THE KEY TO SUCCESS:

HOST PLANT ADAPTATIONS IN THE ROOT HERBIVORE *DIABROTICA VIRGIFERA VIRGIFERA*

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The key to success : Host plant adaptations of a root hervibore, Diabrotica virgifera virgifera

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SUMMARY AND INTRODUCTION

SUMMARY

Antagonistic interactions between plants and insects are likely the drivers of a fascinating coevolutionary arms race between the two trophic levels. Plants- and plant breeders- are continuously developing traits that allow them to fend-off herbivores, while phytophagous insect keep inventing counter-adaptations to withstand plant defenses. *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is a specialist root herbivore of maize, *Zea mays*. Known as the billion dollar bug in the USA, the rootworm causes important crop damage annually, and no pest management strategy seems to effectively restrain its spread and voracity.

This thesis aimed at investigating the interactions of *D. virgifera* larvae with their plant host to underpin the mechanisms of their remarkable ecological success.

My results show that the root herbivore is able to exploit plant volatiles such as (*E*)-β-caryophyllene and ethylene, to assess the host plant quality at a distance and to orient towards optimal hosts (Chapter 1). I also found that *D. virgifera* can exploit direct plant defenses: The herbivore is able to detect the most nutritious root tissues using differences in 1,4-benzoxazin-3-one profiles (Chapter 3). Furthermore, I show that *D. virgifera* induces a reconfiguration of the plant primary metabolism and an attenuation of its defensive inducibility, resulting in induced susceptibility (Chapter 4). Induced susceptibility may explain the benefits for the larvae to aggregate in field. By investigating the aggregative behavior of the larvae, I found that *D. virgifera* uses (*E*)-β-caryophyllene in a dose-dependent manner to evaluate the density of conspecifics feeding on a plant. The perception of the sesquiterpene allows the insect to aggregate on plants infested with optimal densities of conspecifics, thereby avoiding intraspecific competition and overexploitation (Chapter 4).

Maize plants seem to be maladapted to *D. virgifera*. Yet, plant breeders have grown maize plants in the presence of *D. virgifera* for almost 10 000 years now. It is therefore hardly conceivable that breeding would not have led to selection of resistant germplasm. For instance, the emissions of (*E*)-β-caryophyllene was altered and lost in American maize varieties, possibly to reduce the capacity of *D. virgifera* to aggregate. My work highlights the ecological and physiological costs associated with the emission of this compound, and proposes a novel scenario to explain the evolution of (*E*)-β-caryophyllene (Chapter 2). Finally, one resistance trait that would not exert any pressure on the pest that would cause to adapt is herbivore-induced tolerance. I investigated this trait in maize plant and highlight an unexpected role of stems as storage organs for plants under attack (Chapter 5). Focusing on tolerance mechanisms rather than resistance may be a promising avenue to reduce the impact of *D. virgifera* on maize yield and food production.

INTRODUCTION

Plant-insect interactions

Plant-insect interactions dominate the world. This statement, as strange as it may sound at first, is not far from reality: Insects are the most species rich class of higher organisms on the planet, and the majority of them are herbivores (Strong *et al.*, 1984). The countless antagonistic interactions between plants and insects have likely led to a fascinating coevolutionary arms race between the two trophic levels, resulting in remarkable radiation patterns of host plants (driven by the evolution of novel plant defenses) and phytophagous insect species, which may have evolved counter-adaptations (Ehrlich & Raven, 1967; Schoonhoven *et al.*, 1998; Thompson & Cunningham, 2002).

Plant defenses against phytophagous insects

Plants possess a wide spectrum of strategies that allow them to resist or to tolerate herbivory (Nunez-Farfan *et al.*, 2007). Resistance is governed by the plant immune system, which can be innate, and therefore, constitutively expressed, or adaptive, involving induced responses elicited by herbivory. Constitutive defenses include physical barriers (cuticle, trichomes, callose, and lignified cell walls), deterrent and toxic chemicals (secondary metabolites and direct biochemical agents such as proteases and proteinase inhibitors) (Walling, 2000). Adaptive responses are triggered by the recognition of elicitors contained in the herbivore saliva, that results in a plasma membrane (V_m) depolarization, followed by an increase in intracellular Ca^{2+} concentrations, induction of kinases, nitric oxide (NO), hydrogen peroxide (H_2O_2) and phytohormones (Bricchi *et al.*, 2010) that trigger the expression of physical and chemical defenses. Induced defenses are commonly separated into direct defenses, which target the herbivore directly, as well as indirect defenses, which help the plant to recruit natural enemies of herbivores (Howe & Jander, 2008; Kessler & Heil, 2011). The induction of defenses generally results in induced resistance against the attacker, which negatively affects herbivore growth and thereby leads to reduced tissue loss (Karban & Baldwin, 1997; Steppuhn *et al.*, 2004a; Erb *et al.*, 2009a). Plant tolerance, on the other hand, relies on the reallocation of resources, leading to their sequestration in tissues that are inaccessible to the herbivore, and later allows compensatory growth (Gómez et al., 2010; Babst et al., 2008, 2005; Schwachtje et al., 2006). Tolerance mechanisms, therefore, do not necessarily include a reduction in herbivore feeding and may be accomplished by an increase in growth of the herbivore (Strauss & Agrawal, 1999).

Did domestication "disarm" plants?

The selection for increased yield, which has been the main goal of many plant breeders over the last centuries, may be associated with reduced defenses against phytophagous insects. This hypothesis is based on two assumptions: First, that defenses may be costly to produce and divert photoassimilates from growth (Herms & Mattson, 1992; Gershenzon, 1994) and second, that yield increases generally result from reallocation of photoassimilates rather than from an increased photosynthesis rate (Gifford *et al.*, 1984; Evans, 1993). Although shifts in resource allocation selected for by plant breeders may depend on the probability of insect attack, the intensity of damage and its consequences for the plant fitness in a given cultivation environment, many examples have reported weakened defenses in cultivated plants compared to their wild relatives (Massei & Hartley, 2000; Lindig-Cisneros *et al.*, 2002; Chen & Welter, 2003; Chen & Welter, 2005; Chen & Welter, 2007; Mondolot *et al.*, 2008; Rodriguez-Saona *et al.*, 2011), but see (Yahiaoui *et al.*, 2006).

Herbivore counterresistance to plant defenses

Demonstrating that herbivore traits, which counteract plant defenses, have coevolved with the latter is a challenging task, as it requires information about the environment, the genetic variability and heredity of those traits as well broad comparisons between species (Reznick & Travis, 1996). Nevertheless, phytophagous insects have evolved an impressive set of strategies that reduce the detrimental effects of plant defenses and increase their fitness (Karban & Agrawal, 2002). Some herbivores for instance are specialists, with their host range restricted to a few plant species. Specialists often possess highly specialized strategies that allow them to withstand the defenses of their specific host plants. Generalists on the other hand, feed on a number of different plant species. Although they perform less well than specialist insects on a given plant species, generalists have developed counterresistance strategies that enable them deal with or tolerate a broad spectrum of plant defensive mechanisms (Agrawal, 1999; Agrawal, 2000; Karban & Agrawal, 2002).

The strategies of both specialists and generalists involve behavioral, morphological and physiological traits. Behavioral traits include the ability to balance nutritional value and the defensive status of host plant tissues via host selection and compensatory feeding (Simpson & Simpson, 1990; Slansky, 1993). Also, the effectiveness of host defenses can be reduced by feeding in groups (Berryman *et al.*, 1989; Raffa, 2001; Wallin & Raffa, 2001; Kane & Kolb, 2010), covering physical defenses with silk (Rathcke & Poole, 1975), draining defensive liquids from their storage sites (Dussourd, 1993; Dussourd, 1999; Wallin & Raffa, 2001), or even deactivating plant defenses by depriving leaves of light by rolling or tying them up (Sagers,

1992). Morphological traits, such as herbivore's mouthparts, are also associated with particular strategies of consumption, and may be responsive to dietary changes (Bernays, 1986; Bernays & Janzen, 1988; Thompson, 1992). Physiological traits of insect adaptations include enzymes, usually present in the insects saliva or gut, that can suppress plant defenses (Felton & Eichenseer, 1999; Musser *et al.*, 2002; Sarmento *et al.*, 2011), or detoxify plant secondary metabolites and biochemical agents (Jongsma *et al.*, 1995; Feyereisen, 1999; Engler *et al.*, 2000; Agrawal *et al.*, 2002; Hirayama *et al.*, 2007; Daimon *et al.*, 2008; Glauser *et al.*, 2011b; Schuler, 2011). Various phytophagous insects are also able to tolerate plant secondary metabolites and may even sequester them in their body tissues or integuments, resulting in enhanced defences of the insect against its own natural enemies (Duffey, 1980; Blum *et al.*, 1990; Pennings & Paul, 1993; Muller & Brakefield, 2003; Opitz & Muller, 2009). Interestingly, plant defenses have also been reported to be exploited by specialist herbivore in order to locate their host plants or the most nutritious tissues (Hopkins *et al.*, 1998; Agrawal & Sherriffs, 2001; Smallegange *et al.*, 2007; Howe & Jander, 2008). Furthermore, herbivores may divert plant resources to their feeding sites (Way & Cammell, 1970; Price *et al.*, 1987; Larson & Whitham, 1991; Larson & Whitham, 1997; Wool *et al.*, 1999; Stone & Schrönrogge, 2003; Giron *et al.*, 2007; Compson *et al.*, 2011).

Taken together, these strategies do not only allow the insect to resist plant defences, but can also result in induced susceptibility of the plant to further attack (Karban & Agrawal, 2002).

Belowground plant-insect interactions: a neglected field of study

Although root feeders play a crucial role in agricultural and natural ecosystems (Blossey & Hunt-Joshi, 2003; Wardle *et al.*, 2004), being "out of sight" kept them largely "out of mind" of entomologists and plant biologists (Hunter, 2001). Soil dwelling herbivores affect entire plant communities (De Deyn *et al.*, 2003), as well as micro- and macro-organisms communities below- (Wardle, 2006) and aboveground (Bezemer & van Dam, 2005; Erb *et al.*, 2009b; Soler, JJ *et al.*, 2009; Soler *et al.*, 2010). The average number of root feeders in a given volume of soil in temperate grasslands exceeds the number of herbivores that can be found aboveground (Ilia Sonnemann, personal communication), and root damage effects on plant fitness are equivalent to those following aboveground herbivory (Brown & Gange, 1989; Maron, 1998). Yet, little is known about plant defense belowground. Recent studies suggest that plants also possess a potent defensive system to withstand herbivory (Rasmann & Agrawal, 2008). Although the first steps of recognition of herbivory and ensuing defense responses belowground appear to be similar to early herbivory-induced events in leaves (Vadassery & Oelmuller, 2009), the resulting defensive pattern is different in roots (Erb, 2012).

The model system

Understanding belowground plant-herbivore interactions is of fundamental importance, as it may lead to new discoveries about plant physiology and ecology that could be incorporated into an integrated theory of plant response to herbivory. Furthermore, the acquired knowledge may be immediately transferrable to applied ecology, especially in an agricultural context. Only a few belowground plant-insect models have been developed during the past decade.

Maize plants (*Zea mays* L.) suffer from root herbivory by the western corn rootworm, *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae). Maize is one of the most important crop worldwide (Rice, 2004; Gray *et al.*, 2009) and serves as food for cattle and, both indirectly and directly, for humans (Fedoroff, 2003). Maize cultivation started about 9000 years ago in Mexico with the domestication of the wild grass *Zea mays* spp. *parviglumis* (Piperno & Flannery, 2001; Matsuoka *et al.*, 2002). Breeding for high yielding maize lines resulted in weakened pant defenses against aboveground herbivores (Klenke *et al.*, 1986; Rosenthal & Dirzo, 1997). The root herbivore *D. virgifera* has co-evolved with maize since the beginning of the domestication process (Branson & Krysan, 1981) and has become largely specialized on the crop (Oyediran *et al.*, 2004). By feeding on maize roots, *D. virgifera* larvae threaten the plant's functional and structural integrity (Vidal *et al.*, 2004), resulting crop losses that cost more than one billion dollar annually in the US alone (Krysan & Miller, 1986). Recently, *D. virgifera* was introduced in Europe through multiple events that caused different outbreaks (Miller *et al.*, 2005).

Nowadays, pest management strategies against *D. virgifera* include crop rotation, pesticides, and genetically modified plants, none of which provide satisfactory protection. Crop rotation can break the insects life cycle, but its implementation has led to the appearance of *D. virgifera* strains that can also feed on soybean, which enables them to tolerate a two-year rotation (Sammons *et al.*, 1997). Pesticides are frequently accompanied by groundwater contamination, phytotoxic interactions with herbicides, toxicity to applicators and non-target organisms (Journey & Ostlie, 2000), and are only of limited efficiency against *D. virgifera* larvae that are hidden in the soil. Genetically modified maize strains produce a complex of two Bt (*Bacillus thuringiensis*) proteins against *D. virgifera* (Masson *et al.*, 2004), but the latter is able to adapt within a few generations, resulting in larvae that readily survive exposure to the toxins in greenhouse and in field (Meihls *et al.*, 2008; Gassmann *et al.*, 2011). The acquired resistance of *D. virgifera* to pest management strategies reveals its remarkable adaptive abilities and may contribute to its ecological success in maize agroecosystems.

Thesis outline

Given the high degree of specialization and adaptive ability of *D. virgifera*, I hypothesized that the root feeder should have evolved distinct strategies to i) locate suitable host plants, ii) orient itself within the root system, and iii) overcome plant defenses. As yet, such traits have hardly been studied for belowground herbivores in general, and little is known about the contribution of these types of behavioral adaptations to the success of *D. virgifera* as a pest.

In Chapter 1, I investigated host selection mechanisms of *D. virgifera* larvae, specifically their ability to detect and select host plants that are optimal for their development. As I found the induced sesquiterpene (*E*)-β-caryophyllene (EβC) to be attractive to the herbivore, I next evaluated the physiological and ecological benefits and costs associated with the constitutive emission of this volatile in a series of additional field and laboratory experiments (Chapter 2). In Chapter 3, I investigated the foraging behavior of *D. virgifera* in detail, in an attempt to understand how it selects the best roots within a given root system. Based on the observation that *D. virgifera* larvae aggregate in field, I then evaluated the advantages of aggregating for the larvae and possible mechanisms by which this behavior allows it to overcome plant defenses (Chapter 4). In the last chapter, I describe how root-attacked maize plants reprogram their primary metabolism and reveal a putative mechanism of plant tolerance to belowground herbivory, which, when facing the "perfect pest", may be an appropriate strategy to minimize fitness loss.

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CHAPTER I

Herbivore-induced plant volatiles mediate host selection by a root herbivore

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ABSTRACT

In response to herbivore attack, plants mobilize chemical defenses and release distinct bouquets of volatile organic compounds. Aboveground herbivores are known to use changes in leaf-volatile patterns to make foraging decisions, but it remains unclear if belowground herbivores also use volatile emissions to select suitable host plants. We therefore investigated how above- and belowground infestation affects the performance of the root feeder *Diabrotica virgifera virgifera* LeConte, and whether the larvae of this highly specialized beetle are able to use volatile cues to assess from a distance if a potential host plant is already under herbivore attack. *D. virgifera* larvae grew better on roots previously attacked by conspecific larvae, but performed worse on roots of plants whose leaves had been attacked by larvae of the moth *Spodoptera littoralis*. Fittingly, *D. virgifera* larvae were attracted to plants that were infested with conspecifics, while they avoided plants that were attacked by *S. littoralis*. We identified *(E)*-β-caryophyllene, which is induced by *D. virgifera,* and ethylene, which is suppressed by *S. littoralis,* as two signals that *D. virgifera* larvae use to orient towards plants that are most suitable for their development. Our study demonstrates that soil dwelling insects can use herbivore-induced changes in root volatile emissions to locate suitable host plants.

INTRODUCTION

Different herbivores can interact through physiological changes in shared host plants (van Dam *et al.*, 2003; Erb, 2009; Erb *et al.*, 2009a; Gray *et al.*, 2009; Poelman *et al.*, 2010; Pierre *et al.*, 2011). The outcome of those plant-mediated interactions depends on the herbivore species (Wurst & Van der Putten, 2007) and their sequence of arrival (Erb *et al.*, 2011c). Herbivore-induced changes in plant volatile patterns in particular have been found to influence oviposition and larval choice aboveground (Carroll *et al.*, 2006; Carroll *et al.*, 2008; Soler, R *et al.*, 2009; Soler *et al.*, 2010). For instance, female moths can use herbivore-induced volatiles to avoid plants that are already infested, probably to avoid competition and/or plants that have otherwise upregulated their defenses (De Moraes *et al.*, 2001b; Anderson *et al.*, 2011). For soil dwelling herbivores, effects of herbivore-induced changes in plant volatiles on their foraging behavior have not yet been studied, despite the fact that the performance of soil herbivores is affected by the presence of other insects on the same plant (Hausmann & Miller, 1989; Erb *et al.*, 2011c).

Insect larvae can disperse in the soil and locate plants by using semiochemical cues (Johnson & Gregory, 2006). Carbon dioxide for instance, which is released by roots and diffuses rapidly in the soil, is known to be a common attractant for soil insects (Johnson & Gregory, 2006). Because emissions of carbon dioxide by roots is ubiquitous and non-specific, it is not surprising that several studies identified additional, non-volatile chemical signals that enable specialized root herbivores to recognize their host plant (Johnson & Gregory, 2006; Bernklau *et al.*, 2009) and host species of high quality (Johnson *et al.*, 2005). However, it remains unclear whether root herbivores are able to use induced changes in plant volatiles to distinguish host plants. Given the considerable physiological variation of plant quality due to genetic and environmental factors, including systemic resistance induced by other plant feeders (Moran & Whitham, 1990; Masters, 1995; Erb *et al.*, 2011c; Pierre *et al.*, 2011), root herbivores should be able to assess host quality even among closely related plants within a population. Because movement of insects through the soil matrix is costly, we hypothesized that root herbivores may make use of long-range signals to assess the suitability of host plants from a distance.

To test this hypothesis, we explored the interaction between the specialist root feeder *Diabrotica virgifera virgifera* and its main host plant, *Zea mays*. *D. virgifera* females oviposit at the end of the vegetation period, and their eggs diapause in the bare soil during winter, waiting for a new generation of maize plants to germinate in spring. Therefore, it is impossible for females to assess the quality of the host plants that their offspring will eventually encounter. To assess whether, instead, *D. virgifera* larvae are able to select and orient towards maize plants that are best suited for their development we performed a series of performance and preference experiments.

D. virgifera larvae performed better on plants that were infested with conspecifics than on healthy plants, while they performed worse when feeding on plants infested by the leaf-herbivore *Spodoptera littoralis*. By analyzing changes in the volatile bouquets emitted by roots from plants attacked by below- or aboveground herbivores and performing choice experiments with pure compounds and different maize varieties, we identified ethylene, a gaseous phytohormone (Yang & Hoffman, 1984), and *(E)*-β-caryophyllene, a sesquiterpene emitted by maize upon root infestation (Rasmann et al., 2005) as two distinct signals that are used by *D. virgifera* larvae to evaluate plant quality from a distance.

METHODS

Plants and insects

Maize seeds (*Zea mays* L, varieties "Delprim", "Pactol", "Ronaldinho", and "C"; Delley DSP, Delley, Switzerland) were sown in plastic pots (11 cm high, 4 cm diameter) by placing them on a layer of moist washed sand (0-4 mm, Jumbo, Switzerland). The seeds were then covered with 2 cm of commercial soil (Aussaaterde, Ricoter, Aarberg, Switzerland). Seedlings were grown in a climate chamber (23 ± 2 °C, 60% relative humidity, 16:8h L/D, and 50'000 mmol*m^{-2}), and MioPlant Vegetable and Herbal Fertilizer (Migros, Switzerland) was added every two days after plant emergence. Twelve-day old plants were used for the experiments. Larvae of *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) were reared on freshly germinated maize seedlings until use. *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuid) larvae were reared on artificial diet.

D. virgifera *performance*

To determine whether infestation by other herbivores influences host-plant quality for *D. virgifera*, we conducted two performance experiments. First, to measure *D. virgifera* larval performance on plants that were previously infested with conspecifics, maize root systems were infested with five second-instar *D. virgifera* larvae. Control plants remained uninfested. After two days, all the larvae were carefully removed by gently washing the roots with tap water. Control roots were washed under the same conditions. Maize roots were then repotted in 10% (v/v) moist white sand (Migros, Switzerland) and infested with five new second-instar *D. virgifera* larvae for six hours. The induction of root defenses by *D. virgifera* happens within 4-5h after onset of feeding (Hiltpold, I. *et al.*, 2011b), and the chosen time window was thus deemed optimal to compare the performance of the larvae on induced and uninduced plants. The weight of the larvae was recorded before and after infestation, and average individual relative weight gain was calculated.

In the second experiment, *D. virgifera* larval performance on plants previously infested with *S. littoralis* was measured by first adding 20 second-instar *S. littoralis* larvae on maize leaves, a density comparable to field infestations (Martins, 2000). Control plants were left uninfested. Transparent 1.5 L PET bottles were placed upside down over the aboveground part of the plants to confine leaf herbivores as described elsewhere (Erb *et al.*, 2011c). Forty-eight hours after leaf infestation, single pre-weighed second-instar *D. virgifera* larvae were placed on the soil of infested and uninfested plants and left to feed on the roots for 5 days, after which they were recovered and weighed again.

Host plant selection by D. virgifera

To investigate if *D. virgifera* larvae can locate good hosts from a distance using volatile cues, the attraction of larvae to infested and uninfested plants was tested in dual-choice olfactometers. Maize seedlings were potted in glass pots (5 cm diameter, 11 cm deep) with a horizontal connector (29/32 mm) at 0.5 cm height and filled with moist (10% water) white sand (Migros, Switzerland). Pots without plants were filled with moist white sand only. Pots were wrapped in aluminum foil to keep the root systems in the dark and avoid visual cues for the larvae. After 48 h, the pot connectors were linked using a glass tube (24/29 mm, 8 cm long) with a vertically connected access port in the middle, and one Teflon connector at both sides of the glass tube (24/29 mm to 29/32 mm). The Teflon connectors contained a fine metal screen (2,300 mesh; Small Parts Inc., Miami Lakes, FL, US), which prevented the larvae from reaching the roots. The system was left connected for half an hour before introducing six second or third-instar *D. virgifera* larvae via the vertical port of the central connector. As soon as the six *D. virgifera* larvae had moved from the glass connector into one of the Teflon connector, the system was disassembled and the position of the larvae was recorded. Larvae that had not entered a Teflon connector after 10 minutes were scored as "no choice". In preliminary control experiments, larval choice assays with healthy plants *versus* pots with sand only and healthy plants *versus* plants with infested roots were conducted using central connectors filled with moist sand. As the use of empty connectors gave similar results, but enabled a more efficient larval recovery (Figure S1), all subsequent experiments were performed using empty central connectors. This type of setup was used to test *D. virgifera* attraction to healthy *vs. D. virgifera* infested seedlings and healthy *vs. S. littoralis* infested plants. For this, maize plants were either infested with five second-instar *D. virgifera* larvae, 20 second-instar *S. littoralis* larvae, or left uninfested. Aboveground feeders were confined to the leaves by placing transparent 1.5 L PET bottles over the leaves as described (Erb *et al.*, 2011c). To confirm that the roots were the source of volatiles used by the root herbivore to distinguish between healthy and leaf infested plants, leaves, leaf herbivore larvae, frass and soil were removed from the system and isolated roots from healthy and leaf-infested plants were offered to *D. virgifera*.

Volatile collection and analysis

To find potential signals that *D. virgifera* can use to distinguish between infested and uninfested plants, volatiles emitted by roots from healthy, *D. virgifera* and *S. littoralis* infested plants were measured in several independent experiments. Twelve day-old maize plants were infested with either five second-instar *D. virgifera* larvae, 20 second-instar *S. littoralis* caterpillars, or left uninfested. Transparent 1.5 L PET bottles were placed upside down over the aboveground part of all the plants as described above. After 48 hours, roots were washed with tap water and frozen in liquid nitrogen, and root volatile production was determined using SPME-GC-MS analysis following a previously described protocol (Erb *et al.*, 2011a). CO_2 emission was evaluated in a second experiment by connecting the belowground glass pots to an additional glass vessel (28 cm long, 5 cm diameter) via the female connector and a glass male joint. The glass vessel was closed using parafilm and left to stabilize for two hours. A CO_2 gas meter (Voltcraft, CM-100, Conrad Electronics, Dietlikon, Switzerland) was then introduced into the connected vessel for 3 minutes, and CO_2 levels were recorded. Ethylene measurements were performed by removing the roots from the pots and gently washing them with tap water. The entire root systems were then placed in 20 mL gastight vials (Gerstel GmbH, Mülheim, Germany) and incubated at room temperature for 12 h. One mL headspace samples were withdrawn from the vials with a 2.5 mL gastight syringe and injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Hewlett Packard HP 6890 GC). The GC-FID was operated in split-mode (2:1) with a liner temperature of 60° C, a column temperature of 50° C and a detector temperature of 300°C. For separation, a GS-Alumina column was used at a constant flow-rate of 4.8ml/min. Ethylene was identified by comparison of the retention time with that of the pure compound. Absolute quantification was based on a standard-curve obtained by injecting different concentrations of pure ethylene.

Identification of attractants

The attractiveness of *(E)*-β-caryophyllene (EβC) and ethylene to *D. virgifera* larvae was evaluated in three different assays using the dual-choice setup as described above. For all of these assays, healthy *vs. D. virgifera* infested plants or healthy vs. *S. littoralis* infested plants were included as positive controls. In a first experiment, *D. virgifera* larvae were given the choice between a *D. virgifera* infested plant and a healthy plant whose rhizospace was complemented with synthetic *(E)*-β-caryophyllene (Sigma Aldrich Chemie GmbH, Buchs SG, Switzerland). *(E)*-β-caryophyllene was added using slow-release capillary dispensers as described previously (Mérey *et al.*, 2011). To verify that the dispensers release *(E)*-β-caryophyllene at a similar rate as infested maize roots, we performed a series of quantification experiments. Using SPME-GC-MS

as described above, we first established a calibration curve with different doses of pure *(E)*-β-caryophyllene (0, 12, 25 and 50 ng) dissolved in 50 µl 0.1% ethanol (v/v). Second, we measured *(E)*-β-caryophyllene emissions from dispensers with a 1µl capillary every hour over a period of 8 hours, and calculated the release rate in ng/h. To compare the release of dispensers with real maize plants, twelve day-old plants were infested with six *D. virgifera* larvae for 48h. After this period, the root system was gently washed with tap water, excised from the stem and placed in an SPME vial. To minimize effects of removing the leaves, *(E)*-β-caryophyllene emissions were measured immediately after cutting with SPME.

For the behavioral experiments, the dispensers with a 1 µl capillary were placed upside down into a small cavity in the sand surface for 24h. Dispensers continuously released up to 40 ng.h-1 of *(E)*-β-caryophyllene, which is well within the physiological range of infested maize roots (Figure S4). Empty dispensers were added to *D. virgifera* infested plants. In a second experiment, *D. virgifera* selection between healthy and *D. virgifera* infested plants was tested with plants of the variety C, which does not emit *(E)*-β-caryophyllene (Erb et al., 2011). In a third experiment, larvae were offered uninfested plants with empty control dispensers and uninfested plants with *(E)*-β-caryophyllene filled dispensers. Finally, to test the effect of previous *(E)*-β-caryophyllene exposure during the rearing of the larvae, *D. virgifera* larvae were reared either on maize seedlings that emit *(E)*-β-caryophyllene (variety "Ronaldinho", Landi Lyss, Switzerland) or not (variety "Pactol", Delley Semences DSP SA, Delley, Switzerland) upon wounding. Choice experiments were then performed for "naïve" and "experienced" larvae as described above. The role of ethylene was investigated using similar complementation experiments as above. *D. virgifera* larvae were given a choice between plants whose rhizospace was enriched with 2 ppm of ethylene and plants who received ambient air. This increased ethylene concentrations corresponds to approximately half the amount that maize roots release over 12h (see results). To achieve the enrichment, 10 nl of ethylene in 10 µl of ambient air or ambient air only were injected into the soil with a gastight syringe 10 minutes before the experiment started.

Statistical analysis

All analyses were performed using the software package R, version 2.8.1. Data was first analyzed with Levene's and Kolmogorov-Smirnov tests to determine heteroscedasticity of error variance and normality. *D. virgifera* performance was compared using Student's t-tests. Host selection was analyzed using a log linear model as described (D'Alessandro & Turlings, 2006) and the proportion of choosing larvae were compared to control experiments using chi-square tests. When volatile emission data passed the Levene's and Kolmogorov-Smirnov tests, root volatiles were compared using student tests (t-test) and one-way ANOVAs. Pairwise comparisons following

ANOVAs were conducted using Tukey HSD tests. If the data did not pass the Levene's and the Kolmogorov-Smirnov tests, nonparametric Mann-Whitney U-tests or Kruskal-Wallis analysis of variance on ranks (H-tests) were carried out. Pairwise comparisons were realized by performing Dunn's tests. The correlation between CO_2 emission and larval preference was tested using Pearson's correlation coefficients.

RESULTS

Performance of D. virgifera *larvae on infested plants*

D. virgifera larvae gained over 30% more weight on plants that had been infested with conspecifics for two days compared to healthy plants (n=7; Student's t-test, t=-2.675, *df*=12, p=0.020; Fig, 1a). On the other hand, weight gain of *D. virgifera* was only a fourth on plants that were infested with *S. littoralis* larvae, as compared to healthy plants (control plants: n=14; infested plants: n=9; Student's t-test, t=2.515, *df*=21, p=0.020; Figure 1b).

D. virgifera *detects optimal host plants using volatile signals*

As in accordance with current literature (Bernklau & Bjostad, 1998), *D. virgifera* clearly preferred pure CO_2 or maize roots over controls (Figure S2a). When *D. virgifera* larvae were given a choice between healthy plants and plants infested with conspecifics, they significantly preferred the latter (n=20; glm, F= 7.418, *df*=38, p=0.009; Figure 1c). *D. virgifera* larvae were not attracted by conspecifics alone (n=9; Figure S2b). When offered control or leaf-infested plants, *D. virgifera* larvae preferentially selected healthy plants over *S. littoralis* infested plants (n=15; glm, F= 6.4257, *df*=28, p=0.017; Figure 1d). Removing leaves, larvae, frass and soil from the set-up did not change this preference (n=20; glm, *df=38*, F=7.7377, p=0.008; Figure 2).

Above- and belowground herbivory induce changes in root volatile emission

Maize roots infested with *D. virgifera* produced a distinct bouquet of volatiles compared to healthy roots: Infested roots released significant amounts of *(E)*-β-caryophyllene (EβC), a compound that was not detected in uninfested roots (Kruskal-Wallis One-way analysis of variance on ranks, *df=2*, H=21.083, p<0.001). Furthermore, α-humulene (Kruskal-Wallis One-way analysis of variance on ranks, *df*=2, H=7.499, p=0.024), hexadecanal (One-way ANOVA, *df*=20, F=13.655, p<0.001) and tetradecanal (One-way ANOVA, *df*=20, F=8.812, p=0.002) were emitted in greater quantities from infested plants (n=9; Figure 3a). Plants infested with *D. virgifera* were also found to emit less CO_2 than healthy plants (n=8; Student's t-test, *df*=14, t=2.767, p=0.015; Figure 3b), an effect that could be attributed to a loss of root biomass following herbivory (Figs. S3a and b).

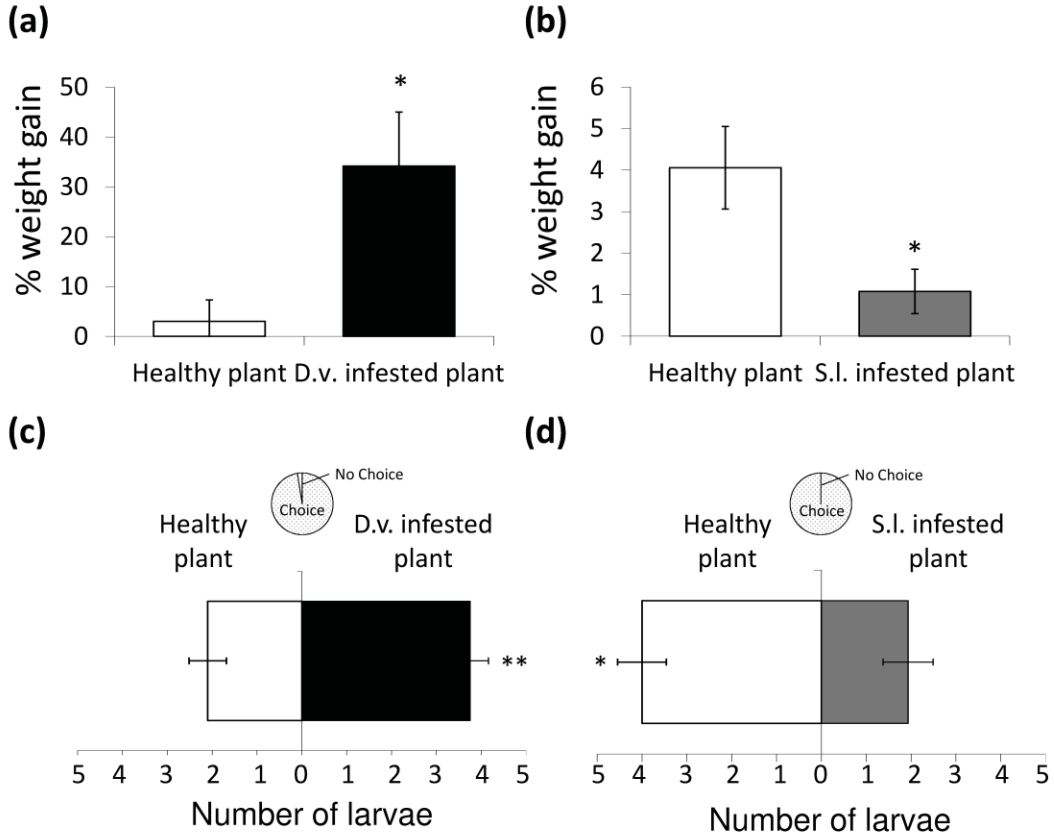

Figure 1: D. virgifera *selects optimal host plants*. Average (±SE) individual relative weight gain of *D. virgifera* larvae after 6 h of feeding on (a) healthy or *D. virgifera* infested plants, or (b) healthy or *S. littoralis* infested plants. (c) Average number (±SE) of larvae that chose volatiles from a healthy or a *D. virgifera* infested plant, and (d) volatiles from a healthy or a S. *littoralis* infested plant. Pie charts show the proportion of larvae that entered an arm. Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

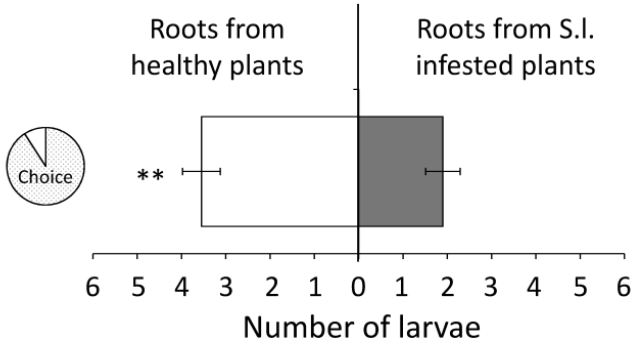

Figure 2: D. virgifera *larvae detect leaf-herbivore induced changes in root volatiles.* Average number (±SE) of larvae that chose isolated roots of uninfested plants or isolated roots of *S. littoralis* infested plants. The pie chart shows the percentage of larvae that entered an arm. Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

No difference in ethylene emission was noted between *D. virgifera* attacked and healthy plants (n=12; Student's t-test, *df*=22, t=1.309, p=0.204; Figure 3d). The GC-MS root volatile profile of plants whose leaves were infested by *S. littoralis* was not different to healthy plants (Figure 3a). There was also no effect of *S. littoralis* on root CO_2 emission (n=12; Student's t-test, *df*=22, t=0.814, p=0.424; Figure 3c). However, roots of *S. littoralis* infested plants emitted 50% less ethylene than root systems of healthy plants (n=14; Student's t-test, *df*=26, t=247.5, p=0.043; Figure 3e).

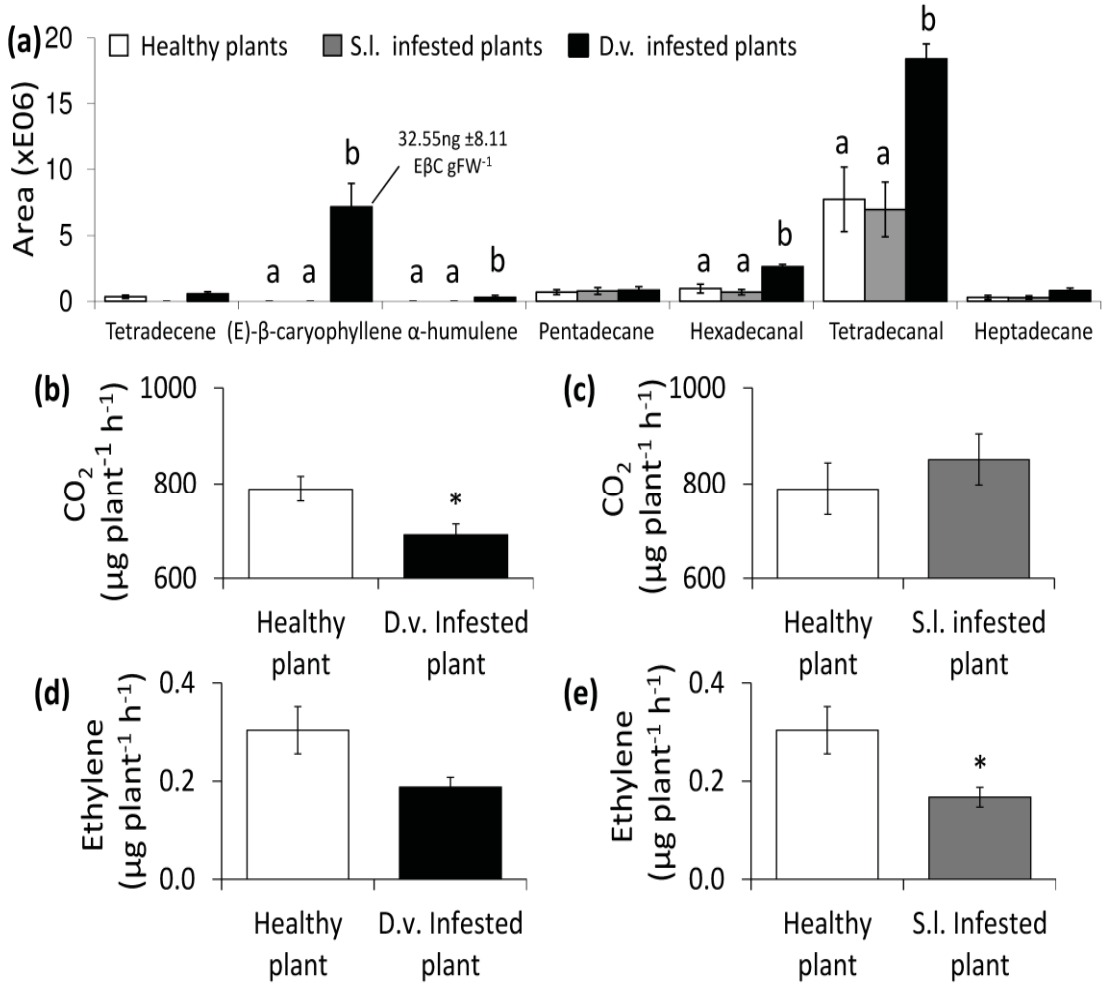

Figure 3: *Root volatile emission changes after leaf- and root attack.* (a) Average relative amounts (±SE) of root volatile compounds detected with solid phase micro extraction (SPME) GC-MS analysis. Different letters indicate significant differences ($p<0.05$). Average CO_2 emissions (±SE) of healthy and *D. virgifera* infested plants (b) and healthy and *S. littoralis* infested plants (c). Average ethylene emissions (±SE) of healthy and *D. virgifera* infested plants (d) and healthy and *S. littoralis* infested plants (e). Asterisks indicate significant differences between treatments (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

D. virgifera *can use* (E)-*β-caryophyllene and ethylene to locate optimal hosts*

Following the above results, we carried out a set of behavioral experiments to investigate the role of *(E)*-β-caryophyllene and ethylene in the attraction of *D. virgifera* to infested maize plants. We found that the preference of *D. virgifera* larvae for plants infested with conspecifics could be

counter-balanced by adding capillary dispensers releasing synthetic *(E)*-β-caryophyllene at a rate of approximately 40 ng h^{-1} to healthy roots (n=32; glm, *df*=62, F=0.0047, p=0.954; Figure 4a). When a maize variety that does not emit *(E)*-β-caryophyllene (variety "C") (Erb *et al.*, 2011a) was offered to the larvae, they did not distinguish between plants infested with conspecifics and healthy plants any more (n= 17; glm, *df*=32, F=0.0383, p=0.846; Figure 4a). Also, *D. virgifera* larvae were found to selectively orient towards healthy plants with *(E)*-β-caryophyllene diffusing dispensers rather than to healthy plants with control dispensers (n=10; glm, df=18, F= 20.696, p<0,001; Figure 4a). Interestingly, *(E)*-β-caryophyllene in the absence of plants did not attract *D. virgifera* larvae, even in a context of moderate CO_2 levels (Figure S5a and b). Adding *(E)*-β-caryophyllene releasing dispensers did not alter the emission of other root volatiles (Figure S6).

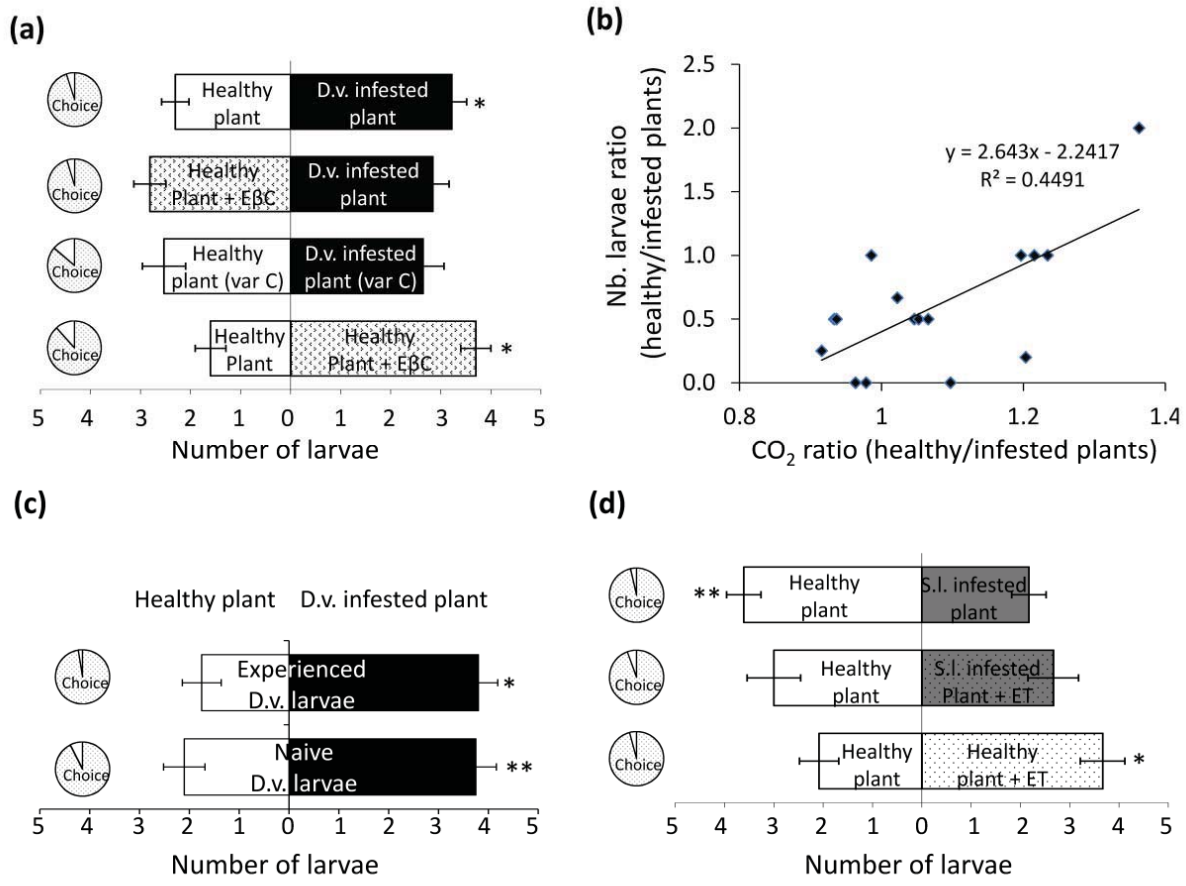

Figure 4: D. virgifera *larvae use root-derived cues to locate good quality hosts.* (a) *D. virgifera* choice between healthy (white bars) and *D. virgifera* infested plants (black bars) without (solid bars) or with added (*E*)-β-caryophyllene (speckled bars). Var C refers to a maize variety that does not emit *(E)*-β-caryophyllene. (b) Correlation between larval choice and CO_2 emission. Ratios between the numbers of larvae that preferred healthy plants over infested plants plotted against the ratio of CO_2 emission between healthy and infested plants are shown. (c) Average number (±SE) of experienced or naïve larvae choosing a healthy plant (white bars) or a plant infested with conspecifics (black bars). (d) *D. virgifera* host preference between healthy (white bars) and *S. littoralis* infested plants (grey bars) without (solid bars) or with ethylene addition (speckled bars). Pie charts show the percentage of larvae that entered an arm. Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

Because in a few individual cases, *D. virgifera* larvae preferred healthy over infested plants, we explored the relative role of CO_2 and *(E)*-β-caryophyllene in attracting *D. virgifera* in more detail. We found that the ratio of larval choice was positively correlated with the ratio of CO_2 between healthy and infested plants (n=16; Pearson's product moment correlation, *df*=14, Q_{obs}=3.376, p=0.004; Figure 4b). When the two source plants emitted similar amounts of CO_2, *D. virgifera* larvae oriented towards the infested plant (Figure 4b). This preference was reversed whenever healthy plants emitted more than 1.2 times more CO_2 than infested plants (Figure 4b). In an additional experiment, we found that the attraction of *D. virgifera* to infested plants is innate and not dependent on previous feeding experience in the presence of *(E)*-β-caryophyllene, as larvae that were reared on seedlings that did not emit the compound were equally attracted towards infested plants as experienced larvae that were reared on an emitting line (n=20 per treatment; glm, *df*=76, plant status: F=19.2850, p<0.001, larvae experience: F=0.1245, p=0.7252, Figure 4c).

To test the impact of ethylene on host selection behavior, we increased ethylene concentrations by 2 ppm by direct injection into the sand surrounding the roots 10 minutes prior to the preference assays. In the control experiment, *D. virgifera* larvae again preferred healthy plants over *S. littoralis*-infested plants (n=23; glm, *df*=44, F=8.048, p=0.007; Figure 4d). The addition of ethylene to the rhizosphere of *S. littoralis*-infested plants counterbalanced this effect, resulting in similar attractiveness of both odor sources (n=15; glm, *df*=28, F=0.199, p=0.659; Figure 4d). Furthermore, the injection of ethylene into the rhizosphere of a healthy plant made it more attractive for *D. virgifera* (n=12; glm, *df*=32, F=7.4381, p=0.012; Figure 4d).

DISCUSSION

This study demonstrates that soil dwelling herbivores can use herbivore induced plant volatiles to select the most suitable host plants. Previous infestation of maize plants by root or leaf herbivores changes the host quality for *D. virgifera*: Aggregation of the root feeder on the same host plant for instance was beneficial for the insect (Figure 1a). Similar effects have been documented for a number of leaf-feeding beetles (Dickens, 2006; Weed, 2010). Previous experiments show that *D. virgifera* is entirely resistant to benzoxazinoids, the major defensive secondary metabolites in maize roots (Robert *et al.*, 2012), and the results presented here add to the growing evidence that maize roots do not possess any effective defenses against this specialist feeder. Contrary to root herbivory, leaf-infestation by *S. littoralis* reduced the growth of *D. virgifera* via systemically induced changes in root physiology (Figure 1b). This result confirms earlier laboratory and field studies showing that leaf-feeders have a general negative impact on root herbivores (Erb *et al.*, 2009a; Gill *et al.*, 2011; Pierre *et al.*, 2011). Thus, even in a genetically uniform plant population,

D. virgifera larvae encounter plants of different suitability, which could have led to the evolution of efficient host-location and selection strategies.

Indeed, *D. virgifera* larvae were able to select host plants from a distance by using herbivore induced volatile signals: The root feeder was more strongly attracted to root-infested plants than to uninfested plants (Figure 1c). Changes in plant volatiles rather than larval cues were responsible for the observed differential attraction (Figure S2b). Similarly, *D. virgifera* oriented towards control plants rather than leaf-infested plants (Figure 1d). This preference was still there after removing leaves, *S. littoralis* larvae, frass and soil from the set-up (Figure 2), demonstrating that *D. virgifera* is able to detect systemic changes in root volatile emissions to avoid leaf-infested plants. This remarkable capacity to detect changes in root volatile signals to orient towards the most suitable hosts is likely to be adaptive for this highly specialized root feeder, as it optimizes its growth and fitness.

Leaf- and root herbivory resulted in distinct volatile patterns. Infested roots released large amounts of *(E)*-β-caryophyllene, a sesquiterpene that was not detected at all in uninfested roots (Figure 3a). *(E)*-β-caryophyllene is known to be emitted upon *D. virgifera* attack in maize (Rasmann *et al.*, 2005; Hiltpold, I. *et al.*, 2011b) and diffuses well through the soil (Hiltpold & Turlings, 2008). Plants infested with *D. virgifera* larvae also produced more α-humulene, hexadecanal and tetradecanal and less CO_2 than control plants (Figure 3). The reduction of CO_2 emission may be explained by a decrease of metabolically active root mass following root herbivore attack (Figure S3). Leaf-herbivory by *S. littoralis* did not change the abundance of most detected root volatile compounds, with the exception of ethylene, which was emitted in lower amounts by leaf-infested plants (Figure 3). Ethylene is a gaseous phytohormone involved in root growth (Yang & Hoffman, 1984), and the reduced emission may reflect leaf-herbivore-induced changes in elongation or branching, as they are known to occur in *Nicotiana attenuata* upon wounding (Hummel *et al.*, 2007). Determining ethylene emissions in the roots *in vivo* remains a technical challenge, as the sensitivity of GC-FID methods is insufficient to detect ethylene over short sampling intervals. The use of highly sensitive photo acoustic lasers may eventually make it possible to test the observed effect in real time and to exclude possible artifacts that may arise from i) removing the shoots of maize plants to measure root emissions and ii) the relatively long incubation period.

The choice-assays demonstrate that *D. virgifera* larvae can use *(E)*-β-caryophyllene as a signal to locate *D. virgifera* infested plants (Figure 4a) and ethylene to distinguish uninfested from *S. littoralis* infested plants (Figure 4d). As *(E)*-β-caryophyllene in the absence of plants did not attract *D. virgifera* larvae (Figure S5a and b), we suggest that the attraction of *D. virgifera* larvae to plants

infested with conspecifics stems from an attractive effect of *(E)*-β-caryophyllene within a plant volatile background. Our study adds to the growing evidence that semiochemicals, including sesquiterpenes, are only active in the presence of a plant background odor (Mumm & Hilker, 2005; Schroeder & Hilker, 2008). In addition to *(E)*-β-caryophyllene, CO_2 is a well-known attractant for *D. virgifera* (Strnad *et al.*, 1986; Johnson & Gregory, 2006) that may be used by the larvae to locate a host plant. In our assays, this volatile did apparently not serve by itself to distinguish root-infested plants from healthy plants, as emissions were lower in the more attractive plants (Figure 3b). Interestingly, however, the preference for root-infested plants was reversed whenever healthy plants emitted large amounts of CO_2 (Figure 4b). As previously demonstrated, high emissions of CO_2 can override the attractiveness of other volatile signals (Bernklau & Bjostad, 1998), possibly because *D. virgifera* larvae will be better off in some cases to feed on inferior roots that are close by rather than venturing over longer distances to reach a higher quality plant. Nevertheless, our results demonstrate that *(E)*-β-caryophyllene is an attractant for *D. virgifera*. Its attractiveness does not depend on previous feeding experience (Figure 4c) and is therefore innate.

D. virgifera larvae are frequently found to be clustered in maize fields (Ellsbury *et al.*, 1999), but this effect has not been attributed to plant-produced volatile signals, as it is known for aboveground coleopterans (Sakuma, 1994; Loughrin *et al.*, 1996; Soroka *et al.*, 2005; Dickens, 2006; Beran *et al.*, 2011). Based on our results, it seems possible that *(E)*-β-caryophyllene is one of the signals that *D. virgifera* can use to aggregate. As *(E)*-β-caryophyllene also attracts entomopathogenic nematodes to herbivore-infested plants (Rasmann *et al.*, 2005), it is tempting to speculate about the evolution of its induced emission. In the light of our results, a possible scenario is that *(E)*-β-caryophyllene initially served to protect wounded sites of maize roots against opportunistic and pathogenic microorganisms in the soil. Several studies suggest that *(E)*-β-caryophyllene acts as an antibiotic (Alma *et al.*, 2003; Lourens *et al.*, 2004; Pichette *et al.*, 2006; Huang *et al.*, in press), and we have previously shown that it is released directly from the wounded tissue rather than systemically (Hiltpold, I. *et al.*, 2011b), which supports the notion that it serves an antimicrobial role at the site of injury. Over evolutionary time, the signal may then have been hijacked by *D. virgifera*, as a host location and aggregation kairomone, and by entomopathogenic nematodes as a cue to locate root herbivores. The attraction of the beetle larvae to this compound could also explain why it is no longer emitted by American maize cultivars (Köllner *et al.*, 2008), as breeders may have unknowingly selected for less attractive maize lines.

The ethylene complementation experiments reveal that this compound is attractive to *D. virgifera* as well (Figure 4d). Ethylene has previously been described as an attractant for a variety of insects such as moths (Raina *et al.*, 1992) and beetles (Arita *et al.*, 1988; Gonzalez & Campos, 1996), and the experimental evidence presented here suggests that *D. virgifera* can use ethylene to locate plants

that are leaf-herbivore free. It remains to be determined how specific this signal is as an indicator for the presence of leaf-feeders. From a physiological perspective, it seems likely that ethylene is a general belowground indicator for plant growth and quality (Pierik *et al.*, 2006), and we hypothesize that *D. virgifera* integrates this signal as a general cue for healthy and vigorously growing plants rather than using it as a specific signal to detect leaf-herbivores.

So far, the dispersal and distribution of *D. virgifera* in the field has been shown to be determined by soil texture (Ellsbury *et al.*, 1994), moisture (Ellsbury *et al.*, 1994), porosity (Gustin & Schimacher, 1989), and plant density (Toepfer *et al.*, 2007). The distribution of other root herbivores is known to depend on vegetation cover (Toepfer *et al.*, 2007) and non-volatile plant metabolites (Johnson *et al.*, 2005). Our study reveals an additional important role of plant volatiles in the distribution of soil insects. The maize specialist *D. virgifera* seems to have evolved recognition mechanisms to detect specific changes in volatile emissions from roots in order to locate plants of superior quality and avoid plant-mediated competition from a distance. Although it has been suggested that *D. virgifera* has poor sensory capabilities to use volatiles for orientation (Bernklau & Bjostad, 1998), our assays show that the beetle larvae can recognize at least two additional specific signals apart from CO_2 and use them for successful host location.

CONCLUSION

From an ecological perspective, our study shows that the distribution and abundance of belowground herbivores is influenced by their capacity to locate and evaluate plant quality from a distance, and suggests that both above- and belowground herbivory can influence the structure of soil dwelling communities via indirect, plant-volatile mediated effects. From an applied perspective, the results may be relevant for the development of push-pull approaches in crop protection, which combine attractive and repellent plants to lure herbivores away from the crops and attract natural enemies into the field (Cook *et al.*, 2007). Our results could help to establish a basis for such an approach against *D. virgifera*. Identifying plants that have an increased turnover of root mass resulting in high emissions of CO_2 and ethylene, combined with either natural or engineered production of *(E)*-β-caryophyllene (Degenhardt *et al*., 2009) may be the ideal trap crop for *D. virgifera* larvae. The fact that entomopathogenic nematodes are also attracted by *(E)*-β-caryophyllene would further increase the efficacy of the approach, as this would result in aggregation of *D. virgifera* larvae on roots together with the biocontrol agent.

ACKNOWLEDGEMENTS

We thank Roland Reist from Syngenta (Stein, CH) for providing *S. littoralis* eggs. Wade French and Chad Nielson (USDA-ARS-NACRL Brookings, USA) supplied *D. virgifera* eggs. Research activities by C.A.M.R., M.E., M.D., C.Z., G.R.D., N.V., and T.C.J.T. were supported by the Swiss National Science Foundation (FN 31000AO-107974). This project was partially funded by the National Centre of Competence in Research (NCCR) 'Plant Survival', a research program of the Swiss National Science Foundation.

SUPPLEMENTARY FIGURES

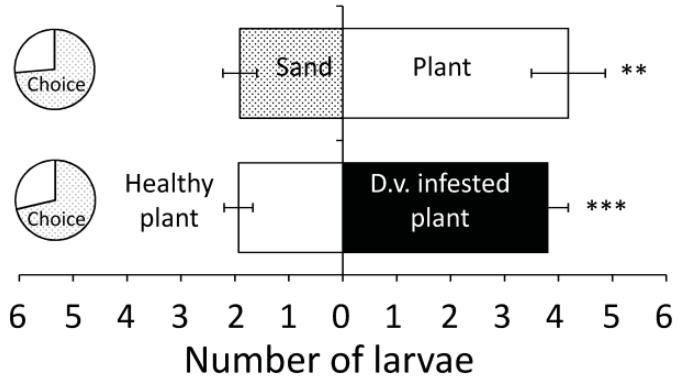

Figure S1: Attraction of *D. virgifera* larvae. (a) Average number (±SE) of larvae that chose sand vs. a healthy plant (n=11, glm, *df*=20, F=10.583, p=0.004) or a healthy plant vs. a root-infested plant (n=15, glm, *df*=28, F=16.533, p<0.001) in dual choice olfactometers with sand-filled central connectors. Pie charts show the percentage of larvae that entered an arm. Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

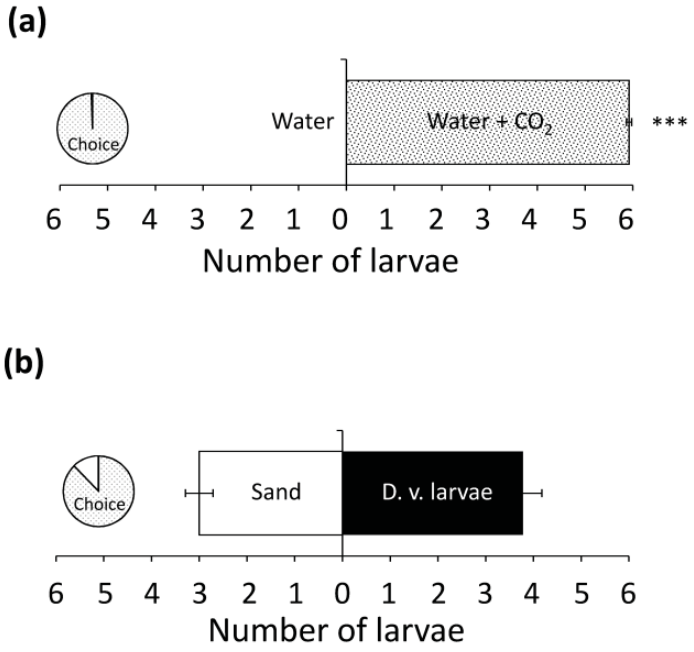

Figure S2: *Attraction of* D. virgifera *larvae*. (a) Larval attraction to 7 ml of still water vs. 7 ml of sparkling water (resulting in a CO_2 emission of 3000 ppm, n=25, glm, *df=48*, F=31 686, p<0.001). (b) Attraction of *D. virgifera* larvae to sand (white bar) and *D. virgifera* larvae (black bar, n=9, glm, *df=16*, F=2.5462, p=0.130). Pie charts show the percentage of larvae that entered an arm. Asterisks indicate significant differences between treatments (*: p<0.05, **: p<0.01, ***: p<0.001).

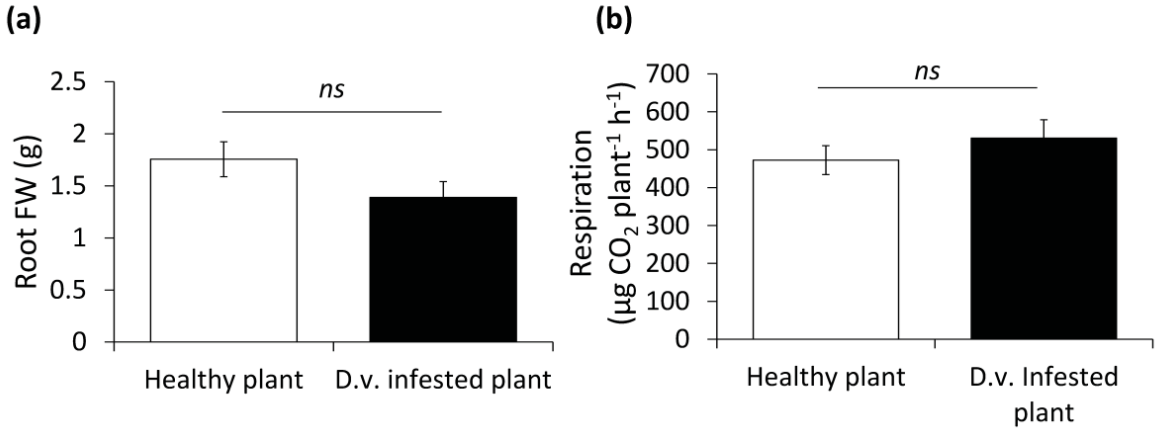

Figure S3: Biomass and CO_2 emission of infested roots. (a). Average fresh weight (FW) (±SE) of the root systems of healthy or *D. virgifera* infested plants (n= 8, Student's t test, t=1.618, *df*=14, p=0.1279). (b) CO2 emission per gram of fresh root tissue for healthy and infested plants (n=8, Student's t test, *df*=14, t=-0.945, p=0.360).

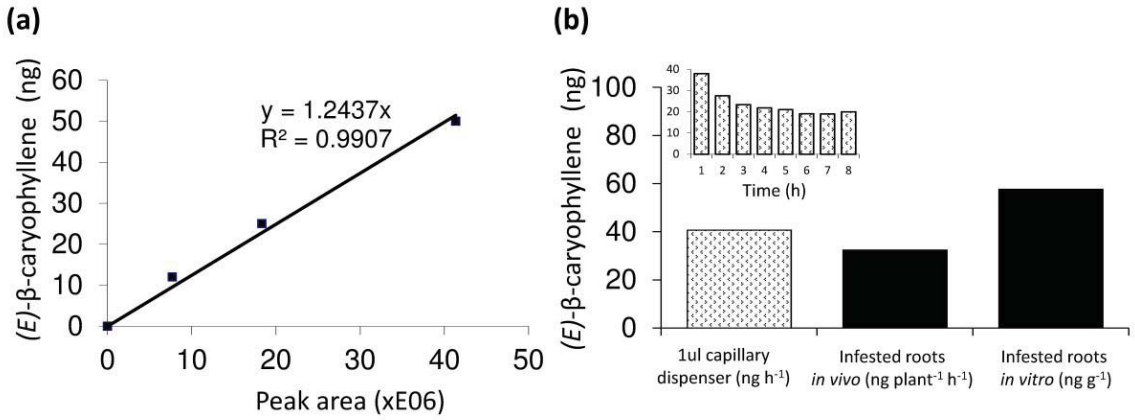

Figure S4: Quantification of *(E)*-β-caryophyllene emissions. (a) A Standard curve for *(E)*-β-caryophyllene was obtained by diluting known amounts of pure *(E)*-β-caryophyllene in 0.1% EtOH (v/v) and determining the detector response after SPME-GC extraction and separation. (b) *(E)*-β-caryophyllene release by 1µl capillary dispensers (speckled bars) and roots infested with *D. virgifera* larvae (black bars) were measured. Release was measured from roots *in vivo* (whole root system placed in vial) and *in vitro* (roots ground to fine powder). Inset: *(E)*-β-caryophyllene emission of the capillary dispenser over a period of 8 hours. All treatments were repeated 2-3 times, and the highest detected values are shown.

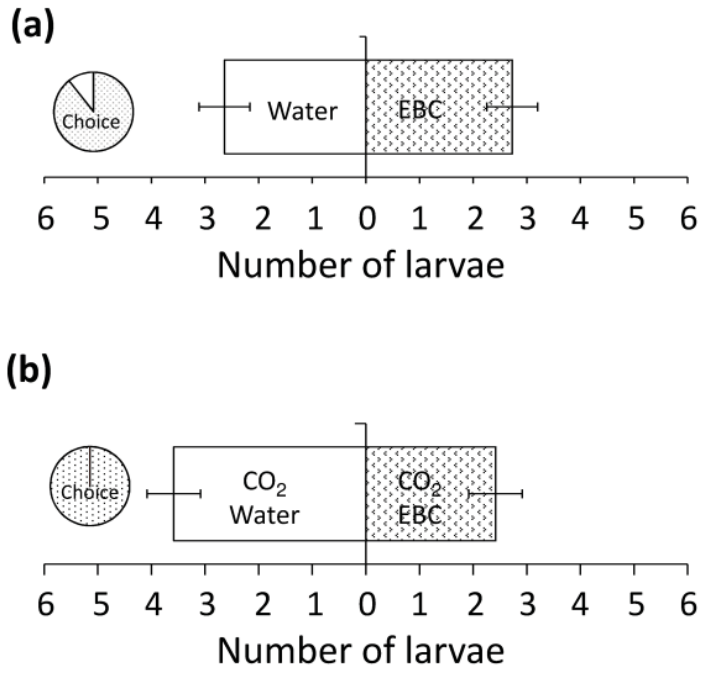

Figure S5: *(E)*-β-caryophyllene is not attractive by itself, even in the context of moderate CO2 levels. (a). Attractiveness of dispensers with *(E)*-β-caryophyllene or water (n=11, glm, *df*=20, F=0.0222, p=0.883). (b). Attractiveness of dispensers with *(E)*-β-caryophyllene or water in the context of moderate CO_2 levels provided by 10mL of sparkling water (1000ppm) (n=12, glm, *df*=22, F=2.642, p=0.118). The pie charts indicate the percentage of larvae that entered an arm.

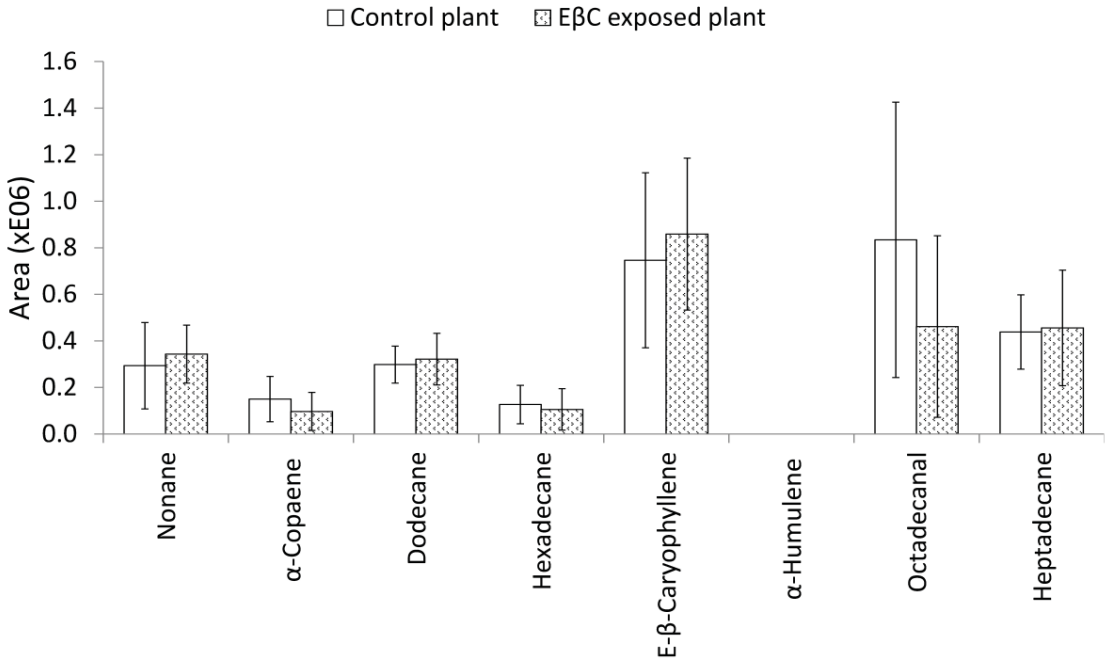

Figure S6: (E)-*β-caryophyllene (EβC) does not affect volatile production in roots*. Average (±SE) peak area (x10^6) of root volatiles for healthy plants (white bars, n=7) and infested plants exposed to *(E)*-β-caryophyllene (n=5) for 24 hours. No statistically significant differences were detected.

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CHAPTER II

Genetically engineered maize plants reveal distinct costs and benefits of constitutive volatile emissions in the field

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ABSTRACT

Genetic manipulation of plant volatile emissions is a promising tool to enhance plant direct and indirect defences against herbivores. However, the potential ecological and physiological costs associated with the manipulation of specific volatile synthase genes are unknown. Therefore, we investigated the physiological and ecological effects of transforming a maize line with an oregano terpene synthase gene in field and laboratory assays, both above- and below-ground. The transformation, which resulted in the constitutive emission of (*E*)-β-caryophyllene and α-humulene, was found to compromise seed germination, plant growth and yield. These physiological costs provide a possible explanation for the inducibility of an (*E*)-β-caryophyllene-synthase gene in wild and cultivated maize. The overexpression of the terpene synthase gene did not impair plant resistance nor indirect defenses, suggesting that genetic engineering of volatile production does not compromise other defenses. However, terpenoid emission increased plant apparency to herbivores, including adults and larvae of the aboveground pest *Spodoptera frugiperda*, resulting in an increase in leaf-damage. The opposite effect was observed below-ground: Although terpenoid producing lines were attractive to the specialist root herbivore *Diabrotica virgifera virgifera*, they did not suffer more root damage in the field, possibly because of the enhanced attraction of entomopathogenic nematodes. Furthermore, fewer adults of the root herbivore *Diabrotica undecimpunctata howardii* were found to emerge near plants that emitted (*E*)-β-caryophyllene and α-humulene. Yet, overall, under the given field conditions, the costs of constitutive volatile production overshadowed its benefits. This study highlights the need for a thorough assessment of the physiological and ecological consequences of genetically engineering plant signals in order to determine the potential of this approach for sustainable pest management strategies. For the specific transformation that we studied here, the results are encouraging in light of current efforts to insert an herbivore-inducible promoter in front of the (*E*)-β-caryophyllene-synthase gene.

INTRODUCTION

Upon herbivory, plants emit a blend of volatile organic compounds (herbivore-induced plant volatiles, HIPVs) that act as direct and indirect defence against phytophagous arthropods. HIPVs may repel adult Lepidoptera and Hemiptera (Turlings & Wackers, 2004) and act as oviposition deterrents for many herbivores (Karban & Baldwin, 1997; De Moraes *et al.*, 2001a; Kessler & Baldwin, 2001; Sanchez-Hernandez *et al.*, 2006). Furthermore, they are attractive for natural enemies of herbivores (Dicke & Sabelis, 1988; Turlings *et al.*, 1990; Paré *et al.*, 1999; Paré & Tumlinson, 1999; Rasmann *et al.*, 2005; Heil, 2008). Altogether, direct and indirect effects of HIPV emissions reduce herbivore loads and feeding damage (Gomez & Zamora, 1994; Karban *et al.*, 1997; Agrawal, 1999; van Loon *et al.*, 2000; Hoballah & Turlings, 2001; Kessler & Baldwin, 2001; Rasmann *et al.*, 2011), but see (Hare, 2011).

Defensive traits can be costly if they drain resources that could otherwise be invested in reproductive output (Herms & Mattson, 1992; Heil & Baldwin, 2002; Strauss *et al.*, 2002; Moore *et al.*, 2003; Agrawal, 2011). Metabolic costs of HIPV production are expected to be minor due to their low molecular weight and the small quantities in which they are emitted (Dicke & Sabelis, 1990; Halitschke *et al.*, 2000). Indeed, Hoballah et al. (2004) measured only a transient cost to growth in maize plants that were artificially induced to emit HIPVs. Similarly, trade-offs between HIPV production and direct defence have rarely been observed (Koricheva *et al.*, 2004; Erb *et al.*, 2011a; Rasmann *et al.*, 2011), but see (Ballhorn *et al.*, 2008), possibly because the defensive traits do not share limiting resources and synergistically enhance plant protection (Agrawal & Fishbein, 2006; Rasmann & Agrawal, 2009). However, HIPV emission may pose ecological costs by increasing plant apparency to herbivores (Turlings & Wackers, 2004; Halitschke *et al.*, 2008), stimulating feeding behaviour of lepidopteran larvae (Halitschke *et al.*, 2004; Carroll *et al.*, 2006), synergizing with beetle derived aggregation or sex pheromones (Loughrin *et al.*, 1995; Reddy & Guerrero, 2004) and disrupting the plant's interactions with beneficial organisms such as pollinators (Kessler *et al.*, 2011; Lucas-Barbosa *et al.*, 2011) (for reviews about costs of induced resistance, see (Heil, 2002; Walters & Heil, 2007).

Several recent attempts to manipulate plant volatile emissions through genetic engineering have been successful (Degenhardt *et al.*, 2003; Kappers *et al.*, 2005; Beale *et al.*, 2006; Degenhardt *et al.*, 2009). Terpenoid production has been the most frequently target in this context, as terpenoids dominate HIPV blends of many plants (Schnee *et al.*, 2002; Degenhardt *et al.*, 2003; Dudareva *et al.*, 2004; Cheng *et al.*, 2007; Mumm & Dicke, 2010; Tholl *et al.*, 2011) and are assumed to play a major role in the attraction of natural enemies of herbivores (Paré & Tumlinson, 1999). Furthermore, induction of terpenoid release is often much more pronounced in response to

herbivory than to mechanical damage (Turlings *et al.*, 1998) and their emission is often systemic and prolonged (Paré & Tumlinson, 1999).

Engineering plants that constitutively emit terpenoids has resulted in a series of promising experiments that demonstrate repellency to herbivores and attraction of their natural enemies in both the laboratory and the field. For instance, *Arabidopsis thaliana* plants overexpressing a dual linalool/nerolidol synthase (FaNES1) repel aphids (Aharoni *et al.*, 2003) and tobacco plants genetically engineered to release cembatriene-ol also show reduced aphid colonization (Wang *et al.*, 2001). *A. thaliana* plants engineered to release (3S)-(*E*)-nerolidol and (*E*)-β-farnesene attract more predatory mites, and ladybugs have been found to spent significantly more time on such plants (Kappers *et al.*, 2005; Beale *et al.*, 2006). The transformation of *A. thaliana* with a maize *tps10* gene, a terpene synthase that produces (*E*)-β-farnesene and (*E*)-β-bergamotene, was found to increase the attraction of the transformed *A. thaliana* to parasitic wasps, but only after wasps had learned to associate the volatiles with host presence (Schnee *et al.*, 2006).

Of specific interest to the current study is an (*E*)-β-caryophyllene (EβC) synthase gene from oregano that was used to restore the emission of this sesquiterpene in an American maize line (Degenhardt et al., 2009). Most American lines have lost the ability to synthesize EβC and α-humulene, whereas the roots from most non-American maize lines release these compounds in considerable amounts in response to rootworm feeding, thereby attracting entomopathogenic nematodes (Rasmann *et al.*, 2005; Kollner *et al.*, 2008). In field experiments, it was found that transformed lines with the restored emission of EβC and α-humulene were indeed better protected against root damage through the enhanced attraction of nematodes (Degenhardt et al., 2009). Such engineered plants provide powerful models to evaluate both physiological and ecological costs associated with constitutive emission of volatiles. Knowing these costs could provide the basis for a better understanding of the evolution of inducibility and would be important in order to fully evaluate the potential of the manipulated plants in agriculture.

Yet, no study has comprehensively addressed this important issue. To do so, we used terpenoid engineered maize lines that had undergone the above-mentioned transformation to constitutively emit EβC and α-humulene (Degenhardt et al., 2009) and compared them to their isogenic counterparts in both field and laboratory assays in order to estimate the physiological and ecological costs and benefits of constitutive emission of the terpenoids.

METHODS

Plants, insects and entomopathogenic nematodes

Plants. Maize plants (*Zea mays L.,* variety HiII, and transformed lines) were provided by the Institute of Pharmacy, Halle, Germany. The plant transformation procedure was followed as previously described (Frame *et al.*, 2002). Briefly, the maize variety HiII was transformed with an EβC synthase gene from *Origanum vulgare* L. (Crocoll *et al.*, 2010), under the control of a maize ubiquitin promoter (Christensen & Quail, 1996). The transgenic maize lines 201-L1, 202-L2, 201-L3, and 202-L5 were obtained from independent transgenic calli. Selfed T_1 generations of the maize lines, including HiII as non-transformed controls, were used for both field and lab experiments.

Field experiments were conducted with the three transformed lines 201-L2, 202-L2, 202-L5, that constitutively overexpress the EβC synthase, and the non-transformed control HiII in 2007 and with 201-L1, 202-L2, 201-L3 and HiII in 2009. Plants of the variety Pioneer 33M16 were used as buffer plants in both years.

Laboratory assays were performed with 201-L1, 202-L2, and 202-L5 because 201-L3 seeds were not available at the time of experiments. Isogenic HiII was used as the non-emitting control. Maize plants were sown in plastic pots (11 cm high, 4 cm diameter) with washed sand (0-4 mm, Jumbo, Switzerland) and topped with 2 cm of commercial soil (Aussaaterde, Ricoter, Aarberg, Switzerland). Seedlings were grown in a climate chamber (23 ± 2 °C, 60% relative humidity, 16:8h L/D, and 50'000 $mmol.m^{-2}$) and MioPlant Vegetable and Herbal Fertilizer (Migros, Switzerland) was added every two days after plant emergence. Twelve-day old plants with two fully expanded leaves were used for the experiments.

Insects. *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) eggs were provided by French Agricultural Research Inc. (Lamberton, MN, USA). *D. undecimpunctata howardii* Barber (Coleopteran: Chrysomelidae) eggs were provided by Crop Characteristics, Inc. (Farmington, MN, USA). Second-instar larvae were used for all experiments. *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) larvae were collected from maize fields in Brazil in 2008 and reared in the laboratory on artificial diet (Greene *et al.*, 1976) until pupation. Pupae were transferred to cages (30 x 30 x 30 cm). Adults were fed on water solution containing 10% honey (v/v) and oviposited egg masses were collected daily. The rearing colony was maintained at 25 ± 2°C, 70% R.H., 14:10h L/D. Second-instar larvae were used in all laboratory assays.

Entomopathogenic nematodes (EPN). *Heterorhabditis bacteriphora* Poinar and *H. megidis* Poinar, Jackson & Klein (Rhabditida: Heterorhabditidae) were provided by Andrew Brown from Becker Underwood Ltd (Littlehampton, West Sussex, UK).

Field experiments

Field experiments were conducted in 2007 (also see (Degenhardt et al., 2009)) and 2009, at the Bradford Research Extension Center (Columbia, MO, USA). In both years, plants were sown with 43-cm spacing between plants and 76-cm spacing between rows. All plots were surrounded by two rows of buffer plants. Because of low germination, two weeks after sowing, seedlings were transplanted where needed to fill the gaps in the 2007 and 2009 plots. In 2007, experimental plots (n=21 per line) consisted of one rows of eight maize seedlings of the same maize line. All plants were sown on May, 22. Fifteen of the plots were infested with 600 *D. virgifera* eggs, and EPN (*H. megidis*) were applied along 10 of those plots. The remaining plots were left uninfested. In 2009, experimental plots (n=24 per line) consisted of two rows of three plants of the same maize line. Plants were sown on June, 23. Plots were separated by two buffer plants. Half of the 2009 plots were infested with 600 *D. virgifera* eggs 15 days after germination. Fourteen days later, entomopathogenic nematodes (*H. bacteriophora*) were released in the centre of each plot.

Plant growth and yield. In 2007, the height of all plants was measured 15 days after sowing. At the end of the season, corn cobs were harvested. Husks were removed before the individual fresh weight was recorded. In 2009, the germination rate of the different line was evaluated 15 days after planting.

Leaf damage and leaf herbivore identification in field. In 2009, leaf damage was recorded on July 30, using a homemade transparent plastic scale with different shapes and surface areas (that ranged from 0.1 cm^2 to 20 cm^2). The entire surface damage of a plant was recorded. Average damaged surface and scores per plant per plot was calculated. On July 30, leaf feeders were collected and kept in vials filled with absolute alcohol until identification.

Root damage and soil-dwelling herbivore collection from field. In 2009, three plants were removed from each plot on August, 4^{th}. The entire root system of each of those plants was individually placed into onion bags and the bags were suspended in a greenhouse to collect *D. virgifera* larvae from the roots. A water bowl was positioned under each onion bag to collect *D. virgifera* larvae that fell down and to prevent them from escaping. Larvae were collected twice a day until no larva was recovered anymore for four consecutive days. After this period, root damage was rated using Oleson's node injury score (Oleson *et al.*, 2005), and average damage per

plant per plot was calculated. Modified emergence cages (78 x 36 cm) (Pierce & Gray, 2007) were placed on individual plants left in field. Adult *D. virgifera* and *D. undecimpunctata howardii* (Coleoptera: Chrysomelidae) were collected once a week or 7 weeks. The average number of emerging adults per plant was calculated for each plot.

Plant phenotype identification. In order to characterize the plant phenotype of each individual (EβC producing or non-producing), the second youngest leaf of every plant was sampled and immediately frozen in liquid nitrogen (Field experiments 2007 and 2009). The presence of EβC in leaves was investigated using SPME-GC-MS analysis following a previously described protocol (Erb *et al.*, 2009a): Leaves were ground in liquid nitrogen using a pestle and a mortar. The obtained powder (0.3 g) was placed in a glass vial (20 mL, Supelco, Sigma-Aldrich Co. LLC, US) with a septum in the lid. A 100 µm poly-dimethylsiloxane (PDMS) solid phase micro extraction (SPME; Supelco c/o Sigma-Aldrich Chemie GmbH Buchs, Switzerland) fibre was inserted in the vial and exposed for 20 min at 35 °C. The fibre was then automatically inserted into the injector port of a gas chromatograph (Agilent 6890 series GC system G1530A) heated at 250 °C. The sample was injected on a non-polar column (DB1-MS, 30 m, 0.25 mm internal diameter, 0.25 µm film thickness; J &W Scientific) under constant flow of helium (50.6 kPa). Following the injection, the column temperature was maintained at 60 °C for 1 minute before ramping to 220°C at a rate of 10 °C per minute followed by a post-run of 3 minutes at 250 °C. The gas chromatograph was coupled to a quadrupole type mass selective detector (Agilent 5973; transfer line 230 °C, source 230 °C, ionization potential 70 eV). The obtained peaks were analysed and identified using the CMS data analysis software (Agilent Technologies Inc.) by comparing volatile retention times and mass spectra with those of the NIST05 Mass Spectra Library and those of pure compounds. Plots of terpenoid-engineered plants that counted less than three EβC producing plants were excluded from all analyses, with the exception of *D. virgifera* adult emergence, as too few replicates were left per treatment after excluding such plots.

Laboratory experiments

Plant phenotype identification. The presence of EβC in the volatile bouquet of transformed plants was controlled prior to any laboratory experiment using gas chromatography coupled to mass spectrometry as described in (Turlings *et al.*, 2004). Briefly, plant leaves were placed in glass bottles and connected to an air delivery system. Purified air was pulled in the bottles at a rate of 1.1 $L.min^{-1}$ and pulled through a Super-Q filters (25 mg, 80-100 mesh; Alltech Associates, Deerfield, IL, USA) at a rate of 0.7 $L.min^{-1}$ for four hours. Volatiles were extracted from the filters with 150 µL dichloromethane (Super solvent, Merck, Dietikon, Switzerland) and 200 ng of n-octane and n-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µL dichloromethane

were added as internal standards. Aliquots of 2 µL of each sample were injected into a gas chromatograph (Agilent 7890A) coupled to a mass spectrometer (Agilent 5975C VL MSD with Triple-Axis Detector; transfer line 230 °C, source 230 °C, ionization potential 70 eV). Samples were injected on a non-polar column (HP-1 MS, 30 m, 0.25 mm ID, 0.25 mm film thickness, Alltech Associates, Inc) under constant flow of helium (0.9 $mL.min^{-1}$) as carrier gas. After injection, the temperature was maintained at 40°C for 3.5 min before ramping to 100 °C at 8 $°C.min^{-1}$, then to 200 °C at 5 °C.min-1, followed by a post-run of 5 min at 250°C. Volatiles were identified using the CMS data analysis software (Agilent Technologies Inc.) by comparing volatile retention times and mass spectra with those of the NIST05 Mass Spectra Library and those of pure compounds. The amount of volatiles emitted was corrected for the leaf fresh biomass. Transformed seedlings that did not release any EβC were not used in the experiments.

Plant biomass. The plant biomass was determined under laboratory conditions. The leaf fresh weight of 15 day-old plants was measured and leaves were then wrapped in aluminum foil and dried into an oven at 100 °C for 48 hours. After this period, leaves were left in a desiccator for an hour. Leaf dry weight was measured and water content was calculated.

Spodoptera frugiperda *larval performance.* The herbivore performance on terpenoid engineered plants was assessed by adding one pre-weighed first instar larva on transformed maize plants or on the non-transformed line, HiII. Transparent 1.5 L PET bottles were placed upside down over the aboveground part of the plants as described elsewhere (Erb *et al.*, 2011c), to prevent the larvae from escaping. After a week, *S. frugiperda* larvae were collected and their relative weight gain calculated.

S. frugiperda *larvae host selection. S. frugiperda* attraction to the different maize lines was investigated using a 6-arm olfactometer (Turlings *et al.*, 2004). Briefly, six glass bottles were each connected to one arm of the olfactometer. The position of each plant was randomly determined for each olfactometer. The central chamber was turned upside down to prevent the larvae from escaping. Purified and humidified air was blown through the system via ports of the glass bottles at a rate of 0.9 $L.min^{-1}$. Twenty first instar larvae were released in the central part of the system for 30 minutes. After this delay, the number of larvae in each arm was counted. Four consecutive releases of larvae were conducted for each olfactometer.

Spodoptera frugiperda *oviposition preference.* To assess the preference of adult *S. frugiperda* to oviposit on terpenoid engineered plants, one plant of each line was placed in a plastic pyramidal transparent tent (55 x 55 x 55 cm, MegaView Science Education Services Co., Ltd, Taichung, Taiwan). The four plants were positioned at vertexes of a 25 cm length square. The

position of each line was randomly determined in each tent. Two days after pupation, twelve *S. frugiperda* pupae were added in the centre of the square together with a 10% honey solution as a food source for the adult moths. The presence of eggs on the plants was checked every day after adult emergence. The experiment ended the first day eggs were found on a plant, and the number of egg masses on each plant was recorded.

D. virgifera larval performance. The performance of *D. virgifera* larvae was assessed by infesting maize seedlings with six pre-weighed larvae. Two days later, the larvae were collected and weighed again. Their relative weight gain was calculated.

D. virgifera *and* D. undecimpunctata howardii *host plant selection*. The host selection by the root herbivores was conducted using belowground 6-arm olfactometer (Rasmann *et al.*, 2005). Briefly, one plant of each line (control, 201-L1, 202-L2 and 202-L5) was potted in a glass pot with a horizontal connector (29/32 mm) at 0.5 cm height and filled with moist (10% water) white sand (Migros, Switzerland). Pots without plants were filled with moist white sand only. The position of the pots in the system was randomly determined for each olfactometer. The pots were connected to an empty central chamber (8 cm in diameter, 11 cm deep) using glass tubes (24/29 mm, 8 cm long) with Teflon connectors at both sides (24/29 mm to 29/32 mm). The Teflon connectors contained a fine metal screen (2,300 mesh; Small Parts Inc., Miami Lakes, FL, US), which prevented the larvae from reaching the roots. The system was wrapped in aluminum foil to keep the root systems in the dark and avoid visual cues for the larvae. The system was connected for one hour before adding 20 larvae in the middle of the central chamber. Larvae were allowed to choose for 15 minutes. For each species, four to six releases of larvae was performed per olfactometer.

Statistical procedures

All analyses were performed using the software package R, version 2.8.1. Data was first analyzed with Levene's and Kolmogorov-Smirnov tests to determine heteroscedasticity of error variance and normality. If volatile emission, plant growth and yield, and herbivore performance data passed the Levene's and Kolmogorov-Smirnov tests, the data were compared using one-way ANOVAs; else, nonparametric Kruskal-Wallis analysis of variance on ranks (H-tests) were carried out. Pairwise comparisons were conducted using post-hoc Holm-Sidak tests and Tukey Honest Significant Differences (HSD) or Dunn's tests respectively. Additionally, transgenic lines were compared to the non-transformed line using t contrasts. Herbivore preference for plant phenotype was analyzed using a log linear model as described elsewhere (D'Alessandro & Turlings, 2006).

RESULTS

Developmental and fitness costs of (E)-*β-caryophyllene synthase overexpression*

Genetically engineered plants clearly suffered developmental costs: While non-transformed plants had a germination rate of 73.6%, only half of transformed maize plants germinated (n=24; Kruskal-Wallis on ranks, *df*=3, H=31.813, p<0.001; Tukey HSD: HiII *vs*. 202-L2: p <0.001, HiII *vs*. 202-L3: p <0.001 and HiII *vs*. 201-L1: p< 0.001; t contrast on ranks: *df*=95, t=-6.648, p<0.001; Figure 1a).

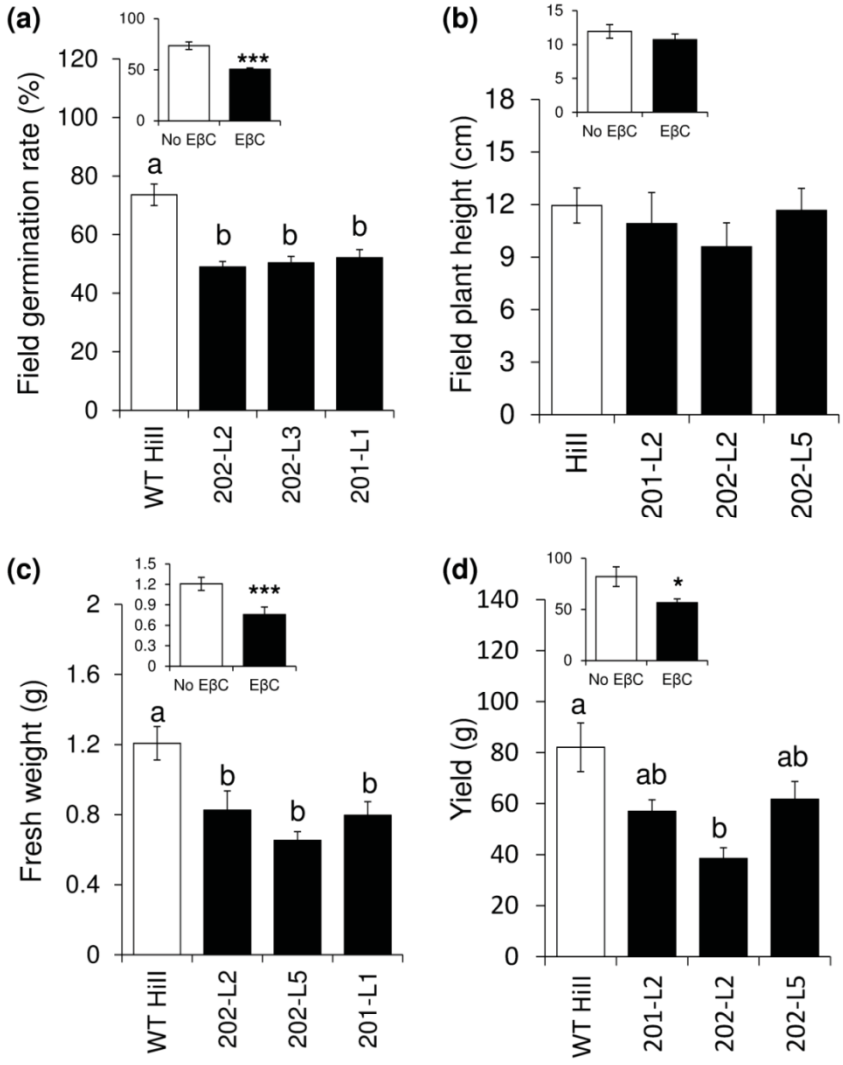

Figure 1: *Overexpression of the* (E)-*β-caryophyllene synthase products,* (E)-*β-caryophyllene and α-humulene, affect plant development and yield.* (a) Field germination rate (mean ±SE) of the non-transformed line and individual (main) or combined (inset) EβC synthase-overexpressing lines. (b) Fourteen day-old plant height in field (mean ±SE) of the non-transformed line and individual (main) or combined (inset) EβC synthase-overexpressing lines. (c) Fourteen day-old fresh weight (mean ±SE) of the non-transformed line and individual (main) or combined (inset) EβC synthase-overexpressing lines under laboratory conditions. (d) Ear wings fresh weight (mean ±SE) of the non-transformed line and individual (main) or combined (inset) *tps2*- overexpressing lines in field. The white bar corresponds to the non-transformed line, while black bars refer to EβC synthase-overexpressing maize lines. Different letters indicate significant differences (p<0.05). Stars indicate significant differences (*: p<0.05, **: p< 0.01, ***: p<0.001).

Even though in the field no difference in plant height was observed (n_{HiII} =21,$n_{201\text{-}L2}$=20, $n_{202\text{-}L2}$=19, $n_{202\text{-}L5}$=21; one-way ANOVA, *df*=77, F=1.796, p=0.155; t contrast, t=0.392, *df*=79, p=0.700; Figure 1b), the overexpression of the introduced EβC-synthase gene reduced the leaf biomass by 25% compared to non-transformed plants under laboratory conditions (n_{HiII} =8, $n_{201\text{-}L1}$=13, $n_{202\text{-}L2}$=15, $n_{202\text{-}L5}$=12; one-way ANOVA, *df*=44, F=8.627, p<0.001; Tukey HSD: HiII *vs*. 202-L2 and 201-L1: p<0.01; HiII *vs*. 202-L5: p<0.001; t contrast: *df*=46, t=-4.834, p<0.001; Figure 1c). Water contents were similar for all lines (n_{HiII} =8, $n_{201\text{-}L1}$=13, $n_{202\text{-}L2}$=15, $n_{202\text{-}L5}$=12; Kruskal-Wallis on ranks, *df*=3, H= 4.335, p=0.227; t contrast on ranks: *df*=46, t=0.770, p=0.445). Furthermore, the transformed plants achieved lower yields in the field than the non-transformed control HiII (n_{HiII} =3, $n_{201\text{-}L2}$=5, $n_{202\text{-}L2}$=4, $n_{202\text{-}L5}$=5; One-way ANOVA, *df*=78; F=3.212, p=0.058; t contrast, *df*=15, t=2.882, p=0.011; Figure 1d).

Constitutive and herbivore-induced plant volatile production is not disturbed by the (E)-*β-caryophyllene synthase overexpression*

Engineered plants only emitted more EβC and α-humulene than the non-transformed line, independently of the collection period (Healthy plants: n_{HiII} =14, $n_{201\text{-}L1}$=6, $n_{202\text{-}L2}$=9, $n_{202\text{-}L5}$=6; EβC: n_{HiII} =14, $n_{201\text{-}L1}$=6, $n_{202\text{-}L2}$=9, $n_{202\text{-}L5}$=6; Kruskal-Wallis on ranks: *df*=3, H=26.434, p<0.001; Dunn's tests: HiII *vs*. 202-L2, 201-L1 and 202-L5: p<0.001; t contrast on ranks, *df*=33, t=10.384, p<0.001; Humulene: Kruskal-Wallis on ranks, *df*=3, H=11.729, p=0.008; t contrast on ranks, *df*=33, t=4.012, p<0.001; 4 hours of infestation: n_{HiII} =10, $n_{201\text{-}L1}$=6, $n_{202\text{-}L2}$=10, $n_{202\text{-}L5}$=8; EβC: Kruskal-Wallis on ranks, *df*=3, H=17.665, p<0.001; Dunn's test: HiII *vs*. 202-L2 and 202-L5: p<0.001; t contrast on ranks: *df*=32, t=4.931, p<0.001; Humulene: Kruskal-Wallis on ranks, *df*=3, H=11.302, p=0.010; t contrast, *df*=32, t=3.091, p=0.004; 8 hours of infestation: n_{HiII} =10, $n_{201\text{-}L1}$=7, $n_{202\text{-}L2}$=9, $n_{202\text{-}L5}$=8; EβC: Kruskal-Wallis on ranks: *df*=32, t=5.410, p<0.001; Humulene: Kruskal-Wallis on ranks, *df*=3, H=8.331, p=0.040; t contrast on ranks, *df*=33, t=2.733, p=0.01; Table 1).

Table 1: *Constitutive and induced volatile profiles of non-transformed and* (E)-*β-caryophyllene synthase-overexpressing plants (pg/h/plant).* Healthy plant volatiles were collected (0h). After the first volatile collection, plants were infested with 20 second-instar *Spodoptera frugiperda* larvae. Volatile bouquets were then collected 4 and 8 hours after infestation. Bold values and different letters indicate significant differences between the lines (p<0.05).

	0h				4h				8h			
volatile	WT HiII	202-L2	201-L1	202-L5	WT HiII	202-L2	201-L1	202-L5	WT HiII	202-L2	201-L1	202-L5
(Z)-3-Hexenal	0	0	0	0	470.20 (±163.85)	492.09 (±217.62)	299.81 (±136.33)	739.71 (±378.33)	956.70 (±272.64)	768.13 (±354.12)	972.21 (±180.80)	603.09 (±182.43)
(E)-2-hexenal	0	0	0	0	117.97 (±60.41)	83.87 (±50.62)	0	0	557.22 (±195.36)	428.37 (±132.99)	482.17 (±53.27)	437.05 (±118.46)
(Z)-3-hexen-1-ol	0	0	0	0	241.53 (±56.68)	133.20 (±52.22)	169.15 (±35.89)	807.72 (±280.85)	329.54 (±114.70)	248.00 (±96.27)	298.96 (±69.36)	184.59 (±63.90)
Acetate 2-Hexen-1-ol	0	0	0	0	0	0	0	0	17.61 (±12.35)	16.53 (±12.11)	69.64 (±27.89)	106.68 (±30.05)
(Z)- acetate 2-Penten-1-ol	0	0	0	0	0	0	0	0	61.71 (±34.58)	72.45 (±29.18)	35.16 (±22.82)	16.16 (±16.16)
(Z)-3-Hexenyl acetate	0	0	0	0	789.57 (±281.42)	655.88 (±253.02)	781.05 (±273.33)	1762.91 (±582.84)	1998.62 (±503.20)	1491.30 (±491.90)	2640.98 (±674.85)	1673.76 (± 496.32)
Indole	21.53 (±22.34)	0	0	214.98 (±56.68)	169.88 (±147.01)	151.39 (±76.33)	31.06 (±19.68)	755.79 (±446.94)	911.29 (±472.31)	695 (±377.84)	561.79 (±253.55)	147.03 (± 79.88)
Linalool	29.45 (±28.85)	645.99 (±506.42)	0	98.00 (±97.99)	417.00 (±383.59)	19.80 (±19.80)	0	970.86 (±630.19)	643.14 (±473.39)	259.37 (±193.37)	0	412.84 (±157.54)
DMNT	0	0	0	0	298.47 (±169.97)	104.26 (±75.43)	22.48 (±22.48)	1016.33 (±576.74)	799.60 (±373.89)	533.42 (±276.06)	514.98 (±239.28)	650.57 (±226.06)
β-myrcene	28.22 (±17.56)	37.33 (±14.75)	12.92 (±12.92)	368.31 (±200.66)	32.67 (±22.21)	22.80 (±13.36)	32.66 (±22.21)	34.73 (±22.23)	60.08 (±25.60)	20.91 (± 14.43)	12.03 (±12.03)	32.93 (±18.47)
(E)-β-ocimene	0	0	0	0	8.49 (±8.49)	0	0	31.77 (±23.28)	20.83 (±13.89)	0	0	11.52 (±7.57)
***(E)*-β-caryophyllene**	**0 *(a)***	**542.44 (±121.6) *(b)***	**2024.52 (±1394.21) *(b)***	**1078.11 (±1038.35) *(b)***	**0 *(a)***	**597.02 (±153.10) *(b)***	**1272.71 (±1193.37) *(ab)***	**1938.52 (±1119.87) *(b)***	**0 *(a)***	**639.18 (±184.61) *(b)***	**1747.28 (±1560.25) *(ab)***	**1790.80 (±638.49) *(b)***
(E)-β-farnesene	0	0	0	0	0	0	0	1285.50 (±874.30)	80.08 (±33.71)	68.59 (±68.59)	20.08 (±13.08)	76.73 (±35.75)
α-humulene	**0**	**54.34 (±23.97)**	**313.59 (±231.05)**	**32.23 (±32.23)**	**0**	**82.05 (±28.88)**	**194.78 (±194.78)**	**347.63 (±212.54)**	**0**	**74.09 (±41.84)**	**257.53 (±239.90)**	**261.30 (±102.16)**
α-copaene	0	0	0	0	0	0	0	28.69 (±19.03)	0	0	0	0
β-bergamotene	0	0	0	0	0	0	0	798.02 (±535.86)	92.22 (±34.18)	53.21 (±21.24)	104.09 (±59.21)	0

Terpenoid engineered plants are subjected to more aboveground herbivory in field

Plants that constitutively emitted EβC and α-humulene were more damaged than non-transformed controls, independent of the scoring method (n_{HiII} =16, $n_{201\text{-}L1}$=13, $n_{202\text{-}L2}$=18, $n_{202\text{-}L3}$=13; Damage score: One-way ANOVA, *df*=56, F=4.950, p=0.004, Tukey HSD: HiII *vs*. 201-L1 and 202-L3: p<0.05; t contrast: *df*=58, t=-3.621, p<0.001; Damaged surface: Kruskal-Wallis on ranks, *df*=3, H=10.335, p=0.016; Dunn's tests: HiII *vs*. 201-L1: p<0.05; t contrast on ranks, *df*=58, t=3.351, p=0.001; Figure 2a and b).

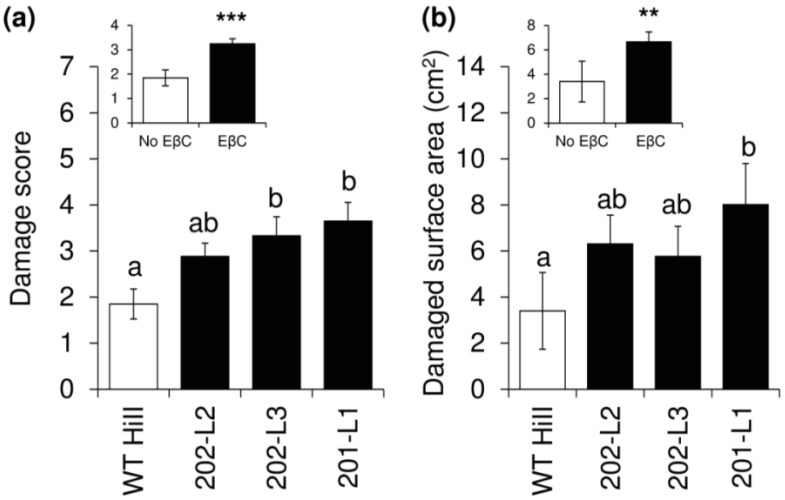

Figure 2: *Overexpression of the terpene synthase increases leaf damage*. (a) Leaf damage score (0: healthy plant to 5: all leaves damaged) (mean ±SE) of non-transformed plants and plants of each (main) or all (inset) EβC synthase overexpressing lines in field. The white bar corresponds to the non-transformed line, while black bars refer to EβC synthase-overexpressing maize lines. (b) Leaf damage area (mean ±SE) of non-transformed plants and plants of each (main) or combined (inset) EβC synthase-overexpressing lines in field. The white bar corresponds to the non-transformed line, while black bars refer to EβC synthase-overexpressing maize lines. Different letters indicate significant differences (p<0.05).

Constitutive emission of (E)-*β-caryophyllene and α-humulene increases plant apparency to herbivores*

When given the choice, *S. frugiperda* larvae strongly preferred to orient towards the transgenic line 201-L1 compared to all the other lines (n=6; glm, *df*=26, F=6.458, p<0.001; Tukey: 201-L1 *vs*. HiII and 202-L2: p<0.001, 201-L1 *vs*. 202-L5: p=0.004; t contrast: *df*=28, t=16.464, p=0.619; Figure 3a). *S. frugiperda* larvae were also more attracted to plants infested with conspecifics compared to healthy plants (Figure S1a), but synthetic EβC alone was not responsible for this preference (Figure S1b). Furthermore, the adult female moths preferentially oviposited on EβC-emitting plants, especially on 202-L5 and 201-L1, as compared to non-transformed control plants (n=14; glm, *df*=52, F=3.768, p=0.017; Tukey: 202-L5 *vs*. HiII: p=0.016, 202-L5 *vs*. 202-L2: p=0.016; t contrast, *df*=54, t=2.343, p=0.023; Figure 3b).

Constitutive production of (E)-*β-caryophyllene and α-humulene does not affect the leaf feeder,* S. frugiperda*, performance*

The larval weight gain of the leaf feeder *S. frugiperda* was similar on all plant lines, independent of the transformation (n_{HiII} =10, $n_{201\text{-}L1}$=10, $n_{202\text{-}L2}$=16, $n_{202\text{-}L5}$=17; One-way ANOVA, *df*=42, F= 0.238, p=0.869; t contrast: *df*=44, t=0.160, p=0.874; Figure 3c).

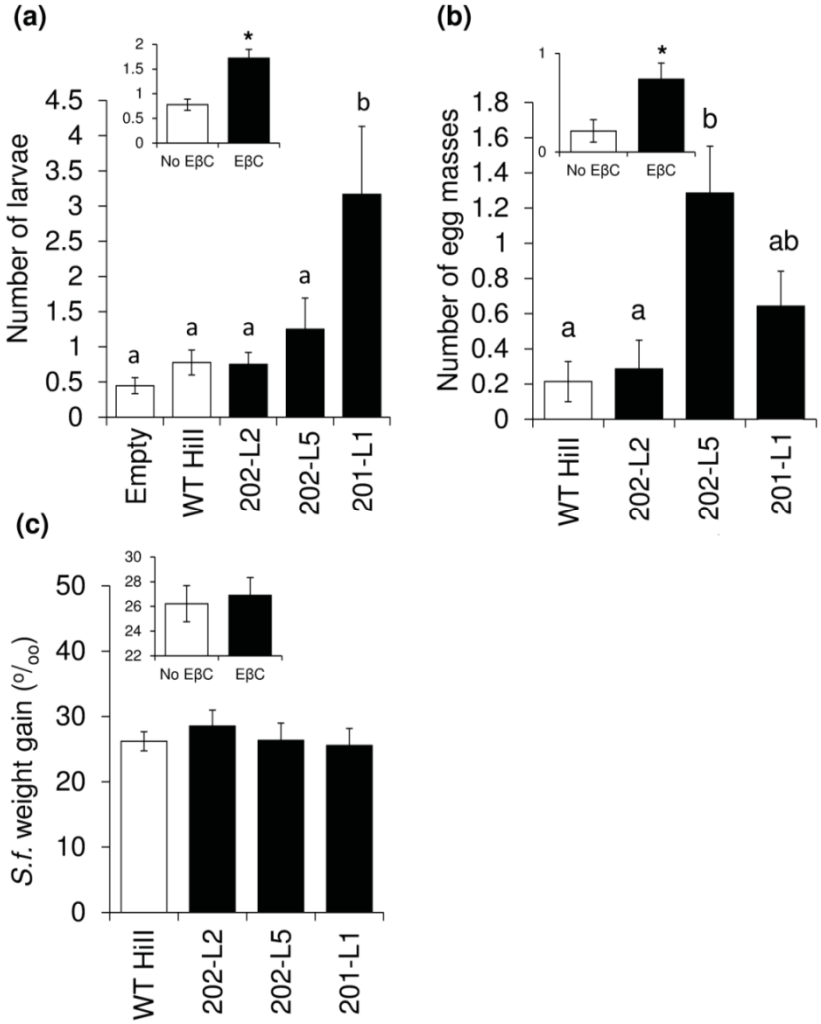

Figure 3: *Overexpression of the terpene synthase increases plant apparency to leaf herbivores.* (a) Attraction of *S. frugiperda* larvae to the non-transformed line and individual (main) or combined (inset) EβC synthase-overexpressing-lines in 6-arm olfactometers. The white bar corresponds to the two empty arms and to the non-transformed plants, while black bars refer to EβC synthase-overexpressing maize lines. (b) Number of egg masses (mean ±SE) laid by *S. frugiperda* females on non-transformed plants and on plants from each (main) or pooled (inset) EβC synthase-overexpressing lines in tents that presented one plant of each line simultaneously. (c) *S. frugiperda* larval weight gain was measured over 7 days of feeding on the non-transformed line (white bar) and EβC synthase-overexpressing lines (black bars). The white bar corresponds to the non-transformed line, while black bars refer to EβC synthase-overexpressing maize lines. Different letters indicate significant difference ($p<0.05$). Stars indicate significant differences (*: $p<0.05$, **: $p< 0.01$, ***: $p<0.001$).

Constitutive emission of (E)-*β-caryophyllene and α-humulene reduces root herbivory*

In field, roots of terpenoid-engineered plants displayed a slight, albeit non-significant, decrease in root damage (n_{HiII} =11, $n_{201\text{-}L1}$=10, $n_{202\text{-}L2}$=9, $n_{202\text{-}L3}$=8; Kruskal-Wallis on ranks, *df*=3, H=1.920, p=0.589; t contrast: *df*=36, t=-1.203, p=0.237; Figure 4a). The emergence of *D. undecimpunctata howardii* was strongly affected by the plant genotype, with twice as less adults emerging from EβC synthase-overexpressing lines compared to control lines (n_{HiII} =18, $n_{201\text{-}L1}$=14, $n_{202\text{-}L2}$=14, $n_{202\text{-}L3}$=16; Kruskal-Wallis on ranks, *df*=3, H=4.449, p=0.217; t contrast: *df*=60, t=-2.545, p=0.014; Figure 4b). On the other hand, the emergence of adult *D. virgifera*, was not affected by the plant phenotype, independently of the adding of *D. virgifera* eggs or not earlier in the season (n_{HiII} =16, $n_{201\text{-}L1}$=14, $n_{202\text{-}L2}$=13, $n_{202\text{-}L3}$=15; Kruskal-Wallis on ranks, *df*=3, H=0.657, p=0.883; t contrast: *df*=54, t=0.796, p=0.430; Figure 4c).

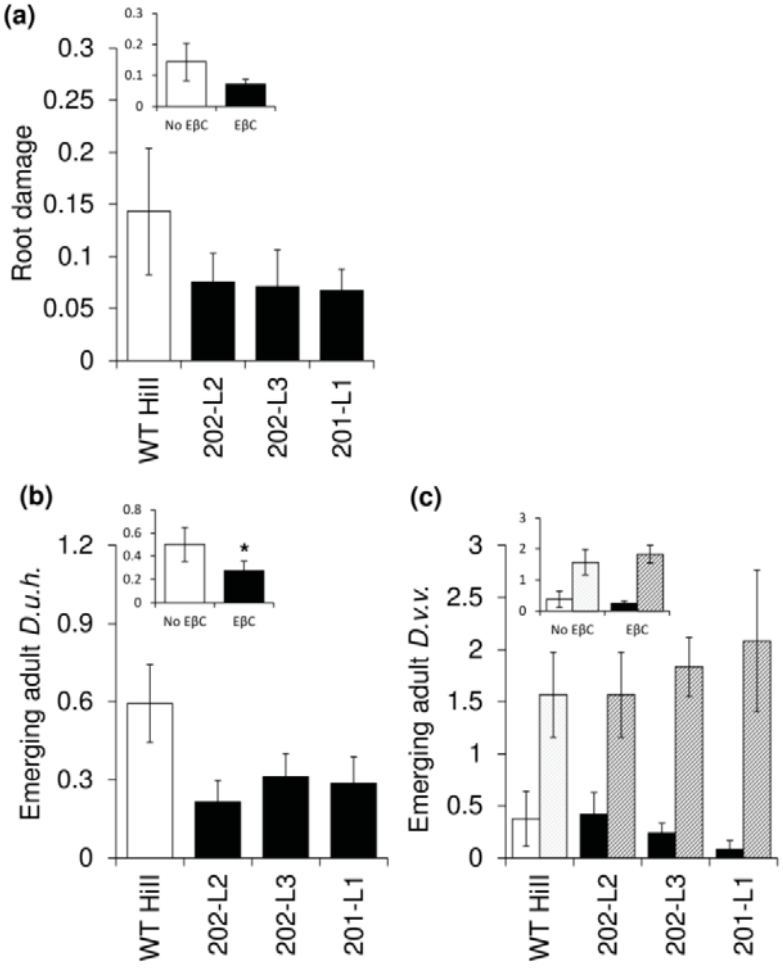

Figure 4: *Constitutive emission of* (E)-*β-caryophyllene and α-humulene protects the plant below-ground.* (a) Root damage score (mean ±SE) was evaluated using a node injury scale (Oleson *et al.*, 2005) of non-transformed plants and of plants of each (main) or all (inset) EβC synthase-overexpressing lines in field. (b) Number of *D. undecimpunctata howardii* adults (mean ±SE) trapped in emergence cages on each non-transformed plants and of plants of each (main) or all (inset) EβC synthase-overexpressing lines in field. (c) Number of *D.virgifera* adults (mean ±SE) on non-transformed plants and of plants of each (main) or all (inset) EβC synthase-overexpressing lines in field. Filled bars correspond to natural populations of the insects while hatched bars correspond to experimentally infested plots. The light bar corresponds to the non-transformed line, while dark bars refer to EβC synthase-overexpressing maize lines. Stars indicate significant differences (*: p<0.05, **: p< 0.01, ***: p<0.001).

Plant genotype had no effect on *D. undecimpunctata howardii* larval host selection (n=5 olfactometers; glm, *df*=4, F=5.172, p<0.001; t contrast: *df*=28, t=0.692, p=0.491; Figure 5a). In contrast, terpenoid-engineered plants were more attractive to *D. virgifera* than the non-transformed control line (n=8 olfactometers; glm, *df*=4, F=11.664, p<0.001; t contrast: *df*=38, t=0.692, p=0.491; Figure 5b). Yet, *D. virgifera* larvae grew similarly on plants overexpressing the EβC synthase-transformed and on isogenic plants (n_{HiII} =7, $n_{201\text{-}L1}$=5, $n_{202\text{-}L2}$=7, $n_{202\text{-}L5}$=6; One-way ANOVA, *df*=21, =2.509, p=0.087; t contrast: *df*=23, t=-0.468, p=0.645; Figure 5c).

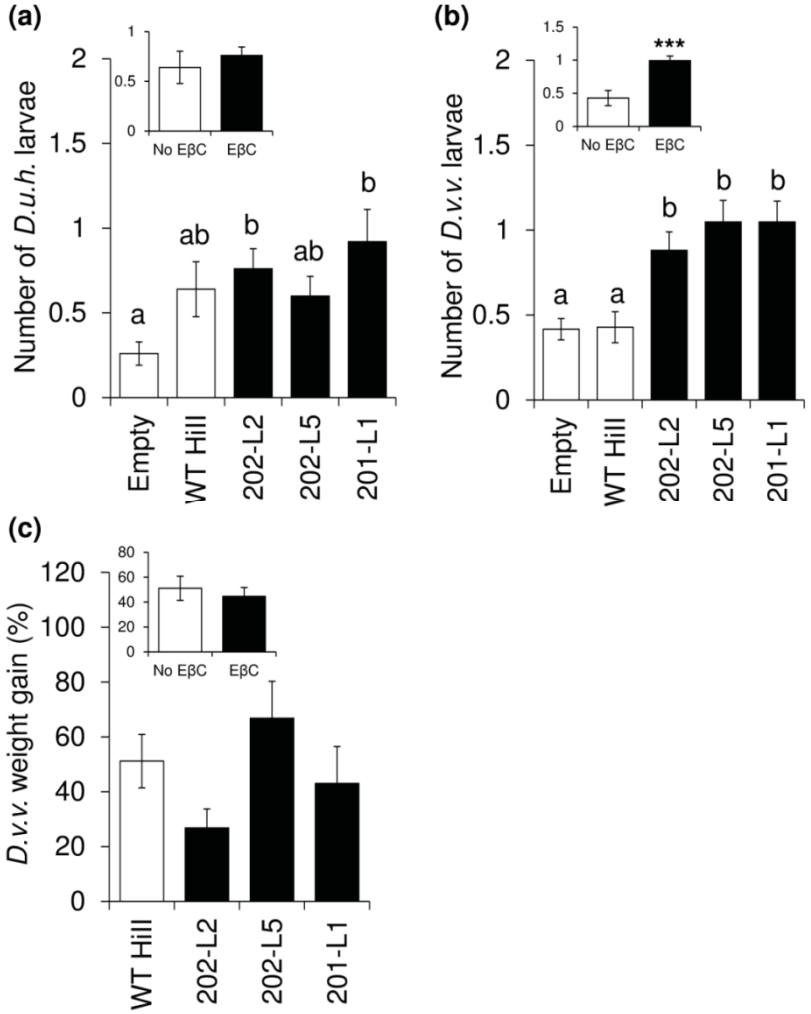

Figure 5: (E)-*β-caryophyllene and α-humulene emissions are attractive to the specialist herbivore,* D. virgifera. (a) Number of *D.undecimpunctata howardii* larvae (mean ±SE) that oriented towards transformed and non-transformed plants in 6-arm olfactometers. (b) Number of *D.virgifera* larvae (mean ±SE) that oriented towards transformed and non-transformed plants in 6-arm olfactometers. (c) Relative weight gain (mean ±SE) of *D. virgifera* larvae feeding on non-transformed and EβC synthase-overexpressing lines. The white bar corresponds to the non-transformed line, while black bars refer to EβC synthase-overexpressing maize lines. Different letters indicate significant differences (p<0.05). Stars indicate significant differences (*: p<0.05).

Overexpression of EβC synthase increases tolerance to belowground herbivory

In absence of *D. virgifera*, control plants showed higher yields than terpenoid engineered plants (see above). Yet, in plots in which *D. virgifera* was introduced, control plants' yield was negatively affected, while it was not in EβC emitting lines (Control: : n_{HiII} =3, $n_{201\text{-}L2}$=5, $n_{202\text{-}L2}$=5, $n_{202\text{-}L5}$=5; *D. virgifera* : n_{HiII} =3, $n_{201\text{-}L2}$=5, $n_{202\text{-}L2}$=5, $n_{202\text{-}L5}$=4; *D. virgifera* and entomopathogenic nematodes (EPN): n_{HiII} =15, $n_{201\text{-}L2}$=10, $n_{202\text{-}L2}$=9, $n_{202\text{-}L5}$=11; Two-way ANOVA, *df*=68, factor: plant variety: F=3.741, p=0.015; factor: infestation: F=5.163, p=0.008; factor: variety*infestation: F=0.953, p=0.464; t contrast on WT HiII: control *versus D. virgifera* infested plants: *df*=19,t=-2.113, p=0.049; Figure 6).

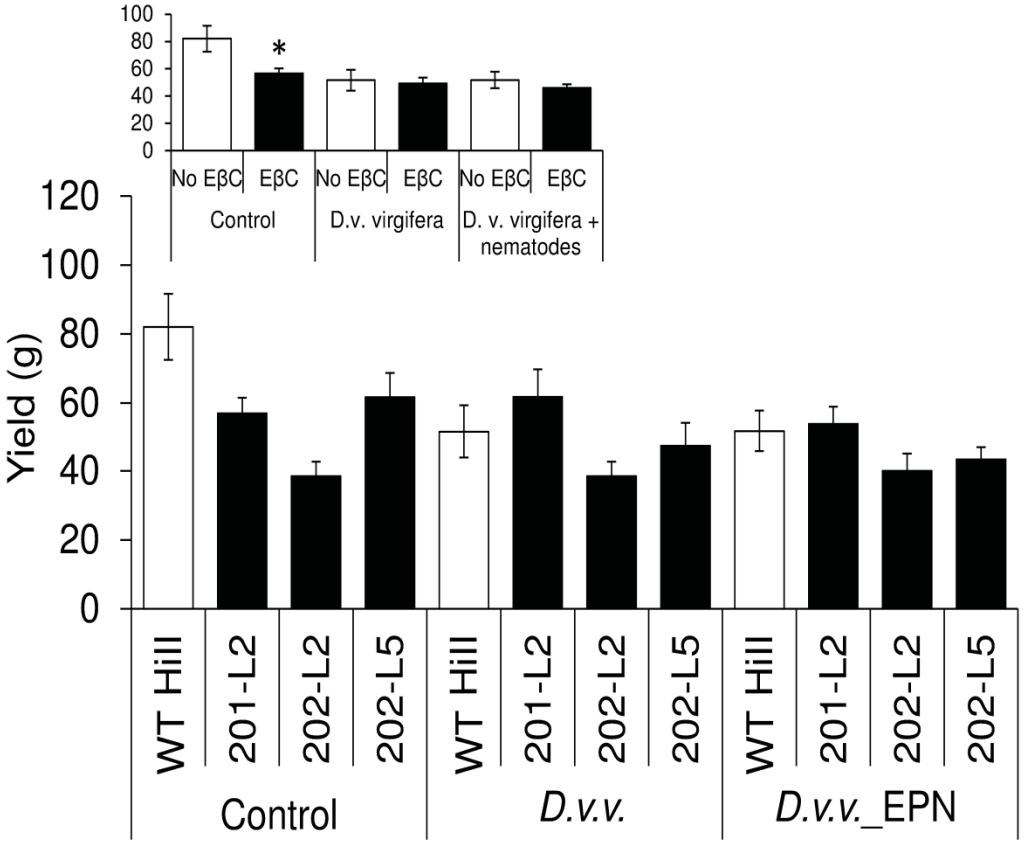

Figure 6: *Yield of EβC synthase-transformed plants is not affected by the presence of the root herbivore,* D. virgifera**.** Ear wings fresh weight (mean ±SE) of non-transformed plants and of plants of each (main) or combined (inset) EβC synthase-overexpressing lines in field was measured in presence or absence of the western corn rootworm, *D. virgifera* with or without addition in soil of their natural enemies, the entomopathogenic nematodes (EPN), *Heterorhabditis megidis*. The white bar corresponds to the non-transformed line, while black bars refer to EβC synthase-overexpressing maize lines. Stars indicate significant differences (*: p<0.05).

DISCUSSION

This study demonstrates that genetically engineering terpenoid emission in plants can induce significant physiological and ecological costs that may outweigh the potential benefits. Physiological costs of HIPV production have previously been assumed to be minor due to their low molecular weight and the relatively low concentration in which the volatiles are emitted (Dicke & Sabelis, 1990; Halitschke *et al.*, 2000). However, our study shows that the transformation of maize with an oregano terpene synthase gene may compromise germination, growth and yield in the laboratory and the field (Figure 1). This is in line with previous studies that found that terpenoids can inhibit seed germination, root and shoot growth (Fischer, 1986; Fischer, 1991; Aharoni *et al.*, 2003; Aharoni *et al.*, 2006) and that terpenoid content is negatively correlated with plant growth (Hanover, 1966; Mathur *et al.*, 1988; Adzet *et al.*, 1992). Possibly, the constitutive production of a major sesquiterpene in the range of a few ng per hour (EβC: 1.35 ng/hour=6.61pmoles), as was the case for the transformed plants, is already costly to a maize plant. Due to their high degree of chemical reduction, EβC and α-humulene production requires considerable supplies of substrate (AcetylCoA) and co-factors (ATP and NADPH). About 3.54 g of glucose is required to provide all substrates and co-factors consumed to produce one gram of the terpenoids (Dehal & Croteau, 1988; Gershenzon, 1994), diverting those glucose molecules away from primary metabolism. Moreover, terpenoids can be autotoxic to plants (Shomer & Erner, 1989; Loveys *et al.*, 1992). The transformation process by itself is unlikely to have imposed any cost on the plant as all the transformed lines showed similar growth effects (Figure 1). We therefore conclude that the production of EβC and α-humulene itself is costly to the plant, which indicates that physiological costs may, at least in part, explain the evolution of the inducibility of sesquiterpenes in nature.

We did not find any trade-off between terpenoid production and direct or indirect defense against phytophagous insects, as both above- and belowground herbivores grew equally on the different lines (Figures 3c and 5c), and HIPV bouquets between transformed and non-transformed lines were similar (Table 1). This is consistent with several recent studies reporting no trade-offs between volatile production and direct defense traits (Anderson & Alborn, 1999; Koricheva *et al.*, 2004; Erb *et al.*, 2011a; McCallum *et al.*, 2011; Rasmann *et al.*, 2011), and suggests that different defensive traits do not rely on the same limiting resources (Agrawal & Fishbein, 2006; Rasmann & Agrawal, 2009). Diverting resources away from processes involved in plant growth rather than from other defensive traits is likely to be adaptive, since different defensive traits may act in a complementary, non-redundant fashion (Agrawal & Fishbein, 2006; Rasmann & Agrawal, 2009; Rasmann *et al.*, 2011).

Constitutive emission of EβC and α-humulene was found to also have ecological costs aboveground, as indicated by the increased leaf damage in transformed plants in the field (Figure 2). Olfactometer experiments in the laboratory confirmed that *S. frugiperda* larvae preferentially oriented towards plants that were infested with conspecifics (Figure S1a). *S. frugiperda* larvae were also attracted towards healthy transformed 201-L1 plants that constitutively emit EβC and α-humulene (Figure 3a). That one transformed line was more attractive than the others can be explained by the fact that all plant lines were simultaneously offered to the larvae in the olfactometers: *S. frugiperda* larvae may have oriented towards the most attractive line, which may have somewhat masked the attractiveness of the other transformed lines. The overexpression of the EβC synthase also seemed to result in enhanced apparency of plants to adult *S. frugiperda* moths: Females deposited more egg masses on the EβC-emitting line 201-L5 (Figure 3b). Our study suggests a role of sesquiterpenes in host selection by *S. frugiperda* larvae and adults. However, EβC was only attractive within the context of a plant background odor, as EβC by itself was not attractive (Figure S1). It cannot be excluded that α-humulene also play a role in the attractiveness of the transformed maize lines although it is produced in much lesser amount that EβC. Our study complements the growing evidence that whole plant volatile bouquets are required as a background to make semiochemicals attractive (Mumm & Hilker, 2005; Schroeder & Hilker, 2008; Robert *et al.*, in press). Attractiveness of other terpenoids such as linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*)-α-farnesene to lepidopteran larvae has been reported before (Landolt *et al.*, 2000; Carroll *et al.*, 2006; Carroll *et al.*, 2008).

The restoration of the signal in the roots also led to beneficial effects: Transformed lines showed a slight decrease in root damage in *D. virgifera* infested fields compared to the non-transformed line (Figure 4a), and had a significantly reduced number of emerging adult *D. undecimpunctata howardii* (Figure 4b). These effects may be attributed to the higher attractiveness of EβC-emitting lines to entomopathogenic nematodes (Degenhardt et al., 2009). HIPVs can repel generalist species, as it was previously demonstrated for several aboveground herbivore, such as moths (Anderson *et al.*, 2001; De Moraes *et al.*, 2001a; Kessler & Baldwin, 2001; Xu *et al.*, 2002), and aphids (Bernasconi *et al.*, 1998). On the other hand, HIPVs can also attract phytophagous insects above-ground (Kalberer *et al.*, 2001; Carroll *et al.*, 2008; Halitschke *et al.*, 2008; Sun *et al.*, 2010). Our results demonstrates that the constitutive terpenoid emission does not affect the generalist *D. undecimpunctata howardii* host selection, but is attractive to larvae of the specialist herbivore *D. virgifera* belowground (Figure 5a and b). This attraction may be mediated by the emission of EβC (Robert *et al.*, in press).

Yet, our study did not show any difference in the root infestation by this specialist root herbivore between transformed and non-transformed plants (Figure 4c). Furthermore, while the presence of *D. virgifera* reduced yield of non-transformed plants independently of whether or not the plots were treated with entomopathogenic nematodes, the yield of EβC synthase-transformed lines was not affected by the root herbivore (Figure 6). This phenomenon can be explained by the attractiveness of the transformed plants to natural populations of entomopathogenic nematodes that reduced root damage during the field season (Degenhardt et al., 2009) Overall, our results show that EβC synthase overexpression belowground had positive consequences for the plant under the tested field conditions. So far, plants that were genetically engineered to release terpenoids have been found to have enhanced direct and indirect defences against herbivores (Wang *et al.*, 2001; Aharoni *et al.*, 2003; Kappers *et al.*, 2005; Beale *et al.*, 2006; Schnee *et al.*, 2006; Degenhardt *et al.*, 2009). However, most of these studies focused on a well-defined bi- or tritrophic system above- or belowground.

CONCLUSION

Our study reveals that, despite the significant progress in our abilities to manipulate plant volatiles, the overall outcome of the manipulation remains unpredictable, and a thorough evaluation of possible side effects of plant genetic engineering in the field is necessary. Physiological cost of such manipulations depend on their direct impact on plant metabolism and regulation, while ecological costs depend on the community composition of herbivores and natural enemies (Poelman *et al.*, 2008). For instance, plants that overexpress the EβC synthase may be attacked more by leaf herbivores, but this may render them more resistant to root herbivory due to plant-mediated interactions between the herbivores (Erb *et al.*, 2011d). While the release of EβC indirectly protects the maize root systems against herbivory, the net outcome of the transformation appears to be negative since constitutive EβC emission is associated with considerable physiological and ecological costs that decreases plant performance and increases the apparency to aboveground herbivores. Such costs may explain the evolution of the inducibility of sesquiterpenes, as well as the loss of signal during plant breeding in most of American maize varieties (Köllner *et al.*, 2008). The results support the idea that the inclusive benefit of manipulated EβC release can be greatly improved by making it inducible by inserting an herbivore inducible root specific promoter in front of the EβC-synthase gene (Degenhardt *et al*., 2009). This would prevent the attraction of herbivores by healthy plants and only lead to recruitment of natural enemies when the plant is attacked by herbivores. Alternatively, the continuous emission of EβC aboveground may also be applied in a push-pull strategy to pull aboveground herbivores away from the crop. Our study concurs with the notion that a thorough assessment of the "double-edged sword" of plant volatiles (Dicke & Baldwin, 2010) is essential for their application in sustainable pest management strategies.

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SUPPLEMENTARY FIGURE

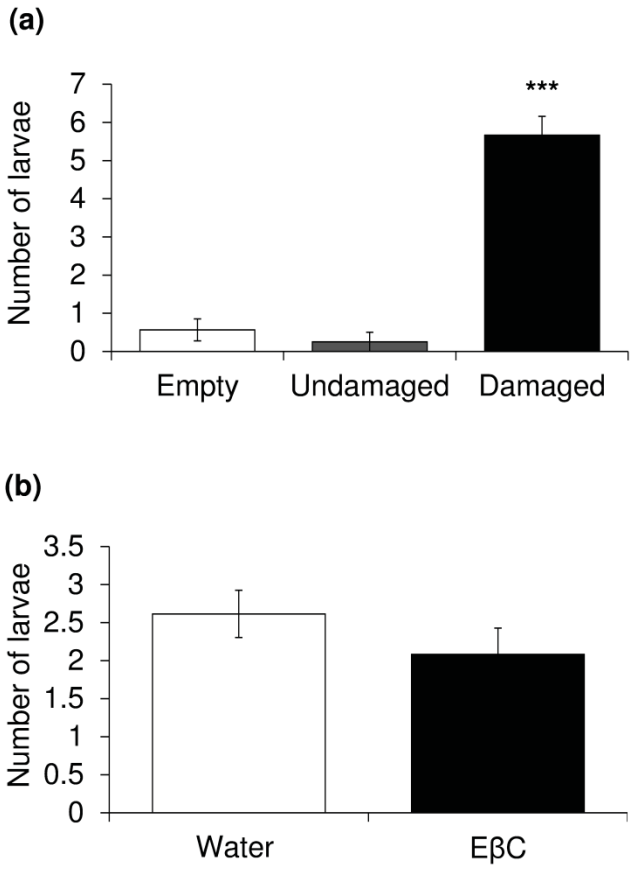

Figure S1: *Attraction of* Spodoptera frugiperda *larvae to herbivore induced volatiles (HIPVs).* (a) Number of larvae (mean ±SE) attracted to plants (var. Delprim) infested with conspecifics or left uninfested in 6-arm olfactometer assays (n=12; glm, *df*=71, F=25.995, p<0.001; Tukey HSD: Damaged *vs*. undamaged plant: p<0.001, empty arm *vs*. damaged plant: p<0.001, empty arm *vs*.undamaged plant: p=0.793). (b) Number of larva attracted to capillary dispensers filled with water or synthetic *(E)*-β-caryophyllene in 2-arm olfactometers (n=13; glm, *df*= 24, F=1.739, p=0.200). Stars indicate significant differences (***: p=0.001).

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CHAPTER III

A specialist root herbivore exploits defensive metabolites to locate nutritious tissues

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ABSTRACT

The most valuable organs of plants are often particularly rich in essential elements, but also very well defended. This creates a dilemma for herbivores that need to maximize energy intake while minimizing intoxication. We investigated how the specialist root herbivore *Diabrotica virgifera* solves this conundrum when feeding on wild and cultivated maize plants. We found that crown roots of maize seedlings were vital for plant development and, in accordance, were rich in nutritious primary metabolites and contained higher amounts of the insecticidal 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and the phenolic compound chlorogenic acid. The generalist herbivores *Diabrotica balteata* and *Spodoptera littoralis* were deterred from feeding on crown roots, whereas the specialist *D. virgifera* preferred and grew best on these tissues. Using a 1,4-benzoxazin-3-one-deficient maize mutant, we found that *D. virgifera* is resistant to DIMBOA and other 1,4-benzoxazin-3-ones and that it even hijacks these compounds to optimally forage for nutritious roots.

INTRODUCTION

Plants possess a wide arsenal of toxic secondary metabolites to defend themselves against herbivores (Sicker *et al.*, 2000; Steppuhn *et al.*, 2004a; Gershenzon & Dudareva, 2007). The concentrations of these compounds vary considerably between (Kaplan *et al.*, 2008) and within tissues (Shroff *et al.*, 2008), as well as over the course of plant development (Cambier *et al.*, 2000). A proposed evolutionary explanation for this variability is that, because of metabolic costs, the concentrations of secondary defense metabolites in a particular tissue at a given developmental stage should reflect its relative fitness value (Rhoades & Cates, 1976). As a part of the "optimal defense" hypothesis, this concept has found considerable support in the literature (Stamp, 2003). However, as plant organs with a high reproductive value such as young leaves or developing flowers receive a substantial amount of nutrients and photo-assimilates (Pommel *et al.*, 2006; Li *et al.*, 2009), these are also the tissues that are potentially valuable food sources for herbivores (Awmack & Leather, 2002). To optimize their intake of energy per unit of time (Macarthur & Pianka, 1966), herbivores should therefore attempt to feed on the most valuable, and, consequently, best defended plant tissues. This creates an intriguing dilemma for the herbivores, which is solved if they can overcome the plant's defenses, thus allowing them to feed on the most nutritious tissues and maximize their fitness. Indeed, specialist herbivores have been shown to be able to develop on highly defended plant organs (Kimmerer & Potter, 1987; Ishimoto & Chrispeels, 1996). The capacity to cope with plant defenses is thought to have favored adaptive radiation of herbivores (Wheat *et al.*, 2007), and is therefore widely recognized as a major driver of plant-insect co-evolution.

While the above interactions have received considerable attention above ground, little is known about how strategies of defense and foraging shape the interplay between plants and herbivores below ground (Yanai & Eissenstat, 2002; Johnson & Gregory, 2006; van Dam, N. M., 2009). This prompted us to carry out a series of experiments on the interaction between wild and cultivated maize (*Zea mays* L. *spp*) and its most important root pest, *Diabrotica virgifera virgifera* (L.). By combining behavioral and performance experiments with analytical and molecular methods, we show how *D. virgifera* has successfully solved the optimal foraging dilemma. Our experiments reveal how a counter adaption of a below ground herbivore to a chemical plant defense determines its distribution and abundance in the soil.

METHODS

Plants and insects

The maize hybrid Delprim (*Zea mays* L. *ssp. mays*) was obtained from Delley DSP (Delley, Switzerland). The *bx1* mutant (428G) and its wild type parental line (H88) were ordered from the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu). Plants were grown as described (Erb *et al.*, 2011c) and used when they were 12-14 days old and had three fully developed leaves, unless specified otherwise. Teosinte (*Z. mays L. ssp. mexicana*) seeds were collected from two wild populations near Texcoco (Mexico) in 1998. As the teosinte plants grew more slowly than cultivated maize, they were left in the phytotron for 20 days until they had three fully developed leaves. Maize plants for the $^{11}CO_2$ labeling experiments (see below) were germinated in petri dishes and transplanted into cylindrical glass cells (20 cm height, 10 cm diameter) containing a growth medium consisting of 1.6 g Hoagland modified basal salt mixture (PhytoTechnologiy Laboratories TM) and 0.55 g of 2-(N-Morpholino) ethanesulfonic acid (MES) hydrate (Sigma Life Science) in 1 liter of distilled water. After adjusting the pH to 5.8 with a few droplets of sodium hydroxide, 2.5 g of Gelzan TM CM (Sigma Life Science) was added. *Diabrotica virgifera virgifera* (LeConte) and *Spodoptera littoralis* (Boisduval) were reared as described before (Erb *et al.*, 2011a). *Diabrotica balteata* (LeConte) was reared under identical conditions as *D. virgifera.*

Relative value of root types for the plant

The root systems of maize consists of primary and secondary roots that grow directly from the embryo (also called embryonic roots), as well as crown roots that originate from the stem (also called adventitious or post-embryonic roots; Hochholdinger& Tuberosa, 2009). To determine the relative value of these different root types for the development of maize seedlings, two separate experiments were carried out. In a first assay, either crown-roots or primary and secondary roots were excised from individual maize seedlings (n=11). To remove the different roots without otherwise damaging the plant, the topsoil was washed off under a gentle stream of warm water, until the different roots were visible and could be accessed with surgical scissors. After excision, the missing substrate in the pots was replaced with fresh moist soil. Plants were checked every five days over a period of 30 days, and regrowing roots were cut over the first 20 days to simulate an ongoing herbivore attack by *D. virgifera*. Complete pruning of crown roots by *D. virgifera* occurs regularly in the field, resulting in plants losing their stability (Oleson *et al.*, 2005). Senescence symptoms for each emerging leaf were recorded using a scale from 0 (no senescence) to 4 (leaf yellow and curling). Three weeks after the beginning of the experiment, the leaves of the plants were harvested, and their biomass was determined. A second experiment was carried out

using the same procedure as above, except that the plants were transplanted into 2 l pots before treatment (n=6), which enabled them to grow more vigorously and possibly enabled additional compensatory growth. For this experiment, crown, primary and secondary roots were excised separately, and untreated controls were included. Leaf-growth was determined by measuring the height of the plant every two days.

Determination of free amino acids, total protein, starch, sugars and resource allocation

To measure concentrations of free amino acids in primary and crown roots, 12 day old maize seedlings were harvested, and their roots were gently washed. Crown and primary roots were separated and immediately frozen in liquid nitrogen (n=6). Amino acids were determined following a previously described method (Knill, Tanja *et al.*, 2008). Total soluble protein was determined on crown and primary roots (n=5-6) using an adapted Bradford assay (Jongsma *et al.*, 1994). To determine free sugars in the different roots, a 50mg aliquot of freshly ground material was lyophilized over 48h and analyzed using and a method based on a previously published procedure (Rovio *et al.*, 2007). Starch concentrations (n=8) were determined as published previously (Smith & Zeeman, 2006). To measure the allocation of photo assimilates to the different roots, plants were grown in an agar-based growth medium for 20 days (see above). Single plants were then pulsed with 30 mCi of $^{11}CO_2$ (< 1 ppm CO_2) for 30 sec using the methodology described previously (Babst *et al.*, 2005) (n=8). Two and a half hours after exposure, roots were excised, and the accumulation of radioactivity in individual roots was measured with a beta/gamma-counter. As the plants already had 3 generations of crown roots at this stage, these were separated according to their growth stage. For visualization purposes, an individual plant was treated as described above, the roots were extracted from the gel and the full plant was visualized with beta-imaging.

Extraction and analysis of 1,4-benzoxazin-3-ones

To determine the concentrations of 1,4-benzoxazin-3-one derivatives (BXDs, see Figure S1 in Supporting Information) in different root types, five different experiments were carried out. In the first two experiments, basal BXDs were determined in crown and primary roots of maize plants (n=8; n=9). In a second experiment, we determined BXDs in teosinte (n=11). Maize plants were included as a positive control. In a fourth experiment, maize plants were infested with 6 second instar *D. virgifera* larvae over 24h (n=8). Finally, BXD concentrations in crown, primary and secondary roots of maize were determined in control plants and plants treated with 200 µM jasmonic acid (JA) for 24 h (n=8) following a previously described protocol (Erb *et al.*, 2009a). Roots of all experiments were extracted in 1ml of acidified H_2O/MeOH (50:50 v/v; 0.5% formic acid) as described (Erb *et al.*, 2009a) and analyzed using UPLC-QTOF-MS (Glauser *et al.*,

2011a). To measure concentrations BXDs in root exudates, we developed a method based on liquid extraction surface analysis using the Advion TriVersaNanomatechip-based infusion nanoESI system (Advion bioscience, NY, USA, see Appendix S1). With this system, exudation of benzoxazinoids from crown and primary roots was quantified (n=6).

Extraction and analysis of phenolic compounds and analysis of gene expression

Phenolic compounds were analyzed using 100 mg samples of the roots from the JA experiment (n=8, see above). The extraction and analysis of phenolics were similar to that of BXDs, except for the following modifications: the extraction solvent was MeOH/H_2O (80:20, v/v); The injection volume was 2.5 µl and gradient analysis was performed at 400 µl/min under the following conditions: A= water+formic acid 0.05%, B= acetonitrile+formic acid 0.05%; 2-30% B in 2.5 min, 30-100% in 3 min, 100% B for 2 min, re-equilibration at 2% B for 1 min. The expression of defense marker genes in different roots after JA induction was measured using previously established methods and primers (Erb, Matthias *et al.*, 2010) on the same material as above (n=8).

Herbivore feeding preference

The preference of *D. virgifera* for different root types was determined by infesting 12-day old seedlings with 6 second instar *D. virgifera* larvae (n=11). In the field, egg densities have been estimated to be over 200 per plant, and infestation with 10 or more larvae per plant is not uncommon (Hibbard *et al.*, 2004). Timing of attack varies with climatic conditions, but *D. virgifera* larvae with an extended diapause can hatch as early as April, shortly after maize plants start to develop in the field. Thus, the chosen experimental conditions reflect a possible field situation. After 4 days of feeding, the root systems were washed and the different root types were rated for damage using a scale from 0 (no visible damage) to 3 (pruned or completely tunneled roots). In a second approach, freshly grown, maize or teosinte seedlings with 2-3 fully developed leaves were removed from pots and their roots were gently washed under a stream of warm water. The root systems were then laid onto a moist filter paper embedded in a large petri dish (12cm diameter). The petri dish had a cavity on the side, into which the stem was laid, leaving the leaves of the plant freely outside of the dish. Six second instar larvae were introduced into the dish, which was sealed with its lid and laid out on a table supplied with plant lights. To guarantee moisture saturated air around the exposed roots, water-drenched paper tissue was wrapped around the petri dishes, followed by a layer of aluminium foil to shade the roots from light. Every three hours for 24h, the foil and paper were removed and the position of the larvae was recorded. Four independent experiments were carried out using this procedure: First, the preference of *D. virgifera* for the different root types was determined by exposing larvae to the root systems of maize (n=8) or teosinte (n=18). In a third experiment, the generalist root herbivore *Diabrotica*

balteata and the leaf-herbivore *Spodoptera littoralis* were observed on independent root systems of maize and compared with *D. virgifera* (n=15). *S. littoralis* larvae will accept belowground tissues as food source if leaves are unavailable. Finally, the behavior of *D. virgifera* on wild type (H88) and BXD mutant plants (*bx1*) was compared (n=16). The *bx1* mutant produces only trace amounts of BXDs (Frey, M *et al.*, 1997). An additional choice assay between H88 and *bx1* plants was realized using the same setup, but by combining the root systems of the two genotypes in the same dish before adding *D. virgifera* larvae and observing their behavior (n=25).

Diabrotica virgifera *performance*

To measure the performance of *D. virgifera* on different root types, root systems of maize seedlings were gently washed and either the primary or crown roots were re-potted in 50ml falcon tubes filled with moist soil (n=12). A single 2nd instar *D. virgifera* larva was weighed and introduced into the falcon tube, which was then sealed at the top using plastic film, leaving just a small opening for the roots. The falcon tube together with the rest of the root system was then buried in a bigger pot filled with soil, thereby guaranteeing that all roots of the plants were able to grow in an adequate environment. After 6 days, the larvae were recovered from the falcon tubes and re-weighed. To evaluate growth of *D. virgifera* under more controlled conditions, a second experiment was carried out. Roots of maize plants were washed, and either the primary or a crown root was gently slid into a 200µl micro-capillary (n=6). Twenty µl of water were added to the capillary to guarantee adequate water supply. A single, pre weighed 2nd instar *D. virgifera* larva was then introduced into the capillary, which was sealed at the top and bottom with aluminum foil. The capillary together with the rest of the root system were put on a moist paper tissue and covered with another piece of wetted paper. After 24h, the larvae were removed from the capillaries and re-weighed.

Statistical procedures

Average ratings of leaf-senescence, final biomass of the plants in the root-removal experiments and herbivore performance in the capillary assays were compared using one-way Analysis of Variance (ANOVA). Where data did not conform to normality $\log_{10}+1$ transformation was carried out. Mann-Whitney Rank Sum Tests were used for data that could not be normalized by transformation. Differences in free amino acids, soluble sugar and BXD concentrations in different roots of the same plants were tested using paired T-tests (plant as subject, type of root as treatment). Induction of defense markers, BXDs and phenolic compounds by JA was tested using Two-Way ANOVAs (root type and treatment as factors, roots sampled on independent plants). The amount of radioactivity in the different roots, the damage ratings for *D. virgifera* and *D. virgifera* performance on crown and primary roots were tested using Two-Way ANOVAs (plant

and root type as factors). Holm-Sidak post-hoc tests were used for pairwise comparisons following ANOVAs. Preference of *D. virgifera* for mutant or wild type roots was tested using a Chi^2-test. Herbivore preference for the different root types in the petri-dish assays was determined using average counts of larva for each root type over 24h using a log-linear model as described (Erb, Matthias *et al.*, 2010).

RESULTS

Crown roots are more valuable and nutritious than primary and secondary roots

Thirty days after selective excision of different roots, the leaves of maize plants without crown roots showed stronger symptoms of senescence (Figure 1a) and had a lower leaf biomass than plants without embryonic roots (Figure 1a inset). These results were confirmed in a second experiment showing that plants without crown roots grew significantly less tall (Figure 1b) and accumulated less biomass than control plants or plants without primary or secondary roots (Figure 1b inset).

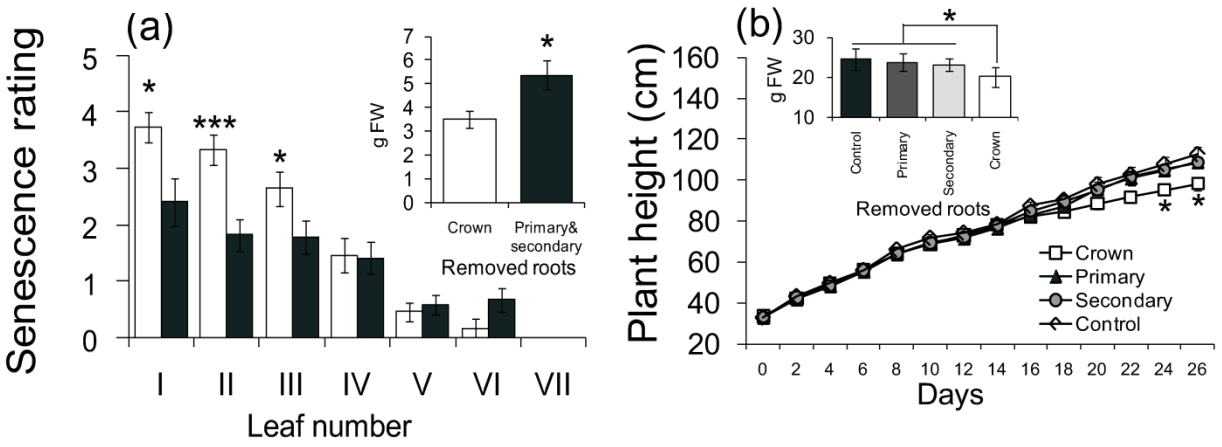

Figure 1: *Crown roots are more valuable for young maize plants than primary and secondary roots.* (a) Average (±SE) leaf senescence (main) and leaf biomass (inset) of maize plants 20 days after the removal of crown (white bars) or primary and secondary roots (black bars). Senescence rating goes from 0 (no visible symptoms) to 5 (leaf yellow and wilting). Leaf numbers correspond to the number of fully developed leaves on maize seedlings from I (oldest leaf) to VII (youngest). (b) Average (±SE) plant height (main) and leaf biomass (inset) of maize plants at different times after removal of different root parts in a separate experiment. FW= fresh weight. Stars indicate significant differences between treatments (*p<0.05, **p<0.01, ***p<0.001).

Crown roots also contained significantly higher amounts of most measured free amino acids (Figure 2a), total soluble protein (Figure 2a inset), sucrose (Figure 2b) and starch (Figure 2b inset) than primary roots. Moreover, crown roots were the primary sink for newly fixed CO_2: Two hours after the administration of $^{11}CO_2$ to source leaves of 20 day-old plants, the highest activity could be detected in the emerging and elongating crown roots (Figure 2c and 2d).

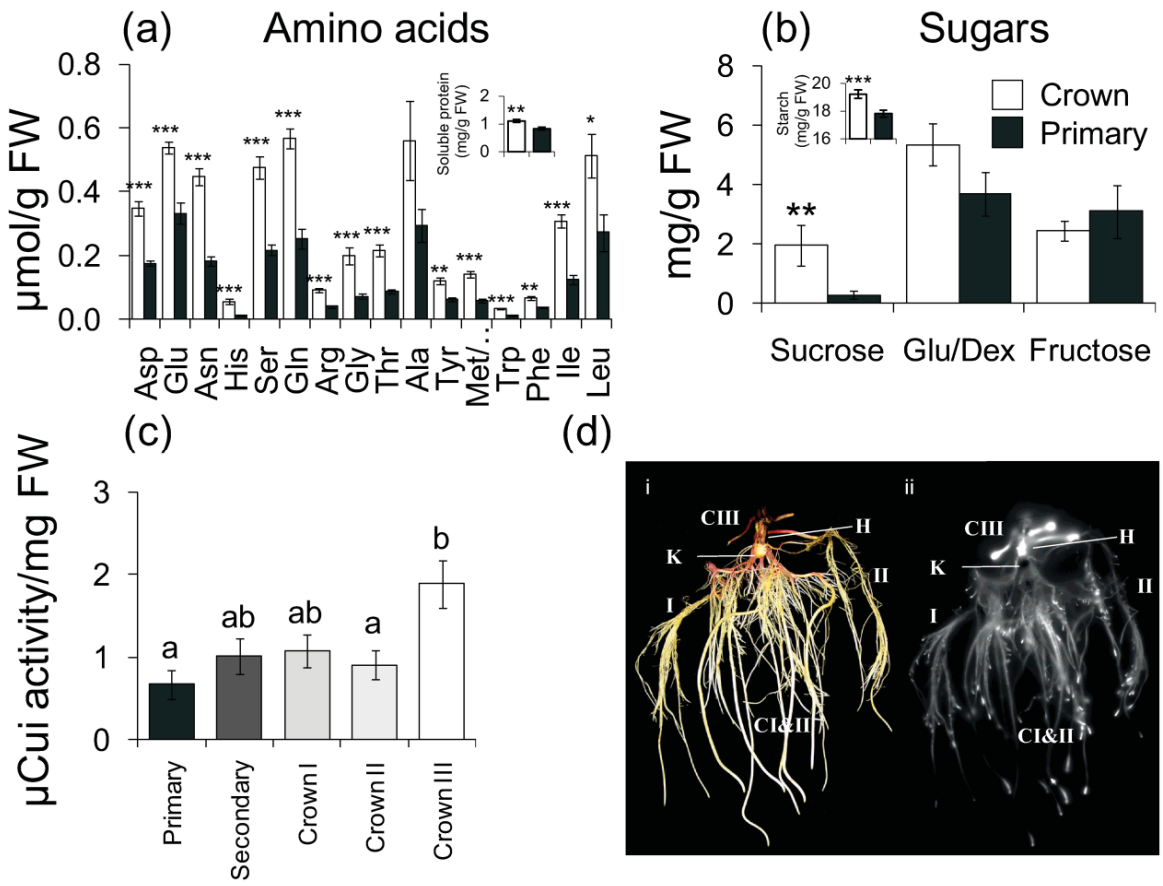

Figure 2: *Crown roots are major sinks for photoassimilates and nutrients.* (a) Average free amino acid concentrations (±SE) in crown (white bars) and primary roots (black bars) of maize seedlings. Inset: Total soluble protein (±SE) in crown and primary roots. (b) Average concentrations (±SE) of sucrose, glucose/dextrose and fructose in the different root types. Inset: Starch concentrations (±SE) in crown and primary roots. Stars indicate significant differences between root types (*p<0.05, **p<0.01, ***p<0.001). (c) Average accumulation of ^{11}C in different root types 90 minutes after $^{11}CO_2$ administration to leaves of 20 day old maize plants. Crown root generations are shown separately from I (oldest) to III (youngest, emerging roots). Different letters indicate significant differences between root types (p<0.05). (d) Photograph (i) and beta-image (ii) of roots of a $^{11}CO_2$ exposed maize plant. Brighter white indicates higher accumulation of ^{11}C assimilates. H: Hypocotyl, K: Kernel, I: Primary root, II: Secondary Root, C: Crown roots from I (oldest) to III (youngest). FW= fresh weight.

Crown roots have higher concentrations of BXDs and phenolics

To test whether embryonic and post-embryonic roots contain different amounts of defensive metabolites, we analyzed crown and primary roots for BXDs, which are key resistance factors of grasses (Niemeyer, 2009). Crown roots had higher total concentrations of BXDs than primary roots (Figure 3a inset). Especially the concentration of the highly toxic aglucone DIMBOA was 5-fold higher in crown roots (Figure 3a). HDMBOA-Glc and HDM2BOA-Glc, were present in slightly lower concentrations, resulting in a root-specific BXD profile. No difference was found between primary and secondary roots (see Figure S2a). Teosinte plants contained 4 times less BXDs than the commercial maize hybrid (Figure 3b, inset). The BXD distribution pattern however was similar, with crown roots containing pronouncedly higher amounts of DIMBOA and

lower amounts of HDMBOA-Glc and HMBOA-Glc (Figure 3b). While *D. virgifera* infestation did not significantly change BXD concentrations in maize (see Figure S2a), JA treatment induced DIMBOA and HDM2BOA-Glc and reduced HDMBOA-Glc in crown roots, but not in embryonic roots (see Figure S2b).

Crown roots also contained 10-times more of the phenolic compound cholorogenic acid than primary and secondary roots (see Figure S2a). Chlorogenic acid concentrations in crown roots were constitutively higher and did not change significantly following JA treatment. The gene *Zm-bx1*, which encodes for the first dedicated enzymatic step of BXD production and *Zm-Pal*, which is involved in the biosynthesis of phenolics, were most strongly expressed in crown roots (Figure S3b). Leaf-defense markers coding for proteinase inhibitor (PI) homologues (Erb *et al.*, 2009a) showed a strong induction after JA treatment. For all tested PIs, crown roots were more inducible by JA than primary and secondary roots, while basal levels were slightly higher in the latter (Figure S3b). We found BXDs to be the dominant compounds in maize root exudates (see Figure S4). In accordance with the endogenous pattern, crown roots of maize plants also exuded significantly more DIMBOA-Glc than primary roots (Figure 3b).

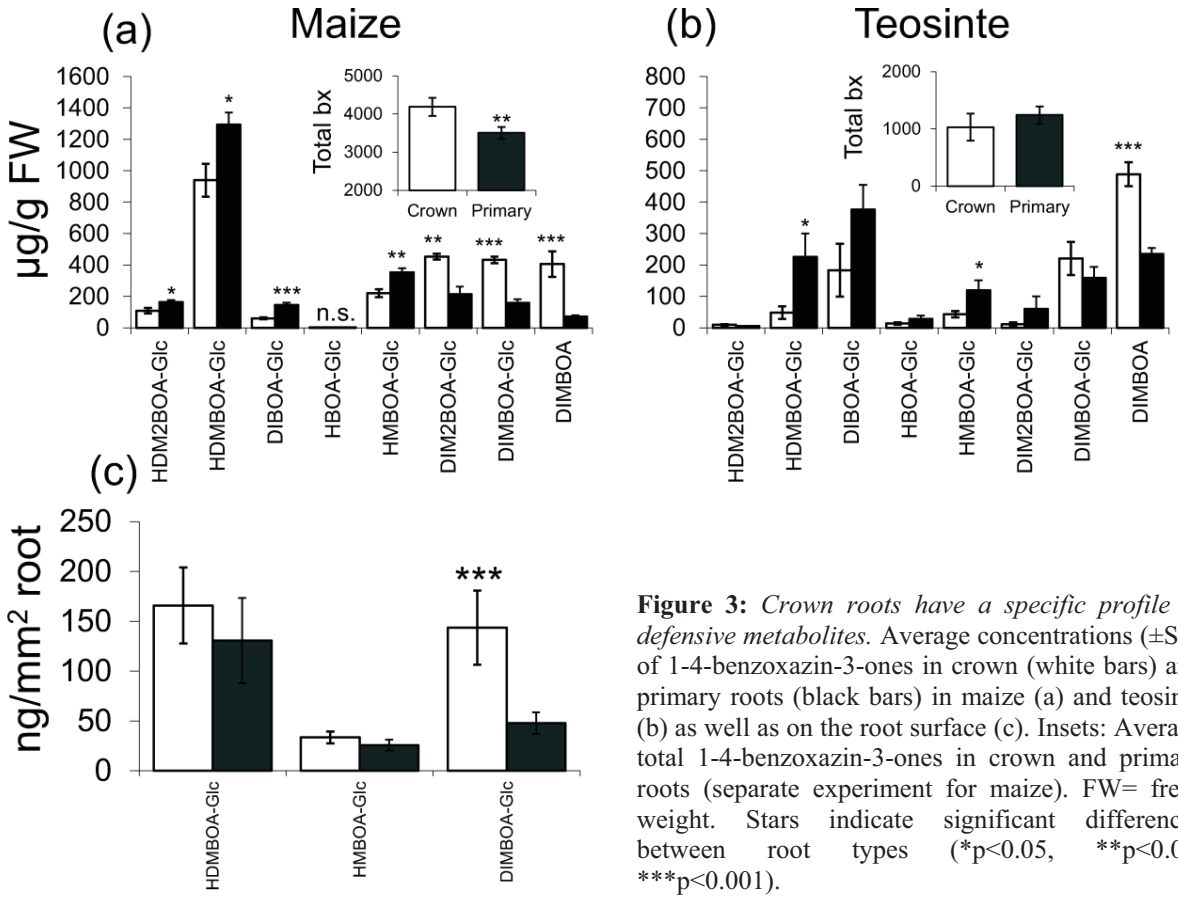

Figure 3: *Crown roots have a specific profile of defensive metabolites.* Average concentrations (±SE) of 1-4-benzoxazin-3-ones in crown (white bars) and primary roots (black bars) in maize (a) and teosinte (b) as well as on the root surface (c). Insets: Average total 1-4-benzoxazin-3-ones in crown and primary roots (separate experiment for maize). FW= fresh weight. Stars indicate significant differences between root types (*p<0.05, **p<0.01, ***p<0.001).

In contrast to generalist herbivores, the specialist Diabrotica virgifera *preferentially feeds on crown roots*

When we infested young maize plants with root-feeding larvae of the maize specialist *D. virgifera*, we observed that second instar larvae preferably fed on crown roots: After four days of infestation, crown roots of infested plants showed obvious signs of damage and were often chewed off, while primary and secondary roots were largely intact (Figure 4a and 4b). *In vivo* observations confirmed the pronounced preference of the larvae for crown roots over other root tissues (Figure 4c) over the full observation period of 24h (Figure 4c inset). This preference pattern was similar on teosinte plants (Figure 4d).

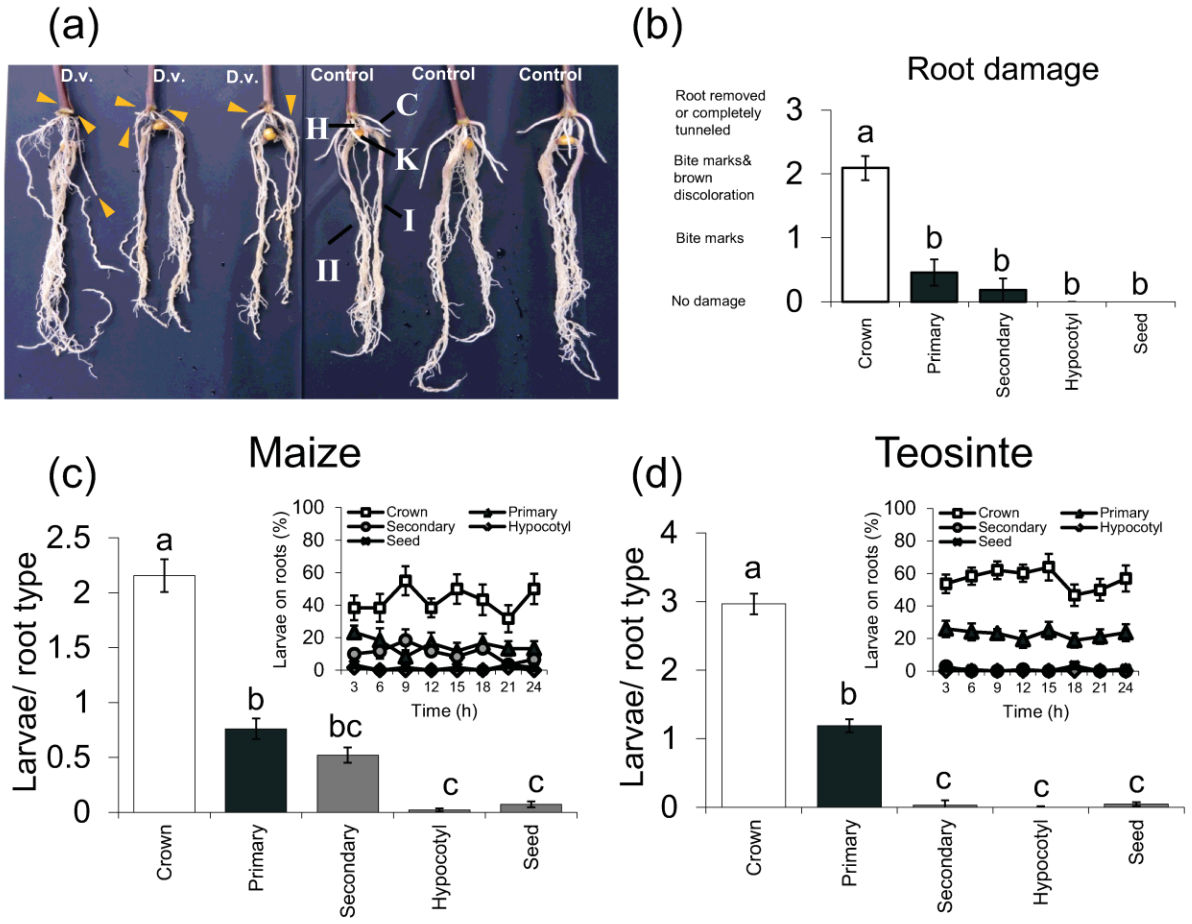

Figure 4: *The specialist* Diabrotica virgifera *feeds preferentially on crown roots*. (a) Representative photographs of 10 day old maize seedlings 4 days after *D. virgifera* attack (left, D.v.) compared to unattacked plants (right, controls). Orange triangles point to pruned or attacked crown roots. H: Hypocotyl, K: Kernel, I: Primary root, II: Secondary Root, C: Crown roots. (b) Average values (±SE) of visual damage rating of roots after *D. virgifera* attack from 0 (no visible damage) to 3 (root removed or completely tunneled). (c) and (d) Average number of larvae on each root type (mean over 24h) of maize and teosinte. Insets: Percentage (±SE) of larvae observed on different root types over an observation period of 24h. Different letters indicate significant differences between root types ($p<0.05$).

In contrast to maize, we rarely observed *D. virgifera* larvae feeding on secondary roots, which were much smaller and sometimes entirely absent from the teosinte seedlings. When comparing the behavior of the specialist with generalist herbivores, *D. virgifera* again showed a pronounced preference for crown roots. When embryonic roots (primary and secondary) were compared with post-embryonic roots (crown roots), the preference of *D. virgifera* for the latter was highly significant (Figure 5a inset). The generalists *S. littoralis* and *D. balteata*, on the other hand, showed a tendency to settle on primary roots and strongly preferred embryonic over post-embryonic roots (Figure 5a). *S. littoralis*, which normally feeds on leaves, had a lower feeding activity compared to the two root-feeders (Figure 5b).

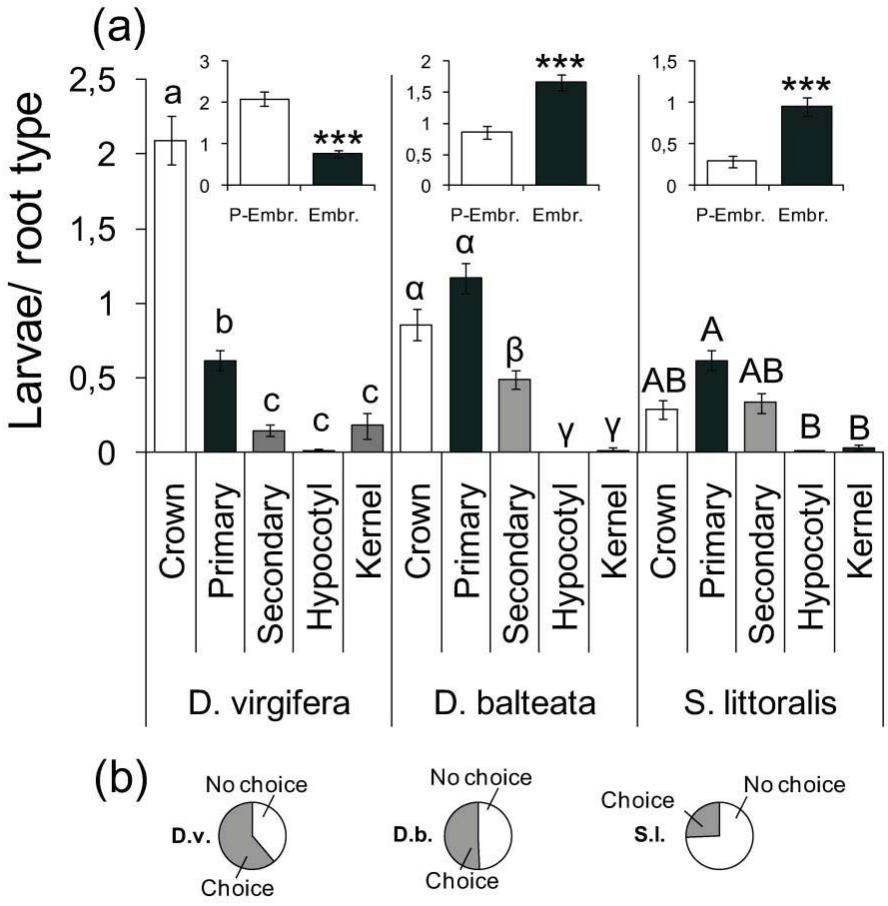

Figure 5: *Unlike the specialist* Diabrotica virgifera*, generalist herbivores prefer embryonic roots*. (a) Average number (±SE) of larvae of *Diabrotica virgifera*, *Diabrotica balteata* and *Spodoptera littoralis* observed on different root types over an observation period of 24h. Different letters indicate significant differences between root types within species ($p<0.05$). Insets: Average number of larvae on post-embryonic (white bars: Crown roots) and embryonic roots (black bars: Primary and secondary roots). Stars indicate a significant preference (***$p<0.001$). (b) Average proportion of larvae feeding on the roots (gray) *vs*. inactive individuals (white).

D. virgifera *uses BXDs as foraging cues*

To test whether *D. virgifera* grows differently on different root types and whether its development and preference are affected by BXDs, we confined larvae to feed on either crown or primary roots of wild-type (H88), or of *bx1* mutant maize plants, which only produce trace amounts of BXDs (Frey, M. *et al.*, 1997). *D. virgifera* larvae gained over 50% more weight on crown than on primary roots on both wild-type and mutant plants (Figure 6a). In an additional experiment, we confirmed that *D. virgifera* also grows better on crown than on primary roots when feeding on the BXD producing maize hybrid Delprim over 24h (Figure 6b).

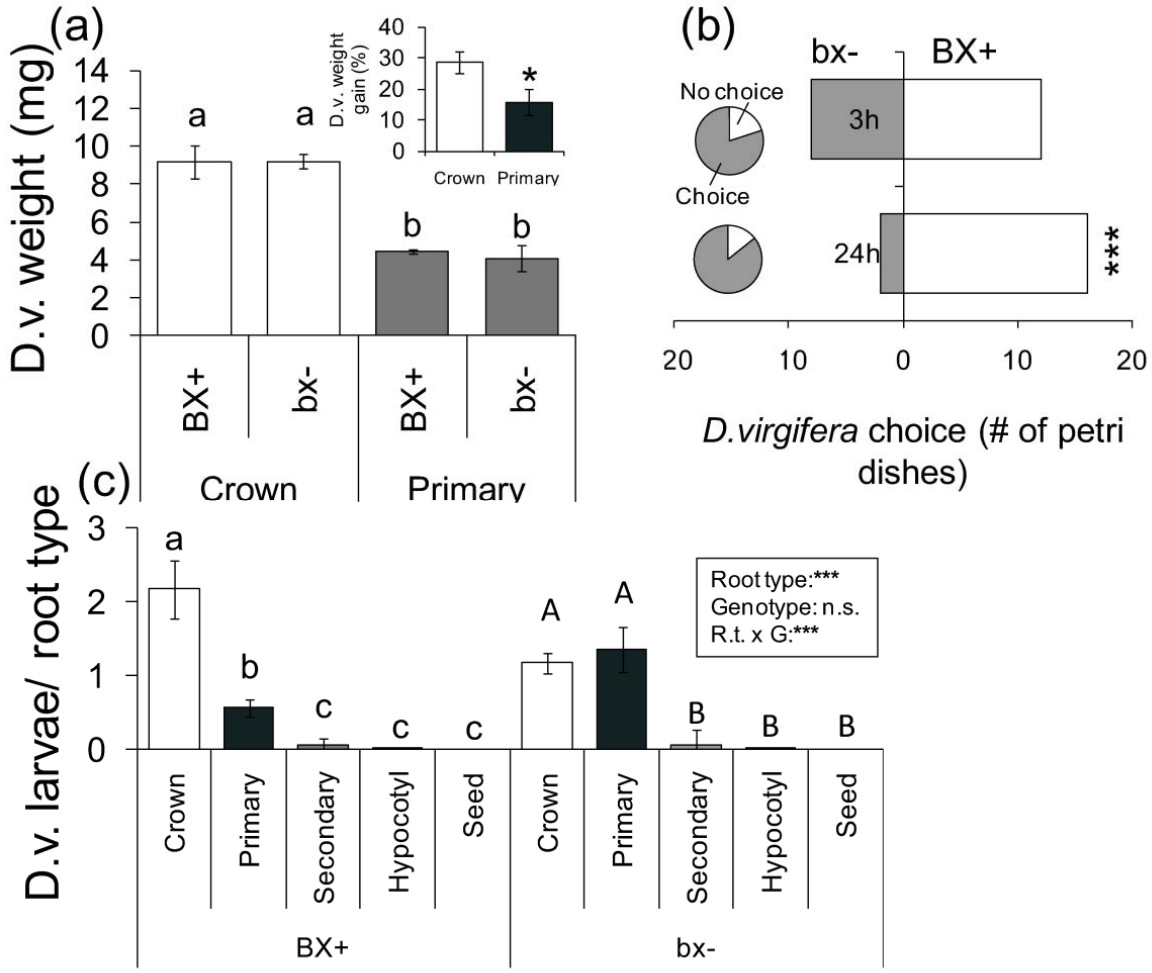

Figure 6: Diabrotica virgifera *requires 1-4-benzoxazin-3-ones to locate optimal roots.*(a) *D. virgifera* weight (±SE) 7 days after feeding on crown or primary roots (no-choice assay) of plants producing 1-4-benzoxazin-3-ones (BX+) or *bx1* mutants without these compounds (bx-). Different letters indicate significant differences between root types and genotypes. Inset: Average relative growth of *D. virgifera* feeding on crown or primary roots of the 1-4-benzoxazin-3-one producing hybrid Delprim over 24h ($p<0.05$). (b) Choice of *D. virgifera* for BX+ (white bars) or bx- roots (gray and black bars) at 3h (top) and 24h (bottom). Numbers of petri dishes with a choice of the larvae for either genotype are shown. Pie charts show the proportion of dishes with equal distribution of larvae (no choice situation, white). Stars indicate a significant preference (***$p<0.001$). (c) Average number of *D. virgifera* larvae on each root type (mean over 24h) in a no-choice situation. Significance levels (***$p<0.001$) of a Two-Way ANOVA are shown for the factors "root type", "genotype" and the interaction term (root type*genotype). Different letters indicate significant differences between root types (Tukey's HSD: $p<0.05$) within the BX+ (small letters) and bx- (big letters) plant genotypes.

From preliminary observations, it appeared that *D. virgifera* showed no preference for crown roots in the mutant plants. We therefore hypothesized that the specialist uses BXDs to distinguish between crown and primary roots. Indeed, while the choice pattern in the WT line H88 was similar to our previous observations with the hybrid Delprim, *D. virgifera* no longer distinguished primary from crown roots when feeding on mutant plants (Figure 6c).

DISCUSSION

The root removal experiments demonstrate that crown roots are more important for maize development than the primary and secondary roots (Figure 1). These results are in accordance with the typical development of many gramineous root systems, including teosinte: While in the first days after germination, primary and secondary roots are responsible for nutrient and water uptake, the plants start to grow multiple layers of shoot-borne crown roots soon after, which take over these functions and become essential for overall plant performance (Hochholdinger & Tuberosa, 2009). Thus, analogous to what has been found aboveground (Ohnmeiss & Baldwin, 2000; Rostás & Eggert, 2008), newly developing belowground tissues have the highest relative value for the plant. That crown roots are metabolically more active than primary and secondary roots becomes evident from our $^{11}CO_2$ allocation data (Figure 2c and 2d) as well as sugar and amino acid measurements (Figure 2a and 2b). These differences are likely to be the result of an increased investment into the growth of these newly forming roots.

For herbivores, newly emerging crown roots would seem an ideal food source, as they are rich in carbohydrates and amino acids as well as soluble proteins (Figure 2). However, our results suggest that differences in defensive chemistry may reduce the quality of post-embryonic roots for non-adapted consumers: Crown roots contained and exuded higher amounts of basal and JA-inducible BXDs (Figure 3 and S2b), which are considered a particularly important resistance factor of grasses (Macias *et al.*, 2009; Niemeyer, 2009). Especially the 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), which was increased 5-fold in crown roots compared to embryonic tissues (Figure 3) has been shown to be toxic against a wide variety of insect herbivores (Rostas, 2007; Niemeyer, 2009; Glauser *et al.*, 2011a). Interestingly, *D. virgifera* infestation, contrary to JA treatment, did not induce DIMBOA in crown roots. It remains to be elucidated if *D. virgifera* has the ability to suppress the production of BXDs, or if the induction by the herbivore, contrary to a systemic JA treatment, is predominantly local (Hiltpold, Ivan *et al.*, 2011), making it hard to detect when analyzing entire roots. Untreated crown roots also contained 10 times more chlorogenic acid than primary roots (see Figure S3a), a phenolic compound that has been implicated in maize resistance (Nuessly *et al.*, 2007) and expressed proteinase inhibitor genes, which have an important role in induced resistance (Cordero *et al.*, 1994; Ton *et al.*, 2007),

at higher induced levels (see Figure S3b). The higher levels of defensive compounds may explain why the generalist *D. balteata* and the leaf-feeder *S. littoralis* avoided crown roots and preferred to feed on embryonic roots instead (Figure 5a insets). From the experiment with teosinte (Figure 3b), it becomes clear that at least the higher levels of DIMBOA in crown roots are not an artifact of plant breeding, but a conserved preferential allocation pattern that may help the plant to defend its most valuable belowground tissues against environmental threats. It is interesting to note that cultivated maize contained over 4 times more BXDs that the wild teosinte plants (Figure 3), and it remains to be determined if this is due to positive selection for insect resistance during cultivation, or if BXDs have other, yet unknown functions in *Zea mays* spp. that were targeted by plant breeders.

Interestingly, the alleged BXD defense strategy of maize and teosinte does not seem to work against the specialist *D. virgifera* : The larvae of the root feeder strongly preferred crown roots over all other parts of the root system (Figure 4). That this behavior may be adaptive for *D. virgifera* is evident from the fact that the herbivore grows pronouncedly better on crown roots (Figure 6a). In artificial diet assays, it was found that the protein source is an important determinant of *D. virgifera* fitness (Pleau *et al.*, 2002), and it is likely that the insect is able to take advantage of the higher concentrations of free amino acids, soluble proteins and photo-assimilates, including sucrose and starch (Figure 2). The performance results are in accordance with an earlier study reporting that *D. virgifera* develops better on growing root systems than older plants in the generative phase (Hibbard *et al.*, 2008). The fact that *D. virgifera* gains more weight on crown roots, despite the fact that they contain more active 1-4-benzoxazin-3-ones begs the question if the herbivore is resistant to this type of defense. Earlier studies found positive correlations between DIMBOA contents and resistance against *D. virgifera* in some, but not all cases in the field (Assabgui *et al.*, 1995; Davis *et al.*, 2000). However, none of these studies took into account the differential distribution of 1-4-benzoxazin-3-ones among root types, and the employed analytical techniques did not permit to separate the full profile of 1-4-benzoxazin-3-ones. In our assays, it was evident that *D. virgifera* was not negatively affected by the presence of 1-4-benzoxazin-3-ones in the roots (Figure 6a). *D. virgifera* is known for its capacity to rapidly evolve resistance or tolerance to pest control methods, including novel compounds like the *bt* toxin (Meihls *et al.*, 2008), and our results show that it possesses effective tolerance or detoxification mechanisms against the naturally occurring 1-4-benzoxazin-3-ones and possibly also the other defensive traits of maize roots, thereby enabling it to fully exploit the higher nutrient content of crown roots. Additional research will be necessary to elucidate the mechanism that enables *D. virgifera* to tolerate BXDs. As yet, nothing is known about BXD detoxification in chrysomelids.

D. virgifera does not only tolerate 1-4-benzoxazin-3-ones, but even uses them to identify the most nutritional roots: When given a choice, the herbivore settled on 1-4-benzoxazin-3-one containing roots rather than those of the *bx1* mutants (Figure 6b), and in the absence of BXDs, it did no longer distinguish between crown and primary roots (Figure 6c). MBOA, a breakdown product of DIMBOA and HDMBOA, has previously been reported to attract *D. virgifera* larvae *in vitro* (Bjostad & Hibbard, 1992): In choice assays, *D. virgifera* larvae preferred volatile compounds coming from MBOA-treated glass wool within a CO_2 background. Another study, however, reported that the application of pure DIMBOA and MBOA to maize roots deterred *D. virgifera* (Xie *et al.*, 1992). The experiments in that case were conducted with BXD producing roots that were dipped into ethanol solutions containing 250-1000 ppm of additional DIMBOA or MBOA, concentrations which are well above the amounts that are typically exuded by maize roots (Figure 3). While the contradictory results of these two studies indicate that *D. virgifera* is highly sensitive to 1-4-benzoxazin-3-ones, they also illustrate how challenging it is to develop biologically meaningful *in vitro* assays to test the role of BXDs in *D. virgifera* feeding behavior. Our experiments involving a BXD deficient mutant circumvent potential problems associated with purification and dose for *in vitro* tests and show that *D. virgifera* is not repelled by the higher DIMBOA concentrations in crown roots, but uses BXD patterns to select optimal roots. That *D. virgifera* also showed a preference for crown roots of teosinte (Figure 4d), despite the fact that the total amount of BXDs was similar between crown and primary roots (Figure 3b inset), suggests that *D. virgifera* may either use single BXDs like DIMBOA or ratios between compounds (e.g. DIMBOA/HDMBOA-Glc) to select roots. In general, it should be noted that knocking out BXD production may also affect the root metabolism and other defensive processes. Using a different set of mutants, it was recently shown that BXDs have a positive effect on callose deposition following plant treatment with fungal elicitors (Ahmad *et al.*, 2011). Wound-induced JA-accumulation in the leaves on the other hand is not affected by the *bx1* mutation (Huffaker *et al.*, 2011).

Future research should aim at determining whether such indirect effects, possibly also in combination with changes in nutritional patterns, may affect the feeding behavior of *D. virgifera*. Generating maize lines that have an altered capacity to express *bx1* downstream genes will make it possible to further disentangle the role of individual BXDs in below ground plant-insect interactions. Overall, our study shows that *D. virgifera* hijacks a plant defense that is most probably targeted at deterring attackers from nutritious tissues. Thereby, it effectively turns the tables on maize to maximize its own fitness.

CONCLUSION

Evolving the capacity to cope with plant defenses is thought to drive radiation of insect species, and, consequently, the co-evolution among plants and insects (Wheat *et al.*, 2007). The capacity of *D. virgifera* to tolerate BXDs may therefore have facilitated its ongoing spread and success through maize growing regions around the world, contrary to other *Diabrotica* species, which have remained much less important. The current study shows that plant defenses may not only determine the large scale abundance of insect herbivores (Johnson & Gregory, 2006), but their distribution within the plant system: Small scale adaptive behavior enabled the generalists *D. balteata* and *S. littoralis* to escape toxic compounds, whereas it facilitated the aggregation of *D. virgifera* on the most nutritious tissues. The distribution of herbivores within a root system is evidently an important determinant of the fitness of both plant (Figure 1) and insect (Figure 6). We therefore conclude that understanding plant-insect interactions at this scale is important to determine the factors that shape natural and agricultural systems.

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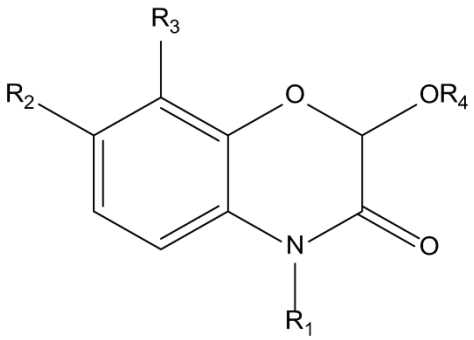
SUPPLEMENTARY FIGURES AND APPENDIX

Appendix S1: *Method for surface extraction and quantification of 1,4-benzoxazin-3-onesusing NanoMate*

To measure concentrations of exuded BXDs, we developed a method based on liquid extraction surface analysis using the Advion TriVersaNanomatechip-based infusion nanoESI system (Advion bioscience, NY, USA) equipped with a 1536 surface sampling plate and a disposable ESI chip with a 200 × 200 array of nozzles. The developed liquid surface extraction method permitted us to target specific points of the root surface at a resolution of 1mm2 and extract the surface compounds using a 1µl solvent droplet. The solvent used for the analysis was MeOH/H2O 1/1 with 0.1 % formic acid. Two µl were aspirated while 1 µl was dispensed at 0.5 mm above the surface sample for 5 seconds to ensure the junction with the tip and the diffusion of compounds through the drop. A drop of 1 µl covers approximately 1 mm² of the surface sample. MS signals were recorded on a triple quadrupole mass spectrometer (TSQ vantage, Thermo Scientific) equipped with an ESI interface using selected reaction monitoring (SRM) in positive ionization mode The following transitions were monitored: for HMBOA-Glc, *m/z* 358.1 → 178; for HDMBOA-Glc, *m/z* 388.1 → 166, DIMBOA-Glc, 374.1 → 166. The optimized collision energy was set to 19 eV for all transitions. Absolute quantities of BXDs were determined using the average value of three standard curves obtained from purified DIMBOA-Glc, HMBOA-Glc and HDMBOA-Glc. The sampling procedure was as follows: Roots of intact 12 day-old maize seedlings were gently removed from the pots, washed and kept on a wet paper for five minutes to allow exudation. The plants were directly placed on the 1536 surface sampling plate. For each measurement, the X, Y and Z position was estimated by doing a blank analysis without solvent. A second analysis at the same coordinate with the extraction solvent was then carried out and the SRM transitions were recorded during 180 seconds. Two sample spots were taken for each root type of each plant (n=6).



Compound	Abbreviation	R1	R2	R3	R4
2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one	DIMBOA	OH	OCH_3	H	H
2-b-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one	DIMBOA-Glc	OH	OCH_3	H	Glc
2-b-D-glucopyranosyloxy-4-hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one	DIM2BOA-Glc	OH	OCH_3	OCH_3	Glc
2-b-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one	HMBOA-Glc	H	OCH_3	H	Glc
2-b-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one	DIBOA-Glc	OH	H	H	Glc
2-b-D-glucopyranosyloxy-1,4-benzoxazin-3-one	HBOA-Glc	H	H	H	Glc
2-b-D-glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one	HDMBOA-Glc	OCH_3	OCH_3	H	Glc
2-b-D-glucopyranosyloxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one	HDM2BOA-Glc	OCH_3	OCH_3	OCH_3	Glc

Figure S1: *Structures and full names of 1,4-benzoxazin-3-ones in maize roots*

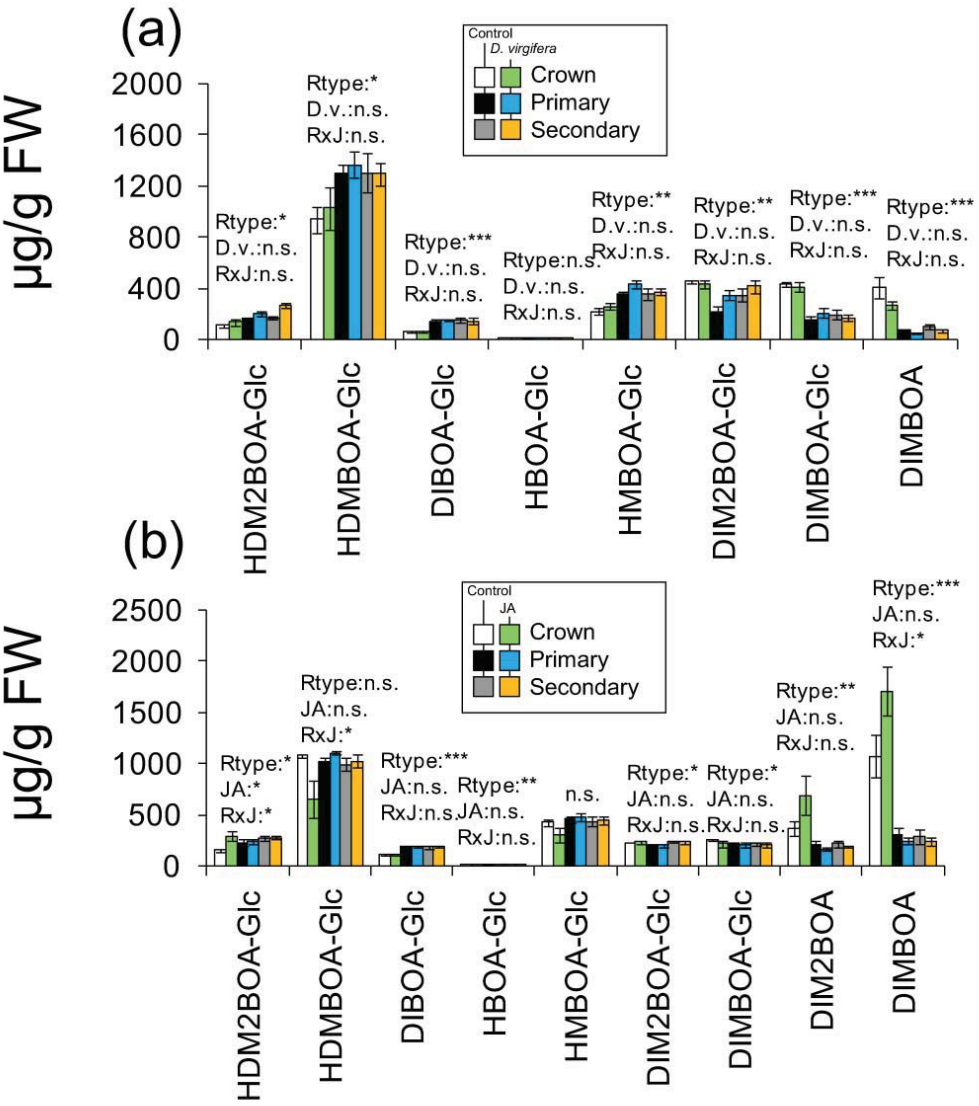


Figure S2: *Induction of 1,4-benzoxazin-3-ones by* Diabrotica virgifera *and jasmonic acid.* (a) Average (±SE) abundance of BXDs in crown (white and green) primary (black and blue) and secondary (gray and orange) roots in controls (gray shaded bars) and *Diabrotica virgifera* infested plants (colored bars). Values of uninduced plants are identical to Figure 3a. (b) Average (±SE) abundance of BXDs in crown, primary and secondary roots in controls (grey shaded bars bars) and JA-treated plants (colored bars). Note that in this experiment, DIM2BOA could also be quantified due to improved chromatographic separation. Results of Two-Way-ANOVAs are shown for each gene (Rtype=root type, JA= JA treatment, RxJ= Interaction term, n.s.=not significant, *p<0.05, **p<0.01, ***p<0.001).

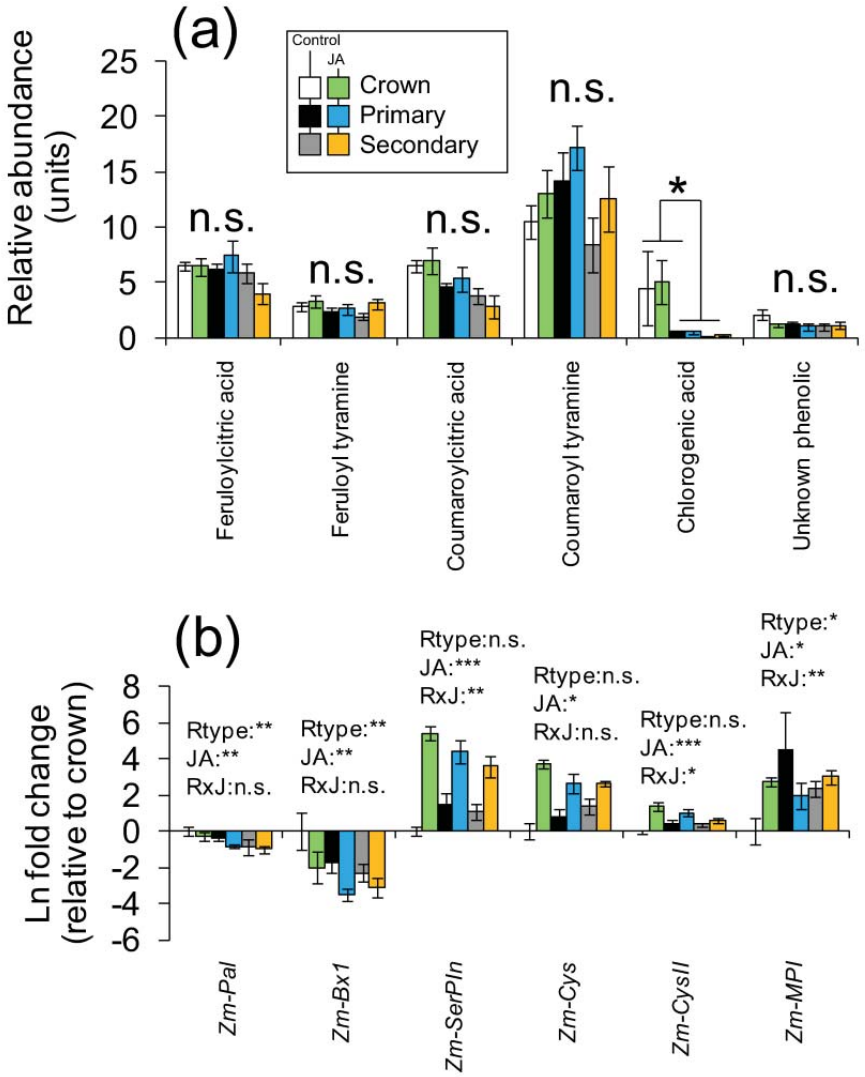


Figure S3: *Basal and induced phenolic compounds and proteinase inhibitors in maize roots.* (a) Average (±SE) abundance of detected phenolic compounds in crown (white and green) primary (black and blue) and secondary (gray and orange) roots in controls (gray shaded bars) and JA-treated plants (colored bars). Stars indicate significant differences between crown and embryonic roots (n.s.=not significant, *p<0.05, **p<0.01, ***p<0.001). (b) Average (±SE) Ln-fold change of gene expression in different roots of control- and JA-treated plants relative to untreated crown roots. Results of Two-Way-ANOVAs are shown for each gene (Rtype=root type, JA= JA treatment, RxJ= Interaction term, n.s.=not significant, *p<0.05, **p<0.01, ***p<0.001).

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CHAPTER IV

Induced susceptibility: Below ground attack reduces root resistance and increases root herbivore attraction in maize

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ABSTRACT

Herbivory usually leads to the induction of resistance responses in plants that negatively affect the attacker. However, growing evidence suggests that specialized insect herbivores can reconfigure a plants' primary and secondary metabolism, thereby increasing plant susceptibility to the benefit of the herbivore. Although induced resistance and susceptibility are well studied and understood aboveground, little is known about the prevalence of induced responses belowground. A recent study suggested that feeding by the specialist root herbivore*, Diabrotica virgifera virgifera*, makes maize roots more susceptible to conspecifics. We conducted field and laboratory experiments to understand the behavioral responses and biochemical mechanisms underlying this phenomenon of induced susceptibility. We found that *D. virgifera* benefits from feeding on a root system in groups of intermediate size, whereas its performance was reduced in large groups. Interestingly, the herbivore was able to select host plants with an optimal density of conspecifics by using induced plant volatiles such as *(E)*-β-caryophyllene and α-humulene in a dose-dependent manner. Using a split root experiment, we show that the plant induced-susceptibility is systemic and, therefore, plant-mediated. Chemical analyses on plant resource reallocation and defenses upon herbivory showed that the systemic induced-susceptibility is likely to stem from a combination of (i) increased free amino acid concentration and (ii) relaxation of defense inducibility. Understanding the mechanisms underlying induced susceptibility in maize will contribute to our understanding of the remarkable ecological success of the pest, and may pave new avenues for pest management.

INTRODUCTION

To withstand herbivory, plants reconfigure their metabolism (Karban & Baldwin, 1997; Walling, 2000; Schwachtje & Baldwin, 2008). This reconfiguration includes the production of toxic secondary metabolites (Steppuhn *et al.*, 2004b; Glauser *et al.*, 2011b), as well as reallocation of primary compounds (Babst *et al.*, 2005; Orians, CM *et al.*, 2011). In many cases, the induced changes increase the plant's resistance against the attacking herbivore. For instance, *Spodoptera spp.* caterpillars induce the production of secondary metabolites such as 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA-Glc) in maize leaves, which deters the larvae from the plant (Erb *et al.*, 2009a; Glauser *et al.*, 2011b). Also, induced nicotine in wild tobacco reduces herbivore damage in the field (Steppuhn *et al.*, 2004b). However, in some cases, herbivore attack can also reduce plant resistance. Most of the time, such plant susceptibility is induced by specialist herbivores, which have, over evolutionary time, adapted to specific host plants.

Several mechanisms have been proposed to contribute to induced susceptibility (Karban & Agrawal, 2002). First, herbivores may be able to suppress plant defenses. For instance, bark beetles, *Ips grandiicollis*, overcome conifer defenses by feeding in massive groups (Berryman *et al.*, 1989; Raffa, 2001; Wallin & Raffa, 2001; Kane & Kolb, 2010): the beetles reduce the flow of defensive resins by severing and blocking transport canals (Wallin & Raffa, 2001). *Helicoverpa zea* and *Spodoptera exigua* caterpillars reduce the induced defense levels with an inhibiting glucose oxidase contained in their saliva (Musser *et al.*, 2002; Bede *et al.*, 2006). Furthermore, spider mite *Tetranychus evansi* suppresses the induction of salicylic and jasmonic acid pathways in their host plants, resulting in decreased levels of proteinase inhibitors and defensive volatiles (Sarmento *et al.*, 2011). Second, specialized herbivores may have evolved resistance to induced secondary metabolites and use them to their own benefit (Ehrlich & Raven, 1964; Rausher, 1996). Such specialists have developed potent mechanisms to cope with host defenses (Berenbaum & Zangerl, 1998; Stout & Bostock, 1999; Glauser *et al.*, 2011b; Robert *et al.*, 2012) and in some cases sequester them for their own defenses against their natural enemies (Tallamy *et al.*, 1998; Muller *et al.*, 2001; Muller *et al.*, 2002; Nishida, 2002; Muller & Brakefield, 2003). Plant defenses can even be used specialist herbivores to locate host plants and the most nutritious tissues (Hopkins *et al.*, 1998; Agrawal & Sherriffs, 2001; Smallegange *et al.*, 2007; Howe & Jander, 2008; Robert *et al.*, 2012), further contributing to the plants' susceptibility. For example, induced plant volatiles attract *Spodoptera frugiperda* caterpillars to attacked maize plants (Carroll *et al.*, 2006), and wound-induced volatiles attract flea beetles to *Nicotiana attenuata* plants (Halitschke & Baldwin, 2003). Third, herbivores can induce plants to reallocate primary metabolites to their advantage. For instance, galling aphids are able to manipulate the sink-source translocation patterns of their host by creating sinks that are preferentially supplied with nutrients (Way &

Cammell, 1970; Larson & Whitham, 1991; Compson *et al.*, 2011). Similarly, the leaf miner, *Phyllonorycter blancardella*, maintains functional "green islands" photosynthetically active in senescent leaves (Giron *et al.*, 2007; Kaiser *et al.*, 2010).

One important aspect of induced susceptibility is density-dependence: herbivores may benefit from changes in their host plant that are induced by conspecifics as long as the resource is sufficiently abundant (Katano *et al.*, 2007). High densities of herbivore may lead to intraspecific exploitation competition that is likely to outweigh these benefits, leading to a net negative effect on herbivore fitness (Ellner *et al.*, 2001). Yet, it remains unclear to what extent specialist herbivores can select host plants on the basis of an optimal density of attacking conspecifics. Herbivore-induced plant volatiles are likely to be implicated as signals in such choices.

It is generally accepted that induced resistance is more common than induced susceptibility in above-ground plant-insect interactions (Karban & Agrawal, 2002), even for specialist herbivores (Agrawal & Kurashige, 2003). Some studies show that root-herbivory also induces plant defenses (van Dam, N.M., 2009), but in general evidence for induced resistance and/or induced susceptibility remains scarce, despite the fact that root herbivores are common in many ecosystems and are among the most important agricultural pests (Hunter, 2001). In a recent study, feeding by larvae of the vine weevil on raspberry plants caused a 19% reduction in growth of subsequently attacking weevil larvae (Clark *et al.*, 2011). On the other hand, slightly damaged onion bulbs support higher survival of *Delia antiqua* larvae (Hausmann & Miller, 1989), and *D. radicum* larvae grow better on previously attacked turnip plants (Pierre *et al.*, 2011). In all cases, the underlying mechanisms remain unclear.

We have found that the specialist root herbivore *Diabrotica virgifera virgifera* induces susceptibility in its host plant, *Zea mays*, and that *D. virgifera* uses induced volatiles to find infested host plants (Robert *et al.*, in press). We hypothesize that the larvae may also be stimulated to feed by these compounds. For the current paper we tested whether *D. virgifera* can use the induced volatiles to select plants with an optimal density of attacking conspecifics. As it has been reported that *D. virgifera* induces water-stress in maize (Godfrey, L.D. *et al.*, 1993; Dunn & Frommelt, 1998b; Erb *et al.*, 2011b), a condition which can lead to an increase in shoot-root assimilate flow, we also tested whether changes in primary metabolism may be responsible for the increase in performance. Finally, we investigated whether, analogous to bark beetles, which "overcome" plant defenses by attacking in high numbers, attack by *D. virgifera* reduces the capacity of maize plants to mobilize defenses in response to subsequent herbivory.

METHODS

Plants and insects

Maize plants (*Zea mays* L., variety Delprim) were sown in plastic pots (11 cm high, 4 cm diameter) by placing them on a layer of moist washed sand (0-4 mm, Jumbo, Switzerland). The seeds were then covered with 2 cm of commercial soil (Aussaaterde, Ricoter, Aarberg, Switzerland). Seedlings were grown in a climate chamber (23 ± 2 °C, 60% relative humidity, 16:8h L/D, and 350 $\mu mol.m^{-2}.s^{-1}$), and MioPlant Vegetable and Herbal Fertilizer (Migros, Switzerland) was added every two days after plant emergence. Twelve-day old plants with two fully developed leaves were used for the experiments. *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) eggs were obtained from the USDA-ARS-NCARL Brookings (SD, USA) and kept on freshly germinated maize until use. Second instar larvae were used in all experiments.

Field experiment

A field study was conducted at the University of Missouri Bradford Research and Extension Center, 9 km east of Columbia, MO in 2005. Field design and soil conditions were described elsewhere (Hibbard et al., 2010). Briefly, plots of 64 plants were planted using at 76.2 cm row spacing and a 17 cm seed spacing. Densities of 25, 50, 100, 300, 600, 1 200, and 2 400 viable *D. virgifera* eggs per 30.5 cm of maize row were applied into the soil and left to develop. Plots were covered with a screen tent (3.05 x 3.66 m, Coleman, Rye, NY) prior adult emergence to avoid them to escape from the plot. Beetles were then collected two to three times weekly using either mouth aspirators (BioQuip, Rancho Dominguez, CA) or battery operated aspirators (BioQuip, Rancho, Dominguez, CA) in tents with many adults. Adults were immediately transferred into 95% ethanol and head capsule widths of the collected beetles were measured.

Density effect on D. virgifera *performance and host selection under laboratory conditions*

To evaluate the density effect on *D. virgifera* performance, maize seedlings were infested with 1, 3, 6, 9 or 12 pre-weighed larvae. After two days, all larvae were collected and weighted again to evaluate their weight gain. The density impact *D. virgifera* host selection, healthy plants and plants infested with 1, 3, 6, 9, or 12 larvae were potted in two-arm belowground olfactometers as described elsewhere (Robert *et al.*, in press). All pots were filled using moist white sand (10% water, Migros, Switzerland) and covered with aluminum foil to restrain the light in the system and soil desiccation. After two days, the two pots were connected via a glass tube with a vertically connected access in the middle and one Teflon connector at both sides of the glass tube as previously described (Robert *et al.*, in press). The Teflon connectors contained a fine metal screen

(2,300 mesh; Small Parts Inc., Miami Lakes, FL., US), that allowed volatile dispersion but avoided any visual cues for the herbivore. Furthermore, Teflon connectors prevented the larvae from reaching the roots. Six larvae were released in the central glass connector of the olfactometer and their first choice towards one or the other plant was recorded according to (Robert *et al.*, in press). Larvae that did not choose after 15 minutes were noted "no choice". Leaf wilting was recorded for all plants and scored from 0 (no symptoms) to 4 (complete loss of turgidity). The experiment was repeated twice. At the end of the first assay, CO_2 emissions were evaluated as described below and root systems were collected and gently washed with tap water to determine their fresh biomass. In the second repetition, root systems were collected at the end of the choice experiment, washed with tap water, and immediately frozen in liquid nitrogen to determine induced volatiles as described below.

Volatile emission

In order to investigate the potential signals that *D. virgifera* uses to assess the infestation level of a plant, volatile emissions of seedlings that were used in the above two-arm olfactometers assays were determined. CO_2 emission was evaluated by connecting the belowground glass pots to an additional glass vessel (28 cm long, 5 cm diameter) via the connector and a glass joint. The glass vessel was closed using parafilm and left to stabilize for one hour. A CO_2 gas meter (Voltcraft, CM-100, Conrad Electronics, Dietlikon, Switzerland) was then introduced into the connected vessel for 3 minutes, and CO_2 levels were recorded.

Induced volatiles were determined using SPME GC-MS following a previously described protocol (Erb *et al.*, 2011a). The obtained peaks were analysed and identified by comparing volatile retention times and mass spectra with those of the NIST05 Mass Spectra Library and those of pure compounds.

Dose response to (E)-*β-caryophyllene*

As *(E)*-β-caryophyllene was previously reported to be attractive for *D. virgifera* larvae (Robert *et al.*, in press), we investigated a possible dose response effect of the induced volatile on *D. virgifera* host selection. Two-arm olfactometers were used as described above. Healthy plants were potted in the two pots of the system using moist white sand (10% water, Migros, Switzerland). Synthetic *(E)*-β-caryophyllene (Sigma Aldrich Chemie GmbH, Buchs SG, Switzerland) was continuously released into the rhizosphere of one of the healthy plants using slow-release capillary dispensers as described in (von Mérey *et al.*, 2011). Capillaries of 0.5, 1, 2, 3, 6, and 25 µL were used. One µL capillary dispenser continuously releases up to 40 $ng.h^{-1}$ (Robert *et al.*, in press). Assuming *(E)*-β-caryophyllene release to be linear with the size of the

capillary, the amount of *(E)*-β-caryophyllene released by the 0.5, 1, and 2 µL are well within the physiological range of infested maize seedlings, while *(E)*-β-caryophyllene released by 3 µL would correspond to extreme values and those emitted by 6 and 25 µL capillary dispensers would be aberrant in nature. All pots were wrapped in aluminum foil to avoid light and desiccation of the system. After 48 hours, the two pots were connected using the glass tube and Teflon connectors. After 30 minutes, six *D. virgifera* larvae were inserted into the belowground olfactometers central part and the larval orientation towards one or the other plant was noted.

D. virgifera *larvae performance on systemic roots of infested plants*

In order to investigate the underlying mechanisms of plant-mediated interactions between *D. virgifera* conspecifics, a split root set-up was designed (Figure 7a). Maize root systems were gently washed with tap water and potted in two separate glass vials (15 cm long, 2 cm diameter) filled with moist white sand (10% water). Five *D. virgifera* larvae were added to one of the glass tube (local roots). Control plants were left uninfested. After four days, a second batch of five *D. virgifera* larvae, was weighted and added to the second (systemic) side of the root system of all plants. Six hours later, larvae were recovered and weighted. The individual larval weight gain was calculated. To evaluate the impact of the plant ability to uptake water, the split root experiment was repeated using 10 and 20% moist sand. Furthermore, the performance of *D. virgifera* larvae was assessed after 48 hours feeding on hydric stress tolerant and susceptible maize lines by weighing the larvae before and after feeding.

Volatile-mediated feeding stimulation

Upon attack, plants emit a broad range of volatiles locally and systemically (Rasmann *et al.*, 2005; Hiltpold, I. *et al.*, 2011a). To determine the impact of induced volatiles on *D. virgifera* feeding performance, two glass pots (5 cm diameter, 11 cm deep) were connected. Each pot had a connector (29mm diameter) at 0.5 cm height. The connecting system between the two pots consisted of one glass tube (24 mm diameter), and Teflon connectors at both sides of the glass tube (24 mm diameter and 5 mm thickness) as previously described in (Robert *et al.*, in press). The Teflon connectors contained a mesh screen (2,300 mesh; Small Parts Inc., USA) to prevent the larvae from escaping. The first pot contained the odor source: a healthy plant, a plant infested with five *D. virgifera* larvae or five larvae feeding on artificial diet (Pleau *et al.*, 2002). After 24 hours, five *D. virgifera* larvae were weighted and allowed to feed on artificial diet in the second pot while exposed to the odor source. One day later, those larvae were collected and weighted. Their average individual relative weight gain was calculated.

Local and systemic root response to herbivory

Resource allocation. The local and systemic response of maize roots following infestation was investigated using the split-root system as described above (Figure 1a). Five *D. virgifera* larvae were added to one of the glass tube (local roots). Control plants were left uninfested. Physiological changes in the systemic roots were assessed four days following the infestation. The two different parts of the root system were collected separately, washed with tap water and immediately frozen in liquid nitrogen and stored at -80°C. To ensure enough material for analyses, roots from three plants were pooled together. Roots were ground into a fine powder under liquid nitrogen using a pillar and a mortar.

Amino acids were determined as previously described (Knill, T. *et al.*, 2008). Sucrose and hexose contents were determined enzymatically using a Sucrose, D-fructose and D-glucose assay kit (Megazyme International Ireland Limited) following the manufacturer's instructions. The concentrations of D-glucose, D-fructose and sucrose were calculated using the megazyme Mega-CalcTM software. The expression of marker genes involved in carbohydrate transport and metabolism was assessed using previously established methods and primers (Erb, M. *et al.*, 2010).

Plant defense. The expression of marker genes involved in plant direct defences, hormonal signalling and volatile production was assessed using previously established methods and primers (Erb, M. *et al.*, 2010).

Plant response to subsequent attack

In order to test if the root infestation impacts the ability of systemic healthy roots to respond to herbivore attack, maize root systems were split by washing and transplanting them in two glass tubes as described above (Figure 1a). Five *D. virgifera* larvae were added in one of the tube. Control plants remained uninfested on both sides. After four days, jasmonic acid (100 µM) diluted in 10 mL water or 10 mL water only was added to the second side of the root system. Twelve hours later, all roots were collected, washed with tap water and immediately frozen in liquid nitrogen. Roots were then ground into a fine powder as described above. The expression of marker genes involved in plant defense, carbohydrate transport and metabolism was assessed following the methods and primers described in (Erb, M. *et al.*, 2010).

Statistical analysis

Analyses were performed on the software package R, version 2.8.1 and SAS Statistical Package (SAS Institute 2004). All data were first analyzed with a Levene's and a Kolmogorov-Smirnov test to determine heteroscedasticity of error variance and normality. The effect of *D. virgifera*

density on their head capsule width in field and performance in laboratory were analysed using Proc Mixed of the SAS Statistical Package. *D. virgifera* host selection was evaluated using a log linear model (glm) using R. As the data did not fit to simple variance assumptions implied in using a binomial distribution, quasi-likehood functions were used to compensate for the over-dispersion of the larvae in the system. The two experiments were included as a co-factor in the analysis. As the repetition of the experiment had no effect on the model, the factor "experiment" was removed from the analysis. Root biomasses and volatile production of healthy and infested plants were compared using one-way ANOVAs followed by post-hoc Tukey HSD tests. If the data did not pass the two tests, Kruskall-Wallis one-way ANOVAs on ranks were performed, followed by pairwise Dunn's tests. The effect of the emitted volatiles on the larval choice was evaluated by performing one-way ANCOVAS. *D. virgifera* performance on healthy or infested plants in the split root design was compared using Student's t-tests. *D. virgifera* growth on artificial diet exposed to different odor sources was analyzed using a Kruskal-Wallis on ranks (H-tests), and the comparison between the growth of larvae exposed to plant odors with the growth of larvae exposed to the volatile bouquet of conspecifics feeding on artificial diet was compared using t-contrast on ranks test. The effect of *D. virgifera* feeding on amino acids and carbohydrate contents as well as marker gene expression in maize roots were investigated using t-tests when the data filled the heteroscedasticity of error variance and normality conditions, otherwise, Mann Whitney rank sum tests (U-tests) were conducted.

RESULTS

D. virgifera *fitness is density dependent*

In field, *D. virgifera* head capsule width was strongly dependent on the applied egg densities: the herbivore had larger head capsule when feeding in medium-sized group than in small or large densities (n_{25}=6, n_{50}=5, n_{100}=5, n_{300}=4, n_{600}=4, n_{1200}=4, n_{2400}=4; Proc mixed, differences of least squares means: 25 eggs per 30.5 cm (I_{25}) and I_{2400} *vs.* I_{100}, I_{300} and I_{600}: $p<0.05$; all other pairwise comparisons: $p>0.05$; Figure 1a).

D. virgifera larvae performance was also density dependent, as larvae grew better when feeding in group of 3, 6 or 9 larvae than alone or in high density groups (12 larvae) (n=7; Proc mixed, *df*=19, F=1.68, p=0.197; differences of least squares means: plants infested with 1 larvae (I_1) *vs.* I_3, I_6 and I_9: $p<0.05$; all other pairwise comparisons: $p>0.05$; Figure 1b).

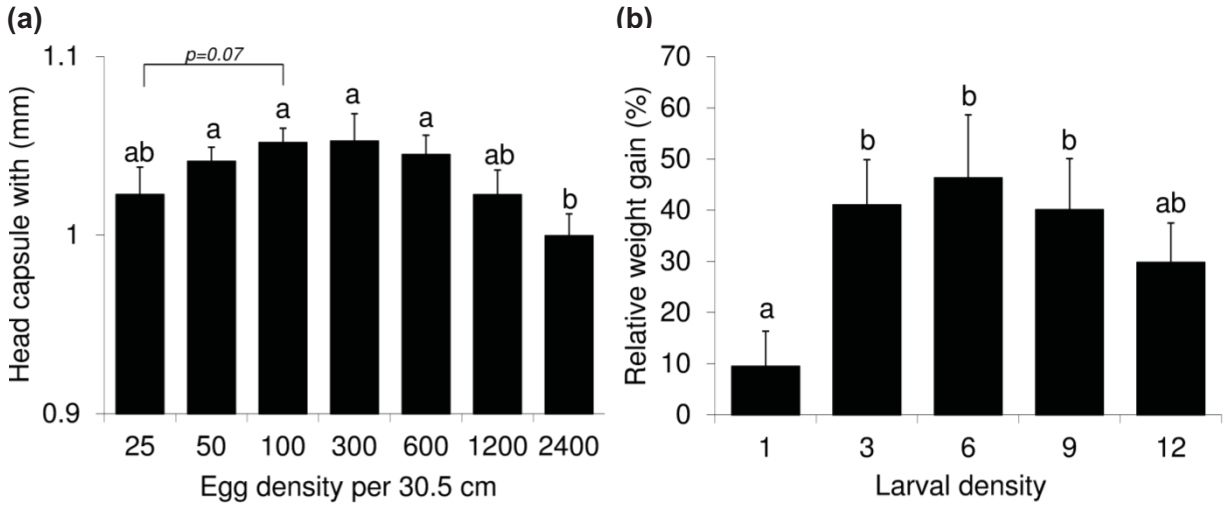

Figure 1: D. virgifera *fitness is density dependent*. (a) *D. virgifera* adult head capsule width (mean±se) after developing on plants infested with different egg densities. (b) *D. virgifera* larvae performance (mean±se) when feeding on plants with different larval densities in laboratory. Different letters indicate significant differences ($p<0.05$).

Host selection relies on the infestation level of the plant

D. virgifera host selection is density dependent: while larvae preferentially oriented towards healthy plants rather than plants that were infested with low (1 larvae) or high (12 larvae) density of conspecifics, they were able to orient towards plants infested with intermediate densities of conspecifics (6 larvae) rather than healthy plants (glm, Healthy (H) *vs.* I_1: n=17, *df*=33, F=5.677, p=0.023; H *vs.* I_3: n=17, *df*=33, F=1.199, p=0.282; H *vs.* I_6: n=17, *df*=33, F=8.050, p=0.008; H *vs.* I_9: n=8, *df*=15, F=0.0251, p=0.876; H *vs.* I_{12}: n=17, *df*=33, F=17.299, p<0.001; Figure 2). Although the density of herbivores strongly influenced the plant leaf wilting (Figure S1a), such above-ground symptoms did not affect *D. virgifera* host selection, with the exception of plants that had completely lost their turgidity that were avoided by the larvae (Figure S1b).

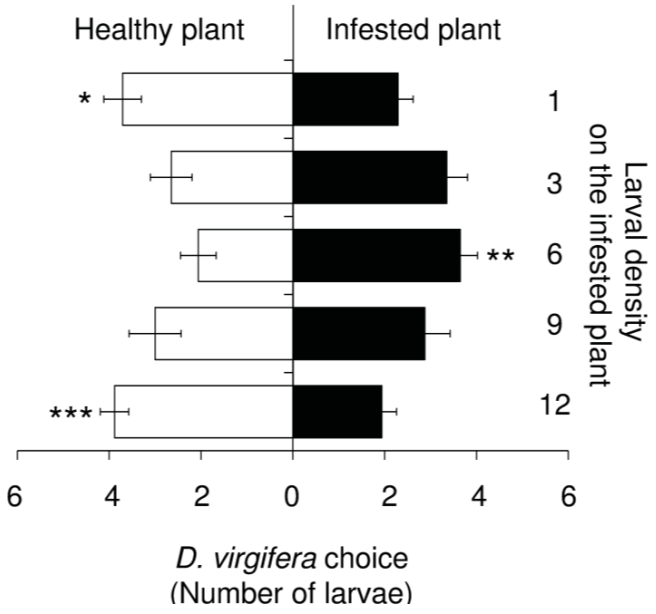

Figure 2: D. virgifera *selectively orients towards optimal hosts*. Number of larvae (mean±se) that oriented towards a healthy plant or a plant infested with different densities of *D. virgifera* larvae. Stars indicate significant differences (*: p≤0.05, **: p ≤0.01; ***: p≤0.001).

D. virgifera *host selection is related to the induced volatile emission of* (E)-*β-caryophyllene and α-humulene*

Upon high infestation densities (9 and 12 larvae per plant), maize root system fresh weight considerably decreased (n=8; Kruskall Wallis on ranks, *df*=5, H=19,918, p=0.001; Dunn's tests: H *vs.* I_9 and I_{12}: p<0.05; all other pairwise comparisons: p>0.05; Figure 3).

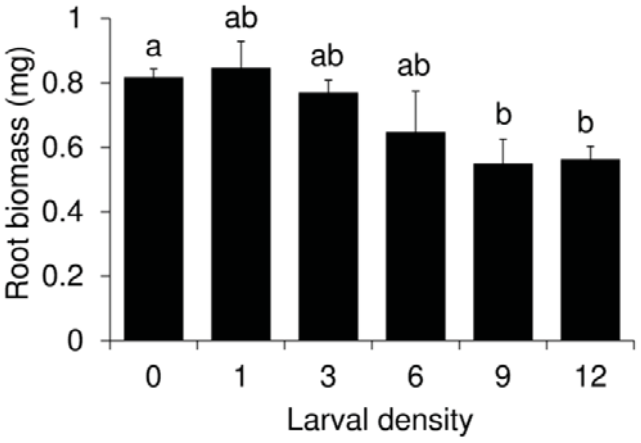

Figure 3: D. virgifera *feeding affects maize root biomass at high densities*. Fresh biomass (mean±se) of maize root systems infested with different densities of *D. virgifera*. Different letters indicate significant differences.

High infestation also increased the CO_2 production per gram of fresh roots (n=8; Kruskall Wallis on ranks, *df*=5, H=20.943, p<0.001; Dunn's test: H *vs.* I_9 and I_{12}: p<0.05; all other pairwise comparisons: p>0.05; Figure 4a). Taken together, the infestation level did not affect the total amount of CO_2 released by roots was independent of the number of larvae feeding on the plant (n=8; one-way ANOVA, *df*=72, F=0.318, p=0.901; Figure 4b).

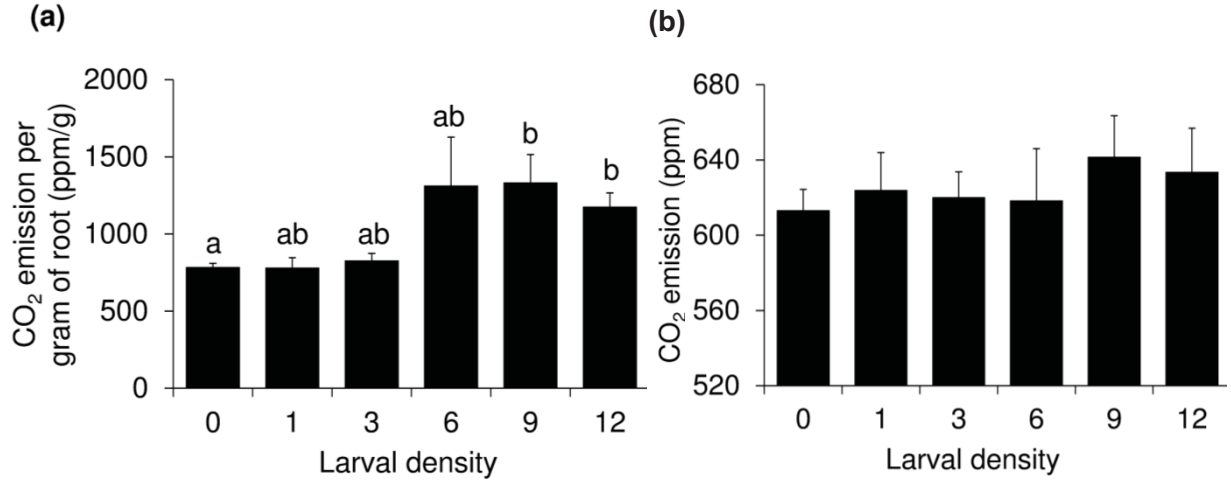

Figure 4: *Total CO_2 emissions are not influenced by the density of feeding herbivores*. (a) CO_2 emissions per gram (mean±se) of fresh root. (b) CO_2 emissions per plant (mean±se) infested with different densities of *D. virgifera* larvae. Different letters indicate significant differences.

Root herbivory induced the production of *(E)*-β-caryophyllene, α-humulene, α-copaene, tetradecane, heptadecane, 4-methyl nonane, 4-methyl heptane and tetradecene (n=8; Kruskal-Wallis one-way analysis of variance on ranks, *df*=4, *(E)*-β-caryophyllene: H=51.969, p<0.001, Dunn's tests: H *vs.* I_3, I_6 and I_{12}: p<0.05; all other pairwise comparisons: p>0.05; α-humulene: H=23.242, p<0.001, Dunn's tests: all pairwise comparisons: p>0.05; α-copaene: H=17.182 , p=0.002, Dunn's tests: H *vs.* I_3 : p<0.05; all other pairwise comparisons: p>0.05; tetradecane: H=29.262, p<0.001, Dunn's tests: H *vs.* I_1 and I_3 : p<0.05; all other pairwise comparisons: p>0.05; heptadecane: H=13.336, p=0.010; all pairwise comparisons: p>0.05; 4-methyl nonane: H=11.321,

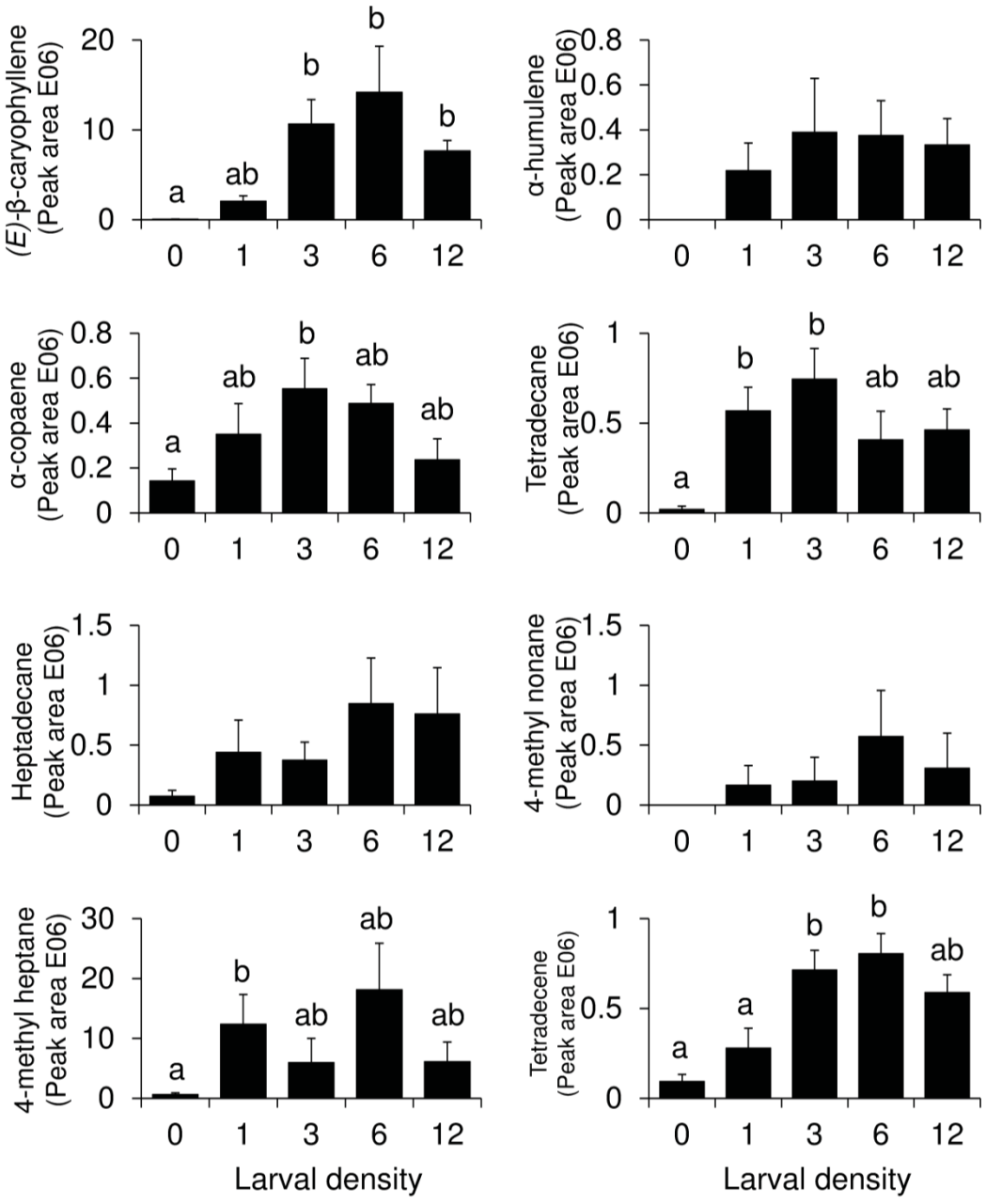

Figure 5: D. virgifera *induces plant volatiles*. SPME GC-MS peak areas (xE06) (mean±se) of plant induced volatiles upon *D. virgifera* attack. Different letters indicate significant differences.

p=0.023, all pairwise comparisons: p>0.05; 4-methyl heptane: H=26.946, p<0.001, H *vs.* I_1 : p<0.05; all other pairwise comparisons: p>0.05; tetradecene: H=40.038, p<0.001, Dunn's tests: I_3 *vs.* H, and I_1, H *vs.* I_6: p<0.05, all other pairwise comparisons: p>0.05; Figure 5). Volatiles such *(E)*-β-caryophyllene and α-humulene were correlated to larval attraction to infested plants (One-way ANCOVAs, k=4, *df*=3, *(E)*-β-caryophyllene: F=4.84, p=0.008; α-humulene F=3.34, p=0.03; α-copaene F=0.63, p=0.603; Figure 5).

D. virgifera *response to* (E)-*β-caryophyllene is dose dependent*

D. virgifera attraction towards *(E)*-β-caryophyllene was dose mediated as they tended to orient towards plants whose rhizosphere was complemented with 0.5 µL *(E)*-β-caryophyllene dispensers (n=11; *df*=20, F=3.8472, p=0.064; Figure 6), strongly oriented towards plants with 1µL *(E)*-β-caryophyllene dispensers (n=10; glm, *df*=18, =20.696, p<0.001; Figure 6), and were not attracted by higher amount of released *(E)*-β-caryophyllene (2 µL dispensers: n=11, *df*=20, F=0.332, p=0.571; 3 µL dispensers: n=10, *df*=18, F=0.300, p=0.591; 6 µL dispensers: n=10, *df*=18, F=0.042, p=0.840; 25 µL: n=10, *df*=18, F=0.606, p=0.446; Figure 6).

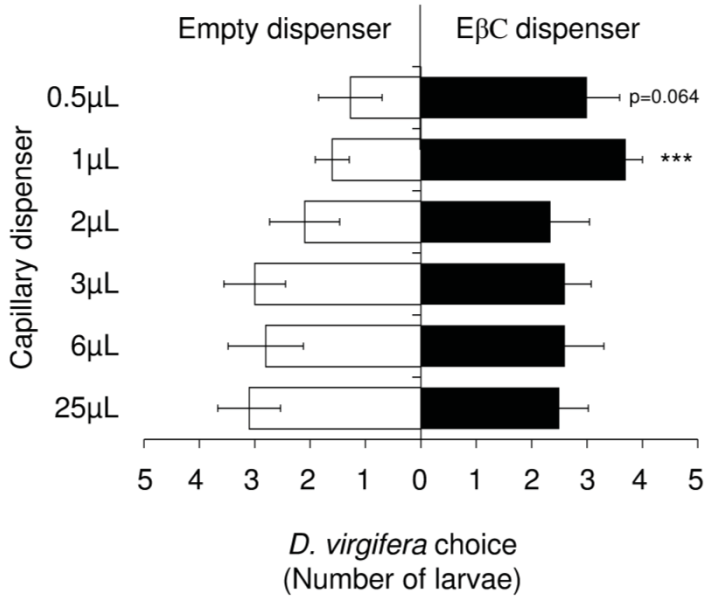

Figure 6: D. virgifera *attraction to* (E)-*β-caryophyllene is dose dependent*. Number of larvae (mean±se) that oriented towards a healthy plant or a healthy plant whose rhizosphere was complemented with different amounts of (E)-β-caryophyllene using slow release capillary dispensers. Stars indicate significant differences (*: p≤0.05, **: p ≤0.01; ***: p≤0.001).

Systemic plant physiological changes upon infestation benefits to D. virgifera *larvae*

Larvae feeding on the systemic side of infested roots grew more than five times better than larvae feeding on a healthy plant (n=7; t-test, *df*=11, t=-3.247, p=0.008; Figure 7b).

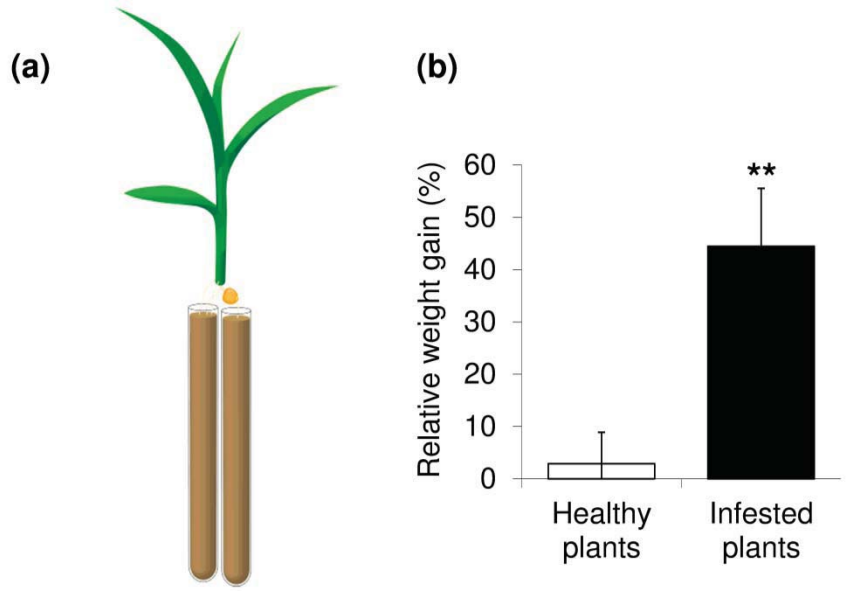

Figure 7: D. virgifera *larvae benefit from plant-mediated interactions with spatially separated conspecifics*. (a) Split root set up: maize roots were potted in two glass tubes. (b) *D. virgifera* larvae performance (mean±se) over a six hour feeding period on healthy plants or on the systemic undamaged roots of a plant that had been infested for four days with conspecifics. Stars indicate significant differences (*: p≤0.05, **: p ≤0.01; ***: p≤0.001).

The effect was still present when the sand moisture was increased to 20% (Figure S2a), and that *D. virgifera* performance was not related with the plant tolerance to water stress (Figure S2b). Since the emission of some induced volatiles is not only restricted to the damaged site (Hiltpold, I. *et al.*, 2011a), we investigated their influence on *D. virgifera* feeding behavior. Although the exposure to plant volatiles stimulated *D. virgifera* larvae to feed (n_{diet}=6, n_{plants}=21; t contrast on ranks, *df*=25, t=-2.066, p=0.050; Figure 8), no difference was noticed between the performance of larvae exposed to the volatile bouquet of healthy or infested plants (n_{diet}=6, $n_{healthy}$=9, $n_{infested}$=12; Kruskal-Wallis on ranks, *df*=2, H=6.508, p=0.039; Dunn's test: Diet *vs*. infested plant: p<0.05; all other pairwise comparisons: p>0.05; Figure 8).

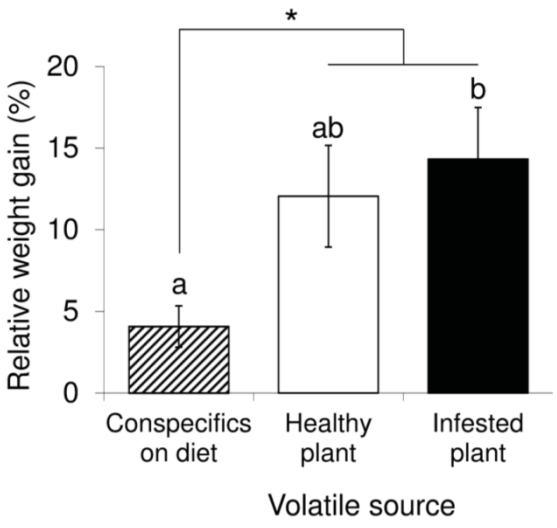

Figure 8: *Exposure to* D. virgifera *induced plant volatile does not stimulate larval performance*. *D. virgifera* larvae relative weight gain (mean±se) when feeding for 24 hours on artificial diet (Pleau *et al.*, 2002) and exposed to volatiles from conspecifics feeding on diet, healthy plant or *D. virgifera* infested plant volatiles. Stars indicate significant differences (*: p≤0.05, **: p ≤0.01; ***: p≤0.001). Different letters indicate significant differences.

Root resource allocation is altered both locally and systemically upon root herbivory

Amino acid concentrations were drastically enhanced upon herbivory in the roots. Locally, asparagine, aspartic acid, glutamine, histidine, phenylalanine, and tryptophane significantly increased, and leucine, serine and tyrosine showed similar trends (n=6; t-tests, *df*=10; ala: t=1.214, p=0.252; arg: t=-0.0194, p=0.985; asp: t=4.054, p=0.002; gln: t=4.326, p=0.001; glu: t=2.866, p=0.017; gly: t=0.748, p=0.472; his: t=3.001, p=0.013; ile: t=-0.615, p=0.552; leu: t=1.920, p=0.084; met/val: t=1.187, p=0.263; ser: t=1.830, p=0.097; thr: t=-0.444, p=0.666; trp: t=3.851, p=0.003 ; tyr : t=2.110, p=0.061 ; Mann Whitney rank sum test, *df*=1, asn: U=0, T=57, p=0.002; phe: U=1, T=56, p=0.004; Figure 9a). Systemically, amino acids concentrations of histidine, phenylalanine, tryptophan and tyrosine increased upon infestation. Asparagine, aspartic acid and glutamic acid concentrations also showed similar trends upon infestation (n=6; t-tests, *df*=10, ala: t=-1.587,p=0.144; arg: t=-1.374, p=0.200; asn: t=-1.881, p=0.089; asp: t=-1.974, p=0.077; gln: t=-2.237, p=0.049; glu: t=-2.117, p=0.060; gly: t=-1.551, p=0.152; his: t=-2.878, p=0.016; ile: t=-1.596, p=0.142; leu: t=-1.327, p=0.214; met/val: t=-1.723, p=0.116; phe: t=-2.292, p=0.045; ser: t=-1.739, p=0.113; thr: t=-1.715, p=0.117; trp: t=-2.334, p=0.042; tyr: t=-2.607, p=0.026; Figure 9b). Although amino acid composition in roots changed drastically in infested plants, leaves amino acid profiles were similar between healthy and infested plants (Figure S3).

Carbohydrate partitioning in roots upon infestation was locally affected: glucose content was reduced twice and sucrose accumulated (n=6; sucrose: t-test, *df*=10, t=-2.343, p=0.041; fructose: Mann Whitney rank sum test, *df*=1, U=12, T=38, p=0.432; glucose: t-test, *df*=10, t=4.061, p=0.002; Figure 9c). On the opposite, the partitioning of sucrose and hexoses was not affected in the undamaged systemic roots of infested plant (n=6; sucrose: Mann Whitney rank sum test, *df*=1, U=15, T=36, p=0.399; fructose: Mann Whitney rank sum test, *df*=1, U=17.5, T=38, p=0.937; glucose: t-test, *df*=10, t=0.368, p=0.721; Figure 9d).

The lower sink strength of infested roots may be mediated by invertases and sugar transporters

Locally, both vacuolar (ivr) and cell wall (incw) invertases marker genes expression was down regulated upon herbivory, with the exception of *ivr1*, whose expression was enhanced (n=7; t-tests, *df*=9, *ivr1*: t=-2.222, p=0.053 ; *ivr2* : t=4.101, p=0.003 ; *incw2* : t=5.879, p<0.001 ; *incw3* : t=2.456, p=0.036 ; *incw4* : t=2.072, p=0.068 ; Figure 10a). The expression of carbohydrate transporter marker gene was mainly upregulated upon infestation, except for c4 that was less expressed (n=7 ; t-tests, *df*=9, *stp1* : t=-3.376, p=0.008 ; *zifl2* : t=2.157, p=0.059 ; *mtrans* : t=-3.726, p=0.005 ; *mss1* : t=-7.187, p<0.001 ; Figure 10b).

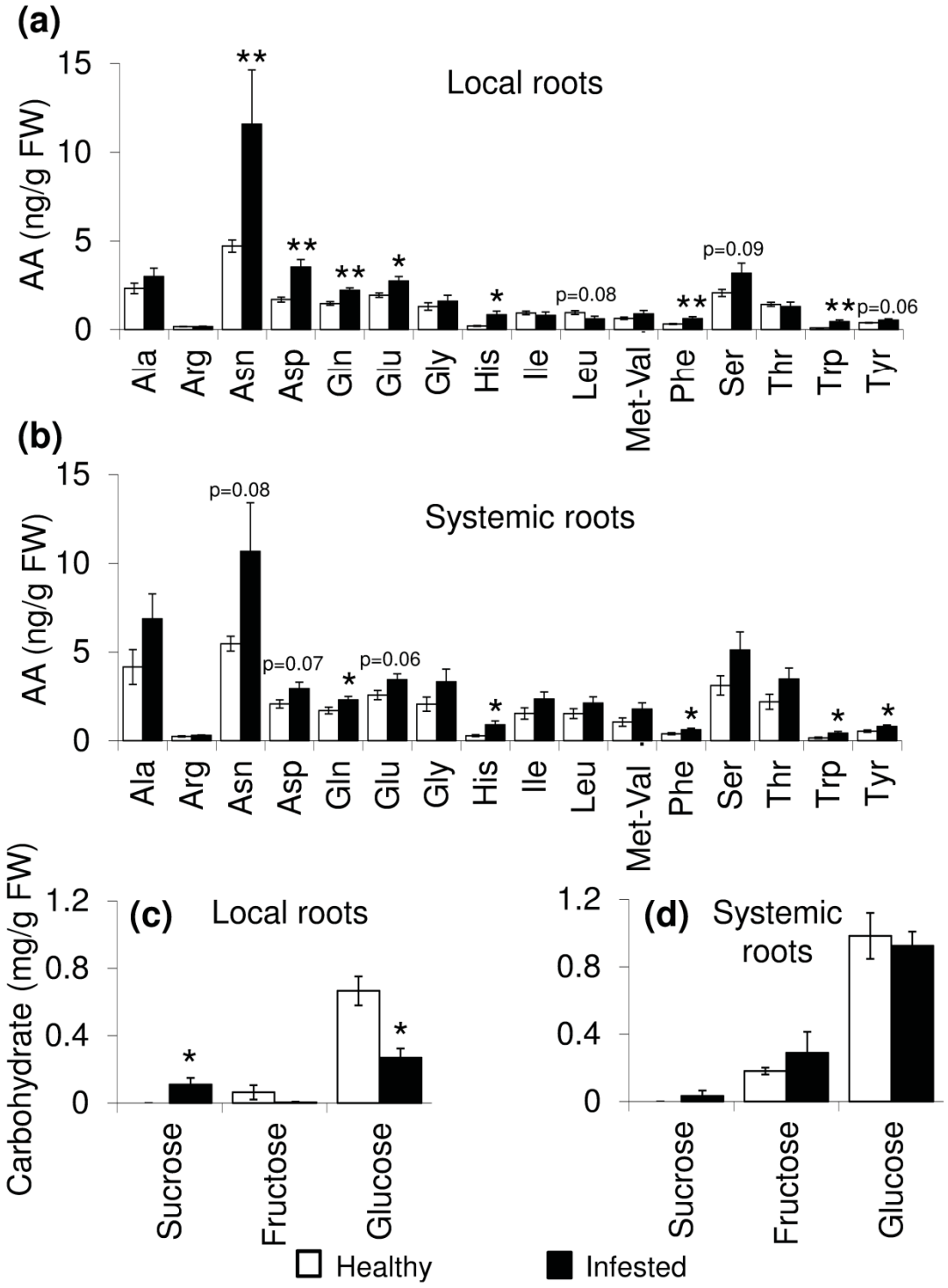

Figure 9: *Root herbivory leads to reconfiguration of the primary metabolism*. (a) Local amino acid contents (mean±se) in healthy or infested roots. (b) Systemic amino acid contents in roots of healthy or infested plants. (c) Local carbohydrate contents (mean±se) in healthy or infested roots. (d) Systemic carbohydrate contents in roots of healthy or infested plants. Stars indicate significant differences (*: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$).

No change in invertases and carbohydrate transporters was detected in the systemic undamaged roots of infested plants (n=7; t-tests, *df*=11, ivr1: t=0.086, p=0.932; ivr2: t=1.311, p=0.217; incw2: t=0.477, p=0.643; incw3: t=0.793, p=0.444; incw4: t=-.-0.527, p=0.608 ; stp1 : t=-0.312, p=0.761 ; zifl2 : t=-0.188, p=0.854; mtrans : t=-0.044, p=0.965; mss1: t=-0.933, p=0.371; Figures 10c and d).

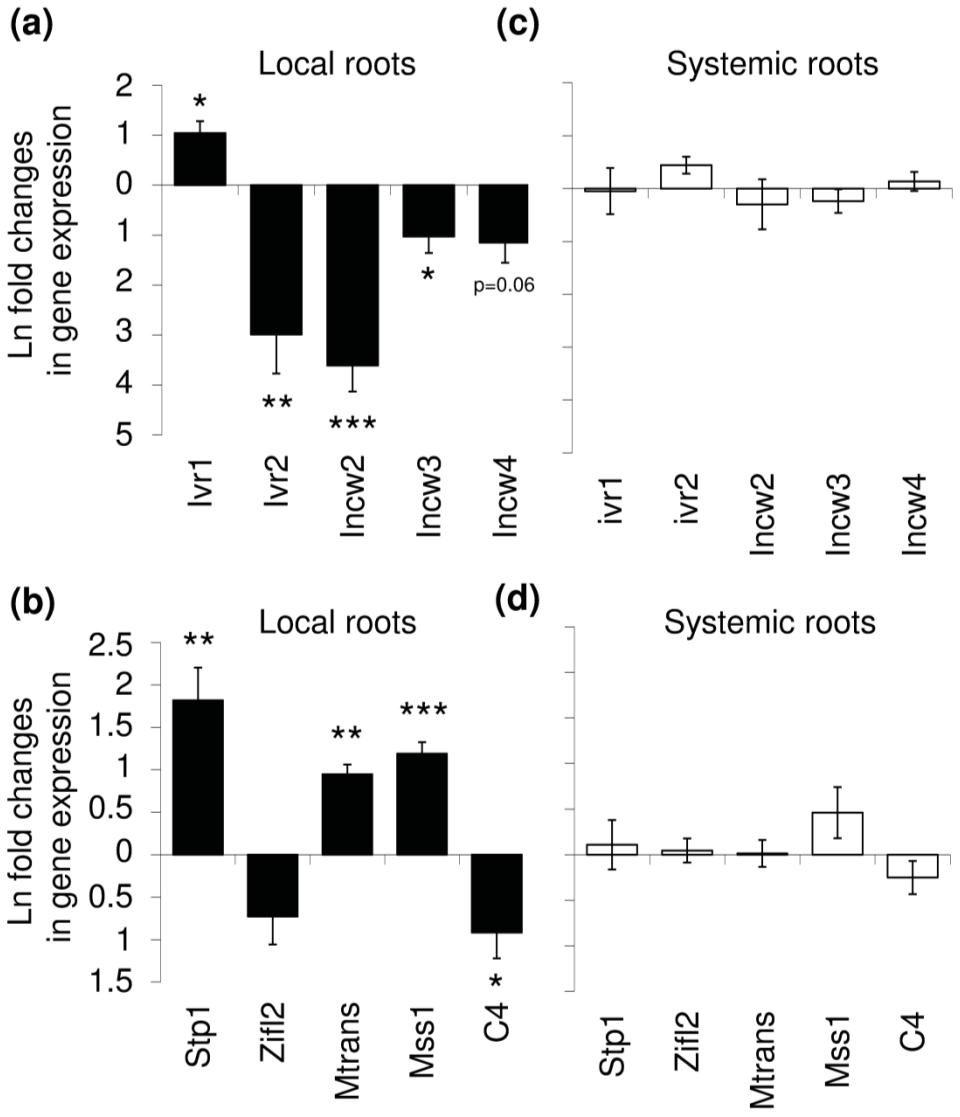

Figure 10: *Local carbohydrate metabolism changes upon root infestation*. (a) Local ln fold changes (mean±se) in expression of vacuolar (ivr) and cell wall (incw) invertases. (b) Local ln fold changes (mean±se) in expression of carbohydrate transporters. (c) Systemic ln fold changes (mean±se) in expression of vacuolar (ivr) and cell wall (incw) invertases. (d) Systemic ln fold changes (mean±se) in expression of carbohydrate transporters. Stars indicate significant differences (*: p≤0.05, **: p ≤0.01; ***: p≤0.001).

Defense response is localized

Roots responded locally to the infestation by *D. virgifera* larvae. Marker genes of volatiles (such as *(E)*-β-caryophyllene (*tps23*) and indole (*igl*)), lipoxygenases (*lox3*, *lox5* and *lox8*), direct defenses (proteinase inhibitors: *cysII*, *cyst*, *serpin*, *mpi*; phenylalanine ammonia lyase: *pal* and benzoxazinones: *bx1*) and pathogenesis related proteins (*pr1* and *pr5*) were upregulated upon herbivory (n=7; t-tests, *df*=11, *tps23*: t=-7.073, p<0.001; *igl*: t=-5.382, p<0.001; *lox3*: t=-8.079, p<0.001; *lox8* : t=-4.257, p=0.002; *lox10*: t=0.508, p=0.624; *pr1*: t=-4.500, p=0.001; *pr5*: t=-6.559, p<0.001; *cysII* : t=-6.781, p<0.001; *cyst*: t=-1.020, p=0.332; *serpin*: t=-6.467, p<0.001; *mpi*: t=-10.186, p<0.001; *pal*: t=-1.357, p=0.217; Mann Whitney rank sum test: *df*=1, *lox5*: U=0, T=45, p=0.004; *bx1*: U=0, T=42, p=0.030; Figure 11a). On the other hand, the expression of marker genes of hormones like ethylene (*acs6*), auxin (*saur2*), abscisic acid (*nced*) and jasmonic acid (*opr7*) remained unaffected upon infestation (n=7; t-tests, *df*=11, *acs6*: t=1.180, p=0.272; *saur2*: t=2.222, p=0.053; *nced*: t=-0.648, p=0.533; *opr7*: t=-0.326, p=0.752; Figure 11a). Systemically, a few marker genes only responded to the herbivory: igl, acs6 and lox8 were upregulated while all the other tested marker genes expression remained unchanged (n=7; *df*=11, *tps23*: t=-1.642, p=0.129; *igl*: t=-3.001, p=0.012; *saur2*: t=2.222, p=0.053; *nced*: t=-0.275, p=0.789; *opr7*: t=-0.850, p=0.413; *lox3*: t=-1.319, p=0.214; *lox5*: t=0.375, p=0.715; *lox8* : t=-2.539, p=0.028; *lox10*: t=-1.192, p=0.258; *pr1*: t=0.886, p=0.395; pr5: t=1.534, p=0.153; *cysII* : t=-1.026, p=0.327; *cyst*: t=-1.129, p=0.283; *serpin*: t=-0.681, p=0.511; *mpi* : t=-0.897, p=0.389; *pal* : t=-0.054, p=0.958 ; Mann Whitney rank sum test, *df*=1 : *acs6*: U=1, T=16, p=0.005; *bx1*: U=1, T=38, p=0.628; Figure 11b).

A first infestation decreases the defense abilities of systemic roots

Following the infestation of one side of the root system by *D. virgifera* larvae, the undamaged systemic root side response to jasmonic acid application was significantly reduced compared to healthy plants (n=8; t-tests, *df*=14, *cysII*: t=2.150, p=0.050; *cyst*: t=4.086, p=0.001 ; *serpin* : t=1.179, p=0.258 ; *mpi* : t=2.138, p=0.051 ; *bx1* : t=3.238, p=0.006 ; *pal* : t=-0.359, p=0.725 ; Figure 12).

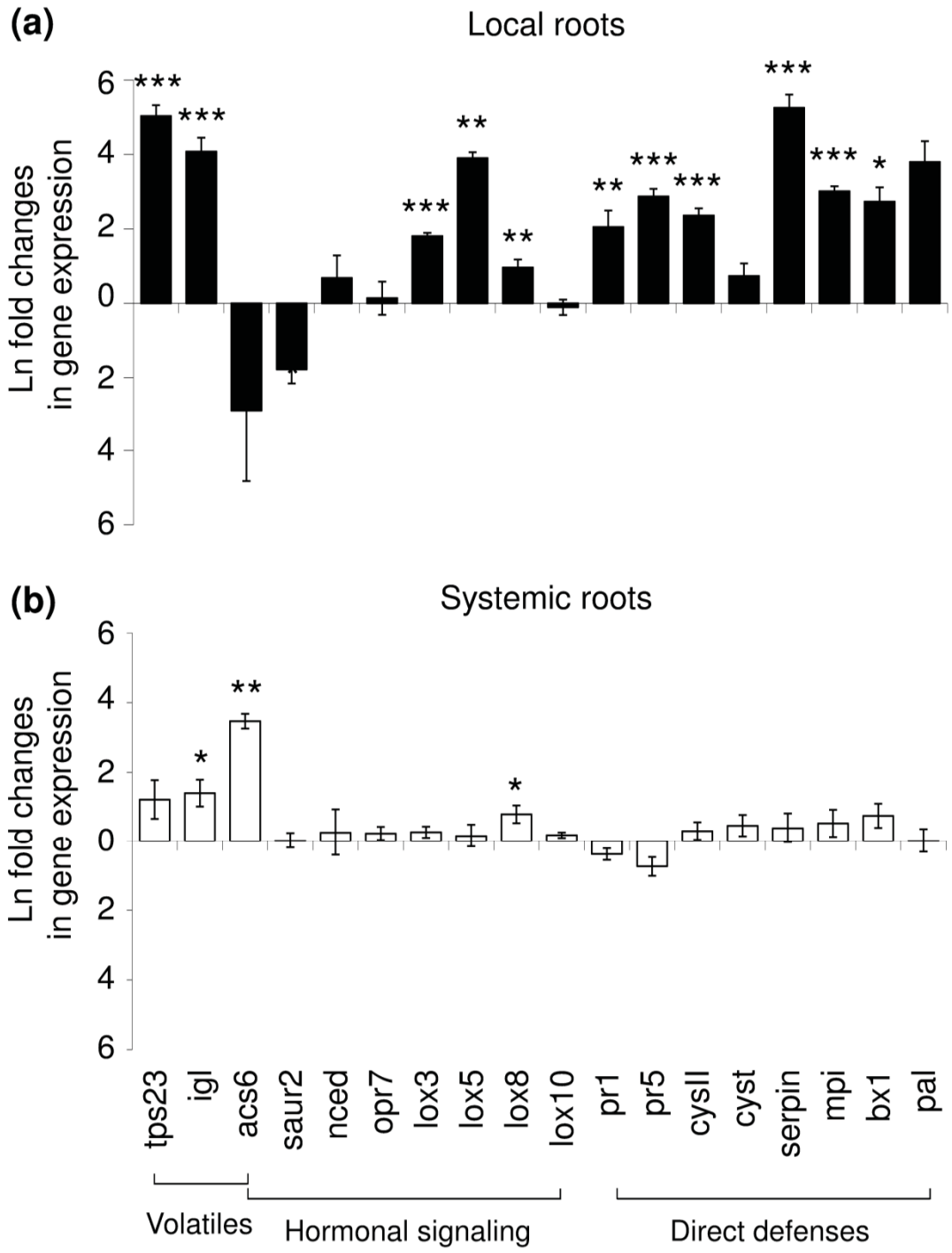

Figure 11: *Plant defenses are strongly induced locally*. (a) Local ln fold changes (mean±se) in expression of volatile, hormone signaling and direct defense marker genes. (b) Systemic ln fold changes (mean±se) in expression of volatile, hormone signaling and direct defense marker genes. Stars indicate significant differences (*: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$).

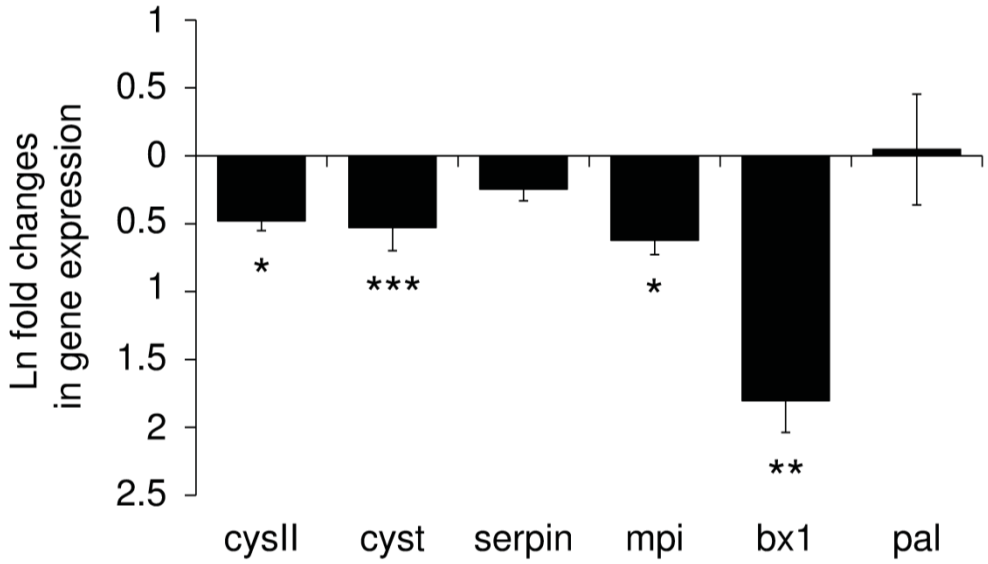

Figure 12: *A first infestation by* D. virgifera *attenuates subsequent defense responses*. Ln fold changes (mean±se) in expression direct defense marker genes in systemic roots of healthy or infested plants induced with 100µM of jasmonic acid for 12 hours. Stars indicate significant differences (*: p≤0.05, **: p ≤0.01; ***: p≤0.001).

DISCUSSION

This study demonstrates that plant-mediated facilitation occurs between *D. virgifera* conspecifics sharing a maize plant, a phenomenon which is likely due to a combination of volatile-mediated host location, plant resource reallocation and weakened plant defenses.

D. virgifera larvae benefits from feeding in groups, as they performed better on plants infested with other larvae in laboratory and field conditions (Figure 1). Yet, the observed feeding facilitation was reversed when *D. virgifera* fed in large groups: in our assays, both larval performance and head capsule width of emerging adults decreased at high densities (Figure 1). Negative density dependent effects are likely due to competition for limiting plant-resources. For instance, in our assays, high densities of larvae considerably decreased the root biomass available for conspecifics (Figure 3). Such negative interactions were also reported from field, where adult *D. virgifera* emergence decreases at high egg density (Onstad *et al.*, 2006; Hibbard *et al.*, 2010). Interestingly, the specialist *D. virgifera* was able to select host plant with an optimal density of attacking conspecifics: When given a choice between a healthy plant and a plant infested with different densities of conspecifics, *D. virgifera* larvae preferentially oriented towards plants infested with an intermediate number of conspecifics (6 larvae), but were not attracted to plants infested with low and high densities of conspecifics (Figure 2). This behavior was optimal for the root herbivore, because it enabled it to locate the best host plants (Figure 1b). Together with our

previous study showing that *D. virgifera* can distinguish good from bad host plants (Robert *et al.*, in press), these results demonstrate its remarkable ability for optimal host selection.

We propose here that *D. virgifera* uses plant volatiles to find plants with an optimal density of conspecifics. Although CO_2 emissions are known to be attractive to the herbivore (Bernklau & Bjostad, 1998), our results suggest that other plant volatiles are involved in the host selection by *D. virgifera*, as CO_2 emissions remained constant upon infestation by different densities (Figure 4). On the other hand, several induced volatiles showed a density dependent pattern (Figure 5). The production of *(E)*-β-caryophyllene, α-humulene, α-copaene, tetradecane and tetradecene in particular showed a parabolic pattern, with peak emission occurring at medium densities of infestation (Figure 9). It should be taken into account that, as root biomass decreases upon infestation in higher densities (Figure 3), our analyses likely over-estimated the total amounts emitted from plants infested by 9 or 12 larvae. *In vivo* analyses of root volatile emission of *D. virgifera* infested plants show that, among the compounds detected in the present study, only *(E)*-β-caryophyllene, α-humulene and α-copaene are actually released into the rhizosphere (Chapter 2). Analyses of covariance (ANCOVA) using these three compounds as covariates showed that *(E)*-β-caryophyllene and α-humulene, but not α-copaene, can improve the fit of the model of larval choice (see results), suggesting that these two compounds may be used by *D. virgifera* to distinguish plants infested by different densities of conspecifics. *(E)*-β-caryophyllene and α-humulene are both products of a single terpene synthase, *tps23*, and well-known to be induced upon infestation by the root herbivore (Rasmann *et al.*, 2005). Since *(E)*-β-caryophyllene is emitted in higher amount than α-humulene (Rasmann *et al.*, 2005) and was shown to be an attractant for *D. virgifera* larvae (Robert *et al.*, in press), we focused on that compound to investigate its dose-dependent effect on the root herbivore. *(E)*-β-caryophyllene was attractive to *D. virgifera* only when released at a rate of 40 $ng.h^{-1}$ (1µL capillary dispensers), which corresponds to the release rate of plants infested with 6 conspecifics (Robert *et al.*, in press). Therefore, it is highly probable that *D. virgifera* uses *(E)*-β-caryophyllene in a dose-dependent manner to locate optimal host plants.

The feeding facilitation of *D. virgifera* when feeding in medium-sized group may be attributed to either plant-mediated effects or the direct influence of conspecifics. Our split root experiment shows that spatially separated larvae grew five times better than larvae feeding on healthy plants (Figure 7b), showing that plant-mediated effects are sufficient to explain the positive density-dependence observed in the field. As upon belowground attack, *(E)*-β-caryophyllene is produced both locally and systemically (Hiltpold, I. *et al.*, 2011a), we first tested the hypothesis that this sesquiterpene may directly stimulate feeding. Many lepidopteran leaf-herbivores for example are stimulated by green leaf volatiles released from fresh wounds (Meldau *et al.*, 2009) or by volatile

breakdown products of induced secondary metabolites like glucosinolates (Agrawal & Sherriffs, 2001; Nielsen *et al.*, 2001). We found that larvae exposed to *D. virgifera* induced volatiles grew similarly than larvae exposed to the volatile bouquet of healthy plants (Figure 8), suggesting that induced plant volatiles do not stimulate larvae to feed. Interestingly, exposure to plant volatiles in general increased larval weight gain (Figure 8), suggesting that constitutive volatile compounds like ethylene or CO_2 may have a stimulatory effect on *D. virgifera*.

D. virgifera attack led to changes in the primary metabolism of maize roots. Larval feeding induced the accumulation of free amino acids both locally and systemically (Figure 9a and b). Free amino acids can be involved in (i) osmotic adjustment (Navari-Izzo *et al.*, 1990; Marur *et al.*, 1994) in response to the water stress imposed by the root herbivore (Dunn & Frommelt, 1998b; Erb *et al.*, 2011b), (ii) defense (D'Auria & Gershenzon, 2005; Tzin & Galili, 2010; Vogt, 2010), or (iii) nitrogen transport away from the roots (Trumble *et al.*, 1993; Baldwin & Ohnmeiss, 1994b; Strauss & Agrawal, 1999; Tiffin, 2000; Schwachtje *et al.*, 2006; Schwachtje & Baldwin, 2008). We also suggest a possible role of the stem in storing additional resources to tolerate *D. virgifera* attack (Chapter 5), and the higher concentrations observed after attack may reflect nitrogen reallocation into the stem. Leaves are unlikely to receive the free amino acids, as their concentrations were unaffected (Figure S3).

At the same time, amino acids are known to be the growth limiting factor of herbivorous insects (Behmer, 2006) and their accumulation in the roots of infested plants may, therefore, explain the better performance of *D. virgifera*. Apart from nitrogen metabolism, *D. virgifera* also affected carbon distribution. Upon infestation, attacked roots accumulated more sucrose, but less glucose than roots of healthy plants (Figure 9c). Three putative carbohydrate transporter genes were more strongly expressed in attacked roots, while invertases were generally down regulated (Figure 10). Invertases play a key role in regulating root sink strength (Weil & Rausch, 1990; Miller & Chourey, 1992; Kim *et al.*, 2000; Roitsch *et al.*, 2003; von Schweinichen & Buttner, 2005). Vacuolar invertases such as ivr1 and ivr2 are considered to be more important than cell wall invertases, incw2, incw3, and incw4, in maintaining the sucrose gradient (Duke *et al.*, 1991; Sturm *et al.*, 1995). Upon water-stress, vacuolar invertases were reported to be strongly induced in maize roots, resulting in higher ratios between hexoses and sucrose that contributes to the osmotic adjustment (Kim *et al.*, 2000). The general down regulation of the invertase marker genes observed in our assays suggests that *D. virgifera* attack may reduce the plants' capacity to react to the accompanying water stress conditions (Erb *et al.*, 2009a). The expression of some vacuolar invertases, such as ivr2, is restricted to root tips (Kim *et al.*, 2000), which were likely removed by herbivory in our experiments. However, the capacity of the plant to withstand herbivore-induced water stress is not crucial for *D. virgifera* growth, as the larvae grew similarly on water stress

resistant and susceptible lines (Figure S2a) and differentially watered plants (Figure S2b). On the other hand, the local down-regulation of invertases may lead to a decrease in root sink strength that could lead to resource allocation away of the damaged site (Trumble *et al.*, 1993; Baldwin & Ohnmeiss, 1994b; Strauss & Agrawal, 1999; Tiffin, 2000; Schwachtje *et al.*, 2006; Schwachtje & Baldwin, 2008). However, as the changes in carbohydrate concentrations were limited to the local tissue (Figure 9), they are unlikely to explain the differential performance of *D. virgifera* on systemic roots.

D. virgifera attack induced a pronounced local defense response (Figure 11a), as indicated by the increased expression of marker genes involved in hormonal signaling, such as lipoxygenases (*lox* genes), direct defenses, such as proteinase inhibitors (*mpi*, *serpin*, *cysII*), benzoxazinoids (*bx1*), pathogenesis related proteins (*pr1* and *pr5*), and indirect defenses such as volatile production (*igl* and *tps23*). These results show that *D. virgifera* does not strongly, if at all, manipulates its host defenses as it has been shown to exist in aboveground feeding herbivores (Zarate *et al.*, 2007; Sarmento *et al.*, 2011). On the other hand, the undamaged part of the root system was barely induced (Figure 11b), with the exception *igl*, *acs6*, and *lox8* whose expression was slightly upregulated (Figure 11b). Interestingly, a second induction of the undamaged part of infested root systems by jasmonic acid resulted in a lower induction of direct defense marker genes compared to plants that had not been previously infested (Figure 12). This could indicate that *D. virgifera* larvae that attack an already infested root system will encounter a plant immune system that is less inducible, and consequently, less resistant. It remains to be determined whether this relaxation of inducibility can explain the higher performance of *D. virgifera* larvae on attacked plants. Testing this hypothesis would however need a more detailed understanding of the mechanisms of induced resistance in maize roots.

CONCLUSION

Overall, our study shows that *D. virgifera* attack changes the root metabolism of maize plants, leading to systemically induced susceptibility. *D. virgifera* was able to use *(E)*-β-caryophyllene as a signal to locate plants with an optimal density of conspecifics. The presented experiments allow us to rule out volatile-mediated stimulation of feeding and direct effects of larval behavior as explanations for the increase in larval growth. The hypotheses that either the higher amino acid levels or the relaxation of inducibility may be responsible for the enhanced *D. virgifera* performance remain to be tested. Understanding the mechanisms behind induced susceptibility is likely to improve our understanding of the extraordinary success of *D. virgifera* as a maize pest.

AKNOWLEDGEMENTS

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SUPPLEMENTARY FIGURES

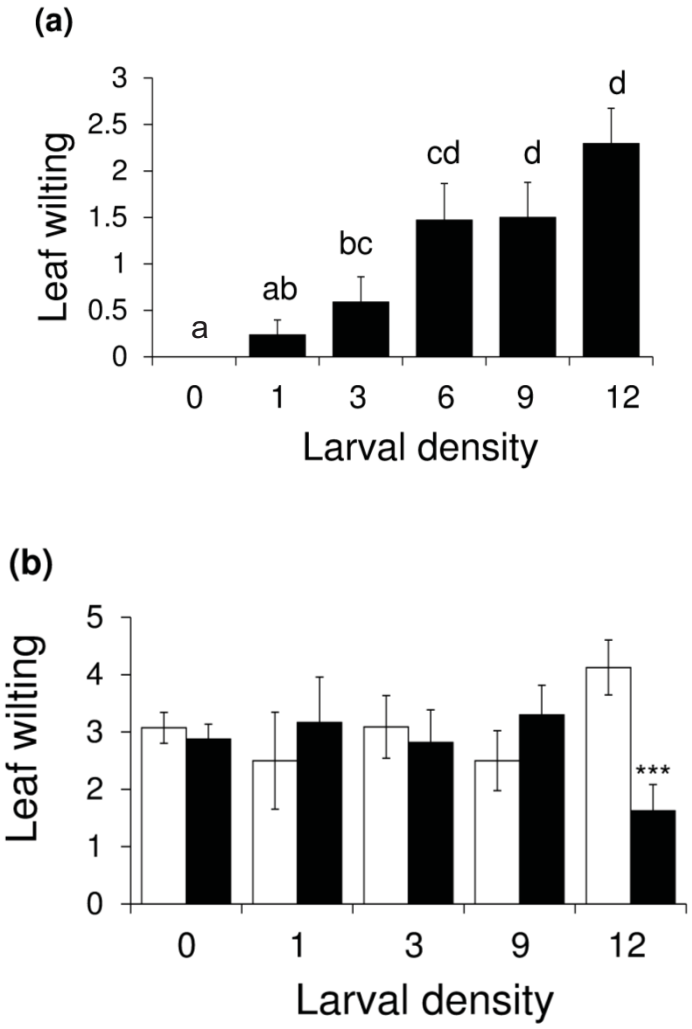

Figure S1: *Root-herbivory induced leaf wilting does not influence* D. virgifera *host selection.*(a) Leaf wilting is related to the density of larvae feeding on the plant ($n_{healthy}$=71, n_{1larva}= $n_{3larvae}$ = $n_{6larvae}$ = $n_{12larvae}$ 17, $n_{9larvae}$=8; One-way ANOVA on ranks, *df*=146, F=27.523, p<0.001; Dunn's test: H *vs* I_1: p=0.786; H *vs* I_3 : p=0.017; H vs I_6 : p<0.001; H vs I_9 : p<0.001; H vs I_{12} : p<0.001; I_1 *vs* $I_{3:}$ p=0.639; I_1 *vs* $I_{3:}$ p=0.639; I_1 *vs* $I_{6:}$ p<0.001; I_1 *vs* I_9: p <0.001; I_1 *vs* I_{12}: p <0.001; I_3 *vs* $I_{6:}$ p=0.117; I_3 *vs* I_9: p=0.023; I_3 *vs* I_{12}: p<0.001; I_6 *vs* $I_{9:}$ p=0.875; I_6 *vs* $I_{12:}$ p=0.150; I_9 *vs* $I_{12:}$ p=0.960). (b) Leaf wilting (L_w) is not a good indicator of the larval host selection (glm, L_0: n=41, *df*=81, F=0.272, p=0.603; $L_{1:}$ n=6, glm, *df*=11, F=0.324, p=0.582; L_2: n=11, glm, *df*=21, F=0.119, p=0.734; L_3: n=10, glm, *df*=19, F=1.165, p=0.295; L_4: n=8, glm, *df*=15, F=12.072, p=0.003).

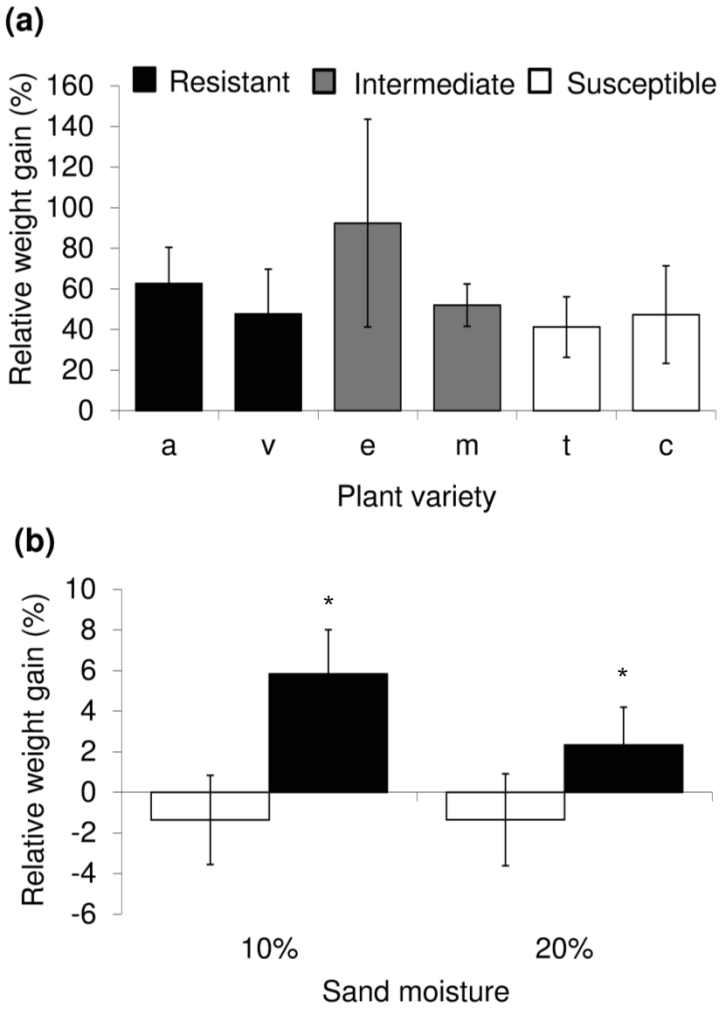

Figure S2: D. virgifera *larval growth is not affected by the host plant resistance to hydric stress.* (a) Larval growth (mean ±SE) after feeding for 48 hours on resistant, intermediately resistant and susceptible maize lines (n=6; one-way ANOVA, *df*=5, F=0.611, p=0.692). (b). *D. virgifera* larvae growth (mean ±SE) after feeding six hours on healthy roots (in white) or on the undamaged part of roots that had been inested for four days (black) at 10 and 20% moisture (v/v) (n=8; *df*=30, two-way ANOVA, infestation: F=3.22, p=0.05, soil moisture: F=0.855, p=0.36).

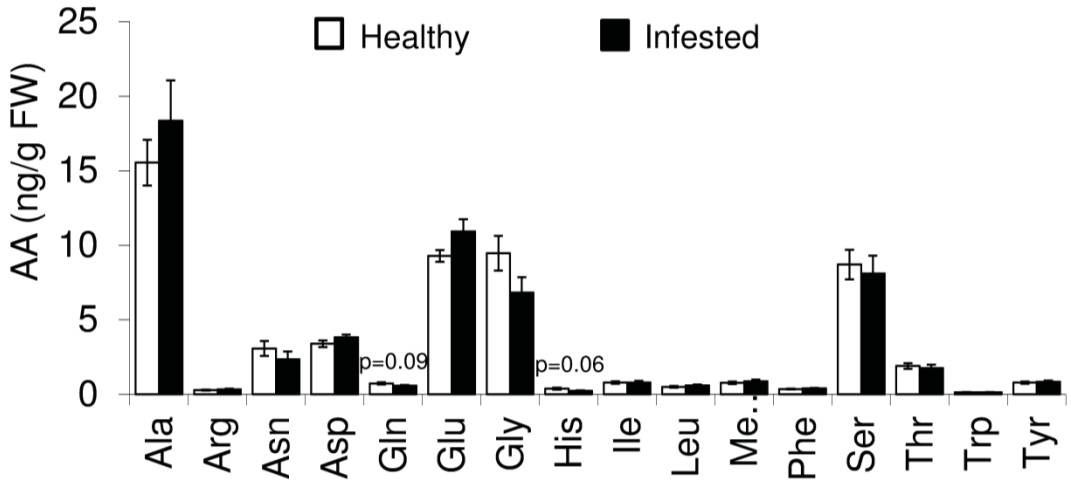

Figure S3: *Amino acid contents in leaves* (n=6, t-tests, *df*=10, Ala: t=-0.901, p=0.389; Arg: t=-0.844, p=0.419; Asp: t=-1,517, p=0.160; Gln: t=1.864, p=0.092; Glu: t=-1.828, p=0.097; Gly: t=1.695, p=0.121; His: t=2.077, p=0.065; Ile: t=0.025, p=0.981; Leu: t=-0.845, p=0.418; Met/Val: t=-0.644, p=0.534; Phe: t=-1.409, p=0.189; Ser: t=0.387, p=0.707; Thr: t=0.476, p=0.644; Trp: t=0.398, p=0.699; Tyr: t=-0.493, p=0.633; Mann Whitney Rank Sum test: Asn: T=48, p=0.18).

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CHAPTER V

Does maize tolerate root herbivory by increasing resource allocation to the stem?

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ABSTRACT

Upon attack by leaf-herbivores, plants reallocate photo-assimilates to the roots. It has been suggested that this behavior is a tolerance response, as the carbon resources stored in below ground tissues may be used for later regrowth. But how do plants react when the alleged "carbon bunker" is under attack itself? We investigated this question by profiling photoassimilate flows in maize plants that are attacked by the specialist root herbivore *Diabrotica virgifera virgifera*. Using $^{11}CO_2$ labelling combined with beta-imaging, scintillation counting and PET-scans, we demonstrate that root-infested plants assimilate more carbon and export it more quickly from the source leaves. Root meristematic activity and carbon allocation were significantly decreased by *D. virgifera* attack, leading to a higher proportion of assimilates that remain in the above ground part of the seedlings. Root-attacked plants had thicker stems than healthy plants, indicating that root-infested plants reallocate carbon resources to the stem. We did not find any increase in labeled glucose, fructose or sucrose in above ground tissues, suggesting that the additional photo-assimilates were directly converted into storage compounds. We propose that carbon reallocation may be a tolerance strategy of maize plants to root herbivory, as after the attack, compensatory root growth in the form of adventitious roots could be initiated directly from the base and internodes of the stem. Comparing carbon allocation of maize genotypes with different degrees of *D. virgifera* tolerance is likely to shed light on this hypothesis.

INTRODUCTION

Plants are able to perceive and respond to a broad spectrum of biotic and abiotic stimuli in an integrated manner (Sultan, 2000; de Kroon *et al.*, 2005; Metlen *et al.*, 2009), which allows them to optimize resource allocation, and, ultimately, fitness, in a continuously changing environment (Karban et al., 1997). Defensive strategies against herbivores, for instance, include resistance traits that repel, deter or kill the attacker (Karban *et al.*, 1997; Howe & Jander, 2008), but they can also activate tolerance mechanisms that allow regrowth and reproduction after tissue loss (Tschaplinski & Blake, 1989b; Tschaplinski & Blake, 1989a; Karban & Baldwin, 1997; Strauss & Agrawal, 1999; Tiffin, 2000). While resistance mechanisms have been extensively studied, much less is known about the mechanistic basis of tolerance (Stowe et al., 2000).

Tolerance to herbivory relies on the activation of dormant meristems, the increase of photosynthetic activity, as well as the diversion of resources away from the attacked tissues into storage organs that are inaccessible for the foraging herbivores (Trumble *et al.*, 1993; Baldwin & Ohnmeiss, 1994a; Strauss & Agrawal, 1999; Tiffin, 2000; Schwachtje *et al.*, 2006; Schwachtje & Baldwin, 2008). Resource reallocation following real or simulated leaf attack has been found to occur in numerous plant species, including tomato, tobacco, maize, barley, and poplar seedlings, all of which increase the export of photosynthate from the leaves to the stem and roots upon herbivory (Holland *et al.*, 1996; Babst *et al.*, 2005; Schwachtje *et al.*, 2006; Babst *et al.*, 2008; Henkes *et al.*, 2008; Gomez *et al.*, 2010; Hanik *et al.*, 2010a). Similarly, acquisition of nitrogen was found to increase in roots of tomato plants that were subject to simulated herbivory aboveground (Gomez et al., 2010). Yet, shifts in resource allocation do not necessarily coincide with tolerance *per se*. For instance, free amino acids are allocated to the leaves of tobacco plants to be used for the biosynthesis of defensive secondary metabolites rather than regrowth (Hanik et al., 2010a; Hanik et al., 2010b). Also, increased resource flow to the roots can lead to more carbon exudation into the rhizosphere, with no net storage of assimilates (Holland et al., 1996). One of the few examples where herbivore-induced resource allocation was correlated with tolerance comes from wild tobacco (*Nicotiana attenuata*), where silencing of a herbivore-suppressed SNF1-like kinase delayed senescence and prolonged flowering (Schwachtje et al., 2006). Clearly, understanding plant tolerance to herbivory requires a thorough evaluation of the mechanisms behind resource partitioning and allocation to storage and defense (Orians, C *et al.*, 2011).

A largely neglected aspect of tolerance-related resource diversion belowground is the fact that roots may not be a “safe-haven” for photo-assimilates after all (van Dam, N. M., 2009; Orians, C *et al.*, 2011), because roots also are under constant attack by various consumers, including insects, nematodes and microorganisms. So how do plants reallocate resources when the storage organs

themselves are under attack? We investigated this question by studying the response of maize plants to *Diabrotica virgifera virgifera,* an agricultural pest that threatens maize production in the U.S. and in Europe. *D. virgifera* is a specialist herbivore that easily overcomes maize defenses (Robert et al., 2012). Tolerant maize genotypes have been selected to reduce the negative impact of *D. virgifera* on yield via increased root growth following attack (Prischmann et al., 2007). We therefore hypothesized that maize should possess effective inducible tolerance mechanisms, including resource reallocation away from the attacked roots. Using radioactive $^{11}CO_2$, we explored the dynamics of carbon partitioning between leaves and roots following root herbivory. In addition, chemical analyses of primary and secondary metabolites were performed to understand the metabolic fate of the reallocated photoassimilates.

METHODS

Plants and insects

Maize plants (*Zea mays* L., variety Delprim) used in radiography assays and positron emission topography were germinated in petri dishes until at least 3cm of roots were formed. Maize seedlings were then sown in cylindrical glass cells (74 mm ID x 150 mm length, Q glass Co, Towaco, NJ, USA) containing a growth medium. Briefly, the growth medium was obtained by adding 1.6 g of Hoagland modified basal salt mixture (*Phyto*Technology Laboratories ™) and 0.55 g of 2-(N-Morpholino) ethanesulfonic acid (MES) hydrate (Sigma Life Science) to 1 liter of distilled water. After adjusting the pH to 5.8 with a few droplets of sodium hydroxide, 2.5 g of Gelzan ™ CM (Sigma Life Science) was added. All media and glass cells were autoclaved prior to use. Plants used for experiments had three to four fully developed leaves. Maize seedlings that were used to trace ^{11}C and ^{12}C soluble photosynthates were sown in plastic pots (12 cm^3) with sand (Sakrete ® multipurpose medium coarse sand, Bonsal America, Charlotte, NC, USA) and covered with 2 cm of potting soil (Pro-Mix BX, Premier Horticulture LTEE, Quebec, Canada). Plants with two to three fully developed leaves were used for the analyses. All plants were grown under metal-halide lamps (23 ± 2 °C, 60% relative humidity, 16:8h L/D, and 350 $\mu mol.m^{-2}.s^{-1}$). Maize plants that were used for the characterization of the stem morphology and physiology upon root infestation were sown in plastic pots (11 cm high, 4 cm diameter) by placing them on a layer of moist washed sand (0-4 mm, Jumbo, Switzerland). The seeds were then covered with 2 cm of commercial soil (Aussaaterde, Ricoter, Aarberg, Switzerland). Seedlings were grown in a climate chamber (23 ± 2 °C, 60% relative humidity, 16:8h L/D, and 350 $\mu mol.m^{-2}.s^{-1}$), and MioPlant Vegetable and Herbal Fertilizer (Migros, Switzerland) was added every two days after seed germination. Twelve-day old plants with two fully developed leaves were used for the experiments.

Eggs of *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), were obtained from the USDA-ARS Columbia (MO, USA) and USDA-ARS-NACRL Brookings (SD, USA). After hatching they were kept on freshly germinated maize roots until we used them as second instar larvae.

Radiotracer production and administration

$^{11}CO_2$ production was achieved via the $^{14}N(p,\alpha)^{11}C$ nuclear reaction (Ferrieri & Wolf, 1983) by irradiation of high-purity nitrogen gas target with 17 MeV protons from the Brookhaven National Laboratory TRi-19 (Ebco Industries Ltd, Richmond, BC, Canada) cyclotron, and captured on a molecular sieve (4 A°) (Ferrieri et al., 2005). ^{11}C has a half-life ($t_{½}$) of 20.4 min, and decays by emission of positrons via the $^{11}C(\beta+)^{11}B$ reaction. $^{11}CO_2$ was pulse-fed into a leaf chamber made of Plexiglas on a split clamshell hinge for 30 seconds. Red and blue LED lights provided an irradiance of 900 $\mu mol.m^{-2}.s^{-1}$. A PIN diode gamma radiation detector (Bioscan, Inc., Washington, DC, USA) was installed in the leaf chamber to record $^{11}CO_2$ administration, tissue fixation and ^{11}C photosynthate phloem translocation. To prevent inadvertent escape of radioactive gas, the Department of Energy safety regulations required special containment of the $^{11}CO2$, therefore limiting the analysis to four plants per day (two couples of healthy and infested plants per day). The second and third true leaves were sealed in the leaf chamber between neoprene rubber gaskets. The plant and leaf cell were contained in a climatic chamber, at 22 ± 2°C and under 300 $\mu mol.m^{-2}.s^{-1}$ light irradiance at 16h: 8h light: dark photoperiod. All data were processed using the software Peak Simple 3.56 (SRI instruments, Torrance, CA, USA).

Transport and partitioning of ^{11}C photosynthates

Plants were infested with twelve *D. virgifera* larvae or left uninfested for four days before radiotracer exposure. The PIN diode gamma radiation detector (Bioscan, Inc., Washington, DC, USA) installed in the leaf chamber recorded $^{11}CO_2$ administration, tissue fixation and ^{11}C photosynthate export from leaves. Two hours after tracer administration, shoots were excised and an average of six individual roots was cut before removing the whole root system out of the growth medium. Fresh weight of the plant tissues was determined immediately after excision. ^{11}C assimilates in roots, shoots and *D. virgifera* larvae were quantified using a beta counter (Capintec, Inc., Ramsey, NJ, USA). ^{11}C photosynthate distribution in leaf tissue, individual roots, meristems and larvae was also evaluated by using phosphorplate imaging of positron emissions, and quantified using Science Laboratory 99 Image Gauge software (Fuji Photo Film Co., Ltd, Tokyo, Japan). Individual roots were dried under red light overnight. The shoot and the remaining root tissues were dried in an oven at 70°C for 24 hours and weighted to determine their dry biomass.

Positron Emission Tomography (PET) imaging.

After exposure, three healthy plants and three infested plants were removed from the exposure chamber to determine ^{11}C photosynthate transport speed in roots. Distribution of ^{11}C in maize roots was determined by detection of gamma rays that are emitted as a result of positron annihilation *in situ* using a microPET R4 (Siemens Preclinical Solutions, Knoxville, TN, USA). The PET scanner was used in its default configuration as outlined in (Alexoff *et al.*, 2003; Alexoff *et al.*, 2011). Image reconstruction was achieved using MicroPET Manager 2.3.3.0 and image display was realized using the microPET software ASIPro VM 6.2.5.0. ^{11}C assimilates transport speed was determined by counting gamma ray emissions for 90 minutes at five minutes intervals at two regions of interest (ROIs) along the root system. Plants were then treated following the same procedure as described above to evaluate ^{11}C partitioning among plant tissues, exudates and larvae.

Stem morphology

To evaluate the possible role of the stem as storage tissue upon root herbivory, maize seedlings were infested with six *D. virgifera* larvae. Control plants were left uninfested. After seven days, the stem length (from the soil to the first leaf) and circumference were measured. All tissues were excised separately and the stem mass was noted. The stem density ($g.cm^{-3}$) was calculated by assuming the stem was cylindrical.

Soluble ^{11}C photosynthate identification

Maize seedlings were infested for two days with six *D. virgifera* larvae, and healthy plants were left uninfested. One hour after $^{11}CO_2$ exposure, the leaf and crown roots were collected and ground into a fine powder under liquid nitrogen. 100 µL of methanol were added to 100 mg of plant tissue, and centrifuged 2 minutes at 15000 rpm. Radioactivity of both supernatant and residue of leaf and root tissues was evaluated with a beta counter (Capintec, Inc., Ramsey, NJ, USA). Supernatants were used to assess soluble carbohydrates and amino acid profiles. Carbohydrate contents were determined as follow: A drop (2 µL) of the supernatant of each extract was spotted on a silica thin layer chromatography (TLC) strip (Fisher Scientific, Inc., Fairlawn, NJ, USA). Solutions of mixed sucrose, glucose and fructose were used as standards (5, 3, 1, and 0.5mM). Methanol extracts were then developed on TLC plates using acetonitrile/water as the mobile phase (75/25). The distribution of radiolabelled photosynthate was detected one hour after ^{11}C administration using phosphorplate imaging of positron emissions as described above. TLC plates were sprayed with 1-naphtol-sulfuric acid reagent (11.5% H2SO4 in 160 mL ethanol and 13 mL water containing 5g of 1-Naphtol) and heated in an oven at 80°C until color

development. ^{12}C assimilates appeared reddish under visible light. Digital image was analyzed using Image Gauge software (Fuji Photo Film Co., Ltd, Tokyo, Japan) for quantification of spot intensity, and calculation of extract concentrations.

Statistical analyses

All analyses were performed using the software package R, version 2.8.1. Data were first analyzed with Levene's and a Kolmogorov-Smirnov test to determine heteroscedasticity of error variance and normality. ^{11}C transport and partitioning data were corrected for the decay of the radioisotope over the time course of the experiment. Healthy and infested plants were compared within each couple using paired t-tests. If the data did not pass the Levene and Kolmogorov-Smirnov tests, nonparametric Wilcoxon signed rank tests. Stem morphological traits of root-infested plants were compared to uninfested plants using Student's t-tests.

RESULTS

Maize plants respond dynamically to root herbivory

Leaves of root-infested plants fixed slightly more $^{11}CO_2$ than healthy plants (n=7; paired t-test, *df*=6, t=2.269, p=0.064; Figure 1a). Furthermore, infested plants translocated slightly more ^{11}C photosynthates from leaves through the phloem (n=12; Wilcoxon signed rank test, *df*=1, W=54, $Z_{statistic}$=2.118, p=0.034; Figure 1b). Infested plants also had a root transport that was twice as fast as in healthy plants (n=5; paired t-test, *df*=4, t= -2.976, p=0.041; Figure 1c), and a lower allocation of ^{11}C photosynthates to meristematic zones than healthy plants (n=6; paired t-test, *df*=5, t=3.924, p=0.011; Figure 1d and figure S1).

^{11}C photosynthates accumulates in the shoot of infested plants

Despite the significant reduction of shoot and root fresh biomass upon herbivory (Figure S2), the total amount of ^{11}C photosynthates remaining in the whole plant one hour after administration was slightly raised in infested plants (n=5; paired t-test, *df*=4, t=2.346, p=0.079; Figure 2a). The partitioning of the photosynthates was drastically affected by the root herbivory, as the shoot ratio increased by 40% in favor of leaves and stem in infested plants (n=6; paired t-test, *df*=5, t=2.893, p=0.034; Figure 2b) and the absolute amount of ^{11}C assimilates was higher in the shoot of infested plants than in healthy ones, while it was similar in roots and exudates (n=6; shoots: Wilcoxon signed rank test, *df*=1, W=-21, $Z_{statistic}$=-2.201, p=0.031; Roots: paired t-test, *df*=5, t=0.630, p=0.556; Exudates: paired t-test, *df*=4, t=-0.261, p=0.807; larvae: Wilcoxon signed rank test, *df*=1, $Z_{statistic}$=-2.201, p=0.031; Figure 2c). Similar results were observed when we corrected for the plant dry weight (Figure S3).

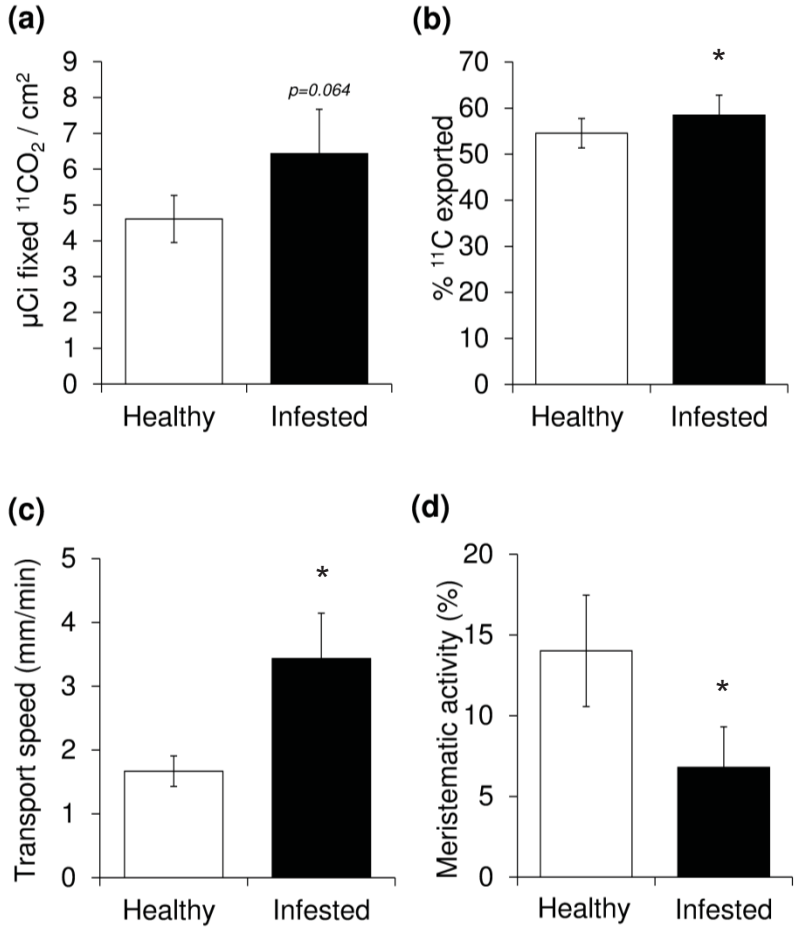

Figure 1*: Maize plants respond to root infestation by* D. virgifera *larvae with dynamic changes in carbon flow*. (a) $^{11}CO_2$ fixation (mean±se) by healthy and root-infested plants. (b) ^{11}C leaf export (mean±se) in healthy and root-infested plants. (c) ^{11}C root transport speed (mean±se) in healthy and infested plants. (d) Meristematic activity (mean±se) of healthy and root-infested plants. Stars indicate significant differences (*: $p \leq 0.05$).

Consistent with these results, the relative distribution of newly acquired photosynthates increased in shoot and decreased in roots of infested plants compared to healthy plants (n=6; shoot: paired t-test, *df*=5, t=2.380, p=0.063; roots: paired t-test, *df*=5, t=-2.303, p=0.069; Exudates: paired t-test, *df*=4, t=-1.092, p=0.336; larvae: paired-test: *df*=5, t=-2.908, p=0.033); Figure 2d). Finally, the ^{11}C volatile emissions did not differ between healthy and infested plants (Figure S4).

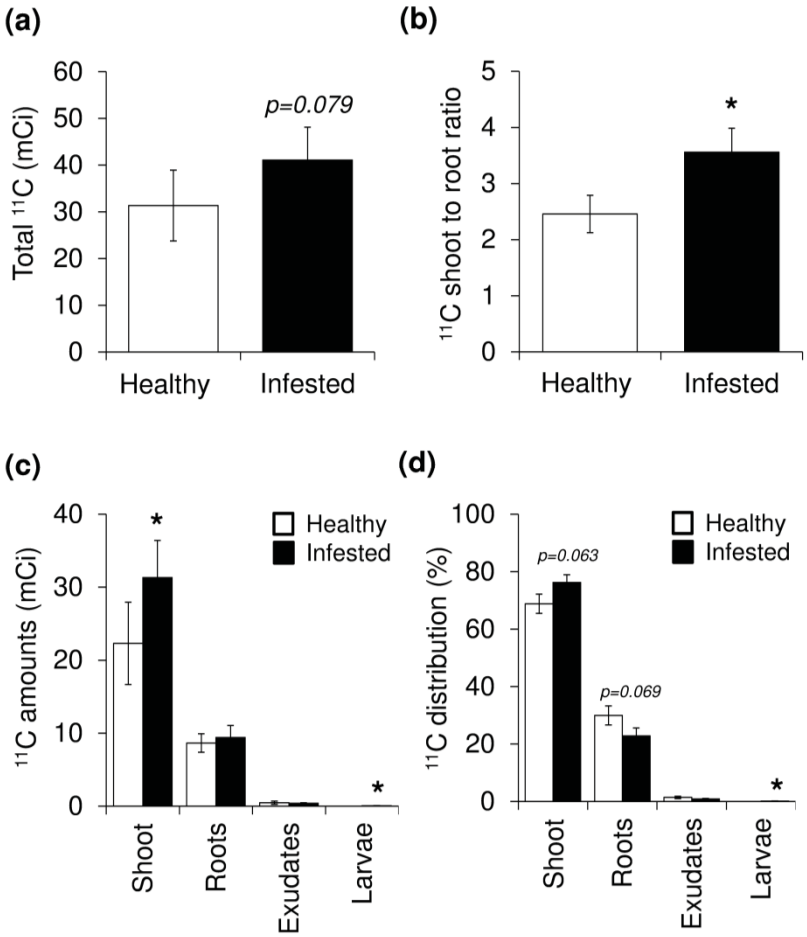

Figure 2: *Root-infested plants store ^{11}C photosynthates in the stem*. (a) Total amount (mean±se) of ^{11}C fixed by healthy and *D. virgifera* infested plants. (b) ^{11}C shoot to root ratio (mean±se) of healthy and infested plants. (c) ^{11}C partitioning in plant (shoot and roots), exudates and the herbivore larvae. (d) Relative distribution of the ^{11}C assimilates among the plant (shoot and roots), exudates and larvae. Stars indicate significant differences (*: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$).

The stem of infested plants grows larger upon root herbivory

Root herbivory affected stem morphological traits: The length of infested plants stems was reduced (n_{ctl}=18, n_{inf}=17; Student's t-test, *df*=33, t=2.206, p=0.034; Figure 3a), while their circumference increased (n_{ctl}=18, n_{inf}=17; Student's t-test, *df*=32, t=-2.848, p=0.008; Figure 3b). The total mass of the stem was enhanced upon herbivory ($n_{healthy}$=18, $n_{infested}$=17; Student's t-test, *df*=33, t=2.083, p=0.045; Figure 3c). However, the stem density was similar between infested and healthy plants ($n_{healthy}$=15, $n_{infested}$=14; Student's t-test, *df*=27, t=0.543, p=0.591; Figure 3d).

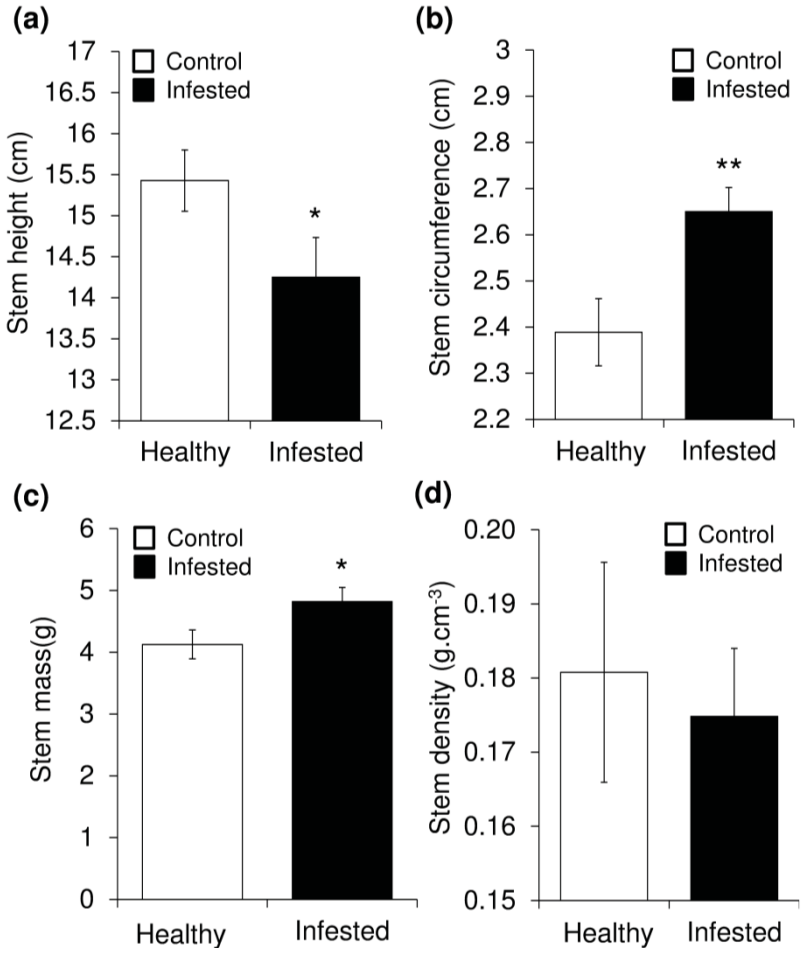

Figure 3: *Stems can serve as storage organs upon root infestation*. (a) Stem height (mean±se) from the base to the first leaf node of healthy and infested plants. (b) Stem circumference (mean±se) of healthy and infested plants. (c) Stem mass (mean±se) of healthy and infested plants. (d) Stem density (mean±se) of healthy and infested plants. Stars indicate significant differences (*: p≤0.05, **: p ≤0.01; ***: p≤0.001).

^{11}C photosynthates profiles differ slightly in infested root

Both the amount of newly formed carbohydrates (^{11}C) and the stored pools of carbohydrates (^{12}C) remained unchanged in the leaves of root-infested plants (n=6; paired t-tests, *df*=5, ^{11}C Maltose/Raffinose: t=-0.831, p=0.444; ^{11}C Sucrose: t=1.101, p=0.321; ^{11}C Glucose: t=-1.324, p=0.2143; ^{11}C Fructose: t=-0.727, p=0.500; ^{11}C Xylose: t=-0.120, p=0.909; ^{12}C Sucrose: t=0.202, p=0.848, ^{12}C Glucose: t=-1.698, p=0.150; ^{12}C Fructose: t=-0.237, p=0.822; Figure 4a and b). Also belowground the partitioning of newly formed carbohydrates was similar between healthy and infested plants (n=6; paired t-tests, *df*=5: maltose/raffinose: t=-1.673, p=0.155; sucrose: t=0.551, p=0.605; glucose: t=0.534, p=0.616; fructose: t=0.321, p=0.761; xylose: t=-1.096, p=0.323; Figure 4c).

On the other hand, pools of fructose decreased by more than 50% in infested plants compared to healthy plants, while pools of sucrose and glucose remained unchanged (n=6; paired t-tests, *df*=5:

sucrose: t=0.710, p=0.509; glucose: t=1.773, p=0.136; fructose: t=3.722, p=0.014; Figure 4d). Finally, the ratio between soluble and insoluble ^{11}C contents was similar between healthy and infested plants (Figure S5).

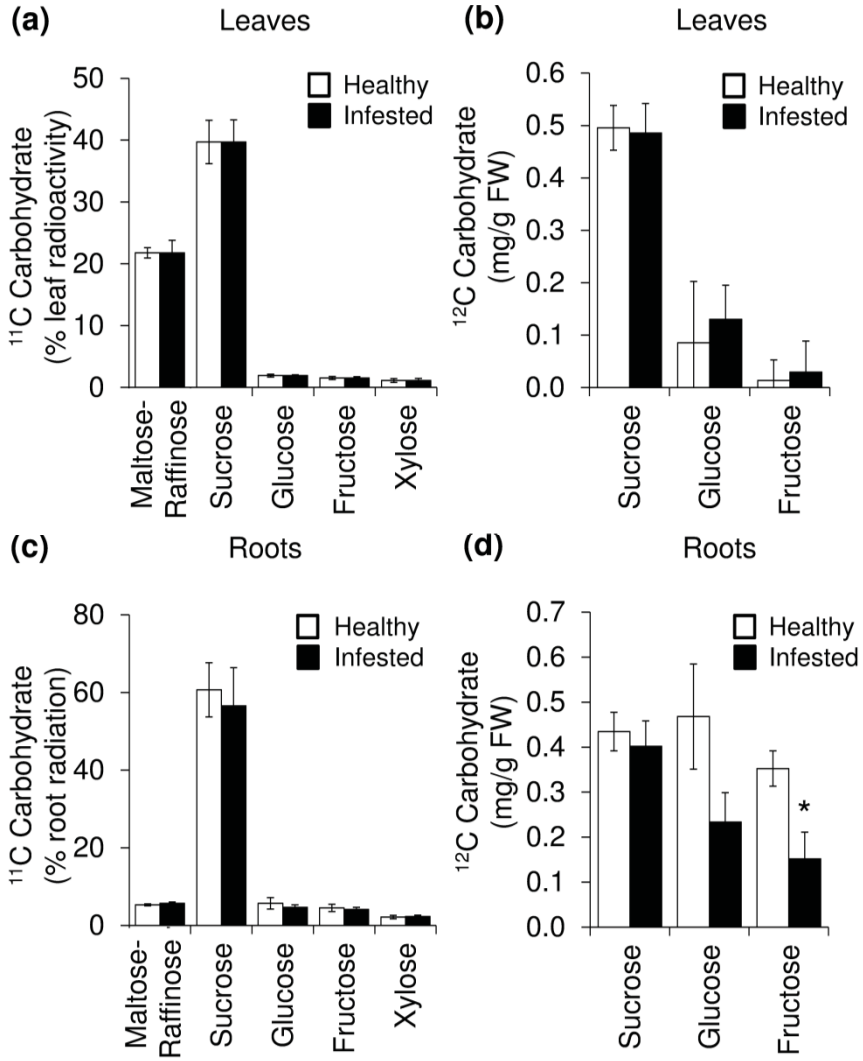

Figure 4: *Root herbivory affects ^{12}C carbohydrate pools in roots only*. (a) Relative amounts (mean±se) of ^{11}C carbohydrates in healthy and infested plants. (b) ^{12}C carbohydrate amounts (mean±se) in healthy and infested plants. (c) Relative amounts (mean±se) of ^{11}C amino acids in healthy and infested plants. (d) ^{12}C amino acid amounts (mean±se) in healthy and infested plants. (c) Relative amounts (mean±se) of ^{11}C carbohydrates in healthy and infested roots. (d) ^{12}C carbohydrate amounts (mean±se) in healthy and infested roots. Stars indicate significant differences (*: p≤0.05, **: p ≤0.01; ***: p≤0.001).

DISCUSSION

This study reveals an unexpected role of stems as storage organs, allowing a plant to divert resources away from herbivore-infested roots and sequester them elsewhere. The experiments with radioactive $^{11}CO_2$ show that the whole maize plant responds dynamically to *D. virgifera* attack by i) increasing photosynthetic rates in the leaves, ii) increasing photoassimilate export from a source leaf, iii) intensifying carbon allocation to the shoot and iv) increasing root transport

speed (Figures 1 and 2). In the past, variable effects of *D. virgifera* attack on photosynthetic rates have been documented: In the greenhouse, well-watered maize plants did not show any change in photosynthetic rates upon infestation, while infested water-stressed plants had lower photosynthetic activity than their non-infested controls (Dunn & Frommelt, 1998). In a field study, on the other hand, higher photosynthetic activity was measured in the leaves of *D. virgifera* infested plants (Godfrey, L. D. *et al.*, 1993; Dunn & Frommelt, 1998a), a phenomenon which was interpreted as a component of the compensatory root-growth response of *D. virgifera* tolerant maize lines. Our experiments confirm the notion that maize plants can increase the assimilation of CO_2 in the leaves following root attack by *D. virgifera*. The effect of root attack on photosynthesis is likely to be even higher than observed in our assays, as CO_2 assimilation in the gas-exchange chambers was above 90% even in control plants, indicating that some plants would have been able to accept even more CO_2, if available. The fact that some studies report a decrease in photosynthetic activity following *D. virgifera* attack may be explained by a reduction in water supply from the roots: Especially under water-limiting conditions, root herbivory increases water stress in maize leaves, which may lead to stomatal closure and reduction of CO_2 assimilation (Erb *et al.*, 2011b). In our experiments, plants were grown in supplemented water agar and did not suffer from any water stress following *D. virgifera* attack, as was reflected in similar levels of relative water contents between control and infested plants.

Interestingly, *D. virgifera*-attacked maize plants seemed to accumulate more newly acquired photosynthates in the stem than uninfested controls: Infested plants produced more ^{11}C assimilates than healthy plants (Figure 2a) and exported them more quickly from the source leaves (Figure 2b, c and d). As the excess assimilates did not accumulate in the roots, exudates, volatiles or *D. virgifera* larvae themselves (Figure 2c), we conclude that they must have accumulated in the sink leaves or stem. Beta-imaging showed that sink leaves receive similar amounts of labeled photosynthate (data not shown), indicating that they are unlikely to be the "missing sink". On the other hand, although infested plants suffered from a reduction of fresh mass below- and aboveground (Figure S2 and 5a), their stems became thicker, but were not reduced in density or mass, lending support to the conclusion that more assimilates were allocated to this tissue following root-attack (Figure 3b, c and d). The fact that the stems became thicker also explains why we were not able to see an increase in ^{11}C assimilates in the stem using beta-imaging: The increase in tissue mass is likely to have led to an attenuation of the positron signals, thereby masking possible treatment effects. Previous studies measured tissue biomass of *D. virgifera* infested plants and found a lower leaf and stalk biomass (Dunn & Frommelt, 1998b). Unfortunately, leaves and stalk were weighed together, making it impossible to assess changes in resource partitioning between these tissues. Our study therefore seems to be the first one to

investigate carbon allocation following root herbivory, and the results are consistent with previous studies that show remobilization of resources away from the herbivore damage site and sequestration into inaccessible organs (Babst *et al.*, 2005; Schwachtje *et al.*, 2006; Babst *et al.*, 2008; Schwachtje & Baldwin, 2008; Gomez *et al.*, 2010; Hanik *et al.*, 2010a).

In the specific case of maize, increased allocation of resources to the stem following *D. virgifera* attack may serve three purposes: Firstly, *D. virgifera* larvae may be deprived of assimilates from the leaves, which may reduce their fitness or prompt them to move away from infested plants. Secondly, maize plants may be able to use the sequestered resources to grow new adventitious roots after the attack is over. Until now, we have little evidence to support the first possibility, as *D. virgifera* larvae aggregate on host plants and perform better on already attacked root systems (Robert *et al.*, in press). That resource reallocation to the stem helps maize to compensate for the loss of root biomass is a hypothesis that needs to be tested. Several *D. virgifera* tolerant maize lines have been developed by plant breeders (Prischmann et al., 2007), and comparing their allocation behavior to intolerant maize genotypes may provide further insights into the relevance of increased stem-allocation. Measuring changes in stem circumference of tolerant and non-tolerant genotypes may be a simple way of evaluating whether reallocation to the stem is associated with the plants' ability of root regrowth.

Another question that remains open is in what form the photoassimilates are stored in the stem of *D. virgifera* attacked plants. We did not find any significant differences in ^{11}C and ^{12}C mono- and disaccharide concentrations between *D. virgifera* infested and control leaves (Figure 4), suggesting that at least these carbohydrates do not change significantly in abundance, and that the increased $^{11}CO_2$ is either distributed to more cells (giving equal concentrations) or rapidly converted into storage carbohydrates. The fact that stems of infested plants did not have a greater biomass (Figure 3c), and that cell density was not significantly changed (Figure 3d) makes the second possibility more likely. Measuring the concentration of carbohydrate storage molecules in the stem following root herbivory will therefore be a priority for future experiments.

In our assays, the root sink strength decreased, as we observed a reduction of meristematic activity (Figure S1), as well as a reduction of ^{12}C glucose and fructose concentrations belowground (Figure 4d). This was true for both attacked as well as non-attacked roots from infested plants (Figure S1), suggesting a systemic reaction rather than a physical disruption of meristem transport by herbivory. This observation is in tune with the suppression of invertase transcription in *D. virgifera* attacked roots (Chapter 4), and can be interpreted as a component of the plants tolerance response: In order to increase the allocation of assimilates to the stem, reducing root sink strength would appear to be a logical consequence.

CONCLUSION

In conclusion, we show first evidence for a possible scenario of how plants may tolerate root herbivory: Following belowground attack, the photosynthetic activity in the leaves is increased, and the produced photoassimilates are diverted away from the attacked roots and stored in the stem. After the attack, these compounds may then be used for vigorous regrowth of the lost belowground tissue, a mechanism which is likely to reduce the adverse effects of root herbivory. As such, this mechanism may be important for plant survival in natural systems, but may be exploitable in an agricultural context to secure yields in years of high root herbivore pressure.

AKNOWLEDGMENTS

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SUPPLEMENTARY FIGURES

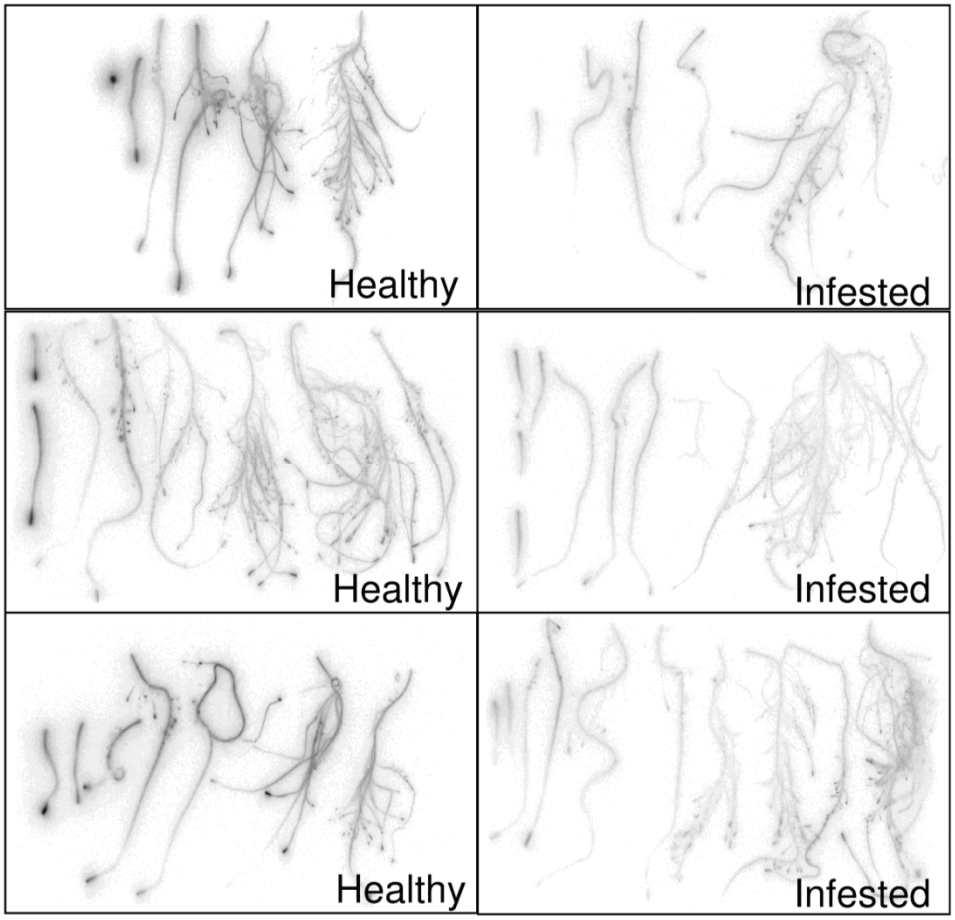

Figure S1: *^{11}C photosynthate distribution in healthy and* D. virgifera *infested roots.*

Figure S2: *Fresh and dry biomass* (mean±se) *of healthy and* D. virgifera *infested shoots and roots* (Fresh biomass: leaves: paired t-test, n=10, *df*=9, t=2.626, p=0.028; roots: n=7 *df*=6, t=2.884, p=0.028; dry biomass: leaves: paired t-test, n=11, *df*=10, t=-0.097, p=0.924; roots: Wilcoxon signed rank test, *df=1*, n=7, W=5, $Z_{statistic}$=0.255, p=0.846). Stars indicate significant differences ($p \leq 0.05$).

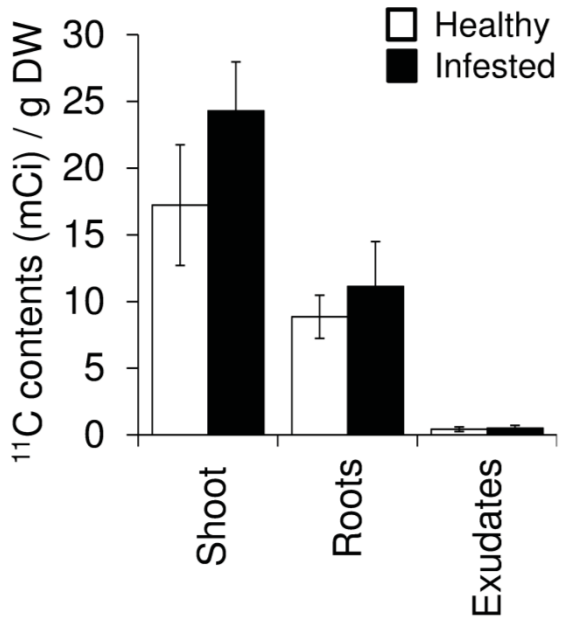

Figure S3: *Metabolism activity (mean±se) in tissues of of healthy and* D. virgifera *infested plants* (n=6, paired t-test, *df*=5, shoots: t=1.851, p=0.123; roots: n=5, paired t-test, *df*=4, t=0.926, p=0.407; exudates: n= n=5, paired t-test, *df*=4, t=0.222, p=0.835).

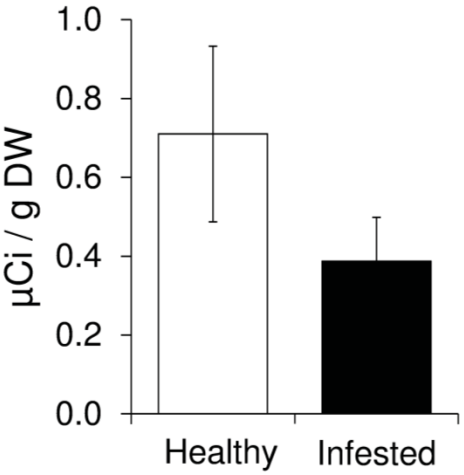

Figure S4: *^{11}C root volatile emission (mean±se) from healthy and* D. virgifera *infested plants* (n=2; paired t-tests, *df*=1, t=1.391, p=0.397).

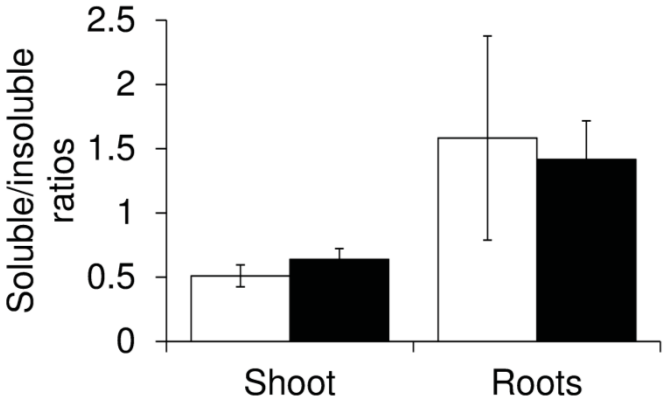

Figure S5: Ratio (mean±se) between soluble and insoluble ^{11}C contents in shoot and roots of healthy and *D. virgifera* infested plants (leaves: n=6, paired t-test, *df*=5, t=-1.747, p=0.141; roots: n=2, paired t-test, *df*=1, t=1.500, p=0.374).

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CONCLUSIONS AND OUTLOOKS

CONCLUSIONS

The results of my thesis provide new insights into the mechanisms of interaction between the specialist root feeder *D. virgifera* and its host plant, maize. In the following section, I will take two approaches to synthesize the different thesis chapters. First, I take an herbivore-centered perspective and illustrate how specific behavioral adaptations contribute to the success of *D. virgifera* as "a perfect pest". Second, I discuss possible evolutionary constraints and defense strategies of plants that face such a well-adapted enemy. Finally, I highlight open questions and future directions of research in this fascinating system.

Diabrotica virgifera- the perfect pest?

As I show here, *D. virgifera* has developed effective offensive traits to optimize its own fitness at the cost of its host plant. First, *D. virgifera* larvae are able to use plant volatiles to locate and orient towards host plants that are beneficial for their development (Chapters 1 and 2). Second, once arrived on a host plant, the larvae have developed the capacity to tolerate and even exploit plant defensive metabolites to orient within the root system and select the optimal site of foraging (Chapter 3). Third, *D. virgifera* larvae aggregate on host plants, which results in a favorable reconfiguration of the plants' metabolism (Chapter 4).

Host location and selection are of crucial importance for *D. virgifera* larvae. Adult females do not lay eggs on a plant directly, but in the soil at the end of the vegetation period. These eggs overwinter and larvae hatch at the beginning of the growing season. The survival of *D. virgifera* larvae therefore relies entirely on their own ability to find a suitable host plant for their development. Orienting and moving in the soil can be costly for the newly hatched larvae, and the resulting selection pressure may have resulted in a highly effective ability to detect host plants. Plant CO_2 is known to be a common attractant for root herbivores (Strnad *et al.*, 1986; Johnson & Gregory, 2006), as it indicates the presence of respiratory activity, for example from plant roots. Yet, CO_2 emissions are a poor indicator of host quality (Chapter 1). This thesis revealed that *D. virgifera* was able to orient towards the plant on which it grew best by exploiting herbivore-induced volatiles such as ethylene and *(E)*-β-caryophyllene (Chapter 1). Ethylene is known to be an attractant for insects such as moths (Raina *et al.*, 1992) and beetles (Arita *et al.*, 1988; Gonzalez & Campos, 1996). The reduced emission of ethylene by the roots of leaf-infested plants may be a general belowground indicator for plant growth and fitness (Pierik *et al.*, 2006), and may therefore be a reliable cue for long-distance assessment of host quality (Chapter 1). The sesquiterpene *(E)*-β-caryophyllene was previously found to attract entomopathogenic nematodes to damaged roots (Rasmann et al., 2005) and to have antibacterial properties (Huang *et al.*, in press). My results reveal an additional role of this compound as an attractant for *D. virgifera*

(Chapters 1 and 2). Given that *D. virgifera* benefits from aggregating on host plants (Chapter 3), it is not surprising that the larvae also exploit herbivore-induced volatiles like *(E)*-β-caryophyllene as an indicator of infested, and hence more suitable, host plants. It is particularly noteworthy that *D. virgifera* uses the sesquiterpene in a dose-dependent manner to assess an optimal density at which to infest a root system and to avoid intraspecific competition and host overexploitation (Chapters 1, 2 and 3). Interestingly, I also found that the generalist root herbivore, *D. undecimpunctata howardii*, is not attracted by plants that emit *(E)*-β-caryophyllene (Chapter 2), suggesting that attraction to *(E)*-β-caryophyllene is specific for *D. virgifera*.

Once *D. virgifera* larvae have located a suitable host plant, their host selection behavior does not stop: The herbivore has also evolved a remarkable ability to locate the most nutritious tissue within the root system, namely the growing crown roots, and prefers to feed on these roots, despite the fact that these tissues were more highly defended by 1,4-benzoxazin-3-ones and phenolic compounds, which effectively deterred two generalist herbivores, *S. littoralis* and *D. balteata* (Chapter 3). I found that 1,4-benzoxazin-3-ones (BXDs) (Macias *et al.*, 2009; Niemeyer, 2009) did not affect the performance of *D. virgifera* larvae, showing that they are resistant to BXDs (Chapter 3). On the contrary, 1,4-benzoxazin-3-ones are exploited by the root herbivore as indicators for tissue quality (Chapter 3). It is very likely that the ability of *D. virgifera* to the turn tables on the plant and to exploit a main line of defense for its own benefit contributes to its success as a pest.

As mentioned above, *D. virgifera* larvae grow better when aggregating on host plants (Chapter 4). This remarkable phenomenon is based on the fact that, upon attack, maize roots do not become more resistant against *D. virgifera*, but actually more susceptible. The specialist, therefore, does not only succeed in finding the best plants and the best tissues within a root system, but also seem to reprogram the metabolism of maize roots for its own benefit. I propose several non-exclusive explanations for this observation: First, *D. virgifera* is obviously adapted and resistant against maize defenses, including 1,4-benzoxazin-3-ones (Chapter 3). Second, the inducibility of a plant's defense response is attenuated following a first attack, which may further reduce the effectiveness of induced root defenses (Chapter 4). Third, the infestation of maize roots by *D. virgifera* leads to a reconfiguration of the plants' primary metabolism, including an increase in free amino acid concentrations in the roots, which may benefit the larvae by improving the nutritional value of their diet. Induced susceptibility and aggregation of *D. virgifera* may therefore represent a further piece in the puzzle that explains its ecological success.

In summary, from the perspective of the herbivore, its extraordinary behavioral and physiological adaptations clearly make it a voracious and hard to combat pest. I identified only few traits that

could be characterized as a "weaknesses". Does that mean *D. virgifera* is the winner, and that the game for maize plants, and plant growers, is lost?

***Zea mays*, a defenseless "lettuce"?**

Indeed, maize seems to be utterly maladapted to this specialist root herbivore, as both its constitutive and induced responses are circumvented or exploited by *D. virgifera*. In the past, it was suggested that the release of volatiles like *(E)*-β-caryophyllene from attacked roots is an alternative weapon of the plant to fight *D. virgifera*, as it may be used by entompathogenic nematodes to find and kill the herbivore (Rasmann et al., 2005). But why would a plant evolve a signal that facilitates host finding by one of its worst enemies? It is highly unlikely that the release of *(E)*-β-caryophyllene initially evolved in the wild ancestor of maize as an indirect anti-herbivore defense signal. I suggest an alternative evolutionary scenario in which *(E)*-β-caryophyllene first evolved as an antimicrobial compound (Alma *et al.*, 2003; Lourens *et al.*, 2004; Pichette *et al.*, 2006; Huang *et al.*, in press) that protects wounded sites from soil microorganisms that could use them as entry points into the plant. *D. virgifera* could have evolved to use *(E)*-β-caryophyllene as a signal to aggregate on host plants. Entomopathogenic nematodes could then in turn have evolved to exploit the sesquiterpene to locate *D. virgifera*. Depending on the net outcome of these conflicting effects, both the plant and the herbivore may have been under selective pressure to change their behavior, or not. At low abundance of entomopathogenic nematodes, the benefit of *D. virgifera* to use the signal to aggregate may still have outweighed the cost of a higher risk of nematode attack, thereby stabilizing the trait in the herbivore. The presence of the signal in the wild ancestor and the ideal diffusion properties of this specific sesquiterpene (Hiltpold & Turlings, 2008) suggest that from the plants perspective, releasing *(E)*-β-caryophyllene is beneficial in nature. Because However, *D. virgifera* pressure is high in cultivated maize, and American maize breeders may still have selected host plants with reduced attractiveness to *D. virgifera*, which could explaine the fact that many American varieties no longer produce *(E)*-β-caryophyllene (Köllner *et al.*, 2008). My experiments also show additional ecological and physiological costs of *(E)*-β-caryophyllene emission, including attraction of aboveground herbivores (Chapter 2), which further adds to the notion that selecting for plants that do not produce *(E)*-β-caryophyllene may not have been a coincidence. For the moment, the exact evolutionary scenario remains speculative. What becomes clear from my experiments is however that American maize lines, apart from not having any effective direct defenses against *D. virgifera*, do not possess any indirect defensive mechanisms either. So, is maize indeed a "lettuce" without any functional resistance mechanisms against *D. virgifera*?

Indeed, farmers and plant breeders have grown maize in the presence of *D. virgifera* and other insect pests for almost 10 000 years now. It would seem unthinkable this would not have led to the selection of germplasm that is able to resist insects. Even selecting for higher grain yield would hardly have been possible without retaining at least some insect resistance traits. The fact that modern maize still contains BXD levels that are comparable to that of its ancestor (Chapter 3) nicely reflect this notion. One other trait that may allow maize to deal with herbivore is induced tolerance: As tolerance mechanisms rely on effective regrowth rather than herbivore resistance, they do not exert any pressure on the pest that would cause it to adapt. From an agronomic perspective, the high phenotypic plasticity of *D. virgifera* and its extraordinary ability to exploit endo- and exogenous plant defenses (Meihls *et al.*, 2008) could be circumvented through tolerance in order to stabilize plant yield. Chapter 5 indeed shows that maize plants reconfigure their primary metabolism upon root infestation: While the root tissues are being removed by the herbivore, aboveground tissues slow down their expansion. Yet, photosynthetic activity and stem diameter are increasing, suggesting that attacked maize plants increase their carbon reserves in the stem. Although the nature of the stored compounds remains unknown, these could be used for regrowth after the attack, thereby helping the plant to tolerate root herbivory by *D. virgifera*. I conclude that exploring such tolerance mechanisms rather than resistance may be the more promising way to reduce the negative impact of *D. virgifera* on maize yield and food production.

OUTLOOK AND FUTURE DIRECTIONS

Several important questions have arisen during this thesis, and some important points for future research are as follows:

- **Why do plants produce root volatiles?** I suggest that the sesquiterpene *(E)*-β-caryophyllene may have evolved as an antibiotic before being exploited as a foraging cue by the specialist herbivore (Chapter 2). Even though testing the evolution of a signal remains a challenging task, the use of terpene synthase transformed plants that constitutively emit *(E)*-β-caryophyllene above- and belowground may help to elucidate potential antibiotic properties of the sesquiterpene.
- **What is the impact of belowground volatiles root herbivore communities?** Chapter 1 revealed that root volatiles may be used by root herbivores to assess host plant quality at a distance. It is tempting to speculate that volatiles induced by root herbivores are attractive to specialist herbivores only and may repel generalist herbivores, leading to volatile-mediated root herbivore assemblages. Clearly, detailed field experiments would be interesting to understand such effects in natural and managed ecosystems.

- **What are the mechanisms that allow *D. virgifera* to resist to BXDs?** Following the metabolic transformation of individual isotopically-labeled BXDs *in vivo* would be a first approach to unravel the mechanisms that allow *D. virgifera* to tolerate these phytochemicals. Based on the generated metabolic model, it would then become possible to identify putative genes that regulate *D. virgifera* resistance to BXDs. Functional genomics via RNAi may be a promising tool to understand the proposed mechanisms.

- **How herbivore-specific is induced susceptibility in maize roots?** In chapter 4, I demonstrated that *D. virgifera* feeding makes roots more susceptible to subsequent attack. It remains however unclear whether the plant reaction is specific to *D. virgifera* and whether it can be exploited by other herbivores. Comparing induced susceptibility among different root herbivores by alternating inducer and responder would provide evidence whether *D. virgifera* indeed manipulates the plant, or whether root wounding in general increases plant susceptibility.

- **How do plants tolerate root herbivory?** Chapter 5 suggests a possible reallocation of resources to the plant stem upon root herbivory. Testing the hypothesis that this reaction is a tolerance response could be accomplished by comparing maize varieties that differ in their tolerance capacity to *D. virgifera*.

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