ASSESSING THE IMPORTANCE OF SPECIFIC VOLATILE ORGANIC COMPOUNDS IN MULTITROPHIC INTERACTIONS

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Summary – Plants interact with a multitude of beneficial and harmful organisms of different trophic levels and it is generally accepted that such multitrophic interactions are highly relevant for various defence responses in plants. One of the most intriguing responses in plants is induced by herbivore feeding and results in the release of large amounts of volatile organic compounds (VOCs). These herbivore-induced plant volatiles (HIPVs) may serve as indirect defence signals by attracting natural enemies of herbivorous arthropods, but also function as key regulators of plant physiological changes. Some HIPVs even trigger defence responses in neighbouring plants or prime these plants to respond faster to subsequent herbivore attacks. However, the function of individual compounds within complex blends of HIPVs has largely remained uninvestigated. The current thesis aimed to understand the role of specific VOCs in multitrophic interactions from the perspective of plant defence responses.

In a first part (*Chapters II and III*), we focused on indirect defence responses by maize seedlings (*Zea mays* var. Delprim). We developed two new methods that allowed us to study the importance of various HIPVs for the attraction of *Cotesia marginiventris* and *Microplitis rufiventris*, two parasitoids that attack larvae of various *Spodoptera* moths. It was found that some major HIPVs were not important in the attraction of these parasitoids, whereas several minor compounds seemed to be essential and highly attractive. Moreover, the two parasitoid species were differently attracted towards these HIPVs and their responses strongly depended on previous experiences with specific HIPVs.

In a second part (*Chapter IV*), we investigated the role of soil-born micro-organisms in defence responses by maize seedlings and we found that they too affected the attraction of the parasitoids. Analyses of VOC-blends revealed that seedlings grown in soil with micro-organisms released additional compounds, mainly isomers of 2,3-butanediol, and these compounds were produced by the γ-proteobacterium *Enterobacter aerogenes* that we isolated from maize seeds. Both bacteria and synthetic versions of the bacteria-derived VOCs induced systemic resistance in maize seedlings against the fungal pathogen *Setosphaeria turcica*, but not against the herbivore *Spodoptera littoralis*. Molecular tools were employed to study possible mechanisms of induction.

The results of these studies not only revealed novel roles that organisms and VOCs play in plant defence responses, but also present new methodological approaches that can be used to

identify key compounds affecting multitrophic interactions. The advances and challenges in the identification of such key compounds are reviewed in *Chapter V*.

Overall, this work aims to contribute to a better understanding of the role of specific VOCs in interactions between plants and other organisms, which seems a fundamental first step towards the application of VOCs in alternative crop protection strategies against agricultural pests.

Résumé – Les plantes interagissent avec une multitude d'organismes bénéfiques et nuisibles des niveaux trophiques différents, et il est généralement admit que de telles interactions multitrophiques jouent un rôle dans de nombreux mécanismes de défense utilisés par les plantes. Une des réponses les plus fascinantes des plantes ayant subit l'attaque d'un herbivore est la libération de grandes quantités de composés organiques volatils (Volatile Organic Compounds = VOC). Ces substances volatiles végétales induites par les herbivores (Herbivore Induced Plant Volatiles = HIPV) peuvent servir de signaux indirects de défense afin d'attirer certains ennemis des arthropodes phytophages, mais ils fonctionnent également comme régulateurs principaux des changements physiologiques des plantes. Quelques composés HIPV peuvent même induire des réponses de défense chez les plantes voisines ou activer ces dernières, qui répondront alors plus rapidement à une attaque future d'un herbivore. Cependant, les fonctions individuelles de différents composés constituant ces bouquets complexes d'HIPV n'ont, jusqu'à aujourd'hui, pas été considérés. Cette thèse met en évidence le rôle que jouent certains VOCs lors d'interactions multitrophiques dans le contexte des stratégies de défense des plantes.

Dans la première partie (*chapitres II et III*), nous nous sommes concentrés principalement sur les stratégies de défenses indirectes des jeunes plantes de maïs (*Zea mays* var. Delprim). Nous avons développé deux nouvelles méthodes qui nous ont permises d'étudier l'importance de divers composés HIPV dans l'attraction de *Cotesia marginiventris* et *Microplitis rufiventris*, deux guêpes parasitoïdes qui parasitent les larves de divers papillons du genre *Spodoptera*. On a constaté que certains des composés HIPV principaux n'influençaient pas l'attraction de ces parasitoïdes, tandis que plusieurs composés mineurs semblaient être essentiels et fortement attractifs. En plus, on a montré que les deux espèces de parasitoïdes réagissaient différemment à certains composés HIPV et que leurs réponses dépendaient fortement de leurs expériences précédentes avec des composés spécifiques.

Dans une deuxième partie (*chapitre IV*), nous avons étudié l'influence des micro-organismes du sol sur les stratégies de défense des jeunes plantes de maïs et nous avons constaté que ces derniers aussi affectaient l'attraction des parasitoïdes. Les analyses de bouquets de substances VOC ont indiqué que les jeunes plantes qui grandissent dans un sol avec des micro-organismes émettent des composés supplémentaires, principalement des isomères de 2,3-butanediol. Ces composés sont produits par la y-proteobactérie *Enterobacter aerogenes* que nous

avons isolée des graines de maïs. Les bactéries et les substances bactériennes synthétiques induisent une résistance systémique des jeunes plantes de maïs contre le champignon pathogène *Setosphaeria turcica*, mais pas contre le ravageur *Spodoptera littoralis*. Des mécanismes possibles ont été analysés avec des outils moléculaires.

Cette étude et ses résultats n'ont pas seulement mis en évidence de nouvelles fonctions que ces organismes et leurs substances volatiles peuvent avoir dans la défense des plantes, mais ont aussi permis le développement et l'affinement de la méthodologie visant à identifier les principales molécules affectant les interactions multitrophiques. Les progrès et les défis dans l'identification de tels composés-clés ont été passés en revue en *chapitre V*.

En conclusion, ce travail va contribuer à une meilleure connaissance du rôle des substances VOC spécifiques dans les interactions entre les plantes et les autres organismes, ce qui semble une première étape fondamentale vers l'application de ces substances dans des stratégies de protection alternatives contre les parasites et les pathogènes des cultures.

Resumaziun – Plantas interageschan cun blers organissems nizevels e nuschevels tgi dareivan da divers nivels trofics, e generalmaintg ègl accepto tgi chellas interacziuns multitroficas èn fitg impurtantas per las differentas reacziuns da defensiuns dallas plantas. Egna dallas raspostas las pi fascinontas da plantas tgi èn attatgedas dad erbivors è l'emissiun d'ena gronda quantitad da substanzas organicas volatilas (Volatile Organic Compounds = VOC). Chellas substanzas volatilas, indutgeidas digls erbivors dallas plantas (Herbivore Induced Plant Volatiles = HIPV), èn betg angal signals indirects da defensiun tgi èn attractivs per inimeis naturals digls erbivors artropods, mabagn er regulatours da midadas fisiologicas ainten las plantas. Tschertas substanzas HIPV inizieschan perfign reacziuns da defensiun ainten las plantas vischinantas u preparan chellas sen attatgas eventualas dad erbivors. La funcziun da substanzas singulas da chellas masdadas cumplexas da HIPV è però anfignen oz per gronda part betg neida examinada. La dissertaziun preschainta ò ampruo dad ancleir la rolla tgi substanzas VOC particularas on ainten las interacziuns multitroficas or dalla perspectiva dallas reacziuns da defensiun dallas plantas.

Ainten l'amprema part (*tgapetels II e III*) ans ischans surtot concentros sen las reacziuns indirectas da defensiun dallas plantas giovnas da furmantung (*Zea mays* var. Delprim). Nous vagn sviluppo dus novas metodas d'analisa tgi ans on lubia da stibgier l'impurtanza da diversas substanzas HIPV per l'attracziun da *Cotesia marginiventris* e da *Microplitis rufiventris*, dus vespras parasiticas, tgi attatgan las larvas da diversas pullas digl gener *Spodoptera.* Nous vagn observo tgi tschertas substanzas HIPV principalas eran betg impurtantas per l'attracziun da chellas vespras parasiticas, pero otras substanzas minoras eran essenzialas e fitg attractivas. Pinavant vainsa musso tgi las dus spezias da vespras parasiticas eran attiradas diversamaintg da chellas substanzas HIPV e tgi l'attracziun era dependenta fermamaintg dallas experientschas precedentas dallas vespras cun tschertas da chellas substanzas.

Ainten la sagonda part (*tgapetel IV*) vainsa surtot examino tge rolla tg'igls micro-organissems dalla tera gioian ainten las defensiuns dallas plantas da furmantung, e nous vagn observo tgi er chels organissems influenzeschan l'attracziun dallas vespras parasiticas. Analisas dallas substanzas volatilas on musso tgi las plantas tgi creschan ainten teras cun igls micro-organissems relaschan substanzas supplementaras, surtot isomers dalla substanza 2,3-butanediol. Chellas substanzans eran produtgeidas dalla y-proteobacteria *Enterobacter aerogenes*,

tgi nous vagn savia isolar or digls sems da furmantung. Schibagn las bacterias scu er las substanzas VOC sinteticas dallas bacterias on indutgia la resistenza sistemica ainten las plantas giovnas da furmantung cunter igl bulia infectous *Setosphaeria turcica* pero betg cunter igl erbivor *Spodoptera littoralis*. Igls mecansissems prubabels èn nias analisos cun metodas molecularas.

Chels studis e resultats on betg angal contribuo allas differentas funcziuns tgi differents organissems e substanzas VOC on sen la reacziun da defensiun dallas plantas, mabagn er allas metodas tgi pon neidas duvradas ainten l'identificaziun dallas substanzas impurtantas per las interacziuns multitroficas. Igls progress e las difficultads dad identifitgier chellas substanzas èn discutadas ainten igl *tgapetel V*.

Chesta lavour contribuescha ad ena miglra tgapientscha dalla rolla da subtanzas VOC specificas ainten las interacziuns dallas plantas cun oters organissems, e chegl è en amprem pass fundamental per eventualmaintg far adiever da chellas substanzas volatilas ainten strategias alternativas per cumbatter igls parasits ed igls patogens dallas plantas cultivadas.

Zusammenfassung – Pflanzen gehen mit vielen nützlichen und schädlichen Organismen unterschiedlicher Trophieebenen Wechselwirkungen ein, und es ist allgemein anerkannt, dass solche multitrophischen Wechselwirkungen für die pflanzlichen Abwehrreaktionen von zentraler Bedeutung sind. Eine der wohl faszinierendsten Reaktionen der Pflanzen auf Herbivorenfrass ist die Emission grosser Mengen flüchtiger organischer Verbindungen (Volatile Organic Compounds = VOC). Diese sogenannten Herbivoren-induzierten, flüchtigen Verbindungen der Pflanzen (Herbivore Induced Plant Volatiles = HIPV) dienen einerseits als Substanzen der indirekten Abwehr, indem sie die natürlichen Feinde pflanzenfressender Gliederfüsser anlocken, andererseits auch als wichtige Regulatoren pflanzenphysiologischer Prozesse. Einige dieser HIPV-Substanzen können auch Abwehrreaktionen in benachbarten Pflanzen auslösen oder diese auf einen eventuellen zukünftigen Herbivorenbefall vorbereiten. Die Funktion einzelner Verbindungen innerhalb komplexer Gemische solcher HIPV-Substanzen ist bis heute jedoch noch weitgehend unbekannt. Die vorliegende Doktorarbeit versucht die Rolle spezifischer VOC-Substanzen, welche innerhalb multitrophischer Wechselwirkungen von Bedeutung sind, aus dem Blickwinkel der Pflanzenabwehrreaktionen zu verstehen.

Im ersten Teil (*Kapitel II und III*) konzentrierten wir uns auf die indirekten Abwehrreaktionen junger Maispflanzen (*Zea mays* var. Delprim). Wir entwickelten zwei neue Methoden, welche uns erlauben, die Wichtigkeit unterschiedlicher HIPV-Substanzen für die Anlockung von *Cotesia marginiventris* und *Microplitis rufiventris* zu bewerten. Diese Schlupfwespen parasitieren die Larven unterschiedlicher *Spodoptera* Falter. Wir beobachteten, dass mehrere Hauptverbindungen keine Bedeutung für die Anlockung dieser Schlupfwespen haben, während andere, unscheinbare Verbindungen notwendig sind und sehr anziehend wirken. Ferner zeigten wir, dass die beiden Schlupfwespenarten unterschiedlich angelockt werden und dass ihre Reaktion stark von vorhergehenden Erfahrungen mit einzelnen dieser flüchtigen Verbindungen abhängt.

In einem zweiten Teil (*Kapitel IV*) untersuchten wir die Rolle von Mikroorganismen im Boden auf die Abwehrreaktionen der Maispflanzen, und wir beobachteten, dass auch diese einen Effekt auf die Anlockung der Schlupfwespen ausüben. Duftstoffanalysen zeigten, dass Pflanzen, die in Böden mit Mikroorganismen wachsen, zusätzliche Verbindungen freisetzen, nämlich Isomere der Substanz 2,3-Butanediol. Diese Substanzen werden vom y-Proteobakterium *Enterobacter aerogenes* produziert, welches wir aus Maissamen isolieren konnten. Sowohl die Bakterien als

auch die bakteriellen flüchtigen Verbindungen induzieren in jungen Maispflanzen systemische Resistenz gegen den pilzlichen Krankheitserreger *Setosphaeria turcica*, jedoch nicht gegen den Herbivoren *Spodoptera littoralis*. Mögliche Mechanismen wurden mittels molekularbiologischen Methoden untersucht.

Diese Studie zeigt nicht nur neue Funktionen auf, die Organismen und spezifische VOC-Substanzen in der Abwehrreaktionen von Pflanzen haben, sondern auch neue methodologische Ansätze, die genutzt werden können, um die wesentlichen Verbindungen in multitrophischen Wechselwirkungen zu identifizieren. Die Fortschritte und die Herausforderungen bei der Identifikation solcher Verbindungen werden in *Kapitel V* diskutiert.

Diese Arbeit trägt zu einem besseren Verständnis der Rolle spezifischer VOC-Substanzen bei, welche in Wechselwirkungen zwischen Pflanzen und anderen Organismen essentiell sind. Dies stellt einen ersten grundlegenden Schritt für eine eventuelle Anwendung solcher flüchtigen organischen Verbindungen in alternativen Bekämpfungsstrategien gegen Pflanzenschädlinge in der Landwirtschaft dar.

Riassunto – Le piante interagiscono con un gran numero di organismi benefici e nocivi appartenenti a livelli trofici differenti, e generalmente è accetato che queste interazioni multitrofiche sono di grande importanza per le diverse risposte di difesa delle piante. Tra queste risposte delle piante, una delle più interessanti è l'emissione di grandi quantità di sostanze organiche volatili (Volatile Organic Compounds = VOC) in sequito all'attacco di erbivori. Queste sostanze volatili delle piante, indotte dagli erbivori (Herbivore Induced Plant Volatiles = HIPV), non solo possono servire da segnali per una difesa indiretta della pianta, poiché attirano i nemici naturali degli artropodi erbivori, ma funzionano anche da regolatori dei cambiamenti fisiologici nelle piante. Inoltre, alcune sostanze HIPV inducono delle risposte di difesa nelle piante vicine o innescano in esse dei meccanismi che permettono loro di rispondere più velocemente ai successivi attacchi di erbivori. Tuttavia, la funzione di singoli composti all'interno delle complesse miscele che costituiscono le sostanze HIPV, è rimasta in gran parte inesplorata. In questa tesi si è cercato di chiarire quale ruolo sostanze VOC possano avere sulle interazioni multitrofiche, nel quadro delle risposte di difesa della pianta.

In un primo tempo (*capitoli II ed III*), ci siamo concentrati sulle risposte indirette di difesa nelle giovani piante di mais (*Zea mays* var. Delprim). A questo scopo abbiamo sviluppato due nuovi metodi che hanno permesso di studiare l'importanza delle diverse sostanze HIPV nell'attrazione di *Cotesia marginiventris* e di *Microplitis rufiventris*, due parassitoidi che attaccano le larve di diversi lepidotteri del genere *Spodoptera*. Parecchie tra le principali sostanze HIPV sono risultate non significative nell'attrazione di questi parassitoidi, mentre alcuni composti minori sono risultati essenziali ed altamente attrattivi. Inoltre, abbiamo scoperto che le due speci di parassitoidi sono attratte in modo diverso delle sostanze HIPV e che le loro risposte dipendono altemente dalle loro precedenti esperienze con delle specifiche sostanze volatili.

In un secondo tempo (*capitolo IV*), abbiamo studiato il ruolo dei micro-organismi del suolo nelle risposte di difesa delle piante di mais, ed abbiamo osservato che anch'essi sono in grado di modificare l'attrazione dei parassitoidi verso i loro ospiti. L'analisi delle sostanze VOC ha dimostrato che le piante cresciute nel terreno con i micro-organismi hanno emesso delle sostanze supplementari, principalmente isomeri del 2,3-butanediolo. Queste sostanze sono state prodotte dal y-proteobatterio *Enterobacter aerogenes*, che abbiamo isolato dai semi del mais. Inoltre, sia i batteri che le loro sostanze volatili sintetiche, hanno indotto una resistenza sistematica nelle

giovani piante di mais contro il fungo patogeno *Setosphaeria turcica*, ma non contro l'erbivoro *Spodoptera littoralis*. I meccanismi di difesa possibili sono stati analizzati con dei metodi molecolari.

Questo studio non soltanto aggiunge alle diverse funzioni che diversi organismi e le sostanze VOC hanno nelle risposte di difesa delle piante, ma propone anche dei nuovi approcci metodologici per l'identificatiazione delle sostanze chiave che modificano le interazioni multitrofiche. I progressi e le diverse sfide nell'identificazione di tali sostanze sono trattati nel *capitolo V*.

In generale, questo lavoro contribuisce ad una migliore comprensione del ruolo delle sostanze VOC specifiche nelle interazioni tra le piante e gli altri organismi, cio che sembra essere un primo passo fondamentale verso l'utilizzo di questi composti nelle strategie alternative di protezione delle piante coltivate contro i diversi parassiti e patogeni.

Chapter I

General introduction and thesis outline

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General introduction - Most of us are well aware of the wonderful fragrances and flavours produced by lilac trees during the first warm nights in spring, which not only attract pollinating moths, but also awaken people's desire for love and passion. Less well known and less pleasing are the odours emitted by the leaves of common weeds and crop plants, but they too elicit exciting responses, at least in a variety of insects and neighbouring plants. In fact, quite often the chemical composition and intensity of these plant odours carry information on the plants' physiological state and on the stresses that they are being subjected to. For example, in response to feeding by arthropods, many plants actively and systematically emit volatile organic compounds (VOCs) and natural enemies of these herbivorous arthropods have evolved ways to exploit these volatiles to locate their hosts and preys (Turlings and Wäckers, 2004). The ecology, evolution, and physiological mechanisms underlying these so called herbivore-induced plant volatiles (HIPV) has been studied to great detail over the last decade and have been summarised in various recent review papers (Dicke et al., 2003; Dudareva et al., 2004; Pichersky, 2004; Arimura et al., 2005).

Interestingly, a lot of the knowledge on herbivore-induced plant volatiles (HIPVs) is derived from studies with agricultural crop plants, probably because of the potential to apply such volatiles as novel tools to enhance the control of agricultural pests (Turlings and Ton, 2006). Indeed, there is increasing evidence that plant volatiles can be applied to reduce the damage done by herbivorous insects and pathogens. In field studies, for example, Khan and colleagues (1997) nicely demonstrated that intercropping maize fields with the odourous grass *Melinis minutiflora*, which emits a compound that is typically released by maize in response to caterpillar damage, resulted in largely reduced damage by a lepidopteran stemborer, partly because the pest was repelled by the odour of the grass, but also because one of its parasitoids was attracted to the mixed fields, leading to high parasitism rates. Promising approaches to reduce the damage by insect pests and diseases might also be the application of synthetic plant VOCs (James, 2003; Neri et al., 2006) or the genetically engineering of plants to alter the release of attractive VOCs (Degenhardt et al., 2003; Aharoni et al., 2005). This later approach has recently been applied to transform *Arabidopsis thaliana* plants to overexpress genes involved in the terpenoid synthesis, which resulted in increased attractiveness of natural enemies. In a first study, a linalool/nerolidol synthase gene from strawberry (*FaNES1*) was introduced into *Arabidopsis*, causing the transformed plants to constitutively release two additional sesquiterpenoids, which rendered them attractive to predatory

mites (Kappers et al., 2005). In another study, *Arabidopsis* was transformed with a maize terpene synthase gene (*TPS10*), and this resulted in the emission of a blend of sesquiterpenoids, which is typically release be herbivore-infested maize seedlings and increased the attraction of parasitoid females that did experience such terpenoid blends before (Schnee et al., 2006).

Some plant volatiles, including some HIPVs, might also affect defence responses in neighbouring plants (Baldwin and Schultz, 1983; Arimura et al., 2000; Farmer, 2001). Although a detailed understanding of the mechanisms behind this so called 'plant-plant-communication' is still in its infancy, a recent finding that maize plants that were exposed to a volatile blend of neighbouring plants responded stronger and faster with the release of HIPVs, indicates that VOCs do not induce commonly known plant defences but rather 'prime' plants to respond stronger and faster to subsequent herbivore attack (Engelberth et al., 2004). Priming by VOCs was since then found in various other studied systems (Heil and Kost, 2006; Kessler et al., 2006; Paschold et al., 2006), and could also be measured by a faster and stronger activation of some defence genes (Ton et al., 2006).

Although these pioneering studies show good potential for successful application of plant VOCs to enhance the control of insect pests, little is known about the role and specificity of individual compounds within the complex volatile environment. The reason for this lack of knowledge might be explained by the enormous variability and diversity of plant VOCs. Plants are known to emit more than 30'000 divergent foliar compounds, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters and carboxylic acids (Niinemets et al., 2004) and HIPVs are derived from at least three different biosynthetic pathways (Paré and Tumlinson, 1999; Pichersky et al., 2006) (Figure 1). Moreover, many traits in plants and insects show great genetic variability and phenotypic plasticity. For example, the release of HIPVs not only depends on the genotype (Loughrin et al., 1995; Degen et al., 2004), but also on other biotic and abiotic factors (Turlings and Wäckers, 2004) and similarly, the detection of HIPVs by natural enemies is not only affected by the animals' genotype (Wang et al., 2003), but also by the physiological state and previous experiences with volatile blends (Takasu and Lewis, 1993; Vet et al., 1995; Faria, 2005).

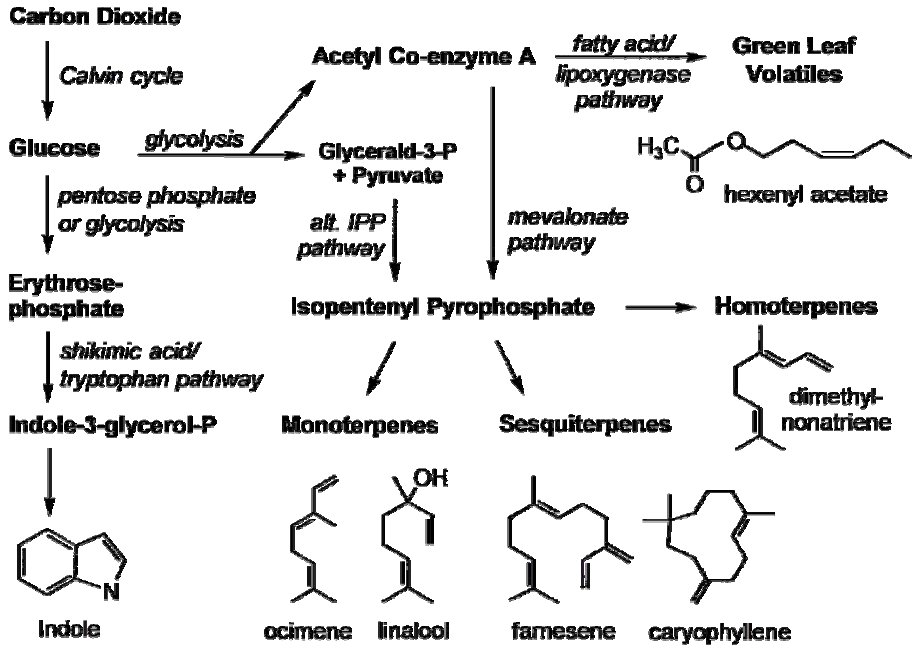

Figure 1. Major biosynthetic pathways leading to the release of HIPVs.

Volatile indole, a product of the shikimic acid pathway, is formed from indole-3-glycerol-P via the enzyme indole-3-glycerol phosphate lyase, which differs form the enzymes BX1 that produces indole as an intermediate for direct defence compounds (e.g. 2,4-dihydroxy-7-methoxy-2*H*-,1,4-benzoxazin-3(4*H*)-one), or tryptophane synthase, which produces the amino acid tryptophane (Frey et al., 1997; Frey et al., 2000). Sesquiterpenes are synthesised via the isopentenyl pyrophosphate (IPP) intermediate following the classical mevalonate pathway, whereas monoterpenes and diterpenes are synthesised via an alternative IPP pathway with glyceraldehyde-3-P and pyruvate identified as the direct precursors of IPP (Lichtenthaler et al., 1997). The mevalonate pathway is localised in the cytosol and reactions for the non-mevalonate pathway are localised in plastids. The homoterpene (e.g. (*E*)-4,8-dimethyl-1,3,7-nonatriene) is derived from farnesylpyrophosphate by a series of enzymatic steps with the overall loss of four carbon units (Donath and Boland, 1994). The green-leaf volatiles are derived from linolenic acid via a 13-hydroperoxylinolenic acid intermediate (Blee, 1998). This oxidised linolenic acid, instead of losing water and committing the molecule down the defense signaling jasmonic acid pathway, is cleaved to form two fragments of 12 and six carbon units. The variety of green-leaf volatiles are formed from this second pathway by multiple rearrangement steps of the six-carbon (*Z*)-3-hexenal. Modified after (Paré and Tumlinson, 1999).

The current thesis aims to address the question of the specificity of VOCs affecting defence responses in maize plants (*Zea mays* var. Delprim). HIPVs in maize have been studied over the last twenty years in at least five laboratories around the globe, and various aspects of

these maize volatiles are well characterised, ranging from the genetics, biosynthesis, induction, and release to the ecological significance of these compounds in tritrophic interactions (Turlings et al., 1990; Alborn et al., 1997; Bernasconi et al., 1998; Degenhardt and Gershenzon, 2000; Frey et al., 2000; Shen et al., 2000; Turlings et al., 2000; Hammack, 2001; Hoballah and Turlings, 2001; Gouinguené and Turlings, 2002; Schnee et al., 2002; Schmelz et al., 2003; Degen et al., 2004; Köllner et al., 2004; Lawrence and Novak, 2004; Lou et al., 2005; Rasmann et al., 2005; Williams et al., 2005). Earlier studies with the same model system as in the current thesis (Figure 2) already demonstrated that several parasitoids that attack lepidopteran larvae are strongly attracted to HIPVs released by maize seedlings (Fritzsche Hoballah et al., 2002; Gouinguené et al., 2003). In fact, these compounds have been considered to be the most important host locations cues used by these parasitoid species, but the attractiveness was strongly influenced by prior experience of the wasps with such volatile blends in association with hosts. Interestingly, the effects of this so-called associative learning differ considerably between different parasitoid species, suggesting that they have evolved different strategies to exploit these signals (Hoballah and Turlings, 2005; Tamò et al., 2006). Recent studies on maize HIPVs revealed an important role of belowground herbivores and aboveground pathogens on the induction of such compounds (Rasmann, 2006; Rostás et al., 2006) and this opens the questions on the importance of specific VOCs for multitrophic species interactions.

Figure 2. Picture showing the main organisms used for the current thesis: *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae and one of its potential parasitoids, *Cotesia marginiventris* (Hymenoptera: Braconidae), on a maize (*Zea mays* var. Delprim) leaf.

Thesis outline - In the first section (*chapters II and III*) of this thesis we study the role of maize volatiles induced by *Spodoptera littoralis* in the host location by two parasitic wasps, *Cotesia marginiventris* and *Mircroplitis rufiventris*, and we specifically focus on the importance of individual and groups of compounds for the attraction of the wasps and the role that these compounds may play in associative learning. In a second section (*chapter IV*) we look at how these tritrophic interactions and various defence responses in maize plants are affected by soil-born micro-organisms and their volatile metabolites. Finally we review the advances and the challenges in the identification of VOCs that mediate interactions among plants and arthropods (*chapter V*).

Chapter II and III – The role of specific *Spodoptera littoralis* induced maize VOCs in the host location by two parasitic wasps, *Cotesia marginiventris* and *Mircroplitis rufiventris*.

A sound way of studying the importance of individual VOCs within a complex blend is to compare the attractiveness of volatile blends differing in only one or few known compounds. The tremendous knowledge on HIPVs released by maize seedlings (see introduction) opens new ways to manipulate and modify blends. We divide such approaches in 'subtractive' methods, whereby the complexity of normally induced volatile blends is reduced, and 'additive' methods, which generate blends with increasing complexity (Figure 3). In both cases manipulations can be done (1) in the headspace of the plant by either filtering out compounds from a blend or by adding compounds to a blend (volatome modification; volatome = sum of all released VOCs over a specific time and space), (2) at the plant phenotype level by either inhibiting or inducing VOC-pathways (phenotype modification), and finally (3) at the genotype level by either silencing genes or transforming plants with constitutively expressed genes involved in the VOC biosynthesis (genotype modification).

Here we introduce novel ways to employ the first two of these approaches, which were used to study the role and specificity of HIPVs in the host location of parasitoids. In *chapter II* we use different adsorbing materials to filter out compounds from an induced blend and in *chapter III* we use an inhibitor to disrupt the shikimate pathway, which is an important biosynthetic pathway involved in the production of aromatic HIPVs. In both approaches we test the attractiveness of the

modified blends to parasitoids in olfactometer experiments and we estimate the importance of the eliminated compounds for the attraction of the wasps by adding back the missing compounds to the modified blends.

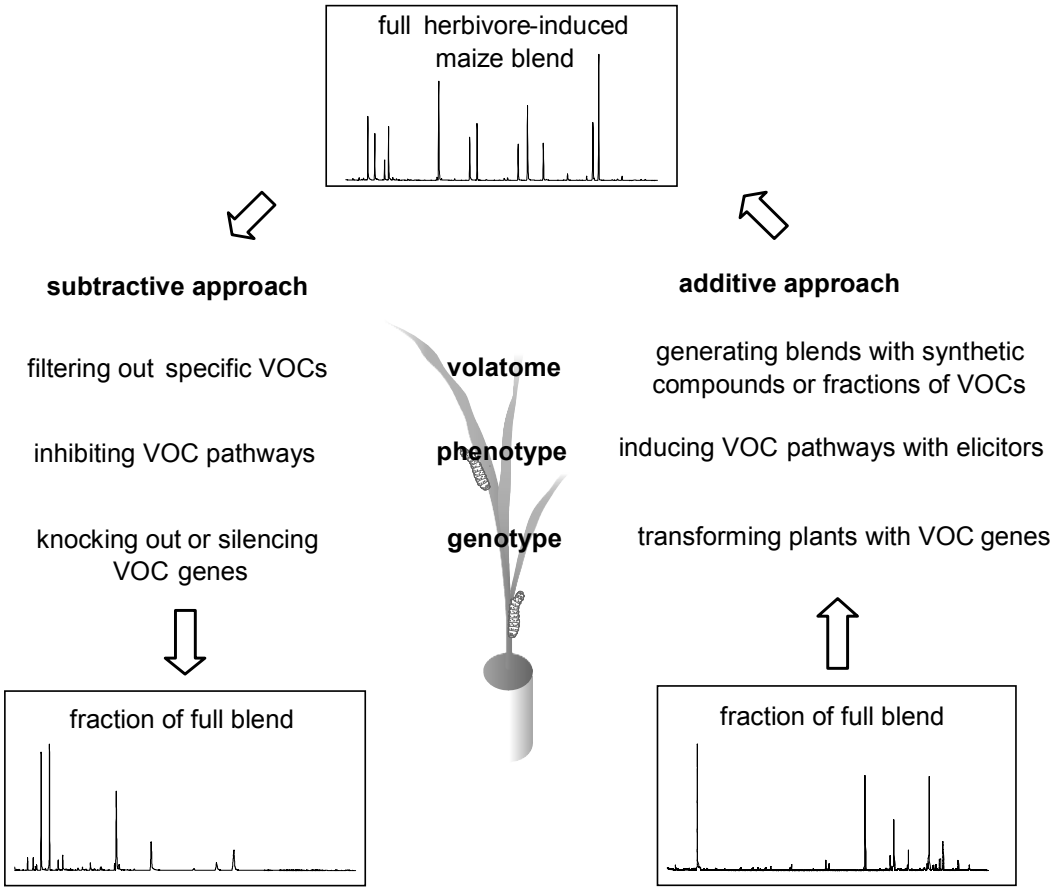

Figure 3. Schematic representation of experimental approaches used to modify and generate blends of herbivore-induced maize VOCs. Typical chromatographic traces of volatile blends are indicated in the boxes. To study the importance of individual and group of compounds, such blends can be compared and tested for attractiveness to parasitoids in behavioural assays.

Chapter IV - The role of soil-born micro-organisms and their volatile metabolites in defence responses in maize plants.

Due to the multi-facet nature of chemical ecology, many chemical ecologists tend to bury their heads in the sand when it comes to detailed physiological studies or real ecological approaches. But sometimes burying one's head into the sand may lead to new exciting discoveries.

For example, by analysing roots of maize seedlings attacked by *Diabrotica* larvae, Rasmann and colleagues (2005) revealed that roots under attack by insects also release VOCs. The major compound, (*E*)-β-caryophyllene, not only attracted entomopathogenic nematodes in laboratory bioassays in sand, but also in ecologically more relevant field experiments, showing that VOCs also play important roles in signalling belowground. Besides belowground interactions with herbivorous insects, the rhizosphere of plants is also a zone of intense microbial activity (Figure 4) and these micro-organisms may release additional VOCs that affect the defence response of plants (e.g. Ryu et al., 2004). During the experiments conducted for *chapters II* and *III* we occasionally detected VOCs typically known to be produced by soil-born micro-organisms. In *chapter IV* we show that endophytic bacteria are responsible for these emissions and that the volatiles may modify defences in maize seedlings against pathogens and insects.

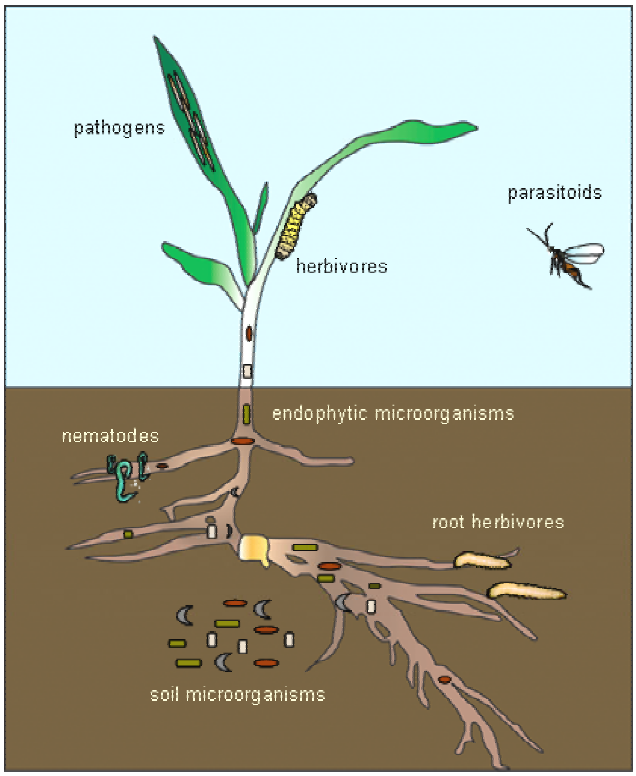

Figure 4. A cartoon illustrating the major aboveground and belowground interactions of plants with organisms of other throphic levels.

Chapter V - Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods.

Enormous progress has been made over the last two decades in understanding ecological significance of HIPVs. Although molecular and genetic approaches are frequently applied to elucidate the mechanisms of plant-mediated interactions, the chemical analyses of HIPVs remain an integral part of virtually all studies. *Chapter V* first presents a brief overview of the physiological and ecological role of HIPVs in interactions between plants and other organisms, and further reviews the current methods that are commonly used by biologists to collect and analyse HIPVs that are biologically relevant. Finally it identifies the challenges that remain to be tackled in this area of research.

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Chapter II

In-situ modification of herbivore-induced plant odours: a novel approach to study the attractiveness of volatile organic compounds to parasitic wasps

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In Situ Modification of Herbivore-Induced Plant Odors: A Novel Approach to Study the Attractiveness of Volatile Organic Compounds to Parasitic Wasps

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Abstract

Many parasitic wasps (parasitoids) exploit volatile organic compounds (VOCs) emitted by herbivore-infested plants in order to locate their hosts, but it remains largely unknown which specific compounds within the volatile blends elicit the attractiveness to parasitoids. One way of studying the importance of specific VOCs is to test the attractiveness of odor blends from which certain compounds have been emitted. We used this approach by testing the attraction of naive and experienced females of the two parasitoids *Cotesia marginiventris* and *Microplitis rufiventris* to partially altered volatile blends of maize seedlings (*Zea mays* var. Delprim) infested with *Spodoptera littoralis* larvae. Adsorbing filter tubes containing carbotrap-C or silica were installed in a four-arm olfactometer between the odor source vessels and the arms of the olfactometer. The blends breaking through were tested for chemical composition and attractiveness to the wasps. Carbotrap-C adsorbed most of the sesquiterpenes, but the break-through blend remained attractive to naive *C. marginiventris* females. Silica adsorbed only some of the more polar VOCs, but this essentially eliminated all attractiveness to naive *C. marginiventris*, implying that among the adsorbed compounds there are some that play key roles in the attraction. Unlike *C. marginiventris*, *M. rufiventris* was still attracted to the latter blend, showing that parasitoids with a comparable biology may employ different strategies in their use of plant-provided cues to locate hosts. Results from similar experiments with modified odor blends of caterpillar-infested cowpea (*Vigna unguiculata*) indicate that key VOCs in different plant species vary greatly in quality and/or quantity. Finally, experienced wasps were more strongly attracted to a specific blend after they perceived the blend while ovipositing in a host. Considering the high number of distinct adsorbing materials available today, this *in situ* modification of complex volatile blends provides a new and promising approach pinpointing on key attractants within these blends. Advantages and disadvantages compared to other approaches are discussed.

Key words: host location, indirect plant defense, induced plant odors, olfactometer, parasitoids, tritrophic interactions

Introduction

Herbivore-induced plant volatiles are known to play an important role in the interactions between plants and arthropods (Dicke *et al.*, 2003b; Turlings and Wackers, 2004; van Poecke and Dicke, 2004; Arimura *et al.*, 2005). These highly detectable volatile organic compounds (VOCs) may act either directly, for example, by deterring oviposition by lepidopteran herbivores (Landolt, 1993; De Moraes *et al.*, 2001; Kessler and Baldwin, 2001), or indirectly, by attracting natural enemies of herbivores (Dicke and Sabelis, 1988; Turlings *et al.*, 1990). In addition, there is growing evidence that herbivore-induced VOCs are involved in chemical information transfer between plants (Arimura *et al.*, 2000; Baldwin *et al.*, 2002; Engelberth *et al.*, 2004).

Plants are known to emit more than 1000 different VOCs, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters, and carboxylic acids (Dudareva *et al.*, 2004; Niinemets *et al.*, 2004). Some VOCs are taxon specific, such as the glucosinolate breakdown products in *Brassica* species (Mattiacci *et al.*, 1995), whereas others appear to be common to many different plant families (Van Den Boom *et al.*, 2004). These common VOCs include the "green-leaf volatiles" (C6 aldehydes, alcohols, and derivatives), cyclic and acyclic terpenes, phenolic compounds, and nitrogenous compounds (Dicke, 1999b; Paré and Tumlinson, 1999). The induction and release of such compounds is dependent on the interaction of biotic factors, such as plant hormones (de Bruxelles and Roberts, 2001; Thaler *et al.*, 2002; Farmer *et al.*, 2003; Rojo *et al.*, 2003; Schmelz *et al.*, 2003; Ament *et al.*, 2004; van Poecke and Dicke, 2004), herbivore-derived elicitors (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Halitschke *et al.*, 2001; Spiteller and Boland, 2003; Merkx-Jacques and Bede, 2004), and associated microorganisms (Cardoza *et al.*, 2002), and abiotic factors, such as wounding (Schmelz *et al.*, 2001; Howe, 2004; Mithöfer *et al.*, 2005), O_3 and CO_2

concentration (Vuorinen *et al.*, 2004a,b), UV radiation (Johnson *et al.*, 1999), heavy metals (Mithöfer *et al.*, 2004), temperature, and light (Takabayashi *et al.*, 1994; Gouinguené and Turlings, 2002). In addition, there is great variability in the composition of volatile blends among different plant genotypes within a plant species (Gouinguené *et al.*, 2001; Degen *et al.*, 2004). It is unlikely that every VOC emitted by plants serves as an ecological or physiological signaling compound (Penuelas and Llusia, 2004), but in only a few systems behavioral active compounds of the total blend have been identified (Du *et al.*, 1998; Powell *et al.*, 1998; de Boer and Dicke, 2004; de Boer *et al.*, 2004).

In this study, we address the question whether in complex odor blends emitted by *Spodoptera littoralis*–infested maize and cowpea seedlings there are key VOCs that mediate the attraction of two parasitoid species, *Cotesia marginiventris* and *Microplitis rufiventris*. Both species have previously been shown to be highly attracted by herbivore-induced VOCs, which are the main cues used by these parasitoids to locate their host habitat (Turlings *et al.*, 1991a,b, 2004; Fritzsche Hoballah *et al.*, 2002; Gouinguené *et al.*, 2003). Still, the use of induced volatiles differs between the two species. While naive *C. marginiventris* preferred blends with high amounts of green-leaf volatiles over blends with high amounts of sesquiterpenes, *M. rufiventris* did not show such a preference (Hoballah and Turlings, 2005). Here, we study the role of herbivore-induced VOCs for the attraction of these parasitoids in more detail. One way of studying the importance of individual VOCs is to compare the attractiveness of volatile blends differing in only few known compounds. These blends can be obtained by using different chemical elicitors (Dicke *et al.*, 1999; Turlings *et al.*, 2000) or by silencing genes involved in indirect defenses (Degenhardt *et al.*, 2003; van Poecke and Dicke, 2003; Kessler *et al.*, 2004). Confirmation of the importance of the missing VOCs can then be obtained by adding back synthetic compounds to the incomplete blends (de Boer and Dicke, 2004).

Here, we introduce a novel approach to obtain volatile blends of only partially different composition. Volatile blends were passed over adsorbing filters, which resulted in the adsorption of some VOCs, while others broke through and were measured and tested for attraction to naive and experienced parasitoid females. The results show that *C. marginiventris* and *M. rufiventris* use different cues and that some commonly induced VOCs have little or no impact on attraction, whereas other, minor, compounds are essential and highly attractive.

Materials and methods

Plants and plant treatments

Maize (*Zea mays* var. Delprim) and cowpea (*Vigna unguiculata*, Haeflinger, Herzogenbuchsee, Switzerland) were sown in plastic pots (10 cm high, 4-cm diameter) with fertilized commercial soil (Balkonerde, Coop, Switzerland) and grown at 27 ± 2°C, 60% relative humidity, 16:8 h light:dark (16L:8D), and 50,000 lm/m^2. Maize plants used for the experiments were 10–12 days old and had three fully developed leaves. Cowpea plants were 14–16 days old and had the cotyledons and six small leaves.

The evening before the experiments, plants and pots were introduced into the odor source vessels of an olfactometer (described by Turlings *et al.*, 2004) and infested with 20 second-instar *S. littoralis* larvae by releasing them in the whorl of the youngest leaf. After infestation, plants were kept under laboratory conditions with supplemented light (26 ± 3°C, 40 ± 10% relative humidity, 16L:8D, and 10,000 lm/m^2) and were used for the experiments the day after, between 11:00 AM and 4:00 PM.

Insects and insect treatments

The caterpillar *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the solitary endoparasitoids *C. marginiventris* (Cresson) (Hymenoptera: Braconidae) and *M. rufiventris* (Kokujev) (Hymenoptera: Braconidae) were reared as described before (Turlings *et al.*, 2004). Adult parasitoids were kept in plastic cages at a sex ratio of approximately 1:2 (male:female) and were provided with moist cotton wool and honey as food source. The cages were kept in incubators (*C. maginiventris*: 25 ± 1°C; *M. rufiventris*: 23 ± 1°C; 16L:8D) and transferred to the laboratory 30 min before the experiments. We tested mated 2- to 4-day-old naive and experienced females. The latter were given experiences by allowing them to oviposit three to five times into second-instar *S. littoralis* larvae while simultaneously being exposed to the complete blend ("no filter", see subsequently). Experienced wasps were kept separately in small plastic boxes with moist cotton wool and honey and released in the olfactometer 1–3 h after their oviposition experience.

Olfactometer bioassays

To test the attractiveness of various herbivore-induced volatile blends to *C. maginiventris* and *M. rufiventris*, we used a four-arm olfactometer (Figure 1), which was modified after the six-arm olfactometer used in earlier studies (Turlings *et al.*, 2004). The olfactometer consisted of a central glass chamber [6-cm internal diameter (ID), 5-cm length] with four arms (15-mm ID, 5-cm length), each with a glass elbow (5-cm length) and an upward connection for an insect-trapping bulb (50 ml). Each glass elbow had a horizontal opening for a volatile collection trap (see subsequently) and was connected via a glass tube (4-mm ID, 8-cm length) to a glass vessel that contained the odor source. This connecting tube was either empty (controls) or contained an adsorbing material to filter out specific compounds from the blend emitted by the odor source. All parts were connected either via male/female ground glass connectors or via Teflon-coated GL-screw cap fittings.

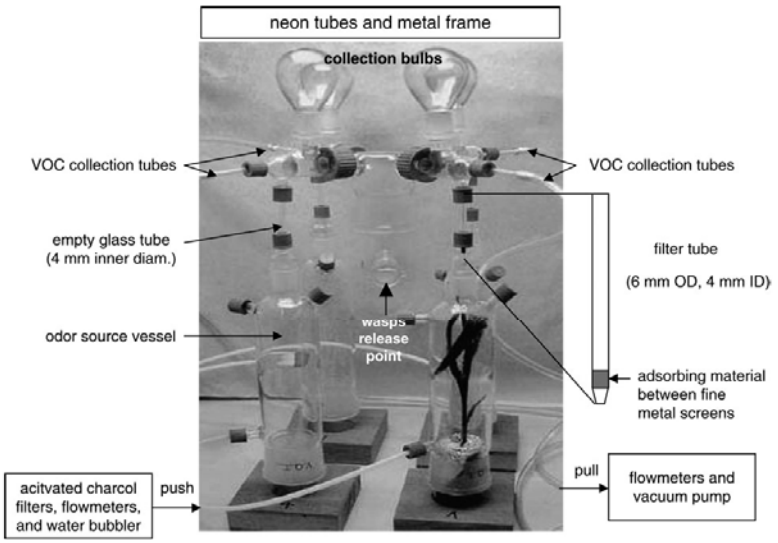

Figure 1 Picture and schematic representation of the four-arm olfactometer and the adsorbing filter tube.

Purified and humidified air entered each odor source vessel at 1.2 l/min (adjusted by a manifold with four flow meters; Analytical Research System, Gainesville, FL) via Teflon tubing and carried the VOCs through the connector tube to the elbows of the olfactometer. In these elbows, half of the air (0.6 l/min) was pulled out via the volatile collection traps (see subsequently) and the other half entered the central glass chamber.

Wasps were released in groups of six into the central glass chamber via a horizontally attached glass tube (6-cm ID, 10-cm length) with a 2.5-cm-ID opening. Wasps that entered an arm reached the elbow, where a stainless steel screen blocked their path. Eventually, they walked up in the direction of the light source above the olfactometer and into a trapping bulb, where they could easily be counted and removed. Ten neon tubes attached on a metal frame above the olfactometer provided approximately 7000 lm/m^2 at the height of the odor source vessels. To eliminate any visual distractions, a white cardboard cylinder was placed around the central chamber between the four odor source vessels (not shown in Figure 1). Wasps that did not enter a bulb or an elbow after 30 min were removed and considered having made "no choice." A total of four groups of six wasps were tested during a 2-h period, with naive and experienced groups released alternately. All experiments were run between 11:00 AM and 4:00 PM and repeated several times as indicated in Table 1.

Adsorbing filters and odor sources

Odour blends differing quantitatively and qualitatively in specific VOCs were obtained by passing air at 1.2 l/min containing the natural herbivore-induced blend (see Plants and Plant Treatments) over adsorbing filters of carbotrap-C (20–40 mesh, Supelco, Bellefonte, PA) or silica (63–200 mesh, 60 Å, Brunschwig, Basel, Switzerland). The adsorbing filters were positioned as indicated in Figure 1, and the resulting blends were tested in various experiments against positive controls, which consisted of the full odor blend of herbivore-infested seedling (no filter), and against negative controls, which consisted either of an empty vessel only (empty) or of an empty vessel with an adsorbing filter (e.g., empty and carbotrap filter) or with solvent on a filter paper (solvent) (Table 1). To obtain well-defined modified VOC blends, we selected different amounts of adsorbing materials, and we passed the whole blend for a certain prerun time (Table 1) over the filter before testing and sampling the blend for 2 h. Filters were prepared by filling various amounts (Table 1) of the adsorbents into the connection tube (8-cm length, 4-mm ID) sealed on both sides with a stainless steel screen mesh. Prior to each experiment, filters were rinsed with 3 ml of dichloromethane (Suprasolv, GC-grade, Merck, Dietikon, Switzerland) and baked for 4 h at 200°C. To standardize the adsorption of water, silica filters were rinsed with 100 µl Milli-Q water and dried in the humidified air stream of the olfactometer for 15 min before installing them into the olfactometer. Carbotrap-C filters were not rinsed with water because of the hydrophobic properties of this material. The silica extract consisted of VOCs that were extracted with 300 µl of dichloromethane from a 25-mg silica filter of a previous experiment. An aliquot of 100 µl of this extract was placed on a filter paper (1/2 disk, 50-mm diameter, Nr. LS 14, Schleicher and Schuell, Bottmingen, Switzerland) and

Table 1 Odour sources and experimental design

Experiment	Plant	Odor sources				Wasp	Replications of experiment
		Arm 1	Arm 2	Arm 3	Arm 4		
1	Maize	No filter (whole blend)	Carbotrap low (30 mg, prerun 3.5 h)	Carbotrap high (150 mg, prerun 0.5 h)	Empty	*C. marginiventris*	6
2	Maize	Carbotrap high (150 mg, prerun 0.5 h)	Empty and carbotrap filter (150 mg, prerun 15 min)	Empty	Empty	*C. marginiventris*	6
3	Maize	No filter (whole blend)	Silica low (12.5 mg, prerun 3.5 h)	Silica high (25 mg, prerun 0.5 h)	Empty	*C. marginiventris*	6
4	Maize	Silica high (25 mg, prerun 0.5 h)	Empty and silica filter (25 mg, prerun 15 min)	Empty	Empty	*C. marginiventris*	6
5	Maize	Silica high (25 mg, prerun 0.5 h)	Empty and silica filter (25 mg, prerun 15 min)	Empty	Empty	*M. rufiventris*	6
6	Maize	Silica extract (100 µl, prerun 2 h)	Solvent (100 µl, prerun 2 h)	Empty	Empty	*C. marginiventris*	4
7	Maize	Restored blend (silica high and extract)	Silica high and solvent	Empty	Empty	*C. marginiventris*	6
8	Cowpea	Silica high (25 mg, prerun 0.5 h)	Empty and silica filter (25 mg, prerun 15 min)	Empty	Empty	*C. marginiventris*	6

Further details on odor sources, number, and treatment of wasps are described in the text. Amounts of adsorbing materials or extracts and prerun time (time that the VOCs were passed over the filter before sampling and testing the blends) are indicated in parentheses.

introduced into an empty glass tube (4-mm ID, 8-cm length) that connected the odor vessel with the central chamber (Figure 1). The "restored blend" was obtained with a combination of the "silica high" filter and a silica extract, which was placed on filter paper after the silica filter. The positions of the odor sources were randomly chosen for different replications of the experiments.

Collection and analyses of VOCs

VOCs of each odor source were collected on a Super-Q trap (25 mg, 80–100 mesh, Alltech Associates, Inc., Deerfield, IL, described by Heath and Manukian, 1992) that was attached horizontally to the elbow of an olfactometer arm (Figure 1) and connected via Tygon tubing to a flow meter (Analytical Research System) and a vacuum pump. Air carrying the VOCs was pulled through each trap during the 2-h bioassay period at a rate of 0.6 l/min. Afterward, the traps were extracted with 150 µl dichloromethane, and 200 ng each of *n*-octane and *n*-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µl dichloromethane was added to the samples as internal standards. Traps were washed with 3 ml of dichloromethane before reusing them for a next collection. VOCs adsorbed on the silica filters were extracted with 150 µl dichloromethane and VOCs on the carbotrap filters with 300 µl dichloromethane for subsequent analyses. Internal standards were added as described earlier. All solutions were stored at −76°C until analyses or bioassays.

VOCs were analyzed with a Hewlett Packard HP 6890 series gas chromatograph equipped with an automated column injection system (HP G1513 A) and a flame ionization detector. A 3-µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25-mm ID, 0.25-µm film thickness, Alltech Associates, Inc.). Helium at constant pressure (18.55 psi) was used as carrier gas flow. Following injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min followed by a postrun of 5 min at 250°C. The detected VOCs were quantified based on a comparison of their peak areas with those of the internal standards (*n*-octane for compounds 1–14, *n*-nonyl acetate for compounds 15–27) and identified by comparison of retention times with those from previous analyses (Turlings *et al.*, 1998; Gouinguené *et al.*, 2001; Fritzsche Hoballah *et al.*, 2002). To confirm these identities, at least one sample per odor source was analyzed using a gas chromatograph (Agilent 6890 Series GC system G1530A), with the same kind of apolar column (HP-1) and an identical temperature program, coupled to a mass spectrometer operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33–280 amu). Mass spectra were compared with those of the NIST 02 library, and where necessary, spectra and retention times were compared with those of authentic standards. Compounds that were not identified by comparing retention times and spectra with those of pure standards are indicated in Table 2 and are labeled with a superscript N in the text, and their identification should be considered tentatively.

Statistical analyses

The functional relationship between parasitoids' behavioral responses and the different odor sources offered in the four-arm olfactometer was examined with a log linear model (a generalized linear model, GLM). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of wasps within the olfactometer (Turlings *et al.*, 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (version 1.9.1), and its adequacy was assessed through likelihood ratio statistics and examination of residuals. We tested treatment effects (=odor sources) for naive and experienced wasps individually. In addition, we tested if there was a significant effect of the experience and an interaction between treatment and experience.

The amounts of VOCs were analyzed using analyses of variance (ANOVAs) and *t*-tests. Amounts of VOCs that were not normally distributed were $\log(x + 1)$ transformed prior to analysis. Differences between the treatments were analyzed using the Tukey's test. All analyses were run on SigmaStat (version 2.0).

Results

Modification of induced maize blends over carbotrap-C filters

We detected 27 VOCs in quantifiable amounts in the unfiltered induced maize blend (no filter) (Figure 2A, Table 2). Terpenes (compounds 5, 8–10, 16–27) were the most abundant VOCs and made up more than 80% of the whole blend. Within the terpenes, the sesquiterpenes (*E*)-α-bergamotene and (*E*)-β-farnesene were the most dominant ones and made up more than 65% of all quantified terpenes. Furthermore, green-leaf VOCs (compounds 1–4, 6, 7), shikimic acid–derived compounds (11–13, 15), and an unknown compound (14) were detected in quantifiable amounts. (*Z*)-Jasmone, two oximes, and some other compounds were detected by gas chromatography–mass spectrometry in low quantities after concentrating the extract over nitrogen (data not shown). The blend "carbotrap low" (Figure 2B, Table 2) was lacking two unknown minor sesquiterpenes (21, 23) and (*E*)-nerolidol (26) and contained only trace amounts of the minor terpenes (compounds 17, 27) and of the unknown compound (14). Additionally, there was a significant reduction in the amounts of the two major sesquiterpenes, (*E*)-α-bergamotene (*t*-test, $t_{16} = 2.64$, $P = 0.018$) and (*E*)-β-farnesene ($t_{16} = 4.06$, $P < 0.001$), of the sesquiterpenes 18 ($t_{16} = 2.82$, $P = 0.012$), 24 ($t_{16} = 5.31$, $P < 0.001$), and 25 ($t_{16} = 4.69$, $P < 0.001$), and of geranyl acetate ($t_{16} = 4.66$, $P < 0.001$). The blend "carbotrap high" (Figure 2C, Table 2) was lacking all sesquiterpenes (compounds 16–26) and the homoterpene (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) and contained only trace amounts of (*Z*)-β-ocimene, benzyl acetate, and phenethyl acetate. One-way ANOVA indicated significant differences in the amounts of β-myrcene ($F_{2,27} = 9.19$, $P < 0.001$), linalool ($F_{2,27} = 15.32$, $P < 0.001$), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) ($F_{2,27} = 58.71$, $P < 0.001$), and indole ($F_{2,27} = 7.13$, $P = 0.003$). No VOCs in quantifiable amounts were detected in the clean air or in clean air passed over filter tubes. Therefore, these blends are not shown in Figure 2.

In a first experiment, wasps had the choice between the whole blend, the modified blends, and clean air (experiment 1, Table 1). GLM indicates a significant difference between the four treatments for naive wasps ($F_{3,33} = 13.20$, $P < 0.001$) as well as for experienced wasps ($F_{3,33} = 25.67$, $P < 0.001$). Both groups of wasps were strongest attracted to the unfiltered blend no filter (Figure 3A). Naive wasps were also strongly attracted toward carbotrap low, and this attraction was not significantly different from attraction to the unfiltered blend ($P = 0.35$). In a following experiment, carbotrap high was tested alone against three arms with clean air (experiment 2, Table 1), and both naive and experienced wasps were still clearly attracted to this modified maize blend (naive: $F_{2,34} = 16.59$, $P < 0.001$; experienced: $F_{2,34} = 21.43$, $P < 0.001$) (Figure 3B). However, the overall responsiveness (=wasps that entered an arm) was relatively low (naive: 54%, experienced: 54%).

Modification of induced maize blends over silica filters

The VOCs detected in the blend no filter (Figure 2A, Table 2) were similar to those from the experiment with carbotrap filters described previously. The blend "silica low" (Figure 2D, Table 2) did not contain geranyl acetate, (*E*)-nerolidol, and a minor unknown compound (14). The blend silica high (Figure 2E, Table 2) was lacking the same compounds as well as methyl anthranilate and contained only trace amounts of linalool and phenethyl acetate. There was also a significant difference in the amounts of β-myrcene (one-way ANOVA, $F_{2,47} = 4.00$, $P = 0.025$) and TMTT ($F_{2,47} = 25.87$, $P < 0.001$). No VOCs were detected in quantifiable amounts in the clean air.

As in the first experiment with carbotrap filters, wasps had the choice between the whole blend, the modified blends, and clean air only (experiment 3, Table 1). The GLM revealed a significant difference between the four treatments for the choice of naive wasps ($F_{3,33} = 13.20$, $P < 0.001$) as well as for those of experienced wasps ($F_{3,33} = 25.67$, $P < 0.001$), and both groups of wasps were strongest attracted to the whole blend (Figure 4A). In a following experiment (experiment 4, Table 1), with only the silica high blend versus three arms with clean air, neither naive nor experienced wasps were attracted to the modified maize blend, and there was no significant difference between the treatments (Figure 4B). Less than 35% of all tested wasps entered an arm, confirming the absence of attraction toward this blend.

Unlike *C. marginiventris*, naive and experienced females of *M. rufiventris* were attracted to the blend silica high

Table 2 Overview of VOCs and quantities (ng ± SE) collected during the bioassays

Nr.	Compounds	Experiments 1 and 2			Experiments 3, 4, and 5			Experiments 6 and 7			Experiment 8
		No filter (12)	Carbotrap low (6)	Carbotrap high (12)	No filter (12)	Silica low (12)	Silica high (24)	Silica extract (12)	Restored blend	Silica high solvent (6)	Silica high (6)
1	(*Z*)-3-Hexenal	134.9 ± 18.9	160.9 ± 41.8	110.4 ± 11.3	134.9 ± 18.9	91.8 ± 8.6	123.8 ± 14.2	8.2 ± 2.3	141.4 ± 24.6	131.0 ± 20.0	39.0 ± 8.2*
2	(*E*)-2-Hexenal	22.7 ± 4.5	18.8 ± 3.4	25.3 ± 3.5	22.7 ± 4.5	21.8 ± 1.9	27.6 ± 3.6	tr	32.4 ± 9.0	21.2 ± 3.4	10.8 ± 3.2
3	(*Z*)-3-Hexenol	25.6 ± 3.6	29.4 ± 5.9	19.7 ± 1.6	25.6 ± 3.6	19.7 ± 1.9	21.1 ± 2.5	4.4 ± 0.6	23.8 ± 6.6	20.4 ± 4.0	18.8 ± 5.8
4	(*Z*)-2-Penten-1-ol acetate[N]	3.8 ± 0.5	5.5 ± 0.9	4.8 ± 0.5	3.8 ± 0.5	3.5 ± 0.2	4.4 ± 0.4	tr	3.8 ± 0.4	4.6 ± 0.6	nd
5	β-Myrcene	12.1 ± 1.2 (a)	19.4 ± 2.6 (b)	9.2 ± 1.2 (a)	12.1 ± 1.2 (a)	16.5 ± 0.7 (ab)	18.5 ± 1.6 (b)	tr	20.8 ± 1.4	21.8 ± 3.0	tr
6	(*Z*)-3-Hexenyl acetate	96.9 ± 13.1	144.5 ± 27.8	102.1 ± 16.6	96.9 ± 13.1	86.5 ± 6.7	84.0 ± 11.4	51.0 ± 7.5	77.2 ± 11.8	85.6 ± 17.0	29.0 ± 6.0*
7	(*E*)-2-Hexenyl acetate	5.5 ± 1.1	7.1 ± 1.5	11.6 ± 2.7	5.5 ± 1.1	5.5 ± 0.6	7.7 ± 1.3	4.1 ± 1.4	6.8 ± 2.6	6.8 ± 2.4	tr
8	(*Z*)-β-Ocimene	3.9 ± 0.9	5.3 ± 1.0	tr	3.9 ± 0.9	5.3 ± 0.6	5.2 ± 0.6	nc	5.4 ± 1.2	5.4 ± 1.2	tr
9	Linalool	207.7 ± 31.4 (a)	379.7 ± 67.4 (b)	86.0 ± 17.5 (c)	207.7 ± 31.4	332.9 ± 10.1	tr	517.5 ± 65.7	tr	tr	nd
10	DMNT	112.7 ± 22.3 (a)	194.7 ± 51.8 (a)	3.2 ± 1.8 (b)	112.7 ± 22.3	163.9 ± 14.3	196.8 ± 25.9	2.9 ± 0.5	166.8 ± 26.4	187.2 ± 36.6	71.6 ± 20.2*
11	Benzyl acetate	3.3 ± 0.9	5.3 ± 1.8	tr	3.3 ± 0.9	5.1 ± 0.8	4.2 ± 1.0	8.7 ± 1.1	5.6 ± 1.4	4.0 ± 1.8	nd
12	Phenethyl acetate	26.8 ± 4.9	41.7 ± 8.4	tr	26.8 ± 4.9	15.4 ± 1.6	tr	74.9 ± 11.0	tr	tr	nd
13	Indole	125.9 ± 37.0 (a)	269.2 ± 66.7 (a)	41.1 ± 24.7 (b)	125.9 ± 37.0	119.4 ± 11.4	278.7 ± 41.7	104.1 ± 23.3	296.0 ± 78.8	290.4 ± 92.2	37.2 ± 16*
14	Unknown	3.2 ± 1.8	tr	nd	3.2 ± 1.8	nd	nd	5.9 ± 2.0	nd	nd	nd
15	Methyl anthranilate	12.2 ± 5.0	13.8 ± 4.4	nd	12.2 ± 5.0	7.5 ± 1.0	nd	37.6 ± 11.5	nd	nd	nd
16	Geranyl acetate	76.4 ± 10.5 (a)	21.5 ± 3.1 (b)	nd	76.4 ± 10.5	nd	nd	262.9 ± 42.9	nd	nd	nd
17	Unknown sesquiterpenoid	6.1 ± 0.9	tr	nd	6.1 ± 0.9	7.3 ± 0.4	7.1 ± 0.9	nc	6.2 ± 0.4	7.0 ± 1.4	nd
18	Unknown sesquiterpenoid	14.3 ± 2.2 (a)	5.2 ± 0.6 (b)	nd	14.3 ± 2.2	16.2 ± 1.0	15.8 ± 2.0	2.2 ± 0.4	17.0 ± 3.0	15.4 ± 4.0	nd
19	(*E*)-β-Caryophyllene	143.1 ± 18.8	163.7 ± 16.9	nd	143.1 ± 18.8	174.0 ± 9.9	189.4 ± 27.2	19.0 ± 3.8	142.2 ± 22.4	144.0 ± 38.0	6.9 ± 3.0*
20	(*E*)-α-Bergamotene	501.9 ± 70.0 (a)	234.4 ± 19.1 (b)	nd	501.9 ± 70.0	548.5 ± 33.5	571.1 ± 78.1	59.3 ± 11.9	453.6 ± 44.8	537.8 ± 101.0	tr
21	Unknown sesquiterpenoid	13.6 ± 2.2	nc	nd	13.6 ± 2.2	17.5 ± 1.1	12.0 ± 2.2	3.6 ± 0.8	13.0 ± 1.4	13.0 ± 3.2	nd
22	(*E*)-β-Farnesene	998.4 ± 141.2 (a)	172.7 ± 26.9 (b)	nd	998.4 ± 141.2	1099.5 ± 69.1	943.1 ± 139.7	505.3 ± 110.7	778.0 ± 77.0	874.0 ± 184.8	15.8 ± 5.6*
23	Unknown sesquiterpenoid	10.0 ± 1.4	nc	nd	10.0 ± 1.4	12.8 ± 0.9	10.2 ± 1.7	3.6 ± 1.4	7.8 ± 0.8	8.8 ± 2.0	nd
24	Unknown sesquiterpenoid	21.7 ± 2.5 (a)	2.4 ± 1.1 (b)	nd	21.7 ± 2.5	26.2 ± 1.5	22.1 ± 3.0	9.0 ± 1.8	19.4 ± 2.2	20.8 ± 4.4	nd
25	β-Sesquiphellandrene[N]	59.8 ± 7.4 (a)	9.9 ± 1.3 (b)	nd	59.8 ± 7.4	66.4 ± 4.2	56.1 ± 8.4	25.8 ± 5.6	46.4 ± 4.8	50.6 ± 11.4	nd
26	(*E*)-Nerolidol	6.0 ± 1.5	nc	nd	6.0 ± 1.5	nd	nd	9.2 ± 2.6	nd	nd	nd
27	TMTT	20.6 ± 2.1	tr	nd	20.6 ± 2.1 (a)	16.8 ± 1.1 (a)	5.1 ± 1.2 (b)	33.0 ± 5.2	6.2 ± 1.2	4.6 ± 1.0	nd

Superscript N = compound identified by comparison with the NIST 02 library only; tr = compound found in trace amounts only (average peak area below 1% of internal standard) or in less than half of the samples; nd = compound not detected during the 2-h sampling period. Same treatments were pooled from different experiments, and the number of replicates is given in parentheses. Letters in parentheses indicate significant differences between the treatments within one experiment (see Materials and Methods for statistical procedures). The amounts of the silica extract were not taken into statistical consideration. The amounts of the cowpea experiment were compared to the amounts of the same filter treatment in the silica experiments (maize), and significant differences are indicated by asterisks. Only cowpea VOCs identified also in maize blends are indicated in the table; additional VOCs and VOCs found in trace amounts are reported in the text.

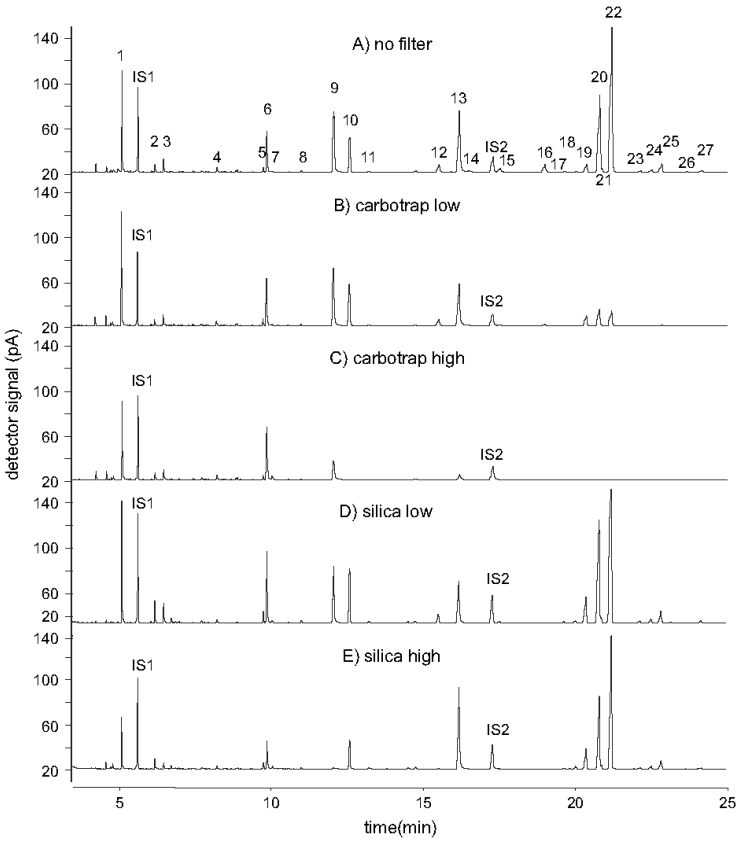

Figure 2 Chromatograms of *Spodoptera*-induced maize VOCs: **(A)** whole blend without a filter, **(B)** after filtration over a 30-mg carbotrap-C filter, **(C)** after filtration over a 150-mg carbotrap-C filter, **(D)** after filtration over a 12.5-mg silica, and **(E)** after filtration over a 25-mg silica filter. Main peaks are labeled in graph A. A complete list and mean quantities of individual compounds are given in Table 2. IS1 and IS2 correspond to internal standards.

(Figure 5; experiment 5, Table 1), resulting in a significant difference between the treatments (naive: $F_{2,45} = 71.21$, $P < 0.001$; experienced: $F_{2,45} = 57.76$, $P < 0.001$). In addition, there was a significant treatment × experience effect ($F_{2,90} = 4.20$, $P = 0.018$).

Attractiveness of VOCs adsorbed by silica

Earlier, we found that *C. marginiventris* females are not attracted to the modified induced maize blend silica high (Figure 4B), which was a surprising result considering that many volatiles readily break through the filter. We extracted the VOCs that were adsorbed on the silica filters during these experiments and found that the extracts indeed contained the compounds that were missing in the breakthrough as well as the compounds that were found in reduced amounts in the breakthrough (Table 2, silica extract). In experiment 6 (Table 1), we tested this extract on filter paper in the olfactometer and found that it was extremely attractive to naive *C. marginiventris* females (Figure 6A, GLM; $F_{2,46} = 65.30$, $P < 0.001$). We also tested if the missing attraction of the blend silica high could be restored by adding silica extract to this blend (experiment 7, Table 1). Wasps were highly attracted to this restored blend (Figure 6B), and the difference between the treatments was significant ($F_{2,70} = 33.36$, $P < 0.001$). Analyses of the VOCs collected during this experiment indicated that the restored blend was similar to silica high (Table 2). Indeed, no significant differences were found in the amounts of individual VOCs between the two blends, indicating that most VOCs of the silica extract evaporated fast from the filter paper.

Modification of induced cowpea blends over silica filters

The modified cowpea blend was qualitatively and quantitatively very different from the modified maize blend (Table 2).

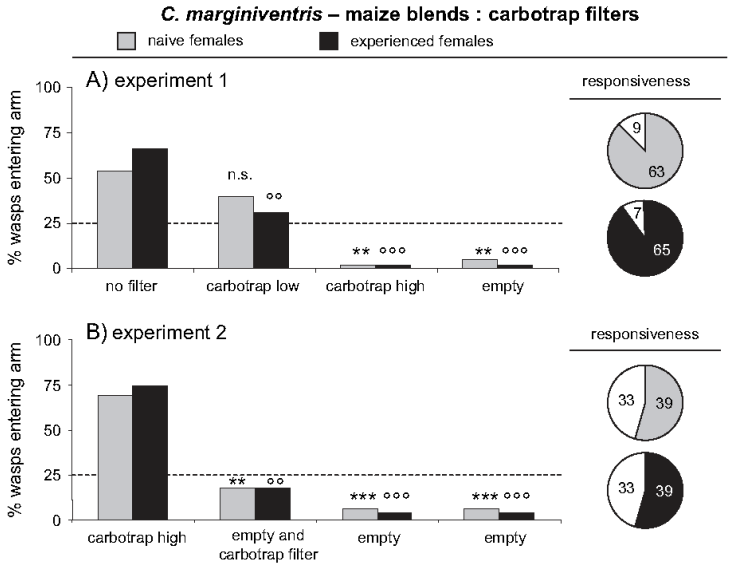

Figure 3 Response of naive and experienced *Cotesia marginiventris* females to whole and modified *Spodoptera*-induced maize blends. Carbotrap-C filters were used to modify the blends. **(A)** Wasps had the choice between three plant-derived blends and clean air only (=empty). **(B)** Wasps had the choice among one plant-derived blend, clean air passed over an empty filter, and clean air only. Composition of the plant-derived blends is given in Figure 2 and Table 2. The responsiveness (proportion of wasps choosing an arm) is indicated by the pie charts with the white part showing the total number of wasps that did not enter any olfactometer arm. Data were analyzed using a GLM, and symbols indicate significant differences between the odor sources within one treatment of wasps (* for naive and ° for experienced wasps: 1 symbol $P < 0.05$, 2 symbols $P < 0.01$, 3 symbols $P < 0.001$) based on comparisons to a reference odor source (=odor source with highest attraction).

It contained significantly less (*Z*)-3-hexenal (*t*-test: $t_{10} = 2.63$, $P = 0.026$), (*Z*)-3-hexen-1-ol acetate ($t_{10} = 2.33$, $P = 0.042$), DMNT ($t_{10} = 3.18$, $P = 0.010$), indole ($t_{10} = 4.14$, $P = 0.002$), (*E*)-β-caryophyllene ($t_7 = 6.43$, $P < 0.001$), and (*E*)-β-farnesene ($t_7 = 12.37$, $P = 0.001$) and only trace amounts of β-myrcene, (*E*)-2-hexenyl acetate, (*Z*)-β-ocimene, and (*E*)-α-bergamotene. In addition to the VOCs listed in Table 2, we also detected the sesquiterpene α-cubebene[N] and trace amounts of (*E*)-2-hexen-1-ol, methyl salicylate, eucalyptol[N], and some unknown compounds. Analyses of the VOCs adsorbed on the filter (not shown in Table 2) showed that the silica filter adsorbed mainly (*Z*)-3-hexen-1-ol acetate, indole, and (*E*)-nerolidol. In addition, we detected trace amounts of (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol benzaoate[N], methyl anthranilate, methyl salicylate, (*Z*)-jasmone, eucalyptol[N], and (*E*)-β-farnesene in the filter extract.

Unlike the maize blend, *C. marginiventris* females were readily attracted to the modified cowpea blend by passing it over the silica filter (Figure 7; experiment 8, Table 1) (GLM: $F_{2,70} = 41.64$, $P < 0.001$).

Discussion

The complexity and variability of VOC blends emitted by herbivore-infested plants have proven to greatly complicate the identification of the principal compounds mediating interactions between the emitting plants and associated organisms. Here, we introduce a novel approach to study the attractiveness of herbivore-induced plant VOCs to parasitoids. Typical blends of VOCs released by herbivore-infested plants were altered by filtration over adsorbing filters that were installed in-line between the odor source vessels and the arms of a four-arm olfactometer. This resulted in the adsorption of several VOCs, while others broke through and were tested simultaneously for chemical identity and for attractiveness to the wasps.

Attractiveness of herbivore-induced VOCs to parasitoids

Our results show that a partial reduction of the sesquiterpenes of *Spodoptera*-induced maize blends did not have a significant effect on the attraction of naive *C. marginiventris* females, while experienced females preferred the unfiltered blend with higher amounts of sesquiterpenes (Figures 2 and 3, Table 2). These findings are consistent with earlier studies (Turlings and Fritzsche, 1999; Hoballah and Turlings, 2005) in which, after oviposition experiences in the presence of *Spodoptera*-induced maize VOCs, the wasps were highly attracted to blends that contained sesquiterpenes. During contact with hosts, many parasitoids are known to associate the perceived odor with the presence of hosts and subsequently exhibit an attraction to the experienced

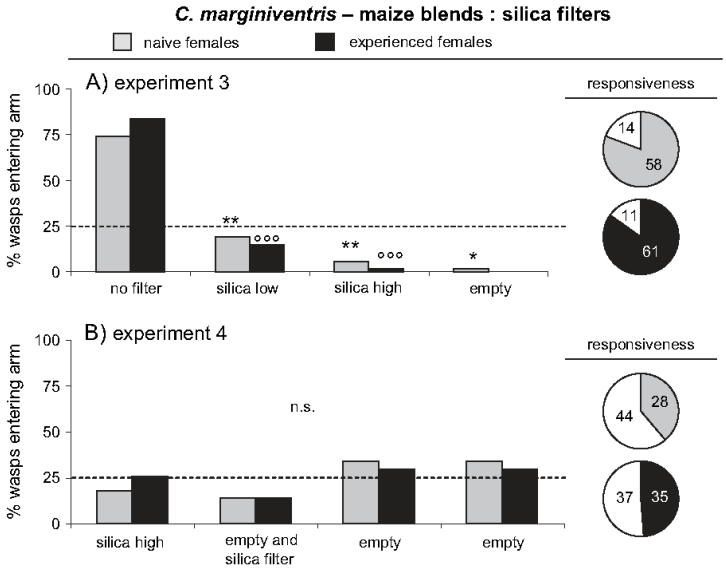

Figure 4 Response of naive and experienced *Cotesia marginiventris* females to whole and *Spodoptera*-induced maize blends. Silica filters were used to modify the blends. **(A)** Wasps had the choice between three plant-derived blends and clean air only (=empty). **(B)** Wasps had the choice among one plant-derived blend, clean air passed over an empty filter, and clean air only. Composition of the plant-derived blends is given in Figure 2 and Table 2. See the caption of Figure 3 for further explanations.

odor (Turlings *et al.*, 1993b; Vet *et al.*, 1995). Studies on associative learning by the parasitoid *M. croceipes* (Meiners *et al.*, 2003) and by honeybees (Laloi *et al.*, 2000) have shown that, after conditioning to a complex mixture, these insects established a hierarchy among various components, with some of them accounting for a major part of the behavioral activity evoked by the mixture. In our experiments, it remains to be determined whether the stronger attraction toward the blends with high amounts of sesquiterpenes is due to an association of these compounds during oviposition or due to increased attraction to compounds correlated with the sesquiterpenes. Interestingly, in the current study, both naive and experienced females were still attracted to a blend that did not contain any detectable amounts of sesquiterpenes and only 20% of the total quantified VOCs compared to the no filter blend. These results imply that the sesquiterpenes are not essential for the attraction of *C. marginiventris* females.

In contrast, a reduction of a few rather polar compounds strongly affected the attraction of this parasitoid species (Figures 2 and 4, Table 2). The blend silica high that still contained more than 80% of all VOCs and about 70% of the total quantity detected in the unfiltered blend had completely lost its attractiveness to naive and experienced wasps. This suggests that some compounds that are essential for the attraction of the wasps were filtered out. Support for this notion comes from the experiment that tested the attractiveness of the VOCs that were filtered out by the silica filter. Dichloromethane extracts of these compounds on filter paper were highly attractive to the wasps, and adding the extract to the unattractive silica high blend completely restored its attractiveness to naive females (Figure 6). Barely detectable amounts of VOCs were collected from the headspace of the filter paper (Table 2), suggesting that the implicated compounds are behaviorally active at very low doses. Indeed, arthropod chemoreceptors are much more sensitive than the detectors of analytical instruments (Dicke, 1999a; Rains *et al.*, 2004), and responses can be triggered by fewer than six molecules of a specific VOC (Angioy *et al.*, 2003). Further studies will attempt to identify which compounds in the silica extract attract the wasps at such low doses.

Unlike *C. marginiventris*, *M. rufiventris* females were readily attracted to the blend silica high (Figure 5). Hence, different parasitoid species exploit different VOCs to locate their hosts. Although *M. rufiventris* is less of a generalist than *C. marginiventris*, the biology and host range of these wasps imply that there is an overlap in the potential plant cues that they could use (Hegazi and El-Minshawy, 1979; Maes, 1989). The difference between the two species is consistent with earlier studies (Hoballah and Turlings, 2005), showing that *M. rufiventris* responds differently to induced maize VOCs than *C. marginiventris*. Differences in the use of plant cues to locate the hosts have also been found for other generalist and specialist parasitoid species (Röse *et al.*, 1998; De Moraes and Lewis, 1999) and even for closely related species (Geervliet *et al.*, 1998; Smid *et al.*, 2002).

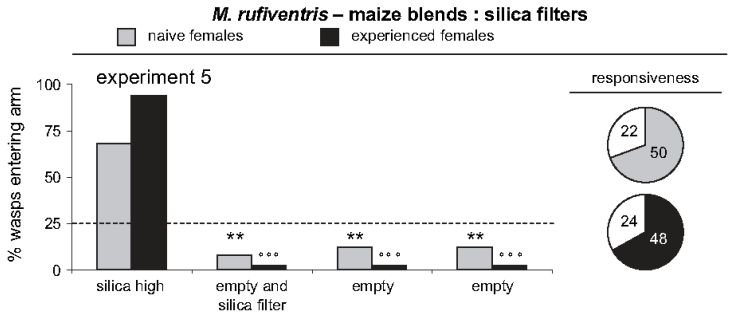

Figure 5 Response of naive and experienced *Microplitis rufiventris* females to a modified *Spodoptera*-induced maize blend. See the caption of Figure 3 for further explanations.

Interestingly, filtration of an induced cowpea blend over silica resulted in a blend that contained only 15% of the total amount of VOCs compared to the similarly modified maize blend, but this blend was still very attractive to naive *C. marginiventris* females (Figure 7). This indicates that cowpea contains different or larger amounts of highly attractive compounds, which supports the conclusion of an earlier study comparing the attractiveness of nonmodified cowpea and maize blends (Fritzsche Hoballah *et al.*, 2002). Indeed, the modified cowpea blend contained some VOCs that were not detected in the maize blend. Specifically, the behavioral importance of the trace amounts of methyl salicylate found in the cowpea blend should be further investigated. Gas chromatography electroantennogram detector analyses using *C. marginiventris* females showed that this compound was electrophysiologically active at very low dosages (Gouinguené and Turlings, 2005). Furthermore, methyl salicylate has been shown to be attractive to several carnivorous arthropods in the laboratory (Dicke *et al.*, 1990; Pickett *et al.*, 1999; de Boer and Dicke, 2004), as well as to parasitic wasps in the field (James and Price, 2004).

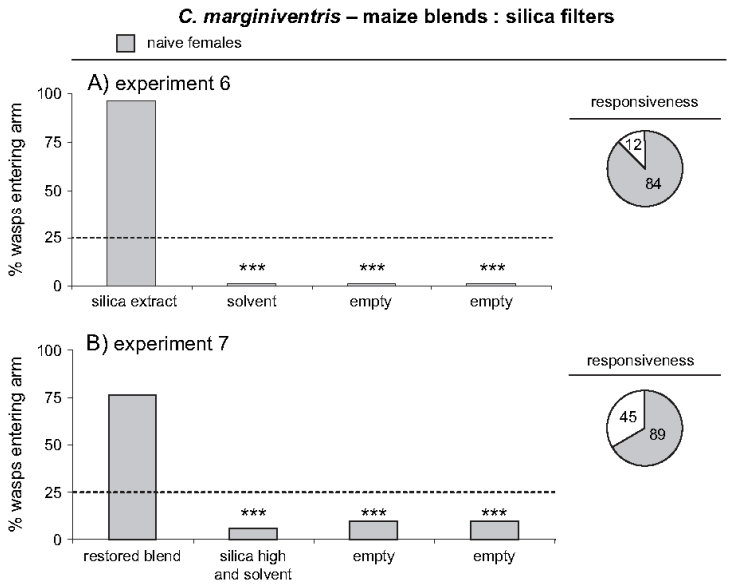

Figure 6 **(A)** Response of naive *Cotesia marginiventris* females to an extract of *Spodoptera*-induced maize VOCs adsorbed by a silica filter in a prior experiment. **(B)** Response of naive *C. marginiventris* females to modified *Spodoptera*-induced maize blends. Silica filters were used to modify two maize blends, and to one such blend an extract of silica-adsorbed volatiles was added on filter paper. Compositions of the blends are given in Table 2. See the caption of Figure 3 for further explanations.

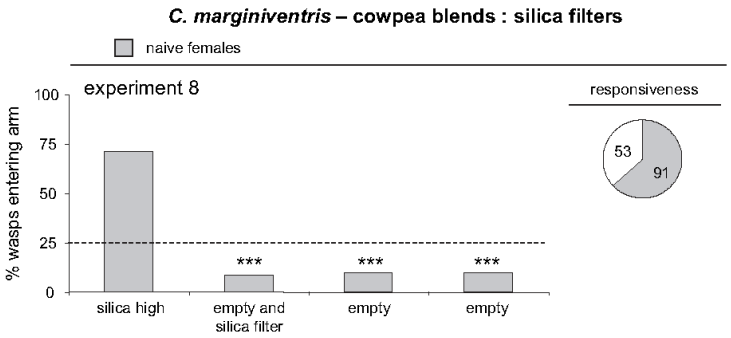

Figure 7 Response of naive *Cotesia marginiventris* females to a modified *Spodoptera*-induced cowpea blend. The composition of the cowpea blend is given in Table 2. See the caption of Figure 3 for further explanations.

Advantages and disadvantages of *in situ* modification of VOC blends

The differential attraction of the two parasitoid species tested in our study as well as the differential response of naive and experienced wasps and the differential attractiveness of VOCs from two plant species illustrate the complexity of the exploitation of plant-derived VOCs by parasitoids for host location. The approach we used here takes this complexity into account. It is a top-down approach starting with the whole herbivore-induced VOC blend and reducing its complexity by selectively adsorbing some compounds. Simultaneous testing and collecting of VOCs allow a direct linking of the VOC profile to the wasp behavior. We used two different adsorbing materials, carbotrap-C and silica. Carbotrap-C is a graphitized carbon that is usually used for adsorptive enrichment and thermal desorption of VOCs in the sampling range of C12 to C20 (Dettmer and Engewald, 2002), whereas silica is mainly used to adsorb very polar compounds (Harper, 2000). The breakthrough of VOCs from an adsorbing bed depends on many factors, including vapor concentration, air flow and volume, bed geometry, flow rates, and temperature (Harper, 2000; Dettmer and Engewald, 2002). We ran our experiments at room temperature for a relatively short bioassay period (2 h), and we adjusted the flow rates, amount of adsorbent, and prerun times to obtain VOC blends with well-defined quantitative and qualitative differences from a natural blend. In only two of 12 experiments, we found a significant "release time × treatment" effect (statistical test not shown), suggesting that the blends tested in this study remained more or less equally attractive over the 2-h bioassay period. Furthermore, this *in situ* modification of plant-emitted VOC blends has little impact on the interaction between the plant and the herbivore, and it avoids pleiotropic effects, which might occur in studies using genetically modified organisms (van Poecke and Dicke, 2003).

Other studies have used bottom-up approaches by, for example, identifying VOC profiles and testing individual or blended synthetic compounds (Dicke *et al.*, 1990; Whitman and Eller, 1990; Turlings *et al.*, 1991b). Such studies face the problem that plants emit numerous different compounds (Dudareva *et al.*, 2004; Niinemets *et al.*, 2004) with various isomeric forms. Each of these compounds could be of key importance, but it is unfeasible to study them all. Many of the minor compounds will not have been identified, and not all are readily available for individual testing. In addition, insect responses to different VOCs in a blend are often of a nonadditive nature (Visser and de Jong, 1988). For example, neither nonanal nor gernaylacetone alone attracts females of *Apanteles carpatus*, a parasitoid of the cloth moth *Tinea pennionella*, but a one-to-one blend of both compounds is as attractive as an extract of all volatiles from moth-infested beaver pelt (Takacs *et al.*, 1997). Synergistic effects have also been found in field experiments (Hammack, 2001), and the attraction of insects to VOCs can be influenced by background odors as well (Reddy *et al.*, 2002; Dicke *et al.*, 2003a; Mumm and Hilker, 2005). Moreover, compounds that normally attract insects can be repellent or even toxic at elevated concentrations (Read *et al.*, 1970). Releasing different fractions of VOCs from filter papers (Udayagiri and Jones, 1992; Turlings and Fritzsche, 1999) allows virtually no control of release rates and might lead to ratios of VOCs that are far different from natural. Relative ratios are important in the attraction of many insects, which is particularly evident from studies on pheromones, but may also be important for specific recognition of herbivore-induced VOCs (Turlings *et al.*, 1993a; De Moraes *et al.*, 1998; Bruce *et al.*, 2005). The approach we suggest in this study significantly altered the odors, but the compounds that broke through the filter had similar concentrations and ratios as in the natural blend (Table 2).

On the other hand, this approach is faced with the problem that VOCs on certain types of adsorbent material may create artifacts by causing reactions with reactive atmospheric species (Hoffmann, 1995; Kleno *et al.*, 2002), and compounds might be rearranged or decomposed (Rothweiler *et al.*, 1991). Although we did not detect additional peaks in the modified blends, we cannot exclude the possibility that

some minor artifacts were produced while passing VOCs over the adsorbents. We specifically tested for this possibility by passing clean air over adsorbing filters (negative control) and by adding back the adsorbed fraction to a nonattractive blend, which restored the attraction (positive control). Furthermore, the specificity and efficacy of the technique could be improved by using adsorbent materials coated with a specific reagent. Such microchemical reactions have played crucial roles in the determination of the structure of insect pheromones (Attygalle and Morgan, 1988; Jones and Oldham, 1999) and could easily be adapted to study the importance of plant-derived VOCs *in situ*. We are currently testing silica filters coated with 2, 4-dinitrophenylhydrazine (Supelco), which selectively adsorb compounds with carbonyl groups.

Conclusions

The *in situ* modification of herbivore-induced VOC blends appears to be an effective new approach to study the importance of specific VOCs involved in tritrophic interactions. Considering the large number of different adsorbing materials that are commercially available, this approach could easily be adapted to study the role of VOC blends in other biological systems, including VOCs involved in attracting pollinators or herbivores. To our knowledge, only one study has used a similar approach to assess the attraction of insects toward different fractions of plant-derived VOCs (Natale *et al.*, 2003), but the breakthrough VOCs were not recollected and identified in that study. Information on the relative attractiveness of individual VOCs within complex blends is highly desired, not only as it may aid in the development of crop varieties with odor emissions that facilitate biological control of pests and diseases (Degenhardt *et al.*, 2003; Wei *et al.*, 2004) but also for a comprehensive understanding of insect olfaction.

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Chapter III

The role of indole and other shikimic acid derived maize volatiles in the attraction of two parasitic wasps

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Abstract – After herbivore attack, plants are known to release a plethora of different volatile organic compounds (VOCs), which results in odour blends that are attractive to predators and parasitoids of these herbivores. The VOCs in the odour blends emitted by maize plants (*Zea mays*) infested by lepidopteran larvae are well characterized. They are derived from at least three different biochemical pathways, but the relative importance of the different pathways for the production of VOCs that attract parasitic wasps is unknown. Here, we studied the importance of shikimic acid derived VOCs for the attraction of females of the parasitoids *Cotesia marginiventris* and *Microplitis rufiventris.* By incubating caterpillar-infested maize plants in glyphosate, an inhibitor of the 5-enolpyruvylshikimate-3-phospate (EPSP) synthase, we obtained induced odour blends with only minute amounts of shikimic acid derived VOCs. In olfactometer bioassays, the inhibited plants were as attractive to naive *C. marginiventris* females as control plants that released normal amounts of shikimic acid derived VOCs, whereas naive *M. rufiventris* females preferred inhibited plants to control plants. By adding back synthetic indole, the quantitatively most important shikimic acid derived VOC in induced maize odours, to inhibited plants, we show that indole had indeed no effect on the attraction of *C. marginiventris* and that *M. rufiventris* preferred blends without synthetic indole. Exposing *C. marginiventris* females either to odour blends of inhibited or control plants during oviposition experiences shifted their preference in subsequent olfactometer tests in favour of the experienced odour. Further learning experiments with synthetic indole showed that *C. marginiventris* can indeed learn to respond to this compound, but that this does not affect its choices between natural induced blends with or without indole. We hypothesize that for naïve wasps the attractiveness of an herbivore-induced odour blend is reduced due to masking by non attractive compounds and that during oviposition experiences in the presence of complex odour blends, parasitoids strongly associate some compounds, while others are largely ignored.

Key Words - *Cotesia marginiventris, Microplitis rufiventris, Spodoptera littoralis, Zea mays,* parasitoids, volatile organic compounds (VOCs), herbivore-induced plant volatiles (HIPVs), host location, associative learning, tritrophic interactions, indole, shikimic acid, glyphosate, induced defences.

INTRODUCTION

Plants that are attacked by herbivorous arthropods are known to release a complex blend of volatile organic compounds (VOCs). These herbivore-induced plant volatiles (HIPVs) are exploited by predators and parasitoids as foraging signals that help them to locate their herbivorous prey or hosts (Arimura et al., 2005). At present, more than 1,000 low molecular weight organic compounds have been reported to be emitted from plants, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters and carboxylic acids (Niinemets et al., 2004;Dudareva et al., 2004). Although some of these compounds are constitutively emitted by undamaged, healthy plants, considerably higher amounts are emitted after herbivore damage and various HIPVs may even be synthesised *de novo* in response to damage (Paré and Tumlinson, 1997; Turlings et al., 1998). Some HIPVs are specific to certain plant taxa, as for example sulphur containing compounds in *Allium* plants (Dugravot et al., 2004) or glucosinolate breakdown products in Brassicaceae species (Scascighini et al., 2005), but other compounds are common to many plant species (Van Den Boom et al., 2004). Common compounds include “green-leaf volatiles” (C6 aldehydes, alcohols and derivatives), cyclic and acyclic terpenoids, phenolic compounds and nitrogenous compounds (Dicke, 1999; Paré and Tumlinson, 1997). These compounds derive from at least three biochemical pathways. Green leaf volatiles are products of the enzymatic activity of hydroperoxide lyase (HPL), a component of the lipoxygenase (LOX) pathway, which results in multiple rearrangement of (*Z*)-3-hexenal (Bate and Rothstein, 1998; Blee, 1998). Terpenoids are synthesised via the isopentenyl pyrophosphate (IPP) intermediate following the classical mevalonate pathway or via an alternative IPP pathway with glyceraldehyde-3-phosopate and pyruvate identified as the direct precursors of IPP (Lichtenthaler et al., 1997). Finally, aromatic compounds, such as methyl salicylate and indole are formed via the shikimic acid pathway (Bennett and Wallsgrove, 1994; Paré and Tumlinson, 1996).

In maize plants, the mechanisms of biosynthesis, induction and release of HIPVs are well characterized (Turlings et al., 1998; Degenhardt and Gershenzon, 2000; Frey et al., 2000; Shen et al., 2000; Gouinguené and Turlings, 2002; Schnee et al., 2002; Gouinguené et al., 2003; Schmelz et al., 2003; Schmelz et al., 2003; Schmelz et al., 2003; Degen et al., 2004; Köllner et al., 2004; Lawrence and Novak, 2004; Ruther and Kleier, 2005) and the ecological significance of these

compounds in tritrophic signalling has been demonstrated in laboratory and field experiments (Rasmann et al., 2005). Especially the role of green leaf volatiles and terpenoids in attracting natural enemies of the herbivores has been investigated in various experiments (D'Alessandro and Turlings, 2005). Yet, it remains unclear which compounds are essential for attraction (D'Alessandro and Turlings, 2006). One group of compounds that has hardly been studied in the context of parasitoid attraction are the shikimic acid derived VOCs.

The main shikimic acid derived VOC released by maize seedlings after infestation with larvae of *Spodoptera* moths is indole (Turlings et al., 1998; D'Alessandro and Turlings, 2005). This compound has also been shown to be induced after treatment of maize seedlings with volicitin [*N*-(17- hydroxylinolenoyl)-L-glutamine], a fatty acid-amino acid conjugate found in the regurgitate of *Spodoptera* larvae (Alborn et al., 1997; Turlings et al., 2000). Indeed, jasmonic acid, which has been shown to be involved in the induction of HIPVs in maize (Schmelz et al., 2003), also appears to be an integral part of volicitin-mediated induction of indole (Frey et al., 2004) identified an enzyme in maize, indole-3-glycerol phosphate lyase (Igl), which converts indole-3-glycerol phosphate to free indole. This differs from the enzyme BX1, which catalyses the conversion of indole-3-glycerol phosphate to indole to form the direct defence compounds DIBOA (2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one) and DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-,1,4-benzoxazin-3(4*H*)-one), or tryptophane synthase, which produces the amino acid tryptophane (Frey et al., 1997). The selective activation of the evolutionarily similar genes *igl* and *bx1* suggests that the plants are capable of selecting direct or indirect defence mechanisms depending on the type of stress they are exposed to. Therefore, volatile indole was expected to be a key compound in the attraction of natural enemies of the herbivores.

Here we study the importance of shikimic acid derived HIPVs, in particular indole, in attracting females of two parasitoid species, *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) and *Microplitis rufiventris* (Kokujev) (Hymenoptera: Braconidae). Both parasitoids attack early instar larvae of numerous lepidopteran moths, including many pests, and are known to use plant-provided VOCs in host location (Gouinguené et al., 2003; Hoballah and Turlings, 2005). We manipulated the volatile blend emitted by maize seedlings (*Zea mays* var. Delprim) that had been fed upon by larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) by incubating the plants in glyphosate [*N*-(phosphonomethyl)-glycine]. This compound inhibits the enzyme 5-

enolpyruvylshikimate-3-phospate (EPSP) synthase (Haslam, 1993; Schönbrunn et al., 2001) and thus strongly reduces the amounts of shikimic acid derived VOCs. Attraction of the odour from these inhibited plants was compared to that of the natural blends emitted by control plants. Subsequently, we tested an inhibited blend against an inhibited blend to which we added back a natural amount of synthetic indole, the major shikimic acid derived HIPVs.

C. marginiventris females, like many other female parasitoids, are able to associate plant VOCs with the presence of suitable hosts during oviposition experiences (Turlings et al., 1993; D'Alessandro and Turlings, 2005). We therefore compared the responses of naïve and experienced female parasitoids, and in a series of learning experiments with *C. marginiventris* females we estimated how well the wasps can learn to associate indole with host presence.

MATERIAL AND METHODS

Insects and Insect Treatments – The caterpillar *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the solitary endoparasitoids, *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) and *Microplitis rufiventris*, (Kokujev) (Hymenoptera: Braconidae) were reared as described before (Turlings et al., 2004). Adult parasitoids were kept in plastic cages at a sex ratio of approximately 1:2 (male:female) and were provided with moist cotton wool and honey as food source. The cages were kept in incubators (*C. maginiventris*: 25±1 °C; *M. rufiventris*: 23±1 °C; 16L:8D) and transferred to the laboratory 30 min before the experiments. We tested mated 2-4 days old naive and experienced females. The latter were given experiences by allowing them to oviposit 3-5 times into second instar *S. littoralis* larvae. A metal screen was attached to the top opening (2 cm diam.) of an odour source vessel of the same type as used in the olfactometer (see below). Approximately 20 larvae were placed on the screen and individual wasps were allowed to oviposit in one or two larvae, while they were exposed to the odour of the source that was placed inside the vessel. This source was either an infested control plant, an infested inhibited plants, or synthetic indole only. Airflow and concentrations of volatiles were the same as during the olfactometer bioassays (see below). Naïve females were neither exposed to the volatiles nor given any oviposition experience before testing. The different groups of wasps were kept separately in small plastic boxes with moist cotton wool and honey and released into the olfactometer 1-3 hr after the oviposition experiences.

Plants and Odour Sources – Maize (*Zea mays*, var. Delprim) was sown in plastic pots (10 cm high, 4 cm diam.) with commercial potting soil (Ricoter Aussaaterde, Aarberg, Switzerland) and placed in a climate chamber (23±2 °C, 60 % r.h., 16L:8D, and 50000 lm/m^2). Maize plants used for the experiments were 10-12 days old and had three fully developed leaves. The evening before the experiments, plants were cut with a razor blade at soil level, while the stem was held under water to prevent air entering the vascular system. Subsequently, they were placed in a vial (8 mL) filled with either deionised water (**control plant**) or in a 1 mM glyphosate ([*N*-(phosphonomethyl)-glycine], Fluka, Switzerland) solution (**inhibited plant**). Vials were wrapped in aluminium foil and one vial containing a single plant was placed in an open odour source vessel of the olfactometer

(described by Turlings et al., 2004). Two hours after incubation, the plants were infested with 20 second instar *Spodoptera* larvae, which were released in the whorl of the youngest leaf. After infestation, plants were kept under laboratory conditions (25±2°C, 40±10% r.h., 16L:8D, and 8000 lm/m^2) and were used for the experiments the following day, between 10 am and 4 pm.

To check whether the treatment of the plants with the inhibitor affected the larval feeding behavior, plants were collected after the bioassays and the leaves were scanned into Adobe Photoshop 6.0. The total leaf area that was removed during the 24 hr feeding period was compared for the different treatments based on differences in pixels that indicated tissue removal.

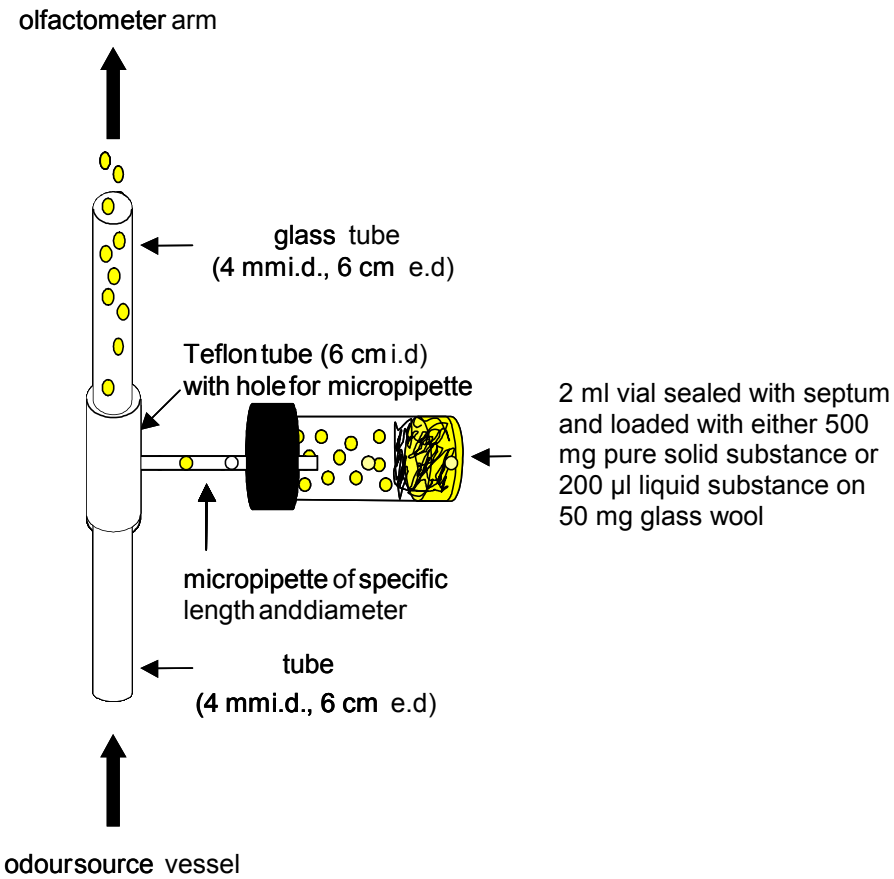

FIG. 1. Schematic representation of the odour delivery system for synthetic VOCs.

Indole (purity ≥ 99 %, Fluka, Switzerland) was released from a device consisting of a 2 mL glass vial that contained 500 mg synthetic indole (Fig. 1), which was connected, via a glass capillary, to a Teflon tube placed between two glass tubes connecting the top of an odour source

vessel to an olfactometer arm. Preliminary experiments with various synthetic volatile compounds (e.g. methyl salicylate, linalool, indole) showed that at room temperature this device allowed the constant release of pure compounds and that their release rates could be controlled by adjusting the length and the diameter of the capillary tube (Duran, Hirschmann EM). The release rate was calibrated to the lower range of amounts of indole that was found for infested maize plants. Vials were prepared freshly the evening before the experiments and connected the following morning to the odour source vessels used for training or testing.

Olfactometer Bioassays – All odour sources were tested for attractiveness to the parasitoids in a 4-arm olfactometer (described by D'Alessandro and Turlings, 2005) as indicated in table 1. Cleaned and humidified air entered the odour source vessel at 1.2 l/min (adjusted by a manifold with 4 flow-meters; Analytical Research System, Gainesville, Florida, USA) via Teflon tubing and carried the VOCs through to the olfactometer compartment. Half of the air (0.6 l/min/olfactometer arm) was pulled out via a volatile collection trap that was attached to the system above the odour source vessels (see *collection and analyses of VOCs*). Incoming and outgoing air were balanced by a Tygon tube connected to a vacuum pump via another flow meter and a pressure gauge. Empty arms were connected to empty vessels and carried clean, humidified air only.

Wasps were released in groups of 6 into the central part of the olfactometer and after 30 min the wasps that had entered an arm of the olfactometer were counted and removed. Wasps that did not enter an arm after this time were removed from the central part of the olfactometer and considered as "no choice". Experiments were replicated on 8 different days and for each replicate a total of 6 groups of 6 wasps were tested during a 3 hr sampling period, alternating between groups of naive and experienced wasps in experiments were three different groups were tested (total of 96 wasps/group), or only naïve wasps in the other experiments (total 288 wasps). Ten neon tubes attached to a metal frame above the olfactometer provided approximately 7000 lm/m^2 at the height of the odour source vessels. All bioassays were carried out between 10 am and 4 pm.

TABLE 1: ODOUR SOURCES AND EXPERIMENTAL DESIGN

figure	odour sources				wasp	wasp treatment	replications
	arm 1	arm 2	arm 3	arm 4			of experiment
3 A)	control plant (infested)	empty	inhibited plant (glyphosate, infested)	empty	*C. marginiventris*	naive, control inhibited	8
3 B)	control plant (infested)	empty	inhibited plant (glyphosate, infested)	empty	*M. rufiventris*	naive, control inhibited	8
4 A)	inhibited plant & indole (gylphosate, infested)	empty	inhibited (glyphosate, infested)	empty	*C. marginiventris*	naive	8
4 B)	inhibited plant & indole (gylphosate, infested)	empty	inhibited (glyphosate, infested)	empty	*M. rufiventris*	naive	8
5 A)	indole	empty	empty	empty	*C. marginiventris*	naive, indole control	8
5 B)	control plant (infested)	empty	inhibited plant (glyphosate, infested)	empty	*C. marginiventris*	naive, indole control	8

Further details on odour sources, number, and treatment of wasps are described in the text and in the figures. Treatments of the plants are given in parenthesis.

Collection and Analyses of VOCs – VOCs of each odour source were collected during the olfacotemeter bioassay on a Super-Q trap (25 mg, 80-100 mesh, Alltech, Deerfield, Illinois, USA, described by Heath and Manukian ,1992). Each trap was attached horizontally to the elbow of the olfactometer and connected via Tygon tubing to a flowmeter (Analytical Research System, Gainesville, Florida, USA) and a vacuum pump. Air carrying the volatiles was pulled through each trap for 3 hr at a rate of 0.6 l/min during each behavioral bioassay. Afterwards, the traps were extracted with 150 µl dichloromethane (Suprasolv., Merck, Switzerland), and 200 ng of n-octane and n-nonyl acetate (Sigma, Switzerland) in 10 µl dichloromethane were added to the samples as internal standards. All extracts were stored at –76°C until analyses. Traps were washed with 3 mL of dichloromethane before reusing them for a next collection.

VOCs of the experiments with control and inhibited plants were analysed using a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectrometer that operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33-280 amu). A 2 µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness, Alltech Associates, Inc, USA). Helium at constant flow (0.9 mL/min) was used as carrier gas. Following injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min followed by a post-run of 5 min at 250°C. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 02 library, by comparison of their spectra and retention times with those of authentic standards and by comparison of retention times with those in previous analyses

(D'Alessandro and Turlings, 2005). Compounds that were not identified by comparing retention times and spectra with those of pure standards are indicated in figure 2 with the label ^{N}, and their identity should be considered tentative. Twelve samples per treatment were analysed in the SIM mode (ion 117, qualifier 95) and and indole was quantified based on a calibration curve with known amounts of synthetic indole. All other compounds were only quantified in the full scan range based on comparison of their peak area with those of the internal standards (n-octane for compounds 1-14, n-nonyl acetate for compounds 14-27). A total of 18 samples were injected per treatment.

Statistical Analyses – The functional relationship between parasitoids' behavioral responses and the different odor sources offered in the 4-arm olfactometer was examined with a log linear model (a generalized linear model, GLM). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of wasps within the olfactometer (Turlings et al., 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (R: A language and Enviornment for Statistical Computing, Version 1.9.1, Vienna, Austria, 2006, ISBN 3-900051-07-0 http://www.R-project.org) and its adequacy was assessed through likelihood ratio statistics and examination of residuals. We tested 'treatment' effects (= odor sources) for naive and experienced wasps separately, and we included 'release' as an explanatory variable to avoid 'pseudo-replications'. In addition we tested if there was a significant effect of 'experience' and an interaction between 'treatment x experience'.

The amounts of VOCs were analysed using *t*-tests. Amounts of VOCs that were not normally distributed were log(x+1) transformed prior to analysis. The amounts of indole quantified in the single ion mode were analysed using a Kruskal-Wallis test. Differences between the treatments were analysed using the Tukey's test. Differences between the removed leaf areas after caterpillar feeding were analysed using a *t*-test. All analyses were run on SigmaStat (Version 2.03).

RESULTS

VOCs of Control and Inhibited Plants – Herbivore-infested maize seedlings that were incubated with their cut stem in water (control plants) released a volatile blend consisting of 24 detectable VOCs, including green leaf volatiles, terpenoids and shikimic acid derivatives (Figure 2). Seedlings that were incubated in a 1 mM glyphosate solution released only trace amounts of indole and methyl anthranilate and had strongly reduced amounts of other shikimic acid derived VOCs (*t*-Test: benzyl acetate, t_{34} = 1.741, P = 0.092; phenethyl acetate, t_{34} = 11.218, P < 0.001). The amounts of the VOCs derived from other biochemical pathways were similar to those of the control plants, except for the somewhat reduced amounts of (*Z*)-3-hexen-1-ol (t_{34} = 2.306, P = 0.027) and (*Z*)-3-hexen-1-ol acetate (t_{34} = 2.577, P = 0.014). In addition to the compounds quantified in figure 2 we also detected trace amounts of (*E*)-nerolidol, (*Z*)-jasmone, and some minor, unidentified compounds. These VOCs were not included in quantification analyses.

The single ion mode analyses of indole revealed that during the 3 hr bioassay periods we collected 835.44 ± 135.91 ng from control plants and only 0.55 ± 0.17 ng from inhibited plants. In bioassays where synthetic indole was added to the air stream with the odour of an inhibited plant we detected 192.77 ± 11.46 ng. There was a significant difference between the amounts released by these three treatments (Kruskal-Wallis followed by Tukey test, H_2 = 30.294, P < 0.001).

Inhibitor treatment did not affect the larvae's feeding rate; the leaf areas removed by the larvae during a 24 hr feeding period were similar for control plants (4.73 ± 0.40 cm^2) and for inhibited plants (4.81 ± 0.38 cm^2) (*t*-Test: t_{20} = - 0.15, P = 0.882).

Attractiveness of Inhibited versus Control Plants – Neither naïve nor experienced *Cotesia marginiventris* females significantly distinguished between VOC-blends emitted by *Spodoptera*-induced maize seedlings with strongly reduced amounts of shikimic acid derived VOCs (see above) and VOCs emitted by control plants (Figure 3A, GLM, naïve: $F_{1,15}$ = 0.78, P = 0.39; experienced on inhibited blend: $F_{1,15}$ = 3.91, P = 0.067; experienced on control blend: $F_{1,15}$ = 0.43, P = 0.52). Yet, the type of experience had a significant effect on the choice of the wasps ($F_{1,60}$ = 4.566, P = 0.0367), implying that *C. marginiventris* females were able to detect the difference between the two odour sources. The responsiveness (the proportion of wasps entering an arm with one of the two

treatments) was high and similar for all treatments of the wasps and only few wasps entered an empty arm (Figure 3A).

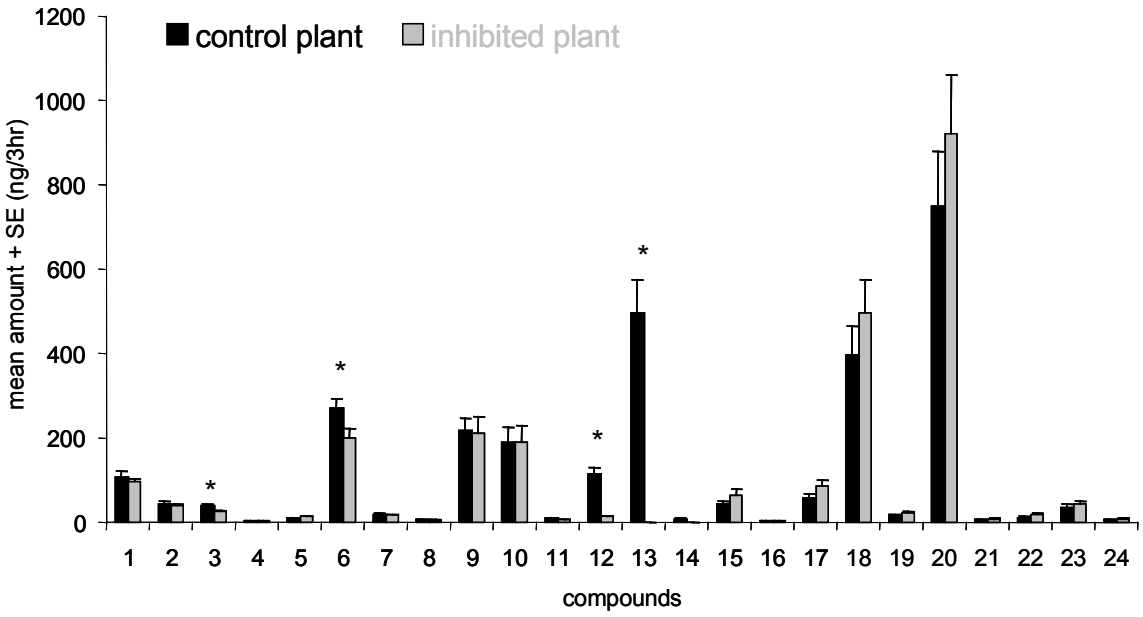

FIG. 2. Mean amount (ng + SE) of major VOCs recollected from cut *Spodoptera*-induced maize seedlings during 3 hr. Control plants were incubated in water, inhibited plants were incubated in a 1 mM glyphosate solution. Asterisks above bars indicate significant differences (*t*-Test on log (x +1) transformed data, $P < 0.05$) in the amount of a specific compound. N = 18 per treatment. The compounds are: 1 = (*Z*)-3-hexenal, 2 = (*E*)-2-hexenal , 3 = (*Z*)-3-hexen-1-ol, 4 = (*Z*)-2-penten-1-ol acetate, 5 = β-myrcene, 6 = (*Z*)-3-hexenyl acetate, 7 = (*E*)-2-hexenyl acetate, 8 = (*Z*)- β-ocimene^{N}, 9 = linalool, 10 = (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), 11 = benzyl acetate, 12 = phenethyl acetate, 13 = indole, 14 = methyl anthranilate, 15 = geranyl acetate, 16 = unknown sesquiterpenoid, 17 = (*E*)-β-caryophyllene, 18 = (*E*)-α-bergamotene, 19 = unknown sesquiterpenoid, 20 = (*E*)-β-farnesene, 21 = unknown sesquiterpenoid, 22 = unknown sesquiterpenoid, 23 = β-sesquiphellandrene^{N}, 24 = (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). The compounds are ordered in accordance with their retention on a non-polar capillary column.

Naïve and experienced females of *Microplitis rufiventris* significantly preferred the inhibited blend (Figure 3B, GLM, naïve: $F_{1,15}$ = 11.04, P = 0.005; experienced on inhibited blend: $F_{1,15}$ = 26.19, P < 0.001; experienced on control blend: $F_{1,15}$ = 6.19, P = 0.025). As with *C. marginiventris*, the type of experience had a significant effect on the choice of the wasps ($F_{1,60}$ = 7.073, P = 0.010). The responsiveness was high and none of the *M. rufiventris* wasp entered an empty arm.

The Role of Indole – Indole was the major shikimic acid derived VOC released by *Spodoptera*-induced maize seedlings (Figure 2). We tested its role in the attraction of the two parasitoid species by comparing the attractiveness of HIPV-blends released by two inhibited plant, whereby we added synthetic indole back to one of the blends. The amounts of indole added (192.77 ± 11.46 ng / 3 hr) fell within the lower ranges of indole detected in a natural induced maize blend (see above). *C. marginiventris* did not distinguish between the two blends (Figure 4A, GLM: $F_{1,47}$ = 0.87, P = 0.36). In contrast, *M. rufiventris* significantly preferred the inhibited blend without synthetic indole (Figure 4B, $F_{1,47}$ = 43.16, P < 0.001). The results for both wasps are consistent with those from the previous experiment: an insignificant role of shikimate-derived compounds for *C. marginiventris* attraction, whereas they have a repellent effect on *M. rufiventris*.

Learning of Indole – When *C. marginiventris* females were given a choice between one arm with synthetic indole (192.77 ± 11.46 ng / 3 hr) and 3 arms with clean air only *C. marginiventris* females did neither show an innate (naive wasps) attraction towards indole (Figure 5A, GLM: $F_{1,47}$ = 0.60, P = 0.44), nor were they attracted to indole after having experienced a natural blend that contained similar amounts of indole ($F_{1,47}$ = 0.29, P = 0.60). However, if they were exposed to pure indole during oviposition experiences they significantly preferred an arm carrying indole over arms with clean air ($F_{1,47}$ = 11.62, P = 0.001), but there was no significant learning effect ($F_{1,124}$ = 2.919, P = 0.090) and the overall responsiveness was rather low.

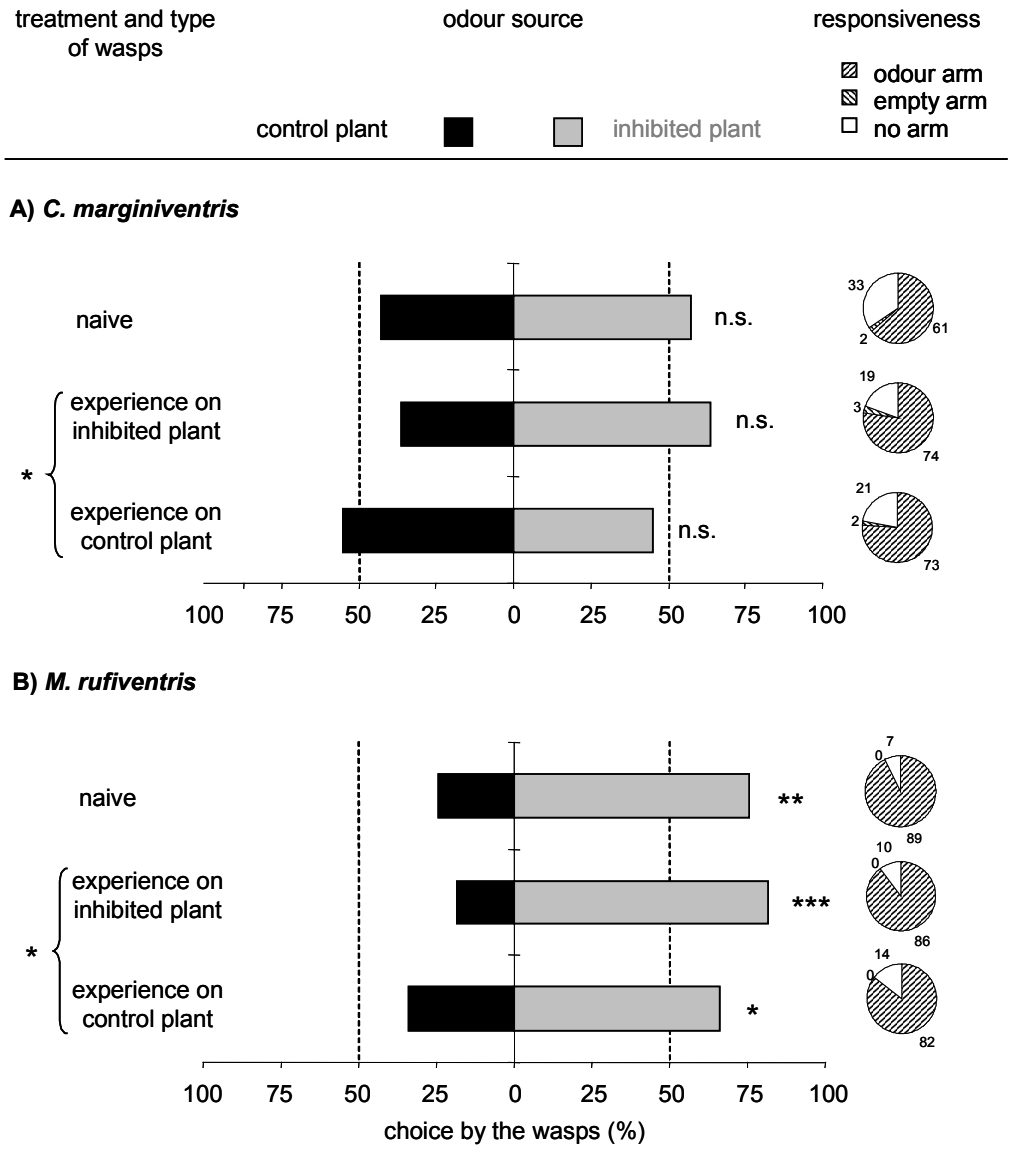

FIG. 3 The importance of shikimic acid derived VOC for the attraction of two parasitoid species. A) Choice of *C. marginiventris* females and B) *M. rufiventris* females between arms carrying VOCs of *Spodoptera*-induced maize seedlings that were either incubated in water (control plants) or in a 1 mM glyphosate solution (inhibited plants). Pre-treatment of the wasps (= type of experience) is indicated on the left. The pie charts indicate overall responsiveness (= number of wasps entering the different types of arms). GLMs were performed in order to test for differences between the two odour arms within one group of wasps as well as to compare the types of experiences. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, n. s. = no significant difference $P > 0.05$.

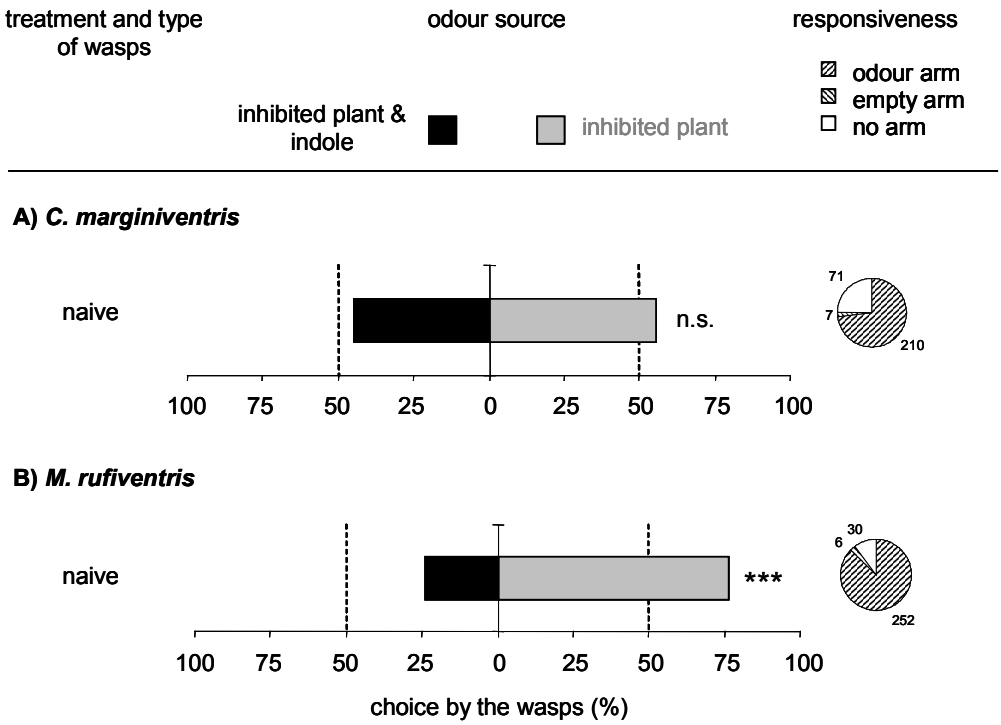

FIG. 4. The importance of indole for the attraction of two parasitoid species. A) Choice of naïve *C. marginiventris* females and B) naïve *M. rufiventris* females between arms carrying *Spodoptera*-induced maize VOCs of inhibited plants and of inhibited plants to which synthetic indole was added. See figure 3 for further explanations.

In an additional experiment we tested if exposing the wasps to indole during ovipositions increases the attraction towards an induced maize blend containing indole (control plant) compared to a blend with only trace amounts of indole (inhibited plant). As in the experiments above, naïve wasps and wasps that had experienced the control blend did not distinguish between the two blends (Figure 5B, GLM: $F_{1,15}$ = 0.062, P = 0.81; $F_{1,15}$ = 1.92, P = 0.19, respectively). Interestingly even an experience with pure synthetic indole did not result in a change in preference ($F_{1,15}$ = 3.70, P = 0.074) and the type of experience did not have a significant effect on the wasps' choice ($F_{1,60}$ = 0.214, P = 0.646).

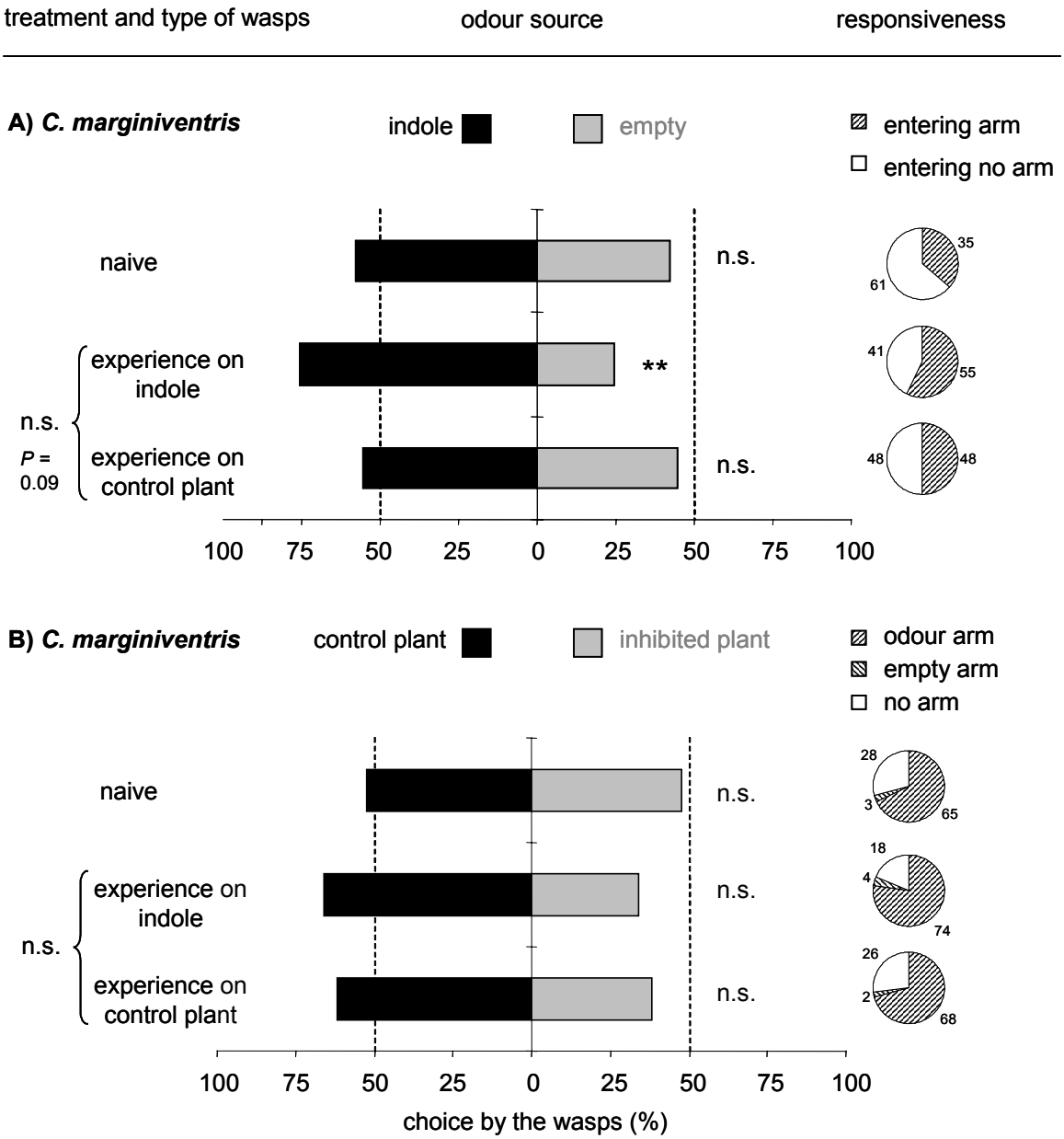

FIG. 5. The importance of indole in learning experiments by *C. margininventris*. A) Choice of females with different learning experiences between arms carrying synthetic indole and empty arms with clean air only. B) Choice of females with different learning experiences between arms carrying *Spodoptera*-induced maize VOCs of control and inhibited plants. See figure 3 for further explanations.

DISCUSSION

One way of studying the importance of individual VOCs for the attraction of natural enemies is to compare the attractiveness of an incomplete with a normal blend of HIPVs (D'Alessandro and Turlings, 2005). By restoring the incomplete blend with synthetic compounds that are missing, the importance of the added compounds can be confirmed (de Boer and Dicke, 2004). Here we used glyphosate an inhibitor of the enzyme 5-enolpyrovylshikimate-3-phosphate (EPSP) synthase (Haslam, 1993; Schönbrunn et al., 2001), to inhibit the production of shikimic acid derived HIPVs. As expected, this inibitor treatment resulted in a strong reduction of the emission of indole, methyl anthranilate, phenethyl acetate and benzyl acetate, while the amounts of most other compounds, except two green-leaf volatiles were not significantly affected by the inhibitor treatment (Figure 2). Treating plants with glyphosate normally results in lower amounts of chorismate, a precursor for the production of salicylic acid (SA) (Wildermuth et al., 2001; Shah, 2003). SA has been shown to synergistically or antagonistically interact with jasmonic acid (Thaler et al., 2002; Bostock, 2005) an important hormone involved in the induction of maize VOCs (Schmelz et al., 2003c). We speculate that SA does not play a major role in induced volatile emissions in this maize variety. Indeed, methyl salicylate (MeSA), the volatile form of SA, was only occasionally detected in trace amounts in our control plants.

Glyphosate treated plants are also expected to contain lower levels of phenolic compounds, which should have a positive effect on herbivores. However, we did not observe any difference in the amount of leaf-damage inflicted by *Spodoptera* larvae between glyphosate treated and untreated plants. A possible negative effect of glyphosate through direct toxicity has been ruled out in ecotoxicological risk assessment studies with arthropods (Giesy et al., 2000). Furthermore, glyphosate is not readily volatilized and is degraded primarily by microbial metabolism in the soil (Tu et al., 2001). Indeed, we did not detect additional VOCs from glyphosate treated seedlings, making glyphosate a suitable inhibitor to study the attractiveness of shikimic acid derived VOCs.

Attractiveness of shikimic acid derived VOCs - It is generally assumed that HIPVs are exploited by natural enemies of the herbivores in order to locate their host or prey (Dicke, 1999; Turlings and Wäckers, 2004), but which compounds of a volatile blend are actually important in the foraging behavior of natural enemies is not yet known for most tritrophic systems (Dicke and van

Loon 2000). Previous studies on caterpillar-induced maize volatiles showed that qualitative differences in the odour blend may be more important for the attraction of parasitic wasps than quantitative differences (Fritzsche Hoballah et al., 2002). Indeed, a blend of induced maize volatiles with reduced amounts of the three major sesquiterpenoids, (*E*)-β-caryophyllene, (*E*)-α-bergamotene and (*E*)-β-farnesene was equally attractive to naive *C. marginiventris* as control blends with high amounts of these compounds, whereas removing some minor, polar compounds from the blend rendered it completely unattractive to *C. marginiventris* (D'Alessandro and Turlings, 2005). Here we provide another example that shows that some common HIPVs, i.e. shikimic acid derived volatiles, are not involved in the initial attraction of two parasitoids to host infested plants (Figure 3 and 4). Indole, which is one of the dominating compounds in *Spodoptera*-induced maize volatiles (Turlings et al., 1998; D'Alessandro and Turlings, 2005), might even be repellent or masking the attractiveness of compounds used for host location. Interestingly, several studies on maize volatiles indicate specific induction of volatile indole by herbivore-derived elicitors and not by excision stress or mechanical damage (Frey et al. 2000; Schmelz et al. 2003b). However, in other studies, many of the common herbivore-induced VOCs, including indole and several terpenoids, have been detected in analyses of VOCs released by plants exposed to other forms of stresses, as for example mechanical wounding (van den Boom et al., 2004), exposure to other VOCs (Ruther and Fürstenau, 2005), or infection by micro-organism (Huang et al., 2003). Hence, the emission of various volatiles can be induced by a number of enemies and stresses, yet natural enemies are able to discriminate between different forms of stresses (Takabayashi et al., 1995; De Moraes et al., 1998; de Boer et al., 2004; Vuorinen et al., 2004). Selection must have favoured parasitoids with an ability to distinguish between host and non-host related compounds and an innate response to compounds that are specifically correlated with host presence is the most likely mechanism that allows them to make such distinctions (Vet and Dicke, 1992). We hypothesize that naïve females of generalist parasitic wasps are attracted only to a few key compounds within a complex blend of volatiles and that most other compounds within such a blend contribute little to its initial attractiveness, may mask the attractive compounds, or may even be repellent. Still, these compounds may become attractants when the wasps have associated them with host presence.

Importance of Learning – One way for parasitoids to deal with highly complex and variable blends is their ability to learn by association (Turlings et al., 1993; Vet et al., 1995). It is assumed that such learning processes are specifically important for generalist wasps like *C. marginiventris* and *M. rufiventris*, parasitizing various host, feeding on different plant species (Vet and Dicke, 1992; Steidle and van Loon, 2003). Indeed, *C. marginiventris* shows a keen ability of associative learning, whereas this form of learning is less clear for *M. rufiventris* (D'Alessandro and Turlings, 2005; Hoballah and Turlings, 2005; Tamò et al. 2006). Here again *C. marginiventris* showed a significant shift in its preference in favour of the blend that it had experienced during multiple ovipositions (Figure 3a). The response of *M. rufiventris* also changed significantly after experience, but it maintained a significant preference for the odour of inhibited plants even after having experienced the odour of control plants (Figure 3b).

The predatory mites, *Phytoseiulus persimilis* has been found to strongly associate methyl salicylate (MeSA) with the presence of their prey if they are reared in the presence of a complex blend of herbivore-induced VOCs that includes MeSA (de Boer et al. 2004). Similarly, Vet et al. (1998) found that the *Drosophila* parasitoid *Leptopilina heterotoma* learned to discriminate between odours from substrates that were qualitatively different, but failed to discriminate when differences were small, unless unrewarding experiences provided evidence of the absence of hosts in one of the substrates. That some compounds are more important than other for associative learning was found for the parasitoid *Microplitis croceipes*. After conditioning to a complex mixture, females of this species established a hierarchy among various components, with some of them accounting for a major part of the behavioral activity evoked by the mixture (Meiners et al., 2003). In our study, *C. marginiventris* was able to learn and subsequently respond to pure synthetic indole, but this learning of indole had no effect on the females' responses to natural, complex blends with or without indole (Figure 5). Apparently, indole is not a compound that is strongly associated during learning processes, especially not if offered in a complex volatile environment. Although indole is strongly induced after *Spodoptera* infestation on maize plants, it is also found in a variety of other stress-induced plant volatile blends (see above) and may not provide foraging parasitic wasps with specific information on the presence or absence of hosts.

Conclusions – We studied the role of shikimic acid derived VOCs, in particular indole, in the host searching behavior of two parasitoid species, *Cotesia marginiventris* and *Microplitis rufiventris.* This group of VOCs forms a substantial part of the volatile blend released by maize seedlings in response to feeding by lepidopteran larvae, but the results show that they are not important for the attraction of the two wasp species tested here. Attraction of *C. marginiventris* was not affected by presence or absence of indole, the major shikimic acid derived VOC, whereas this compound was repellent rather than attractive to *M. rufiventris*. Learning of indole during oviposition experiences did not greatly alter these responses. Hence, this study suggests that parasitoids do not use all herbivore-induced VOCs for habitat and host location to a similar degree, but rather pay selective attention to a few compounds. Identifying these key compounds seems crucial for a good understanding of the host searching process in parasitoids and for the development of strategies to increase the efficiency of natural enemies for the control of pest insects (Turlings and Ton, 2006).

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Chapter IV

Volatile organic compounds produced by soil-born endophytic bacteria modify direct and indirect defences in maize seedlings

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Abstract – Plants are constantly challenged by a multitude of herbivorous arthropods and pathogens against which they have evolved complex inducible direct or indirect defence strategies. Studies have focused mostly on inducible defences in the phyllosphere (aboveground), few have investigated defences in the rhizosphere (belowground), and relatively little is known about how interactions with belowground biota affects aboveground plant defences or *vice versa*. Here we first show that soil-born micro-organisms modify indirect defences of maize plants (*Zea mays*) aboveground by adding the compound 2,3-butanediol to the volatile blends released by maize seedlings in the phyllosphere, thereby increasing the attractiveness of the seedlings to the parasitic wasp *Cotesia marginiventris*, a natural enemy of herbivorous caterpillars. Subsequently, we demonstrate that 2,3-butanediol is produced by *Enterobacter aerogenes*, a bacterium which we isolated from germinated maize seeds grown in the presence of soil-born micro-organisms. Adding this endophytic bacterium to maize seedlings not only resulted in the release of high amounts of 2,3-butanediol in the phyllosphere, but also induced systemic resistance against the northern corn leaf blight *Setosphaeria turcica*, while the herbivorous larvae of *Spodoptera littoralis* grew slightly better on plants containing the bacterium. By using synthetic 2,3-butanediol and its precursor acetoin, it was shown that these bacteria-derived volatiles were at least partially responsible for the observed differences. Moreover, gene expression profiling of pathogen- and/or herbivore- inducible marker genes suggests that the observed effects of *E. aerogenes* and its volatile metabolites on the resistance of maize seedlings are not caused by defence mechanisms that are controlled by jasmonic acid or salicylic acid. This work demonstrates that soil-born micro-organisms capable of colonising plants can form connecting links between interactions in the rhizosphere and in the phyllosphere, and that their volatile metabolites may act as key signalling compounds affecting defence responses across trophic levels.

Key words – endophytic bacteria, parasitoids, herbivores, pathogens, maize, inducible defences, induced systemic resistance, 2,3-butanediol, volatile organic compounds (VOCs)

Introduction

Terrestrial plants are exposed to at least three of the four 'empedoclian elements', soil, water, and air, and therefore they are constantly interacting with a multitude of organisms in the rhizosphere (belowground) and in the phyllosphere (aboveground). Some of these organisms are beneficial symbionts, whereas others are antagonists, like herbivores and pathogens, against which plants have evolved various defence strategies. These defences might act directly against these attackers, as for example defensive proteins or toxic secondary plant compounds negatively affecting herbivores and pathogens, while others might act indirectly, as for example herbivore-induced plant volatiles attracting natural enemies of the herbivores. Both defence strategies can be either constitutively expressed or induced after the plant has been challenged by an invader (Agrawal et al., 1999; Karban and Baldwin, 1997). Inducible defences have received widespread attention over the last decades and recent studies challenging plants with multiple stressors and applying holistic approaches including metabolomic and genomic analyses have revealed the ecophysiological complexity of such defence mechanisms (Schmelz et al., 2004; Rodriguez-Saona et al., 2005; Kant et al., 2004; Kessler and Baldwin, 2004). A major outcome of these studies was the appreciation of the tremendous plasticity of inducible defences in plants, depending on belowground and aboveground biotic and abiotic factors, as for example, availability of nutrients (Schmelz et al., 2003a; Lou and Baldwin, 2004; Gouinguené and Turlings, 2002), type and site of attacking organisms (Walling, 2000; Dicke and Hilker, 2003), plant genotype (Degen et al., 2004; Krips et al., 2001; Loughrin et al., 1995; Takabayashi et al., 1991), or even exposure to volatile organic compounds (VOCs) from neighbouring plants (Farmer, 2001; Engelberth et al., 2004; Baldwin et al., 2006, Ton et al., 2006). Currently, ecologists are trying to link these factors (Blossey and Hunt-Joshi, 2003; Wardle et al., 2004; Bezemer and van Dam, 2005), and there is growing evidence that both aboveground and belowground interactions are driving forces for the evolution of inducible defences (Van der Putten et al., 2001; van Dam et al., 2003; Rasmann et al., 2005).

One important aspect, which has so far largely been neglected, especially in studies addressing plant inducible defences against insects, are non-pathogenic micro-organisms that are associated with plants, as for example, plant growth promoting rhizobacteria (PGPR), mycorrhiza, rhizobia, and other fungal or bacterial endophytes. Such organisms are known to manipulate the

plant resistance against a broad range of pathogens (Sturz et al., 2000; Pieterse et al., 2003), and they might also affect the resistance against insects (Zehnder et al., 2001). The outcome and mechanisms of such associations strongly depends on the plant species and on the type of micro-organism and the defence responses are probably as diverse as the studied systems themselves. Some micro-organisms improve the plant resistance as they enhance the nutrient availability to plants by fixing nitrogen, excreting phosphatases, or chelating iron via siderophores, while others synthesise complex secondary metabolites with antimicrobial proprieties or even hormones and hormone-like compounds, as for example auxin analogues, gibberellins or cytokinins, interacting with the plant defence responses (Whipps, 2001; Ping and Boland, 2004). Recently VOCs, released by specific strains of the PGPR *Bacillus subtilis* and *B. amyloliquefaciens* have been reported to trigger systemic resistance in *Arabidopsis* plants against the pathogen *Erwinia carotovora* (Ryu et al., 2004). Such volatile metabolites released by soil-born micro-organisms (soil MOs) add a new layer of complexity to plant defence responses. Considering the fact that one gram of soil can contain between 5000 and 10,000 different species of micro-organisms (Torsvik et al., 1990) it is evident, that soil and soil MOs, some of which are capable of colonizing plant tissue, must be of key importance for plant defence responses.

Here we investigate the effects of soil MOs on aboveground indirect and direct defences in maize (*Zea mays* var. Delprim). First we compare VOC profiles of herbivore-infested seedlings grown in uncontaminated autoclaved soil to those emitted by herbivore-infested seedlings grown in autoclaved soil to which we added an extract of non-autoclaved soil (with soil MOs). The attractiveness of these blends was tested to the parasitic wasp *Cotesia marginventris* (Cresson) (Hymenoptera: Braconidae). Subsequently, we localise the site of differentially released VOCs and we isolate endophytic bacteria that were responsible for this difference from germinated maize seeds. Finally, we show that one of the bacterial isolates, *Enterobacter aerogenes,* induces the release of 2,3-butanediol in maize seedlings, which affects the resistance of the plants against the northern corn leaf blight, *Setosphaeria turcia*, and against the herbivore, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). Possible mechanisms by which these enodphytically produced VOCs modify the plant's defence responses are discussed.

Materials and Methods

Plants, micro-organisms, and insects - All maize (*Zea mays*, var. Delprim) seeds were rinsed in 70 % ethanol followed by rinsing in sterile deionised water. Seeds were placed individually in plastic pots (10 cm high, 4 cm diam.) with either autoclaved (121 °C for 1 hr, repeated after 24 hr) potting soil only (Aussaaterde, Ricoter, Aarberg, Switzerland) or with autoclaved soil to which we added 25 mL of a water extract with soil-born micro-organism (soil MOs). The extract was prepared by incubating 50 mg potting soil (1:1 mixture of Ricoter Zimmerpflanzenerde and Ricoter Aussaaterde, Aarberg Switzerland) for 4-5 hr into 250 mL of tap water. Afterwards, the soil with water containing the MOs was passed over a household sieve and 25 mL of this extract was added to pots containing the maize seeds (treatment: with soil MOs). For the control treatments (treatment: without soil MOs) we added 25 mL tap water instead of soil extract. Seeds used to grow plants for the induced systemic resistance and larval growth experiments were first incubated for 3-4 hr in an autoclaved 10 mM $MgSO_4$ solution containing approx. 10^8 CFU/mL of an overnight culture (see below) of *Enterobacter aerogenes* (treatment: bacteria) before placing them in autoclaved soil as described above. All seedlings were grown at 30 °C, 60 r.h., 16L:8D, and 25000 lm/m^2 and watered daily with tap water except some seedlings (as indicated below), which were grown in the green house under controlled conditions (60 RH., 16 h day/ 8 h night, 26 °C day /22 °C night, with a maximum light intensity of 25000 lm/m^2) and watered every second day with tap water. Maize seedlings used for olfactometer experiments and for the quantification of the VOCs were 10-12 days old and had three fully developed leaves. All seedlings were screened (see below) for the absence or presence of 2,3-butanediol prior to the experiments by collecting and analysing the VOCs of non-infested seedlings. Only seedlings releasing 2,3-butanediol were selected for the treatments “with soil MOs”.

The necrotrophic fungus *Setosphaeria turcica* (anamorph: *Exserohilum turcicum*, Ascomycota: Pleosporaceae) was kindly provided by Michael Rostás (University of Würzburg, Würzburg, Germany) and cultivated on V8-Agar in darkness under laboratory conditions.

The caterpillars *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the solitary endoparasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) were reared as described before (Turlings et al., 2004). We used 2^{nd} instar larvae to infest maize seedlings and we

tested mated 2 to 4 day-old naive and experienced females. The latter were given experiences as described by D'Alessandro et al. (2006) by allowing them to oviposit 3-5 times into second instar *S. littoralis* larvae, while simultaneously being exposed either to the odour of an herbivore-infested maize seedling grown in autoclaved soil only or of a maize seedling grown in autoclaved soil with added soil MOs. The different groups of wasps were kept separately in small plastic boxes with moist cotton wool and honey and released in the olfactometer 1-3 hr after their oviposition experience.

Collection and analyses of VOCs – VOCs were trapped during the 3 hr olfactometer bioassay by passing half of the air (0.6 l/min) over Super-Q traps (25 mg, 80-100 mesh, Alltech, Deerfield, Illinois, USA, described by Heath and Manukian, 1992) that where attached to the arms of the olfactometers (described by D'Alessandro and Turlings 2005). Additionally, VOCs released by non-infested plants with or without bacteria (*E. aerogenes*) were collected on Super-Q traps for 3 hr by using the MAD-VOC-collection system (described by Ton et al., 2006). All VOCs were desorbed from the traps with 150 µl dichloromethane, separated by GC on an apolar column (HP-1MS) and quantified and analysed by FID and MS as described by D'Alessandro and Turlings (2005). The right isomeric composition of 2,3-butanediol was identified by analysing VOC-extracts on a chiral column (CycloSil-B, 30 m, 0,25 mm ID, 0.25 µm film thickness, Agilent, USA) installed in the GC-MS and compared to authentic standards (isomeric mixture and meso (RS)-2,3-btutanediol from Fluka, (2*R*,3*R*)-(-)- and (2*S*,3*S*)-(+)-butandediol from Aldrich) (see *appendix II*). To locate the production of bacteria-derived VOCs, maize seedlings grown in the presence of soil MOs were washed with autoclaved water and different parts of the plants (third leaf, sheath, and root, each approx 200 mg, and the whole germinated seed) were excised and placed into a glass vial (75.5 x 22.5 mm, LDZ, Marin-Epagnier, Switzerland) with a septum in the lid. Similar 200 mg of soil from the upper and lower part of the pot were introduced into a vial. A solid phase micro extraction (SPME) fibre (75 µm, carboxen-polydimethylsiloxane, Supelco) was inserted through the septum and exposed for 20 min at 40 °C to the VOCs in the vials. Subsequently, the fibre was automatically inserted in the injector port (250 °C), which was connected to a polar column (Innowax, 30 m, 0.25 mm ID, 0.25 µm film thickness, Agilent, USA) of a GC-MS (Agilent 5973; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33-250 amu). Helium at

constant flow (0.9 mL/min) was used as carrier gas. Following injection, the column temperature was maintained at 40 °C for 3 min and then increased to 250 °C at 8 °C/min followed by a post-run of 5 min at 250 °C. Mass spectra were compared with those of the NIST 02 library and retention times and spectra were compared with those of authentic standards. Compounds that were not identified by comparing retention times and spectra with those of pure standards are labelled with an ^{N}, and their identification should be considered tentative. Prior to each experiment plants with MOs were screened for the release of 2,3-butanediol by using the MAD-VOC-collection system. Collections were run for 1.5 – 3 hr and VOCs were analysed by GC as described above, but without quantification and with a faster program (starting temperature 40 °C, ramp 8 °C/min to 60 °C, post run 280 °C for 5 min). All solutions were stored at –76°C until analyses.

Olfactometer bioassays – The attraction of he parasitoid *C. marginiventris* to volatile blends was tested in a 4-arm olfactometer (described by D'Alessandro and Turlings, 2005). As indicated in figure 2, wasps had the choice between arms carrying volatiles from caterpillar-infested or non-infested plants, with or without MOs, and two empty arms carrying purified air only. In additional experiments, wasps had the choice between blends released by uninfested plants without MOs, with or without an isomeric mixture of synthetic 2,3-butanediol (Fluka, purity ≥ 99%). This compound was either directly released into the air flow of the olfactometer via a microcapillary dispenser (described by D'Alessandro et al. 2006) or added to the soil (approximately 25 mL of 2,3-butanediol in water: 2 mg/mL, isomeric mixture, Fluka, ≥ 99%) the evening before the experiment. The concentrations of 2,3-butanediol in the headspace in both treatments corresponded to the ones found in the natural blend released by a plant growing in autoclaved soil with added MOs (Table 2). Pure humidified air entered each odour source vessel at 1.2 l/min (adjusted by a manifold with 4 flow-meters; Analytical Research System, Gainesville, Florida, USA) via Teflon tubing and carried the VOCs through the arms to the olfactometer compartment. Half of the air (0.6 l/min/olfactometer arm) was pulled out via a volatile collection trap that was attached to the system above the odour source vessels (see *collection and analyses of VOCs*).

Wasps were released in groups of 6 into the central part of the olfactometer and after 30 min the wasps that had entered an arm of the olfactometer were counted and removed. Wasps that did not enter an arm after this time were removed from the central part of the olfactometer and

considered as “no choice”. Six groups each of 6 wasps were tested during a 3 h sampling period alternating between groups of naive and experienced wasps. Each experiment was replicated at six different days with freshly prepared odour sources placed in different positions of the ofactometer. All experiments were carried out between 10 am and 4 pm.

Isolation, cultivation, and identification of Enterobacter aerogenes - Seeds of 10-day old maize seedlings grown in the presence or absence of soil MOs (see above) were harvested and rinsed with water. Subsequently, seeds were vapour-phase sterilised for 3-5 hr by placing them in a 10 L desiccator with a 250 mL beaker containing 100 mL bleach (10 % $Ca(ClO)_2$) and 3 mL concentrated HCl. After sterilization, seeds were rinsed with sterile water. Seeds (6 of each treatment) were cut into two parts and one part of each seed was placed on a LB-agar plate (Difco, LB Broth, Miller, Le Pont de Claix, France) and the other one on a PDA-agar plate (Potato Dextrose Agar, Difco, Brunschwig, Basel, Switzerland) and incubated for 24 hr at 28 °C (LB plates) or room temperature (approx 25 °C, PDA plates). Subsequently, bacteria from both types of plates were cultivated by inoculation of 20 mL LB medium (Difco, LB Broth, Miller, Le Pont de Claix, France) filled in plastic tubes (50 mL 114 x 28 mm, PP, Sarstedt, Nümbrecht, Germany) over night at 28 °C and 250 rpm (Innova 4230, New Brunswick Scientific, Edison, NJ, USA). These overnight cultures were again plated on LB-plates and PDA plates and incubated as described above. Twenty-four single colonies were randomly selected and transferred into liquid LB medium. All bacteria were stored in 25 % glycol solution at – 76 °C.

DNA of the isolated bacterial strains was extracted with the Wizard Genomic DNA purification kit (Promega) and the 16S rRNA gene was PCR amplified using universal eubacterial primers (GM3f, GM4r, Muyzer et al., 1995). Based on their restriction pattern with *HaeIII* and *TaqI* and the potential for 2,3-butanediol production in tyndallised maize seeds (*see appendix III*) five strains were selected for identification. Purified PCR products (Wizard PCR Clean-up, Promega) of the 16S rRNA gene were sent for sequencing (MWG-Biotech, Germany). Resulting rDNA sequences (between 960 and 1350 bp length) were aligned in the RDP II online Sequence Aligner (http://rdp.cme.msu.edu/) and compared to the RDP II and the NCBI (http://www.ncbi.nlm.nih.gov/) databases using the Sequence Match and the Blastn algorithms. All 5 isolates turned out to be closely related and belong to the *Enterobacteriaceae* within the γ-proteobacteria. However, the

phylogenetic information contained in the 16S rRNA sequence is often insufficient for an unambiguous identification of *Enterobacteria* to the species level. Isolate 8, which was later used in most experiments, was therefore further characterised by physiological tests using the *Enterobacteria*-specific API 20E test strip (Bio Merieux). Morphology and motility of Isolate 8 was determined by microscopy of cells grown at 35 °C on Nutrient Agar and LB plates.

Effects of E. aerogenes *and its major volatile metabolites on the resistance of maize seedlings against* S. turcica – In a first experiment we added 25 mL of 2,3-butanediol in water (2 mg/mL, isomeric mixture, Fluka, ≥ 99%) to 7-day old seedlings grown in autoclaved soil only the evening before an experiment (synth. BD). The following day, these seedlings would release similar amounts of 2,3-butanediol as maize seedlings with soil micro-organisms. Additional seedlings were prepared with similar amounts of (±)-acetoin (3-hydroxy-2-butanone, Fluka, purum, mixture of monomer and dimer, ≥97.0%) (synth. AC), (±)-2-butanol (Fluka, ≥ 99.5%) (synth. BO), or water only (control) (n = 12 for each treatment). In addition, we selected (after screening their volatile emissions, see below) seedlings with added *E. aerogenes* that released substantial amounts of 2,3-butanediol (bacteria, BD releasing, n = 12) and some that did not (bacteria no BD, n = 6). The following day, an 8-week old Petri-dish culture of *S. turcica* was flooded with approx. 10 mL of an autoclaved 10 mM $MgSO_4$ solution containing 0.015% Silwet L-77 and then brushed gently with a small paintbrush in order to detach the spores from the mycelium. The density of the spore suspension was determined by a Neubauer chamber and adjusted to approx. 5 x 10^4 spores/mL. Maize seedlings (8 days old) of the different treatments were inoculated by applying 100 µl spore suspension to the first, second and third leaf, respectively. The spores were then spread homogeneously using a paintbrush. Seedlings of the same treatment were together placed in a moistened, closed plastic box (30 x 70 x 50 cm) for 16 hr at > 90% r.h. and ambient temperatures. The following morning all plants were transferred to a climate chamber (23°C, 60% r.h., and LD 16:8 h, 25000 lm/m^2). Disease symptoms were allowed to develop for 3 days after which the strength of infection was estimated by scanning the diseased leaves into Photoshop 7.0 (Adobe) and measuring the necrotic and chlorotic areas with Surface (© C. Thiemann, Berlin, Germany) as described by Rostás et al. (2006).

In a second experiment we grow seedlings in a green house and we inoculated 8-day old seedlings with spores of an 8-week old Petri-dish culture of *S. turcica* as described above. As described above, seedlings were either treated with water only (control), with synthetic 2,3-butanediol (synth. BD), or with *E. aerogenes* bacteria (bacteria) and disease symptoms were measured 3 days after inoculation as described above. In addition, to assess the resistance against *S. turcica* colonization, 5 leaves per treatment were collected, stained with lactophenol trypan-blue (Koch and Slusarenko, 1990). The lengths of *S. turcica* germination hyphae were examined under a microscope (Olympus BX50W1) and quantified using AnalySIS-D software (Soft Imaging System GmbH, Germany).

Larval performance measurements - To determine whether the addition of soil MOs affected the larval feeding rate, 12 plants of each treatment were collected after the olfactometer bioassays and the leaves were scanned into Adobe Photoshop (7.0). The total leaf area that was removed during the 24 hr feeding period was calculated as described above with 'Surface'. We further compared the weight of *S. littoralis* larvae feeding on maize seedlings that were either grown in autoclaved soil with *E. aerogenes,* in autoclaved soil with synthetic 2,3-butanediol or in autoclaved soil only (see above). For this, six second instar *S. littoralis* larvae were weighed (Mettler Toledo MX5 micro-balance, Greifensee, Switzerland) and placed into the whorl of a potted 10 to 12 days old maize seedling. A cellophane bag (Celloclair, Liestal, Switzerland) over each plant prevented caterpillars from escaping while permitting gas exchange. A total of 12 plants per treatment were prepared in this way and subsequently, infested seedlings were placed in a completely randomised design in an incubator and kept at 30 °C, 60 r.h., 16L:8D, and 25000 lm/m^2. Over five days, larvae were weighed daily and maize seedlings were replaced daily with fresh, uninfested seedlings of the same treatment.

Gene expression studies – Expression studies of stress inducible genes were performed in two independent experiments. In a first experiment, total RNA was extracted from pooled shoot samples (n = 3–5) of maize seedlings that were grown in a green house in autoclaved soil only (control), in autoclaved soil with synthetic 2,3-butanediol (synth. BD), or in autoclaved soil with *E. aerogenes* (bacteria), as well as from seedlings grown in autoclaved soil on different time-points (1,

2 and 3 days) after inoculations with *S. turcica* (fungus) or application of *S. littoralis* regurgitant (regurgitant). For the latter treatment, which mimics *S. littoralis* infestation, leaves of 10-day old maize seedlings were scratched with a razor blade and treated with regurgitant of *S. littoralis* as described by Ton et al. (2006). Seedlings were harvested and frozen in liquid nitrogen at 0 (no regurgitant), 1.5, 3, 5 and 24 hr after application of regurgitant.

In a second experiment total RNA was extracted from pooled shoot samples (n = 3) of 10-day old maize seedlings that were grown in autoclaved soil and treated with (±)-jasmonic acid (JA; 200 mM, Sigma, soil drenched on day 9), with benzothiadiazole (BTH 5 mM, active ingredient of Bion, Novartis, soil drenched on on day 8) autoclaved soil with synthetic 2,3-butanediol (as above), or in autoclaved soil with *E. aerogenes.*

In both experiments frozen leaf tissue was homogenised in extraction buffer (0.35 M glycine, 0.048 N NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 mL/g leaf tissue), extracted with phenol/chloroform, and RNA was precipitated using LiCl, as described by Sambrook et al. (1989). Q-RT-PCR was performed essentially as described previously by Ton et al. (2006). Defence marker genes were selected based on previously identified maize genes with putative functions in plant defence, or based on ESTs that had been identified in a previously performed differential hybridization screen for *Spodoptera littoralis*-inducible maize genes (Ton et al., 2006). Primers for RT-qPCR reactions are listed in (Table 1). Each reaction contained 1 µL of cDNA, 0.5 µL of each of the gene-specific primers (10 pmol. μL^{-1}), 7 µL of 2x IQ SYBR Green Supermix reagent (Bio-Rad, Switzerland), and 9 µL water (final volume: 18 µL). The following PCR program was used for all Q-RT-PCR reactions: 95°C for 3 min; 40 cycles of 95°C for 30 sec, 59.5 °C for 30 sec, and 72 °C for 30 sec. C_T values were calculated using Optical System Software, version 1.0 for MyIQ^{TM} (Bio-Rad, Switzerland). C_T values were normalised for differences in dsDNA synthesis using GAPC and Actin1 C_T values. Gene expression patterns between treatments were statistically compared using MeV software (Saeed et al., 2003).

Statistical analyses – The behavioural responses of the parasitoids to different odour sources were analysed with a log-linear model (a generalised linear model, GLM) corrected for the expected distribution of the wasps within the olfactometer as described earlier (Turlings et al., 2004; D'Alessandro et al., 2006). "No choice" and "empty arms" wasps were not included in the

TABLE 1. Primer sequences of 33 different defence-related marker genes used for RT-qPCR.

gene	gene bank no.	putative function	left primer '5 -- 3'	right primer '5 -- 3'
Zm-serPIN	BM382058	serine proteinase inhibitor	gagcagggcatattcgagga	cggatgccgtagaacttcgt
Zm-STC1	AF296122	sesquiterpenecyclase	agggatctgctgagccttca	atctcgagcgcacgctttat
Zm-cyst	BM072984	cystatin proteinase inhibitor	caaggagcacaacaggcaga	ggacatgagctggcgatttt
Zm-dehydrin	X15290	dehydrin	accagtacggcaacccagtc	gccggtcttgtgctcctc
Zm-PR2	DQ417752	pathogenesis-related gene 2	gtgactcgacggagctgttc	gccgtctcaagcttctcctt
Zm-PR1	U82200	pathogenesis-related gene 1	ctgggtgtccgagaagcagt	cgggttgtagctgcagatgat
Zm-L6E	AY103559	L6E ribosomal protein (*O. sativa*)	tcaagtctggcctgctcctt	acttggcgacatcaacacca
Zm-CPK10	AJ007366	calcium-dependent protein kinase	gagcagggcatattcgagga	cggatgccgtagaacttcgt
Zm-lipase	AI820221	lipase/esterase (*O. sativa*)	ccaagagcctcatcatcgtg	cgtggtagtggtccgtgttg
Zm-Bx1	AY254103	DIMBOA biosynthesis gene	cccgagcacgtaaagcagat	cttcatgcccctggcatact
Zm-IGL	AF271383	indole-3-glycerol phosphate lyase	gcctcatagttcccgacctc	gaatcctcgtgaagctcgtg
Zm-PR5	U82201	pathogenesis related gene	tgcatgcatgggctagtgat	cgcacacaaatccagctacg
Zm-cysII	D38130	cystatin II proteinase inhibitor	tgccctgctcatactgcttg	gcgagttcctggaggtgaag
Zm-cyst I-like	CK827737	cystatin-like proteinase inhibitor	agggcttgttcggttaggtg	tgcagaataaggagccatgc
Zm-ERF1	AY672654	homology to ERF1 transcription factor	aaggtggaggcacagactca	taagggatgccgaggaagtt
Zm-Px5	BG837605	peroxidase	ggattgatcctgcgctgag	gactcgaagaggcccaggtt
Zm-HPL	AY540745	hydroperoxide lyase	acttcggcttcaccatcctg	gtagtagcccggccagatga
Zm-FPS	AF330036	homology to farnesyl Pi phosphatase	cgtgctgatgagagccaaaa	ctgggcttcaatgtctgcaa
Zm-AOS	AY488135	allene oxide synthase	acctgttcacgggcacctac	cgaggagcgaggagaagttg
Zm-B73Lox	AF465643	B73 lipoxygenase	gcgacaccatgaccatcaac	gctcggtgaagttccagctc
Zm-GAPC	X07156	glyceraldehyde phosphate dehydrogenase	gcatcaggaaccctgaggaa	catgggtgcatctttgcttg
Zm-thiolase 2	BQ618947	thiolase	ttcgcccaagtttcaaggag	gccgcatctgcatatcctct
Zm-Actin1	J01238	actin1	ccatgaggccacgtacaact	ggtaaaacccccactgagga
Zm-MPI	X78988	maize proteinase inhibitor	ggataactcggcggattttg	acgtttcggggtgtttgttt
Zm-Cyp6c	T15323	cytochrome P450 monooxygenase	gagagcaaggagcagcagaa	tgcctatctggagcaggttg
Zm-AOC	AY488136	allene oxide cyclase	ccccttcaccaacaaggtgt	accgagatgtggccgtagtc
Zm-GRP unknown EST	-	glycine-rich protein	ggcgacgataaatttgaatgc	tcaaaagccagacacatgcac
Zm-SAUR2	X79211	auxin biosynthesis gene	gtgccttagcacccctgtct	ggctcctctcctgagcaaac
Zm-MFS1	CA452753	multiflux efflux synthase	cactgtgggctgtgagcagt	gcaggccgaaatgtcttgat
Zm-TPS1	AF529266	sesquiterpenecyclase	tgctggcaccatgttctctc	tcgtccacatcttcaaccaa
Zm-ABI	X12564	homology to glycin rich protein	gcgagatcctcgactccaag	gggcttggttaacggtgatg
Zm-lectin	CF032590	lectin (*T. aestivum*)	tcgtcgtccttggagagctt	catctgccaagtccccttct
Zm-PR10	AY953127	pathogenesis-related gene 10	gtcatgccgttcagcttcat	tgttcttgcactcgcacttg

analyses. The model was fitted by maximum quasi-likelihood estimation in the software package R (Version 1.9.1; R-Project, Vienna), and its adequacy was assessed through likelihood ratio statistics and examination of residuals. We tested treatment effects (= odour sources) for naive and experienced wasps individually. In addition we tested if there was a significant effect of the experience and an interaction between treatment x experience.

The amounts of VOCs were analysed using *t*-tests and one-way analysis of variance (ANOVA). The disease symptoms on maize seedlings after inoculations with *S. turcica* and the weight of the *S. littoralis* larvae were analysed using a one-way ANOVA. Differences between groups were analysed using the Tukey's post-hoc test. Data that did not fulfil assumptions for parametric statistics were log-transformed prior to analysis. All comparisons were run on SPSS (11.0) or SPSS (14.0). Due to only 3 to 4 replicates per treatment for the gene-expression studies, no statistical test were carried out with these data.

Results

Effect of soil-born micro-organisms (soil MOs) on indirect defences– We compared the attractiveness of VOC-blends released by *Spodoptera*-infested maize seedlings grown in the presence or absence of an extract with soil MOs to females of the parasitic wasp *Cotesia marginiventris*. Plants of both treatments emitted similar amounts of typically herbivore-induced VOCs, including green-leaf volatiles, shikimic acid derivates, as well as mono-, homo-, and sesquiterpenoids (*t*-Test: $P > 0.05$ for all compounds). However, the blend from plants treated with the soil-extract contained an additional mixture of (2*R*,3*R*)-(-)-, (2*S*,3*S*)-(+)-, and meso (*R,S*)-2,3-butanediol (figure 1 and *appendix II*). The odours of both plant types were attractive to the wasps, however naïve females significantly preferred the odour released by infested plants with MOs (Figure 2 A; GLM: naïve, $F_{1,11} = 5.39$, $P = 0.04$). This preference was even more pronounced after they had been given an oviposition experience in the presence of the odour released by herbivore-infested seedlings with MOs, whereas wasps that had had an experience in the presence of the odour released by seedlings without soil MOs did not show any preference (with soil MOs, $F_{1,11} = 11.74$, $P = 0.006$; without soil MOs, $F_{1,11} = 4.01$, $P = 0.07$). Yet, the shift in the choices made by the two experience types was not statistically significant (GLM for experience x treatment: $F_{1,44} = 53.38$, $P = 0.34$). The responsiveness to plant volatiles (= proportion of wasps that entered an olfactometer arm with plant-derived VOCs) was similar for all groups tested (naïve 72.2%:, without soil MOs: 63.9 %, with soil MOs: 65.3 %).

Healthy, uninfested plants with MOs also released large amounts of the isomeric mixture of 2,3-butanediol (figure 1), but the amount of the optically active isomers was significantly lower compared to the one released by herbivore-infested plants (*t*-Test; (2*R,*3*R*)-(-)- and (2*S,*3*S*)-(+)-butanediol: $t_{22} = 3.32$, $P = 0.003$; meso (*R,S*)-2,3-butanediol: $t_{22} = 1.11$, $P = 0.32$). In addition, uninfested seedlings released substantial amounts β-myrcene and linalool, but these amounts were significantly lower than the amounts released from herbivore-infested seedlings (One-way ANOVA; β-myrcene: $F_{3,44} = 4.82$, $P = 0.006$; linalool: $F_{3,44} = 31.89$, $P < 0.001$). As in the first experiment, naïve *C. marginiventris* females showed a significant preference for a blend released by seedlings with soil MOs (GLM: $F_{1,35} = 5.97$, $P = 0.02$) but the responsiveness was relatively low (50 %) (Figure 2 B).

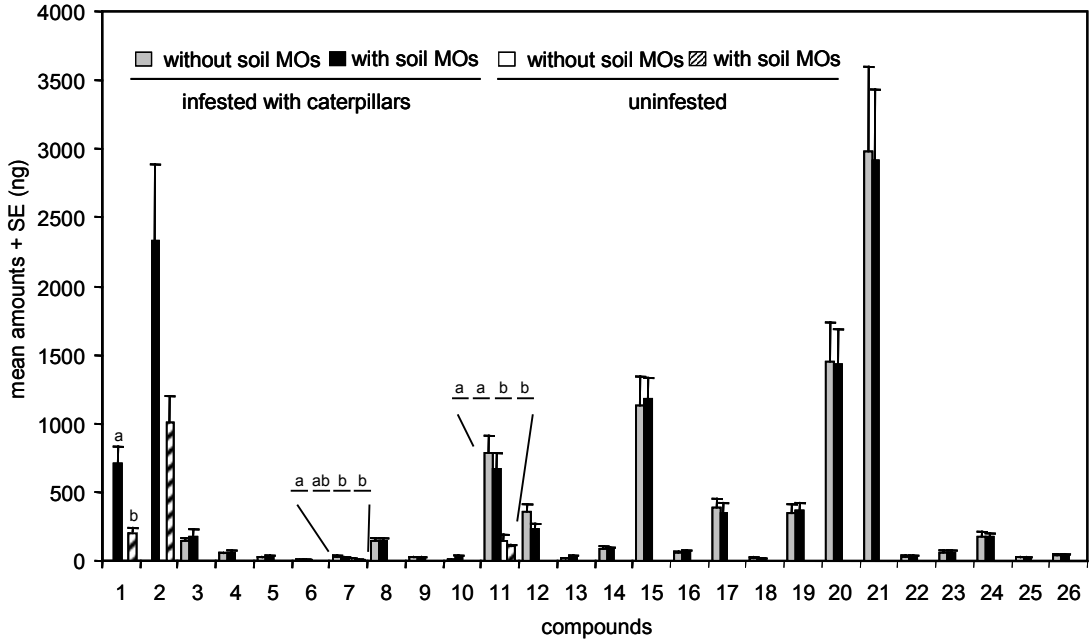

FIG. 1. Mean amount (ng + SE) of VOCs of *Spodoptera*-infested and non-infested maize seedlings grown in the presence and absence of soil-born MOs recollected during the 3 hr olfactory bioassays. Different letters above bars indicate significant differences in the amount of a specific compound (*t*-Test or one-way ANOVA on log - transformed data, $P < 0.05$). N = 12 per treatment. The compounds are: 1 = sum of (2*S*,3*S*)-(+)- and (2*R*,3*R*)-(-)-butanediol, 2 = meso (*R,S*)-2,3-butanediol, 3 = (*Z*)-3-hexenal, 4 = (*E*)-2-hexenal , 5 = (*Z*)-3-hexen-1-ol, 6 = (*Z*)-2-penten-1-ol acetate, 7 = β-myrcene, 8 = (*Z*)-3-hexenyl acetate, 9 = (*E*)-2-hexenyl acetate, 10 = (Z)- β-ocimene, 11 = linalool, 12 = (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), 13 = benzyl acetate, 14 = phenethyl acetate, 15 = indole, 16 = methyl anthranilate, 17 = geranyl acetate, 18 = unknown sesquiterpenoid, 19 = (*E*)-β-caryophyllene, 20 = (*E*)-α-bergamotene, 21 = (*E*)-β-farnesene, 22 = unknown sesquiterpenoid, 23 = unknown sesquiterpenoid, 24 = β-sesquiphellandrene^{N}, 25 = (*E*)-nerolidol, 26 = (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). The compounds are ordered in accordance with their retention time on a non-polar capillary column (HP-1MS). (2*S*,3*S*)-(+)- and (2*R*,3*R*)-(-)-butanediol were separated on a chiral column and identified by comparing the retention times with those of authentic synthetic standard (see *Appendix II*). ^{N} = compounds identified by comparison of with NIST 02 library only.

To test whether the increased attractiveness of volatile blends released by seedlings with soil MOs was due to 2,3-butanediol, wasps were given a choice between the odour of a seedlings grown in autoclaved soil only and of a similarly treated seedling to which a synthetic isomeric mixture of 2,3-butanediol was added directly to the headspace of the plants. The amount of the added compound was in the same range as found in the natural blend (Table 2). No difference in attractiveness could be observed between the control plants and plants that had been complemented with 2,3-butanediol (Figure 2 C, GLM: $F_{1,35} = 0.02$, $P = 0.90$, responsiveness = 68.1

%). In contrast, when the isomeric mixture of 2,3-butanediol was added to the soil in amounts that resulted in similar concentration in the headspace (Table 2), the wasps were significantly more attracted to plants emitting the 2,3-butanediol than to control plants (Figure 2 D, $F_{1,35}$ = 14.22, P < 0.001). The amounts of the plant-derived compounds were similar as in the blends of healthy uninfested seedlings grown in autoclaved soil only (Table 2) and the responsiveness was 67.6 %.

TABLE 2. Mean amounts (± SE) of VOCs released by non-infested maize seedlings grown in presence or absence of micro-organisms and 2,3-butanediol.

experiment	compounds	treatment			N
Fig 2B		without MOs	with soil MOs		
	(R,R)- and (S,S)-2,3-butanediol	0.0 ± 0.0	154.6 ± 25.2		12
	(*R,S*)-2,3-butanediol	0.0 ± 0.0	853.6 ± 142.5		12
	ß-myrcene	11.2 ± 2.2	9.0 ± 0.7	n.s.	12
	linalool	99.9 ± 17.3	98.5 ± 13.0	n.s.	12
Fig 2C		without MOs	without MOs & BD in airflow		
	(R,R)- and (S,S)-2,3-butanediol	0.0 ± 0.0	304.6 ± 53.7		6
	(R,S)-2,3-butanediol	0.0 ± 0.0	1151.2 ± 503.0		6
	ß-myrcene	11.7 ± 3.4	8.7 ± 1.6	n.s.	6
	linalool	94.7 ± 23.0	79.9 ± 14.7	n.s.	6
Fig 2D		without MOs	without MOs & BD in soil		
	(R,R)- and (S,S)-2,3-butanediol	0.0 ± 0.0	261.1 ± 86.2		6
	(R,S)-2,3-butanediol	0.0 ± 0.0	384.1 ± 109.2		6
	ß-myrcene	16.5 ± 1.9	12.2 ± 2.6	n.s.	6
	linalool	121.0 ± 32.4	115.6 ± 35.2	n.s.	6
Fig 3B		without bacteria	with bacteria		
	(R,R)- and (S,S)-2,3-butanediol	0.0 ± 0.0	152.9 ± 58.5		12
	(R,S)-2,3-butanediol	0.0 ± 0.0	465.6 ± 131.1		12
	ß-myrcene	15.4 ± 4.0	5.6 ± 1.0	*	12
	linalool	144.9 ± 45.6	61.7 ± 12.0	*	12

Values correspond to the mean amounts recollected during different experiments as indicated in the figures. * = significant difference in the amounts of a specific compound between two treatments (*t*-Test, P < 0.05), n. s. = no significant difference P > 0.05.

Isolation and identification of the 2,3-butanediol producing bacterial endophyte Enterobacter aerogenes – To localise the MOs responsible for the induction of 2,3-butanediol, we did detailed analyses of the volatile blends collected from different parts of healthy, 2,3-butanediol releasing seedlings, to which we added the soil-born MOs (Figure 3). Major compounds known from fermentation pathways of bacteria were mainly present in germinated seeds and were ethanol (1), acetoin (5) and (2*R*,3*R*)-(-)- and (2*S*,3*S*)-(+)-butanediol (6), and meso (*R,S*)-2,3-butanediol (7). Sheath and leaves of the seedlings released an isomeric mixture of 2,3-butanediol and some acetoin. With the exception of trace amounts of acetoin in roots, we did not detect any of these compounds in soil samples (chromatograms not shown).

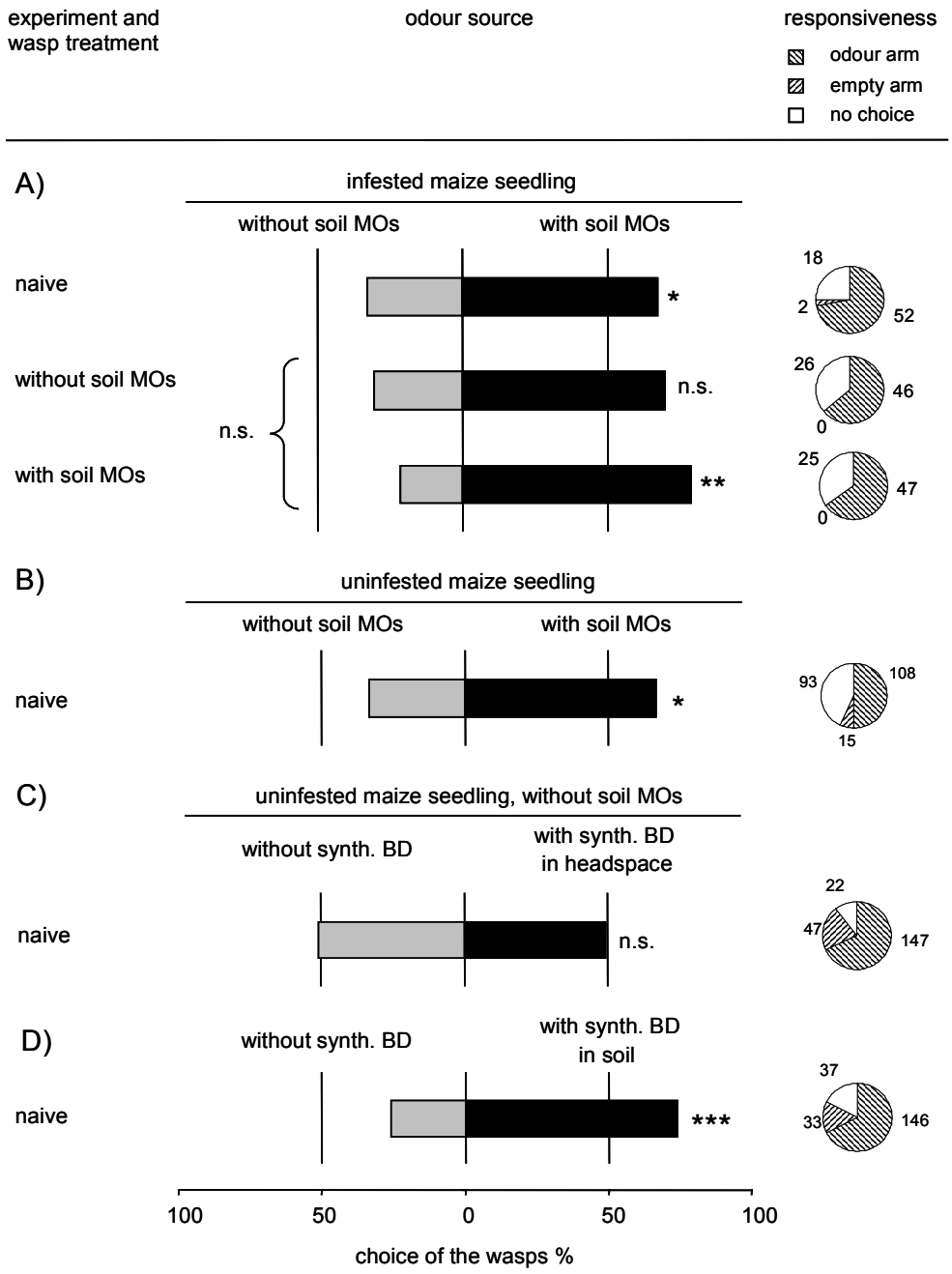

FIG. 2. Responses of *C. marginiventris* females to VOC-blends released by maize seedlings. Wasp treatment is given on the left site of the graphs, and pie charts on the right of the graphs indicate the number of wasps entering arms carrying plant derived odours, arms with clean air only or not entering an arm at all (no choice). A) *Spodoptera*-infested seedlings grown in the presence or absence of soil-born MOs, B) uninfested seedlings grown in the presence or absence of soil-born MOs, C) uninfested seedlings grown in the absence of soil-born MOs with or without an isomeric mixture of synthetic 2,3-butanediol added to the headspace, and D) as C) but synthetic 2,3-butanediol added to the soil. Composition of the blends of experiment A) and B) are given in Figure 1 and of experiments B), C) and D) in Table 2. GLMs were performed in order to test for differences between the two odour arms within one group of wasps as well as to compare the types of experiences. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, n. s. = no significant difference $P > 0.05$.

The additional compounds in the graph were also detected in seedlings without soil-born MOs. The graphs suggest a high microbial activity in germinated seeds and therefore, we used surface sterilised maize seeds to isolate bacteria. One bacterial isolate that was able to produce 2,3-butnaediol from maize seeds (see *appendix III*) was identified as *Enterobacter aerogenes* (synonymous with *Klebsiella mobilis*) based on its 16S rRNA gene sequence, physiological tests and microscopy. Incubating surface-disinfected maize seeds into a suspension of these bacteria (~ 10^8 CFU/mL) produced maize seedlings that released substantial amounts of (2*R,*3*R*)-(-)- and (2*S,*3*S*)-(+)-butanediol and meso (*R,S*)-2,3-butanediol 10 days after planting (Figure 3 B). The amounts of the bacteria-derived VOCs were comparable to those released by seedlings to which we added the soil-born MOs. However, the bacteria-treated plants contained significant lower amounts of β-myrcene and linalool compared to control plants (*t*-Test; β-myrcene: t_{22} = 2.37, P = 0.027; linalool: t_{22} = 2.30, P = 0.031) (Table 2). Interestingly, adding bacteria or the extract of the soil micro-organisms to the seedlings resulted not always in the release of 2,3-butanediol. In an additional time dependent experiment (data not shown), some seedlings released 2,3-butanediol already at the first sampling day (six days after planting), while others released 2,3-butanediol later, at day 8, 10 or 12. In approximately 20 % of all sampled seedlings to which we added the bacteria we did not detect 2,3-butanediol at all during the 14 days of sampling, while we occasionally detected 2,3-butanediol in control seedlings.

Effects of soil-MOs, E. aerogenes*, and 2,3-butanediol on direct defences of maize against the herbivore* Spodoptera littoralis – The feeding rate of the larvae of the noctuid moth *S. littoralis* was similar on seedlings grown in the presence or absence of soil-born MOs; the mean leaf areas removed by the larvae during a 24 hr feeding period were 5.95 ± 0.41 cm^2 on plants without MOs and 6.43 ± 0.86 cm^2 on plants with soil MOs (*t*-Test: t_{20} = - 0.15, P = 0.882). However, larvae grow slightly bigger on maize seedlings with *E. aerogenes* than on maize seedlings grown in autoclaved soil only (Figure 4). This weight difference was significant only 3 and 4 days after continuous feeding on these seedlings (one-way ANOVA: day 3, $F_{2,203}$ = 4.42, P = 0.013; day 4, $F_{2,193}$ = 4.05, P = 0.019) but not before or after these time periods. Larvae feeding on seedlings to which we added synthetic 2,3-butanediol showed an intermediate weight (P > 0.05). The mortality of the larvae after 4 days of feeding was similar for all treatments, reaching 11.1% on seedlings with bacteria,

5.6 % on seedlings with synthetic 2,3-butanediol, and 9.7 % on seedlings in autoclaved soil only, and increased to 18.1% on seedlings with the bacteria, 8.3 % on seedlings with synthetic 2,3-butanediol, and 12.5% on seedlings in autoclaved soil at the end of the experiment.

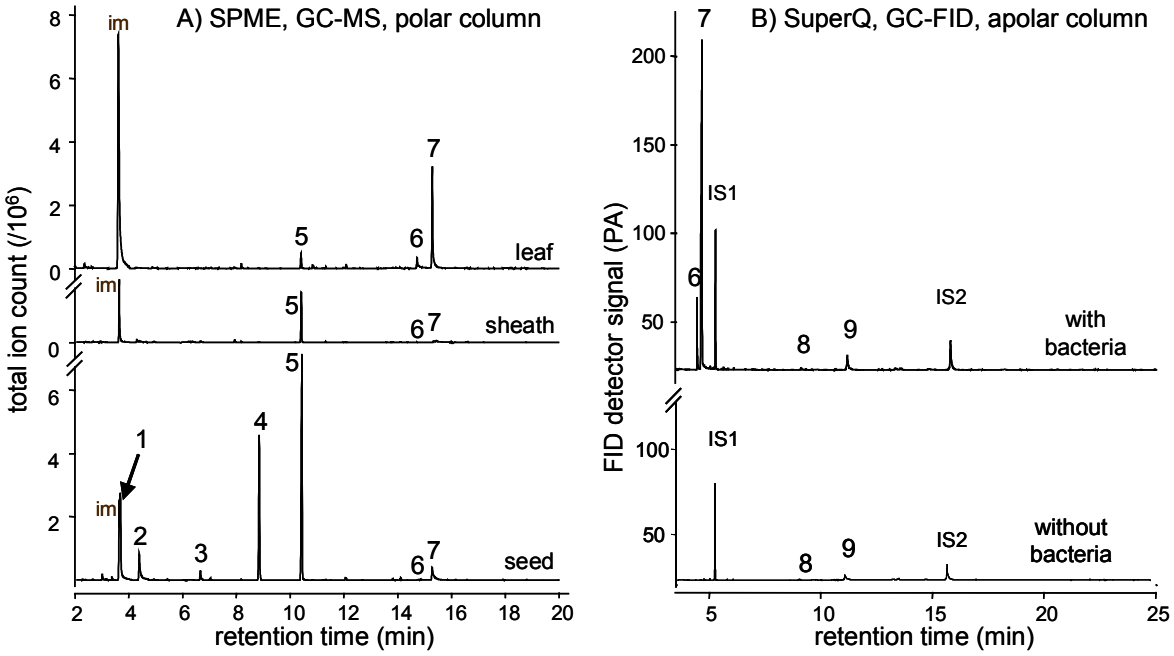

FIG. 3. Typical chromatographic traces of VOCs released by 10-day old maize seedlings A) Volatile profiles of various parts of maize seedlings grown in the presence of soil-born MOs. VOCs were adsorbed on a SPME fiber and analysed by GC-MS on a polar column. B) Volatile profiles of intact, non damaged maize seedlings in the presence or absence of *E. aerogenes* (10^8 CFU/mL). VOCs were adsorbed on SuperQ traps and analysed by GC-MS on an apolar column. The compounds are 1 = ethanol, 2 = 2,3-butandione^N, 3 = 2-methyl-1-propanol^N, 4 = 3-methyl-1-butanol^N, 5 = acetoin, 6 = sum of (2*S*,3*S*)-(+)- and (2*R*,3*R*)-(-)-2,3-butanediol, 7 = meso (*R*,*S*)-2,3-butanediol, 8 = *β*-myrcen, 9 = linalool. im. = impurity from the system.

Effects of E. aerogenes *and its major volatile metabolites on the resistance of maize seedlings against the pathogen* Setosphaeria turcica – In a first experiment, we found significant differences in disease symptom severity between maize seedlings grown in the presence or absence of the bacterium *E. aerogenes* and its volatile metabolites three days after exposing the seedlings to spores of a ~ 8-week old *S. turcica* Petri dish culture (Figure 5, one-way ANOVA: $F_{5,58}$ = 16.64, P < 0.001). Tukey's test indicated a significant reduction of the necrotic and/or chlorotic leaf-

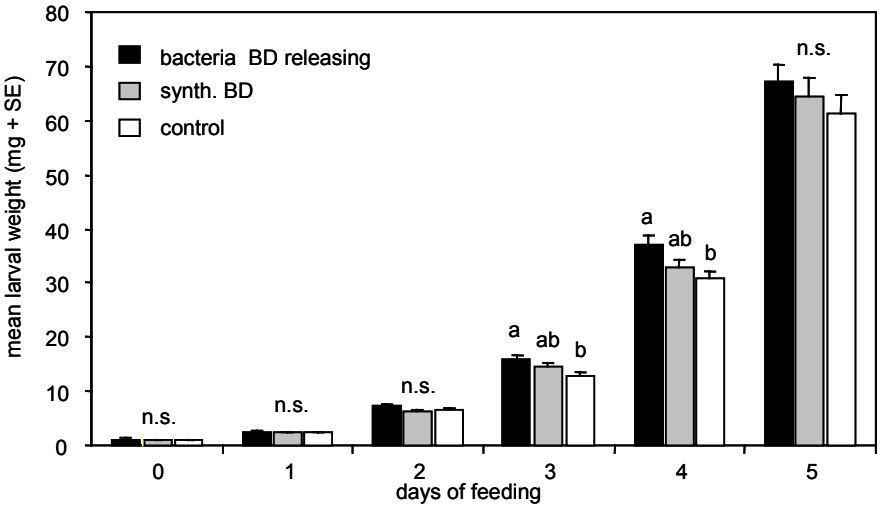

FIG. 4. Mean weight (± SE) of *S. littoralis* larvae feeding on maize seedlings grown in autoclaved soil only (control), on seedlings with added synthetic 2,3-butanediol, or with added *Enterobacter aerogenes* bacteria. Larvae were weighed and transferred to fresh plants with the same treatments daily. Different letters indicate significant differences of the larval weights at one specific day (one-way ANOVA, $P < 0.05$). N = 72 per treatment.

area of 2,3-butanediol releasing maize seedlings with *E. aerogenes* compared to maize seedlings grown in autoclaved soil only ($P < 0.001$). However, this difference was less pronounced for the few seedlings inoculated with the bacteria that did not release 2,3-butanediol one day before treatment with the pathogen ($P = 0.090$). Isomeric mixtures of 2,3-butanediol and acetoin also had a strong effect on the reduction in diseased leaf-area compared to seedlings without these volatiles ($P < 0.001$ for both treatments). A structural mimic of 2,3-butanediol, 2-butanol had no effect on the resistance of maize seedlings against the fungus compared to the control seedlings ($P = 0.909$).

In a second experiment, using spores of a younger pathogen culture (~ 3 weeks old), we measured slightly bigger diseased leaf areas than in the first experiment (Figure 6 A). Although there was still a significant difference between the three treatments (one-way ANOVA: $F_{2,28} = 3.454$, $P = 0.047$), compared to the control the necrotic and/or chlorotic leaf area was only significantly reduced by adding synthetic 2,3-butanediol ($P = 0.049$). However, the spore tubes were significantly shorter in the treatments with the bacteria and with synthetic 2,3-butanediol, indicating that both the bacteria and the synthetic 2,3-butanediol induced resistance against leaf colonization by *S. turcica* (Figure 6 B and C, one-way ANOVA: $F_{2,129} = 14.75$, $P < 0.001$).

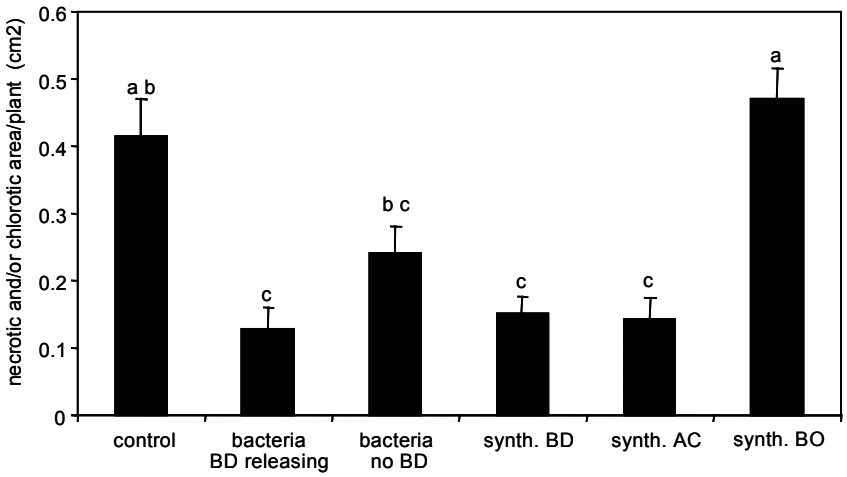

FIG. 5. Mean (± SE) necrotic and chlorotic leaf area on maize seedlings with and without *E. aerogenes* or synthetic compounds 3 days after inoculations with spores of the pathogen *S. turcica*. BD = 2,3-butanediol, AC = acetoin, BO = 2-butanol, synth. = synthetic compounds. Different letters indicate significant differences between the treatments (one-way ANOVA, $P < 0.05$). N = 12 for all treatments, except for plants with *E. aerogenes* that did not release 2,3-butanediol: N = 6.

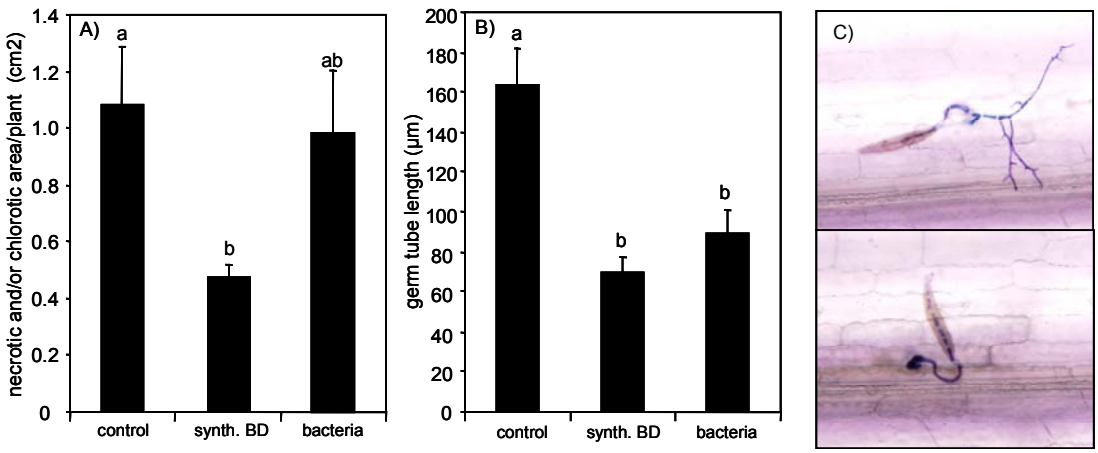

FIG. 6. Disease symptoms induced by the fungal pathogen *S. turcica* on maize seedlings gown in the presence of *E. aerogenes*, synthetic 2,3-butanediol, or autoclaved soil only. Different letters above bars indicate a significant difference between the treatments (one-way ANOVA: $P < 0.05$). A) Mean (± SE) necrotic and chlorotic leaf area as in figure 5. N = 11 control, N = 8 other treatments. B) Mean (± SE) spore tube length on trypan blue stained maize leaves 3 days after inoculations with spores of the pathogen. N = 46 control, N = 42 other treatments. C) Typical pictures of germinated spores of *S. turcica* on maize leaves three days after inoculation. Leaf disks were stained with lactophenol trypan-blue and sum of tube lengths were examined and measured microscopically.

Effects of E. aerogenes *and 2,3-butanediol on the expression of pathogen and herbivore inducible genes* – In a first experiment we found that the pathogen *S. turcica* strongly induced the expression of the pathogenesis related gene, *Zm-PR5*, but not of the herbivore-inducible proteinase inhibitor genes, *Zm-SerPI* and *Zm-MPI*, during the three days of infestation (Figure 7A). In contrast, mimicking larval feeding by scratching maize leaves with a razor blade and applying regurgitant of *S. littoralis* resulted in a strong induction of *PI* genes, but not of the *Zm-PR5* gene during the 24 hr sampling period. Interestingly, synthetic 2,3-butanediol (5 mM) and the addition of *E. aerogenes* bacteria had no effect on the expression levels of these three selected defence genes. In a second experiment, we quantified gene expression of a dedicated set of 33 typically stress-inducible marker genes of maize (J. Ton and D. Karlen, unpublished results). In this transcriptional profiling, the expression of *Zm-SerPI* and *Zm-MPI* was up-regulated after application of the plant hormone jasmonic acid (JA, 200 µM), but suppressed after treatment with benzothiadiazole (BTH, 5 mM), a mimic of the plant hormone salicylic acid (SA) (Figure 7B). In contrast, the SA-inducible *Zm-PR1 and Zm-PR5* genes were more strongly up-regulated upon BTH treatment. Furthermore, a range of other typically stress-inducible genes were differentially up- or downregulated after BTH and JA treatments, but neither 2,3-butanediol (5 mM) nor *E. aerogenes* bacteria had strong effects on the expression level of these defence-related genes. Cluster analysis of the Euclidian distance between the four different gene expression patterns revealed that the gene expression pattern of treatment with synthetic 2,3-butanediol closely resembles the gene expression pattern of the *E. aerogenes* treatment. This suggests that the effects of synthetic 2,3-butanediol and *E. aerogenes* bacteria on the resistance against *S. littoralis* and *S. turtica* are not based on activations of the JA- and/or SA-dependent defence pathways.

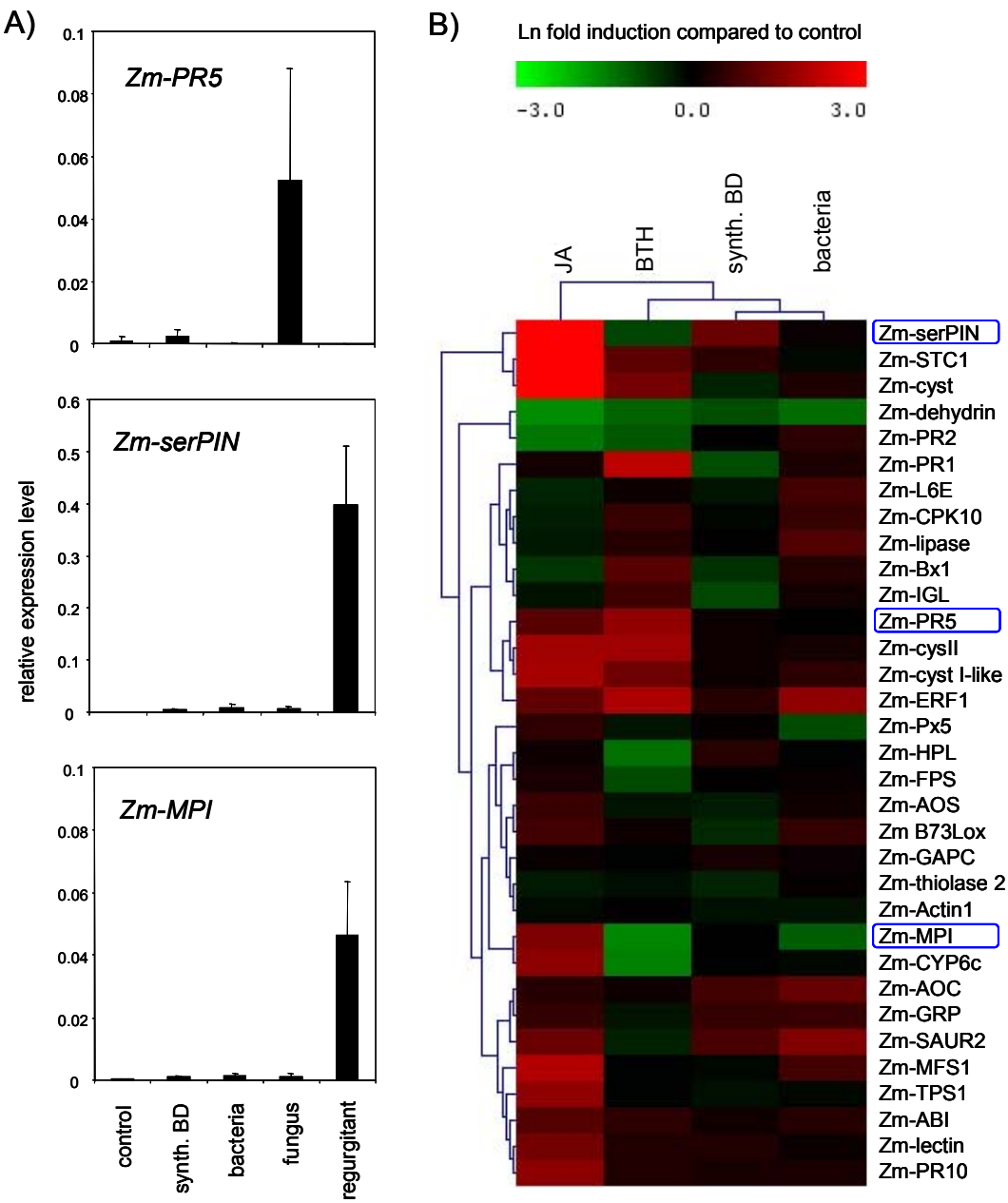

FIG. 7. Gene expression studies of major stress inducible genes in maize seedlings. A) Relative (± SE) transcription levels of selected stress inducible genes in plants that were left undamaged (control), treated with synthetic 2,3-butanediol (synth. BD, 5 mM), with the endophyte *E. aerogenes* (bacteria), with the pathogen *S. turcica* (fungus), or with *S. littoralis* regurgitant (regurgitant). N = 3-4 for all treatments. B) Fold induction (Ln scale) of selected stress inducible genes in maize seedlings treated with JA (200 µM), with BTH (5 mM), with synth. BD (5 mM) or with bacteria compared to expression levels of water treated control seedlings (one biological replicate, 3 pooled maize seedlings per treatment).

Discussion

From recent studies it has become increasingly clear that inducible plant defences are highly complex and variable depending on various aboveground and belowground factors (van Dam et al., 2003; Van der Putten et al., 2001, Turlings and Ton, 2006). The current study adds to this complexity and reveals an important role of soil-born, endophytic micro-organisms and their volatile metabolites in defence responses of maize plants against herbivorous insects and fungal pathogens.

Effect of soil-born micro-organisms (soil MOs) on indirect defences – In a first series of experiments, we show that soil MOs have an effect on indirect defence responses of maize seedlings by increasing the attractiveness of the seedlings to the parasitic wasp *Cotesia marginiventris* (Figure 2). The same wasp, which strongly relies on herbivore-induced VOCs to locate its hosts (Turlings et al., 1990), has previously been shown to be more attracted to herbivore-infested peanut plants that were also infected by white mould than to herbivore-infested plants without the mould (Cardoza et al., 2003). Diseased peanut plants released 3-octanone and methyl salicylate in addition to the herbivore-induced VOCs, showing that pathogenic MOs can indeed interact with indirect plant defences. In contrast, a study with maize seedlings infected with the pathogenic fungus *Setosphaeria turcica* found no effects of the infection on the attraction of the parasitoids *C. marginiventris* and *Microplitis rufiventris* (Rostás et al., 2006). Soil MOs also have been studied in this context. For example, tomato plants with arbuscular mycorrhizal fungi were more attractive to aphid parasitoids than plants without mycorrhiza (Guerrieri et al., 2004), and in field studies and laboratory experiments Gange et al. (2003) demonstrated varying effects of plant species with different types of arbuscular mycorrhizal fungi on the parasitism of *Chromatomyia syngenesiae* by its parasitoid *Diglyphus isaea*. Moreover, beneficial endophytic fungi can also modify the attractiveness of volatile blends to natural enemies (Feath and Bultman, 2002). Yet, these studies did not identify any particular volatile compound that might be responsible for the observed effects of such beneficial micro-organisms on indirect defences.

The novelty of our study lies in the detection of the isomeric mixture of 2,3-butanediol in the phyllosphere of seedlings grown in the presence of soil MOs. This compound was released by

herbivore-infested and non-infested maize seedlings and was the only major difference between volatile blends of seedlings grown in the presence or absence of the soil MOs (Figure 1). Interestingly, the addition of a synthetic mixture of the isomers of 2,3-butanediol to the headspace of non-infested seedlings without MOs did not result in enhanced attractiveness of the supplemented blends, but adding the synthetic compounds in similar concentrations to the soil significantly increased the attractiveness (Figure 2 C and D). This suggests that the parasitoids are not attracted to 2,3-butanediol itself, but rather to other subtle changes in volatile profiles induced by soil MOs or application of 2,3-butanediol to the soil. We did not detect any changes in the constitutively released compounds (Table 2), implying that minor changes, below the threshold level of the chemical analyses, can be perceived by the wasps and affect their responsiveness towards the volatile blends. It further remains to be investigated whether this enhanced attraction of *C. marginiventris* to volatiles of seedlings with added MOs would be ecologically relevant, as in the field all plants can be expected to be exposed to soil MOs. For the same reason, the enhanced attractiveness of the plants could merely reflect the fact that exploitation of volatile host location cues by parasitoids has evolved in the consistent presence of micro-organisms, and therefore plants growing in the presence of soil MOs are more attractive than plants growing in autoclaved soil only.

Minor changes in volatiles blends could become ecologically relevant for parasitoids that are able to associate volatile cues with the presence of their hosts during oviposition experiences (Turlings et al. 1993; Vet et al. 1995). *C. marginiventris* females, for example, have been shown to learn differences in volatile blends during oviposition experiences, and afterwards they were more attracted to the learned odours (Turlings et al., 1993; D'Alessandro and Turlings, 2005; Tamò et al., 2006). Here we found that the attractiveness of the blends released by seedlings with soil MOs was slightly more pronounced after the wasps experienced the volatiles released by seedlings with MOs (Figure 2A). However, the non-significant shift in preference suggests that 2,3-butanediol is not strongly associated with host presence during oviposition experiences. A previous study already indicated that not all compounds in the parasitoid's complex volatile environment are strongly associated with host presence during oviposition experiences (D'Alessandro and Turlings, 2006). Like for the innate responses, it remains to be determined which compounds are of key importance for learned responses in this and other parasitoids.

Isolation and identification of the 2,3-butanediol producing bacterium Enterobacter aerogenes – 2,3-Butanediol is typically produced by a wide range of micro-organisms as a fermentation product under limited oxygen availability and low pH levels (Syu, 2001). To localise the production of 2,3-butanediol in maize seedlings grown in soil with MOs, we sampled VOCs from different aboveground and belowground parts of 10-day old uninfested maize seedlings. Highest amounts of the 2,3-butanediol were emitted by leaves, and less by sheaths, and germinated seeds (Figure 3 A). Acetoin, the precursor of 2,3-butanediol, was found to be predominately emitted by germinating seeds. With the exception of trace amounts of acetoin in the roots, we did not detect any 2,3-butanediol or acetoin in the roots or in the soil. Hence, the VOC analyses indicated a high microbial activity in the germinated seeds exposed to micro-organisms and, indeed, we were able to cultivate several endophytic bacterial isolates from surface-sterilised, germinated maize seeds. Based on 16S ribosomal DNA sequences and metabolic test strips one of the bacterial isolates was identified as the y-proteobacterium *Enterobacter aerogenes* (synonymous with *Klebsiella mobilis*). This bacterium readily fermented thyndallised (sterilization by 3 x boiling seeds in autoclaved H2O) maize seeds to 2,3-butanediol (see *Appendix III*) and caused the release of 2,3-butanediol in maize seedlings (Figure 3 B). Several strains of *E. aerogenes* are known to produce 2,3-butanediol (Johansen et al., 1975; Syu, 2001) and various endophytic bacteria, including *Enterobacter* spp., have previously been isolated from maize plants (Hinton and Bacon, 1995; McInroy and Kloepper, 1995; Fisher et al., 1992; Bressan and Borges, 2004; Zinniel et al., 2002). Interestingly, the stereochemistry of 2,3-butanediol production is strain and species specific and *E. aerogenes* has been predominantly found to synthesise the meso (*R,S*)-2,3-butanediol (Hohnbentz and Radler, 1978), the same major isomer that we detected in the maize volatile blend. Thus, although acetoin forming enzymes have been identified in maize cell cultures (Forlani, 1999), our data imply that 2,3-butanediol released by maize seedlings is produced by endophytic bacteria, as for example *E. aerogenes*, rather than by the plant itself.

Effects of E. aerogenes *and its major volatile metabolites on direct defences* - There are numerous examples of how endophytic micro-organisms interact with plant resistance against insects and pathogens and depending on the type of endophyte the effects can be negative, neutral or positive (for reviews see Hallmann et al., 1997; Sturz et al., 2000; Selosse et al., 2004).

Our study showed that incubating maize seeds with the bacterium *E. aerogenes* resulted in seedlings that were more resistant against the northern corn leaf blight *Setosphaeria turcica*, while *Spodoptera littoralis* larvae grow slightly better on the bacteria-treated seedlings compared to non-treated seedlings (Figure 4, 5, and 6). The mechanism of these bacteria-induced changes in resistance appears to be linked to the release of 2,3-butanediol and its precursor acetoin. Synthetic versions of both compounds had similar effects on pathogen resistance, while 2-butanol did not show any effect at all. In the few cases where the addition of the bacteria did not result in of the release of 2,3-butanediol by seedlings they were less resistant to the pathogen. Hence, 2,3-butanediol is at least partially responsible for the observed differences in the defence responses. Moreover, 2,3-butanediol has previously been identified in a volatile blend released by the plant growth promoting rhizobacteria (PGPR), e.g. *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a, and was found to promote growth and induce systemic resistance in *Arabidopsis* plants against the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* (Ryu et al., 2003, Ryu et al., 2004). Recently, 2,3-butanediol has also been identified in a culture filtrate of the plant-beneficial rhizobacterium, *Pseudomonas chlororaphis* 06, and the (2*R*,3*R*)-(-)-butanediol promoted growth and induced systemic resistance in tobacco to *E. carotovora* subsp. *carotovora*, but not to *Pseudomonas syringae* pv. *tabaci* causing wildfire (Han et al., 2006). Apparently, the isomeric form of 2,3-butanediol was important in this study because (2*S*,3*S*)-(+)-butanediol did not affect the plant. Moreover, 2,3-butanediol might only affect the resistance against some pathogens. We detected all three isomers of 2,3-butanediol and it remains to be determined which of the isomers were biological active. Yet, the current results provide further evidence that bacteria-derived C_4-VOCs, such as 2,3-butanediol and acetoin, are important regulators of plant defence responses (Ping and Boland, 2004; Paré et al., 2005). Furthermore, we show that such 2,3-butanediol producing bacteria might be endophytic and that the volatiles they emit may contribute to their symbiotic interactions with plants.

Possible mechanism of VOC-induced plant defence responses - Already back in the seventies Dennis and Webster (1971) suggested that some VOCs of endophytic *Trichoderma* species have antimicrobial activity. More recently, the endophytic fungus *Muscodor albus* was found to produce a mixture of VOCs that act synergistically to kill a wide variety of plant and human

pathogenic fungi and bacteria (Strobel, 2006). Although we cannot entirely rule out possible direct effects of VOCs from *E. aerogenes* on the pathogen *S. turcica,* our olfactometer experiments also suggest that 2,3-butanediol is a plant bioactive compound inducing physiological changes in maize seedlings, which can be detected by parasitic wasps in the emitted volatile blends. Indeed, various VOCs have the potential to affect defence responses even in neighbouring plants (Farmer, 2001; Engelberth et al., 2004; Baldwin et al., 2006, Ton et al. 2006).

Mechanisms behind modified resistances in plants due to interacting micro-organisms are very diverse and range from the activation of pathogenesis related genes (PR-genes), as for example found in systemic acquired resistance (Morris et al., 1998), to 'priming' effects typically found in induced systemic resistance (Conrath et al., 2002). Despite a multitude of possible mechanisms, it is generally accepted that plant hormones, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) play key roles in orchestrating plant defence responses (Spoel et al. 2003; Thaler and Bostock. 2004; De Vos et al., 2005; Jalali et al., 2006). We measured the expression levels of defence-related genes, which typically are induced by pathogens, herbivores, and the plant hormones JA and SA (Figure 7). Neither the addition of the bacteria *E. aerogenes* nor synthetic 2,3-butanediol had any major effect on the expression of these genes, indicating that the observed difference in the defences probably depended on other signalling compounds. One candidate might be ET, which is believed to be involved in the enhanced resistance after exposing *Arabidopsis* to the VOCs of *Bacillus subtilis* GB03 (Ryu et al., 2004). ET is known to synergise VOC-emission in maize seedlings after exposure to green leaf volatile (*Z*)-3-hexenol, JA or volicitin, an elicitor found in the regurgitant of *Spodoptera*-larvae, but in contrast, it suppresses the amounts of constitutively emitted VOCs in untreated healthy maize seedlings (Schmelz et al., 2003b; Ruther and Kleier, 2005). In our experiments the amounts of amounts of β-myrcene and linalool that were constitutively emitted by uninfested maize seedlings, were significantly reduced in seedlings to which we added *E. aerogenes* compared to seedlings without bacteria, indicating a possible involvement of ET (Table 2). However, there was no such effect in the seedlings exposed to the extract of MOs or 2,3-butanediol in the soil. An alternative explanation for the induced resistance after exposure to 2,3-butanediol comes from studies on maize exposed to herbivore-induced VOCs of neighbouring plants. It was found that theses plants, although they were temporarily more resistant against the herbivore *S. littoralis*, did not have

enhanced expression levels of typically inducible defence genes, but rather they were ‘primed’, which enabled them to respond much faster to a future herbivore attack (Ton et al, 2006). It would be interesting to investigate, whether such priming effects also exist in plants harbouring endophytic 2,3-butanediol releasing bacteria.

Finally, it has been proposed that VOCs and their effects on plant defences might be exploitable to enhance the control of agricultural pests (D'Alessandro and Turlings, 2006; Turlings and Ton, 2006). A good understanding of how volatile metabolites of endophytic bacteria affect plant defence responses and the interactions with other organisms across trophic levels can be expected to contribute to such novel pest control strategies.

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Chapter V

Advances and challenges in the identification of volatile compounds that mediate interactions between plants and arthropods

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Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods

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The relatively new research field of Chemical Ecology has, over the last two decades, revealed an important role of plant-produced volatile organic compounds (VOCs) in mediating interactions between plants and other organisms. Of particular interest are the volatile blends that plants actively emit in response to herbivore damage. Various efforts are underway to pinpoint the bioactive compounds in these complex blends, but this has proven to be exceedingly difficult. Here we give a short overview on the role of herbivore-induced plant volatiles in interactions between plants and other organisms and we review methods that are currently employed to collect and identify key volatile compounds mediating these interactions. Our perspective on future directions of this fascinating research field places special emphasis on the need for an interdisciplinary approach. Joint efforts by chemists and biologists should not only facilitate the elucidation of crucial compounds, but can also be expected to lead to an exploitation of this knowledge, whereby ecological interactions may be chemically manipulated in order to protect crops and the environment.

Introduction

Until recently, it was not common knowledge that plants have a way to express themselves. They mainly do so by emitting odours and the chemical composition and intensity of these odours can carry information on the plants' physiological status and on the stresses that they are being subjected to. In fact, plants emit an enormous spectrum of volatile organic compounds (VOCs). At present, more than 1000 of these low molecular weight organic compounds are known, ranging from terpenoids, fatty acid derivatives, benzenoids and phenylpropanoids, C5-branched compounds and various nitrogen and sulfur containing compounds.[1] Some of these VOCs are constitutively emitted by undamaged, healthy plants, but herbivore damage commonly induces plants to emit much larger amounts and may even cause several VOCs to be synthesised *de novo*[2 4] (Fig. 1). These herbivore-induced plant volatiles (HIPVs) are known to be emitted by various parts of the plant, including leaves[4 7] and flower buds[8] as well as roots.[9] Not only feeding by a herbivorous insect induces the release of HIPVs; even deposition of eggs by herbivorous insects has been shown to induce the plant to emit HIPVs.[10 12] In addition, HIPV emission is not limited to the site of damage but occurs systemically throughout the plant, also from undamaged leaves.[13 17] Many insects are well aware of this fragrant lingo of plants and have evolved ways to exploit it (Fig. 2). Recently, researchers have also become aware of this and now HIPV emissions have been the subject of an increasing number of investigations that have revealed an important role of HIPVs in communication and self-protection. Although molecular and genetic approaches are now frequently applied to reveal the intricacies of

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Marco D'Alessandro is currently finishing his PhD thesis in Chemical Ecology at the University of Neuchâtel under the supervision of his co-author Ted Turlings. During his research Marco has developed and employed different methods to identify key volatile organic compounds that attract parasitic wasps. He previously studied at the University of Zurich and at the Swiss Federal Institute of Technology Zurich, and worked

as a research assistant at the USDA research station, Tifton, Georgia, USA and at the Swiss federal research station in Reckenholz (FAL), where he studied physiological and ecological aspects of the interactions between plants and insects. Currently he is also working on belowground interactions between plants and microbes and on the effects of these interactions on other organisms aboveground.

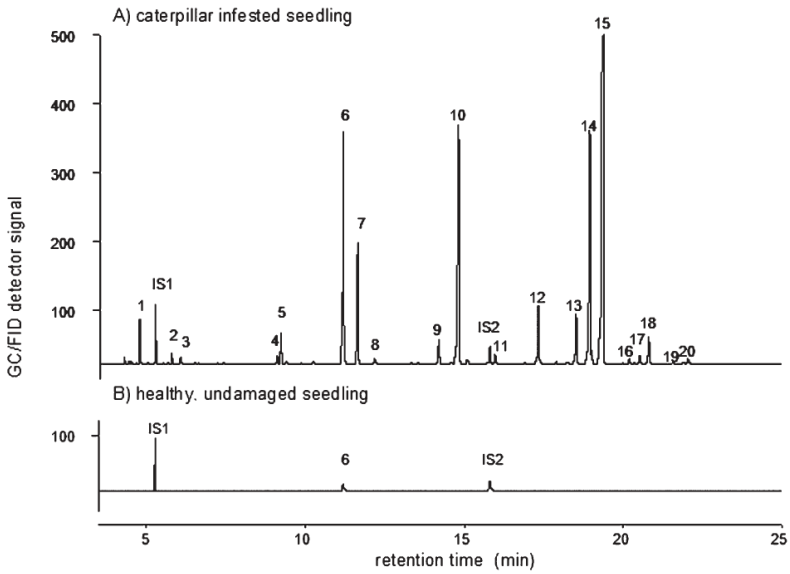

Fig. 1 Example of GC-FID chromatograms obtained with the collected volatiles of 10-day old maize seedlings, (A) seedlings infested with *Spodoptera littoralis* larvae (B) undamaged seedlings. Further details on material and methods are given by D'Alessandro and Turlings.[103] Compounds are: 1 = (*Z*)-3-hexenal, 2 = (*E*)-2-hexenal, 3 = (*E*)-3-hexen-1-ol, 4 = β-myrcene, 5 = (*Z*)-3-hexen-1-ol acetate, 6 = linalool, 7 = (3*E*)-4,8-dimethyl-1,3,7-nonatriene, 8 = benzyl acetate, 9 = phenethyl acetate, 10 = indole, 11 = methyl anthranilate, 12 = geranyl acetate, 13 = (*E*)-β-caryophyllene, 14 = (*E*)-α-bergamotene, 15 = (*E*)-β-farnesene, 16 = unknown sesquiterpenoid, 17 = unknown sesquiterpenoid, 18 = β-sesquiphellandrene, 19 = (*E*)-nerolidol, 20 = (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. IS1 and IS2 = internal standards.

plant-mediated interactions, the chemical analyses of HIPVs remain an integral part of virtually all studies.

Below we provide a short overview of what is known about the physiological and ecological role of HIPVs in interactions between plants and other organisms (for recent reviews see ref. 18–21), we further review methods commonly used by biologists to collect and analyse HIPVs and highlight the challenges that remain to be tackled in this area of research. The focus is on techniques used to study the importance of such HIPVs for the attraction of arthropods aboveground, and we propose some methods that could be useful in future studies, including the analysis of HIPVs belowground. Further details on these techniques and on techniques used to analyse plant VOCs in general have been described and reviewed elsewhere (*e.g.* ref. 22–26). Finally, we discuss and stress the importance of testing the biological relevance of HIPVs with appropriate behavioural bioassays and we propose that the remarkable sensitivity of insect chemoreceptors to VOCs make them exploitable as tools for future research on plant odours.

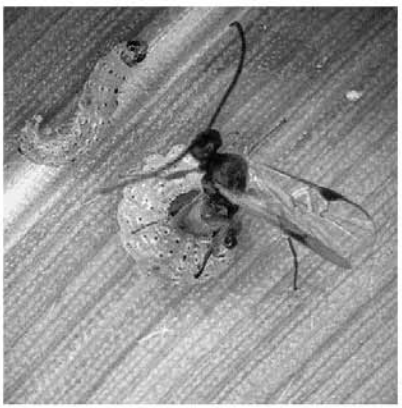

Fig. 2 A female of the parasitic wasp *Cotesia marginiventris* lays an egg in a caterpillar. The wasp found its victim with the use of volatiles emitted by the plant in response to being damaged by the caterpillar. Picture by Matthias Held.

Physiology, ecology and application of biologically relevant HIPVs

HIPV-blends are complex and consist of a variety of divergent VOCs, including alkanes, alkenes, aldehydes, alcohols, ketones, ethers, esters and carboxylic acids. It is unlikely that every VOC emitted by plants serves an ecological or physiological role[27] and there is still disagreement about the evolutionary history and function of plant VOCs.[20,27,33]

Undoubtedly, HIPVs play a central role in mediating interactions between plants and herbivores, between herbivores and their natural enemies, between plants and microorganisms, but also between plants themselves.[21,34,35] For example, laboratory studies have shown that HIPVs deter oviposition by arthropod herbivores,[36,37] attract natural enemies of these herbivores[4,5,9] and even induce defence genes and VOC emission in neighbouring plants[38–40] or prime these plants to respond faster to future herbivore attack.[41] In field studies, HIPVs have been shown to have the potential to

reduce the damage by pests of various plants including crop plants.[9,42–45] This opens the possibility of exploiting HIPVs for the development of novel strategies in crop protection. Indeed, an experiment whereby a hop yard was baited with methyl salicylate (MeSA) resulted in a significant increase in the numbers of beneficial predatory insects and in a dramatic reduction in spider mite numbers, the major arthropod pest of hops.[46] Recent advances in the biochemistry and molecular genetics of terpene biosynthesis in various plant families[47–59] should enable breeders to engineer plants to emit odours that more effectively attract the enemies of herbivores and thus reduce herbivory.[60,61] That this is a realistic possibility was demonstrated with *Arabidopsis thaliana* plants that were transformed with sesquiterpenoid synthase genes, making them release two additional terpenoid compounds that were attractive to the predatory mites, *Phytoseiulus persimili*.[62] Further field studies with synthetic HIPVs and genetically modified plants releasing such HIPVs will provide additional indications as to whether such manipulations could be included in agricultural practices to protect crops and the environment.

Various studies have provided detailed knowledge on the physiological and molecular basis of plant volatile synthesis and indirect defence responses in plants (reviewed by ref. 1,18,19). Still, our understanding of how the biosynthetic pathways are induced and interact with each other is rudimentary, probably because the induction and emission of HIPVs depends on the interaction between biotic factors, such as plant hormones,[19,63–68] herbivore-derived elicitors,[69–74] associated microorganisms[75,76] and abiotic factors, such as wounding,[77–80] O_3 and CO_2 concentration,[81–84] temperature and light,[85–87] UV-radiation[88] and many other factors.[89,90] Applying several stresses simultaneously to get a better understanding of how different pathways interact, will be one of the challenges in future studies on the physiology of plant volatiles.

There is a current boom in interest among ecologists to include the belowground interactions in studies on plants and associated organisms,[91–94] and VOCs have been found also to be involved in tritrophic signalling belowground.[16,95–97] Chen *et al.*[98] characterized a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole, which is possibly involved in belowground interactions. Recently, Rasmann *et al.*[9] identified a sesquiterpene, (*E*)-β-caryophyllene, as a belowground herbivore-induced volatile signal that attracts entomopathogenic nematodes, which infect and kill larvae of the corn rootworm, *Diabrotica virgifera virgifera*, a voracious pest of maize. Another challenge in future studies will be to determine how such belowground interactions might influence aboveground volatile emissions and *vice versa*.

Collection and analysis of HIPVs

Sampling aboveground

The literature on HIPVs released by vegetative plant parts is vast and is continuously growing. In most cases it is unknown which compounds have biological activity and therefore biologists mostly try to sample and analyse the full range of HIPVs. This is usually achieved by collecting VOCs in the headspace of herbivore-induced plants that are enclosed in collection chambers using an adsorbing material with a relatively broad spectrum of adsorption (*e.g.* ref. 4,99). Subsequently the collected volatiles can be analysed by gas-chromatography (GC) and mass-spectrometry (MS) or a combination of both (GC/MS).

Fig. 3 shows the frequency with which the most commonly used sampling methods have been applied in the research of HIPVs during the last 10 years. Although this literature survey probably includes only part of all studies on herbivore-induced volatiles, it is clear that collection on adsorbents followed by desorption with solvents has been the most commonly used method. One advantage associated with solvent desorption is that it results in liquid samples that can be stored, which then can be used several times. This can be very useful for repeated analyses in GC, GC/MS and GC-EAD (gas-chromatography and electroantennogram-detection),[100] for further fractionation,[101] for peak enhancement coinjections[102] and for bioassays.[103–105] Adsorbents and solvents used in these studies vary, but the porous polymer Super Q[106] (80–100 mesh, Alltech, Deerfield, Illinois, USA) and the solvent dichloromethane are widely used by many groups. Although this adsorbent–solvent combination has proven highly effective in HIPV adsorption and desorption, Harper[107] argues that more than one adsorbent might be required to cover different classes of compounds, such as amines, aldehydes and aromatic hydrocarbons. There exist a wide range of adsorbent materials that can be used for this purpose, including activated charcoal, Anasorb 747, carboxens, silica gel, carbon molecular sieves and porous polymers such as Tenax, Chromosorb.[107,108] Choosing a combination of appropriate adsorbents may increase the number of compounds found in HIPV-blends. Still, only a small volume of the desorbed compounds can be injected into the GC, leaving minor compounds undetected, and the solvent peak can mask some highly volatile compounds. These problems can be avoided by using a desorption method without solvent, like thermal desorption, or solid-phase microextraction (SPME).[22,24,108–113]

The SPME technique employs a small fibre coated with an adsorbing material and has found widespread application in many fields, mostly for qualitative and semi-quantitative analyses in environmental and food analyses, but also

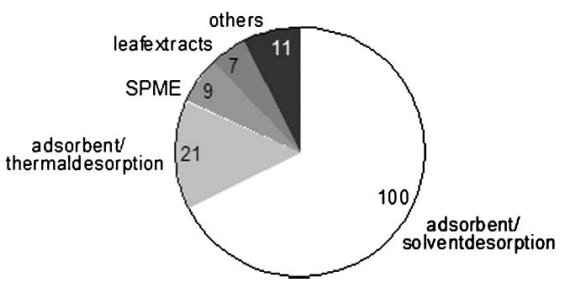

Fig. 3 The most commonly used HIPV collection methods during the last ten years. The numbers represent the number of studies found with a search on the "Web of Science" by entering the terms: "induc* and volatil* and plant*" from 1995–2004.

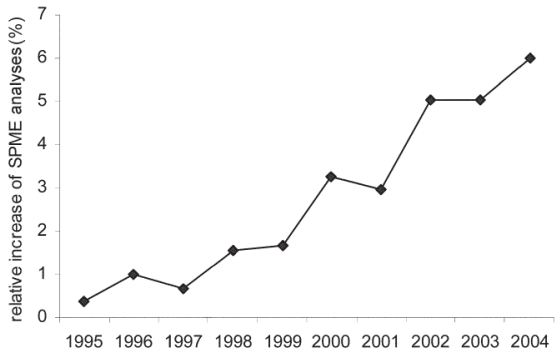

Fig. 4 Relative increase of the use of SPME to collect plant volatiles. The graph depicts the percentage of all surveyed plant volatile collection studies that employed the SPME technique. This information was obtained with a search on the "Web of Science" using the terms: "SPME and plant* and volatil*" and "volatil* and plant*".

quantitatively in analytical chemistry.[22] SPME has also gained increasing popularity in analyses of living biological samples, including plant VOCs[111] (Fig. 4). It has a number of advantages, like simplifying sample preparation, increasing reliability, selectivity and sensitivity and reducing analysis time. Various coating materials for the SPME fibres are available,[22,24,111] and choosing the appropriate fibre and sampling conditions are crucial for this technique to be effective. Still, the biggest limitation of SPME in studies on HIPVs is that the sample is lost after analysis and cannot be used for further analyses, GC-EAD, bioassays or fractionation.

Higher selectivity and sensitivity could also be obtained by using techniques specifically adapted for compounds with known functional groups. For example, damaged *Allium* plants produce and release sulfur containing VOCs, presumably to prevent insect herbivory.[114] To analyse true onion volatiles, Arnault *et al.*[115] used a combination of SPME-GC/MS and solvent extraction followed by GC/MS analyses specifically developed for these compounds. Other compounds might be trapped by using microchemical techniques such as derivatization and degradation methods.[116,117] Such techniques have played crucial roles in the determination of the structure of insect pheromones[118,119] and are widely used in ambient air analyses.[110,120 122] In pheromone studies, micro-reactions have three main uses; derivatization to aid chromatographic properties, functional group modification to help with MS structure determination, and assignment of absolute configuration to chiral centres.[118] In analyses of HIPVs these techniques are rarely used, despite the fact that such techniques seem ideal for sampling trace amounts or highly reactive VOCs, which are difficult to sample with any other technique. For example, aldehydes can be sampled using a 2,4-dinitrophenylhydrazine (DNPH)-coated filter, and amines with a filter coated with 1-naphthyl isothiocyanate.[122] Pre-treated filters are available from various companies (*e.g.* Supelco, SKC) and can easily be adapted to selectively adsorb HIPVs with a specific functional group. In our research, we used DNPH-coated silica (Supelco) to selectively adsorb 3-(*Z*)-hexenal and 2-(*E*)-hexenal, the two major aldehydes emitted by maize plants under caterpillar attack (see below and Fig. 5).

An additional problem faced in analyses of HIPVs is that the release of some volatiles is highly dynamic, depending on the time after the plant is damaged,[3,123] but also on the time of day[36,124] and even season.[89,125,126] Insects can perceive minor changes in odour quantity and quality. Therefore, a good understanding of the kinetics of formation and release of HIPV is highly desired. Specifically designed and automated headspace collection systems and GC-analyses allow time dependent collection of volatile plant compounds,[3,99,127] and novel techniques even allow real time analyses of the emission of VOCs. For example, proton-transfer-reaction mass spectrometry (PTR-MS)[128 130] and portable artificial noses[131] permit fast sampling and real time (one measurement per second) analyses of plant volatiles and thus provide new insights into the kinetics of plant volatile releases. These techniques have the limitation that they do not distinguish between different VOCs with the same mass. Yet, Penuelas, Filella and coworkers[132] nicely show that the PTR-MS technique can be used to monitor small volatiles, like methanol released by *Succisa pratensis* leaves infested with caterpillars of *Euphydryas aurinia*. Such highly volatile compounds are likely to break through from sorbent tubes or are masked by the solvent peak in liquid sample GC-analyses and are seldom reported in HIPV studies using sorbent trapping.

To fully understand how HIPVs are produced and released, more comprehensive metabolomic approaches are needed. Schmelz *et al.*[66,133] have introduced an elegant technique based on vapour phase extraction and analysis by chemical ionization-GC/MS that simultaneously analyses phytohormones, phytotoxins, and VOCs in plants. Derivatization techniques are widely employed in metabolic profiling (reviewed in ref. 134) and combining metabolic and transcript

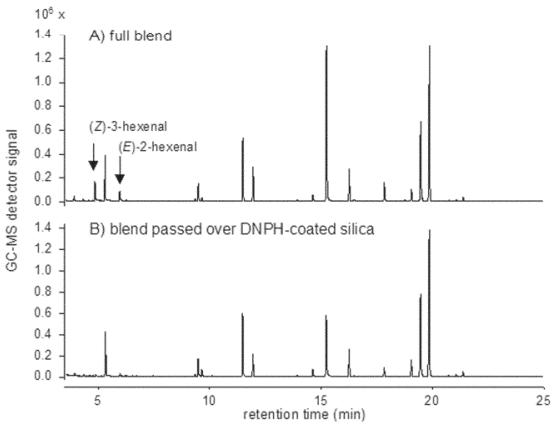

Fig. 5 GC-MS chromatograms obtained with the collected volatiles of caterpillar-infested maize seedlings. (A) Complete caterpillar-induced maize blend adsorbed on Super-Q filters and desorbed with dichloromethane. (B) Volatiles from the same blend after it was passed over a DNPH-coated silica filter, which selectively adsorbs carbonyl compounds. This breakthrough blend of HIPVs was collected and analysed as in (A). Further details on material and methods are given by D'Alessandro and Turlings.[103]

analyses might provide new insights into which genes are involved in the production of HIPVs.[135]

Deciding on which technique to use remains difficult, as several comparisons of different sampling techniques can result in quantitative and qualitative differences.[136 138] We suggest that the most reliable results in HIPV-analyses can be expected from the use of a combination of different approaches, including techniques specifically developed and calibrated for quantification of compounds with known functional groups. Knowing the exact relative quantities can be essential because often ratios among different compounds can be important in determining the attractiveness of odour blends to insects.[139 141]

Sampling belowground

Studies on belowground interactions bring new challenges to develop collection and analytical methods. So far, research on belowground allelochemicals mainly used extracts of root exudates,[142 144] but other methods to analyse VOCs from soil samples are also available.[130,145 150] As in aerial VOC analyses, comparing various sampling methods can lead to different quantities of VOCs.[148] It is therefore pertinent to include more than one method in the belowground collections. The soil is much more heterogeneous than the air and large differences in soil properties and VOC concentrations can occur over extremely short distances, which must be taken into account during sampling.[151] Biotic and abiotic factors that are known to affect the quantity and quality of HIPV-blends aboveground are likely to significantly affect belowground volatiles as well. Therefore, a major challenge for the analyses of belowground HIPVs will not only be to choose appropriate sampling and analysis tools, but also to obtain detailed knowledge on their kinetics, concentrations and distribution in the soil. Probably a good way to start is to analyse HIPVs in relatively well-defined, homogeneous soil types and then to repeat the measurements in more complex soils as well as in field experiments. For example, to determine the role of insect-induced (*E*)-β-caryophyllene from maize roots as an attractant for nematodes, an initial comparison was made between insect-damaged and undamaged maize roots, using pulverized roots. In subsequent tests, it was confirmed that (*E*)-β-caryophyllene rapidly diffuses through sand and attracts the nematodes, and finally the attractiveness of this sesquiterpene was tested in field experiments under natural conditions.[9]

Bioassays and bioassay-linked HIPV analyses

Using HIPVs as novel tools in crop protection implies not only a need for detailed knowledge about the biosynthesis and release of plant VOCs, but also about the perception and exploitation of these chemical signals by the animals. The identification of the specific, behaviourally active compounds within a complex blend has proven to be difficult. Commonly, such compounds are identified with a combination of behavioural studies and chemical analyses of the tested odour blends.[36,103,105,152 156] Another frequently practised method is gas chromatography combined with electroantennogram detection (GC-EAD) or with single cell recordings (GC-SCR),[157] whereby the end of the GC column is split and one part of the effluent passes into the normal flame ionization detector (FID) and the other part is passed over an antenna or a single olfactory sensillum of the study insect. With the use of electrodes and amplifiers the responses of the antennal receptors can be measured and registered. Correlating these responses with the chromatogram obtained with FID detection reveals which compounds are perceived by the antennae.[100,158 162] Advances in this methodology that allow recordings to be made inside the central nervous system in combination with the novel technique of *in vivo* calcium-imaging have helped neurobiologists to start to understand how plant VOCs are detected and processed by insects.[163 165]

While methods such as GC-EAD or GC-SCR might give information on whether a compound is perceived by the olfactory system of an animal,[157] only behavioural tests will show if the animals are indeed attracted or repelled by a particular compound. So far, methods for testing the attractiveness of odour blends to arthropods consisted mainly of dynamic air bioassays using olfactometers,[166,167] wind tunnels,[4,168,169] or static air bioassays.[170,171] In these experiments arthropods are released at a defined distance from an odour source and their behaviour and attraction is recorded. In most cases chemical information on the odour sources had to be obtained in separate analytical studies. However, natural HIPV-blends exhibit not only high interspecific and intraspecific variability,[172 174] but quantity and quality of the compounds depends also on the degree and time after infestation.[3,175] To account for such variation, newer approaches attempt to standardize odour blends and to closely link chemical profiles with the insect behaviour.[175,176] For example, we have developed an olfactometer in which six odour sources can be tested simultaneously for their relative attractiveness, and during the assays part of each odour blend can be trapped for further analyses.[175] This device not only provides a direct link between the chemical profiles and the insect behaviour, but it also allows direct comparison of the attractiveness of a multitude of odour sources with multivariate statistical approaches, including principal component analysis (PCA) and redundancy analysis (RDA). Multivariate statistical analyses are widely applied in analyses of food volatiles[177 179] and might also provide additional information in the analyses of HIPVs[172,180] and reveal key compounds that attract arthropods.

Another way of studying the importance of individual VOCs within a complex blend is to compare the attractiveness of volatile blends differing in only a few known compounds. These blends can be obtained by using different chemical elicitors and inhibitors[181 183] or by silencing genes involved in indirect defences.[184,185] Adding back missing VOCs to incomplete blends is a sound way to study the importance of individual compounds. For example the predatory mite *Phytoseiulus persimilis* prefers a methyl salicylate (MeSA)-containing volatile blend, induced by the spider mite *Tetranychus urticae* to a similar but MeSA-free blend, induced by jasmonic acid.[152] Adding synthetic MeSA to the MeSA-free blend significantly increased attraction to this odour, suggesting an important role for MeSA as a foraging cue for this predatory arthropod.[152,186]

We used an additional approach to obtain blends differing in only a few known compounds,[103] for which we modified the six-arm olfactometer described by Turlings *et al.*[175] in order to install adsorbing filter tubes between the odour source vessels and the arms of the olfactometers. By passing the induced volatile blend over these filter tubes, some compounds can be retained by the adsorbent material, while others break through and can be tested for attraction (Fig. 5). Furthermore, synthetic compounds can readily be added to the HIPV-blend and thus we are able to evaluate the importance of individual or groups of specific compounds. This novel technique has revealed an unexpected importance of minor compounds for the attraction of parasitic wasps to the complex HIPV-blends, whereas several of the dominating compounds appear to be only important after the wasps learn to associate them with the presence of hosts.[103] These experiments once more showed that responses towards HIPVs are not fixed. Indeed, it is well known that responses of insects to VOCs are highly plastic, depending on the physiological state of the animals and on previous experiences with odour sources.[187 189] Different forms of learning may modify the perception of chemical compounds and the response of insects towards odour sources.[190 193] This flexibility in the insects' responses, as well as the great variability in the odours produced[172,174,194 196] duly complicates the elucidation of key attractants, and learning experiments should be included in behavioural assays.

Conclusions and prospects

HIPVs play crucial roles in the interactions among various organisms. A good understanding of the key compounds involved in these interactions will not only provide important fundamental ecological knowledge, but should also allow us to manipulate certain of these interactions to our advantage, especially in agriculture. The complexity and variability of volatile blends emitted by herbivore-infested plants have proven to greatly complicate the identification of the principal compounds mediating interactions between the emitting plants and associated organisms. Renewed efforts should be made to integrate and adapt the latest techniques in analytical chemistry for this purpose. Choosing appropriate methods for sampling and analysing HIPVs is crucial. It might be possible to directly exploit insects' olfaction and behaviour to develop new selective and sensitive biosensors.[197 201] Insect chemoreception is known to be more sensitive and specific than any chemical detection system available today.[189] For example, in the moth *Spodoptera littoralis*, a change in heartbeat frequency can be triggered when fewer than six molecules of a key pheromone component hit the antennae of the insect.[202] Thus, the insects themselves might be most capable of informing us on which are the key substances that they use in their behaviour. Including well-designed electrophysiological and behavioural bioassays can provide essential additional information about bioactive compounds that cannot or can barely be detected in chemical analyses. In future studies biosensing techniques including electronic noses[203 206] might add to classical chemical analyses of HIPVs. If biologists, chemists and engineers join forces in this effort they will undoubtedly be able to fully unravel the fascinating world of chemically-mediated interactions between plants and their biotic environment. Such insight will then open the way to manipulate the interactions for our own benefit.

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Chapter VI

Synthesis and outlook

Marco D'Alessandro

2006

Synthesis – This thesis once more highlights the enormous importance of volatile organic compounds (VOCs) as signalling compounds, not only in plant physiological processes, but also at an ecological scale across trophic levels.

In a first part (*Chapters II and III*), we addressed the question of the role of single or specific groups of herbivore-induced plant volatiles (HIPVs) in the attraction of two parasitoid species, *Cotesia marginiventris* and *Microplitis rufiventris* (both Hymenoptera: Braconidae). These parasitoids have previously been shown to use such volatile cues to locate their hosts, the larvae of various *Spodoptera* moths (Lepidoptera: Noctuidae) (Gouinguené et al., 2003 ; Hoballah and Turlings, 2005; Tamò et al., 2006). As expected, we found substantial differences in the attractiveness of various HIPV-blends, but, surprisingly, some compounds that are typically emitted by insect-damaged plants (e.g. indole, *Chapter III*) actually reduced the attractiveness of HIPV-blends, whereas others, minor compounds (e.g. compounds retained by silica, *Chapter II*) appeared to be essential and highly attractive to the parasitoids. In fact, these minor compounds, which made up less than 30 % of all HIPVs released by maize seedlings, not only were more attractive to *C. marginiventris* than compounds that broke through the silica filters, but were also significantly more attractive than the combination of the breakthrough and the retained compounds (*Appendix I*). However, a synthetic mixture of the 5 major compounds that were retained by the silica filter did not attract the wasps, if they were applied on a filter paper at similar concentrations as found in the highly attractive silica extract (*Appendix I*). Even by testing different fractions of the silica extract that were obtained by preparative GC (data not shown), we were not able to identify any key compound that could explain the enormous attractiveness of the silica extract. The reason for this surely is the enormous complexity of HIPV-blends. Even modern collection and analytical techniques probably allowed us to only detect the tip of the iceberg in terms of the compounds that the plants released. On the other hand, many plant VOCs are ubiquitous and recent studies done with sophisticated neurophysiological methods suggest that odour recognition in insects is ratio-specific and not just compound-specific (Bruce et al., 2005). In *Drosophila melanogaster*, for example, some olfactory receptor neurons (ORNs) for plant odours have been shown to be narrowly tuned responding to only one or few chemical compounds, while others seem to be broadly tuned, responding to various numbers and classes of chemical compounds (Hallem and Carlson, 2006). In addition, there is a functional organization of co-located ORNs for plant VOCs in

the same olfactory sensillum on the antennae of *Drosophila* (Stensmyr et al., 2003). These findings provide evidence for a fine-scale spatio-temporal resolution of olfactory input to the CNS, which could explain why the correct ratios of different compounds in plant odours are important (Bruce et al., 2005). It is likely that ratios are also important for the parasitoids tested here, making it exceedingly difficult to identify key compounds that make a blend highly attractive.

Whatever the mechanisms behind the odour recognition are, our experiments demonstrated that HIPV-blends differ in their attractiveness and this opens the possibility to modify plants or their environment in order to attract more parasitoids. A fascinating but complicating factor in order to do so is the keen learning ability of volatile cues by many parasitoids (Vet et al., 1995). It can be expected that some compounds will be learned better than other ones. Indole, for example, a major compound of *Spodoptera*-induced maize VOCs was not a compound that *C. marginiventris* strongly associated during learning experiments, which suggests that this compound is not an important host location cue, even not for experienced wasps (*Chapter III*). Understanding how individual compounds within complex blends of VOCs are learned by these parasitoids could help to assess the importance of the manipulated compounds in an ecological context, in which learning has been shown to affect the parasitoids' foraging success an fitness (Vet, 1999; Olson et al., 2003).

The second part of this thesis (*Chapter IV*) revealed an important role of soil-born micro-organisms in defence responses in maize seedlings. Such micro-organisms interact with indirect defences by adding biological active VOCs to the blend of HIPVs. These microbial VOCs were also released after adding the maize endophytic bacteria *Enterobacter aerogenes* to the seedlings and this induced systemic resistance against the fungal pathogen *Setosphaeria turcica*, but not against the lepidopteran herbivore *Spodoptera littoralis*. Hence, such bacteria-derived VOCs add a new layer of complexity to plant defence responses. The findings imply that the soil, which is a major source of many endophytic bacteria and symbiotic fungi, is of central importance in plant defence responses. However, it remains to be established how these micro-organisms and their volatile compounds interact with plant defence responses on a physiological level.

To conclude, this work demonstrates that single VOCs can have important consequences for plant defence responses by affecting multitrophic interactions. A good understanding of the role of VOCs in these interactions may lead to their exploitation in the development of novel,

sustainable crop protection strategies. Indeed choosing appropriate soil management strategies might not only affect the endophytic community in crop plants, but also enhance plant growth and resistance against a various pests (Sturz and Christie, 2003). This is particularly pertinent for maize, which is one of the most important crops worldwide, and the losses due to insect pests and diseases still lies above 30 % despite current crop protection practices (Oerke, 2006). Alternative crop protection strategies are desperately needed. Addressing the following unresolved problems and questions that arose out of this thesis might contribute to this aim.

Outlook – *Chapters II and III* clearly showed differences in the attractiveness of various HIPV-blends to two parasitoids species. Interestingly, some major compounds were found not to be very important for the attraction, whereas certain minor compounds were essential and highly attractive (*Appendix I*). In future studies the role of such minor compounds for the attraction of parasitoids should be thoroughly investigated and their identities determined. Such studies should also explore the differences in the attraction of chiral compounds, some of which we identified during this thesis (*Appendix II*).

Results from *Chapter III* indicate that, despite the fact that indole can be learned by the parasitoid *C. marginiventris,* it was not strongly associated with the host presence if it was offered to the wasps in a complex volatile environment. Other compounds can be expected to play a more important role in associative learning. Knowledge on such compounds is also missing in most other studied systems. Their eventual identification will contribute considerably to the understanding of the insect's olfaction and learning abilities.

In *Chapter IV* it was revealed that bacteria-derived VOCs induced systemic resistance in plants against the pathogen *Setosphaeria turcica.* Molecular analyses of defence genes further suggested that the mechanisms behind this enhanced resistance probably induced pathways that were not dependent on jasmonic acid or salicylic acid. Further investigations in this area might identify novel plant defence pathways, possibly leading to additional means of crop protection.

We have speculated on the possibility that VOC-releasing bacterial endophytes may manipulate the odour emission of the seedlings in order to attract insects. Phloem or mesophyll

feeding insects, such as planthoppers, could serve as vectors for the bacteria in the horizontally transmission of these bacteria to neighbouring, uninfected plants. Research in this area could be initiated and can be expected to lead to new insight into the interactions between plants, insects, and micro-organisms.

In *Chapter V* we reviewed advances in the identification of volatiles that mediate interactions among plants and arthropods, which also include new sensitive and fast methods to analyse VOCs. One specifically interesting method that allows real time analyses of plant volatiles is the proton-transfer-reaction mass spectrometry (PTR-MS). This method could be applied in our system to analyse the dynamics of the release of specific HIPVs, including highly volatile compounds that can hardly be detected with any other method.

Last but not least, although there is increasing evidence that VOCs can be applied to control various pests of crop plants, field experiments that tested the potential of this approach and measure the actual reduction in the damage are largely missing. Such field studies are highly desirable and might encourage farmers and policy makers that are aiming for sustainable agriculture to adopt novel VOC-management approaches as alternative methods to control pests.

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Attractiveness of the silica extract and its major compounds

In *chapter II* we passed *Spodoptera*-induced maize volatiles over an absorbing filter that contained silica and showed that the resulting breakthrough blend lost all of its attractiveness to *Cotesia marginiventris* females, even though it still contained more than 70 % of all VOCs. In contrast, the VOCs adsorbed on the silica filter (silica extract) were highly attractive to this parasitoid and adding them back to the breakthrough VOCs restored the attraction towards this blend. Additional experiments were performed in order to determine possible 'key' compounds that make this silica extract so attractive.

First we compared the silica extract to the breakthrough VOCs that we collected and desorbed from Super Q (breakthrough extract) and to the combination of these two extracts (breakthrough & silica extract). As a control we used solvent only. All extracts were placed on filter papers as described in *Chapter II* (50 µl of each extract). Our results indicated that the silica extract is in fact much more attractive than the breakthrough extract, but surprisingly also significantly more attractive that the combination of these two extracts, which basically consisted of all VOCs released by *Spodoptera*-induced maize seedlings (Figure A-1). This indicates that the silica extract, which made up less than 30 % of all VOCs released by *Spodoptera*-induced maize seedlings, was more attractive than the whole blend. Most likely the silica extract contained not only most of the highly attractive compounds, but also less of the non-attractive ones. Another explanation would be that placing this extract on filter paper resulted in different ratios of the compounds, which can make a blend more or less attractive.

In a second experiment we selected the five major compounds of the silica extract that were specific to this blend, which were linalool, phenethyl actetate, methyl anthranilate, geranyl acetate, and (*E*)-nerolidol (see *chapter II*) and we applied them in similar concentrations as in the silica extract on the filter paper in the olfactometer. This blend did not significantly attract naive wasps ($F_{2,58}$ = 1.26, P = 0.29), indicating that these compounds were not important for the attraction of the wasps (Figure 2). An alternative explanation would be that synthetic compounds differ from naturally produced compounds. Linalool, for example, is present in two enantiomers, (-)-linalool and (+)-linalool, in the induced blend but only as the (-)-linalool in the non induced blend (see also *Appendix II*). Interestingly, (-)-linalool (5 µg in 50 µl solvent) was significantly attractive to the wasps

if tested against solvent as controls ($F_{1,71}$ = 8.55, P = 0.005, Figure 3 A), but (+/-)-linalool was rather repellent than attractive ($F_{1,47}$ = 4.61, P = 0.037; Figure 3 B). Although we have no explanation for this observation, these results clearly demonstrate the importance of testing the right isomers of synthetic compounds. The possibility to use synthetic compounds to mimic the plants' volatile emission is however limited, because many compounds are not commercially available in the right isomeric composition.

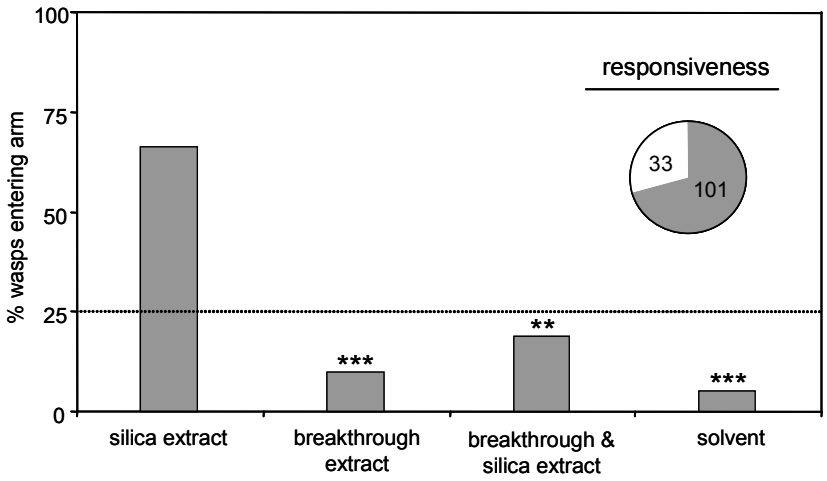

Figure 1. Olfactometer responses of naïve *Cotesia marginiventris* females to different fractions of the *Spodoptera induced* maize blends. The responsiveness (total number of wasps choosing an arm) is indicated by the pie chart with the white part showing the total number of wasps that did not enter any olfactometer arm. Data were analysed using a GLM and stars indicate significant differences between the odour sources based on comparison to a reference odour source (= odour source with highest attraction, * = P < 0.05, ** = P < 0.01, *** P < 0.001). See *Chapter II* for more methodological and statistical details.

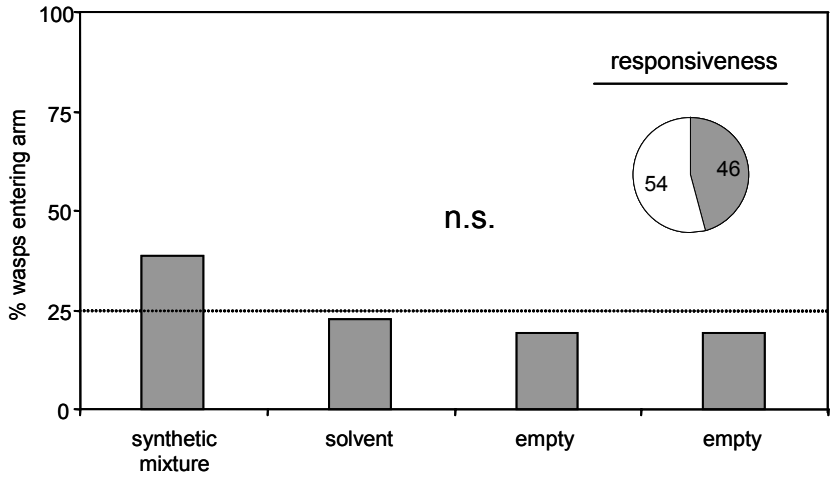

Figure 2. Olfactometer responses of naïve *Cotesia marginiventris* females to a synthetic mixture of the 5 five major compounds that were best trapped by silica filters (linalool, phenethyl actetate, methyl anthranilate, geranyl acetate, and (*E*)-nerolidol). See *Chapter II* for more methodological and statistical details.

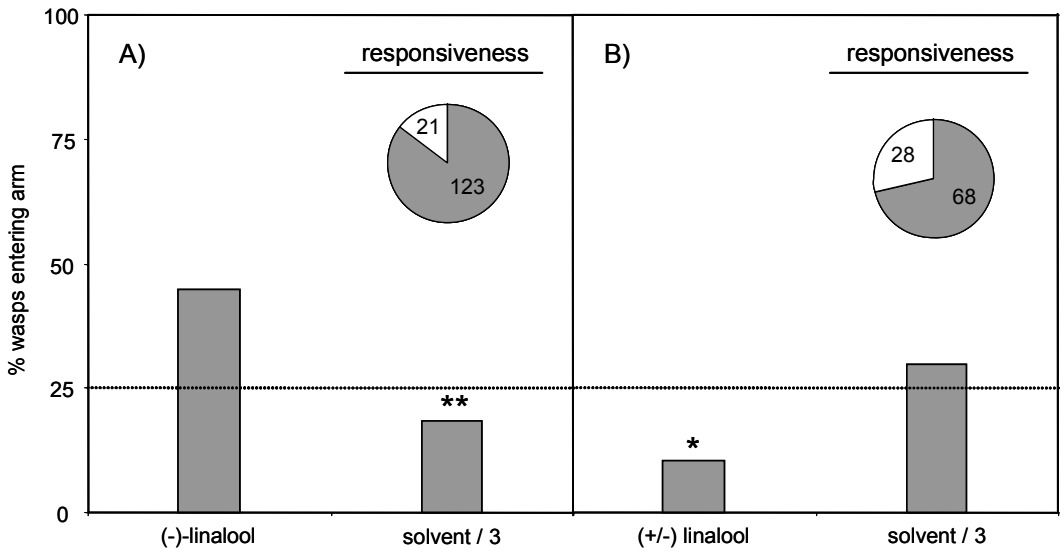

Figure 3. Olfactometer responses of naïve *Cotesia marginiventris* females to different enantiomers of linalool. A) One arm with (-)-linalool was tested against three control arms with solvent only. B) One arm with a mixture of (-)-linalool and (+)-linalool (each about 50 %) was tested against 3 control arms with solvent only. See *Chapter II* for more methodological and statistical details.

Stereochemistry of maize and bacterial volatiles

Many volatile organic compounds (VOCs) have chiral centres and therefore the right enantiomers can not be identified by separation of the compounds on commonly used polar and non-polar columns (e.g. HP1, Innowax, Agilent, USA). To identify the stereochemistry of some of the major chiral compounds of maize and bacteria-derived VOCs we injected collected volatiles on a chiral column (CycloSil-B, 30 m, 0,25 mm ID, 0.25 µm film thickness, Agilent, USA) and we analysed the compounds by GC-MS as described in *Chapter IV* (initial temperature 40 °C for 3 min, ramp up to 250 °C at 8 °C/min, post-run of 5 min at 250 °C). We detected all three isomers of 2,3-butanediol in both caterpillar-induced and in non-induced volatile blends, but the amount of the meso (*R,S*)-2,3-butanediol was considerably higher than the amounts of the optical active isomers (2*R*,3*R*)-(-)- and (2*S*,3*S*)-(+)-butanediol (Figure 1). Interestingly in *Arabidopsis* plants only the (2*R*,3*R*)-(-)-butanediol is biological active (see reference in chapter IV), stressing the importance of determining the stereochemistry in studies that address the role of VOCs in interactions of plants with other organisms.

Another compound that was present in two isomeric forms and that we could easily identify with this method was linalool. In fact, we detected both enantiomers, (-)-linalool and (+)-linalool, in the induced blend, while the non-induced blend contained only (-)-linalool. This indicates that the enzyme involved in the production of (-)-linalool differs from the enzyme for the production of the (+)-linalool and that these enzymes are differentially activated following herbivore-attack. As already shown in *Appendix I*, *C. marginiventris* clearly distinguished between the different enantiomers of this compound. It would be interesting to determine whether the ratios between these to compounds might provide important information to host searching parasitic wasp.

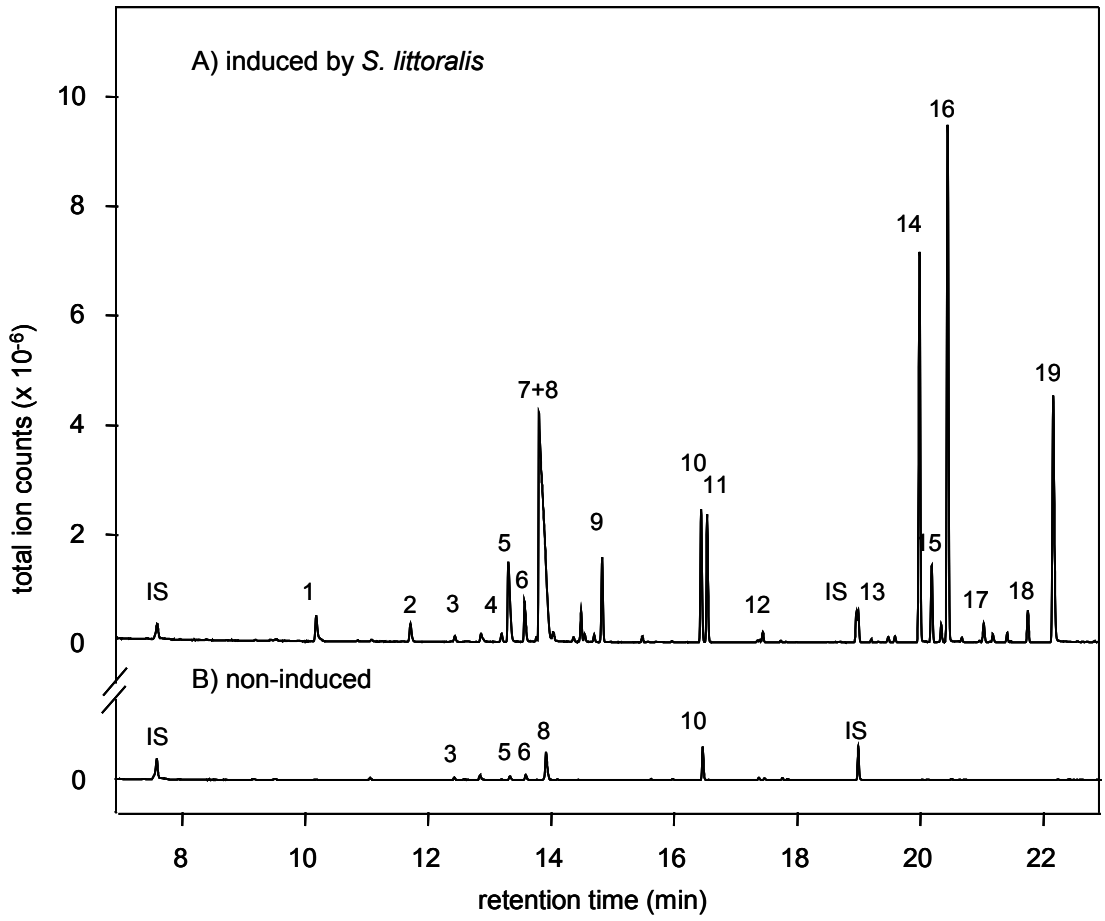

Figure 1. Chromatographic traces of A) *Spodoptera*-induced and B) non-induced volatile blends released by maize seedlings grown in the presence of the bacteria *Enterobacter aerogenes*. Major compounds are: 1 = (*Z*)-3-hexenal, 2 = (*E*)-2-hexenal , 3 = β-myrcene, 4 = (*Z*)-3-hexen-1-ol, 5 = (2*S*,3*S*)-(+)-butanediol, 6 = (2*R*,3*R*)-(-)-butanediol, 7 = (*Z*)-3-hexenyl acetate, 8 = meso (*R*,*S*)-butanediol (major peak, small amounts of compound 7), 9 = (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), 10 = (-)-linalool, 11 = (+)-linaool, 12 = benzyl acetate, 13 = phenethyl acetate, 14 = (*E*)-α-bergamotene, 15 = (*E*)-β-caryophyllene, 16 = (*E*)-β-farnesene, 17 = methyl anthranilate, 18 = β-sesquiphellandrene, 19 = indole, IS = internal standards.

Production of acetoin and 2,3-butanediol by *Enterobacter aerogenes*

In *chapter V* we described the isolation and identification of the γ-proteobacterium *Enterobacter aerogenes* (synonym *Klebsiella mobilis*) from germinated maize seeds and we showed that adding these bacteria to maize seeds before planting resulted in the release of high amounts of 2,3-butanediol. Here we provide further experimental evidence that these bacteria are indeed able to produce acetoin and 2,3-butanediol.

For the first experiment we used tyndallised maize seeds, i.e. seeds that had been sterilised by three periods of boiling at 100 °C for 30 min with 24 h resting time between each boiling step. The first boiling step eliminates most vegetative bacteria and denatures plant proteins. The second and third boiling steps kill vegetative cells of spore forming bacteria which may have germinated after the first boiling step. We show that incubating these seeds in a culture of *E. aerogenes* resulted in the release of 2,3-butanediol and its precursor acetoin three days after incubation (Figure 1). Interestingly, maize seeds that were not incubated with the bacteria also released small amounts of acetoin, the precursor of 2,3-butanediol, especially after only one boiling step. This could be explained by the fact that maize seeds may contain endophytic bacterial spores (e.g. *Bacillus* sp.), which germinated after only one boiling step and which initiated fermentation.

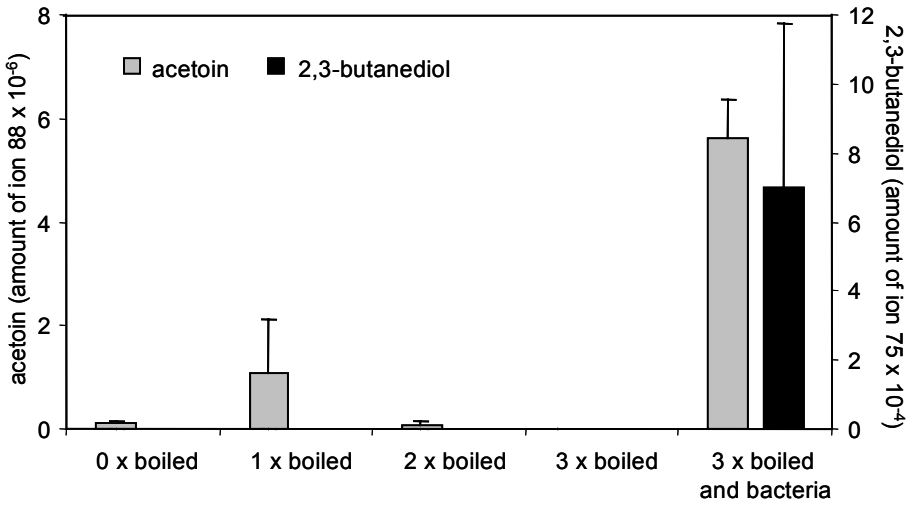

Figure 1. Relative amounts (mean of selective ion counts ± SE) of acetoin and 2,3-butanediol detected in the headspace of maize seeds. Seeds were sterilised by boiling (0 to 3 times) and incubated in an overnight culture of *E. aerogenes* (10^8 CFU/mL) or in autoclaved water only for 5 hr. Single maize seeds were placed in vials sealed with a Teflon caps and VOCs were sampled 3 days after incubation by SPME and analysed by GC-MS similar as described in chapter V, but in the selective ion mode. N = 6, note the different scales for acetoin and 2,3-butanediol.

In a second experiment we incubated surface sterilised seeds of different plants in a solution of the isolated *E. aerogenes* strain. In all seeds we were able to detect 2,3-butanediol and its precursor acetoin. However, 2,3-butanediol was neither detected in seeds to which we added sterile water only, nor in the bacterial culture grown in LB-medium (data not shown). This indicates that the bacteria *E. aerogenes* are able to produce 2,3-butanediol directly by fermenting seed material of a variety of different plant species, including both monocotyledons and dicotyledons. Indeed, 2,3-butanediol has occasionally been detected in the headspace of barley seedlings (M. Rostás, University of Würzburg, personal communication) and it is probably commonly produced by plants that are colonised by endophytic bacteria.

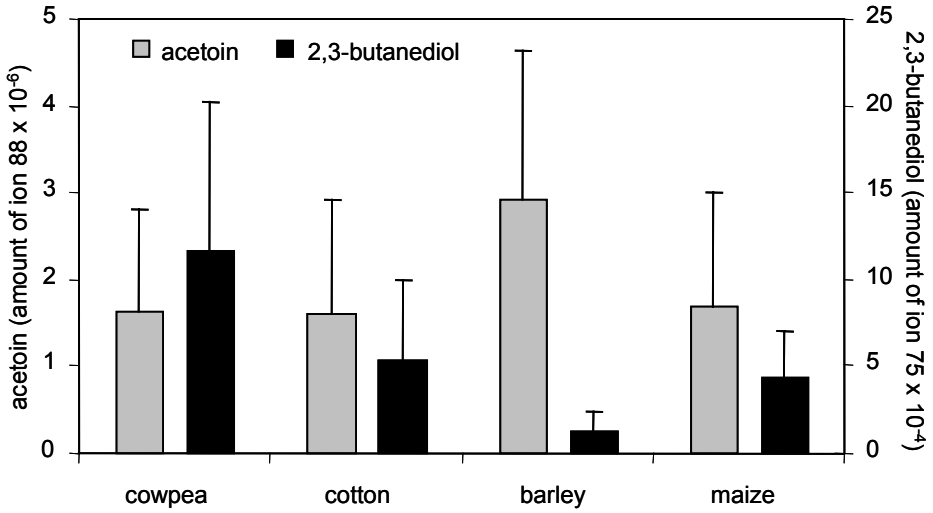

Figure 2. Relative amounts (mean of selective ion counts ± SE) of acetoin and 2,3-butanediol detected in the headspace of seeds from different plant species. Surface sterilised seeds were incubated in an overnight culture of *E. aerogenes* (10^8 CFU/mL). VOCs were sampled and analysed as described in figure 1. N = 5, note the different scales for acetoin and 2,3-butanediol.

In a third experiment we placed surface sterilised maize seeds in SPME-vials (see Chapter IV) and added either 2 mL overnight culture (10^8 CFU/mL LB-medium) of commercially available *Bacillus subtilis* strains (FZB24 Bayer and BD170 Biopro Adermatt) or cultures of different bacterial strains that we had previously isolated from germinated maize seeds. As a control we added 2 mL of a solution containing antibiotics (0.4 mg Streptomycinsulfate and 0.2 mg Novobiocin) or

autoclaved water only to the vials containing the seeds. Three days after incubation we collected the headspace VOCs on a SPME-fibre and we analysed the VOCs by GC-MS similar as described in chapter IV. Figure 1 shows that various amounts of acetoin were present in the headspace of all treatments with added bacteria while the control treatments contained only trace amounts of acetoin. 2,3-Butanediol was also present in all but one treatment (*Bacillus subtilis* BD170) but it was completely absent in the control treatments. This data suggest, that many bacterial strains and species are able to produce 2,3-butanediol and its precursor acetoin by fermentation of maize seeds.

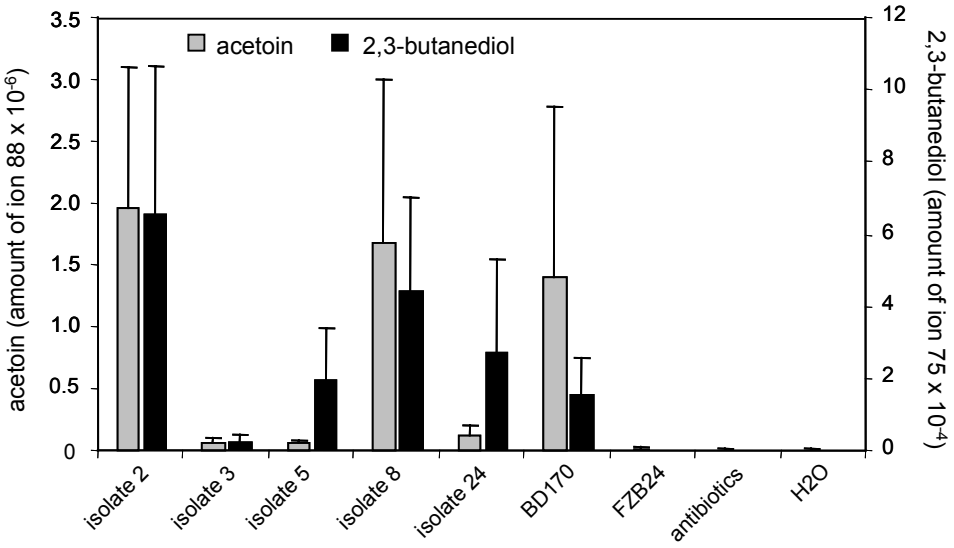

Figure 3. Relative amounts (mean of selective ion counts ± SE) of acetoin and 2,3-butanediol detected in the headspace of maize seeds. Surface sterilised seeds were incubated in overnight cultures (10^8 CFU/mL LB) of different bacterial isolates and strains of *Bacillus subtilis* (BD170 and FZB24) or in antibiotics or autoclaved water only. VOCs were sampled and analysed as described in figure 1. Isolate 8 was later identified as *Enterobacter aerogenes (*synonym *Klebsiella mobilis)* and used for further experiments described in chapter V. N = 6, note the different scales for acetoin and 2,3-butanediol.

Curriculum vitae

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Formation

2003 - 2006	**PhD in Chemical Ecology** at the University of Neuchâtel, Switzerland. Title: Assessing the importance of specific volatile organic compounds in multitrophic interactions. Supervision: Prof. Dr. T. J. Turlings.
1995 - 2001	**Student in Biology** at the University of Zürich, Switzerland. **Masters** in Plant Science at the ETH Zürich. Title: Parasitoids' innate responses to herbivore induced synomones. Supervision: Prof. Dr. S. Dorn, Dr. L. Mattiacci.
1998 - 1999	**Exchange year** at the University of Exeter, UK. Research project in Entomology, Title: Hitch-hiking behaviour in the leaf cutting ant *Atta cephalotes*. Honourable mention: Dean's commendation for exceptional performance in Biology modules.
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Work experiences

since 2006	**Federal Office for the Environment FOEN**, Bern, Switzerland. Division of Substances, Soil, Biotechnology. Scientific officer. Major tasks and activities: Convention on Biological Diversity, access to genetic resources and benefit sharing, biosecurity of biocontrol agents.
2002 - 2003	**Agroscope FAL Reckenholz**, Zürich. Research assistant. Research in the group of Biosafety and Ecotoxicology. Effects of transgenic plants on non-target organisms. Supervision: Dr. F. Bigler and Dr. A. Dutton.
2001 - 2002	**USDA-ARS**, Tifton, Georgia, USA. Research assistant. Research in the group of Crop Production and Management. Development of a biosensor by using parasitic wasps. Supervision: Dr. W. J. Lewis and Dr. G. Rains.
2001 - 2001	**CABI, Delémont**. Internship. Literature research in the group of Biological Weed Control. Supervision: Dr. A. Gassmann.

Languages

German	mother tongue
Rumantsch	mother tongue
English	very good skills written and oral
French	good skills written and oral
Italian	good skills written and oral

Publications

2006 **D'Alessandro M**, Held M, Triponez Y, Turlings TCJ. The role of indole and other shikimic acid derived maize volatiles in the attraction of two parasitic wasps. *Journal of Chemical Ecology*, in press.

2006 Ton J, **D'Alessandro M**, Jourdie V, Jakab G, Karlen D, Held M, Mauch-Mani B, Turlings TCJ. Priming by air-borne signals boosts direct and indirect resistance in maize. *The Plant Journal*, in press.

2006 Held M, **D'Alessandro M**, Turlings TCJ. Methods to study the role of individual volatile organic compounds (VOCs) in indirect defences of plants against herbivorous arthropods. *IOBC Bulletin*, in press.

2006 **D'Alessandro M**, Turlings TCJ. Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods. *The Analyst,* 131: 24-32.

2005 **D'Alessandro M**, Turlings TCJ. In situ modification of herbivore-induced plant odors: A novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chemical Senses,* 30: 739-753.

2005 Scascighini N, Mattiacci L, **D'Alessandro M**, Hern A, Rott AS, Dorn S. New insights in analysing parasitoid attracting synomones: early volatile emission and use of stir bar sorptive extraction. *Chemoecology,* 15: 97-104.

2004 Rains GC, Tomberlin JK, **D'Alessandro M**, Lewis WJ. Limits of volatile chemical detection of a parasitoid wasp, Microplitis croceipes, and an electronic nose: A comparative study. *Transactions of the Asae,* 47: 2145-2152.

2004 Dutton A, **D'Alessandro M**, Romeis J, Bigler F. Assessing expression of Bt-Toxin (Cry1Ab) in transgenic maize under different environmental conditions. *IOBC/wprs Bulletin,* 27: 49-56.

2004 Dutton A, Obrist L, **D'Alessandro M**, Diener L, Müller M, Romeis J, Bigler F. Tracking Bt-toxin in transgenic maize to assess the risks on non-target arthropods. *IOBC/wprs Bulletin,* 27: 57-64.

2001 Mattiacci L, Rocca BA, Scascighini N, **D'Alessandro M**, Hern A, Dorn S. Systemically induced plant volatiles emitted at the time of "danger". *Journal of Chemical Ecology,* 27: 2233-2251.

Participation in Editorial Work

Since 2006 Reviewer for BioControl

Since 2005 Editorial Board of Plant Signalling and Behavior

Since 2003 Reviewer for Entomologia Experimentalis et Applicata

Attended international congresses and seminars

2006 Julius von Sachs Seminar, University of Würzburg, Germany, July 6. Title: Towards the identification of key volatile compounds affecting multitrophic interactions in maize plants.

2006 IOBC-meeting: Breeding for inducible resistance against pests and diseases. Heraklio, Crete, Greece, April 27 – 29. Paper: Endophytic bacteria modify defences of maize plants against insects and pathogens.

2005 Seminar of the section Phytopathology, Utrecht University, Utrecht, the Netherlands, November. Title: How soil micro-organisms and microbial volatile organic compounds affect a tritrophic signalling network

2005 The 21st Annual Meeting of the International Society of Chemical Ecology, Washington D.C., USA, July 23-27. Paper: Possible effects of soil micro-organisms and microbial volatile organic compounds on a tritrophic signaling network. Travel Grant awarded.

2005 Insect Chemical Ecology for PhD students ICE, Alnarp, Sweeden, March 7-18. Title: Towards the identification of key compounds for the attraction of parasitoids. Travel Grant awarded.

2005 NCCR Plant Survival International Conference, Leysin, Switzerland, March 31 – April 3. Poster: Effects of soil micro-organisms and microbial volatile organic compounds on a tritrophic signaling network.

2004 IOBC-meeting: Methods in Research on Induced Resistance agains Insects and Diseases. Delémont, Switzerland, November 2-4. Paper: Towards the identification of key compounds in tritrophic interactions - In situ and in vivo modification of herbivore-induced plant odours.

2003 12th International Symposium on Insect-Plant Relationships SIP, Berlin, Germany, 07-12 August 7-12. Paper: In situ modification of herbivore-induced plant odours: a new approach to study the attractiveness of volatile organic compounds to parasitoids.

2001 Annual meeting of the Entomological Society of America ESA, San Diego, USA, December 9-12. Paper: Parasitoids as chemical biosensors: Capacity to detect and discriminate indicator chemicals.