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

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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

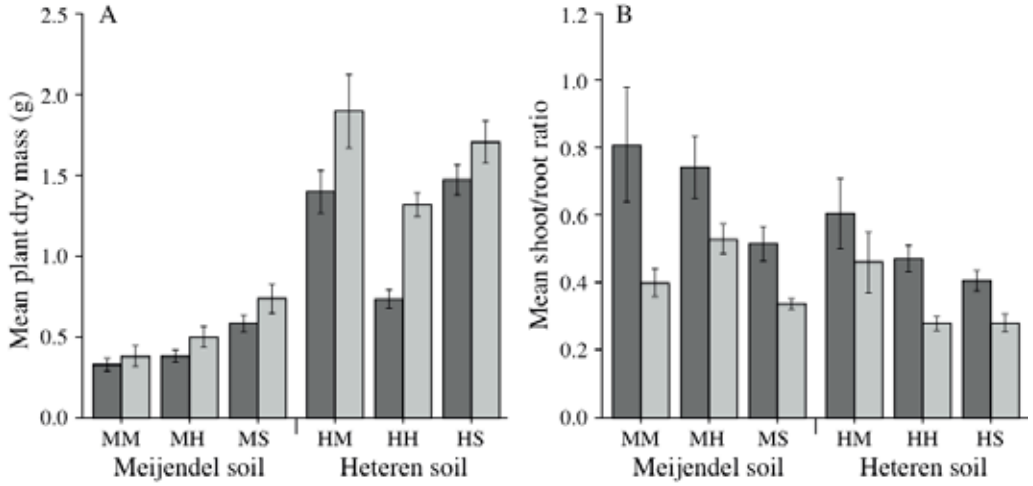

Figure 1. A Plant dry mass (Mean±SE, n=8) per soil-type per inoculum treatment of both *Jacobaea vulgaris* genotypes. B Shoot/root ratio (Mean±SE, n=8) per soil-type per treatment of both *Jacobaea vulgaris* genotypes. Left bar = genotype A; Right bar = genotype B; MM = sterilized Meijendel soil inoculated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

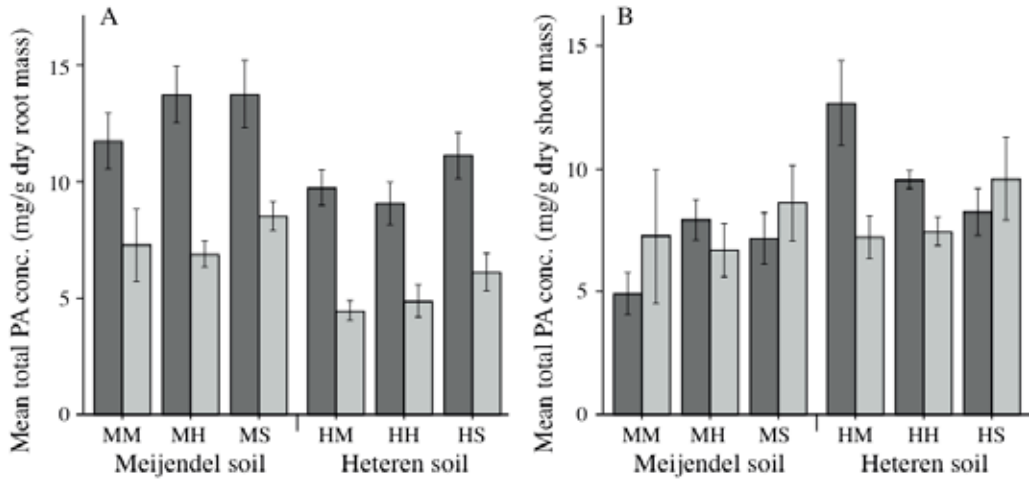

Figure 2. A Total PA concentration (Mean±SE, n=8) for root per soil-type per inoculum treatment of both *Jacobaea vulgaris* genotypes. B Total PA concentration (Mean±SE, n=8) for shoot per soil-type per inoculum treatment of both *Jacobaea vulgaris* genotypes. Red = genotype A; Blue = genotype B; MM = 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.

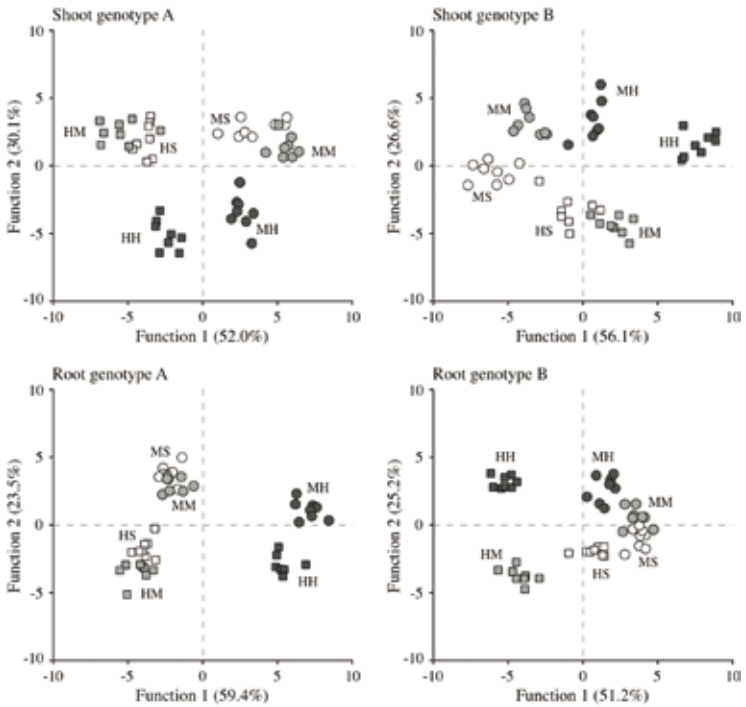

Figure 3. Four combined-group scatterplots showing the discriminant scores of the cases on discriminant function 1 and 2 generated by Classify Discriminant Analysis per genotype per plant part. MM = Sterilized Meijendel soil inoculated with Meijendel soil; MH = Sterilized Meijendel soil inoculated with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

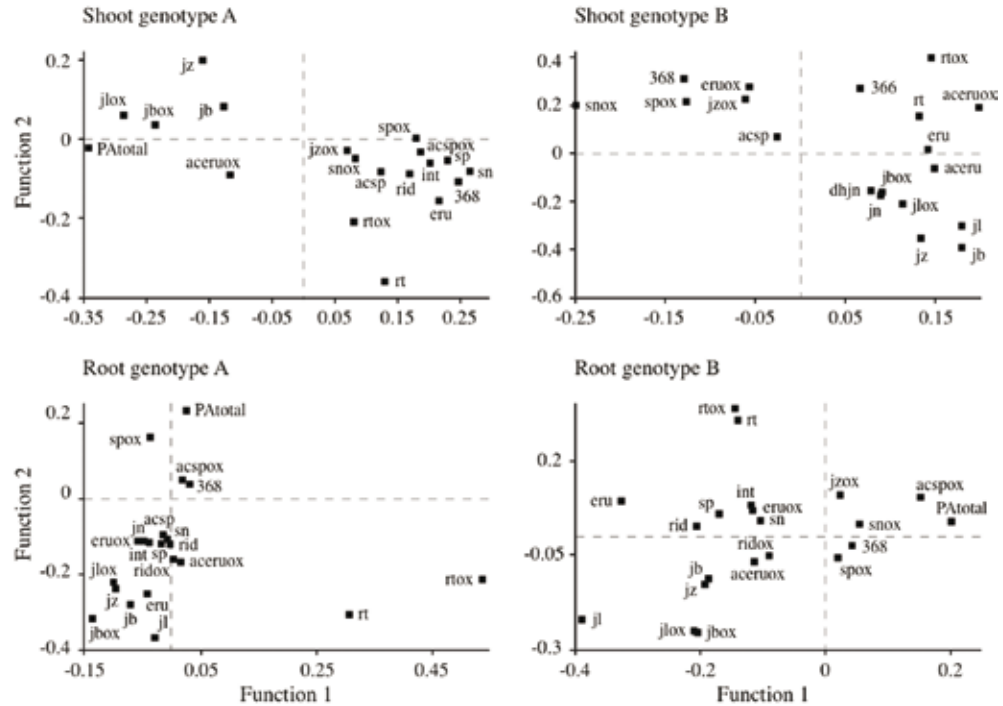

Figure 4. The correlations of each individual PA with the first two discriminant functions. All PAs shown in these structure matrixes showed significantly different group means (ANOVA $P<0.05$). PAtotal = total PA concentration, sn = senecionine, snox = senecionine *N*-oxide, sp = seneciphylline, spox = 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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