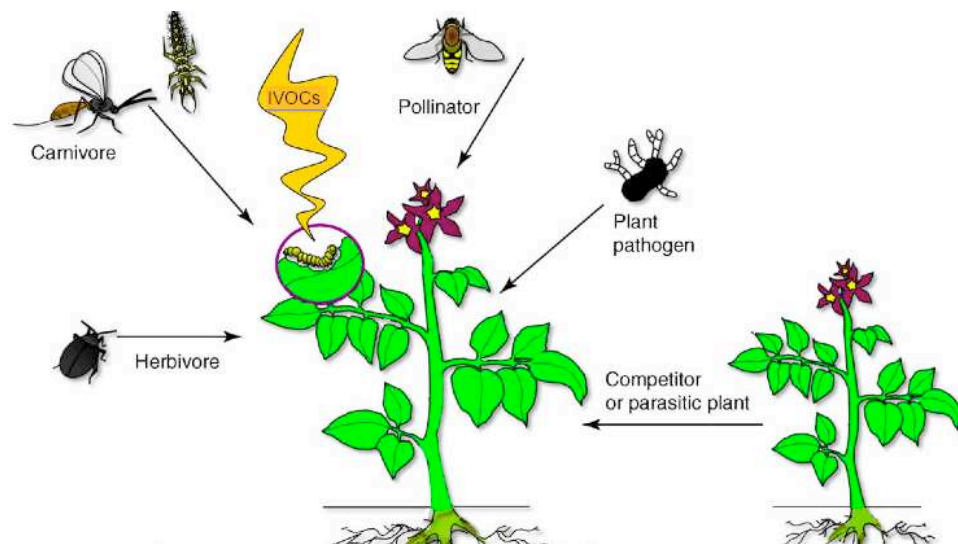
Figure 10. Plant damage and IVOCs. A plant that is locally damaged by herbivore emits induced volatiles systematically. The IVOCs can affect various community members that each exert different selection pressures on the plant (Source: Trends in Plant Science (Dicke and Baldwin, 2010))

Olfactory environment

In the nature, host cues are never perceived alone but in the context of background odour as many non-host plants in the ecosystem are constantly emitting blends of physiologically active ubiquitous compounds. In the nature the host plant volatiles and the background odours vary substantially throughout the diel cycle, through phenological development, and are influenced by environmental and biotic changes. Moreover the volatile signal of any individual plant is blurred into the background odour released from surrounding vegetation. The recognition of correct host plant signal against the background noise is a formidable task for an insect.

Insects have to be capable to monitor VOCs ratios and blends and to distinguish between host and non-host plants and to choose plants in a suitable phenological or physiological state. The same VOCs can function as both host and non-host cues, depending upon the context in which they are perceived. The background volatiles are generally considered as noisy volatiles that mask the perception of host cues. However, the background odour may provide by contrast a sharper view of the sought volatile blend and recent studies have revealed that background odours have crucial role in host localization for parasitoids. For example, volatiles functioning as host cues in a blend become non-host cues when presented alone to the black bean aphid (*Aphis fabae* Scopoli, Hemiptera: Aphididae) (Webster et al., 2010). Or, for example, a synthetic blend of VOCs of the headspace of spruce logs (*Picea abies* Karst, Pinaceae) infested by bark beetles *(Ips typographus* Latreille, Coleoptera: Curculionidae) is attractive to parasitoids when the blend is offered at the background of odour from uninfested logs (Pettersson et al., 2001). The Lima beans (*Phaseolus lunatus* L., Fabaceae) infested by the Spider mites (*Tetranychus urticae* Koch, Trombidiformes: Tetranychidae) produce MeSA, which attracts predatory mites (De Boer and Dicke, 2004). MeSA at low and intermediate concentrations and VOCs from uninfested lima bean plants are unattractive to predatory mites. However, when MeSA is combined with background odour from uninfested plants, the blend becomes attractive to the predatory mites. All these examples show the importance of background odours in host-localisation to parasitoids, but not much information is available on host-seeking moths under natural condition. Majority of studies on plant-moths interactions were conducted under laboratory conditions where the insects are stimulated with single synthetic VOCs or with simple blends and all the background

odours are eliminated and the plant preference of moths is tested by proposing single plants to moths. The laboratory conditions allow to have controlled conditions, but eliminate the background in which the moths navigate under natural conditions. The attraction to plant compounds obtained under "sterile" laboratory conditions does not always translate into attraction in the field and *vice versa*. The importance of background odour was shown to affect for example the codling moth (*Cydia pomonella* L., Lepidoptera: Tortricidae). The males of this species have two kairomonal attractants: trans-β-farnesene and pear ester that attract males in apple orchards but have no effect on the moth behaviour under laboratory conditions in the wind tunnel (Knight and Light, 2005). Furthermore the trans, trans-α-farnesene has a synergistic effect on attraction to trans-β-farnesene in the wind tunnel but not in the field (Coracini et al., 2004). Similar discrepancy was observed between laboratory and field attraction of apple fruit moth (*Argyresthia conjugella* Zeller, Lepidoptera: Yponomeutidae) to its host plant volatiles. The attraction to single synthetic volatiles anethole and 2-phenyl ethanol was reversed in the laboratory and in the field (Knudsen et al., 2008). These examples demonstrate that interaction with the background odour contributes to the behavioural effect of plant volatile signal. The discrepancies in insect behaviour in the field and in the laboratory accentuate the need to study plant–insect communication in ecologically realistic settings and the importance of background odours in host plant localization.

In the nature neither the background odours nor the host plant specific blend are not stable and fixed, but dynamic in both space and time (Murlis et al., 2000) and are influenced by biotics and abiotics factors. They change depending on plant species, the geno-and phenotype variability, wind speed, humidity, temperature and luminosity, diurnal cycles and even on herbivore species attacking the plant. Interestingly the studies on plant VOCs implicated in plant-insect interactions are recurrently done under laboratory condition and the plant volatile data is obtained by extraction of plant material or from headspace samples of induced, cut or otherwise injured plants. However, headspace method from intact plants should be preferred as it gives a more accurate picture of the ratios produced by plants. Furthermore, as the plant volatile emissions are known to change diurnally, the time period of insect activity has to be taken account when the host plants VOCs are collected. Insects are more active at certain times of the day, for example the nocturnal ECB moths stay

hidden in dense vegetation during the daytime and fly out to oviposit at dusk. Since the oviposition flight occurs within a specific and very narrow time frame, it crucial to collect the potential host-cue during that specific time. Till now most of the ECB host-cue studies are made under laboratory conditions without taking account the activity period of insect and thus the VOCs profiles obtained have unfortunately little ecological relevance.

On the other hand there is great amount of information about the olfactory environment of different types of forest, and especially about boreal forest VOCs emissions. Studies where forest ambient air and branches headspace VOCs are sampled in parallel observe an interesting discrepancy between the samples composition. Recurrently the headspace samples are dominated by SQT, whereas they are almost never detected in the ambient air. The olfactory environment is predominantly composed of MT and of GLV. The absence of SQT in the ambient air is due to their chemical proprieties and high reactivity under atmospheric conditions and its undergoing oxidation by $\cdot$OH, O_3 and NO_3. The SQT are extremely unstable and their average chemical lifetime in ambient air is few minutes, whereas the GLV and MT are rather stable and are detected hours after the emission from plants (Table 7). Atmospheric concentrations of VOCs range between a few ppt and several ppb. It has not yet been clarified whether or not the VOCs oxidized compounds or other breakdown products have ecological functions. The instability of VOCs must be taken into consideration when their ecological functions are discussed (Pinto et al., 2007).

The main studies on ambient air VOCs composition are on forest biotopes and no comparable information is available on agricultural or on other herbaceous biotopes. The information about the host-cues perceived by host seeking insect comes from studies under laboratory conditions. Considering the importance of background odours in host localization and the disparity of VOCs composition obtained from headspace and ambient air samples, there is a great need to take the experiences out in the field to obtain the ecologically relevant information about the olfactory cues perceived by host seeking insect. We studied the olfactory environment of a host-seeking ECB moth. During the ECB first generation oviposition flight, which occurs in the end of June in Ile de France region, we collected the VOCs from maize field air and from individual maize plants (headspace) using SPME fibres. We began by

characterizing the maize field olfactory environment by comparing it to forest biotope (Paper I). Subsequently we investigated the diurnal variations of VOCs emissions that occur at the time of ECB oviposition flight in maize field and maize plant (Paper II). Both of the studies were conducted under natural condition and during the ECB oviposition flight period. These studies give insight into the olfactory environment of host seeking ECB moth and provide ecologically relevant information on the chemical signals released by maize under natural conditions.

Table 7. Calculated atmospheric lifetimes of divers VOCs.

	Life span for reaction with*			Life span	
	OH	O_3	NO_3	Day	Night
Green leaf volatiles (GLV, C_6)					
Z3-6:OH	1.3 hrs	6.2 hrs	4.1 hrs		
Z3-6:Ac	1.8 hrs	7.3 hrs	4.5 hrs		
MeSA					
Monoterpenes (MT, C_{10})					
Camphene	2.6 hrs	18 day	1.7 hrs		
3-Carene	1.6 hrs	11hrs	7 min		
Limonene	49 min	2.0 hrs	5 min	40-80 min	5-20 min
Myrcene	39 min	50 min	6 min	40-80 min	5-20 min
Linalool	52 min	55 min	6 min		
Ocimene	33 min	44 min	3 min	40-80 min	5-20 min
α-Phellandrene	27 min	8 min	0.9 min	15-20 min	< 1 min
β-Phellandrene	50 min	8.4 hrs	8 min		
α-Pinene	2.6 hrs	4.6 hrs	11 min	2-3 hrs	5-30 min
β-Pinene	1.8 hrs	1.1 day	27 min	2-3 hrs	5-30 min
Sabinene	1.2 hrs	4.8 hrs	7 min	2-3 hrs	5-30 min
α-Terpinene	23 min	1 min	0.5 min	< 5 min	< 2 min
γ-Terpinene	47 min	2.8 hrs	2 min		
Terpinolene	37 min	13 min	0.7 min	15-20 min	< 1 min
Sesquiterpenes (SQT, C_{15})					
β-Caryophyllene	42 min	2 min	3 min	< 4 min	<2 min
α-Cedrene	2.1 hrs	14hrs	8 min		
α-Copaene	1.5 hrs	2.5 hrs	4 min		
α-Humulene	28 min	2 min	2 min		
Longifolene	2.9 hrs	> 33 day	1.6 hrs		

All data from Atkinson and Arey (2003), Bouvier-Brown and others (2009) .and Kesselmeier and Staudt (1999). *Assumed OH radical concentration: 2×10^6 molecule cm^{-3}, 12 hrs daytime average. Assumed O_3 concentration: 7×10^{11} molecule cm^{-3}, 24 hrs average. Assumed NO_3 radical concentration: 2.5×10^8 molecule cm^{-3}, 12 hrs nighttime average. Carbon content of VOCs is indicated in parentheses after the chemical class.

Paper I

The chemical landscape of maize field for host-seeking moth

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Abstract

Most of the crop pests find the suitable host through chemical cues released from the plants, but little is known on the chemical landscape encountered by the foraging insects. In this study, the volatile organic compound (VOC) composition of the maize (*Zea mays*, L.) field, in which the host-seeking pest navigates, was characterized and was compared with the forest chemical landscape. VOCs from the maize field and the forest atmosphere were collected by solid phase microextraction (SPME). Analyses revealed clear differences in the VOC composition of the maize field and the forest chemical landscapes. Each of them was composed of ubiquitous VOCs in specific ratio. The chemical landscape of the maize field was found to be mainly a mixture of cyclic monoterpenes (MT), whereas the chemical landscape of the forest was characterized by sesquiterpenes (SQT) and green leaf volatiles (GLV), which both of which are missing in the maize field atmosphere. The results suggest that the different biotopes produce specific chemical signatures that insects may use as host cues. To our knowledge the present paper is the first report on the nature of chemical landscapes encountered by host-seeking insects under field conditions. The role of the chemical landscape in host plant detection and host selection are discussed.

1. Introduction

Insects, especially Lepidoptera, have established specialized relations with the cultivated plants, which often involve chemical cues released by the plants and olfaction sense for the insects. Several studies have demonstrated that chemicals released by host plants play a crucial role in oviposition (Binder et al., 1995; Calatayud et al., 2008) and as a synergist of sex pheromone in mate finding behavior (Ochieng et al., 2002; Schmidt-Büsser et al., 2009). Among moths, and particularly in stem borers, the mated females choose the suitable host for brood development. Females rely on volatile cues for host plant location and acceptance. Behavioral studies in wind tunnels demonstrate that olfactory cues steer these two steps and suggest a response to a specific host chemical blend (Bengtsson et al., 2006; Tasin et al., 2007). Not much is known on the VOC composition encountered by flying insects in the natural environment. Although many studies report on maize VOCs (Bengtsson et al., 2006; Buttery and Ling, 1984; D'Alessandro and Turlings, 2005; Degen et al., 2004; Köllner et al., 2004; Solé et al., 2010; Takabayashi et al., 1995; Turlings et al., 1998) and on chemical components perceived by the insect antennae (Bruce et al., 2005), nothing is known about the VOC composition of the maize field atmosphere, especially during the lapse of time dedicated to oviposition. Moreover, little is known how a change of biotope influences the VOCs composition. Insects navigate in a constantly changing chemical landscape composed of VOCs, are mainly released by plants but also by other organisms developing in the biotope. The chemical landscape is sometimes referred to as a veritable aerial "soup" of active compounds with background odors (Bruce et al., 2005; Knudsen et al., 2008; Solé et al., 2010). The system would appear dynamic, unstable and complex. Shortly after the release in to the atmosphere, VOCs are mixed and react with natural hydroxyl radicals (OH) and ozone (O_3) present in the atmosphere (Atkinson and Arey, 2003; Pinto et al., 2007). There is an extensive amount of research published on forest VOCs emissions, and especially in boreal environments (Bonn et al., 2006; Tarvainen et al., 2005). To our knowledge, no study exist on biotopes of herbaceous cultivated plants. The main studies on chemical landscape are principally focused on estimating the vegetation-related VOC emission to the atmosphere and on the anthropogenic impact on air quality (Das et al., 2003; Kesselmeier and Staudt, 1999). Most of the studies on plant-

insect relationships are conducted under simplified laboratory conditions with stressed or damaged plants and are focused on the role of herbivore-induced volatiles (Dicke and Baldwin, 2010). Furthermore, for behavioral and electrophysiological tests all background odors are eliminated and insects are stimulated with single compounds or with a simple blends of VOCs.

In this study, we aimed to gain insight into the chemical landscape in which the Lepidoptera maize pests- the European Corn Borer (ECB) (*Ostrinia nubilalis*, Hüb.) and Corn Stalk Borer (*Sesamia nonagrioides*, Lefebvre) seek a suitable host plant to lay eggs. The oviposition flight of these two nocturnal moths occurs shortly after dusk. During that specific time-period, when the nocturnal moths are active, we collected VOCs from two different biotope atmospheres. We identified the VOC composition from the maize field atmosphere and compared it with deciduous forest, considering the latter as an unsuitable biotope for maize moths. VOC profiles are a reflection of the natural chemical landscapes encountered by host-seeking nocturnal moths. The VOCs were collected by using SPME fibers and were identified by gas-chromatography (GC) linked mass spectrometry (MS). Findings provide ecologically relevant information on the chemical landscape in which the host-seeking moths navigate.

2. Materials and methods

2.1 Sampling site

The experiments were conducted in two maize fields. One was located in the southwest of France near Pau (Pyrenees Atlantique; 43°35'N, -0.3°60'E) and the other was in Normandy (Orne; 48°63'N, 0.7°25'E). The majority of maize plants in the fields were at the silking stage (Lancashire, 1991). The Pau site consisted of approximately 15 ha continuous cropland without major industrial sources of VOCs. The Orne site was a rather small maize field (5 ha) amidst grassland fields. The forest VOCs were collected in the Ile de France region, in a mixed deciduous forest, mainly composed of beech (*Fagus* sp. L.) and oak (*Quercus* sp. L.). The collections were conducted from 30 July to 10 August 2010 from dusk to dawn to capture the main VOCs released during at night, the period during which the Lepidoptera pests are active. Temperature from dusk till dawn varied from 9°C to 18°C and RH% from

74% to 100%. Throughout the collection periods, there was neither precipitation, nor strong winds.

2.2 VOCs collection

Maize field VOCs were collected by placing SPME fibers in open-air condition in the middle of the maize field. The fiber was attached at the top part of a maize stem. The forest VOCs were collected by placing SPME fibers in open-air conditions on the lower branches of trees at 1.50 m above the ground. The sampling was conducted at least 5 meters inside of the forest.

2.3 SPME fiber

SPME fibers DVB/CARBOXEN/PDMS 50/30 μm (Supelco) were conditioned by heating in the gas chromatograph injector at 250 °C for 5·min with helium as carrier flow. Cleaned fibers were then wrapped in aluminum foil and stored in individual screw-capped Pyrex glass tubes until use.

2.4 Chemical analysis

After volatile collection, SPME fibers were desorbed in a Varian 3400 GC injector held at 250°C. The GC was coupled to a MS detector Varian QIMS. Compound separation was carried out using a Rxi-5ms column (Restek, France) 30 m × 0.32 mm i.d., film thickness 1.0 μm). The column was programmed from 50 °C for after 3 minutes at 8 °C/min to 300 °C. Helium was used as carrier gas. Mass spectra were obtained in electron impact mode (70 eV) with the ion source at 230 °C.

Compounds eluted from SPME collections were identified according to their mass spectra and retention index (RI). RIs were computed using C_{10} to C_{24} n-alkanes, eluted under the same conditions as the samples. Every compound spectrum and RI was compared with the RI and spectrum of standard and NIST 1998 library as reference using deconvolution software AMDIS32.

The calibration curves of green leaf volatiles (GLV), homoterpenes (HT), monoterpenes (MT) and sesquiterpenes (SQT) were obtained by injection of standards: α-linalool, α-pinene, α-humulene, methyl salicylate (MeSA), cis-3-hexenol (Sigma-Aldrich), β-farnesene (Chemtech), ocimene (Fluka), cis-3-hexenyl acetate (Lancaster) and (E)-4,8-dimethyl-1,3,7- nonatriene (DMNT) (gift from Jarmo K.

Holopainen (University of Kuopio, Finland). Each compound was injected at least three times at given concentrations: 5, 10 25, 50 ng/μl.

2.5 Data analysis

All the raw GC-MS data of VOCs peak areas were transformed into nanograms (ng) using the calibration curves. Relative amounts of each compound was calculated by dividing the compound amounts (ng) by the sum of detected compounds amount of the same analyze and expressed as a percentage. Since there were seven replicates of VOCs profiles taken from the maize field and four from the forest landscape; percentage composition of each product is the mean ± standard error.

3. Results

The analyses showed that the chemical landscape of the maize fields and the forest were clearly different. Both chemical landscapes were composed of VOCs, widespread in plant taxa (Figure 1). The maize field atmosphere was a blend of 13 VOCs with low variation between samples, whereas in the forest atmosphere, only seven VOCs were detected (Table 1). The major component of the VOC blend emitted in the maize field was limonene. In the forest, the most abundant component of the VOC blend was linalool (Figure 2). Interestingly, in the maize field chemical landscape neither GLVs, nor SQTs were detected. In addition, the maize fields were characterized by a blend of terpenes. Eight terpenes detected in the maize field were absent in the forest VOC collections (Figure 3). The forest chemical landscape was composed of seven VOCs: four monoterpenoids, one homoterpene, MeSA and one SQT (Figure 1, 2). A set of five VOCs was released in both biotopes but in different ratios (Figure 2). The main difference between the two chemical landscapes occurs due to the presence of eight MTs in maize field VOC collections not present in the forest, and to the presence of MeSA and α-copaene (SQT) in the forest VOC collection that were not detected in the maize field atmosphere (Figure 2).

4. Discussion

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Our results revealed a difference between chemical composition of the maize field and forest atmospheric landscapes. Both of the chemical landscapes were characterized by a specific blend of VOCs produced in the ecosystem. Even though the two biotopes have a certain number of VOCs in common, their ratios were very different. Moreover, the main components released into each biotope were also different. The maize field chemical landscape was mainly composed of limonene, whereas the forest chemical landscape was composed of linalool. Both limonene and linalool are detected by the ECB antennae (Solé et al., 2010). Interestingly, limonene is described only once as the major component of maize VOCs (Solé et al., 2010) and nothing is known on the biological activity of this product on maize moth pests. Concerning other moths, there is a report on limonene in a mixture with carvone serving as an oviposition repellent for the cabbage moth (*Plutella xylostella*, L.) (Ibrahim et al., 2005) and in a mixture with phenylacetaldehyde it is attractive to noctuid and pyralid moths (Meagher and Landolt, 2008).

The five VOCs common to the maize field and the forest atmosphere are known to induce electroantennogram responses from the ECB antennae (Solé et al., 2010). Additionally, electrophysiological studies on Lepidoptera maize pests have demonstrated that most of the VOCs identified from the maize and forest biotopes evoke antennogram responses (Bengtsson et al., 2006; Birkett et al., 2006; Bruce et al., 2005; Solé et al., 2010) but it does not necessarily mean that they act on pest host recognition behavior. The distinct blend of these five VOCs in specific ratios should generate two distinct responses in host-seeking insect. The maize field blend is probably perceived as a positive signal while the forest blend is most likely a negative signal of an inappropriate host to be avoided.

There is no information available on the perception by insects of the monoterpenoids, which were detected solely in the maize field VOC collections. It is likely that these compounds enable or participate in the specific recognition of the host plant by gravid moths and reinforce the specificity of the maize field chemical signal. Further studies on the perception of these compounds by the maize pests should be undertaken. Our results are consistent with previous studies suggesting that host plant recognition is induced by specific ratios of widespread volatiles, rather than by plant species-specific compounds (Birkett et al., 2004; Bruce et al., 2005; Dufour and Frérot, 2008; Natale et al., 2003). The two biotopes released VOCs in biotope-characteristic blends

that were rather consistent between repetitions and therefore constitute probably a reliable host-characteristic cue.

Maize plants are reported to emit around 40 VOCs, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters and carboxylic acids (Degen et al., 2004). Surprisingly, the myriad of VOCs previously identified as maize VOCs under controlled conditions in the laboratory were undetectable in the maize field chemical landscape. Our results showed that under natural conditions the chemical landscape in which the insect navigates is rather poor in volatiles. The difference between the simplicity of chemical landscape and the complexity of VOCs collected from individual maize plants is likely to originate from the sampling methods and environmental conditions. Previous studies on the forest ecosystem have shown that VOC profiles are different depending on the collection method. While headspace of enclosed branches are composed mainly of SQT, the forest atmosphere VOC blend was composed principally of MT (Bouvier-Brown et al., 2009). In nature, once the VOC are released, they are mixed and diluted in the atmosphere and react with ambient oxidants (O_3, OH and NO_3) (Bonn and Moortgat, 2003). Plants VOC atmospheric lifetimes vary from several hours to days and SQTs are more reactive than other common plant volatiles. Within minutes, SQTs are completely destroyed by O_3 and are therefore present at low concentrations and rarely detected in atmosphere analyses (Atkinson and Arey, 2003; Bonn et al., 2006). However uncertainty remains as to whether SQTs or other VOCs are emitted in quantities below technical levels of detection.

The results of the present study provide insight into the chemical landscapes that host-seeking moths encounters. We assume that nocturnal and crepuscular maize pests seeking for an oviposition site rely on the specific atmosphere of the maize field and avoid non-suitable biotopes, *e.g.* forest. The maize field and forest chemical landscapes were composed of ubiquitous VOCs released in biotope-characteristic ratios. Our results suggest that each biotope releases a chemical signature composed of distinct blend of compounds in a specific ratio that is implicated in host recognition and in nonhost avoidance. The present study opens a way to future research on the chemical landscape encountered by insects and on host plant recognition under field conditions.

5. Abbreviations

(E)-4,8-dimethyl-1,3,7- nonatriene (DMNT) ; Divinylbenzene (DVB); Electron volt (eV); European Corn Borer (ECB); Gas-Chromatography (GC); Green leaf volatiles (GLV); Homoterpene (HT); Hydroxyl radicals (OH); Mass-Spectrometry (MS); Monoterpene (MT); The National Institute of Standards and Technology (NIST); Nitrate radical (NO_3); Ozone (O_3); Polydimethylsiloxane (PDMS); Relative humidity (RH); Retention index (RI); Retention time (rt); Sesquiterpene (SQT); Solide Phase Microextraction (SPME); Volatile Organic Compounds (VOCs).

6. Acknowledgements

FIGURE CAPTIONS

Figure 1. The chemical structures of VOCs detected in the maize field and the forest chemical landscapes

Figure 2. The set of 5 VOCs common to the maize field and the forest chemical landscapes. The mean relative amount of each VOC is expressed in percent of the 5 VOCs total emission with the corresponding standard error (SE). Black bars represent the field (n=7) and grey bars the forest chemical landscape (n=4)

Figure 3. VOCs detected in the maize field and forest chemical landscapes

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Table 8. VOCs detected in maize fields and the forest chemical landscapes. VOCs are listed in order of their elution on the gas chromatographic column.

			VOCs of the chemical landscape					
			Maize field (n=7)			Forest (n=4)		
RI	VOCs	Identification	Mean	SE	O	Mean	SE	O
934	α-thujene	NIST	0,5	0,1	2	-	-	-
942	α-pinene	rt/MS	12,2	2,0	7	-	-	-
960	Camphene	rt/MS	0,5	0,0	6	-	-	-
982	Sabinene	NIST	6,7	6,2	3	-	-	-
989	β-pinene	rt/MS	2,2	0,7	3	-	-	-
993	β-myrcene	rt/MS	3,0	0,4	7	13,2	3,5	4
1013	α-phellandrene	rt/MS	0,5	0,1	6	-	-	-
1020	3-carene	rt/MS	4,8	1,8	6	-	-	-
1032	P-cymene	rt/MS	9,6	1,4	7	21,8	12,	3
1038	Limonene	rt/MS	61,3	2,0	7	15,1	3,8	3
1103	α-linalool	rt/MS	3,4	0,2	7	42,5	2,1	2
1118	Thujone	rt/MS	1,7	0,4	6	-	-	-
1119	DMNT	rt/MS	0,4	0,1	5	2,7	0,5	4
1209	Methylsalicylate	rt/MS	-	-	-	29,6	6,4	4
1395	α-copaene	rt/MS	-	-	-	10,9	0,3	2

RI - Retention index; NIST - identification by the National Institute of Standards and Technology Mass Spectra database of 1998; rt/MS - identification by retention time and mass spectrum; Mean - mean relative amount, SE - standard error; O - occurrence (i.e. the number of analyses in which the compound was detected).
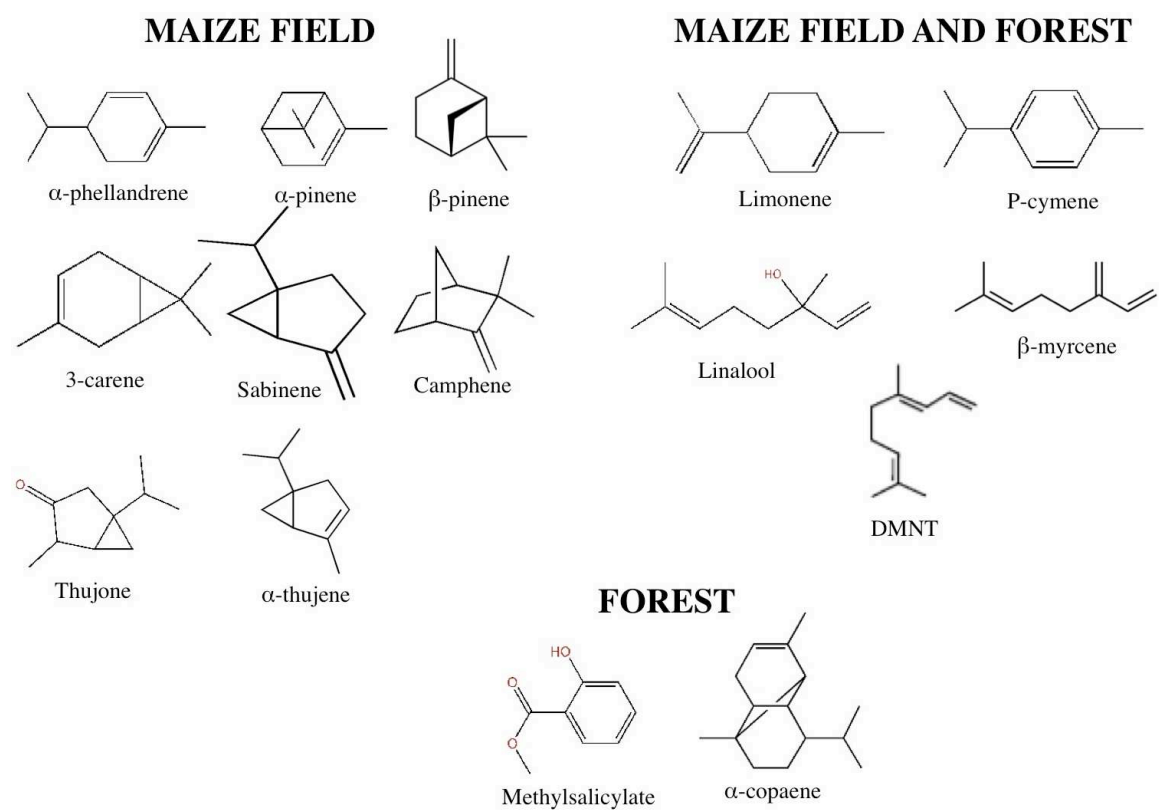
Figure 1. The chemical structures of VOCs detected in the maize field and the forest chemical landscape.
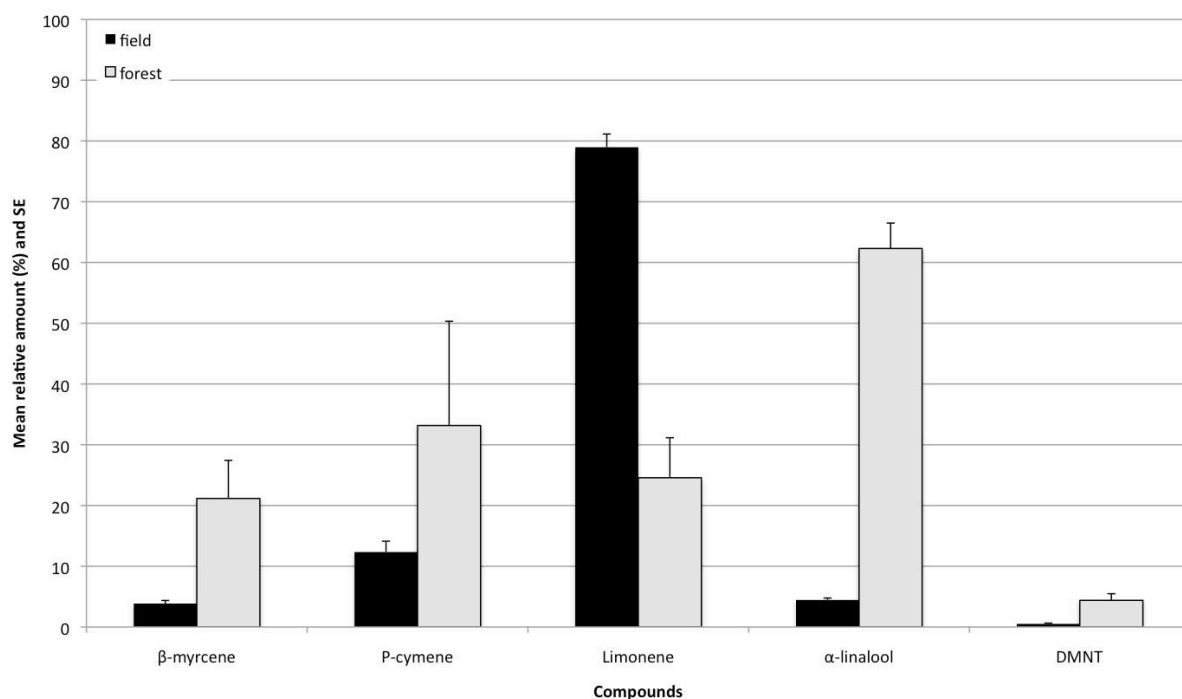
Figure 2. The common set of VOCs released by the maize field and the forest chemical landscapes. The mean relative amount of each VOC is expressed in percent of the 5 VOCs total emission, with the corresponding standard error (SE). Black bars represent the field (n=7), and grey bars the forest chemical landscape (n=4).
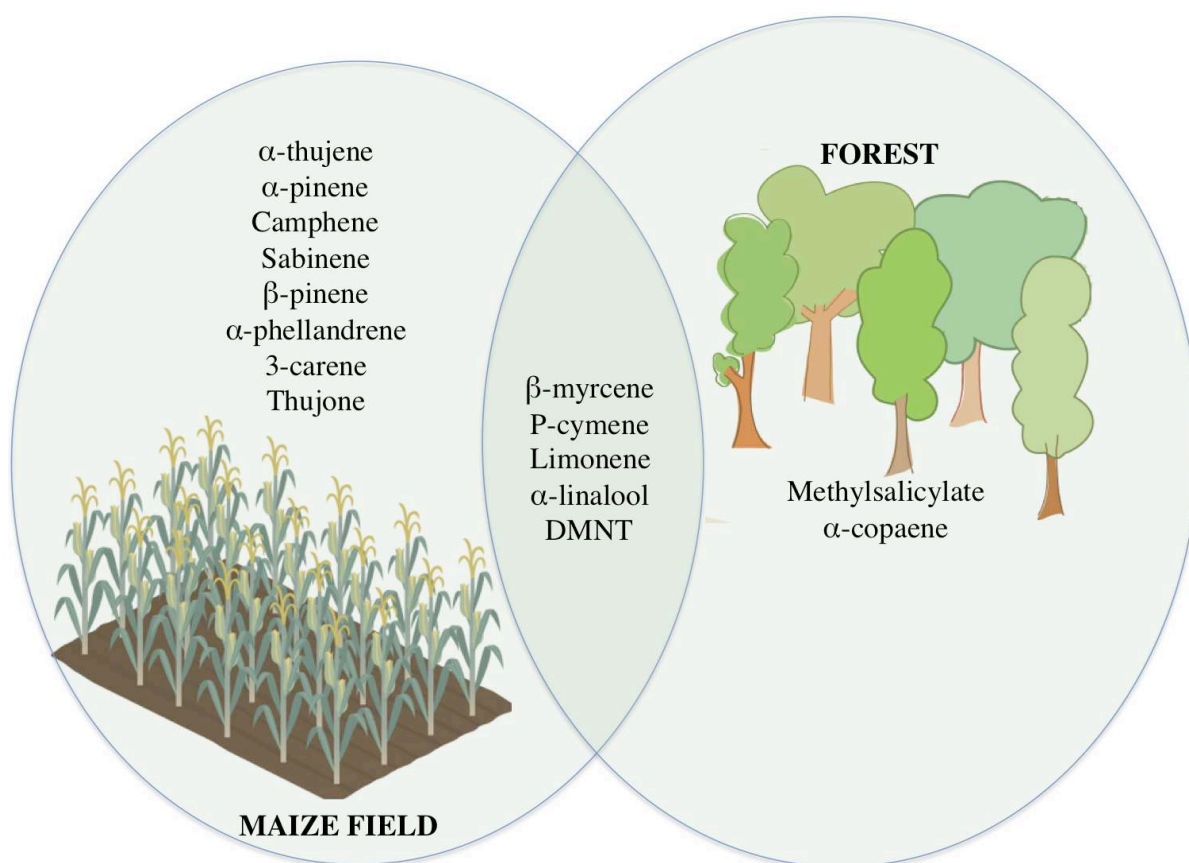
Figure 3. VOCs detected in the maize field and in the forest chemical landscape.

Paper II

"Diel patterns of volatile organic compounds released by maize plants: The chemical
environment of the *Ostrinia nubilalis* moth"
Ene Leppik, Toomas Tammaru and Brigitte Frérot

Diel patterns of volatile organic compounds released by maize plants: The chemical environment of the *Ostrinia nubilalis* moth

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Abstract

Volatile organic compounds (VOCs) released by host plants attract gravid European corn borer (ECB) female moths for oviposition. Despite extensive studies, little is known about VOCs emitted by maize under natural conditions or the chemical atmosphere of a maize field, particularly at the time of ECB oviposition. Here, we characterised VOCs released by undamaged maize plants and VOCs in the maize field atmosphere. VOCs were collected throughout the diel cycle with solid-phase microextraction fibres and were identified by GC-MS and quantified with calibration curves. Four replicates per time period were collected; *i.e.,* dusk, night, dawn, and day. VOC patterns differed between the maize plants and the maize field atmosphere throughout the diel cycle. At night, the period of ECB oviposition, the VOC pattern was characterised by an increase in monoterpenes, a decrease in sesquiterpenes, and the presence of methyl salicylate, α-copaene, and Z-3-hexenyl acetate. An apparent discrepancy between maize plant and field VOC compositions was observed. Key compounds were identified as putative host-cues, including methyl salicylate, α-pinene, 3-carene, p-cymene, limonene, and dimethyl nonatriene. This study showed that VOCs were released by maize in a diel pattern, and host-characteristic cues were present for nocturnal ECB oviposition.

1. Introduction

Insects and plants have forged close ties through co-evolution. This was often based on the perception by insects of chemical signals emitted by plants (Metcalf and Metcalf, 1992; Schoonhoven et al., 2005). Although not native to Europe, cultivated maize *(Zea mays*, L.) has been colonised by the native herbivore Lepidoptera, the European corn borer (ECB). The yearly cultivation of maize over large areas provides an inexhaustible food resource, and often leads to uncontrolled ECB population growth.

ECB is a nocturnal insect that mates in densely vegetated areas, where they rest and hide during the day (Caffrey and Worthley, 1927; Showers et al., 1976). Host plant recognition is achieved by gravid ECB females that seek oviposition sites shortly after sunset (Showers et al., 1976). Field observations have clearly shown that gravid ECB females reach maize fields by flights from a resting area (Frérot & Leppik, unpublished data), and they are carried on the prevailing wind, which is likely to carry scents from maize fields.

Previous studies on volatile organic compounds (VOCs) released by maize were mostly conducted under laboratory conditions. Those studies used isolated or cut plants to identify VOCs induced by herbivore-damaged or wounded plants that attracted natural enemies (D'Alessandro and Turlings, 2005; Köllner et al., 2004; Takabayashi et al., 1995; Turlings and Tumlinson, 1992). Other studies that focused on identifying maize VOCs as attractants for ECBs were conducted during the photophase (Bengtsson et al., 2006). Only two studies reported a diel pattern of VOC release; those VOCs were collected under laboratory conditions from water-stressed maize (Solé et al., 2010) and herbivore-damaged maize (Turlings et al., 1995). To our knowledge, all other studies on maize VOCs investigated genetic variations (Degen et al., 2004), environmental conditions, and growth stages (Köllner et al., 2004). They led to the identification of 40 maize VOCs and showed that maize was rather poor in volatiles compared to other ECB host plants, like hops or mugwort (Leppik & Frérot, submitted).

Despite the numerous studies on maize-emitted VOCs, little is known about maize VOCs released under field conditions. VOC profiles acquired under photophase

conditions are probably not relevant to the study of nocturnal host-seeking moths. The natural chemical environments encountered by nocturnal host-seeking ECBs remain unknown. The aim of this study was to provide ecologically relevant insight into natural, plant-produced VOCs at the levels of the plant and the field during the period of ECB activity. VOCs were collected from maize plant headspaces and from the field atmosphere. The collected VOCs were identified and quantified. The differences between VOC blends from maize plant headspaces and the field atmosphere are discussed, based on the present knowledge of the chemistry of emitted VOCs and their ecological significance.

2. Results

2.1. Maize headspace VOC

From maize headspace VOC collections, 21 components were detected and identified based on comparing the RI and mass spectra to authentic samples or appropriate data bases (Table 1a). Nineteen components out of 21 had previously been identified. Two VOCs were newly identified as maize VOCs: p-cymene, and a compound tentatively identified as selina-3,7 (11) diene (SQT).

The maize scent was found to be a mixture of three GLVs, six MTs, two homoterpenes (HTs), and 12 SQTs (Table 1a). The amounts of each component varied considerably throughout the 24-h cycle. The relative ratios of MT and SQT changed from day to night (Fig. 2). The peak SQT emission occurred during the day. In contrast, the peak MT emission was observed at night and dawn. HT was always present in low quantities, and the quantity did not change substantially over time. The diel GLV composition was characterized by the absence of Z3-6:Ac during the day and at dawn, but the relative amount of Z3-6:OH increased at dusk.

MeSA (an induced GLV) and α-copaene, were the two main compounds in maize headspace collections; they accounted for half of all the VOCs detected (Table 1a). The relative amounts of MeSA and α-copaene varied over time. The diel variations in these two VOCs were clearly separate at dusk and dawn. The ratio of MeSA to α-copaene in the headspace varied from 0.39 at dusk to 2.32 at dawn. The ratio between day and night was less impressive; it ranged from 1 during the day to 0.68 at night (Fig. 3). During the day, the emission rates of α-copaene and MeSA were nearly

equal. At dusk, however, individual maize plants emitted about 2.5 times more α-copaene than MeSA. At night, they tended to be emitted at similar levels At dawn, the emission rates were the reverse of those at dusk, and maize plants emitted about 2.3 times more MeSA than α-copaene.

The three PCs calculated from the measured amounts of VOCs to compare time periods explained 35, 21, and 14 % of the variance, respectively. A MANOVA performed on the three PC values revealed significant differences in the ratios of maize VOCs among the four time periods (Wilk's lambda = 0.071, p = 0.0015). When the three PC's were analysed separately, only the scores of PC2 differed significantly between time periods (two-way ANOVA, with date as an additional factor: $F_{3,11}$ = 15.7, p = 0.0002) and captured the time-related variance of the VOC ratio (Fig. 4). Pairwise comparisons of PC2 scores among the four periods showed no significant differences between the maize VOCs ratios for day and dusk, or night and dawn. All other comparisons showed a significant difference (Tukey test: p<0.02).

Separate analyses were performed on the headspace VOC data for different classes of compounds. We found that the SQT profiles differed among time periods (Wilk's lambda = 0.060, p = 0.006). Again, the profiles for night and dawn, and for day and dusk were similar; but pairwise comparisons among other time points attained significance. In contrast, no among-period differences could be shown for the other compound classes (Wilk's lambda = 0.27, p = 0.15); i.e., GLV, HT, MT.

The among-period differences for individual VOCs was analysed with the Kruskal-Wallis test (Table 1a). In the individual maize static headspace collections, the diel relative amounts of three VOCs out of 21 changed significantly (Z3-6:Ac [df=3, N=16, Z=8.45, p=0.038], trans-α-bergamotene [df=3, N=16, Z=10.25, p=0.017], and α-cadinene [df=3, N=16, Z=12.91, p=0.005]). Pairwise comparisons for the GLV, Z3-6:Ac, showed no significant changes in the relative ratio over time (p≥0.05). However, Z3-6:Ac was detected only at dusk and night in maize VOC collections. Trans-α-bergamotene and α-cadinene were not detected at night or dawn; they were present only during the photophase, with a significantly higher amount at dusk (p=0.038, Z=8; p=0.013, Z= 8.500 respectively).

2.2 Maize field atmosphere VOCs

In the maize field atmosphere, a total of 13 VOCs were detected and identified (Table 1b). The VOCs profile was dominated by MeSA and a complex of p-cymene with limonene. There was also a constant low level of dimethyl nonatriene (DMNT). The ratio of MeSA to the complex of p-cymene-limonene did not change with diel periods; α-pinene, 3-carene, linalool, α-copaene, β-farnesene, and trans-nerolidol were detected in random amounts in the atmosphere without a clear diel pattern.

None of the 13 VOCs detected in the maize field significantly changed in amount over time. Furthermore, when the 13 VOCs were grouped into chemical classes, no significant diel variation was observed for the relative amounts (Kruskal Wallis test, $p \geq 0.05$ for all cases).

There were 12 VOCs in common between samples from the maize field atmosphere and the maize headspace. Out of the 13 VOCs detected in the maize field atmosphere, the MT alcohol, linalool, although repeatedly detected in the atmosphere, was the only VOC that was not found in the maize headspace. The VOC profiles from maize headspaces were considerably richer than the profiles from the field air samples. The headspace maize VOC blend mainly comprised SQT; in contrast, the maize field atmosphere mainly comprised MT. In general, the diel pattern changes in the VOC composition for individual maize plants did not match the patterns observed in the maize field atmosphere. Only MeSA (GLV) had the same diel pattern of emission in the field and the headspace collections; the emission peaked while it was light over the 24-h cycle, and it decreased at dusk and night.

3. Discussion

This work, performed in the field on healthy maize plants, has generated a new description of volatiles released by maize. This study was the first to provide insight on what a flying insect might encounter in a maize field. The diel variations observed in this study supported our hypothesis that pests, like ECB moths, are likely to be tuned to maize plant VOCs, and probably use specific volatile cues released by plants at the beginning of the night to reach an appropriate oviposition site.

At the plant level, the relevant changes were the Z3-6:Ac levels, which was released only during the dark period, and the increase in limonene and p-cymene, which were newly identified in the maize headspace. The SQTs were absent during the dark

period, and only detected during the day, in contrast to the MT emission. Other studies showed that the diel variations in MT and SQT emissions from other plants were influenced by abiotic factors and photosynthesis (Duhl et al., 2008; Fall et al., 1999; Fuentes et al., 2000; Grote and Niinemets, 2008; Ibrahim et al., 2010; Sharkey et al., 2008). Interestingly, MeSA was identified as one of two main components in the headspace of healthy maize plants. The second main component, α-copaene, showed opposite fluctuations in the diel pattern. Moreover, our results showed that MeSA was not strictly an herbivore-induced component; instead, it appeared to be a constituent of the maize headspace. This result was reinforced with the results for the maize field atmosphere, where MeSA was identified in large amounts. MeSA can be considered a key compound for maize plant recognition by ECB, because it can elicit strong responses from female antennae (Bengtsson et al., 2006), and it acts on female attraction behaviour (Solé et al., 2010). The most frequently detected VOCs in maize headspace analyses were MeSA, α-pinene, p-cymene with (S)-limonene, and α-copaene. The volatile compound pattern described here differed from those of water-stressed plants (Solé et al., 2010) and herbivore-damaged plants (Turlings et al., 1995). The global changes in VOCs over time may be sensed as different signals to host-seeking moths.

Analyses of maize field atmosphere VOCs showed that the natural chemical environment of host-seeking insects is poor in volatiles and comprises mainly MeSA and MT. SQT were detected rarely and without any apparent pattern. The most consistently detected VOCs in the maize field atmosphere were MeSA, α-linalool, and β-myrcene; these were previously reported as VOCs from maize, but after herbivore damage, (D'Alessandro and Turlings, 2005; Ozawa et al., 2008; Turlings et al., 1998). Moreover, limonene, α-pinene, 3-carene were also frequently detected, but little is known about their biological activity on ECBs.

Linalool, a previously identified compound from stressed or damaged maize plants, was not detected in our VOCs collected from healthy plants in the field. Thus, this emission may be part of a chemical signal for plant stress. We speculated that the linalool identified in the field atmosphere was derived from the woods at the edges of the fields. Large amounts of this compound are generally released in deciduous forests (Ciccioli et al., 1999; Owen et al., 2001).

The apparent discrepancy between VOC profiles of maize headspaces and field atmosphere was primarily due to the different patterns of MT and SQT. In the atmosphere VOC collections, SQT were rarely detected. This distortion in the SQT:MT ratios between the atmosphere and plants was similar to that previously described (Bouvier-Brown et al., 2009; Ciccioli et al., 1999). The SQTs are highly reactive to atmospheric O_3, and they are often completely destroyed in the atmosphere before they can be detected (Atkinson, 1990; Bonn and Moortgat, 2003). The average lifetime of an MT molecule in the air is about 1 h; that for SQT is only 2-4 min (Kesselmeier and Staudt, 1999). The differences might also be explained by the experimental design. For the headspace VOCs collection, we created a limited space, where the volatiles were concentrated; in contrast, for the atmospheric VOCs collection, the SPME fibres were exposed to field air with extremely low concentrations of VOCs. Indeed, similar results were obtained when maize field VOCs were compared to VOCs in a deciduous forest atmosphere (Leppik & Frérot, submitted).

4. Conclusion

As expected, the chemical signal released by maize plants differed with diel periodicity. The chemical signal encountered by nocturnal flying insects would be specific to the dark period. The VOCs blends from the maize headspace mainly comprised MeSA and α-copaene. However, Z3-6:Ac, MeSA, α-pinene, 3-carene, p-cymene, limonene, and DMNT were found in both the maize headspace and the atmosphere. These can be considered candidate compounds for host plant recognition. Further study is merited on their relevance to insect behaviour.

5. Experimental

5.1. Sampling site
The experiments were conducted in a maize field situated in the Ile-de-France region near Grignon (Yvelines) (48°85'N, 1.9°68'E) in France. The maize plant was represented by the Troubadour variety, commonly cultivated in the northern part of France. The maize field consisted of approximately 15 ha of continuous cropland with

no industrial sources of VOCs. The collections were conducted from 10 June to 16 June 2010; from 22 June to 23 June 2010; and from 25 June to 26 June 2010. Maize plants were at the four-leaf phenological stage (Lancanshire et al., 1991). The temperature from dusk to dawn varied from 9 °C to 18 °C and the RH% varied from 74 to 100%. Throughout the collection sessions, there was neither precipitation nor strong winds.

5.2. VOC collections

The non-destructive, static, headspace sampling mode was chosen for collecting maize VOCs. Plants were chosen that had approximately equal leaf surfaces; headspaces were enclosed in individual Teflon bags (50 × 25 cm). A small hole was pierced in the Teflon bag to introduce a solid phase microextraction (SPME) fibre. Control samples, one per time period, constituted an empty closed Teflon bag, with a SPME fibre introduced under the same conditions as used for the plants. Headspace VOCs were collected over two hours at four distinct time periods: dusk (22:00 - 00:00), night (01:00 - 03:00), dawn (05:00 - 07:00), and day (13:00 - 15:00). After VOC collection, SPME fibres were packed individually into aluminium foil and stored at -20 °C, until analysis by gas chromatography (GC) and mass spectrometry (MS). A different plant was chosen for every collection session. Two replicates were collected for each time period on two different days and nights; thus, VOC profiles were recorded from a total of 16 plants.

Maize field atmosphere VOCs were collected by placing SPME fibres in the open-air, in the middle of the maize field. The fibres were attached to the top part of a maize stem and VOCs were collected under the same conditions as described above for the headspaces. Maize field atmosphere VOCs were collected for two hours at four distinct time periods: dusk (22:00 - 00:00), night (01:00 - 03:00), dawn (05:00 - 07:00), and day (13:00 - 15:00) from the same field where the headspace VOCs were collected.

5.3. SPME fibres

SPME fibres were DVB/CARBOXEN/PDMS 50/30 μm (Supelco). Prior to each sampling, fibres were cleaned by placing them in the GC injector at 250 °C for 5 min,

with a helium gas flow. Cleaned fibres were wrapped in aluminium foil and stored in screw-capped individual Pyrex glass tubes until use.

5.4. Chemical analyses

To identify VOCs from the maize headspace and field atmosphere, the SPME fibres were desorbed in the Varian 3400 GC injector at 250 °C. The GC was linked to a MS detector (QIMS, Varian). Compound separation was carried out with a Rxi-5ms column (30 m × 0.32 mm i.d., film thickness 1.0 μm; Restek, France). The column was programmed to maintain a temperature of 50 °C for 3 min and to increase the temperature by 8 °C/min to a maximum of 300 °C. Helium was used as the carrier gas. Mass spectra were obtained in electron impact mode (70 eV) with the ion source at 230 °C.

VOCs eluted from SPME collections were identified according to their mass spectra and retention indexes (RI). The RIs were computed by comparing elution times to those of n-alkanes (C10 to C24) under the same conditions. For every compound, the RI and spectra were compared with the RI and spectra of laboratory and NIST 1998 libraries with deconvolution software (AMDIS32).

The calibration curves for green leaf volatiles (GLV), monoterpenes (MT), and sesquiterpenes (SQT) were obtained by injecting the following synthetic samples onto the GC-MS: α-linalool, α-pinene, α-humulene, α-copaene, MeSA, cis-3-hexenol (Z3-6:OH; Sigma-Aldrich), β-farnesene (Chemtech), ocimene (Fluka), and cis-3-hexenyl acetate (Z3-6:Ac; Lancaster). Each compound was injected in at least three replicates at concentrations of 5, 10, 25, and 50 ng/μl. Linear calibration curves (Fig. 1) were created for each compound to estimate the GC-MS ion trap detector response. The GC-MS detector response for α-linalool, ocimene, and myrcene were used to quantify the acyclic MT, α-pinene for cyclic MT, α-humulene and α-copaene for cyclic SQT, and β-farnesene for acyclic SQT. The GC-MS MeSA, Z3-6:OH, and Z3-6:Ac responses were used to quantify the different GLVs.

5.5. Data analyses

All the raw GC data for VOCs were converted to nanograms (ng) with the respective authentic calibration curves. Relative amounts of compounds were expressed as a percentage by dividing the amount of compound (ng) by the amount of total

compounds detected in the same analysis. The measurements of emitted VOCs during the diel cycle were expressed as the averages of four replicates of VOC profiles for each time period and two replicates of air collections per time period from the maize fields. To test for differences in VOC compositions among time periods, it was not feasible to perform a straightforward MANOVA approach (i.e., treating the amount of each compound as a dependent variable), due to an insufficient number of degrees of freedom. To reduce the dimensionality, we conducted a principal component (PC) analysis for the total spectrum of VOCs. A MANOVA was then performed on the values of the PC scores. Taken separately, the first three PCs were tested with two-way ANOVAs to identify significant differences between time periods. Analogous analyses were performed separately; one for SQT, and one for all the other compounds. For all ANOVAs, distributions of residuals were checked, and we found no substantial deviations from normality. The differences in individual compounds were compared among the four time periods with the non-parametric Kruskal-Wallis test, because evidence of clear deviations from normality precluded a parametric ANOVA approach. Analogous MANOVA PC analysis and Kruskal-Wallis tests were conducted on field air VOCs data.

6. Acknowledgements
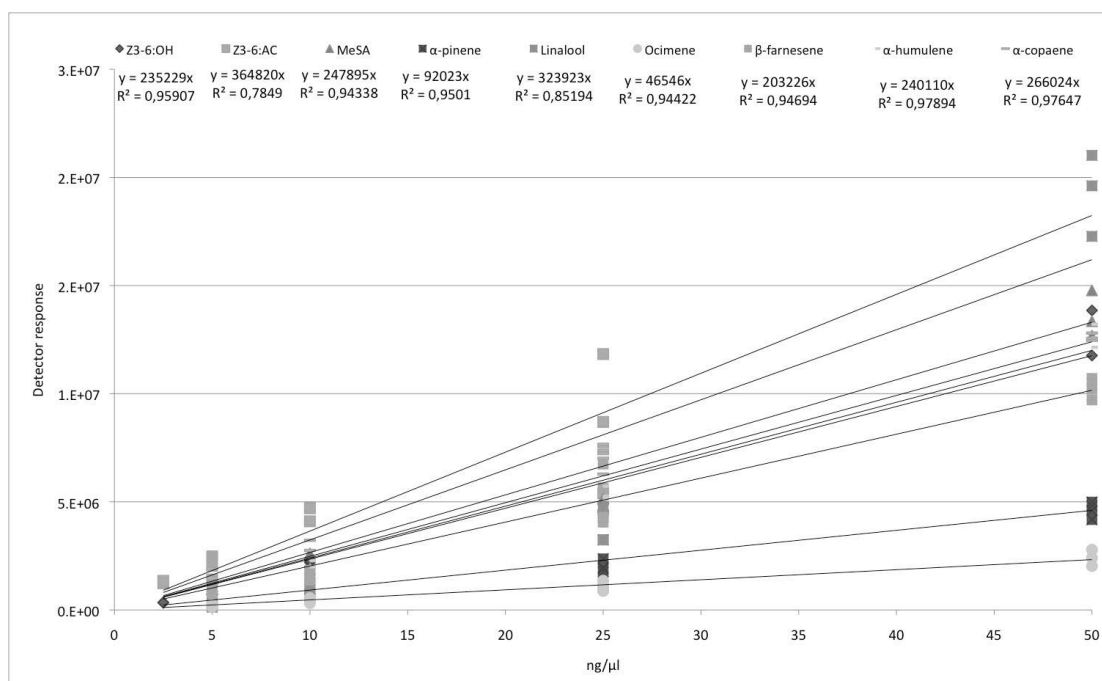
Figure 1. Linear calibration curves were created to estimate the GC-MS detector response to different plant VOCs. Green leaf volatiles (GLV), monoterpenes (MT), and sesquiterpenes (SQT) were quantified with MeSA, Z3-6:OH, and Z3-6:Ac (GLVs); α-linalool, ocimene, and myrcene (acyclic MT); α-pinene (cyclic MT); α-humulene and α-copaene (cyclic SQT); and β-farnesene (acyclic SQT).
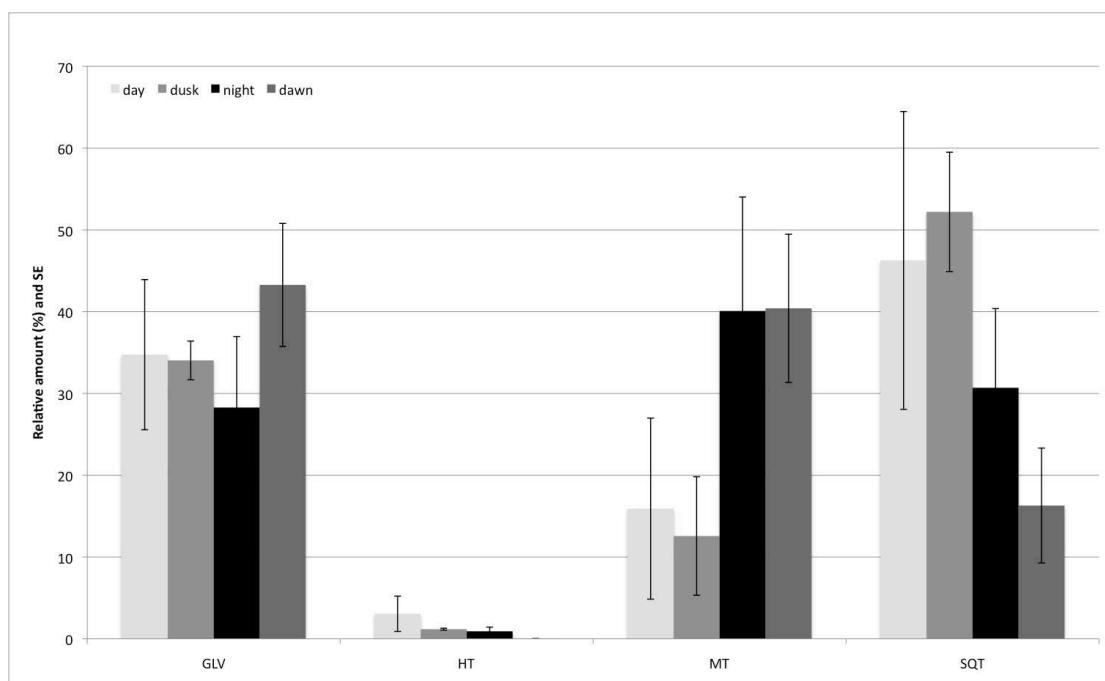
Figure 2. The diel changes in relative amounts of green leaf volatiles (GLV), homoterpenes (HT), monoterpenes (MT), and sesquiterpenes (SQT) from maize headspace samples (n=4).
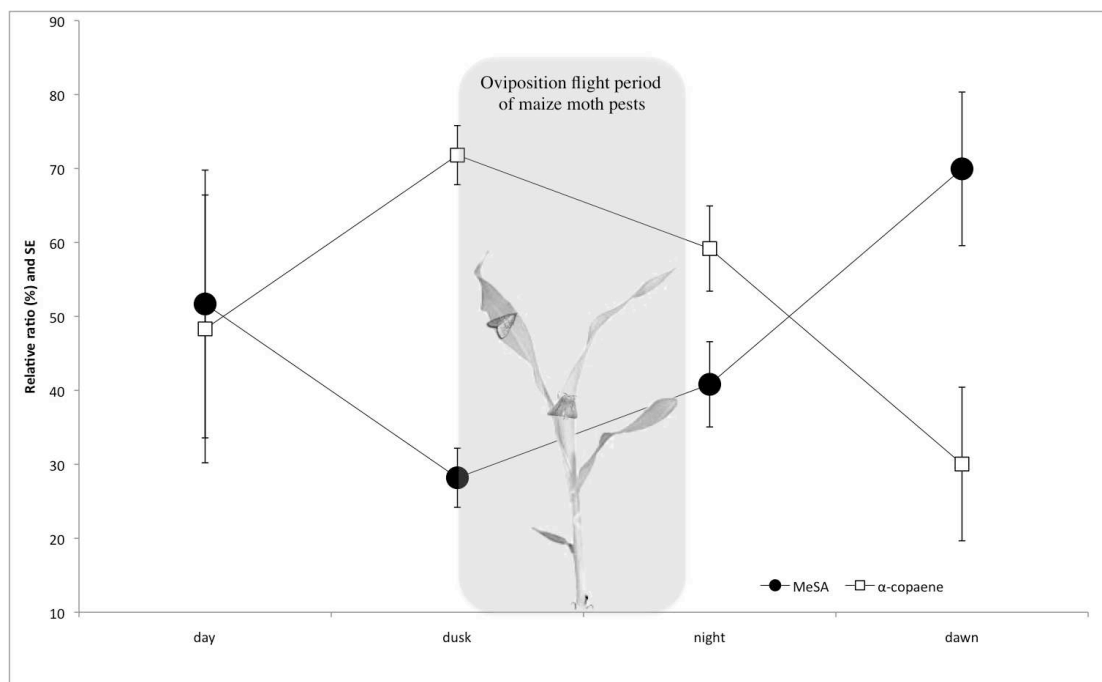
Figure 3. The diel changes in relative amounts of MeSA and α-copaene from maize headspace samples (n=4). The oviposition flight of maize moth pests occurs shortly after sunset (represented by the grey area).
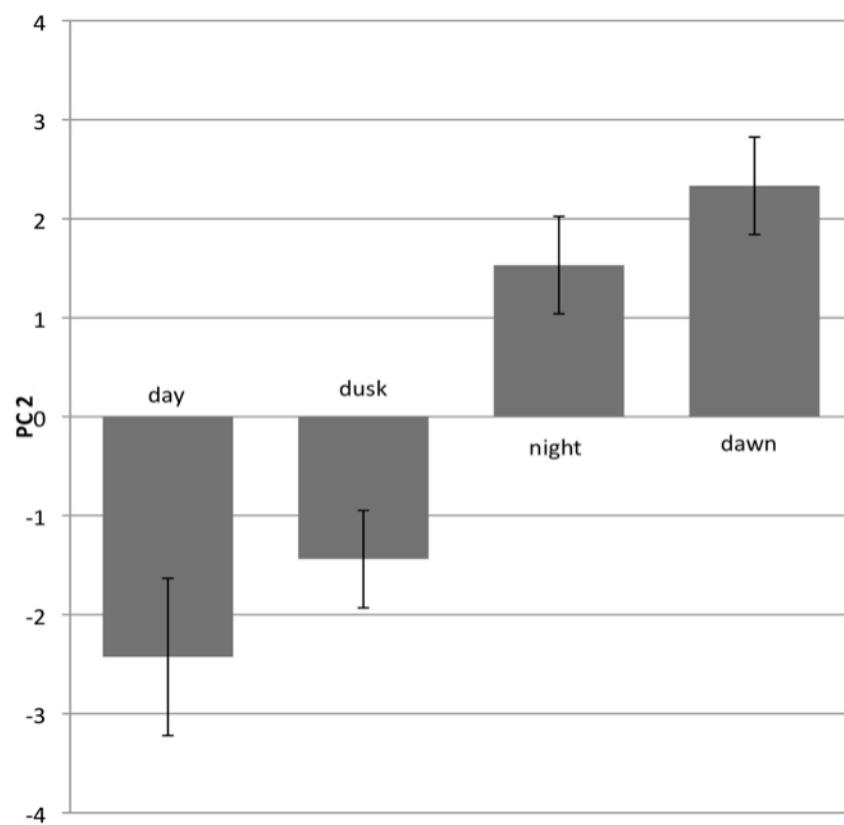
Figure 4. Principal component bi-plot of maize headspace VOCs. The variations in VOC composition and relative amounts distinguish day and dusk from night and dawn.

Table 1a. Mean percentages of VOCs identified in individual maize plants relative to the total number of VOCs.

| | | Relative amount (%) of VOCs in maize headspace | | | | | | | | | |
| | | Day (n=4) | | Dusk (n=4) | | Night (n=4) | | Dawn (n=4) | | Kruskal Wallis | |
RI	VOCs	Mean±SE	O	Mean±SE	O	Mean±SE	O	Mean±SE	O	χ^2	p–value
	Green leaf volatiles										
860	Cis-3-hexenol[1-3] *	6.82±5.35	2	20.58±3.31	4	6.86±6.86	1	9.11±9.11	1	3.366	0.339
1007	Cis-3-hexenyl acetate [1,3-5]*	–	0	1.15±0.48	3	3.28±2.61	3	–	0	8.454	0.038
1209	MeSA[2,3,5] *	27.92±11.21	4	12.30±0.77	4	18.13±3.14	4	34.15±4.22	4	6.199	0.102
	Monoterpenes										
942	α-pinene[5] *	4.20±3.04	2	4.08±1.76	3	8.12±3.21	3	12.91±4.76	3	3.295	0.348
993	β-myrcene[1,2] *	0.25±0.25	1	–	0	–		–		3.000	0.392
1017	3-carene*	–	0	–	0	2.85±1.67	2	1.71±1.71	1	3.845	0.279
1032	p-cymene*	6.92±5.2	3	7.05±2.75	2	11.09±2.86	4	12.55±1.95	4	4.993	0.172
1038	S(-) limonene*	4.54±2.94	3	4.96±3.23	2	18.02±9.6	3	13.22±5.68	3	2.568	0.463
1098	α-linalool[1,3-5]*	–	0	–		–		–	0	–	
	Homoterpenes										
1117	DMNT[1,2] *	2.29±2.29	1	1.77±0.11	4	0.92±0.5	3	0.02±0.02	0	6.058	0.109
1128	TMTT[1,4]	0.77±0.77	2	–	0	–	0	–	0	6.400	0.094
	Sesquiterpenes										
1395	α-copaene[3] *	22.01±9.47	3	33.65±6.18	4	30.7±9.68	4	16.29±7.02	3	3.474	0.324
1415	Ylangene[3,4]	0.46±0.46	1	–	0	–	0	–	0	3.000	0.392
1450	Trans-α-bergamotene[1,2,4,6]	2.30±1.76	2	6.14±0.09	4	–	0	–	0	10.252	0.017
1452	β-caryophyllene[1-6] *	1.37±1.37	1	–	0	–	0	–	0	3.000	0.392
1464	β-farnesene[1-4,6]*	0.10±0.10	1	0.02±0.02	1	–	0	–	0	2.150	0.542
1516	GermacreneD[3,4,6] *	0.46±0.46	1	–	0	–	0	–	0	3.000	0.392
1527	γ-cadinene[6]	–	0	1.25±1.25	1	–	0	–	0	3.000	0.392
1535	δ-cadinene[6]	0.47±0.47	1	2.57±1.48	2	–	0	–	0	4.604	0.203
1544	Selina-3,7 (11) diene	0.42±0.42	1	0	0	–	0	–	0	3.000	0.392
1549	α-cadinene[6]	0.43±0.43	1	5.28±0.17	4	–	0	–	0	12.913	0.005
1592	Trans-nerolidol[1,2,4]	18.20±11.48	2	3.27±3.27	1	–	0	–	0	4.604	0.203

Table 1b. Mean percentages of VOCs identified in the field collections relative to the total number of VOCs

| | | Relative amount (%) of VOCs in maize field atmosphere | | | | | | | | | |
| | | Day (n=2) | | Dusk (n=3) | | Night (n=4) | | Dawn (n=3) | | Kruskal Wallis | |
RI	VOCs	Mean±SE	O	Mean±SE	O	Mean±SE	O	Mean±SE	O	χ^2	p–value
	Green leaf volatiles										
1007	Cis-3-hexenyl acetate [1,3-5]*		0	–	0	0.11±0.11	1	–	0	2.000	0.572
1209	MeSA[2,3,5] *	41.57±26.68	2	30.10±7.46	3	25.21±8.63	4	34.35±6.24	3	0.737	0.864
	Monoterpenes										
942	α-pinene[5] *	–	0	13.05±7.50	2	3.65±2.13	2	9.68±4.85	2	3.389	0.335
993	β-myrcene[1,2] *	–	0	2.00±2.00	1	9.63±5.58	2	2.59±2.59	1	1.942	0.584
1017	3-carene*	3.18±3.18	1	3.80±3.80	1	1.63±1.07	2	–	0	1.838	0.607
1032	p-cymene*	28.95±2.31	2	11.70±6.04	2	18.86±9.37	4	13.84±4.19	3	3.013	0.390
1038	S(-) limonene*	2.95±2.95	1	19.90±2.25	3	9.99±4.20	3	19.35±6.94	3	6.550	0.088
1098	α-linalool[1,3-5]*	19.97±19.97	1	12.32±12.32	1	12.62±8.33	2	14.87±14.87	1	0.295	0.961
	Homoterpenes										
1117	DMNT[1,2] *	3.34±2.88	2	0.81±0.68	2	1.87±1.00	3	1.77±1.16	2	1.768	0.622
	Sesquiterpenes	–				–					
1395	α-copaene[3] *	–	0	3.42±3.42	1	–	0	3.51±3.51	1	2.212	0.530
1464	β-farnesene[1-4,6]*	–	0	–	0	0.21±0.12	2	–	0	4.364	0.225
1535	δ-cadinene[6]	–	0	2.86±2.86		–	0	–	0	3.000	0.392
1592	Trans-nerolidol[1,2,4]	–	0	–	0	16.16±10.01	2	–	0	4.364	0.225

RI-Retention index; SE-standard error; O- occurrence (i.e., the number of analyses in which the compound was detected). Mean- mean relative amount of each compound is expressed as the percent of the total emission (i.e., the sum of 21 VOCs from maize headspace samples, or the sum of 13 VOCs from maize field atmosphere samples). *compounds detected by female ECB antennae according to Solé et al., (2010) and Bengtsson et al., (2006). Superscript numbers are citations of papers that previously reported the VOC in the maize headspace. [1] D'Alessandro and Turlings (2005), [2] Turlings et al., (1998), [3] Bengtsson et al., (2006), [4] Degen et al., (2004), [5] Buttery and Ling, (1984), [6] Solé et al., (2010).

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Chapter 3: Host plant specialization

Host plant choice and recognition

Different sensory modalities such as smell, taste, touch and sight direct a host-seeking insect and are integrated by the central nervous system. Phytophagous insects use principally visual and odour stimuli to localize the host. The nature of what is perceived by contact is necessary to decide if the plant is acceptable for oviposition, or as food. Plants release mostly ubiquitous volatile compounds that are detected by host-seeking insects and used to identify the plant. There are mainly two hypotheses to explain how an insect uses plant volatiles in host recognition (Visser, 1986).

The first assumes that host-plant odour recognition relies on highly specific volatiles not found in unrelated plant species. This is the "token stimulus" theory of Fraenkel (1959), which proposes that specific compounds are used for host detection by herbivorous insects. The alternative hypothesis supposes that plant odor specificity is achieved by a particular ratio between constituent volatiles distributed generally among plant species. Examples for the "token stimulus" theory are rare, whereas most of the plant-insect studies indicate that phytophagous insects recognize host by the species-characteristic combinations of these ubiquitous plant volatiles (Visser and Avé, 1978) and the mixtures are more attractive than a single compounds (Natale et al., 2003). This phenomenon is valid for specialist (Birkett et al., 2004) as well as for oligophagous insects (Thiéry and Marion-Poll, 1998). Nevertheless, plant volatiles, which are not emitted in species-characteristic ratios, might still provide information to the insect if they form a specific blend. The mixture of plant volatiles are perceived via olfactory receptor neurons (ORN) in different types of sensilla on the insect antenna, palps and ovipositor sharing the same general structure. Furthermore, most phytophagous insects have ORN on the antennae that respond specifically to ubiquitous volatile compounds. Comparative studies of specialist and generalist insects ORNs show that at the level of peripheral nervous system there is a high maintenance of ORNs that respond to ubiquitous volatiles (Ramachandran et al., 1990; Raguso and Light, 1998). The central processing of peripheral signals is crucial as insects with different host preference can have similar peripheral response to common plant volatiles but nevertheless express different behavioural response (Bruce et al., 2005). The use of ORNs for common plant volatiles allows an insect the flexibility to adapt to any change in volatile cues and evaluate a wider range of

potential hosts (Mustaparta, 1990).

European corn borer host plants

ECB is commonly considered to be an extremely polyphagous (Caffrey and Worthley, 1927) and to attack any herbaceous plants with stems large enough for the larva to enter (Bourguet et al., 2000). Its host range has more than 200 different plant species belonging to 40 different botanical families including both agronomic and horticultural crops and many common weeds (Hodgson, 1928). It would not be reasonable to consider all these 200 plant species as the real host plants. Some of them are probably accidental host (Guennelon, 1972) as only plants where an insect is able to complete its life cycle can be considered as host plants and this is not proven for all of the reported host plants of ECB. Nevertheless the polyphagie remains still ECB most striking trait. ECB larvae cause large yield losses in divers crops (Table 9).

Table 9. European Corn Borer (ECB) most common documented host plants.

Host plant	Family	Reference:
Monocots		
Maize, *Zea mays*	Poaceae	(Hodgson, 1928)
Broomcorn, *Sorghum vulgare*	Poaceae	(Caffrey and Worthley, 1927)
Wheat, *Triticum spp*	Poaceae	(Willson, 1980)
Leek, *Allium porrum*	Alliaceae	(Stoth, 2009)
Dicots		
Hop, *Humulus lupulus*	Cannabinaceae	(Frolov, 1998; Bourguet et al., 1999)
Hemp, *Cannabis sativa*	Cannabinaceae	(Frolov, 1998)
Sunflower, *Helianthus annus*	Asteraceae	(Legg et al., 1986)
Mugwort, *Artemisia vulgaris*	Asteraceae	(Frolov et al., 2007)
Pepper, *Capsicum sp*	Solanaceae	(Clerc et al., 2002)
Potato, *Solanum sp*	Solanaceae	(Nault et al., 1996; Noronha et al., 2008)
Tobacco, *Nicotiana tabacum*	Solanaceae	(Hudon and LeRoux, 1986)
Raspberry, *Rubus strigosus*	Rosaceae	(Hudon and LeRoux, 1986)
Apple, *Malus sp*	Rosaceae	(Straub et al., 1986)
Melon, *Cucumis sp*	Cucurbitaceae	(Clerc et al., 2002)
Snap bean, *Phaseolus vulgaris*	Fabaceae	(Eckenrode and Webb, 1989)
Cotton, *Gossypium hirsutum*	Malvaceae	(Savinelli et al., 1988)
Common lambsquarters, *Chenopodium album*	Amaranthaceae	(Hodgson, 1928)

ECB host plants are divided principally into two groups: monocots (maize, cereals, millet, sorghum) and dicots (mugwort, hemp, hop). Monocots are infested mainly by Z pherotype and dicots by E pherotype (Ponsard et al., 2004). ECB consists of at least two genetically differentiated host races: one feeding on maize, the other feeding on mugwort and hop. Indeed, the bioassays on European ECB populations reveal that the Z pherotype females express high oviposition preference for maize, while E pherotype females attracted mugwort and hop over maize and sorghum (Savinelli et al., 1988; Bethenod et al., 2005; Frolov et al., 2007; Malausa et al., 2008). Allozyme-based studies indicate that ECB populations collected on hop and mugwort are genetically separated from sympatric larval populations collected on maize (Bourguet et al., 2000). There are at least two distinct pherotype populations of ECB, with each displaying successful species isolation and, in areas where they are in sympatry, there is sufficient genetic compatibility between them to produce fertile hybrids (Roelofs et al., 1987). The field studies as well as laboratory bioassays have shown that these hybrid offspring between the maize and native host plant population moths are exceptional and their reproductive success decreased (Pelozuelo et al., 2004; Bethenod et al., 2005). Behind the extremely polyphagous species may be hidden in fact a mosaic of ECB host races. The two pherotypes of ECB are found, with various degrees of sympatry, from western to central Eurasia (Frolov et al., 2007). The host plant specialization seems to be guaranteed mainly by female choice of oviposition site. The behavioural events leading to oviposition by a gravid moth involves: searching, orientation, encounter, landing, host evaluation by contact and finally acceptance or rejection of a host (Jaenike, 1990). Semiochemicals released by plants have a significant role in the selection of suitable hosts for feeding or oviposition (Renwick and Chew, 1994). The oviposition site choice is particularly crucial as the hatching larvae are fragile and relatively immobile and thus depend on the judicious choice of food plant by the gravid moth (Renwick, 1989).

Paper III

Volatile organic compounds and host-plant specialization in European corn borer E and Z pheromone races

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Volatile organic compounds and host-plant specialization in European corn borer E and Z pheromone races

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Abstract

Plant volatile signals are considered the main source of information for ovipositing moths, which use chemical information to locate and recognize the host plant. In Europe, two sympatric populations of European corn borer (ECB; *Ostrinia nubilalis*, Hübner), the Z and E pheromone races, feed mainly on maize and hop/mugwort, respectively. We studied the mechanisms of host-plant recognition and fidelity in ECB pheromone races by testing the attractiveness of host plants to gravid females in a flight tunnel and by analyzing the volatiles released from maize, mugwort, and hop during the scotophase, when the ovipositing flight of the ECB females occurs. In the wind tunnel bioassay, the Z-race and E-race females engaged in upwind flight and expressed a strong host fidelity to their respective main host plants; all three of these host plants possess distinctive volatile profiles specific as to blend and ratio. The host plants shared a certain number of ubiquitous volatiles present in various ratios that likely constitute a species-specific cue to host-seeking ECB moths. Our observations therefore suggest that ECB host fidelity is steered by plant volatiles that are present in species-specific ratios of ubiquitous volatile organic compounds.

Introduction

The European corn borer (ECB; *Ostrinia nubilalis* Hübner: Lepidoptera, Crambidae) is a major maize pest with a worldwide distribution. The species is polyphagous and may develop on other crops and other herbaceous plants (Lewis 1975) with stems large enough to encompass developing larva. Recent studies have indicated that the ECB is actually composed of two genetically differentiated and reproductively isolated pheromone races (Bethenod et al. 2005; Malausa et al. 2007a) that are found in sympatry over a wide geographical range in Eurasia (Frolov et al. 2007). One of the major factors behind the reproductive isolation is the sex-pheromone communication system that leads to assortative mating of individuals belonging to the same race (Pelozuelo et al. 2007).

The ECB sex pheromone is a binary blend of Z11-14:Ac and E11-14:Ac. The so-called Z pheromone race uses Z11-14:Ac as the major component in a 97:3 ratio, while the E pheromone race has adopted an inverse ratio of 4:96 in favor of the E isomer of Δ11-14:Ac (Klun et al. 1973; Pelozuelo et al. 2004). Furthermore, the isolation of ECB pheromone races appears to be reinforced by specialization to host plants (Pelozuelo et al. 2004). The E race develops exclusively on wild native plants such as hop (*Humulus lupulus* L.) and mugwort (*Artemisia vulgaris* L.), while the Z race feeds on maize (*Zea mays* L.). Colonization of this host plant must be relatively recent, since maize is a newly introduced crop to Europe that has only been cultivated on a large scale since the mid-20th century.

However, pleiotropy between the host plant and mating choice is unlikely in moths such as ECB in which mating behaviour does not depend directly on cues from the host plant (Tregenza and Butlin 1999). The ECB mating behaviour does not require the presence of the host plant; the two pherotypes mate assortatively in grassy places where they aggregate (Showers et al. 1976) and remain hidden during the day. Mating occurs at the beginning of the night, when the gravid ECB females fly out of the grassy location to find their respective host plant (Pelozuelo et al. 2004). For the Z pherotype, host-plant location relies mainly on olfaction (Bengtsson et al. 2006), but to our knowledge nothing is known about differences in host-plant discrimination between the E and Z races. Plants produce complex blends of volatile organic compounds (VOCs) with different distributions across plant taxa, some widespread

and others taxonomically restricted (Knudsen et al. 2006; Knudsen et al. 1993). The emission of VOCs from maize plants varies greatly depending on the plant part, developmental stage, photoperiod, and environmental condition (D'Alessandro and Turlings 2006; Degen et al. 2004; Gouinguené et al. 2001; Turlings et al. 1998). Even subtle differences in plant VOCs can provide important information to host-seeking pests regarding plant suitability as a host. Host-plant recognition and fidelity in specialized insects has often been associated with taxonomically specific plant compounds (Visser 1986), although recent studies have demonstrated that a blend of ubiquitous VOCs can be also highly specific (Bruce and Pickett 2011).

The current investigation was undertaken to shed light on the mechanisms of host-plant recognition and fidelity in ECB pheromone races. Does the specialization to host plants rely on the VOCs released by those host plants? Do the two E-race host plants share common chemical signals? We tested the attractiveness of host plants to ECB moths in a flight tunnel; ovipositing ECB females were allowed to choose between maize, mugwort, and hop in a dual-choice test. To address the second question, the VOCs released from these three main hosts were identified and characterized. We assembled a biologically relevant representation of host cues perceived by the host-seeking ECB moths by collecting the VOCs from host plants during the ECB oviposition period at the beginning of the scotophase.

Materials and Methods

Insects

Diapausing ECB E-race larvae were collected from mugwort in France near Amiens (49°86'N, 2°29'E). Z-race gravid females were captured near Grignon (48°85'N, 1°96'E) and near Darvoy (47°85'N, 2°10'E), France. The Z-race larvae, the offspring of field-collected females, were reared on an artificial agar-based diet. In the E-race artificial agar-based diet, 30% of the maize flour was replaced with mugwort stem powder. The larvae were reared under 16 hours of light and 8 hours of dark. Pupae were sexed and maintained in separate Plexiglass containers. Intra-race mating pairs

were formed on the second day after adult emergence, and mated females were set aside for subsequent use in wind-tunnel bioassays.

Insect race confirmation

The pheromone races of the female moths were confirmed by Solid Phase Micro Extraction (SPME; (Frérot et al. 1997) using carbowax/divinylbenzene fibres (65 μm, Supelco Inc.). The pheromone gland was extruded by gentle pressure on the abdomen and kept in this position with metallic forceps. A SPME fibre was gently rubbed on the gland and then either directly analyzed or wrapped in aluminum foil and stored at -20 °C until analysis. Pheromones were identified using a gas chromatograph (GC; Varian 3400Cx) equipped with a split-splitless injector and an Rtx ®-Wax column (Restek; 30 m x 0.32 mm i.d., film thickness 0.25 μm). The compounds absorbed on the fibre were thermally desorbed in the injector (splitless mode), which was maintained at 240 °C. The oven temperature was programmed from 50 °C to 100 °C at 15 °C/min; 100 °C to 245 °C at 5 °C/min; helium (15 psi) was the carrier gas. Pheromone compounds were identified by comparison of the retention times of the natural compounds with those of the synthetic reference samples. The ratios of the various compounds were calculated from the peak areas of the products.

Wind tunnel bioassays

The experiments were carried out in a half-cylinder wind tunnel (190 cm long × 80 cm wide × 45 cm high) with an airflow of 0.6 m/s at 23±2 °C and with ~60% relative humidity. A constant red incandescent light source above the tunnel allowed observations and video recording. Three-to-five-day-old mated females in individual capped wire mesh cages (3×6 cm) were released from a 12 cm-high platform 150 cm downwind of the source. Bioassays were carried out between the third and the sixth hour after the onset of scotophase. Plant odour came from potted maize, mugwort, or hop plants that were kept under the same photoperiod as the insects. Plants were placed in the upwind part of the tunnel. Pairwise oviposition choice-tests of maize *vs.* mugwort and maize *vs.* hop were performed in a randomized fashion. Each test lasted 15 min or ended earlier if the female began to oviposit. Flight behaviour was recorded

on a hard-drive (Archos AV500) with a COHU Solid State Camera equipped with an Avenir TV lens 4.8 mm F1.8 (Japan). We recorded the following behavioral steps of the female moths: activation, taking flight, upwind orientation, landing, and oviposition. Each female was tested once, and a total of 51 E-race moths and 49 Z-race moths were tested.

Plant material

Plants for VOC collections and wind tunnel bioassays were grown in pots in a greenhouse. Three ECB host plants were chosen: maize (Anjou 258 cultivar), mugwort, and hop. Mugwort and hop were collected from fields in the Versailles area of France. All plants were reared in a greenhouse at 23 °C and 60% relative humidity with a photoperiod of 16 light hours and 8 dark hours. Maize plants were used at the four-leaf phenological stage to match the first ECB oviposition flight (Balachowsky 1966). The mugwort and hop plants were ~10-15 cm high.

SPME

Plants VOCs were collected using SPME divinylbenzene/ carboxen/ polydimethylsiloxane 50/30 μm (Supelco Inc.) fibres. The fibres were cleaned before each sampling in the GC injector at 250 °C for 5 min with helium. Cleaned fibres were wrapped in aluminum foil and stored in individual screw-cap Pyrex glass tubes.

VOC collection from plants

The non-destructive static headspace-sampling mode was chosen for collecting plant volatiles. Plants were placed individually in 25x25 cm Teflon bags. The leaf areas of the samples were estimated to be equivalent for all tested species. A small hole was pierced in the Teflon bag and the SPME fibre was exposed to the headspace of the sample. VOC collections took place simultaneously for all three host plants in the greenhouse during the dark portion of the photoperiod. Five replicate VOC collections were carried out for maize, and six replicates each were obtained for mugwort and hop.

Chemical analysis

Plant VOC analysis was performed using a Varian 3400 GC coupled to a Varian QIMS mass spectrometer (MS) detector. SPME fibres were desorbed in the GC injector held at 250 °C. Compound separation was carried out using an Rxi-5ms column (Restek, France) 30 m × 0.32 mm i.d., film thickness 1.0 μm. The column was programmed to hold at 50 °C for 3 min and to increase at 8 °C/min to 300 °C. Helium was used as the carrier gas. Mass spectra were obtained in electron impact mode (70 eV) with the ion source at 230 °C. VOCs were identified according to their mass spectra and retention indexes (RIs). The RIs were computed using n-alkanes from C10 to C24 that were eluted under the same conditions as the samples. Each compound spectra and RI was compared with the RI and spectra of laboratory and NIST 1998 libraries using the deconvolution software AMDIS32. The calibration curves of the green leaf volatiles GLV, homoterpenes (HT), monoterpenes (MT), and sesquiterpenes (SQT) were obtained by injection of the following synthetic samples: cis-3-hexenol (GLV), methyl salicylate (MeSA) (GLV), α-linalool (MT), α-pinene, (MT) α-humulene (SQT), α-copaene (SQT) (Sigma-Aldrich), β-farnesene (SQT) (Chemtech), myrcene (MT), ocimene (MT) (Fluka), cis-3-hexenyl acetate (GLV) (Lancaster Synthesis), and (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (HT) (a gift from Jarmo K. Holopainen of Kuopio, Finland). All of these compounds were injected at least in triplicate at concentrations of 5, 10, 25, and 50 ng/μl.

GC-MS analyses

The raw GC data from the areas of the VOCs were transformed into nanograms using the respective authentic calibration curves. The relative amounts (%) of the VOCs were calculated by dividing the compound amount by the sum of the amounts of the compounds detected in the same analysis.

Statistical analyses

The distributions of the VOCs in the three host plants were analyzed by the non-parametric Kruskal-Wallis test. Principal component analysis (PCA) was used to visualize the differences in the total VOC ranges of the three host plants. To study the

similarity of the VOC profiles, cluster analysis of the detected VOCs were performed via Ward's method. For wind tunnel data a Chi-square (χ^2) test was used to test the hypothesis of a significant difference from a random 50:50 choice. PASW Statistics 18.0 for Mac was used for all statistical analyses.

Results

The wind tunnel bioassay

In the dual-choice tests carried out in the wind tunnel, 69% of gravid ECB females activated and flew out of the cage, and ~52% engaged in an upwind-oriented flight toward the plants. Females of both pheromone races were attracted to their respective host plants, Z-race females to maize and E-race females to mugwort or hop (Table 1). Within the 15-min test period, on average 41% of E-race females and 40% of Z-race females landed on their respective host plants. The E-race females significantly favored mugwort and hop, their native host plants, over cultivated maize plants (χ^2=4.17-14, df=1, n=13-14, p<0.05), whereas the Z-race females landed preferentially on maize (χ^2=5.33, df=1, n=12, p<0.021) in the choice test between maize and mugwort. When Z-race females had the choice between maize and hop plants, 71% of the females that engaged in upwind-oriented flight toward the plants chose maize over hop, although this value was not statistically different from a random 50:50 choice (χ^2=2.57, df=1, n=14, p<0.109).

Identification of ECB host-plant VOCs

The VOC emissions detected from maize, mugwort, and hop differed both in quality and quantity. In all cases, complex mixtures of terpenoids and GLV formed the VOC profiles. A total of 59 VOCs were detected in the total set of collections from the three host-plant species: 7 GLV, 21 MT, 25 SQT, one HT, and five VOCs from other chemical classes (esters, hydrocarbon, nitrogen containing compound, ketone) (Table 2). Of these 59 VOCs, seven compounds remained unidentified, three of which had SQT mass spectra. Thirty-three compounds were detected in the maize VOC

emissions, whereas 44 VOCs were identified in the hop analyses and 35 in the mugwort analyses. Bicyclic SQT were the main VOCs for the three plants, representing 71% of the maize, 76% of the mugwort, and 72% of the hop VOC relative ratios (Fig. 1). The presence and the relative amounts of 45/59 of the VOCs differed significantly among the three species (Kruskal-Wallis, df=2, Z=7-15, p<0.005).

VOCs shared among the three host plants

Fifteen VOCs were shared by maize, mugwort, and hop, all of which were widespread plant volatiles such as the GLV and the common MT and SQT found throughout the plant taxa. These compounds accounted for 11% of maize, 14% of mugwort, and 5% of hop total volatile signal. Of these shared VOCs, the relative amounts of six VOCs significantly differed among the three plants (Table 3). Pairwise comparisons revealed that the differences in the relative amounts of VOCs occurred mainly between maize and mugwort (cis-3-hexenol, α-pinene, trans-β-ocimene, and α-cubebene; Kruskal-Wallis, df=2, Z=8, p<0.05). Only the relative amounts of cis-3-hexenyl acetate and p-cymene were significantly different between maize and hop (df=2, Z=8.533, p<0.016) and between mugwort and hop (df=2, Z=-8.333, p<0.013), respectively (Table 3).

Species-specific VOCs

We observed that each of the three host-plant species released species-specific VOCs. The SQT Z-α-trans-bergamotol and β-cubebene, and the MT borneol and cis-carveol were identified exclusively in maize VOC collections (Table 2). These four compounds accounted for ~16% of the total maize VOC relative ratio. Santolina triene, camphene, 2-hexanol, n-hexanol, 1-tridecene, and one unknown compound were exclusively detected in mugwort, composing only ~5% of the total mugwort VOC relative ratio (Table 2). Eight SQT and one MT were found exclusively in the hop headspace collections and accounted for ~70% of the total hop VOC relative ratio: α-guaiene, germacrene D, isoledene, (+)-epi-bicyclosesquiphellandrene, γ-muurolene, α-amorphene, β-chamigrene, α-trans-bergamotene, and α-thujene (Fig. 1).

VOCs grouped by chemical classes

SQT accounted for 68-83%, MT for 5-15%, and HT for less than 1% of the total VOC relative amounts in the maize, mugwort, and hop headspace collections. GLV composed 0.3-3% of the total VOC relative amount for all three plants. The relative amounts of all the HT and SQT grouped together were not significantly different among the three plant species (Kruskal-Wallis, $p < 0.285$ and $p < 0.644$, respectively). The relative amount of total MT significantly differed between maize and mugwort (df=2, Z=6.018, $p < 0.047$), but there were no significant differences between maize and hop ($p < 0.293$) or between mugwort and hop ($p < 1.00$). The GLV varied significantly between hop and maize (df=2, Z=2.671, $p < 0.023$), but not in the other pairwise comparisons (hop-mugwort $p < 0.250$, maize-mugwort $p < 0.901$).

PCA and cluster analysis

To visualize the differences in total VOC range among the three host plants, we performed a PCA on the amounts of the total pool of 59 VOCs. PC1 and PC2 accounted for 53% of the variability in the original data (Fig. 2a). The three plant species were clearly separated by the VOC relative amounts. An analogous analysis performed on the 15 shared VOCs also separated the three plant species, with PC1 and PC2 together explaining 56% of the variance (Fig. 2b). The three host plants were separated into three groups using Ward's method in a cluster analysis of all 59 VOCs (Fig. 3). Interestingly, the maize and hop VOC profiles were more similar to each other than to the mugwort profile. This similarity of the maize and hop VOC blends originates from the observation that the relative ratios of the 10 VOCs shared by maize and hop did not significantly differ (Kruskal-Wallis, $p > 0.05$).

Discussion

We analyzed VOCs from maize, mugwort, and hop plants and tested the oviposition preference of two pheromone races of ECB moths. The Z-race and E-race females flew upwind to their respective main host plants (Table 1). Our observations of Z-

99

race females are consistent with the previous work of Bengtsson et al. (2006) indicating that these females discriminate maize from hop and mugwort. Taken together, these observations point to a high specificity in host-plant choice in the studied ECB pheromone races and demonstrate that the ovipositing females rely on the VOCs released by host plants for host choice. In the wind tunnel the oriented flight to the plant is mostly steered by chemo-anemotaxy; the red light and visual cues included in our bioassay were not likely to have been involved in host location.

The rise of host-associated populations (host races) has been observed in other Lepidoptera such as the diamondback moth (*Plutella xylostella* L.; (Rossbach et al. 2005) and the larch budmoth (*Zeiraphera diniana* Gn.; (Emelianov et al. 2001), both of which exhibit a strong pleiotropy between host-plant choice and assortative mating. Since ECB mating behaviour is independent of the host plants, the mechanism underlying the divergence of the ECB pheromone races relies on assortative mating and on selective host-plant choice for oviposition.

Phylogenetic studies of host choice in Lepidoptera have demonstrated that host choice is often related to plant chemistry and taxonomic affinities; pests prefer plants belonging to the same botanical family (Thompson and Pellmyr 1991). We expected maize and the two native host plants to share similar volatile compositions that would have facilitated the host shift. The three main host plants belong to different families, grow in different habitats, and exhibit different morphologies and physiologies (maize is a C4 plant, whereas mugwort and hop are C3). Our measurements of the VOCs from maize, mugwort, and hop indicate that these host plants also release distinctive volatile profiles. Nevertheless, they share a certain number of ubiquitous terpenoids and GLV that differed in their specific ratios from species to species, and PCA of the ratios of the 15 shared VOCs separated the three host plants. According to the principle of host plant location proposed by Bruce and Pickett (2011), we conclude that discrimination among the three plants relies on ratio-specific odour recognition, even for shared components. These 15 components elicit responses in the Z-race female antennae, and very little is known regarding their impact on behaviour or their biological significance (Bengtsson et al. 2006; Solé et al. 2010). No similar studies have been undertaken on the E pheromone race.

The 15 VOCs shared by all three host plants included MeSA, β-farnesene, cis-3-hexenyl acetate, and β-ocimene, all of which are important in insect communication

and biology. Most of these compounds are considered to be induced plant VOCs emitted after herbivore damage that attract predators and parasitic wasps or deter herbivores directly (De Moraes et al. 2001; Kessler and Baldwin 2001; Ozawa et al. 2008; Paré and Tumlinson 1997; Pichersky and Gershenzon 2002; Turlings et al. 1991). Our non-destructive headspace VOC collection technique revealed that intact plants release these induced VOCs.

We also detected a number of taxonomically characteristic volatiles, principally bicyclic SQT, that give each plant species a distinctive VOC profile. However, there is no information available on their biological relevance and importance in host-seeking and acceptance by ECB. Although these compounds could theoretically provide an effective means for host recognition, their use by ovipositing insects is rarely reported; only a few pest species are known to use token stimuli in host recognition (Fraenkel 1959; Renwick 2001; Schoonhoven et al. 2005). The host specialization and host fidelity of the ECB pheromone races are likely to rely on perception of species-specific plant volatile signals.

The ECB females exhibited high host fidelity in our wind tunnel bioassay. Based on the differences in the plant VOC profiles and female oviposition choice, we suggest that behind the extremely polyphagous behaviour of ECB there lies a mosaic of host plant races, especially for the E pheromone race. We tested insects that originated from populations living naturally on mugwort and maize. It is thus possible that ECB moths originating from hop plants – not tested in this study – do not possess the same host preferences as the moths reared from mugwort plants, and that these two host races may also be reproductively isolated. Recent molecular studies based on nuclear loci show that the Z race from maize plants and the E race from mugwort plants are genetically divergent, and more interestingly that the E-race ECB from hop populations are more closely related to Z-race ECB than to E-race ECB from mugwort populations (Malausa et al. 2007b). Our cluster analyses of host-plant VOCs revealed that maize and hop are grouped into one cluster separate from mugwort, suggesting that a portion of an ancestral ECB hop population shifted to maize, while another population remained isolated on mugwort. The ECB sex pheromone races reinforce hypotheses of three host-plant populations, since Pelozuelo et al. (2004) found that E-race females that originated in hop or in mugwort plants exhibited slightly different pheromone compositions; the mugwort-race females produced a

classical E pheromone blend of Z11-14:Ac and E11-14:Ac in a 4:96 ratio in addition to Z11-16:Ac, which was never detected in the hop-race females. Further studies need to be undertaken to investigate the possible reproductive isolation between the different host races of the E-pherotype ECB.

Acknowledgements
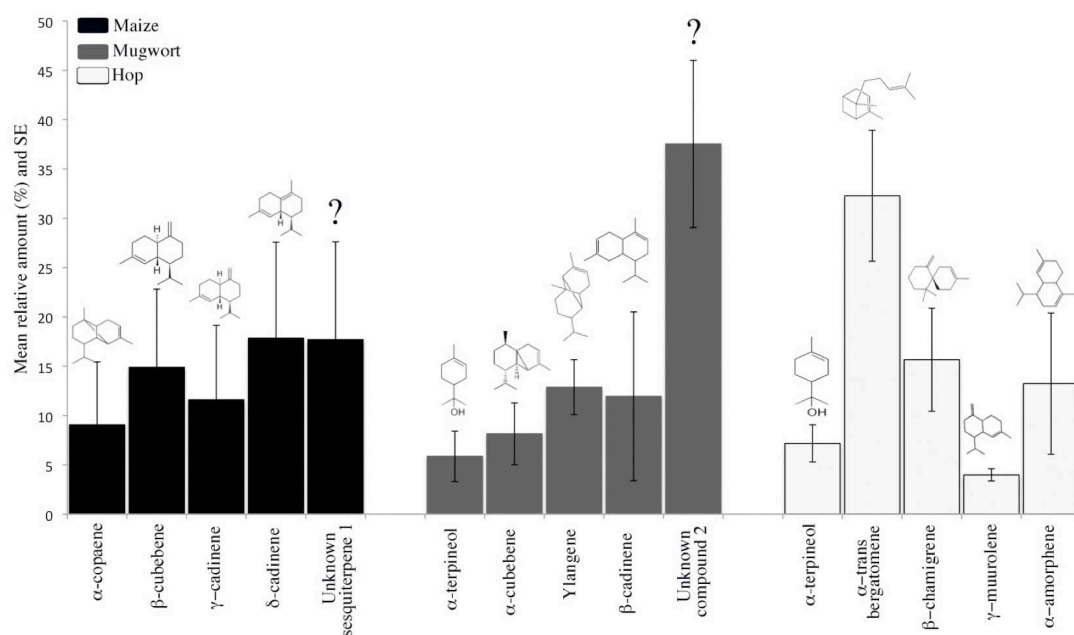
Figure 1. Odor profiles of the five most abundant VOCs in the maize, mugwort, and hop collections. The amount of each compound is expressed as percent of the total emission (the sum of all detected VOCs *per* species).
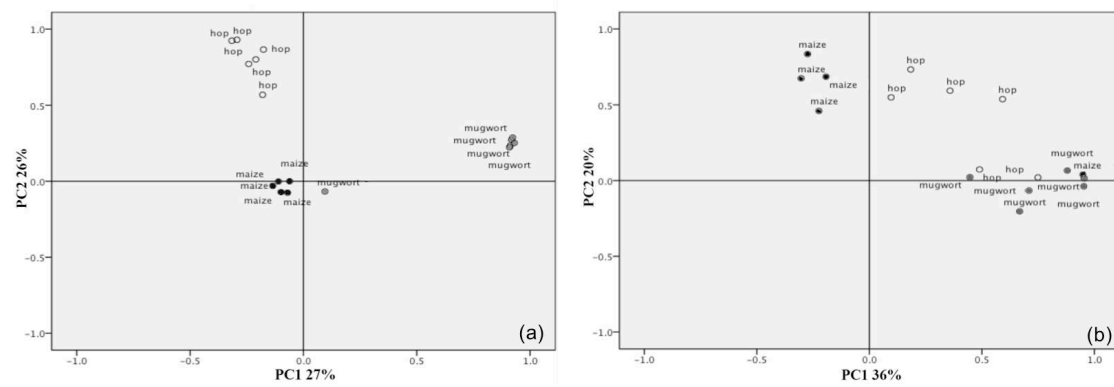
Figure 2. 2 PCA bi-plot demonstrating the separation of host plants, accounting for the variation in 59 total VOCs (a) and in 15 VOCs shared by all three host plants (b). Black dots represent maize, grey dots represent mugwort, and white dots represent hop.
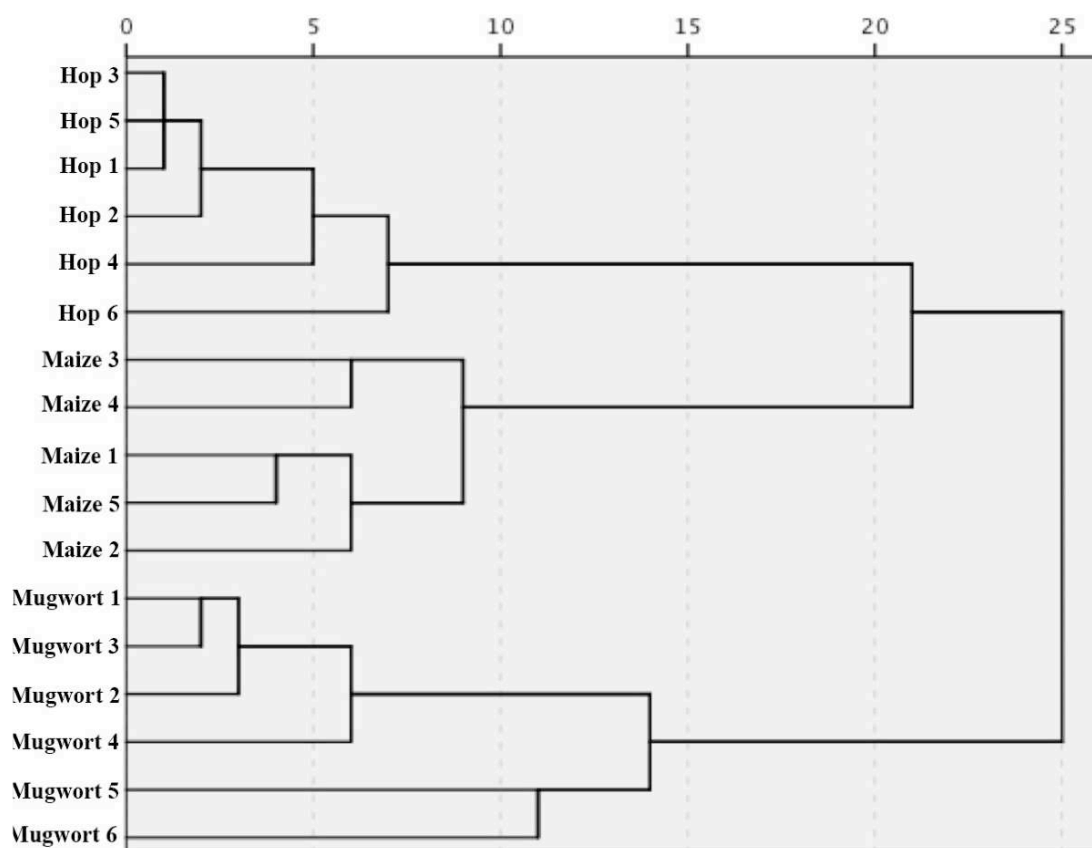
Figure 3. Dendogram using Ward linkage, rescales distance cluster combine. Similarities in the compositions of the VOC profiles from the three ECB host plants. Maize and hop are grouped into one cluster and are separated from mugwort.

Table 1. Attraction of ECB gravid females in a dual-choice test to maize, mugwort, and hop potted plants in a wind tunnel.

Number of females tested		Activation %	Upwind flight followed by landing (%)				X^2	p-value
			Maize	Mugw	Hop	Wall		
Choice test maize-mugwort								
Z race	25	72	40	8	-	24	5.33	0.021
E race	26	69	12	38	-	19	4.17	0.041
Choice test maize-hop								
Z race	24	64	40	-	16	8	2.57	0.109
E race	25	71	8	-	46	17	7.14	0.008

Table 2. Mean relative ratios of the VOCs detected in maize (n=5), mugwort (n=6), and hop (n=6) *via* headspace analysis.

RI	VOCs		Maize (n=5) Mean±SE	Mugwort (n=6) Mean±SE	Hop (n=6) Mean±SE
802	2-hexanol*	rt/MS	–	0.03±0.02	–
860	Cis-3-hexenol [1-4] *	rt/MS	2.72±1.49	0.82±0.69	0.18±0.07
892	n-hexanol [3]	NIST	–	0.09±0.05	–
909	Santolina triene [5]	NIST	–	0.03±0.01	–
934	α-thujene [6,7]	NIST	–	–	0.36±0.17
942	α-pinene [1,3-8]	rt/MS	2.60±1.52	0.26±0.15	0.57±0.32
960	Camphene+ [3,5]	rt/MS	–	0.21±0.95	–
982	Sabinene [3,5]	NIST	–	2.74±1.05	0.35±0.20
989	β-pinene [3-8] *	rt/MS	–	0.46±0.24	0.17±0.11
993	β-myrcene [1,2,4-7,9] *	rt/MS	0.11±0.07	0.10±0.03	0.22±0.08
1007	Cis-3-hexenyl acetate [1-4,10]*	rt/MS	0.41±0.27	0.21±0.20	0.01±0.01
1032	p-cymene [3,5-7]	rt/MS	0.12±0.09	0.07±0.03	0.97±0.40
1038	Limonene [4,7,9]*	rt/MS	0.32±0.26	0.82±0.66	0.21±0.11
1039	Cis-β-ocimene [3,5-8]	rt/MS	–	0.03±0.01	0.01±0.01
1051	Trans-β-ocimene [3,5,7,8]	NIST	1.17±0.27	0.01±0.01	0.48±0.22
1061	γ-terpinene	rt/MS	–	0.55±0.17	0.17±0.12
1103	α-linalool [1,4-7,9-11] *	rt/MS	0.09±0.06	0.18±0.18	0.27±0.20
1105	Methylbenzoate	rt/MS	0.09±0.01	1.02±0.48	–
1119	DMNT [1,2,4]	rt/MS	0.09±0.04	0.08±0.06	0.07±0.03
1128	Terpinolene [3,6,7]	rt/MS	–	1.06±0.48	2.26±1.01
1131	Borneol	NIST	0.39±0.23	–	–
1137	α-terpineol [7]	NIST	–	5.87±2.56	7.18±1.89
1148	γ-terpineol	NIST	–	0.08±0.04	0.41±0.24
1209	MeSA [2,4] *	rt/MS	0.02±0.01	0.13±0.06	0.08±0.02
1233	β-citronellol	rt/MS	0.28±0.11	0.24±0.19	±
1238	Cis-3-hexenyl-2-methyl butanoate[12] *	rt/MS	0.28±0.11	0.21±0.11	0.11±0.06
1261	Trans-carveol [3]	NIST	0.15±0.08	1.03±0.79	–
1280	Indole [1,2,4,10]	NIST	0.50±0.27	–	0.65±0.57
1293	1-tridecene	NIST	–	1.23±0.42	–
1296	Cis-carveol	NIST	0.46±0.23	–	–
1370	Isoledene	NIST	–	–	1.40±0.18
1395	α-copaene [4,6,7,13] *	rt/MS	9.07±6.37	–	2.12±0.23
1401	α-cubebene [3,7]	NIST	3.06±3.06	8.15±3.14	0.60±0.07
1415	Ylangene [6,7]*	NIST	–	12.88±2.78	0.38±0.12
1439	α-trans-bergamotene [6,7,11]*	NIST	–	–	32.28±6.64
1450	β-cubebene [1,2,10,13]	NIST	14.89±7.92	–	–
1452	β-caryophyllene [1-6,8,10,13] *	rt/MS	0.16±0.06	0.33±0.22	0.01±0.01
1464	β-farnesene*[1-3,5-8,10,13]	rt/MS	0.19±0.10	1.34±0.61	0.08±0.01
1473	β-chamigrene	NIST	–	–	15.66±5.23
1479	γ-muurolene [6,7,11]	NIST	–	–	3.98±0.61

| 1488 | α-humulene [3-5] * | rt/MS | 0.42±0.22 | 3.42±2.69 | 0,0±0,0 |

Continuing table 2.

RI	Compound	ID	Mean±SE	Mean±SE	Mean±SE
1497	(+)-Epi-bicyclosesquiphellandrene	NIST	–	–	2.00±0.56
1503	Bicyclogermacrene [3,5,8]	NIST	–	2.73±1.08	0.53±0.09
1511	α-amorphene [7]	NIST	–	–	13.24±7.16
1516	GermacreneD [7] *	rt/MS	–	–	0.31±0.07
1516	β-cadinene [3]	NIST	2.44±1.31	11.95±8.55	–
1527	γ-cadinene [6,7,11,13]	NIST	11.60±7.55	–	1.48±0.58
1535	δ-cadinene [6,7,13]	NIST	17.84±9.73	–	3.05±0.87
1544	Selina-3,7 (11) diene [6,7]	NIST	2.49±1.22	–	0.58±0.09
1549	α-cadinene [6,13]	NIST	2.25±1.39	–	0.48±0.07
1566	Unknown sesquiterpene 1	–	17.70±9.91	–	3.06±1.31
1586	Trans-nerolidol [1,2,10]	rt/MS	0.03±0.02	1.02±0.87	0.69±0.31
1605	Unknown compound 1	–	–	1.07±0.32	–
1612	Unknown sesquiterpene 2	–	0.12±0.09	–	–
1626	Unknown sesquiterpene 3	–	1.10±0.54	–	0.09±0.05
1631	Unknown compound 2	–	–	37.53±8.48	1.55±0.50
1641	Unknown compound 3	–	4.31±2.51	–	1.63±1.00
1654	Unknown compound 4	–	2.51±1.28	–	0.02±0.01

RI-retention index; NIST- identification by the National Institute of Standards and Technology Mass Spectra database of 1998; rt/MS- identification by retention time and mass spectra; Mean- mean relative amount of each compound is expressed in percent of the total emission, i.e. the sum of all 33 VOCs for maize, 35 for mugwort and 44 for hop; SE-standard error. Numbers in superscript refer to publication where the compound has been already cited as maize, mugwort or hops VOCs. [1] D'Alessandro and Turlings 2005, [2] Turlings et al. 1998, [3] Tellez et al. 1999, [4] Solé et al. 2010, [5] Judžentienė and Buzelytė 2006, [6] Bernotiene et al. 2004, [7] Eri et al. 2000, [8] Liu et al. 2010, [9] Steinhaus et al. 2007, [10] Degen et al. 2004, [11] Kovacevic and Kac 2001, [12] Bengtsson et al. 2006 [13] Köllner et al. 2004, * Compounds active on female ECB antennae according to Bengtsson et al. 2006 and Solé et al. 2010.

Table 3. Relative mean (± standard error, SE) amounts of 15 VOCs common to all three host plants.

VOCs	Maize (n=5) Mean±SE	Mugwort (n=6) Mean±SE	Hop (n=6) Mean±SE	Kruskal Wallis p-value
Cis-3-hexenol	2.72±1.49*	0.82±0.68*	0.18±0.06	0.020
Cis-3-hexenyl acetate	0.41±0.27**	0.20±0.19	0.01±0.008**	0.017
MeSA	0.02±0.01	0.13±0.06	0.08±0.02	0.204
Z3-hexenyl 2me-butanoate	0.28±0.11	0.21±0.11	0.11±0.06	0.352
α-pinene	2.61±1.52*	0.26±0.15*	0.57±0.32	0.046
β-myrcene	0.11±0.07	0.09±0.03	0.22±0.08	0.461
P-cymene	0.12±0.08	0.07±0.02*	0.97±0.39*	0.010
Limonene	0.32±0.26	0.82±0.66	0.21±0.11	0.922
Trans-β-ocimene	1.17±0.27**	0.01±0.01**	0.48±0.22	0.002
α-linalool	0.09±0.06	0.18±0.18	0.27±0.20	0.354
DMNT	0.09±0.03	0.08±0.06	0.07±0.03	0.285
α-cubebene	3.05±3.05**	8.15±3.14**	0.59±0.07	0.014
β-caryophyllene	0.16±0.06	0.33±0.22	0.008±0.002	0.105
β-farnesene	0.19±0.10	1.34±0.61	0.08±0.01	0.891
Trans-nerolidol	0.03±0.02	1.02±0.87	0.69±0.31	0.220

The statistically significant different means in pairwise comparison are assigned with asterisk;

* $p<0.01$-0.05, ** $p<0.001$-0.01

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Chapter 4: Assortative mating
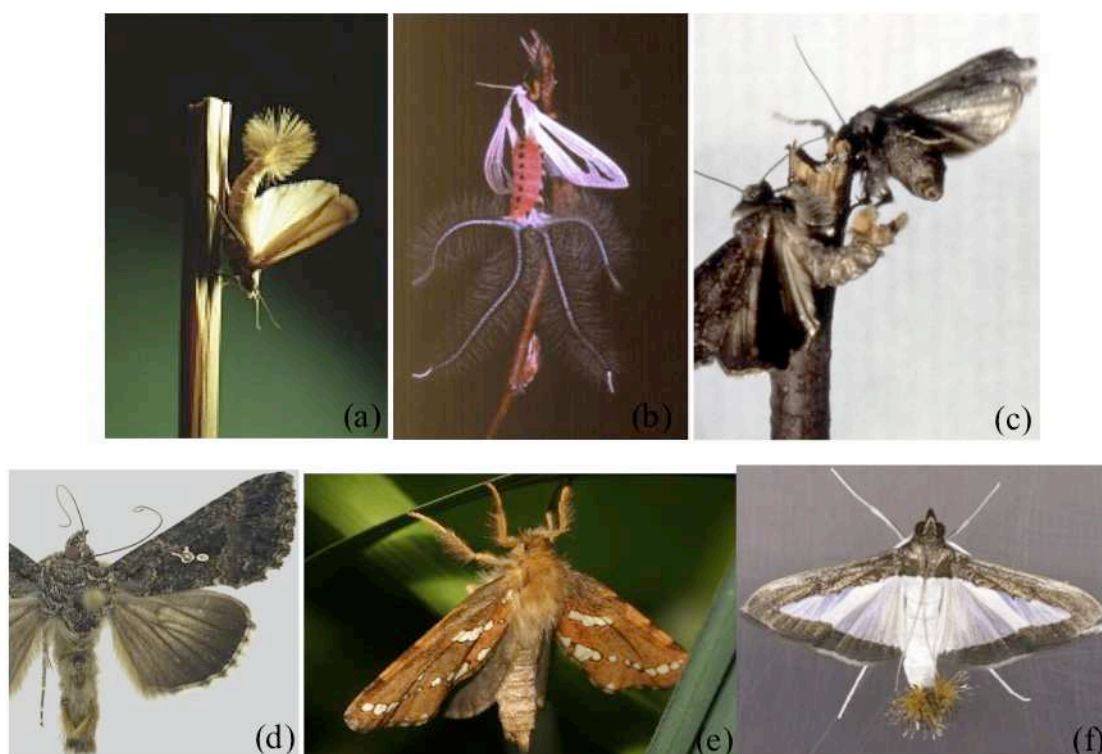
Male scent organs

Figure 11. Examples of male scent organs from different Lepidoptera families which are everted and used during the courtship to release pheromones. (a) The sugarcane borer (Eldana saccharina, Pyralidae) with unfolded hair pencils that attract conspecific females. (Photo: P. Zagatti) (b) Creatonotos gangis, an arctiid moth with everted coremata. (Photo: unknown) (c) The cabbage moth (Mamestra brassicae, Noctuidae) pair with the male everted abdominal hair pencils. (Photo: B. Frérot) (d) The male cabbage looper (Trichoplusia ni, Noctuidae) with well developed brushes at the base of the abdomen. (Photo: G. Fauske) (e) The Gold swift (Phymatopus hecta, Hepialidae) with tibial scent organs on their hind legs. (Photo: H. Gröschl) (f) The melonworm (Diaphania hyalinata, Pyralidae) with brushy hair pencils at the tip of the abdomen. (Photo: http://www.lesfruitsdemer.org/)

Males of many species of nocturnal Lepidoptera bear special organs associated with scent glands and situated on the wings, legs, abdomen and thorax (Table 10). These special organs generally originate in hypertrophied trichogen cells. The scent organs exhibit striking morphological diversity across the Lepidoptera; the structures vary from androconial scales to eversible hair pencils and are sometimes perceptible even for human eyes (Figure 11). Cells of the glands typically occur in groups, and the associated scent scales or hairs form a brush or a hair pencils which are be concealed within a pocket and are everted by means of sclerotized levers. Such glands include

coremata (Figure 11b), androconia (scent scales on the wings), and hairs (Figure 11a) (Birch et al., 1990). Males of some nocturnal Lepidoptera are equipped with a whole system of scent organs. For example the Stored nut moth (*Paralipsa gularis* Zeller, Lepidoptera: Pyralidae*)* has wing and abdomen glands, whereas the Cabbage looper (*Trichoplusia ni* Hübner, Lepidoptera: Noctuidae) has brushes at the base of abdomen and on genitalia. The types of androconia are not related to the systematic classification in Lepidoptera. In some moth species, volatile chemicals are emitted from these organs at close vicinity of females and in only a few cases they have been shown to have a pheromonal long-range function. The presence of pheromone emitting structures in Lepidoptera is often associated with courtship sequence that allows their presentation to the female before mating. The male pheromones were shown to have crucial role in male mating success (Jacquin et al., 1991). It was proposed that the male scent organs have arisen as an adaptive response to mating mistakes between differentially adapted populations and evolve through sexual selection (Phelan and Baker, 1987).
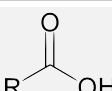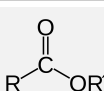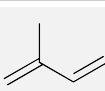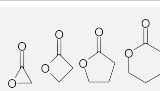
Table 10. Examples of scent organs in different moth families.

Family	Scent organs in moth family	Example	Long-range attraction	Reference:
Arctiidae	Coremata, eversible sacs or tubes covered with scales or hairs	Asian arctiid moth (*Creatonotos gangis*)	Males attract females ♂ ← ♀	(Wunderer et al., 1986)
Crambidae	Hair pencils	European corn borer (*Ostrinia nubilalis*)	Females attract males ♂ → ♀	(Royer and McNeil, 1992)
Geometridae	Wing-glands and brushes, coremata	Clouded silver moth *Bapta temerata)*	Females attract males ♂ → ♀	(Aplin and Birch, 1970)
Noctuidae	Diverse morphological types: hair pencils, brushes, Occur on the legs, thorax, abdomen	Cabbage moth (*Mamestra brassicae)*	Females attract males ♂ → ♀	(Jacquin et al., 1991)
Pyralidae	Hair pencils, wing-glands, abdominal brushes	Tobacco moth (*Ephestia elutella*) Lesser wax moth (*Achroia grisella*)	Females attract males ♂ → ♀	(Dahm et al., 1971; Krasnoff and Vick, 1983)
Tortricidae	Modified scales, pockets, and eversible structures, coremata on wings, legs, abdomen	Oriental fruit moth (*Grapholita molesta*)	Females attract males ♂ → ♀	(Baker and Cardé, 1979)

Male pheromones

Male pheromones are rather poorly studied compared to female pheromones in Lepidoptera and other insects. Their emission and production was shown to be affected by the presence of host plant in a various ways. Insect sex pheromones are sometimes present in plants as pheromone sources (Hendry et al., 1975). Host plants have an important role in the biosynthesis of some phytophagous insects sex pheromone through the acquisition of bioactive chemicals and is a required chemical precursors *via* consumption, absorption, or inhalation of host plant. Male moth pheromone biosynthetic precursors may be obtained from food or derived from larval host plant compounds, as it was well shown in some Arctiidae and Nymphalidae families (Schneider et al., 1975; Schneider et al., 1982). The males of the Oriental fruit moth (*Grapholita molesta* Busck, Lepidoptera: Tortricidae) sequester ethyl-trans-cinnamate from host plant as larva and use this chemical in hairpencil displays as a courtship pheromone (Baker et al., 1981; Löfstedt et al., 1989). The male pheromones are often structurally identical or similar to chemicals commonly found in plants (Baker et al., 1981; Landolt and Heath, 1990) (Table 11). For many species and families their composition is relatively specific.

Table 11. Identified male pheromones derived from host plants.

Aldehydes	Acids	Alcohols	Esters	Terpenes	Lactones
		R—OH			
Benzaldehyde	Benzoic acid	Benzol	Diethyle malonate	Linalool	Decalactone
Carboxy aldehydes	Butanoic acid	E-phytol	Ethyl cinnamate	Methyl heptenone	Dimethylallyl lactone
Octadecanal	Cinnamic acid	Methyl heptenol	Methyl jasmonate	Pinocarvone	γ-decalactone
Phenyl acetaldehyde	Isobutyric acid	Phenol	Phenyl ethanol acetate	Vanillin	γ-undecalactone
Undecanal	Jasmonic acid	Phenyl ethanol			

From evolutionary point of view it would be reasonable to think that the herbivorous insects may have developed plant-related male pheromone communication system.

Since the females have receptors to perceive plant volatiles, they are probably capable also to detect plant-related male pheromones. The plant volatile sensitive system would be thus also available to male pheromones and may have evolved in response to pheromone chemistry. Additionally, insect natural body odour is conditioned by the larval host plant composition and may be used as male pheromone (Baker, 1989).

The male pheromone compositions vary and include cyclic alcohols and aldehydes, unsaturated aldehydes, lactones and vanillin (Table 13). Males of non-related species may have similar pheromone composition or even identical structure (Krasnoff and Dussourd, 1989; Boppré, 1990). Generally, male pheromones have significantly different chemical structure compared with conspecific females. In moths, the female and male pheromones appear to have different origins and to be under different evolutionary constraints, thus they might be considered as independently evolving traits. Indeed, the female pheromones are biosynthesized *de novo* and have different enzymatic machinery than the males (Blomquist and Vogt, 2003). However, both sexes of the Australian grapevine moth (*Phalaenoides glicinae* Lewin, Lepidoptera: Noctuidae) (Heath et al., 1988) and the Tobacco budworm (*Heliothis virescens* Fabricius, Lepidoptera: Noctuidae) (Jacobson and Adler, 1984) share some compounds in their pheromone composition. Recently it was evidenced that the male pheromone of ECB is analogous to the female signal in that structurally similar compounds are being used by both sexes (Table 12) (Lassance and Löfstedt, 2009).

In moths the male pheromones may be very complex containing up to 50 compounds (Schulz, 1987). Some compounds may be precursors (propheromones) or intermediate products in the biosynthesis of active components, while others might be neutral, inhibitory, attractive or synergistic compounds (Birch et al., 1990). The male pheromones usually (with exceptions) are active only over a short range (Baker and Cardé, 1979). The role of male pheromone may vary with species. In some moth families (Arctiidae, Tortricidae, Pyralidae) the male pheromones have long-range attraction to females (Table 10). The short-range attraction of females by male pheromone has been demonstrated in the Cabbage looper (*Trichoplusia ni* Hübner, Lepidoptera: Noctuidae), the Stored nut moth (*Paralispa gularis* Zeller, Lepidoptera: Pyralidae), the Greater wax moth (*Galleria melonella* Fabricius, Lepidoptera: Pyralidae) and the Oriental fruit moth (*Grapholita molesta* Busck, Lepidoptera: Tortricidae) species. However, electrophysiological and behavioural tests reveal that

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female do respond to compounds from male scent organs (Birch, 1971; Jacquin et al., 1991). Odours from male scent organs inhibit sexual activity of conspecific males or deter them and simultaneously encourage nuptial behaviour in females (Alpin and Birch, 1968; Phelan and Baker, 1986; Teal and Tumlinson, 1989). The male pheromone may also inhibit female rejection behaviour (Fitzpatrick and McNeil, 1989) or suppress emission of sex pheromone by females (Hendricks and Shaver, 1975). The male pheromone may also be an indicator of male fitness as it has been shown to be the case in the Cabbage moth (*Mamestra brassicae* L., Lepidoptera: Noctuidae). Jacquin et al., (1991) showed that females preferred males according to their capacity to produce pheromone. Similarly, the sexual selection has been shown to operate in Tobacco moth (Hübner, Lepidoptera: Pyralidae). The females of this species mate preferentially with larger males because apparently they produce significantly more pheromone (Phelan and Baker, 1986).

Courtship behaviour

Insects are excellent analytical chemists, they perceive the environment through semiochemicals. A male moth is capable of detecting a female-produced sex pheromone with inordinate sensitivity. Pheromones are perceived through highly selective olfactory system and the courtship behaviour in Lepidoptera can be viewed as a chemical dialogue between male and female.

Mate attraction and localization usually involve sex pheromones in two stages, with long-distance attraction *via* sex pheromones, followed by utilization of close-range courtship pheromones produced by male prior to mating. The sex pheromones implicated in successful mate attraction and in close-range courtship are often different (Costanzo and Monteiro, 2007). In Lepidoptera, it is mostly females that release the sex pheromones to attract a mate, although exemptions exist (Heath et al., 1992). The male, attracted by the pheromone signal, initiates an upwind orientation to the source, often in zigzag track. Pheromones released by females are regulated by abiotic factors and by female physiological state. The sex pheromone emission may be influenced by different factors like age, reproductive status, time of day, temperature and humidity or vicinity of host plant *etc* (Webster and Cardé, 1982; McNeil, 1991). As the male approaches the pheromone source, visual, acoustic, chemical signals may be involved in close-up courtship behaviour. Even though the

long-range attraction mechanisms reduce the number of species present at the mating site, usually there remains overabundance of potential partners and additional discrimination takes place during the courtship behaviour. Specific courtship behaviour may have a role to prevent interbreeding between closely related sympatric species, but also to evaluate the fitness of the male. The courtship behaviour has a crucial place in sexual selection and may include visual displays (movements of antennae, eyestalks, wings, and ritualized movements) and tactile stimulation (rubbing, stroking). Mating that may take from few minutes to few hours follows the successful courtship sequence.

European corn borer courtship behaviour

Our study has contributed to better the sporadic extant knowledge on the ECB courtship behaviour. Like many other moths, ECB mates in the middle of the night. The female initiates the courtship behaviour by emitting sex pheromone (Figure 12). Positioned on vegetation, the female emits a sex pheromone that drifts downwind and attracts males from distance. The males seek out the sexually receptive females by flying upwind of the pheromone gradient. Courtship in ECB involves more than mere attraction of males by females. After location of the female the male performs a series of courtship behaviours.

Figure 12. Calling female ECB moth under laboratory conditions. (Photo: E. Leppik)
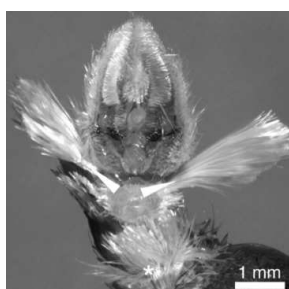
Figure 13. ECB male hairpencils. (Photo: J-M Lassance)

Figure 14. Mating ECB moths. (Photo: E. Leppik)

Figure 15. Sequence of events occurring during courtship behaviour in ECB male. Pictures were taken through transparent Plexiglas in ventral view. (Photos: E. Leppik)
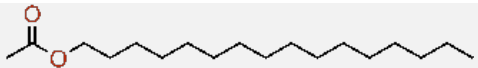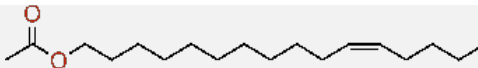
At close range, the male extrudes hair pencils located ventrally on the 8th sternite and on the claspers (Figure 13). The male wing fanning propels volatiles present on the hair pencils toward the female. After landing next to the female the male attempts to copulate by bending its abdomen (Figure 15). The male pheromone released from the hair pencils play a crucial role in closer interaction and in final female choice (Royer and McNeil, 1992). If the female accepts courting male, the genitals are clasped together and the moths copulate in *tête-bêche* position for hours (Figure 14). In the case of unsuccessful courtship the female either moves away or retrieves the gland and stops emitting pheromone. The female rejection behaviour during mating attempts suggests that females exercise an active mate choice. It has been suggested that females evaluate the quality and identity of potential mates through male pheromones, as they have been shown to have considerable importance for male mating success in moths (Jacquin et al., 1991).

The ECB females may be considered polyandric - they mate with more than one male, that is as the numbers of laid eggs is correlated positively with number of copulations (Royer and McNeil, 1993) and most of the females captured in the light traps at the end of the flight periods have more than two spermatophores (Roth and Derron, 1985).

Bioassay tests under laboratory conditions show a strong reproductive isolation between the two ECB pherotypes. The isolation is mainly ensured by assortative matings mediated by long-range pheromone communication (Pelozuelo et al., 2007). Royer and McNeil (1992) showed clearly that the scent released from male hair pencils at close range have a crucial role in the courtship success. The ablations of hair pencils decrease significantly the mating success of males. Lassance and Löfstedt (2009) identified the ECB male sex pheromone as a mixture of 16-carbon acetates that are structurally similar to those used by females (Table 2). The Z pherotype male pheromone was found to be namely a mixture of Z9-16:Ac, Z11-16:Ac, Z14-16:Ac and 16:Ac (Table 12). The composition of the E pherotype bouquet was found to be similar but lacked Z11-16:Ac. The behavioural activity and significance of these identified compounds remains to be conclusively demonstrated. Moreover, the synergy between two or more close range signals might exist. The female mate acceptance may be based for example on the combination of male sex pheromone

quality, ultrasonic courtship signal and wing ornamentation or some other unidentified close range signal that informs female on the male identity and quality.

Table 12. ECB male pheromone composition identified by Lassance and Löfstedt (2009).

Compound	Chemical structure	E pherotye	Z pherotype
16:Ac		Absent	Present
Z9-16:Ac		Present	Present
Z11-16:Ac		Present	Present
Z14-16:Ac		Present	Present
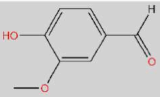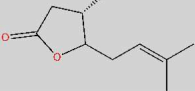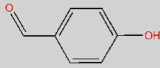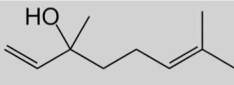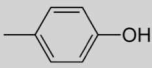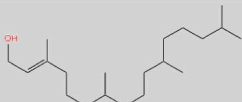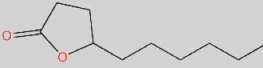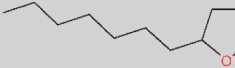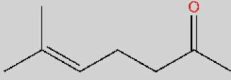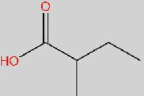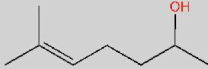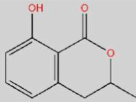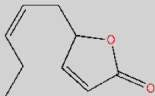
The possibility that male ECB moth use ultrasonic courtship should not be casted aside, since a closely related Asian corn borer (*Ostrinia furnicalis,* Lepidoptera: Crambidae*)* males are shown to emit weak ultrasonic courtship signals in close proximity to a female. The ultrasonic sound is produced with specific vibrations of uprightly raised wings and significantly increases the mating success of the courting male (Nakano et al., 2006; Nakano et al., 2008).

As a logical continuation to study of Royer and McNeil (1992) that showed the importance of male hairpencils in successful courtship in Z pherotype males and to the work of Pelozuelo and others (2007) that showed a strong host fidelity and reproductive isolation between the E and Z pherotypes, we investigated the role of male hairpencil scent in the reproductive isolation and attempted to identify the scent released from male hair pencils (Paper IV). The work was undertaken when the research of Lassance and Löfstedt (2009) on the male pheromone composition and biosynthesis was not yet published. Since ECB mate assoratively and express strong host fidelity, we have hypothesized that the male scent, which is involved in close range courtship behaviour, is probably derived from larval host plant composition, as it has been observed to be the case in many moths families. The E and Z pherotype populations are genetically diverged and their separation time is dated between 75 000 and 150 000 years ago, well before maize was introduced into Europe (Malausa et al., 2007b).

The influence of larvae food plant on the male pheromone composition was investigated by rearing E and Z pherotype larvae on fresh maize, mugwort and spearmint (*Mentha spicata*) plants. We chose spearmint plants as an extreme host plant, since the leaves and stems contain terpenes such as carvone, which give the distinctive and strong smell. We reasoned that if the males retrieve the active compounds of male scent from the larval host plant, the ECB males alimented on fresh spearmint, mugwort and maize plants should all have different scent composition. Probably due to leaves high alkaloid composition and mold problems, the mortality of larvae on mugwort and spearmint plants was very high. Only about 10 % of larvae survived and developed to moths. The male hair pencil composition from 3-4 old virgin males was collected by SPME method and analyzed in GC-MS. We did not detect difference between the male hair pencil composition from individuals reared on maize, spearmint or mugwort or between pherotypes. We concluded that either the male hair pencil scent did not derive from larval food or the compounds collected were thermolabiles and thus destructed in the injector (isotherm at 250°C) of GC-MS or were not captured on SPME fibres used. The latter two hypotheses were rejected as result of testing the total range of commercially available SPME fibres that gave no significant result and by injecting fibres at different temperatures. We did not detect any difference between the pherotypes or host plant groups. Given that this long series of physicochemical tests on the influence of larval host plant on male scent composition did not give any "positive" result, they are not presented in this thesis and are not subject of a publication for a moment.

Table 13. Examples of male produced pheromones identified in the Lepidoptera.

	Rice moth © Photo by K. Walker	*Corcyra cephalonica* **Pyralidae**	Reference: (Hall and others 1987)
Z,E-farnesal	E,E-farnesal	Phytone	Undecanal
Nonanal	Undecanol	Nonanol	
	Oriental fruit moth © Photo by V. Neymorovets	*Grapholita molesta* **Tortricidae**	Reference: (Nishida and others 1982)
Mellein	Methyl jasmonate	Methyl epi-jasmonate	Ethyl trans-cinnamate
	Oil palm bunch moth Photo from http://www.jpmoth.org/	*Tirathaba mundella* **Pyralidae**	Reference: (Sasaerila and others 2003)
Phytone	Z-pyranoid linalool oxide	Vanillin	Trimethylpentadecanol
	Smoky wainscot © Photo by P. Davoust	*Mythimna impura* **Noctuidae**	Reference: (Aplin and Birch 1968; Aplin and Birch 1970)
Methyl propionate	Benzoic acid	Benzaldehyde	Isobutyric acid
	Cabbage moth © R. Zóralski	*Mamestra brassicae* **Noctuidae**	Reference: (Jacquin and others 1991)
2-phenylethanol	2-Methylbutanoic acid	Benzaldehyde	Phenylmethanol
Isobutyric acid	Phenol		

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	African sugarcane borer © Photo by K. Walker	*Eldana saccharina* **Pyralidae**	Reference: (Burger and others 1993; Zagatti and others 1981)
Vanillin	Dimethylallyl lactone	4-hydroxybenzaldehyde	
	Cabbage looper © Photo by G. Fauske	*Trichoplusia ni* **Noctuidae**	Reference: (Heath and others 1992)
S-linalool	m-cresol	p-cresol	
	Tobacco moth ©Photo by M. Wall	*Ephestia elutella* **Pyralidae**	Reference: (Phelan and Baker 1986)
E-phytol	γ-decalactone	γ-undecalactone	
	Angleshade moth © Photo by R. Noel	*Phlogophora meticulosa* **Noctuidae**	Reference: (Aplin and Birch 1970; Birch 1970)
Sulcatone	2-Methylbutanoic acid	Sulcatol	
	Bee moth ©Photo by Sarefo	*Aphomia sociella* **Pyralidae**	Reference: (Kunesch and others 1987)
Mellein	Z-2,6-Nonadien-4-olide		

Speciation

At the heart of evolutionary ecology is the attempt to describe how descent with modification occurs within species and how these processes can, at times, produce a new group of individuals different enough from the others in their group that they are viewed as new species. Speciation is the evolutionary process by which new biological species arise. It has never been directly observed in nature, primarily because speciation can take a long time to occur. Most biologists consider that speciation is a more or less continuous process in which genetic variation becomes segregated between populations. For the speciation to occur, the two incipient species must have genetic differences that are expressed in some way that causes mating between them to either not occur or to be unsuccessful. A small change in the timing, location, or rituals of mating could be enough to provoke the isolation. The genetic differences between the separated populations might evolve by natural selection or genetic drift. Reduced gene flow probably plays a crucial role in speciation. There are four geographic modes of speciation observed in nature, based on the range that isolates the populations from one another: allopatric, peripatric, parapatric and sympatric (Table 14), (Figure 16).

Table 14. The geographic modes of speciation observed in the nature.

	Geographic modes of speciation	Example
Allopatric Allo = other patric = habitat	Occurs when the populations of the same species become isolated due to geographical barriers such as mountain range or social changes such as emigration.	*Charis cleonus* complex (Lepidoptera : Riodinidae) (Harvey and Hall, 2002)
Peripatric peri = near patric = place	Special version of the allopatric speciation mode and it happens when one of the isolated populations has very few individuals.	Fruit fly (*Drosophila santomea*, Diptera) (Lachaise et al., 2000)
Parapatric para = beside patric = place	No specific external barriers between populations exist. The population is continuous, but nonetheless the individuals do not mate randomly: they are more likely to mate with their geographic neighbors than with individuals in a different part of the population range.	Greenish warbler (*Phylloscopus trochiloides*) (Irwin et al., 2001)
Sympatric sym = same patric = place	Process through which new species evolve from a single ancestral species while they occupy the same geographic region.	European corn borer (*Ostrinia nubilalis*, Lepidoptera)
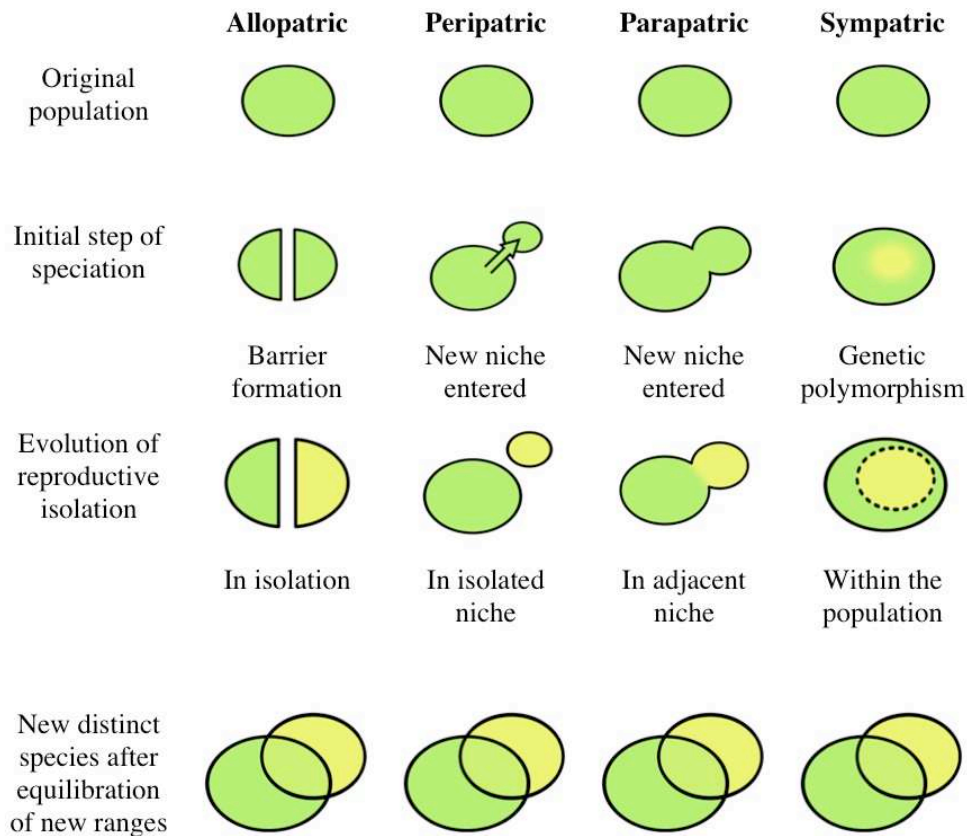
Figure 16. Spatial aspect of speciation. Comparaison of allopatric, peripatric, parapatric and sympatric speciation. (Figure drawn by I. Karonen)

Sympatric speciation

In sympatric speciation, species diverge while inhabiting the same place. It results from disruptive selection for alternative adaptive models and provokes behavioural, ecological, temporal, mechanical, temporal or hybrid isolation. Changes in host, food or habitat preference and resource partitioning may initiate sympatric speciation.

The theory of sympatric speciation is one of the most controversial and has a long history. The idea of sympatric speciation appears for the first time in the writings of Darwin (1859). According to his theory, the sympatric speciation is driven by disruptive, frequency-dependent natural selection caused by competition for diverse resources. Individuals, which diverged most strongly, might have been more suited to survive in a competition between individuals within a population (natural selection). This competition may be for example for the food resources: the individuals most

suited to its research or its assimilation will be selected and preserved. The sympatric speciation was associated with entomology when Benjamin Walsh (1867) proposed that certain host-specific phytophagous insects could speciate in the absence of geographic isolation in the process of shifting and adapting to new host plants.

Sympatric speciation can take place by disruptive sexual selection or/and by disruptive natural selection. It has been argued that disruptive sexual selection alone can also cause sympatric speciation. The model of disruptive sexual selection is driven by selective mating, or by assortative mating, that is, non-random mating leading to differential mating successes of different genotypes. It makes the assumption that heritable variation in female preferences within a single population exerts disruptive selection on male traits, such that males with intermediate trait value obtain fewer matings than males with high trait value of either sign (Higashi et al., 1999). Disruptive sexual selection is a rare and poorly understood mode of selection (Endler, 1986; Smith, 1993) and there are few convincing examples from the nature. It has been shown to occur for male pheromones in the Orange sulphur butterflies (*Colias eurytheme* Boisduval, Lepidoptera: Pieridae) (Sappington and Taylor, 1990) and for a sexually selected trait (body size) in pacific salmon (*Oncorhynchus* spp., Suckley, Salmonidae) (Gross, 1985). The evolution of ECB host races is an example of sympatric speciation where the reproductive isolation between the host races is ensured by assortative mating. It is not clear whether the assortative mating is selected *per se*, *via* reinforcement (Noor, 1999), or as a by-product of host specialization (pleiotropy) (Rice, 1987). Our hypothesis is that in addition to the female sex pheromone communication system, the host plants have a role in the reproductive isolation through male pheromone composition. It has been found that in Lepidoptera, when there is a high probability of mating mistakes between closely related species or host races, the males produce pheromones that provide information on the identity of the male (Phelan and Baker, 1987). Since in Lepidoptera the male pheromone composition is usually strongly related to the larva alimentation, we hypothesize that ECB females prefer males that produce male pheromones with compounds sequestrated from the female host plant. Thereby the ECB females from mugwort would prefer males that have sequestrated mugwort related chemicals and females from hop and maize would prefer males from their respective host plants, since their male pheromone contains chemicals from the female larval host plant.

The second theory of sympatric speciation is the disruptive natural selection that involves habitat preference that varies inside of the population and is determined by the natural selection acting on loci that affect adaptation to the environment. Habitat preference is an especially significant behaviour, since it leads to the differences in food choice, the place of oviposition and place of meeting the sexual partners. In Lepidoptera it has been observed that closely related sympatric insect species generally have different host plants while closely related allopatric species use the same or similar plants.

Prezygotic mechanisms (level of gene flow)		COPULATION	Postzygotic mechanisms (stabilizing or disruptive selection)
•Temporal isolation •Different host plants •Geographic isolation	•Female sex pheromone •Assortative attraction •Specific courtship behavior •Differentiation of the genitalia		•Hybrids inviability •Hybrid sterility •Low hybrid fitness
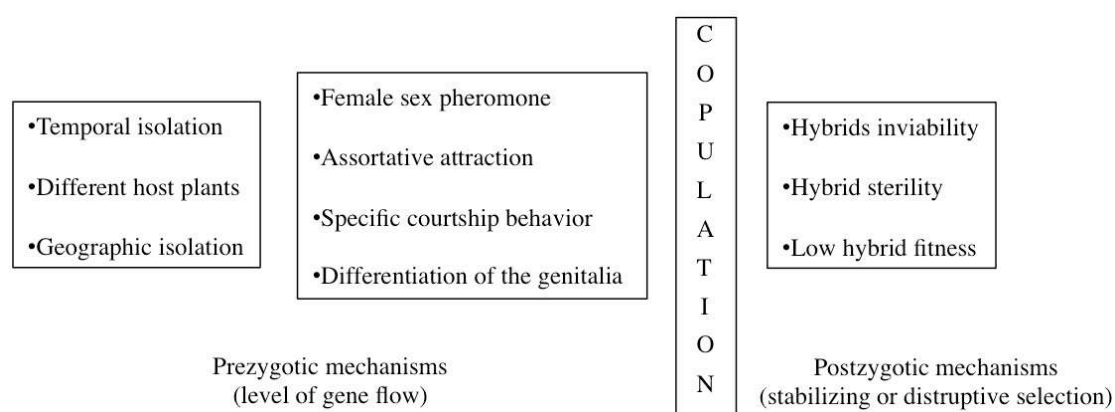
Figure 17. Pre-and postzygotic mechanisms in reproductive isolation in Lepidoptera.

Mechanisms that are behind the differentiation are classified into two large categories of pre- and postzygotic isolation mechanisms, each of which can be further subdivided (Figure 17). Most models of sympatric speciation through disruptive natural selection concern phytophagous insects, in which speciation is driven by host shifts and subsequent host adaptations. Over the years, the ECB E and Z pherotypes reproductive isolation has become a model system for investigating the role of pheromone communication and host plant specialization in speciation. Even though ECB has become a textbook example of sympatric speciation, it is an exception to typical sympatric model. Often in the sympatric speciation models, the life cycle and mating behaviour of phytophagous insect is strongly affected by their host plant and the gene flow between the populations is restricted *via* pleiotropy (Via, 2001). In the case of ECB pherotypes reproductive isolation, there is no evident pleiotropy with the host plant. The ECB moths have very little contact with the host plant outside of the specific period of oviposition flight, when the females fly out to lay egg on their host

Table 14. Pre- and postzygotic mechanisms implicated in reproductive isolation of European corn borer pherotypes.

Taxon	*Ostrinia nubilalis*, Lepidoptera	Reference
Host-races	Mugwort, hop, maize (introduced)	(Pelozuelo et al., 2004)
Age of host-race	<500 years. The Z race colonized maize after its introduction into Europe about 500 years ago.	(Malausa et al., 2007)
Time genetic of divergence between pherotypes	75 000 to 150 000 years. The E and Z pherotypes diverged recently but probably long before the introduction of maize into Europe.	(Malausa et al., 2007)
Oviposition differences	Females emerging from larvae collected on maize show a preference for oviposition on maize rather than on mugwort or hop, whereas females emerging from larvae collected on mugwort show the opposite preference.	(Bethenod et al., 2005), (Paper III)
Prezygotic mechanism in ECB pheromone and host races		
Temporal isolation	Thomas et al., found that moths of the E race emerged earlier from mugwort and hop than those of the Z race from maize. A year later on the same populations Malausa et al., did not find evidence of temporal isolation between races in terms of flight periods.	(Thomas et al., 2003; Malausa et al., 2005)
Different host plants	Z race colonizes maize. E race is found on mugwort and hop.	(Pelozuelo et al., 2004)
Geographic isolation	It is uncertain whether ECB host races originally diverged in sympatry; however at present day they co-occur over a large geographical range in Northern Hemisphere.	(Frolov et al., 2007)
Assortative meeting and mating	The assortative meeting is promoted by long-range pheromone communication system and an additional close range mechanism is implicated.	(Royer and McNeil, 1992)
Female sex pheromone	Both pherotypes use Δ11-14:Ac isomer as sex pheromones. Females of the Z pherotype produce a blend with an E:Z ratio of 1:99 to 3:97 whereas E pherotype females produce approximately the opposite blend.	(Klun et al., 1973), (Klun and Brindley, 1970; Kochansky et al., 1975).
Specific courtship behaviour	The female moth halts the otherwise successful courtship behaviour if a male with extruded hair-pencils from opposite pherotype is placed in to close vicinity of the pre-mating pair.	(Paper IV)
Differentiation of genitalia	Male genitalia morphology clearly shows some polymorphism but there is no clear evidence that male genitalia are not as, or nearly as, polymorphic within inter-race as they are intra-race.	(Frolov et al., 2007)
Postzygotic mechanism		
Hybrids inviability	Under laboratory conditions the hybrids are viable and fertiles.	Personal observations
Hybrid sterility	Hybrids between ECB pherotypes are obtained in experimental settings but are rarely found in nature.	(Bethenod et al., 2005)
Low hybrid fitness	The hybrids have low reproductive success due to incorrect sex pheromone composition.	(Thomas et al., 2003)
Gene flow in the field	There is no gene flow between E and Z race under field conditions (less than 1%)	(Malausa et al., 2005)

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plants: Z race on maize and E race on mugwort and hop. The mating behaviour of ECB species is not related directly to their host plant, since it has been observed that the moths aggregate in dense grassy areas, often hundreds of meters away from nearest host plant. It is not clear what triggered exactly the host plant specialisation and reproductive isolation in ECB pherotypes. Based on the genetical studies on different ECB populations feeding on maize, mugwort and hop show that the host plant populations have high level of genetic divergence, with gene flow less than 1% *per* generation (Malausa et al., 2007a). It is pretty clear that the female pheromone communication system is responsible for assortative meeting and mating, but it is not clear why the pherotypes have specialized on different host plants. Pélissié *et al.* (2010) advanced an idea that the introduction of maize in Europe and its large scale cultivation since 20th century, provided ECB larvae enemy-free space. They found that on mugwort parasitism was twice as high as it was on maize, and parasitoid-related mortality was 8 times as high. Even though the introduction of maize created a parasite free niche to ECB, it is still not clear why only the Z pherotype shifted and why the E pherotypes is only found on wild and native host plants. The identity of ancestral host plant of Z pherotype before the introduction of maize in Europe and the mechanism that triggered the host plant specialization remains unclear.

Paper IV

"Assortative mating between European corn borer pheromone races- the role of male hair-pencil in the reproductive isolation"
Ene Leppik and Brigitte Frérot

Male hairpencils and assortative mating in European corn borer pherotypes

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Abstract

The existence of two pherotypes in ECB specialised on different host plant lead to numerous studies dealing with assortative mating, host plant fidelity. The ECB did not mate on the specific host plant but in grassy area where both pherotype mate assortatively in sympatry conditions addressing the question of assortative mating process. There is not doubt on the role of female sex pheromone that specifically attract the conspecific male but the fact that inter pherotype mating are scarce even under artificial condition in narrow space lead to hypothesise that an additive barriers might occurred to reinforce the reproductive isolation between to pherotype populations. Taking into account that the ECB males bear hairpencils and that male sex pheromone mainly originate in compound sequestered from the food intake, we hypothesise that the hairpencils display during courtship behaviour release a specific chemical signal contributing to reinforce reproductive isolation.

The role of hairpencil display in courtship behaviour was studied and the identification of behaviourally male pheromones was attempted. The male hairpencil compounds were collected by Solide Phase Micro Extraction method and analyzed by gas chromatography (GC) linked to mass spectrometry (MS). The hairpencil extracts and previously identified male pheromone perception by ECB antennae was tested by electroantennography.

The crucial role of hairpencil in assortative mating at close-range was demonstrated. The hairpencil secretions convey a chemical signal to female that enhance the female acceptance.Within the pherotype populations display of male hairpencils from one pherotype inhibited mating success in pairs belonging to other type. We failed to detect the previously identified 16-carbon acetates in the hairpencil extracts of the ECB populations we worked on. Electroantennogram experiments demonstrated candidate compounds for the antennae of females of both pherotypes did not perceive reproductive isolation. The behavioural and electrophysiological tests provide strong evidence that hairpencils have a crucial role in close-range behaviour and contribute to to reinforce the assortative mating. The behaviourally active male sex pheromone composition needs to be further investigated.

Keywords: *Ostrinia nubilalis,* mating behaviour, sex pheromone.

1 Introduction

Assortative mating in ECB was extensively studied during the last decade and discussed as a key mechanism in processes leading to sympatric speciation, in absence of geographic and temporal isolation. In nocturnal Lepidotera, assortative mating is clearly the consequence of reproductive isolation between taxa achieved by the production of the female sex pheromone that elicits in the conspecific males a specific response. In ECB, two different pherotypes were identified. The Z pherotype female produce a blend with an E:Z ratio of 1:99 to 3:97 (Klun et al., 1973), whereas E pherotype females produce approximately the opposite blend (Kochansky et al., 1975). Males of the two pherotypes are selectively attracted to the respective blends produced by females and are reproductively isolated (Pelozuelo et al., 2004).

In France the ECB pherotypes exhibited a host plant specialisation (Pelozuelo et al., 2004) leading to the so called hop-mugwort-E race feeding exclusively on native plants such as hop (*Humulus lupulus* L.) and mugwort (*Artemisia vulgaris* L.) and the maize-Z race feeding on the introduced maize (*Zea mays* L.). Each races display a strong host fidelity for oviposition (Leppik and Frérot, *submitted*). The genetic approach and the use of stable carbon isotopes evidenced strong barriers to gene flow and demonstrates that the genetic differences are maintained in sympatry conditions (Ponsard et al., 2004), even though both pherotypes mate at the same time in the grassy meeting places (Showers et al., 1976). Thus the host plant presence apparently does not reinforce the reproductive isolation of races as shown in other moths (Drès and Mallet, 2002; Via, 2001). The reproductive isolation of E and Z races is achieved by assortative mating resulting from their long-range sex pheromone communication system. However, an additional mate-recognition mechanism to long-range attraction is suspected to be involved in the assortative mating since under laboratory condition - when moths are confined into a small box and males and females are forced to meet - the mating success of inter-pherotype pairs is lower than of same-pherotype pairs (Bethenod et al., 2005; Pelozuelo et al., 2007). Royer and McNeil (1992) described the ECB male hairpencils and demonstrate on the Z pherotype that they are displayed during the courtship behaviour. Their ablations have a critical negative impact on male mating success showing the crucial role of hairpencils display for male acceptance. The author did not succeed to identify any relevant chemical component.

The chemicals produced by male hairpencils are related with larvae food (Baker et al., 1981; Blum, 1987; Jacquin et al., 1991). They are biosynthesized from sequestration of plants compounds or amino acids. The ECB Z and E pherotypes feed on different host plants that belong to different botanical families, known to release very distinct VOCs (Leppik and Frérot, *submitted*) and to bear different alkaloids and specific compounds (Bernotiene et al., 2004; Judžentienė and Buzelytė, 2006; Pino et al., 1999). Thus hairpencils chemicals of host plant specialised males of ECB we hypothesised that hairpencil chemicals of host plant specialised ECB males would be different and that they are a putative candidate as a reinforcement agent of reproductive isolation, acting at short range during the courtship behaviour.

Lassance and Löfstedt (2009) identified for the first time the ECB hairpencils pheromone showing that males produce structurally analogous compounds to female sex pheromones with a difference in the composition of Z and E males.

The purpose of the present study was firstly to investigate the role of ECB male hairpencils in reproductive isolation of two pherotypes. The courtship behaviour was observed and described. The role of hairpencil chemical signals as a reinforcement of the reproductive isolation was examined by placing a male from opposite pherotype with extruded hairpencils at close vicinity of courting pair. We aimed to confirm the presence of male pheromones on hairpencils and their perception by female antennae. The male pheromones from hairpencils were extracted by two alternative methods: Solide Phase Micro Extraction (SPME) and hexane extraction and additionally hexane extracts of whole male moths was made. The extracted compounds were analyzed in gas chromatograpgy (GC) linked to mass spectrometry (MS). The perception of extracts, previously identified male and female pheromone compounds were tested in electroantennography (EAG). Results are discussed in term of sympatric reproductive isolation.

2 Material and methods

2.1 Insects

Diapausing ECB E-pherotype larvae were collected in France near Amiens (49°86'N, 2°29'E) from mugwort plants. Z-pherotype gravid females were captured near Grignon (48°85'N, 1°96'E) and near Darvoy (47°85'N, 2°10'E). The Z-pherotype larvae, issued from the captured gravid moths, were reared on an artificial maize-based diet. In the E-pherotype artificial agar-based diet, 30% of the maize flour was replaced with mugwort stem powder. The larvae were reared under a 16 hours light and 8 hours of dark periods. Pupae were sexed and kept in separate Plexiglas containers.

2.2 Behaviour tests

To study the role of male hairpencils in the assortative mating, we conducted behaviour tests. The courtship behaviour was recorded on Archos AV500 with Hitachi KP161, CDD black and white camera, equipped with Nikon objective AF Micro Nikon 60 mm 1:2.8 D. Every experiment lasted 15 minutes under 23°C, 60% RH and red light. The behaviour sequences were analyzed with The Observer 5.0 software (Noldus, Wageningen, The Netherlands, 2004). On the sixth hour of scotophase two day old female moths were placed in the Plexiglas box (22x14x12cm) with wire mesh sides that open. The air was continually sucked out from one side on the wire mesh wall. When the female expresses a calling behaviour, a conspecific male was placed in the box. As soon as the male extruded the hairpencils and started a courtship behaviour sequence, another male from opposite pherotype was introduced in the box with the hairpencils mechanically extruded by metallic forceps. The second male was placed next to mating pair. The female calling behaviour, oriented flight of the male, hairpencils extrusion, mating attempts and female response to the male courtship was recorded. The control experiment was conducted by placing a conspecific male with extruded hairpencils next to the mating pair from the same pherotype.

2.3 Female moth pherotype confirmation

The pherotypes of female moths were confirmed by SPME-technique (Frérot et al., 1997) using Carbowax-Divinylbenzene fibres (CW/DBV) 65 μm, (Supelco Inc.). The pheromone gland was extruded by gentle pressure on the abdomen and kept in this position with metallic forceps. A SPME fibre was gently rubbed on the gland. Each fibre was then either directly analyzed or wrapped in aluminium foil and stored at −20°C until analysis. Pheromones were identified using a GC Varian 3400Cx. GC was equipped with split–splitless injector and a Rtx ®-Wax column (Restek; 30 m x 0.32 mm ID, 0,25 μm film). The compounds absorbed on the fibre were thermally desorbed in the injector used in splitless mode and maintained at 240°C. The oven temperature was programmed from 50 to 100°C at 15°C min^{-1}; 100 to 245°C at 5°C min^{-1}, helium (15 psi) was the carrier gas. Pheromone blend composition was indentified by comparison of retention times of the natural compounds with those of synthetic reference samples. The ratios of the compounds were calculated from the peak areas.

2.4 Male pheromone extraction

Hexane extraction

The hairpencils of 3 to 4-day old virgin ECB moths were removed with metallic forceps and placed in 50μl of hexane for extraction. One set of extraction contained the hairpencils of two males from the same pherotype. Additionally whole male moth and male moth wings hexane extraction were made, by submerging the moth or the wings in a vial of 100 μl of hexane and briefly vortexing the vial. The hairpencils, whole moth and wing extractions were tested in EAG and analyzed on a GC-MS (Varian QIMS). Total of 8 hairpencils, 8 whole male moths and 6 wing hexane extraction samples for each pherotype was collected to GC-MS analyses.

SPME method

Compounds from male hairpencils were collected using PDMS/DVB and CW/DVB 50/30 μm (Supelco Inc.,) SPME fibres. The phase of the fibre was rubbed against the mechanically extruded hairpencils and desorbed in the GC-MS (Varian QIMS) injector for identification. Total of 20 samples of hairpencil SPME extraction was collected from each pherotype.

2.5 Male pheromone identification

The hexane extracts and SPME fibres were analyzed on GC-MS detector Varian QIMS. The GC injector was held at 250 °C. Compound separation was carried out using a fused silica column Rxi-5ms, 30 m × 0.32 mm i.d., film thickness 1.0 μm (Restek, France). The column temperature was programmed as follow 50 °C for 3 minutes and then at 8°C/min to 300 °C. Helium was used as carrier gas (5 psi). Mass spectra of compounds were obtained in electron impact mode (70 eV, 40 to 350 amu) with the ion source at 230 °C. Identification of compounds collected from male hairpencils was achieved according to their mass spectra and retention indexes (RI). The RI were computed using n-alkanes from C_{10} to C_{24}, eluted under the same conditions as the collections. Every compound spectra and RI were compared with those of authentic samples as well with NIST 1998 libraries using deconvolution software AMDIS32.

2.6 EAG

EAG recordings were performed at room temperature on 3 to 4-day males and females moths. Reference and recording glass capillary electrodes were filled with 6.4 mM KCl, 340 mM Glucose, 10 mM HEPES, 12 mM $MgCl_2$, 1 mM $CaCl_2$, 5 NaCl pH = 6.5. The reference electrode was inserted in the neck and the recording electrode covered the cut tip of one antenna. The signal was amplified (× 1000) and low pass filtered online (2 kHz) with a Cyberamp 320 amplifier (Molecular Devices, Union City, CA) and digitized at 1 kHz with a Digidata 1200B acquisition board (Molecular Devices).

A piece of filter paper (10x30 mm) impregnated with 0.5 μl of test solution was inserted into a glass Pasteur pipette were used as stimulus cartridge. Antennae were stimulated during 0.5 s with 0.5 μl of Z9-14:Ac, E11-14:Ac, Z11-14:Ac, 16:Ac, Z11-16:Ac (1μg/μl) and with Z and E pherotype male hairpencil extracts. Tested compounds were HPLC purified (chemical and isomeric purity >99%). Each compound was diluted in n-hexane (analytical purity >95% GLC) (Carlo Erba, France) to give 1 μg/μl solution. The control stimulus was hexane. Analysis of EAGs was carried out under pClamp 10 (Molecular Devices). Responses were normalized with respect to the control stimulus by subtracting average value for the hexane control from the response and multiplying per ten and divided by the average value

for the hexane control.

$$\text{Normalized response} = \frac{(\text{Response - average hexane})*10}{\text{average hexane}}$$

3 Results

3.1 Behavioural assay

Analyses of 57 courtship sequences showed that a fairly fixed pattern of female and male behaviour occurs. Male flew upwind in response to the sex pheromone released by the calling female and extruded the two sets of hairpencils located between 7th and 8th abdominal segments and associated with the claspers. After landing near the female, the male displayed its hairpencils while wing fanning, and bent his abdomen tip towards the female. The male repeatedly extruded and retracted the hairpencils. The contact with female caused the male to attempt copulation. At the last step, the female continues the calling behaviour and antennation. The unsuccessful courtship ended by female flying away or stopping the calling behaviour. In that case the male usually follows female or searches in the area that female vacated. When female departs, the male at some point stops wing fanning and stays on the wall.

We examined whether ECB male mating success was influenced by the close vicinity of the opposite pherotype hairpencils. E-pherotype male mating success was significantly reduced by the presence of the Z-pherotype hairpencils (n=18, χ^2=10.2 df=1, p=0.001) (Figure 1). In the presence of Z-pherotype hairpencils 89% of E-pherotype females refused to mate with the courting E-pherotype male. The same phenomenon was observed in the behaviour of the Z-pherotype females. 90% of Z females refused to mate with the Z-pherotype courting male, when the E-pherotype hairpencils were placed in the close vicinity (n=16, χ^2=9 df=1, p=0.003). When the hairpencils of the same pherotype as the courting pair were introduced in the close vicinity, about 80% of females accepted the courting male (Table 1).

3.2 EAG

The mean EAG responses of ECB antennae to a variety of synthetic pheromone compounds, ranged from 2.4 ± 0.7 (16:Ac) to 51.0 ± 11.8 mV (Z11-14:Ac) in males and from 1.6 ± 4.0 (Z9-14:Ac) to 5.51 ± 4.0 mV (Z11-14:Ac) in females (Table 2). In

males, all the tested 14 and 16 carbon acetates and male extracts elicited a higher electrophysiological responses than the hexane. The highest depolarization was observed when male antennae was stimulated with the two $\Delta11$-14:Ac whereas almost no significantly different response was observed in females (Table 2).

Male hairpencil extracts did not elicit any response on neither female nor on male antennae. Therefore extraction of whole male moth was tested instead. The Z-pherotype male moth extract elicited high response in Z-pherotype males, a moderate depolarization in Z pherotype females and no significant response in E-pherotype males and the E pherotype females antennae. The E-pherotype male moth extract elicited a high depolarization in Z-pherotype males but a moderate response in E-pherotype males. The Z-pherotype females responded moderately to E-pherotype male moth extract, whereas the E-pherotype females to not seem to perceive the extraction at all. The whole male moth extraction lost its property to depolarize antennae about 10 minutes after extraction. Since the whole moth extraction had an electrophysiological activity on female antennae, we tested whether the active compounds are extracts from male wings. The wing extracts failed to elicit any response on both pherotype male and female (Table 2).

3.4 Male pheromone identification

The hairpencil extracts that were tested in EAG were also analyzed in GC-MS. The hairpencil hexane extractions contained relatively small number of compounds. In both pherotype hairpencil extracts two compounds with the mass fragments of a fatty acid (m/z 55, 71, 83, 101) were detected (RI 3787 and 3900). By the mass fragmentation, these compounds were tentatively identified as two isomers of unsaturated fatty acids with about 22 carbon atoms. In the analyses collected by the SPME methods only two compounds were detected repeatedly. They had the same RI and mass fragmentation as the previous two compounds detected in hairpencil extracts.

No pike were detected at the RI of Z9-16:Ac, Z11-16:Ac, 16:Ac and Z14-16:Ac, thus the specific ions of acetates were used in single ion detection. No trace of 16 carbon chain-length acetates were detected neither in SPME nor hexane extraction samples.

4 Discussion

The ECB courtship behaviour appeared as a sophisticated chemical dialogue between the male and the female. The long-range attraction of potential sexual partners in ECB is steered by the female produced sex pheromone perceived by the conspecific males. ECB males are specifically attracted towards the female of their own phertotype and thus the female pheromone blend differences is the basis of the assortative mating of E and Z phertotypes. Our results corroborate with previous results showing that inter-pherotype attraction was not possible between ECB pherotypes .

Once male was specifically attracted and located the pheromone releasing female by flying upwind in pheromone plume, the close-range courtship behaviour, the so called chemical dialogue between calling female and answering male, begins. During the courtship behaviour the ECB male extrudes hairpencils, fans wings and makes copulation attempts. Similar short-range behaviour involving hairpencils, abdominal brushes or other scent-organs has been described in numerous male moth species (Birch et al., 1990). We observed that the close vicinity of the other pherotype hairpencils reduced the mating success of courting pairs. Otherwise successful courtship was stopped by simply placing a male moth, from the other pherotype, with its hairpencils extruded near the conspecific pair. Our observation suggest that the mate-choice and the decision to end the courtship behaviour come from the female moth, indeed in most Lepidoptera, the mate choice is the decision of the females (Zagatti and Castel, 1987). Our results concord with the Royer and McNeil (1992) conclusion that the abdominal and clasper hairpencils of ECB males release a sex pheromone and have a crucial role in mate-acceptance. Our results demonstrate the additive role of hairpencils on reproductive isolation. It is clear that each pherotype male produced chemicals that inhibit female acceptance of the conspecific male. Thus we conclude that the hairpencils secretion contribute to the assortative mating. The nature of the compounds released by hairpencils stayed elusive for almost two decades. Recently Lassance and Löfstedt (2009) identified a blend of 16 carbon length saturated and monounsaturated acetates We failed to detect the aforementioned 16 carbon length acetates in SPME or in hexane extraction samples even though all the mass spectra analyzes were scanned for acetate specific ions.

The response profiles obtained from electrophysiological recordings of female ECB antenna showed that females did not perceive 16:Ac nor Z11-16:Ac, neither did their antennae detect Z9-14:Ac or Δ11-14:AC molecules. In the other hand, these 14 carbon acetates were well detected by male antennae and induced strong depolarization confirming their role as key compound of the female pheromone blend. Consequently, based on our results we concluded that the ECB females from French Z and E-pherotypes did not respond to 16:Ac and Z11-16:Ac identified by Lassance and Löfstedt (2009). This result is puzzling as the presence of Z11-16:Ac was the main difference between the identified hairpencils pheromone blend in E and Z pherotype. This compound was only identified in the Z pherotype. Thus putting together our behavioural test and the Lassance and Löfstedt identification, the negative effect of the Z pherotype hairpencils on the mating acceptance of E pairs is likely to be due to the Z11-16:Ac. The failure in this conclusion is that this compound is released by the E pherotype females and should not have any depressive effect on mating success of E pherotype pairs.

The male hairpencils and wing extracts failed to induce a depolarisation of both sexes antennae, whereas the whole male extracts elicited a strong response on both conspecific sexes antennae indicating that the male pheromone secretions are not located exclusively in hairpencils. We were not able to identify the active compounds in the male moth extractions. They might be chemically instable and undergo decomposition or are extremely volatiles since the extracts lost their electrophysiological activity within minutes after the preparation. We detected only few compounds in hairpencils and whole moth extracts analyzes. Recurrently two isomers of unsaturated fatty acids with about 22 carbon atoms were detected, but their identification stayed tentative devoid of authentic compounds. In Lepidoptera the male pheromones are consistently closely related structurally to compounds taken from diet (Baker, 1985). The ECB females, like most of the phytophagous moths, have receptors for plant volatile detection (Bruce et al., 2005; Solé et al., 2010) and therefore we can postulate that females have appropriate sensory system to detect host plant metabolites from male scent organs. Our results evidenced that further experiments should be undertaken on pheromone identification of ECB male hairpencils. Even more this work point out the crucial role of the hairpencil display for assortative mating.

5 Abbreviations

16:Ac - hexadecanyl acetate; df - degrees of freedom; E11-14:Ac - *trans*-11-tetradecenyl acetate; EAG - Electroantennography; ECB - European Corn Borer; GC - gas chromatography; MS - mass spectroscopy; Z9-14:Ac - *cis*-9-tetradecenyl acetate; Z11-14:Ac - *cis*-11-tetradecenyl acetate; Z11-16:Ac - *cis*-11-hexadecenyl acetate.

6 Acknowledgements

Table 1. The percentages of mate refusal and acceptance in the close vicinity of hairpencils introduced by experimenter.

	Female refusal	Female acceptance	n	Chi^2	p-value
Courtship in close vicinity of pherotype Z hairpencils					
E♀ x E♂	89%	11%	18	10.2	0.001
Z♀ x Z♂	23%	77%	13	4.17	0.041
Courtship in close vicinity of pherotype E hairpencils					
E♀ x E♂	20%	80%	10	3.6	0.005
Z♀ x Z♂	88%	12%	16	9	0.003

Table 2. The mean EAG responses and the corresponding SE of ECB antennae elicited by pheromone compounds and hairpencil extracts.

|	Males		Females	
	Z pherotype	E pherotype	Z pherotype	E pherotype
Compounds	n=12	n=9	n=8	n=7
Z9-14:Ac	24.5±10.6	18.7±5.6	1.6±4.0	3.4±1.1
E11-14:Ac	24.5±4.0	32.1±12.9	4.0±3.4	2.6±2.6
Z11-14:Ac	51.0±11.8	39.0±13.6	5.5±4.0	4.3±1.9
16:Ac	3.3±1.0	2.4±0.4	2.4±2.4	–
Z11-16:Ac	11.8±2.5	7.7±1.8	–	3.9±2.0
Z male extract	29.4±24.3	7.7±3.2	16.1±9.8	–
E male extract	40.6±29.1	13.5±9.9	12.1±11.8	–
Z hairpencil extract	–	–	–	–
E hairpencil extract	–	–	–	–
Z male wing extract	–	–	–	–
E male wing extract	–	–	–	–
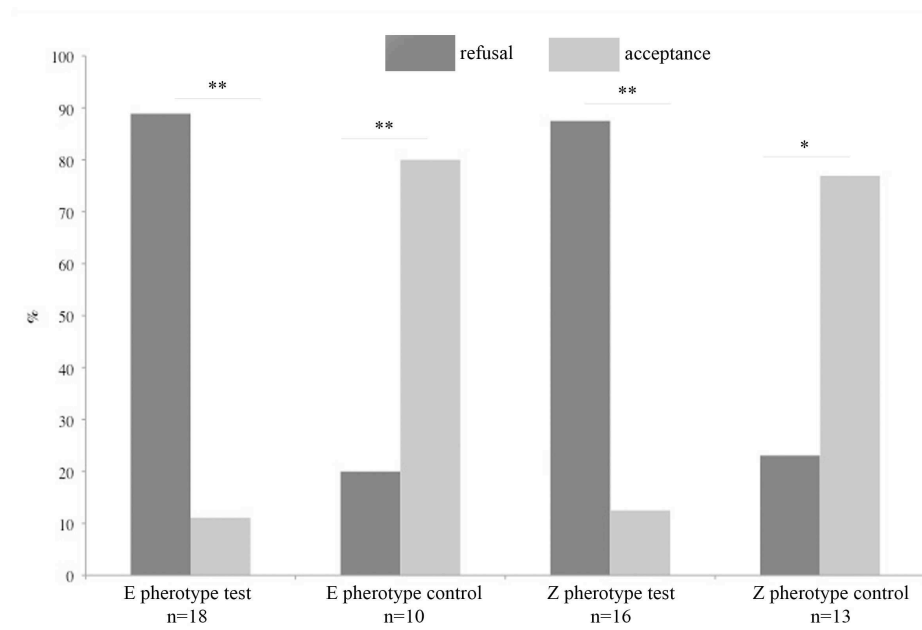
Figure 1. The percentage of mate acceptance and refusal in the close vicinity of introduced hairpencils.

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Discussion and perspectives

Discussion and perspectives

The present study is one of the first to give ecologically relevant information about the chemical signals that are emitted by ECB host plants and by maize field in concordance with the insect biology and behaviour. Two noticeable points were mainly considered: host plant colonisation and oviposition. To take full advantage of the results obtained on the volatile composition released by ECB host plants, further studies should be conducted at the levels of olfactory receptors and behaviour. The aim of such studies will be to determine the key compounds used by host seeking moth, amidst of all the identified VOCs. The screening of identified volatiles by EAG method would narrow down the myriad of volatiles to volatiles that are really perceived by female antennae. These volatiles should receive particular interest and should be subject of further behavioural tests to determine the behaviourally active key compounds or to propose artificial volatile blends.

Deciphering the signal perceived and used by host-seeking moth is highly relevant in developing alternative methods of ECB population control. The behaviourally active host plant blends are potential kairomones that could be used to monitor the ECB populations. Furthermore, the identification of key compounds in host recognition would open a way for maize breeders and genetic engineering to develop maize varieties in which certain VOCs biosynthesis is suppressed or released in different quantity that are unattractive to host-seeking ECB moth.

Comparing the volatile profiles collected from maize plant headspace samples and from maize field olfactory environment, we observed a puzzling phenomenon. There is a discrepancy between the complexity of VOCs in plant headspace samples and the actual olfactory environment that a host-seeking moth encounters: the headspace samples of individual maize plants is composed mainly of SQT, whereas the open-air samples of maize field is composed chiefly of MT. The VOCs released by plants are readily oxidized by O_3 which is an important oxidant of the low atmosphere. SQT and some oxygenated compounds have short atmospheric life times that range from seconds to hours. We think that the O_3 degradation of SQT and GLV is one of the main reasons why the SQT are rarely detected in the open-air. The insects have probably learned to use oxidation products of plant VOCs that are more stable in nature to locate their host plant. To understand better the chemical signal perceived by

host-seeking moths, it would be necessary to investigate the perception of oxidized plant VOCs and other O_3 stable compounds by female antennae and oxidized VOCs behavioural activity on moths.

The results on host fidelity leads us to hypothesize that the host plant population in ECB could be in fact a mosaic of isolated and specialized populations. Still little is known about what criteria determine the suitability of a host and in particular, what are the cues that make a host attractive to gravid females. Better knowledge on the host range and on host preference of ECB is needed, since it is not clear whether ECB is a truly opportunistic polyphagous species, or rather a mosaic of host-plant races hidden under the same name.

Our work on assortative mating of ECB pherotypes showed that the male pheromone and hairpencil display have crucial role in the close range chemical communication. The females clearly perceived a chemical signal from males since they systematically refused a conspecific male when hairpencils of other pherotype were place in the close vicinity. Further physicochemical research is required to identify the behaviourally active compounds. One of the hypotheses is that the compounds are thermolabils and are destroyed in the injector of GC. The derivation of hairpencils compounds would transform them to stable and detectable form even when subjected to high temperatures.

The electrophysiological and biological activity of ECB male pheromone composition, identified by Lassance and Löfstedt (2009), should be also further investigated. The perception of Δ16:Ac by ECB antennae should be confirmed by EAG, since our results did not show any activity of the candidate component (Z11-16:Ac) on the female ECB antennae.

The research on ECB chemical communication continues to fascinate scientists, since it is both an excellent model of sympatric speciation and an agricultural pest against which new tools of monitoring and control are needed. The ECB communication system is complex and much is yet to discover mainly on female host choice and on mechanism that ensure the assortative mating of pherotypes. The present work has given an insight into the chemical environment that host-seeking ECB moth encounters and opens a way to future research on host cues that are ecologically relevant to insects.

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