Publication 5396 Netherlands Institute of Ecology (NIOO-KNAW)

EFFECTS OF ROOT HERBIVORY ON PYRROLIZIDINE ALKALOID CONTENT AND ABOVEGROUND PLANT-HERBIVORE-PARASITOID INTERACTIONS IN *Jacobaea Vulgaris*

OLGA KOSTENKO[1*], PATRICK P. J. MULDER[2], T. MARTIJN BEZEMER[1]

[1]*Netherlands Institute of Ecology (NIOO-KNAW), Department of Terrestrial Ecology, PO Box 50, 6700 AB Wageningen, The Netherlands*

[2]*RIKILT-Institute of Food Safety, Wageningen University and Research Centre, P.O. Box 230, 6700 AE Wageningen, The Netherlands*

**Correspondence: ***Olga Kostenko

Telephone: +31(0)317-473627

Fax: + 31 (0)317-473675

E-mail: o.kostenko@nioo.knaw.nl

Abstract – The importance of root herbivory is increasingly recognized in ecological studies and the effects of root herbivory on plant growth, chemistry and performance of aboveground herbivores have been relatively well studied. However, how belowground herbivory by root feeding insects affects aboveground parasitoid development is largely unknown. In this study, we examined the effects of root herbivory by wireworms (*Agriotes lineatus*) on the expression of primary and secondary compounds in the leaves and roots of ragwort (*Jacobaea vulgaris*). We also studied the effects of root herbivory on the performance of a generalist aboveground herbivore, *Mamestra brassicae* and its parasitoid *Microplitis mediator*. In contrast to what most other studies have reported, root herbivory in *J. vulgaris* had a strong negative effect on the total concentration of pyrrolizidine alkaloids (PAs) in shoot tissues. The composition of PAs in the shoots also changed after root herbivory. In particular, the concentration of less toxic *N*-oxide PAs decreased. There was no significant effect of root herbivory on PA composition and concentration in the roots. Although the concentration of PA in the leaves decreased, *M. brassicae* tended to grow slower on the plants exposed to root herbivory. Parasitoid performance was not affected by root herbivory, but parasitoids developed faster when the concentration of jacobine-type PAs in the foliage was higher. These results point at a putative role of individual PAs in multitrophic interactions and emphasize that generalizations about aboveground-belowground effects should be made with great caution.

Key Words – Belowground herbivory, chemical defense, parasitoid, plant-mediated interactions, ragwort, secondary metabolites.

INTRODUCTION

Root feeding insects can be very abundant in natural and agricultural systems and the importance of root herbivory is increasingly recognized in ecological studies (Whittaker 2003; Blossey and Hunt-Joshi 2003; Rasmann and Agrawal 2008; Van Dam 2009). Roots are essential for acquiring water and nutrients from the soil, and damage to the roots often results in decreased plant growth (Brown and Gange 1990). Besides the direct damage to the roots, belowground herbivory can also lead to changes in the concentration and composition of primary and secondary compounds in the roots. Due to root-shoot signaling, these changes frequently do not only occur in the roots, but also in the aboveground parts of a plant (Bezemer and Van Dam 2005; Blossey and Hunt-Joshi 2003; Johnson et al. 2008; Erb et al. 2009; Soler et al. 2012). Root herbivory can result in increases (e.g., Bezemer et al. 2003; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2008) or decreases in concentrations of aboveground secondary plant compounds (e.g., Kaplan et al. 2008a), although increased concentrations have been reported much more frequently than decreases (Kaplan et al. 2008b). As a result, this variation in plant responses to root herbivory may have important consequences for aboveground communities associated to the plant and interactions between aboveground and belowground herbivory.

Root herbivore-induced changes in aboveground plant chemistry can subsequently affect the performance of aboveground herbivores feeding on the plant (e.g. Bezemer et al. 2005; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2011). Moreover, via these changes in the plant and in the herbivores, root herbivory can affect the performance and the behavior of consumers of these herbivores such as parasitoids (Soler et al. 2012). A number of studies has shown that the level of parasitism or the host location behavior of parasitoids is affected by whether or not the herbivorous host is feeding on a plant that is also exposed to root herbivory (Masters et al. 2001; Rasmann and Turlings 2007; Soler et al. 2007; Staley et al. 2007; Olson et al. 2008). In contrast, the effects of belowground herbivory by root feeding insects on aboveground parasitoid development are less well studied. As far as we are aware, the impact of root feeding insects on aboveground parasitoid development have only been studied for *Cotesia glomerata*, a parasitoid of the specialist herbivore *Pieris brassicae*. In this system, root herbivory or even jasmonic acid application to the roots increases the glucosinolate contents in the leaves of *Brassica* plants and results in increased developmental times and reduced pupal weights of the parasitoid (Soler et al. 2005; Qiu et al. 2009). In the present study we examine the effects of root herbivory on aboveground multitrophic interactions for another plant-herbivore-parasitoid system. We exposed ragwort plants (*Jacobaea*

vulgaris Gaertn., Asteraceae) to root herbivory by wireworms (*Agriotes lineatus* L., Coleoptera: Elateridae), and examined the influence of root herbivory on the concentration and composition of pyrrolizidine alkaloids in roots and in foliar tissues, and on the performance of a generalist aboveground insect herbivore, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and its parasitoid *Microplitis mediator* Haliday (Hymenoptera: Braconidae).

Pyrrolizidine alkaloids (hereafter PAs) in *J. vulgaris* are root produced secondary metabolites (Hartmann 1999). PAs are a well studied group of plant allelochemicals due to their important role in plant-insect interactions. They serve as feeding and oviposition stimulants to specialist herbivores and are known to deter generalist insect herbivores (reviewed in Macel 2011). In the roots, the basic alkaloid structure senecionine *N*-oxide is produced, and this is transformed into several related senecionine-type PAs. These PAs are transported exclusively via the phloem path to the aboveground plant parts where additional diversification takes place, resulting in the formation of jacobine- and erucifoline-type PAs (Hartmann 1999; Cheng et al. 2011b). PAs generally occur in plants in tertiary amine (free base) form and in *N*-oxide form. Tertiary amines are regarded as degradation products of *N*-oxides (Hartman and Dierich 1998). A number of studies have shown that tertiary amines are more toxic for herbivorous insects than their corresponding *N*-oxides (Van Dam et al. 1995; Macel et al. 2005). Even though the importance of PAs in plant-insect interactions has been studied in great detail, little is known about the role of PAs in interactions between aboveground and belowground organisms (e.g., Hol et al. 2004; Joosten et al. 2009; Reidinger et al. 2011; Kostenko et al. 2012). Furthermore, the effects of PAs on parasitoid development and performance are not yet well ascertained (reviewed in Trigo 2011).

In a greenhouse experiment, we investigated the effects of root herbivory on the expression of primary and secondary compounds in the leaves and roots of ragwort. We further examined whether the survival and performance of the foliar feeding generalist herbivore and its parasitoid differed between plants exposed to root herbivory and control plants. Finally, we tested whether aboveground insect performance correlated with qualitative and quantitative characteristics of the chemistry of the leaves or roots. In line with what has been reported in other studies (e.g. Bezemer et al. 2003; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2008), we hypothesized that root herbivory (1) will increase total PA concentration in the shoots of *J. vulgaris*, and consequently (2) will have a negative effect on aboveground herbivore and parasitoid performance.

METHODS AND MATERIALS

Insects. Wireworms are larvae of the click beetle *A. lineatus* and considered to be generalist root feeders. *A. lineatus* larvae were obtained commercially from Applied Plant Research (PPO-WUR), Lelystad, The Netherlands. Larvae of *M. brassicae* are generalist leaf-chewing insects that feed on a wide variety of food-plants, including *J. vulgaris* (de Boer, 1999; Hol et al. 2004). *M. mediator* is a solitary larval endoparasitoid of *M. brassicae* (Harvey and Gols 2011). This parasitoid develops in first to fourth instar larvae of its host. Larvae of *M. mediator* feed solely on host hemolymph, and thus can be directly exposed to the plant allelochemicals ingested into hemolymph by host. *M. brassicae* and *M. mediator* were obtained from an insect culture at the Laboratory of Entomology of Wageningen University, The Netherlands. Cultures of *M. brassicae* and *M. mediator* were maintained on Brussels sprouts cv. Cyrus in climate rooms at 22±2°C, with a light regime of 16:8 L/D.

Experimental set-up. Seeds of *J. vulgaris* were collected from a single population at a semi-natural grassland in the Mossel nature restoration area (Ede, The Netherlands, 52°03'38"N, 5°45'04"E) where cropping ceased in 1995. Seeds were surface sterilized (1 min in a 0.1% sodium chloride solution and rinsed with water) and germinated on glass beads. Three *J. vulgaris* seedlings were planted in each of 80 one-liter pots filled with a mixture of sterilized and non-sterilized field soil (1:1 ratio). The sandy-loam soil (particle size distribution: < 2 µm, 3%; 2–63 µm, 17%; > 63 µm, 80%) was collected from the same area as the seeds and contained 4.5% organic matter. In the laboratory, the soil was sieved through a 0.5 cm mesh to remove stones and large arthropods and was subsequently homogenized. Half of the soil was sterilized using gamma irradiation (> 25 KGray gamma irradiation, Isotron, Ede, The Netherlands). The plants were grown in a greenhouse (21/16°C day/night, 16 hours photoperiod). Natural daylight was supplemented by 400 W metal halide lamps (1 lamp per 1.5 m^2). Plants were watered three times per week and randomly redistributed within the greenhouse once a week. After one week, the seedlings were randomly thinned to two seedlings per pot.

Six weeks after transplantation, two late-instar wireworm larvae were introduced into each of 40 randomly chosen pots assigned to the root herbivory treatment. Wireworms were placed into a small hole (1 cm deep) made in the soil. The larvae immediately burrowed into the soil. Similar holes were also made in the soil of the remaining 40 control pots. Prior to their introduction, wireworm larvae were starved for three days in moist soil at room temperature. Two weeks later, all pots were placed individually into a fine meshed cylindrical cage (70

cm height, 25 cm diameter). Two second- instar larvae of *M. brassicae* were then introduced to 20 control and 20 root herbivory pots. The remaining pots received two parasitized *M. brassicae* larvae. Larvae were introduced onto the plant by carefully placing them with a small brush on the youngest fully mature leaf of the plant. Parasitized larvae were parasitized individually using freshly mated *M. mediator* female parasitoids and then immediately introduced on the plant. The two larvae could move freely on the plants within each cage. Insects were kept on the plant for four weeks. Once a week, starting two weeks after introducing them on the plant, all larvae were collected from the plants, weighed on the microbalance, and returned to the same cage. Unparasitized larvae remained in the larval stage throughout the entire experiment. Cages with parasitized *M. brassicae* larvae were checked daily for egression of cocoons. Parasitoid cocoons were carefully collected from the plant and placed individually in Petri dishes until adult emergence. To record adult parasitoid emergence cocoons were checked twice a day. At emergence, the date of eclosion was recorded and parasitoids were sexed. Hind tibia length was recorded as a measure of adult size (Godfray 1994), using a calibrated slide and a stereomicroscope. Development time was calculated as days between parasitism and adult emergence. At harvest, shoots were clipped and roots were carefully removed from the soil and rinsed. Shoot and root biomass of each pot was oven-dried at 70ºC for three days and weighed. All wireworm larvae were recovered alive from the soil.

Chemical analysis. Eight weeks after germination, just prior to the introduction of the unparasitized and parasitized *M. brassicae* larvae, the third youngest leaf of 20 control plants and 20 plants with root herbivory was removed with a razor blade immediately freeze-dried and finely ground. The root samples were taken from the oven-dried root material for the same plants and pulverized. For both treatments there were 10 plants allocated for unparasitized and 10 for parasitized larvae. Carbon (C) and Nitrogen (N) content were determined only for leaf samples using a Flash EA1112 CN analyzer (Interscience, Breda, The Netherlands). PA composition and content was determined using a Waters Acquity ultra performance liquid chromatographic system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MS, USA); see also Cheng et al. (2011a, b). For each sample, 10 mg of ground plant material was mixed with 1.0 ml 2% formic acid solution. Heliotrine was added to the extraction solvent as an internal standard at a concentration of 1 µg/ml. The mixture was centrifuged and filtered through a 0.2 µm nylon membrane filter (Acrodisc, Pall Life Sciences, MI, USA). An aliquot of 25 µl of the extracted filtrate was diluted with 975 µl of 10 mM ammonium hydroxide solution and injected in the LC-MS/MS system. PAs were separated on a Waters BEH C18 UPLC column (150 x 2.1 mm, 1.7

µm particles) applying 5 mM ammonium hydroxide as mobile phase and using acetonitrile as organic modifier (0-50%) in a 12-min linear gradient. The mass spectrometer was operated in positive electrospray mode and the samples were screened for a total of 37 PAs. Details on the mass spectrometric settings are described in Cheng et al. (2011a). PAs were quantified against a calibrant of PA standards added to *Tanacetum vulgare* plant extract (which itself is free of PAs) to minimize matrix effects that otherwise could play a role when using standards in solvent only. The calibrant solution was injected every 20 samples to monitor for variations in detector response. Samples were injected in a randomized order. Data were processed using Masslynx 4.1 software (Waters, Milford, MA, USA).

Statistics. The impact of root herbivory on plant biomass, chemistry, herbivore and parasitoid performance was assessed using a Welch's robust *t-test* which does not require homogeneity of variances. In the robust Welch t-test the degrees of freedom are corrected with the Welch-Satterthwaite modification (Welch 1947). The percentage difference in individual PA concentrations was calculated as: (mean PA concentration of plants subjected to root herbivory treatment – mean PA concentration of control plants)/ mean PA concentration of control plants. The overall difference in the concentration of *N*-oxides and tertiary amines was compared using a paired *t-test*. The relative concentration of *N*-oxides was calculated as: % *N*-oxide = *N*-oxide concentration/(*N*-oxide concentration + the corresponding tertiary amine concentration)×100. Percentage data were arcsine square-root transformed prior to statistical analysis. For graphical representation we calculated the natural logarithm of the ratio between *N*-oxides and tertiary amines that is symmetrical around the 1:1 ratio point. The relationship between plant characteristics and herbivore and parasitoid performance were analyzed using Pearson's product-moment correlation. As the number of replicates was relatively low, significance in multiple statistical tests was not corrected (Moran 2003). To examine whether root herbivory influenced the PA composition aboveground or belowground we used multivariate principal component (PCA) and redundancy (RDA) analyses. The choice of linear methods was justified by the short length of gradients (less than 2.0). RDA was also used to test the relationship between the shoot PA composition and herbivore or parasitoid performance. Significances in multivariate analyses were tested using a Monte Carlo permutation test with 999 permutations. Univariate analyses were performed in R statistical language, ver. 2.15.0 (R Development Core Team 2012) and multivariate analyses in CANOCO version 4.55 (Ter Braak and Šmilauer 2002).

RESULTS

Plant Responses. Plant shoot and root biomass did not differ significantly between treatments (Table 1). Root herbivory did also not influence leaf nitrogen concentrations and leaf C:N ratios. The total PA concentration in shoots of plants exposed to root herbivory was significantly lower (38%) than in control plants (Table 1). The total PA concentration in roots was slightly higher (12%) in plants exposed to root herbivory than in control plants but this was not statistically significant (Table 1). Twenty-nine PAs were detected in shoots and 33 PAs in roots of *J. vulgaris* (Table 2). The detected PAs belonged to four structural groups: erucifoline-type, jacobine-type, senecionine-type and otosenine-type (Table 2). Otosenine-type PAs were only identified in roots. In shoots, dehydrojaconine was detected in trace amounts and only occurred as tertiary amine. All other PAs were found in *N*-oxide and in tertiary amine form. In roots, senecivernine, senkirkine, otosenine, onetine and desacetyldoronine were only present as tertiary amines. The concentration of tertiary amines in shoots was not affected by root herbivory ($t_{35.5}$=0.61, P=0.54), whereas the overall levels of *N*-oxides in shoots decreased by 52% in the plants exposed to root herbivory ($t_{29.5}$=3.24, P=0.003; Figure 1). In roots, there was no significant difference in the concentration of tertiary amines ($t_{35.2}$=0.81, P=0.42) and *N*-oxides ($t_{37.5}$=-1.28, P=0.21; Figure 1) between treatments, although the levels of *N*-oxides were 14% higher in roots exposed to root herbivory. The contribution of tertiary amines increased from 34% to 48% in the total shoot PA concentration while in the total root PA concentration it decreased from 9% to 7% (Figure 1)

In shoots, independent of root herbivory, jacobine and jacobine *N*-oxide were present in the highest concentrations in all plants (35% and 33% respectively of the total PA concentration) and the total concentration of jacobine-type PAs decreased after root herbivory (Table 2). The total concentration in shoots of senecionine-type PAs was lower in plants exposed to root herbivores, but the total concentration of erucifoline-type PAs did not differ between the treatments, although levels of acetylerucifoline (+1024%) and acetylerucifoline *N*-oxide (+337%) responded most strongly to root herbivory (Table 2). In roots, the total concentrations of none of the four groups of PAs was affected by root herbivory (Table 2).

Overall, the relative concentration of *N*-oxides was higher than that of tertiary amines (shoots: t_{39}=-2.58, P=0.014; roots: t_{39}=-32.15, P<0.001, Figure 1). In shoots, for erucifoline- and senecionine-type PAs the relative concentration of *N*-oxides was much higher than the concentration of tertiary amines (P<0.01 in all cases), while for jacobine-type PAs concentrations of *N*-oxides were equal or lower than concentrations of tertiary amines

(Figure 2). In roots, the relative concentration of *N*-oxides was much higher for all compounds except for jaconine (Figure 2). Root herbivory significantly decreased the relative concentration of *N*-oxides for senecionine ($t_{28.4}$=-2.63, P=0.014), erucifoline ($t_{28.5}$=2.73, P=0.011) and integerrimine ($t_{33.2}$=2.98, P=0.005) in shoots, and increased the relative concentration of *N*-oxides for acetylseneciphylline in roots ($t_{38.0}$=-3.05, P=0.005, Figure 2).

Principle component analyses of the shoot PA composition showed that most of the variation in PA profiles could be explained by three principle component axes (74.3% cumulative explained variation). Shoot PA profiles differed significantly between plants exposed to root herbivory and control plants (RDA: F=4.50, P=0.002; 10.6% explained variation). Shoot PA profiles of plants exposed to root herbivory and control plants clearly separated in an unconstrained analysis (PCA; Figure 3). In the PCA, the levels of acetylerucifoline, acetylerucifoline *N*-oxide, jaconine *N*-oxide were higher in plants with root herbivory, whereas levels of jacobine *N*-oxide, jacoline *N*-oxide, erucifoline *N*-oxide, senecionine *N*-oxide, integerrimine *N*-oxide, usaramine *N*-oxide, seneciphylline *N*-oxide and retrorsine *N*-oxide were higher in control plants (Figure 3). The PA composition in roots was not affected by root herbivory (RDA: F=0.62, P=0.74; data not shown).

Herbivore and Parasitoid Performance The relative growth rates of unparasitized *M. brassicae* larvae tended to be lower on plants with root herbivory, but this was only marginally significant (P=0.054; Table 1). Mortality of *M. brassicae* did not differ significantly between the two treatments (Table 1). Herbivore growth rate and survival were not significantly related to leaf nitrogen concentration, C:N ratio, total shoot PA concentration, levels of individual PAs in the shoots, or shoot PA composition (P>0.05 in all cases). Parasitoid performance, measured as hind tibia length, % successful cocoon egression, % adult emergence, and development time, also did not differ between the two treatments (Table 1). However, there was a negative relationship between parasitoid development time and total shoot *N*-oxide concentration (R^2=55.0%, P=0.033). Analyses of individual shoot PA compounds revealed that parasitoid development time negatively correlated with concentrations of jacoline *N*-oxide (R^2 = 89.5%, P = 0.006), jacobine *N*-oxide (R^2 = 64.6%, P = 0.016), and usaramine (R^2 = 76.4%, P = 0.046).

DISCUSSION

In our study, root herbivory greatly affected the concentration and composition of PAs in the leaves of *J. vulgaris*. However, in contrast with our hypothesis, total PA concentration in the shoots of *J. vulgaris* decreased strongly (38%) when plant roots were exposed to herbivory by *A. lineatus*. In a previous study, Hol et al. (2004) found that mechanical root damage caused an increase in PA concentrations in the roots of *J. vulgaris* but mechanical damage to roots had only weak and inconsistent effects on shoot PA concentrations. Clearly, mechanical tissue damage may not elicit the same effect on the expression of allelochemicals as actual herbivory (Bezemer et al., 2004; Kaplan et al., 2008a). The majority of studies that have examined the effects of root damage by real herbivores on concentrations of aboveground secondary plant compounds for other plant species report increases in the amount of secondary metabolites following root herbivory (e.g. Bezemer et al. 2003, 2004; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2008; Wurst et al. 2008; Kaplan et al. 2008b). One of the reasons for the discrepancy between the results of these studies and ours may be that PAs are synthesized in the roots whereas many of the secondary compounds included in the other studies can be produced in the shoots. Similar to our results, root herbivory by the nematode *Meloidogyne incognita* in tobacco plants causes a decline in the concentrations of the alkaloid nicotine in the foliage, and nicotine is also synthesized in the roots (Hanounik and Osborne 1977; Kaplan et al. 2008a). However, Hanounik and Osborne (1977) also showed that root herbivory by *M. incognita* caused an increase in nicotine in leaves of a resistant tobacco cultivar showing that the effects of root herbivory can greatly vary even within a single plant species. In the study of Kaplan et al. (2008a), even though root herbivory caused a decline in the concentrations of nicotine aboveground, concentrations of other secondary plant compounds that are not exclusively produced in the roots increased in the foliage. In another study, terpenoid aldehydes in cotton (*Gossypium* sp.), which are also synthesized in roots, increased in the foliage of cotton following root herbivory by wireworms (Bezemer et al. 2004). Synthesis of gossypol is also known to occur in the foliage of cotton plants but in lower concentrations (Bezemer et al. 2004). Therefore, it is plausible that the synthesis of gossypol was enhanced in the shoots rather than in the roots by belowground herbivory. However, a more likely explanation for the different responses observed among the different plant species is that there are various mechanisms by which belowground herbivory can lead to changes in aboveground plant chemistry (reviewed in Soler et al. 2012). These results therefore emphasize that generalizations about aboveground-belowground effects should be made with great caution.

An important question that requires further study is whether root herbivory in ragwort negatively interferes with PA synthesis in the roots, or whether the negative effects of root herbivory on aboveground PA concentrations

result from a difference in allocation of PAs to aboveground tissues. PA production in *J. vulgaris* is closely linked to root growth (Frischknecht et al. 2001). Interestingly, in our study, root biomass was not significantly affected by the belowground herbivory. Such a lack of a response in root biomass to root herbivory has also been observed in other experiments in which *J. vulgaris* was exposed to root herbivory (M. Bezemer, unpublished data) and can be the result of compensatory growth or a reallocation of resources from shoots to roots. For *J. vulgaris* roots are more essential organs than shoots, because roots accumulate resources that are used by plant for regrowth after complete defoliation (Van der Meijden et al. 2000). As root biomass did not change after root herbivory this suggests that the production of PAs in the roots could be maintained at the same level. Indeed in our study, total root PA concentration and composition were not significantly affected by belowground herbivory. Although, the effect of root herbivory on total root PA concentration was not significant, the total amount of PAs in the roots tended to increase (12%) in presence of root herbivory whereas the total amount of PAs in shoots decreased significantly (38%). This suggests that root herbivory caused a reallocation of PAs from the shoots to roots, or that less PAs were transported from the roots to the shoots in plants exposed to root herbivory. Overall, concentrations of PAs were much higher in roots than in leaves. These results, in line with other studies (Hol et al. 2004; Van der Meijden et al. 2000) suggest that roots are more important to *J. vulgaris* than shoot tissues. However, it is important to note that, in our study, the root samples were collected later than the leaf samples, and after a period of aboveground herbivore feeding.

The use of the LC-MS/MS procedure allowed us to detect both the tertiary amine and *N*-oxide forms of PAs, as well as PAs that are present only in extremely low concentrations in the plant (Joosten et al. 2009). Earlier studies were restricted to the major PAs that are present in plants and in these studies the authors were not able to discriminate between the two forms of PAs (e.g., Hol et al. 2004; Macel and Klinkhamer 2010). Our results in line with other more recent studies (e.g. Joosten et al. 2011) show that the concentration of tertiary amine forms in jacobine-type PAs is higher than in other PA groups (for a discussion on the selective formation of jacobine tertiary amines see Joosten et al 2011). Interestingly, in our study most of the individual PAs in plant shoots that responded to the root herbivory treatment were *N*-oxides. As a result, the ratio of *N*-oxides to tertiary amines in the shoots changed from 2:1 in control plants to 1:1 in plants exposed to root herbivores. At the same time, there was a slight increase in the *N*-oxide concentration in the roots, mostly due to an increase in the concentration of senecionine *N*-oxide, while the total tertiary amine concentration in the roots remained constant. The concentration of *N*-oxides of major PAs such as jacobine *N*-oxide, jacoline *N*-oxide and erucifoline *N*-oxide did

not increase in the roots in response to root herbivory, suggesting that it is unlikely that *N*-oxides are actively back-transported from shoots to roots when the plant is exposed to root herbivory. Therefore, our data suggest that plants when they are exposed to root herbivory, alter PA concentrations in shoots and roots via restrictions in the flow of *N*-oxides from root to shoot tissues. As a result, if PA transport from roots to shoots is restricted, over time the PA concentration in the shoots will decrease, because the plant continues to grow (dilution effect). At the same time the conversion from *N*-oxides to tertiary amines continues to take place in the shoots. This conversion further reduces the concentration of *N*-oxides in the shoots, but stabilizes the tertiary amines concentrations.

Apart from affecting the total PA concentration in the plant, root herbivory also caused a change in the relative composition of PAs in the leaves. Traditionally, it was assumed that PAs are produced in the root as senecionine *N*-oxide only, and that diversification of this compound then occurs in the foliage (Harmann and Dierich 1998). Recent studies, however, have shown that PA diversification may already start in the roots, where besides senecionine *N*-oxide, considerable amounts of compounds that are closely related to senecionine *N*-oxide, such as seneciphylline *N*-oxide, acetylseneciphylline *N*-oxide and integerrimine *N*-oxide have been detected (Joosten et al. 2009; Cheng et al. 2011b). Further conversion of PAs takes place in the leaves and this process is highly plastic and depends on a number of physiological processes in the plant (reviewed in Hartmann 1999). The exact mechanism of PA diversification remains unclear. Interestingly, in our study the concentration of acetylerucifoline and acetylerucifoline *N*-oxide in shoots increased greatly in plants exposed to root herbivory, while the concentration of erucifoline *N*-oxide significantly decreased. At the same time, the overall concentration of erucifoline-type PAs remained constant between the treatments. Acetylerucifoline *N*-oxide can be formed by acetylation of erucifoline *N*-oxide or by conversion of acetylseneciphylline *N*-oxide to acetylerucifoline *N*-oxide. Acetylseneciphylline *N*-oxide was not found in significant amounts in the shoots indicating that this compound is not transported well from roots to shoots perhaps due to its chemical properties. Therefore, we hypothesize that root herbivory causes an increase in the acetylation of erucifoline *N*-oxide in aboveground plant parts. Similarly, acetylseneciphylline *N*-oxide is synthesized by introducing an acetyl functional group to seneciphylline *N*-oxide in the root system (Cheng et al. 2011b). Acetylseneciphylline *N*-oxide also slightly increased in the roots of plants exposed to belowground herbivory. The ecological functions of acetylerucifoline and acetylseneciplylline are not yet known. More studies are needed that further explore how

environmental stresses such as root herbivory affect the diversification and what the ecological consequences are of changes in plant PA composition for other organisms in natural communities.

The performance of the aboveground generalist herbivore *M. brassicae* was not significantly affected by root herbivory although unparasitized larvae tended to grow faster on undamaged plants containing higher concentrations of PAs in the shoots. This is a rather unexpected result that may be explained by the differences in the ratios between *N*-oxides and tertiary amines. *N*-oxide and tertiary amine forms of PAs are known to differently affect herbivorous insects. Several studies have shown, for example, that PAs in the form of *N*-oxides have less deterrent or toxic effects on generalist insect herbivores than tertiary PAs (Dreyer et al. 1985; Van Dam et al. 1995; Macel et al. 2005). In addition, individual PAs differ in their effects on herbivores. For example, jacobine tertiary amine has been shown to adversely affect the performance of non-specialized herbivorous insects (Leiss et al. 2009; Macel and Klinkhamer 2010, Cheng et al. 2011a). In our study, jacobine was one of the major PAs present in leaves and the ratio of *N*-oxide to tertiary amine of this compound changed from 1.19 in control plants to 0.63 in plants exposed to root herbivory. Therefore, *M. brassicae* caterpillars feeding from root damaged plants may have suffered from the higher concentration of more toxic compounds that were present in the leaves even though the total PA concentration decreased. Furthermore, in our study larval mortality was high, and none of the unparasitized caterpillars pupated, even though they were kept on the plants for four weeks. The caterpillars performed much worse on *J. vulgaris* plants than on artificial diet (Kostenko, unpublished data), and this suggests that PA levels may already have been too high for this herbivore, independent of whether the plant was exposed to root herbivory or not. However, in a choice experiment where the individual and combined effects of six PAs were tested in an artificial diet, Macel et al. (2005) did not find a deterrent effect of PAs on *M. brassicae*. These authors concluded that *M. brassicae* is a generalist herbivore that is relatively insensitive to various secondary metabolites in its diet. Alternatively, root herbivory may have caused an increase in other defensive compounds in *J. vulgaris* such as phenolics or may have induced changes in morphological characteristics such as trichomes that can increase physical resistance of the plant to herbivory.

Clearly, besides plant defenses, other plant characteristics may also have affected the performance of *M. brassicae* on *J. vulgaris* plants. In line with the plant-stress hypothesis (White 1984), Masters et al. (1993) proposed that stress induced by root herbivory will cause an increase in the concentrations of nitrogen and carbohydrates in foliar tissues of a plant. For the majority of herbivorous insects, the amount of nitrogen in the

diet is the major limiting nutritional factor determining insect growth (Awmack and Leather 2002) and root herbivory would therefore lead to increased performance of aboveground herbivores. However, in our study, feeding by *A. lineatus* did not affect leaf nitrogen concentrations or C:N ratios in *J. vulgaris* plants.

The diet of a herbivorous host may also affect parasitoids that develop in this host by exposing them to unmetabolized defensive chemicals (Ode 2006). In our study, root herbivory did not affect the performance of the parasitoid *M. mediator*. Interestingly, although we did not detect a relationship between PA concentrations and *M. brassicae* performance, in our study parasitoids developed faster when the concentration of jacobine-type PAs, such as jacobine *N*-oxide and jacoline *N*-oxide in the plant was higher. This suggests that *N*-oxides indeed could have less adverse effects on the performance of insects than tertiary amines. Future studies should examine whether there is a true causal positive relationship between jacobine-type *N*-oxides and parasitoid performance, or whether this is merely a coincidental correlation, and what the mechanisms are that underlie these interactions.

In summary, this study shows that root herbivory by wireworms has a strong negative effect on the concentration of PAs in the leaves of *J. vulgaris* possibly via the mechanism of restricted transport of PA *N*-oxides from roots to leaves. However, this does not result in a positive effect on the performance of the generalist insect herbivore *M. brassicae* or its parasitoid *M. mediator*. In contrast, *M. brassicae* tends to grow slower on plants exposed to root herbivory. This decline in herbivore performance can be explained by changes in foliar PA composition in plants exposed to root herbivory whereby the relative concentration of less toxic PAs decreases. Moreover, in our study the performance of parasitoids was also positively correlated with the concentration of less toxic PAs. Further research should aim at elucidating the putative role of individual PAs in aboveground-belowground multitrophic interactions.

Acknowledgments – We thank Rieta Gols and Jeff Harvey for providing *Mamestra brassicae* and *Microplitis mediator*; Wiecher Smant, Joop Woelke and Ciska Raaijmakers for technical assistance. This work was funded by the Netherlands Organization of Scientific research (NWO, VIDI grant no. 864.07.009 to TMB). Publication XXXX Netherlands Institute of Ecology (NIOO-KNAW).

REFERENCES

AWMACK, C. S. and LEATHER, S. R. 2002. Host plant quality and fecundity in herbivorous insects. *Annu. Rev. Entomol.* 47:817-844.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., and WÄCKERS, F. L. 2003. Interactions between above- and belowground insect herbivores as mediated by the plant defense system. *Oikos* 101:555-562.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., VAN DER PUTTEN, W. H., and WÄCKERS, F. L. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *J. Chem. Ecol.* 30:53-67.

BEZEMER, T. M., DE DEYN, G. B., BOSSINGA, T. M., VAN DAM, N. M., HARVEY, J. A., and VAN DER PUTTEN, W. H. 2005. Soil community composition drives aboveground plant-herbivore-parasitoid interactions. *Ecol. Lett.* 8:652-661.

BEZEMER, T. M. and VAN DAM, N. M. 2005. Linking aboveground and belowground interactions via induced plant defenses. *Trends Ecol. Evol.* 20:617-624.

BLOSSEY, B. and HUNT-JOSHI, T. R. 2003. Belowground herbivory by insects: Influence on plants and aboveground herbivores. *Annu. Rev. Entomol.* 48:521-547.

DE BOER, N. J. 1999. Pyrrolizidine alkaloid distribution in *Senecio jacobaea* rosettes minimizes losses to generalist feeding. *Entomol. Exp. Appl.* 91:169-173.

BROWN, V. K. and GANGE, A. C. 1990. Insect herbivory below ground. *Adv. Ecol. Res.* 20:1-58.

CHENG, D. D., KIRK, H., VRIELING, K., MULDER, P. P. J., and KLINKHAMER, P. G. L. 2011a. The relationship between structurally different pyrrolizidine alkaloids and western flower trips resistance in F_2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. *J. Chem. Ecol.* 37:1071-1080.

CHENG, D. D., KIRK, H., MULDER, P. P. J., VRIELING, K., and KLINKHAMER, P. G. L. 2011b. Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and *Jacobaea aquatica*. *New Phytol.* 192:1010-1023.

DREYER, D. L., JONES, K. C., and MOLYNEUX, R. J. 1985. Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrthosipon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. *J. Chem. Ecol.* 11:1045-1051.

ERB, M., ROBERT, C. A. M., HIBBARD, B. E., and TURLINGS, T. C. J. 2011. Sequence of arrival determines plant-mediated interactions between herbivores. *J. Ecol.* 99:7-15.

ERB, M., LENK, C., DEGENHARDT, J., and TURLINGS, T. C. J. 2009. The underestimated role of roots in defense against leaf attackers. *Trends Plant Sci.* 14:653-659.

ERB, M., TON, J., DEGENHARDT, J., and TURLINGS, T. C. J. 2008. Interactions between arthropod-induced aboveground and belowground defenses in plants. *Plant Physiol.* 146:867-874.

FRISCHKNECHT, P. M., SCHUHMACHER, K., MÜLLER-SCHÄRER, H., and BAUMANN, T. W. 2001. Phenotypic plasticity of *Senecio vulgaris* from contrasting habitat types: growth and pyrrolizidine alkaloid formation. *J. Chem. Ecol.* 27:343-358.

GODFRAY, H. C. J. 1994. Parasitoids: Behavioral and Evolutionary Ecology. Princeton University Press, Princeton.

HANOUNIK, S. B. and OSBORNE, W. W. 1977. The relationships between population density of *Meloidogyne incognita* and nicotine content of tobacco. *Nematologica* 23:147-152.

HARTMANN, T. and DIERICH, B. 1998. Chemical diversity and variation of pyrrolizidine alkaloids of the senecionine type: Biological need or coincidence? *Planta* 206:443-451.

HARTMANN, T. 1999. Chemical ecology of pyrrolizidine alkaloids. *Planta* 207: 483-495.

HARVEY, J. A. and GOLS, R. 2011. Population-related variation in plant defense more strongly affects survival of an herbivore than its solitary parasitoid wasp. *J. Chem. Ecol.* 37:1081-1090.

HOL, W. H. G., MACEL, M., VAN VEEN, J. A., and VAN DER MEIJDEN, E. 2004. Root damage and aboveground herbivory change concentration and composition of pyrrolizidine alkaloids of *Senecio jacobaea*. *Basic Appl. Ecol.* 5:253-260.

JOHNSON, S.N., BEZEMER, T.M., and JONES T.H. 2008. Linking aboveground and belowground herbivory, pp. 153-170, in S. N. Johnson and P. J. Murray (eds.). Root Feeders. An Ecosystem Perspective. CABI, UK.

JOOSTEN, L., MULDER, P. P. J., KLINKHAMER, P. G. L., and VAN VEEN, J. A. 2009. Soil-borne microorganisms and soil-type affect pyrrolizidine alkaloids in *Jacobaea vulgaris*. *Plant Soil* 325:133-143.

JOOSTEN, L., CHENG, D. D., MULDER, P. P. J., VRIELING, K., VAN VEEN, J. A., and KLINKHAMER, P. G. L. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. *Phytochemistry* 72:214-222.

KAPLAN, I., HALITSCHKE, R., KESSLER, A., REHILL, B. J., SARDANELLI, S., and DENNO, R. F. 2008a. Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecol. Lett.* 11:841-851.

KAPLAN, I., HALITSCHKE, R., KESSLER, A., SARDANELLI, S., and DENNO, R. F. 2008b. Effects of plant vascular architecture on aboveground-belowground-induced responses to foliar and root herbivores on *Nicotiana tabacum*. *J. Chem. Ecol.* 34:1349-1359.

KOSTENKO, O., VAN DE VOORDE, T. F. J., MULDER, P. P. J., VAN DER PUTTEN, W. H., and BEZEMER, T. M. 2012. Legacy effects of aboveground–belowground interactions. *Ecol. Lett.* 15:813-821.

LEISS, K. A., CHOI, Y. H., ABDEL-FARID, I. B., VERPOORTE, R., and KLINKHAMER, P. G. L. 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. *J. Chem. Ecol.* 35:219-229.

MACEL, M., BRUINSMA, M., DIJKSTRA, S. M., OOIJENDIJK, T., NIEMEYER, H. M., and KLINKHAMER, P. G. L. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. *J. Chem. Ecol.* 31:1493-1508.

MACEL, M. and KLINKHAMER, P. G. L. 2010. Chemotype of *Senecio jacobaea* affects damage by pathogens and insect herbivores in the field. *Evol. Ecol.* 24:237-250.

MACEL, M. 2011. Attract and deter: A dual role for pyrrolizidine alkaloids in plant-insect interactions. *Phytochem. Rev.* 10:75-82.

MASTERS, G. J., JONES, T. H., and ROGERS, M. 2001. Host-plant mediated effects of root herbivory on insect seed predators and their parasitoids. *Oecologia* 127:246-250.

MASTERS, G. J., BROWN, V. K., and GANGE, A. C. 1993. Plant mediated interactions between above- and belowground insect herbivores. *Oikos* 66:148-151.

MORAN, M. D. 2003. Arguments for rejecting the sequential bonferroni in ecological studies. *Oikos* 100:403-405.

ODE, P. J. 2006. Plant chemistry and natural enemy fitness: Effects on herbivore and natural enemy interactions. *Annu. Rev. Entomol.* 51:163-185.

OLSON, D. M., DAVIS, R. F., WÄCKERS, F. L., RAINS, G. C., and POTTER, T. 2008. Plant-herbivore-carnivore interactions in cotton, *Gossypium hirsutum*: Linking belowground and aboveground. *J. Chem. Ecol.* 34:1341-1348.

QIU, B. L., HARVEY, J. A., RAAIJMAKERS, C. E., VET, L. E. M., and VAN DAM, N. M. 2009. Nonlinear effects of plant root and shoot jasmonic acid application on the performance of *Pieris brassicae* and its parasitoid *Cotesia glomerata*. *Funct. Ecol.* 23:496-505.

RASMANN, S. and AGRAWAL, A. A. 2008. In defense of roots: A research agenda for studying plant resistance to belowground herbivory. *Plant Physiol.* 146:875-880.

RASMANN, S. and TURLINGS, T. C. J. 2007. Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies. *Ecol. Lett.* 10:926-936.

R DEVELOPMENT CORE TEAM. 2012. R: A language and environment for statistical computing. R foundation for Statistical Computing, Vienna, Austria. URL http://www.r-project.org/.

REIDINGER, S., ESCHEN, R., GANGE, A. C., FINCH, P., and BEZEMER, T. M. 2012. Arbuscular mycorrhizal colonization, plant chemistry, and aboveground herbivory on *Senecio jacobaea*. *Acta Oecol*. 38:8-16.

SOLER, R., VAN DER PUTTEN, W. H., HARVEY, J. A., VET, L. E. M., DICKE M., and BEZEMER, T. M. 2012. Root herbivore effects on aboveground multitrophic interactions: patterns, processes and mechanisms. *J. Chem. Ecol.* 38: 755-767.

SOLER, R., HARVEY, J. A., KAMP, A. F. D., VET, L. E. M., VAN DER PUTTEN, W. H., VAN DAM, N. M., STUEFER, J. F., GOLS, R., HORDIJK, C. A., and BEZEMER, T. M. 2007. Root herbivores influence the behaviour of an aboveground parasitoid through changes in plant-volatile signals. *Oikos* 116:367-376.

SOLER, R., BEZEMER, T. M., VAN DER PUTTEN, W. H., VET, L. E. M., and HARVEY, J. A. 2005. Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. *J. Anim. Ecol.* 74:1121-1130.

STALEY, J. T., MORTIMER, S. R., MORECROFT, M. D., BROWN, V. K., and MASTERS, G. J. 2007. Summer drought alters plant-mediated competition between foliar- and root-feeding insects. *Glob. Change Biol.* 13:866-877.

TER BRAAK, C. J. F. and ŠMILAUER, P. 2002. CANOCO reference manual and Cano Draw for Windows user's guide. Software for canonical community ordination, version 4.5. Microcomputer Power, Ithaca, New York.

TRIGO, J. R. 2011. Effects of pyrrolizidine alkaloids through different trophic levels. *Phytochem. Rev.* 10:83-98.

VAN DAM, N. M., VUISTER, L. W. M., BERGSHOEFF, C., DEVOS, H., and VAN DER MEIJDEN, E. 1995. The "raison d'être" of pyrrolizidine alkaloids in *Cynoglossum officinale*: deterrent effects against generalist herbivores. *J. Chem. Ecol.* 21:507-523.

VAN DAM, N. M., RAAIJMAKERS, C. E., and VAN DER PUTTEN, W. H. 2005. Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol. Exp. Appl.* 115:161-170.

VAN DAM, N. M. 2009. Belowground herbivory and plant defenses. *Annu. Rev. Ecol. Evol. Syst.* 40:373-391.

VAN DER MEIJDEN, E., DE BOER, N. J., and VAN DER VEEN-VAN WIJK, C. A. M. 2000. Pattern of storage and regrowth in ragwort. *Evol. Ecol.* 14:439-455.

WELCH, B. L. 1947. The generalization of "student's" problem when several different population variances are involved. *Biometrika* 34: 28-35.

WHITE, T.C.R. 1984. The abundance of invertebrate herbivores in relation to the availability of nitrogen in stressed food plants. *Oecologica* 63: 90-105.

WHITTAKER, J. B. 2003. Root-animal interactions, pp. 363-385, in H. de Kroon and E. J. W Visser (eds.). Root Ecology. Springer, New York.

WURST, S., VAN DAM, N. M., MONROY, F., BIERE, A., and VAN DER PUTTEN, W. H. 2008. Intraspecific variation in plant defense alters effects of root herbivores on leaf chemistry and aboveground herbivore damage. *J. Chem. Ecol.* 34:1360-1367.

TABLE 1 Effects of root herbivory by wireworms on plant, herbivore and parasitoid performance parameters. Means (±SE) are shown for control plants (-RH) and plants exposed to the root herbivory by wireworms (+RH) and results of a statistical test

	-RH	+RH	*N*		*P*[a]
Shoot biomass	0.42 ± 0.01	0.42 ± 0.01	80	$t_{77.5}$=0.40	0.69
Root biomass	1.30 ± 0.05	1.24 ± 0.04	80	$t_{69.3}$=0.89	0.38
Leaf nitrogen concentration (%)	1.26 ± 0.05	1.30 ± 0.03	40	$t_{31.0}$=-0.76	0.45
C:N ratio	34.04 ± 1.43	32.25 ± 0.86	40	$t_{31.0}$=1.07	0.29
Total shoot PA concentration (µg/g dw)	1.44 ± 0.14	0.89 ± 0.07	40	$t_{35.4}$=3.27	0.0024
Total root PA concentration (µg/g dw)	3.49 ± 0.24	3.91 ± 0.28	40	$t_{37.3}$=-1.26	0.22
Herbivore RGR (mg/day)	0.10 ± 0.009	0.07 ± 0.008	31	$t_{28.9}$=2.01	0.054
Herbivore mortality (%)	43.0 ± 7.5	38.0 ± 9.0	40	$t_{37.1}$=0.43	0.67
Parasitoid tibia length (mm)	0.87 ± 0.02	0.82 ± 0.08	10	$t_{2.1}$=0.59	0.62
Successful pupation (%)	15.0 ± 5.26	12.5 ± 6.15	40	$t_{37.1}$=0.31	0.76
Adult emergence (%)	12.5 ± 4.97	10.0 ± 5.85	40	$t_{37.0}$=0.33	0.75
Parasitoid development time (days)	31.20 ± 1.02	34.33 ± 1.45	10	$t_{1.4}$=-1.27	0.38

[a] Differences between the two treatments were tested using a Welch robust *t-test* (t) which does not require homogeneity of variances.

TABLE 2 Mean concentration (±SE, mg g^{-1} dw) of individual PAs detected in shoots and roots of control (-RH) plants and plants exposed to belowground herbivory (+RH). The % difference in mean concentration between –RH and +RH is also presented. The "-" sign indicates that the concentration of specific PA decreased when plants were exposed to root herbivore compare to control plants. AcEr – Acetylerucifoline, AcSp – Acetylseneciphylline, DADn – Desacetyldoronine, DHJn – Dehydrojaconine, Er – Erucifoline, Ir – Integerrimine, Jb – Jacobine, Jl – Jacoline, Jn – Jaconine, Jz – Jacozine, On – onetine, Ot – otosenine, Rd – Riddelliine, Rt – Retrorsine, Sk – Senkirkine, Sn – Senecionine, Sp – Seneciphylline, St – Spartioidine, Sv – senecivernine, Us – Usaramine, -ox – *N*-oxide form of the corresponding PA

PA	Shoot					Root				
	-RH	+RH	% difference	*t*	*P*[a]	-RH	+RH	% difference	*t*	*P*[a]
Erucifoline-type										
AcEr	0.2 ± 0.1	2.3 ± 0.7	1024	-3.20	**	0.4 ± 0.1	0.2 ± 0.1	-41	1.48	ns
AcEr-ox	8.0 ± 4.7	34.9 ± 11.4	337	-2.18	*	26.2 ± 7.3	18.7 ± 2.7	-28	0.96	ns
Er	6.8 ± 1.6	6.2 ± 1.2	-8	0.28	ns	7.0 ± 1.2	8.0 ± 2.0	15	-0.45	ns
Er-ox	114.2 ± 18.3	49.9 ± 7.9	-56	3.23	**	45.2 ± 10.9	36.1 ± 4.7	-20	0.77	ns
Total	129.2 ± 20.9	93.3 ± 11.5	-28	1.1	ns	78.7 ± 18.4	63.0 ± 7.9	-20	0.05	ns
Jacobine-type										
DHJn	0.04 ± 0.01	0.08 ± 0.02	114	-1.79	ns	-	-	-	-	ns
Jb	439.5 ± 58.6	382.3 ± 37.8	-13	0.82	ns	21.7 ± 2.1	22.6 ± 2.1	4	-0.32	ns
Jb-ox	522.6 ± 101.6	239.5 ± 38.9	-54	2.60	*	202.7 ± 24.0	205.2 ± 28.0	1	-0.07	ns
Jl	32.8 ± 4.7	26.9 ± 2.9	-18	1.06	ns	37.2 ± 3.7	33.0 ± 2.3	-11	0.97	ns
Jl-ox	14.8 ± 2.4	8.0 ± 1.1	-46	2.59	*	48.0 ± 4.7	50.0 ± 6.0	4	-0.27	ns
Jn	1.2 ± 0.2	1.6 ± 0.2	35	-1.23	ns	16.5 ± 1.7	13.0 ± 1.2	-21	1.7	ns
Jn-ox	0.08 ± 0.03	0.20 ± 0.1	156	-2.02	ns	8.7 ± 1.0	6.0 ± 0.6	-31	2.32	*
Jz	2.7 ± 0.4	2.5 ± 0.4	-9	0.40	ns	0.3 ± 0.1	0.4 ± 0.1	40	-1.07	ns
Jz-ox	2.8 ± 0.6	1.7 ± 0.3	-40	1.59	ns	3.0 ± 0.5	3.8 ± 0.6	27	-0.99	ns
Total	1016.4 ± 112.9	662.7 ± 67.4	-35	2.73	**	337.9 ± 33.0	333.8 ± 37.1	-1	0.07	ns
Senecionine-type										
AcSp	0.06 ± 0.02	0.12 ± 0.1	84	-0.94	ns	17.6 ± 4.1	9.1 ± 1.4	-48	1.98	ns
AcSp-ox	0.5 ± 0.1	0.4 ± 0.1	-33	1.37	ns	274.8 ± 32.2	378.1 ± 47.5	38	-1.80	ns
Ir	0.4 ± 0.2	0.3 ± 0.1	-17	0.36	ns	17.2 ± 1.7	14.7 ± 1.8	-14	1.02	ns
Ir-ox	40.7 ± 7.1	17.7 ± 2.7	-57	3.01	**	357.8 ± 31.0	380.6 ± 22.8	6	-0.59	ns
Rd	0.06 ± 0.02	0.03 ± 0.01	-38	0.93	ns	1.6 ± 0.4	1.4 ± 0.3	-14	0.45	ns

Rd-ox	2.7 ± 0.5	2.2 ± 0.3	-21	0.95	ns	65.6 ± 10.7	79.6 ± 8.2	21	-1.04	ns
Rt	0.3 ± 0.1	0.2 ± 0.04	-27	1.07	ns	13.5 ± 2.0	13.0 ± 1.8	-4	0.19	ns
Rt-ox	12.1 ± 2.2	6.2 ± 0.8	-49	2.48	*	238.0 ± 30.9	270.8 ± 40.9	14	-0.64	ns
Sn	2.9 ± 0.7	2.9 ± 0.5	-1	0.02	ns	120.9 ± 10.0	113.4 ± 11.1	-6	0.51	ns
Sn-ox	164.1 ± 34.3	66.6 ± 12.6	-59	2.67	*	1471.1 ± 123.0	1616.0 ± 121.6	10	-0.84	ns
Sp	1.8 ± 0.5	1.6 ± 0.3	-10	0.33	ns	27.5 ± 2.4	34.4 ± 5.5	25	-1.14	ns
Sp-ox	64.8 ± 12.2	31.2 ± 4.6	-52	2.59	*	418.0 ± 56.6	543.3 ± 62.6	30	-1.49	ns
Sv	-	-	-	-	-	4.7 ± 0.5	4.4 ± 0.8	-5	0.25	ns
St	0.05 ± 0.01	0.06 ± 0.01	17	-0.54	ns	0.7 ± 0.1	0.7 ± 0.1	3	-0.20	ns
St-ox	2.3 ± 0.4	1.8 ± 0.2	-23	1.19	ns	9.2 ± 1.3	9.4 ± 0.8	1	-0.08	ns
Us	0.15 ± 0.03	0.08 ± 0.02	-45	1.89	ns	1.8 ± 0.2	1.5 ± 0.2	-17	1.13	ns
Us-ox	2.8 ± 0.5	1.1 ± 0.2	-61	3.13	**	22.7 ± 3.4	22.9 ± 4.8	1	-0.03	ns
Total	295.8 ± 55.2	132.4 ± 19.0	-55	2.51	*	3062.7 ± 215.9	3493.1 ± 218.4	14	-1.34	ns
Otosenine-type										
Sk	-	-	-	-		7.3 ± 5.0	11.2 ± 10.0	53	-0.35	ns
Ot	-	-	-	-		4.2 ± 1.8	5.3 ± 3.3	26	-0.28	ns
On	-	-	-	-		2.3 ± 1.0	2.7 ± 1.6	13	-0.17	ns
DADn	-	-	-	-		1.0 ± 0.5	1.3 ± 0.8	27	-0.30	ns
Total	-	-	-	-		14.9 ± 8.1	20.5 ± 15.6	37	-0.04	ns

[a]Asterisks indicate significant differences analyzed by *t-test* *** $P<0.001$; ** $P<0.01$; * $P<0.05$; ns – not significant

Fig. 1 Mean *N*-oxide and tertiary PAs concentration (N =40; ± SE, mg g^{-1} dw) of *J. vulgaris* shoots (bars without pattern) and roots (hatched bars) in plants kept without root herbivory (-RH, white bars) and plants exposed to root herbivory by *A. lineatus* (+RH, grey bars). Asterisks indicate a significant difference based on a Welch's robust *t-test* ****** $P<0.01$

Fig. 2 Ratio of *N*-oxides to tertiary amines (± SE) of individual pyrrolizidine alkaloids of *J. vulgaris* shoots and roots in plants kept without root herbivory (-RH, white bars) and plants exposed to root herbivory by *A. lineatus* (+RH, grey bars). The ratio was calculated as ln ([total *N*-oxide concentration]/[total tertiary amine concentration]). Values larger than 0 indicate that the concentration of the *N*-oxide form of a PA is higher than that of the tertiary amine form, and values less than 0 indicate that the concentration of the *N*-oxide form is lower than that of the tertiary amine form. Dehydrojaconine and otosenine-type PAs occurred only as tertiary amine and therefore were not included in the figure. For the legend of PA names see Table 2

Fig. 3 Biplot showing the first and second axis of a principal component analysis (PCA) of the shoot pyrrolizidine alkaloid profiles. The mean (± SE) sample scores of undamaged control plants (-RH, open circles) and plants exposed to root herbivory by *A. lineatus* (+RH, filled circles) are shown, and all PAs with more than 30% fit. The numbers between brackets show the amount of variation explained by each axis. For the legend of PA names see Table 2

FIG. 1

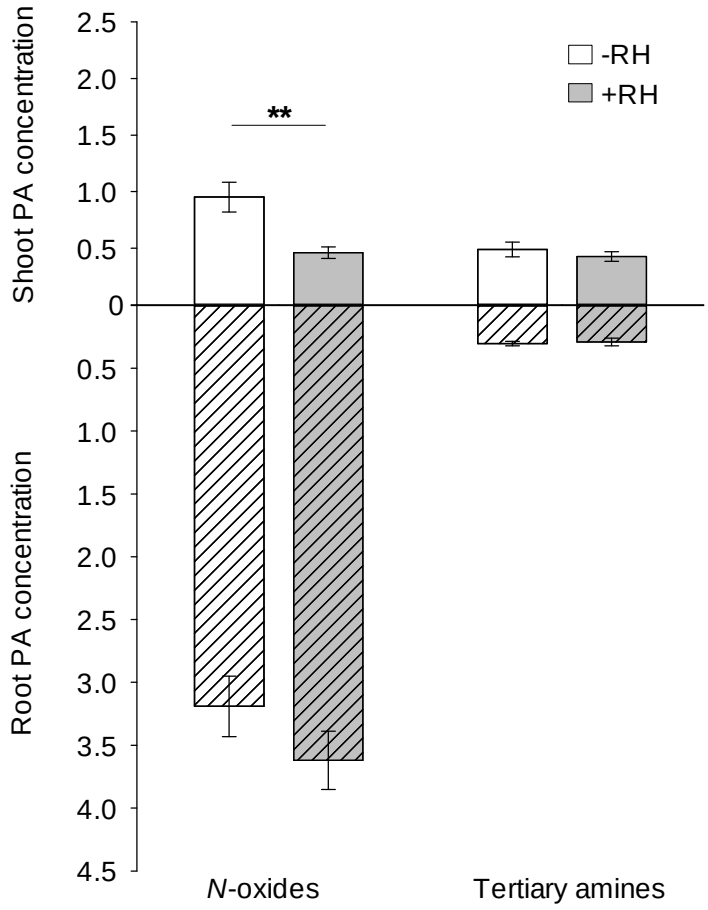
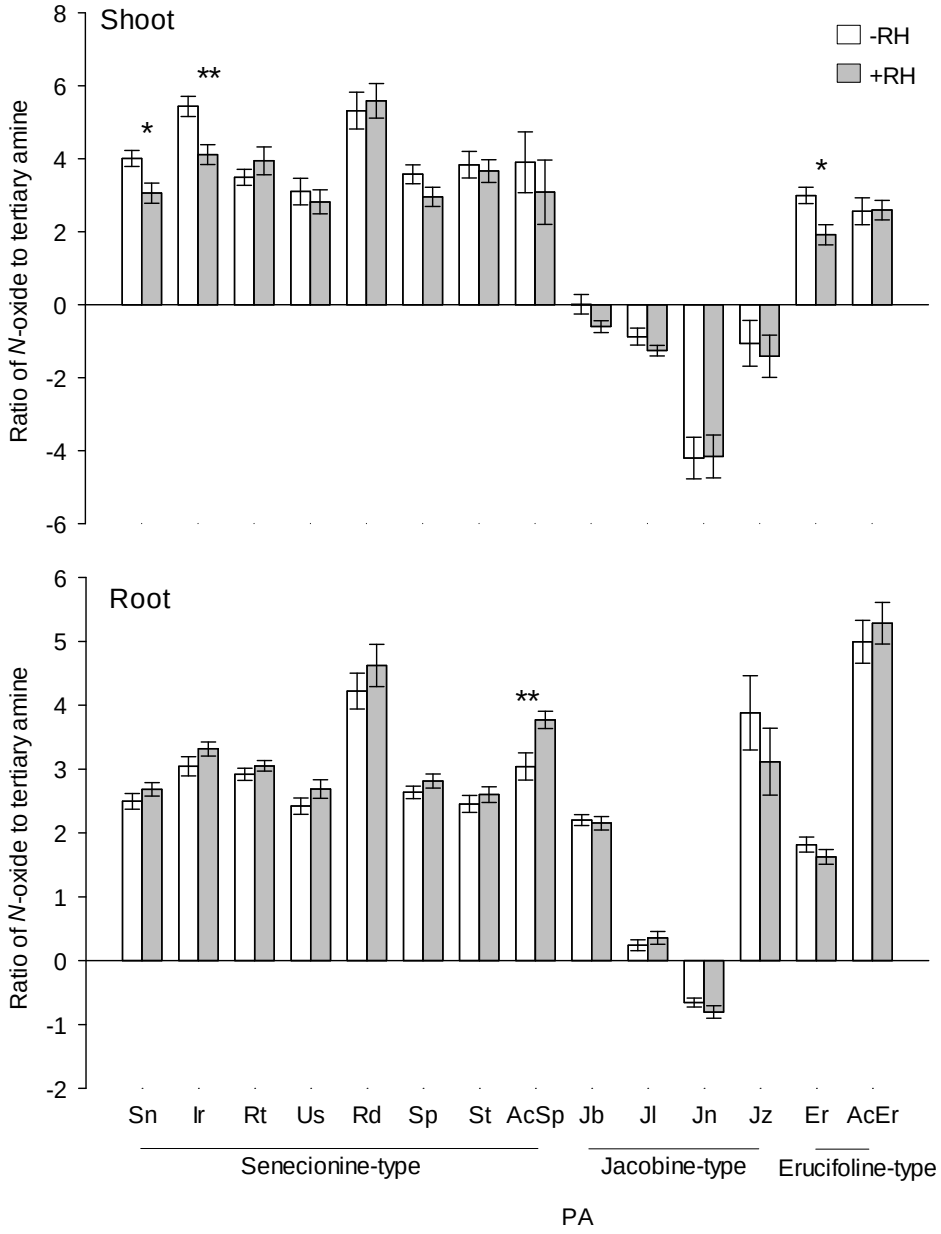
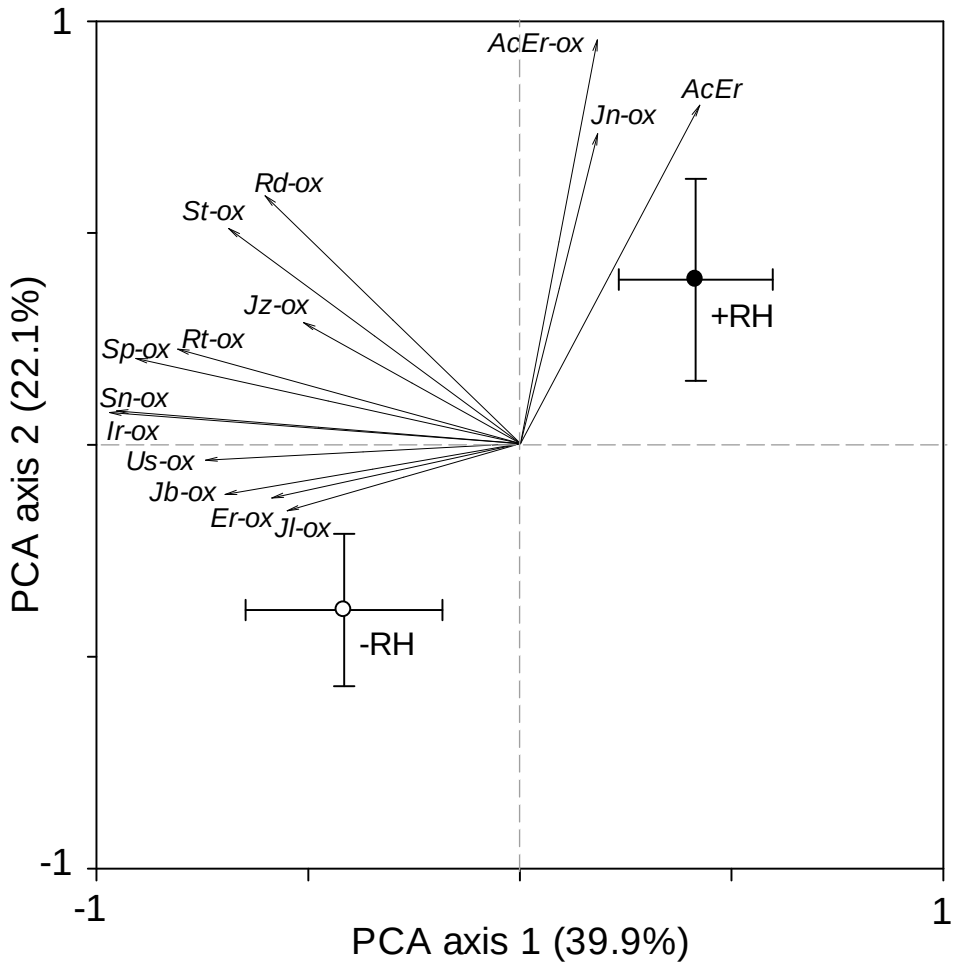

Shoot PA concentration
Root PA concentration
2.5
2.0
1.5
1.0
0.5
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
-RH
+RH
**
N-oxides
Tertiary amines

FIG. 2


Shoot
-RH
+RH
Ratio of N-oxide to tertiary amine
8
6
4
2
0
-2
-4
-6
*
**
*
Root
Ratio of N-oxide to tertiary amine
6
5
4
3
2
1
0
-1
-2
**
Sn
Ir
Rt
Us
Rd
Sp
St
AcSp
Jb
Jl
Jn
Jz
Er
AcEr
Senecionine-type
Jacobine-type
Erucifoline-type
PA

1 FIG. 3


1
-1
-1
1
PCA axis 2 (22.1%)
PCA axis 1 (39.9%)
AcEr-ox
AcEr
Jn-ox
Rd-ox
St-ox
Jz-ox
Sp-ox
Rt-ox
Sn-ox
Ir-ox
Us-ox
Jb-ox
Er-ox
Jl-ox
+RH
-RH

2