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

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Abstract

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

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

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

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

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



Introduction

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

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

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

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

Material and Methods

Plants and soils

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

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

Pyrrolizidine alkaloid analysis

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

Thrips experiment

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

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

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

After two weeks feeding damage caused by the thrips was scored as visual damage on the leaf (mm²). In addition hairiness and toughness of the leaf as well as plant dry mass were measured. Toughness of the leaf was measured with a penetrometer. We did not find any significant effect of inoculum on these morphological resistance factors. Earlier findings by Leis et al. (2009) in *J. vulgaris* showed that morphological traits did not influence thrips resistance. Therefore our data on these traits will not be shown in the remainder of this manuscript. The plants were stored at -20°C before being freeze-dried for 72 hours under vacuum with a collector temperature of -55°C (Labconco Free Zone® 12 I Freeze Dry System). Before and after freeze-drying the biomass of the plant was measured.

Repeated thrips experiment with more replicates

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

Data analysis

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

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

Results

Plant dry mass

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

Total pyrrolizidine alkaloid concentration aboveground

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

Inoculum effect on pyrrolizidine alkaloid composition

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

Feeding damage

Genotype and Inoculum effect on Feeding damage

Genotype had a significant effect on feeding damage (Table 3). Genotype Kassel had, with a mean of 72,0 mm², the highest amount of feeding damage. The two jacobine-genotypes; Meijendel A and B, had the lowest amount of feeding damage, 39,8 and 32,2 mm² respectively (Figure 4). Inoculum treatment did not affect feeding damage (Table 3). When analyzing the inoculum effect per genotype we found that only the Wageningen genotype showed a significant inoculum effect on feeding damage ($F=4.9$, $df=2$, $Df=18$, $P=0.02$). Unfortunately, we cannot draw any firm conclusions on this observation, as we did not find a significant interaction between genotype and inoculum (Table 3).

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

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

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

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

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

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

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

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

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

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

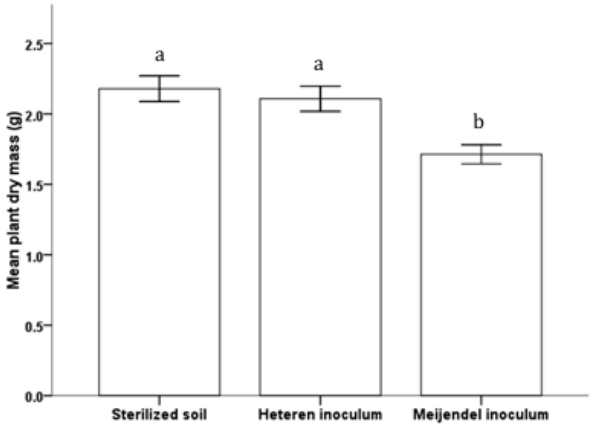


Figure 1. Mean plant dry mass per inoculum treatment (n = 40) of *Jacobaea vulgaris* plants. Error bars ±1SE. ANOVA with Post Hoc Bonferroni test in SPSS 17.0. Different letters above the bars indicate significant differences between inoculation treatments (P<0.05).

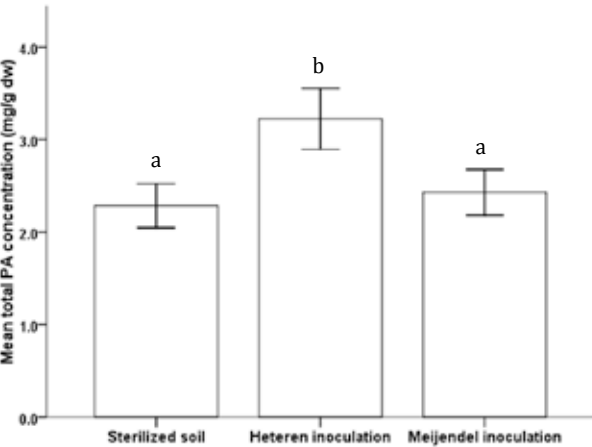


Figure 2. Mean total PA concentration in the shoots per inoculum treatment (n = 40) of *Jacobaea vulgaris* plants. Error bars ±1SE. ANOVA with Post Hoc Bonferroni test in SPSS 17.0. Different letters above the bars indicate significant differences between inoculation treatments (P<0.05).

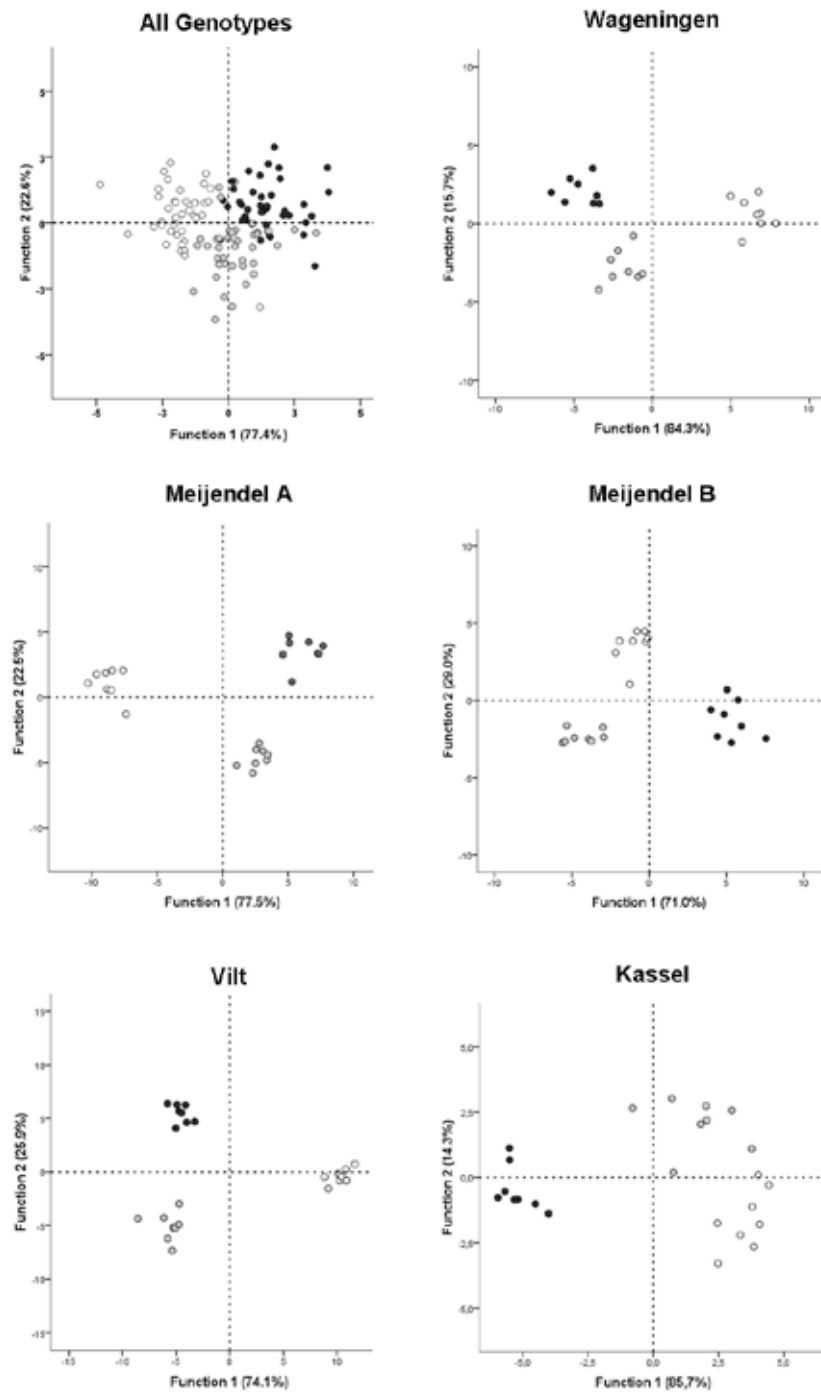


Figure 3. Scatterplots of plants grown on three different soil treatments, showing the discriminant scores of the cases on discriminant function 1 and 2 generated by Classify Discriminant Analysis for all genotypes plotted together and separately. White is sterilized Meijndel soil; Black is sterilized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

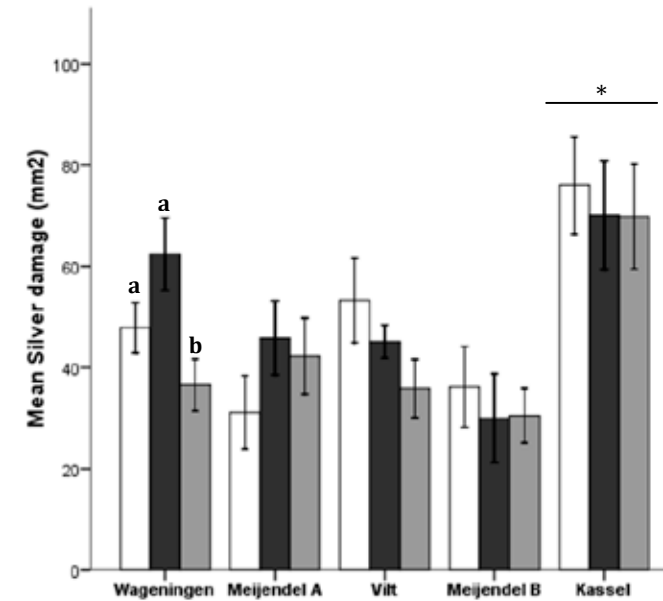


Figure 4. Mean of the total feeding damage per inoculum and genotype of *Jacobaea vulgaris*. White is sterilized soil (n = 35); Black is sterilized soil inoculated with 5% Heteren soil (n = 33); Grey is sterilized soil inoculated with 5% Meijndel soil (n = 34). Genotypes Wageningen & Meijndel A (n =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

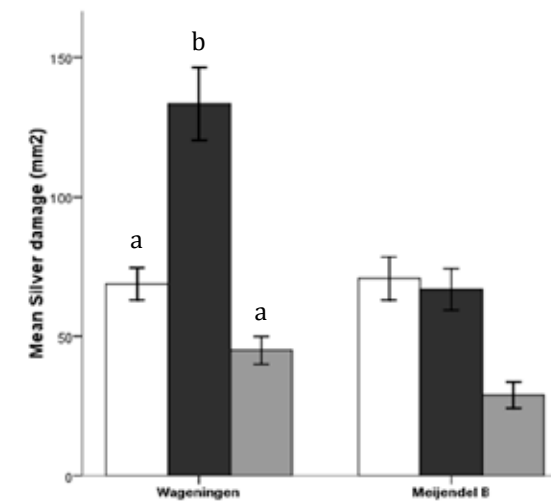


Figure 5. Mean of the total feeding damage per inoculum of two *Jacobaea vulgaris* genotypes. White is sterilized soil (n = 30); Black is sterilized soil inoculated with 5% Heteren soil (n = 30); Grey is sterilized soil inoculated with 5% Meijndel soil (n = 26). Genotype Wageningen (n = 44); Genotype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

Total and individual PA concentration effect on feeding damage

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

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

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

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

Discussion

Inoculum treatment had a great impact on the mean plant dry mass, which implies that plant growth was influenced by the soil-borne microorganisms. Plants grown on sterilized soil had the highest dry mass whereas plants grown on sterilized soil inoculated with 'own' Meijendel soil had the lowest dry mass. This supports the findings of for instance van der Putten et al. (1993), Bever et al. (1994 and 1997) and Klironomos (2002) that plant growth may considerably be reduced when grown in soils containing the microorganisms from their own natural habitat. Joosten et al. (2009) suggested that this could be the result of a negative effect by the introduced microorganisms, which in case of an inoculation with the same soil as the sterilized soil, may have been selected for plant specific pathogens (Putten et al. 1993; Bezemer et al. 2006). The decrease in plant mass may also be explained by a 'nutrient competition' between plant roots and a selective population of microorganisms. Jackson et al. (1989) showed that on a short timescale, soil microorganisms do compete better than plants, particularly for NH₄⁺: its uptake by microorganisms was five times faster than that by plants.

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

The second experiment confirmed that the microbial community can affect herbivores

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

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

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

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