



Universiteit
Leiden
The Netherlands

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

Joosten, L.

Citation

Joosten, L. (2012, September 20). *Pyrrolizidine alkaloid composition in the plant and its interaction with the soil microbial community*. Retrieved from <https://hdl.handle.net/1887/19839>

Version: Not Applicable (or Unknown)

License: [Leiden University Non-exclusive license](#)

Downloaded from: <https://hdl.handle.net/1887/19839>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/19839> holds various files of this Leiden University dissertation.

Author: Joosten, Lotte

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

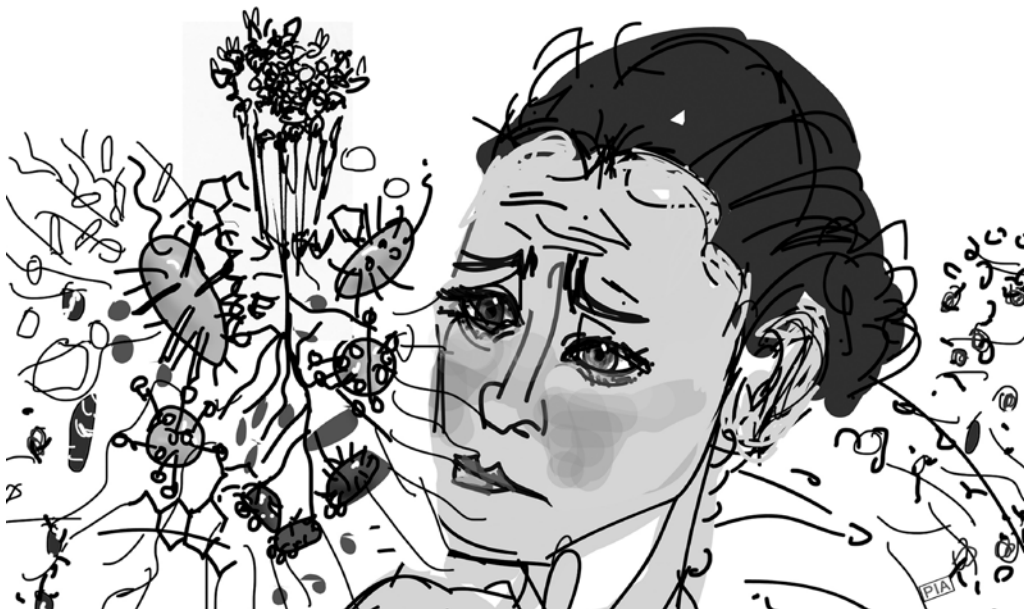
Issue Date: 2012-09-20

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 jacobine 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 Meijndel 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 Meijndel, 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 I 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 electrophoresis 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 pre-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 $H = -\sum P_i \ln P_i$ (Shannon and Weaver 1963). For PCR-DGGE analysis, P_i is the relative abundance, and is measured as $P_i = n_i/N$, where n_i 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, Meijndel, Wageningen and Vilt) as fixed factor. DGGE profiles per were translated in a matrix based on both abundance (P_i values per band) and microbial richness (presence/absence of P_i 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: Meijndel 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, jacozone, and dehydrojacozone; 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
	integerrimine
	retrorsine
	seneciphylline
	acetyl-seneciphylline
	riddelliine
	senecivernine
	senkirkine
Jacobine-like	jacobine
	jacoline
	jaconine
	dihydrojacozone
	jacozone
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 Meijndel and Wageningen, both contain jacobine-like PAs in the roots while Kassel and Vilt lack jacobine and its derivatives.

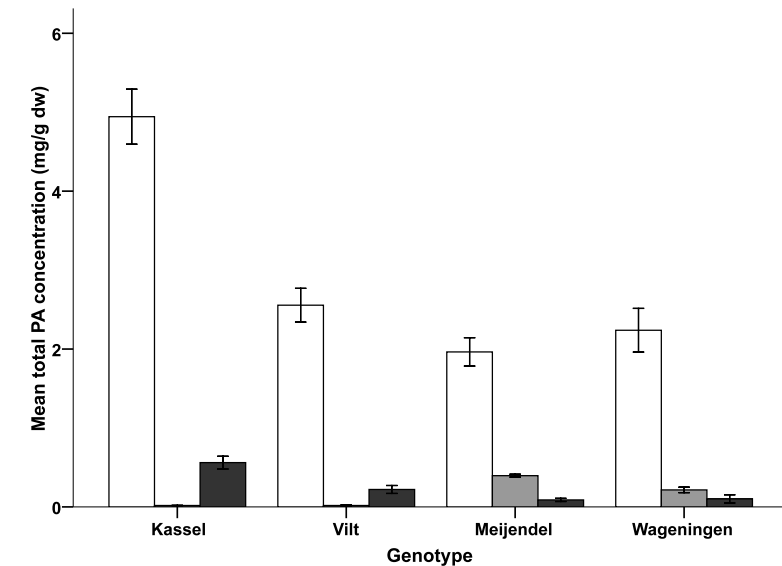


Figure 1. Mean total PA concentration in the roots per genotype grown on Sand and Löss soil combined. White bar = senecionine-like PAs, grey bar = jacobine-like PAs and black bar = erucifoline-like PAs. Error bars $\pm 1SE$, $n = 6$.

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 Meijndel genotype ($3.93 \pm SE 0.11$) was significantly lower compared to the Wageningen genotype ($4.51 \pm SE 0.11$, overall mean for all genotypes: $4.15 \pm SDEV 0.28$).

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 löss (Figure 2). The root bacterial community of Vilt genotypes separated in both soil-types from Wageningen, Meijndel and Kassel genotypes (Figure 2A and B). Plant genotype showed no clear effect on the rhizospheric 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).

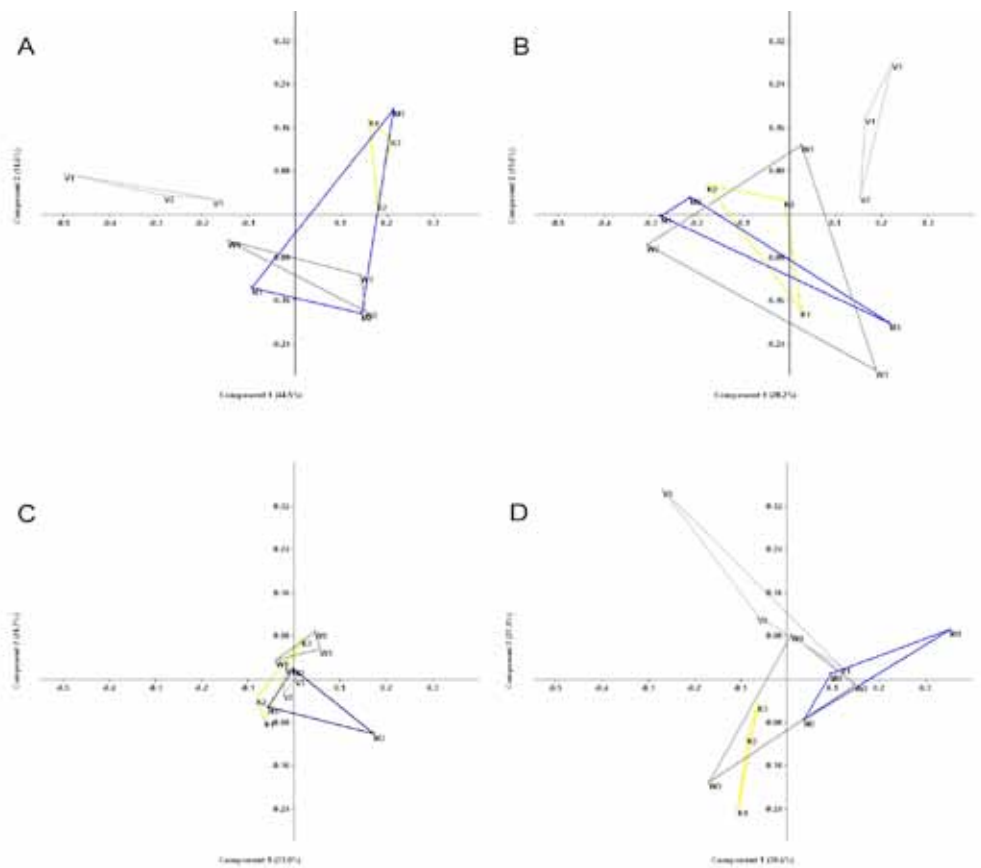


Figure 2. PCA scatterplot of the means of scores of the different genotypes grown on Sand (A and C) and Löss (B and D), for the first two PC-axes with the bacterial community matrix of the Roots (A and B) and Rhizosphere (C and D) as dependent variable. Light grey V = Vilt; yellow K = Kassel; blue M = Meijndel; 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 ($2.98 \pm SE 0.05$) was significantly higher compared to Kassel ($2.34 \pm SE 0.05$), Meijndel ($2.55 \pm SE 0.05$) and Vilt ($2.49 \pm SE 0.05$) grown on sand. While on löss, the mean root fungal diversity of Kassel ($3.33 \pm SE 0.05$) was significantly higher compared to Meijndel ($3.06 \pm SE 0.05$) and Vilt ($3.02 \pm SE 0.05$).

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, Meijndel 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, Meijndel 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

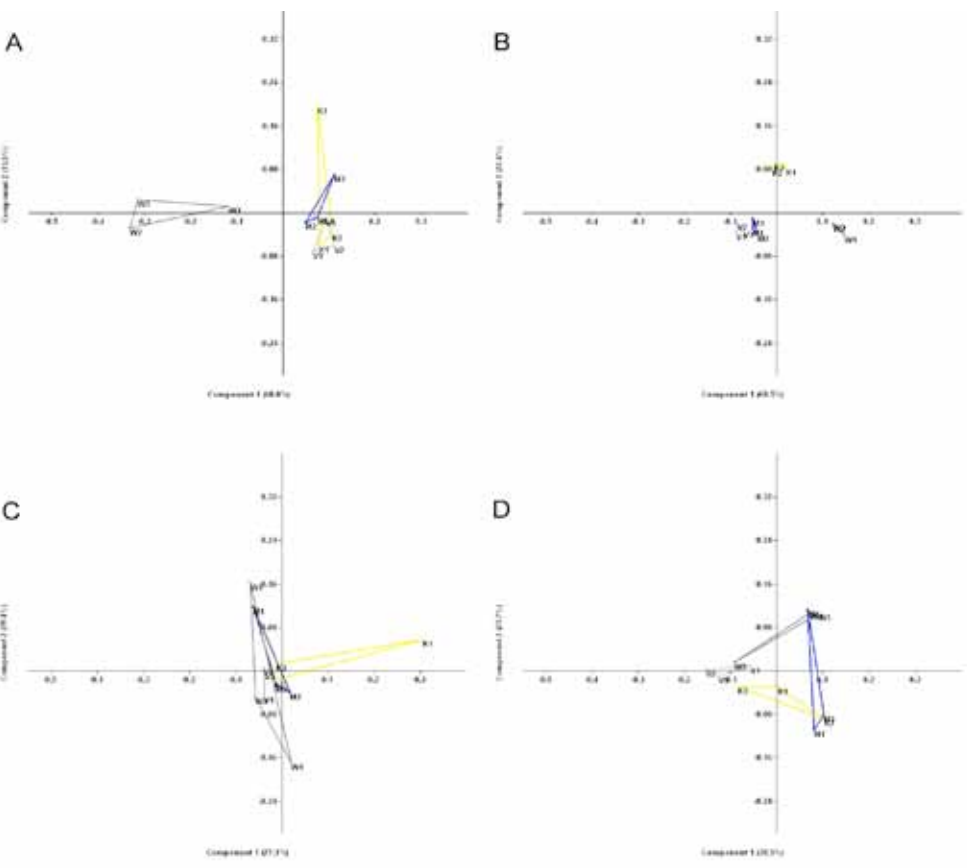


Figure 3. the PCA scatterplot of the means of scores of the different genotypes grown on Sand (A and C) and Löss (B and D), for the first two PC-axes with the fungal community matrix of the Roots (A and B) and Rhizosphere (C and D) as dependent variable (n = 3). Light grey V = Vilt; yellow K = Kassel;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

Microorganisms	Sample treatment		ANOVA (based on H-data)	
	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 rhizosphere of both soil-types ($df = 1$, $DF = 10$, $F \leq 1$, $P > 0.05$), 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 ($2.42 \pm SE 0.08$) was significantly lower compared to Jacobine-chemotypes ($2.77 \pm SE 0.08$) 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 ($27.3 \pm SE 2.8$) was higher compared to Jacobine-chemotypes ($20.3 \pm SE 2.3$) 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).

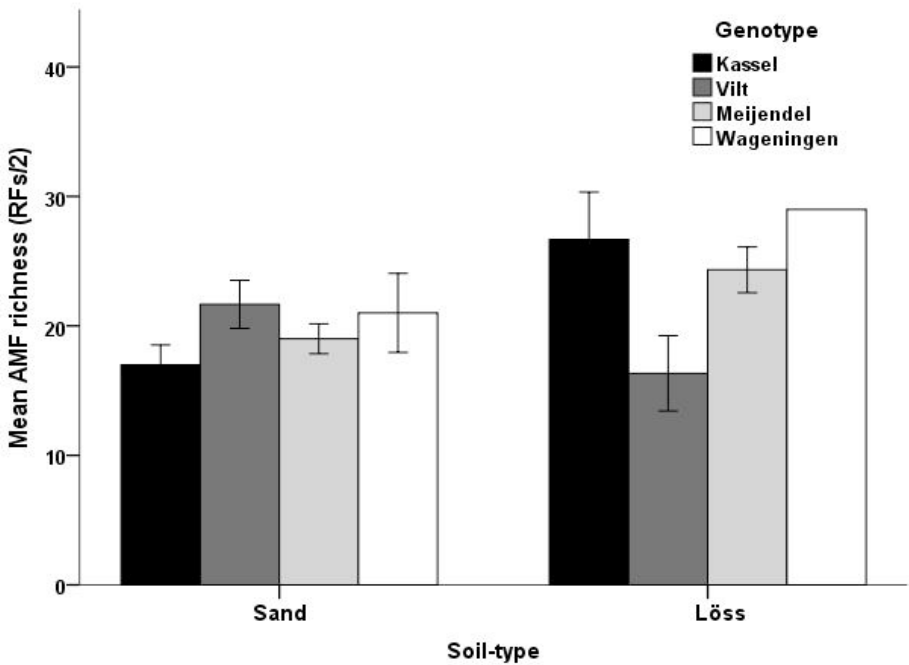


Figure 4. Mean AMF richness per genotype for AluI, TaqI and MboI digestion enzymes combined. Error bars $\pm 1SE$ $n = 3$ except for Wageningen Löss $n = 1$

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*, Thébaud 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-Echeverría et al. 2008). Plant host identity (VandenKoornhuyse et al. 2002 and 2003; Golotte 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

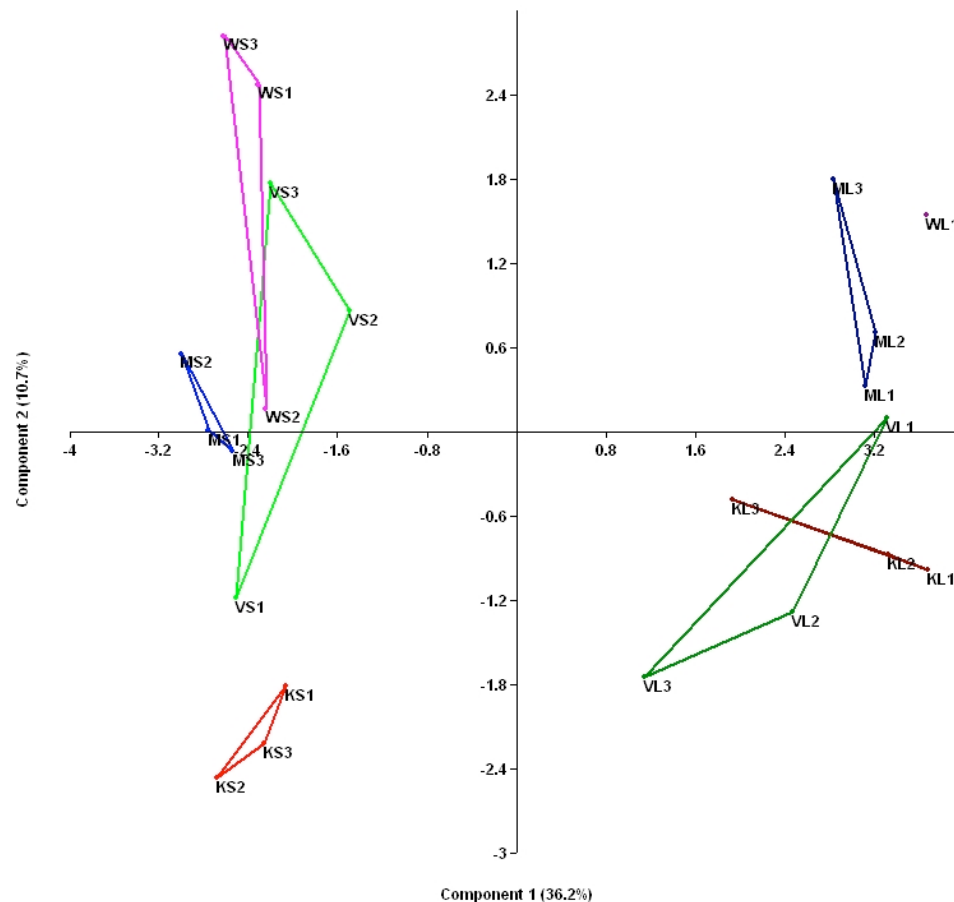


Figure 5. the PCA scatterplot of the means of scores; genotypes and soil-types, for the first two PC-axes with T-RFs binary matrix as dependent variable (n = 3). Dark colors = Löss soil; light colors = Sandy soil. Green = Vilt; Red = Kassel; blue = Meijendel; purple = Wageningen.

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 ± 0.13 mg/g jacobine-like/dw root) were around 4 times lower than in the studied Jacobine-chemotypes of Kowalchuk et al. 2006 (mean 1.30 ± 0.39 mg/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.

Acknowledgements

We thank Agaat Pijl, Ciska Raaijmakers, Cilke Hermans, Karin van der Veen and Henk Nell for their technical assistance, Patrick Mulder the PA analysis and Hannes A. Gamper for performing the T-RFLP analysis.

References

- Ampe F, Miambi E (2000) Cluster analysis, richness and biodiversity indexes derived from denaturing gradient gel electrophoresis fingerprints of bacterial communities demonstrate that traditional maize fermentations are driven by the transformation process. *Int. J. Food Microbiol.* 60:91-97
- Badri DV, Vivanco JM (2009) Regulation and function of root exudates. *Plant Cell Environ.* 32:666-681
- Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57:233-266
- Berg G, Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68:1-13
- Bower E (1997) Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects. PhD thesis. University of London, London
- Broeckling CD, Broz AK, Bergelson J, Manter DK, Vivanco JM (2008) Root exudates regulate soil fungal community composition and diversity. *Appl. Environ. Microbiol.* 74:738-744
- Brundrett MC (2002) Co-evolution of roots and mycorrhizas of land plants. *New Phytol.* 154:275-304
- Candrian U, Luethy J, Schmid P, Schlatter C, Gallasz E (1984) Stability of pyrrolizidine alkaloids in hay and silage. *J. Agric. Food Chem.* 32:935-937
- Cheng D, Vrieling K, Klinkhamer PGL (2011) The effect of hybridization on secondary metabolites and herbivore resistance: implications for the evolution of chemical diversity in plants. *Phytochem. Rev.* 10:107-117
- Cheng D, Kirk H, Mulder PPJ, Vrieling K, Klinkhamer PGL (2011) b Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. *New Phytol.* 192:1010-1023
- Clapp JP, Young JPW, Merryweather JW, Fitter AH (1995) Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytol.* 130:259-265
- Crews C, Driffield M, Berthiller F, Krska R (2009) Loss of pyrrolizidine alkaloids on decomposition of ragwort (*Senecio jacobaea*) as measured by LC-TOF-MSJ. *Agric. Food Chem.* 57:3669-3673
- Culman, SW, Bukowski R, Gauch HG, Caliddo-Quiroz H, Buckley DH (2009) T-REX: software for the processing and analysis of T-RFLP data. *BMC Bioinformatics* 10:171-181
- de Ridder-Duine AS, Kowalchuk GA, Klein Gunnewiek PJA, Smant W, van Veen JA, de Boer W (2005) Rhizosphere bacterial community composition in natural stands of *Carex arenaria* (sand sedge) is determined by bulk soil community composition. *Soil Biol. Biochem.* 37:349-357
- Drigo B, Pijl AS, Duyts H, Kielak AM, Gamper HA, Houtekamer MJ, Boschker HTS, Bodelier PLE, Whiteley AS, van Veen JA, Kowalchuk GA (2010) Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO₂. *PNAS* 107:10938-10942
- Duineveld BM, Kowalchuk GA, Keizer A, Van Elsas JD, van Veen JA (2001) Analysis of the bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR amplified 16S ribosomal RNA and DNA fragments. *Appl. Environ. Microbiol.* 67: 172-178
- Egerton-Warburton LM, Johnson NC, Allen EB (2007) Mycorrhizal community dynamics following nitrogen fertilization: a cross-site test in five grasslands. *Ecol. Monogr.* 77:527-544
- Falk LR, Doran PM 1996 Influence of inoculum morphology on growth of *Atropa belladonna* hairy roots and production of tropane alkaloids. *Biotech. Lett.* 18:1099-1104
- Fitzsimons MS, Miller RM, Jastrow JD (2008) Scale-dependent niche axes of arbuscular mycorrhizal fungi. *Oecologia* 158:117-127
- Gange AC, Brown VK, Farmer LM (2002) Differential effects of insect herbivory on arbuscular mycorrhizal colonization. *Oecologia* 131:103-112
- Gollotte A, van Tuinen D, Atkinson D (2004) Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment. *Mycorrhiza* 14:111-117
- Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeont. Elec.* 4:1-9
- Hausmann NT, Hawkes CV (2009) Plant neighborhood control of arbuscular mycorrhizal community composition. *New Phytol.* 183:1188-1200
- Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? *Nature* 394:431
- Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl. Environ. Microbiol.* 63:3233-3241
- Hiltner L (1904) Über neue Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderes Berücksichtigung der Grundungen und Brauche. *Arb. Dtsch. Landwirt Ges. Berl.* 98:59-78
- Hol WHG, van Veen JA (2002) Pyrrolizidine alkaloids from *Senecio jacobaea* affect fungal growth. *J. Chem. Ecol* 28:1763-1772

- Hol WHG, Vrieling K, van Veen JA (2003) Nutrients decrease pyrrolizidine alkaloid concentrations in *Senecio jacobaea*. *New Phytol.* 158:175-181
- Jain S, Sharma R (1987) Antimicrobial activity of pyrrolizidine alkaloids from *Heliotropium ellipticum*. *Chem. Pharm. Bull.* 55:3487-3489
- Johnson D, Vandenkoornhuyse PJ, Leake JR, Gilbert L, Booth RE, Grime JP, Young JPW, Read DJ (2004) Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. *New Phytol.* 161:503-515
- Joosten L, Mulder PPJ, Klinkhamer PGL, van Veen JA (2009) Soil-borne microorganisms and soil-type affect pyrrolizidine alkaloids in *Jacobaea vulgaris*. *Plant Soil* 325:133-143
- Joosten L, Cheng D, Mulder PPJ, Vrieling K, van Veen JA, Klinkhamer PGL (2011) The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. *Phytochemistry* 72:214-222
- Kirk H, Vrieling K, van der Meijden E, Klinkhamer PGL (2010) Species by environment interactions affect pyrrolizidine alkaloid expression in *Senecio jacobaea*, *Senecio aquaticus*, and their hybrids. *J. Chem. Ecol.* 36:378-387
- Klironomos JN, McCune J, Hart MM, Neville J (2000) The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. *Ecol. Lett.* 3:137-141.
- Klironomos JN, Hart MM (2002) Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. *Mycorrhiza* 12:181-184
- Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PGL, van Veen JA (2002) Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie Van Leeuwenhoek* 81:509-520
- Kowalchuk GA, Hol WHG, van Veen JA (2006) Rhizosphere fungal communities are influenced by *Senecio jacobaea* pyrrolizidine alkaloid content and composition. *Soil Biol. Biochem.* 38:2852-2859
- Lekberg Y, Koide RT, Rohr JR, Aldrich-Wolfe L, Morton JB (2007) Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. *J. Ecol.* 95:95-105
- Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere. *Plant Soil* 129:1-10
- Marschner P, Yang CH, Lieberei R and Crowley DE (2001) Soil and plant specific effects on bacterial community structure in the rhizosphere. *Soil Biol. Biochem.* 33:1437-1445
- Marschner P, Neumann G, Kania A, Weiskopf L, Lieberei R (2002) Spatial and temporal dynamics of the microbial community structure in the rhizosphere of cluster roots of white lupin (*Lupinus albus* L.). *Plant Soil* 246:167-174
- Marquina G, Laguna A, Franco P, Fernandez L, Perez P, Valiente O (1989) Antimicrobial activity of pyrrolizidine alkaloids from *Heliotropium bursiferum* W. ex Grisebach. *Pharmazie* 44:870-871
- Merryweather J, Fitter A (1998) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* L. Diversity of fungal taxa. *New Phytol.* 138:117-129
- Mummey DL, Rillig MC, Holben WE (2005) Neighboring plant influences on arbuscular mycorrhizal fungal community composition as assessed by T-RFLP analysis. *Plant Soil* 271:83-90
- Mummey DL, Rillig MC (2007) Evaluation of LSU rRNA-gene PCR primers for analysis of arbuscular mycorrhizal fungal communities via terminal restriction fragment length polymorphism analysis. *J. Microbiol. Methods* 70:200-204
- Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695-700
- Reidinger S, Eschen R, Gange AC, Finch P, Bezemer TM (2012) Arbuscular mycorrhizal colonization, plant chemistry, and aboveground herbivory on *Senecio jacobaea*. *Acta Oecologica* 38:8-16
- Reina M, Mericli A, Cabrera R, Gonzalez-Coloma A (1995) Pyrrolizidine alkaloids from *Heliotropium bovei*. *Phytochemistry* 38:355-358
- Reina M, Gonzalez-Coloma A, Gutierrez C, Cabrera R, Henriquez J, Villarroel L (1997) Bioactive saturated pyrrolizidine alkaloids from *Heliotropium floridum*. *Phytochemistry* 46:845-853
- Reina M, Gonzalez-Coloma A, Gutierrez C, Cabrera R, Henriquez J, Villarroel L (1998) Pyrrolizidine alkaloids from *Heliotropium megalanthum*. *J. Nat. Prod.* 61:1418-1420
- Rodríguez-Echeverría S, Hol WHG, Freitas H, Eason WR, Cook R (2008) Arbuscular mycorrhizal fungi of *Ammophila arenaria* (L.) Link: spore abundance and root colonisation in six locations of the European coast. *Eur. J. Soil Biol.* 44:30-36
- Scheublin TR, Ridgway KP, Young JPW, van der Heijden MGA (2004) Nonlegumes, legumes, and root nodules harbor different arbuscular mycorrhizal fungal communities. *Appl. Environ. Microbiol.* 70:6240-6246
- Shannon CE, Weaver W (1963) *The Mathematical Theory of Communications*. University of Illinois Press, Urbana, IL
- Singh B, Sahu PM, Singh S (2002) Antimicrobial activity of pyrrolizidine alkaloids from *Heliotropium subulatum*. *Fitoterapia* 73:153-155
- Thébault A, Frey B, Mitchell EAD, Buttler A (2010) Species-specific effects of polyploidisation and plant traits of *Centaurea maculosa* and *Senecio inaequidens* on rhizosphere microorganisms. *Oecologia* 163:1011-1020
- Thoden TC, Boppré M, Hallmann J (2009) Effects of pyrrolizidine alkaloids on the performance of plant-parasitic and free-living nematodes. *Pest Manag. Sci.* 65:823-830
- Trouvelot S, van Tuinen D, Hijri M, Gianinazzi-Pearson V (1999) Visualization of ribosomal DNA loci in spore interphasic nuclei of *glomalean* fungi by fluorescence *in situ* hybridization. *Mycorrhiza* 8:203-206
- Vandenkoornhuyse P, Husband R, Daniell TJ, Watson IJ, Duck JM, Fitter AH, Young JPW (2002) Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. *Mol. Ecol.* 11:1555-1564
- Vandenkoornhuyse P, Ridgway KP, Watson IJ, Fitter AH, Young JPW (2003) Co-existing grass species have distinctive arbuscular mycorrhizal communities. *Mol. Ecol.* 12:3085-3095
- van de Voorde TFJ, van der Putten WH, Gamper HA, Hol WH, Bezemer TM (2010) Comparing arbuscular mycorrhizal communities of individual plants in a grassland biodiversity experiment. *New Phytol.* 186:746-754
- van der Meijden E, Wijn H, Verkaar J (1988) Defence and regrowth, alternative plant strategies in the struggle against herbivores. *Oikos* 51:355-363
- van der Meijden E, de Boer NJ, van der Veen-van Wijk CAM (2000) Pattern of storage and regrowth in ragwort. *Evol. Ecol.* 14:439-455
- van Tuinen D, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. *Mol. Ecol.* 7:879-887
- Vaini EJ, Hantula J (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycol. Res.* 104:927-936
- Verbruggen E, Rölting WFM, Gamper HA, Kowalchuk GA, Verhoef HA, van der Heijden MGA (2010) Positive effects of organic farming on below-ground mutualists: large-scale comparison of mycorrhizal fungal communities in agricultural soils. *New Phytol.* 186:968-979
- Wink M (1988) Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Genet.* 75:225-233