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Pyrrolizidine alkaloid composition in the plant and its interaction with the soil microbial community

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Pyrrolizidine alkaloid composition of the plant and its interaction with the soil microbial community

by Lotte Joosten

Joosten, Lotte

Pyrrolizidine Alkaloid Composition of the Plant and its Interaction with the Soil Microbial Community

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Pyrrolizidine alkaloid composition of the plant
and its interaction with the soil microbial community

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Contents

Chapter	title	page
1	General Introduction	7
2	Defensive Properties of Pyrrolizidine Alkaloids against Microorganisms; a Review	15
3	The Analysis of Pyrrolizidine Alkaloids in *Jacobaea vulgaris*; a Comparison of Extraction and Detection Methods	27
4	The Genotype Dependent Presence of Pyrrolizidine Alkaloids as Tertiary Amine in *Jacobaea vulgaris*	41
5	Soil-borne Microorganisms and Soil-type affect Pyrrolizidine Alkaloids in *Jacobaea vulgaris*	61
6	Soil-borne Microorganisms affect Aboveground Metabolic Profiles and Defence against Thrips in *Jacobaea vulgaris*	75
7	Microbial Community Structure in Rhizosphere Soil and Roots of Different Genotypes of *Jacobaea vulgaris*	89
8	Summary and Discussion	107
	Nederlandse samenvatting	117
	Acknowledgements	131
	Curriculum Vitae	133
	Publications	135
	Notes	137

General introduction

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 pants 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.

Pyrrolizidine 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 pyrrolizidine alkaloids (PAs) and quinolizidine alkaloids.

Pyrrolizidine 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). Senecionine-chemotypes contain mainly senecionine-like PAs and largely lack jacobine- and erucifoline-like PAs, Erucifoline-chemotypes contain mainly senecionine- 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; Ehmke 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 (Boppré1986; 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.

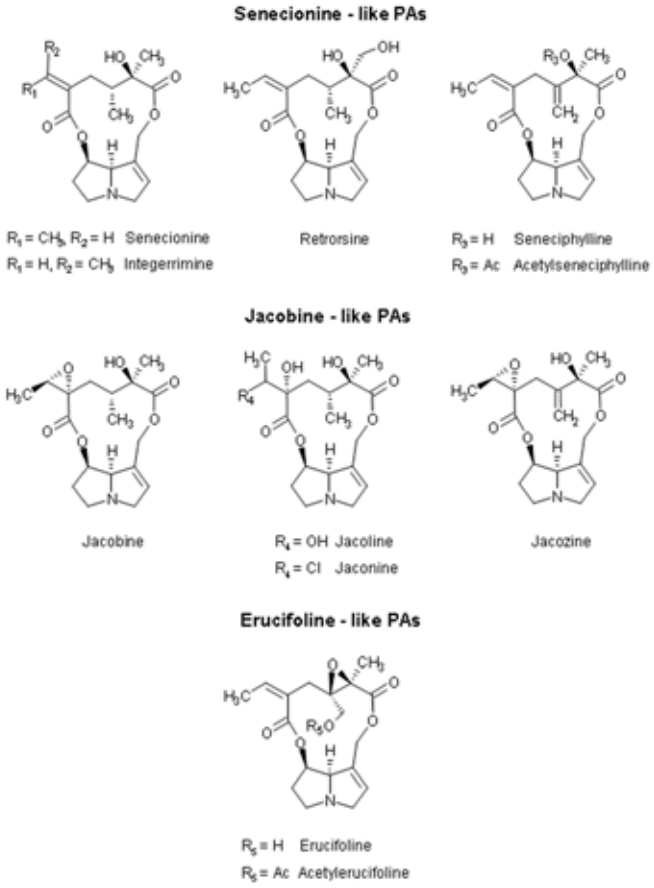

Figure 1. The structure of the major PAs detected in *Jacobaea vulgaris* plants and which occur in both tertiary amine and *N*-oxide form. Only the structures of the tertiary amine form are shown.

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 metabisulfite ($Na_2S_2O_5$) 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 F_2 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* chemotypes 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 depended?

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 assessed 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, löss and sand. General bacterial and fungal community structures were analysed by DGGE and AMF by T-RFLP.

The results of the different studies are summarized, discussed and evaluated in **Chapter 8**.

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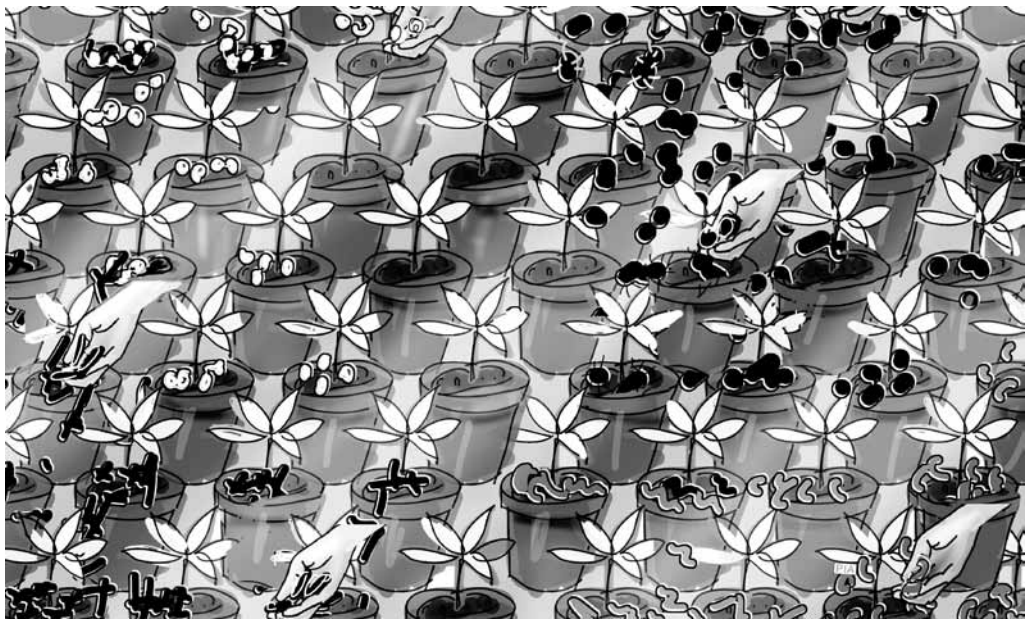
Defensive Properties of Pyrrolizidine Alkaloids against Microorganisms; a Review

Lotte Joosten & Johannes A. van Veen

Phytochemical Reviews (2009) 325: 133-143

Abstract

The understanding of the selection factors that drive chemical diversification of secondary metabolites of constitutive defence systems in plants, such as pyrrolizidine alkaloids (PAs), is still incomplete. Historically, plants have always been confronted with microorganisms. Long before herbivores existed on this planet, plants had to cope with microbial pathogens. Therefore, plant pathogenic microorganisms may have played an important role in the early evolution of the secondary metabolite diversity. In this review, we discuss the impact that plant-produced PAs have on plant-associated microorganisms. The objective of the review is to present the current knowledge on PAs with respect to anti-microbial activities, adaptation and detoxification by microorganisms, pathogenic fungi, root protection and PA induction. Many *in-vitro* experiments showed effects of PAs on microorganisms. These results point to the potential of microorganisms to be important for the evolution of PAs. However, only a few *in-vivo* studies have been published and support the results of the *in-vitro* studies. In conclusion, the topics pointed out in this review need further exploration by carrying out ecological experiments and field studies.



Introduction

Many plants synthesize a range of diverse secondary metabolites that are toxic and/or deterrent for herbivores and pathogens. This diversity might be one of the strategies that plants use in order to defend themselves against the great variety of potential environmental threats. Species of several families within the angiosperms are known for their production of nitrogen-based secondary metabolites called pyrrolizidine alkaloids (PAs). PAs have toxic, deterrent and/or repellent effects on a wide range of generalist herbivores in order to reduce or prevent damage (van Dam et al. 1995; Hartmann 1999; Hartmann and Ober 2000; Ober 2003). Our understanding of the selection factors that drive chemical diversification of PAs in plants is still incomplete. It has been suggested that the diversity of PAs in plant species have evolved under selective pressures by generalist herbivores (Hartmann and Dierich 1998; Macel et al. 2005). However, long before herbivores existed, plants already had to cope with the attack of microorganisms. Therefore, we suggest that also microbial pathogens played an important role in the evolution of the secondary metabolite diversity. If so there should be differences in effectiveness of the individual PA types against microorganisms as well as differences in adaptation of microorganisms to the different PAs.

In this review we will discuss the current knowledge on the anti-microbial activities of PAs and the ecological consequences of this activity. More specifically we will ask; (1) Do PAs show anti-microbial activities in *in-vitro* tests; (2) Do microorganisms adapt to PAs? (3) Do PAs have an effect on aboveground pathogenic fungi?; (4) Do PAs play a role in root protection against soil-borne microorganisms?; (5) Is the PA defence system inducible by microorganisms?

Ecologically relevant *in-vitro* anti-microbial activity of alkaloids

A Web of Science search for "+Topic=(alkaloid*) AND Topic=(microorganism*) AND Topic=(plant*) Timespan=All Years" throws back 153 hits while when replacing "Topic=(microorganism*)" for "Topic=(insect*)" 602 hits are shown. This already indicates that more attention is paid to the effects of PAs on insect herbivores. In addition, most studies on the anti-microbial activities of alkaloids have pharmaceutical purposes and deal with PA toxicity in mammals or are based on PAs not produced by plants. Nevertheless, toxic anti-bacterial or anti-fungal activities of plant alkaloids have been shown in a number of studies. Recently Erdemoglu et al. (2007 and 2009) reported that quinolizidine alkaloid extracts (inhibitory concentrations of 62.2-500 µg/ml) from the aerial parts of *Lupinus angustifolius* and *Genista vuralii* showed activity against several different bacterial species. Anti-fungal effects by alkaloids also have been found for several plant associated fungi by bioassay experiments (Wippich and Wink 1985; Zhao et al. 1998; Ma et al. 1999; Zhou et al. 2003). In the study by Wippich and Wink's (1985), several quinolizidine alkaloids were mentioned that inhibited the germination of conidia of *Erysiphe graminis*. Zhao et al. (1998) reported that two furoquinoline alkaloids showed activity against the phytopathogenic fungus *Cladosporium cumcumerinum*. The inhibitory concentrations of the two alkaloids, dictamnine and haplopine, was 25 µg/ml and originated from root bark of *Dictamnus dasycarpus*. Ma et al. (1999) showed that the isoquinoline alkaloids, corynoline and acetylcorynoline, inhibited the fungal growth of *Cladosporium herbarum* at a concentration of 3 µg/ml. Zhou et al. (2003) reported that steroidal alkaloids from the rhizomes and roots of *Veratrum taliense* inhibited the growth of the phytopathogenic fungi, *Phytophthora capisis* and *Rhizoctonia cerealis* in concentrations of 80-200 µg/ml.

The role that PAs (as a specific group of alkaloids) play in plant protection against microorganisms is still rather unclear. A Web of Science search for "Topic=(pyrrolizidine) AND Topic=(microorganism* OR fung* OR bacteri*) AND Topic=(plant*) Timespan=All Years" throws back 81 hits. Around 75% of all studies on the anti-microbial activities of PAs, are not related to plant defence. When replacing "Topic=(microorganism* OR fung* OR bacteri*)" by "Topic=(insect*)", than 162 hits are shown.

Table 1 shows an overview of studies on PA effects on the growth of microorganisms. From a total of 43 bioassay-tests with different bacterial species 65% showed a negative sensitivity to the different PAs (Jain and Sharma 1987; Marquina et al. 1989; Singh et al. 2002). Anti-bacterial effects of PAs produced by *Heliotropium* species were investigated in a few *in-vitro* studies (Jain and Sharma 1987; Marquina et al. 1989; Singh et al. 2002). In these studies the growth of bacterial species, mostly human pathogens, such as *E. coli*, *Streptococcus pneumoniae*, *B. subtilis*, *Bacillus anthracis* and *S. aureus*, was inhibited in the presence of different pure PAs and PA plant extracts (Table 1).

Table 1. Overview of studies on pyrrolizidine alkaloids and the effect on the growth of microorganisms. PAs studied showed a significant negative effect on at least one of the microorganisms tested and vice versa.

Pyrrolizidine alkaloids	Alkaloid origin	Microorganisms	Inhibition concentration (mg/ml)	Reference
Europine Heliotridine Lasiocarpine Lasiocarpine *N*-oxide PA extract	Aerial parts *Heliotropium ellipticum*	Bacteria *Escherichia coli* *Streptococcus pneumoniae* *Bacillus subtilis* *Bacillus anthracis* *Staphylococcus aureus* Fungi *Candida albicans* *Curvularia lunata* Phytopathogenic fungi *Drechslera tetramera* *Aspergillus flavus* *Fusarium moniliforme*	Bacterial and fungal growth 100	Jain and Sharma 1987
9-Angeloylretronecine *N*-oxide Supinine Heliotrine (no sign. effect) Lasiocarpine PA extract	Aerial parts *Heliotropium bursiferum*	Bacteria *Bacillus subtilis* Phytopathogenic fungi *Candida tropicalis* *Aspergillus niger*	Bacterial and fungal growth 50	Marquina et al. 1989
Europine 7-acethyleuropine (no sign. effect)	Aerial parts *Heliotropium bovei*	Phytopathogenic fungi *Fusarium moniliferme*	Fungal growth 0.01-0.25	Reina et al. 1995
3'-Acetyltrachelanthamine Floridinine PA extract Plant extract	Aerial parts *Heliotropium floridum*	Phytopathogenic fungi *Fusarium oxysporum* *Fusarium moniliferme* *Fusarium avenaceum* *Fusarium solani*	Fungal growth 0.5	Reina et al. 1997
Megalanthonine (no sign. effect) Lycopsamine (no sign. effect)	Aerial parts *Heliotropium megalanthum*	Phytopathogenic fungi *Fusarium monoliferme*	Fungal growth 0.5	Reina et al. 1998
Subulacine *N*-oxide 7-Angeloyl heliotrine Retronecine Heliotrine	Aerial parts *Heliotropium subulatum*	Bacteria *Escherichia coli* *Streptococcus pneumoniae* *Bacillus subtilis* *Bacillus anthracis* *Staphylococcus aureus* Phytopathogenic fungi *Aspergillus fumigatus* *Aspergillus niger* *Rhizoctonia phaseoli* *Penicillium chrysogenum*	Bacterial and Fungal growth 2 mg/disk	Singh et al. 2002
Monocrotaline Retrorsine Retrorsine *N*-oxide PA extract	Aerial parts *Jacobaea vulgaris*	Phytopathogenic fungi *Fusarium oxysporum* *Fusarium sambucinum* *Trichoderma* spp.	Fungal growth 0.01-1.08	Hol and van Veen 2002
Monocrotaline Senecionine Retrorsine Integerrimine Integerrimine + Retrorsine Bulk extract *Jacobaea vulgaris*	Aerial parts *Senecio brasiliensis* *Jacobaea vulgaris*	Phytopathogenic fungi *Fusarium oxysporum* *Fusarium sambucinum* *Mortierella* sp. *Plectosphearella cucumerina* *Rhizoctonia* sp. *Broomella acuta* *Pestalotiopsis* sp.	Fungal growth 0.01-1.08	Hol 2003

More is known about the effect of PAs on fungi. Also here most studies were carried out *in-vitro*. Of the 145 bioassay-tests with different fungal species, 61% showed significant growth inhibition caused by the different PAs (Jain and Sharma 1987; Marquina et al. 1989; Reina et al. 1995, 1997 and 1998; Singh et al. 2002; Hol and van Veen 2002; Hol et al. 2003). Early work of Jain and Sharma (1987) and Marquina et al. (1989) reported anti-yeast and anti-fungal activities (Table 1). Reina et al. (1995) found anti-fungal activity against *Fusarium moniliferme* by the PA europine produced by *Heliotropium bovei*. PAs produced by *Heliotropium subulatum* showed to be active against *Aspergillus fumigatus*, *Aspergillus niger*, *Rhizoctonia phaseoli* and *Penicillium chrysogenum* (Singh et al. 2002).

PAs from *Heliotropium* species are open chain diesters and differ in structure from the PAs in *Senecio* and *Jacobaea* species, which are macrocyclic diesters (Figure 1). Hol and van Veen (2002) investigated the growth-reducing effects of PAs from *Jacobaea vulgaris* on different plant-associated fungi. The growth of five soil fungal strains of *Fusarium oxysporum*, *Fusarium sambucinum* and *Trichoderma* sp. was temporarily inhibited by different purified PAs among which monocrotaline, retrorsine, retrorsine *N*-oxide and PA plant extract. The concentrations 0.33 mM and 3.33 mM (equal to ca. 0.1 mg/ml and 1.08 mg/ml used in *in-vitro* tests), at which the most inhibitory effects were found, are comparable to PA concentrations (0.3 mM – 3 mM fresh weight) found in plant tissue under natural conditions for *J. vulgaris* (Hol et al. 2003; Kowalchuk et al. 2006). PA plant extracts from *J. vulgaris*, containing a bouquet of different PAs, was the most active inhibitor. The tested PAs never stopped fungal growth totally. After initial growth-delay the fungi were able to adapt and to grow normally within 30 days. Hol and van Veen (2002) hypothesized that the observed temporary mycelium growth-delay may have serious ecological consequences for the affected fungi, as it may impact the competition between microorganisms in the rhizosphere and will give plant roots extra time to raise its defences or escape (Hol and van Veen 2002). Mycelium growth was not in all cases negatively influenced by the PA treatments but depended on where a particular fungal line originated from. The potential adaptation of isolated fungi from PA-producing plants will be further discussed below.

Based on the published literature the majority of *in-vitro* studies showed a negative effect of PAs on microorganisms. Although results of *in-vitro* studies cannot be translated easily to ecologically relevant conditions, these studies indicate that PAs can play an important role in the defence against microorganisms in natural systems.

Adaptation by microorganisms to alkaloids

The diversity in plant defence compounds is thought to result from the arms race between the plant and its attacker (Ehrlich and Raven 1964). This biochemical co-evolutionary theory assumes that, in response to adaptation by the attacker, plants need to synthesize new defence compounds by modification of the original compound. Crucial in this hypothesis is the ability of the attackers to adapt to defence compounds. For insects it has been shown that indeed they can (Lindigkeit et al. 1997). For microorganisms the evidence is less clear. However, given their short generation time and the extremely high numbers of individuals one might expect an even higher ability to adapt to chemical defence compounds. In addition it has been suggested (Hol 2003) that microbial pathogens may be stronger selective agents than herbivore attackers because they are more likely to kill hosts in contrast to herbivores, which often abandon their host after defoliation. Although potentially an interesting hypothesis, the evidence to support this is weak. Adaptation is known in microbiology as a development of resistance. Many resistance mechanisms are known, such as making it more difficult for the toxin to enter the microorganism, and chemical modification of the toxic compound which results in inactivation of the toxin. Adaptation by fungi to secondary

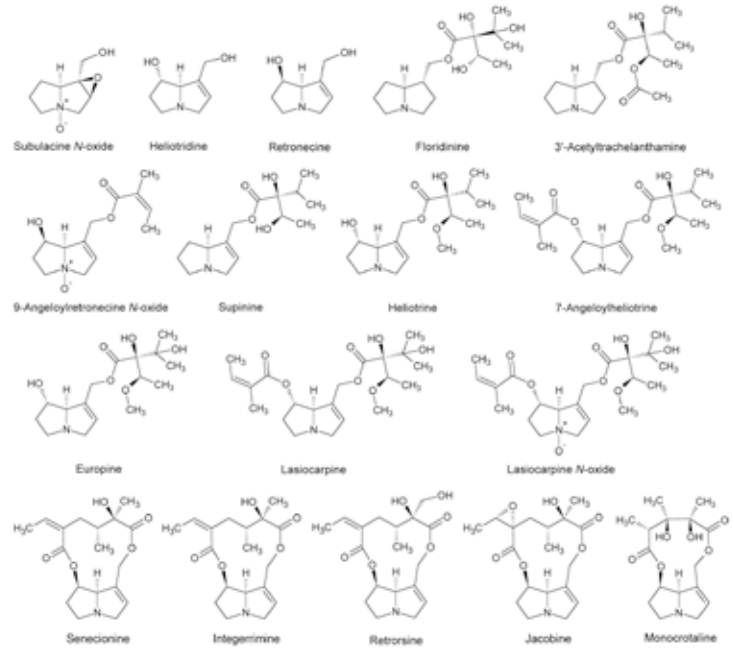


Figure 1. Effective chemical structures of pyrrolizidine alkaloids against microorganism mentioned in this manuscript

metabolites is known for a variety of compounds such as steroidal glycoalkaloids (Osbourn 1996; Morrissey and Osbourn 1999) and phytoalexins (Soby et al. 1996). Adaptation by inactivating the toxin could be followed by the ability to utilize the compounds as substrate for energy and biomass production or storage for their own defence against attackers (McGonigle and Hyakumachi 2001). Endophytic symbionts, for instance, need to cope with the secondary metabolites of their host plant, like *Rhizobium* specimens. They form symbioses with members of the leguminoseae, such as lupines, which produce large amounts of alkaloids, in particular, quinolizidine alkaloids (Wink 1988; Harborne 2003). Werner et al. (1997) showed that endophytic fungi were able to metabolize the polyamine alkaloid aphelandrine. Several fungi were capable to use part of the polyamine alkaloid as nitrogen source. Nearly all fast-growing endophytes of the family of *Nectriaceae* were able to degrade and to grow on this alkaloid while slow-growing endophytes could only partially degrade aphelandrine.

A study by Hol and van Veen (2002) showed that there is a remarkable difference in fungal sensitivity in relation to the host plant. Five *F. oxysporum* lines, isolated from different host plants (2x *J. vulgaris*, *Carex arenaria*, *Senecio vulgaris* and unknown host plant) were stimulated by the PA bulk treatments. The stimulation of some isolates by the PA bulk suggests that adaptation may have taken place (Hol and van Veen 2002). Hol (2003) showed that fungi isolated from *J. vulgaris* roots were initially inhibited but, later, their growth was stimulated by different concentrations of a bulk PA mix extracted from the plant. This

suggests that adaptation took place. They suspected that the increased growth could be the result of the ability to use PAs as nutrition. In these experiments 75% of the nitrogen was present in the form of PAs (Hol 2003). However, the idea that PAs are used as nutrition is merely an assumption because the study was not targeted at finding direct evidence that fungi were able to metabolize and use PAs as a source of nitrogen. It would be very interesting to test if the fungi actually inactivate PAs and utilises PAs for further growth. Three fungi, which were sensitive to retrorsine, were grown on 5 mM retrorsine during 10 days. Hol (2003) found that after these 10 days of pre-culturing fungi on water agar containing retrorsine, *Pestalotiopsis* sp. was significantly less sensitive to the PAs retrorsine and integerrimine, while *Rhizoctonia* sp. and *Broomella acuta* showed the same sensitivity to PAs as after pre-culturing on water agar alone. Hol (2003) suggested that the increased performance of *Pestalotiopsis* sp. was probably a phenotypic change. Apparently rapid phenotypic adaptation does occur. When it is this easy for fungi to adapt to PAs, we might question whether PAs are effective as antifungal defence. Tolerance to PAs was shown to be species specific and a variation was shown in PA activities. The fact that some fungi were tolerant or even stimulated by PAs does not deny the antifungal activity of PAs against other fungi. Antifungal effect of major PAs such as senecionine, seneciphylline, erucifoline and jacobine in the roots of PA-producing plants have not yet been tested.

There are also other microorganisms in an entire different biotope that are able to cope with PAs. Ruminal bacteria living in the stomach of some mammals can transform PAs before absorption into the body of the host may occur (Lanicang 1970). This may result in detoxification of the ingested PAs, which may be life saving for the host mammals. It is well known that PA-containing plants cause extensive livestock losses all over the world (Craig et al. 1991). When parts of these plants are ingested in substantial quantities, PAs can cause acute and chronic liver damage in large mammals including humans (Mattocks 1986). Sheep and goats are relatively resistant to the toxic effects of PAs thanks to bacterial decomposition of PAs in their stomach (Cheeke 1988; Stegelmeier et al. 1999). The microorganisms that detoxify PAs have not yet been identified but Wachenheim et al. (1992) found that jacobine biotransformation involves multiple bacterial species and they suggested that gram-positive bacteria play a key role. The PA biotransformation was inhibited when ruminal antibiotics, especially against gram-positive bacteria, where used. These results might be very helpful for the identification and isolation of PA-transforming bacteria and can be applied to induce and enhance resistance to PAs in mammals (Lodge-Ivey et al. 2005). From the few studies that exist we can conclude that there are indications that adaptation of microorganisms to PAs occur. However, the evolutionary ecological consequences of this remains unclear. More research needs to be done to get a better insight on the adaptation mechanisms and strategies by microorganisms.

Ecological effects on pathogens; *aboveground*

Aboveground pathogens like rusts are common parasites of many plants among which PA-producing *Asteraceae*. *Puccinia dioicae*, for instance, is a common rust species on *J. vulgaris* (Harper and Wood 1957) and *Puccinia lagenophorae* is common on *S. vulgaris* (Paul and Ayres 1986a and b). *S. vulgaris* plants infected by *P. lagenophorae* are strongly hampered in their growth and reproduction. Reproduction was negatively influenced by rust infection because fewer plants flowered and per plant fewer buds, mature capitula and seeds were produced (Paul and Ayres 1986a and b). Studies on the effects of PAs on aboveground pathogens are very scarce. Infection of *S. vulgaris* by *P. lagenophorae* was enhanced at high-nutrient conditions (Tinney et al. 1998). This could have a relation with the PA production in the plant. Hol et al. (2003) found that high-nutrient conditions decreased the PA concentrations in *J. vulgaris*. This may imply that the increased mycelium growth of pathogenic fungi such as *P. lagenophorae* may be related to lowering of the PA concentration in the host at higher nutrient concentrations. However in contrast with this suggestion, *P. dioicae* was found to cause most damage on *J. vulgaris* plant containing high PA levels including the alkaloid jacobine. The relationship between PA-producing plants and their aboveground pathogens in the field need further investigation before any conclusions can be drawn on their relation with PAs.

Ecological effects on pathogens; *belowground*

Perennial plant species contain high food reserves in the roots for vegetative reproduction and re-growth. Also, this helps plants to tolerate aboveground herbivory such as complete defoliation by specialists like *T. jacobaeae* on *J. vulgaris* (van der Meijden et al. 1988 and 2001). The roots of these plant species are used for production and storage of PAs (Hartmann and Ober 2000). The concentration of PAs in the root crown of fully grown *J. vulgaris* plants in the field can be up to 4 mg/g dry weight (Kowalchuk et al. 2006). Thus, one could expect that these roots may strongly be protected against attackers.

The tissue in which plant defence compounds are stored is often crucial for their effectiveness. Hol et al. (2003) studied the distribution of PAs over different root parts. They observed that the concentration in the main root cortex was five times higher than the concentrations in the vascular cylinder. This suggests that a first line of defence against microbial attackers from the outside world may be created by tissue containing the highest concentration of PAs. The mature root parts are probably well protected because of its importance for re-growth after damage. Therefore from an evolutionary point of view it is reasonable that they contained the highest PA concentrations.

Recent observations point to certain clues on what type of PAs in *J. vulgaris* act as key players in root protection (Hol et al. 2003; Hol et al. 2004; Kowalchuk et al. 2006). When the roots or shoots of this species were damaged, jacobine and seneciphylline levels increased in the roots (Hol et al. 2004). This suggests that these PAs are important for root protection when the plant is under attack belowground. Hol et al. (2003) also found that an increase of nutrients in the soil decreased the PA concentrations in *J. vulgaris* probably due to a dilution effect. The biomass of the plant increased when nutrient levels rose mainly due to increases in the aboveground biomass, whereas the belowground plant biomass did not change significantly. Since the PA production is closely linked to root growth (Frischknecht et al. 2001) the total plant PA amount remained constant, therefore the concentration decreased as the total plant biomass increased. The concentrations of all PAs decreased with one exception. The concentration of jacobine remained constant in the shoots and increased in the roots when the total plant biomass increased. This emphasises the potential importance of jacobine for the defence of the plant (Hol et al. 2003). A field study on the role of root PAs in relation to fungi present in the rhizosphere of *J. vulgaris* pointed in the same direction. High-PA plants (1.13-3.92mg/g dw) with jacobine as the major root PA suppressed the development of microorganisms by inducing a lower diversity of fungi in the rhizosphere compared to low-PA plants (0-0.53mg/g dw) or high-PA plants lacking jacobine in the root (Kowalchuk et al. 2006).

The presence of defence compounds such as PAs plays a role in the selection processes that shape the soil-borne microbial community present in the rhizosphere as shown in the above described study by Kowalchuk et al. (2006). This selection might favour those microorganisms that are tolerant or resistant to these defence compounds or in some cases even can degrade or utilize them. As described above, the highest PA concentrations in the roots were found in root cortex and the lateral roots instead of the vascular cylinder (Hol et al. 2003). Thus, we may expect that at least by root damage and sloughed of root cells PAs leak into the rhizosphere. Plants may also actively secrete PAs into the soil but, to our knowledge, this has never been tested neither have the exact PA levels in the rhizosphere been measured. The exact

role that defence compounds play in plant protection against root-infecting bacteria and fungi is still not fully understood. Measuring low levels of chemical compounds that occur in the rhizosphere of the plant is still a challenge study area because of inadequate methods for analyses (Bais et al. 2006).

PA induction by microorganisms

Many studies on the diversity and effectiveness of PAs as defence compounds have been performed with *J. vulgaris* and *S. vulgaris* of the *Asteraceae* family, especially in relation to insects. The concentration and composition of PAs in plant species is genotype dependent but also affected by the environment (Vrieling et al. 1993; Hol et al. 2003; Macel et al. 2004; Hol et al. 2004; Joosten et al. 2009; Macel and Klinkhamer 2010). Less is known about PA induction by pathogens aboveground. Tinney et al. (1998) found no significant effect of *P. lagenophorae* on the total PA concentration of *S. vulgaris* and hardly any effect on the PA composition, although infection caused a reduction in growth. The root dry weight of the infected plants was significantly lower compared to the uninfected control plants, while no significant difference was found for the vegetative tissue aboveground. We would expect that the PA concentration in the infected plants should be lower since PA production is closely linked to root growth (Hol et al. 2003). When focussing on particular plant parts instead of the whole plant, a significantly lower PA concentration in the rust infected plants were found in capitula and roots compared to the uninfected plants but not in the vegetative tissue which contained 90% of the total PA concentration. Although the rust infection caused reduction in growth for *S. vulgaris*, it had little influence on the overall PA concentration. PA synthesis was not induced by the aboveground rust infection but changes in PA distribution could be one of the effects (Tinney et al. 1998). This result is consistent with the conclusions from a study on *Cynoglossum officinale* (van Dam and Vrieling 1994), where mechanical wounding in leaves did not induce PA synthesis.

Bezemer et al. (2006) found that aboveground herbivory was related to the fungal community belowground. They suggested that the fungal community directly (Joosten et al. 2009) or indirectly (Hol et al. 2003) changed the concentration of different PAs in the shoots and in this way affected the aboveground insect community. Unfortunately, the PA concentrations were not measured. 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. Low nutrient levels in soil (Hol et al. 2003) and root damage (Hol et al. 2004) has been shown to result in an increased PA concentration in the shoots of *J. vulgaris*. Joosten et al. (2009) found a strong effect of soil-type and soil-borne microorganisms on the composition of PAs in roots and shoots of this plant species. Clonal plants of two genotypes were grown on two sterilized soil and sterilized soils inoculated with with 5% of non-sterilized soil of either of the two soil-types. Statistically, the first two discriminant functions classified around 80% of all the combinations of soil-types and inoculation treatments correctly based on the PA expression in roots and shoots of both genotypes. In particular the levels of retrorsine and retrorsine *N*-oxide were case specific in response to specific soil inoculation. In the study of Hol and van Veen (2002), retrorsine and retrorsine *N*-oxide had inhibitory effects on mycelium growth of several plant-associated fungi. The levels of jacobine and jacobine *N*-oxide were raised in the shoots of plants grown on specific soils. This influence of soil-type and soil-borne microorganisms could have major ecological consequences as changes in the concentration of individual PAs aboveground may attract specialist herbivores while deterring generalists (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 for the success of invasive plants and for the biological control of plants.

Conclusions

The existing evidence on the role PAs play in plant defence against microorganisms is scarce especially in comparison to ecological studies on insects. However, the studies that are available do suggest a potential role of PA in the plant's defence strategy against microorganisms. Unfortunately hardly any field studies are performed on this topic, which is remarkable, given the results of the *in-vivo* experiments. *In-vitro* experiments show potential effects of plant-produced PAs on microorganisms. This idea is supported by *in-vivo* experiments, although in limited numbers. PA mixtures affect microorganisms *in-vivo* and *in-vitro* and variation exists in anti-microbial effects of different PAs (Hol et al. 2003). Several PAs tested showed that structurally related PAs differ in their effects on microorganisms but some PAs were more effective than others. High levels of jacobine were associated with lower fungal diversity in the rhizosphere, but the primary PA senecionine, did not have this effect (Kowalchuk et al. 2006). There are indications that adaptation of microorganisms to PAs occur, in particular microorganisms that were isolated from PA-containing plants showed a higher tolerance and more rapid adaptation (Hol 2003). The relationship between PAs and microorganisms need further investigation before the strength of microorganisms as selection factor can fully be assessed.

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The Analysis of Pyrrolizidine Alkaloids in *Jacobaea vulgaris*; a Comparison of Extraction and Detection Methods

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Abstract

Pyrrolizidine alkaloids (PAs) serve an important function in plant defence. In this study different extraction methods and detection techniques were compared, namely gas chromatography with nitrogen phosphorus detection (GC-NPD) and liquid chromatography tandem mass spectrometry (LC-MS/MS) with quadrupole analysers, for analysing PAs in *Jacobaea vulgaris*.

Both formic acid and sulfuric acid were tested for PA extraction from dry plant material. For GC-NPD, reduction is required to transform PA *N*-oxides into tertiary amines. Zinc and sodium metabisulfite were compared as reducing agents.

The lowest PA concentration measured with GC-NPD was approximately 0.03 mg/g and with LC-MS/MS 0.002 mg/g. The detection of major PAs by both techniques was comparable but a number of minor PAs were not detected by GC-NPD. With the LC-MS/MS procedure higher concentrations were found in plant extracts, indicating that losses may have occurred during the sample preparation for the GC-NPD method. Zinc proved a more effective reducing agent than sodium metabisulfite. The sample preparation procedure for LC-MS/MS analysis using formic acid extraction without any reduction and purification steps is far less complex and less time consuming compared to GC-NPD analysis with sulfuric acid extraction and PA *N*-oxide reduction with zinc and purification.

In terms of sensitivity and discrimination, formic acid extraction in combination with LC-MS/MS detection is the method of choice for analysing PAs (both free and *N*-oxide forms) in plant material.

Introduction

Pyrrolizidine alkaloids (PAs) are regarded as chemical defence substances against herbivores (Bentley et al. 1984; Dreyer et al. 1985; Vrieling et al. 1991; Leiss et al. 2009) and pathogenic fungi (Hol and van Veen 2002). Plants containing PAs such as *Jacobaea vulgaris* (also known as *Senecio jacobaea*) are an important food source for numerous insect species (Harper and Wood 1957). *Jacobaea vulgaris* originates from Europe and Asia (Cameron 1935; Harper and Wood 1957) and was introduced into North America, New Zealand and Australia (Pemberton and Turner 1990). In the introduced areas it is considered a pest species as it causes extensive livestock losses and is a potential contaminant in milk and honey. When parts of these plants are ingested in substantial quantities, PAs can cause acute and chronic liver damage in mammals including humans. It is therefore of great importance to develop analytical methods including sample preparation, extraction and detection techniques to get a better insight in the physiology, ecology and toxicology of PA containing plants. The mode of action in invertebrate species is not well known, but Frei et al. (1992) showed that PAs caused DNA damage. In general PAs deter generalist herbivores (van Dam 1995; Macel et al. 2005); however several specialist herbivores have adapted to PAs in their diet. In fact some specialists even prefer plants containing PAs (Boppré 1986; Hartmann and Witte 1995; Macel and Klinkhamer 2010).

PAs occur in free (tertiary amine) or *N*-oxide forms. Until now it has been generally assumed that in plants PAs are mainly present as *N*-oxide except in some seeds. However, recently we found that some PAs of *J. vulgaris* also occur in substantial amounts as tertiary amine (Chapter 4). This is of importance for understanding the role of PAs as plant defence compounds, as the tertiary PA form is known for its strong negative influence on several plant attackers. The *N*-oxide form is considered to be non-toxic (Lindigkeit et al. 1997).

For many years, PAs were typically isolated by acid-base extraction in combination with zinc reduction (Hartmann and Zimmer 1986; Witte et al. 1993). Gas chromatography (GC) with flame ionisation detection (FID) or nitrogen phosphorus detection (NPD) have been typically used as analytical methods (Hartmann and Toppel 1987; Witte et al. 1992; Hartmann and Dierich 1998; Kowalchuk et al. 2006). Over the years several other PA extraction methods (Pieters et al. 1989; Betteridge and Colegate 2005; Joosten et al. 2009; Jiang et al. 2009) and techniques for analysing PAs have been applied such as spectrophotometry (Mattocks 1967; de Boer 1999), TLC, HPLC-UV, GC-MS (Hartmann and Toppel 1987; Witte et al. 1992; Hartmann and Dierich 1998) and NMR (Pieters et al. 1989, Leiss et al. 2009). Recently liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been introduced for measuring PAs in plant material (Wuilloud et al. 2004; Betteridge and Colegate 2005; Zhang et al. 2008; Jiang et al. 2009; Joosten et al. 2009). Unlike GC-related methods, LC-MS/MS and NMR can detect both tertiary amines and *N*-oxides without the time-consuming and tedious reducing step.

The aim of this study was to compare two different methods for the analysis of PAs; the traditional method consisting of sulfuric acid extraction, reduction of PA *N*-oxide with zinc and then purification by liquid-liquid extraction followed by GC-NPD analysis. The other LC-MS/MS method involves a formic acid extraction without reduction and further purification steps. In addition we also compared some critical steps involved in the two methods (Figure 1). For example we investigated whether, irrespective of the sample preparation procedure, detection of (reduced) PAs with GC-NPD and LC-MS/MS (procedure 4, 6, 8 vs 5, 7, 9) give similar results with respect to the concentration and composition in plant material. Furthermore, we tested the efficiency of formic acid extraction as an alternative to sulfuric acid extraction (procedures 6, 7 vs 8, 9). We also evaluated another reducing agent, sodium metabisulfite ($Na_2S_2O_5$) as an alternative to the commonly used zinc dust (procedures 2 vs 3 and 4, 5 vs 6, 7) for GC-based analysis.

Finally we investigated whether the traditional and the novel analytical methods give similar results with regards to PA concentration and composition in plant material (procedure 1 vs 8).

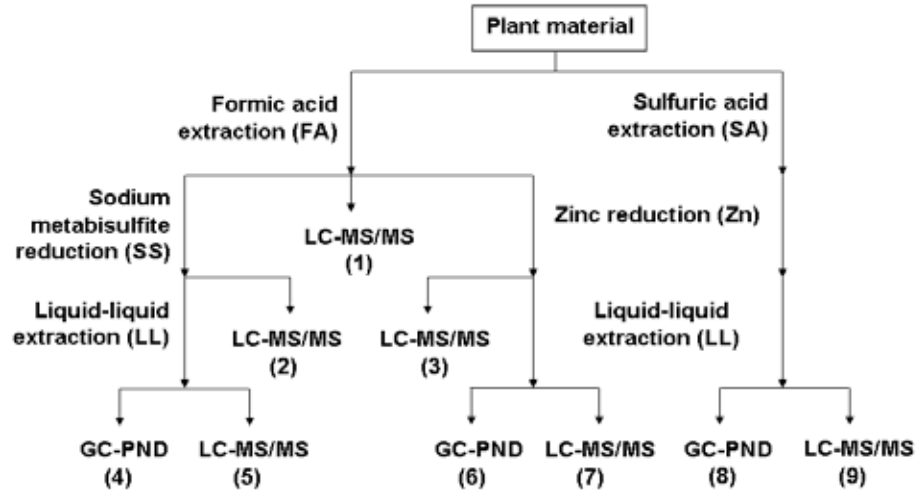

Figure 1. Extraction and detection of PAs from dried plant material and the different procedures applied. Dry plant material was extracted with formic acid (1-7) or sulfuric acid (8-9). The formic acid extract was either directly measured on the LC-MS/MS (1) or reduced with sodium metabisulfite (2, 4, 5) or reduced with zinc (3, 6, 7). The sulfuric acid extract was only reduced with zinc (8-9). The reduced extracts were purified by liquid-liquid extraction and analysed with GC-NPD (4, 6, 8) or LC-MS/MS (5, 7, 9). Reduced extracts were also analysed directly by LC-MS/MS before liquid-liquid purification (2-3). Procedure 8 is the traditional method and procedure 1 the relatively novel method.

Material and Methods

Plant material samples

Five dried *J. vulgaris* samples were used for PA analysis. One of the samples was used as a reference as it contained a mixture of several different *J. vulgaris* plants (aerial parts) collected in Meijendel (the Netherlands) in July, 1997. This sample had been used in the past for identification of PAs detected by GC-NPD. The other four samples (two roots and two shoots) were made from a genotype that originated from a dune population in Meijendel in our tissue culture laboratory. Two plants were propagated from the genotype by tissue culture and grown for six weeks in a climate room (relative humidity 70%, light 16h at 20°C, dark 8h at 20°C). After 6 weeks the two plants were harvested and cut above the root crown by scissors. The

plants were divided into roots (R1 and R2) and shoots (S1 and S2) and freeze-dried for 72 h under vacuum with a collector temperature of -55ºC (Labconco Free Zone® 12 l Freeze Dry System, Kansas City, MO, USA).

Pyrrolizidine alkaloid analysis

Formic acid extraction (procedures 1-7)

Ten milligrams of dried and ground plant material was extracted with 2% formic acid in a 1.2:100 w/v ratio. The plant extract solution was shaken for 1 h. Solid plant material was removed by centrifugation at 2600 rpm for 10 min. Heliotrine (100 µg/mL in methanol) was added as an internal standard to a concentration of 1 µg/mL. An aliquot of the extract (25 µL) was diluted with water (975 µL) and injected in the LC-MS/MS system (procedure 1 in Figure 1).

Sulfuric acid extraction with zinc reduction (procedures 8 and 9)

Ten milligrams of dried and ground plant materials was extracted with 0.25 M sulfuric acid (H_2SO_4) in a 1.5:100 w/v volume ratio. The plant extract solution was shaken for 1 h and subsequently the plant material was removed by centrifugation at 2600 rpm for 10 min. Zinc dust was added to the extraction solution and shaken for 2 h to reduce the PA *N*-oxides. Sulfuric acid extraction and zinc reduction (procedures 8 and 9 in Figure 1) were performed following the 'standard' extraction method of Hartmann and Zimmer (1986) and adapted for small quantities by de Boer (1999) for GC analysis.

Sodium metabisulfite reduction (procedures 2, 4 and 5)

One molar Sodium metabisulfite was added to the formic acid extract solution in a 1:80 v/v ratio and shaken for 2 h to reduce the PAs *N*-oxides. At this stage an aliquot was taken for LC-MS/MS analysis (25 µL), diluted with water (975 µL) and heliotrine (100 µg/mL in methanol) was added as an internal standard to a concentration of 1 µg/mL (procedure 2 in Figure 1).

Zinc reduction (procedures 3, 6 and 7)

A small "knife tip" of zinc dust was added to the formic acid extract solution and shaken for 2 h to reduce the PAs *N*-oxides. If during shaking no visible hydrogen bubbles were formed anymore, additional zinc was added. At this stage an aliquot (25 µL) was diluted with water (975 µL) and after addition of heliotrine (100 µg/mL in methanol) as an internal standard to a concentration of 1 µg/mL (procedure 3 in Figure 1), it was analysed by LC-MS/MS.

Purification by liquid-liquid extraction (procedures 4-9)

To the reduced extract, 25% ammonium hydroxide was added to reach a pH 9-10. The extract was purified by applying it over a 0.60 x 30 mm Extrelut® NT 1 column (Merck, Darmstadt, Germany). The column was eluted with dichloromethane. After elution the dichloromethane fraction was evaporated overnight and the residue was redissolved in 150 µL methanol (MeOH) containing 0.5 mg/mL heliotrine as internal standard.

Analysis by LC-MS/MS (procedures 1-3, 5, 7 and 9)

The aqueous extracts obtained from procedures 1-3 were analysed. From the methanolic extracts obtained after liquid-liquid purification (procedures 5, 7 and 9 in Figure 1) aliquots (10 µL) were diluted with water (990 µL) and injected into the LC-MS/MS system. The LC-MS/MS system consisted of an Agilent HP1100 HPLC equipped with a binary pump system, an autoinjector and a column oven, coupled to a Waters Micromass Micro tandem mass spectrometer.

Chromatographic separation was achieved on a Waters Xbridge 150 x 3.0 mm HPLC column, run with a water-acetonitrile linear gradient containing 0.05% ammonia (pH 11 ± 0.5) at a flow rate of 0.4 mL/min. The gradient started at 100% water (2 min) and during analysis the acetonitrile percentage was raised to 70% in 16 min. The column was kept at 40°C and the injection volume was 5 µL.

An electrospray ion source of the quadrupole mass analyser was used in positive mode, with the following instrument settings: capillary voltage, 2.7 kV; source temperature, 100°C; cone gas (N_2) flow, 50 L/h; desolvation gas (N_2) flow, 600 L/h; desolvation temperature, 400°C. Argon was used as collision gas at a pressure of 3.0×10^{-3} mbar. Data were recorded in multiple monitoring mode (MRM) using one selected precursor to product ion transition per compound. Based on the retention times of the individual PAs the transitions could be combined into three MRM windows. One window contained the early eluting PAs, and a second window the late eluting PAs. The PAs with precursor mass *m/z* 352 were combined into a third window, because they overlapped with the other two windows. In this way the number of simultaneously monitored transitions (channels) could be limited to 10 (dwell time 120 ms/transition) (Figure 2). Cone and collision energy settings were optimised for the individual compounds (Table 1). Obtained peak areas were internally calibrated using the internal standard heliotrine and the individual compounds were quantified against a standard solution of the PAs in water. Fourteen individual PA standards were available for this study, representing over 90% of the total amount of PAs present in the plant extracts. The remaining PAs, being tertiary amines as well as *N*-oxides, were quantified by using the mean response of the tertiary amine standards and the *N*-oxide standards, respectively. Data processing was conducted with Masslynx 4.0 software (Waters, Milford, MA, USA).

Analysis by GC-NPD (procedures 4, 6, 8)

The methanol extracts of procedure 3, 6 and 8 (Figure 1) were determined with GC-NPD (Hewlett Packard 6890 and a 30 m x 0.25 µm, HP-1) under the following conditions: injector 250°C, temperature programmed from 220°C (3 min) to 250 at 5°C/min, in the split mode (1:20), carrier gas flow (N_2) 0.9 mL/min, pressure 560 mbar, detector NPD. The injection volume was 1 µL. Quantitative analysis were performed via the NPD signals using heliotrine as internal standard.

Data processing was conducted with Microsoft Office Excel 2003 for Windows (Microsoft Corporation, Redmond, WA, USA). For identification of the tertiary PAs, retention times were compared with a reference sample (Table 2). The reference sample ('M') contained a mix of dried shoot material of several different *J. vulgaris* plants collected in Meijendel.

Data analysis

Data were all normally distributed. The correlation between the PA concentration measured by GC-NPD and LC-MS/MS was tested by the Pearson correlation coefficient with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Regression was based on 15 data points (5 samples x 3 different extraction methods). The regression coefficient was estimated by linear regression model II (Sokal and Rohlf 1981; de Jong and Klinkhamer 2005). We used 'two-factor ANOVA without replication' to analyse if the different methods and techniques did have a significant influence on the estimates of the total PA concentration and the estimates of the concentration of different major PAs. Regression and 'two-factor ANOVA without replication' were conducted with Microsoft Office Excel 2003 for Windows (Microsoft Corporation, Redmond, WA, USA).

Table 1. MS/MS fragmentation conditions and LC retention times for PA identified in *Jacobaea vulgaris* extracts. The mean PA concentration determined with the LC-MS/MS method.

Pyrrolizidine alkaloid	Abbreviation	Precursor to product ion transition (m/z)	Cone voltage (V)	Collision Energy (V)	Retention time (min)	Mean PA concentration (µg/g)	Occurrence in samples
Heliotrine	Hel	314.2 > 138.0	30	25	14.51		
Seneciphylline	Sp	334.2 > 120.0	40	30	15.35	56.3	5/5
Seneciphylline *N*-oxide	Spox	350.2 > 120.0	40	30	11.96	398.2	5/5
Spartioidine*	St	334.2 > 120.0	40	30	15.10	1.1	2/5
Integerrimine	Ir	336.2 > 120.0	40	30	16.00	13.2	5/5
Integerrimine *N*-oxide	Irox	352.2 > 120.0	40	30	12.52	153.6	5/5
Senecionine	Sn	336.2 > 120.0	40	30	16.24	90.9	5/5
Senecionine *N*-oxide	Snox	352.2 > 120.0	40	30	12.69	923.7	5/5
Jacozine*	Jz	350.2 > 94.0	40	40	13.19	7.6	5/5
Erucifoline*	Er	350.2 > 94.0	40	40	13.39	12.8	5/5
Erucifoline *N*-oxide*	Erox	366.2 > 94.0	40	40	9.97	33.3	5/5
Riddelliine	Rd	350.2 > 94.0	40	40	13.78	1.9	5/5
Riddelliine *N*-oxide	Rdox	366.2 > 94.0	40	40	10.83	21.4	5/5
Jacobine	Jb	352.2 > 120.0	40	30	13.81	482.5	5/5
Jacobine *N*-oxide	Jbox	368.2 > 94.0	40	40	10.88	600.7	5/5
Retrorsine	Rt	352.2 > 120.0	40	30	14.44	1.4	3/5
Retrorsine *N*-oxide	Rtox	368.2 > 94.0	40	40	11.53	3.15	2/5
Jacoline*	Jl	370.2 > 120.0	40	30	11.50	21.1	5/5
Jacoline *N*-oxide*	Jlox	386 > 94.0	40	40	9.20	9.6	5/5
Acetyl-seneciphylline	Acsp	376.2 > 120.0	40	30	18.54	6.0	5/5
Acetyl-seneciphylline *N*-oxide	Acspox	392.2 >120.0	40	30	14.43	6.7	5/5
Jaconine*	Jn	388.2 > 120.0	40	30	14.82	20.0	5/5
Jaconine *N*-oxide*	Jnox	404.2 > 94.0	40	40	11.14	5.3	3/5
Acetyl-erucifoline*	Acer	392.2 >120.0	40	40	16.65	8.1	5/5
Acetyl-erucifoline *N*-oxide*	Acerox	408.2 > 94.0	40	40	13.05	38.9	5/5

Table 2. GC-NPD retention time of individual PAs and the mean PA concentration (tertiary amines + reduced *N*-oxides) in *Jacobaea vulgaris* extracts, determined with the traditional method.

Pyrrolizidine alkaloid	Abbreviation	Retention time (min)	Mean PA concentration (µg/g)	Occurrence in samples
Heliotrine internal standard	Hel	5.4		
Senecionine	Sn	7.3	485.9	5/5
Seneciphylline	Sp	7.6	282.1	5/5
Integerrimine	Int	8.1	92.1	5/5
Jacobine	Jb	9.5	433.4	5/5
Jacozine	Jz	9.9	23.9[a]	2/5
Jacoline	Jl	10.4	55.2[a]	1/5
Erucifoline	Er	11.0	8.7[a]	2/5

[a] Concentration may be an underestimate, because in some samples the amount could not be quantified.

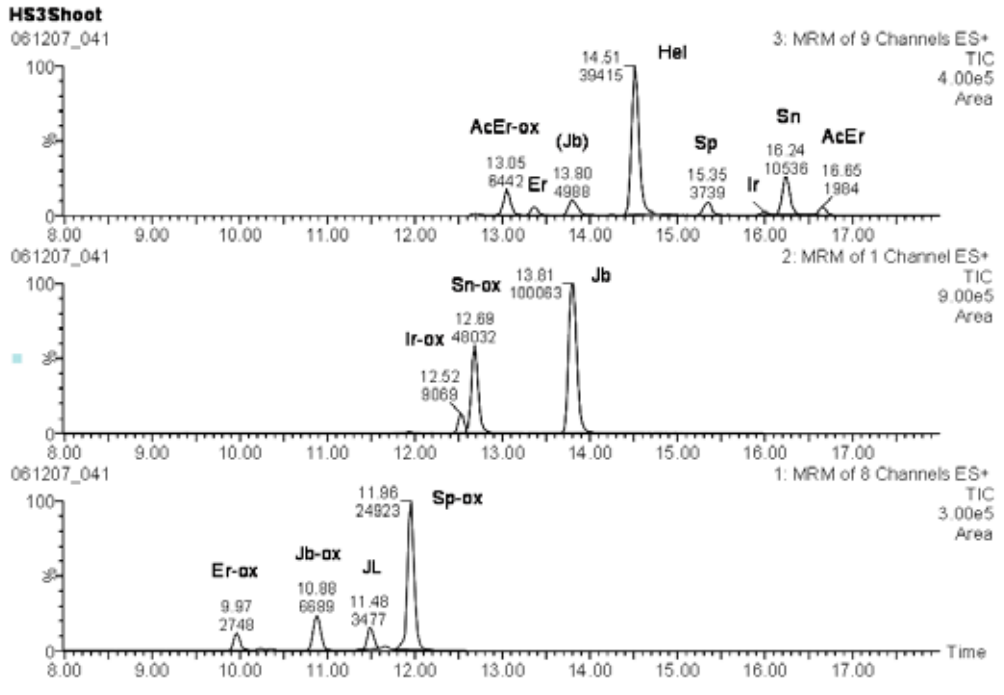

Figure 2. LC-MS/MS chromatograms of pyrrolizidine alkaloids in a *Jacobaea vulgaris* shoot extract. The various precursor to product ion transitions (channels) have been combined in this figure into three MRM windows. Mass spectrometer settings and abbreviations of individual PAs are as explained in Table 1. Some of the minor PAs are not visible in the combined MRM chromatograms. These PAs can be seen when the individual transitions are plotted (data not shown)

Results and Discussion

Detection of PAs by GC-NPD and LC-MS/MS

The GC-NPD and LC-MS/MS detection techniques for the determination of PAs in plant material were compared by the analysis of a number of extracts of dried *J. vulgaris* (procedures 4, 6, 8 vs 5, 7, 9). In this study the lowest PA concentration in dry plant material that could be quantified with GC-NPD was approximately 30 µg/g and with LC-MS/MS around 2 µg/g. The introduction of a tandem mass spectrometer as a detector in combination with liquid chromatographic separation thus greatly improves the possibilities of determination of individual PAs by lowering the detection limits (Betteridge and Colegate 2005; Wuilloud et al. 2004). It should be noted that, for LC-MS/MS analysis, the plant extracts had to be diluted 40 times, to keep the peak responses of the most abundant PAs within the linear dynamic range of the mass spectrometer, while for GC-NPD analysis the final extracts had to be concentrated approximately four times. As a result LC-MS/MS detected up to 13 different tertiary PAs in *J. vulgaris* extracts while GC-NPD detected only 7 PAs (Tables 1 and 2). With GC-NPD only four major PAs (senecionine, seneciphylline, integerrimine and jacobine) were detected in all samples (Figure 3). The concentrations of the other three PAs were sometimes just below the quantitation limit of the GC-NPD (Table 2).

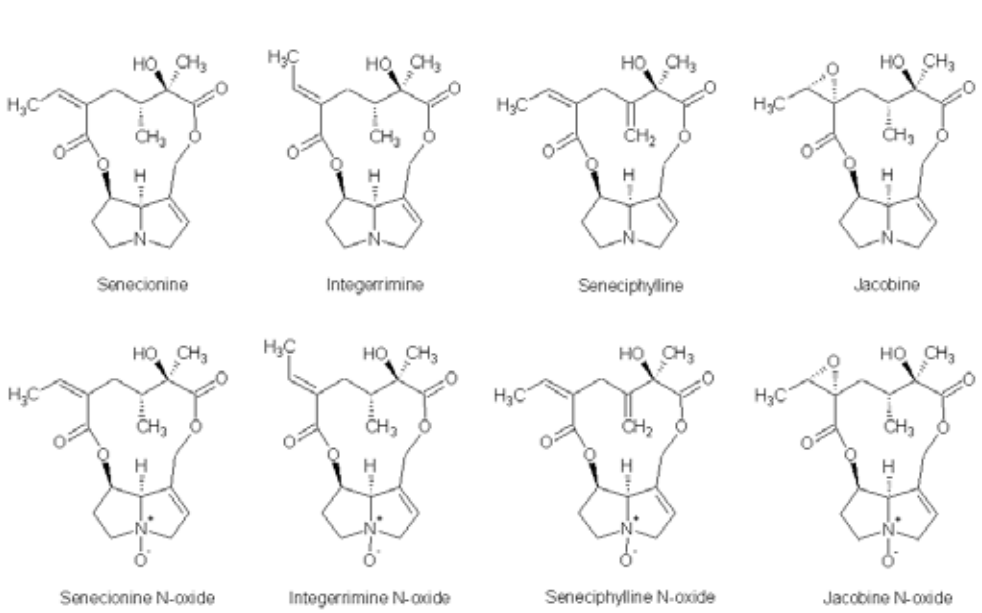

Figure 3. The chemical structures of tertiary amine and *N*-oxide forms of four major PAs present in the *Jacobaea vulgaris* extracts

Omission of the reduction step resulted in the additional detection of 11 different PA *N*-oxides by LC-MS/MS. Thus the number of PAs detected is almost doubled. It is a major advantage of LC-MS/MS that it can determine both *N*-oxides and tertiary amines directly, without the necessity of reduction of *N*-oxides to the corresponding tertiary amines, as is required for GC-based methods.

GC-NPD and LC-MS/MS measurements of the reduced extracts (procedures 4-9) were found to be highly correlated with respect to the total PA concentration (r^2=0.98) as well as the four major individual PA concentrations separated (Figure 4).

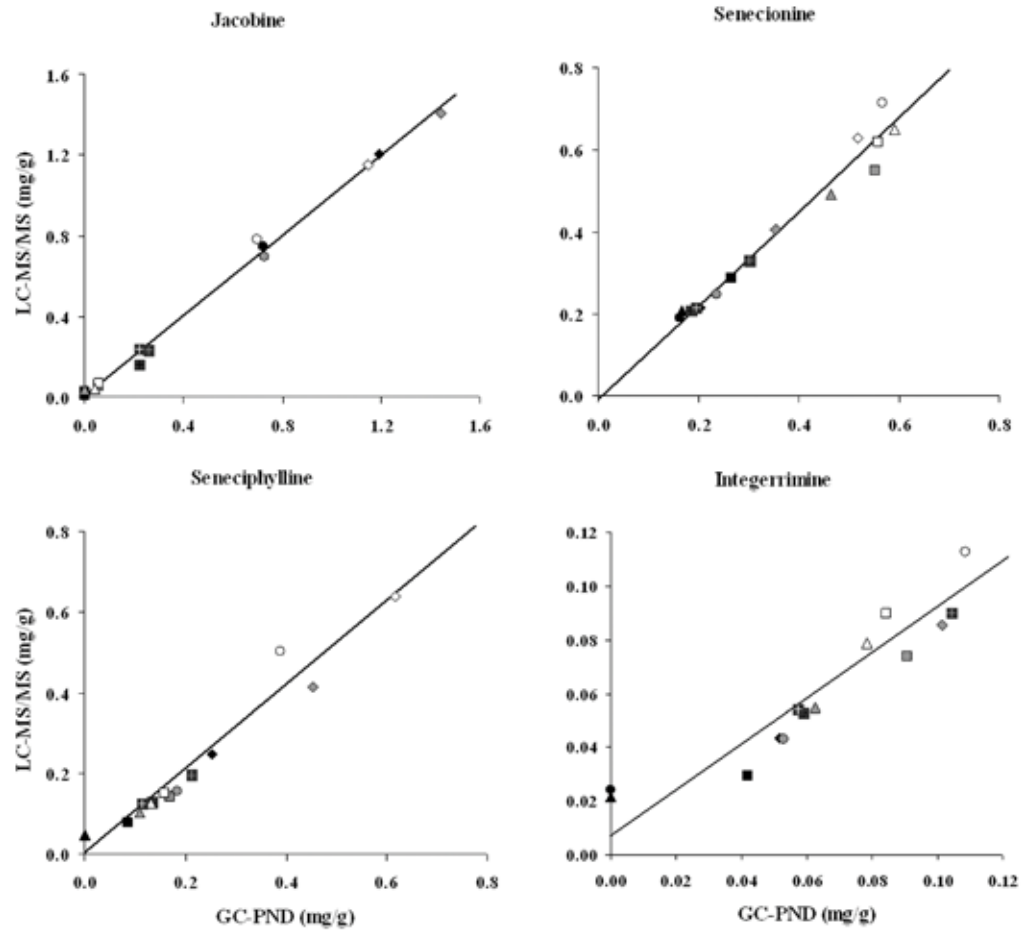

Figure 4. Relationship between the concentrations of jacobine (y=0.995x+0.006 r^2=0.996 p<0.01 n=15), senecionine (y=1.146x-0.008 r^2=0.972 p<0.01 n=15), seneciphylline (y=1.057x-0.009 r^2=0.955 p<0.01 n=15) and integerrimine (y=0.854x+0.007 r^2=0.904 p<0.01 n=15) concentration (mg/g dry plant material) in samples analysed by GC-NPD and LC-MS/MS. Fifteen different points represent five samples (■= Root 1, ◆= Shoot 1, ▲= Root 2, ●= Shoot 2 and + = reference sample) and three extraction/reduction methods (black = 4 and 5, grey = 6 and 7 and white = 8 and 9; Figure 1)

Formic acid versus sulfuric acid as extraction solvent

Formic acid is often used in analytical methods with LC-MS detection. Formic acid forms volatile salts when combined with a mobile phase that contains ammonia as modifier. Sulfuric acid, on the other hand, will form non-volatile salts that can precipitate in the source of the mass detector, and in the long run will affect sensitivity and reproducibility. For this reason direct injection of (diluted) sulfuric acid extracts into the LC-MS/MS system is not recommended. The formic acid and sulfuric acid extractions were compared (procedure 6 vs 8 and 7 vs 9 in Figure 1 and Figure 5). Exchange of sulfuric acid as extraction solvent by formic acid did not lead to significant differences in concentration of the total PAs or the individual major PAs when measured with GC-NPD or LC-MS/MS.

Zinc versus sodium metabisulfite as reduction method

Reduction of PA *N*-oxides with zinc dust is commonly used when the analysis is performed by GC-based methods. Hartmann and Toppel (1987) described the use of sodium dithionite ($Na_2S_2O_4$) in sulfuric acid as alternative to zinc as reducing agent. We observed that reduction with sodium dithionite in combination with formic acid proceeded very slow. As an alternative we tested sodium metabisulfite ($Na_2S_2O_5$). A 10 mM solution of sodium metabisulfite in dilute formic acid efficiently reduced PA *N*-oxides. To compare sodium metabisulfite reduction with zinc, the total PA concentration of formic acid extraction before purification was measured with LC-MS/MS (procedure 2 vs 3 in Figure 1). Zinc reduction gave a higher total PA concentration compared with sodium metabisulfite, respectively 1.96 and 1.43 mg/g dry plant material ($F_{1,4} = 2.1$, $p = 0.224$), but this was not significant due to the high levels of jacobine in the two shoot samples (Figure 6a). For jacobine no significant difference was found between the two reducing agents ($F_{1,4} = 0.7$, $p = 0.451$). The concentrations of other major PAs nearly doubled when zinc was used instead of sodium metabisulfite, respectively for senecionine 1.9 and 1.2 mg/g ($F_{1,4} = 11.7$, $p < 0.05$), seneciphylline 0.4 and 0.2 mg/g ($F_{1,4} = 16.4$, $p < 0.05$) and integerrimine 0.15 and 0.08 mg/g ($F_{1,4} = 22.6$, $p < 0.01$) dry plant material. A similar rest was obtained when the total PA concentration among the three different extraction/reduction combinations (procedures 4-5, 6-7 and 8-9) was compared (Figure 5). Formic acid extraction in combination with sodium metabisulfite reduction resulted in the lowest mean total PA concentration, respectively 0.78 and 0.81 mg/g dry plant material according to GC-NPD and LC-MS/MS. Sulfuric acid extraction with zinc reduction produced the highest mean total PA concentration, 1.29 and 1.42 mg/g dry plant material, respectively. It can be concluded that on the whole zinc dust is somewhat more efficient in transforming PA *N*-oxides to the corresponding tertiary amines.

Comparison between GC-NPD and LC-MS/MS method

Sulfuric acid extraction in combination with zinc reduction of the *N*-oxides followed by liquid-liquid purification and GC-NPD analysis can be regarded as the traditional method of PA analysis (procedure 8 in Figure 1). Formic acid extraction without reduction and purification steps, followed by LC-MS/MS detection is the newer method (procedure 1 in Figure 1). For comparison of the LC-MS/MS data with those of the GC-NPD, the individual PA concentrations of the *N*-oxides and tertiary amines obtained for the LC-MS/MS samples were summed.

The LC-MS/MS method gave a significantly higher total PA concentration compared with the traditional method (Figure 6b), 2.18 and 1.29 mg/g dry plant material ($F_{1,4} = 10.7$, $p < 0.05$), respectively. For jacobine no significant difference was found between the two methods ($F_{1,4} = 4.4$, $p = 0.105$), but for the other three major PAs the concentration nearly doubled using the LC-MS/MS method. It should be noted that this difference was only a trend close to significance, for senecionine 0.49 and 1.01 mg/g ($F_{1,4} = 6.0$, $p = 0.07$), seneciphylline 0.28 and 0.45 mg/g ($F_{1,4} = 7.7$, $p = 0.05$) and integerrimine 0.09 and 0.17 mg/g ($F_{1,4} = 6.3$, $p = 0.07$) were obtained in dry plant material, respectively.

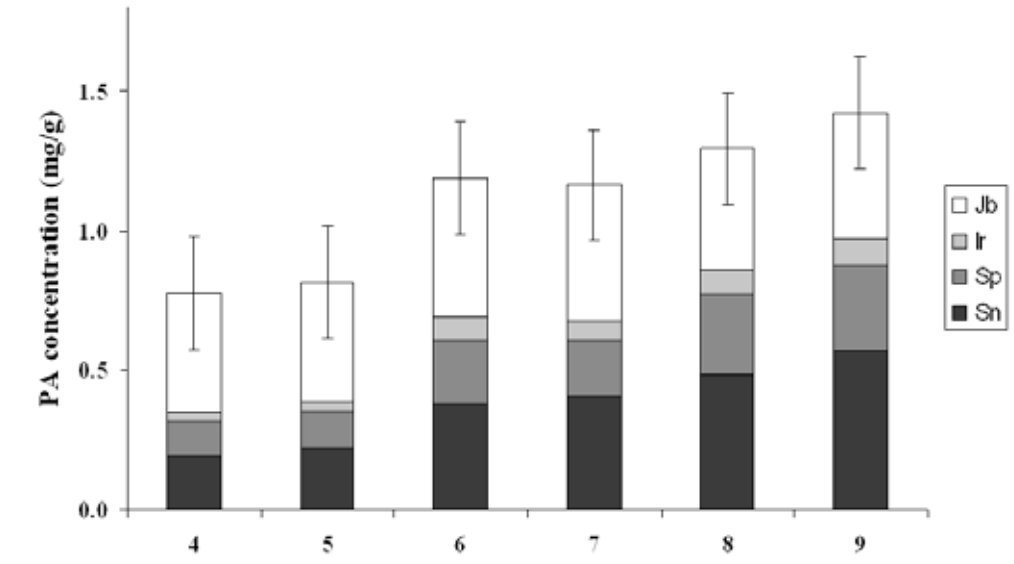

Figure 5. The mean PA concentration (±SE-bar of total PA concentration) of formic acid extracts reduced by zinc analysed with GC-NPD (4) or LC-MS/MS (5). Formic acid reduced by sodium metabisulfite with GC-NPD (6) or LC-MS/MS (7). Sulfuric acid extracts reduced with zinc analysed with GC-NPD (8) or LC-MS/MS (9)

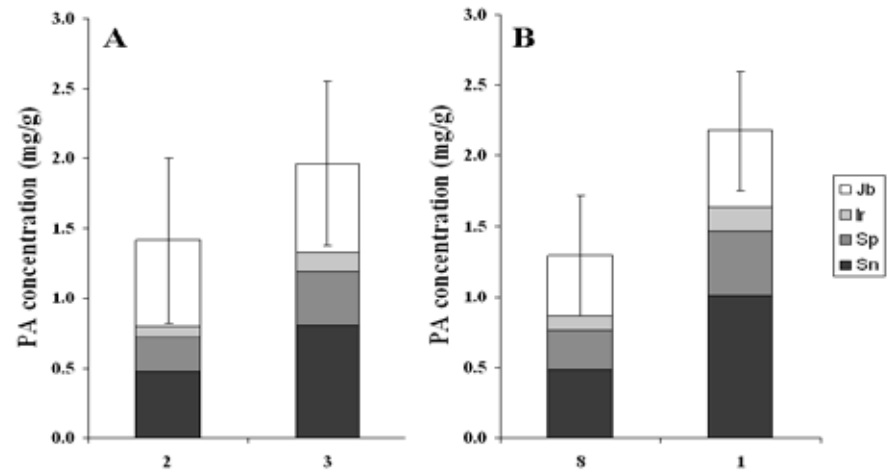

Figure 6. (A) The mean PA concentration (±SE-bar of total PA concentration) analysed with LC-MS/MS of formic acid extracts reduced by sodium metabisulfite (2) and by zinc (3). (B) The mean PA concentration obtained with the traditional method: sulfuric acid extraction, PA *N*-oxide reduction with zinc, purification and GC-NPD analysis (8) and the newer method: formic acid extraction with LC-MS/MS analysis (1)

The PA concentrations obtained by the two different extraction methods (formic acid vs sulfuric acid) and the two detection techniques (GC-NPD vs LC-MS/MS) were more or less comparable as described above. Nevertheless with the LC-MS/MS method a higher PA concentration was obtained than with the traditional method. This can only be explained by the fact that losses occur during zinc reduction and/or in the liquid-liquid purification step of the traditional method. Therefore it is preferable to use the novel LC-MS/MS method; without reduction and purification to minimize PA loss.

For a high sample throughput it is desirable to minimize the complexity of the extraction procedure and to minimize the number of analytical steps to obtain the final extract. The extraction method of the novel technique, namely with formic acid without any extra steps (except a simple dilution step), is far less complex and time consuming compared with the traditional method: extraction with sulfuric acid, reduction with zinc and purification for GC-NPD analysis.

The traditional method for PA analysis by GC-NPD and the newer method for PA analysis by LC-MS/MS were compared by measuring a number of dried *J. vulgaris* samples. The latter technique performed better also with respect to sensitivity, simplicity and selectivity as analysis by LC-MS/MS tolerates a much simpler sample treatment procedure than the classical method with GC analysis. Reduction of analytical steps will in the end result a higher sample throughput and this will allow more comprehensive studies on PA analysis of plant material. The simultaneous detection of PA *N*-oxides and tertiary amines in extracts widens the possibility of investigation of these two forms in biological matrices (plants as well as invertebrates and mammals). LC-MS/MS intrinsically offers a much higher sensitivity than analysis by GC. It is not necessary to use this higher sensitivity to the full in case of plant extracts where there is a plentiful supply, but it will be very valuable when minute amounts of material have to be analysed or when trace levels in biological samples need to be determined. This study shows that the concentrations measured in plant material by LC-MS/MS are higher than those measured by GC-NPD. This strongly indicates that losses occur during the reduction and purification steps required for GC-NPD analysis. These losses are minimised in the LC-MS/MS method.

Based on the simple and rapid sample preparation, sensitivity and discrimination between the two PA forms, formic acid extraction in combination with LC-MS/MS is the method of choice for determining PAs in plant material.

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The Genotype Dependent Presence of Pyrrolizidine Alkaloids as Tertiary Amine in *Jacobaea vulgaris*

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Lotte Joosten and Dandan Cheng contributed equally to this work.

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Abstract

Secondary metabolites such as pyrrolizidine alkaloids (PAs) play a crucial part in plant defence. PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide. PA extraction and detection are of great importance for the understanding of the role of PAs as plant defence compounds, as the tertiary PA form is known for its stronger influence on several generalist insects, whereas the *N*-oxide form is claimed to be less deterrent. We measured PA *N*-oxides and their reduced tertiary amines by liquid chromatography–tandem mass spectrometry (LC-MS/MS). We show that the occurrence of tertiary PAs is not an artifact of the extraction and detection method. We found up to 50% of tertiary PAs in shoots of Jacobine-chemotype plants of *Jacobaea vulgaris*. Jacobine and its derivatives (jacoline, jaconine, jacozine and dehydrojaconine) may occur for more than 20% in reduced form in the shoots and more than 10% in the roots. For 22 PAs detected in F_2 hybrids (*J. vulgaris* × *Jacobaea aquatica*), we calculate the tertiary amine percentage (TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100). We found that the TA% for various PAs was genotype-dependent. Furthermore, TA% for the different PAs were correlated and the highest correlations occurred between PAs which share high structural similarity.

Introduction

Pyrrolizidine 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 are present as mixtures of the tertiary alkaloids and the respective *N*-oxides in plants (Rizk 1991). It is generally accepted that in *Senecio* and *Jacobaea* plants PAs occur mainly or even exclusively in *N*-oxide form (Hartmann and Toppel 1987; Hartmann et al. 2004; Cao et al. 2008; Kempf et al. 2010).

In several *Senecio* and *Jacobaea* species, such as *Senecio 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 by specific enzymes, further diversification into different individual PAs takes place (Hartmann and Dierich 1998). The water soluble *N*-oxide form is considered to be ideal for phloem transport (Hartmann et al. 1989) and storage in cell vacuoles (von Borstel and Hartmann 1986; Ehmke et al. 1988).

Generalist insect herbivores reduce *N*-oxides in the gut to tertiary PAs, where these tertiary PAs are passively taken up into the body and when converted into pyrroles they are toxic by acting as highly reactive alkylating agents in mammals and fruit flies (Mattocks 1986; Frei et al. 1992). Since the PA *N*-oxides are reduced in the herbivore's gut, we could expect that it displays the same degree of toxicity as the respective tertiary amines. However in several studies was shown that individual PA *N*-oxides showed less deterrent or toxic effects for some generalist insect herbivores compared to the tertiary PAs (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005). Van Dam et al. (1995) found that three PAs from *Cynoglossum officinale* equally deterred feeding by *Spodoptera exigua* larvae, but the tertiary PA form deterred feeding more efficiently than the corresponding PA *N*-oxides. Macel et al. (2005) showed that retrorsine *N*-oxide was significantly less repellent to the locust *Locusta migratoria* compared to the corresponding tertiary PA. After 6 days on a diet of retrorsine *N*-oxide 60% of the thrips *Frankliniella occidentalis* survived against 0% on the tertiary PA. Specialist insects, i.e., some butterflies and moths (Lepidoptera), certain chrysomelid leaf beetles (Coleoptera) and the grasshopper *Zonocerus variegates* are adapted to PAs, sequestrate the tertiary PAs and specifically convert them into *N*-oxides which they store and utilize for their own chemical defence (Boppré 1986; Lindigkeit et al. 1997; Dobler 2001; Nishida 2002; Narberhaus et al. 2003).
For many years, PAs were typically isolated by acid-base extraction in combination with zinc reduction. Gas chromatography (GC) with flame ionisation detection (FID), nitrogen phosphorus detection (NPD) or mass spectrometric detection (MS) have typically been used as analytical methods. Recently liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been introduced for measuring PAs in plant material. Unlike GC-related methods, LC-MS/MS and NMR can detect both tertiary amines and *N*-oxides without an additional reducing step (Crews et al. 2010; Joosten et al. 2010). However, NMR needs relatively high concentrations of PAs for detection. LC-MS/MS is therefore a suitable and sensitive method to detect both forms of PAs.

We used LC-MS/MS to detect both forms of PAs. We found consistently large amounts of tertiary amines in *Jacobaea vulgaris* plants (Joosten et al. 2009 and 2010). However, the general tendency in literature is that tertiary amines are present only in very small amounts and maybe are due to artifacts during extraction or detection (Hartmann and Toppel 1987; Hartmann 1999; Hartmann and Ober 2000). PA *N*-oxides from *Senecio* plants are relatively unstable and are easily converted into their reduced form, the pre-toxic tertiary PAs under various experimental conditions. For example, the reduction increased upon prolonged heating of the sample (e.g. soxhlet extraction), when the amino acid cysteine was added and in the presence of plant material (Hartmann and Toppel 1987; Hösch et al. 1996). Therefore we tested our method for possible artifacts by several PA reduction and oxidation experiments with chemical agents and plant material.

Further proof of the presence of tertiary amines in living plant tissue can be obtained by showing that the concentrations of tertiary amines have a genetic basis and result from transformations by specific enzymes. It is already known that variation in composition and concentration of PAs in *J. vulgaris* has a large genetic component (Vrieling et al. 1993; Macel et al. 2004). In order to assess the genetic basis in the variation, the occurrence of the tertiary amine form, we conducted a crossing of *J. vulgaris*, which has high levels of tertiary amines, with the closely related *Jacobaea aquatica* (syn. *Senecio aquaticus*), which has low levels of tertiary amines (Cheng et al. 2011).

Here we report on studies to obtain a better understanding of the (bio)chemistry of PAs in above- and belowground plant parts of *J. vulgaris* and hence on the mechanisms of their activity as defence compounds against herbivores. Thus, we investigated: (1) the chemical reduction of three different PA *N*-oxides (representatives of the three structural groups) to assess the chemical PA (in)stability towards two different reducing agents; (2) the chemical oxidation of three different tertiary PAs to assess the chemical PA (in)stability towards an oxidation agent; (3) the spontaneous reduction of three different PA *N*-oxides in the presence of possibly reducing agents as well as the spontaneous *N*-oxidation of three different tertiary PA in the presence of possibly oxidation agents naturally occurring in plant material of several different *Asteraceae* species; (4) the spontaneous reduction of PAs during freeze-drying compared to immediate PA extraction from freshly ground material under liquid nitrogen; (5) the PA distribution in five different *J. vulgaris* genotypes by using an LC-MS/MS method for simultaneous measurement of PA *N*-oxides and tertiary PAs and (6) the genotype effect on the tertiary alkaloid relative content (TA%) for different PAs in the hybrids and the correlation between the TA% of different PAs.

Material and Methods

Standard PA extraction for LC-MS/MS

Freeze-dried plant material (approximately 10 mg) was extracted in 1 ml 2% formic acid. Heliotrine was added as internal standard to the extraction solvent at a concentration of 1 µg/ml. The plant extract solution was shaken for 30 min. After centrifugation the residual plant material was removed by filtering the extraction solution through a 0.2 µm nylon membrane (Acrodisk® 13 mm syringe filter). An aliquot of 25 µl filtered solution was diluted with 975 µl water and 10 µl was injected in the LC-MS/MS system.

Standard PA analysis by LC-MS/MS

A Waters Acquity ultra performance liquid chromatographic (UPLC) system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, PA, USA) was used for PA determination. Chromatographic separation was achieved on a Waters Acquity BEH C18 150 x 2.1 mm, 1.7 µm, UPLC column, kept at 50 °C and ran with a water/acetonitrile linear gradient containing 6.5 mM ammonia at a flow of 0.4 ml/min. The gradient started at 100% water and during analysis the acetonitrile percentage was raised to 50% in 12 min.

The MS system was operated in positive electrospray mode and data were recorded in multiple monitoring mode using two selected precursor ion to product ion transitions per compound. Cone and collision energy settings were optimized for the individual compounds. Obtained peak areas were

internally calibrated using the internal standard and the individual compounds were quantified against a standard solution of the PAs in an extract of the non-PA containing asterid *Tanacetum vulgare* to mimic the plant matrix. Seventeen individual PA standards were available for this study, representing over 90% of the total amount of PAs present in the plants extracts. Senecionine, seneciphylline, retrorsine and their *N*-oxides as well as senkirkine were available from commercial sources (Phytolab, Vestenbergsgreuth, Germany; Phytoplan, Heidelberg, Germany). Integerrimine was obtained as a kind gift of Dr. Trigo (UNICAMP, Campinas, Brazil). Riddelliine and its *N*-oxide were obtained as a kind gift from Dr. Chou (NCTR, Jefferson, AR, USA). Acetylseneciphylline was obtained by acetylation of seneciphylline with acetic anhydride and pyridine. Jacobine and erucifoline were isolated from *J. vulgaris* plant material (PRISNA, Leiden, the Netherlands). The identity of the standards isolated was confirmed by 1H-NMR and LC-MS analysis. *N*-oxides of integerrimine, jacobine, erucifoline and acetylseneciphylline were prepared by *N*-oxidation according to the method of Christie et al. (1949), adapted by Chou et al. (2003). The remaining PAs, being tertiary PAs as well as *N*-oxides, were quantified by using the response of a structurally related standard. Data processing was conducted with Masslynx 4.1 software.

Chemical reduction of PA *N*-oxides

A mixture of three PA *N*-oxides (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide, 1 µg /ml) was exposed to the reducing agent sodium metabisulfite ($Na_2S_2O_5$) in a range of 5 concentrations (0, 0.01, 0.03, 0.1, 0.3, 1 mM) in 2% formic acid solution. After 1, 4 and 24 h of incubation at room temperature the solutions were diluted 10-fold with water and injected in the LC-MS/MS system. The same mixture of standards was also exposed to the amino acid cysteine at three concentrations (1, 10 and 1000 mM), in two different solutions, 2% formic acid and water.

The relative amount of tertiary PA present in a sample was calculated as the measured concentration of tertiary PA divided by the sum of the concentration of tertiary PA and corresponding PA *N*-oxide.

A three-way ANOVA with two replications was used to analyze if PA type (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide), reducing agent concentration (0, 0.01, 0.03, 0.1, 0.3, 1 mM) and incubation time (1, 4, 24 h) have a significant influence on the relative concentration of tertiary PAs formed by reduction of the added PA *N*-oxides. The analysis was made by General Linear Model (GLM) univariate analyses procedure with the relative concentration of tertiary PAs as the dependent variable and PA type, reducing agent concentration and incubation time as fixed factors. All tests were conducted with SPSS 17.0 for Windows.

Chemical *N*-oxidation of tertiary PAs

The three individual tertiary PAs (senecionine, jacobine and erucifoline), were added to five concentrations (0.01, 0.03, 0.1, 0.3, 1 mM) of the oxidation agent hydrogen peroxide (HOOH) in 2% formic acid solution. After 1, 4 and 24 h of incubation at room temperature the solutions were diluted 10-fold with water and injected in the LC-MS/MS system.

The relative amount of *N*-oxide present in the sample was calculated as the measured concentration of the PA *N*-oxide divided by the sum of the concentration of PA *N*-oxide and the corresponding tertiary PA.

The same statistical test was used as for the chemical reduction experiment described above, to analyze if PA-structural group (senecionine, jacobine and erucifoline), reducing agent concentration (0,

0.01, 0.03, 0.1, 0.3, 1 M) and incubation time (1, 4, 24 h) did have a significant influence on the relative concentration of PA *N*-oxides formed by *N*-oxidation of the added tertiary PAs.

PA *N*-oxide reduction and PA *N*-oxidation in the presence of plant material

Three PA *N*-oxides (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide) and three tertiary PAs (senecionine, jacobine and erucifoline) were added separately to dry plant material of five different flowering *Asteraceae* species collected in the field, *S. gigantea*, *E. cannabinum*, *S. sylvaticus, J. erucifolia* (syn. *Senecio erucifolius*) and *J. vulgaris* (erucifoline chemotype). *S. gigantea* contains no PAs, *E. cannabinum* contains the lycopsamine type of PAs, *S. sylvaticus* contains the triangularine type of PAs, and *J. erucifolia* contains the senecionine type of PAs. Dried, ground plant material of the shoot (approximately 100 mg) was wetted with 500 µl distilled water containing senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide (10 µg of each PA) in 3-fold and incubated for 1 h. As a control, from each plant species one sample was wetted with water containing no PA *N*-oxides. After adding 10 ml 2% formic acid and heliotrine as internal standard (1 µg/ml), the plant extract was shaken for 30 min. After centrifugation, the residual plant material was removed by filtering the extraction solution through a 0.2 µm nylon membrane. An aliquot of 25 µl filtered solution was diluted with 975 µl water and injected in the LC-MS/MS system.

Species description

J. vulgaris (syn. *Senecio jacobaea*) is a suitable system to study PAs. This species is native in Europe and West Asia but invasive in North America, Australia and New Zealand. In previous studies, up to 30 different PAs were detected in *J. vulgaris* (Witte et al. 1992; Macel et al. 2004; Kowalchuk et al. 2006; Joosten et al. 2009). Based on their structural features, major PAs in *J. vulgaris* can be divided into 3 structural groups: senecionine-like, comprising senecionine, integerrimine, retrorsine and (acetyl)seneciphylline; jacobine-like, comprising jacobine, jacoline, jaconine jacozine, and dehydrojaconine; erucifoline-like, comprising erucifoline and acetylerucifoline (Table 2).

Based on the PA composition, 4 chemotypes of *J. vulgaris* were distinguished: Senecionine-chemotype, largely lacking jacobine- and erucifoline-like PAs; Erucifoline-chemotype, lacking jacobine-like PAs; Jacobine-chemotype, containing high levels of jacobine-like PAs; mixed chemotype, containing both jacobine- and erucifoline-like PAs in similar amounts (Witte et al. 1992; Macel et al. 2004).
J. aquatica is a close relative but not a sister species to *J. vulgaris* (Pelser et al. 2003). These two species naturally hybridize in some areas and the hybrids can backcross into the parental populations (Kirk et al. 2004 and 2005)

Effect of freeze-drying on the tertiary PA content

Freeze-drying is a general used method to dry plant material before analyzing PAs in plant material. In this way enzymatic activity can be prevented or at least strongly reduced. We tested if the freeze-drying can lead to spontaneous reduction of PAs. To compare freeze-dried material to the original plant condition we extracted PAs from fresh plant material as control treatment. Liquid nitrogen was used to ground fresh plant material under deep frozen conditions.

Plant material

One genotype of *J. vulgaris* originating from a population near Wageningen was used to study if reduction can take place during freeze-drying. The plants were propagated by tissue culture. In total eight clones per treatment (PA extraction of fresh material versus PA extraction of freeze-dried material) were

used. The plants were potted in 1.3 l pots filled with potting soil (Slingerland Potgrond, Zoeterwoude, the Netherlands). The plants were kept in a climate room for 6 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C) and randomly distributed every 8-10 days.

PA extraction from fresh and freeze-dried material

The shoot of each plant was cut lateral in two pieces with scissors so each part had an equal number of leaves of similar size, one shoot part for the control treatment and the other part for the freeze-drying treatment. The control part of the shoot was weighted, immediately ground under liquid N_2 and in frozen condition mixed in 20 ml of 2% formic acid containing 0.2 µg/ml heliotrine as internal standard. From this point on the standard PA extraction for LC-MS/MS was performed as described above. After weighting, the other half of the shoot was immediately stored at -20 °C before being freeze-dried. After freeze-drying, the standard PA extraction for LC-MS/MS was performed.

Data analysis

The 9 major PAs and their corresponding *N*-oxides were included in the analysis. We excluded the minor PAs which had a concentration close to detection limit and for which the ratios were not reliable. The relative concentration of tertiary amine (TA%) were calculated as: TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100. To calculate the percentage of *N*-oxides in fresh material transformed to tertiary amines during freeze-drying, the following formula was used to calculate the relative reduction amount of the *N*-oxides: (tertiary amine concentration in freeze-dried material – tertiary amine concentration in fresh material)/*N*-oxide concentration in fresh material. The difference of total PA, individual PAs and relative concentration of tertiary amines between the two methods were evaluated by paired *t*-test, with the absolute concentration of total PA, individual PA and TA% as the dependent variable, respectively. To test whether different individual PAs had a different amount of reduction from *N*-oxides to tertiary amines, a one-way ANOVA was performed with the relative reduction amount as variable and individual PA as group factor. All tests were conducted with SPSS 17.0 for Windows.

PA analysis for *J. vulgaris*

Plant material and PA analysis

Five different genotypes of *J. vulgaris* were used representing two chemotypes: three Jacobine-chemotypes and two Erucifoline-chemotypes. Two Jacobine-chemotypes originated from two different populations in Meijendel near The Hague and the third originated from a population near Wageningen. The two Erucifoline-chemotypes originated from a Dutch population near Vilt (Limburg) and a German population near Kassel. The five different genotypes were propagated by tissue culture. In total eight clones per genotype were used. The plants were potted in 1.3 l pots filled with calcareous sandy soil collected from Meijendel, a coastal dune area North of The Hague. The plants were kept in a climate room for 5 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C) and randomly distributed every 8-10 days.

After 5 weeks the plants were harvested in order to determine the PA concentration and composition. The plants were cut with scissors just above the root crown and roots and shoots were immediately stored at -20 °C for 4 days before being freeze-dried for 1 week under vacuum with a collector temperature of -55 °C (Labconco Free Zone® 12 l Freeze Dry System). PAs were extracted by formic acid, as described above. An aliquot of 25 µl filtered solution was diluted with 975 µl water and injected in the LC-MS/MS system.

Data analysis

A two-way ANOVA was used to analyze if chemotype and plant part (root and shoot) have a significant influence on the TA%. The ANOVA was performed by GLM (General Linear Model) univariate analyses procedure with TA% as the dependent variable, chemotype and plant part as fixed factors. The tests were conducted with SPSS 17.0 for Windows.

Relative concentration of tertiary amine analysis for *Jacobaea hybrids*

Plant material and PA analysis

F_2 hybrids of two different species were used in this study; *J. vulgaris* subs. *dunensis* and *J. aquatica* subs. *aquatica*. Seeds were collected for *J. vulgaris* at Meijendel, a coastal dune area north of The Hague (the Netherlands) and for *J. aquatica*, a coastal dune area at Zwanenwater Reserve (the Netherlands). Crossings were performed by rubbing flower heads together. This cross resulted in numerous seeds which were germinated. Both species are self incompatible and all F_1 and F_2 seeds are true crosses confirmed by molecular analysis (unpublished data). Two F_1 individuals with rayed flowers were chosen and crossed reciprocally with each other resulting in offspring. The two parental, two F_1 and >100 F_2 individuals were maintained in tissue culture.

The plants used in this study were cloned from the tissue culture material. Beside the two parental genotypes (*J. vulgaris* and *J. aquatica*) and two different F_1 hybrids, 102 different F_2 hybrid genotypes were used. On average 6 cloned replicates per F_2 genotype and 12 cloned replicates per parental and F_1 genotype were grown. In total, 609 plants were used in this study, among which 562 were F_2 individuals. The plants were potted in 1.3 l pots filled with 95% sandy soil, collected from Meijendel, 5% potting soil (Slingerland Potgrond, Zoeterwoude, the Netherlands) and 1.5 g/l Osmocote (Scotts®, Geldermalsen, the Netherlands, N:P:K = 15:9:11). The plants were randomly distributed and kept in a climate room for 6 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C). After 6 weeks the plants were harvested and prepared for LC-MS/MS analysis as described above.

Data analysis

Of the 37 detected PAs, 9 were otonecine structural group PAs for which no corresponding *N*-oxide exists and 6 were absent or close to the detection limit in some samples. The remaining 22 PAs were used to calculate the relative concentration of tertiary amine as TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100.

The genotype effect on TA% was statistically analyzed by a Kruskal-Wallis test with the TA% as the independent variable and genotype (including parental, F_1 and F_2, 106 genotypes in total) as the grouping variable. Spearman correlation matrix between the 11 kinds of TA% was calculated based on the mean TA% per genotype in root and shoot. *P*-values of the correlations were adjusted by Holm's method (Holm 1979). To determine if different type of plant material (root/shoot) had a different degree of the correlation between TA%, a paired *t*-test was done with the correlation values as the independent variable. Depending on the PA-structural group the specific PAs belong to, the correlations were divided into 6 categories: Category 1, correlation between the PAs of the senecionine-like PAs; Category 2, correlation between the PAs of the jacobine-like PAs; Category 3, correlation between the PAs of the erucifoline-like PAs; Category 4, correlation between the PAs of the senecionine- and jacobine-like PAs; Category 5, correlation between the PAs of the senecionine- and the erucifoline-like PAs; Category 6, correlation between the PAs of the jacobine- and the erucifoline-like PAs. Differences between the correlation values belonging to the different categories were analyzed with a one-way ANOVA with the correlation values as the

independent variable and correlation category (Category 1-6) as fixed factor. All tests were conducted with SPSS 17.0 for Windows, except for the correlation matrix and adjustment by Holm's method, which was conducted with R 2.10.0 for Windows.

Results

Chemical reduction of PA *N*-oxides

The chemical reduction of the three PA *N*-oxides, senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide, with sodium metabisulfite into their tertiary amines showed a significant difference ($F_{2, 87} = 10.8$, $P < 0.001$) in rate of reduction at any concentration of sodium metabisulfite added. Averaged over all incubation times (1, 4, 24 h) and reducing agent concentrations (0.01, 0.03, 0.1, 0.3, 1 mM) 42.2% (SE ± 0.63) of the jacobine *N*-oxide was reduced while 45.8% (SE ± 0.63) for both senecionine *N*-oxide and erucifoline *N*-oxide (Supplementary data Figure 1). However, the difference is not significant due to the analytical error, which is estimated at 10%.

Exposure of the three PA *N*-oxides to 1 M cysteine produced no measurable amount of tertiary amines after 24 h under acidic conditions (2% formic acid). However, under neutral conditions (water) with 1 M cysteine a very slow reduction occurred: after 24 h the production of senecionine, jacobine and erucifoline was respectively 1.9%, 4.2% and 2.7% (data not shown). The amounts of tertiary amines formed were too low to draw definitive conclusions about a difference in reactivity of the PA *N*-oxides towards cysteine and other potential sulfur-containing plant components. It should be pointed out that under the extraction conditions used in this study, the PA *N*-oxides displayed no measurable reactivity whatsoever towards cysteine. Interestingly, we found that 1 M cysteine catalyzed the isomerisation of senecionine *N*-oxide into integerrimine *N*-oxide notably under acidic conditions. After 24 h approximately 30% of senecionine *N*-oxide has isomerised to integerrimine *N*-oxide, under neutral condition this was only 14%. In the absence of cysteine the isomerisation in formic acid was less than 1% after 24 h.

Chemical *N*-oxidation of tertiary PAs

For the chemical oxidation under acidic conditions of the three macrocyclic tertiary PAs, senecionine, jacobine and erucifoline, with hydrogen peroxide (HOOH) into their *N*-oxides, relatively high concentrations of peroxide were required to induce oxidation at a measurable rate. Oxidation with HOOH proceeded much faster under neutral conditions (data not shown). Averaged over all incubation times (1, 4, 24 h) and oxidation agent concentrations (0.01, 0.03, 0.1, 0.3, 1 mM), 2.8% (SE ± 0.14) of the jacobine was oxidized while 1.0 (SE ± 0.14) and 1.1% (SE ± 0.14) for senecionine and erucifoline, respectively. The chemical oxidation of senecionine and erucifoline takes place with approximately the same rate, but that the oxidation of jacobine significantly proceeded faster ($F_{2, 105} = 48.6$, $P < 0.001$). The difference in rate was irrespective to the HOOH concentration. After 24 h with 1 M peroxide approximately 22.2% (SE ± 1.5) of jacobine had been converted to its *N*-oxide, while for senecionine the conversion was only 6.4% (SE ± 1.5) and for erucifoline 7.5% (SE ± 1.5) (Supplementary data Figure 2).

Extraction of tertiary PAs and PA *N*-oxides in the presence of dried plant material of five different Asteraceae species

The three PA *N*-oxides, senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide, in presence of dry plant material of 5 flowering *Asteraceae* species showed no measurable induced formation of tertiary amine PAs by naturally reducing agents if present (data not shown). All PA *N*-oxides added were recovered with LC-MS/MS after extraction. Only a very small amount (2%) of the added senecionine *N*-oxide was reduced in the presence of *Solidago gigantea* and *Eupatorium cannabium* plant material, but the concentrations measured were close to the detection limit. In the presence of *Senecio sylvaticus* no reduction was observed for all three PA *N*-oxides. In the control samples (no PA *N*-oxides added) of *Jacobaea erucifolia* and *J. vulgaris* senecionine *N*-oxide, erucifoline *N*-oxide and its tertiary PAs were already present in the plant material but jacobine or jacobine *N*-oxide were not present in detectable amounts. Since senecionine *N*-oxide and erucifoline *N*-oxide were naturally present in the plant, we could not draw any conclusions on the reduction of these PAs, as the added *N*-oxide volumes were negligible. For the jacobine *N*-oxide added it could be shown that there was no reduction by naturally occurring reducing agents present in *J. erucifolia* and *J. vulgaris*.

The three tertiary PAs, senecionine, jacobine and erucifoline, in presence of dry plant material of several flowering *Asteraceae* species showed no detectable induced oxidation of PAs by naturally occurring oxidation agents (data not shown). All PAs added were recovered after extraction.

Effect of freeze-drying on the tertiary PA content

The total PA concentration and the concentration of the individual PAs was not significantly different comparing the freeze-dried with fresh plant material (Table 1). The freeze-dried (lyophilized) materials had a higher TA% for all individual PAs compared to the corresponding fresh materials, which illustrates that the freeze-drying process caused some reduction from *N*-oxide to tertiary amine. The reduction is not PA specific, because the relative reduction amount was not significantly different between the PAs (Table 1, ANOVA, $F_{8, 63} = 0.69$, $P = 0.70$).

Table 1. Effect of sample treatment on the observed concentration of total PA, individual PA, relative concentration of tertiary amines (TA%), and relative reduction amount.

PA[a]	Concentration[b] (mg/g dry wt)			TA%[c]			Relative reduction amount[d] (%)
	Freeze-dried	Fresh	Paired t- test	Freeze-dried	Fresh	Paired t- test	
total PA	0.654	0.794	ns	22	13	*	4
sn	0.042	0.047	ns	6	1	*	3
ir	0.014	0.017	ns	5	1	*	2
sp	0.095	0.108	ns	7	2	*	3
acsp	0.012	0.008	ns	4	2	*	5
jb	0.435	0.560	ns	28	17	**	4
jl	0.007	0.008	ns	43	29	*	12
jz	0.011	0.011	ns	20	11	ns	6
er	0.020	0.020	ns	9	3	ns	6
acer	0.014	0.014	ns	5	1	*	3

[a] Abbreviations: sn = senecionine; ir = integerrimine; sp = seneciphylline; acsp = acetylseneciphylline; jb = jacobine; jl = jacoline; jz = jacozine; er = erucifoline; acer = acetylerucifoline
[b] Concentration was the absolute concentration of PAs as tertiary amines and *N*-oxides
[c] TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100.
[d] Relative reduction amount = (concentration of tertiary amines in freeze-dried material – concentration of tertiary amines in fresh material)/ concentration of the corresponding *N*-oxides in fresh material.
ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

PA distribution in *Jacobaea vulgaris*

A total of 27 different PAs (*N*-oxides + tertiary amines) were found in roots and shoots of the five genotypes. Dehydrojaconine, spartioidine and senecivernine were found in trace amounts and did only occur in detectable amounts as tertiary PA, while all other individual PAs were found in both forms.

The mean TA% in the roots of Jacobine-chemotypes and both plant parts of Erucifoline-chemotypes were all below 6.2%, while the TA% in the shoots of Jacobine-chemotypes was approx. 6 times higher, resulting in a significant chemotype $_{x}$ plant part interaction (ANOVA, $F_{1, 78} = 53.07$, $P<0.001$). In the roots no significant difference between the chemotypes (Mean TA% roots Jacobine and Erucifoline-chemotype = 5.3% and 5.7%, respectively) was found while in the shoots the difference was highly significant (Mean TA% shoots Jacobine and Erucifoline-chemotype = 37.0% and 6.1%, respectively).

In the roots of all genotypes on average 94.7% of all PAs were in *N*-oxide form (Figure 1). Senecionine *N*-oxide, seneciphylline *N*-oxide and acetylseneciphylline *N*-oxide were the most abundant PAs in the roots with on average 71.0% of the total PA root concentration (Figure 4). The Jacobine-chemotypes from Meijendel (Meijendel A and B) contained jacobine *N*-oxide as one of the dominant root PAs, while the Erucifoline-chemotypes (Vilt and Kassel) contained erucifoline *N*-oxide as a dominant PA (Figure 2), with respectively 14.3% (for jacobine) and 14.9% (for erucifoline) of the total PA root concentration.

The four most dominant PAs in the shoots of the Erucifoline-chemotypes were senecionine, seneciphylline, erucifoline and acetylerucifoline. In the shoots of this chemotype, a lower concentration of PAs were in the tertiary PA form as compared to the Jacobine-chemotypes only 3.6% and 8.2% of the total shoot PA concentration for Vilt and Kassel, respectively (Figure 1).

The TA% in the shoots was higher in the Jacobine-chemotypes. In particular, the chemotypes from Meijendel contained a high percentage of tertiary PAs (Figure 1). In the shoots of this chemotype, on average 45.5% of the total shoot PA concentration occurred as tertiary PA. In the Jacobine-chemotype from Wageningen, tertiary forms comprised nearly 20% of the total shoot PA concentration (Figure 1).

The TA% is in fact only determined by the presence of the jacobine-like PAs. Jacobine and its derivatives jaconine, jacoline, jacozine and dehydrojaconine showed the highest percentage in reduced form (Figure 2). In the two Jacobine-chemotypes from Meijendel on average only 17.0% of the total senecionine and seneciphylline concentration was present as tertiary PAs while for jacobine this was 54.1%.

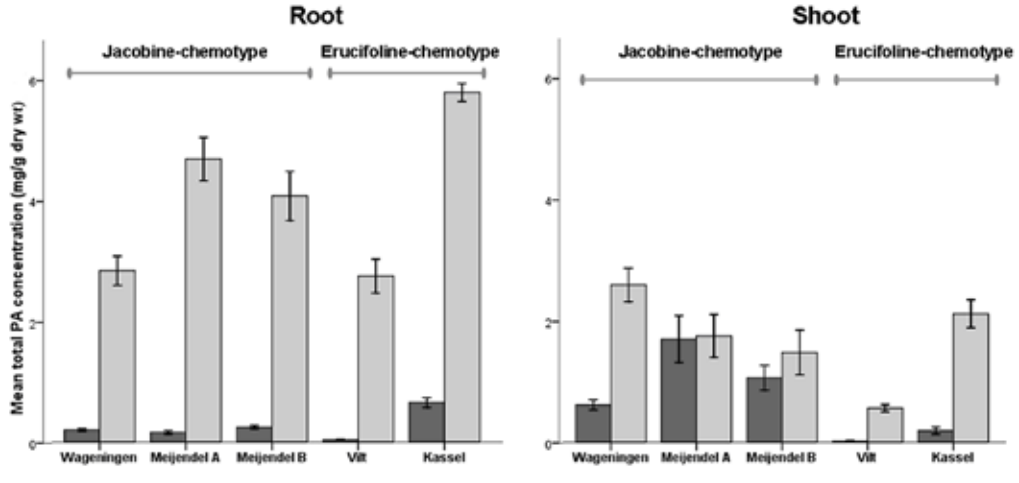

Figure 1. The absolute mean total PA concentration in dry root and shoot material per genotype (n = 8). Light bar = PA *N*-oxides and dark bar = tertiary PAs. Error bars: ±1SE. Above the bars, the genotype the chemotype is indicated. Jacobine = Jacobine chemotype, Erucifoline = Erucifoline chemotype.

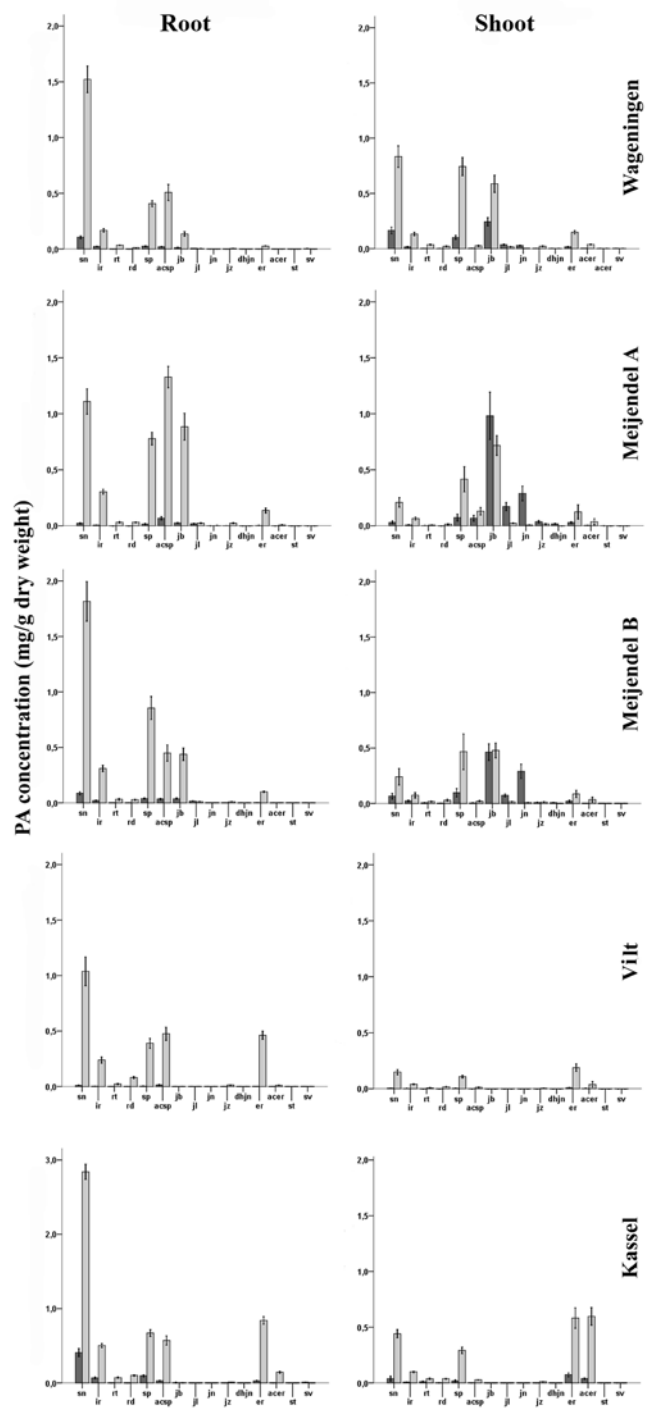

Figure.2 The PA composition of *J. vulgaris* in absolute concentration per individual PA of dry root and shoot material (n = 8) Light bar = PA *N*-oxides and dark bar = tertiary PAs. For abbreviations see legend Table 2. Error bars: ±1SE.

Relative tertiary amine concentration in *Jacobaea* hybrids

Of the 37 detected PAs in the *Jacobaea* hybrids, 9 were otonecine-group PAs with no corresponding *N*-oxides and 6 were absent or close to the detection limit in some samples. The remaining 22 PAs were used to calculate the relative concentration of tertiary amine as TA%.

The TA% of the senecionine-like and erucifoline-like PAs in the roots were lower than 10%, which demonstrates that more than 90% of these PAs were present in *N*-oxide in the roots. But the jacobine-like PAs had TA% ranging from 10% till 56%. Except for senecionine, integerrimine and acetylerucifoline, the TA% of all the other PAs was genotype dependent in the roots. In the shoots, the TA% were higher than those in the roots (for all 11 PAs, paired *t*-test, df = 608, P < 0.001). Particularly for jaconine, the TA% was up to 80% in the shoots. The TA% of all the individual PAs were genotype dependent in the shoots (Table 2). Generally there was a significant positive correlation between the TA% both in the roots and in the shoots. The correlation coefficients were not significantly different between the shoots and roots (paired *t*-test, df = 54, t = -0.393, P = 0.696), but correlation coefficients differed between structural groups (ANOVA, $F_{5, 104} = 10.69$, P < 0.001). Correlation coefficients of TA% within structural groups are always higher as TA% correlation between different structural groups (Supplementary data Figure 3).

Discussion

We observed that the tertiary amine proportion was different among PAs and genotypes. Two possible and nonexclusive hypotheses may explain this pattern. Firstly, the chemical transformation and perhaps allocation of PA *N*-oxides, is accompanied by a continuous slow reduction of the original *N*-oxides. Thus, the most peripheral "on a time scale oldest" PAs like jacoline and jaconine, which are far down the pathway (Supplementary data Figure 4), show the highest TA% and the "youngest" PAs, i.e. senecionine or integerrimine, have the lowest TA%. The observation that the TA% values in shoots are always higher than the values for the respective PAs in roots goes in the same direction (Hartmann 2010, personal communication). Secondly, specific (re-)oxidation of the tertiary PAs might explain the pattern. The reduction of PA *N*-oxides in the plant is an unspecific, chemical process induced by the presence of endogenous reducing compounds and (traces of) transition metal salts. Meanwhile, there is a, biochemically based, process operating to re-oxidize the reduced tertiary amines for PA transport. Enzyme(s) that may be involved seem to work well for senecionine-like and erucifoline-like PAs but work less well for jacobine-like PAs. Possibly, the substrate specific enzyme is affected when alterations at positions 15 and 20 (addition of O, H_2O, HCl, Supplementary data Figure 5) are made. This perhaps makes the epoxidized PAs less accessible for the enzyme, which results in a lower conversion rate. So, the second hypothesis could explain the TA% difference among the PAs and the genotypes. Furthermore, it may get more support from a biochemical point of view, since the plant has to use an enzyme to produce the back-bone senecionine *N*-oxide at the beginning of the PA pathway. Senecionine *N*-oxygenase (SNO) was isolated (from the larvae of specialist insect *Tyria jacobaeae*, less relevant for plants) and *Crotalaria scassellatii* seedlings. The enzymes were tested with different PAs as substrates and showed that they specifically oxidized tertiary PAs (Lindigkeit et al. 1997; Chang and Hartmann 1998). These enzymes might be highly preserved and similar in the various PA containing plants. A very interesting follow-up of this study could be the identification, isolation and characterization of this putative *N*-oxidation enzyme(s) and exploration of genetic variation concerning these enzymes. It would also be interesting to see if the TA% can be influenced by external factors, like a high metal content in the soil, or by application of reducing compounds to the leaves.

Table 2. The concentration of tertiary and *N*-oxide PA, TA% and the genotype effect on the TA% in two parental, two F1 and 102 F2 genotypes from a cross between *J. vulgaris* and *J. aquatica*.

Plant Part	Structural group	Pyrrolizidine alkaloid	Code	Concentration (mg/g dry wt)		TA%[a]	$x^{2\,b}$	P[c]
				Tertiary amine	*N*-oxide			
Roots	Senecionine-like	senecionine	sn	0.053	1.435	4	111.7	ns
		intergerrimine	ir	0.007	0.232	3	97.9	ns
		retrorsine	rt	0.002	0.037	5	131.8	*
		seneciphylline	sp	0.025	0.601	4	144.6	**
		acetylseneciphylline	acsp	0.047	0.996	5	133.9	*
	Jacobine-like	jacobine	jb	0.029	0.250	13	245.2	***
		jacoline	jl	0.009	0.013	45	252.1	***
		jaconine	jn	0.033	0.025	56	166.7	***
		jacozine	jz	0.001	0.009	10	268.2	***
	Erucifoline-like	erucifoline	er	0.003	0.039	9	144.5	**
		acetylerucifoline	acer	0.000	0.009	6	98.4	ns
Shoots	Senecionine-like	senecionine	sn	0.011	0.177	8	144.2	**
		integerrimine	ir	0.003	0.063	7	132.6	**
		retrorsine	rt	0.001	0.009	17	163.6	***
		seneciphylline	sp	0.038	0.513	9	134.2	**
		acetylseneciphylline	acsp	0.009	0.148	10	147.5	**
	Jacobine-like	jacobine	jb	0.077	0.234	24	311.9	***
		jacoline	jl	0.023	0.016	55	354.8	**
		jaconine	jn	0.252	0.047	80	376.4	***
		jacozine	jz	0.003	0.012	31	343.3	***
	Erucifoline-like	erucifoline	er	0.015	0.138	15	203.5	***
		acetylerucifoline	acer	0.004	0.052	11	134.9	*

[a] TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100.
[b] Kruskal-Wallis test with the concentration data from two parental, two F1 and 102 F2 genotypes. Ca. 12 replicates per parental and F1 and ca. 6 replicates per F2 hybrid, in total n = 609 plants.
[c] ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001

Our results showed that jacobine-like PAs had a higher TA% than the other PAs. This coincides with the role of jacobine-like PAs as important defence compounds. Several studies showed that jacobine and jaconine were especially feeding deterrent for generalist insect herbivores (Macel et al. 2005; Leiss et al. 2009), while some specialists, preferred plants containing high concentrations of jacobine (Macel and Klinkhamer 2010). From an evolutionary and ecological point view, it represents a next step in the arm-race between plants and herbivores as a number of studies show that tertiary amines are more toxic than their respective *N*-oxides (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005). Further research on the chemistry and biology of PA *N*-oxides and tertiary PAs and their influence on generalist and specialist insects are required to better understand the ecological significance of these highly interesting compounds.

Conclusion

We showed that the occurrence of tertiary PAs is not an artifact of the freeze drying, extraction or detection method. The three main PA *N*-oxides of *J. vulgaris* showed no significant differences during the reduction experiments. Jacobine was significantly more reactive compared to senecionine and erucifoline towards chemical *N*-oxidation with oxidation agent HOOH.

These results strongly indicate that the high levels of free bases found for jacobine and other jacobine-like PAs are not caused by an intrinsic structural instability of the PA molecule or by chemical attack. Also it was not observed that naturally occurring agents in plant material caused reduction or oxidation of the added PAs during our extraction method. Plant material of different species did not induce any transformation of PAs from one form into the other. From our results we can conclude that the high percentages in tertiary form for jacobine-like PAs are not due to instability or higher sensitivity for reducing agents in the extraction and analytical process, but likely are the result of a change induced by (bio)chemical processes in the plant itself. We cannot exclude that a minor amount of reduction occurs during harvesting and the freeze-drying, but it seemed to affect all PA *N*-oxides to the same extent. We did find that in the Jacobine-chemotype plants a much higher level of tertiary PA present compared to the Erucifoline-chemotypes. By crossing *J. vulgaris* Jacobine-chemotype with the closely related *J. aquatica*, which lacks jacobine, and measuring PA *N*-oxide and tertiary amine concentrations, we showed that the TA% was genotype-dependent. This means that the variation found for relative tertiary amine content has a genetic base, since the environmental conditions of the plants during growth and analysis were kept equal for all plants.

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Supplementary data

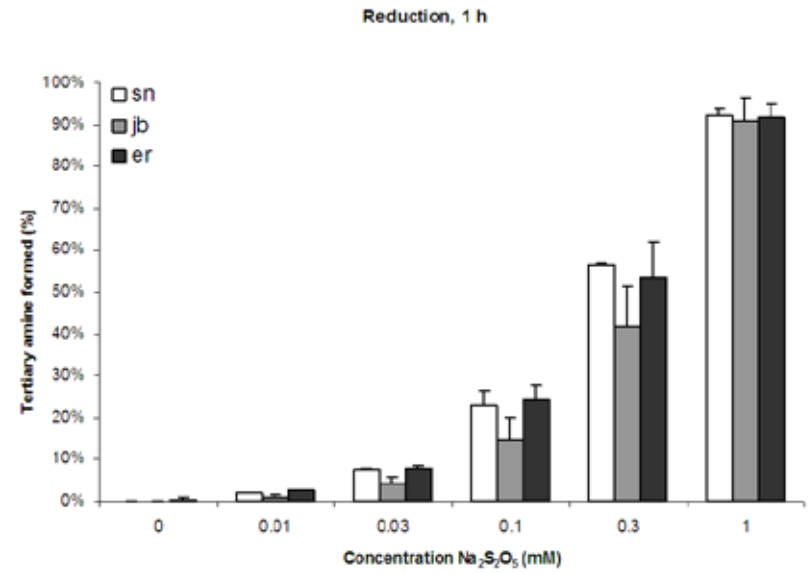

Figure S1. Reduction of PA *N*-oxides after incubation (1h, n = 2) in 2% formic acid solution with sodium metabisulfite ($Na_2S_2O_5$) in five different concentrations (0.01, 0.03, 0.1, 0.3, 1 mM). sn = senecionine; jb = jacobine; er = erucifoline. Error bars: ±1SD. The tertiary amine formed is the relative amount of tertiary PA present in a sample, which was calculated as the measured concentration of tertiary PA divided by the sum of the concentration of tertiary PA and corresponding PA *N*-oxide.

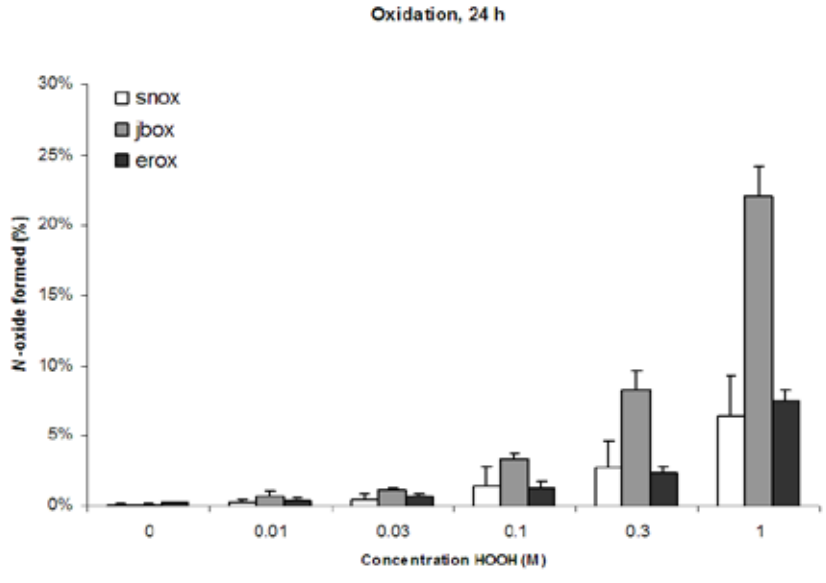

Figure S2. Oxidation of PA tertiary amines after incubation (24h, n = 2) in 2% formic acid solution with hydrogen peroxide (HOOH) in 5 different conurecentrations (0.01, 0.03, 0.1, 0.3, 1 M). snox = senecionine *N*-oxide, jbox = jacobine *N*-oxide, erox = erucifoline *N*-oxide. Error bars: ±1SD. The *N*-oxide formed is the relative amount of *N*-oxide present in the sample which was calculated as the measured concentration of the PA *N*-oxide divided by the sum of the concentration of PA *N*-oxide and the corresponding tertiary PA.

sn	0.85***	0.38**	0.79***	0.68***	0.25ns	0.17 ns	0.17 ns	0.15 ns	0.45***	0.46***
0.89***	ir	0.35**	0.77***	0.60***	0.32*	0.21 ns	0.21 ns	0.06 ns	0.46***	0.44***
0.55***	0.55***	rt	0.42***	0.38***	0.37**	0.35**	0.40***	0.48***	0.55***	0.49***
0.83***	0.80***	0.58***	sp	0.66***	0.35**	0.23 ns	0.24 ns	0.29*	0.68***	0.63***
0.81***	0.84***	0.56***	0.80***	acsp	0.27*	0.28*	0.29*	0.34**	0.5***	0.59***
0.43***	0.38**	0.32*	0.51***	0.50***	jb	0.82***	0.86***	0.46***	0.6***	0.44***
0.43***	0.37**	0.26 ns	0.52***	0.49***	0.80***	jl	0.87***	0.49***	0.56***	0.48***
0.51***	0.43***	0.34**	0.54***	0.55***	0.79***	0.82***	jn	0.53***	0.59***	0.49***
0.15 ns	0.10 ns	0.31*	0.20 ns	0.18 ns	0.19 ns	0.07 ns	0.17 ns	jz	0.58***	0.43***
0.50***	0.41***	0.51***	0.52***	0.44***	0.43***	0.45***	0.52***	0.31*	er	0.75***
0.21 ns	0.18 ns	0.14 ns	0.15 ns	0.15 ns	0.02 ns	0.03 ns	0.17 ns	0.28 ns	0.21 ns	acer

FigureS3 The correlations between genotype mean TA% of the PAs. Two parental, two F1 and 102 F2 genotypes were used. Numbers in the cells are the correlation coefficients. The background color of the cells is related to the number: black (>0.75); dark grey (0.50~0.75); light grey (0.25 ~ 0.50); white (< 0.20 and (or) p-value is not significant). ns: not significant, * p<0.05,**p<0.01, ***p<0.001. In the cells along the diagonal line are the codes for PAs. Sn = senecionine; ir = integerrimine; rt = retrorsine; sp = seneciphylline; acsp = acetylseneciphylline; jb = jacobine; jl = jacoline; jz = jacozine; er = erucifoline; acer = acetylerucifoline. Correlation coefficients above the diagonal line are for shoots, below the diagonal for roots.

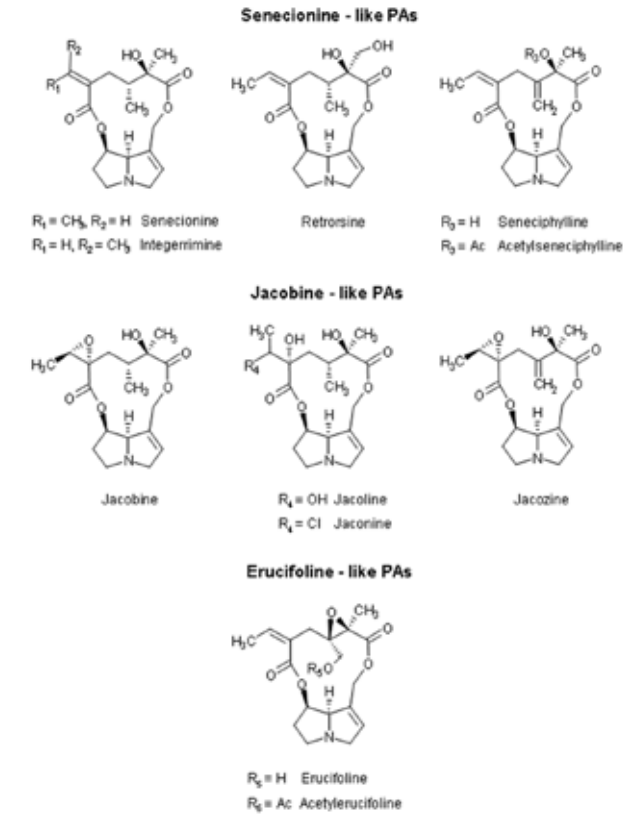

Figure 5. The structure of the major PAs detected in Jacobaea plants and which occur in both tertiary amine and *N*-oxide form. Only the structures of the free base form are shown.

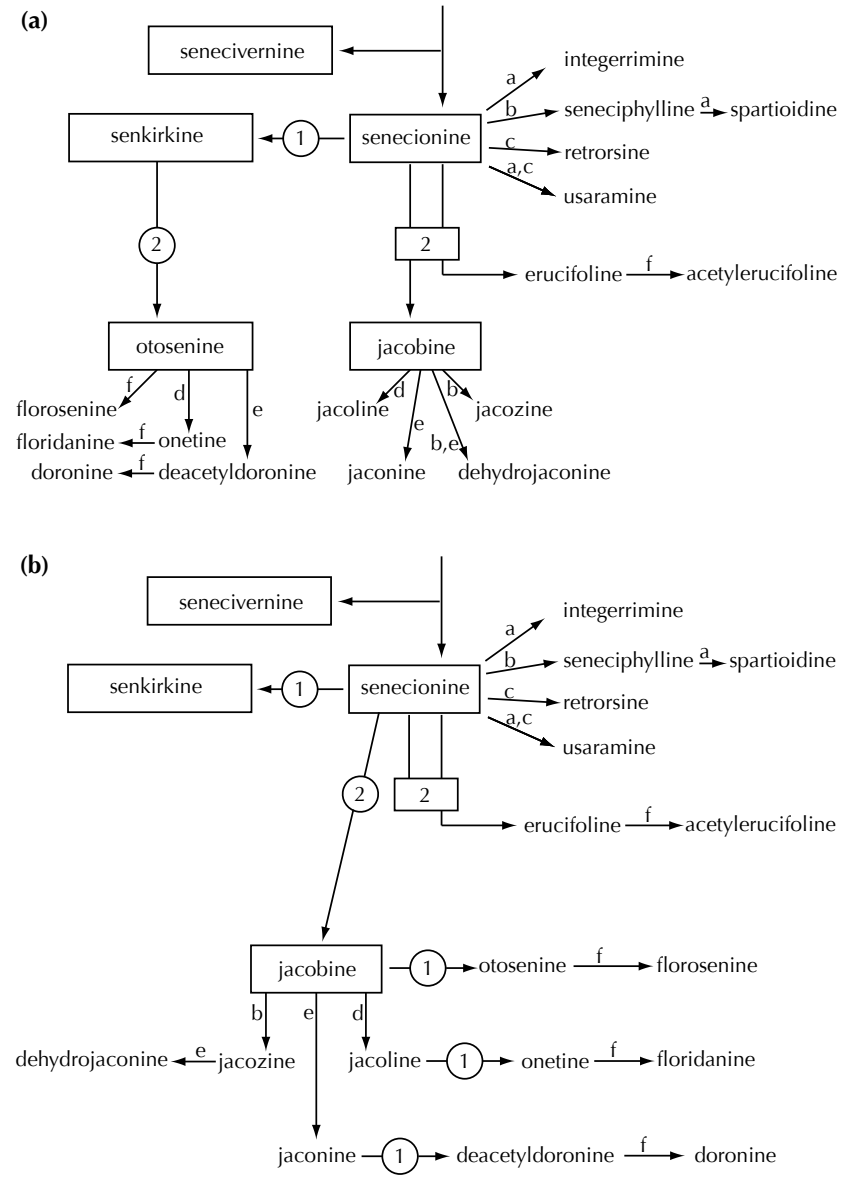

Figure 4. Possible biosynthetic pathways of diversification of PAs in the *Jacobaea* section. With the exception of senecivernine, senecionine is the common precursor of all other PAs. Since the substrate specificity of the enzymes involved is not known, two scenarios are illustrated: (a) = senkirkine is assumed as common precursor of all otonecine derivatives; (b) = the otonecine derivatives originate independently from the respective retronecine derivatives. Two main reactions exist: conversion of retronecine to otonecine (reaction 1) and site-specific epoxide formation (reaction 2). Further structural diversification requires six simple one-step-reactions marked by letters a–f: a = Z/E-isomerization at C20; b = 13, 19-dehydrogenation; c = site-specific hydroxylations; d = hydrolysis of 15, 20-epoxide; e = chlorolysis of 15, 20-epoxide; f = site-specific O-acetylations. (Adapted from Pelser et al., 2005)

5

Soil-borne Microorganisms and Soil-type affect Pyrrolizidine Alkaloids in *Jacobaea vulgaris*

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Abstract

Secondary metabolites like pyrrolizidine alkaloids (PAs) play a crucial part in plant defence. We studied the effects of soil-borne microorganisms and soil-type on pyrrolizidine alkaloids in roots and shoots of *Jacobaea vulgaris*.

We used clones of two genotypes from a dune area (Meijendel), propagated by tissue culture and grown on two sterilized soils and sterilized soils inoculated with 5% of non-sterilized soil of either of the two soil-types.

Soil-borne microorganisms and soil-type affected the composition of PAs. By changing the composition rather than the total concentration below and aboveground, plants have a more complex defence strategy than formerly thought.

Interestingly, a stronger negative effect on plant growth was found in sterilized soils inoculated with their 'own' microbial community suggesting that pathogenic and/or other plant inhibiting microorganisms were adapted to their 'own' soil conditions.

Introduction

Many plants synthesize a diversity of compounds as a defence against herbivores and pathogens. This diversity seems to be one of the strategies of the plant to cope with the great variety of potential environmental threats. The composition of defence compounds depends on the genotype of the plants, showing variation within species and even among individual plants within a population. All this genetically based variability can also be influenced by the abiotic and biotic environment (Karban and Baldwin 1997; Agrawal 1998; Macel and Klinkhamer 2010).

When the root system is exposed to belowground organisms (e.g. herbivore insects, nematodes, root pathogens and mycorrhizal fungi) plants can show several direct defence responses in the shoots that may affect aboveground herbivores and, thus plant fitness (van Loon et al. 1998; van der Putten et al. 2001; Paul et al. 2002; Gange et al. 2002; Dicke and Hilker 2003; van Dam et al. 2003; Bezemer et al. 2005; Bezemer and van Dam 2005).

Jacobaea vulgaris (syn *Senecio jacobaea*) is a suitable system to study chemical defence, because it contains a well studied group of defence compounds; pyrrolizidine alkaloids (PAs). The concentration and composition of PAs in *J. vulgaris* depend on the genotype and environment (Vrieling et al. 1993; Hol et al. 2003; Macel et al. 2004). Macel and Klinkhamer (2010) noticed, in a field experiment, that in genotypes of *J. vulgaris* the composition of defence compounds (pyrrolizidine alkaloids) changed compared to the initial composition of clones in the laboratory. The composition also differed between the aboveground parts of clones grown on two different experimental field sites. This raises the question if soil-type and/or soil-borne microorganisms could have an impact on defence compounds in below- and aboveground plant parts.

We expect that if the net effect of the soil-borne microbial community on plant growth is negative, the plant responds by increasing its PA concentration in order to suppress the effect of pathogens. However, because the production of these defence compounds takes place in the roots, the interactions with soil-borne pathogens can also reduce the production of PAs by inducing root damage and increasing the shoot/root ratios of the plants (Frischknecht et al. 2001; Hol et al. 2003) in combination with reduced plant growth (Bever 1994; Klironomos 2002).

In addition the PA composition could be changed by activating particular transforming enzymes that are responsible for the diversification of the PAs (Hartmann and Dierich 1998).

The most recent study on the defence of *J. vulgaris* indicated that plants with high jacobine levels suppress the growth of microbes by inducing a lower diversity of fungi in the rhizosphere compared to plants lacking high levels of jacobine PAs (Kowalchuk et al. 2006). This implies that the PA composition may have an important influence on fungi in the rhizosphere. Interestingly, the *J. vulgaris* alkaloids; retrorsine and retrorsine *N*-oxide showed to have inhibitory effects on mycelium growth of several plant-associated fungi (Hol and van Veen 2002). Apart from the studies mentioned above, so far little is known about the specific effects of different PAs on soil-borne microorganisms.

In the present study we tested if soil-borne microbial communities effect PA concentration and composition in *J. vulgaris*. In a laboratory experiment we grew cloned plants of two genotypes, on two different sterilized soils and sterilized soils inoculated with 5% of non-sterilized soil of either of the two soil-types. To obtain a detailed picture of the PA concentration and composition of the plants after 6 weeks, LC-MS/MS was used to analyze the root and shoot extracts.

Material and Methods

Experimental design

We selected two different genotypes with high (A) and low (B) total plant PA concentration, which originated from different populations at Meijendel. The genotypes were propagated by tissue culture. Eight cloned replicates per genotype per treatment were planted in two different sterilized soil-types in 1.3 l pots. The two soil-types used were 'Meijendel soil', calcareous sand from a coastal dune area in the North of The Hague (52°9'N, 4°22'E) and 'Heteren soil', a mixture of sand and potting soil from an experimental garden that has been in use since 1994 (51°57'N, 5°44'E) in the Netherlands. For each soil we compared three treatments 1. control (sterilized soil) 2. sterilized soil treated with inoculum (5%) of non-sterilized soil of the same origin 3. sterilized soil treated with inoculum (5%) of the other soil-type. In total this resulted in 96 plants (8 replicates * 2 genotypes * 2 soils * 3 treatments). Soil sterilization was done by 25 kilo Gray gamma-radiation (Isotron, Ede, the Netherlands). Soil for the inocula was collected within a radius of one meter from naturally occurring *J. vulgaris* plants.

Plants were randomly distributed and grown for 6 weeks in a climate room (relative humidity 70%, light 16 h at 20°C, dark 8 h at 20°C). Demi-water was given three times a week with additions of 20 ml of Steiner nutrient solution (Steiner 1968) once every fortnight. After 6 weeks the plants were harvested and cut above the root crown by a scissor. Harvested plant parts (shoots and roots) were kept at -25°C for approximately two weeks before being freeze-dried for 72 hours under vacuum with a collector temperature of -55°C (Labconco Free Zone® 12 l Freeze Dry System). Plant dry mass was measured as a proxy for the net effect of the inoculum on plant growth.

Pyrrolizidine alkaloid analysis

Freeze-dried plant material (approximately 10 mg) was extracted with 2% formic acid in a 1 to 100 weight to volume ratio. Heliotrine, monocrotaline and monocrotaline *N*-oxide were added as internal standards to the extraction solvent at a concentration of 1 µg /ml. After centrifugation an aliquot of the solution (10 µl) was diluted with water (990 µl) and injected in the LC-MS/MS system (an Agilent HP1100 HPLC system coupled to a Waters Micromass Micro tandem mass spectrometer).

Chromatographic separation was achieved on a Waters Xbridge 150 x 3.0 mm HPLC column, run with a water/acetonitrile linear gradient containing 0.05% ammonia at a flow of 0.4 ml /min. The gradient started at 100% water and during analysis the acetonitrile percentage was raised to 70%.

The MS system was operated in positive electrospray mode and data were recorded in multiple monitoring mode using one selected precursor ion to product ion transition per compound. Cone and collision energy settings were optimized for the individual compounds. Obtained peak areas were internally calibrated using the internal standards and the individual compounds were quantified against a standard solution of the PAs in water. Fourteen individual PA standards were available for this study, representing over 90% of the total amount of PAs present in the plants extracts. The remaining PAs, being tertiary base as well as *N*-oxides, were quantified by using the mean response of the tertiary amine standards and the *N*-oxide standards, respectively. Data processing was conducted with Masslynx 4.0 software.

Data analysis

Plant dry mass, shoot/root ratio and total PA concentrations in shoots and roots were statistically analyzed by GLM (General Linear Model) univariate analyses procedure. With PA concentration as the dependent variable, genotype (Genotype A and B), soil-type (Meijendel and Heteren) and inoculum (Sterilized,

Meijendel and Heteren soil inoculum) as fixed factors and plant dry mass and shoot/root ratio as covariates. In order to assess the effects of soil-type and inoculum treatments on the composition of the PAs in roots and shoots for each genotype we used discriminant analyses to predict to which treatment a sample belonged on basis of its alkaloid pattern. An F-test (Wilks' lambda) was used to test if the four discriminant models (roots and shoots of both genotypes) as a whole were significant. The relative concentrations (expressed as the percentage of the total PA concentration) and the total PA concentration were included as independents variables. All tests were conducted with SPSS 15.0 for Windows.

Results

Plant dry mass

Soil-type had a greater impact on plant dry mass than genotype or inoculum (Table 1). The mean dry mass of plants grown on the two soil-types, Heteren and Meijendel was across treatments, 1.42 and 0.48 g respectively (Figure 1A). Mean plant dry mass of genotype A was 0.82 g, while that of genotype B was 1.09 g. Mean dry mass was highest on sterilized soils (HS and MS) indicating an overall negative effect of the soil-borne microbial community. Plant dry mass was lowest on sterilized soils inoculated with non-sterilized soil of the same origin (HH and MM) leading to a significant soil-type x treatment interaction. In addition the effects on plant dry mass depended on the three-way interaction between genotype, soil-type and inoculum treatment.

Shoot/root ratio

The largest difference in shoot/root ratio was found between the two genotypes (Table 1). Mean shoot/root ratio of genotype A was 0.59, while that of genotype B was 0.38 (Figure 1B). Shoot/root ratio was significantly higher in Heteren soils than in Meijendel soil, 0.42 and 0.55 respectively. Inoculation lowered the shoot/root ratio of the plants especially when soils inoculated were inoculated with non-sterilized soil of the other soil-type (HM and MH). In addition the effects on shoot/root ratio depended on the three-way interaction between genotype, soil-type and inoculum treatment.

Total pyrrolizidine alkaloid concentration

Soil-type, did not significantly affect the mean total PA concentration of roots and shoots (Table 2). In contrast to our expectation, shoot and root dry mass and shoot/root ratio did not significantly affect total PA concentration either. Genotype had the largest impact on the total PA concentration in the roots (Figure 2A). Across soil-type and treatments, the total PA concentration in the roots of genotype A and B, was 11.3 mg and 6.6 mg /g dry root material respectively. The mean total PA concentrations in the shoots of genotypes A and B, was 8.5 mg and 7.7 mg /g dry shoot material, respectively. The PA concentration of the two genotypes was affected differently by the two soil-types and the combination of soil-type and treatment as can be seen by the two and three way interactions (Table 2). Inoculation with non-sterilized soils decreased the total PA concentration in the roots of both genotypes. In addition inoculation decreased the total PA concentration in the shoots of genotype B. For genotype A, the effect of inoculation was less clear.

Table 1. ANOVA of the effect of genotype, soil-type and inoculum treatment on plant dry mass, shoot/root-ratio of *Jacobaea vulgaris*

Dependant variables	Fixed factors	df (k-1)	Df (N-k)	F	P
Plant dry mass	Genotype	1	94	88.413	0.000
	Soil-type	1	94	1041.942	0.000
	Inoculum Treatment	2	93	63.355	0.000
	Genotype x Soil-type	1	94	31.732	0.000
	Genotype x Inoculum Treatment	2	93	2.506	0.088
	Soil-type x Inoculum Treatment	2	93	49.908	0.000
	Genotype x Soil-type x Inoculum Treatment	2	93	4.731	0.011
	Error	84			
	Total	96			
Shoot/root ratio	Genotype	1	94	99.704	0.000
	Soil-type	1	94	43.309	0.000
	Inoculum Treatment	2	93	26.397	0.000
	Genotype x Soil-type	1	94	7.192	0.009
	Genotype x Inoculum Treatment	2	93	2.966	0.057
	Soil-type x Inoculum Treatment	2	93	8.437	0.000
	Genotype x Soil-type x Inoculum Treatment	2	93	3.308	0.041
	Error	84			
	Total	96			

Table 2. ANOVA of the effect of shoot dry mass, root dry mass, genotype, soil-type and inoculum treatment on the mean total PA concentration of roots and shoots.

Dependant variables	Fixed factors	df (k-1)	Df (N-k)	F	P
Total PA conc. root	Root dry mass (covariate)	1	94	0.925	0.339
	Shoot dry mass (covariate)	1	94	1.314	0.255
	Genotype	1	94	128.023	0.000
	Soil-type	1	94	0.407	0.525
	Inoculum Treatment	2	93	9.296	0.000
	Genotype x Soil-type	1	94	3.282	0.074
	Genotype x Inoculum Treatment	2	93	0.368	0.693
	Soil-type x Inoculum Treatment	2	93	2.170	0.121
	Genotype x Soil-type x Inoculum Treatment	2	93	3.484	0.035
	Error	82			
	Total	96			
Total PA conc. shoot	Root dry mass (covariate)	1	94	1.244	0.268
	Shoot dry mass (covariate)	1	94	1.762	0.188
	Genotype	1	94	4.038	0.048
	Soil-type	1	94	0.425	0.516
	Inoculum Treatment	2	93	0.854	0.430
	Genotype x Soil-type	1	94	17.370	0.000
	Genotype x Inoculum Treatment	2	93	8.410	0.000
	Soil-type x Inoculum Treatment	2	93	1.122	0.330
	Genotype x Soil-type x Inoculum Treatment	2	93	11.740	0.000
	Error	82			
	Total	96			

Table 3. The concentration of different PAs in roots and shoots (Mean±SE, n=96)

	Root		Shoot	
PAs (mg/g plant dry mass)	**N-oxide**	**Tertiary amine**	**N-oxide**	**Tertiary amine**
1. Senecionine	6.1332 ± 0.2632	0.0768 ± 0.0056	2.1148 ± 0.0973	0.1933 ± 0.0145
2. Seneciphylline	1.1884 ± 0.0394	0.0023 ± 0.0011	1.7267 ± 0.0995	0.1674 ± 0.0120
3. Acetyl-seneciphylline	0.4370 ± 0.0211	0.0077 ± 0.0006	0.0257 ± 0.0025	0.0072 ± 0.0014
4. Integerrimine	0.5707 ± 0.0273	0.0046 ± 0.0003	0.3166 ± 0.0133	0.0163 ± 0.0013
5. Retrorsine	0.0992 ± 0.0102	0.0035 ± 0.0004	0.0811 ± 0.0049	0.0066 ± 0.0007
6. Jacobine	0.1961 ± 0.0111	0.0658 ± 0.0047	0.9370 ± 0.0823	1.8734 ± 0.1063
7. Jacoline	0.0050 ± 0.0003	0.0031 ± 0.0001	0.0232 ± 0.0020	0.0375 ± 0.0027
8. Jacozine	0.0067 ± 0.0003	0.0003 ± 4.9E-5	0.0169 ± 0.0007	0.0161 ± 0.0010
9. Jaconine	0.0002 ± 4.1E-5	-	0.0036 ± 0.0004	0.0072 ± 0.0012
10. Dehydro-jaconine	-	-	-	0.0003 ± 5.9E-5
11. Erucifoline	0.0610 ± 0.0017	0.0071 ± 0.0005	0.1459 ± 0.0087	0.0462 ± 0.0026
12. Acetyl-erucifoline	0.0075 ± 0.0006	-	0.2490 ± 0.0119	0.0176 ± 0.0011
13. Riddelliine	0.0339 ± 0.0011	0.0002 ± 3.9E-5	0.0674 ± 0.0003	0.0027 ± 0.0002
14. 368 (eruciflorine)	0.0188 ± 0.0009	-	0.0111 ± 0.0004	-
Total PA concentration	8.7577 ± 0.3362	0.1919 ± 0.0116	5.7187 ± 0.2228	2.3921 ± 0.1134

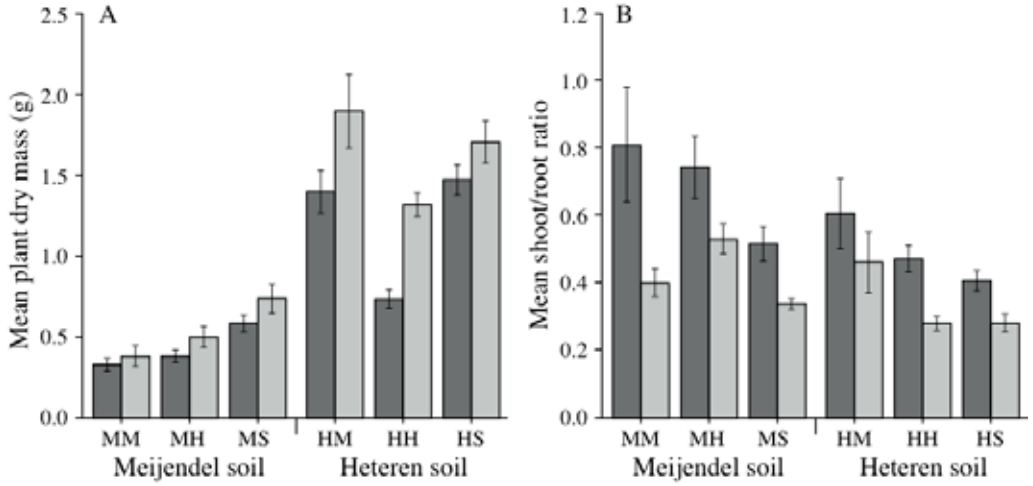

Figure 1. A Plant dry mass (Mean±SE, n=8) per soil-type per inoculum treatment of both *Jacobaea vulgaris* genotypes. B Shoot/root ratio (Mean±SE, n=8) per soil-type per treatment of both *Jacobaea vulgaris* genotypes. Left bar = genotype A; Right bar = genotype B; MM = sterilized Meijendel soil inoculated with non-sterilized Meijendel soil; MH = sterilized Meijendel soil inoculated with non-sterilized Heteren soil; MS = sterilized Meijendel soil; HM = sterilized Heteren soil inoculated with non-sterilized Meijendel soil; HH = sterilized Heteren soil inoculated with non-sterilized Heteren soil; HS = sterilized Heteren soil.

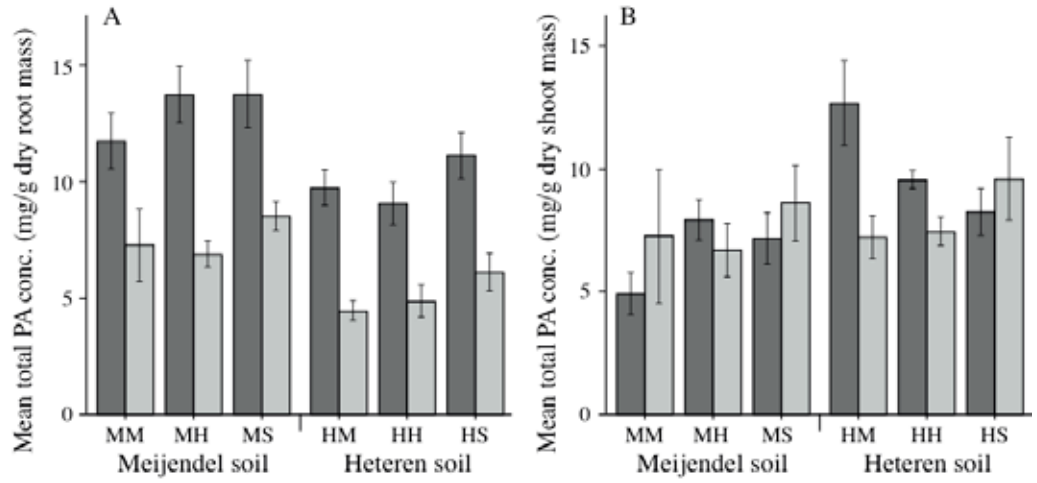

Figure 2. A Total PA concentration (Mean±SE, n=8) for root per soil-type per inoculum treatment of both *Jacobaea vulgaris* genotypes. B Total PA concentration (Mean±SE, n=8) for shoot per soil-type per inoculum treatment of both *Jacobaea vulgaris* genotypes. Red = genotype A; Blue = genotype B; MM = Sterilized Meijendel soil inoculated with non-sterilized Meijendel soil; MH = Sterilized Meijendel soil inoculated with non-sterilized Heteren soil; MS = Sterilized Meijendel soil; HM = Sterilized Heteren soil inoculated with non-sterilized Meijendel soil; HH = Sterilized Heteren soil inoculated with non-sterilized Heteren soil; HS = Sterilized Heteren soil.

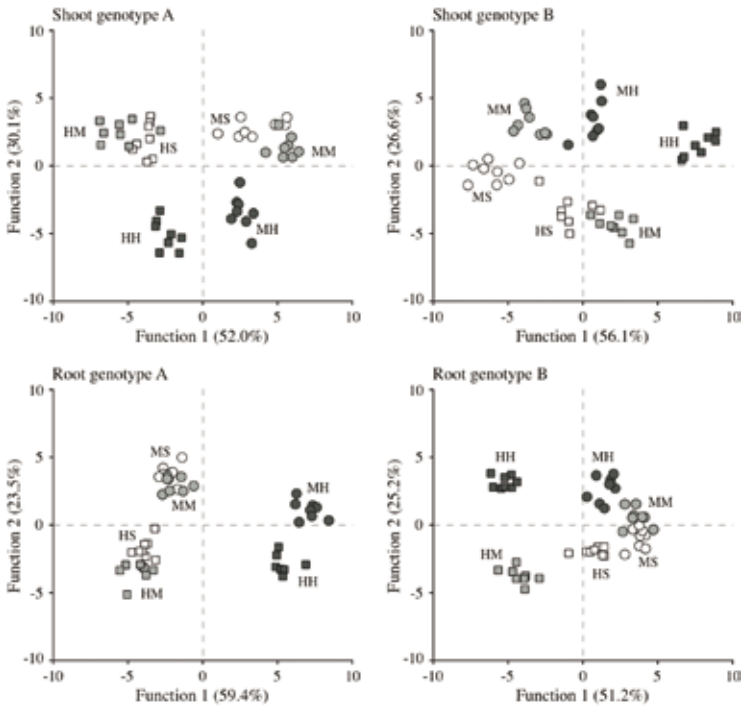

Figure 3. Four combined-group scatterplots showing the discriminant scores of the cases on discriminant function 1 and 2 generated by Classify Discriminant Analysis per genotype per plant part. MM = Sterilized Meijendel soil inoculated with Meijendel soil; MH = Sterilized Meijendel soil inoculated with Heteren soil; MS = Sterilized Meijendel soil; HM = Sterilized Heteren soil inoculated with Meijendel soil; HH = Sterilized Heteren soil inoculated with Heteren soil; MS = Sterilized Heteren soil.

Pyrrolizidine alkaloid composition

In this study in total 13 different PA *N*-oxides and 13 different tertiary amines were detected in root and shoot extracts (Table 3). These PAs have all been described for *Jacobaea vulgaris* (Witte et al. 1992).

However, compared to previous studies on *J. vulgaris* plants (Witte et al. 1992; Macel et al. 2002; Macel et al. 2004; Kowalchuk et al. 2006), a high number of PAs was detected simultaneously within a single genotype. This is in part due to the low concentrations of PAs that can be detected with LC-MS/MS in plant material compared to previously used GC-NPD and GC-MS techniques (Wuilloud et al. 2004; Betteridge and Colegate 2005). Another advantage of LC-MS/MS is that it can determine both *N*-oxides and tertiary amines directly, without the necessity of reduction of *N*-oxides to the corresponding tertiary amines, as is required for GC-based methods. As a result the number of PAs detected in the extracts is effectively doubled. Moreover, the relative proportion of PA *N*-oxides and tertiary amines can be accurately determined for each individual PA. One individual PA-type could not be identified with certainty but based on its retention time and molecular mass (367, $M+H^+$: 368) it is probably eruciflorine *N*-oxide. In the roots of both genotypes nearly 98% of all alkaloids were *N*-oxides. Senecionine *N*-oxide was the most abundant PA with a percentage up to 76% of the total PA concentration in the roots. The average percentage of *N*-oxides in the shoots was much lower and the PA composition was more diverse. In the shoot

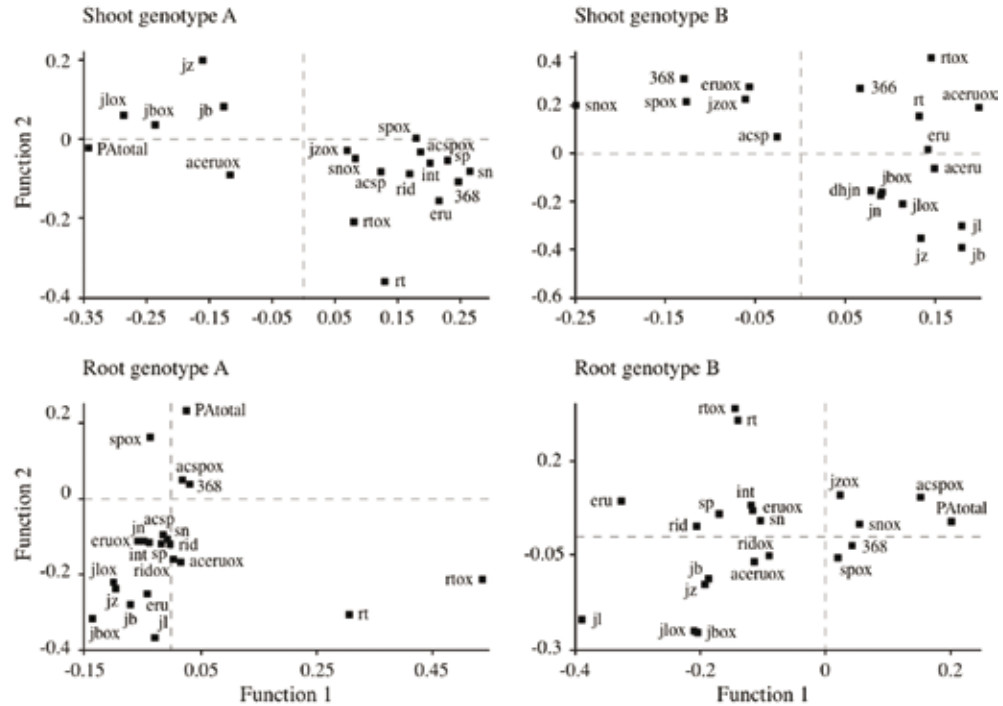

Figure 4. The correlations of each individual PA with the first two discriminant functions. All PAs shown in these structure matrixes showed significantly different group means (ANOVA $P<0.05$). PAtotal = total PA concentration, sn = senecionine, snox = senecionine *N*-oxide, sp = seneciphylline, spox = seneciphylline *N*-oxide, acsp = acetyl-seneciphylline, acspox = acetyl-seneciphylline *N*-oxide, int = integerrimine, intox= integerrimine *N*-oxide, rt = retrorsine, rtox = retrorsine *N*-oxide, eru = erucifoline, eruox = erucifoline *N*-oxide, aceru = acetylerucifoline, aceruox = acetylerucifoline *N*-oxide, jb = jacobine, jbox = jacobine *N*-oxide, jz = jacozine, jzox = jacozine *N*-oxide, jl = jacoline, jlox = jl *N*-oxide, jn = jaconine, jnox = jaconine *N*-oxide, dhjn = dehydrojaconine, rid = riddelliine, ridox = riddelliine *N*-oxide, 368 = PA *N*-oxide with molecular mass 368 (*eruciflorine*)

of genotype A, 35% of the PAs occurred as tertiary amines and in genotype B, nearly 25%. Senecionine *N*-oxide, seneciphylline and jacobine were the three major PAs present in the shoots. Jacobine is mainly responsible for the relatively high amount of tertiary amines found in the shoots. Less than 10% of senecionine and seneciphylline is present in the shoots as tertiary amine, for jacobine this is 65%.

Effect of soil-type and inoculum treatment on the pyrrolizidine alkaloid composition

In order to assess the effects of the treatments on the composition of the PAs in shoots and roots we performed four discriminant analyses (roots and shoots of two genotypes). In all four analyses the relative concentration of different PAs discriminated the six treatment groups significantly (Meijendel soil inoculated with Meijendel soil Heteren soil or sterile: MM, MH, MS and Heteren soil inoculated with Meijendel soil Heteren soil or sterile: HM, HH, HS). In all four analyses all eight replicates per treatment clustered together (Figure 3). In total five functions were needed to classify all cases correctly. The first two functions classified around 80% of all the cases correctly for all four discriminant analyses. Figure 4 shows the structure matrixes, presenting the correlations of each individual PA with the first two discriminant functions. Roots of genotype A: discrimination of the three treatments (sterile and 2 inocula) was mainly explained

by function 1 and discrimination of the two soil-types by function 2. Function 1 classified 59.4% correctly and together with function 2 the discriminant analysis classified in total 82.9% of the cases correctly (Figure 3). So, the inoculum treatment had more effect on the belowground PA composition than soil-type. Shoots of genotype A: discrimination on soil-type was mainly explained by function 1 (52.0% classified correctly) and discrimination on inoculum treatment by function 2 (30.1% classified correctly), meaning that soil-type had more influence on the aboveground PAs than the inoculum.

Roots and Shoots of genotype B: soil-type and inoculum treatment were discriminated by both functions to the same extend. Thus the effects of soil-type and inoculum treatment on the alkaloid composition in the plant were equally strong.

The relative concentration of jacobine, jacobine *N*-oxide, jacoline, jacoline *N*-oxide and jacozine, was significantly higher in Heteren soil (HS, HM, HH), as is shown by combining the information provided in Figures 3 and 4. For example, the mean relative shoot concentration of both jacobine and jacobine *N*-oxide in genotype A on Heteren soils was around 51% of the total PA concentration while on Meijendel soils around 32% of the total PA concentration consisted of this PA. The mean relative root concentration of both jacobine and jacobine *N*-oxide in genotype A was more than twice as high in Heteren soils compared to Meijendel soils, 4.4 and 1.8% respectively.

Remarkably, in roots and shoots of both genotypes, the concentration of retrorsine *N*-oxide was highest in the soils treated with Heteren inoculum (MH and HH). In genotype A grown on MS and HS soil the mean relative concentration was 0.4% while for MH and HH soils the mean relative concentration was significantly higher, 2.7 and 2.4% respectively. Also the tertiary amine retrorsine was higher in both sterilized soils treated with non-sterilized Heteren inoculum.

Discussion

We found that the PA composition below and aboveground was significantly affected by both soil-type and soil-borne microbial community. The effect on total PA concentration was, however, relatively small. Plant dry mass was also influenced by both soil-type and soil-borne microorganisms but the changes in the relative concentrations of individual PAs were not associated with changes in dry mass. For instance, the difference between plant dry mass of plants grown on Meijendel soils was largest between the sterile soil (MS) and the soil inoculated with Meijendel soil (MM). However, the discrimination between these two treatments based on the relative concentration of the individual PAs was not very strong (Figure 3). Plant dry mass was highest on sterilized soils (HS and MS) while it was lowest on soils inoculated with the non-sterilized same soil. The negative influence of the 'own' inoculum treatment on plant growth might be the result of a pathogenic effect or the result of competition for nutrients by the introduced microorganisms. The increased occurrence of microorganisms that act as plant pathogens and/or inhibitor microorganisms in the 'own' soil might be due to adaptations to local soil conditions.

After addition of only a small inoculum (5%) into the 'biologically empty' sterilized own soil, microorganisms may have developed into a community that is capable of reducing plant growth. Inoculation with the other soil may have also introduced potential pathogens, but these pathogens may be less adapted to these new soil conditions compared to potentially pathogen suppressive agents in that same inoculum. This also holds for the sterilized soils that were not inoculated. The soil probably did not remain sterile in the course of the experiment, but will have been inoculated randomly by air-borne microorganisms.

At this point we cannot draw any hard conclusions on the above mentioned since the soil microbial community is a black box. Although we clearly show an overall effect of the microbial community on PA composition of the plant we cannot pinpoint which microorganisms caused these effects.

Soil-type and soil-borne microorganisms influenced the composition of defence compounds in the roots and shoots of the plant primarily by changing the concentration of specific PAs. Changes in the concentration of individual PAs aboveground may attract specialist herbivores while deterring generalists (Macel and Vrieling 2003; Macel et al. 2005, Macel and Klinkhamer 2010). In our study, the levels of retrorsine and retrorsine *N*-oxide were raised in the plants grown on soils inoculated with non-sterilized Heteren soil. Retrorsine *N*-oxide is formed by the addition of a hydroxy group to senecionine *N*-oxide. In a previous study, retrorsine and retrorsine *N*-oxide showed to have inhibitory effects on mycelium growth of several plant-associated fungi (Hol and van Veen 2002). In addition to changes in retrorsine and retrorsine *N*-oxide, the levels of jacobine and jacobine *N*-oxide were raised in shoots of plants grown on Heteren soils, especially sterilized Heteren soil inoculated with Meijendel soil (HM). Jacobine is especially interesting because jacobine was mainly responsible for the relative high amounts of tertiary amines found in the shoots. Tertiary amines are considered as the pre-toxic state (Lindigkeit et al. 1997), by acting as highly reactive alkylating agents (Mattocks 1986). While being toxic for generalist herbivores, several specialists prefer plants containing high levels of jacobine (van Dam et al. 1995; Macel and Klinkhamer 2010). A previous study on soil-borne microorganisms showed that *J. vulgaris* plants containing high levels of jacobine alkaloids had a lower fungal diversity in the rhizosphere than *J. vulgaris* plants lacking high levels of jacobine alkaloids (Kowalchuk et al. 2006). Apart from the above, at this stage there is still too less known about the functions of specific alkaloids to predict the ecological consequences of the change in alkaloid composition. Further research on the influence of specific PAs on fungal and bacterial growth *in vivo* is warranted.

In conclusion this study shows that plants have a more complex chemical defence strategy as previously thought. Our results demonstrate that inoculating sterilized soils with only 5% of non-sterilized soil has a great impact on the plant growth and the plant's defense system. This may have considerable ecological consequences for instance for the invasive success and biological control of plants. In addition this has many practical implications for the design of experiments on plant defence. We will continue to investigate this functional response and the consequences of these changes in chemical defence for plant fitness and the influence on herbivore and pathogen pressure above- and belowground.

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Soil-borne Microorganisms affect Aboveground Metabolic Profiles and Defence against Thrips in *Jacobaea vulgaris*

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Abstract

Secondary metabolites play a crucial role in plant defence. The metabolic profile of plants is determined by a complex set of interacting factors. It has been suggested that soil-borne microorganisms may trigger the plant's defence system thereby influencing aboveground defence against herbivores. We studied (1) the effects of belowground soil-borne microorganisms on pyrrolizidine alkaloids (PAs) in the shoots of different *Jacobaea vulgaris* genotypes and consequently, (2) the effect on aboveground herbivory by thrips.

We used clonal plant individuals, propagated by tissue culture, of five genotypes originating from different West European populations; these genotypes included three Jacobine- and two Erucifoline-chemotypes. Plants were grown on sterilized soil from Meijendel, a dune area near The Hague (the Netherlands). Plants were subjected to three different soil treatments: sterilized soil, sterilized soil inoculated with 5% of non-sterilized dune soil from Meijendel and sterilized soil inoculated with 5% of a different humus-rich soil collected in Heteren in the east of the Netherlands.

We found that the PA concentration and composition aboveground were significantly affected by the belowground microbial community for all genotypes. The amount of feeding damage depended on genotype; plants containing jacobine-like PAs showed a higher degree of resistance. Resistance to thrips was affected by inoculation with unsterilized sandy dune soil in one genotype. However, neither of the main effects of inoculum nor the interaction with genotype were significant. Because of these inconclusive results we repeated the experiment with more individual plants (15 instead of 7 replicates) of two selected genotypes, the genotype that showed a significant effect of inoculum on thrips resistance and one genotype that did not. This repeated experiment qualitatively confirmed the results of the first experiment. Soil inoculum had a highly significant effect on thrips resistance in one genotype but not in the other.

Interestingly, we also found a strong negative effect on plant growth in sterilized Meijendel soils inoculated with its 'own' microbial community as we found in previous studies. This seems to suggest that pathogenic and/or other plant inhibiting microorganisms were adapted to the 'own' soil conditions, and ,thus, were more effective in plant growth reduction than in "foreign" soil.

We found conclusive evidence that shifts in defence compounds can be induced by soil-borne microorganisms. This can have ecological consequences for the plant as, depending on genotype, thrips resistance was affected by the microbial soil community.

Introduction

Plants are exposed to many threats during their lifespan. These treats are not only found aboveground, but also belowground, and they may occur simultaneously. Threats include both abiotic and biotic factors, such as herbivores and pathogens. Plants synthesize a range of defence compounds that are toxic and/or deterrent to these biotic stressors. The metabolic profile of a plant is determined in a complex interaction between genetic and environmental conditions both above- and belowground.

Plant defence can be constitutive, thus being effective prior to plant attack but in many cases it is adjusted to the actual threat and induced as a consequence of physical and/or chemical interaction between the plant and its attacker (Karban and Baldwin 1997; Argawal et al. 2002). The classification of compounds into constitutive or induced defence is often not straightforward. Pyrrolizidine alkaloids (PAs), for instance, are a class of well-known constitutive defence compounds but they are also inducible by herbivores or pathogens (Hartmann and Ober 2000; Hol et al. 2004; Joosten et al. 2009). The concentration and composition of PAs in plant species such as *Jacobaea vulgaris* depend on their genetics (Vrieling et al. 1993; Macel et al. 2004) but also on the environment (Hol et al. 2003; Hol et al. 2004; Bezemer et al. 2006; Joosten et al. 2009; Macel and Klinkhamer 2010). Low nutrient levels in soil (Hol et al. 2003) and root damage (Hol et al. 2004) resulted in an increased PA concentration in the shoots. Macel and Klinkhamer (2010) found that the composition of PAs in genotypes of *J. vulgaris* changed in the field compared to the initial composition in laboratory clones. The composition also differed between the aboveground parts of clones grown on two different experimental field sites. Joosten et al. (2009) found that induced responses in the roots triggered by soil-borne microorganisms had an effect on the concentration of individual PAs in the shoots. Although it is known that plants induce their aboveground defence, there is little known on the role of roots in this process.

Plant roots are responsible for the synthesis and storage of several defence compounds. Furthermore, they play an active role in environmental sensing, defence signalling and may serve as a dynamic storage place for regrowth and vegetative reproduction (van der Meijden et al. 1988; van der Meijden and van der Veen-van Wijk 2000; Erb et al. 2009). In this way the root system is of great importance for the degree of attractiveness and tolerance for and defence against herbivores and pathogens, both below- and aboveground (Bonte et al. 2010; Vandegehuchte et al. 2010). The possibility to induce defence mechanisms belowground may result in aboveground changes as well. Thus, the change in defence compound concentration, induced belowground, may affect herbivores aboveground (Bezemer and van Dam 2005).

We studied the effect of soil-borne microorganisms on the plant defence system aboveground and tested the indirect effect on thrips feeding by measuring the feeding damage on the leaves. We addressed the following questions: (1) does the soil-borne microbial community affect plant growth?, (2) does the soil-borne microbial community affect PA concentration and PA composition in shoots?, and as a consequence (3) does the soil-borne microbial community affect resistance against thrips?, and (4) are the effects of the soil-borne microbial community on growth, PAs and thrips resistance, genotype depended?

Material and Methods

Plants and soils

Five genotypic tissue culture lines of *J. vulgaris* were used originating from five different populations: Wageningen, Vilt, Kassel and two populations from Meijendel. One of the genotypes (Meijendel A) has been used previously and described by Joosten et al. (2009; Chapter 5). To get an overview of the PA composition of these genotypic lines see Joosten et al. 2011 (Chapter 4).

For each genotype per soil treatment 15 clonal replicates were grown. Eight replicates were used for growth and PA analyses, and seven replicates for a whole plant-thrips bioassay. The plants were propagated by tissue culture and grown on soil in a climate room (relative humidity 70%, light 16h at 20ºC, dark 8h at 20ºC). We used two soil-types to inoculate (5%) sterilized sandy dune soil from Meijendel. Together with the sterilized control soil this gave three treatments. The first inoculum was 'Heteren-soil' from an experimental garden that is in use since 1994, the second inoculum was the same 'Meijendel-soil', from the dune system of the coast of the Netherlands. The soils for the inocula were collected within a radius of one meter from a *J. vulgaris* plant. The composition of the two soils differs; 'Meijendel-soil' is composed of calcareous dune sand, while 'Heteren-soil' is a mixture of potting soil and sand. Sterilization was performed by exposure the soil to 25 kiloGray gamma-radiation (Isotron Nederland, Ede, the Netherlands). After five weeks, eight plants per soil treatment were harvested and cut above the root crown for further analyses. The plants were freeze-dried for 72 hours under vacuum with a collector temperature of -55ºC (Labconco Free Zone® 12 l Freeze Dry System). The dry biomass was determined, as well as the PA concentration and composition of the plant parts after freeze drying. The remaining 7 plants per soil treatment were subjected to thrips infestation.

Pyrrolizidine alkaloid analysis

Dried, ground, plant material (approximately 10 mg accurately weighted) was extracted with 2% formic acid (HCOOH) in a 1.2 to 100 weight to volume ratio. Heliotrine (100 µg/ ml in methanol) was added as internal standard to the extract to a concentration of 1 µg /ml. The plant extract solution was shaken for 1 hour. Solid plant material was removed by centrifugation at 2600 rpm for 10 min and filtered with an Acrodisc LC 13 mm Syringe Filter. An aliquot of the extract (25 µl) was diluted with water (975 µl) and injected in the LC-MS/MS system. A Waters Acquity ultra performance liquid chromatographic (UPLC) system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, PA, USA) was used for PA determination as described in Joosten et al. 2011 (Chapter 3).

Thrips experiment

The western flower thrips (*Frankliniella occidentalis*) has been used as a model to study plant resistance for different plant species including *J. vulgaris* (Leiss et al. 2009; Macel and Klinkhamer 2010; Cheng et al. 2011a and 2011b). *F. occidentalis* are cell feeders ingesting whole plant cell content with piercing mouthparts, including the vacuoles (Hunter and Ullman 1989). As cell vacuoles are the storage compartments for PAs in the plant, *F. occidentalis* also ingests PAs when obtaining the fluids from the vacuoles. After feeding, pierced cells are empty and feeding damage is visible on the plant.

Plants were placed in individual thrips-proof cages. These cages consist of plastic cylinders (80 cm height, 20 cm diameter) that can be closed on the top with a ring of thrips-proof gaze. In every cage 20 adult female western flower thrips are added and left for two weeks to forage the plant (humidity 70%,

light 16h at 20°C, dark 8h at 20°C) as described in Leiss et al. (2009).

After two weeks feeding damage caused by the thrips was scored as visual damage on the leafs (mm^2). In addition hairiness and toughness of the leaf as well as plant dry mass were measured. Toughness of the leaf was measured with a penetrometer. We did not find any significant effect of inoculum on these morphological resistance factors. Earlier findings by Leis et al. (2009) in *J. vulgaris* showed that morphological traits did not influence thrips resistance. Therefore our data on these traits will not been shown in the remainder of this manuscript. The plants were stored at -20°C before being freeze-dried for 72 hours under vacuum with a collector temperature of -55°C (Labconco Free Zone® 12 l Freeze Dry System). Before and after freeze-drying the biomass of the plant was measured.

Repeated thrips experiment with more replicates

When analyzing the inoculum effect per plant genotype we found that the Wageningen genotype showed a significant inoculum effect on feeding damage (see results experiment above). However, in an ANOVA including all five genotypes, neither the main effect of inoculum nor the interaction with genotype was significant (Table 3). Because of these inconclusive results we repeated the above described experiment with more replicates, (15 instead of 7 individual plants per treatment per genotype). We selected two genotypes with a similar overall thrips resistance; Wageningen genotype which showed a significant inoculum effect and Meijendel B which showed no inoculum effect on feeding damage.

Data analysis

The effect of genotype and inoculum treatment on plant dry mass was analyzed by GLM (General Linear Model) univariate analyses procedure. With plant dry mass as the dependent variable, and genotype and inoculum (Sterilized, Meijendel and Heteren soil inoculum) as fixed factors. The effect of genotype and inoculum treatment on the total PA concentration was also analyzed by GLM (General Linear Model) univariate analyses procedure, with PA concentration as the dependent variable, and genotype and inoculum (Sterilized, Meijendel and Heteren soil inoculum) as fixed factors. In order to determine the effect of the soil inoculum on the composition of PAs in the shoot we used discriminant analyses for all genotypes together and for all individual genotypes. An F-test (Wilks' lambda) was used to test if the 6 discriminant models as a whole were significant (95% confidentiality). The relative concentrations of individual alkaloids (expressed as the percentage of the total PA concentration) and the total PA concentration were included as independent variables. The effect of genotype and inoculum on feeding damage was statistically analyzed by GLM (General Linear Model) univariate analyses procedure, with feeding damage as the dependent variable, and genotype and inoculum as fixed factors. In all analyses of thrips resistance shoot biomass was included as a covariate. In cases where the effects on shoot biomass were not significant (P>0.05) it was removed from the final model.

The relationships between the absolute total PA concentration and feeding damage and the relative individual PA concentrations and feeding damage were analysed with two-tailed Pearson correlation. For this analyses six individual PAs were selected namely jacobine, jacobine *N*-oxide, jaconine, jaconine *N*-oxide senecionine *N*-oxide and retrorsine *N*-oxide. Jacobine, jaconine and the corresponding *N*-oxides were chosen based on previous research by Leiss et al. (2009) and Cheng et al. (2011a; 2011b). They found a relation between these PAs and feeding damage. Senecionine *N*-oxide is the most basal PA, produced in the roots as primary PA and was therefore selected as well. Joosten et al. (2009) found a shift in retrorsine *N*-oxide between treatments so for that reason retrorsine *N*-oxide was included in this study. The P-values were Bonferroni corrected. All tests for data analyses were conducted with SPSS 17.0 for Windows.

Results

Plant dry mass

Both genotype and inoculum had a high significant impact on plant dry mass (Table 1). The effect of inoculum on the 5 different genotypes was similar as can be seen by the non-significant two-way interaction. Mean plant dry mass was highest on sterilized soil and sterilized soil inoculated with Heteren soil. Plant dry mass was lowest on soil inoculated with Meijendel soil (Figure 1).

Total pyrrolizidine alkaloid concentration aboveground

Genotype and inoculum had a significant effect on the mean total PA concentration of the shoots (Table 2). The genotypes Meijendel A, Meijendel B and Wageningen had significantly higher PA concentration in the shoots than the genotypes Vilt and Kassel. Inoculation with Heteren soil resulted in a significantly higher PA concentration in the shoots as compared to the sterilized control treatment and the inoculum of Meijendel soil (Figure 2). The PA concentration in the shoots was affected by inoculum in a similar way for all five genotypes as indicated by the non-significant genotype x inoculum interaction.

Inoculum effect on pyrrolizidine alkaloid composition

We detected in total 12 different PA *N*-oxides and 15 different tertiary amines in the shoot extracts. On the basis of discriminant analyses, we observed that the relative concentration of different PAs in the shoot differed significantly between the 3 inoculum treatments for all genotypes (Figure 3). In total two functions were needed to classify all cases correctly. The discrimination of inoculum treatment for all genotypes were mainly explained by function 1 (>71% classified correctly). The first graph in Figure 3 (All Genotypes) shows that the sterilized control and the Heteren inoculum treatment had the highest discrimination by function 1, while the Meijendel inoculum treatment was discriminated from the other two by function 2. This discrimination was less strong because function 2 explained only 22.6% of the data. For the genotypes, Wageningen, Meijendel A and Vilt, the sterilized control was discriminated strongly from the inoculated treatments by function 1, while the Heteren and Meijendel inoculation treatments were discriminated by function 2. In Meijendel B and Kassel genotypes, the Meijendel inoculation and the sterilized control had a stronger similarity in PA composition compared to the Heteren inoculation treatment, this discrimination was mainly explained by function 1. Of all PAs in particular the relative concentration of retrorsine *N*-oxide was significantly higher in inoculated soils, especially belowground (data not shown).

Feeding damage

Genotype and Inoculum effect on Feeding damage

Genotype had a significant effect on feeding damage (Table 3). Genotype Kassel had, with a mean of 72,0 mm^2, the highest amount of feeding damage. The two jacobine-genotypes; Meijendel A and B, had the lowest amount of feeding damage, 39,8 and 32,2 mm^2 respectively (Figure 4). Inoculum treatment did not affect feeding damage (Table 3). When analyzing the inoculum effect per genotype we found that only the Wageningen genotype showed a significant inoculum effect on feeding damage (F=4.9, df=2, Df=18, P=0.02). Unfortunately, we cannot draw any firm conclusions on this observation, as we did not find a significant interaction between genotype and inoculum (Table 3).

Table 1. ANOVA of the effect of genotype and inoculum treatment on plant dry mass of *Jacobaea vulgaris*

Dependent variable	Fixed Factors	df (k-1)	Df (N-k)	F	P
Plant dry mass	Genotype	4	115	15.03	<0.001
	Inoculum	2	117	13.79	<0.001
	Genotype*Inoculum	8	111	1.54	0.151
	Error	105			
	Total	120			

Table 2. ANOVA of the effect of genotype and inoculum treatment on the total PA concentration in the shoots of *Jacobaea vulgaris*

Dependent variable	Fixed Factors	df	Df	F	P
Total PA concentration	Genotype	4	115	19.48	<0.001
	Inoculum	2	117	5.70	0.004
	Genotype x Inoculum	8	111	1.71	0.105
	Error	105			
	Total	120			

Table 3. ANOVA of the effect of genotype and inoculum treatment on feeding damage on *Jacobaea vulgaris*

Dependent variable	Fixed Factors	df	Df	F	P
Feeding damage	Genotype	4	97	11.45	<0.001
	Inoculum	2	99	1.383	0.256
	Genotype x Inoculum	8	93	1.076	0.387
	Error	88			
	Total	102			

Table 4. Two-tailed Pearson correlation of the mean feeding damage (mm2) with the absolute total PA concentration and the relative PA concentration of previous selected PAs in the shoots of *Jacobaea vulgaris* plants. n = 15 is based on the main PA and feeding damage values of 5 genotypes x 3 soil treatments

	Mean feeding damage			
Independent variables	**n**	**R**	**P**	**Bonferroni**
Jacobine	15	-0.59	0.02	0.06
Jacobine *N*-oxide	15	-0.59	0.02	0.08
Senecionine *N*-oxide	15	0.46	0.08	0.42
Retrorsine *N*-oxide	15	0.37	0.18	1.25
Jaconine	15	-0.63	0.01	0.03
Jaconine *N*-oxide	15	-0.80	<0.001	<0.001
Total PA	15	-0.46	0.08	0.50

Table 5. ANOVA of the effect of genotype and inoculum treatment on feeding damage on *Jacobaea vulgaris* with shoot biomass as covariate

Dependent variable	Fixed Factors with covariate	df	Df	F	P
Feeding damage	Genotype	1	84	0.3	0.56
	Biomass shoot **(covariate)**	1	84	18.5	<0.001
	Inoculum	2	83	5.6	0.005
	Genotype x Inoculum	2	83	13.2	<0.001
	Error	79			
	Total	86			

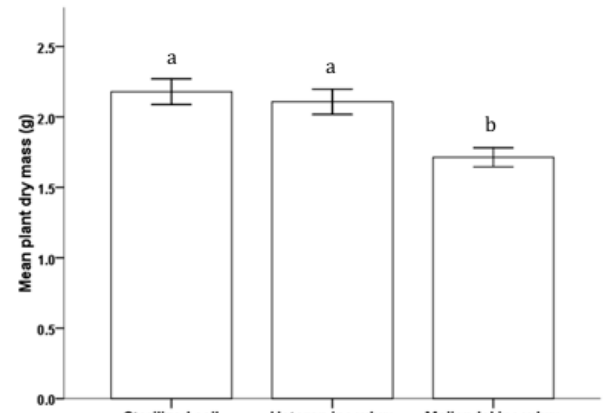

Figure 1. Mean plant dry mass per inoculum treatment (n = 40) of *Jacobaea vulgaris* plants. Error bars ±1SE. ANOVA with Post Hoc Bonferroni test in SPSS 17.0. Different letters above the bars indicate significant differences between inoculation treatments (P<0.05).

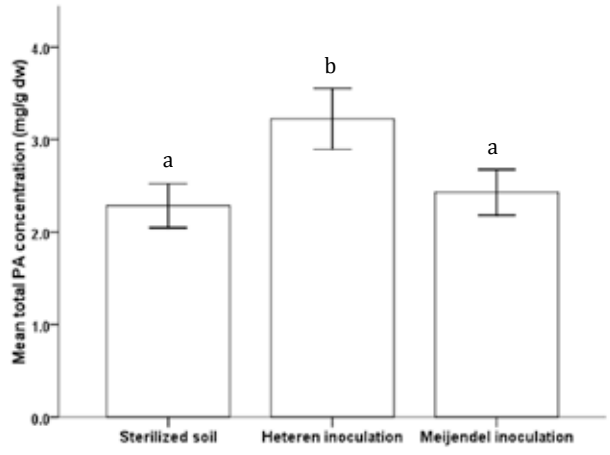

Figure 2. Mean total PA concentration in the shoots per inoculum treatment (n = 40) of *Jacobaea vulgaris* plants. Error bars ±1SE. ANOVA with Post Hoc Bonferroni test in SPSS 17.0. Different letters above the bars indicate significant differences between inoculation treatments (P<0.05).

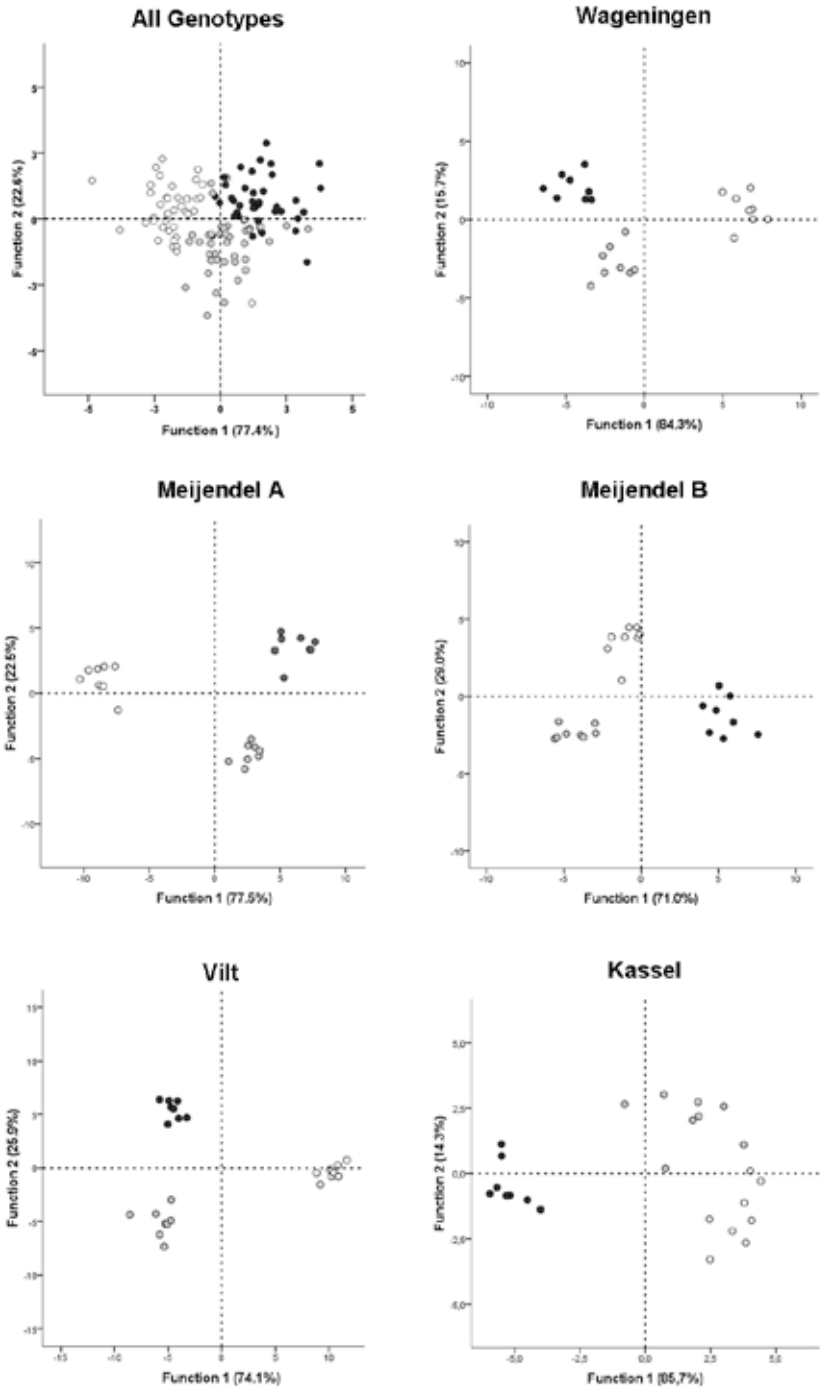

Figure 3. Scatterplots of plants grown on three different soil treatments, showing the discriminant scores of the cases on discriminant function 1 and 2 generated by Classify Discriminant Analysis for all genotypes plotted together and separately. White is sterilized Meijendel soil; Black is sterililized Meijendel soil inoculated with 5% non-sterilized Heteren soil; Grey is sterilized Meijendel soil inoculated with 5% non-sterilized Meijendel soil.

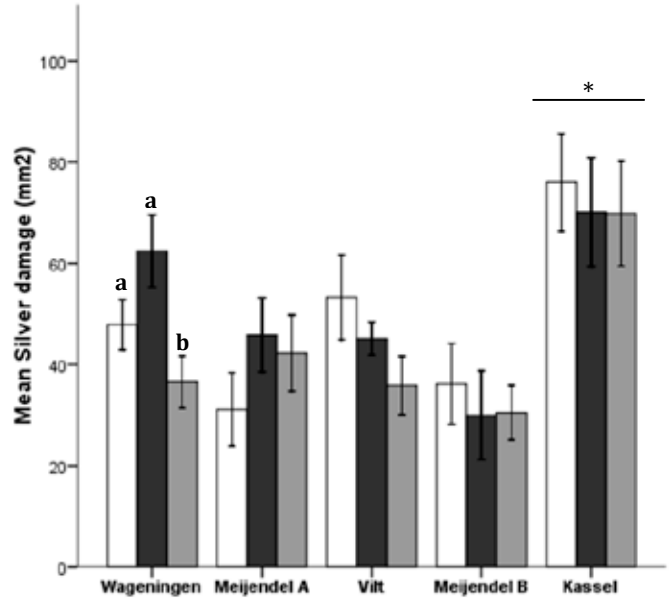

Figure 4. Mean of the total feeding damage per inoculum and genotype of *Jacobaea vulgaris*. White is sterilized soil (n = 35); Black is sterilized soil inoculated with 5% Heteren soil (n = 33); Grey is sterilized soil inoculated with 5% Meijendel soil (n = 34). Genotypes Wageningen & Meijendel A (n = 21); Genotypes Vilt, Meijendel B & Kassel (n = 20). Error bars ±1SE. ANOVA with Post Hoc Bonferroni test in SPSS 17.0. Different letters above the bars indicate significant differences between inoculation treatments within the genotypes (P<0.05) and * above plant genotype indicate significant differences to the other genotypes as a whole (P<0.05).

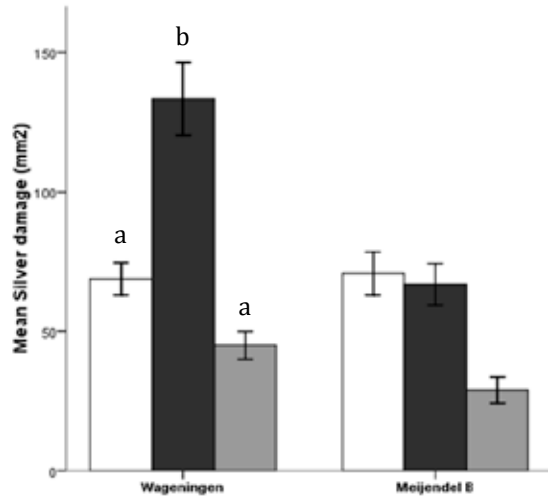

Figure 5. Mean of the total feeding damage per inoculum of two *Jacobaea vulgaris* genotypes. White is sterilized soil (n = 30); Black is sterilized soil inoculated with 5% Heteren soil (n = 30); Grey is sterilized soil inoculated with 5% Meijendel soil (n = 26). Genotype Wageningen (n = 44); Genotype Meijendel B (n = 42). Error bars ±1SE. ANOVA with Post Hoc Bonferroni test in SPSS 17.0. The ANOVA model was corrected by adding shoot biomass as covariate since shoot biomass had a significant effect on thrips resistance in this experiment. Different letters above the bars indicate significant differences between inoculation treatments within the genotype (P<0.05).

Total and individual PA concentration effect on feeding damage

For the total PA concentration and most of the individual PAs like senecionine *N*-oxide and retrorsine *N*-oxide we did not find significant correlations with feeding damage inflicted by thrips (Table 4). However, jacobine and jaconine and the corresponding *N*-oxides were both significantly negatively correlated with feeding damage. Only jaconine and jaconine *N*-oxide still had a significant effect on thrips herbivory after Bonferroni correction, while jacobine and jacobine *N*-oxide were only marginally significant.

Genotype and inoculum effect on feeding damage repeated with more replicates for two genotypes

Genotype and inoculum both significantly affected feeding damage (Table 5) in the repeated experiment with more replicates on the two selected genotypes. In this experiment, shoot biomass had a significant effect on the thrips resistance (Table 5) and therefore, as correction to the model, we added shoot biomass as covariate. When analysing the inoculum effect per genotype we found that again only the Wageningen genotype showed a significant inoculum effect on feeding damage (Figure 5). The inoculum effect on feeding damage of genotype Meijendel B was not significant with shoot biomass as covariate (Figure 5). The results of this repeated experiment are therefore as we predicted on basis of the results of the previous experiment in which we also only found effects of inoculation on feeding damage for genotype Wageningen.

Feeding damage on the Wageningen genotype decreased with inoculation of Heteren soil, while the total PA concentration independent of genotype increased (Figure 5) but did not raise the levels of jacobine-like PAs (data not shown). So unfortunately, this belowground-aboveground interactions cannot be explained based on the changes in PA concentration and composition.

Discussion

Inoculum treatment had a great impact on the mean plant dry mass, which implies that plant growth was influenced by the soil-borne microorganisms. Plants grown on sterilized soil had the highest dry mass whereas plants grown on sterilized soil inoculated with 'own' Meijendel soil had the lowest dry mass. This supports the findings of for instance van der Putten et al. (1993), Bever et al. (1994 and 1997) and Klironomos (2002) that plant growth may considerably be reduced when grown in soils containing the microorganisms from their own natural habitat. Joosten et al. (2009) suggested that this could be the result of a negative effect by the introduced microorganisms, which in case of an inoculation with the same soil as the sterilized soil, may have been selected for plant specific pathogens (Putten et al. 1993; Bezemer et al. 2006). The decrease in plant mass may also be explained by a 'nutrient competition' between plant roots and a selective population of microorganisms. Jackson et al. (1989) showed that on a short timescale, soil microorganisms do compete better than plants, particularly for NH 4+: its uptake by microorganisms was five times faster than that by plants.

The PA composition aboveground was significantly affected by the soil-borne microbial community for all genotypes as previous results (Joosten et al. 2009) including the two genotypes lacking jacobine-like PAs (Vilt and Kassel). In contrast with this previous study there was a significant effect of inoculum on the total PA concentration in the shoots as well. However, the effect of genotype was far more important. The possibility to induce defence mechanisms belowground may result in aboveground changes as well. Thus, the change in defence compound concentration, induced belowground, may affect herbivores aboveground (Bezemer and van Dam 2005).

The second experiment confirmed that the microbial community can affect herbivores aboveground and that this belowground-aboveground interaction is genotype dependent. For one genotype, the microbial soil community significantly affected aboveground plant defence. Most likely this is through changes in the metabolic profile of the plant. However, changes in the concentration of PAs cannot explain the subsequent differences in thrips damage and so the relation to the metabolic profile of the plant. Inoculation of Heteren soil increased total PA concentration independent of genotype (Figure 5). Feeding damage in the Wageningen genotype decreased with inoculation of Heteren soil.

Feeding damage on the plants was significantly different between the genotypes. Kassel (Erucifoline-chemotype) had the highest amount of feeding damage and the two Jacobine-chemotypes from Meijendel had the lowest amount of feeding damage. The effects of the total PA concentration and the relative concentration of individual PAs on the resistance to *F. occidentalis* are in accordance with the results of Cheng et al. (2011a and 2011b), Macel and Klinkhamer (2010) and Leiss et al. (2009). The concentrations of jacobine-like PAs significantly influenced the amount of feeding damage inflicted by thrips. Jacobine-like PAs are mainly responsible for tertiary amines in the plant (Joosten et al. 2011; Chapter 4), the more toxic form of Pas, especially in the shoots. However, the increased resistance of the Wageningen genotype inoculated with Heteren soil could not be explained by increased levels of jacobine-like PAs.

This study showed that in different *J. vulgaris* genotypes originating from several different populations, the PA concentration and composition, was triggered by soil-borne microorganisms. The belowground-aboveground interactions in plant defence were genotype dependent and could not be explained on basis of the observed changes in the concentration and composition of PAs. At this point we do not know which factors are involved in this interaction. Studies with a non-targeted approach with respect to the plant's metabolome (e.g. NMR studies) are clearly needed.

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7

Microbial Community Structure in Rhizosphere Soil and Roots of Different Genotypes of *Jacobaea vulgaris*

Lotte Joosten and Johannes A. van Veen

Abstract

The microbial community nearby root system of the plant is selected from the microorganisms present in the bulk soil by the plant and other factors. Rhizodeposition and secretion of defence compounds may stimulate or suppress the success of decomposing soil microorganisms, pathogens and symbiotic microorganisms in the rhizosphere. The defensive role that compounds such as pyrrolizidine alkaloids (PAs) play in plant protection against root-infecting bacteria and fungi is not fully understood (Chapter 2). Here we report on the impact of plant genotype, differing in PA composition, on the community structure of general (1) fungi and (2) bacteria in the rhizosphere; (3) fungi and (4) bacteria in the roots; and (5) arbuscular mycorrhizal fungi in the roots. Four different *Jacobaea vulgaris* genotypes were used in this study differing in the PA composition. They were chosen based on the presence or absence of jacobine-like PAs and were grown on two different soil-types; löss and sand. The general microbial community structures in the roots and rhizosphere were characterized by using Denaturing Gradient Gel Electrophoresis (DGGE) and for AMF in the roots by using Terminal Restriction Fragment Length Polymorphism (T-RFLP). The fungal community in both, rhizosphere soil and plant roots was clearly genotype dependent. However, we found no indications that the genotype effect was related to the PA composition except for the fungal diversity in the roots. We found a significant negative correlation between erucifoline-like PAs and the fungal diversity in the roots of the plant. There were no overall significant differences in bacterial community structure in roots and rhizosphere among the genotypes. Soil-type affected the AMF community structure in the roots but plant genotype did not.There were no overall significant differences in bacterial community structure in roots and rhizosphere among the genotypes. Soil-type affected the AMF community structure in the roots but plant genotype did not.

Introduction

Plant species contain high food reserves in their root system for vegetative reproduction and re-growth to survive complete defoliation by specialist herbivores (van der Meijden et al. 1988; van der Meijden and van der Veen-van Wijk 2000). Therefore it is to be expected that these species protect their roots strongly against belowground herbivores and pathogens. One of the protection mechanisms is the production of secondary metabolites, toxic or deterrent for attackers (Wink et al. 1988; Falk and Doran 1996; Hol et al. 2003; Thoden et al. 2009). Hol et al. (2003) found that the concentration of pyrrolizidine alkaloids (PAs), a class of secondary metabolites typical for *Senecio* and *Jacobaea* species, in the main root cortex of *J. vulgaris* was five times higher than the concentration in the vascular cylinder. These high PA levels in the outer parts of the mature roots may serve as a first line of defence against attackers.

Soil-borne plant pathogens are part of soil-borne microbial communities. The microbial community occurring nearby the plant root system is determined by the pool of microorganisms that are present in the surrounding bulk soil in which the plant grows but is also driven by the plant itself (Kowalchuk et al. 2002, de Ridder-Duine et al. 2005, Berg and Smalla 2009). The direct influence of living roots on the abundance, activity and composition of the soil-borne microbial community is known as the rhizosphere effect (Duineveld 2001). The rhizosphere is generally defined as the volume of soil that is in the narrow root zone of approximately 2 mm and under the influence of the living root system of the plant (Hiltner 1904). The increased biological activity in the rhizosphere is mainly due to the release of compounds such as exudates, secretions, plant mucilage, mucigel and lysates that may serve as substrate (Lynch and Whipps 1990; Broeckling et al. 2008). Plant roots also secrete and/or leak defence compounds that may suppress or stimulate the growth of particular microbial populations in the rhizosphere (Marschner et al. 2001 and 2002; Bais et al. 2006; Badri and Vivanco 2009). The defensive role that secondary plant metabolites play in plant protection against root-infecting bacteria and fungi is still not fully understood because of inadequate methods for analysing low concentrations of secondary metabolites released in the rhizosphere (Bais et al. 2006).

In this study we focussed on the effect of plant genotype on the microbial community in the rhizosphere with special reference to the PA composition of the plant. A field study by Kowalchuk et al. (2006) showed that PAs affected the fungi present in the rhizosphere of *J. vulgaris*. High jacobine-production in plants (1.13-3.92 mg/g PA/dw root) was associated with a lower diversity of fungi in the rhizosphere compared to low PA-producing plants (0-0.53 mg/g PA/dw root) or high PA-producing plants lacking jacobine in the root (Kowalchuk et al. 2006). This study implies that the PA composition of the plant may have an important influence on fungal community in the rhizosphere. Interestingly, there are quite a number of reports that showed that PAs had inhibitory effects on mycelium growth of several plant-associated fungi and bacteria (Jain and Sharma 1987; Marquina et al. 1989; Reina et al. 1995, 1997, 1998; Singh et al. 2002; Hol and van Veen 2002; Hol et al. 2003) in in-vitro systems.

The mechanisms of the influence of PAs on microbial communities in the rhizosphere are unknown. We may expect that PAs may leak into the rhizosphere by root damage and sloughed off root cells. Plants may also actively secrete PAs into the soil but, to our knowledge, this has never been proven as the exact PA levels in the rhizosphere have never been measured. Since PAs are very recalcitrant against decomposition (Candrian et al. 1984; Crews et al. 2009), there is a high probability that defence compounds such as these particular type of PAs are persistent in the rhizosphere.

Another mechanism is the effect of PAs inside the root tissue on endophytes such as arbuscular mycorrhizal fungi (AMF; Glomeromycota). AMF form symbiotic associations with most terrestrial plant species and play an important role in nutrient cycling and plant productivity (Klironomos et al. 2000, Klironomos and Hart 2003, Drigo et al. 2010). Members of the genus *Senecio* and *Jacobaea* are facultative mycorrhizal plants (Bower 1997 in Gange et al. 2002 and Reidinger et al. 2012). Gange et al. (2002) suggested that AMF are parasitic in *J. vulgaris* species. However, it is unknown whether the effect of AMF colonization on *J. vulgaris* is always negative, or depends on the biotic or abiotic environment of the plant (Reidinger et al. 2012). Associations between *Glomales* fungi and *Jacobaea* plants are to be expected in the poor sandy dune soil since mineral nutrients as phosphorus and nitrogen are limiting. PAs are nitrogen-based defence compounds. PA-producing plants could benefit from the extra nitrogen uptake by AMF by using it for the production of more chemical compounds.

Since *J. vulgaris* is a low mycorrhizal host plant and no common partner of AMF, we suggest that many AMF species, colonizing this plant species, are generalists and not adapted to the PA defence system. Less is known about the plant factors, like secondary metabolites, that determine colonization and the final AMF composition in the roots of the plant (Bais et al. 2006). When AMF encounter PAs, we expect no equal sensitivity of AMF species to the different PAs. Reidinger et al. (2012) found the total PA and the individual PA jacoline to be negatively related to root colonisation by vesicles and as Kowalchuk et al. (2006) found that the general fungi diversity in the rhizosphere was negatively related by high levels of the jacobine PAs, we expect similar results for AMF in the roots.

In this study we will focus on genotype and PA-group effects on the diversity within general; (1) fungal and (2) bacterial soil communities in the rhizosphere; (3) fungal and (4) bacterial communities in the roots; and (5) AMF in the roots of four different *J. vulgaris* genotypes grown on two different soil-types; löss and sand. We characterized the general microbial community structure in the roots and rhizosphere by using Denaturing Gradient Gel Electrophoresis (DGGE) and for AMF in the roots by Terminal Restriction Fragment Length Polymorphism (T-RFLP).

Material and methods

Plant origin and growth

We used four different genotypes of *J. vulgaris* (Joosten et al. 2011; Chapter 4). Two Jacobine-chemotypes originated from a coastal population in Meijendel near The Hague and from a population in Wageningen, both in the Netherlands. Two Erucifoline-chemotypes originated from a Dutch population in Vilt and a German population in Kassel. The genotypes were propagated by tissue culture. In total three clonal replicates per genotype were used in each of two soils, giving a total of 24 plants. We used calcareous sandy soil collected from Meijendel, a coastal dune area North of The Hague and löss soil from a meadow in Vilt, Limburg.

Plants were potted in 1.3 L pots and kept in a climate room (humidity 70%, light 16h at 20°C, dark 8h at 20°C). After every time period of 8 days plants were randomly replaced within the climate room. After 8 weeks the plants were harvested in order to determine the PA concentration and composition. The closely adhering soil from the roots was collected as rhizosphere soil. The soil was brushed from the root surface with a fine paintbrush after shaking the whole plant. The plants were cut with scissors just above the root crown and roots and shoots were immediately stored at -20°C for 4 days before being freeze-dried for 1 week under vacuum with a collector temperature of -55°C (Labconco Free Zone® 12 l Freeze Dry System). PAs were extracted by 2% formic acid. An aliquot of the filtered solution (25 µl) was diluted with water (975 µl) and injected in the LC-MS/MS system. The PA detection was performed as described by Joosten et al. (2011; Chapter 4).

DNA extraction

DNA was extracted from 0.25 g wet weight soil samples and 0.05 g dry weight root samples with the Powersoil™ DNA Isolation Kit according to the manufacturer's specifications (Mo Bio Laboratories, Carlsbad, CA, USA). DNA was eluted in 50 µl 10 mM Tris (pH 8) and stored at -20°C until use.

PCR-DGGE

PCR-DGGE technique was used to assess the composition of bacterial, fungal and mycorrhizal communities. The primer sequences, DGGE gradient and references for general fungal and bacterial communities are summarized in table 1. Forward primers were combined with a GC rich region at the 5'end, called GC-clamp, which is necessary for DGGE separation (Muyzer et al. 1993).

Table 1. The primer sequences, amplification protocol and references for general fungal and bacterial communities

Target	Primers PCR	Sequence	Product size (bp)	DGGE gradient (% denaturant)	Reference
Fungi	FR1-GC FF390	gc-AIC CAT TCA ATC GGT AIT CGA TAA CGA ACG AGA CCT	~390	40-55	Vainio and Hantula 2000 Kowalchuk et al 2006
Bacteria	968-GC 1378	Gc-CGG GGG GAA CGC GAA GAA CCT TAC CGG TGT GTA CAA GGC CCG GGA ACG	~410	45-65	Heuer et al 1997

PCR amplifications were performed in 25 µl reaction volumes using 30 pmol of each of the primers and Fast-Start PCR System high-fidelity DNA polymerase (Roche Diagnostics; Nederland b.v. Almere, the Netherlands), while using the manufacturer's recommended buffer conditions. All reactions were performed in a PTC200 Peltier thermal cycler (MJ Research; Waltham, MA, USA). PCR product quality was examined by standard 1.5% (w/v) agarose gel electroforesis with ethidium bromide staining.

DGGE analysis was performed using the method of Muyzer et al. (1993) incorporating the modifications described below. Gradient gels contained 8% (w/v) polyacrylamide (37:1 acrylamide:bis-acrylamide), 0.5 x TAE and were 1.5 mm thick (20 x 20cm). The linear gradient used for fungi was from 40 to 55% denaturant, for bacteria was 45 to 65% denaturant. The 100% denaturing acrylamide was defined as containing 7 M urea and 40% formamide (Muyzer et al. 1993). To ensure well-polymerized slots, a 10 ml top gel layer containing no denaturants was added before polymerization was complete. All DGGE analysis were run using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at a constant temperature of 60°C. Electrophoresis was for 10 minutes at 200 V, after which the voltage was lowered to 75 V for an additional 18 hours. Gels were stained for 20 minutes with MilliQ water containing 0.5 ml/l ethidium bromide; destaining was applied for 10 minutes in MilliQ water prior to UV-transillumination. Gel images were digitally captured using the ImaGo Compact Imaging System (B and L System, Maarssen, the Netherlands). DGGE images were examined within the ImageMaster Elite program (version 4.20, Amersham Pharmacia Biotech). The background was first subtracted by using a rolling circle algorithm (circle diameter 10) and the lanes were normalized so they contained the same amount of total signal. Bands were called automatically and controlled visually. Band positions were then converted to Rf values between 0 and 1 by using the uppermost and lowermost bands in the marker lanes as boundaries.

PCR and T-RFLP mycorrhiza

AMF community structure in the roots was determined by T-RFLP as described by Mummey and Rillig (2007) adjusted according to Verbruggen et al. (2010). This method involved nested PCR with primer pair LR1 and LR2 (van Tuinen et al. 1998; Trouvelot et al. 1999) and a nested reaction with the AMF-specific primer pair FLR3 and FLR4 (Gollotte et al. 2004) which were duel end-labelled with fluorescent dyes respectively, 6-FAM and NED (Applied Biosystems), followed by enzyme digestion of these fragments with the restriction enzymes. The product of the first nested PCR was diluted 1:100 before the second PCR. The 5 µl of PCR product was digested with three different restriction enzymes for 3 hours (TaqI at 65˚C, MboI and AluI at 37˚C; New England Biolabs) in an appropriate buffer with Bovine Serum Albumin (BSA). Multiple enzymes were chosen to improve the discrimination of T-RFLP and have been successfully used in AMF analyses by Mummey and Rillig (2007).

Digested products were purified by a post-reaction cleanup with sodium acetate and ethanol precipitation and diluted in water (1:30 diluted). Of this solution, 0.5 µl was added to 10 µl HiDi formamide (Applied Biosystems; ABI, Nieuwekerk a/d IJssel, the Netherlands) and 2 µl (1:600 diluted) GeneScan 500LIZ sizer (ABI), denatured and run on an ABI 3130 Genetic Analyser. The fragments were electrophoretically separated according to their size and presence/absence was scored. After running on the automated sequencer the results were analysed. The quality of T-RFLP data was first visually inspected in Gene-Mapper Software v4.1 (Applied Biosystems). The threshold for peak recognition was set at 50 relative fluorescence units (rfu). The resulting profiles were subjected to the procedure as described in Verbruggen et al. (2010). The resulting T-RF profiles were uploaded to the T-REX web application (Culman et al. 2008; 2009) for final dataset-wide T-RF binning with a clustering threshold of 0.5 bp. A binary matrix was formed based on T-RF presence. There were two missing values, as the AMF community of two clonal replicates of Wageningen plants on löss soil could not be successfully fingerprinted with the TaqI enzyme. These plants were not included in the analyses in which all three enzyme datasets were combined.

Data analysis

The microbial diversity index was expressed in terms of Shannon-Weaver diversity index and calculated using the formula $H = -\Sigma Pi \ln Pi$ (Shannon and Weaver 1963). For PCR-DGGE analysis, Pi is the relative abundance, and is measured as $Pi = ni/N$, where ni is the intensity of band "i" based on the normalized peak volume per band and N is the sum of all band intensities in the same sample (Ampe and Miambi 2000).

Genotype

The effect of genotype on the microbial diversity (H) was analysed by GLM (General Linear Model) univariate analyses procedure with H values per sample as the dependent variable, and genotype (Kassel, Meijendel, Wageningen and Vilt) as fixed factor. DGGE profiles per were translated in a matrix based on both abundance (Pi values per band) and microbial richness (presence/absence of Pi value) for further analyses.

T-RFLP profiles were translated in a binary matrix only based on AMF richness (presence/absence) for further analyses. AMF richness is set as the number of T-RFs divided by two (each sequence digested by one enzyme gives two peaks). All T-RFs of the different enzymes are listed together in one binary matrix. Principal component analyses (PCA) were performed to compare the communities isolated from the roots and rhizosphere soil of different genotypes grown in two different soil-types, by using DGGE bands and T-RFs of each individual genotype as data input. Two samples were excluded from the AMF analysis because they did not contain any T-RFs.

Chemotype

J. vulgaris plants can be distinguished into different chemotypes based on the PA composition (Witte et al. 1992 and Macel et al. 2004). Senecionine-chemotypes contain mainly senecionine-like PAs and largely lack jacobine- and erucifoline-like PAs, Erucifoline-chemotypes contain mainly senecionine- 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. The effect of chemotype on the microbial diversity (H) was analysed by GLM (General Linear Model) univariate analyses procedure with H values per sample as the dependent variable, and Chemotype (Erucifoline-chemotype: Kassel and Vilt; Jacobine-chemotype; Meijendel and Wageningen) as fixed factor.

PA-groups

In previous studies, up to 30 different PAs were detected in *J. vulgaris* (Witte et al. 1992; Macel et al. 2004; Kowalchuk et al. 2006; Joosten et al. 2009). Based on their structural features, major PAs in *J. vulgaris* can be divided into 3 structural groups: senecionine-like, comprising senecionine, integerrimine, retrorsine and (acetyl)seneciphylline; jacobine-like, comprising jacobine, jacoline, jaconine jacozine, and dehydrojaconine; erucifoline-like, comprising erucifoline and acetylerucifoline (Table 2). The relationships between the root PA concentration of the three structural PA groups and microbial diversity (H) or AMF richness (RFs/2) were analysed with two-tailed Pearson correlation. Correlations and ANOVAs were conducted with SPSS 17.0 for Windows and PCAs with PAST (Hammer et al. 2001).

Table 2. Main pyrrolizidine alkaloids divided into three structural groups

Structural group	Pyrrolizidine alkaloid
Senecionine-like	senecionine
	intergerrimine
	retrorsine
	seneciphylline
	acetylseneciphylline
	riddelliine
	senecivernine
	senkirkine
Jacobine-like	jacobine
	jacoline
	jaconine
	dihydrojaconine
	jacozine
Erucifoline-like	erucifoline
	acetylerucifoline

Results

PA composition in roots of the different genotypes

The total PA concentration of genotype Kassel was twice as high as that of the other three genotypes. Especially the concentration of senecionine- and erucifoline-like PAs was higher (Figure 1 and Table 2). Genotypes Meijendel and Wageningen, both contain jacobine-like PAs in the roots while Kassel and Vilt lack jacobine and its derivates.

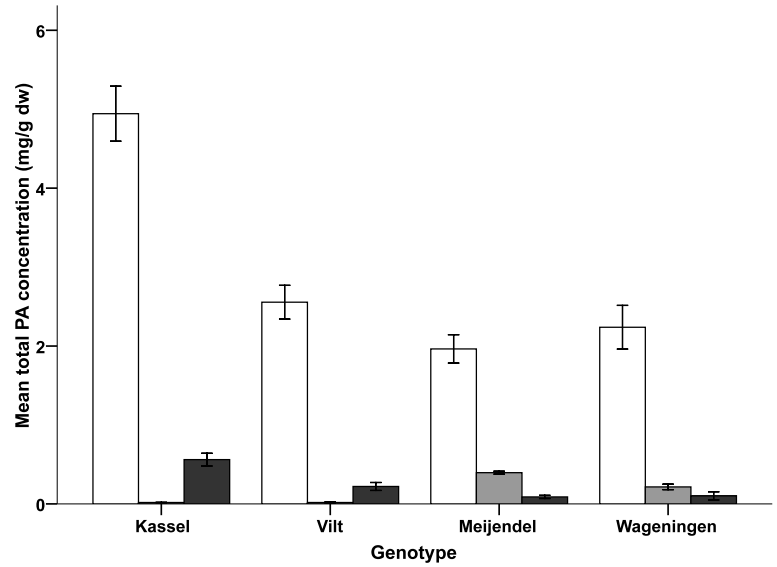

Figure 1. Mean total PA concentration in the roots per genotype grown on Sand and Lösss soil combined. White bar = senecionine-like PAs, grey bar = jacobine-like PAs and black bar = erucifoline-like PAs Error bars ±1SE. n = 6.

Plant genotype effect on the bacterial community structure

Plant genotype showed no significant effect on the bacterial diversity in the roots of both soil-types (Table 3). There was a significant genotype effect on the bacterial diversity in the sand rhizosphere. The mean bacterial diversity (H) in the sand rhizosphere of the Meijendel genotype (3.93 ± SE 0.11) was significantly lower compared to the Wageningen genotype (4.51 ± SE 0.11, overall mean for all genotypes: 4.15 ± SDEV 0.28).

The difference in bacterial community structure for roots and rhizosphere between the four different genotypes was visualized by PCA scatterplot for both soil-types separately; sand and lösss (Figure 2). The root bacterial community of Vilt genotypes separated in both soil-types from Wageningen, Meijendel and Kassel genotypes (Figure 2A and B). Plant genotype showed no clear effect on the rhizospheral bacterial community structure of both soil-types (Figure 2C and D). From the PCA we can see that although we found genotype effects on bacterial diversity this is not reflected in the overall bacterial rhizosphere composition because genotypes do not clearly separate in the PCA (Figure 2D).

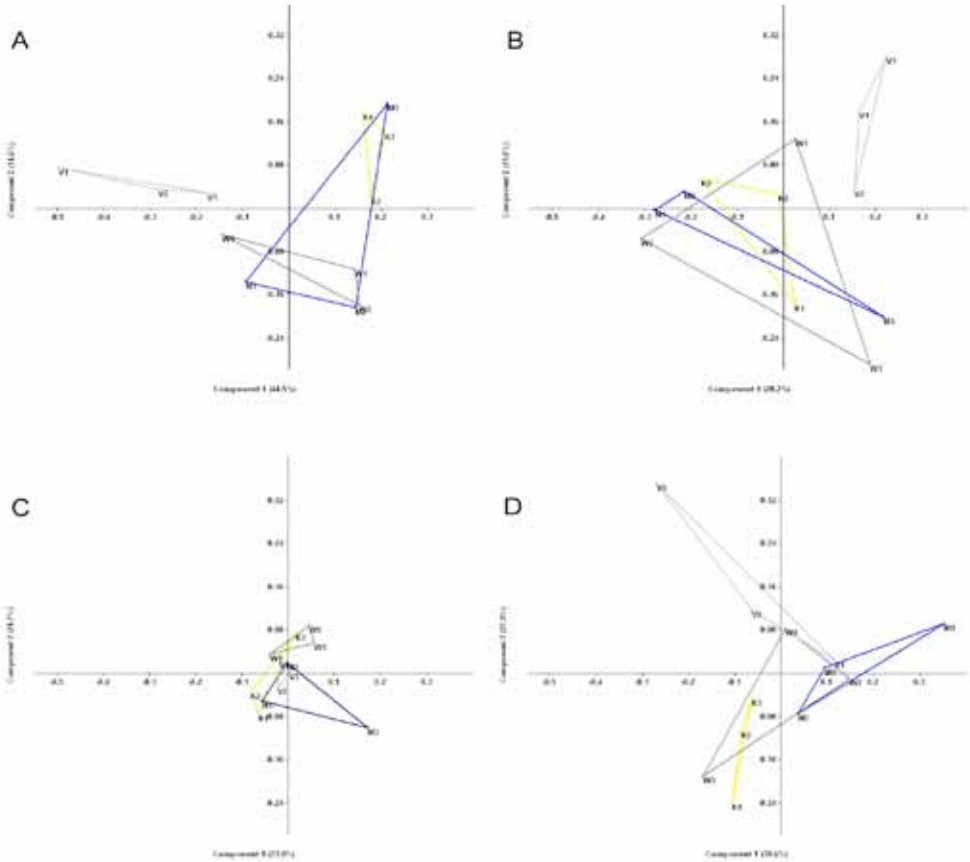

Figure 2. PCA scatterplot of the means of scores of the different genotypes grown on Sand (A and C) and Löss (B and D), for the first two PC-axes with the bacterial community matrix of the Roots (A and B) and Rhizosphere (C and D) as dependent variable . Light grey V = Vilt; yellow K = Kassel; blue M = Meijendel; dark grey W = Wageningen

Plant genotype effect on the fungal community structure

Plant genotype showed a highly significant effect on the fungal diversity in the roots of both soil-types and the löss rhizosphere but no significant effect on the fungal diversity in the rhizosphere of sand (Table 3: ANOVA on H). The mean root fungal diversity (H) in the roots of Wageningen (2.98 ± SE 0.05) was significantly higher compared to Kassel (2.34 ± SE 0.05), Meijendel (2.55 ± SE 0.05) and Vilt (2.49 ± SE 0.05) grown on sand. While on löss, the mean root fungal diversity of Kassel (3.33 ± SE 0.05) was significantly higher compared to Meijendel (3.06 ± SE 0.05) and Vilt (3.02 ± SE 0.05).

The difference in root fungal community structure between the four different genotypes was visualized by PCA for both soil-types separately; sand and löss (Figure 3A and B). For both soil-types, Wageningen genotypes separated clearly from Vilt, Meijendel and Kassel, based on the first principal component explaining almost 70% and 60% of the variation in the matrix data, respectively. In addition Kassel genotype clearly separated from Vilt, Meijendel and Wageningen on the second principal component explaining 22.4% of variation in the matrix data of roots in löss soil (Figure 3B). This is in line with the ANOVA results based on the fungal diversity (H) in the roots described above. The difference in fungal community structure in the rhizosphere among the four different genotypes was visualized by PCA

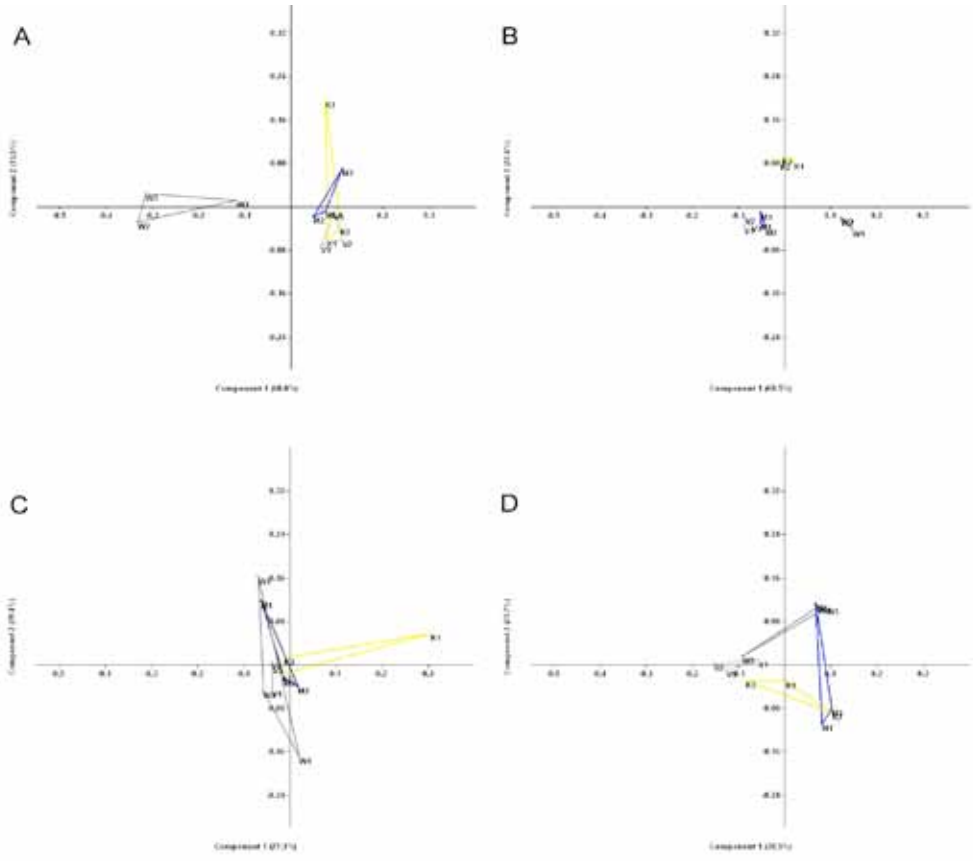

Figure 3. the PCA scatterplot of the means of scores of the different genotypes grown on Sand (A and C) and Löss (B and D), for the first two PC-axes with the fungal community matrix of the Roots (A and B) and Rhizosphere (C and D) as dependent variable (n = 3). Light grey V = Vilt; yellow K = Kassel; blue M = Meijendel; dark grey W = Wageningen

for both soil-types separately; sand (Figure 3C) and löss (Figure 3D). The genotype-effect on the fungal community structure in the rhizosphere was weaker compared to the roots. The first three principal components were needed for both sand and löss, to explain 63% and 72% of the variance, respectively.

Table 3. ANOVA on Shannon diversity index on the abundance-matrix of microbial communities in roots and rhizosphere of *Jacobaea vulgaris* with genotype as fixed factor Df (k-1)/ (N-k)=3/ 8

	Sample treatment		ANOVA (based on H-data) Fixed factor genotype	
Microorganisms	**Soil-type**	**Sample origin**	**F**	**P**
Bacteria	Sand	root	2.3	n.s.
		rhizosphere	5.6	0.02
	Löss	root	0.1	n.s.
		rhizosphere	0.2	n.s.
Fungi	Sand	root	25.3	<0.0001
		rhizosphere	0.4	n.s.
	Löss	root	8.9	0.006
		rhizosphere	9.1	0.006

Chemotype and PA-group effect on the microbial diversity in roots and rhizosphere

The two genotypes, Wageningen and Meijendel B, are Jacobine-chemotypes and Kassel en Vilt are Erucifoline-chemotypes (Joosten et al. 2011; Chapter 4). Plant chemotype showed no significant effect on the microbial diversity in the roots and rhizospere of both soil-types (df = 1, DF = 10, F ≤ 1, P > 0.05), except for the fungal community in the roots of sandy soil (df = 1, DF = 10, F = 9.8, P = 0.01). The mean fungal diversity (H) in the roots of Erucifoline-chemotypes (2.42 ± SE 0.08) was significantly lower compared to Jacobine-chemotypes (2.77 ± SE 0.08) in sandy soil.

On plant basis, across genotypes and soil-type, we also did not find any correlation between the three structural PA groups (senecionine- and erucifoline- and jacobine-like PAs) and bacterial and fungal diversity in the roots and rhizosphere for sand or löss soil (N = 24, P > 0.05 for all correlations), except for the total concentration of erucifoline-like PAs, which was significantly negatively correlated with the fungal diversity in the roots (N = 24, R = -0.45, P = 0.03).

Effect of soil-type and genotype on AMF richness in the roots

In this study in total 111 different TRFs were detected in the root extracts by combining the results of all three digesting enzymes. The total AMF richness over all samples was around 56 with a mean AMF richness per sample of 21. On average, digestion enzyme AluI reproduced 42%, MboI 36% and TaqI 22% of the detected T-RFs.

Soil-type had a significant effect on the AMF richness in the roots (Table 4). Löss soil had a significantly higher AMF richness in the roots compared to sandy soil, with a mean of 24.1 and 19.7 respectively, with the exception for genotype Vilt which was significantly lower compared to the other genotypes within the löss soil treatment (Figure 4). Plant genotype had no significant effect on the AMF richness (Figure 4) but we did find a genotype soil-type interaction (Table 4). AMF community structure in roots of *J. vulgaris* was compared between 4 different genotypes grown in two different soil-types by PCA. The means for each genotype in the two different soil-types (n = 3) for the first two PCA components are shown for all soil samples (Figure 5). This suggests that soil-type may explain a large degree of differences among the analyzed AMF community structure.

Genotypes were separated mainly on the second component, which explained only 10% of the variation. This all suggests that genotype may play no role in the differences among the analyzed AMF community structure. The separation of the genotypes within the two different soil-types were in both cases again not significant.

Table 4. ANOVA of the effect of genotype and soil-type on AMF richness in roots of *Jacobaea vulgaris.* ANOVA with Post Hoc Bonferroni test in SPSS 17.0

Dependent variable	Fixed Factors	Df (k-1)	Df (N-k)	F	P
AMF richness	Genotype	3	18	1.41	0.282
	Soil-type	1	20	5.27	0.038
	Soil-type*Genotype	3	18	3.63	0.040
	Error	14			
	Total	22			

Chemotype and PA-group effect on the AMF richness in roots

The two genotypes, Wageningen and Meijendel B, are Jacobine-chemotypes and Kassel en Vilt are Erucifoline-chemotypes (Joosten et al. 2011; Chapter 4). Plant chemotype showed no significant effect on the AMF richness in the roots of both soil-types (sand: df = 1, DF = 10, F = 0.4, P > 0.05 and löss: df = 1, DF = 8, F = 3.6, P = 0.09). The mean AMF richness in the roots of Erucifoline-chemotypes (27.3 ± SE 2.8) was higher compared to Jacobine-chemotypes (20.3 ± SE 2.3) in löss soil.

On plant basis across genotypes and soil-type we did not find any correlation between the three structural PA groups (senecionine- and erucifoline- and jacobine-like PAs) and AMF richness in the roots for sand or löss soil (N = 22, P > 0.05 for all correlations).

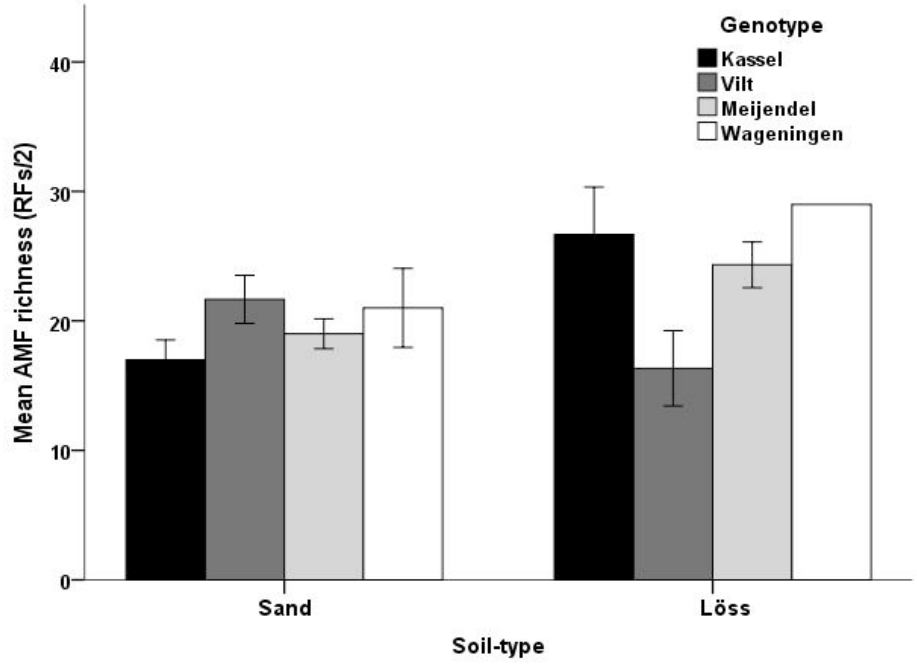

Figure 4 . Mean AMF richness per genotype for AluI, TaqI and MboI digestion enzymes combined. Error bars ±1SE n = 3 except for Wageningen Löss n = 1

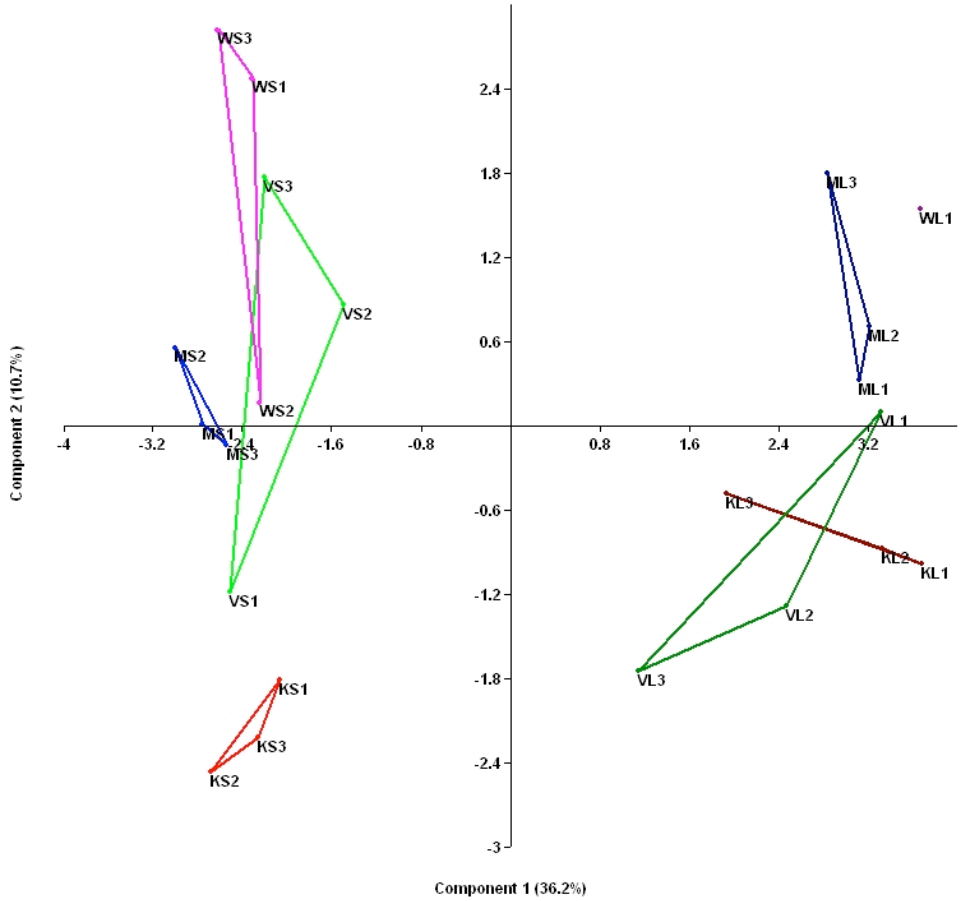

Figure 5. the PCA scatterplot of the means of scores; genotypes and soil-types, for the first two PC-axes with T-RFs binary matrix as dependent variable (n = 3). Dark colors = Löss soil; light colors = Sandy soil. Green = Vilt; Red = Kassel; blue = Meijendel; purple = Wageningen.

Discussion

The genotypes used in this study differed in PA composition based on the presence or absence of jacobine-like PAs. Although other plant compounds and characteristics may have influenced the structure of the microbial community of roots and rhizosphere soil, we hypothesized that the main genotype effect was caused by the PA composition. It is known that PAs may have an inhibitory effect on the growth of microorganisms. In particular fungi were shown to be sensitive for PAs (Hol and van Veen 2002; Kowalchuk et al. 2006). So, it is not surprising that we found the strongest genotype effects on the fungal community, especially in the roots, where endophytes and pathogens live in direct contact with the plant. The outer parts of the mature roots of *J. vulgaris* contain higher PA levels compared to the vascular inner part of the root (Hol et al. 2003). This may serve as a first line of defence against attackers to protect the plant and therefore cause a higher selection pressure for microorganisms entering the roots system. The fungal community in both, roots and rhizosphere soil was clearly plant driven but there were no indications that the genotype effect was directly related to the PA composition, except that the fungal diversity in the roots was lower in Erucifoline-chemotypes compared with Jacobine-chemotypes grown in sandy soil. Also a negative correlation was found between the total erucifoline-like PAs and the fungal diversity in the roots. This is not in line with the findings of Kowalchuk et al. (2006). This study implied that the PA composition of the plant has a strong influence on fungal community in the rhizosphere. Plants lacking jacobine-like PAs in the roots had a higher fungal diversity in the rhizosphere soil compared to plants containing high levels of jacobine in the roots.

Genotype affected the bacterial community less strongly compared to the fungal community. There was no overall significant difference found based on bacterial community structure in rhizosphere soil between the genotypes. Although one genotype; Vilt, separated from the other three genotypes based on the bacterial community in the roots of the plant, we did not find an overall significant difference in bacterial community diversity in roots among the genotypes. For a related PA-producing species, *Senecio inaequidens*, Thébault et al. (2010) found neither significant changes in bacterial community structure based on geocytotypes (i.e. individuals of a species that have a different origin and chromosomal factor to another). They mentioned in the discussion that polyploidisation could influence PA composition and concentration and therefore affect plant-soil interactions. Thereby, PA levels were not measured in this study to prove differences in PA defence between the geocytotypes.

From our study there are no indications that the microbial communities were directly related to the PA composition. In a previous study we found evidence that the other way around, the soil microbial community affected the PA composition in roots and shoots of *J. vulgaris* plants (Joosten et al. 2009; Chapter 5). AMF spore community composition in the soil is known to be influenced by soil properties like soil-type, chemistry and disturbance history (Helgason et al. 1998; Egerton-Warburton et al. 2007; Lekberg et al. 2007; Fitzsimons et al. 2008). Studies have shown that spore community does not predict the AMF community composition colonising the plant root system (Clapp et al. 1995; Merryweather and Fitter 1998; Rodríguez-Echeverríia et al. 2008). Plant host identity (VandenKoornhuyse et al. 2002 and 2003; Gollotte et al. 2004; Scheublin et al. 2004; Hausmann and Hawkes 2009) and vegetation (Johnson et al. 2004; Mummey et al. 2005; van de Voorde et al. 2010) are known factors that affect AMF community composition in the roots of the plant. Our study showed that soil-type had a significant influence on the AMF composition in the roots, while genotype had no effect on the AMF composition in the roots. So the soil selected much stronger for AMF colonization in the plant than the individual plant itself. The effect of soil-type on AMF community in the roots may be caused by the original soil differences such that two different soil-types

contained a different AMF spore and hyphae composition at the start of the experiment which was greater than the selective force on it. Scheublin et al (2004) showed that individual plant species can show preferences for specific AMF taxa. This was not clearly shown in our study based on individual plant genotypes, which could be explained by the fact that *J. vulgaris* is known as facultative mycorrhizal. So the pool of AMF that was present nearby the plant root system in the surrounding bulk soil in which the plant grew was the factor influencing the AMF community colonizing the roots (de Ridder-Duine et al. 2005). For all genotypes the löss treatment had the highest AMF richness except for genotype Vilt; that originates from the same area where löss soil was collected (Figure 4). For the three structural PA groups we did not find any relation with the AMF richness in the roots of the plant.

The present results on the role of PAs in shaping the microbial community structure in the rhizosphere and roots are, highly probably, affected by low concentrations of PAs in the present genotypes. We had hoped to confirm the results of Kowalchuk et al. (2006) but with a more uniform genetic background by using cloned replicates. However the jacobine-like PA concentrations in the roots of the present studied Jacobine-chemotypes (mean 0.32±0.13mg/g jacobine-like/dw root) were around 4 times lower than in the studied Jacobine-chemotypes of Kowalchuk et al. 2006 (mean 1.30±0.39mg/g jacobine-like/dw root). In future studies also needs to be prevented that unknown physiological traits influence the experiment by using, genotypes of *Jacobaea* F2 hybrids (Kirk et al. 2010) instead of *J. vulgaris*. The frequently occurring transgressive segregation in F2 hybrids offers potentially large variation in PA concentration and composition of secondary chemistry, while at the same time being genetically close related by sharing the same (grand-)parents (Cheng et al. 2011a and 2011b). So, F2 genotypes can be selected with extreme differences in PA composition.

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8

Summary and Discussion

Lotte Joosten

The aim of the research described in this thesis was to obtain better understanding on the PA composition of plants and its interaction with soil-borne microorganisms. In order to do so, a more sensitive, PA extraction and detection method was developed and applied, which enabled us to obtain a better picture of the PA composition both above- and belowground, and it allowed us to distinguish between PAs in tertiary amine and *N*-oxide form.

Traditional versus novel PA analysis and the consequences of using a more sensitive methodology

Until recently, the study on the role of PAs as plant defence compounds, was hampered by the low sensitivity of the extraction and detection methods and by the fact that no easy distinction could be made between tertiary amine and *N*-oxide form. The traditional method consists of PA extraction with sulfuric acid, reduction with zinc and purification for gas chromatography with nitrogen phosphorus detection (GC-NPD). The novel method consists of PA extraction with formic acid with only a simple dilution step to allow for liquid chromatography tandem mass spectrometry (LC-MS/MS). The two different methods were compared by measuring a number of dried *Jacobaea vulgaris* samples (Joosten et al. 2010; Chapter 3). The concentrations measured in plant material by LC-MS/MS were substantially higher than those measured by GC-NPD. This indicates that losses may have occurred during the reduction and purification steps required for the traditional method. These losses are minimized in the LC-MS/MS method. Moreover, the formic acid extraction for the LC-MS/MS is far less complex and time-consuming compared with GC analysis by tolerating a much simpler sample treatment procedure. The introduction of a tandem mass spectrometer as a detector in combination with liquid chromatographic separation greatly improved the possibilities to detect different PAs because of lower detection limits (Betteridge and Colegate 2005; Wuilloud et al. 2004). As a result LC-MS/MS detected up to 13 different tertiary PAs in the *J. vulgaris* extracts while GC-NPD detected only 7 PAs. With GC-NPD only 4 major PAs (senecionine, seneciphylline, integerrimine and jacobine) were detected in all samples (Chapter 3). The concentrations of the other 3 PAs were in a number of cases just below the quantitation limit of the GC-NPD.

When plant material was analysed previously by the traditional method only a tip of the iceberg of the PA bouquet was visualized, while with this novel technique also PAs, which were previously under detection limit or close to detection limit, are detected. This underestimation of the PA composition of plants may have consequences for the interpretation of previous research.

Witte et al. (1992) and Macel et al. (2004) used the traditional methods for PA detection. Based on their PA composition data, individual *J. vulgaris* plants were distinguished into different chemotypes. Senecionine-chemotypes contain mainly senecionine-like PAs and largely lack jacobine-like PAs and erucifoline-like PAs, Erucifoline-chemotypes contain mainly erucifoline-like PAs and lack jacobine-like PAs and its derivatives, 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. As a result of the more sensitive LC-MS/MS technique, the discrimination between different chemotypes has become more vague, because we find all 3 structural PA groups; senecionine-, erucifoline- and jacobine-like PAs, frequently in all plant material. Therefore, many genotypes categorized previously as Senecionine-, Jacobine- or Erucifoline-chemotypes could actually well be mixed-chemotypes.

Pelser et al. (2005) used the less sensitive GC-MS method for his study on the evolution of PA formation in *Senecio* plants sect. *Jacobaea* and reconstructed the evolutionary history of PA variation. This was partly achieved by optimizing additive presence/absence data of PAs. Besides showing large intra- and interspecific variation, PA distribution appeared to be largely incidental within the whole clade. It would be very interesting to repeat this study with the novel PA extraction and detection method, because now we are aware that far more PAs are present than previously assumed and as a consequence the incidence of disappearing and evolving of PAs may be far less than calculated on basis of the old detection method. Thus, the distribution of PAs should be far less incidental within the clades.

In conclusion, the novel method, formic acid extraction in combination with LC-MS/MS, was the method of choice for determining PAs in plant material throughout the research described in this thesis, because of the simple and rapid sample preparation, sensitivity and discrimination between the two PA forms (PA *N*-oxides and its reduced tertiary amines).

Tertiary amines occur in plant material, what are the ecological consequences?

PAs may occur in two forms: tertiary amine and *N*-oxide. The tertiary PA form is known to have a more negative effect on generalist insects (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005; Thoden et al. 2009). The simultaneous detection of PA *N*-oxides and tertiary amines in extracts by using the novel extraction and detection method widens the possibility to investigate these two forms in plant material. Our study clearly showed that the high levels of tertiary amines found for jacobine and other jacobine-like PAs are not caused by an intrinsic structural instability of the PA molecule or a higher sensitivity for reducing agents in the extraction and analytical process by chemical or naturally occurring agents in plant material as suggested in previous studies (Hartmann and Toppel 1987; Hartmann 1999; Hartmann and Ober 2000). Therefore we conclude that for specific PAs, high levels of tertiary amines may occur in the plant, as a result of a change induced by (bio)chemical processes in the plant itself (Joosten et al. 2011; Chapter 4). We observed that Jacobine-chemotypes have a much higher level of tertiary PAs compared to the Erucifoline-chemotypes, due to the fact that especially jacobine-like PAs occur in the reduced form. Besides that, we showed that the proportion of tertiary amines is PA specific and genotype dependent.

Two possible and non-exclusive hypotheses may explain the observed pattern. Firstly, the chemical transformation and perhaps allocation of PA *N*-oxides, might be accompanied by a continuous slow reduction of the original *N*-oxides (Hartmann 2010, personal communication). Secondly, specific (re-)oxidation of the tertiary PAs might partly explain the pattern as well. The reduction of PA *N*-oxides in the plant is an unspecific, chemical process induced by the presence of endogenous reducing compounds and (traces of) transition metal salts. This part supports the first hypothesis, but meanwhile, there is a, biochemically based, process operating to re-oxidize the reduced tertiary amines for PA transport. Enzyme(s) that may be involved seem to work well for senecionine-like and erucifoline-like PAs but work less well for jacobine-like PAs. Therefore, the second hypothesis could explain the difference in tertiary amine proportion among individual PAs and genotypes. Furthermore, this hypothesis is supported also by the fact that the plant has to use an enzyme to produce the back-bone senecionine *N*-oxide at the beginning of the PA-pathway. The discovery of high levels of reduced PAs in some specific groups of *J. vulgaris* genotypes is very interesting from an evolutionary and ecological point view. The presence of reduced PAs may represent a next step in the arm-race between plants and herbivores, as a number of studies show that tertiary amines are more toxic than their respective *N*-oxides (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005). The fact that especially jacobine-like PAs occur in a large proportion as tertiary form coincides with the role of jacobine-like PAs as important defence compounds. Several studies showed that jacobine and jaconine are especially feeding deterrent for generalist insect herbivores (Macel et al. 2005; Leiss et al. 2009; Macel 2010), while some specialists prefer plants containing high concentrations of jacobine (Macel and Klinkhamer 2010). Further research on the chemistry and biology of PA *N*-oxides and tertiary PAs and their influence on generalist and specialist insects are required for a better understanding of the ecological significance of these highly interesting defence compounds.

The impact of soil-borne microorganisms on plant growth and PA composition; the ecological consequences

Macel and Klinkhamer (2010) noticed, in a field experiment, that in genotypes of *J. vulgaris* the PA composition changed compared to the initial composition of clones in the laboratory. The composition also differed between the aboveground parts of clones grown in two different experimental field sites. This

raised the question if environmental conditions and in particular soil-type and/or soil-borne microorganisms could have an systemic impact on the concentration of individual defence compounds in the plant. Therefore we tested if soil-borne microorganisms affect PA concentration and composition in *J. vulgaris* (Joosten et al. 2009; Chapter 5). In a laboratory experiment we grew cloned plants of two genotypes, on two different sterilized soils and sterilized soils inoculated with 5% of non-sterilized soil of either of the two soil-types.

Inoculum treatment had a great impact on the plant dry mass, which implies that plant growth is influenced by the soil-borne microorganisms. Plants grown on sterilized soil have the greatest dry mass whereas plants grown on sterilized soil inoculated with 'own' non-sterilized soil have the lowest dry mass. So, after addition of only a small soil inoculum (5%) into the 'biologically empty' sterilized same soil, microorganisms may develop into a community that is capable to reduce plant growth. This supports the findings of for instance van der Putten et al. (1993), Bever et al. (1994 and 1997) and Klironomos (2002) that there is often a negative feedback of the natural soil-borne community on plant growth.

This negative effect may be caused by pathogens or through 'nutrient competition' between plant roots and microorganisms (Jackson et al. 1989). The latter explanation seems in our experiment, however, less likely since sterilized soil, inoculated with 'other' soils except its own non-sterilized soil, resulted in a higher dry mass compared to inoculation with 'own' non-sterilized soil. Inoculation with another soil-type may have also introduced potential pathogens, but these pathogens may be less adapted to these 'new' soil conditions compared to potentially pathogen suppressive agents of the 'own' inoculum. This also holds for the sterilized soils that were not inoculated. These soils probably did not remain sterile in the course of the experiment, but will have been inoculated randomly by air-borne microorganisms, without developing a pathogenic community.

We also found that the PA composition below and aboveground was significantly affected by both soil-type and inoculum. On the contrary, the effect on the total PA concentration was, relatively small. When we repeated this study (Chapter 6), with more genotypes with different PA compositions, we again found that the PAs composition aboveground was significantly affected by the soil-borne microorganisms. The changes caused by this induction was similar for all genotypes, originating from several different populations, including the Erucifoline-chemotypes, and similar to the results published in Joosten et al. (2009; Chapter 5) on two Jacobine-chemotypes from Meijendel.

The levels of retrorsine and retrorsine *N*-oxide were raised in the plants grown on soils inoculated with non-sterilized Heteren soil. Retrorsine *N*-oxide is formed by the addition of a hydroxy group to senecionine *N*-oxide. Our conclusion is that this process was stimulated by the Heteren inoculum. In addition to changes in retrorsine and retrorsine *N*-oxide, the levels of jacobine and jacobine *N*-oxide was raised in shoots of plants grown on Heteren soils, especially sterilized Heteren soil inoculated with Meijendel soil. Changes in the concentration of individual PAs aboveground may attract specialist herbivores while deterring generalists (Macel and Vrieling 2003; Macel et al. 2005; Macel and Klinkhamer 2010). Jacobine is especially interesting because jacobine is mainly responsible for the relative high amounts of tertiary amines found in the shoots (Joosten et al. 2011; Chapter 3). Hol et al. (2004) showed that jacobine may be a key player in root protection in *J. vulgaris*. When the roots or shoots of this species were damaged, jacobine levels increased in the roots. This suggests that this PA are important for root defence when the plant is under attack belowground. A previous study on soil-borne microorganisms showed that *J. vulgaris* plants containing high levels of jacobine PAs had a lower fungal diversity in the rhizosphere than *J. vulgaris* plants lacking high levels of jacobine PAs (Kowalchuk et al. 2006). Apart from the above information on jacobine, in general there is hardly anything known about the functions of specific PAs to predict accurately the ecological consequences of the change in PA composition.

One of the possible ecological consequences has been investigated in this research project. It has been suggested that belowground interactions may impact the plant's defence system and thereby influence the aboveground defence against herbivores. (van Loon et al. 1998; van der Putten et al. 2001; Paul et al. 2000; Gange et al. 2002; Dicke and Hilker 2003; van Dam et al. 2003; Bezemer et al. 2005; Bezemer and van Dam 2005). Thus, we studied the effect of changes in the PA composition induced by soil-borne microorganisms on the resistance of the plant against thrips feeding (*Frankliniella occidentalis*) by measuring the feeding damage on the leaves. Feeding damage on the plants is significantly different between the genotypes. Genotypes without jacobine-like PAs had the highest amount of feeding damage while genotypes with high levels of jacobine-like PAs had the lowest amount of feeding damage. Although, the amount of feeding damage depends basically on the genotype, the resistance to thrips was significantly affected by inoculum in one out of five genotypes. In this genotype plants grown on Heteren inoculated soil had a significant higher feeding damage aboveground. The effects of the total PA concentration and the relative concentration of individual PAs on the resistance to *F. occidentalis* are in accordance with the results of Cheng et al. (2011a and 2011b), Macel and Klinkhamer (2010) and Leiss et al. (2009). The concentrations of jacobine and jacobine *N*-oxide and its derivates influenced the amount of feeding damage inflicted by thrips. However, in an ANOVA neither the main effects of inoculum nor the interaction with genotype was significant. Because of these inconclusive results we repeated the experiment with more replicates (15 instead of 7) of two selected genotypes, the genotype that did show a significant effect of inoculum on thrips resistance and one genotype that did not. In this repeated experiment, the results of the first experiment were confirmed. Inoculum has a highly significant effect on thrips resistance in one genotype but not in the other. The feeding damage on plants grown on Heteren inoculated soil was twice at high compared to the other two treatments (Chapter 6). However, at this stage there is still not enough known about the functions of specific PAs to predict feeding damage by the change in concentration of individual PAs induced by microorganisms.

PA defence system affects fungal community but has less or no effect on bacterial and mycorrhizal communities in roots and rhizosphere soil

Many plant species contain high food reserves in their root system for vegetative reproduction and regrowth to survive complete defoliation by specialist herbivores (Verkaar 1987; van der Meijden et al. 1988). Therefore it is to be expected that these species protect their roots strongly against belowground herbivores and pathogens. One of the protection mechanisms is the production of secondary metabolites, which are toxic or deterrent for attackers (Falk and Doran 1996; Hol et al. 2003; Thoden et al. 2009). Soil-borne microorganisms occur nearby the plant root system. These microbial communities are shaped by selection from the pool of microorganisms present in the surrounding bulk soil (de Ridder-Duine et al. 2005) for instance by the plant itself (Kowalchuk et al. 2002). Rhizodeposition and secretion of defence compounds suppress or stimulate the success of root colonizing microorganisms, pathogens and symbiotic microorganisms (Marschner et al. 2001 and 2002; Bais et al. 2006; Badri and Vivanco 2009). The defensive role that compounds such as PAs play, in plant protection against root-infecting bacteria and fungi is still not fully understood.

In chapter 7 we report on the impact of plant genotype, differing in PA composition, and PA-type on the community structure of fungi and bacteria in the rhizosphere and in the roots, and of arbuscular mycorrhizal fungi (AMF) in the roots. In this experiment four different *J. vulgaris* genotypes were used that differed in the PA composition. They were chosen based on the presence or absence of jacobine-like PAs

(Kowalchuk et al. 2006) and were grown on two different soil-types; löss and sand.

The fungal community, in both roots and rhizosphere soil, was clearly genotype dependent. However, we found no indications that the genotype effect was related to the PA composition, except that the fungal diversity in the roots was lower in Erucifoline-chemotypes compared with Jacobine-chemotypes grown in sandy soil. Also a negative correlation was found between the total erucifoline-like PAs and the fungal diversity in the roots. This is not in line with the findings of Kowalchuk et al. (2006). This study implied that the PA composition of the plant has a strong influence on fungal community in the rhizosphere. Plants lacking jacobine-like PAs in the roots had a higher fungal diversity in the rhizosphere soil compared to plants containing high levels of jacobine in the roots.

There were no overall significant differences in bacterial community structure in roots and rhizosphere between the genotypes. Soil-type affected the AMF community structure in the roots but plant genotype did not. So the soil selected much stronger for AMF colonization in the plant than the individual plant itself. The effect of soil-type on AMF community in the roots may be caused by the original soil differences such that two different soil-types contained a different AMF spore and hyphae composition at the start of the experiment, which apparently, was greater than the selective force on it. For the three structural PA groups we did not find any relation with the AMF richness in the roots of the plant.

The present results on the role of PAs in shaping the microbial community structure in the rhizosphere and roots are, highly probably, affected by low concentrations of PAs in the present genotypes. We had hoped to confirm the results of Kowalchuk et al. (2006) but with a more uniform genetic background by using cloned replicates. However the jacobine-like PA concentrations in the roots of the present studied Jacobine-chemotypes were around 4 times lower than in the studied Jacobine-chemotypes of Kowalchuk et al. 2006.

Chapter 2 presents the current knowledge on PAs with respect to anti-microbial activities, adaptation and detoxification by microorganisms (Joosten and van Veen 2010). Many *in-vitro* experiments showed effects of PAs on microorganisms (Hol and van Veen 2002, Hol 2003). These results point to the potential of microorganisms to be important for the evolution of PAs. When different individual PAs affect different microbial species, and adaptation occurs, selective pressure makes plants, which synthesize new effective defence compounds, more successful. However, only a few *in-vivo* studies have been published and support the results of the *in-vitro* studies (Kowalchuk et al. 2006).

In conclusion, the results on PA composition and their effect on soil microbial communities and vice versa, presented in this thesis, are very interesting such as the presence of tertiary amines in the plant. The results also point out that further exploration is needed, especially on microbes, by carrying out ecological experiments and field studies. For instance by using genotypes of *Jacobaea* F2 hybrids (Kirk et al. 2010) instead of *J. vulgaris*. The frequently occurring transgressive segregation in F2 hybrids offers a potentially large variation in PA concentration and composition of secondary metabolites, while at the same time being genetically close related by sharing the same (grand-)parents (Cheng et al. 2011c). So, F2 genotypes can be selected with extreme differences in PA composition, without interference of unknown physiological traits (Kirk et al. 2010).

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Directe chemische afweer van planten

Planten worden continu aangevallen door een verscheidenheid aan (micro)organismen. Veel planten produceren diverse chemische stoffen die werken als afweer tegen ziekten en plagen. Waarom produceren planten die diversiteit aan nauw verwante afweerstoffen zoals alkaloïden, fenolen of terpenoïden? Vanuit evolutionair perspectief zijn er verschillende theorieën opgesteld om deze vraag te beantwoorden. Eén van de oudste theorieën is de "arms race hypothesis", waarvan de voortdurende ontwikkeling van nieuwe afweerstoffen in de plant het resultaat is. Deze hypothese stelt namelijk dat nieuwe afweerstoffen evolueren en een fitness voordeel hebben voor de plant. Door fitnessvoordeel breidt de nieuwe afweerstof zich uit in de populatie, waarmee de stof algemeen wordt. Vervolgens kan er bij de belager een verandering plaatsvinden waardoor deze aangepast raakt aan de nieuwe stof, genaamd adaptatie. De plant is hierdoor hernieuwd kwetsbaar voor zijn belager. Deze hernieuwde kwetsbaarheid biedt een mutant, die een nieuwere stof produceert, een fitnessvoordeel waardoor alles weer van voren af aan begint.

De tweede hypothese veronderstelt dat een breed scala aan verschillende afweerstoffen een sterkere bescherming biedt tegen een belager, dan de productie van één stof in een hele hoge concentratie. De verschillende stoffen samen hebben dan een synergistische effect (Adams and Bernays 1978, Lindroth et al. 1988, Berenbaum et al. 1991). Een derde hypothese stelt dat verschillende afweerstoffen een verschillende uitwerking hebben tegen specifieke belagers. Dit maakt dat, omdat er veel verschillende belagers zijn, ook veel verschillende stoffen evolueren.

Een algemene opvatting is dat het evolueren van de diversiteit aan afweerstoffen onder andere wordt gedreven door herbivore insecten. Populaties van insecten zijn namelijk erg veranderlijk met als gevolg dat de selectieve druk op het chemische afweersysteem van de plant ook aan verandering onderhevig is (Macel et al. 2005). Generalisten, de minder kieskeurige insecten, zijn minder goed aangepast aan hun voedselplanten. Zij zijn naar verwachting meer gevoelig voor verschillen tussen afweerstoffen in de plant dan gespecialiseerde insecten. Specialisten hebben zich toegelegd op een beperkte groep waardplanten en zich daaraan aangepast. Hierdoor zijn ze niet tot minder gevoelig voor de soms kleine chemische verschillen tussen de diverse afweerstoffen in hun specifieke waardplant (Miller and Feeny 1983, Lindroth et al. 1988, Agrawal 2000, Macel et al. 2002, Macel et al. 2005). Specialisten gebruiken afweerstoffen om hun waardplant te lokaliseren. In sommige gevallen gebruiken ze de afweerstoffen voor eigen verdediging. Een voorbeeld is *Tyria jacobaeae*, de sint-jacobsvlinder. Deze vlinder is gespecialiseerd op enkele kruiskruidsoorten en slaat de aanwezige afweerstoffen, pyrrolizidine alkaloïden, uit de plant op met een afstotende werking tegen predatoren (Boppre 1986; Dobler 2001).

Ziekteverwekkers zoals bacteriën en schimmels zijn ook een serieuze bedreiging

voor planten evenals een aantal soorten insecten. Bovendien hebben ze een zeer korte generatietijd waardoor snelle evolutie mogelijk is. Om die redenen zouden micro-organismen mede van invloed kunnen zijn op de selectie van afweermechanismen (Hol and van Veen 2002). De invloed die micro-organismen hebben uitgeoefend op het ontstaan van nieuwe afweerstoffen in de plant is tot op heden veel minder duidelijk dan de invloed van insecten. Historische gezien zijn planten altijd al geconfronteerd met belagers. Lang voordat er herbivore insecten en zoogdieren bestonden op deze planeet moesten planten al een manier vinden om zich staande te houden tegen micro-organismen. Om het ontstaan van het hele pallet aan nauw verwante afweerstoffen in planten te verklaren is het cruciaal dat de belagers zich kunnen aanpassen aan de werking van afweerstoffen. Juist micro-organismen zijn uitstekend in staat zich aan te passen aan hun omgeving. De extreem hoge aantallen individuen en snelle voortplanting van ziekteverwekkende micro-organismen zouden adaptatie tegen de negatieve werking van afweerstoffen in de plant mogelijk maken. Hoe dan ook, bevindingen op basis van ecologische studies over de rol die afweerstoffen spelen in het verdedigen van de plant tegen micro-organismen zijn schaars, zeker in vergelijking met insecten.

Studiesysteem-plant *Jacobaea vulgaris*

Jacobaea vulgaris (synoniem *Senecio jacobaea*) is een uitermate geschikt systeem om de chemische afweer van de plant te bestuderen. Deze soort, in het Nederlands ook wel bekend als jacobskruiskruid (JKK), bevat een goed bestudeerde groep afweerstoffen, genaamd pyrrolizidine alkaloïden (PAs). JKK is een belangrijke voedingsbron voor een verscheidenheid aan insectensoorten (Harper and Wood 1957) en zeer toxisch voor vertebraten. JKK heeft maar weinig te lijden onder ziekteverwekkers in het veld. De meest frequente schimmelinfectie aangetroffen op JKK is de algemene roestsoort *Puccinia dioicae* (Harper and Wood 1957).

Pyrrolizidine alkaloïden

PAs hebben toxicologische en/of afstotende werking op generalistische herbivoren zoals plantetende insecten (van Dam et al. 1995; Hartmann 1999; Hartmann and Ober 2000; Ober 2003; Macel et al. 2005).

In kruiskruidsoorten, zoals JKK of kleinkruiskruid (*Senecio vulgaris*), worden PAs gevormd in de wortels van de plant met als basisstof senecionine *N*-oxide (Hartmann and Toppel 1987; Toppel et al. 1987). Senecionine *N*-oxide wordt getransporteerd via de stengel naar de bladeren en de bloemen, waar het door specifieke enzymen wordt omgezet in verschillende andere PAs (Hartmann and Dierich 1998). Er zijn ongeveer 14 verschillende PAs gedetecteerd in JKK in eerdere studies (Witte et al. 1992; Macel et al. 2004; Kowalchuk et al. 2006). De concentratie en de samenstelling van PAs in JKK is genotype-afhankelijk (Vrieling et al. 1993) en kan beïnvloed worden door de omgeving van de plant (Hol et al. 2003; Macel et al. 2004; Hol et al. 2004; Macel and Klinkhamer 2010).

Twee PA-vormen in de plant

PAs komen in de plant in twee chemische vormen voor: *N*-oxide en in de gereduceerde vorm als vrije base. De wateroplosbare *N*-oxide vorm maakt het mogelijk om PAs te distribueren tussen weefsels, te transporteren via het floëem transport (Hartmann et al. 1989) en op te slaan in de vacuole van de plantencel (von Borstel and Hartmann 1986; Ehmke et al. 1988). De omzetting van de *N*-oxide vorm naar de vrije base vorm vindt plaats door reductie; een niet specifiek chemisch proces dat geïnduceerd wordt door de aanwezigheid van reducerende stoffen in de plant. Generalistische herbivore insecten reduceren de *N*-oxides in de maag tot vrije base na het eten van JKK. De vrije basen worden vervolgens opgenomen in het lichaam en omgezet in pyrrolen (Lindigkeit et al. 1997; Hartmann 1999). Pyrrolen (heterocyclische aromatische stikstofverbindingen) zijn toxisch omdat ze zich gedragen als zeer reactieve stoffen die zich bijvoorbeeld kunnen binden aan DNA (Mattocks 1986; Frei et al. 1992).

PA *N*-oxides hebben minder afstotende of toxische werking voor generalistische herbivoren dan de bijbehorende vrije basen (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005). Specialistische insecten, aangepast aan PAs, nemen vrije basen op en zetten ze actief om in *N*-oxides (Boppre 1986; Dobler 2001; Nishida 2002; Narberhaus et al. 2003; Lindigkeit et al. 1997).

JKK heeft een ander mechanisme om zich toch te kunnen weren tegen de vraat van specialisten. De plant bevat een hoge voedselreserve in het wortelstelsel voor vegetatieve reproductie en krachtige hergroei om na volledige ontbladering te kunnen overleven (Verkaar 1987; van der Meijden et al. 1989). Te verwachten is dat planten als JKK, die gebruik maken van deze overlevingsstrategie, hun wortels goed verdedigen tegen ondergrondse bodem(micro-)organismen.

Inductie van PAs door bodemmicro-organismen

Planten worden door verschillende organismen zowel boven- als ondergronds tegelijkertijd aangevallen. Wanneer de wortels blootgesteld worden aan ondergrondse belagers (zoals herbivore insecten, nematoden, bacteriën en schimmels) kunnen planten reageren door verschillende soorten directe afweerreacties te geven. Dit kan bovengrondse afweer beïnvloeden doordat afweerstoffen worden getransporteerd en verspreid over de hele plant (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). Er is tot nu toe weinig bekend over de inductie van PAs door (micro)organismen. Macel and Klinkhamer (2010) ontdekten dat de PA samenstelling in JKK planten veranderde in het veld in vergelijking met de oorspronkelijke samenstelling van dezelfde genotypen opgekweekt onder laboratoriumomstandigheden. Ook bleek, voor deze genotypen, de PA samenstelling afhankelijk van het natuurlijke habitat waarin ze werden uitgeplant. Verder merkte Bezemer et al. (2006) op dat de bovengrondse schade aan JKK door insecten mogelijk te relateren is aan de schimmelgemeenschap in de grond.

Het doel

Het doel van dit proefschrift is om meer informatie te vergaren over het PA afweersysteem in het bijzonder en de interactie met micro-organismen uit de bodem. Het accent ligt met name op de impact die bodemtype en micro-organismen hebben op de ondergrondse en bovengrondse PA samenstelling en de ecologische gevolgen die daaruit voortvloeien. Moleculaire technieken zijn toegepast om een beter inzicht te krijgen in de effecten van

PAs op de microbiële gemeenschappen in de wortels en rhizosfeer van de plant.

Een vernieuwde PA extractie- en analysemethode is ontwikkeld (Patrick Mulder, Rikilt Wageningen) die het mogelijk maakt een beter inzicht te krijgen in de PA concentratie en samenstelling van boven- en ondergrondse plantendelen en de chemische vorm waarin PA voorkomen.

Een verbeterde analysemethode voor PAs

Voor het begrijpen van de rol die specifieke plantenstoffen spelen bij de afweer van planten is het allereerst van belang dat we ze op de juiste wijze kunnen meten. In veel gevallen is de extractie- of analysemethode van grote invloed op wat we (kunnen) meten.

De traditionele methode bestaat uit een PA extractie met zwavelzuur, een PA reductie met zink en een zuiveringsstap voor gaschromatografie gekoppeld aan een stikstof-fosfor detector (GC-NPD). Tijdens de toepassing van de traditionele methode maakt men gebruik van een aantal onvriendelijke stoffen zoals zwavelzuur en zink. Een ander nadeel van de traditionele methode is dat een reductiestap essentieel is omdat de GC geen PA *N*-oxiden kan detecteren. Mede door deze twee negatieve eigenschappen van de traditionele methode is een vernieuwde methode ontwikkeld. De nieuwe methode bestaat uit PA extractie met mierenzuur met maar één eenvoudige verdunningsstap gevolgd door de analyse met vloeistofchromatografie gekoppeld aan een dubbele massaspectrometer (LC-MS/MS). Deze twee verschillende methoden zijn vergeleken door de PAs in een aantal gevriesdroogde JKK monsters te meten (zie hoofdstuk 3). De PA concentraties waren hoger in de metingen met de LC-MS/MS dan met de GC-NPD. De introductie van de dubbele massaspectrometer als detector in combinatie met de scheiding van PAs met behulp van de vloeistofchromatograaf vergroot de mogelijkheden om individuele PAs te identificeren en te meten (Betteridge and Colegate 2005; Wuilloud et al. 2004). De LC-MS/MS detecteerde tijdens de test tot dertien verschillende vrije basen in de JKK extracten terwijl GC-NPD maar zeven vrije basen detecteerde. Met de GC-NPD waren maar vier grote PAs (senecionine, seneciphylline, integerrimine and jacobine) consequent gedetecteerd in alle JKK monsters. De concentratie van de andere drie PAs was in een aantal gevallen simpelweg net beneden de meetgrens van de GC-NPD.

De nieuwe methode , mierenzuurextractie in combinatie met LC-MS/MS, is de gekozen methode om de PAs te analyseren tijdens het onderzoek beschreven in dit proefschrift.

Vrije basen aanwezig in de plant

Het simultaan kunnen detecteren van PA *N*-oxides en vrije basen in JKK extracten met de nieuwe methode biedt voor het eerst de mogelijkheid om het relatieve belang van deze twee vormen voor de afweer van de plant te bestuderen. In dit hoofdstuk tonen wij aan dat vrije basen aanwezig zijn in het plantenextract en dat dit geen artefact is van de nieuwe methode. Wij concluderen daarom dat voor specifieke PAs, hoge concentraties aan vrije basen aanwezig zijn in de plant in tegenstelling tot wat nu toe in de literatuur werd aangenomen. Wij ontdekten dat Jacobine-chemotypes bovengronds een veel hogere concentratie aan vrije basen hebben in vergelijking met de Erucifoline-chemotypes, omdat het vooral de jacobine-achtige PAs zijn die in gereduceerde vorm aanwezig zijn in de plant.

Daarnaast hebben we, door het analyseren van alle PA *N*-oxides en vrije basen in de wortels en bladeren van F_2 hybriden (*J. vulgaris* en *J. aquatica*), aangetoond dat het aandeel vrije basen sterk afhankelijk is van de PA soort en het genotype.

Twee mogelijke hypothesen, die elkaar niet noodzakelijkerwijs uitsluiten, kunnen de bovengenoemde waarnemingen verklaren. Allereerst zou de chemische transformatie van de ene naar de andere PA *N*-oxide, en verspreiding van *N*-oxides over de weefsels van de plant, vergezeld kunnen gaan van een continue langzame reductie van PA *N*-oxides. Dit passieve proces zou de aanwezigheid van lage hoeveelheden vrije basen verklaren (Hartmann 2010, persoonlijke communicatie). Daarnaast zou specifieke (re-)oxidatie van vrije basen ook onze resultaten kunnen verklaren. De reductie van PA *N*-oxides in de plant is een niet-specifiek chemisch proces dat geïnduceerd wordt door de aanwezigheid van endogene reducerende stoffen en (sporen van) overgangsmetalen zoals magnesium en ijzer. Dit deel komt nog overeen met de eerste hypothese van Hartmann (2010, persoonlijke communicatie), maar tegelijkertijd is er een biochemisch proces aan de gang, dat de gereduceerde vrije basen re-oxideert, speciaal voor transport van PAs en de verspreiding over de weefsels. Mogelijk zijn hier enzymen bij betrokken die goed werken voor senecionine-achtige en erucifoline-achtige PAs maar minder goed werken voor jacobine-achtige PAs. Deze tweede hypothese verklaart niet alleen de aanwezigheid van vrije basen in de plant maar ook het verschil in het aandeel vrije basen dat sterk afhankelijk is van de PA soort en het genotype. De tweede hypothese wordt ook ondersteund door het feit dat het enzym dat nodig is voor de re-oxidering zeer waarschijnlijk een sterke verwantschap heeft met het enzym dat nodig is om de primaire PA, senecionine *N*-oxide, aan het begin van de PA-pathway te vormen. Aangezien de plant een soortgelijk re-oxidatie-enzym produceert, is de stap om een enzym te ontwikkelen om vrije basen om te zetten naar *N*-oxides voor transport en verspreiding over de plant, niet ondenkbaar.

Het ontdekken van de hoge concentraties vrije basen in sommige specifieke JKK genotypen is vanuit een evolutionair en ecologisch oogpunt zeer interessant aangezien meerdere studies hebben laten zien dat vrije basen meer toxisch zijn dan hun respectievelijke *N*-oxides (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005). Het feit dat voornamelijk de jacobine-achtige PAs in hoge concentraties aan vrije basen in de plant aanwezig zijn is, vanuit een ecologisch standpunt, in overeenstemming met de resultaten van ander onderzoek dat laat zien dat jacobine-achtige PAs de belangrijkste PA afweerstoffen zijn van JKK tegen generalisten zoals tripsen (Macel et al. 2005; Leiss et al. 2009; Macel 2010), terwijl sommige specialisten, zoals *Tyria jacobaeae*, juist de voorkeur geven aan JKK planten met hoge concentraties jacobine (Macel and Klinkhamer 2009). Er is echter meer onderzoek nodig naar de chemie en de biologie van PA *N*-oxides en vrije basen en de invloed op generalisten en specialisten, om de ecologische betekenis van deze hoogst interessante afweerstoffen volledig te kunnen begrijpen.

De impact van bodemmicro-organismen op plantengroei en PA samenstelling

De mogelijke impact die micro-organismen uit de bodem hebben op het bovengrondse chemische afweersysteem van de plant is bestudeerd door gebruik te maken van reproduceerbare genotypen. Twee verschillende JKK genotypes, oorspronkelijk van een duingebied (Meijendel), zijn vermeerderd met behulp van weefselkweek. De planten

hebben daarna zes weken tijd gehad te groeien in twee gesteriliseerde bodemtypen (zandgrond uit de duinen in Meijendel en humusrijke grond afkomstig uit een proeftuin in Heteren) al dan niet geïnoculeerd met 5% ongesteriliseerde grond van één van de twee bodemtypen. In totaal waren er dus zes behandelingen. De planten die groeiden in gesteriliseerde grond hadden het hoogste drooggewicht, terwijl planten die groeiden in gesteriliseerde grond geïnoculeerd met niet-gesteriliseerde grond van het eigen bodemtype, het laagste drooggewicht hadden. Dit betekent dat na het toevoegen van een kleine hoeveelheid grond-inoculum (5%) in de 'biologisch lege' gesteriliseerde grond van hetzelfde bodemtype, de micro-organismen zich tot een gemeenschap ontwikkelen die in staat is de plantengroei te remmen. Dit resultaat ondersteunt eerdere literatuur waarin aangetoond wordt dat plantengroei aanzienlijk kan afnemen wanneer planten in een grond groeien die micro-organismen bevat die uit hun eigen natuurlijke omgeving komen (van der Putten et al. 1993; Bever et al. 1997; Klironomos 2002).

Inoculatie met 'de andere' niet-gesteriliseerde grond, resulteerde in een hoger drooggewicht van de plant in vergelijking met inoculatie met 'eigen' niet-gesteriliseerde grond. Inoculatie met 'de andere' niet-gesteriliseerde grond heeft waarschijnlijk ook potentiële pathogenen geïntroduceerd maar mogelijkerwijs waren deze pathogenen minder aangepast aan het 'nieuwe' bodemtype.

De onder- en bovengrondse PA samenstelling in de plant bleek significant beïnvloed te worden door het bodemtype en de inoculumbehandeling (zie hoofdstuk 5). Het effect op de totale PA concentratie was relatief klein. Ook toen het bovenstaande experiment gedeeltelijk herhaald werd (met meer genotypes met verschillende PA samenstellingen) bleek wederom dat de bovengrondse PA samenstelling significant beïnvloed werd door bodemmicro-organismen (zie hoofdstuk 6). De veranderingen in het PA afweersysteem van planten tijdens het tweede experiment kwamen sterk overeen met de planten uit het eerste experiment. Ook de genotypen onderling vertoonden een soortgelijke reactie tijdens het tweede experiment, waar naast Jacobine-chemotypes ook Erucifoline-chemotypes gebruikt zijn. Dit waren alle genotypes uit weefselkweek die oorspronkelijk afkomstig waren van verschillende populaties uit verschillende gebieden.

Bodemtype en bodemmicro-organismen beïnvloedden de PA samenstelling in de wortels en spruit van JKK voornamelijk door de concentratie van specifieke PAs te veranderen. Bijvoorbeeld de concentratie retrorsine en retrorsine *N*-oxide was verhoogd in planten die groeiden op grond geïnoculeerd met niet-gesteriliseerde Heteren grond. Retrorsine *N*-oxide wordt gevormd door aan senecionine *N*-oxide een hydroxy-groep toe te voegen. Dit proces wordt blijkbaar gestimuleerd door het Heteren inoculum. Naast de veranderingen in retrorsine en retrorsine *N*-oxide, waren de concentraties jacobine en jacobine *N*-oxide verhoogd in de spruit van planten die groeiden op Heteren grond, voornamelijk in gesteriliseerde Heteren grond geïnoculeerd met niet-gesteriliseerd Meijendel grond. De verandering in concentraties bovengronds voor jacobine is extra interessant omdat juist deze specifieke PA verantwoordelijk is voor de hoge concentraties vrije basen in de plant, die uit eerder verschenen onderzoek afstotelijker en/of giftiger bleken te zijn dan de corresponderende *N*-oxides. Eerder onderzoek naar micro-organismen toonde aan dat JKK planten, met hoge concentraties jacobine in de wortels, een lagere diversiteit aan rhizosphere schimmels had in vergelijking met JKK planten met zeer lage concentraties jacobine in de wortels (Kowalchuk et al. 2006).

Het effect van de bodemmicrobiële samenstelling op bovengrondse resistentie

Meerdere onderzoekers geven aan dat ondergrondse interacties tussen bodemorganismen en de plant van invloed kunnen zijn op het afweersysteem in bovengrondse delen van de plant en daarmee ook effect hebben op de herbivore insecten (van Loon et al. 1998; van der Putten et al. 2001; Paul et al. 2000; Gange et al. 2002; Dicke and Hilker 2003; van Dam et al. 2003; Bezemer et al. 2005; Bezemer and van Dam 2005). Om die reden hebben wij het effect door bodemmicroorganismen op de resistentie van de plant tegen trips (*Frankliniella occidentalis*) onderzocht door de zilverschade op de bladeren te meten. Zilverschade is de schade aan de plant die ontstaat doordat tripsen plantencellen leegzuigen. Zilverschade aan de plant verschilde sterk tussen de verschillende JKK genotypes. Genotypes met zeer lage concentraties jacobine-achtige PAs hadden de grootste zilverschade terwijl genotypes met hoge concentraties aan jacobine-achtige PAs de laagste zilverschade vertoonden.

Hoewel de hoeveelheid zilverschade grotendeels afhankelijk was van het JKK genotype, bleek de resistentie tegen trips ook beïnvloed te worden door het inoculum. Echter dit was slechts voor één van de vijf gebruikte genotypen significant. We hebben het experiment daarom herhaald met twee genotypen; het genotype waarvoor het grondinoculum een effect had op bovengrondse zilverschade en een genotype waarbij dat niet het geval was. Om de statistische power te verhogen vond dit tweede experiment plaats met het dubbele aantal replica's. De resultaten van het eerdere experiment werden hierdoor bevestigd. Het inoculum heeft een significant effect op de tripsresistentie maar dit effect is genotype afhankelijk.

PA afweersysteem beïnvloedt de schimmelgemeenschap maar heeft vrijwel geen effect op de bacterie- en mycorrhizagemeenschap in de wortels en rhizosfere grond

Veel plantensoorten hebben een hoge voedselreserve in de wortels. Dit is ten behoeve van vegetatieve reproductie en krachtige hergroei om te kunnen overleven na volledige ontbladering door specialistische herbivoren (Verkaar 1987; van der Meijden et al. 1989). Te verwachten is dat de soorten die gebruik maken van deze overlevingsstrategie hun wortels goed verdedigen tegen ondergrondse hebivoren en pathogenen. Planten gebruiken afweerstoffen als beschermingsmechanisme. Deze afweerstoffen hebben een giftige en/of afstotende werking op generalistische belagers (Falk and Doran 1996; Hol et al. 2003; Thoden et al. 2009).

Veel micro-organismen leven in de rhizosfeer; dit is de grond die onder directe invloed van de wortels van de plant is (Hiltner 1904). De microbiële gemeenschappen worden in de basis gevormd door een selectie uit micro-organismen die aanwezig zijn in de omliggende grond buiten de rhizosfeer (Kowalchuk et al. 2002; de Ridder-Duine et al. 2005). Plantenwortels scheiden stoffen uit die de kolonisatie van micro-organismen, zoals pathogenen en symbionten, onderdrukken en stimuleren (Marschner et al. 2001 and 2002; Bais et al. 2006; Badri and Vivanco 2009). Over de afwerende werking van stoffen, zoals PAs, om de plant te beschermen tegen pathogene micro-organismen is nog maar weinig bekend.

In hoofdstuk 7 rapporteer ik over de invloed van plant genotype, verschillen in PA compositie en PA type op micro-organismen. In het bijzonder op de

gemeenschapsstructuur van schimmels en bacteriën in de plantwortels en rhizosfeer. Ook het effect op arbuscular mycorrhiza schimmels (AMF) in de wortels wordt beschreven. In het experiment zijn vier verschillende JKK genotypes gebruikt met verschillende PA samenstellingen. Deze planten zijn uitgekozen op de aan- en afwezigheid van jacobine-achtige PAs (Kowalchuk et al. 2006) en na weefselkweek, opgepot in twee soorten bodemtypes: löss and zand.

De schimmelgemeenschap was duidelijk genotype afhankelijk, zowel in plantenwortels als in rhizosfeer. Er zijn weinig indicaties dat het genotype-effect gerelateerd was aan de PA samenstelling. Enkel de schimmeldiversiteit in de wortels was lager in Erucfoline-chemotypes in vergelijking met Jacobine-chemotypes op zandgrond. Daarnaast is ook een negatieve correlatie gevonden tussen de totale erucifoline-achtige PAs en de schimmeldiversiteit in de wortels. Dit ligt niet in lijn met de resultaten van Kowalchuk et al. (2006). Deze studie impliceert dat de PA samenstelling in de plant invloed heeft op de schimmelgemeenschap in de rhizosfeer. Planten met een lage concentratie aan jacobine-achtige stoffen in de wortels van de plant hadden een hogere schimmeldiversiteit in de rhizosfeer dan planten met een hoge concentratie aan jacobine-achtige PAs in de wortels. Er zijn geen significante verschillen gevonden tussen de genotypes in de bacteriegemeenschappen in wortels en rhizosfeer.

Het bodemtype beïnvloedde de AMF-gemeenschap in de wortels van de plant maar het plantengenotype niet. Hieruit kan geconcludeerd worden dat de grond sterker selecteert voor AMF-kolonisatie in de plant dan de individuele plant zelf. Het effect van bodemtype op de AMF-gemeenschap in de wortels kan veroorzaakt zijn door de originele grondverschillen. Dit kan geconcludeerd worden uit het feit dat aan het begin van het experiment twee verschillende bodemtypes een verschillende samenstelling aan AMF schimmeldraden en sporen bevatten. Dit verschil was kennelijk groter dan de selectiedruk van de plant op de AMF kolonisatie. Ook voor de drie structurele PA groepen (senecionine-, erucifoline- en jacobine-achtige PAs) vonden wij geen relatie met de AMF soortenrijkdom in de wortels van de plant.

We hoopten de resultaten van Kowalchuk et al. (2006) te bevestigen met een meer uniforme genetische achtergrond. Dit door gekloonde weefselkweek planten te gebruiken in plaats van individuele planten te selecteren uit veldpopulaties. Het huidige resultaat dat PAs weinig invloed hebben op het vormen van de microbiële gemeenschapsstructuur in de rhizosfeer en wortels van de plant, is mogelijkerwijs een effect van te lage jacobine concentratie in de gebruikte planten tijdens dit experiment. De totale PA concentratie van de jacobine-achtige PAs in de wortels van de planten gebruikt tijdens het experiment, lagen vier keer lager dan de totale PA concentratie van de jacobine-achtige PAs in de wortels van de planten gebruikt door Kowalchuk et al. 2006.

Hoofdstuk 2 behandelt de huidige kennis van PAs in relatie tot antimicrobiële activiteiten, adaptatie en detoxificatie door micro-organismen (zie hoofdstuk 2). Veel in-vitro experimenten hebben effecten van PAs op micro-organismen aangetoond (Hol and van Veen 2002; Hol 2003). Deze resultaten laten zien dat micro-organimsen in potentie belangrijk kunnen zijn geweest in het ontstaan van de diversiteit aan PAs in planten als JKK. Wanneer de verschillende individuele PAs invloed hebben op de verschillende microbiële soorten en er adaptatie plaatsvindt, zullen planten die nieuwe effectieve afweerstoffen synthetiseren, onder selectieve druk van belagers, meer succesvol zijn dan anderen. Er zijn nog maar enkele in-vivo studies gepubliceerd die de resultaten van in-vitro studies ondersteunen (Kowalchuk et al. 2006).

Samenvattend worden in dit proefschrift de resultaten over de PA samenstelling in JKK en het effect op bodemmicro-organismen en vice versa gepresenteerd. Opvallend is de aanwezigheid van vrije basen in de plant. Verder is duidelijk dat meer onderzoek wenselijk is, met name ecologische experimenten en veldonderzoek naar de interactie tussen micro-organismen en PAs in de plant. Voor volgend onderzoek aan PAs is het aan te bevelen F2 kruiskruid-kruisingen (Kirk et al. 2010) te gebruiken in plaats van JKK planten. Deze kruiskruid-kruisingen bieden een potentieel hogere variatie in PA concentratie en PA samenstelling. Daarnaast zijn deze kruisingen genetisch sterk verwant, doordat ze dezelfde (groot-)ouders delen (Cheng et al. 2011). Hierdoor kunnen genotypes geselecteerd worden met extreme verschillen in PA compositie; dit zonder grote effecten van onbekende fysiologische eigenschappen van de plant (Kirk et al. 2010).

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Curriculum Vitae

Lotte Joosten was born on May 26, 1978 in Boxtel, a village in a beautiful area in the South of the Netherlands. Her study career began at the secondary school named Bracbant HAVO in Boxtel in 1990 and she finished secondary school at the Willem I college in s-Hertogenbosch. In 1998 she received her Propedeuse diploma in Nutrition & Dietician (Voeding & Diëtetiek) in Nijmegen. In 1999 she chose for the study Biology at the Wageningen University. After receiving the Propedeuse and Bachelor degree she started her Master at the department of Biosystematics. In this group she studied Succession of arthropod fauna on carrion: an evaluation of arthropod sampling methods using Wild Boar carrion. In Spring of 2003 she stayed for a few months at the research group of Richard Stouthamer at the Riverside University in California USA, to perform field-work on parasitoid wasps in the Mojave Desert California focussing on their sex-ratio distorters. During the spare time of her Master she finished several projects at the department Entomology namely on the imperfect vertical transmission of parthenogenesis-inducing Wolbachia in natural populations of a parasitoid wasp, the learning behaviour of malaria mosquitos and the identification of Wolbachia in sheep. In March 2005 she received her Master degree on Animal Biology and Behaviour.

For a few months she worked as guest worker at the department of Entomology to study relation between Wolbachia and Borrelia burgdorferi in sheep ticks. During the six months before she started her PhD, she performed a literature study on pest organisms and pathogens during plant production in horticulture for Hortimea Group in Lent.

In February 2006 she started her Phd project at the section Plant Ecology of the Institute of Biology Leiden (IBL) and Microbial Ecology of the Netherlands Institute of Ecology Research (NIOO-KNAW).

At the beginning of 2010 she started a part-time job as technician at the department of Biosystematics at the Wageningen University. Here she serves the technical organisation and teaching, together with several colleges of the Biosystematic group, on the two most interesting and exciting courses in Life Science, namely Flora & Fauna in the Netherlands and the European Flora & Fauna in the Pyrenees. When insects hibernate, she participates as a team coach in the Master course Academic Consultancy Training for students who work on an cross-disciplinary assignment for an external client.

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Notes