The Role of Indole in Maize-Herbivore Interactions

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SUMMARY

In order to counter herbivore attacks, plants have developed a multitude of defence strategies, including the release herbivore-induced plant volatiles (HIPVs). HIPVs can be used as foraging cues by natural enemies of the herbivores, including predators and parasitoids. In addition, they can also be exploited by herbivores themselves to localize their host plants. Some HIPVs even prime non-attacked plant tissues or neighbouring plants to respond faster and more strongly to subsequent attacks. Whereas some HIPVs have been well studied, the role of many others remains unclear. For instance, little is known about indole, a major constituent of the herbivore-induced volatile blend. In the present thesis, we studied the role of indole in direct and indirect defences in maize using indole deficient mutants and synthetic indole.

In Chapter 1 we investigated the role of indole as a plant defence signal. We provide evidence that indole is essential for within-plant priming of other HIPVs and acts as a between-plant signal that primes non-attacked neighbours. In Chapter 2, we investigated the impact of indole on the generalist herbivore *Spodoptera littoralis*. We demonstrate that volatile indole acts as a direct defence in maize by repelling *S. littoralis* moths and caterpillars and by reducing the survival of early instar caterpillars and the reproductive output of adults. In Chapter 3, we studied the importance of indole on the third trophic level. We found that, although indole attracts certain parasitoids, indole-exposure protects *S. littoralis* caterpillars by increasing their resistance against parasitism. In Chapter 4, we investigated the specificity of the effects found in chapters 2 and 3. We found that neither the degree of host plant specialization nor the phylogenetic origin or the association with indole-producing plants determines the response of herbivores and natural enemies to the volatile, and that the role of indole is highly species-specific.

Overall, this thesis contributes to a better understanding of the role of indole in interactions between plants, herbivore insects and natural enemies and highlights the diverse roles of HIPVs in tritrophic interactions.

Key words: Indole, herbivore-induced plant volatiles, natural enemies, priming, herbivores, *Spodoptera littoralis,* maize, tritrophic interactions, host plant specialization.

RÉSUMÉ

Afin de se protéger contre les attaques d'insectes herbivores, les plantes ont développé de multiples moyens de défense, dont la libération de composés volatils induits par les herbivores (HIPVs). Ces composés volatils peuvent être utilisés par les ennemis naturels des herbivores tels que les prédateurs et les parasitoïdes. D'autre part, ils peuvent être exploités par les herbivores eux-mêmes pour localiser leurs plantes hôtes. Certains HIPVs peuvent aussi avertir les tissus non attaqués d'une même plante ou les plantes voisines d'un risque d'attaque. Le terme employé est "priming". Les plantes averties pourront ainsi répondre plus rapidement et de manière plus efficace lorsque l'attaque se produira. Tandis que certains HIPVs ont été bien étudiés, le rôle de beaucoup d'autre reste à trouver. Par exemple, nous n'avons que peu de connaissances en ce qui concerne l'indole, un composé dominant du mélange de volatils émis par les plantes. Dans la thèse présentée ici, nous avons étudié le rôle de l'indole dans les défenses directes et indirectes du maïs grâce à l'utilisation de plantes mutantes dans la production d'indole et d'indole synthétique.

Dans le premier chapitre, nous avons étudié le rôle de l'indole en tant que signal de défense. Nous fournissons la preuve que l'indole est essentiel pour le "priming" d'autres HIPVs au sein d'une même plante mais qu'il agit aussi comme signal de communication entre différentes plantes afin de les préparer à une possible attaque. Dans le deuxième chapitre, nous avons étudié l'effet de l'indole sur un insecte herbivore généraliste, *Spodoptera littoralis*. Nous démontrons que l'indole agit en tant de défense directe chez le maïs en repoussant les adultes et les chenilles de cette espèce et en réduisant la survie des chenille et le succès reproducteur des adultes. Dans le troisième chapitre, nous avons étudié l'importance de l'indole au niveau du troisième niveau trophique. Nous avons trouvé que malgré une attraction de certains parasitoïdes, une exposition à l'indole protège les chenilles de l'espèce *S. littoralis* en augmentant leur résistance contre les parasitoïdes. Dans le quatrième chapitre, nous avons étudié la spécificité des effets trouvés dans les deux chapitres précédents. Nous avons trouvé que ni le degré de spécialisation pour les plantes hôtes, ni l'origine phylogénétique, ni l'association avec des plantes produisantde l'indole ne déterminent la réponse des insectes herbivores et des ennemis naturels à l'indole. Nous concluons que le rôle de l'indole est dépendant des espèces.

D'une manière générale, cette thèse contribue à une meilleure compréhension du rôle de l'indole dans les intéractions entre les plantes, les insectes herbivores et les ennemis naturels; elle confirme le rôle multiple des composés volatils dans les intéractions tri-trophiques.

Mots-clés: Indole, composés volatils induits par les herbivores, ennemis naturels, priming, herbivores, *Spodoptera littoralis,* maïs, interactions tritrophiques, spécialisation plantes-hôtes

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GENERAL INTRODUCTION AND THESIS OUTLINE

INTRODUCTION

Since more than 400 millions years, plants have coexisted and interacted with insects. Even though some of these interactions such as pollination are beneficial, many of them are detrimental for the plants. Herbivorous insects are the most abundant and diverse group of insects, and almost every plant species is attacked by at least one insect species (Strong *et al.* 1984). Insects can strongly reduce the survivorship and/or the reproduction of plants. To survive in nature, plants therefore have to defend themselves against herbivore attacks.

Over evolutionary time, plants have developed a wide range of defence strategies. Plant defences can affect herbivore preference, including feeding behaviour or host plant selection and herbivore performance, including growth rate and development. The first line of defence in plants is of physical nature. Morphological features such as spines, trichomes, rough leaf-surfaces or resin ducts can reduce food accessibility (Schoonhoven *et al.* 2005). The second line of defences is chemical. It include repellent or toxic compounds such as phytoalexins (Grayer *et al.* 1993) and digestibility reducers (Pearce *et al.* 1991) that are stored in plant cells. Despite their effectiveness, the continuous production of defences is costly and may lead to growth trade-offs under limiting conditions. This is the reason why plants have developed the ability to activate defences only when they are needed. The benefits of induced defences are well documented (Dicke & Loon 2000; Dicke *et al.* 2003; Heil 2004; Karban & Baldwin 2007).

In addition to direct defences, plants can also target their enemies indirectly by recruiting organisms from higher trophic levels. Mechanisms that enhance the effectiveness of carnivorous natural enemies are termed indirect defences. Constitutive indirect defences include special structures that provide refuge for organisms such as ants and mites or the production of extra-floral nectar or nutritive structures that are used as food sources by natural enemies of the herbivores. Induced indirect defences include volatile organic compounds (VOCs) that are used by natural enemies of the herbivores to locate their preys or hosts (Turlings & Benrey 1998). These herbivore-induced plant volatiles (HIPVs) are often herbivore specific and are only triggered by specific elicitors (Dicke *et al.* 1990; Turlings *et al.* 1990; Paré & Tumlinson 1999), which enable plants to distinguish between different herbivores (De Moraes *et al.* 2001; Arimura *et al.* 2004). Several elicitors that trigger the production of HIPVs have been found and isolated from the regurgitant of herbivorous insects. Conjugation of plant and herbivore-derived precursors results in the formation of fatty acid amino conjugates (FACs) (Hopke *et al.* 1994; Mattiacci *et al.* 1995; Alborn *et al.* 1997; Schmelz *et al.* 2007). N-17-hydroxylinolenoyl-L-glutamine, also called volicitin, is a common FAC found in the oral secretions of lepidopterans (Turlings et al. 1993a; Alborn et

al. 1997; Halitschke et al. 2001). Application of volicitin after mechanical damage in maize activates the formation and release of the same attractive blend of HIPVs produced after an attack by herbivores (Turlings *et al.* 2000).

The role of HIPVs in multitrophic interactions

Third trophic level

The role of HIPVs in tritrophic interactions have been characterized in a wide range of plant (D'Alessandro *et al.* 2006; Snoeren *et al.* 2007; Dicke *et al.* 2009). HIPVs are not only released by the damaged site of the plant, but also systemically (Turlings et al. 1991; Dicke 1994) which increases their detectability by natural enemies (De Moraes *et al.* 1998; Hilker *et al.* 2002; Dicke & Baldwin 2010). For instance, maize plants infested by the African cotton leaf worm *Spodoptera littoralis* emit a blend of over 30 different HIPVs that are attractive for *Cotesia marginiventris* females (Schnee *et al.* 2006). Because the blend of HIPVs depends on the herbivore species and plant developmental stage (Takabayashi *et al.* 1995; De Moraes *et al.* 1998; Turlings *et al.* 1998), beneficial insects are able to distinguish damaged from undamaged plants, but also plants infested by different herbivore species or different stages of the same species (Dicke 1994). It has been shown that natural enemies often rely on odour signals provided by the plant, the host or from a combination of both the plant and the host (Stowe *et al.* 1995). Indeed, parasioids are able to associate the odours from the plant-herbivore complex with the suitability of the host (Turlings *et al.* 1993a). This associative learning helps to overcome problems with the reliability of HIPV signals (Vet & Dicke 1992).

Second trophic level

HIPVs may also directly affect the behaviour of herbivores. For instance, many females of noctuid moths use HIPVs to search for appropriate oviposition sites (Renwick & Chew 1994). It has been shown that females preferentially choose to oviposit on undamaged plants, probably in order to limit exposure of their offspring to competition by conspecifics, particularly from older larvae (Kakimoto *et al.* 2003) or to avoid costs associated with induced plant defences (Landolt 1993; Anderson & Alborn 1999; De Moraes *et al.* 2001; Singer & Stireman 2001). Moreover, olfaction also plays an important role for larval behaviour. Ablation experiments showed that caterpillar antennae mediate olfactory attraction to odours located 10–30 cm away (Schoonhoven 1987). Studies conducted with silkworm *Bombyx mori* (Lepidoptera: Bombycidae) have shown that some odorant receptor genes are expressed only in the larval stages (Mooney *et al.* 2009). In certain cases, larvae may have to disperse from the oviposition site and look for a new host plant, for instance when the oviposition

choice made by the female is inappropriate or when the conditions at the oviposition site are becoming suboptimal (Roitberg & Mangel 1993; Berdegué *et al.* 1998; Doak 2000). For example, resources can be insufficient or severely degraded by the presence of conspecifics (Chapman *et al.* 1999; Singer & Stireman 2001), or competition can be too intense (Kakimoto *et al.* 2003). In 2006, Caroll *et al*., showed that *Spodoptera frugiperda* larvae preferred odours from damaged maize by conspecifics over odours from undamaged maize (Carroll *et al.* 2006).

First trophic level

Plants themselves are also able to detect HIPVs. HIPVs can for instance act as airborne signals between neighbouring plants or between different parts of the same plants (Baldwin & Schultz 1983; Arimura *et al.* 2001; Agrawal & Kurashige 2003; Engelberth *et al.* 2004; Heil & Kost 2006; Karban *et al.* 2006). Initially, it was believed that HIPVs might directly activate defence responses in plants, but recently it was shown that they do not induce any defence, as no differences in level of defences was found in absence of herbivory. Rather, they prime HIPV-exposed plant parts in order to respond stronger and faster to subsequent herbivore attack (Engelberth *et al.* 2004; Kost & Heil 2006). Priming has been reported under both laboratory and field conditions (Engelberth et al. 2004; Baldwin et al. 2006; Kessler et al. 2006; Ton et al. 2007; Frost et al. 2008b). Exposure to HIPVs induces the expression of defence related genes (Arimura et al. 2000) and defence metabolites such as phytohormones (Engelberth *et al.* 2004), phytoalexins (Bate & Rothstein 1998; Matsui 2006), extra-floral nectar (Heil & Kost 2006) or the production of HIPVs themselves (Heil & Kost 2006; Kessler et al. 2006; Heil & Bueno 2007; Ton et al. 2007; Frost et al. 2008a). In maize, studies on HIPVs-induced priming have shown that priming leads to stronger and earlier induction of a subset of genes after subsequent herbivore attack but also to a stronger attraction of natural enemies and a reduced caterpillar feeding and development (Ton et al. 2007).

The biosynthesis and function of major HIPV classes

The HIPVs blend is composed of many classes of compounds emitted at different time points after infestation (Turlings *et al.* 1998). They include the six-carbon (C6)-volatiles, also called green leaf volatiles (GLVs), aromatic compounds, mono-, homo- and sesquiterpenes (Paré & Tumlinson 1999; D'Alessandro & Turlings 2005). At least three biosynthetic pathways are involved in the production of HIPVs (Paré & Tumlinson 1999) (Figure 1).

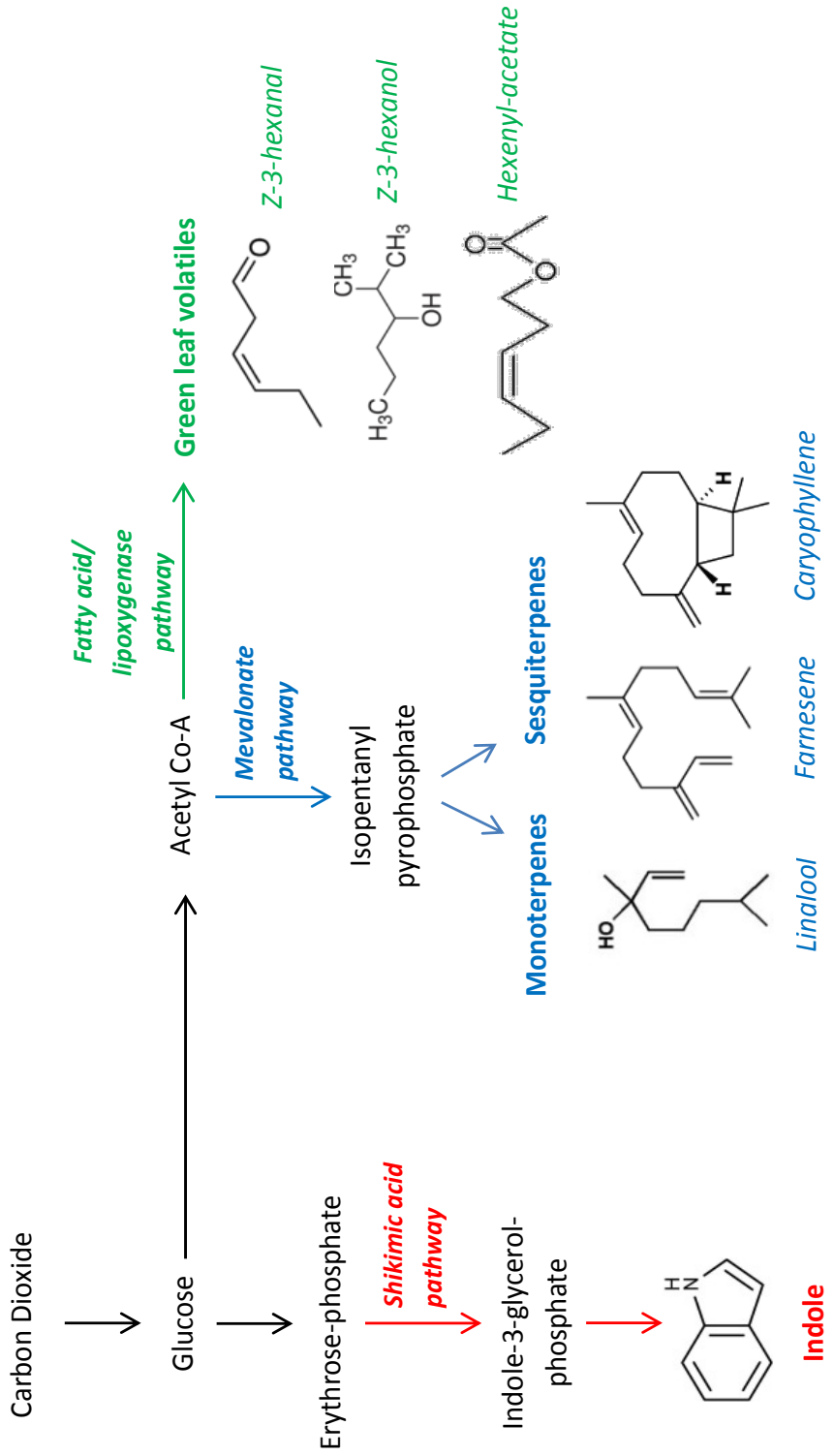

Figure 1: Three biosynthetic pathways are involved in the production of different classes of VOCs (Modified after (Pare and Tumlinson 1997). The fatty acid-lipoxygenase pathway generates green leafy volatiles, the isoprenoid pathway produces monoterpenes and sesquiterpenes and the shikimic acid-tryptophan pathway results in several compounds, including indole.

Green leaf volatiles

The fatty acid-lipoxygenase (LOX) pathway generates green leaf volatiles. GLVs include isomers of hexenol, hexenal and hexenyl-acetate. With the exception of *(Z)*-3-hexenyl-acetate which is produced a few hours after feeding (Turlings *et al.* 1995), they are released immediately after feeding starts and their release slowly fades out after feeding stops (Matsui *et al.* 2000). GLVs seem to be involved in several plant processes, including the induction of defence gene expression, the accumulation of endogenous jasmonic acid (JA) and the production of terpenoids (Bate & Rothstein 1998b; Farag & Pare 2002; Engelberth *et al.* 2004). Moreover, it was shown that GLVs act as signal in plant-plant communication (Arimura et al. 2000) and are implicated in the attraction of parasitoids (Hoballah & Turlings 2005).

Terpenoids

Terpenoids include monoterpenes (C10), homoterpenes (C11, C16), and sesquiterpenes (C15). The isoprenoid pathway produces monoterpenes and sesquiterpenes. 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. Homoterpenes are derived from farnesylpyrophosphate. They are released less rapidly than GLVs upon herbivore feeding. In maize, their release starts 2 to 4 hours after damage (Turlings *et al.* 1998); in cotton the delay is between 12 and 24 hours (Loughrin *et al.* 1995). Similarly to GLVs, terpenoids are implicated in the attraction of natural enemies (Takabayashi et al. 1995; D'Alessandro & Turlings 2005; Hoballah & Turlings 2005; D'Alessandro et al. 2006; Fontana et al. 2011) and in the attraction of many herbivore insects (Hanson *et al.* 1999; Halitschke & Baldwin 2004; Halitschke *et al.* 2008; von Mérey *et al.* 2011).

Aromatic compounds

An important class of secondary metabolites that actively participate in plant direct defence are aromatic metabolites. While most of the aromatic compounds are usually non volatile, the volatile subset is represented by benzenoid, phenylpropanoid and phenylpropanoid-related compounds and by products synthesized from the shikimic acid pathway and containing an aromatic ring such as indole and methyl anthranilate. A biological activity toward insects has been shown for few aromatic volatile compounds. A repellent effect of methyl salicylate has been shown on aphids both in field and laboratory (Pettersson

et al. 1994; Ninkovic et al. 2003, Hardie et al. 1994). Another aromatic compound Eugenol is repellent to the four beetle species (Obeng-Ofori and Reichmuth 1997). In addition, insecticidal activities have been shown for a large range of other aromatic compounds such as benzyl salicylate, benzyl benzoate, isoeugenol or methyl-eugenol (Ngoh et al. 1998; Bin Jantan et al. 2005).

Many other aromatic compounds are emitted from herbivore-infested plants, but their roles towards herbivore insects and natural enemies remain to be determined. For instance, little is known about the importance and the specific role of the individual compound indole. Indole is a heterocyclic, nitrogen containing metabolite and a major constituent of the herbivore induced volatile blend in many plant species, including maize, cotton, rice, gerbera, peanut and lima bean (Turlings et al. 1991; McCall et al. 1994; Gols et al. 1999; Cardoza et al. 2003; De Boer et al. 2004; Yuan et al. 2008). The biosynthetic pathway of indole has been well studied, especially in maize. Indole is an intermediate in at least three different biosynthetic pathways (Figure 2). First, it is an intermediate in the formation of the amino-acid tryptophan produced by a tryptophan synthase (TS) (Frey *et al.* 1997). TS catalyses the conversion of serine and indole-3-glycerol-phosphate (IGP) to tryptophan by two independent reactions. IGP is converted by the alpha subunit (TSA) to indole and glyceraldehyde-3-phosphate, and then indole is channelled to a beta subunit (TSB) and converted to tryptophan. Free indole is not released from the TS complex, but travels through a tunnel connecting the active sites of both subunits (TSA and TSB). Secondly, the production of indole from IGP by the BX1 enzyme leads to the production of direct defence secondary compounds named benzoxazinoids (e.g. DIMBOA). Finally, the gene *igl* encodes for an indole-3-glycerol-phosphate lyase which converts IGP in volatile indole and glyceraldehyde-3-phosphate (Frey et al. 2000). TSA is inactive as monomer and can only function by interacting with TSB. Contrary to TSA, BX1 and IGL function as monomers (Kriechbaumer *et al.* 2008). The maize BX1 and IGL proteins share an amino-acid sequence identity of more than 60 % to TSA. This suggests that both bx1 and igl genes are evolutionary related to the TSA gene (Schullehner *et al.* 2008). They may have evolved as defensive enhancers. The BX1 enzyme is constitutively expressed in all plant stages. To the contrary, IGL enzyme is expressed only in response to insect damage (Frey *et al.* 1997). The production of indole is also activated by the herbivore derived elicitor volicitin, but not by mechanical damage alone (Frey et al. 2000). Indole production is not pest-species specific and is induced by regurgitates from at least five different caterpillar species as well as grasshoppers (Turlings *et al.* 1993b).

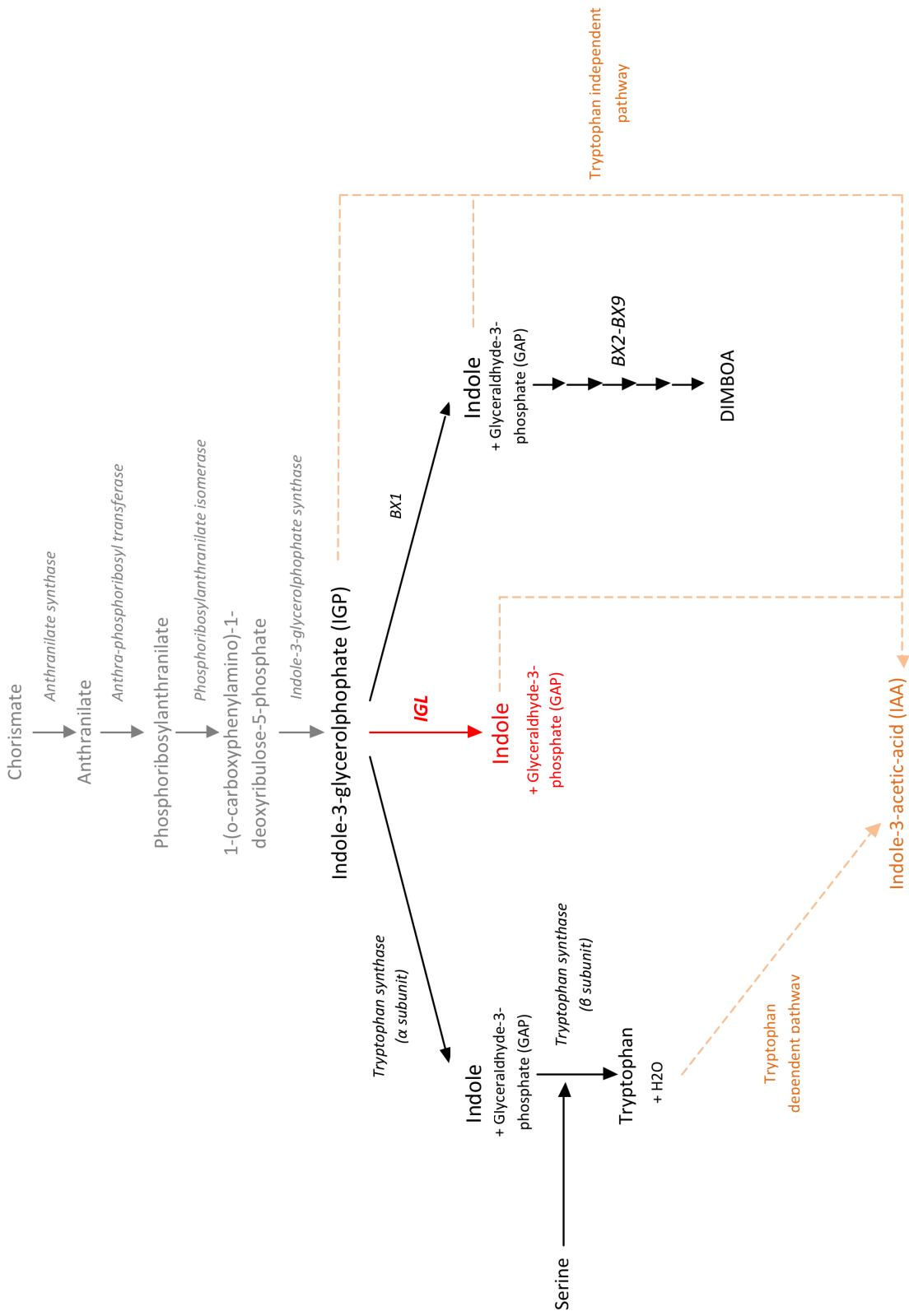

Figure 2: **Biosynthesis of indole**. Indole serves as an intermediate in at least three different biosynthetic pathways. IGL converts IGP in free volatile indole, BX1 and TSA catalysed indole biosynthesis for the formation of benzozaxinoids and tryptophan.

The mechanism of indole biosynthesis is well characterized, but almost nothing is known about its role and contribution in plant defences. Preliminary results suggest a role of indole in indirect defences. In fact, it has been shown that a blend of indole and other three volatile compounds had an attractive effect for *Cotesia kariyai* (Fukushima *et al.* 2002). Howerver, recent studies showed that indole alone is unattractive for *Cotesia marginiventris* wasps and has a repellent effect for *Microplitis rufiventris* (D'Alessandro *et al.* 2006). In addition, some studies have demonstrated an effect of indole in combination with other compounds on the attraction of *Diabrotica virgifera virgifera* and several species of diabrocitite rootworm beetles and corn rootworms (Metcalf & Lampman 1991; Metcalf *et al.* 1995).

Maize as a model to study the role of indole in plant-herbivore interactions

The current thesis aims to elucidate the role of indole in maize multitrophic interactions. Maize is one of the most important crops cultivated worldwide (Sattaur 1989) with 158 million hectares of maize harvested every year (FAO, 2007), maize is of economic importance for both cattle and human consumption (Fedoroff 2003). About 10 000 species of insects are able to attack maize, causing serious management problems. It was estimated that every year, animal pests are responsible for maize yield losses of about 16% (Oerke 2006). Archaeological evidence and molecular data suggest that maize cultivation started in Mexico between 9000 and 6000 years ago (Piperno & Flannery 2001; Matsuoka *et al.* 2002) with the domestication of the wild grass teosinte (*Zea mays spp. Parviglumis*), an ancestor of our modern maize (*Zea mays ssp. Mays*). The process of domestication involved strong selection and breeding for yield. However, maize genetics is highly diverse, and high intraspecific variation in the composition and concentration of herbivore-induced VOCs between different maize varieties are present (Gouinguene *et al.* 2001). Interestingly, indole is produced by all tested varieties, including teosinte and a wide variety of other plants (Turlings et al. 1990; McCall et al. 1993; Gols et al. 1999; Cardoza et al. 2003; De Boer et al. 2004; Zhuang et al. 2012). Because indole is specifically released in response to herbivore-elicitors (Frey *et al.* 2004; Zhuang *et al.* 2012) and is known to be induced by herbivory, the insect-derived elicitor volicitin and MeJA treatment but not by mechanical damage (Frey et al. 2000; Frey et al. 2004), it is a interesting and promising compound in the context of plant defences. Recently, we isolated an igl mutant in a bx1 mutant background (Ahmad *et al.* 2010). This double mutant does no longer release indole upon herbivore induction. In this thesis, we used these genetic resources to test whether indole is involved in direct and indirect defences against herbivores.

Thesis outline

In Chapter 1, we investigated whether indole is involved in airborne priming. By exposing maize plants to herbivore-induced volatiles of *igl* mutants or IGL wild type plants and to synthetic indole released from dispensers at physiologically relevant concentrations, we tested whether volatile indole serve as an essential within-plant and plant-plant priming signal in maize.

In Chapter 2, we investigated the effect of volatile indole as direct defence against the herbivore *Spodoptera littoralis* (Cresson, Hymenoptera: Braconidae). Also called Egyptian cotton leafworm, *S. littoralis* is one of the most destructive lepidopteran pests in agriculture. *Spodoptera littoralis* attacks more than 40 families of plants, containing at least 87 species with considerable economic importance (Salama *et al.* 1971). The larvae are able to feed on all plant organs, even if they prefer young leaves. Serious infestation can lead to a complete defoliation of plants. The distribution of *S. littoralis* occurs throughout Africa and extends into Turkey, eastern Spain, southern France and northern Italy. By using the resources described above, we tested the effect of indole on *S. littoralis* performance and behaviour.

In Chapter 3, we studied the importance of indole in tritrophic interactions. We first investigated the effect of indole on the attraction and the performance of two parasitic wasps *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) and *Microplitis rufiventris* (Kokujev) (Hymenoptera: Braconidae). Both species attack early instar larvae of many lepidopteran moths and are known to use VOCs in host location (Gouinguené et al. 2003; Hoballah & Turlings 2005). We also examined the impact of indole on their performance when parasitizing indole-exposed *S. littoralis* caterpillars.

In Chapter 4, we investigated whether the degree of host plant specialization or the association with indole-producing plants determine the response of herbivores and natural enemies to indole to understand the specificity of the effects observed in chapters 2 and 3.

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CHAPTER 1

Indole is the principal herbivore-induced volatile priming signal in maize

INDOLE IS THE PRINCIPAL HERBIVORE-INDUCED VOLATILE PRIMING SIGNAL IN MAIZE

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ABSTRACT

Herbivore-induced volatile organic compounds prime non-attacked plant tissues to respond more strongly to subsequent attacks. However, the key volatiles that trigger this “primed state” remain largely unidentified. In maize, the release of the aromatic compound indole is herbivore-specific and occurs earlier than other induced defences. We therefore hypothesized that indole may be involved in airborne priming. Using indole-deficient mutants and synthetic indole dispensers, we show that herbivore-induced indole enhances the induced production of defensive mono-, homo- and sesquiterpenes in neighbouring plants. Furthermore, the release of indole was found to be essential for priming of systemic leaves in previously attacked plants. Indole exposure markedly increased the herbivore-induced production of the stress hormones jasmonic acid and abscisic acid, which is likely the mechanism for indole-triggered volatile priming. These results demonstrate that indole functions as a rapid and potent aerial priming agent that warns systemic tissues and neighbouring plants from incoming attack.

INTRODUCTION

In response to herbivore-attack, plants activate a wide array of defences that can reduce herbivore damage (Grayer *et al.* 1993; Schoonhoven *et al.* 2005), including blends of volatile organic compounds (VOCs) that can be used as foraging cues by natural enemies of the herbivores (Dicke *et al.* 1990; Turlings *et al.* 1990; Vet & Dicke 1992; Dicke & Loon 2000). Herbivore induced volatiles (HIPVs) have also been implicated in plant-plant communication, as they can be perceived by neighbouring plants (Dicke *et al.* 2003; Baldwin *et al.* 2006), and prime them for an enhanced response upon subsequent insect attack (Engelberth *et al.* 2004). By targeting JA-inducible genes, HIPVs have been shown to enhance both direct and indirect defence responses (Ton *et al.* 2007; Heil & Ton 2008), which can benefit the receiver by decreasing herbivore damage (Heil & Karban ; Ton *et al.* 2007). However, the benefit for the emitter plant is not evident in this context, leading to the notion that plants do not "communicate" but "eavesdrop" on each other (Karban *et al.* 2003). As an adaptive explanation for why plants emit HIPVs, a role of HIPVs as within-plant signal has been proposed (Farmer 2001). Indeed, HIPV-mediated within-plant communication has been demonstrated in several plant species including sagebrush, lima beans, poplar and blueberry (Karban *et al.* 2006; Frost *et al.* 2007; Heil & Bueno 2007; Rodriguez-Saona *et al.* 2009). In these cases, HIPVs released from an attacked part of the plant primed the healthy parts of the same plant to respond more strongly (Heil & Bueno 2007; Frost *et al.* 2008a). Within-plant communication through HIPVs is especially efficient when the vascular connectivity is limited or when adjacent leaves are spatially but not anatomically close (Heil and Bueno 2007). As discussed by Heil and Ton (2008), herbivorous insects often move from one leaf to another, but adjacent leaves are not always directly connected via the plant's vascular system. Therefore, volatile compounds may reach distal parts of the plant faster than vascular signals.

An important step to understand the mechanistic underpinnings of airborne and vascular systemic priming is the elucidation of the actual messengers. In *Arabidopsis thaliana*, for instance, azelaic acid (AzA), a nine carbon dicarboxylic acid, has been proposed as a mobile vascular priming signal in plant-pathogen interactions (Jung *et al.* 2009). Furthermore, methylated forms of plant hormones, including methyl jasmonic acid (MeJA) and methyl salicylic acid (MeSA) can function as volatile signals in plant defence (Klessig & Malamy 1994; Shulaev *et al.* 1997; Fidantsef *et al.* 1999). In *Arabidopsis thaliana* however, none of these signals are strictly required for systemic acquired resistance (SAR) (Attaran *et al.* 2009) and the existence of other volatile priming agents has been proposed (Yao *et al.* 2011). Other candidate volatiles that may prime systemic tissues are green leaf volatiles (GLVs) and terpenoids. Exposing lima bean (*Phaseolus lunatus)* leaves to volatiles from

spider mite infested lima bean leaves as well as to the terpenoids β-*ocimene,* (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) or (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) resulted in the induction of defence-related genes (Arimura *et al.* 2000; Arimura *et al.* 2001). However in maize, no evidence that terpenoids are able to prime defence responses in the receiver plants has been found (Ruther & Furstenau 2005). Evidence for GLVs as priming signals on the other hand has been found in multiple plant species. Exposure to (*Z)*-3-hexenyl acetate for instance was sufficient to induce extrafloral nectar (EFN) secretion in lima bean plants (Kost & Heil 2006). Treatment of *A. thaliana* seedlings with (*E*)-2-hexenal induced the transcription of several genes involved in the plant's defence response including LOX and PAL (Bate & Rothstein 1998). Furthermore, exposure to *(Z)*-3-hexenol led to an higher production of VOCs in tomato (Farag & Pare 2002). In maize, the role of GLVs is controversial. At least three green leaf volatiles, *(Z)*-3-hexenal, *(Z)*-3-hexen-1-ol, and *(Z)*-3-hexenyl acetate, have been identified to induce the production of sesquiterpenes and JA in neighbouring plants following infestation (Engelberth *et al.* 2004). Also, an induction of HIPV emission was demonstrated after exposure to *(Z)*-3-hexenol, an effect which was enhanced by ethylene application (Ruther & Kleier 2005). However, in another study, exposure to *(Z)*-3-hexenol led to an increased production of *(Z)*-3-hexenyl acetate and methyl salicylate, but not sesquiterpenes (Farag *et al.* 2005). One possible reason why the role of various HIPVs as volatile priming signals has remained unclear is that in most studies, healthy plants were supplemented with synthetic volatiles, a procedure that does not adequately mimic the precise timing and concentrations of HIPV emissions in nature. The use of “deaf” and “mute” plants has therefore been advocated as an alternative approach to study volatile plant-plant communication (Baldwin *et al.* 2006). With this approach, it was for instance demonstrated that neither GLVs nor terpenoids primed the expression of defence genes in *Nicotiana attenuata* (Paschold *et al.* 2006). To date, it remains largely unknown which plant volatiles are the actual drivers of priming responses in plants.

Compared to GLVs and terpenoids, aromatic HIPVs have received little attention as potential priming signals. Indole in particular is a promising candidate in this context, as it is produced by a wide variety of plants (Turlings *et al.* 1990; McCall *et al.* 1993; Gols *et al.* 1999; Cardoza *et al.* 2003; De Boer *et al.* 2004; Zhuang *et al.* 2012) and specifically released in response to herbivore-elicitors (Frey *et al.* 2004; Zhuang *et al.* 2012). Furthermore, indole emission in maize peaks about two hours prior the emission of sesquiterpenes (Turlings *et al.* 1998), which could enable it to act as a fast and reliable synergistic factor in within-plant induced defence signalling. In maize, indole is produced from indole-3-glycerol phosphate and is channelled into different pathways. First, indole can be formed by the tryptophan synthase alpha subunit (TSA), which cannels it directly to TSB for further conversion into the

essential amino acid tryptophane (Frey et al. 1997; Frey et al. 2004). Second, it can be produced by the BX1 enzyme as an intermediate in the production of benzoxazinoids, a class of non-volatile defensive secondary metabolites of the grasses (Frey *et al.* 2009). Finally, indole can be formed by the indole-3-glycerol phosphate lyase IGL, which subsequently releases it as a volatile (Frey *et al.* 2000). The *IGL* gene is known to be induced by herbivory, the insect-derived elicitor volicitin and MeJA treatment (Frey *et al.* 2000; Frey *et al.* 2004). Recently, we isolated an igl mutant in a bx1 mutant background (Ahmad *et al.* 2010). This double mutant does no-longer release indole upon herbivore induction.

Here, we used these genetic resources to test whether indole is involved in HIPV priming. By exposing maize plants to herbivore-induced volatiles of *igl* mutants or IGL wild type plants and to synthetic indole released from dispensers at physiologically relevant concentrations, we show that volatile indole serves as an essential within-plant and plant-plant priming signal in maize.

MATERIALS AND METHODS

Plant cultivation

The maize lines 22 (igl.bx1), 7 (IGL.bx1) (Cross A) 32R (igl.bx1) and 16R (IGL.bx1) (Cross B) were obtained as previously described by (Ahmad *et al.* 2011b). Seeds of the hybrid Delprim, which are homozygous for both BX1 and IGL wild type alleles, were obtained from Delley Semences et Plantes SA., Delley DSP, Switzerland. All maize lines were grown individually in plastic pots (10 cm high, 4 cm diameter) with commercial potting soil (Aussaaterde, Ricoter, Aarberg, Switzerland) and placed in a climate chamber (23°C ± 2°C, 60% relative humidity, 16:8 h L/D, 50'000 lm/m^2). Maize plants used for the experiments were ten to twelve days old and had three fully developed leaves. The evening before the experiments, plants were transferred and kept under laboratory conditions (25 ± 2°C, 40 ± 10% relative humidity, 16h light/8h dark, and 8000 lm/m^2).

Within-plant priming with supplementation of synthetic indole

To test whether volatile indole is a key compound in within-plant priming in maize, we exposed different maize lines to synthetic indole. Delprim plants, igl-mutant plants (lines 22 and 32R) or IGL-wild type plants (lines 7 and 16R) were subjected to an elicitation treatment and put into clean odour vessels in the presence of a control- or indole- dispenser. Plants were connected to a multiple air-delivery system via Teflon tubing. This system consisted of

a central wooden tray with six or twelve glass odour source vessels (Turlings et al., 2004), a metal frame with eight neon tubes (four Osram 18W/21-810 alternated with four Sylvania Gro-Lux F18W/GRO-T8), and one or two manifolds with six flow meters (Aalborg Instruments & Controls; Monsey, NY, USA), each followed by charcoal filters and water bubblers filled with MilliQ-water (Model VCS-HADS-6AF6C6B; ARS Analytical Research System, Gainesville, FL, USA). The elicitation treatment was performed by scratching two leaves over an area of approximately 1 cm^2 on both sides of the central vein with anatomical forceps (stainless steel, 14.5 cm) (n=4). Then 10 μL of *Spodoptera littoralis* regurgitant were applied over the scratched leaf areas. Regurgitant had been previously collected from fourth instar *S. littoralis* that had been feeding on maize leaves for 24 hours and was then stored at -80°C until use. Dispensers consisted of 2 mL amber glass vials (11.6x32mm; Sigma-Aldrich, Buchs, Switzerland) containing 20 mg of synthetic indole (> 98 %, GC, Sigma-Aldrich, CH-9471 Buchs, Switzerland). The vials were sealed with a PTFE/rubber septum pierced by a Drummond 1 μL micro-pipette (Drummond, Millan SA, Plan-Les-Ouates, Switzerland) in black polypropylene cap. This device allowed the constant release of volatile indole. The length of the pipette was calibrated to release a 50 ng/h of indole, which corresponds to the amount emitted by IGL-wild type plants (*Zea mays* cv. Delprim). Control dispensers consisted of empty glass vials. Vials were prepared the day of the experiment. VOCs were collected for 600 minutes following elicitation.

Within-plant priming in igl-mutant and wild type plants

To confirm the specific role of indole as a within-plant priming agent, we performed an experiment using igl-mutant and IGL-wild type plants of both genetic backgrounds. Plants used for this experiment consisted of igl-mutant plants (lines 22 and 32R) and IGL-wild type plants (line 7 and 16R). Plants were submitted to three different treatments (n=4). Two groups were subjected to an elicitation treatment as previously described. For one of these groups, a Teflon bag (8x3cm) was placed around the wounded leaf in order to prevent VOCs to act as a volatile priming signal. The second group was wounded and left without Teflon bag. The last group was left undamaged. All plants were put into clean odour vessels. Twelve hours later (6 h light/6 h dark), the second leaf of all groups was subjected to an elicitation treatment as previously described. A new Teflon bag was put on the first leaf of each plant in order to prevent the volatile collection from the first leaf. All plants were put in clean odour vessels and connected to the multiple air-delivery system. VOCs were collected for 600 minutes.

Plant-plant communication: igl-mutant vs. wild type plants

To test whether volatile indole could also prime neighbouring plants, we exposed healthy Delprim plants to VOCs from infested igl-mutant plants or IGL-wild type plants of both genetic backgrounds (n=4). Source and target plants were individually introduced into glass vessels. Source plants consisted of igl-mutant plants (lines 22 and 32R) or IGL-wild type plants (line 7 and 16R). Target plants were either Delprim plants or IGL-wild type plants (line 7 and 16R). Source plants were infested with 20 first-instar *S. littoralis* larvae that were placed into the whorl of the youngest leaves. The glass vessels with the plants were connected to a multiple air-delivery system via Teflon tubing. Four hours later, target plants in similar vessels were exposed to air from herbivore-infested igl-mutant plants; or air from herbivore-infested IGL-wild type plants at a flow-rate of 0.3 L.min-1. After twelve hours of exposure, target plants were subjected to an elicitation treatment as described above and put in clean odour vessels for VOCs collection. VOCs were collected for 600 minutes.

Plant-plant communication: synthetic indole

To confirm the specific role of indole as a priming agent for communication between plants, healthy plants were exposed to control- or indole-dispensers for twelve hours (n=4). The same experiment as described above was performed, but a control- or indole-dispenser was placed in source bottles instead of the source plants. Target plants consisted of Delprim plants, IGL-wild type plants and igl-mutant plants and were expose to air from the neighbouring bottle for 12h. After elicitation treatment, VOCs were collected for 600 minutes.

Volatile collection and analysis

VOC-collections for all experiments were carried out by using a multiple air-delivery system. Purified air from the system entered the source vessels via Teflon tubing at a rate of 1.1 L.min-1 and was pulled out through the Super-Q trap at a rate of 0.7 L.min-1. Super-Q trap consisted of 7 cm glass tubes in which 25 mg of 80–100 mesh Super Q adsorbent (Altech, Deerfield, Illinois) placed and kept in place by one fine mesh metal screen on the one side and a little quantity of fibreglass held by a piece of Teflon (2 mm length) on the other side. In all experiments, a filter was attached to the horizontal port at the top of each odour source vessel. The other ports were sealed with a Teflon-coated septum in the screw cap. A 6 mm i.d. Tygon tube was connected to each collection trap, through which air was pulled out. Before each experiment, the traps were rinsed with 3 mL of methylene chloride. Super-Q traps from vessels containing elicited plants were collected after 45, 90, 180, 300, 500 and 600 min after elicitation treatment. Immediately after each collection, the volatiles collected

on the trapping filters were extracted with 150 µL of methylene chloride and two internal standards (n-octane and nonyl-acetate, each 200 ng in 10 µL methylene chloride) were added to these samples. For the analysis, an aliquot of 3 µl was injected on-column with the use of an automated injection system onto an apolar HP-1 capillary column, which was preceded by a deactivated retention gap (10 m, 0.25 mm I.D., Connex, U.S.A.) and a deactivated precolumn (30 cm, 0.53 mm I.D., Connex, U.S.A.). The columns were housed in a Hewlett Packard model HP 6890 gas chromatograph equipped with a flame ionisation detector. The oven was held at 50 °C for 3 minutes and then programmed at 8 °C/min to 230 °C, where it was maintained for 9.5 min. Helium (24 cm/s) was used as carrier gas. HP GC Chemstation software was used to quantify all major compounds based on the known quantity of internal standards. Initial identification of most compounds was based on comparisons of retention times with those from previous studies. Identities were confirmed with the mass spectrometry analysis of some samples, using the same column and temperature programme (Agilent 5973, transfer line 230 °C, source 230 °C, quadrupole 150 °C, ionization potential 70 eV, scan range 0–400 amu). Total quantities of volatiles were calculated based on their peak areas compared to those of the internal standards. To obtain an estimate of the different classes of VOC, total amounts of the following compounds were summed: GLV: (Z)-3-hexenal, (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate; monoterpenes: linalool; homoterpenes: 4,8-dimethyl-1,3(E), 7-nonatriene and 4,8,12-tri-methyl-1,3(E),7(E),11-tridecatetraene; sesquiterpenes: (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene; aromatic compounds: indole.

Phytohormone quantification

In order to determine the mechanism of the observed indole priming, we quantified phytohormones in two independent experiments. Delprim plants were exposed to volatile indole or infested igl-mutant (lines 22 and 32R) and IGL-wild type (line 7 and 16R) plants for 12 hours as previously described. Leaf material was collected before wounding and 45 minutes, 3 hours and 8 hours after elicitation treatment. Leaf material (1 cm^2) adjacent to the wound-site was collected, flash frozen in liquid nitrogen and stored at -80°C. ABA (abscisic acid), JA (jasmonic acid) and JA-Ile (JA conjugated with amino acid isoleucine) were quantified using an ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC/MS-MS). Hundred mg per samples were in 990 µL of EtOAc: formic acid, 99.5:0.5 (v/v) and 10 µL of an internal standard solution containing isotopically labelled JA and ABA (10 ng/mL). The extracts were centrifuged at 14000 rpm for 3 minutes. The supernatant was transferred in a 2 mL Eppendorf tube and 500 µL of EtOAc: formic acid, 99.5:0.5 (v/v) was added to the pellet and the same procedure was repeated. The extracts were then

evaporated to dryness and re-suspended in 100 µL of aqueous methanol (50:50 v/v). After centrifugation, 5 µL were injected into the UHPLC/MS-MS. The hormones were quantified by calculating a calibration equation obtained by linear regression from 5 calibration points for each compound. Peak areas of the hormones measured in the samples were normalized to the internal standard before applying the calibration equation.

Statistical analyses

Differences in VOC emissions between control- and indole- exposed plants and between igl-mutant and IGL-wild type plants were analysed using analysis of variance, and p-values below 0.05 were considered significant.

RESULTS

Induced indole emission preceeds the release of other HIPVs

An effective priming signal should be emitted rapidly upon herbivore attack. To profile the timing of volatile emission in herbivore-attacked maize, we artificially damaged three leaves of 10 days old maize seedlings (hybrid “Delprim”) and applied *S. littoralis* regurgitate on the scratched leaves. We then collected the HIPVs emitted over a period of 600 minutes. We found that GLVs are emitted within seconds after the herbivore damage. Indole emission started 45 minutes after elicitation and reached a peak at 180 minutes. Terpenoid emission started about 180 minutes after elicitation (Figure 1A). These results confirm that indole emission in maize precedes the release of other HIPVs by more than 2 h (Turlings *et al.* 1998).

Indole increases the release of HIPVs

Based on the fact that indole is specifically induced upon herbivory by caterpillars and it is released earlier than the other HIPVs in maize, we hypothesized that it may act as a signal that enhances the subsequent induction of plant defences. To test this hypothesis, we isolated igl mutant plants in a bx1 mutant background (Ahmad et al. 2011).

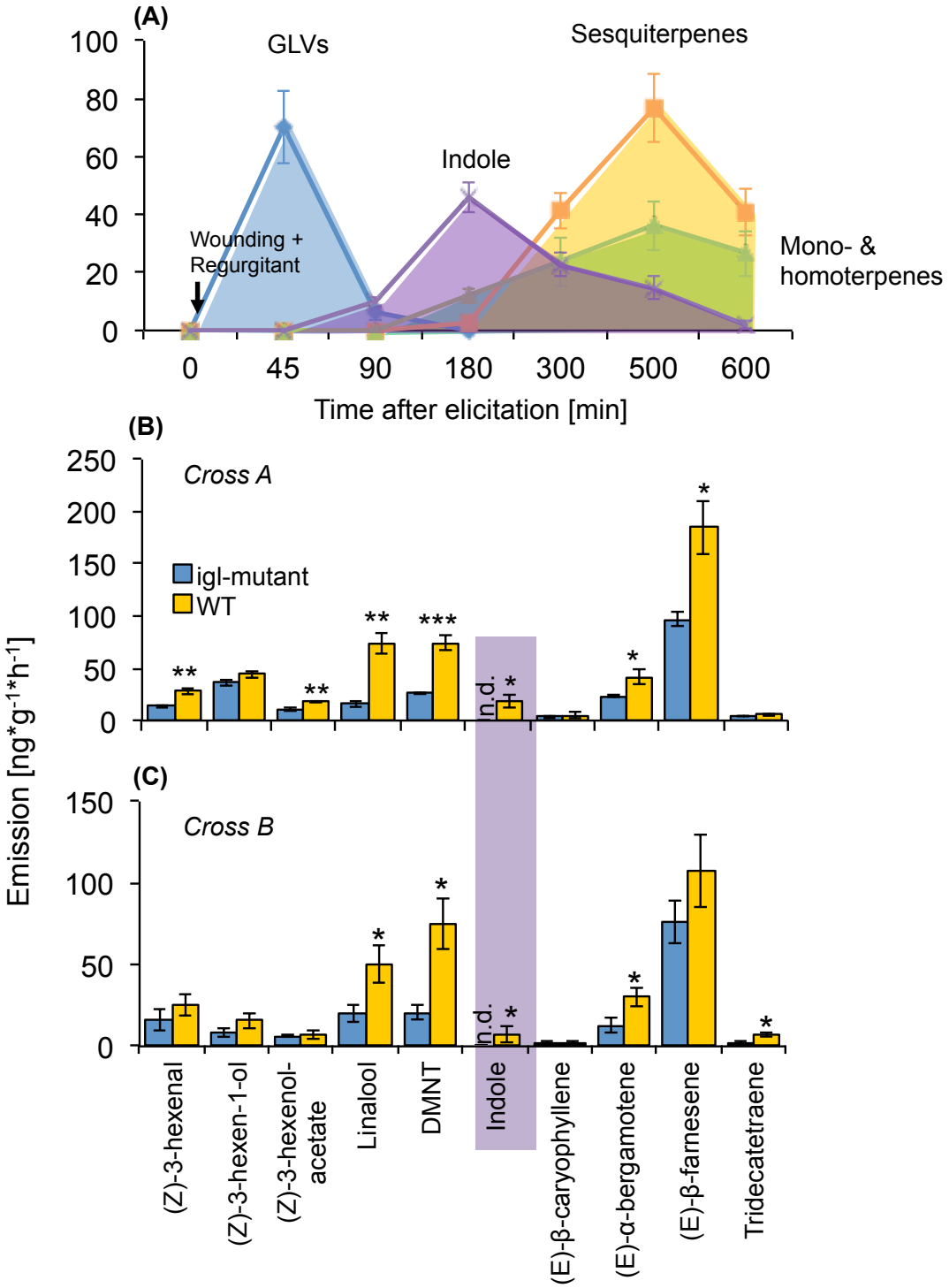

Figure 1: Timing and indole-dependent release of herbivore-induced volatile compounds from maize leaves. Elicitation treatment was performed by scratching three leaves and applying 10 µl of *S. littoralis* larvae regurgitate on the scratched leaf areas. Five major families of VOCs were found: Green leaf volatiles ((Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate)); Monoterpenes (Linalool), Homoterpenes ((E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene); Sesquiterpenes ((E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene) and Aromatic compounds (Indole). **(A):** Time course of volatile release in the hybrid Delprim. **(B)** and **(C)**: volatile release of indole-deficient mutants and wild type plants in two different backgrounds 12 h after elicitation. Stars indicate significant differences between lines (Student's *t*-test: *p<0.05; **p<0.01; ***p<0.001). Error bars correspond to standard errors (±SE).

The double mutant plants are impaired in the emission of indole, while the bx1 single mutant plants release indole at levels comparable with other maize lines. The activity of the bx1 locus varies considerably across maize lines (Butrón *et al.* 2010), and includes naturally inactive forms of the bx1 allele (Frey et al. 1997). Using a bx1 mutant background therefore enabled us to assess the role of volatile indole without any interference from other sources of free indole. Upon wounding and application of *S. littoralis* regurgitant, indole-competent plants produced significantly more HIPVs, including linalool, *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT) and *(E)*-α-bergamotene than indole-mutant plants (Figures 1B, 1C; Student's t-test : $p<0.05$). To test whether volatile indole is sufficient to prime plants for the release of HIPVs, seedlings of the maize hybrid Delprim were exposed to indole- or control-dispensers releasing indole at a physiological dose of 50 ng/h (Figure S1) and subjected them to an elicitation treatment as above. HIPV emissions were measured at different time points after elicitation (Figure 2A). Indole-exposure itself did not induce the release of HIPVs (Figure 2B). The release of GLVs from fresh wounds was not significantly different between control and indole-exposed plants (Figure 2B). However, 500 minutes after defence elicitation, the emission of monoterpenes such as linalool (Figure S2), homoterepenes including DMNT (Figure S2) and sesquiterpenes such as *(E)*-β-caryophyllene (Figure S2) was significantly enhanced in indole-exposed plants (Figure 2B, Student's t-test: $p<0.05$). The release of indole itself was also enhanced in indole-exposed plants (data not shown), possibly due to adsorption and re-release of the synthetic indole. Exogenous application of indole also increased HIPV release in induced igl-mutant and IGL-wild type plants (Figures S3, S4, S5, S6).

Indole is required for within-plant priming

To investigate whether indole is required for systemic priming in unharmed tissues of attacked plants, we performed an experiment in which the first true leaf of igl-mutant and IGL-wild type seedlings was either left intact or elicited by mechanical wounding and application of oral secretions. A subset of the elicited emitter leaves was then wrapped in a small Teflon bag that was sealed around the base of the leaf to minimize HIPV contact of undamaged systemic tissues. All plants were then placed in glass bottles and exposed to a continuous clean airflow of 0.3 L/min to prevent the non-physiological build-up of HIPVs and to isolate the headspace of the different plants. Twelve hours later, all plants were subjected to a new elicitation treatment of the so-far-undamaged leaf 2, and HIPV emissions were measured at different time points (Figure 3A).

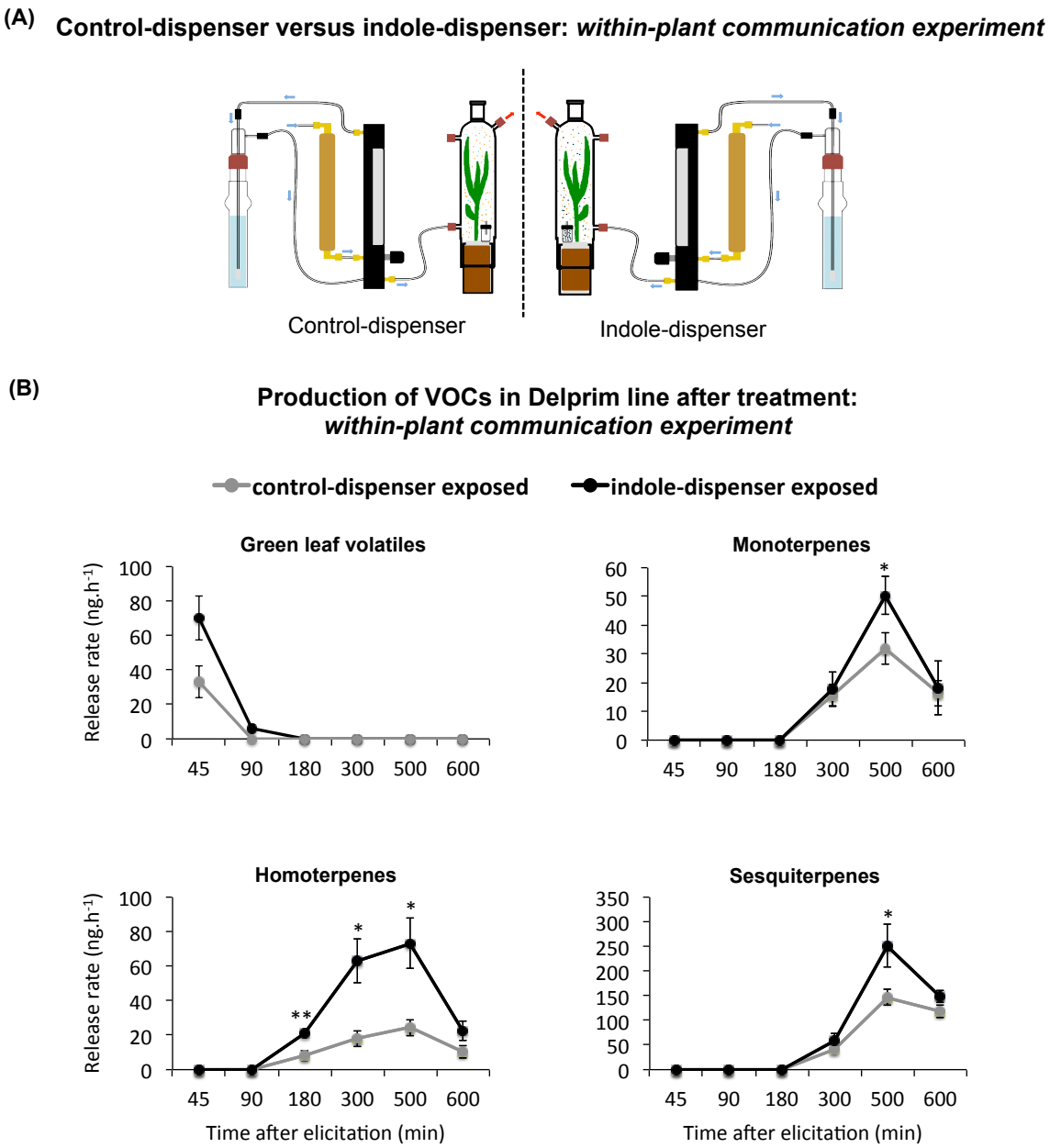

Figure 2: ***Within-plant communication experiment with synthetic indole.***
(A) Experimental conditions. Delprim plants were exposed to control- or volatile indole-dispensers. At the same time, they were subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole induces priming for emission of various VOCs in Delprim line. VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment. Four major families of VOCs were found: Green leaf volatiles; Monoterpenes; Homoterpenes; Sesquiterpenes. Asterisks indicate statistical differences between control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

During these volatile collections, all the first true leaves were enclosed in a clean Teflon bag to ensure that only volatiles from the second elicitation treatment were captured. No HIPVs except GLVs were detected at the beginning of the second elicitation treatment, indicating that 12 h after elicitation, there was no systemic release of HIPVs induced by the first elicitation treatment any more (Turlings & Tumlinson 1992). Five hundred minutes after induction, indole-competent plants that were previously exposed to their own induced headspace released significantly higher amounts of mono- homo- and sesquiterpenes than genetically similar plants that were exposed to constitutive volatiles (Figure 3B). When volatile exposure was interrupted with a Teflon bag, this systemic priming effect disappeared, demonstrating that within-plant systemic priming of HIPVs in maize requires previous HIPVs exposure. Indole-deficient mutant plants on the other hand released the same amounts of HIPVs, irrespective of previous elicitation or exposure to their induced (indole-free) headspace (Figure 3B). Taken together, these results demonstrate that indole is required for the systemic HIPV mediated priming of herbivore-induced defences in maize.

Indole primes neighboring plants

To test whether volatile indole acts as a priming agent between plants, we exposed healthy Delprim plants to HIPVs from igl-mutant and IGL-wild type plants (Figure 4A). HIPV production was on average two to three times higher in IGL-wild type exposed than in igl-mutant exposed plants: At different time points after elicitation, the emission of GLVs, mono-, homo- and sesquiterpenes were significantly enhanced in IGL-wild type exposed seedlings (Figure 4B, Figures S12 and S13; Student's t-test: $p<0.05$). A similar tendency was found when we compared IGL-wild type plants that were either exposed to infested igl-mutant plants or to IGL-wild type plants (Figures S9, S10, S11), even though only the emission of monoterpenes was statistically different (Cross A and 2; Student's t-test: $p<0.05$). To confirm the specific role of indole as a between-plant priming agent, healthy Delprim plants were exposed to control- or indole-emitting dispensers for twelve hours using an identical setup as for the experiment above (Figure 5A). Again, the inducible emission of GLVs and terpenes was significantly enhanced in indole-exposed plants (Figure 5B, Figure S20; Student's t-test: $p<0.05$). Similar results were found for igl-mutant and IGL-wild type plants (Figure S16 to S20), demonstrating that maize plants increase their defensive responsiveness upon perception of herbivore-induced indole from neighbouring plants.

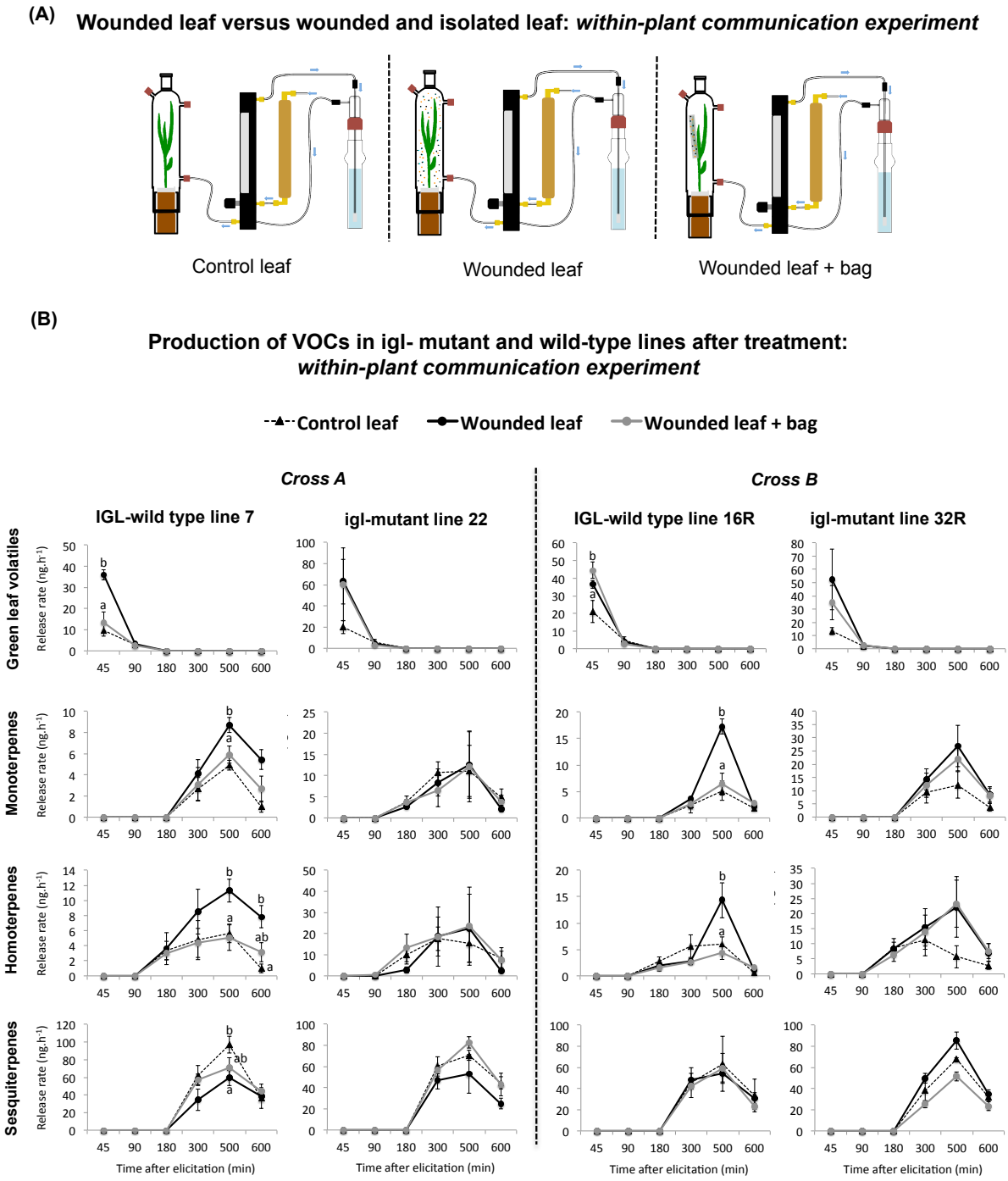

Figure 3: *Within-plant communication experiment with Teflon bag*.
(A) Experimental conditions. Prior to elicitation treatment, the first leaf of each plant was either left intact, wounded or wounded and placed in a Teflon bag for 12 hours. The second leaf of all the plants were then wounded and VOCs were collected for 600 minutes.
(B) Volatile indole released by igl-wild type plants induces within-plant priming for emission of some VOCs after defence elicitation. Four major families of VOCs were found: Green leaf volatiles; Monoterpenes; Homoterpenes; Sesquiterpenes. Asterisks indicate statistical differences between wounded- and wounded and placed in bag plants (ANOVA, different letters indicate significant differences between treatments ($P < 0.05$); n=5)

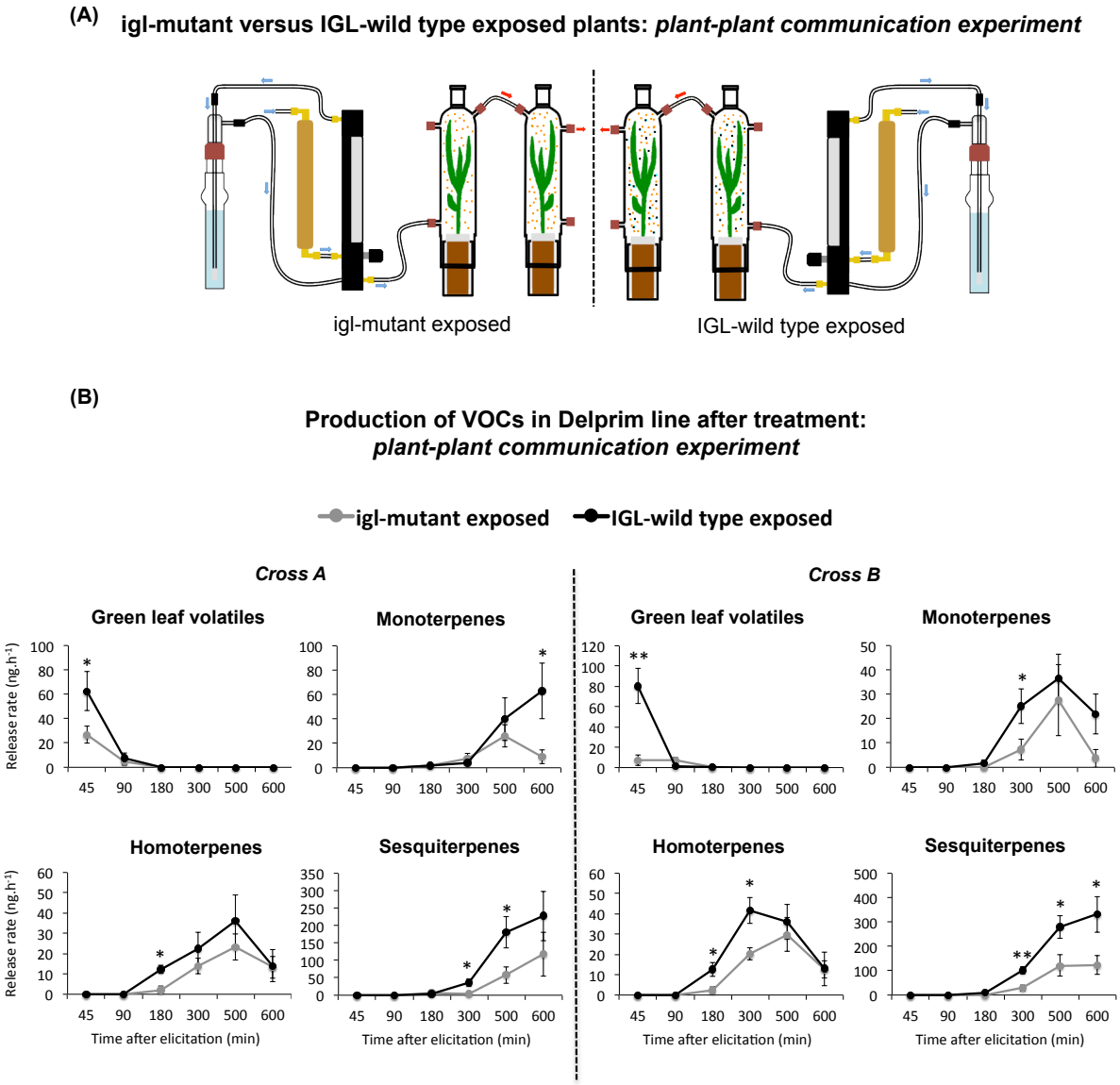

Figure 4: ***Plant-plant communication experiment with igl-mutant and IGL-wild type plants*.**

(A) Experimental conditions. Delprim plants were exposed to igl-mutant or IGL-wild type infested plants of two different genetic backgrounds for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.

(B) Exposure to IGL-wild type plants induces priming for emission of various VOCs in Delprim line. VOCs emissions of igl-mutant (cross A: line 22; cross B: line 32R) or IGL-wild type type (cross A: line 7; cross B: line 16R) exposed plants at different times after elicitation treatment. Five major families of VOCs were found: Green leaf volatiles; Monoterpenes; Homoterpenes; Sesquiterpenes, Aromatic compounds. Asterisks indicate statistical differences between release rates from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

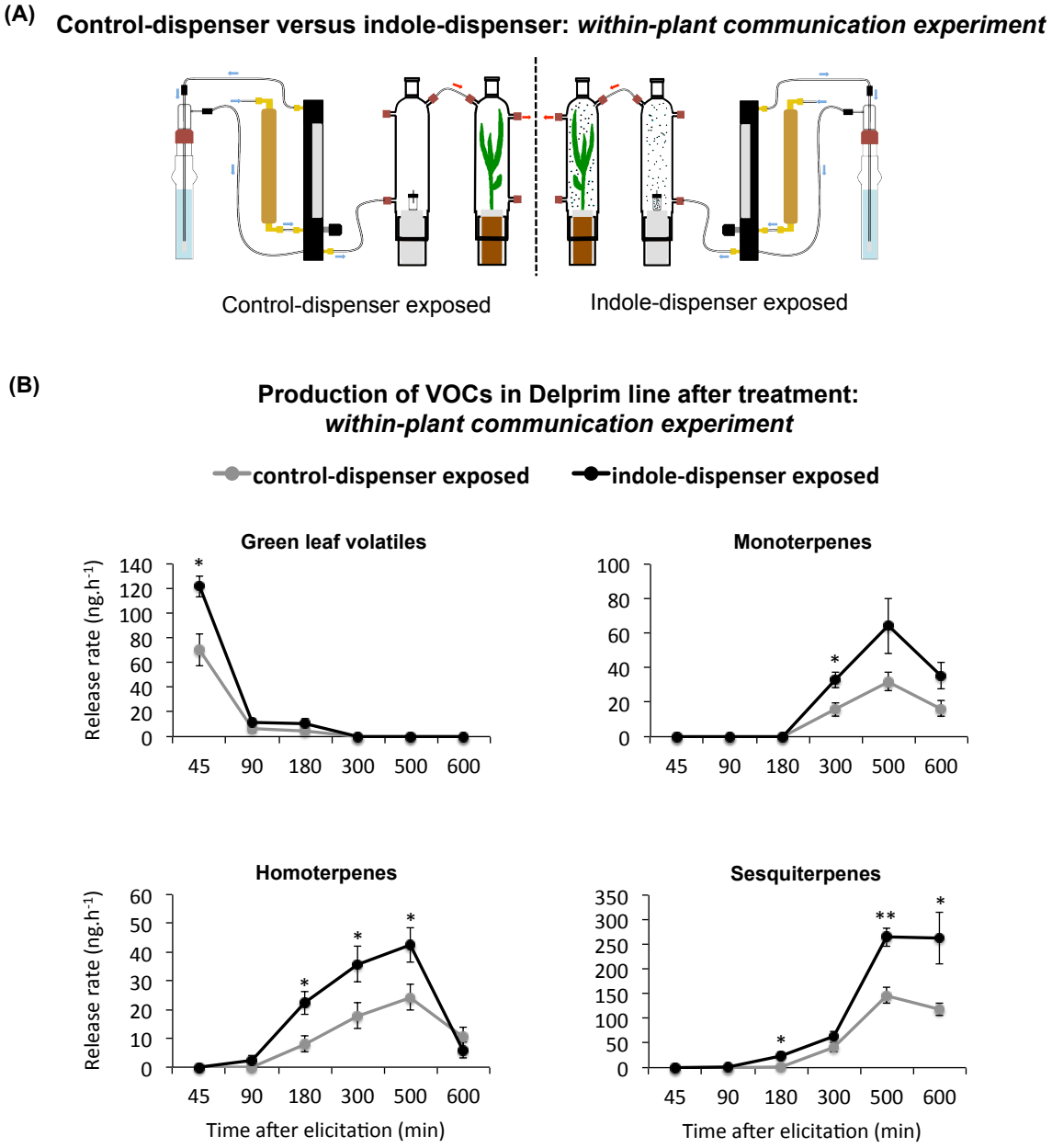

Figure 5: ***Plant-plant communication experiment with synthetic indole.***
(A) Experimental conditions. Delprim plants were exposed to control- or volatile indole-dispensers for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole after elicitation treatment induces priming for emission of various VOCs in Delprim line. VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment. At the time of elicitation treatment, plants were exposed to volatile indole from dispenser. Four major families of VOCs were found: Green leaf volatiles; Monoterpenes; Homoterpenes; Sesquiterpenes. Asterisks indicate statistical differences between release rates from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

Indole exposure increases herbivore induced stress phytohormone concentrations

To study the mechanism of the observed indole priming, we quantified induced defensive phytohormones in indole-exposed maize seedlings. Jasmonic acid in particular is known to regulate herbivore-induced HIPV-release in maize (Schmelz *et al.* 2003a). Leaves of indole-exposed and control-plants were collected at different time points after elicitation treatments in two separate experiments. First, Delprim seedlings were exposed to volatiles from induced igl-mutant and IGL-wild type plants. Second, Delprim plants were exposed to control- or indole-releasing dispensers. Delprim seedlings exposed to indole producing or indole deficient plants had the same constitutive levels of abscisic acid (ABA), jasmonic acid (JA) and jasmonic acid isoleucine (JA-Ile). However, upon elicitation, the levels of all three hormones increased more strongly in indole-exposed seedlings (Figure 6). Forty-five minutes after elicitation, JA and JA-Ile levels were 50% higher in indole-exposed plants. Similar results were obtained by exposing seedlings to realistic concentrations of synthetic indole (Figure 7).

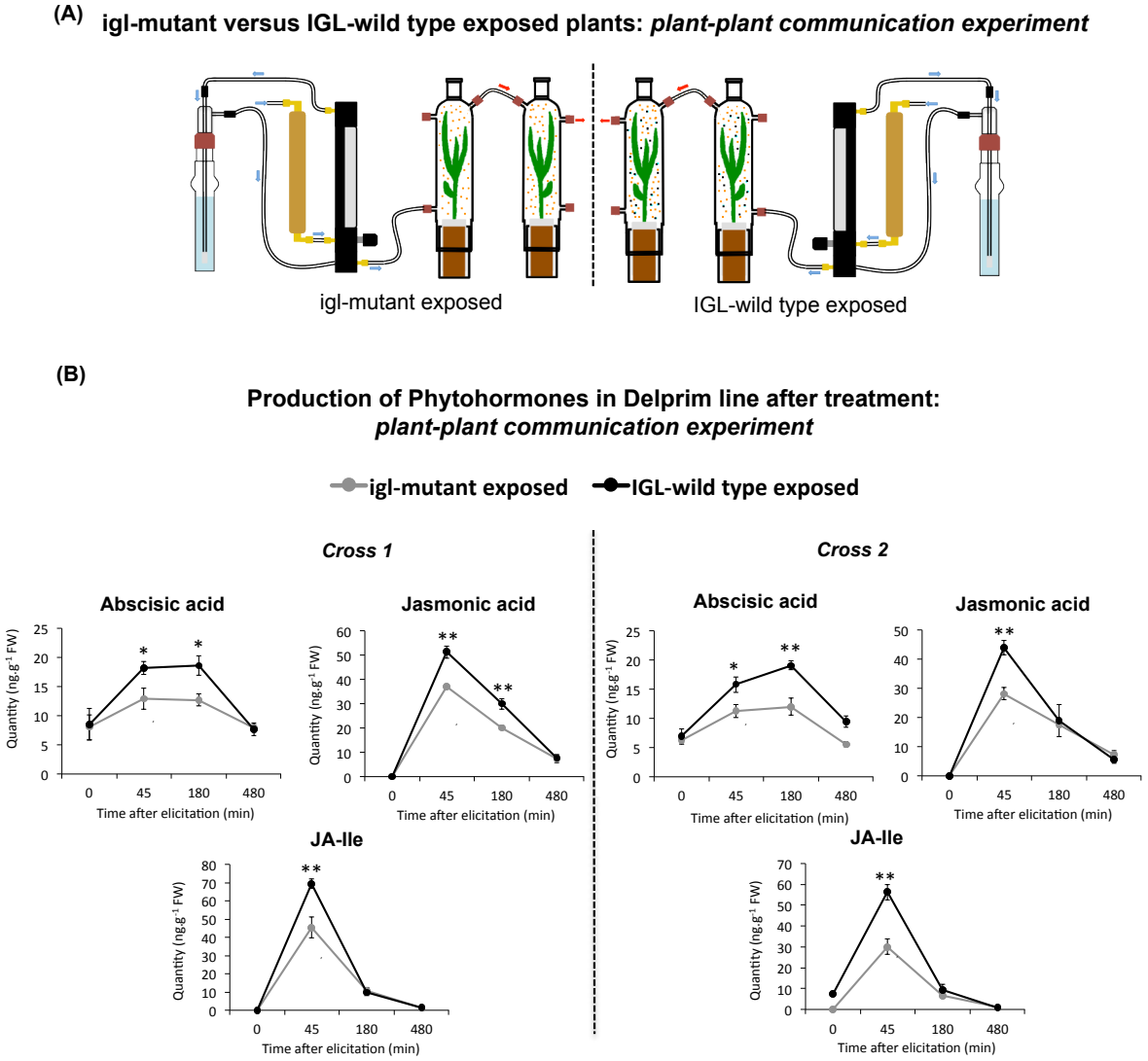

Figure 6: ***Plant-plant communication experiment with igl-mutant and IGL-wild type plants*.**

(A) Experimental conditions. Delprim plants were exposed to igl-mutant or IGL-wild type infested plants of two different genetic backgrounds for 12 hours. Leaf material was taken before and 45, 180 and 480 minutes after elicitation treatment.

(B) Exposure to IGL-wild type plants induces higher level of phytohormones in line Delprim. Average leaf concentrationsAsterisks indicate statistical differences between control- and volatile (+ SE) of abscisic acid (ABA), jasmonic acid (JA) and JA-Ile are shown. indole-dispenser exposed plants (Student's t-test, *:p<0.05, **:p<0.01, ***:p<0.001, n=4)

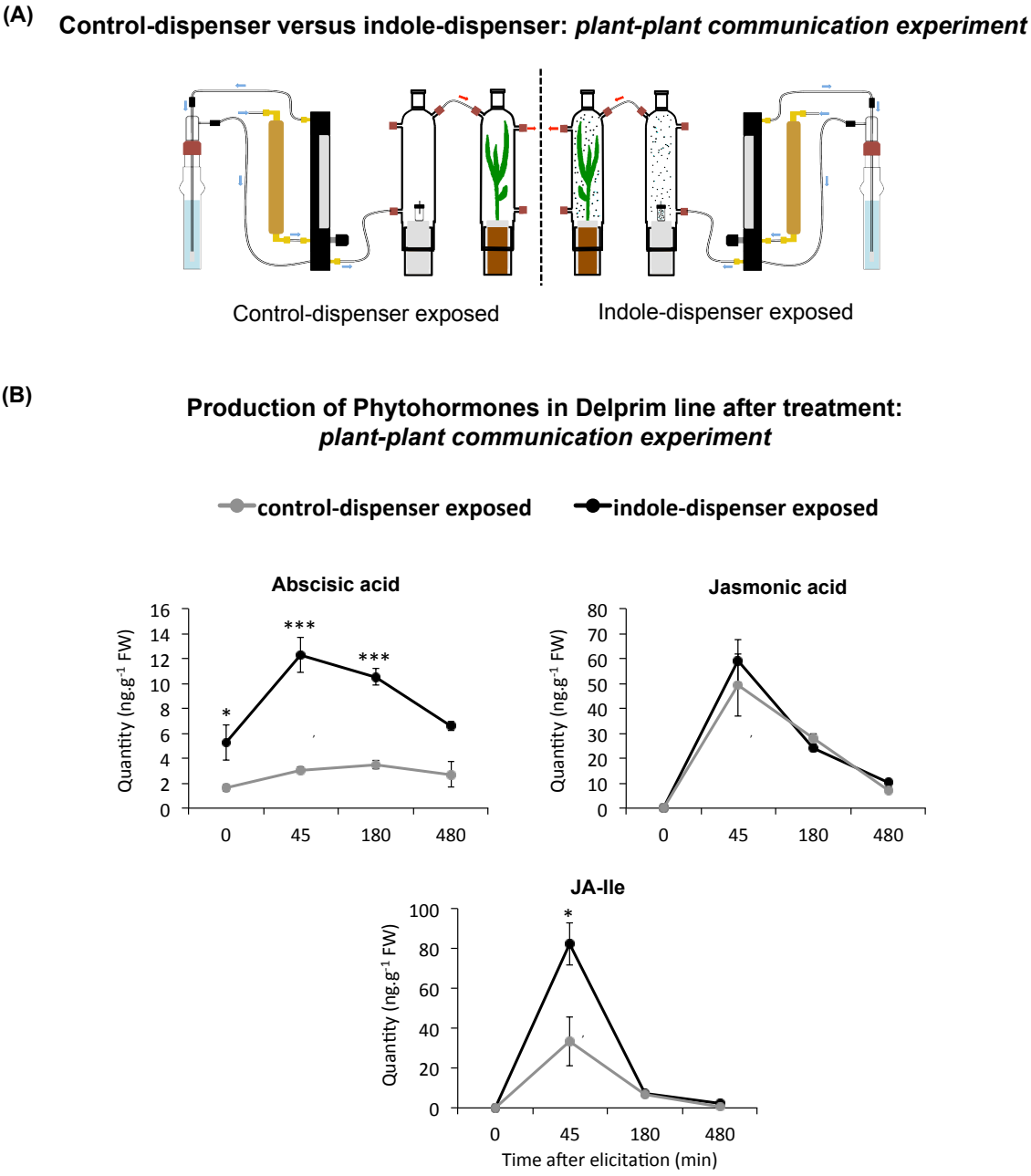

Figure 7: *Plant-plant communication experiment with synthetic indole*.
(A) Experimental conditions. Delprim plants were exposed to control- or volatile indole-dispensers for 12 hours. Leaf material was taken before and 45, 180 and 480 minutes after elicitation treatment.
(B) Exposure to volatile indole induces higher level of phytohormones in line Delprim. Average leaf concentrations (+ SE) of abscisic acid (ABA), jasmonic acid (JA) and JA-Ile are shown. Asterisks indicate statistical differences between control- and volatile indole-dispenser exposed plants (Student's t-test, *:p<0.05, **:p<0.01, ***:p<0.001, n=4)

DISCUSSION

Several studies have shown that HIPVs prime for direct and indirect plant defences (Engelberth *et al.* 2004; Kessler *et al.* 2006; Kost & Heil 2006; Ton *et al.* 2007; Rodriguez-Saona *et al.* 2009). Yet, the identity of the volatile messengers has remained elusive in most cases. The current study reveals an important role of indole in HIPV-induced priming via several lines of evidence: 1) igl-mutant plants impaired in the production of volatile indole produce less volatile compounds after herbivore damage than IGL-wild type plants. 2) Systemic within-plant priming is observed in HIPV-exposed, but not HIPV-unexposed wild type plants and is absent in igl-mutant plants independently of exposure to their own HIPVs. 3) Exposure to synthetic indole or indole-containing HIPVs primes neighbouring plants irrespective of their own capacity to produce indole. Taken together, these results clearly establish that indole has the potential to enhance HIPVs and furthermore suggest that indole is the key-priming agent in within-plant priming in maize.

Several other HIPVs have been proposed as priming signals. Among them, GLVs have been documented repeatedly to possess signalling capacity (Engelberth *et al.* 2004; Farag *et al.* 2005; Ruther & Furstenau 2005; Kost & Heil 2006; Frost *et al.* 2008b). However, the relative contribution of GLVs compared to other potential HIPV signals remains unclear. Kost and Heil (2006) showed that Lima bean plants increase production of EFN after exposure to naturally emitted GLVs and, *(Z)*-3-hexenyl-acetate. However, the weaker effect of the synthetic VOCs blend compared to plant-derived HIPVs indicated that other constituents of the HIPV blend could also have contributed to the priming. Because GLVs are generally emitted after physical leaf damage, they are unreliable signals for an impending herbivore attack. Herbivore-specific volatiles like indole are therefore likely to complement and/or enhance the information value of GLVs. In the case of maize, our experiments consistently indicate that indole, is required for systemic priming. Yet, we cannot exclude that GLVs and other volatiles enhance indole-mediated signaling.

We further found that volatile indole is perceived not only by the emitting plant itself, but also by neighbouring plants. Target plants previously exposed to infested IGL-wild type plants produced larger amounts of volatile compounds shortly after an herbivore attack than plants exposed to infested igl-mutant plants. Similar results were obtained when we exposed target plants to synthetic indole alone. This shows that volatile indole alone is sufficient to induce priming in receiving plants. Given that other volatiles may also possess priming activity (Engelberth *et al.* 2004; Farag *et al.* 2005; Ruther & Furstenau 2005; Kost & Heil 2006; Frost *et al.* 2008b), the within-plant signalling role of indole that leads to enhanced emission of GLVs and terpenoids may trigger a cascading-effect which would further boost

the emitted volatile blend of neighbouring plants that have perceived indole as an early warning.

Previous studies have shown that in absence or in the case of limited vascular connections, HIPVs are sufficient to reduce herbivore feeding (Karban *et al.* 2006; Rodriguez-Saona *et al.* 2009). Here we show that the systemic priming effect is absent when volatile exposure is blocked, further confirming that within-plant systemic priming of HIPVs requires previous HIPV exposure. These results are in accordance with a model that combines vascular and volatile signalling as proposed by Heil and Ton (2008). They suggest that priming involves a two steps regulatory system where airborne signals sensitize distal plant parts for a second vascular signal upon herbivore attack.

The elucidation of the elements of defensive signalling that are enhanced through priming is an important next step to understand the mechanism behind HIPV-mediated priming. The role of JA in HIPVs emission is maize has been well documented (Schmelz *et al.* 2003a; Schmelz *et al.* 2003b; Ozawa *et al.* 2004), and previous studies showed the importance of the octadecanoid pathway in the GLV-mediated priming (Engelberth *et al.* 2004; Farag *et al.* 2005; Ruther & Furstenau 2005). Here we found that the exposure of plants to indole or indole-containing HIPVs enhances the herbivore-induced production of the phytohormones abscisic acid (ABA), jasmonic acid (JA) and jasmonic acid isoleucine (JA-Ile). We therefore propose that indole acts upstream of defence hormonal signalling to increase the production of herbivore-induced HIPVs.

Based on the above results, we propose that indole is a key-priming agent of maize that is essential and far more potent than hitherto proposed signals. Indole is a particularly reliable and suitable signal because its release is greatly enhanced by herbivory as compared to mere mechanical damage (Turlings et al. 1990) and it is released faster than other such compounds (Turlings et al., 1998). Other plant species also emit indole upon herbivory (Turlings and Wäckers, 2004) and we therefore expect that the priming effect of indole will be common across the plant kingdom and may also trigger interspecies responses. The presented findings are likely to facilitate the unravelling the mechanisms of priming and to help testing its ecological relevance in the future.

ACKNOWLEDGEMENTS

We thank Armelle Vallat-Michel and Gaëtan Glauser (chemical analytical service of the University of Neuchâtel) for their help with the phytohormone analysis, Yves Borcard and the students of the University of Neuchâtel for their help with the insect rearing.

SUPPLEMENTARY INFORMATION

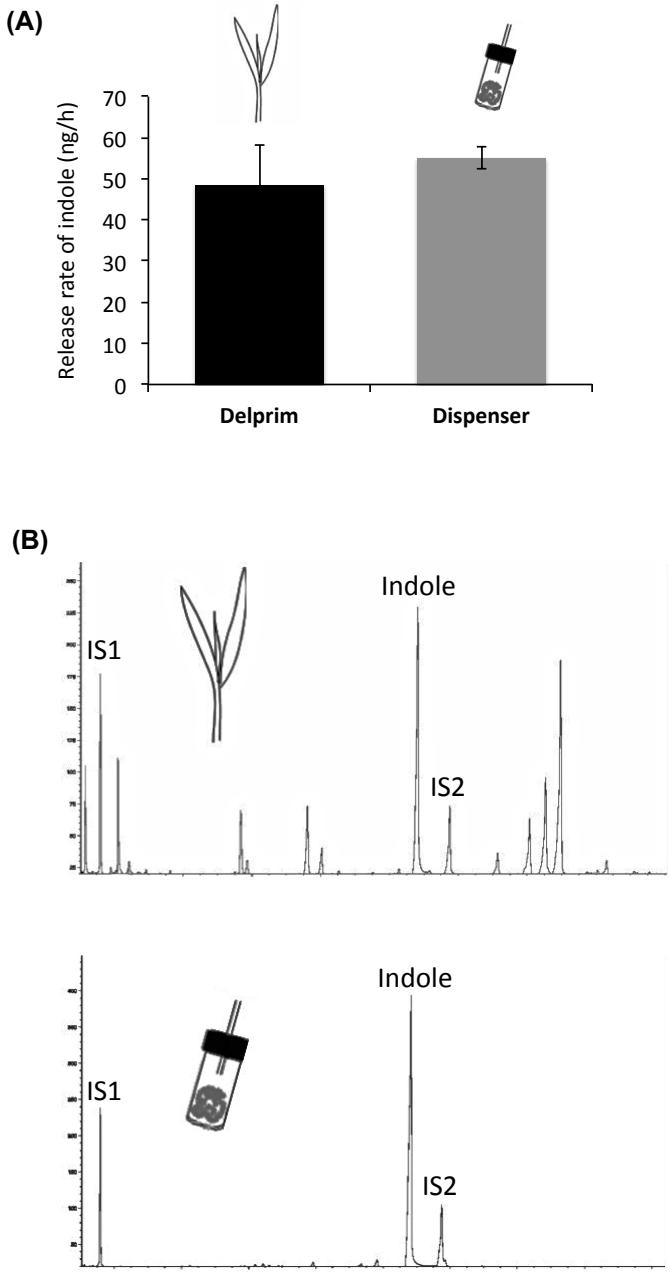

Figure S1: ***Indole releases by infested plants or dispenser***
(A) Release rate of indole from infested Delprim plants and from dispensers.
(B) Chromatograms showing the HIPVs emitted by a Delprim plant after one day of infestation and by an indole-dispenser.

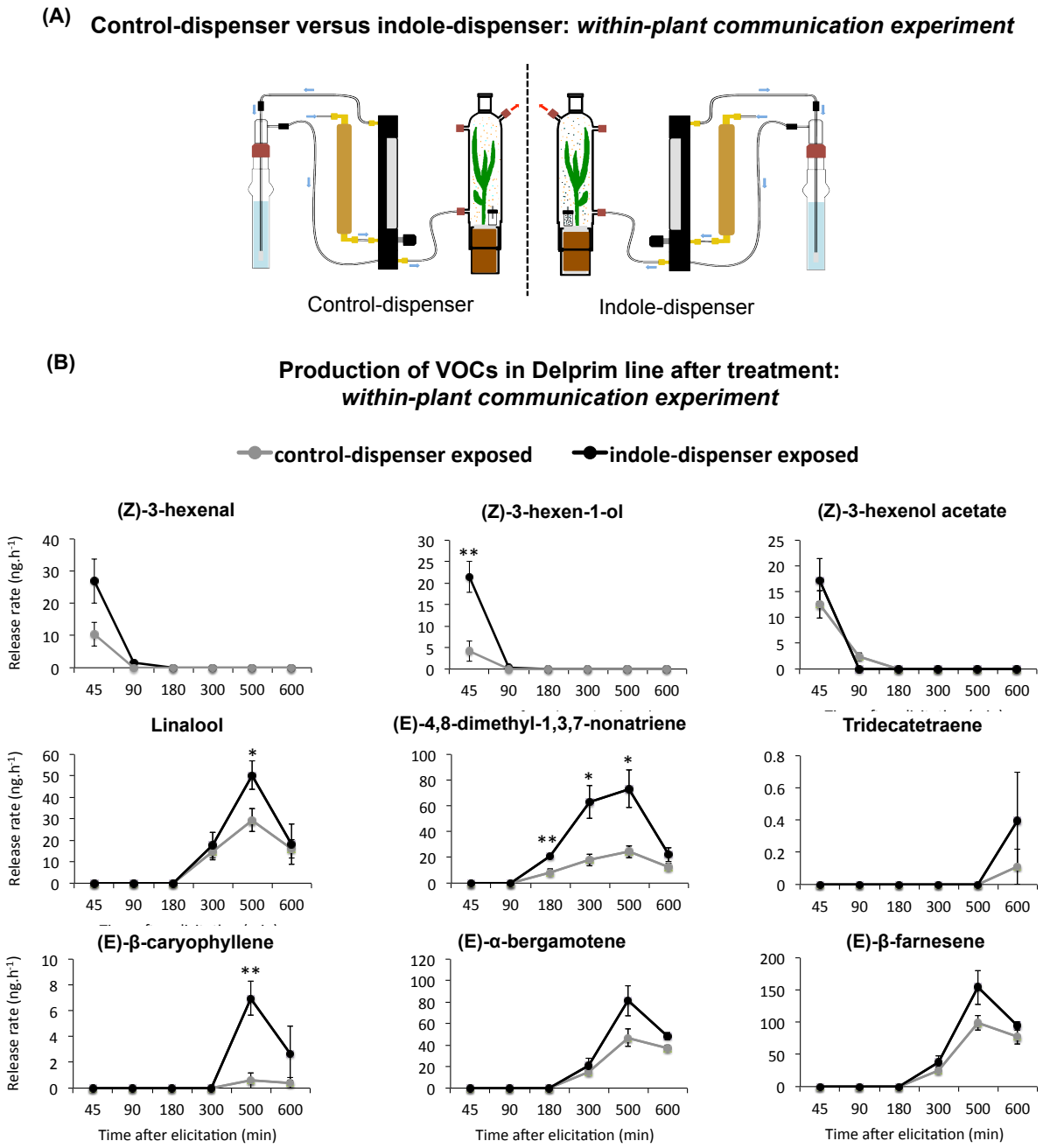

Figure S2: *Within-plant communication experiment with synthetic indole.*
(A) Experimental conditions. Delprim plants were exposed to control- or volatile indole-dispensers. At the same time, they were subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole induces priming for emission of various volatile organic compounds (VOCs) in Delprim line. VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between release rates from control- and indole-exposed plants (Student's t-test, *: p<0.05, **:p<0.01, ***:p<0.001, n=4)

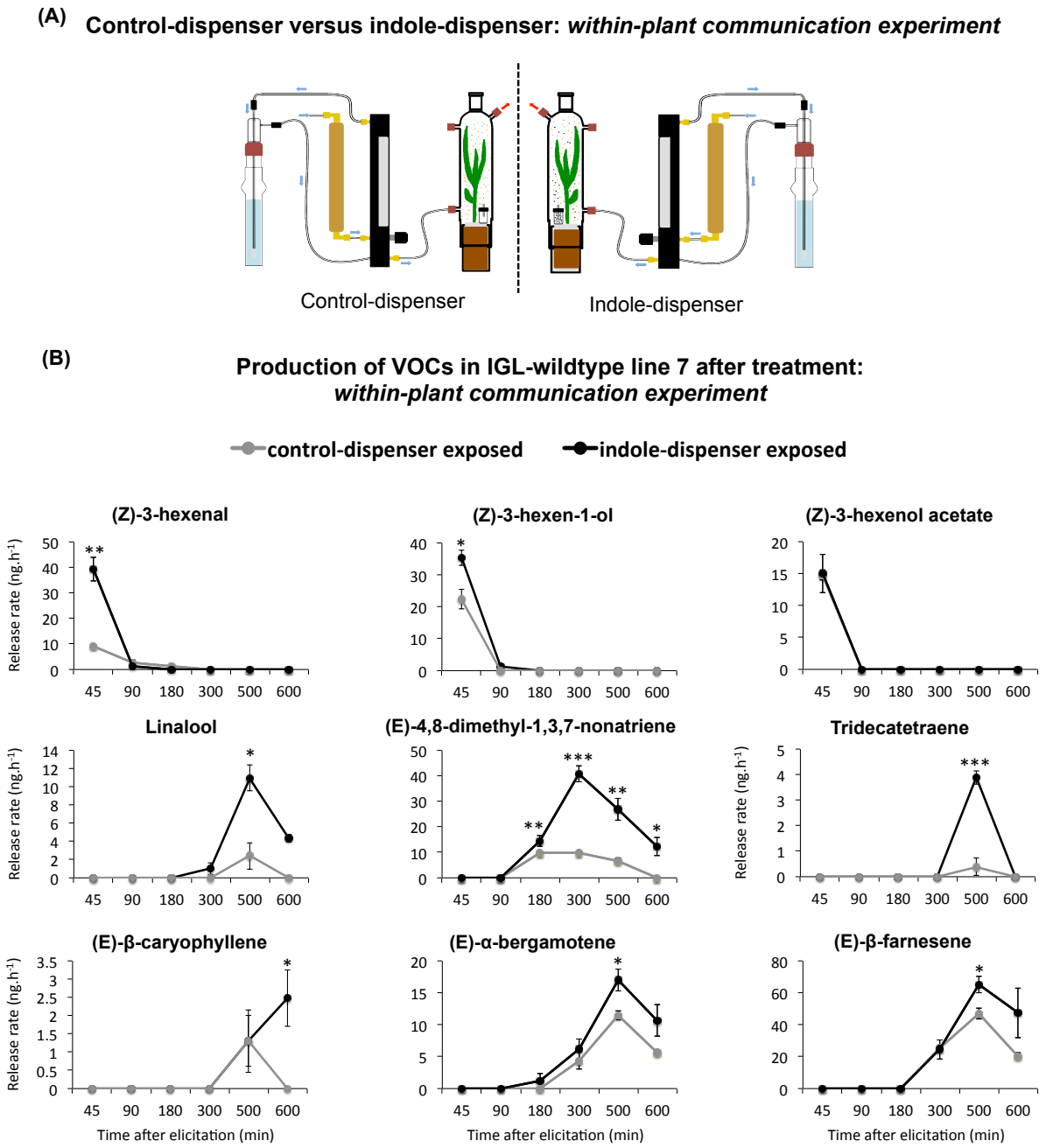

Figure S3: *Within-plant communication experiment with synthetic indole.*
(A) Experimental conditions. IGL-wildtype plants were exposed to control- or volatile indole- dispensers. At the same time, they were subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type line 7. VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between release rates from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

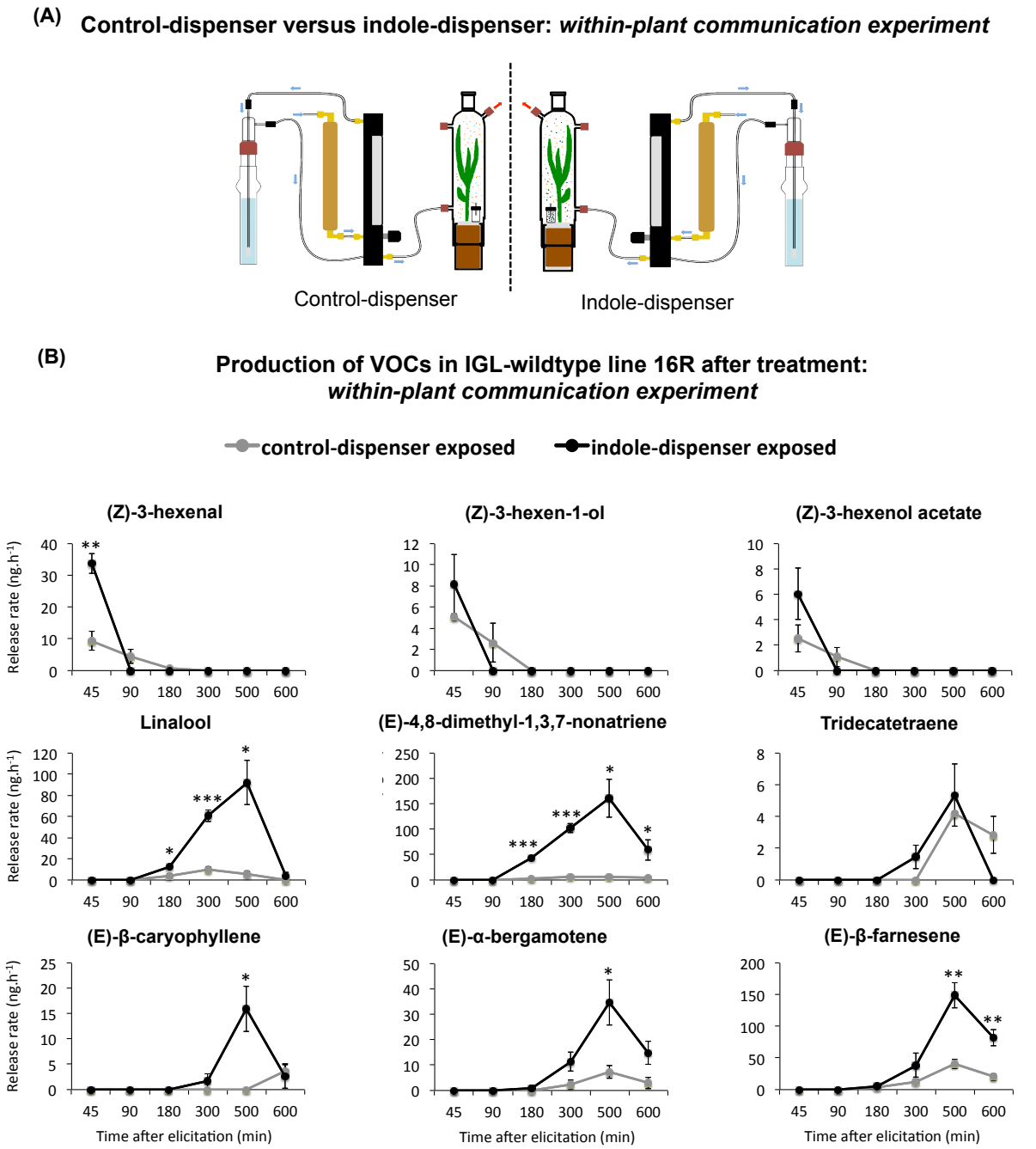

Figure S4: *Within-plant communication experiment with synthetic indole.*
(A) Experimental conditions. IGL-wildtype plants were exposed to control- or volatile indole- dispensers. At the same time, they were subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole induces priming for emission of various volatile organic compounds (VOCs) in IGL-wildtype line 16R. VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between release rates from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

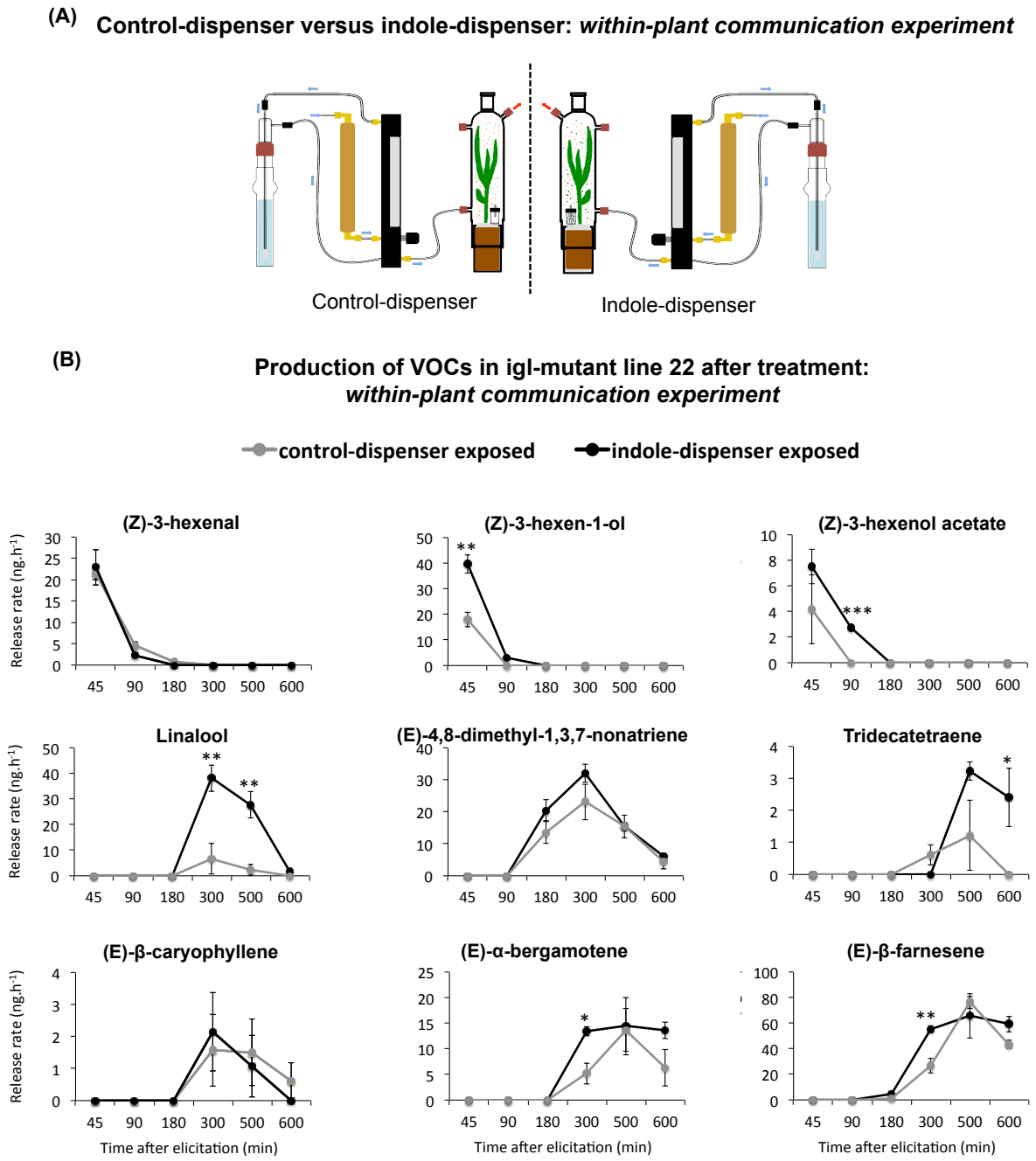

Figure S5: *Within-plant communication experiment with synthetic indole*.
(A) Experimental conditions. igl-mutant plants were exposed to control- or volatile indole-dispensers. At the same time, they were subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole induces priming for emission of various volatile organic compounds (VOCs) in igl-mutant line 22. VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between release rates from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

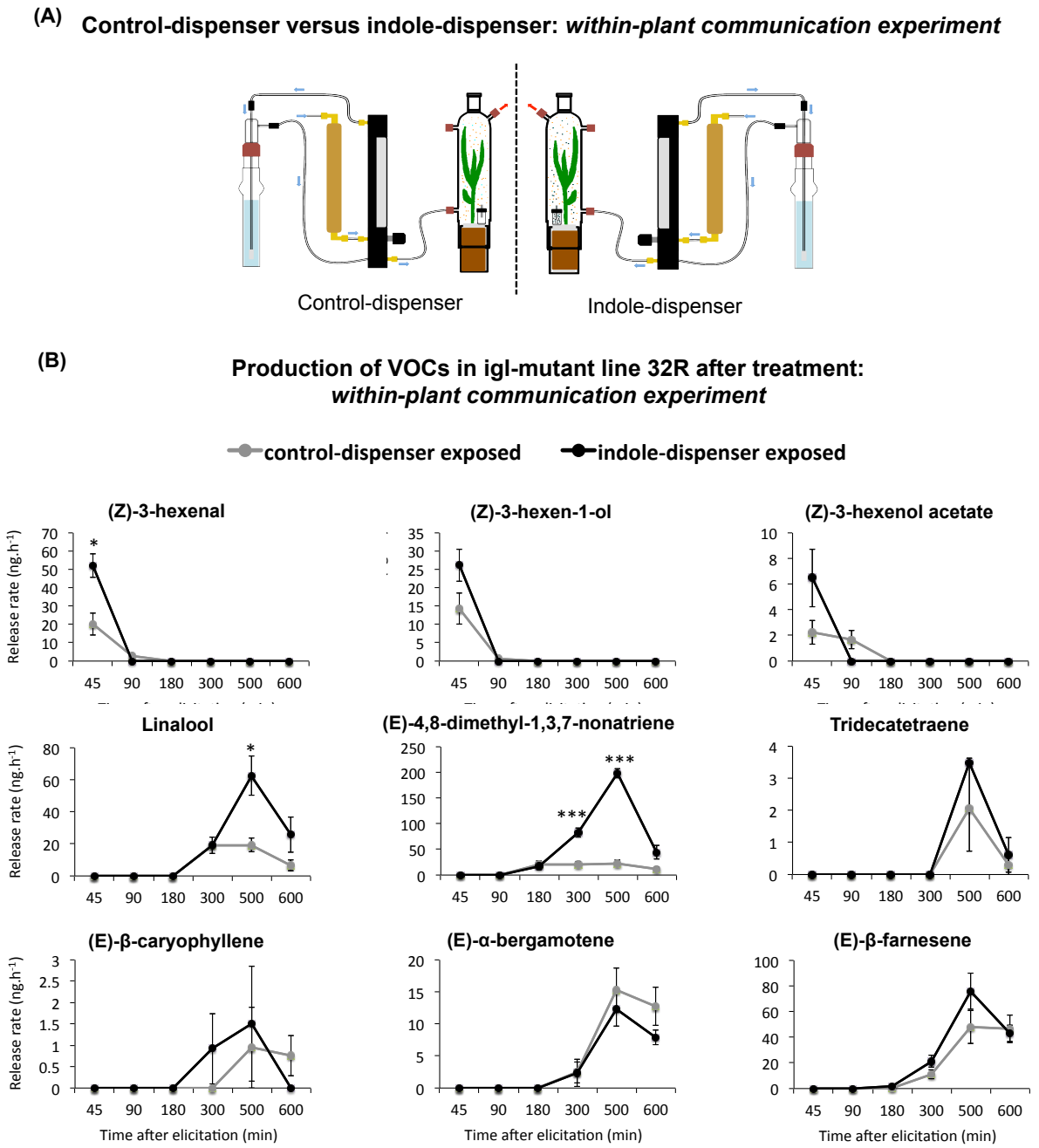

Figure S6: ***Within-plant communication experiment with synthetic indole.***
(A) Experimental conditions. igl-mutant plants were exposed to control- or volatile indole-dispensers. At the same time, they were subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole induces priming for emission of various volatile organic compounds (VOCs) in igl-mutant line 32R. VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between release rates from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

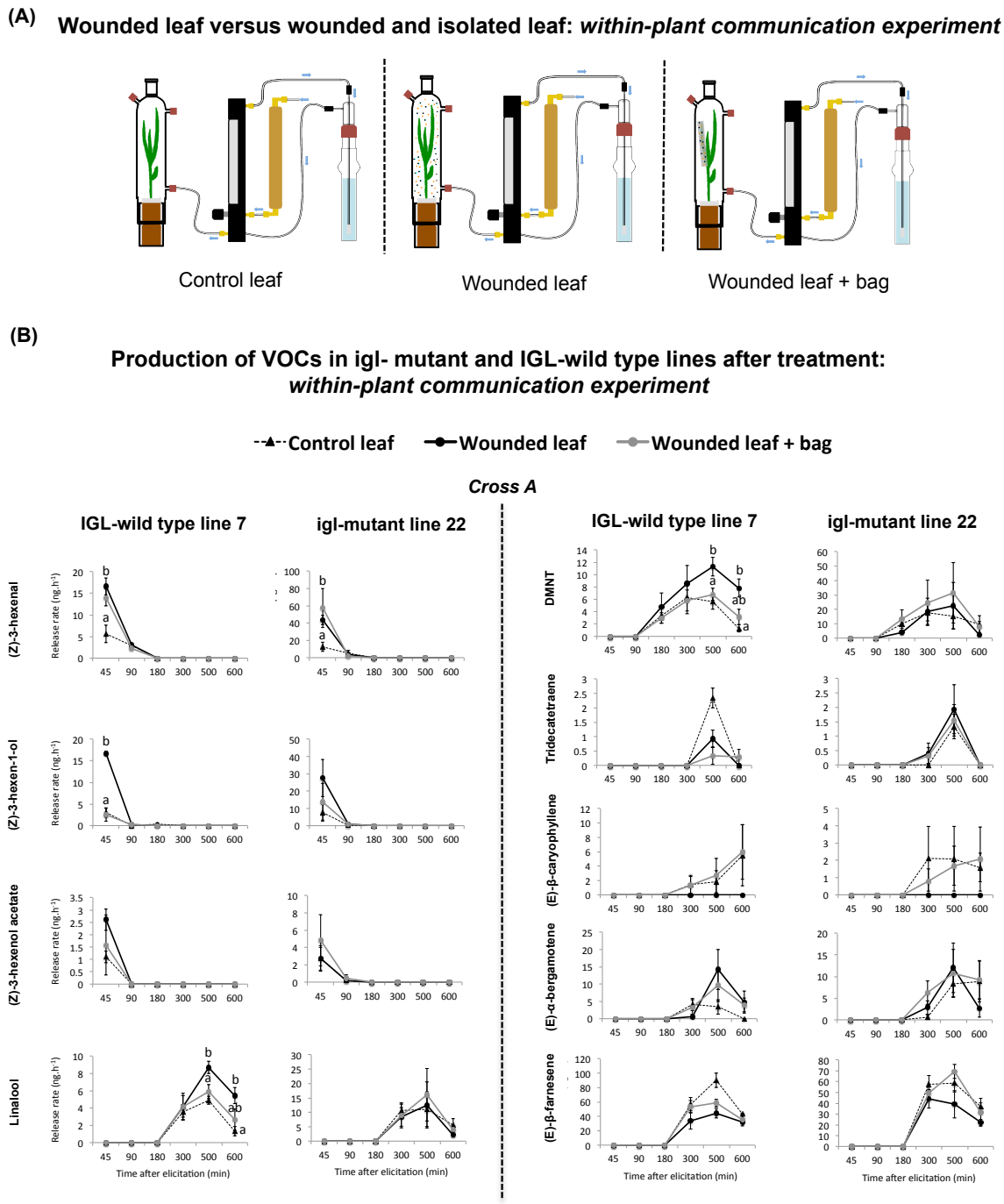

Figure S7: ***Within-plant communication experiment with Teflon bag*****.**
(A) Experimental conditions. Prior to elicitation treatment, the first leaf of each plant was left intact, wounded or wounded and placed in a Teflon bag for 12 hours. The second leaf of all the plants were then wounded and VOCs were collected for 600 minutes.
(B) Volatile indole released by igl-wild type plants induces within-plant priming for emission of some volatile organic compounds (VOCs) after defence elicitation. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between wounded- and wounded and placed in bag plants (ANOVA, different letters indicate significant differences between treatments; n=5)

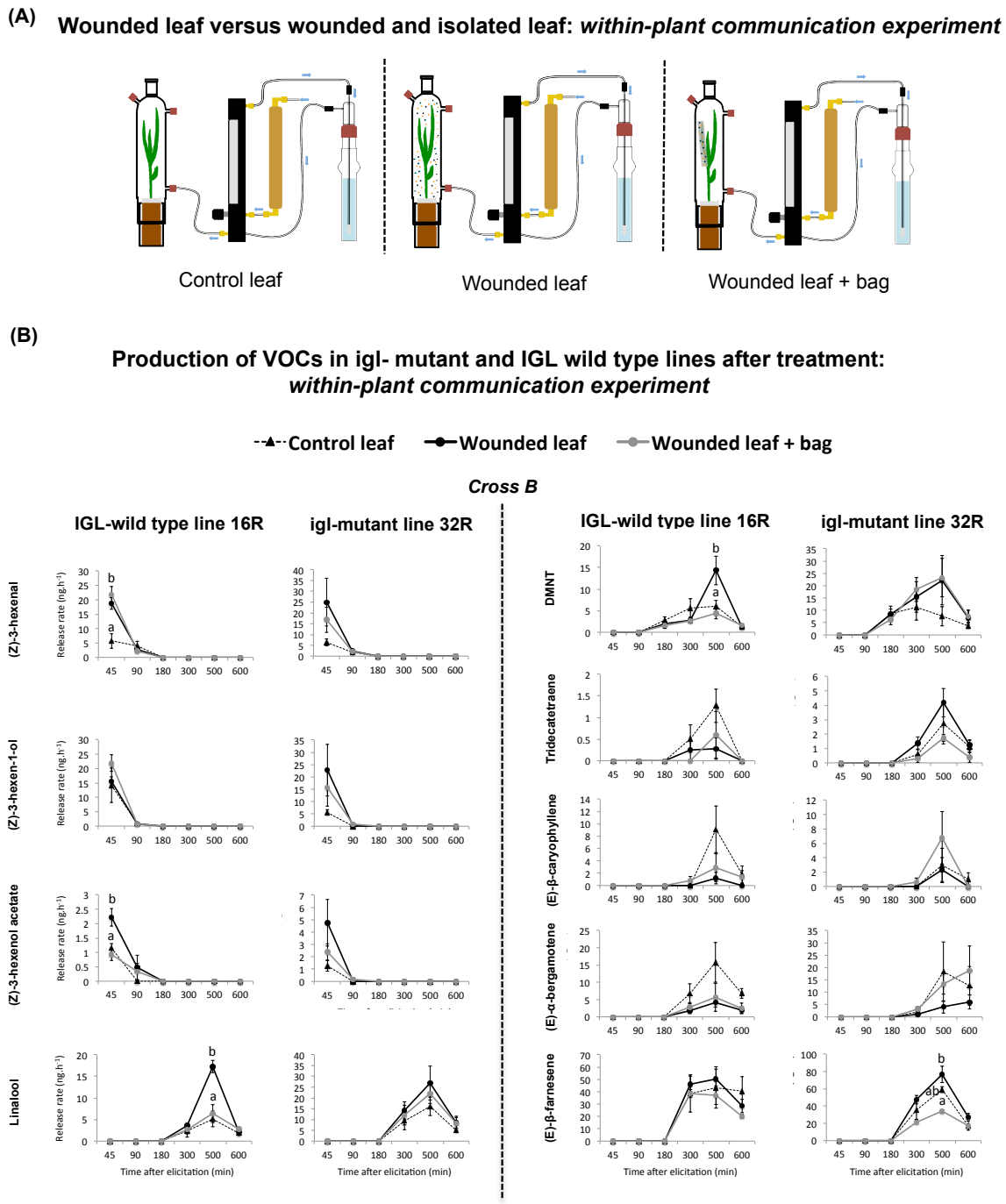

Figure S8: ***Within-plant communication experiment with Teflon bag*****.**
(A) Experimental conditions. Prior to elicitation treatment, the first leaf of each plant was wounded or wounded and placed in a Teflon bag for 12 hours. The second leaf of all the plants were then wounded and VOCs were collected for 600 minutes.
(B) Volatile indole released by igl-wild type plants induces within-plant priming for emission of some volatile organic compounds (VOCs) after defence elicitation. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between wounded- and wounded and placed in bag plants (ANOVA, different letters indicate significant differences between treatments; n=5)

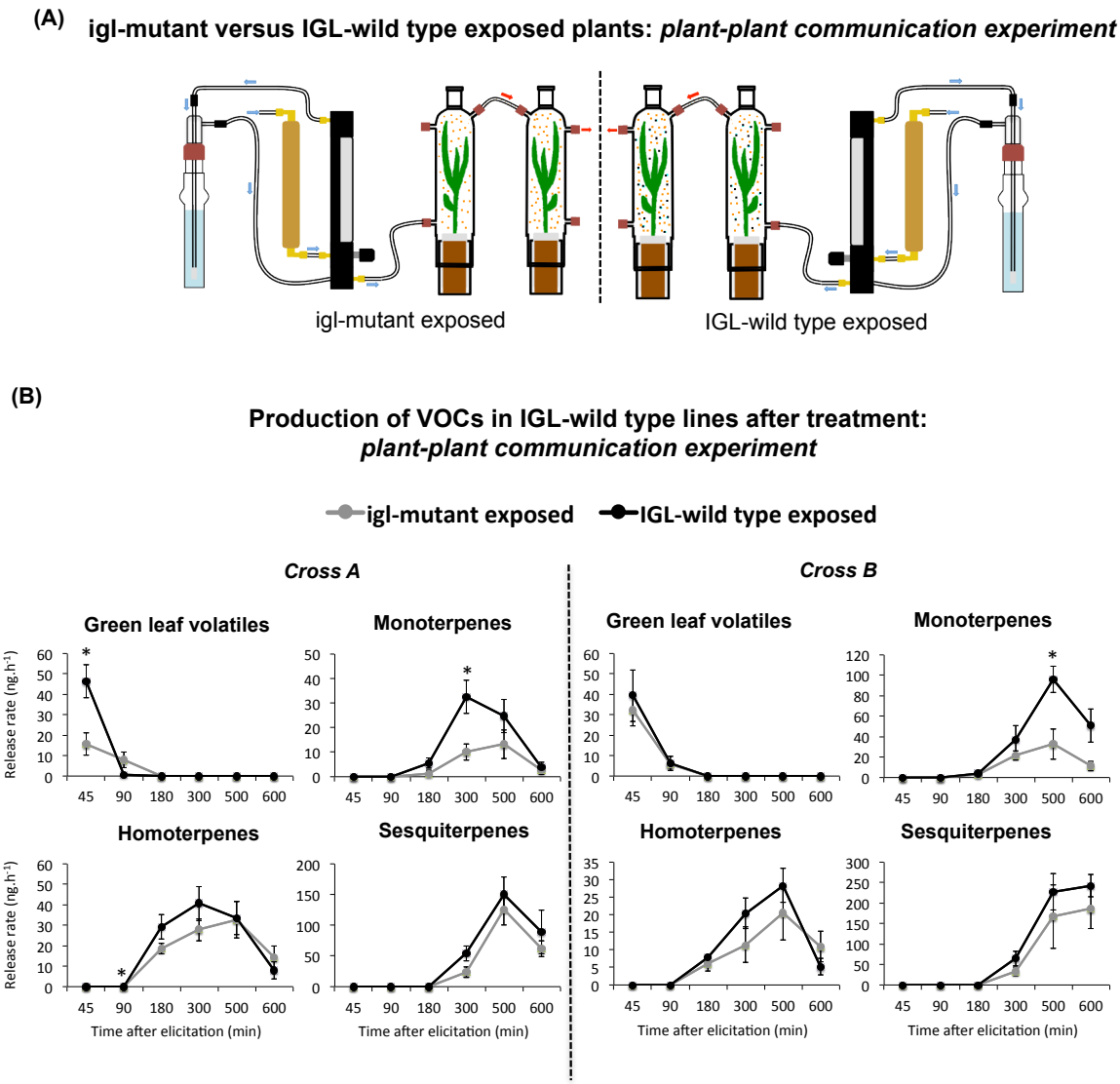

Figure S9: ***Plant-plant communication experiment with igl-mutant and IGL-wild type plants*.**

(A) Experimental conditions. IGL-wild type plants were exposed to igl-mutant or IGL-wild type infested plants of two different genetic backgrounds for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.

(B) Exposure to IGL-wild type plants induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type lines. VOCs emissions of IGL-wild type lines (cross A: line 7; cross B: line 16R) exposed plants at different times after elicitation treatment. Four major families of VOCs were found: Green leaf volatiles; Monoterpenes; Homoterpenes; Sesquiterpenes. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **: $p<0.01$, ***:$p<0.001$, n=4)

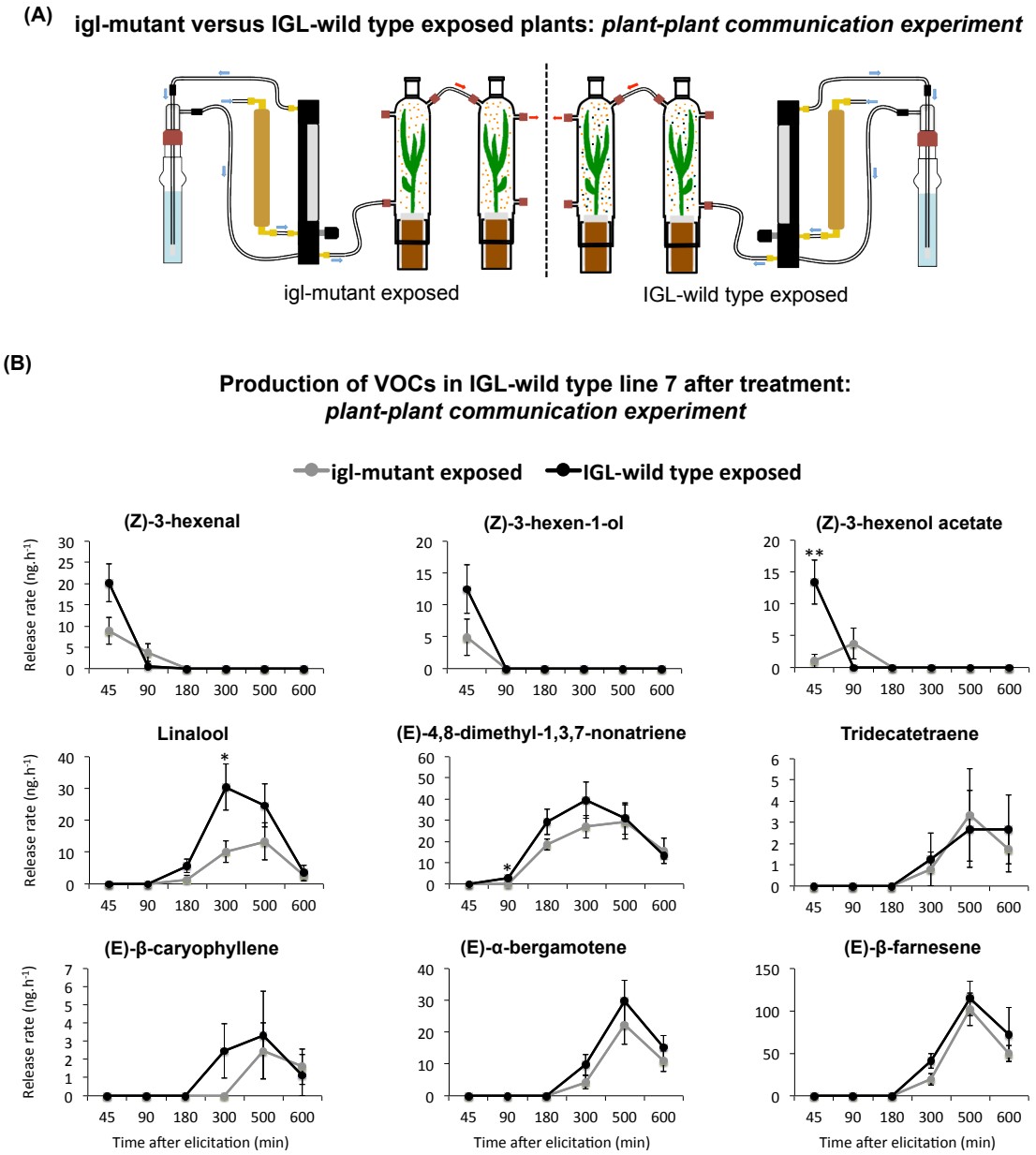

Figure S10: ***Plant-plant communication experiment with igl-mutant and IGL-wild type plants*.**

(A) Experimental conditions. IGL-wild type plants (line 7) were exposed to igl-mutant (line 22) or IGL-wild type (line 7) infested plants of two different genetic backgrounds for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.

(B) Exposure to IGL-wild type plants induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type lines. VOCs emissions of IGL-wild type lines (cross A: line 7) exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *: p<0.05, **: p<0.01, ***:p<0.001, n=4)

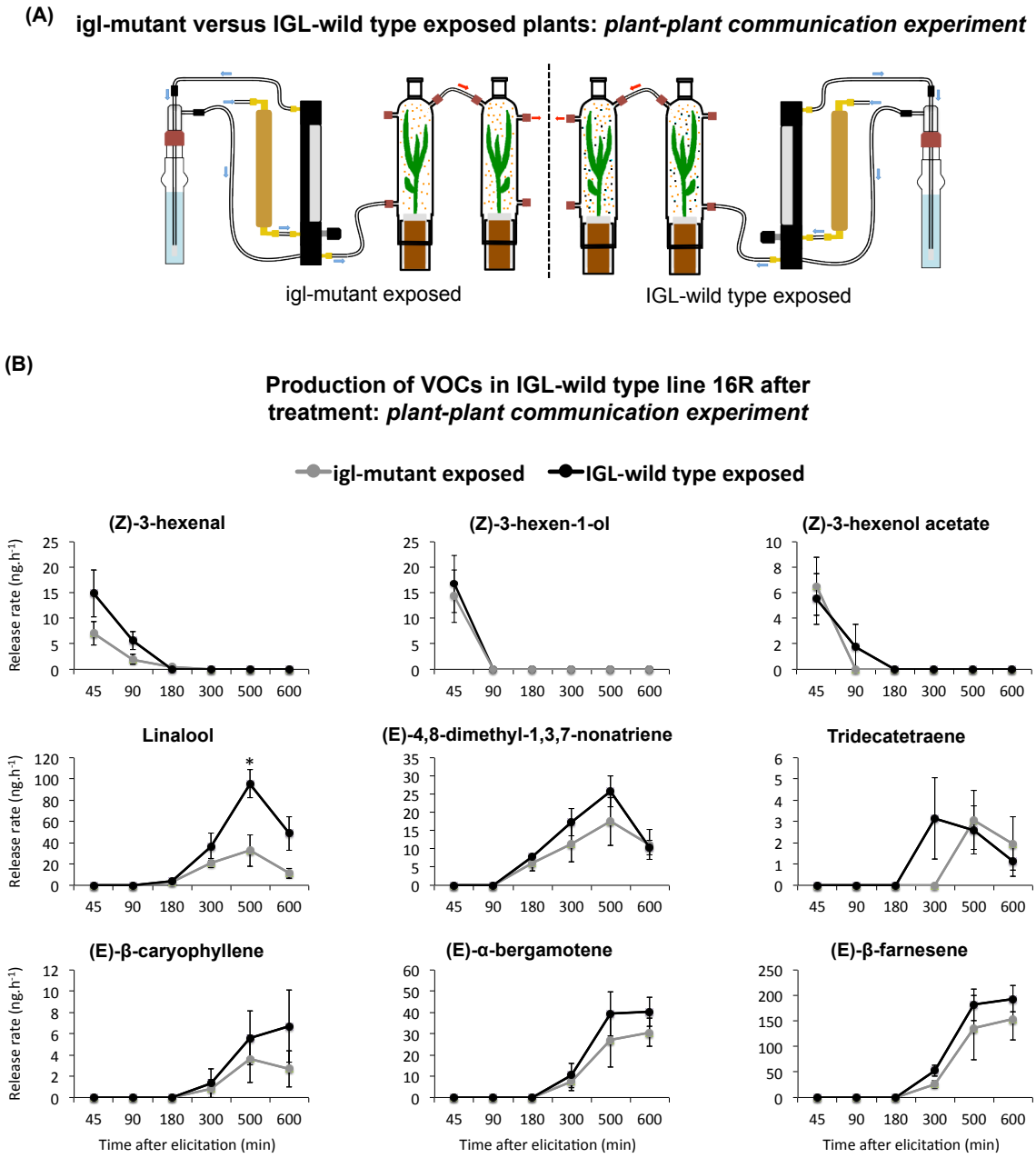

Figure S11: ***Plant-plant communication experiment with igl-mutant and IGL-wild type plants.***

(A) Experimental conditions. IGL-wild type (line 16R) plants were exposed to igl-mutant (line 32R) or IGL-wild type (line 16R) infested plants of two different genetic backgrounds for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.

(B) Exposure to IGL-wild type plants induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type lines. VOCs emissions of IGL-wild type lines (cross B: line 16R) exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *: p<0.05, **: p<0.01, ***:p<0.001, n=4)

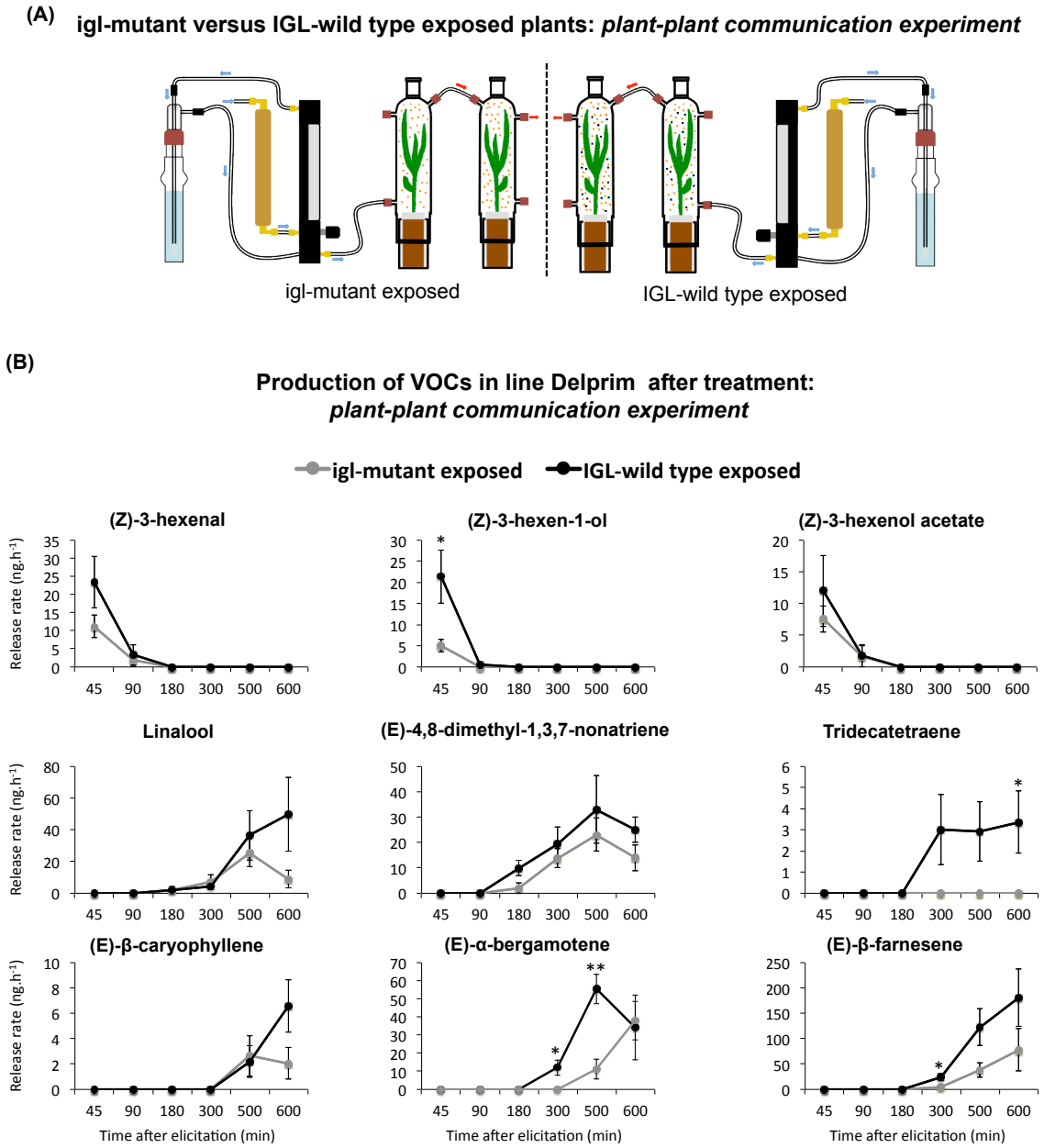

Figure S12: ***Plant-plant communication experiment with igl-mutant and IGL-wild type plants*.**

(A) Experimental conditions. Delprim plants were exposed to igl-mutant (line 22) or IGL-wild type (line 7) infested plants of two different genetic backgrounds for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.

(B) Exposure to IGL-wild type plants induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type lines. VOCs emissions of IGL-wild type lines (genetic backgorund 1: line 7; genetic backgorund 2: line 16R) exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **: $p<0.01$, ***:$p<0.001$, n=4)

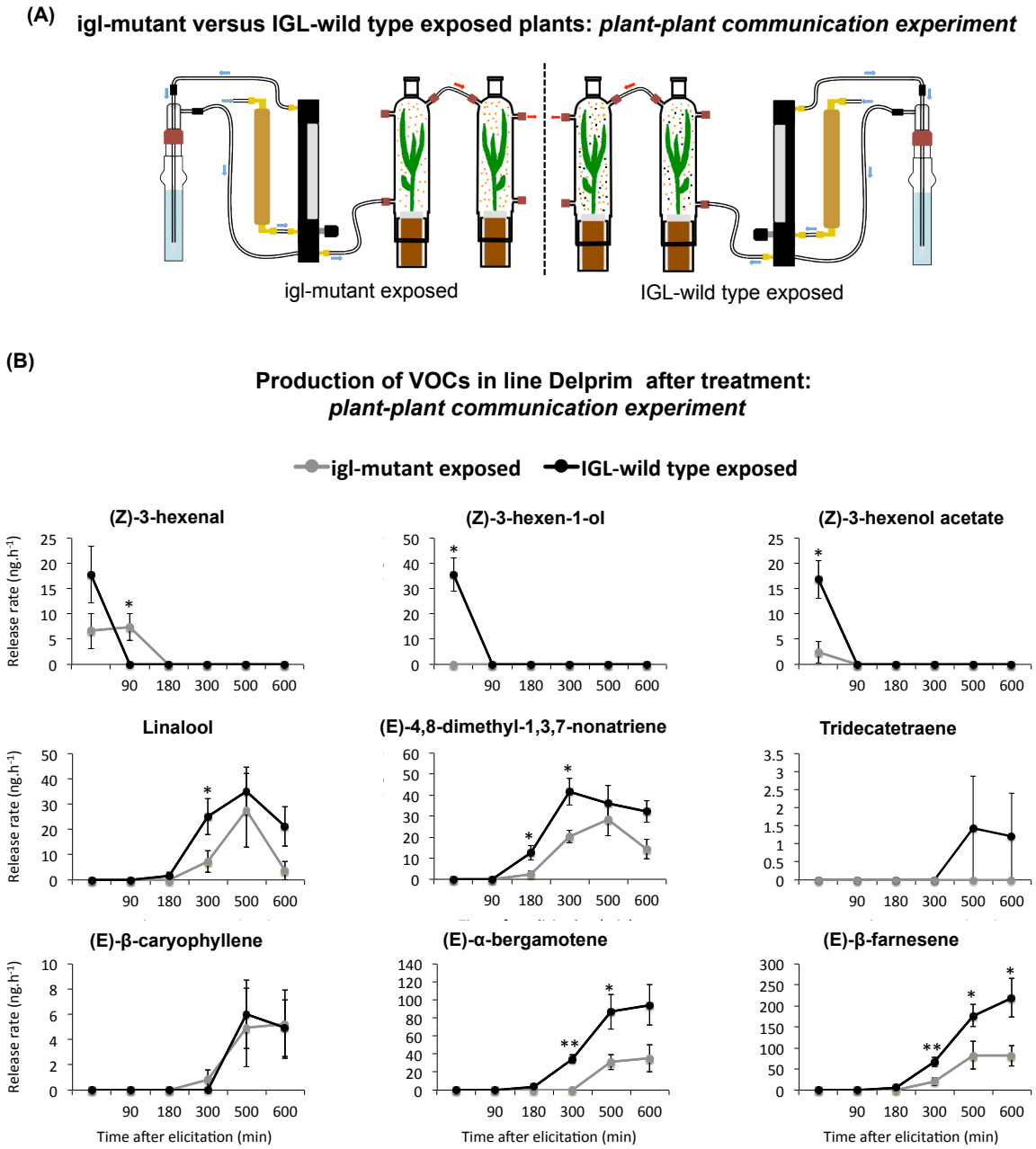

Figure S13: ***Plant-plant communication experiment with igl-mutant and IGL-wild type plants*.**

(A) Experimental conditions. Delprim plants were exposed to igl-mutant (line 32R) or IGL-wild type (line 16R) infested plants for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.

(B) Exposure to IGL-wild type plants induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type lines. VOCs emissions of IGL-wild type lines exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **: $p<0.01$, ***:$p<0.001$, n=4)

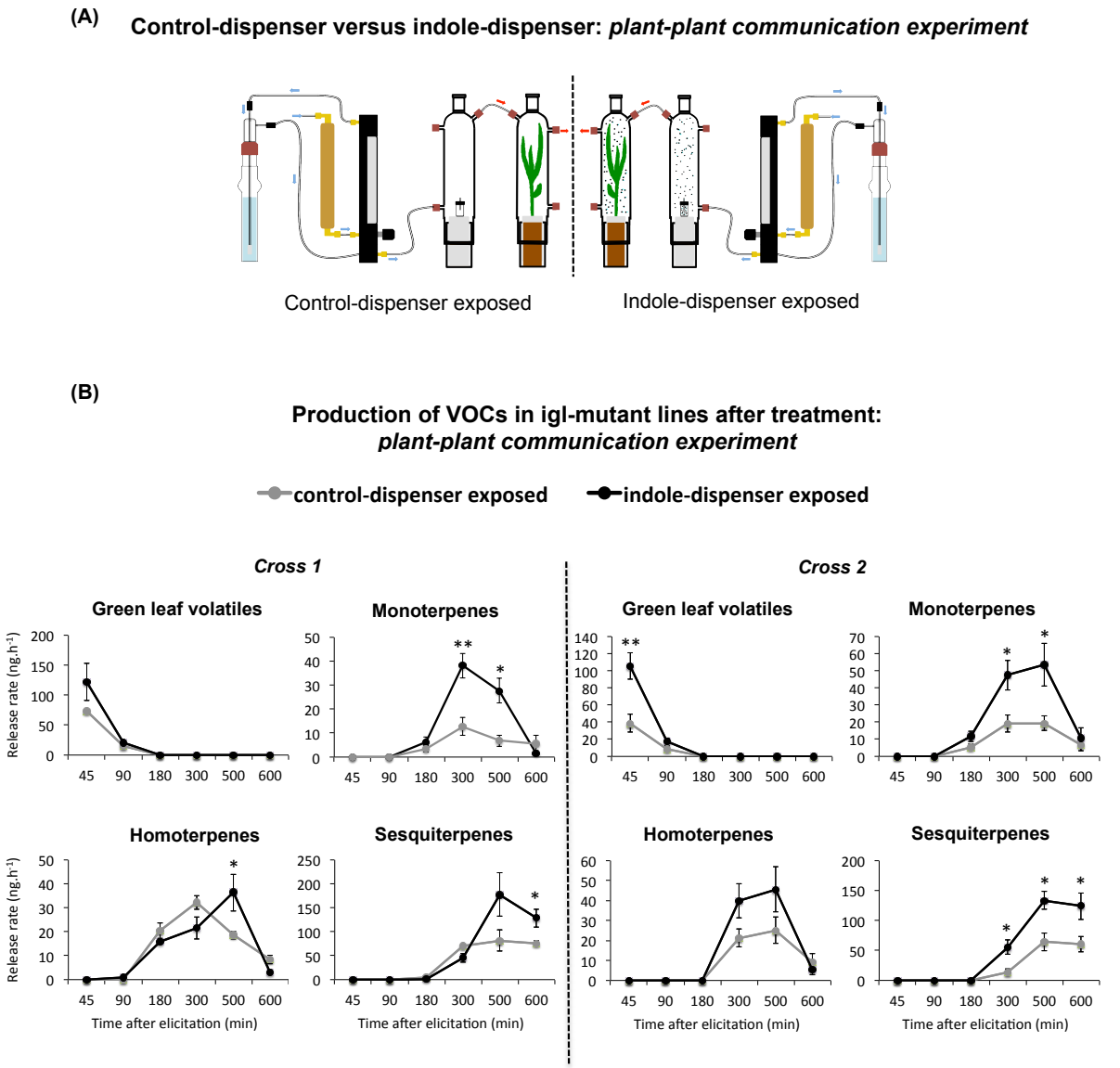

Figure S14: *Plant-plant communication experiment with synthetic indole*.
(A) Experimental conditions. igl-mutant plants of two different genetic backgrounds were exposed to control- or volatile indole- dispensers for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes. **(B) Exposure to volatile indole after elicitation treatment induces priming for emission of various volatile organic compounds (VOCs) in igl-mutant line.** VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment (cross A: line 22; cross B: line 32R). At the time of elicitation treatment, plants were exposed to volatile indole from dispenser. Four major families of VOCs were found: Green leaf volatiles; Monoterpenes; Homoterpenes; Sesquiterpenes. Asterisks indicate statistical differences between release rates from control- and indole-exposed plants (Student's t-test, *: $p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

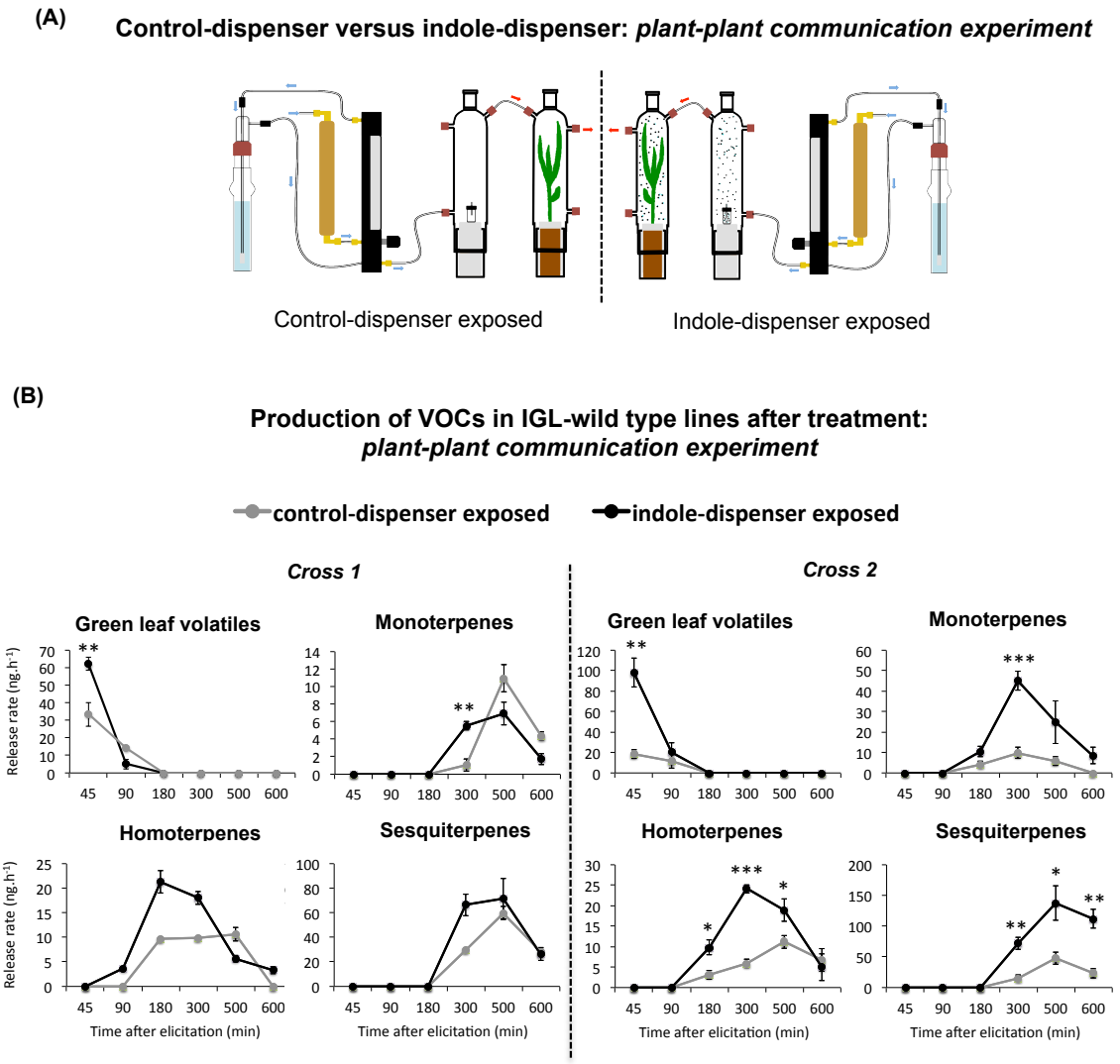

Figure S15: ***Plant-plant communication experiment with synthetic indole.***
(A) Experimental conditions. IGL-wildtype plants were exposed to control- or volatile indole- dispensers for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole after elicitation treatment induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type lines. VOCs emissions of control- and volatile indole-dispenser exposed plants at different times after elicitation treatment (cross A: line 7; cross B: line 16R). At the time of elicitation treatment, plants were exposed to volatile indole from dispenser. Four major families of VOCs were found: Green leaf volatiles; Monoterpenes; Homoterpenes; Sesquiterpenes. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *: p<0.05, **:p<0.01, ***:p<0.001, n=4)

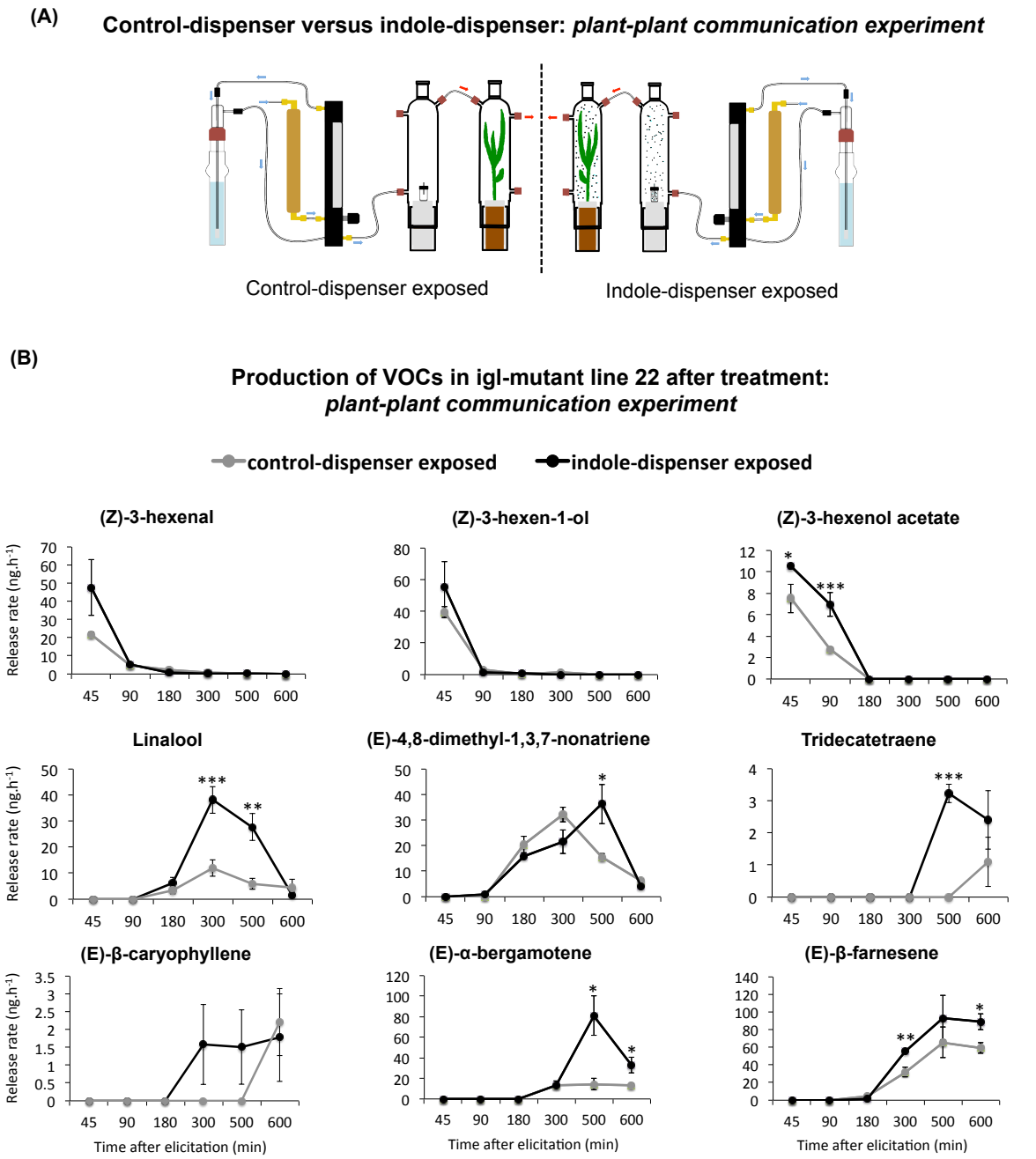

Figure S16: ***Plant-plant communication experiment with synthetic indole.***
(A) Experimental conditions. igl-mutant plants were exposed to control- or volatile indole-dispensers for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole after elicitation treatment induces priming for emission of various volatile organic compounds (VOCs) in igl-mutant line 22. VOCs emissions of control- and volatile indole- exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$, n=4)

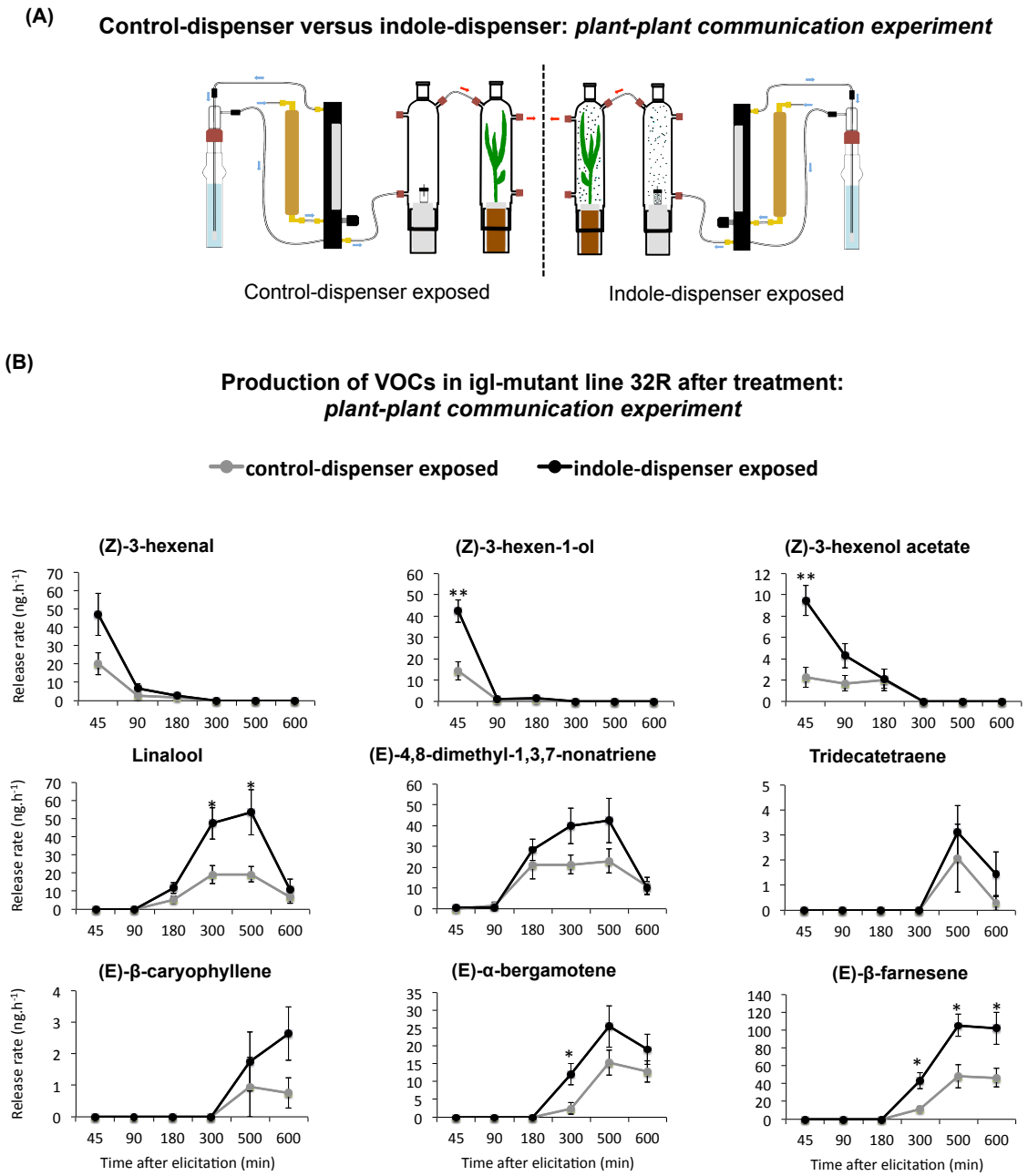

Figure S17: ***Plant-plant communication experiment with synthetic indole.***
(A) Experimental conditions. igl-mutant plants were exposed to control- or volatile indole-dispensers for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole after elicitation treatment induces priming for emission of various volatile organic compounds (VOCs) in igl-mutant line 32R. VOCs emissions of control- and volatile indole- exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *:p<0.05, **:p<0.01, ***:p<0.001, n=4)

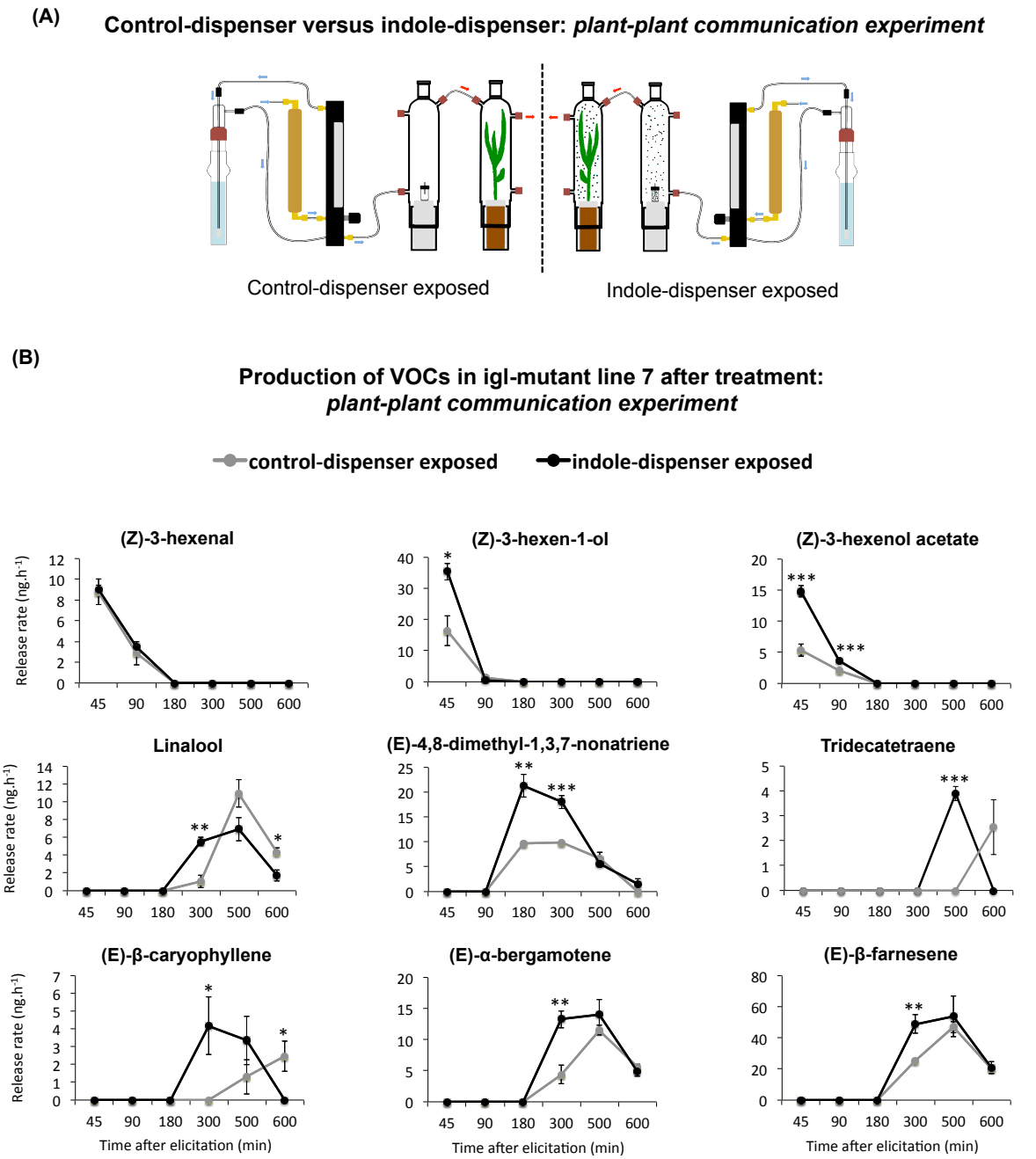

Figure S18: ***Plant-plant communication experiment with synthetic indole*.**
(A) Experimental conditions. IGL-wildtype plants were exposed to control or indole-emitting dispensers for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole after elicitation treatment induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type line 7. VOCs emissions of control- and volatile indole- exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *:p<0.05, **:p<0.01, ***:p<0.001, n=4)

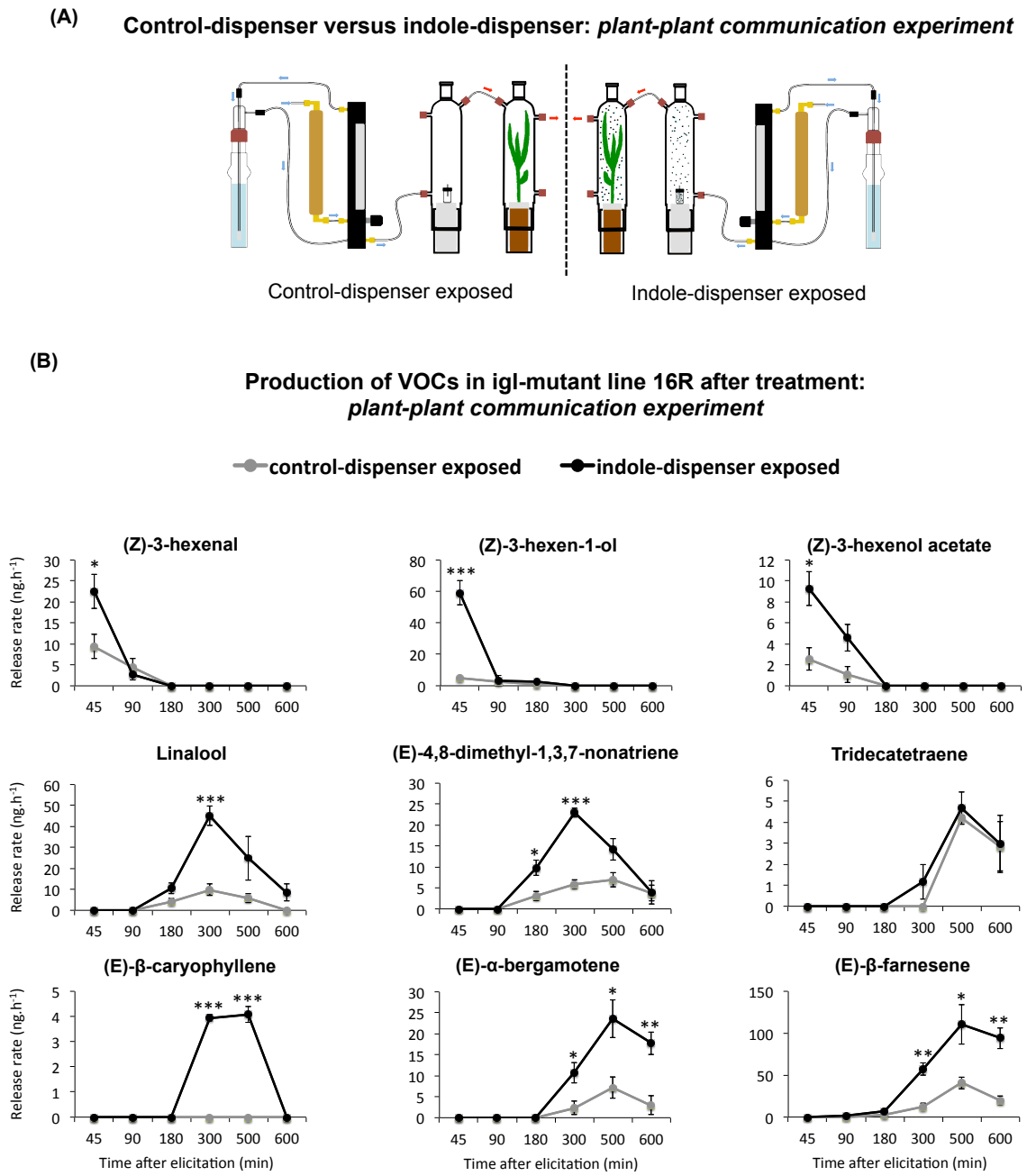

Figure S19: ***Plant-plant communication experiment with synthetic indole.***
(A) Experimental conditions. IGL-wildtype plants were exposed to control or indole-emitting dispensers for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole after elicitation treatment induces priming for emission of various volatile organic compounds (VOCs) in IGL-wild type line 16R. VOCs emissions of control- and volatile indole- exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *:p<0.05, **:p<0.01, ***:p<0.001, n=4)

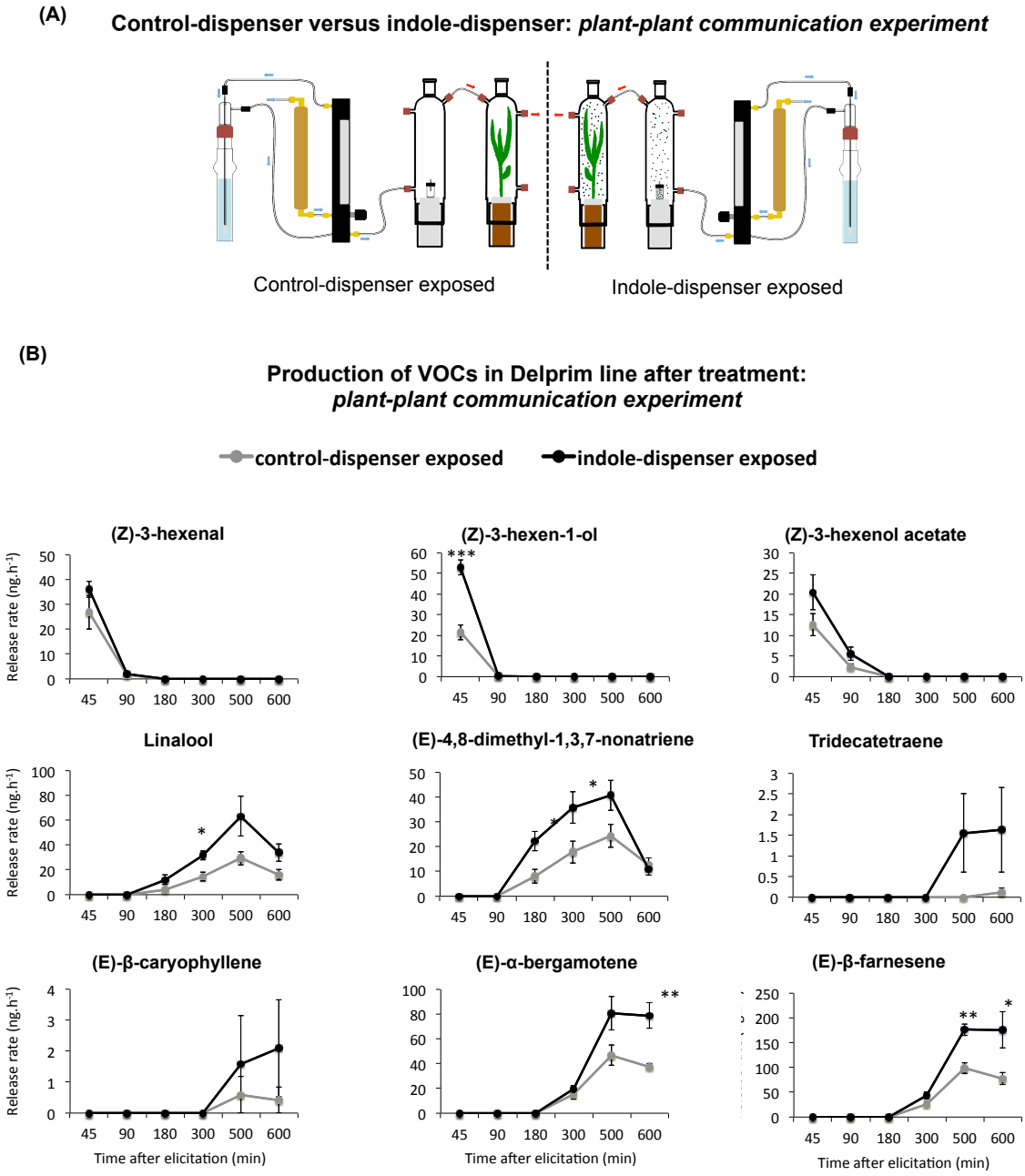

Figure S20: ***Plant-plant communication experiment with synthetic indole.***
(A) Experimental conditions. Delprim plants were exposed to control or indole-emitting dispensers for 12 hours. They were then subjected to an elicitation treatment and put in clean odour vessels. VOCs were collected for 600 minutes.
(B) Exposure to volatile indole after elicitation treatment induces priming for emission of various volatile organic compounds (VOCs) in Delprim line. VOCs emissions of control- and volatile indole- exposed plants at different times after elicitation treatment. Major VOCs found were: (Z)-3-hexenal, (Z)-3-hexen-1-ol, (Z)-3-hexenol acetate, Linalool, (E)-4,8-dimethyl-1,3,7-nonatriene, Tridecatetraene, (E)-β-caryophyllene, (E)-α-bergamotene, (E)-β-farnesene. Asterisks indicate statistical differences between quantity from control- and indole-exposed plants (Student's t-test, *:p<0.05, **:p<0.01, ***:p<0.001, n=4)

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CHAPTER 2

An induced volatile acts as a counter-intuitive direct plant defence that enhances caterpillar growth

AN INDUCED VOLATILE ACTS AS A COUNTER-INTUITIVE DIRECT PLANT DEFENCE THAT ENHANCES CATERPILLAR GROWTH

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ABSTRACT

In order to counter herbivore attacks, plants have developed a multitude of defence strategies, including the release of herbivore-induced plant volatiles (HIPVs) that reduce oviposition by herbivores and attract predators and parasitoids. To date, little is known about the role of HIPVs in direct defence. We investigated the impact of indole, a major constituent of the herbivore-induced volatile blend in many plant species, on the generalist herbivore *Spodoptera littoralis*. Using indole deficient maize mutants and physiologically relevant doses of synthetic indole, we found that *S. littoralis* moths and caterpillars are strongly repelled by indole. Contrary to our expectations, performance did apparently not follow preference. On the contrary, exposure to volatile indole increased caterpillar weight gain and body fat contents. The increased weight gain was associated with a decrease in food consumption, pointing to a direct positive effect of indole on food conversion efficiency. The seemingly contradictory effects of indole on preference and performance were resolved by the discovery that indole exposure reduces the survival of early instar caterpillars as well as the reproductive fitness of adults. Taken together, our results show that volatile indole acts as a direct defence in maize, an effect that is associated with a counterintuitive boost of larval weight gain.

INTRODUCTION

Plants are constantly subjected to insect attack that threatens their reproductive output. As a consequence, they have evolved a wide array of defence strategies (Grayer *et al.* 1993; Schoonhoven *et al.* 2005; Karban & Baldwin 2007). For instance, many plants release herbivore-induced plant volatiles (HIPVs) (Dicke *et al.* 2009) which are used as foraging cues by natural enemies of the herbivores to locate their prey or hosts (Turlings *et al.* 1990; Turlings & Wäckers 2004; Arimura *et al.* 2005). It has been demonstrated that in this way, the emission of HIPVs reduces the number of attackers (Kessler & Baldwin 2001) resulting in reduced damage (Thaler 1999; Karban & Baldwin 2007; Halitschke *et al.* 2008). HIPVs also affect herbivore behaviour directly. For instance, adults of the genus Lepidoptera are repelled by HIPVs and choose to oviposit on healthy plants (De Moraes *et al.* 2001; Kessler & Baldwin 2001; Sánchez Hernández *et al.* 2006), possibly to avoid competition. However, HIPVs may also be attractive to herbivores (Carroll *et al.* 2008; von Mérey *et al.* 2013). Larvae of *Spodoptera frugiperda*, *Ostrinia nubialis* and *Ostrinia furnacalis* for instance are attracted by odours from damaged maize plants (Carroll *et al.* 2006; Huang *et al.* 2009; Piesik *et al.* 2009). This renders the release of volatiles a two-edged sword for the plant, as its apparency is increased to both herbivores and natural enemies (Halitschke *et al.* 2008). Apart from affecting insect behaviour, HIPVs can also directly affect herbivore fitness. For instance, green leaf volatiles (GLVs) negatively affect the growth rate of *Spodoptera littoralis* caterpillars (von Mérey *et al.* 2013). To date, such direct effects of HIPVs on herbivores have received comparably little attention, despite the fact that they may help to explain the initial stabilization of HIPV traits before natural enemies adopted them as foraging signals.

Although the identity and production of HIPVs is well described (Turlings *et al.* 1998; Paré & Tumlinson 1999; D'Alessandro & Turlings 2005), their ecological role is often unclear. HIPVs are commonly divided into three different classes: GLVs, terpenoids and aromatic compounds. Both GLVs and and terpenoids are implicated in the attraction of natural enemies (Takabayashi *et al.* 1995; D'Alessandro & Turlings 2005; Hoballah & Turlings 2005; D'Alessandro *et al.* 2006; Fontana *et al.* 2011) and insect herbivores (Hanson *et al.* 1999; Halitschke & Baldwin 2004; Halitschke *et al.* 2008; von Mérey *et al.* 2011). The role of vegetative aromatic compounds is much less studied. Indole for instance is emitted by maize and many other plants after herbivore attack (Turlings *et al.* 1991; McCall *et al.* 1994; Gols *et al.* 1999; Cardoza *et al.* 2003; De Boer *et al.* 2004; Yuan *et al.* 2008). It is specifically released in response to herbivore-elicitors and is not emitted from physically wounded plants (Frey *et al.* 2004; Zhuang *et al.* 2012). The release of indole can be induced after treatment of maize with volicitin [N-(17- hydroxylinolenoyl)-L-glutamine], an elicitor found in the regurgitant of *Spodoptera* larvae (Alborn *et al.* 1997; Turlings *et al.* 2000). In maize, indole is

produced from indole-3-glycerol phosphate and channelled into three different pathways. First, indole can be formed by the tryptophan synthase alpha subunit (TSA), which channels it directly to the tryptophan synthase beta subunit (TSB) for further conversion into the essential amino acid tryptophan (Frey *et al.* 1997; Frey *et al.* 2004). Second, it can be produced by the BX1 enzyme as an intermediate in the production of benzoxazinoids, a class of non-volatile defensive secondary metabolites of the grasses (Frey *et al.* 2009). Finally, it can be formed by the indole-3-glycerol phosphate lyase IGL, which subsequently releases it as a volatile (Frey et al. 2000). The highly specialized and conserved regulation and function of IGL point to a potentially important role of indole in plant-herbivore interactions. However, only few studies have investigated the impact of this HIPV so far. In an olfactometer study, it was found that the parasitoid *Microplitis rufiventris* is repelled by indole-producing maize plants infested by caterpillars, whereas *Cotesia marginiventris* was not affected by the volatile (D'Alessandro *et al.* 2006).

We therefore investigated the role of indole in the interaction of maize and the generalist herbivore *Spodoptera littoralis*. Using indole-deficient maize mutants and synthetic indole, we document the impact of indole on host plant choice, feeding preference, growth and performance of *S. littoralis*. Our results show that indole protects maize against *S. littoralis* despite its growth promoting effect on caterpillars. This counterintuitive effect adds a novel dimension to our understanding of the direct effects of plant volatiles on plant-herbivore interactions.

MATERIALS AND METHODS

Plants and insects

The maize (*Zea mays* L.) lines 22 (igl.bx1), 7 (IGL.bx1) (cross A) and 32R (igl.bx1), 16R (IGL.bx1) (cross B) were obtained as previously described by (Ahmad *et al.* 2011). Lines 22 and 32 are defective in the indole-3-glycerol-phosphate lyase (IGL) that produces free indole, while lines 32R and 16R carry the wild type allele. All lines have a bx1 mutant background, which enabled us to assess the role of volatile indole without any interference from BX1 derived indole. The activity of the BX1 locus varies considerably across maize lines (Butrón *et al.* 2010), including naturally inactive forms of the BX1 allele (Frey *et al.* 1997). Seeds of the hybrid Delprim, which carry functional variants of the BX1 and IGL wild type alleles, were obtained from Delley Semences et Plantes SA. (Delley DSP, Switzerland). Furthermore, eight different inbred lines of maize known to differ in their capacity to produce volatile indole (Erb *et al.* 2011) were used for an additional performance experiment. The different lines, originally named alphabetically from A to V for convenience were: C, D, E, F (releasing few

volatile indole), and I O, R, S (producing a lot of volatile indole). More information about their genetic background is available from Delley DSP upon request. All maize lines were grown individually in plastic pots (10 cm high, 4 cm diameter) with commercial potting soil (Aussaaterde, Ricoter, Aarberg, Switzerland) and placed in a climate chamber (23°C ± 2°C, 60% relative humidity, 16:8 h L/D, 50'000 lm/m^2). Maize plants used for the experiments were ten to twelve days old and had three fully developed leaves. The evening before the experiments, plants were transferred and kept under laboratory conditions (25 ± 2°C, 40 ± 10% relative humidity, 16h light/8h dark, and 8000 lm/m^2). *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) caterpillars were reared from eggs provided by Syngenta (Stein, Switzerland). The eggs were kept in an incubator at 30.0 ± 0.5°C until emergence of the larvae. Subsequently, they were transferred on artificial diet at room temperature (24 ± 4°C).

Effect of indole on S. littoralis *attraction*

Spodoptera caterpillars are known to be attracted to maize HIPVs. To understand whether indole, as a specific and rapidly induced volatile, attracts *S. littoralis* caterpillars, we measured the attraction of third instar larvae in a modified four-am olfactometer (D'Alessandro & Turlings 2005). The olfactometer consisted of a central glass choice arena (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 a glass bulb (50 mL). Purified and humidified air entered each odour source vessel at 1.1 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, a part of the air (0.7 L/min) was pulled out and the other part entered in the central glass chamber. Ten neon tubes were attached to a metal frame above the olfactometer and provided approximately 7000 lm*m^2 at the height of the odour source vessels. To avoid visual distraction of the larvae, a white cardboard cylinder was placed around the central choice arena and on top of the choice arena. The choice arena was connected to four glass bottles. Two opposite bottles contained an odour source and the two remaining bottles remained empty. The position of the odour sources was changed between each experimental run. All experiments were repeated six times. The system was left connected for half an hour before releasing thirty third-instar larvae in the centre of the choice arena. The larvae would crawl out of the box and enter one of the four arms. After thirty minutes, the number of larvae in each arm was counted. The larvae that did not make the choice arena after thirty minutes were considered as having made "no choice". Six such releases were done for each replicate and pooled for analysis. Using this system, several experiments were carried out. First, we tested the preference of *S. littoralis* for IGL wild type and mutant plants

(n=6). To induce HIPV release, all plants were infested with fifteen second-instar *S. littoralis* larvae that were placed on the plant the evening before the bioassay. Second, we assessed the preference of the larvae for IGL wild type vs. igl mutant plants that were complemented with synthetic indole (n=6). For this purpose, we used volatile dispenser that were constructed as follows: A 2ml amber glass vial (11.6 x 32 mm; Sigma-Aldrich, Buchs, Switzerland) containing 20 mg of synthetic indole (> 98 %, GC, Sigma-Aldrich, CH-9471 Buchs, Switzerland). The vial was sealed with a PTFE/rubber septum (Sigma-Aldrich, Buchs, Switzerland) and pierced with a 2 µL micro-pipette (Drummond, Millan SA, Plan-Les-Ouates, Switzerland). The length of the pipette was calibrated to release a controlled amount of indole, similar to the amount emitted by IGL-wild type plants (approx. 50 $ng.h^{-1}$). In a third experiment, we tested the dose-effect of indole by over complementing the igl mutant with 3 releasers per plant (resulting in three times more indole emanating from the olfactometer arms containing the igl mutant compared to the ones with IGL wild type plants) (n=6). Fourth, we tested the choice of *S. littoralis* when offered igl mutant plants with or without an indole dispenser (n=6). Fifth, we increased the indole-containing blend of the hybrid Delprim by adding an indole dispenser to one arm. Fifth, we tested the role of indole alone by giving *S. littoralis* larvae a choice between arms containing an empty dispenser and arms with an indole dispenser (n=6).

Effect of indole on S. littoralis *feeding preference*

To test whether the repellent effect on *S. littoralis* also affects feeding choice of the larvae, we performed a leaf-disc feeding assay. Leaf-discs (5 mm diameter) from induced igl-mutant or IGL-wild type leaves were placed in a petri dish (diameter 100 mm; 2 leaf discs per dish). Leaf-discs were removed for the second true leaf that was induced previously. The elicitation treatment was performed by scratching the leaf over two areas of approximately 1 cm^2 on both sides of the central vein with anatomical forceps (stainless steel, 14.5 cm). Then 10 µL of *Spodoptera littoralis* regurgitant were applied over the scratched leaf areas. Regurgitant had been previously collected from fourth instar *S. littoralis* that had been feeding on maize leaves for 24 hours and was stored at -80°C until use. One first instar *S. littoralis* larva was put in the centre of each box and was allowed to feed for 18h (n=11). Leaf discs were then scanned and the damaged leaf-area was measured in Photoshop PS6. Using this approach, the preference between wild type and igl mutant plants was measured. Furthermore, in a second experiment, igl mutants were complemented with indole to directly assess the impact of the HIPV on feeding preference. For complementation, the leaf discs were either submerged in H_2O or indole (100 µg/mL H_2O) before the experiment (n=11).

Effect of indole on oviposition preference

To assess the influence of indole on *S. littoralis* oviposition, gravid females that had previously been reared on artificial diet were given a choice between previously infested igl-mutant and IGL-wild-type plants. For each replicate, eight igl mutant and IGL wild type plants were infested with three *S. littoralis* larvae (L1) per plant and placed in an oviposition cage (100cm high, 150cm long, 50 cm deep). To prevent the larvae from escaping and the adult moths to directly touch the plants or larvae, a nylon mesh was placed around the plants. A group of 20 *S. littoralis* pupae (sex ratio=1:1) was then placed in the centre of the cage at equal distance of the infested plants. Seven days later, the number of eggs laid on the nylon mesh and cage walls was counted (n=4). To assess oviposition choice, the cage was divided into three sections of similar size: The side harbouring the igl mutant plants, the side harbouring the IGL wild type plants and the center ("no choice"). Two groups of moths were tested: One group was exposed to volatile indole during the larval development whereas the other one was exposed to control-dispensers. For this purpose, individual *S. littoralis* larvae were reared to pupation in small plastic cages (3 cm x 3 cm x 1 cm) containing a 1 cm^3 cube of wheatgerm-based artificial diet and a control or indole dispenser as described above.

Effect of indole on S. littoralis *performance*

To determine whether indole has an effect on *S. littoralis* growth and feeding, we conducted four performance experiments. In all experiments, individual plants were infested with two first-instar larvae for 7 days. Larvae were prevent from escaping by placing transparent 1.5 L poly-ethylene (PET) bottles with the bottom cut out (30 cm height, cone-shaped, maximum diameter 8 cm) over the leaves. The bottles were placed upside down over the plants and attached to the pots with Parafilm (as described (Erb, Balmer et al. 2011). The weight of the larvae was recorded before and after infestation, and average relative weight gain was calculated for each replicate. In a first experiment, *S. littoralis* larval performance on IGL-wild type and igl-mutant plants was compared (n=12). Second, *S. littoralis* growth on igl-mutants with or without synthetic indole was measured (n=12). Third, the growth of *S. littoralis* was profiled on the hybrid Delprim with and without synthetic indole was determined (n=12). To complement the plants with indole, they were watered with 10 mL of an indole solution of (100 µg/mL H_2O) every day. This concentration of indole was calculated based on physiologically relevant indole emission rates of to 200 $ng*h^{-1}$ per plant, an estimated uptake from the soil of 1% and a release rate of 50%. Fourth, we measured *S. littoralis* growth on eight different inbred lines of maize: Four of them produce a lot of indole upon herbivore attack, and 4 of them producing very little (n=12). The indole release capacity of the different inbred lines was confirmed using the same technique as described (Erb *et al.* 2011).

Effect of volatile indole on S. littoralis *performance*

To separate between direct and plant-mediated effects of indole, we performed a series of experiments within which the larvae were exposed to indole-containing or indole-free volatile blends while feeding on artificial diet. First, the effect of HIPVs from igl mutant and IGL wild type plants on *S. littoralis* was tested (n=15). As a control, we also complemented igl mutants with indole (see below) (n=15). To induce maize plants, they were infested with two first-instar *S. littoralis* larvae that were kept on the leaves by a Teflon bag placed over the leaves. Sixteen hours later, four first-instar *S. littoralis* larvae were transferred to plastic boxes (3 cm x 3 cm x 1 cm, 2 larvae per box) covered with a fine nylon mesh. The plastic boxes were then placed into the Teflon bags with the plant. The larvae in the boxes were provided with a cube of wheatgerm-based artificial diet (1 cm^3) (Turlings *et al.* 2004), which was changed every second day. This setup exposed the test-larvae to all volatile compounds produced by infested-plants while preventing any direct contact. The plants were watered every day from the bottom of the plastic pots. Half of the igl-mutants were watered with a solution of indole dissolved in water (100 µg/mL). The weight of the larvae was recorded before and after seven days of infestation, and average relative weight gain was calculated. To test the effect of volatile indole alone on larval performance, first-instar of *S. littoralis* larvae were kept in small plastic cages (3 cm x 3 cm x 1 cm) containing a 1 cm^3 cube of wheatgerm-based artificial diet and a control or indole dispenser (see above). The weight of the larvae was recorded at 6h after infestation, and average relative weight gain was calculated (n=13). The amount of ingested food was also measured by weighing the diet at the start and the end of the experiment. In a further experiment, *S. littoralis* larvae feeding on artificial diet were exposed to volatile indole from dispensers for a total of 12 days. In this experiment, 6 first instar larvae were placed on diet cubes that were placed on a 10 cm wooden stick within a similar PET bottle setup as the plant performance experiments (see above, n=15). Indole or control dispensers as described above were placed into the bottles, and larval weight was determined 12 days after the start of the experiment. Diet cubes were replaced daily.

Effects of indole on S. littoralis *fitness*

To investigate whether the indole induced increase in weight gain is associated with increased fitness of *S. littoralis*, we determined the survival of *S. littoralis* larvae. The survival data was extracted from larval counts of the experiments described above. To increase the statistical power of the survival analysis, data from the two mutant and wild type lines was combined. First, the survival of larvae feeding on igl mutant and IGL wild type lines for 7 days was determined (n=24). Second, the survival of larvae on igl mutant lines complemented with indole was measured (n=24). Third, the survival of larvae feeding on artificial diet with similar

exposure to induced volatiles from the different mutant and complementation combinations was determined (n=24). Fourth, larval survival of *S. littoralis* feeding on artificial diet with and without indole dispensers was assessed (n=12). Fifth, we performed an additional experiment in which different larval stages of *S. littoralis* were exposed to volatile indole while feeding on artificial diet for 6h. Larvae were either neonates, first instars or third instars (n=12). Larval weight gain, diet consumption and survival were measured. Sixth, we assessed the reproductive output of *S. littoralis* females that were exposed to volatile indole or control dispensers during their larval development. Groups of 20 *S. littoralis* pupae (sex ratio=1:1) were then placed in the centre of oviposition cages (100cm high, 150cm long, 50 cm deep). Seven days later, the number of eggs laid on the nylon mesh and cage walls was counted (n=6).

Physiological effects of indole on S. littoralis

To explain the contradictory effects of indole on *S. littoralis* growth and fitness, we tested several hypotheses. First, we investigated whether indole increases food uptake by *S. littoralis* by measuring leaf-consumption of larvae on igl mutant and IGL wild type plants (see above; n=24) and indole supplemented diet (n=12). Second, we tested if indole changes water retention of *S. littoralis* by measuring water contents of diet fed larvae with or without indole dispensers (7 day feeding period, n=12). Larvae were weighed, killed by flash freezing them in liquid nitrogen and dried at 50°C for 48 h. After two days, the dried larvae were re-weighed to determine water contents. Third, we measured the concentration of triacylglycerols, the main lipid storage molecules of *Spodoptera* (Blacklock & Ryan 1994), in control and indole-dispenser exposed larvae after 7 days of exposure (n=12). The method was adapted from (Schwartz & Wolins 2007). After exposure to a control- or indole-dispenser for seven days, all larvae were weighted and dissected. The fat body was isolated from the body and kept in liquid nitrogen. 500 µL of PBS/10 mM EDTA, pH 7.4, with 0.1% green food coloring was added to each sample. Samples were mixed by vortexing and centrifuged for 10 min to 12 000 rpm. Supernatant was added to 2 mL of isopropanol-hexane-water (80:20:2) was added to a glass tube (100mmx15mm). Samples were covered with aluminium foil and incubated for 30 minutes at room temperature. 500 mL of hexane-diethyl ether (1:1) was added and each tube was mixed by vortexing. Samples were covered with aluminium and incubated at room temperature for 10 minutes. 1 mL of water was then added to the tubes and mixed by vortexing. Samples were covered with aluminium again until phase separation at room temperature. The organic phase was transferred into a 2 mL Eppendorf tube. The organic phase was then evaporated to dryness and re-suspended in 400 mL of triglyceride reagent (Sigma, T2449) diluted 1:5 with free glycerol reagent (Sigma, F6428). Samples were

covered with parafilm and incubated for 90 minutes at 37°C with shaking at 360 rpm. 300 mL of each sample was then transfered to a 96-well plastic microplate. Absorbance was determined at 540 nm using a microplate reader. In parallel, a standard curve from Triolein (Sigma, G7793) was generated for absolute quantification.

Statistics

The functional relationship between larval choice and the different odour sources offered in the four-arm olfactometer was analysed with a generalized linear model (GLM, a log linear model) with the software package R (version 2.13.1). To compensate for the overdispersion of wasps within the olfactometer, we based the models on a quasi-Poisson distribution. For detailed explanations, see (Turlings *et al.* 2004). Differences in larval performance, larval preference, larval survival, adults preference, adults fitness, quantity of eaten diet, water quantities and triolein concentrations between indole- and control-exposed larvae were analysed for significance using analysis of variance (ANOVA), with a significance threshold of 0.05.

RESULTS

S. littoralis *larvae are repelled by indole*

When given a choice in a four-arm olfactometer, *S. littoralis* larvae clearly avoided odor sources containing indole (Figure 1): First, *S. littoralis* larvae were more attracted to induced indole-free igl mutants than to indole-emitting wild type plants (Cross A: n=6, $p<0.001$; Cross B: n=6, $p<0.001$; Figure 1A). Second, complementing igl mutants with synthetic indole recovered the effect (Cross A: n=6, $p=0.0159$; Cross B: n=6, $p=0.093$; Figure 1B). Third, tripling the levels of indole in igl mutants with higher doses of indole rendered them significantly less attractive than the wild type (Cross A: n=6, $p<0.001$; Cross B: n=6, $p<0.001$; Figure 1C. Fourth, *S. littoralis* preferred igl mutants without complementation over igl mutants with indole releasers (Cross A: n=6, glm, $p<0.001$; Cross B: n=6, glm, $p<0.001$; Figure 1D). Similar results were found for induced Delprim plants, which became less attractive when their indole emission was increased by a synthetic dispenser (n=6, $p<0.001$; Figure 1E). Moreover, larvae preferred to enter empty bottles than bottles containing indole dispensers even in the absence of a plant (n=6, $p<0.001$; Figure 1F). Taken together, these results clearly show that *S. littoralis* larvae avoid volatile indole and prefer HIPV blends without the volatile.

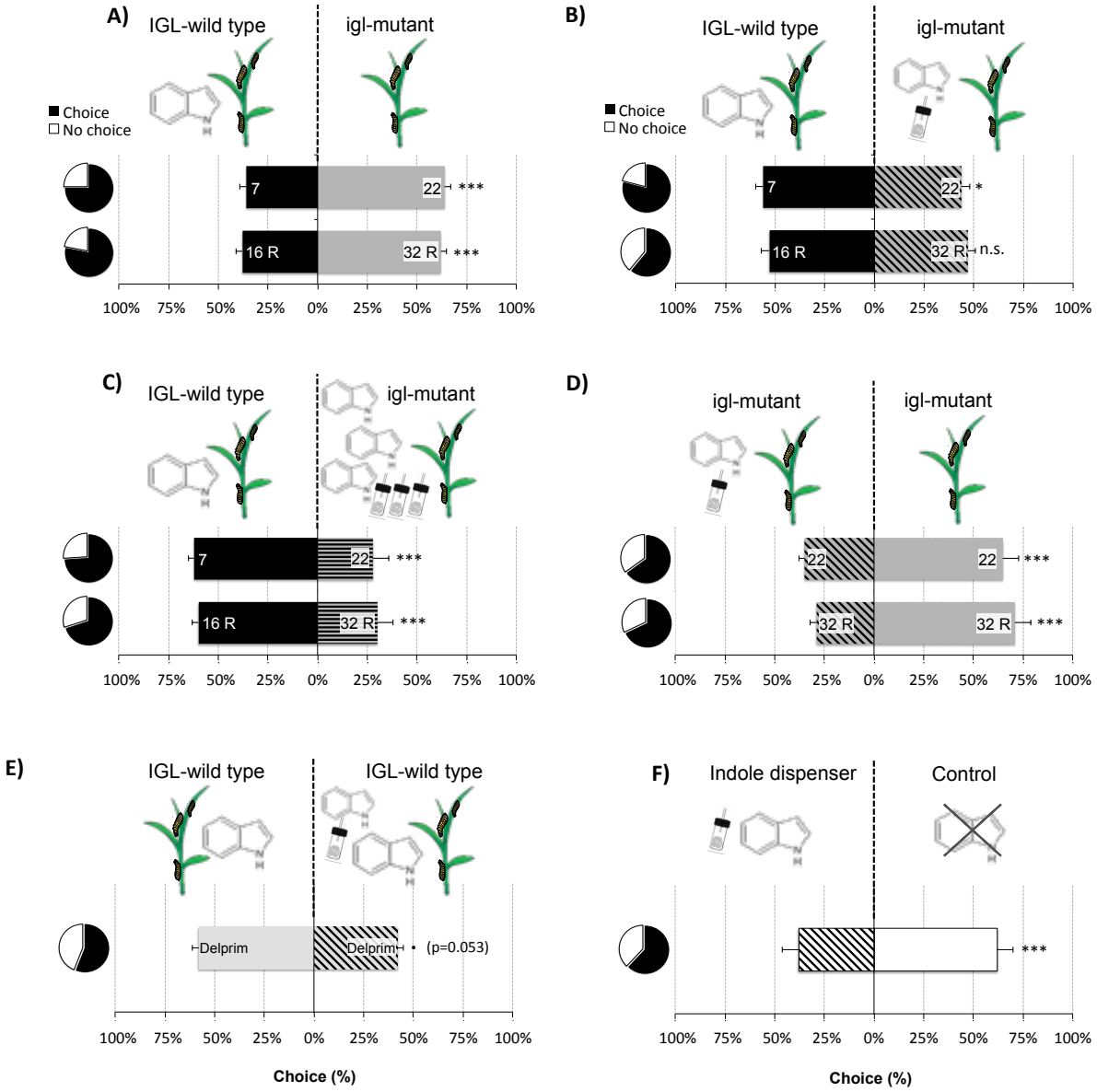

Figure 1: ***Effect of indole on larval attraction***. **(A)** Choice experiment between igl-mutant and IGL wild type plants (n=6). **(B)** Choice experiment between IGL-wild type plants and igl-mutant supplemented with synthetic indole (n=6). **(C)** Choice experiment between IGL wild type plants and igl-mutant plants supplemented with three times more of synthetic indole than normal IGL-wild-type plant (n=6). **(D)** Choice experiment between igl-mutant plants and igl-mutant plants supplemented with synthetic indole (n=6). **(E)** Choice experiment between delprim plants and delprim plants supplemented with synthetic indole (n=6). **(F)** Choice experiment between empty bottles and bottles with a dispenser of volatile indole (n=6). Asterisks indicate significant differences between treatments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

S. littoralis *larvae prefer to feed on indole-free plants*

To test whether indole influences the feeding choice of *S. littoralis*, we offered leaf-disks of indole emitting or mutant plants to the larvae. *S. littoralis* preferred to feed on indole-free mutant over wild-type plants (n=22; p≤0.001 / Cross A: n=11, p≤0.001; Cross B: n=11, Figure 2A). Again, indole supplementation of the mutant confirmed this effect, with the larvae preferring the mutant without supplementation (n=22, p=0.001 / Cross A: n=11, p=0.023; Cross B: n=11, p=0.038; Figure 2B).

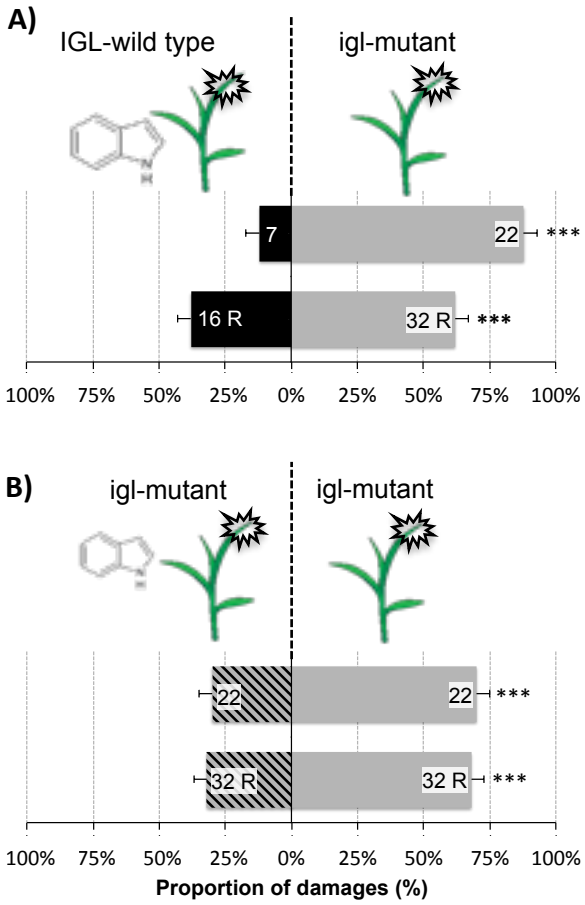

Figure 2: ***Effect of indole on larval feeding preference***. **(A)** Choice experiment in *petri* dishes using igl-mutant and IGL wild type leaf disks (n=11). **(B)** Choice experiment in *petri* dishes using igl-mutant and igl-mutant leaf disks supplemented with synthetic indole (n=11). Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

S. littoralis *adults prefer to oviposit on indole-free plants*

To test whether *S. littoralis* adults also avoid indole-emitting plants, we carried out an oviposition experiment. As did the larvae, *S. littoralis* adults preferred to oviposit on induced igl-mutant over IGL-wild type plants (n=6, p<0.01, Figure 3). Exposure to indole during the larval stage did not change this preference, suggesting that it is innate.

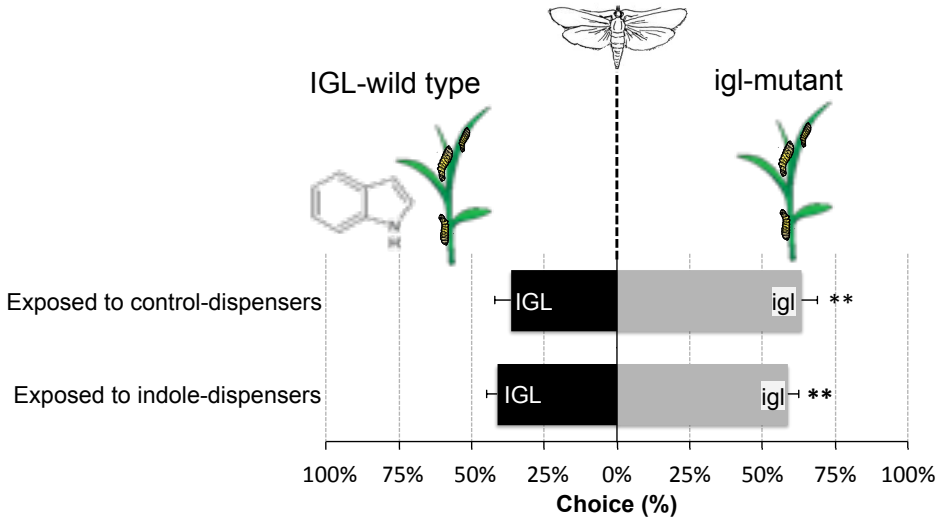

Figure 3: ***Effect of indole on oviposition preference***. Oviposition choice experiment between igl-mutant (lines 22 and 32R) and IGL-wild-type (lines 7 and 16R) plants (n=4). Adults were exposed to indole- or contol-dispensers during their larval and pupal life. Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

Plant indole increases larval growth

To test whether the repellent effect of indole goes together with a reduction of herbivore performance on indole-emitting plants, we measured larval weight gain on indole-mutants and wild type plants. However, after seven days, *S. littoralis* larvae had gained significantly more weight when feeding on indole emitting plants compared to igl mutants (n=26, p≤ 0.001 / cross A: n=13, p=0.035; cross B: n=13, p≤ 0.001; Figure 4A). When we supplemented igl mutants with indole, *S. littoralis* larvae gained more weight than on mutants without supplementation (n=12, p=0.005/ cross A: n=6, p=0.029; cross B: n=12, p=0.057; Figure 4B). Also, *S. littoralis* larvae feeding on indole supplemented-Delprim plants grew more than larvae that fed on Delprim plants without supplementation (n=12, p=0.031; Figure 4C). Finally,*S. littoralis* larvae gained more weight on four high-indole producing maize inbred lines than on low producing lines (n=12, p≤ 0.001; Figure 4D). Taken together, this data shows that indole enhances *S. littoralis* weight gain.

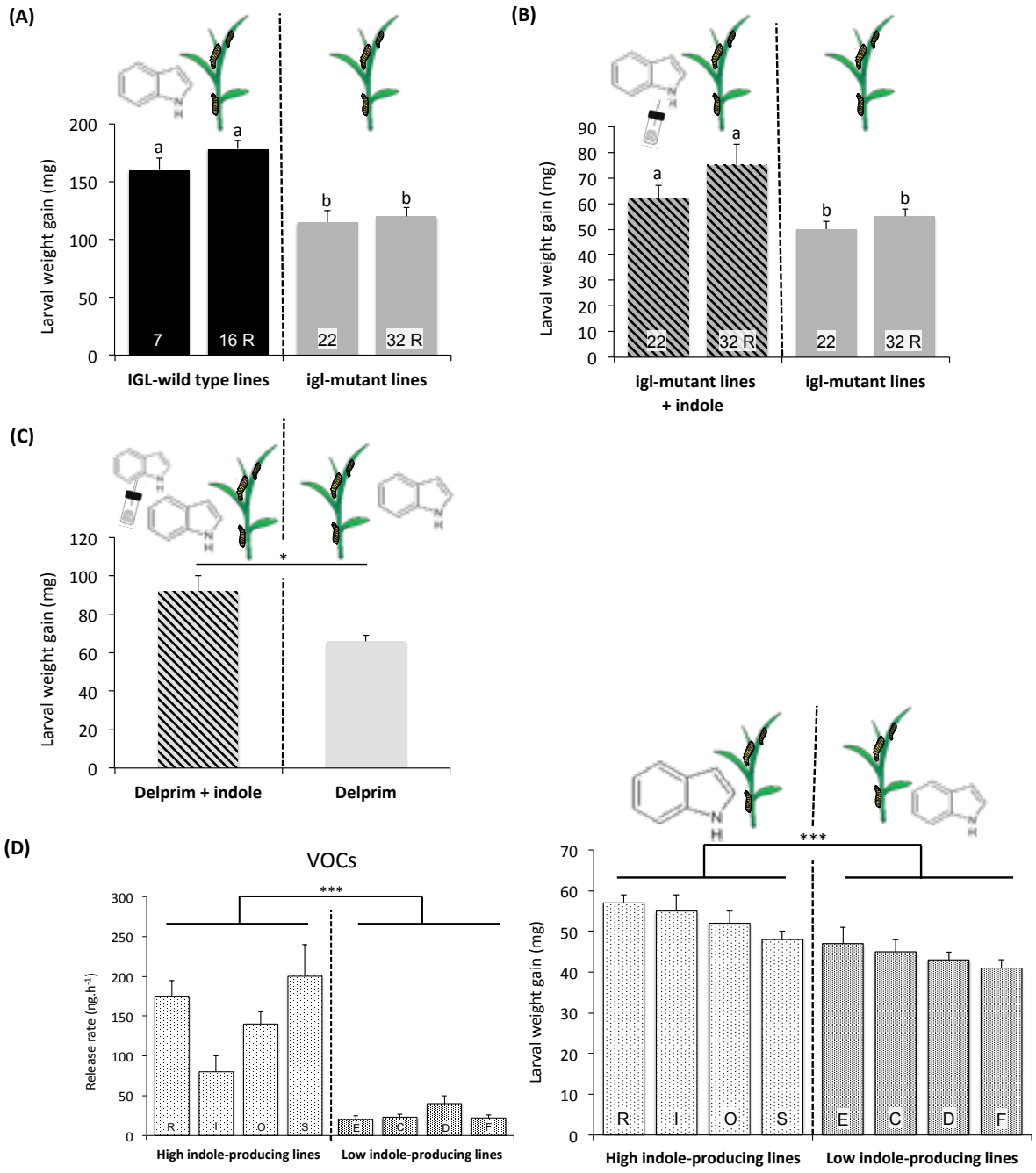

Figure 4: ***Effect of indole on larval performance***. **(A)** Individual weight gain average (+SE) of *S. littoralis* larvae after 7 days of feeding on IGL-wild type and igl-mutant plants (n=12). **(B)** Individual weight gain average (+SE) of *S. littoralis* larvae after 7 days of feeding on igl-mutant plants and igl-mutant plants supplemented with indole (n=12). **(C)** Individual weight gain average (+SE) of *S. littoralis* larvae after 7 days of feeding on Delprim plants and Delprim plants supplemented with indole (n=12). **(D)** Individual weight gain average (+SE) of *S. littoralis* larvae after 7 days of feeding on four maize varieties producing a low concentration of volatile indole (E, C, D, F) and four maize varieties producing a high concentration of volatile indole (R, I, O, S) (n=12). Different letters and asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

Volatile indole is sufficient to trigger weight gain

Indole may increase caterpillar growth directly or via plant-mediated effects. To disentangle these two aspects, we measured the weight gain of *S. littoralis* larvae to indole containing and indole-free volatile blends while feeding on artificial diet. *S. littoralis* larvae feeding on artificial diet gained significantly more weight when they were exposed to volatile compounds from IGL-wild type plants than igl mutants. Indole complementation of the igl-mutants plants was sufficient to recover caterpillar growth to wild type levels (cross A: n=15, p<0.001; cross B: n=15, p=0.010; Figure 5A). Moreover, larvae feeding on artificial diet gained more weight when exposed to indole releasing dispensers after 6h (n=13, p=0.022; Figure 5B) and 12 days of exposure (n=15, p<0.05; Figure 5C).

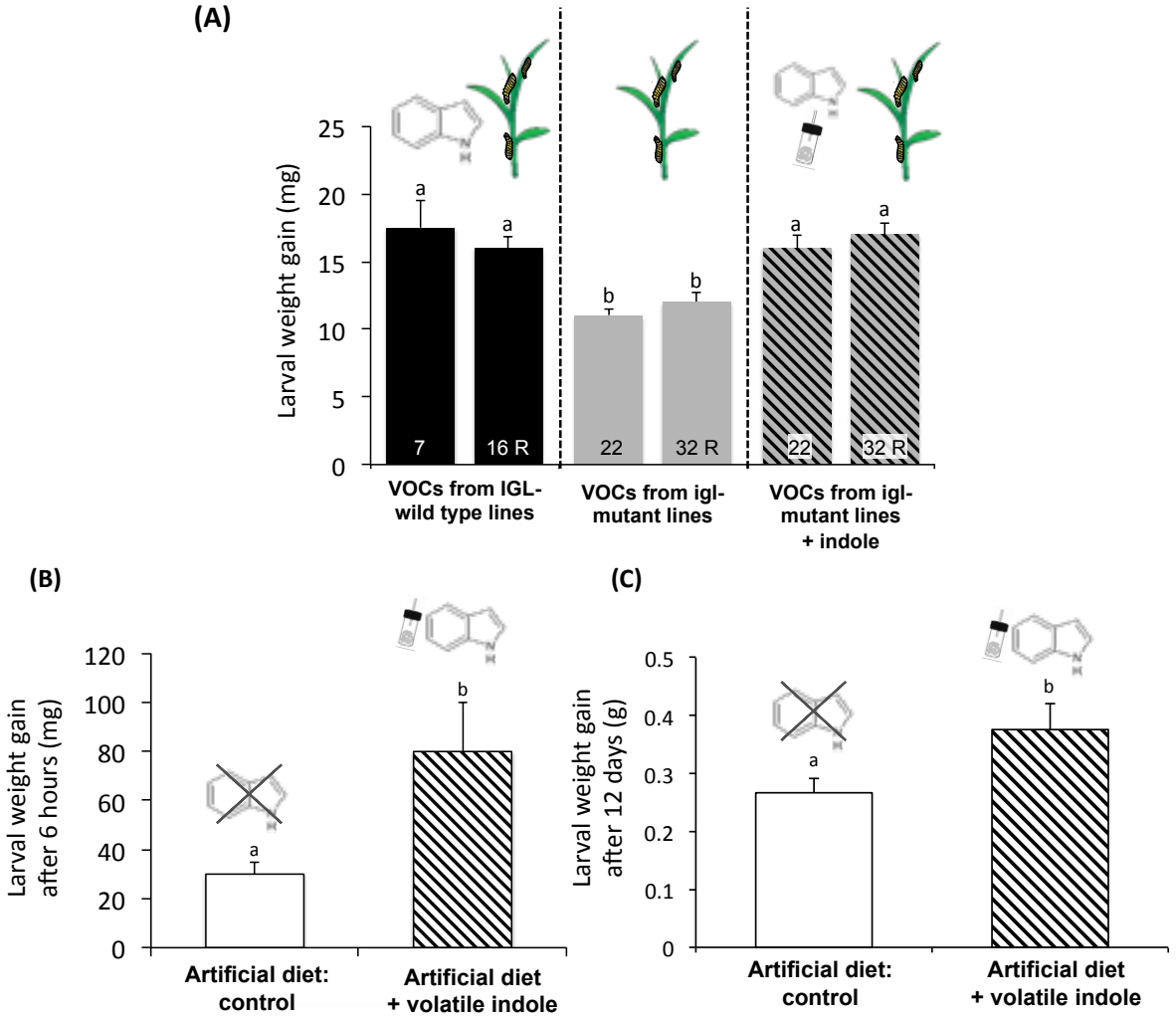

Figure 5: ***Effect of volatile indole on larval performance***. **(A)** Individual weight gain average (+SE) of *S. littoralis* larvae after 7 days of feeding on artificial diet, exposed to IGL-wild type plants, igl-mutant plants or igl-mutants supplemented with a dispenser of volatile indole (n=15). **(B)** and **(C)** Individual weight gain average (+SE) of *S. littoralis* larvae after 6 hours **(B)** (n=13) and 12 days **(C)** (n=15) of feeding on artificial diet or on artificial diet associated to a dispenser of volatile indole. Different letters indicate significant differences between treatments (p<0.05).

Indole reduces S. littoralis *survival and fitness*

To test whether the increased weight gain upon indole exposure results in increased fitness of *S. littoralis*, we measured larval mortality and adult egg production. Contrary to our expection, indole exposure increased larval mortality in several experiments. First, feeding on wild type plants increased S. littoralis larval mortality compared to indole free mutant plants ($n=24$, $p<0.01$; Figure 6A). Second, mortality was higher on igl mutant plants supplemented with indole than on igl mutant plants without supplementation ($n=24$, $p<0.01$; Figure 6B). Third, exposure to HIPVs from indole containing plants was sufficient to increase *S. littoralis* mortality. ($n=24$, $p<0.05$; Figure 6C). Fourth, synthetic indole alone was sufficient to increase larval mortality ($n=15$, $p<0.01$; Figure 6D). Moreover, the reproductive output of *S. littoralis* was lower when females were exposed to indole during their larval development: The number of eggs laid was 2 times lower if larvae were exposed to an indole-releasing dispenser during larval development ($n=6$, $p<0.01$; Figure 6E).

Indole increases food conversion efficiency and fat levels

To test whether indole-exposure increases food consumption or food conversion rates, we measured both parameters in *S. littoralis*. Feeding of *S. littoralis* larvae was increased on indole-deficient mutant plants compared to wild types ($n=24$, $p<0.01$; Figure 7A). Also, indole-exposure increased larval weight gain in neonate and L1 larvae while at the same time decreasing food intake. Early instars were more affected by indole than later stages: Neonates and L1 larvae died more often upon indole exposure compared to the control treatment, while L3 larvae were not affected ($n=12$, $p<0.05$; Figure 7B). We did not find any differences in water retention between *S. littoralis* larvae that were exposed to control- or indole-dispensers ($n=12$, $p>0.05$; Figure 7C). On the other hand, triacylglycerol levels were strongly increased in indole-exposed caterpillars ($n=8$; $p<0.05$; Figure 7D). Taken together, these results suggest that indole increases food conversion efficiency in *S. littoralis*.

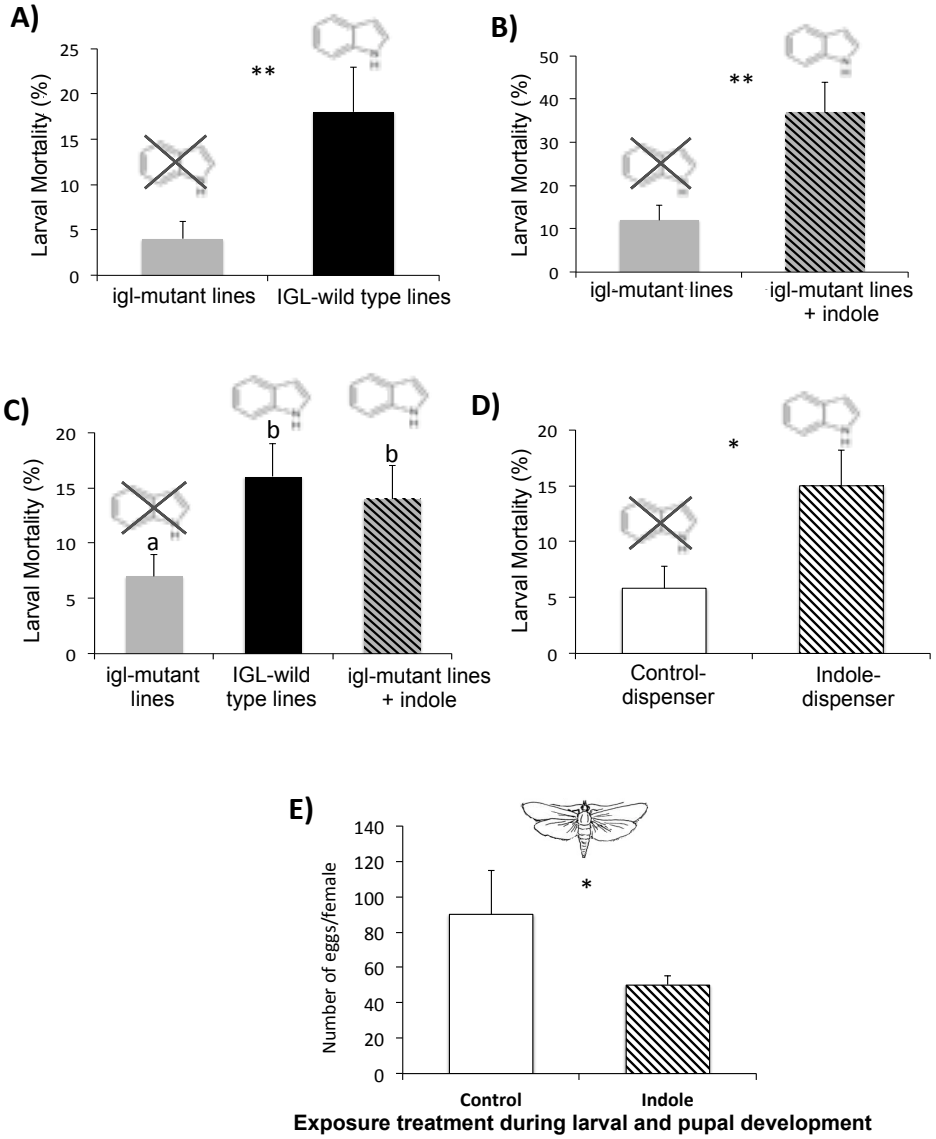

Figure 6: ***Effect of indole on* S. littoralis *fitness***. **(A)** Mortality rate of *Spodoptera littoralis* after 7 days on IGL wild-type plants (7 and 16R) or igl mutant plants (22 and 32R) (n=24). **(B)** Mortalityof *Spodoptera littoralis* after 7 days of feeding on on igl mutant plants (igl-) and igl mutant plants suplemented with synthetic indole (igl- + indole) (n=24). **(C)** Mortality of *Spodoptera littoralis* after 7 days of feeding on artifial diet when exposed to volatile compounds from IGL wild-type plants, igl mutant plants (igl-) and igl mutant plants suplemented with synthetic indole (igl- + indole) (n=24). **(D)** Mortality of *Spodoptera littoralis* after 6 hours of feeding on artificial diet with or without dispenser of volatile indole (n=12). **(E)** Mean number of eggs lay by *Spodoptera littoralis* females after feeding on artifial diet when exposed or not to volatile indole from dispenser during the larval development (n=6). Asterisks and different letters indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

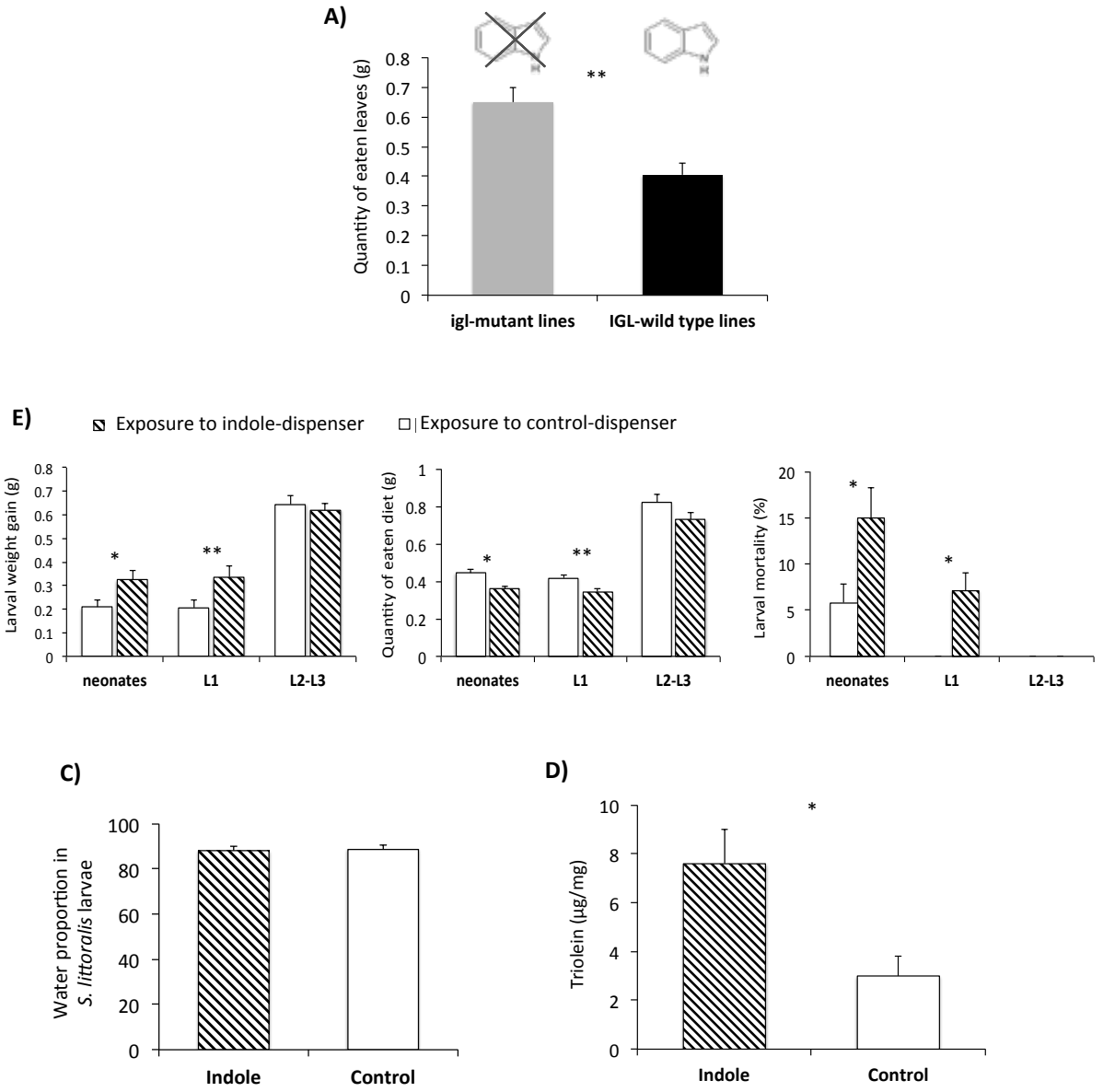

Figure 7: ***Physiological effects of indole on S. littoralis***. **(A)** Feeding rate of *Spodoptera littoralis* after 7 days on IGL wild-type plants (7 and 16R) or igl mutant plants (22 and 32R) (n=24). **(B)** Performance, Feeding rate and Mortality of three different instars of *Spodoptera littoralis larvae* after 6 hours of feeding on artificial diet with or without dispenser of volatile indole (n=12). **(C)** Exposure to volatile indole do not lead to a higher water retention by *S. littoralis larvae.* Water proportion in *S. littoralis* larvae after seven days of exposure to and indole- or control- dispenser (n=12). **(D)** Quantification of triolein (triacylglycerol) in *S. littoralis* larvae after exposure to volaltile indole (n=12). Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

DISCUSSION

Indole is specifically released in response herbivore attack in maize (Frey et al. 2000; Schmelz et al. 2003). This specificity suggests that indole may play an important role in plant resistance to herbivory. Here, we provide evidence for indole as a direct defence that kills and deters a generalist herbivore.

First, we found that *S. littoralis* larvae and adults were repelled by the presence of indole. Using a four-arm olfactometer, we demonstrate that larvae showed a strong preference for the odours of induced indole free igl mutants or inbred lines that emit little indole. Depending on the amount of indole used for complementation, this preference was abolished or reversed. The fact that pure indole alone was sufficient to reduce larval attraction points to a direct repellent effect of the volatile. These results are in contrast with other studies showing that herbivores are attracted by HIPVs. For instance, *Spodoptera frugiperda* larvae are attracted to HIPVs (Carroll *et al.* 2006), and the number of herbivores counted on GLVs-exposed plants was higher than on control-exposed plants in the field (von Merey *et al.* 2012). That adults are repelled by HIPVs and prefer to oviposit on uninfested plants on the other hand is more common (De Moraes *et al.* 2001; Huang *et al.* 2009) An important factor for adult insects is to assess the quality of the plant in order to find a suitable host plant for the growth and development of their offspring (Schoonhoven *et al.,* 2005). Under laboratory and field condition, female *S. littoralis* moths have shown to select plants that are suitable for oviposition and larval feeding and to avoid pants already occupied by other insects or where the risk for predation is high where (Sadek *et al.,* 2010). For instance, *S. littoralis* moths reduce oviposition on cotton plants that have previously been damaged by their own larvae, and able to assess the quality of the plants that has been damaged by a root feeding heterospecific herbivore (Anderson *et al.,* 2011; Anderson & Alborn, 1999). Our results show that, independently of previous experience, *S. littoralis* adults prefer to lay eggs on plants that do not emit indole.

Based on the strong avoidance response of *S. littoralis*, we hypothesized that indole may directly reduce host quality for the caterpillars or act as an indirect signal of poor host suitability. Contrary to this prediction however, we found that *S. littoralis* larvae gained more weight when they fed on indole-producing plants or plants supplemented with indole than on plants producing less or no indole. Subsequent experiments with artificial diet demonstrated that indole itself has a direct positive effect on larval weight gain. These results are surprisingly, as HIPVs are generally expected to negatively affect the performance of the herbivore insects (Kessler & Baldwin 2001; Mandour *et al.* 2013). Subsequent experiments showed that *S. littoralis* grew more and accumulated more fat, despite a reduction in food

intake. This suggests that volatile exposure increases food conversion efficiency.

Increased growth and fat reserves normally results in increased herbivore fitness, and is therefore commonly used as a proxy for host plant quality. However, in our experiments, the opposite was the case: despite an increase in weight gain, caterpillar survival and reproductive output dropped markedly upon exposure to indole. Therefore, together with the reduction in food intake, indole is likely to increase plant resistance to *S. littoralis*, despite its positive effect on growth. This data illustrates that herbivore growth may not always be a good measure for plant resistance, and leads to the intriguing question on how a plant volatile can have such dramatic and counterintuitive effects on herbivore development.

One possible explanation that may reconcile the above phenomena are gut microbes, which affect development, nutrient allocation, and immune responses in animals (Dillon & Dillon 2004; Bäckhed *et al.* 2005). As the gut of Lepidopteran larvae contain no specialized structures, it has been proposed that microorganisms play an important role in nutrition and digestion (Appel 1994; Anand *et al.* 2010). Recently obesity has been associated with changes in the relative abundance of the gut microbiota (Turnbaugh *et al.* 2008). This study suggested that an obesity-associated microbiome is characterized by its increased capacity to deliver energy from the diet to the host digestive system. Interestingly, indole has been identified as a biochemical that is produced by gut microorganisms of *S. littoralis* (Çakici *et al.* 2013). Indole is produced in large quantities by at least 85 species both Gram-positive and Gram-negative bacteria (Lee & Lee 2010). Recent investigations have clearly demonstrated that bacteria also employ their volatiles during interactions with other organisms. Indole as an inter- and intra-cellular signal molecule controls diverse aspects of bacterial physiology, such as spore formation, growth promoting, plasmid stability, drug resistance, biofilm formation, inhibiting agent and virulence in indole-producing bacteria (Kai *et al.* 2009; Lee & Lee 2010). Consequently, exposure to indole could induce changes in the gut microbial community of *S. littoralis* (Kaufman & Klug 1991; Santo Domingo *et al.* 1998) (Chant & Summers 2007; Lee *et al.* 2007). These changes could result in the release of toxic substances which could increase larval mortality, and at the same time change food conversion rates that increase caterpillar growth. Characterizing the gut microbiome of indole exposed *S. littoralis* larvae will shed first light on this novel hypothesis.

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CHAPTER 3

An induced volatile protects *Spodptera littoralis* against its natural enemies

AN INDUCED PLANT VOLATILE PROTECTS *SPODOPTERA LITTORALIS* AGAINST ITS NATURAL ENEMIES

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Abstract

In response to herbivore-attack, maize plants release a blend of herbivore induced plant volatiles (HIPVs) that are used as foraging cues by predators and parasitoids. To date, little is known about direct impacts of HIPVs on herbivores and their resistance against natural enemies. We studied the role of the common aromatic HIPV indole in *Spodoptera littoralis* caterpillar resistance against two parasitoids. Using a combination of indole-free indole-3-glycerol-phosphate maize mutants and indole complementation at physiologically relevant doses, we found that indole enhanced the attraction of the parasitoid *Microplitis rufiventris* to *S. littoralis* induced maize plants. However, in the presence of *S. littoralis* caterpillars in the system, indole had the opposite effect. We subsequently found that indole-exposed caterpillars directly repel *M.* ruvientris through changes in their body odour. Contrary to the *M. rufiventris*, which occurs sympatrically with *S. littoralis*, the non-sympatric parasitoid *Cotesia marginiventris* did not use indole as a discrimination factor. Indole exposure by itself slightly reduced *S. littoralis* survival, but increased caterpillar survival in the presence of parasitoids. Conversely, parasitoid development on indole-exposed caterpillars was impaired. Taken together, our study demonstrates that HIPVs can protect herbivores against their natural enemies, and that adapted parasitoids integrate plant and herbivore odors to avoid inferior hosts.

INTRODUCTION

In response to herbivore-attack, plants activate a wide array of defences which reduce herbivore damage (Grayer *et al.* 1993; Schoonhoven *et al.* 2005; Karban & Baldwin 2007). Depending on their mode of action, induced plant responses have been classified as direct or indirect (Price *et al.* 1980). Direct induced defences have negative effects on the performance of herbivores by reducing the quality of the plant (Karban & Myers 1989). Indirect induced defences affect herbivore by enhancing the performance of their natural enemies (Barbosa & Saunders 1985).

Direct effects of induced defences on herbivores are well described. Following herbivore attack, plants synthetize a broad range of toxic secondary metabolites such as terpenoids, alkaloids or cyanogenic glucosides that can strongly reduce the growth, development and survival of herbivorous insects (Haukioja & Hanhimäki 1985; Broadway *et al.* 1986; Baldwin 1988; Stout *et al.* 1997). However, the fact that toxic secondary metabolites may also negatively affect the fitness of natural enemies has received less attention. Negative effects can occur directly, for instance when the natural enemy encounters toxic plant metabolites in the hemolymph or tissues of its host, or indirectly when retarded host development affects the fitness of the natural enemy (Ode 2006). The development of endoparasitoids in particular is dependent on plant quality, as they develop within herbivore hosts that continue to feed on their host plant. Consequently, allelochemicals can pass from the second to the third trophic level and affect its fitness (Campbell & Duffey 1979; Barbosa *et al.* 1986; Kruse & Raffa 1997; Roth *et al.* 1997; Harvey *et al.* 2005; Harvey *et al.* 2007). Furthermore, specialized herbivores can sequester toxic compounds to defend themselves against natural enemies (Bowers & Collinge 1992; Gauld *et al.* 1992; Dyer 1995; Nishida 2002). Some studies showed that herbivores prefer to select relatively toxic host plants over highly palatable plants when the risk of parasitism is high (Singer & Stireman Iii 2003; Singer *et al.* 2004). Even if only few studies have established a link between host plant chemistry and the performance of parasitoids, many studies showed an impact of the host plant species on the growth and the development of parasitoids (Werren *et al.* 1992; Hay-Roe *et al.* 2013; Lampert & Bowers 2013). For instance, the parasitic wasps *Comperiella bifasciata* and *Habrolepis rouxy* develop successfully when their host herbivore, the California red scale *Aonidiella aurantii* feeds on Citrus, but not on Cago palm (Smith 1957). Additional studies confirmed the negative effect of specific plant allelochemicals on parasitoids by adding them to artificial diet of the herbivores (Barbosa *et al.* 1986; Duffey *et al.* 1986; Havill & Raffa 2000; Harvey 2005; Ode 2006). For instance *Hyposother exigua*, a generalist endoparasite of *Heliothis zea*, had a lower survival, morphological deformities as well as an increased development time, adult weight and longevity when its host fed on artificial diet containing the

glycolakaloid tomatine (Campbell & Duffey 1979, 1981). Another well-known example is the case of the parsitoid *Hyposoter anulipes*, which suffers from lower larval survivorship, longer developmental time and a smaller adult body size when developing on *Spodoptera frugiperda* larvae fed on artificial diet containing nicotine (Osier *et al.* 1996).

The effect of indirect plant defences, including herbivore induced plant volatiles (HIPV) have been well demonstrated (Dicke *et al.* 1990; Turlings *et al.* 1995; Takabayashi & Dicke 1996; Der Geest 2000; Loon *et al.* 2000; Kessler & Baldwin 2001). After an insect attack, plants release a blend of volatiles organic compounds (VOCs) (Dicke *et al.* 2009) that can be used as foraging cues by predators and parasitoids (Turlings *et al.* 1990; Turlings & Wäckers 2004; Arimura *et al.* 2005). A well-studied example is the tritrophic interaction between maize, *Spodoptera caterpillars* and parasitic wasps (Turlings *et al.* 1990). HIPVs blends differ from volatiles emitted by intact or artificially damaged plants. They are specific to plant species, stages or cultivars (Loughrin *et al.* 1995; Krips *et al.* 2001; Degen *et al.* 2004) and can vary with abiotic conditions (Gouinguené & Turlings 2002). In addition, the blend of HIPVs can depend on the herbivore species (De Moraes *et al.* 1998; Powell *et al.* 1998) as well as their developmental stage (Takabayashi *et al.* 1995; Arimura *et al.* 2009; Dicke *et al.* 2009). Differences in the blends of HIPVs make plants differently attractive to the natural enemies, and help them to find specific hosts and host stages. For instance, parasitic wasps can distinguish between a blend induced by a host and one of a non-host. (Takabayashi *et al.* 1995; De Moraes *et al.* 1998; Shiojiri *et al.* 2000; Hoballah *et al.* 2002).

HIPVs are commonly divided into three major classes: GLVs, terpenoids and aromatic compounds. The role of green leaf volatiles and terpenoids in the attraction of natural enemies has been investigated (D'Alessandro & Turlings 2005; Hoballah & Turlings 2005; D'Alessandro *et al.* 2006; Fontana *et al.* 2011). For instance, the green leaf volatile (GLVs) *(E)*-3-hexen-1-ol has been shown to be attractive to the bacronid wasps *Apanteles kariyai* (Watanabe) (Takabayashi *et al.* 1991) whereas the sesquiterpenes γ-bisabolene and *(E)*-β-caryophyllene were found to attract *Cotesia sonorensis*. Apart from attracting natural enemies, HIPVs can also influence the behaviour and performance of the herbivores themselves. HIPVs can be either repellent or attractive to herbivores (Carroll *et al.* 2008; von Mérey *et al.* 2013). In 2006, Caroll *et al.* (2008) showed that *Spodoptera frugiperda* larvae preferred odours from damaged maize plants over odours from undamaged maize (Carroll *et al.* 2006). We previously demonstrated that indole, one of the major constituents of the HIPV blend of maize (Turlings *et al.* 1990; McCall *et al.* 1993) has a direct negative effect on *Spodoptera littoralis* larvae. Even if their growth rate is higher, *S. littoralis* larvae exposed to volatile indole have lower survival rate and reproductive fitness than larvae that are not exposed to the volatile (Veyrat et al. 2014, unpublished). As parasitic wasps are known to be

able to select the most suitable host for their offspring (Godfray 1994; Quicke 1997), we hypothesized that they may use indole as a signal to avoid inferior *Spodoptera* larvae.

Indole is emitted by many plants after an herbivore attack (Turlings *et al.* 1991; McCall *et al.* 1994; Gols *et al.* 1999; Cardoza *et al.* 2003; De Boer *et al.* 2004; Yuan *et al.* 2008). It is specifically released in response to herbivore-elicitors and is not induced by wounding alone (Frey *et al.* 2004; Zhuang *et al.* 2012). Indole is produced from indole-3-glycerol phosphate by the indole-3-glycerol phosphate lyase IGL, which releases it as a volatile (Frey *et al.* 2000). The *igl* gene is induced by herbivory, volicitin and MeJA treatment (Frey *et al.* 2000; Frey *et al.* 2004). Recently, a maize igl-mutant has been isolated in a bx1-mutant background (Ahmad *et al.* 2010). This double mutant does not release any indole upon herbivore induction.

Here, we used *igl*-mutant and IGL-wild type maize plants together with synthetic indole released from dispensers at physiologically relevant concentrations to test whether indole affects the behaviour and performance of two parasitoid species: *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) and *Microplitis rufiventris* (Kokujev) (Hymenoptera: Braconidae). Both species attack early instar larvae of many lepidopteran moths and are known to use HIPVs in host location (Gouinguené et al., 2003; Hoballah and Turlings, 2005). However, contrary to the *C. marginiventris*, *M. rufiventris* occurs sympatrically with *S. littoralis*. Using a four-arm olfactometer, we first investigated the impact of indole on the attraction the parasitoid to maize plants and exposed caterpillars. We then examined the impact of indole on parasitoid performance via herbivore-mediated effects. Our study provides evidence that indole-exposure protects *S. littoralis* caterpillars against parasitoids.

MATERIAL AND METHODS

Plants and insects

The maize lines 22 (igl.bx1), 7 (IGL.bx1) (cross A) and 32R (igl.bx1), 16R (IGL.bx1) (cross B) were obtained as previously described by (Ahmad *et al.* 2011) (Figure S1). Seeds of the hybrid Delprim, which are homozygous for both BX1 and IGL wild type alleles, were obtained from Delley Semences et Plantes SA. (Delley DSP, Switzerland. All maize lines were grown individually in plastic pots (10 cm high, 4 cm diameter) with commercial potting soil (Aussaaterde, Ricoter, Aarberg, Switzerland) and placed in a climate chamber (23°C ± 2°C, 60% relative humidity, 16:8 h L/D, 50'000 lm/m^2). Maize plants used for the experiments were ten to twelve days old and had three fully developed leaves. The evening before the

experiments, plants were transferred and kept under laboratory conditions with additional light supplementation (25 ± 2°C, 40 ± 10% relative humidity, 16h light/8h dark, and 8000 lm/m^2). The caterpillars *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) were reared from eggs provided by Syngenta (Stein, Switzerland). The eggs were kept in an incubator at 30.0 ± 0.5°C until emergence of the larvae. Subsequently, they were transferred on artificial diet at room temperature (24 ± 4°C). Adult *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) and *Microplitis rufiventris* (Kokujev) (Hymenoptera: Braconidae) parasitoids were reared as described before (Turlings *et al.* 2004). For rearing, 25 *Spodoptera littoralis* (Noctuidae: Lepidoptera) larvae were kept in a plastic-box with two mated females for 24h. The parasitized caterpillars were kept at room temperature until cocoon formation. Cocoons were kept in Petri dishes in an incubator at 30°C until adult emergence. Emerging adults were kept in cages at a sex ratio of 1: 2 (male: female) placed in an incubator (25°C, 16L: 8D). The cages were provided with moist cotton wool and honey as a food source. For all bioassays, 2–3-day-old naive females were used. Cages were transferred to the laboratory 30 minutes before the experiments.

Olfactometer bioassays

To test the attractiveness of volatile indole to *C. marginiventris* and *M. rufiventris*, we used a four-arm olfactometer as previously described (D'Alessandro and Turlings 2005). The olfactometer consisted of a central glass choice arena (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 a glass bulb (50 mL). Purified and humidified air entered each odour source vessel at 1.1 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, a part of the air (0.7 L/min) was pulled out and the other part entered in the central glass chamber. Ten neon tubes were attached to a metal frame above the olfactometer and provided approximately 7000 lm/m2 at the height of the odour source vessels. To avoid visual distraction of the parasitoid wasps, a white cardboard cylinder was placed around the central choice arena and on top of the choice arena. The choice arena was connected to four glass bottles. Two opposite bottles contained an odour source and the two remaining bottles remained empty. The position of the odour sources was changed between each experimental assay; all experiments were repeated six times. The system was left connected for half an hour before releasing wasps in the centre of the choice arena. Wasps were released in groups of six in the central glass chamber. Wasps that entered in an arm reached the elbow where a stainless steel screen blocked their path.

They walked up in the direction of the light source above the olfactometer and into a trapping bulb where they were counted and removed. Wasps that did not make the choice arena after thirty minutes were considered as having made “no choice” and all wasps were removed from the olfactometer. Between four and five such releases were done for each replicates.

Odor sources

To induce plants, we either used *S. littoralis* larvae or applied *S. littoralis* oral secretions to wounded leaves. For herbivore infestation, fifteen second-instar *S. littoralis* larvae were placed on the plants the evening before the bioassay. Elicitation treatment was performed by scratching two leaves over an area of approximately 1 cm^2 on both sides of the central vein with anatomical forceps (stainless steel, 14.5 cm). Then 10 μL of *Spodoptera littoralis* regurgitate were applied over the scratched leaf areas. Regurgitate was collected from fourth instar *S. littoralis* that was fed on maize leaves for 24 hours and stored at -80°C until use. To confirm the effect of volatile indole, supplementation tests were performed by adding to the plant an indole-dispenser built up as follows: a 2ml amber glass vial (11.6 x 32 mm; Sigma-Aldrich, Buchs, Switzerland) containing 20 mg of synthetic indole (> 98 %, GC, Sigma-Aldrich, CH-9471 Buchs, Switzerland). The vial was sealed with a PTFE/rubber septum (Sigma-Aldrich, Buchs, Switzerland) pierced with a 2 µL micro-pipette (Drummond, Millan SA, Plan-Les-Ouates, Switzerland). The length of the pipette was calibrated to release a controlled amount of indole, similar to the amount emitted by IGL-wild type maize plants (50 $ng.h^{-1}$). This setup was used to test the preference of both wasp species to the following combinations of odors: 1) Empty bottles vs. empty bottles containing a dispenser of volatile indole (n=6); 2) Wounding and regurgitant-induced IGL-wild type vs igl-mutant plants (n=6); 3) *S. littoralis* infested IGL-wild type vs. infested igl-mutant plants (n=6); 4) *S. littoralis* infested igl-mutant plants vs. infested igl-mutant plants supplemented with volatile indole (n=6); 5) *S. littoralis* infested Delprim plants vs. infested Delprim plants supplemented with an indole dispenser (n=6); 6) *S. littoralis* larvae previously exposed to a control- vs. indole-dispenser (n=6).

Performance experiments

In order to investigate the effect of indole on the fitness of *Cotesia marginiventris* and *Microplitis rufiventris* parasitoids, 20 *Spodoptera littoralis* larvae were kept in non-hermetic plastic boxes (diameter: 8 cm, high : 5 cm) with one mated parasitoid female for 24 hours (n=12). For a better parasitism rate, Neonate *Spodoptera littoralis* larvae were used for the

parasitism by *Cotesia marginiventris* and late L1 *Spodoptera littoralis* larvae were used for the parasitism by *Microplitis rufiventris* (personal communication). Before bringing them together with the parasitoids, *Spodoptera littoralis* larvae were exposed to control- or indole-dispensers for 24 hours. After parasitism, larvae were again subjected to the same treatment until parasitoid emergence. As control treatment, *S. littoralis* larvae were exposed to control- or indole-dispensers without parasitism (n=12). Each day, the number of dead *S. littoralis* larvae was recorded. Characteristic growth depression of *S. littoralis* larvae was also recorded as a sign of successful parasitism and used to determine parasitism rate.

Statistics

The functional relationship between larval choice and the different odour sources offered in the four-arm olfactometer was analysed with a generalized linear model (GLM, a log linear model) with the software package R (version 2.13.1). To compensate for the overdispersion of wasps within the olfactometer, we based the models on a quasi-Poisson distribution. Results from cross A and cross B comparisons were pooled for analysis (n=6 for each plant pair). For a detailed description, see (Turlings, Davison et al. 2004). Differences in mortality, growth depression and parasitism success between control- and indole- treatments were analysed for significance using a *t*-test ($p<0.05$).

RESULTS

Olfactometer bioassays

Both parasitoid species responded well to the treatments. On average 83% of *Microplitis rufiventris* and 78% of *Cotesia marginiventris* made a choice (Figures 1 and 2). *M. rufiventris* adults were significantly attracted by volatile indole alone (Figure 1A, $p<0.001$). Similarly, the parasitoid preferred induced indole-producing plants over indole-mutants (Figure 1B, $p<0.05$). However, in the presence of *S. littoralis* caterpillars, this choice was inverted: suddenly, *M. rufiventris* preferred igl-mutant host-plant complexes (Figure 1C, $p<0.001$). Similarly, more than 90% of wasps preferred *S. littoralis* infested igl-mutant plants over infested igl-mutant plants supplemented with an indole-dispenser (Figure 1D, $p<0.001$). Similar results were found for Delprim plants in which indole-emission was enhanced by a synthetic dispenser (Figure 1E, $p<0.01$). From these results, we hypothesized that *M. rufiventris* may be repelled by volatiles from indole-exposed *S. littoralis* caterpillars. Indeed, more than 75% of the wasps significantly preferred S. littoralis wasps from the control group over *S. littoralis* wasps that had previously been exposed to indole (Figure 1F, $p<0.001$).

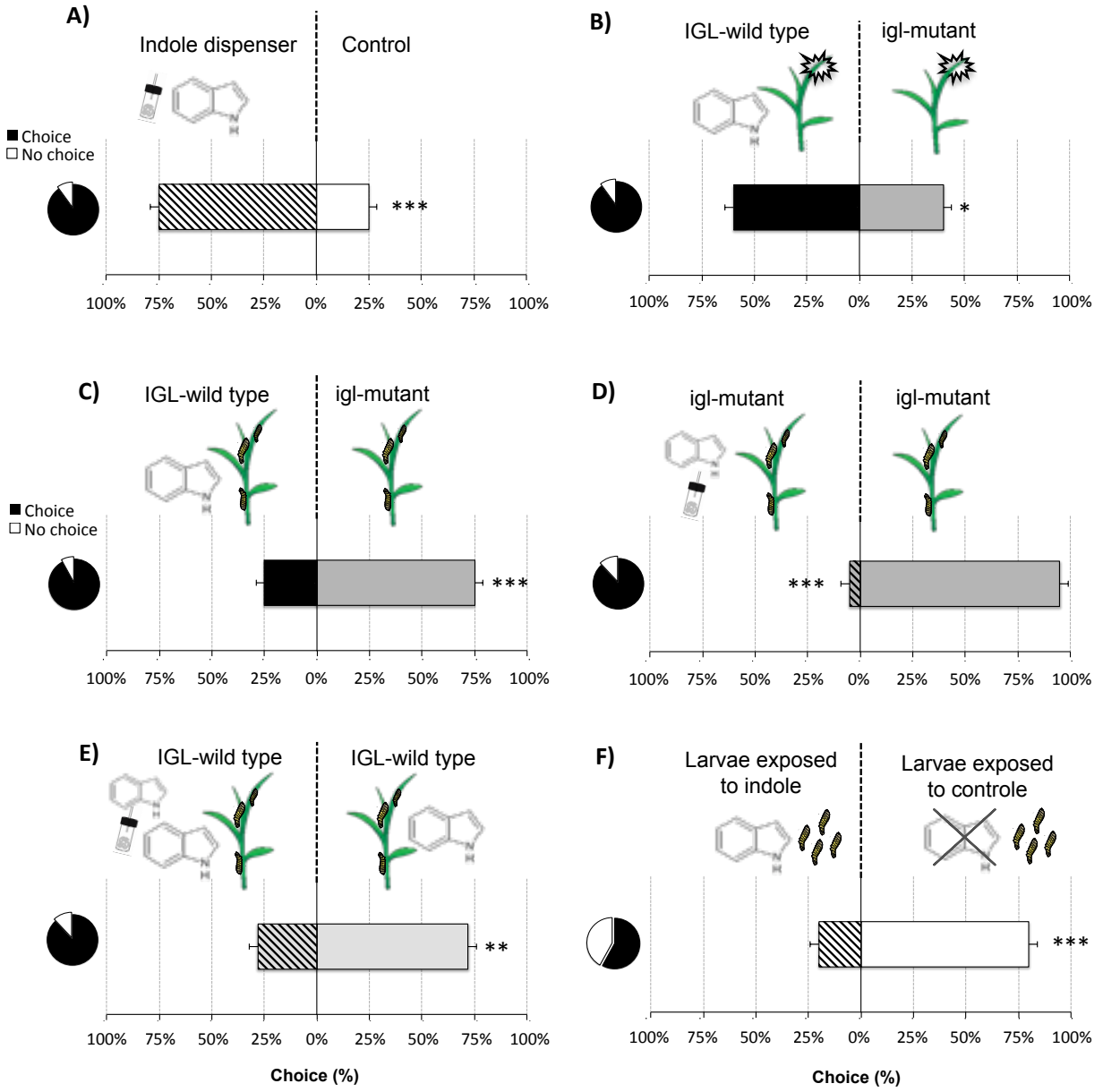

Figure 1: Choice of *Microplitis rufiventris* wasps in a four-arm olfactometer. **(A)** Choice experiment between empty bottles and bottles with a dispenser of volatile indole. **(B)** Choice experiment between artificially damaged igl-mutant and IGL-wild type plants. **(C)** Choice experiment between infested igl-mutant and IGL-wild type plants. **(D)** Choice experiment between infested igl-mutant plants and infested igl-mutant plants supplemented with synthetic indole. **(E)** Choice experiment between infested delprim plants and infested delprim plants supplemented with synthetic indole. **(F)** Choice experiment between *Spodoptera littoralis* larvae exposed to control- or indole-dispensers for 24 hours. Asterisks indicate significant differences between treatments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Compared to *M. rufiventris*, *C. marginiventris* adults did not show any response to synthetic indole (Figure 2A) and generally did not discriminate between induced indole-producing and indole mutant plants and host-plant complexes (Figures 2B-E). The only difference that was found was a stronger attraction to *S. littoralis* infested igl-mutant plants than infested igl-mutant plants supplemented with indole (Figure 2D, $p<0.001$).

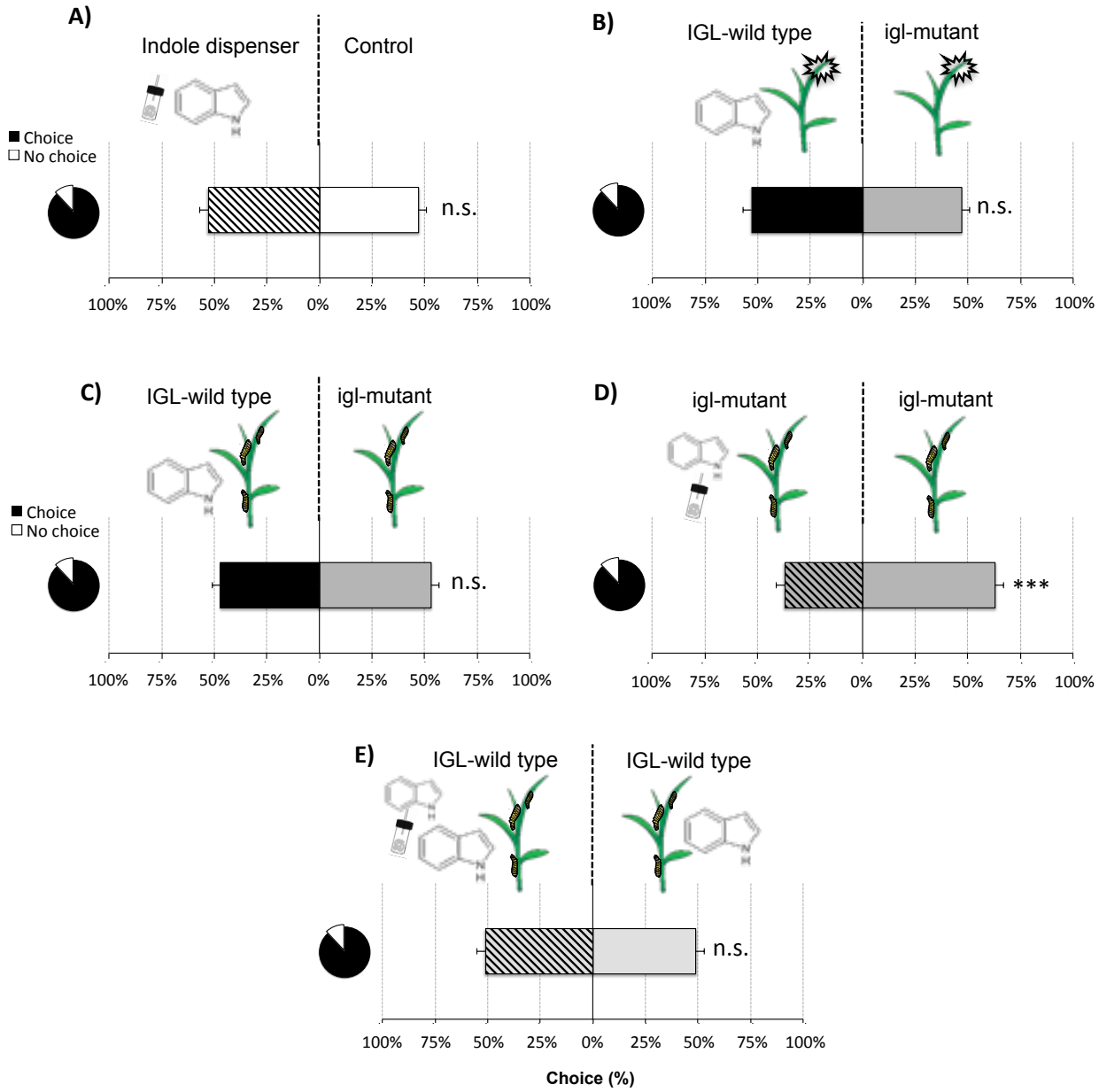

Figure 2: Choice of *Cotesia marginiventris* wasps in a four-arm olfactometer. **(A)** Choice experiment between empty bottles and bottles with a dispenser of volatile indole. **(B)** Choice experiment between artificially damaged igl-mutant and IGL-wild type plants. **(C)** Choice experiment between infested igl-mutant and IGL-wild type plants. **(D)** Choice experiment between infested igl-mutant plants and infested igl-mutant plants supplemented with synthetic indole. **(E)** Choice experiment between infested delprim plants and infested delprim plants supplemented with synthetic indole. Asterisks indicate significant differences between treatments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Herbivore and parasitoid performance

As wasps avoided the presence of *S. littoralis* larvae exposed to volatile indole, we performed a performance experiment to determine whether indole exposure protects the caterpillars against parasitism. We allowed *M. rufiventris* and *C. marginiventris* wasps to parasitize *S. littoralis* larvae that had previously been exposed to control- or indole-dispensers. *S. littoralis* larvae exposed to volatile indole survived slightly less well than larvae exposed to control-dispensers in absence of parasitism: 83% of control-exposed larvae survived whereas only 72% of indole-exposed larvae reached the final larval instar (Figure 3A, $p<0.05$). Similar results were found for the M. rufiventris controls, (exposing L2 larvae to the indole), in which 88% of the larvae survived when exposed to control-dispenser whereas only 78% of indole-exposed larvae reached the final larval instar (Figure 3C, $p<0.05$). Parasitism by *C. marginiventris* increased *S. littoralis* mortality from 20 to 70%. Strikingly, the mortality of indole-exposed *S. littoralis* larvae was three times lower than in the control group (Figure 3A, $p<0.01$). Coversely, *C. marginiventris* parasitoids were significantly more likely to develop on control than on indole-exposed larvae (Figure 3B, $p<0.05$). Similar results were found for *M. rufiventris*: Indole exposure increased *S. littoralis* survival from 30 to 60% (Figure 3C) and decreased *M. rufiventris* survival from 30 to 10% (Figure 3D).

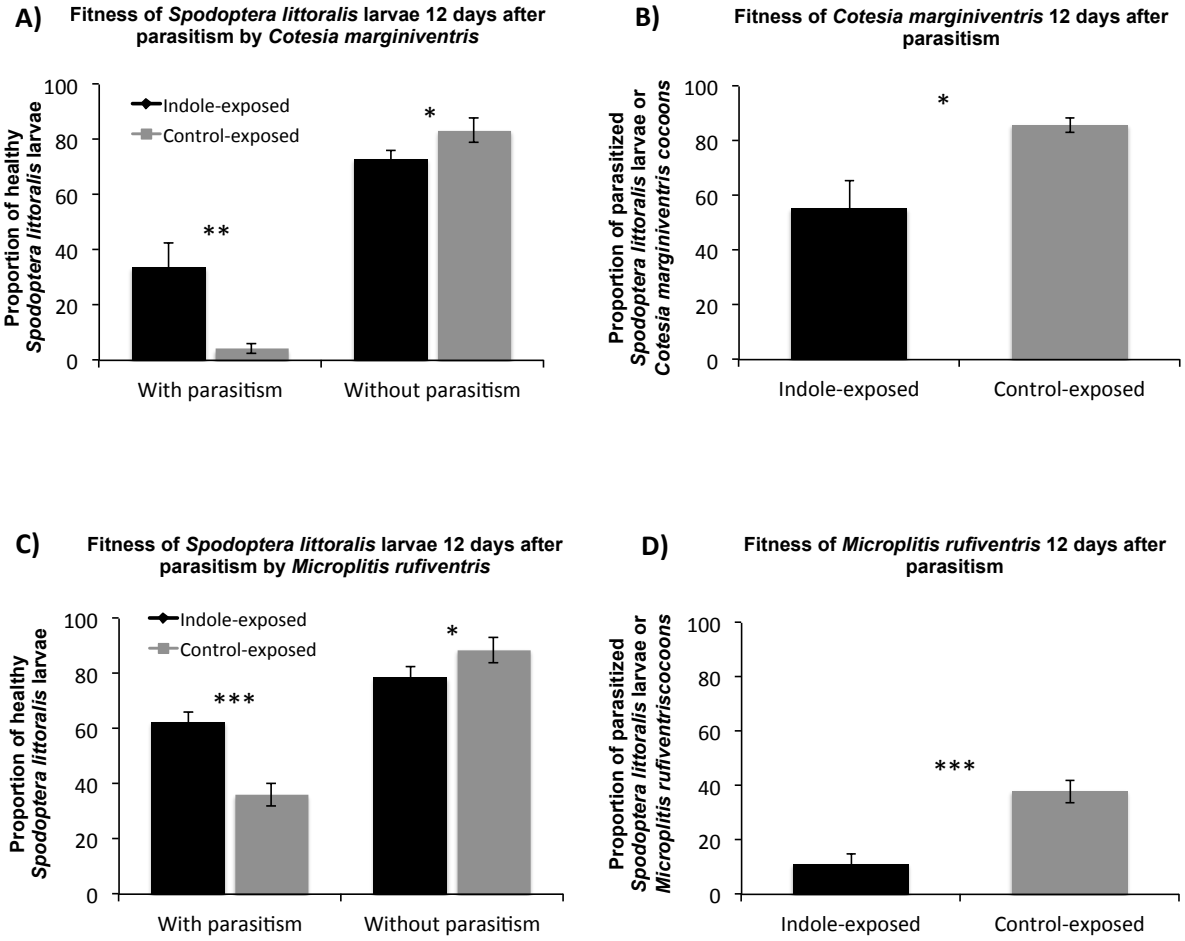

Figure 3: **A)** Fitness of *Spodoptera littoralis* larvae after parasitism by *Cotesia marginiventris* and **B)** Fitness of *Cotesia marginiventris* developing on *Spodoptera littoralis* larvae in presence of indole- or control dispensers. **C)** Fitness of *Spodoptera littoralis* larvae after parasitism by *Microplitis rufiventris* and **D)** Fitness of *Microplitis rufiventris* developing on *Spodoptera littoralis* larvae in presence of indole- or control dispensers. Asterisks indicate significant differences between treatments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

DISCUSSION

Plant allelochemicals affect the behaviour and performance of herbivores but and natural enemies (Harvey 2005). Plant secondary metabolites can increase natural enemy performance by slowing down herbivore development, direct induced defences increase the exposure time of herbivores to parasitoids and predators (Turlings & Benrey 1998). Also, HIPVs are used as foraging cues by predators and parasitoids (Turlings *et al.* 1990; Turlings & Wäckers 2004; Arimura *et al.* 2005),. On the other hand, allelochemicals can also have a negative effect on natural enemies, for instance when there are sequestered for their own defence (Campbell & Duffey 1979) or reduce herbivore development and thereby resource quality (Ode 2006). Until today, little is known about the role of HIPVs in changing host quality parameters for parasitoids. Our study is one of the first to document that herbivore exposure to a HIPV changes both its attractiveness and natural enemy resistance.

In accordance with a previous study (D'Alessandro et al., 2006), M. rufiventris was attracted to volatile indole alone or within a HIPV blend. Although applying caterpillar regurgitate to a mechanically damaged wound leads to the release of the same HIPVs than caterpillar damage (Turlings *et al.* 1990; Turlings & Tumlinson 1992; Turlings *et al.* 1998), when plants were infested by *S. littoralis* larvae, the preference of *M. rufiventris* wasps changed and they were suddently more attracted by HIPVs blends with no or lower concentrations of indole. Subsequent experiments demonstrate that *M. rufiventris* avoids indole-exposed *S. littoralis* larvae, even in the presence of a more attractive plant background. It is still unclear how *M. rufiventris* wasps are able to distinguish between *S. littoralis* larvae exposed to indole, but the olfactometer experiments clearly show that the larvae give off a different blend of odours upon previous exposure to indole. Indole increases larval growth and at the same time induces a slight increase in mortality (Veyrat et al., unpublished data). These changes in growth and development may be associated with differential production of body odours. Alternatively, compounds in the faeces and larvae of herbivores can mediate natural enemy attraction (Jones & Lewis 1971; Lewis & Jones 1971; Auger *et al.* 1989; Reddy *et al.* 2002). For instance *Microplitis demolitor* females are attracted to volatiles from the larval frass of their hosts *Pseudoplusia includens* (Ramachandran *et al.* 1991). It is therefore possible that the quality of *S. littoralis* faeces changes upon indole exposure.

Cotesia marginiventris on the other hand did not respond strongly to indole. Both wasp species are generalist solitary larval endoparasitoids that attack early stages of a wide range of noctuid species. Although both of them are highly attracted by induced maize odours (Turlings *et al.* 1990; Hoballah & Turlings 2005; Tamò *et al.* 2006), some differences in their foraging behaviour are known. *C. marginiventris* wasps show a clear ability to

respond to plant volatiles associated to plant damaged such as green leaf volatiles. The response to other volatiles such as terpenoid compounds occurs only after an association of such compounds with the presence of host (Hoballah & Turlings 2005). However, *M. rufiventris* females appear to use cues that are directly related with the presence of the hosts than the general plant volatiles. This may explain why *M. rufientris* responded differently to the host plant complex. Furthermore, *M. rufiventris* occurs symatrically with *S. littoralis* in Egypt, where cotton is a dominant host plant. Cotton also produces indole as a HIPV, which may explain why the parasitoid can use plant-derived indole as a host-location signal.

The performance experiments with *S. littoralis* and its parasitoids reveal that indole exposure strongly increases *S. littoralis* survival, but decreases parasitoid performance, regardless of their indole-responsiveness. *M. rufiventris* parasitoids may therefore have adapted to avoid unsuitable caterpillars via body odor cues (Godfray 1994; Quicke 1997). From our results, it becomes clear that indole protects S. littoralis against its natural enemy. This counter-intuitive role of a HIPV illustrates the ecological complexity of tritrophic interactions and suggests that general theorems about the primary function of plant HIPVs may not always apply.

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SUPPLEMENTARY INFORMATION

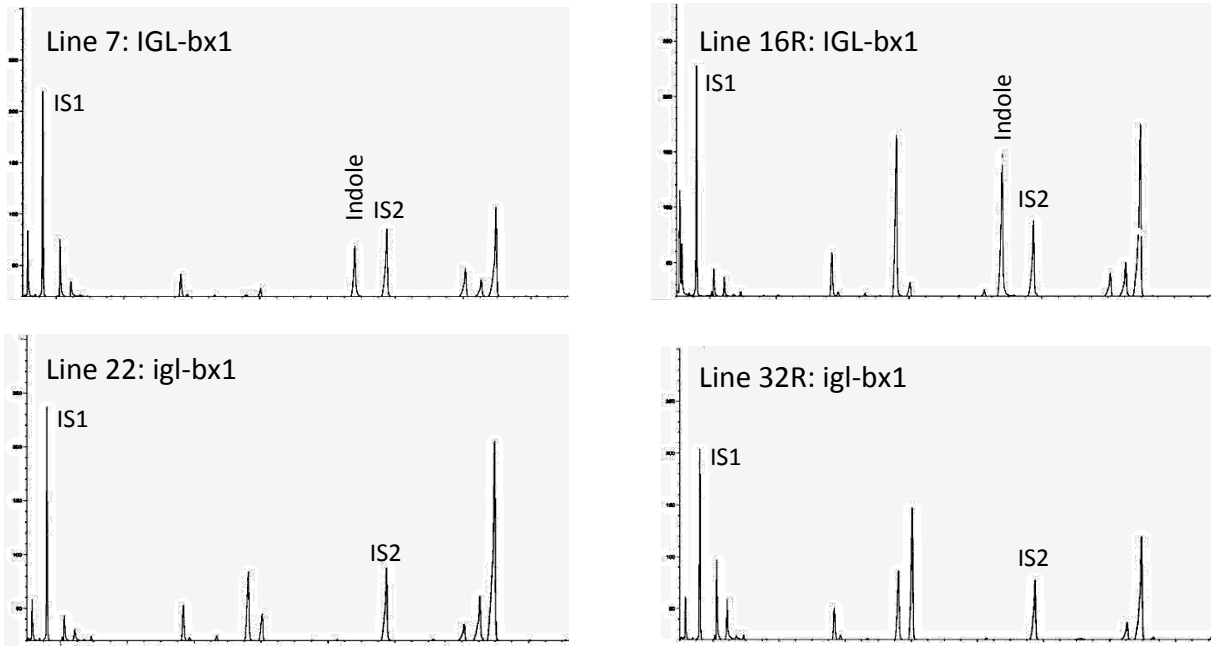

Figure S1: Chromatograms showing the HIPVs production by IGL-wild type plants (Lines 7 and 16R) and igl-mutant plants (Lines 22 and 32R). Plants were infested by 15 L2 *S. littoralis* larvae and volatiles were collected for 3 hours.

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CHAPTER 4

The response of herbivores and parasitoids to indole, a common herbivore-induced volatile is species-specific and independent of host plant specialization

The response of herbivores and parastioids to indole, a common herbivore-induced volatile is species-specific and independent of host plant specialization

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ABSTRACT

In response to herbivore-attack, plants release a blend of herbivore induced plant volatiles (HIPVs) that are commonly used as foraging cues by natural enemies of the herbivores. In addition, HIPVs can be used by herbivore insects themselves to localize suitable host plants. Here we studied the importance of a major compound of the HIPVs blend of many plant species, the aromatic compound indole, for the attraction of a diverse range of species of herbivorous insects and parasitoids. In a four-arm olfactometer, in which the insects had a choice between clean air and physiological relevant concentrations of indole emitted from specially devised dispensers, the responses varied considerable for the different species. Neither the degree of host plant specialization, the phylogenetic origin nor the association with indole-producing plants seemed to determine the response of herbivores and natural enemies. We conclude that the response of herbivores and their natural enemies to this common HIPV is unpredictable and evidently not affected by patterns of convergent evolution and evolutionary inheritance.

INTRODUCTION

In response to an herbivore attack, plants release herbivore-induced plant volatiles (HIPVs) (Dicke *et al.* 2009), which are known to be used as foraging cues by natural enemies of the herbivores to locate their prey or hosts (Dicke and Sabelis, 1988; Turlings *et al.* 1990; Turlings & Wäckers 2004; Arimura *et al.* 2005). This role of HIPVs has been recorded in many tri-trophic systems, including for instance maize plants, *Spodoptera* caterpillars and associated parasitic wasps (Turlings *et al.* 1990).

HIPVs can also affect the behaviour of herbivores that induce them. HIPVs can be repellent to herbivorous, as is for instance the case for the beetles *Cerotoma ruficornis* and *Gynandrobrotica guerreroenois* (Chrysomelidae), which prefer the odour of healthy over infested lima beans (Heil 2004). Similarly, adult lepidoptera are repelled by HIPVs and prefer healthy plants for oviposition (De Moraes *et al.* 2001; Kessler & Baldwin 2001; Sánchez Hernández *et al.* 2006). In other cases, HIPVs have been found to attract herbivores and therefor may pose an ecological cost for the emitting plants. For instance the lepidopteran larvae of *Spodoptera frugiperda*, *Ostrinia nubialis* and *Ostrinia furnacalis* preferred odours from already damaged over odours from undamaged maize plants (Carroll *et al.* 2006; Huang *et al.* 2009; Piesik *et al.* 2009; von Merey et al., 2013). Many beetles such as curculionidae (Heil 2004), scarab beetles (Loughrin et al., 1995a), or buprestid beetles (de Groot *et al.* 2008) are all attracted by odours from damaged plants (Turlings & Wäckers 2004). Thus, the ecological importance of HIPVs and their role in the foraging behaviour of herbivores remains unclear and may vary strongly with plants and herbivore species.

Many experiments have been conducted with full blends, and the identity of the actual behaviourally active HIPVs has remained elusive. HIPVs are commonly divided into three major classes: green leaf volatile (GLVs), terpenoids and aromatic compounds (Paré and Tumlinson, 1999). The respective roles of green leaf volatiles and terpenoids in the attraction of natural enemies have been specifically investigated (D'Alessandro & Turlings 2005; Hoballah & Turlings 2005; D'Alessandro *et al.* 2006; Fontana *et al.* 2011). For instance, the GLV *(E)*-3-hexenol has been shown to be attractive to the braconid wasps *Apanteles kariyai* (Watanabe), whereas the sesquiterpenes γ-bisabolene and *(E)*- β-caryophyllene were found to be attractive to *Campoletis sonorensis* (Takabayashi *et al.* 1991). GLVs have also been shown to play a role in the attraction of many herbivores including flea beetles (Halitschke & Baldwin 2004; Halitschke *et al.* 2008), scarab beetles (Hanson *et al.* 1999), diabrotica beetles and lepidopteran larvae (von Mérey *et al.* 2011).

A compound that has received little attention so far is indole. Indole is an aromatic compound emitted by many plant species after herbivory (Turlings *et al.* 1991; McCall *et al.*

1994; Gols *et al.* 1999; Cardoza *et al.* 2003; De Boer *et al.* 2004; Yuan *et al.* 2008). It is specifically released in response to herbivore-elicitors and is not emitted from physically wounded plants (Frey *et al.* 2004). In maize, indole is produced from indole-3-glycerol phosphate and can be channelled into three different pathways. First, indole can be formed by the tryptophan synthase alpha subunit (TSA), which channels it directly to TSB for further conversion into the essential amino acid tryptophane (Frey *et al.* 1997; Frey *et al.* 2004). Second, it can be produced by the BX1 enzyme as an intermediate in the production of benzoxazinoids, a class of non-volatile defensive secondary metabolites of the grasses (Frey *et al.* 2009). Finally, it can be formed by the indole-3-glycerol phosphate lyase (IGL), which subsequently releases it as a volatile (Frey *et al.* 2000).

We previously found that in its volatile form indole functions as a within and between plant signal that primes maize plants to be more responsive to incoming herbivore attacks (Veyrat et al., unpublished data). We also demonstrated that it has a negative impact on the performance of *Spodoptera* caterpillars, implying multiple functions in direct defence against herbivores (Veyrat et al., unpublished data). Avoidance by non-specialized herbivores would therefore be expected, whereas natural enemies may use the compound as a reliable signal for prey or host location. Although indole is emitted by many plants, its role in tritrophic interactions has not been studied extensively (D'Alessandro *et al.* 2006). A synergistic effect of indole for the attraction of beetles such as western corn rootworms has been demonstrated (Lampman & Metcalf 1987; Barbercheck *et al.* 1995b; Metcalf *et al.* 1995; Herbert Jr *et al.* 1996; Ventura *et al.* 2000; Andrews *et al.* 2007). At the third throphic level we know that the parasitoid *Microplitis rufiventris* is attracted by indole, while *Cotesia marginiventris* is not affected by the presence of indole in an odour blend (D'Alessandro *et al.* 2006; Veyrat et al. 2014 unpublished data).

Given that indole is a common HIPV emitted by many, but not by all plants, we wondered whether the observed patterns of herbivore and parasitoid behaviour are valid across different insect species. It has long been thought that specialist and generalist insects use plant metabolites in different ways (Krieger *et al.* 1971; Whittaker & Feeny 1971). One hypothesis suggests that specialist insects have lost the ability to exploit many host plants, but with their specialization would have gain the ability to tolerate the defences of their specific host plant(s). Consequently they would be less impacted by a specific defence compound than a generalist herbivore (Krieger *et al.* 1971; Whittaker & Feeny 1971). As a consequence of their tolerance to specific compounds, they may evolve the capacity to use these specific compounds in host finding, and exploit these compounds for their own defence against natural enemies. Another theory predicts that generalist insects have resistance mechanisms that are less specific and less powerful, but that may allow for some tolerance

to a large array of plant defences, especially compounds that show highly conserved pathways over may plant taxa (Krieger *et al.* 1971; Whittaker & Feeny 1971; Katsir *et al.* 2008). Because indole is one of the major compounds produced by many plants after an herbivore attack, for instance in maize, cotton or lima bean (Turlings *et al.* 1991; McCall *et al.* 1994; Gols *et al.* 1999b; Cardoza *et al.* 2003a; De Boer *et al.* 2004), we could hypothesize that generalist insects that feed on a large range of indole-emitting host plants or specialist insects that only feed on indole-producing plants may be less likely to avoid indole than insects that feed on plants that do not emit indole. They may even be attracted by indole.

In this study, we investigated the responses to indole in herbivores and parasitoids from different insect families that differ in their host range and degree of specialization. To do so we used a four-arm olfactometer in which we placed custom-made dispensers to release physiologically relevant amount of indole. We chose herbivore species known to feed on plants producing indole in high quantities, as well as herbivores that feed on plants that produce little or no indole and also tested the responses of some of their parasitoids. In total, we compared the responses of eight different species of herbivores, five Lepidoptera, and one each of Coleoptera, Hymenoptera, and Hemiptera. The four different species of parasitoids included two braconids, one pteromalid and one ichneumonid.

MATERIAL AND METHODS

Insects

We tested the following herbivores: *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), *Mamestra brassicae* (Linnaeus) (Lepidoptera: Noctuidae), *Pieris brassicae* (Linnaeus) (Lepidoptera, Pieridae), *Pieris rapae* (Linnaeus) (Lepidoptera: Pieridae), *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae), *Athalia rosae* (Linnaeus) (Hymenoptera : Tenthredinidae) and *Dalbulus maidis* (Delong and Wolcott) (Hemiptera: Cicadellidae). The parasitoids were: *Pteromalus puparum* (Linnaeus) (Hymenoptera Pteromalidae), *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae), *Cotesia glomerata* (Linnaeus) (Hymenoptera: Braconidae), and *Hyposoter ebeninus* (Gravenhorst) (Hymenoptera: Ichneumonidae).

The caterpillars *Spodoptera frugiperda* and *Spodoptera exigua* were reared on artificial diet from eggs provided by Syngenta (Stein, Switzerland). The parasitoid *Microplitis mediator* and the herbivore *Mamestra brassicae* were provided by Oliver Blamer and Elodie Belz (FiBL, Frick, Switzerland). *Hyposoter ebeninus*, *Phyllotreta cruciferae* and *Pteromalus puparum* were collected from different field sites in Switzerland. *Cotesia glomerata* were provided by Jeff Harvey (Wageningen, The Netherlands). *Athalia rosae* were provided by

Caroline Mueller (Bielefeld, Germany). *Pieris brassicae* were provided (Tanja Christoffel (Zurich, Switzerland). *Pieris rapae* were provided by Rieta Gols (Wageningen The Netherlands). *Dalbulus maidis* were obtained from our own rearing colony maintained by Angela Koehler. All eggs were kept in an incubator at 30.0 ± 0.5°C until emergence. Subsequently, they were transferred on artificial diet or on their specific host plant and were kept at room temperature (24 ± 4°C) until use. The cages with parasitoids contained moist cotton wool and honey as a food source. For all bioassays with parasitoids, 2–3-day-old naive females were used. All cages were transferred to the laboratory 30 minutes before the experiments.

Olfactometer bioassays

To test the attractiveness of volatile indole to parasitoids, we used a four-arm olfactometer as previously described (D'Alessandro and Turlings 2005). The olfactometer consisted of a central glass choice arena (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 a glass bulb (50 mL). To test the attractiveness of volatile indole to crawling herbivores, we modified the system and placed the central choice arena upside-down. Purified and humidified air entered each odour source vessel at 1.1 L/min (adjusted by a manifold with four flow meters; Analytical Research System, Gainesville, FL) via Teflon tubing and carried indole (or not) through the connector tube to the elbows of the olfactometer. In these elbows, a part of the air (0.7 L/min) was pulled out and the other part entered in the central glass chamber. Ten neon tubes were attached to a metal frame above the olfactometer and provided approximately 7000 lm/m2 at the height of the odour source vessels. To avoid visual distraction of the insect, a white cardboard cylinder was placed around the central choice arena and on top of the choice arena. The choice arena was connected to four glass bottles. An indole dispenser (see below) was placed in two of the bottles and an empty dispenser was placed in the two other bottles. The position of the odour sources was changed between each experimental assay; all experiments were repeated four to six times. The system was left connected for half an hour before releasing insects. Herbivores and natural enemies were released in groups in the central glass chamber. Insects that entered in an arm reached the elbow where a stainless steel screen blocked their path. They flew or walked up in the direction of the light source above the olfactometer and entered into a trapping bulb where they were counted and removed. Insects that did not make the choice arena after thirty minutes were considered as having made “no choice” and all insects were removed from the olfactometer. All species of natural enemies were released in groups of six. *Spodoptera frugiperda, Spodoptera exigua, Mamestra brassicae, Pieris rapae* and *Pieris brassicae* were

all released in groups of 10; *Phyllotreta cruciferae* were releasd in groups of 15; *Athalia rosae* were released in groups of 4; and *Dalbulus maidis* were released in groups of 30. Between four and six such releases were done for each replicate.

Odor sources

To test the effect of indole on insect's behaviour, we used an indole-dispenser consisting of a 2ml amber glass vial (11.6 x 32 mm; Sigma-Aldrich, Buchs, Switzerland) containing 20 mg of synthetic indole (> 98 %, GC, Sigma-Aldrich, CH-9471 Buchs, Switzerland). The vial was sealed with a PTFE/rubber septum (Sigma-Aldrich, Buchs, Switzerland), which was pierced with a 2 µL micro-pipette (Drummond, Millan SA, Plan-Les-Ouates, Switzerland). The length of the pipette was calibrated to release a 50 ng/h of indole, which corresponds to the amount emitted by IGL-wild type plants (*Zea mays* cv. Delprim). This setup was used to test the preference of all species to empty bottles vs. bottles containing a dispenser of volatile indole.

Statistical analysis

The functional relationship between choices made and the odour sources offered in the four-arm olfactometer was analysed with a generalized linear model (GLM, a log linear model) with the software package R (version 2.13.1). To compensate for the overdispersion of insects within the olfactometer, we based the models on a quasi-Poisson distribution. For detailed explanations, see (Turlings, Davison et al. 2004).

RESULTS

Olfactometer bioassays

The larvae of three of the lepidopteran herbivore species were attracted by volatile indole (Figure 1A, B, C), whereas the two remaining species did not distinguish between indole and control-treatment (Figure 1D, E). About 70% of *Spodoptera fruigiperda* larvae made a choice and 58% preferred to go toward indole-dispensers against 42% toward control-dispensers (Figure 1A, $p<0.05$). More than 50% of *Spodoptera exigua* larvae made a choice and 62% went toward indole-dispensers against 38% toward control-dispensers (Figure 1B, $p<0.01$). Similarly more than 50% of *Mamestra brassicae* larvae made a choice and 68% went toward indole-dispensers against 34% toward control-dispensers (Figure 1C, $p<0.001$). *Pieris brassicae* and *Pieris rapae* larvae did not distinguish between the treatments (Figure 1D, E, $p>0.05$). About 70% of *Pieris rapae* larvae made a choice, while less than 50% of *Pieris brassicae* larvae chose one of the treatments.

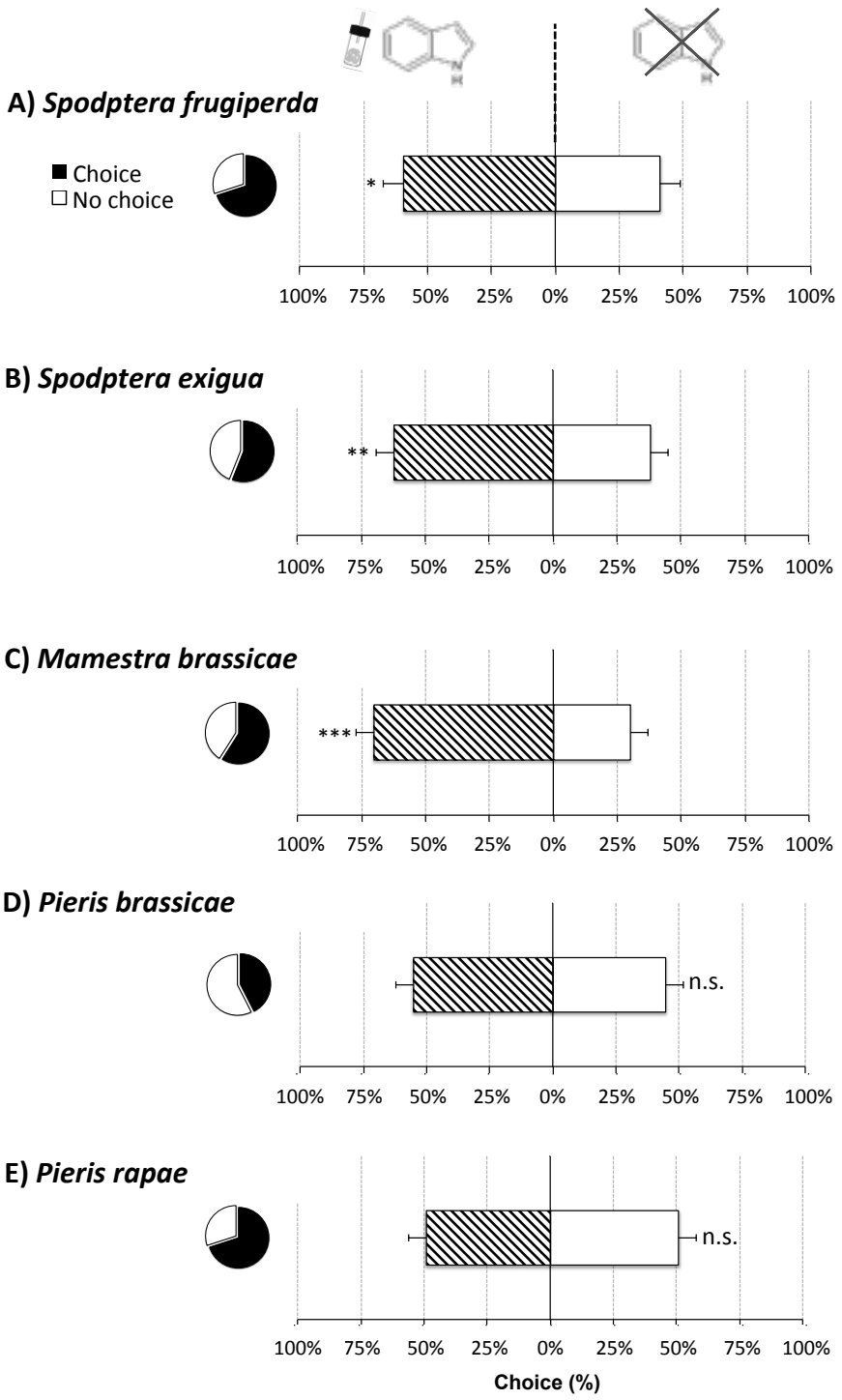

Figure 1: Choices of the larvae of five lepidopteran herbivore species, *Spodoptera frugiperda* **(A)**, *Spodptera exigua* **(B)**, *Mamestra brassicae* **(C)**, *Pieris brassicae* **(D)**, *Pieris rapae* **(E)** in a four-arm olfactometer. Two of the olfactometer arms carried volatile indole emitted from a dispenser; the other two arms had no odour. Asterisks indicate significant differences between treatments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Of *Phyllotreta cruciferae* (Coleoptera) adults, about 70% made a choice, and of those 58% chose for the arms with the indole-dispensers, a significant preference (Figure 2A, $p<0.05$).. In contrast, *Athalia rosae* larvae (Hymenoptera) chose more often for the control- (63%) than for the indole-arms (37%) (Figure 2B, $p<0.001$). *Athalia rosae* larvae responded strongly to the treatment as 89% of larvae made a choice. Similarly, 70% of the leafhoppers *Dalbulus maidis* (Hemiptera) made a choice and among them, 55% went toward control-dispensers (Figure 2C, $p<0.001$).

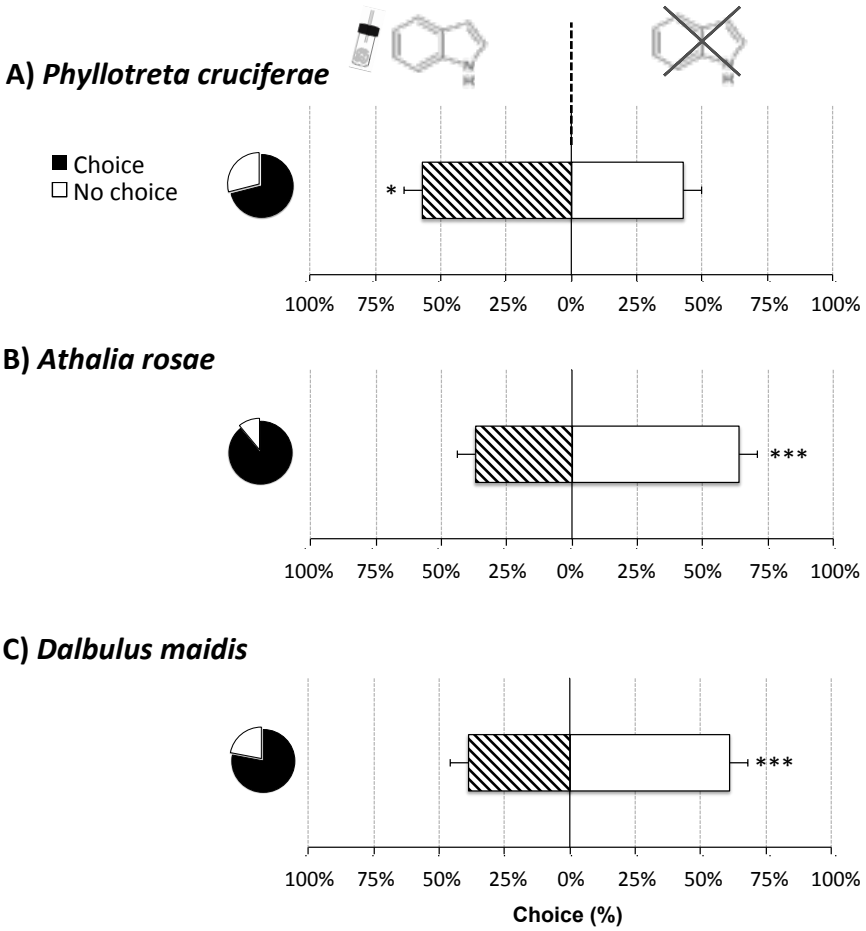

Figure 2: Choices of herbivorous insects of three different orders, *Phyllotreta cruciferae* **(A)**, *Athalia rosae* **(B)***, Dalbulus maidis* **(C)** in a four-arm olfactometer. Two of the olfactometer arms carried volatile indole emitted from a dispenser, the other two arms had no odour. Asterisks indicate significant differences between treatments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

The effect of indole on the attraction of parasitoids was also variable depending on the species. All four species responded well in the olfactometer, with 70% to 96% of wasps making a choice. *Pteromalus puparum* did distinguish between the treatments (Figure 4A, p>0.05). *Hyposoter ebeninus* and *Microplitis mediator* wasps were repelled by volatile indole (Figure 3B, p<0.05; Figure 3C, p<0.001), with 55% and 68% of wasps going toward the indole-dispensers, respectively. In contrast, *Cotesia glomerata* wasps appeared to be attracted by indole, with 65% choosing the indole arms (Figure 3C, p<0.001).

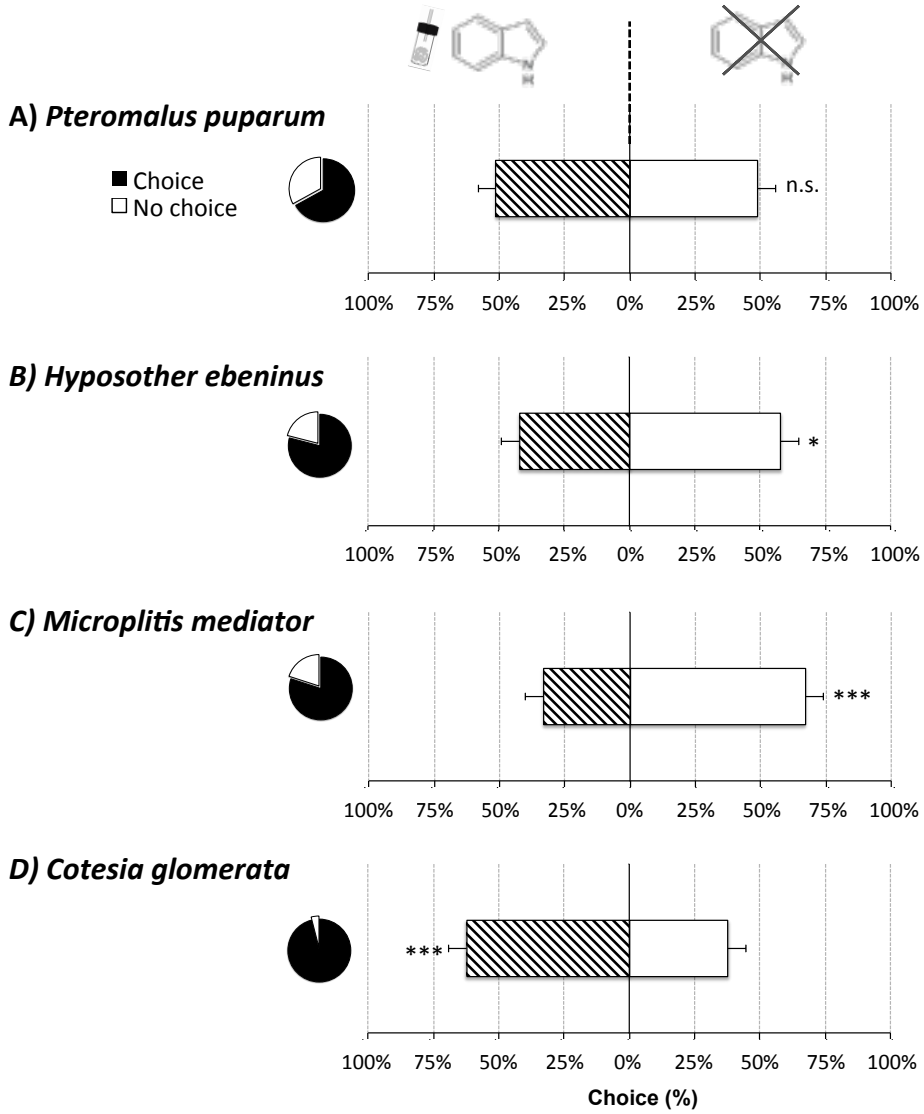

Figure 3: Choices of four different species of parasitoids, *Pteromalus puparum* **(A)**, *Hyposother ebeninus* **(B)**, *Microplitis mediator* **(C)**, *Cotesia glomerata* **(D)** in a four-arm olfactometer. Two of the olfactometer arms carried volatile indole emitted from a dispenser, the other two arms had no odour. Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

Table 1: Comparison of host specialization, specificity of host plants known, phylogeny and the response to indole for each species tested.

Herbivore Insect	Host plant specialisation	Reported host plant	Found on indole producing plant	Phylogeny	Effect of indole
Spodoptera frugiperda	Polyphagous	over 60 species of more than 20 families, including maize (1-2), rice (3-4) and sorghum (5-6)	Yes	Lepidoptera: Noctuidae	Attracted
Spodoptera exigua	Polyphagous	over 60 species of more than 20 families including maize (7-8), tomato (9-10), and cotton (11-12)	Yes	Lepidoptera: Noctuidae	Attracted
Mamestra brassicae	Polyphagous	over 60 species of more than 20 families including cabbage (13-14), tobacco (15) and tomato (16)	Yes	Lepidoptera: Noctuidae	Attracted
Pieris brassicae	Oligophagous	Brassicaceae family (17-19)	No (or in very low quantity)	Lepidoptera: Piridae	Not affected
Pieris rapae	Oligophagous	Brassicaceae family (20-22)	No (or in very low quantity)	Lepidoptera: Piridae	Not affected
Phyllotreta cruciferae	Oligophagous	Brassicaceae family (23-25)	No (or in very low quantity)	Coleoptera: Chrysomelidae	Attracted
Athalia rosae	Oligophagous	Brassicaceae family (26-28)	No (or in very low quantity)	Hymenoptera : Tenthredinidae	Repelled
Dalbulus maidis	Monophagous	Zea mays (29-30)	Yes	Hemiptera: Cicadellidae	Repelled

Natural enemies	Host specialisation	Reported host	Host found on indole producing plant	Phylogeny	Effect of indole
Pteromalus puparum	Generalist	*Pieris* caterpillars including *Pieris rapae* (31-33) and *Pieris brassicae* (34-35)	No	Hymenoptera Pteromalidae	Not affected
Hyposoter ebenius	Generalist	*Pieris* caterpillars (36-37)	No	Hymenoptera: Ichneumonidae	Repelled
Microplitis mediator	Generalist	over 40 different Noctuidae hosts including *Mamestra brassicae* (38-40) and *Helicoverpa armigera* (41-43)	Yes	Hymenoptera: Braconidae	Repelled
Cotesia glomerata	Specialist	*P. brassicae* (44-45) and *P. rapae* (46-48)	No	Hymenoptera: Braconidae	Attracted

1:(Nagoshi & Meagher 2008), 2:(Ni et al. 2008), 3:(Unbehend et al. 2013), 4:(Juárez et al. 2012), 5: (Cheng et al. 2013), 6: (Molina-Ochoa et al. 2001), 7: (Ren et al. 2013), 8: (GÖZÜAÇIK et al. 2009), 9: (Underwood 2010), 10: (Taylor & Riley 2007), 11: (Guo et al. 2010), 12: (Tindall et al. 2009), 13:(Santolamazza-Carbone et al. 2013), 14: (Balmer et al. 2013), 15: (Oka et al. 2013), 16: (Rojas 1999), 17: (Ansari et al. 2012), 18:(Lytan & Firake 2012), 19: (Hasan & Ansari 2011), 20: (Zhang et al. 2013), 21: (Cartea et al. 2009), 22: (Jogar et al. 2009), 23: (Soroka et al. 2011), 23: (Gruber et al. 2009), 24: (Cárcamo & Blackshaw 2007), 25: (Abdalsamee & Müller 2012), 26:(Šedivý & Vašák 2002), 27: (Opitz et al. 2010), 28: (Carloni et al. 2013), 29: (Medina et al. 2012), 30:(Moya-Raygoza et al. 2005), 31: (Zhu et al. 2012), 32: (Zhang et al. 2012), 33: (Zhu et al. 2009), 34: (Harvey et al. 2011), 35: (Razmi et al. 2011), 36: (Mustata & Mustata 2000), 37: (Bhat & Bhagat 2009), 38: (Harvey & Gols 2011a), 39: (Lauro et al. 2005), 40: (Harvey & Gols 2011b), 41: (Wanna et al. 2010), 42: (Li et al. 2006), 43: (Liu et al. 2005), 44: (Pörschmann & Spieth 2011), 45: (Hasan & Ansari 2010), 46: (Van Driesche 2008), 47: (Tagawa et al. 2008), 48: (Perfecto & Vet 2003)

DISCUSSION

The response to indole varied significantly among the different herbivores and parasitoids that were tested. The herbivores *S. frugiperda*, *S. exigua*, *M. brassicae* and *P. cruciferae* were attracted by volatile indole, whereas *A. rosae* and *D. maidis* were repelled. *P. brassicae* and *P. rapae* did not show any preference. *S. frugiperda, S. exigua* and *M. brassicae* are all polyphagous herbivores that feeds on over 60 species of plants of more than 20 families. These three species are very similar in their host plant specialization or their phylogeny and they all showed the same response to indole (Table 1). As they feed on several plants known to produce indole (Table 1), it may therefore be a reliable signal to localize their host plants. Our results are in accordance with previous studies showing that *S. frugiperda* larvae preferred odours from damaged maize seedlings over odours from undamaged seedlings (Carroll *et al.* 2006). In addition, contrary to other adults of lepidoteran species which are usually repelled by HIPVs (De Moraes *et al.* 2001; Huang *et al.* 2009) *M. brassicae* females have been shown to be attracted by cabbage plants infested with conspecific larvae (Rojas 1999). Our results tend to confirm the hypothesis that generalist insect species use a common and highly conserved compound to localize their host. However, *P. cruciferae* on the other hand is an oligophagous coleopteran species that feeds on a small number of plants belonging to Brassicaceae. Only few studies showed an indole production by brassicaceous plants, and in this studies indole does not seem to be a major compound in the HIPV blend produced (Pierre *et al.* 2011; Poelman *et al.* 2011). However, *P. cruciferae* also showed a strong atraction to indole. These results confirm other studies on Coleoptera showing that they are attracted to HIPVs (Loughrin *et al.* 1995; Bolter *et al.* 1997; Halitschke *et al.* 2008). And indole has been previously shown to synergize the beetle attraction (Lewis *et al.* 1990b) (LEWIS *et al.* 1990a; Barbercheck *et al.* 1995a; Metcalf *et al.* 1995; Herbert Jr *et al.* 1996; Ventura *et al.* 2000; Andrews *et al.* 2007). Our results are in accordance with these previous findings. It remains to be determined why *P. cruciferae* follows indole as a cue if their host plant does not release indole. An interesting fact is that flowers from rape belonging to the Brassicaceae family produced specific aromatic volatiles including indole which are attracted to *P. rapae* butterflies (Ômura *et al.* 1999). Consequently, it may be possible that *P. cruciferae* recognize and follow indole produced by flowers of their host plant as a cue.

P. brassicae, *P. rapae* and the turnip sawfly *A. rosae* are three specialist herbivores that mainly feed on plants in the Brassicaceae family (Table 1). They belong respectively to the lepidopteran or hymenopteran family. We found that *P. brassicae* and *P. rapae* were not affected whereas *A. rosae* were repelled by the presence of indole. These results are not surprising as none of these species are known to feed on indole-producing plants. They may

use other volatile cues to localize their host plant. Species in the Brassicaceae plant family all biosynthesize glucosinolates (GS) (Halkier & Gershenzon 2006) and consequently *P. brassicae*, *P. rapae* and *A. rosae* larvae exclusively feed on plants producing GS. It is maybe possible that they use more detectable signals such as GS to localize their host plants. GS are stored in the haemolymph and are released when the larvae are attacked. It is also known that GS and breakdown products of GS play an important role in host finding and host recognition in *A. rosae* (Barker *et al.* 2006).

Finally, the leafhopper *D. maidis* is a specialist on maize from the Hemiptera family. Interestingly, we found that *D. maidis* is strongly repelled by the presence of indole. It is known that chewer insects induced many volatiles in maize, whereas aphids induced no measurable emissions even after heavy infestation (Turlings *et al.* 1998). Even if *D. maidis* do not induce the production of indole, they may use it in order to avoid caterpillar induced plants. Our results are in accordance with studies on aphids attraction. Bernasconi et al. showed that the corn leaf aphid, *Rhopalosiphum maidis* were repelled by herbivore-induced emissions of maize volatiles (Bernasconi *et al.* 1998). By avoiding infested plants, *D. maidis* would then avoid the competition with other herbivores, the production of toxic compounds, and the possible presence of natural enemies.

The response of different parasitoid species was equally diverse and no pattern seems to be observed according to the phylogeny or the host specialisation degree (Table 1). *P. puparum* and *H. ebeninus* are both parasitoids of *Pieris* caterpillars. Whereas *P. puparum* did not show any preferences for one of the treatment, *H. ebeninus* avoided volatile indole. These results are not surprising as *Pieris* caterpillars are found on indole non-producing plants, *P. puparum* and *H. ebeninus* do not use indole as a cue to localize their host. Because *P. puparum* and *H. ebeninus* are parasitoids of herbivore species feeding on GS producing plants, they may use more specific signal from their hosts or the host plant. On the other hand, *C. glomerata* is a specialist gregarious endoparasitoid that attacks first to third instars of *P. brassicae* and a related species, *P. rapae* as well. However we found that *C. glomerata* wasps were attracted by indole. *C. glomerata* wasps are known to strongly discriminate between plant species that are infested with their herbivorous hosts (Geervliet *et al.* 1996). It was shown that *C. glomerata wasps* were able to discriminate between infested and uninfested cabbage plants after only 1 h of feeding by *P. brassicae* and their reponse reached a maximum after 3 h of feeding (Scascighini *et al.* 2005). It remains to be determined why *C. glomerata* is attracted by indole, which is not a dominant signal in the host plants of its host herbivores.

Microplitis mediator on the other hand is a generalist parasitoid of over 40 different Noctuidae hosts, including *M. brassicae* that feeds on indole-producing plants (Table 1).

Interestingly, even if *M. brassicae* larvae showed a strong attraction for indole, *M. mediator* wasps were not attracted by indole. A previous study showed that *M. mediator* wasps were not attracted by mechanically damaged plants or plants treated with *Helicoverpa armigera* oral secretion whereas they were strongly attracted by *Helicoverpa armigera* damaged plants (Yu *et al.* 2010). They also showed that a combination or terpenoids and green leaf volatiles may increase the parasitism rate by *M. mediator. M. mediator* wasps appear to use cues that are directly related with the presence of the hosts and the attractiveness of these cues can be increased by the used of specific HIPVs. This could explain why indole alone is repellent for *M. mediator* wasps.

This study gives an overview of the effect of indole on a variety of herbivore insects and their natural enemies. Evidently, neither the degree of host plant specialization nor the association with indole-producing plants determines the response of herbivores and natural enemies. Also, there was no obvious phylogenetic origin of indole responsiveness. We conclude that the response of both insects and herbivores to single HIPVs is highly variable and unpredictable by commonly used characteristics of convergent evolution and evolutionary inheritance.

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GENERAL CONCLUSIONS AND PERSPECTIVES

GENERAL CONCLUSIONS AND PERSPECTIVES

The results presented in this thesis provide new insights into the role of indole in plants defences and it reveals an unexpected importance of indole for biological interactions at different trophic levels.

It had already been demonstrated that indole is exclusively released in response to herbivore attack, but not after mechanical damages (Frey *et al.* 2000; Frey *et al.* 2004). Therefore, we envisaged a role of volatile indole in direct defence such as it has already been demonstrated for other HIPVs such as GLVs (von Mérey *et al.* 2013). We showed that indole is an efficient direct defence compound against the generalist herbivore *Spodotera littoralis* (Chapter 2). This volatile repels both larval and adult stage of the herbivore and it also leads to a higher mortality of larvae and a lower reproductive fitness of adults. We found that the surviving larvae feeding on indole-producing plants fed ate than the larvae feeding on indole-mutant plants. Interestingly we also demonstrated that despite a decrease in food consumption, larvae feeding on indole-producing plants had a higher weight gain and body fat content. These last results remain surprising and several questions need be addressed for future research. Several explanations could explain why larvae gained more weigh when they were exposed to volatile indole. For instance, indole could have a direct effect on the physiology and metabolism of the larvae. Similar to other products that have insecticidal activity e.g. organophosphates (Wardhaugh, 2005), indole might interfere with physiological processes that regulate specific biochemical pathways involved in the insect growth and development. In addition, indole could interfere with or mimic an insect hormone that regulates insect growth. Therefore, insects exposed to such compounds could die because of an abnormal hormone regulation. It is also probable that adults exposed to such chemicals become sterile or possess abnormally developed genitalia, which reduce their reproductive potential or the capacity to produce fertile offspring. Several natural compounds isolated from plants have been shown to have regulating activity on insect growth (Graf 1993; Vardhini *et al.* 2001; Ikbal *et al.* 2005). For instance andrographolide, a terpenoid isolated from the leaves of *Andrographis paniculata* (Acanthaceae) exhibits a growth regulating activity against the coleoptera *Tribolium confusum* (Duval) (Coleoptera: Tenebrionidae) (Lingampally *et al.* 2012). Taking into account the role of indole in the induction of *S. littoralis* mortality and in the decrease of its reproduction, we speculate that indole interferes with growth regulation. It could be worthwhile to study if larvae exposed to volatile indole show any developmental and moulting perturbation. It could also be interesting to study the development of eggs laid by indole-exposed adults, as well as the survival of the offspring. Although it should be noted that we did not find any differences in the development time or in the pupal weight of insects

that were exposed to indole (data not shown). Therefore we also suggested another possible explanation: that exposure to indole induces changes in the gut microbial community of *S. littoralis*, thereby affecting their food conversion and eventually their performance and survival. The characterization of gut microbiome in indole exposed *S. littoralis* larvae and unexposed larvae could be a first test of this hypothesis.

We showed that larvae feeding on indole-producing plants had a higher weight gain and body fat content. One could speculate that increased weight during the first instars of the larval development could make the larvae more vulnerable to predators and parasitoids, as it could make the larvae more visible and susceptible to predation. Moreover, bigger larvae could represent a better host for parasitoids. Zhudong Liu et al. (2011) showed that females of the parasitoid *Sclerodermus harmandi* prefer to attack large *Monochamus alternatus* larvae rather than small larvae. They also found that more offspring were obtained from larger *M. alternatus* larvae and that the offspring from larger hosts were themselves larger. In the context of host selection, the host quality evaluation by female parasitoids plays a key role in host choice, and parasitoids with an optimal foraging behaviour should select hosts that provide the highest fitness return (Godfray 1994). The nutritional quantity of a host is determined by the host size and the amount of host tissues available for parasitoid development, therefore larger larvae should be selected (Chau & Mackauer 2001; Li & Mills 2004; Chong & Oetting 2006). In this context, *S. littoralis* larvae exposed to indole should be favoured by parasitoid. For instance, in braconid parasitoid larvae which consume most or all host tissues prior to pupation (Gauld & Bolton 1988), the host size is directly correlated with parasitoid body size (Godfray 1994). It should be noted, however, that hosts that feed on superior diets are not only larger, but also physiologically more fit, which also means that their immune system will be better able to resist parasitism by, for instance, encapsulating parasitoid eggs (Turlings and Benrey, 1998).

In Chapter 3, we specifically investigated the effect of indole on the third trophic level and we tested the behaviour and fitness of two braconid species, *Microplitis rufiventris* and *Cotesia marginiventris.* Whereas the foraging behaviour of *C. marginiventris* was not affected by the presence of indole, we found that indole enhanced the attraction of the parasitoid *M. rufiventris* to mechanically induced maize plants. However, if *S. littoralis* caterpillars were present and feeding on the plants, indole had the opposite effect on the wasps' choice. This repellent effect can be explained by our finding that indole exposure increased *S. littoralis* larvae survival in the presence of parasitoids, and, in accordance, the fitness of the parasitoid was reduced. Taking together all these results, we can conclude that indole is attractive to *M. rufiventris and* has a negative effect on the survival of *S. littoralis*. The

reduced attractiveness of the larvae due to indole exposure must therefore be attributed to other chemicals than indole itself. The parasitoid appears to integrate responses to plant and herbivore odours in order to avoid inferior hosts. Our study is one of the first studies showing that exposure to a specific HIPV affects both the attractiveness of and the resistance to a natural enemy. Several points remain to be elucidated. First, it is still unclear how *M. rufiventris* wasps are able to distinguish between *S. littoralis* larvae exposed to indole and unexposed *S. littoralis* larvae. The olfactometer experiments clearly show that the larvae are able to discriminate between odours from larvae previously exposed to indole and non-exposed larave. We also showed that indole increases larval growth and mortality. These changes may be associated with differential production of body odours. Alternatively, it is known that compounds in the faeces of herbivores can mediate natural enemy attraction. Therefore it is possible that the quality of *S. littoralis* faeces changes upon indole exposure. Olfactometer assays that test the attraction of faeces from different larval sources might allow us to determine how *M. rufiventris* wasps are able to distinguish suitable from unsuitable hosts. Secondly, as indole confers resistance to *S. littoralis* larvae against their parasitoids, we can wonder whether the preference of *S. littoralis* larvae between indole-producing or non-producing plants would be affected by the parasitoid pressure. Indeed, we know that in some cases toxic secondary metabolites can be used and sequester by herbivores in order to defend themselves against natural enemies (Bowers & Collinge 1992; Gauld *et al.* 1992; Dyer 1995; Nishida 2002). Some studies showed that herbivores prefer to select relatively toxic host plants over highly palatable plants when the risk of parasitism is high (Singer & Stireman Iii 2003; Singer *et al.* 2004). Performing new choice tests on S. *littoralis* larvae under high and low parasitism pressure will help us to determine whether the benefits of reduced parasitization overcome the direct negative effects of indole exposure. Finally, a remaining question is to know whether indole gives a protection to the plant via the third trophic level, taking in to account that indole itself is attractive to *M. rufiventris* wasps. We know that the volatile blend emitted by infested plants allows long-range detection of hosts by parasitoid. Röse *et al.* (1997) studied the roles of host and plant volatiles used by *M. croceipes* and *C. marginiventris* females in recognizing hosts. They concluded that the host foraging strategies of *C. marginiventris* and *M. croceipes* are similar for long-range host patch orientation, plant volatiles playing a more important role in long-range attraction for both species. However, at close range, the host detection strategies are different between the two species. We speculate that indole may play a role for the long-range host seeking of parasitoids. But at closer range, some parasitoids such as *M. rufiventris* could use volatiles that are directly related to the host, whereas other parasitoids may continue to use plant volatiles as the short-range orientation cues. It should be noted the associative learning by parasitoids was not been addressed in our study. It has largely been demonstrated that

parasitoid wasps are able to learn to associate volatile odours with the presence of suitable host material after a positive experience and incorporate this odour information into their foraging behaviours (Turlings *et al.* 1993). It is also known that the behavioural response of parasitic wasps to chemical cues from their hosts, as well as host plants depends on a learning process. (Gandolfi *et al.* 2003) showed that the response of the codling moth ectoparasitoid *Hyssopus pallidus* to chemical stimuli was dependent of preimaginal learning. They exposed parasitoids to fruit cues at different developmental stages and found that, while parasitoids were not able to learn the fruit cues in the adult stage, exposure to fruit odour at early preimaginal stages significantly increased the adult response to frass from fruit-fed caterpillars. In addition, parasitoids exposed to menthol odour at the egg and larval stages no longer showed negative responses as adults. In our study, all parasitoids were reared on larvae fed on artificial diet. Because lots of parameters can affect the response of parasitoids to indole, more experiments with parasitoids with different rearing conditions should be done in order to determine if experiences during early development affect their subsequent behaviour as adults.

In addition to demonstrating the effects of indole on herbivores and parasitoids, we also showed how an important role for indole in interactions at the first trophic level (Chapter 1). We found that indole is an important airborne signal that, upon release in response to herbivory, systemically primes leaves distant from the attacked tissues within the same plant. Indole-mediate priming also occurred in neighbouring plants. Herbivore-induced indole was found to enhance the induced production of defensive mono-, homo- and sesquiterpenes in receiving leaves. Based on our results, we proposed that indole is a key and essential priming agent of maize. In this context, indole is a particularly reliable and suitable signal because it is specifically released in response to herbivory and not to mechanical damage. Moreover, its release occurs faster than other common inducible compounds such as sesquiterpenes. Besides maize many other plant species emit indole upon herbivory, including cotton, rice, gerbera, peanut and lima bean. Therefore it could be interesting to test whether the priming effect of indole is common across the plant kingdom.

Moreover, we found that the exposure of plants to indole or indole-containing HIPVs enhances the herbivore-induced production of the stress hormones jasmonic acid (JA) and abscisic acid (ABA). Probably this process represents the mechanism for indole-triggered volatile priming and we proposed that indole acts upstream of the defence hormonal signalling pathway to increase the production of herbivore-induced HIPVs. The fact that we found an increased production of the phytohormone ABA in indole-exposed plant is very interesting. Abscisic acid is known as the primary phytohormone that triggers short-term responses such as stomatal closure during drought stress (Finkelstein & Rock 2002; Zhang

et al. 2006). Plants on der herbivore attack may face a trade-off between stomatal closure, which prevents water loss, and stomatal opening, which favours CO2 uptake. It is known that herbivory can profoundly affect plant physiology by decreasing photosynthesis and net primary production as a result of altering the balance between water loss and CO2 assimilation. Complete defoliation, particularly in grasses, often causes an increase of carbon exchange in the remaining or newly formed leaves. In contrast, an incomplete foliar removal often causes a reduction of the rate of net photosynthesis in the remaining leaf tissue (Welter 1989). For instance, chewing damage to soybean leaves by Mexican bean beetle (*Epilachna varivestis* Mulsant) causes substantial losses of photosynthesis in the remaining leaf tissue (Peterson *et al.* 1999). Our ABA results might be an indication that indole primes stomatal closure in exposed-plants in order to avoid dehydratation stress by enhancing water use efficiency during herbivory. Moreover it is known that plant stomata have a function in innate immunity response against bacterial invasion and ABA has been suggested to regulate this process (Melotto *et al.* 2006). Upon contact with microbes, plants actively close stomata to prevent the entry of microbes and the consequent colonization of host tissue. Many bacteria produce indole, therefore they could directly trigger this stomatal defence response (Melotto *et al.* 2006; Melotto *et al.* 2008; Zeng *et al.* 2010; Zeng *et al.* 2011).

Recent studies have suggested that bacterial volatiles play an important role in bacterial-plant interactions. For instance, certain endophytic bacteria may produce volatiles that induce resistance to pathogenic microbes (D'Alessandro et al., 2014). Similarly, plant growth-promoting rhizobacteria (PGPR) elicit induced systemic resistance (ISR) and plant growth promotion in the absence of physical contact with plants via volatile organic compound (VOC) emissions. Bacteria are well-known producers of a diverse blend of volatile compounds (Schulz & Dickschat 2007) and among them indole is a bioactive volatile compounds (Blom *et al.* 2011). The functional role of volatile indole in interaction between rhizobacteria and plant has recently been demonstarted (Yu & Lee 2013). Their results indicated that the plant growth promoting rhizobacterium *Proteus vulgaris* JBLS202 stimulates the seedling growth of Chinese cabbage through indole emission. Both synthetic, as well as biological/bacterial indole increased the growth of cabbage significantly. In association with the discovery of the plant growth promoting properties of bacterial volatiles, a search for possible mechanisms has been initiated. Analysis of *Arabidopsis* has indicated possible involvement of cytokinin, ethylene, auxin, salicylic acid, ABA and JA in the reaction of plants to bacterial volatiles (Ryu *et al.* 2003). Taking together all these results and in the context of the JA-dependent priming results we got in Chapter 1, indole may function in maize as an ISR-eliciting bacterial signal.

Based on the results summarized so far, we concluded that indole plays an important role in interactions at all three trophic levels. We therefore decided in Chapter 4 to investigate the specificity of the consequences of indole exposure found in chapters 2 and 3. We studied the effects of indole on the attraction of a diverse range of species of herbivore insects and parasitoids. The responses were highly variable among the species, ranging from repellency, attraction, or no effect, and there was no correlation with either the degree of the insects' host plant specialization, their phylogenetic origin, or their association with indole-producing plants. We conclude that the response of both herbivores and parasitoids to indole cannot be predicted by commonly used characteristics of convergent evolution and evolutionary inheritance.

Several studies suggest that VOCs can be applied in the field to control pests of crop plants. Taking in to account the indole has an effect on all different trophic levels, this compound could represent a good candidate to use as a chemical agent to enhance, directly and indirectly, the control of pest insects. However, we also show that the effects of indole on insect behaviour and performance are variable and not easily predictable. For this reason its effects will have to be studied for each system separately. It is also important to mention that all the experiments presented in this thesis have been performed in laboratory under controlled conditions. The ecological complexity of tritrophic interactions in nature has not yet been considered for individual compounds. Only by studying the impact of indole on multitrophic interactions under realistic field conditions we will be able to determine whether indole can be used to improve crop protection.

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