

# The Role of Root-Produced Volatile Secondary Metabolites in Mediating Soil Interactions

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## 1. Introduction

Since Darwin's suggestion that natural selection accounts for the diversity of plant morphological and chemical attributes, thousands of papers have been devoted to the ecology and evolution of plant secondary metabolites. Indeed, it is estimated that plants may produce over 200,000 different compounds, the majority of which are classified as secondary metabolites (Pichersky and Gang 2000). The incredible diversity of particular classes of secondary metabolites is stunning. Terpenes, for example, comprise more than 30'000 described compounds (Hartmann 2007). Such incredible diversity of forms can be originated from various enzymes catalyzing the binding of different precursors (Wojciechowski 2003), promiscuity of enzymes (including multiple product and substrate enzyme specificity), changes in cellular compartmentalization patterns (Pichersky and Gang 2000; Bauer *et al.* 2010), or the matrix-like structure of pathways where natural products are formed by elaborate arrays of enzymes, concertedly controlled by the expression of their respective genes (Lewinsohn and Gijzen 2009).

Among early pioneers of plant secondary metabolites as mediators of ecological interactions was Jena botanist Ernst Stahl. Stahl, a fervent follower of Darwin's ideas, suggested some secondary plant metabolites might play protective roles, and thus herbivores may be a primary selecting force for specific biochemical compositions in plants (Stahl 1888). Some seventy years later, Fraenkel's (1959) paper in *Science* resurrected Stahl's pivotal ideas that plants produce an incredible diversity of secondary metabolites for mediating interactions with herbivores, thus providing a *raison d'être* for secondary plant substances. Fraenkel proposed that secondary metabolites have evolved to deter or kill herbivores. Additional coevolutionary frameworks such as Ehrlich and Raven's (1964) seminal paper on

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chemically-mediated coevolution between plants and butterflies, helped to foster a 'new wave' of research focused on plant organic chemistry and evolutionary ecology. Fifty years later, we have finally come to acknowledge the fact that there are ecological and evolutionary reasons for the fact that plants have become true "chemical factories" (Hartmann 2008).

Besides the production of toxic or anti-nutritive secondary metabolites, upon attack, individual plants rely on a matrix-like variety of defense mechanisms, involving physical barriers, and the attraction of "body-guards" to the plant (Schoonhoven, van Loon, and Dicke 2005; Agrawal and Fishbein 2006; Howe and Jander 2008; Karban and Baldwin 1997; Wink 2008; Kessler and Baldwin 2002). Indeed, an additional defence strategy available to plants when 'attacked' by herbivores is to attract mobile enemies of the herbivore. This phenomenon has been referred to as an "indirect plant defence" (Dicke and Sabelis 1988; Turlings, Tumlinson, and Lewis 1990). Natural enemies can be attracted to plants that harbour their host or prey by providing refuges or alternative food sources such as extrafloral nectars (i.e. resource-based indirect defences), or through various visual, chemical and tactile cues (i.e. information-based indirect defences) (Dicke and Baldwin 2010; Heil 2008; Kessler and Heil 2011). Particularly, plant volatile organic compounds (VOCs), which mainly comprise terpenoids, fatty acid derivatives, phenyl propanoids and benzenoids (Dudareva, Pichersky, and Gershenzon 2004) have been the center of intensive studies of plant-herbivore-predator interaction for more than two decades (Dicke and Sabelis 1988; Turlings, Tumlinson, and Lewis 1990). VOCs blends can be complex, comprising hundreds of compounds, some of which are not produced by intact or mechanically damaged plants and others of which are synthesized *de novo* in response to herbivore attack (Mumm and Dicke 2010; Turlings and Wäckers 2004).

Interestingly, the role of secondary metabolites as resistance factors has mainly been studied for aboveground plant parts and their associated communities. With the exception maybe of agricultural pests such as the larvae of various root flies feeding on cabbage, carrot and onion (Johnson and Gregory 2006; Blossey and Hunt-Joshi 2003), and root lesion nematodes *Pratylenchus* spp. (Potter *et al.* 1999), little attention has been paid to the role of secondary metabolites as defences against belowground feeding herbivores (Rasmann *et al.* 2011; van Dam 2009), and how this might shape soil communities (Wenke, Kai, and Piechulla 2010; Bais *et al.* 2006). Roots contain an equally rich variety of plant secondary metabolites as shoots do. Depending on the type of secondary metabolite that is analysed and the ontogenetic stage at which root and shoot levels are compared, the level of secondary plant compounds in the root may be even higher than in the shoot (Kaplan *et al.* 2008; Rasmann and Agrawal 2008). In a compilation of traditional Chinese pharmacopeia, it is surprising to note that more than one-quarter of the preparations are derived from roots and/or rhizomes (Bensky and Gamble 1986). Additionally, the past ten years have seen a surge of work on root exudation and their effect on soil communities. Similarly, as for aboveground herbivores, it has been found that root herbivores use specific chemicals emitted or exuded by the roots as cues to locate their host plant (Johnson and Gregory 2006; Perry and Moens 2006). This and the fact that belowground herbivores can do as much, or sometimes even more, damage to wild plants as aboveground feeding herbivores (Blossey and Hunt-Joshi 2003; Maron 1998; Hunter 2001) indicates that secondary plant metabolites

fulfil similar roles belowground as they do aboveground. The soil surrounding and in close contact with roots of plants is generally termed “rhizosphere” a zone influenced by the plant with high biological activity, influencing many trophic levels (e.g. Bardgett 2005). Plant root exudates have been shown to mediate a wide variety of soil interactions in the rhizosphere (Bais *et al.* 2006). Plants can release up to 20% of the photosynthesized fixed carbon via root exudation (Barber and Martin 1976), affecting both biotic and abiotic soil conditions. Root exudates can chelate inorganic soil contaminants, change rhizosphere pH, and may increase degradation of organic contaminants by microbial metabolism. In addition to soil dwelling organisms, abiotic condition can also spur root exudations. For example, drought stress seem to stimulate the exudation of carbon-based molecules (Henry *et al.* 2007). Mucilage exudation under drought may favor water retention in the soil, and increase rhizosphere stabilization of soil particles (McCully and Boyer 1997). Among the wide variety of exuded molecules ranging from amino acids to complex polysaccharides and proteins, smaller, more volatile compounds have also been shown to directly or indirectly influence soil community of organisms. Thus, through the exudation of plant metabolites, plants may not only protect themselves against root herbivores and pathogens, but contribute to the regulation of the soil microbial community, inhibit growth of neighboring plants, promote the establishment of beneficial symbioses, and regulate soil physic-chemical properties. However, little is known about the biological significance of these metabolites for overall plant physiology and development, as well as their role in mediating soil food webs and interactions (Flores, Vivanco, and Loyola-Vargas 1999).

The role of root produced secondary metabolites in biotic interactions has been reviewed in recent seminal papers (e.g. Badri *et al.* 2009; Bais *et al.* 2001; Flores, Vivanco, and Loyola-Vargas 1999; Wenke, Kai, and Piechulla 2010)}. Here, we will focus our attention to the role of plant VOCs exudation and their effect on various soil organisms including other plants, microorganisms, herbivores and particularly predators of the herbivore. Particularly, volatile terpene production, physiology, emission, and effect on other organisms has been the concern of incredible work on aboveground interactions (Pichersky, Noel, and Dudareva 2006), and a recent trend in research has highlighting their role in belowground interactions and physiology (Degenhardt 2009; Degenhardt *et al.* 2009; Kollner *et al.* 2008). The evolution, detection, and manipulation of such compounds will finally be discussed as a potential tool for biotechnological improvement of resistance against agricultural root feeding pests (Turlings and Ton 2006).

## 2. Root volatile exudates and their effect on soil biota

Plant roots are known to emit an incredible variety of compounds from their roots, which are known to affect interactions between plants and other organism (Flores, Vivanco, and Loyola-Vargas 1999). Here we focus our attention on small lipophilic molecules that are volatile at ambient pressure and temperature. Such molecules have only recently seen a surge of interest as having potential to manipulate soil food-web and dynamics. With a general survey of the available literature, we will below discuss the role of volatile organic compounds (VOCs) in mediating plant-plant, plant-microbe, plant-herbivore, and plant-predator interactions (Fig. 1).

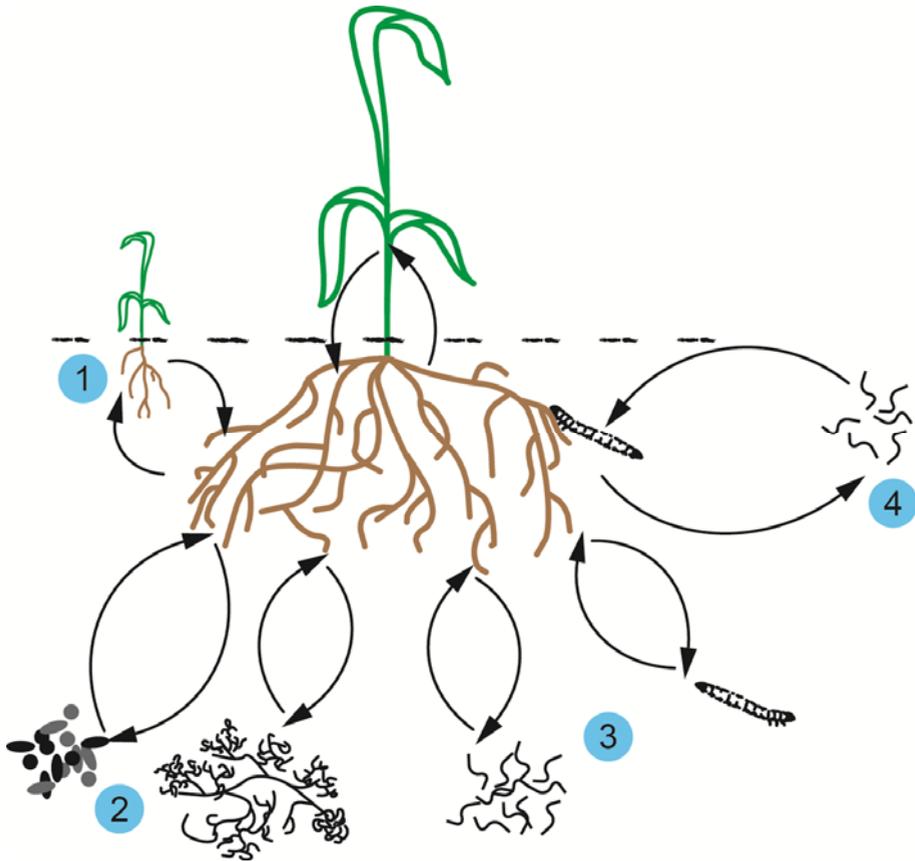


Fig. 1. Diagram showing different soil organisms under possible influence of root emissions of volatile organic compounds (VOCs). From left to right: root emissions of VOCs have been shown to influence 1) neighbouring plants, 2) microbes including bacteria and fungi, 3) herbivores including nematodes and insects, as well as 4) predators of the herbivores such as entomopathogenic nematodes seeking insect larvae for food. In text we highlight each of these interactions. Arrows represent emission of volatile secondary metabolites from roots, as well as animal or micro-organism movement toward roots.

## 2.1 Plant-plant interaction

Root volatiles can mediate allelopathic interaction between neighboring plants. After the first demonstration of seed germination and growth inhibition by plant volatiles (Muller and Muller 1964), several examples of belowground allelopathy have been described and their importance in plant biological invasion is seriously considered. Callaway and Ridenour (2004) proposed the 'Novel Weapon' hypothesis, which states that invading plants may possess novel biochemical weapons that function as unusually powerful allelopathic agents outside their native range, this including VOCs. In support of this, Barney *et al.* (2009) recently demonstrated that monoterpenes volatile emission from the perennial

invasive plant *Artemisia vulgaris* could negatively impact native population of *Solidago canadensis*, hence helping in the invasion of this alien species. Root VOCs are also involved in the interaction between parasitic plants and their host plants (Bouwmeester *et al.* 2003). For instance, Zwanenburg and colleagues (2009) reviewed the bio-activity of the strigolactone family on the germination of parasitic weed germination. The use of such molecules could help in managing parasitic plants such as *Striga spp.* or *Orobancha spp.* population (Zwanenburg *et al.* 2009). Furthermore, root volatiles might also have positive effects on neighboring plants. Indeed, Zwahlen *et al.* (unpublished data) could measure a so-called priming effect of maize damaged roots on neighboring conspecifics. In the laboratory, they have shown that roots in contact with volatiles emitted by an insect induced plant would eventually be faster in responding and emit more volatiles when fed on by the same herbivore (Zwahlen *et al.*, unpublished data). Such examples are still scarce in the literature but may open unsuspected perspective in understanding belowground communities and evolution.

## 2.2 Plants-microbes interaction

Plants are also able to chemically interact with microbe populations using VOCs. Since microbe populations are often limited by carbon availability in soil, root volatiles, especially monoterpenes, might be a relevant carbon source and contribute to the belowground carbon cycle (Owen *et al.* 2007; Zak *et al.* 1994). Some organisms such as *Pseudomonas fluorescens* or *Alcaligenes xylosoxidans* have been able to develop on root monoterpenes as unique source of carbon (Kleinheinz *et al.* 1999). Simpler root VOCs such as carbon dioxide (CO<sub>2</sub>) play an important generic role in plant belowground interactions with other organisms (Johnson and Gregory 2006). However, CO<sub>2</sub> for example, has also been shown to also mediate highly specific interactions. Indeed, Bécard and Piché (1989) could show that carbon dioxide was crucial in the growth of the vesicular-arbuscular fungus *Gigaspora margarita*. The authors showed a synergistic effect of CO<sub>2</sub> and root exudate factors in the hyphal growth of the obligate biotrophic symbiotic fungus. Carbon dioxide and root exudates taken alone had little or no effect, but when mixed together, hyphal growth was significantly stimulated (Bécard and Piché 1989). Further experimentations suggested that in this particular interaction carbon dioxide served as an essential source of carbon for the fungal growth (Bécard and Piché 1989). Since then, numerous plant volatile exudates, mainly belonging to the sesquiterpene lactone family, have been identified potentially mediating plant-microbe interactions. As an example, the strigolactone 5-desoxy-strigol has been isolated from *Lotus japonicus*. This volatile significantly triggered hyphal branching in *G. margarita* (Akiyama, Matsuzaki, and Hayashi 2005).

## 2.3 Plant-herbivore interaction

Two major groups of organisms utilize living roots of plants as their main diet: the insects and the nematodes. A recent survey of the literature indicated that about 17% of all insect families of North America contain species of root feeders (including chewers, sap suckers, and gall makers) (Rasmann and Agrawal 2008). During at least one life stage, a root-feeding phase is common to a variety of species belonging to the orders of crickets (Orthoptera), butterflies (Lepidoptera), flies (Diptera), true bugs (Homoptera), beetles (Coleoptera) and ants or wasps (Hymenoptera) (Brown and Gange 1990). Root-feeding insects play an

important role in both agricultural and natural ecosystems (Blossey and Hunt-Joshi 2003; Wardle *et al.* 2004). Indeed, through physiological and physical changes of roots, belowground herbivores have the potential to shape plant communities (De Deyn *et al.* 2003), belowground microorganism and macroorganism communities (Wardle 2006), as well as aboveground arthropod communities (Bezemer and van Dam 2005).

Volatile organic compounds have been commonly identified as arthropod attractants belowground. A nice review by Wenke *et al.* (2010) highlighted a whole range of compounds that are used by herbivores to locate the food source. Almost ubiquitous signals in the soils are the emissions of CO<sub>2</sub> by roots. A compilation of studies that looked at host attractants for root feeding arthropods underscored CO<sub>2</sub> as a major attractant for at least 20 studies examined (Johnson and Gregory 2006). Detection of CO<sub>2</sub> seems however to be dose-dependent, and soil insect are able to detect very small differences in the concentration of the CO<sub>2</sub> (Johnson and Gregory 2006). Whereas low concentrations are attractive, high concentrations of CO<sub>2</sub> may cause disorientation. Additionally, the orientation of insects within CO<sub>2</sub> gradients could be “masked” by other non-volatile or volatile gustatory and olfactory stimuli exuded by roots (Reinecke, Müller, and Hilker 2008). Besides CO<sub>2</sub>, several disulfides and trisulfides have been identified as potent attractants for the root-feeding larvae of the fly *Delia antiqua* in *Allium cepa* (Carson and Wong 1961). Fatty acids in oaks (*Quercus* sp.) and monoterpenes in carrot (*Daucus carota* ssp. *sativus*) plants triggered the attraction of forest cockchafer larvae, *Melolontha hippocastani* (Weissteiner and Schütz 2006). Volatiles of fresh perennial ryegrass roots attracted larvae of *Costelytra zealandica* (Sutherland and Hillier 1972), and roots of *Medicago sativa* and *Trifolium pratense* attracted larvae of *Sitona hispidulus* (Wolfson 1987).

The second most important group of root feeders encompasses the plant parasitic nematodes. All species are obligate parasites, feeding exclusively on the cytoplasm of living plant cells. The most economically important groups of nematodes are the sedentary endoparasites, which include the genera *Heterodera* and *Globodera* (cyst nematodes) and *Meloidogyne* (root-knot nematodes). Cyst and root-knot nematodes differ in their parasitic life-cycle strategies. Cyst nematodes enter roots and move to the vascular cylinder, before establishing their feeding site; a multinucleate syncytium which results from the breakdown of the cell walls between the initial feeding site cell and its neighboring cells. In contrast to the cyst nematode, the juvenile of the root-knot nematode moves intercellularly after penetrating the root, migrating down the plant cortex towards the root tip. The juveniles then enter the base of the vascular cylinder and migrate up the root, where they establish a permanent feeding site in the differentiation zone of the root by inducing the formation of large, multinucleated cells. Then, the plant cells around the feeding site divide and swell, causing the formation of galls or ‘root knots’ (Williamson and Gleason 2003). A critical step in nematode life-cycle, host searching after hatching or molting into a new life stage, seems to also involve olfactory organs and other sensory organs, which allows sensing chemical gradients in soil (Robinson 2003), plant cell-specific surface determinants, as well as electrical signals (Riga 2004). Plant signals are essential for nematodes to locate hosts and feeding sites, however, besides the general signal furnished by carbon dioxide emissions, what particularly triggers and direct plant parasitic nematode attraction is still largely unexplored. Carbon dioxide was shown to attract *Meloidogyne incognita* (Dusenbery 1987; Pline and Dusenbery 1987), *Ditylenchus*

*dipsaci* (Klinger 1963), and *Caenorhabditis elegans* nematodes (Dusenbery 1980). Aggregation and attraction have been demonstrated in plant parasitic nematodes, for example *M. javanica* and *G. rostochiensis* juveniles respond to tomato (Prot 1980) and potato (Rolfe, Barrett, and Perry 2000) root diffusates, respectively (reviewed in Curtis, Robinson, and Perry 2009). Only very recently, it was shown that phytopathogenic nematodes can also follow gradients of herbivore induced terpene volatile organic compounds. *Tylenchulus semipenetrans* nematodes were more attracted to *Citrus* roots infested by weevil larvae compared to uninfested plants (Ali, Alborn, and Stelinski 2011). A series of terpene compounds were identified as possible attractants for the plant parasitic nematode, including  $\alpha$ -pinene,  $\beta$ -pinene, limonene, geijerene, and pregeijerene (Ali, Alborn, and Stelinski 2011). Interestingly the same compounds were also responsible for the attraction of entomopathogenic nematodes, which function as bodyguards against insect root feeders (Ali, Alborn, and Stelinski 2010, 2011). It seems therefore that a plant parasitic nematode was able to exploit plant signals used for a mutualism.

## 2.4 Belowground tritrophic interactions

Mainly because of methodological constraints, most of the research on plant VOCs released after insect herbivory has so far been conducted mainly aboveground (reviewed in Kessler and Morrell 2010). However, an increasing number of studies are showing that herbivore induced belowground volatiles might also trigger predator attraction in the soil. For example, *Neoseiulus cucumeris* female predatory mites of rust mites (*Aceria tulipae*) responded to belowground volatiles signals of tulip bulbs infested by *A. tulipae* but not to volatiles of untreated or wounded bulbs (Aratchige, Lesna, and Sabelis 2004). Two inspiring papers demonstrated for the first time that unknown emissions of odorous cues were responsible for attracting entomopathogenic nematodes to insect damaged roots (Boff, Zoon, and Smits 2001; van Tol *et al.* 2001). To date, few additional tritrophic interactions implying belowground VOCs signaling have been described both in agricultural systems (Ali, Alborn, and Stelinski 2010, 2011; Rasmann *et al.* 2005) and in wild environment (Rasmann *et al.* 2011). Ali, Alborn, and Stelinski (2010) have demonstrated that citrus roots upon feeding by the root weevil *Diaprepes abbreviatus* emit several terpenes in the surrounding soil. Using belowground olfactometers Ali *et al.* (2010) could show that the entomopathogenic nematode *Steinernema diaprepesi* was significantly more attracted by citrus roots induced by the insect pest larvae than by roots mechanically damaged or by control empty pots. However, Ali *et al.* (2011) recently pointed out that insect induced roots of citrus tree could also attract the phytopathogenic nematode *Tylenchulus semipenetrans* (see above section). Consequently, this may reduce the exploitation of citrus induced VOCs emission in biological control strategies targeting *Diaprepes abbreviatus* where rootstocks are not resistant to *T. semipenetrans*.

Also recently, Rasmann *et al.* (2010) showed that the common milkweed *Asclepias syriaca*, which is generally fed by the specialist root herbivore larvae of the cerambycid beetle *Tetraopes tetraophthalmus*, can release volatiles in the soil. Increased emissions of VOCs after damage were correlated with increased entomopathogenic nematodes *Heterorhabditis bacteriophora* nematodes in lab experiments. Subsequent field trials demonstrated that soil inoculation of nematodes benefitted the plants by restoring their biomass lost due to herbivory to control levels (Rasmann *et al.* 2010). Whether this was correlated with higher

levels of emission in damaged plant was not however assessed. Root emission of *A. syriaca* plants are a very complex mixture of >30 compounds of which only few are described as being in the terpene family (Rasmann *et al.* 2010). Such complex blend, by itself, impedes the assessment of which particular compounds are really responsible for the attraction. A problem that is well known for above-ground systems, where, the emerging picture is that VOCs production in plants is the result of diffuse selection due to multiple players interacting with the plant (Kessler and Heil 2011). As aboveground, the functional role of belowground compounds, individual components or complex blends, would benefit our knowledge of organisms' intimate relationship with the plant, advanced metabolomics and multivariate statistical tools (van Dam and Poppy 2008).

Another example of highly complex volatile blends comes from the roots of cotton (*Gossypium herbaceum*). After feeding by the generalist root feeder larvae of the chrysomelid beetle *Diabrotica balteata*, cotton plants were scored to emit >10 compounds, among which the at least 7 terpenoid volatiles were observed (Rasmann and Turlings 2008). Among all cotton VOCs induced by the chrysomelid larva, the sesquiterpenoid aristolene was discussed as being a good candidate for playing a major role in *H. megidis* nematode attraction. This however remains to be confirmed in future studies (Rasmann and Turlings 2008). In the same study, among corn and cotton, nematode preference was also tested against damaged roots of cowpea (*Vigna unguiculata*) plants. In contrast to corn and cotton, cowpea plants emitted almost undetectable amounts of volatiles, which also resulted in lower nematode attractions (Rasmann and Turlings 2008).

Indeed, by far, corn system, first described by Rasmann *et al.* (2005) is today the best known belowground tritrophic interaction (Fig. 2). Upon attack of the voracious larvae of the western corn rootworm, *Diabrotica virgifera virgifera*, European maize varieties emit in soil the sesquiterpene (*E*)- $\beta$ -caryophyllene (E $\beta$ C) (Kollner *et al.* 2008; Rasmann *et al.* 2005), a highly attractive VOCs to entomopathogenic nematodes *Heterorhabditis megidis* in the laboratory as well as in the field (Hiltpold, Toepfer, *et al.* 2010; Kollner *et al.* 2008; Rasmann *et al.* 2005) (Fig. 2). A series of experiments with various corn lines and synthetic compounds have shown that E $\beta$ C is an ideal compound to diffuse through the complex belowground soil compartment, and that is among the less costly terpenoid that could be travelling within the soil matrix (Hiltpold and Turlings 2008), and that is under selection (Kollner *et al.* 2008; Kollner *et al.* 2004). Its production within the root system appears to be systemic even though the root area upon feeding emits more of the volatile and is more attractive to the *H. megidis* than distal regions (Hiltpold *et al.* 2011).

Recently, Hallem *et al.* (2011) reported positive chemotaxis of the two entomopathogenic nematode *H. bacteriophora* and *Steinernema carpocapsae* to several VOCs such as methyl salicylate, hexanol, heptanol, undecyl acetate or 4,5-dimethylthiazole. Interestingly, they showed that several volatiles repelled the nematodes. Similar effect of VOCs on the behavior of the entomopathogenic nematodes was already observed by (Hiltpold, Toepfer, *et al.* 2010; Rasmann and Turlings 2008) but no volatiles were identified yet. Most of the VOCs involved in belowground tritrophic interactions remain unknown but an increasing effort is invested in this field of research. Understanding more of these complex interactions would not only allow a better understanding of the rhizosphere but could also offer ecologically sound alternatives in pest management in agricultural systems.

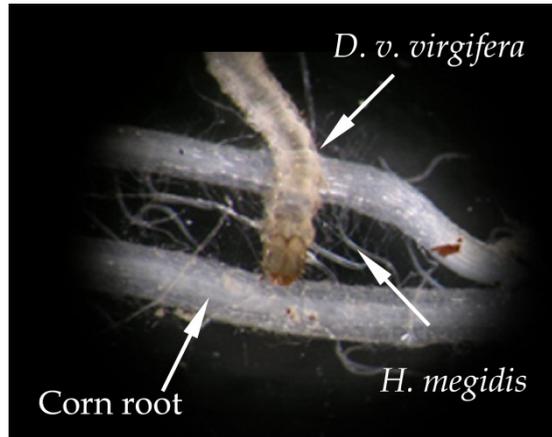


Fig. 2. Root of corn plants, when attacked by the larvae of the western corn rootworm *Diabrotica virgifera virgifera* emits a sesquiterpene volatile organic compound (*E*)- $\beta$ -caryophyllene. This compound was then shown to increase the attractiveness of entomopathogenic nematodes *Heterorhabditis megidis* searching for the host larva (Rasmann *et al.* 2005).

### 3. Evolution of belowground volatile signaling

Similarly to any other adaptive plant traits, belowground emissions of volatiles may evolve if there is heritable variation in their production, which in turn affects fitness. This has barely been studied and little direct evidence indicates that natural selection has shaped root volatile production, and particularly as a defense (direct and indirect) against herbivores.

Nonetheless, root volatile chemistry is certainly heritable. Across 12 genotypes of *A. syriaca* constitutive emissions of total volatiles varied more than four-folds (Rasmann *et al.* 2010). In agricultural systems, corn (*Zea mays*) and *Citrus* plants also showed to display strong genotypic variability in insect-induced volatile production (Rasmann *et al.* 2005; Ali, Alborn, and Stelinski 2010, 2011). Root herbivores have been shown to display strong population level variation in their impact on plants, in turn having strong potential in impacting plant fitness (Maron 1998; Blossey and Hunt-Joshi 2003; Maron and Kauffman 2006). Higher herbivory might therefore lead to higher and more toxic compounds productions. Indeed, plants have been shown to produce some known nematocidal volatile organic compounds in their roots like benzaldehyde, thymol, limonene, neral, geranial, and carvacrol for defending themselves against the attacker in the underground (Bauske *et al.* 1994; Kokalis-Burelle *et al.* 2002; Oka *et al.* 2000; Rohloff 2002). This, however, has not been linked to plant fitness yet.

Allocation to belowground production of VOCs can also be costly for the plant, and classic theory would suggest trade-offs between growth and defense (Herms and Mattson 1992) or between defensive traits (Agrawal, Conner, and Rasmann 2010). Indeed, there is indication that faster growing bitter orange (*Citrus aurantium*) plants produce entomopathogenic nematodes attractive volatiles only after herbivory, whereas the slower growing trifoliolate orange (*Poncirus trifoliata*) produce the same compounds also when undamaged (Ali,

Alborn, and Stelinski 2011), arguing in favor of a cost of producing high amount of VOCs constitutively. Also, in *A. syriaca*, levels of volatile production were negatively correlated with levels of toxic cardenolide production, across 12 genotypes (Rasmann *et al.* 2010), highlighting possible physiological trade-offs between direct and indirect defenses.

In corn, the production of (*E*)- $\beta$ -caryophyllene has been tightly linked to reduction in herbivore performance and in subsequent reduction of root damage (Degenhardt *et al.* 2009) (Fig. 3). However, the actual fitness benefit for the plant has yet to be assessed. Most American cultivars, very likely through human breeding, are lacking the ability to produce this alarm signal (Kollner *et al.* 2008) even though the wild ancestor of maize, Teosinte, is able to synthesize and release this sesquiterpene upon attack of the chrysomelid larvae (Rasmann *et al.* 2005).

#### 4. Methods for detecting volatile emissions in roots

The number of studies focused on belowground multitrophic interactions mediated by root volatile emissions 'pale in comparison' to the amount of research focused on their aboveground counterparts. This is largely due to technical difficulties associated with dynamics of the soil ecosystem. Soil is an opaque, tri-phasic medium making the analysis of individual factors and their interactions difficult. Most research has been based on *in vitro* analysis of individual factors.

Researchers hoping to study factors associated with roots signals are often directed to the study of root diffusates, root leachates, and or root exudates. Such terms are used interchangeably and can often be misleading. Leachate refers to a method of obtaining an extract from the roots, more than it does to the solution itself. Diffusate is used to convey non-volatile substances diffusing through the soil and establishing a gradient. Exudate is most often restricted to liquids that gradually 'ooze' from its source, but can be applied to volatiles as well. Approaches to evaluate roots volatiles have only recently been developed and applied in contexts of chemical and evolutionary ecology.

In 2005, Rasmann *et al.* evaluated the indirect volatile defences of maize roots using solid phase microextraction (SPME). SPME is a method of sampling volatiles without the use of solvents. In short, an adsorbent-coated fused silica fibre with properties similar to a gas chromatography column can collect volatile compound from the headspace of a sample. The volatile compounds once fixed to the SPME fibre can then be thermally desorbed in an injection port of a gas chromatograph and further analysed and/or identified when coupled with known standards or libraries of mass spectroscopy. In order to sample the effects root herbivory had on the plant produced VOCs Rasmann *et al.* (2005) crushed flash-frozen roots, either fed-upon or non-fed-upon into a fine powder. This powder was then exposed to the SPME fibre. This allowed for the volatiles that had accumulated in either treatment to be sampled and compared with GC-MS. SPME is a rapid and simple extraction method that doesn't require the use of solvents. Detection limits can reach parts per trillion (ppt) levels for certain compounds (Pawliszyn, 2009). Although this method is effective it is a destructive method of sampling root material. The plant and herbivore must be separated volatiles from this interaction can only be examined after harvesting and crushing the plant tissues.

Recently, Ali *et al.* (2010) was able to non-destructively sample belowground herbivore induced volatiles from citrus roots using a flow-through dynamic sampling technique

coupled with an adsorbent traps. Volatiles can be collected and extracted by elution of an adsorbent with low boiling point solvents. Adsorbent traps are typically made of glass tubes filled with the granulated adsorbent, held in place by stainless steel mesh, glass wool plugs, or Teflon fitted rings (in pre-made filters, ARS, Gainesville, FL, USA). The most common adsorbents are Hayesep Q® (HayeSeparations Inc., Bandera, TX, USA, the current alternative to Super Q® by Alltech Deerfield, IL, USA), and activated charcoal. By connecting the adsorbent trap to a vacuum pump and pulling air through glass chambers containing intact citrus plants either with or without feeding larvae Ali *et al.* (2010) was able to sample volatiles associated with belowground herbivory non-destructively and *in situ*. The volatiles collected on this trap are rinsed using solvent and analysed with GC-MS. This method allows for the sample to be retained in a solvent, which can be analysed more than once. The solvents containing root volatiles were also tested in sand-filled two-choice bioassays chambers. In this manner, Ali, Alborn, and Stelinski (2010, 2011) found evidence for entomopathogenic nematode attraction to volatiles from infested citrus roots.

Both techniques are effective and informative in different ways. The non-destructive sampling techniques are useful in evaluating belowground interactions *in situ* and may potentially prove useful in additional contexts. However, the properties of the surrounding soil may interfere and make resolution difficult with the potential for significant background. In this way SPME eliminates such background, but can introduce complications from tissue maceration where enzymes or oxidation can rapidly change the chemical profile and might not accurately represent the blend released from intact living organisms (Tollsten and Bergstrom 1988; Heath & Manukian 1992). Perhaps a combination of techniques and refinements of approaches will produce the best resolution for the dynamics of an individual system and the factors of concern (Rasmann, 2010).

## 5. Manipulation of root volatiles for agricultural improvement

The substantial advances in research on molecular mechanisms and ecological signaling of insect herbivore induced VOCs open promising prospects of manipulating the release of these compounds in order to enhance crop protection. Encouraging examples from laboratory and field experiments support this approach to develop novel ecologically sound crop protection strategies.

Manipulating the plant emitting VOCs appears as a first straightforward approach. Aboveground, for instance, Thaler and colleagues (1999) applied jasmonic acid on tomato plants in an experimental field. This treatment resulted in the emission of typical VOC blends that the plant would have produced upon herbivore attack and in a lepidopteran parasitism rate on plant treated with the phytohormone than on control plants (Thaler 1999). Supporting this plant manipulation approach, Rostàs and Turlings (2008) obtained a significant systemic resistance by treating maize plants with salicylic acid-mimic benzo-(1,2,3)-thiadiazole-7-carbothioic acid (S)-methyl ester resulting in an increased resistance against the fungal phytopathogen *Setosphaeria turcica*. Upon caterpillar attack, the treated plants were more attractive for the parasitoid wasp *Microplitis rufiventris* than plants damaged by the insect larvae only. Control experiments showed that these results were due to the plant-mediated effect rather than to the chemical treatment itself (Rostàs and Eggert 2008). So far there is no published example of such manipulation in the belowground compartment. Yet, preliminary results show that root system treated with

alginate, a polysaccharide extracted from brown algae, would emit faster and more VOCs than control plant upon belowground herbivory (Hiltpold, unpublished data). Ali *et al.* (in review) has recently increased mortality of root pests in the field by enhancing host location of naturally occurring entomopathogenic nematodes in Citrus and Blueberry crops with the application of the citrus root volatile, 1, 5-dimethylcyclodeca-1, 5, 7-triene (pregeijerene).

When the induced VOCs blend is clearly identified, it could be considered to genetically manipulate the plant in order to either (I) making the plant more attractive for beneficial predators or parasitoids or (II) to restore a new phenotype that was lost due to natural or human selection that is again attractive for the predators or parasitoids. (I) Again first examples of this approach are aboveground. Indeed, it has been first demonstrated in the model plant *Arabidopsis thaliana* in which a linalool/nerolidol synthase gene FaNES1 from strawberry was introduced. This resulted in the constitutive release of (3S)-(E)-nerolidol rendering the plants attractive to predatory mites *Phytoseiulus persimilis* (Kappers *et al.* 2005). Later, *Arabidopsis thaliana* was transformed with a TPS10 gene, coding for sesquiterpenes typically present in the blends emitted by plant upon caterpillar attacks (Schnee *et al.* 2006). The engineered plant was attractive to the parasitoid wasp *Cotesia marginiventris*, but only after they have learned to associate the HIPV blend to the presence of their hosts. (II) More recently, the terpene synthase gene TPS23 has been identified in maize (Kollner *et al.* 2008). TPS23 is responsible for the synthesis of E $\beta$ C, a key attractant for some entomopathogenic nematodes (Rasmann *et al.* 2005). Most of the European maize varieties and Teosinte produce this sesquiterpene whereas American varieties doesn't (Kollner *et al.* 2008; Rasmann *et al.* 2005), indicating a shift in the gene activity through breeding selection (Kollner *et al.* 2008). In 2009, Degenhardt and colleagues were able to restore the ability of maize to recruit entomopathogenic nematodes by inserting a TPS23 gene from *Origanum vulgare* into a non-producing maize line (Degenhardt *et al.* 2009). In the field, the transformed maize line was significantly more attractive for the entomopathogenic nematode *Heterorhabditis megidis* compared to the wild type leading to a better protection for transformed plants (Fig. 3). Transformed plant received far less damage by the root pest *D. v. virgifera* and the transformation significantly reduced the beetle emergence, and overall this restored indirect defense resulted from the constitutive emission of E $\beta$ C in the soil (Degenhardt *et al.* 2009). This was the first demonstration in the field that plant genotype engineering could enhance biological control. It has to be noticed that the variability in the emission of herbivore-induced volatiles in maize remains high (Degen *et al.* 2004) and therefore, because genetic modifications are still controversial, it should also be possible to incorporate highly attractive volatile compounds into new varieties using classical breeding programs.

In an inundative biological control strategy, the manipulation of the biological control agent can be considered as an option to enhance pest management (Hoy 1976). A classic idea that was put into practice with some relative success (Beckendorf and Hoy 1985; Hoy 2000). In that perspective, entomopathogenic nematodes appear as good candidates. Several studies have succeeded in selecting beneficial traits such as host finding (Gaugler and Campbell 1991; Gaugler, Campbell, and McGuire 1989), virulence (Peters and Ehlers 1998; Tomalak 1994) and tolerance to temperature (Ehlers *et al.* 2005; Grewal, Gaugler, and Wang 1996; Griffin and Downes 1994) or desiccation (Strauch *et al.* 2004).

For specific entomopathogenic nematodes, the knowledge of key attractants is now available (Hiltpold, Toepfer, *et al.* 2010; Rasmann *et al.* 2005). This prompted Hiltpold *et al.* (2010) to evaluate whether selection for enhanced responsiveness to the crucial root signal E $\beta$ C could improve the efficiency of nematodes in controlling the larvae of the chrysomelid beetle *D. v. virgifera*. Using belowground six-arm olfactometers, a strain of the nematode *Heterorhabditis bacteriophora* was successfully selected. Originally, this nematode was not responding to E $\beta$ C (Hiltpold, Baroni, *et al.* 2010) even though its effectiveness in controlling WCR larvae is high (Kurzt *et al.* 2009). The selected strain responded much better to E $\beta$ C in laboratory experiments and was able to significantly control better the pest in the field, in presence of the belowground signal (Hiltpold, Baroni, *et al.* 2010). Because of the strong selective pressure, there were minor trade-offs in the infectiousness of the selected strain. Yet, the higher responsiveness to the HIPV overbalanced these weaknesses (Hiltpold, Baroni, *et al.* 2010). The establishment and the persistence in the field were not influenced by the selection process (Hiltpold, Baroni, *et al.* 2010). These results reflect the great potential of selecting beneficial organisms for a better and faster response, resulting in higher infection rates. Even though some constraints, such as knowledge of key compound/blends, and the laborious selection process, selecting for specific nematode strains could be coupled with selection of more attractive plant genotypes, making biological control of insect pests a success.

Beside from exploiting induced VOCs emitted while and after insect pest attack, luring the foraging insect with volatiles would eventually prevent any damage on the plant. Below ground examples of insect chemical luring are scarce. However, some promising attempts to control belowground pests with such a strategy have been published, notably in maize crops. Since Strnad and Bergman (1986) first reported that *D. v. virgifera* larvae were attracted to CO<sub>2</sub>, disruption of pest foraging behavior with CO<sub>2</sub> has been demonstrated to have high potential method against this maize pest both in the laboratory and the field (Bernklau, Fromm, and Bjostad 2004). Beside this wide spread plant metabolite, more specific compounds, inducing a positive behavior of the beetle larvae, have been identified (Bernklau and Bjostad 2008; Bernklau *et al.* 2009; Bjostad and Hibbard 1992; Hibbard, Bernklau, and Bjostad 1994). Indeed, the authors described a combination of several sugars and fatty acids emitted by maize that serve as feeding stimulants for the *D. v. virgifera* neonate larvae (Bernklau and Bjostad 2008; Bernklau *et al.* 2009; Bjostad and Hibbard 1992; Hibbard, Bernklau, and Bjostad 1994). Recently, Bernklau and colleagues (2011) showed that *D. v. virgifera* larvae were feeding more and staying longer on pesticide-treated filter papers when the right blend of feeding stimulant chemicals was added compare to filter papers only treated with pesticides. The concentration of thiamethoxam required for equivalent kill without feeding stimulants was reduced 100,000 fold (Bernklau, Bjostad, and Hibbard 2011). Exploiting the same idea, capsules based on algae polymer and coated with *D. v. virgifera* feeding stimulants have been produced. When compared to maize root systems, the capsules were as much attractive for the pest larvae. These capsules could contain entomopathogenic nematodes and would then ease their application in the field as well as eventually lure *D. v. virgifera* larvae (Hiltpold *et al.*, unpublished data). These belowground examples support the feasibility of such manipulative approach in the soil compartment even though more research and experiments are needed to achieve a good control of the targeted pest in the field.

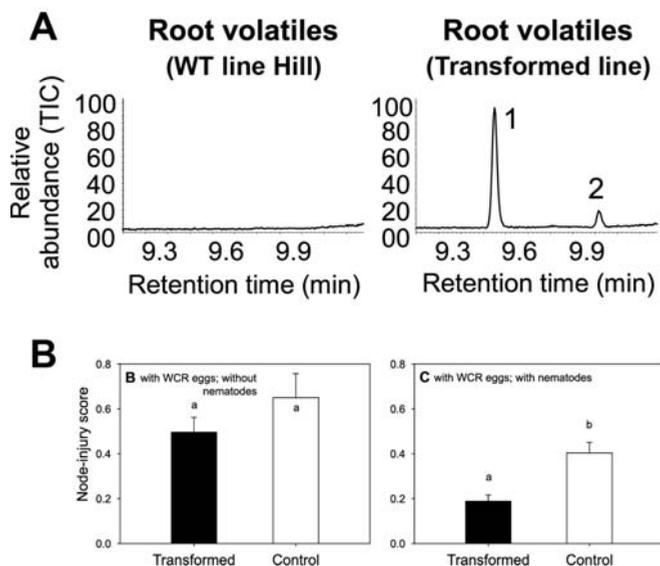


Fig. 3. Introduction of TPS23 genes into American lines of corn increases (*E*)- $\beta$ -caryophyllene production (A) and better protect corn plants (B). Figure (A) shows a typical chromatogram obtained for the volatiles emitted by roots of the hybrid variety Hill line alongside a chromatogram for one of the transformed lines. Peak 1 is (*E*)- $\beta$ -caryophyllene and peak 2 is  $\alpha$ -humulene, a side-product of (*E*)- $\beta$ -caryophyllene synthase. Figure (B) shows that in plants receiving WCR eggs and *H. megidis* nematodes, roots from transformed plants are significantly less damaged than roots from control lines. Modified from Degenhardt *et al.* (2009).

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