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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, seneciophylline, 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 jacobine 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 seneionine *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 derivatives 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 as 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 re-growth 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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