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Solanoeclepin A

Characterization of a rhizosphere communication molecule in tomato and potato

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Chapter 1

General introduction

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Preface

Through the domestication of plants and animals, humans laid the foundation for our modern-day society (Gupta 2004). The concomitant development of agricultural practices, intended to increase field productivity, brought about profound alterations in the soil structure and soil biodiversity with detrimental effects (Postma-Blaauw et al. 2010). For example, agricultural practices have been shown to negatively affect biodiversity, in the soil food web (de Vries et al. 2013; Tsiafouli et al. 2015). To bring this development to a halt, in 2015 the United Nations formulated a Sustainable Development Goal to achieve improved food security with better product quality, but with less influence on the soil ecosystem (Kanter et al. 2018; Stephens et al. 2018; Dias et al. 2019). In order to achieve this goal, a better understanding of the soil ecosystem is needed. Soil is a highly complex entity in which a multitude of interactions between organisms and the soil matrix takes place. All these factors and interactions together constitute the soil ecosystem, the functioning of which determines the availability to plants of mineral nutrients and other abiotic resources, as well as the presence of biotic agents, all of which potentially influence plant fitness.

Plants are not just passengers in all these processes, but actively shape their environment using chemical communication. In the past years, more and more attention is being paid to the interaction of plants with their belowground environment, mainly focussing on the narrow zone of soil that surrounds the plant root and that is called the rhizosphere (Venturi and Keel 2016). Despite this increasing interest in the rhizosphere and the role of metabolites in rhizosphere communication, especially those with potential benefits for agriculture, very little is still known about their role and the regulation of their biosynthesis in plants.

In this introduction, I emphasize the functional role of the chemical compounds that are secreted by plants into this rhizosphere and that affect the physicochemical properties of this root zone or act as chemical signals for other organisms. I discuss the importance of these chemical signals for shaping the soil ecosystem, together with the possibilities to use these chemicals, as leads for the development of new agrochemicals and/or to develop Integrated Pest Management (IPM) strategies, both of which can be used to achieve more sustainable agriculture, is discussed. Finally, I focus my attention on one of these compounds, solanoelepin A, as an example of a chemical signal found in the root exudate of solanaceous species, for which much is still unclear about its role in the rhizosphere. Solanoelepin A has been reported as the major hatching factor for the parasitic Potato Cyst Nematode (PCN) (Schenk et al. 1999). One would assume it also has a beneficial role but if so, that is still unknown. Taking solanoelepin A as a case-study, I want to stress the importance of analytical chemistry in combination with molecular biology techniques as powerful tools to study this molecule and elucidate its biosynthetic pathway in an attempt to fully uncover its full biological importance in the rhizosphere.

2 The soil ecosystem and its importance for agriculture

Soils are complex entities resulting from the interaction of many factors: climate, organisms in the soil, soil matrix and topography (Coleman et al. 2017). Soils are providing the substrate for nature as well as agriculture and in both cases the soil represents a soil ecosystem. This soil ecosystem is not just a mixture of living and non-living matter, but also entails the complex interactions between these components. A better understanding of the relationships between this living and non-living matter is key in grasping the consequences of changes in the sometimes delicate balance, as often occurs in agriculture (Postma-Blaauw et al. 2010).

The soil ecosystem is particularly important for agriculture since it contributes to the decomposition of organic matter and litter, which plays a major role in resource recycling (Frouz 2018), nutrient retention and uptake by the plant, water regulation and biogeochemical cycling (Osler and Sommerkorn 2007; Falkowski et al. 2008). All these processes together potentially enrich the soil with mineral nutrients and redistribute the organic matter that comes from plant residues (Jiao et al. 2018), increasing soil health and fertility and thus improving crop yield. In addition, soil organisms influence many aspects of the plant, from belowground to aboveground. For example, root microbiota can help plants with the uptake of micro- and macronutrients necessary for their growth, such as nitrogen (Hamilton and Frank 2001; Cardoso et al. 2018), and phosphorus (Yoneyama et al. 2007) and thus preventing their loss through greenhouse gas emission and leaching or immobilization, respectively (Wagg et al. 2014). Root associated micro-organisms can also impart resistance to pathogens (Pieterse et al. 2014) or act as rhizoremediators, phytostimulators and stress controllers, as will be discussed below (Shoresh et al. 2010; Antoun 2013).

Although these soil organisms potentially represent a powerful resource to improve the agricultural soil ecosystem and crop yield, agricultural practices such as tillage, crop rotation, fertilizer and pesticide application and monoculture profoundly affect the soil fauna and microbial community composition, which usually results in a loss of biodiversity and/or decrease in biomass (Tsiafouli et al. 2015; Le Guillou et al. 2018). The latter is mainly caused by tillage as it alters soil microhabitats and interrupts the life cycle of organisms with a long life span and larger body size such as earthworms, mites and enchytraeids (Tsiafouli et al. 2015). Le Guillou et al. (2018) confirm that tillage also has a large effect on bacterial and fungal diversity and evenness and destroys mycorrhizal hyphal networks, which results in decreased phosphorous uptake by and availability to the plant (Köhl et al. 2014).

The impact of the application of mineral fertilizers is complex. Geisseler and Scow (2014) showed that as a result of the higher plant productivity there is more organic material secreted into the soil in the form of root exudates and residues, which stimulates growth of the microbial community that uses this pool of carbon as the main resource. On the other hand, fertilizers, in particular urea and ammonium, decrease the soil pH, which negatively affects microorganisms and reduces the solubility of other nutrients. As far as pesticides are concerned, Asad et al. (2017) suggest that in most cases herbicides increase organic acid exudation, which results in the attraction of acidophilic microorganism and

stimulates denitrification. According to Srinivasulu and Ortiz (2017), pesticides, in low concentrations, stimulate bacterial populations, but are detrimental in combination with fungicides at higher doses. As underlined by Satapute et al. (2019) the major concern about pesticides is their accumulation in the field, which influences not only soil organisms, but directly also soil properties such as pH and nutrient content.

Some of the agricultural practices mentioned here are fundamental farming principles that are applied to improve crop productivity and yield. On the other hand, they are among the factors that seem to profoundly affect organisms and processes that are critical for the long-term stability of the soil ecosystem. It will be of fundamental importance to better understand these complex relationships such that the soil ecosystem can be more optimally geared for a sustainable productive agriculture. Critical here is also to understand the interaction between plants and the soil and the organisms living in it through root exuded chemical compounds and how agriculture can benefit from this.

3 How do plants interact with the soil ecosystem

Lyon and Wilson in 1921 were the first to show that crop such as maize, oats, peas and broad bean grown in sterile nutrient solution release organic nitrogen into the medium. These authors focused only on nitrogen-containing molecules, but more recent studies revealed that root exudates contain also carbon-based and organic as well as inorganic compounds (Bais et al. 2006). The latter includes ions, CO₂, protons, H₂, free O₂ and water (Uren 2000), while organic compounds represent the majority of the molecules produced and secreted by roots. They are released into the soil in a process called rhizodeposition and collectively called rhizodeposits and include enzymes, amino acids, organic acids, sugars, proteins, mucilage and secondary metabolites such as: phenolics (mainly benzenoids, flavonols, lignins and anthocyanins), isoprenoids (sterols and terpenoids), alkaloids and sulfur-containing compounds like glucosinolates (Badri and Vivanco 2009).

Lyon and Wilson (1921), linked the presence of organic nitrogen in the growing medium to the sloughing off of root cap cells, since there was no direct evidence that these compounds were released in any other way. However, rhizodeposits are not only derived from the release of dead root cells, but are also actively secreted by the plant itself. Annual crops translocate about 21% of the total fixed carbon to the roots, and grasses about 33% (Pausch and Kuzyakov 2018). From the carbon transported belowground in annual crops and grasses, 3 and 5%, respectively, are released into the soil through rhizodeposition, while 8 and 12% of assimilated C is lost as root-derived CO₂ and the remaining percentage is allocated to the root system itself (Pausch and Kuzyakov 2018). Since plants are investing a substantial amount of carbon into the production and secretion of these metabolites, an important question is: what the fitness benefit is of this process?

Root exudates have both a chemical and biological effect on the surrounding environment, with roles in nutrient acquisition (Marschner et al. 2011) and the interaction with soil organisms (Fig. 1). The biological effect of the root exudate is the chemical signaling between plants and the other organisms living in the soil. Plants can alter their rhizosphere

biome, recruiting protective organisms upon pathogen or insect attack (Berendsen et al. 2012) or attracting useful microbes and fungi to improve nutrient uptake, as will be discussed in more detail below. The chemical effect of root exudates is linked to the complex, physico-chemical characteristics and nutrient availability of soils that affect plant physiology (Philippot et al. 2013). Plants can alter the rhizosphere environment by modifying soil properties such as pH, texture and soil structure in order to improve the physical conditions for root penetration, and nutrient and water uptake (Read et al. 2003; Naveed et al. 2017). For instance, as shown by Read et al. (2003) plants release phospholipids as surfactants that reduce the root tip surface tension and facilitate root growth through the soil. Organic acids and sugars that are present in root exudates influence soil texture by increasing soil dispersion and aggregation, respectively (Naveed et al. 2017). Soil dispersion might increase nutrient release by soil particles, while aggregation might result in a more stable structure around the roots.

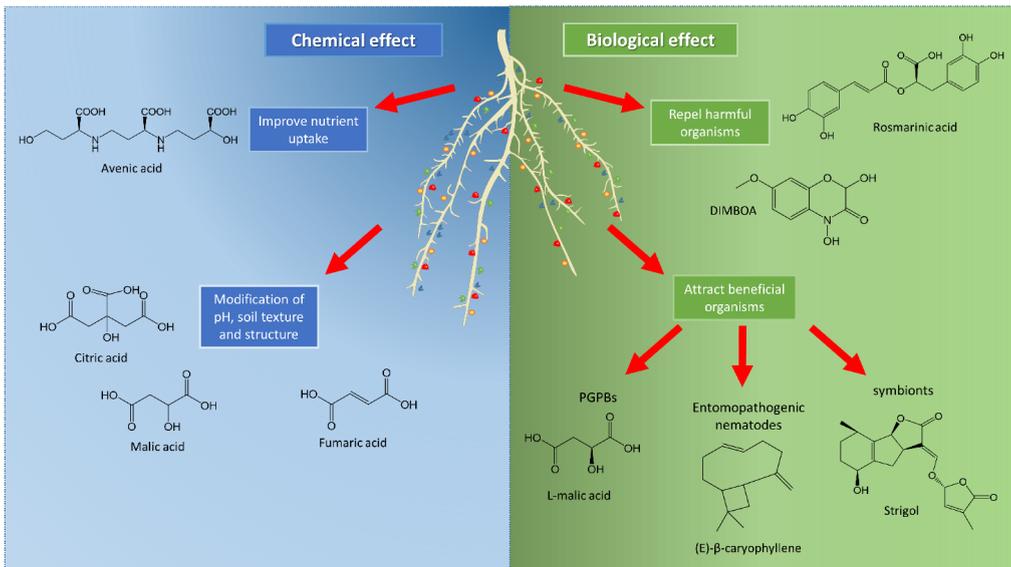


Fig. 1 Schematic representation of the different roles of root exudate compounds (represented by differently colored shapes around the roots). Root exudate compounds are divided into two categories: on the left molecules that exert chemical effects by improving nutrient uptake or modifying soil properties, on the right molecules that exert a biological effect, repelling harmful organisms or attracting beneficial ones.

4 Roles played by plant signaling molecules in the soil ecosystem

4.1 Nutrient acquisition (pH and phytosiderophores)

Phosphorus is an essential element for plant growth. It is part of membrane lipids and phosphate containing molecules such as ATP and NADPH and nucleic acid building blocks (Amtmann and Armengaud 2009). Phosphorus availability is mainly influenced by soil pH: in acidic soils phosphorus reacts with iron and aluminum, while in alkaline or calcareous soil it reacts with calcium, making this element inaccessible for plant uptake (Hinsinger 2001). Agriculture is relying on a non-renewable source of this nutrient that will soon be depleted: rock phosphate (Cordell et al. 2009). Another important element for plant growth is iron, which is a co-factor of many enzymes and is involved in chlorophyll biosynthesis. Despite the abundance of iron in the soil, it is not readily bioavailable due to its low solubility, especially in calcareous soil (Hider and Kong 2010). Some secondary metabolites and organic acids released in the root exudates by plants are able to solubilize these two nutrients or to modify the soil pH in order to increase their solubility and mobility (Dakora and Phillips 2002). For example organic acid anions such as citrate, malate and fumarate are released especially by dicots and non-graminaceous monocots in order to acidify the rhizosphere making Fe, together with P and other micronutrients more available (Jones et al. 1996; Dakora and Phillips 2002). Plants can also chelate and solubilize these nutrients with other strategies, for example by releasing phenolic compounds or using phytosiderophores (Dakora and Phillips 2002; Marschner et al. 2011). This last strategy is mostly used by graminaceous plants for Fe uptake, but also by many bacteria that are able to solubilize and chelate Fe, therefore competing with plants (Marschner et al. 2011). On the other hand, plants assimilate iron also from bacterial siderophores (Ahemad and Kibret 2014), and according to Jin et al. (2010) under iron-deficiency, red clover alters its root microbial community by exuding phenolic compounds in order to promote colonization by siderophore-secreting bacteria.

4.2 Symbiosis

For the uptake of the macronutrients phosphorous and nitrogen, plants also cooperate – and communicate - with microorganisms, such as arbuscular mycorrhizal fungi (AMF) and Rhizobia. An interesting example of the communication between plants and these micro-organisms is presented by the strigolactones (SLs). SLs are powerful inducers of germination of parasitic plants of the Orobanchaceae, which then infect their host to obtain assimilates and nutrients (Al-Babili and Bouwmeester 2015; Mostofa et al. 2018). SLs were later also shown to be plant hormones regulating processes such as shoot branching and root architecture (Al-Babili and Bouwmeester 2015). In 2005, Akiyama et al. applied root exudate fractions of *Lotus japonicus* to the AM fungus *G. margarita*, and showed that the most active fraction inducing hyphal branching contained the SL, 5-deoxystrigol. Intriguingly, under phosphate deficiency, plant species such as tomato, maize and sorghum, produce more SLs. SLs induce hyphal branching in germinating spores of AMF (Akiyama et al. 2005a), which facilitates the initiation of a symbiosis – with over 80% of all land plants - in which fixed carbon from the plant is exchanged for

minerals absorbed from the soil by the fungus (Al-Babili and Bouwmeester 2015). Despite the essential role of SLs in the initiation of the symbiosis, the mechanisms underlying the perception by the fungus has not been elucidated yet and the fungal receptor(s) are still unknown.

The second well-studied case is that of the symbiosis between legumes and the gram-negative soil bacteria defined as 'Rhizobia' (Liu and Murray 2016) that can colonise the roots of legumes and induce the formation of specific structures, called root nodules (Sachs et al. 2018). Within the nodules, bacteria differentiate into bacteroids and through nitrogen fixation, convert atmospheric nitrogen (N_2) into a reduced form and make it available to the host (Masson-Boivin and Sachs 2018). Flavonoids that are secreted by legumes were shown to act as chemo-attractants for Rhizobia and induce the first step in the process required to establish the symbiosis: the induction of the secretion of the bacterial nodulation (nod) factors (Phillips and Tsai 1992). For example, Peters et al. found that 3',4',5,7-tetrahydroxyflavone (luteolin) secreted by *Medicago sativa* induces *nodABC* expression in *Rhizobium meliloti* and that this is required for the induction of the early host responses, cortical cell division and root hair curling (Peters et al. 1986). This symbiosis occurs under low nitrogen conditions and is stimulated under those conditions by the (enhanced) production and secretion of specific flavonoids by the host root (Coronado et al. 1995). Just as for the strigolactones, the flavonoids are not only perceived by the beneficial Rhizobia but are also used as cues by pathogens. For example, in 1992 Morris & Ward, discovered that the isoflavones daidzeina and genistein, exuded by soybean roots are a chemo-attractant to the zoospores of *Phytophthora sojae*, a fungal pathogen (Morris and Ward 1992).

4.3 Molecules attracting beneficial organisms

In addition to beneficial, symbiotic, organisms such as AMF and Rhizobia, a growing body of literature shows that also other micro-organisms, collectively called Plant Growth Promoting Bacteria (PGPBs), can play important roles in the growth, development and survival of plants (Lugtenberg and Kamilova 2009). These PGPBs (but also certain non-AM fungi) help plants to overcome abiotic stresses, such as salinity (Rojas-Tapias et al. 2012; Ali et al. 2014) and drought (Rolli et al. 2015), and increase growth and plant fitness in soils contaminated with heavy metals (Ma et al. 2011; Singh et al. 2018). Despite the fact that many genera of bacteria have been identified as PGPBs, and their roles for the whole plant well studied, for most of these plant-microbe relationships it is still unclear if and how plants recruit them and, if so, if and which compounds in the root exudates are responsible for this.

Already in 1984, Harwood et al. (Harwood et al. 1984) showed that aromatic acids are chemo-attractants for *Pseudomonas putida*, one of the most important and versatile PGPBs. Some strains of this PGPB produce the plant hormone indoleacetic acid (IAA), which enhances the development of the root system in, for example, canola seedlings (Patten and Glick 2002), helping the plant to get better access to soil nutrients (Glick 2012). Maize is another crop that attracts this PGPB. Neal et al. (2012) demonstrated that

the benzoxazinoid, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) producing wild-type maize attracted significantly higher numbers of *P. putida* than the DIMBOA-deficient *bx1* mutant (Neal et al. 2012). Sometimes bacteria are also attracted by a blend of chemo-attractants secreted by plants, for example, the PGPBs *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*, are attracted by amino acids and organic acids produced by cucumber and tomato roots (de Weert et al. 2002; Feng et al. 2018).

These examples suggest rather low specificity of the attraction, but it has been demonstrated that the root exudate composition of plants changes upon pathogen attack and that this results in the attraction of beneficial bacteria. For example, an infection with the foliar pathogen *Pseudomonas syringae* pv *tomato*, resulted in the higher production and secretion of L-malic acid in *Arabidopsis* root exudates, which resulted in turn in the recruitment of the PGPB *Bacillus subtilis* (Rudrappa et al. 2008). The interaction with this beneficial bacterium triggers Induced Systemic Resistance (IRS) as well as the promotion of plant growth, thus giving protection against the infection by *P. syringae* (Rudrappa et al. 2008).

PGPBs are not the only beneficial micro-organisms present in the soil that can be attracted by root exudates and can establish a successful relation with plants. Biocontrol fungi (BCF) such as *Trichoderma* spp. are also an important biological factor in the control of plant diseases. *Trichoderma* spp. interact directly with soil pathogens using efficient mechanisms such as mycoparasitism, antibiotic production and competition for nutrients (Shoresh et al. 2010). A recent study from Lombardi et al. (2018) showed that tomato plants exposed to abiotic and biotic stress attracted the germ tubes of *Trichoderma* spp. Although the attractants were not identified, the authors suggested that peroxidases and oxylipins may be involved.

4.4 Molecules repelling harmful organisms

Through their root system plants interact not only with beneficial organisms, but also with pests and pathogens. For a soil-borne pathogen, roots are the first entry point into the plant and for soil dwelling insects and arthropods roots represent an important food source. Through root exudates plants can release defensive compound, either upon attack (induced defense; phytoalexins), or constitutively (phytoanticipins) (Vanetten et al. 2019). These molecules act as a first line of defense against pathogen infection (Baetz and Martinoia 2014). Some of these molecules change their activity in the rhizosphere when they are modified by other organisms. Benzoxazinoids, for example, attract *Pseudomonas putida* to maize, and are biodegraded by soil microorganisms, to phenoxazinones which have antifungal and antibacterial properties (Neal et al. 2012).

Phenolics and terpenoids often have strong antimicrobial and anti-herbivory properties (Baetz and Martinoia 2014). Rosmarinic acid, for example, which is secreted by roots of sweet basil (*Ocimum basilicum*) challenged by the pathogenic fungus *Pythium ultimum* showed antimicrobial activity against rhizosphere microorganisms (Bais et al. 2002). Lanoue et al. (2010) showed that barley, when attacked by *Fusarium* produces antifungal

phenolic compounds, such as *t*-cinnamic acid which is biosynthesized *de novo* and released by the plant root (Lanoue et al. 2010). *Arabidopsis*, instead, constitutively produces the diterpenoid rhizathalene A, a semi-volatile phytoanticipin that is involved in defense against soil herbivores (Vaughan et al. 2013). The mechanisms underlying the antifungal and antimicrobial activity of such compounds are largely unknown, with some exceptions. Rosmarinic acid, for example, disrupts the fungal cell wall integrity and thus prevents fungal growth. In bacteria, such as *Pseudomonas aeruginosa*, the same compound leads to the proliferation of cell division causing DNA condensation and altered morphology (Bais et al. 2002).

Another strategy used by plants to control the rhizosphere microbiome, in particular the bacteria growing around their root system, is producing molecules that can mimic bacterial communication molecules called Quorum Sensing (QS) signals. QS is based on the synthesis, and detection of and response to bacterial QS signals such as the homoserine lactones (HSLs), that accumulate in the environment as the bacterial population increases (Corral-Lugo et al. 2016a, b). The QS response triggers the expression of genes, a.o. involved in biofilm formation and virulence. In *Agrobacterium tumefaciens*, for example, the transfer of the tumor-inducing plasmid into a plant cell is activated by HSLs (De Kievit and Iglewski 2000). Sensing of and interfering with the QS signaling molecules of pathogenic bacteria potentially provide a fitness benefit to plants. So far, few QS mimicking compounds have been discovered and characterized. Corral-Lugo et al. (2016a) discovered that rosmarinic acid is not only produced after microbial infection as antimicrobial, as already discussed above (Bais et al. 2002), but also interferes with the QS induced activation of virulence factors in *P. aeruginosa*. Binding studies showed that rosmarinic acid binds with high affinity to the *P. aeruginosa* RhlR regulator activating the signaling cascade that normally is activated by the bacterial *N*-butanoyl-homoserine lactone (C4-HLS), stimulating biofilm formation and virulence factors (Corral-Lugo et al. 2016a). This activation of the bacterial QS mechanism when the population density is still low has been proposed as a defense strategy, but this hypothesis is still under debate. This principle could in theory also be used in agriculture to interfere with pathogen QS signaling in the soil and hence prevent plant diseases. Indeed, Pérez-Montaña *et al.*, (2013) suggest that rice and bean produce HSL-mimicking signals that enhance or interfere with the biofilm formation of two plant associated bacteria. They hypothesize that beneficial microorganisms are recognized by the plant and are then stimulated by the plant by the secretion of HSL-mimicking signals, while pathogens would be controlled also through QS mimicking molecules. In both cases, plant QS mimicking compounds showed a higher affinity for the bacterial QS receptors than the bacterial QS molecules (Corral-Lugo et al. 2016a). Therefore, a low concentration of the QS mimicking molecules can already outcompete the bacterial signal.

4.5 Tritrophic interactions

In case of biotic stresses caused by insects and soil-borne pathogens, plants can produce and exude defensive phytoalexins, as discussed above, or produce signaling compounds to attract protective microbes (Mendes et al. 2018) or (other) natural enemies of the pest that is attacking the plant. These so-called tritrophic interactions are particularly important and useful in agriculture since the organisms involved can be applied in Integrated Pest Management (IPM) as biological control agents (Poppy 1997). The central mechanism in tritrophic interaction is that – upon attack - plants produce infochemicals that diffuse through the soil matrix and are perceived by enemies of the attacking pest (Hiltpold et al. 2011). Maize plants under attack from maize corn rootworm (*Diabrotica virgifera virgifera*), one of the most important maize pests that is invading Europe, released the sesquiterpene (*E*)- β -caryophyllene, from their roots (Rasmann et al. 2005). (*E*)- β -caryophyllene attracts the entomopathogenic nematode *Heterorhabditis megidis*, which can efficiently parasitise corn rootworm. Ali et al. (2010, 2011) showed that the hybrid citrus Swingle (*Citrus paradisi* Macf. x *Poncirus trifoliata* L. Raf.) attracts the entomopathogenic nematode *Steinernema diaprepesi*, when attacked by the root-feeding weevil *Diaprepes abbreviatus*, through production of a C12 terpene cue, pregeijerene. Interestingly, pregeijerene also attracted a phytopathogenic nematode: *Tylenchulus semipenetrans*. Just as the strigolactones and flavonoids described above, this represents another example of a pathogenic species that has hijacked a compound with a positive function for the plant (a synomone or allomone) and thus converted it into a pest attractant (kairomone).

A special case of attracting enemies of your enemy, as recently suggested by Eppinga et al. (2006) is the recruitment by plants of soil organisms that damage other plants competing for the same resources. The authors hypothesized that the exotic invasive plant species *Ammophila arenaria* accumulates local pathogens that are not adapted to it, in order to damage neighboring local plant species. This hypothesis is supported by a study of Mangla et al. (2008) who showed that root exudates from the invasive species *Chromolaena odorata* increased the presence of the pathogenic fungus *Fusarium semitectum* at the expense of native plants.

4.6 Allelopathy

Plants are not just competing with other organisms, but also with other plants, either conspecific or with different species. Allelopathy is a long known mechanism by which fitness of plants is increased through the release of allelochemicals, compounds that can interfere with growth or other vital processes, such as germination, in competing plant(s) (species) either directly or upon degradation or transformation in the soil. Well-studied examples of this are phenolics, alkaloids and terpenoids (Latif et al. 2017). Although allelochemicals can be produced in different parts of the plant, here we focus on the ones secreted by roots.

For example, *Ligularia cymbulifera*, a native Chinese herb that is expanding into grasslands and causes a decrease in forage grass yield in the Hengduan Mountains in

China secretes phytotoxic sesquiterpenes to outcompete other plant species, causing cell death in the root tips and consequently inhibiting root elongation (Chen et al. 2017). Phenolic compounds have several beneficial roles in the soil, as discussed above, but can also cause autotoxicity in perennial species such as alfalfa and clover that are mainly used as feed for livestock. In general, phenolic compounds interfere with hormone activity, membrane permeability, photosynthesis and synthesis of organic compounds (Latif et al. 2017), and are mostly produced under nitrogen shortage. Another well-studied allelochemical is sorgoleone, present in the root exudate of sorghum and belonging to the family of benzoquinones. In *in vitro* assays, sorgoleone affects specific processes including photosynthetic and mitochondrial electron transport, while *in vivo* it is a potent inhibitor of PSII (Dayan et al. 2010).

Weston and Mathesius (2013) discuss the fact that autoallelopathy limits the renovation of pastures, since the high amount of phenolic acids and flavonoids released into the soil by the previous plant community can limit the germination and seedling growth of the next generation. Autoallelopathy and autotoxicity also result in replant issues. Yang et al. (2015) for example, showed that the accumulation of ginsenosides produced by *Panax notoginseng*, causes crop replant failure in continuously cultivated ginseng gardens (Yang et al. 2015). Autotoxicity bioassays showed that ginseng seedlings cannot survive in the presence of ginseng root extracts, soil on which ginseng was cultivated, or pure ginsenosides. As underlined by the authors, ginsenosides not only have an autotoxic effect, but also stimulate the growth of soil-borne pathogens such as *Fusarium solani* and *Phytophthora cactorum*, which also contributes to replant failure (Yang et al. 2015).

Allelopathy can also be a resource to protect crops against weeds, for example rice produces diterpene momilactones that suppress the growth of neighbor plants such as *Echinochloa crus-galli* (barnyard grass), one of the rice paddy weeds that infest rice fields (Xu et al. 2012).

4.7 Plant - nematode interaction

Plant pathogenic nematodes such as cyst and root knot nematodes need a host to complete their life cycle, hence they have adapted strategies to detect the presence of their host. Cyst nematodes belong to the families *Heterodera* and *Globodera* and attack many different plant species, including Solanaceae (potato, tomato and eggplant), sugar beet, wheat, rice and soybean (Perry et al. 2018). At the end of their life cycle these parasitic nematodes form a structure called cyst that is released into the soil and can contain over 200 eggs. The cyst is formed by the female body and protects the eggs against biotic and abiotic stresses for up to 20 years (Bohmann 2015; Perry et al. 2018). When a suitable host is nearby, the eggs hatch in response to hatching stimulants produced by the host roots after which the juveniles penetrate the root and induce a feeding site (Lilley et al. 2005). Devine et al. (1996) detected multiple hatching factors in potato root exudate while Byrne et al. (1998) suggested that the glycoalkaloid α -solanine can act as a hatching stimulant or inhibitor depending on the concentration. The most effective hatching stimulants, however, are the nortriterpenoid eclepins that have been reported in several

species (Rasmann et al. 2012). Soybean produces glycinolepin A (Masamune et al. 1982; Fukuzawa et al. 1985a; Masamune et al. 1987), kidney bean glycinolepin B and C (Fukuzawa et al. 1985b), and potato solanolepin A (Schenk et al. 1999).

Root knot nematodes belong to the genus *Meloidogyne* and parasitize the roots of nearly every species of higher plants and thus are considered the most damaging group of plant-parasitic nematodes (Perry et al. 2009). At the site of infection they induce galls or root-knots which affect the nutritional status of the plant causing yield losses and consequently reduction of the product quality (Perry et al. 2009). Volatiles produced by roots of *Capsicum annuum*, such as α -pinene and limonene elicited positive chemotaxis in *Meloidogyne incognita*, with methyl salicylate showing the highest effect in terms of attraction (Kihika et al. 2017). Recently Čepulytė et al. (2018) found in tomato and *Medicago* root exudates from seedling root tips, powerful – non-volatile - attractant(s) for three root knot nematode species, but could not identify them. It is unclear whether root knot nematodes use host-specific cues, or rather a non-specific blend of volatile and non-volatile compounds.

5 Exploitation of belowground signaling in agriculture

Despite the numerous examples of signaling relationships in the soil that we described above, the majority of compounds that are secreted in the root exudates have no known function attributed to them, other than perhaps being a carbon source for microbes (Uren 2000). We think that the latter is a gross underestimation of the importance of root exudates for plants. For those molecules that have been characterized and have at least one clear role in the rhizosphere attributed to them, challenges may remain as demonstrated for the strigolactones for which it took 40 years to discover a second, beneficial, role after the discovery of their parasitic plant seed germination activity in 1965 (Cook et al. 1966; Akiyama et al. 2005a). And finally, the translation of unambiguous results on biological activity in model or even *in vitro* studies to the field is challenging because soil is an unpredictable substrate - catalysing the degradation of organic molecules - it is not homogenous, and it is subject to changes due to weather and other environmental factors such as flooding, agricultural practices and organisms living within it. Nevertheless, for some rhizosphere signaling molecules, research is going on into their potential to be exploited in agricultural systems. Signaling molecules that are present in plant root exudates can be exploited in different ways: through breeding (Fernández-Aparicio et al. 2014), application of compounds (Devine and Jones 2000), intercropping (Khan et al. 2002) or crop rotation (Scholte 2000a; López-Ráez et al. 2009; Cardoso et al. 2011). I will discuss some examples of how these techniques can be applied and in which circumstances they can have drawbacks.

Breeding for the production (or lack thereof) of specific cues is an attractive approach to try to optimize rhizosphere interactions (Zhang et al. 2015). North American maize lines do not produce (or very low amounts of) (*E*)- β -caryophyllene, and thus do not effectively attract entomopathogenic nematodes that can control the corn root worm. These lines have a functional (*E*)- β -caryophyllene synthase, but it is not expressed. Degenhardt et al. in

2009 showed how restoring the production of (*E*)- β -caryophyllene in these lines through transformation with the oregano (*E*)- β -caryophyllene synthase results in the (restored) attraction of entomopathogenic nematodes that parasitize and kill larvae of the western corn rootworm. This resulted in a 60% reduction in adult corn rootworm occurrence in field experiments. Although the production of (*E*)- β -caryophyllene can likely also be restored through classical breeding, as the authors suggest, up to now this objective has not been achieved, due to the long time needed for classical introgression of, even single-gene based, traits.

Fernández-Aparicio et al. (2014) showed that there is genetic variation in faba bean (*Vicia faba*) for broomrape germination stimulant production, offering the possibility to select for this trait in breeding programs. Similarly, Pavan et al. (2016) described the selection of a pea line (*Pisum sativum*), resistant to the parasitic weed *Orobanche crenata* through lower production of strigolactones. Although a lower production of strigolactones seems to be an advantage in these specific contexts, tomato lines in which strigolactone biosynthesis was reduced through an RNAi strategy, displayed severely reduced stem height and increased shoot branching due to the reduced hormonal control by strigolactones, making this an unsuitable strategy for resistance breeding (Kohlen et al. 2012; Guan et al. 2012).

Beneficial compounds can also be applied directly to the soil. Rasmann et al. (2005) applied (*E*)- β -caryophyllene directly into the field and found a more than two-fold decrease in western corn rootworm adult emergence. Similarly, Devine and Jones, (2000) compared the hatching rate of potato cyst nematode in soil and *in vitro* after application of potato and tomato root leachate. They concluded that the direct application of a hatching stimulant (pure or in a mixture) can be used to induce egg hatching in the absence of a host, which would result in the death of the juvenile and a decrease in the PCN population, a procedure called 'suicide hatch' by Devine and Jones (2000).

This strategy has also been suggested for the prevention of parasitic weed infection (Screpanti et al. 2016). Indeed, synthetic strigolactone analogs sometimes display very high germination stimulant activity, but just as for natural strigolactones, the major challenge is their instability in soil and consequent decomposition. Encapsulation using specific formulations to deliver the product on the desired site, protect it against external agents and/or to improve its efficacy could be solutions to this problem. On the basis of these considerations, Zwanenburg et al. (2009) applied the strigolactone analog Nijmegen-1 in the field, using a formulation that included an emulsifier and obtained promising results.

Intercropping is an interesting strategy to interfere with rhizosphere signaling. The most intriguing example of this is the push-pull strategy developed by Khan et al. (2002). In their search for an effective control strategy of maize stem borers they serendipitously found that intercropping maize with the cattle forage legume *Desmodium uncinatum* reduced infection by the parasitic witchweed *Striga hermontica*, and consequently increased maize yield. Hooper et al. (2010) showed that *D. uncinatum* exudes the C-

glycosylflavone isoschaftoside, which acts as an allelochemical and inhibits radicle growth of *Striga* thus reducing and preventing maize parasitism.

Trap cropping is another technique that can be used to prevent infection of crops by pathogenic organisms. Scholte (2000a) investigated this for potato cyst nematode and postulated that a good trap crop should stimulate hatching of juveniles by producing high levels of hatching stimulant, and at the same time being resistant, not allowing infection or the development of the nematodes (Scholte 2000a, b). Scholte (2000a) suggested *Solanum sisymbriifolium* as a trap crop to control PCN, in a system of crop rotation in order to keep nematode populations at low levels, and Dias et al. (2012) suggested this species also for controlling other plant parasitic nematode populations.

6 Future perspectives for rhizosphere signaling molecules

Molecules released in the root exudates of plants can be exploited as antibiotics, allelochemicals, pathogen and pest repellents and for improving nutrient availability, and for their action as signaling molecules, that attract beneficial organisms to the plant. Of utmost interest for agriculture are, for example, the phytoanticipins produced by plants that inhibit growth of and root colonization by pathogens. A better understanding of the efficacy of these molecules and their potential application in the field could perhaps (partially) replace the use of synthetic pesticides. The same consideration holds for allelopathic compounds that could be used in combination with or to replace herbicides in order to reduce the selective pressure on invasive species and other weeds when treated with the same chemical for long periods of time. From the perspective of IPM, using such new molecules could be advantageous, improving the quality of crops without having to use artificial chemistry. Of course, plant derived molecules could be as toxic as synthetic pesticides, and despite all the advantages that these molecules can provide, the normal procedures to assess their safety must be considered. Root exudate molecules can also help in the amelioration of the nutritional status of the plant, for example using organic acids and phytosiderophores in soils where there is poor nutrient availability, or where nutrients are adsorbed to soil particles. The use of plant signaling molecules to stimulate beneficial interactions between plant roots and soil microorganisms, such as PGPBs and (other) symbionts, can lead to preventive protection of crops against pathogens by boosting the plant immune system. Such relationships can bring also nutritional advantages, for example AM fungi and rhizobia interact with plant roots in symbioses that improve the availability of phosphorous and nitrogen, respectively, for the plant.

Many of these beneficial relationships have been known for decades, but still very little is known about the signaling molecules that trigger the association between plants and microorganisms, or the signaling pathways that plants and soil organisms have evolved to perceive and respond to these cues. A better understanding of the mechanisms underlying the myriad interactions that take place in the plant rhizosphere and the signaling molecules produced by both parties, could help us improve current agricultural practices. QS, for example, represents an intriguing area and has been intensively studied in micro-

organisms. The fact that plants produce QS mimicking molecules is highly intriguing and represents a potentially powerful tool to develop strategies against pathogens. Finally, more efforts are needed to find ways of exploiting these molecules for field application, since most of the current knowledge is based on fundamental research that ignores the challenges of scaling-up to industrial application. A connection between fundamental and applied research is therefore needed to link the discovery of new molecules to their potential beneficial role in cultivation.

7 How to improve our knowledge on rhizosphere communication

As discussed above, plants secrete large amounts of different metabolites into the soil, of which the concentrations vary with genotype, developmental stage and nutritional status. The function of most of these metabolites in the rhizosphere, however, is unknown, with just a few exceptions. For example, thalianol, a triterpenes compound found in the root of *Arabidopsis thaliana*, was first discovered in 2004 (Fazio et al. 2004). Only recently, a function for two of its derivatives (thalianin and arabidin) in the rhizosphere was elucidated by demonstrating that these root metabolites shape the *Arabidopsis* root-microbiota by acting as antibiotics or proliferating agents (Huang et al. 2019). However, for every example of the identification of a biological role, there are still many metabolites of which the biological importance in the rhizosphere remains a mystery.

One good example of that is solanoeclepin A. Despite the long history of this compound – already in 1996 it was isolated from potato root exudate as the major hatching factor for Potato Cyst nematode (Mulder et al. 1996) – its biosynthetic pathway and potentially beneficial role in the rhizosphere have not yet been unravelled. This is undoubtedly also due to the extremely low levels produced by plants. Indeed, the isolation of solanoeclepin A from tomato and potato root exudate has been described as a complicated procedure with many time-consuming steps which requires growing of thousands of plants to get a few micrograms of the compound (Schenk et al. 1999). However, new analytical chemistry technologies, such as UHPLC-MRM-MS/MS have rapidly improved bringing the targeted detection of compounds in pmol or even fmol concentrations within reach (Flokova et al. 2020).

The analytical detection of rhizosphere signaling compounds is an important step towards understanding their biological role. However, as also pointed out by Huang et al. in their attempt to study the role of thalianol derivatives in the rhizosphere, the elucidation of the biosynthetic pathway of the signaling molecule, is of great importance too, and is needed to allow for the creation of mutants needed to deliver the final proof-of-concept. The combination of synthetic biology to identify possible candidate genes and the creation of overexpression and knock-down lines, resulted in the characterization of three enzymes involved in thalianin biosynthesis. Moreover, mutants with disrupted thalianin and arabidin pathways, displayed altered root microbiome communities, which proved their role in rhizosphere signaling.

Thesis outline

In my PhD thesis I focus on the detection, biosynthesis and possible biological role of solanoeclepin A. After the General introduction in **Chapter 1**, in **Chapter 2** I describe the method development of solanoeclepin A extraction from root exudates of tomato plants and their detection using UHPLC-MRM-MS/MS. This method allowed me to detect solanoeclepin A in as little as 5 ml of the the root exudate of a single plant. The same method is subsequently used to investigate whether SolA is produced by wild tomato species in **Chapter 2**, and I successfully found this compound in all the analysed samples, indicating that solanoeclepin A is not only present in domesticated species. In **Chapter 3** I investigate the production of solanoeclepin A in wild potato varieties from South America and with hatching assays of species that do not produce solanoeclepin A, I show that this compound is not the only factor involved in Potato Cyst Nematode hatching, but most likely is part of a complex mixture of other hatching factors and inhibitors. In **Chapter 4** I use Virus Induced Gene Silencing (VIGS) to silence genes involved in terpenoid pathways (SSR2 and SMT1) and demonstrate that this technique is an attractive alternative to stable transformation to characterize and validate candidate genes, and can be used to engineer metabolites in both root and root exudates. Moreover, solanoeclepin A level was also effected by the silencing of SSR2 supporting the hypothesis that a terpenoid pathway is involved in solanoeclepin A biosynthesis. In **Chapter 5** I investigate the factors involved in solanoeclepin A production using a custom-built aeroponics system to efficiently analyze root exudates and the effect of biotic and abiotic factors on tomato plants. I discovered that solanoeclepin A production is dependent on nutrient deficiency, in particular nitrogen, and the presence of microorganisms. Using this information, I perform an RNA-seq experiment and I correlate the production of solanoeclepin A with the root transcriptome and identify 4 possible candidate genes involved in solanoeclepin A biosynthesis: one terpene cyclase and three cytochrome P450s that need to be further characterized to validate their role in SolA biosynthesis. In **Chapter 6** I discuss the findings presented in this thesis with regards to future perspectives.

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