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Plants are attacked by a variety of (micro)organisms. In order to cope with the great variety of potential attackers many plants synthesize a diversity of repellent, deterrent and/or toxic compounds as a defence against herbivores and pathogens. The evolution of compound diversity is supposed to be driven by herbivore insects. Herbivore populations change over time and consequently the selection pressure on the defence chemistry of plants will change as well (Macel et al. 2005). Less adapted generalist herbivores are supposed to be more sensitive to differences in chemical compounds. Thus generalists may play a greater role in the evolution and maintenance of diversity of secondary plant metabolites than adapted specialists (Miller and Feeny 1983, Lindroth et al. 1988, van Dam et al. 1995, Agrawal 2002, Macel et al. 2002, Macel et al. 2005).

There are several possible reasons for the variety of secondary metabolites in plants. The first reason is that new compounds are synthesized because of a continuous evolutionary arms race between the production of new defence chemicals by plants and the adaptation to these compounds by attackers. The selective pressure forced by adaptation of the attacker forces the plant to continuously select different compounds. This hypothesis implies that evolutionary older compounds are less effective than more recently developed ones (Berenbaum and Feeny 1981, Miller and Feeny 1983, Berenbaum et al. 1989, Macel et al. 2005).

The second reason for the diversity of compounds might be the synergistically effect of different defence compounds (Adams and Bernays 1978, Lindroth et al. 1988, Berenbaum et al. 1989, Berenbaum et al. 1991). This implies that plants with a more diverse or complex defence compound composition are more successful in deterring attackers than plants with a simpler composition.

The third reason is that this diversity of compounds can be maintained by selection pressure by several attackers (Simms 1990). The plant contains a wide variety of defence compounds, whereby the relative importance of the different compounds differs locally and on the prevailing selective environment. Thereby it is expected that those compounds differ in their toxic and deterrent effects on generalist insects and other enemies such as nematodes (van Dam et al. 1995, Macel et al. 2002; Macel et al. 2005, Dominguez et al. 2008; Thoden et al. 2009).

Pathogens such as fungi and bacteria also are a serious threat to plants and therefore they may also influence the selection of defence mechanisms (Hol and van Veen 2002). Historically, long before herbivores existed on this planet, plants had to cope with microbial pathogens. However, the existing evidence on the role secondary metabolites play in plant defence against microorganisms, like soil pathogens, is scarce especially in comparison to ecological studies with insects on plants. We will try to get a better understanding in the plant chemical defence with the focus on the interaction with soil-borne microorganisms.
Pyrrlizidine alkaloids

Alkaloids represent one of the largest groups of secondary metabolites. From the more than 50,000 secondary metabolites described, ca. 12,000 are alkaloids (Wink and Roberts 1998; Wink 2003). Only around 600 alkaloids are studied in detail on for instance biochemical properties and eco-physiological roles (Wink and Roberts 1998) such as pyrrlizidine alkaloids (PAs) and quinolizidine alkaloids.

Pyrrlizidine alkaloids (PAs) are a well-known class of defence compounds with a wide variety of structures. From several genera of Asteraceae, Boraginaceae, Orchidaceae and Fabaceae, more than 360 structurally different PAs have been isolated (Rizk 1991; Hartmann and Witte 1995). It is known that PAs in plants are present as mixtures of the tertiary alkaloids and the respective N-oxides (Rizk 1991). The number of structurally different PAs actually almost doubles when the PA-state is taken into account since most PAs, but not all, can be present in both forms.

One of the most diverse classes of PAs is the macrocyclic senecionine type with more than 100 structures (Hartmann and Dierich 1998). This type of PAs is abundantly found in species of the tribe Senecioneae of the family Asteraceae (e.g. Genus Senecio and Jacobaea).

PAs have toxic, deterrent and/or repellent effects on a wide range of generalist herbivores, which helps the plant to reduce or prevent damage, but also have attractive effects on specialist herbivores (van Dam et al. 1995; Hartmann 1999; Hartmann and Ober 2000; Ober 2003; Macel et al. 2005). These PA effect on organisms depend on the PA concentration and composition in the plant (Macel 2011).

Jacobaea vulgaris Gaertn. (syn Senecio jacobaea L.) is known to be one of the suitable systems to study the chemical defence mechanisms of PAs in plants. In previous studies about 14 different PAs were detected in J. vulgaris (Witte et al. 1992; Macel et al. 2004; Kowalchuk et al. 2006). The concentration and composition of PAs in J. vulgaris depend on the genotype (Vrieling et al. 1993) and its environment (Hol et al. 2003; Macel et al. 2004; Hol et al. 2004; Macel and Klinkhamer 2010).

In Jacobaea species, such as J. vulgaris, PAs are synthesized in the roots primarily as senecionine N-oxide (Hartmann and Toppel 1987; Toppel et al. 1987). Subsequently, senecionine N-oxide is transported to the shoot, where further diversification into different individual PAs takes place by specific enzymes (Hartmann and Dierich 1998). All PAs are derived from the senecionine N-oxide core-structure and represent end-products, which show no degradation or turnover. Therefore, the total amount of PAs in the plant is controlled by the senecionine N-oxide formation in the roots, which is closely linked to root growth (Hartmann et al. 1988; Sander and Hartmann 1989).

Based on their PA composition individual J. vulgaris plants can be distinguished into different chemotypes (Witte et al. 1992; Macel et al. 2004). Seneconine-chemotypes contain mainly seneconine-like PAs and largely lack jacobine- and erucifoline-like PAs, Erucifoline-chemotypes contain mainly seneconine- and erucifoline-like PAs and lack jacobine-like PAs, Jacobine-chemotypes contain high levels of jacobine-like PAs and mixed-chemotypes containing both high levels of jacobine-like PAs as well as erucifoline-like PAs (Figure 1).

Two PA forms in Jacobaea plants

PAs occur in plants in two forms: N-oxides and its reduced tertiary amines. The water soluble N-oxide form is considered to be the best form for slow allocation between tissues by phloem transport (Hartmann et al. 1989) and storage in cell vacuoles (von Borstel and Hartmann 1986; Ehmkke et al. 1988). Generalist insect herbivores reduce N-oxides in the gut to tertiary PAs, where these are passively taken up into the body (Lindigkeit et al. 1997; Hartmann 1999) and converted into pyrroles. Pyrroles are toxic by acting as highly reactive alkylating agents to organisms like mammals (Mattocks 1986) and fruit flies (Frei et al. 1992). Individual PA N-oxides show less deterrent or toxic effects for some generalist insect herbivores compared to their tertiary PA form (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005). Specialist insects, adapted to PAs, take up the tertiary amines and actively convert them into N-oxides. They even store PA N-oxides and use these compounds for their own defence (Boppré 1986; Lindigkeit et al. 1997; Dobler 2001; Nishida 2002; Narberhaus et al. 2003).

Plants developed other mechanisms to face serious attacks by specialists. J. vulgaris contains high food reserves in the root systems for vegetative reproduction and re-growth to survive complete defoliation by, for instance, specialist herbivores (Verkaar 1987; van der Meijden et al. 1989). Therefore it is to be expected that plant species, using this survival strategy, protect their roots strongly against below-ground herbivores and pathogens.

Figure 1. The structure of the major PAs detected in Jacobaea vulgaris plants and which occur in both tertiary amine and N-oxide form. Only the structures of the tertiary amine form are shown.
Induction of PAs by soil-borne microorganisms

Plants are attacked most times simultaneously above- and belowground. When the root system is exposed to belowground organisms (e.g. herbivore insects, nematodes, root pathogens and mycorrhizal fungi) plants may show several defence responses in the shoots that may also affect aboveground herbivores and, thus plant fitness (van Loon et al. 1998; Paul et al. 2000; van der Putten et al. 2001; Gange et al. 2002; Dicke and Hilker 2003; van Dam et al. 2003; Bezemer et al. 2005; Bezemer and van Dam 2005). Less is known about PA induction by pathogens. Artificial root damage has shown to result in an increased PA concentration in the shoots of J. vulgaris (Hol et al. 2004). Macel and Klinkhamer (2010) noticed that the composition of PAs in genotypes of J. vulgaris changed in the field compared to the initial composition in laboratory clones. The PA composition also differed between the aboveground parts of clones grown on two different experimental field sites. Bezemer et al. (2006) found that aboveground herbivory was related to the fungal community belowground. They suggested that the fungal community directly or indirectly changed the concentration of different PAs in the shoots and in this way affected the aboveground insect community.

The possible impact of soil-borne microorganisms on the defence system of the plant may have ecological consequences. If the aboveground defence compounds are affected this may influence plant resistance by attracting or deterring herbivores (McEvoy et al. 1993; Macel and Vrieling 2003; Macel et al. 2005, Macel and Klinkhamer 2010). It could also have considerable consequences for other relevant processes, for instance, the success of invasive plants and the biological control of plants.

Aims

The aim of this thesis is to gain better understanding on the PA defence system, with the primary focus on the interaction with soil-borne microorganisms. A novel PA analysis has been used that allows for obtaining better knowledge of the composition of PAs in above- and belowground plant parts and the chemical forms in which they occur. Emphasis is on the impact of soil-borne microorganisms on the above- and belowground PA defence system and its ecological consequences. Molecular techniques have been applied to get a better insight in the influence of PAs on the soil-borne microbial community in the rhizosphere and roots of the plant.

The main Research Questions addressed here are:

- Is the novel extraction and detection method reliable, applicable and sensitive enough for studying PAs, by comparing the traditional method with the novel method, in particular with respect to the detection of reduced PAs?
- Are PAs present in tertiary amine form in J. vulgaris? Is the presence of PAs in tertiary amine form in J. vulgaris genotype and/or PA-type dependent?
- Is the PA production inducible by soil-borne microorganisms? Do soil-borne microorganisms affect the aboveground metabolic profiles and so the defence against thrips in J. vulgaris?
- Do PAs affect the soil-borne microbial community living in the rhizosphere and roots of J. vulgaris?

Thesis outline

In Chapter 2, a review is presented on the available literature on defensive properties of PAs against microorganisms.

In Chapter 3, a new technique to extract and detect PAs is described. In this study two different approaches for the analysis of PAs in plant material are compared. The questions that are addressed in this chapter are: (A) does, detection of (reduced) PAs with GC-PND and LC-MS/MS give similar results with respect to the concentration and composition in plant material, irrespective of the sample preparation procedure?, (B) is formic acid extraction an effective alternative to sulphuric acid extraction ?, (C) could PA N-oxide reduction with sodium metabisulphite (NaS2O5) be an alternative to the commonly used zinc dust for analysis by GC based methods? and (D) does the traditional and the novel analytical method give similar results regarding PA concentration and composition in plant material?

In Chapter 4 I tested whether high concentrations of tertiary PAs are present in the plant material of J. vulgaris or just an artefact of this novel approach for PA measurements. By analysing the PAs in both forms in different J. vulgaris genotypes, we observed the PA state to be genotype and PA-type dependent. In order to assess the genetic basis of this variation, we analysed PAs in F1 hybrids of J. vulgaris and Jacobaea aquatica ((Hill) P. Gaertn).

In Chapter 5 information is presented on the impact of soil-type and soil-borne microorganisms on PA concentrations and composition in roots and shoots of J. vulgaris. In Chapter 6 I report that the results in the previous study are reproducible and that the defence system of different J. vulgaris genotypes is changed in a similar way as a response to soil-borne microorganisms. To test if the changed PA composition has any influence on a higher trophic level, I introduced a generalist thrips (Frankliniella occidentalis (Pergande)) on the plants, and measured aboveground herbivore resistance. The following research questions are addressed: (A) does the soil-borne microbial community affect plant growth?, (B) does the soil-borne microbial community affect PA concentration and PA composition in shoots? and as a consequence (C) does the soil-borne microbial community affect resistance against thrips? and (D) are the effects of the soil-borne microbial community on growth, PAs and thrips resistance, genotype dependent? The effects of intra-specific differences in the PA defence system of different J. vulgaris genotypes on the microbial community structure belowground are addressed in Chapter 7. I reported the general fungal and bacterial soil communities in the rhizosphere and in the root tissue as well as Arbuscular Mycorrhizal Fungi, AMF, in the roots of five different J. vulgaris genotypes grown on two different soil-types, loss and sand. General bacterial and fungal community structures were analysed by DGGE and T-RFLP. The results of the different studies are summarized, discussed and evaluated in Chapter 8.