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

4
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15
16 **Abstract** – The importance of root herbivory is increasingly recognized in ecological studies and the effects of
17 root herbivory on plant growth, chemistry and performance of aboveground herbivores have been relatively well
18 studied. However, how belowground herbivory by root feeding insects affects aboveground parasitoid
19 development is largely unknown. In this study, we examined the effects of root herbivory by wireworms
20 (*Agriotes lineatus*) on the expression of primary and secondary compounds in the leaves and roots of ragwort
21 (*Jacobaea vulgaris*). We also studied the effects of root herbivory on the performance of a generalist
22 aboveground herbivore, *Mamestra brassicae* and its parasitoid *Microplitis mediator*. In contrast to what most
23 other studies have reported, root herbivory in *J. vulgaris* had a strong negative effect on the total concentration
24 of pyrrolizidine alkaloids (PAs) in shoot tissues. The composition of PAs in the shoots also changed after root
25 herbivory. In particular, the concentration of less toxic *N*-oxide PAs decreased. There was no significant effect of
26 root herbivory on PA composition and concentration in the roots. Although the concentration of PA in the leaves
27 decreased, *M. brassicae* tended to grow slower on the plants exposed to root herbivory. Parasitoid performance
28 was not affected by root herbivory, but parasitoids developed faster when the concentration of jacobine-type PAs
29 in the foliage was higher. These results point at a putative role of individual PAs in multitrophic interactions and
30 emphasize that generalizations about aboveground-belowground effects should be made with great caution.

1

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

4

INTRODUCTION

1
2
3 Root feeding insects can be very abundant in natural and agricultural systems and the importance of root
4 herbivory is increasingly recognized in ecological studies (Whittaker 2003; Blossey and Hunt-Joshi 2003;
5 Rasmann and Agrawal 2008; Van Dam 2009). Roots are essential for acquiring water and nutrients from the soil,
6 and damage to the roots often results in decreased plant growth (Brown and Gange 1990). Besides the direct
7 damage to the roots, belowground herbivory can also lead to changes in the concentration and composition of
8 primary and secondary compounds in the roots. Due to root-shoot signaling, these changes frequently do not
9 only occur in the roots, but also in the aboveground parts of a plant (Bezemer and Van Dam 2005; Blossey and
10 Hunt-Joshi 2003; Johnson et al. 2008; Erb et al. 2009; Soler et al. 2012). Root herbivory can result in increases
11 (e.g., Bezemer et al. 2003; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2008) or decreases in concentrations
12 of aboveground secondary plant compounds (e.g., Kaplan et al. 2008a), although increased concentrations have
13 been reported much more frequently than decreases (Kaplan et al. 2008b). As a result, this variation in plant
14 responses to root herbivory may have important consequences for aboveground communities associated to the
15 plant and interactions between aboveground and belowground herbivory.

16
17 Root herbivore-induced changes in aboveground plant chemistry can subsequently affect the performance of
18 aboveground herbivores feeding on the plant (e.g. Bezemer et al. 2005; Van Dam et al. 2005; Soler et al. 2005;
19 Erb et al. 2011). Moreover, via these changes in the plant and in the herbivores, root herbivory can affect the
20 performance and the behavior of consumers of these herbivores such as parasitoids (Soler et al. 2012). A number
21 of studies has shown that the level of parasitism or the host location behavior of parasitoids is affected by
22 whether or not the herbivorous host is feeding on a plant that is also exposed to root herbivory (Masters et al.
23 2001; Rasmann and Turlings 2007; Soler et al. 2007; Staley et al. 2007; Olson et al. 2008). In contrast, the
24 effects of belowground herbivory by root feeding insects on aboveground parasitoid development are less well
25 studied. As far as we are aware, the impact of root feeding insects on aboveground parasitoid development have
26 only been studied for *Cotesia glomerata*, a parasitoid of the specialist herbivore *Pieris brassicae*. In this system,
27 root herbivory or even jasmonic acid application to the roots increases the glucosinolate contents in the leaves of
28 *Brassica* plants and results in increased developmental times and reduced pupal weights of the parasitoid (Soler
29 et al. 2005; Qiu et al. 2009). In the present study we examine the effects of root herbivory on aboveground
30 multitrophic interactions for another plant-herbivore-parasitoid system. We exposed ragwort plants (*Jacobaea*

1 *vulgaris* Gaertn., Asteraceae) to root herbivory by wireworms (*Agriotes lineatus* L., Coleoptera: Elateridae), and
2 examined the influence of root herbivory on the concentration and composition of pyrrolizidine alkaloids in roots
3 and in foliar tissues, and on the performance of a generalist aboveground insect herbivore, *Mamestra brassicae*
4 L. (Lepidoptera: Noctuidae) and its parasitoid *Microplitis mediator* Haliday (Hymenoptera: Braconidae).
5
6 Pyrrolizidine alkaloids (hereafter PAs) in *J. vulgaris* are root produced secondary metabolites (Hartmann 1999).
7 PAs are a well studied group of plant allelochemicals due to their important role in plant-insect interactions.
8 They serve as feeding and oviposition stimulants to specialist herbivores and are known to deter generalist insect
9 herbivores (reviewed in Macel 2011). In the roots, the basic alkaloid structure senecionine *N*-oxide is produced,
10 and this is transformed into several related senecionine-type PAs. These PAs are transported exclusively via the
11 phloem path to the aboveground plant parts where additional diversification takes place, resulting in the
12 formation of jacobine- and erucifoline-type PAs (Hartmann 1999; Cheng et al. 2011b). PAs generally occur in
13 plants in tertiary amine (free base) form and in *N*-oxide form. Tertiary amines are regarded as degradation
14 products of *N*-oxides (Hartman and Dierich 1998). A number of studies have shown that tertiary amines are
15 more toxic for herbivorous insects than their corresponding *N*-oxides (Van Dam et al. 1995; Macel et al. 2005).
16 Even though the importance of PAs in plant-insect interactions has been studied in great detail, little is known
17 about the role of PAs in interactions between aboveground and belowground organisms (e.g., Hol et al. 2004;
18 Joosten et al. 2009; Reidinger et al. 2011; Kostenko et al. 2012). Furthermore, the effects of PAs on parasitoid
19 development and performance are not yet well ascertained (reviewed in Trigo 2011).
20
21 In a greenhouse experiment, we investigated the effects of root herbivory on the expression of primary and
22 secondary compounds in the leaves and roots of ragwort. We further examined whether the survival and
23 performance of the foliar feeding generalist herbivore and its parasitoid differed between plants exposed to root
24 herbivory and control plants. Finally, we tested whether aboveground insect performance correlated with
25 qualitative and quantitative characteristics of the chemistry of the leaves or roots. In line with what has been
26 reported in other studies (e.g. Bezemer et al. 2003; Van Dam et al. 2005; Soler et al. 2005; Erb et al. 2008), we
27 hypothesized that root herbivory (1) will increase total PA concentration in the shoots of *J. vulgaris*, and
28 consequently (2) will have a negative effect on aboveground herbivore and parasitoid performance.

29
30

METHODS AND MATERIALS

1
2 *Insects*. Wireworms are larvae of the click beetle *A. lineatus* and considered to be generalist root feeders. *A.*
3 *lineatus* larvae were obtained commercially from Applied Plant Research (PPO-WUR), Lelystad, The
4 Netherlands. Larvae of *M. brassicae* are generalist leaf-chewing insects that feed on a wide variety of food-
5 plants, including *J. vulgaris* (de Boer, 1999; Hol et al. 2004). *M. mediator* is a solitary larval endoparasitoid of
6 *M. brassicae* (Harvey and Gols 2011). This parasitoid develops in first to fourth instar larvae of its host. Larvae
7 of *M. mediator* feed solely on host hemolymph, and thus can be directly exposed to the plant allelochemicals
8 ingested into hemolymph by host. *M. brassicae* and *M. mediator* were obtained from an insect culture at the
9 Laboratory of Entomology of Wageningen University, The Netherlands. Cultures of *M. brassicae* and *M.*
10 *mediator* were maintained on Brussels sprouts cv. Cyrus in climate rooms at $22\pm 2^\circ\text{C}$, with a light regime of 16:8
11 L/D.

12
13 *Experimental set-up*. Seeds of *J. vulgaris* were collected from a single population at a semi-natural grassland in
14 the Mossel nature restoration area (Ede, The Netherlands, $52^\circ 03' 38''\text{N}$, $5^\circ 45' 04''\text{E}$) where cropping ceased in
15 1995. Seeds were surface sterilized (1 min in a 0.1% sodium chloride solution and rinsed with water) and
16 germinated on glass beads. Three *J. vulgaris* seedlings were planted in each of 80 one-liter pots filled with a
17 mixture of sterilized and non-sterilized field soil (1:1 ratio). The sandy-loam soil (particle size distribution: < 2
18 μm , 3%; $2\text{--}63 \mu\text{m}$, 17%; $> 63 \mu\text{m}$, 80%) was collected from the same area as the seeds and contained 4.5%
19 organic matter. In the laboratory, the soil was sieved through a 0.5 cm mesh to remove stones and large
20 arthropods and was subsequently homogenized. Half of the soil was sterilized using gamma irradiation (> 25
21 KGray gamma irradiation, Isotron, Ede, The Netherlands). The plants were grown in a greenhouse ($21/16^\circ\text{C}$
22 day/night, 16 hours photoperiod). Natural daylight was supplemented by 400 W metal halide lamps (1 lamp per
23 1.5 m^2). Plants were watered three times per week and randomly redistributed within the greenhouse once a
24 week. After one week, the seedlings were randomly thinned to two seedlings per pot.

25
26 Six weeks after transplantation, two late-instar wireworm larvae were introduced into each of 40 randomly
27 chosen pots assigned to the root herbivory treatment. Wireworms were placed into a small hole (1 cm deep)
28 made in the soil. The larvae immediately burrowed into the soil. Similar holes were also made in the soil of the
29 remaining 40 control pots. Prior to their introduction, wireworm larvae were starved for three days in moist soil
30 at room temperature. Two weeks later, all pots were placed individually into a fine meshed cylindrical cage (70

1 cm height, 25 cm diameter). Two second- instar larvae of *M. brassicae* were then introduced to 20 control and
2 20 root herbivory pots. The remaining pots received two parasitized *M. brassicae* larvae. Larvae were introduced
3 onto the plant by carefully placing them with a small brush on the youngest fully mature leaf of the plant.
4 Parasitized larvae were parasitized individually using freshly mated *M. mediator* female parasitoids and then
5 immediately introduced on the plant. The two larvae could move freely on the plants within each cage. Insects
6 were kept on the plant for four weeks. Once a week, starting two weeks after introducing them on the plant, all
7 larvae were collected from the plants, weighed on the microbalance, and returned to the same cage.
8 Unparasitized larvae remained in the larval stage throughout the entire experiment. Cages with parasitized *M.*
9 *brassicae* larvae were checked daily for egression of cocoons. Parasitoid cocoons were carefully collected from
10 the plant and placed individually in Petri dishes until adult emergence. To record adult parasitoid emergence
11 cocoons were checked twice a day. At emergence, the date of eclosion was recorded and parasitoids were sexed.
12 Hind tibia length was recorded as a measure of adult size (Godfray 1994), using a calibrated slide and a
13 stereomicroscope. Development time was calculated as days between parasitism and adult emergence. At
14 harvest, shoots were clipped and roots were carefully removed from the soil and rinsed. Shoot and root biomass
15 of each pot was oven-dried at 70°C for three days and weighed. All wireworm larvae were recovered alive from
16 the soil.

17
18 *Chemical analysis.* Eight weeks after germination, just prior to the introduction of the unparasitized and
19 parasitized *M. brassicae* larvae, the third youngest leaf of 20 control plants and 20 plants with root herbivory
20 was removed with a razor blade immediately freeze-dried and finely ground. The root samples were taken from
21 the oven-dried root material for the same plants and pulverized. For both treatments there were 10 plants
22 allocated for unparasitized and 10 for parasitized larvae. Carbon (C) and Nitrogen (N) content were determined
23 only for leaf samples using a Flash EA1112 CN analyzer (Interscience, Breda, The Netherlands). PA composition
24 and content was determined using a Waters Acquity ultra performance liquid chromatographic system coupled to a
25 Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MS, USA); see also Cheng et al. (2011a,
26 b). For each sample, 10 mg of ground plant material was mixed with 1.0 ml 2% formic acid solution. Heliotrine
27 was added to the extraction solvent as an internal standard at a concentration of 1 µg/ml. The mixture was
28 centrifuged and filtered through a 0.2 µm nylon membrane filter (Acrodisc, Pall Life Sciences, MI, USA). An
29 aliquot of 25 µl of the extracted filtrate was diluted with 975 µl of 10 mM ammonium hydroxide solution and
30 injected in the LC-MS/MS system. PAs were separated on a Waters BEH C18 UPLC column (150 x 2.1 mm, 1.7

1 μm particles) applying 5 mM ammonium hydroxide as mobile phase and using acetonitrile as organic modifier (0-
2 50%) in a 12-min linear gradient. The mass spectrometer was operated in positive electrospray mode and the
3 samples were screened for a total of 37 PAs. Details on the mass spectrometric settings are described in Cheng et al.
4 (2011a). PAs were quantified against a calibrant of PA standards added to *Tanacetum vulgare* plant extract (which
5 itself is free of PAs) to minimize matrix effects that otherwise could play a role when using standards in solvent
6 only. The calibrant solution was injected every 20 samples to monitor for variations in detector response. Samples
7 were injected in a randomized order. Data were processed using Masslynx 4.1 software (Waters, Milford, MA,
8 USA).

9
10 *Statistics.* The impact of root herbivory on plant biomass, chemistry, herbivore and parasitoid performance was
11 assessed using a Welch's robust *t-test* which does not require homogeneity of variances. In the robust Welch *t-*
12 test the degrees of freedom are corrected with the Welch-Satterthwaite modification (Welch 1947). The
13 percentage difference in individual PA concentrations was calculated as: (mean PA concentration of plants
14 subjected to root herbivory treatment – mean PA concentration of control plants)/ mean PA concentration of
15 control plants. The overall difference in the concentration of *N*-oxides and tertiary amines was compared using a
16 paired *t-test*. The relative concentration of *N*-oxides was calculated as: % *N*-oxide = $N\text{-oxide concentration} / (N\text{-}$
17 $\text{oxide concentration} + \text{the corresponding tertiary amine concentration}) \times 100$. Percentage data were arcsine
18 square-root transformed prior to statistical analysis. For graphical representation we calculated the natural
19 logarithm of the ratio between *N*-oxides and tertiary amines that is symmetrical around the 1:1 ratio point. The
20 relationship between plant characteristics and herbivore and parasitoid performance were analyzed using
21 Pearson's product-moment correlation. As the number of replicates was relatively low, significance in multiple
22 statistical tests was not corrected (Moran 2003). To examine whether root herbivory influenced the PA
23 composition aboveground or belowground we used multivariate principal component (PCA) and redundancy
24 (RDA) analyses. The choice of linear methods was justified by the short length of gradients (less than 2.0). RDA
25 was also used to test the relationship between the shoot PA composition and herbivore or parasitoid
26 performance. Significances in multivariate analyses were tested using a Monte Carlo permutation test with 999
27 permutations. Univariate analyses were performed in R statistical language, ver. 2.15.0 (R Development Core
28 Team 2012) and multivariate analyses in CANOCO version 4.55 (Ter Braak and Šmilauer 2002).

30 RESULTS

1
2 *Plant Responses.* Plant shoot and root biomass did not differ significantly between treatments (Table 1). Root
3 herbivory did also not influence leaf nitrogen concentrations and leaf C:N ratios. The total PA concentration in
4 shoots of plants exposed to root herbivory was significantly lower (38%) than in control plants (Table 1). The
5 total PA concentration in roots was slightly higher (12%) in plants exposed to root herbivory than in control
6 plants but this was not statistically significant (Table 1). Twenty-nine PAs were detected in shoots and 33 PAs in
7 roots of *J. vulgaris* (Table 2). The detected PAs belonged to four structural groups: erucifoline-type, jacobine-
8 type, senecionine-type and otosenine-type (Table 2). Otosenine-type PAs were only identified in roots. In shoots,
9 dehydrojaconine was detected in trace amounts and only occurred as tertiary amine. All other PAs were found in
10 *N*-oxide and in tertiary amine form. In roots, senecivernine, senkirkine, otosenine, onetine and desacetyldoronine
11 were only present as tertiary amines. The concentration of tertiary amines in shoots was not affected by root
12 herbivory ($t_{35,5}=0.61$, $P=0.54$), whereas the overall levels of *N*-oxides in shoots decreased by 52% in the plants
13 exposed to root herbivory ($t_{29,5}=3.24$, $P=0.003$; Figure 1). In roots, there was no significant difference in the
14 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
15 treatments, although the levels of *N*-oxides were 14% higher in roots exposed to root herbivory. The contribution
16 of tertiary amines increased from 34% to 48% in the total shoot PA concentration while in the total root PA
17 concentration it decreased from 9% to 7% (Figure 1)

18
19 In shoots, independent of root herbivory, jacobine and jacobine *N*-oxide were present in the highest
20 concentrations in all plants (35% and 33% respectively of the total PA concentration) and the total concentration
21 of jacobine-type PAs decreased after root herbivory (Table 2). The total concentration in shoots of senecionine-
22 type PAs was lower in plants exposed to root herbivores, but the total concentration of erucifoline-type PAs did
23 not differ between the treatments, although levels of acetylerucifoline (+1024%) and acetylerucifoline *N*-oxide
24 (+337%) responded most strongly to root herbivory (Table 2). In roots, the total concentrations of none of the
25 four groups of PAs was affected by root herbivory (Table 2).

26
27 Overall, the relative concentration of *N*-oxides was higher than that of tertiary amines (shoots: $t_{39}=-2.58$,
28 $P=0.014$; roots: $t_{39}=-32.15$, $P<0.001$, Figure 1). In shoots, for erucifoline- and senecionine-type PAs the relative
29 concentration of *N*-oxides was much higher than the concentration of tertiary amines ($P<0.01$ in all cases), while
30 for jacobine-type PAs concentrations of *N*-oxides were equal or lower than concentrations of tertiary amines

1 (Figure 2). In roots, the relative concentration of *N*-oxides was much higher for all compounds except for
2 jaconine (Figure 2). Root herbivory significantly decreased the relative concentration of *N*-oxides for
3 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
4 shoots, and increased the relative concentration of *N*-oxides for acetylseneciphylline in roots ($t_{38,0}=-3.05$,
5 $P=0.005$, Figure 2).

6
7 Principle component analyses of the shoot PA composition showed that most of the variation in PA profiles
8 could be explained by three principle component axes (74.3% cumulative explained variation). Shoot PA
9 profiles differed significantly between plants exposed to root herbivory and control plants (RDA: $F=4.50$,
10 $P=0.002$; 10.6% explained variation). Shoot PA profiles of plants exposed to root herbivory and control plants
11 clearly separated in an unconstrained analysis (PCA; Figure 3). In the PCA, the levels of acetylerucifoline,
12 acetylerucifoline *N*-oxide, jaconine *N*-oxide were higher in plants with root herbivory, whereas levels of jacobine
13 *N*-oxide, jacoline *N*-oxide, erucifoline *N*-oxide, senecionine *N*-oxide, integerrimine *N*-oxide, usaramine *N*-oxide,
14 seneciphylline *N*-oxide and retrorsine *N*-oxide were higher in control plants (Figure 3). The PA composition in
15 roots was not affected by root herbivory (RDA: $F=0.62$, $P=0.74$; data not shown).

16
17 *Herbivore and Parasitoid Performance* The relative growth rates of unparasitized *M. brassicae* larvae tended to
18 be lower on plants with root herbivory, but this was only marginally significant ($P=0.054$; Table 1). Mortality of
19 *M. brassicae* did not differ significantly between the two treatments (Table 1). Herbivore growth rate and
20 survival were not significantly related to leaf nitrogen concentration, C:N ratio, total shoot PA concentration,
21 levels of individual PAs in the shoots, or shoot PA composition ($P>0.05$ in all cases). Parasitoid performance,
22 measured as hind tibia length, % successful cocoon egression, % adult emergence, and development time, also
23 did not differ between the two treatments (Table 1). However, there was a negative relationship between
24 parasitoid development time and total shoot *N*-oxide concentration ($R^2=55.0\%$, $P=0.033$). Analyses of individual
25 shoot PA compounds revealed that parasitoid development time negatively correlated with concentrations of
26 jacoline *N*-oxide ($R^2 = 89.5\%$, $P = 0.006$), jacobine *N*-oxide ($R^2 = 64.6\%$, $P = 0.016$), and usaramine ($R^2 =$
27 76.4% , $P = 0.046$).

28 29 DISCUSSION 30

1 In our study, root herbivory greatly affected the concentration and composition of PAs in the leaves of *J.*
2 *vulgaris*. However, in contrast with our hypothesis, total PA concentration in the shoots of *J. vulgaris* decreased
3 strongly (38%) when plant roots were exposed to herbivory by *A. lineatus*. In a previous study, Hol et al. (2004)
4 found that mechanical root damage caused an increase in PA concentrations in the roots of *J. vulgaris* but
5 mechanical damage to roots had only weak and inconsistent effects on shoot PA concentrations. Clearly,
6 mechanical tissue damage may not elicit the same effect on the expression of allelochemicals as actual herbivory
7 (Bezemer et al., 2004; Kaplan et al., 2008a). The majority of studies that have examined the effects of root
8 damage by real herbivores on concentrations of aboveground secondary plant compounds for other plant species
9 report increases in the amount of secondary metabolites following root herbivory (e.g. Bezemer et al. 2003,
10 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
11 reasons for the discrepancy between the results of these studies and ours may be that PAs are synthesized in the
12 roots whereas many of the secondary compounds included in the other studies can be produced in the shoots.
13 Similar to our results, root herbivory by the nematode *Meloidogyne incognita* in tobacco plants causes a decline
14 in the concentrations of the alkaloid nicotine in the foliage, and nicotine is also synthesized in the roots
15 (Hanounik and Osborne 1977; Kaplan et al. 2008a). However, Hanounik and Osborne (1977) also showed that
16 root herbivory by *M. incognita* caused an increase in nicotine in leaves of a resistant tobacco cultivar showing
17 that the effects of root herbivory can greatly vary even within a single plant species. In the study of Kaplan et al.
18 (2008a), even though root herbivory caused a decline in the concentrations of nicotine aboveground,
19 concentrations of other secondary plant compounds that are not exclusively produced in the roots increased in
20 the foliage. In another study, terpenoid aldehydes in cotton (*Gossypium* sp.), which are also synthesized in roots,
21 increased in the foliage of cotton following root herbivory by wireworms (Bezemer et al. 2004). Synthesis of
22 gossypol is also known to occur in the foliage of cotton plants but in lower concentrations (Bezemer et al. 2004).
23 Therefore, it is plausible that the synthesis of gossypol was enhanced in the shoots rather than in the roots by
24 belowground herbivory. However, a more likely explanation for the different responses observed among the
25 different plant species is that there are various mechanisms by which belowground herbivory can lead to changes
26 in aboveground plant chemistry (reviewed in Soler et al. 2012). These results therefore emphasize that
27 generalizations about aboveground-belowground effects should be made with great caution.
28
29 An important question that requires further study is whether root herbivory in ragwort negatively interferes with
30 PA synthesis in the roots, or whether the negative effects of root herbivory on aboveground PA concentrations

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

18
19 The use of the LC-MS/MS procedure allowed us to detect both the tertiary amine and *N*-oxide forms of PAs, as
20 well as PAs that are present only in extremely low concentrations in the plant (Joosten et al. 2009). Earlier
21 studies were restricted to the major PAs that are present in plants and in these studies the authors were not able to
22 discriminate between the two forms of PAs (e.g., Hol et al. 2004; Macel and Klinkhamer 2010). Our results in
23 line with other more recent studies (e.g. Joosten et al. 2011) show that the concentration of tertiary amine forms
24 in jacobine-type PAs is higher than in other PA groups (for a discussion on the selective formation of jacobine
25 tertiary amines see Joosten et al 2011). Interestingly, in our study most of the individual PAs in plant shoots that
26 responded to the root herbivory treatment were *N*-oxides. As a result, the ratio of *N*-oxides to tertiary amines in
27 the shoots changed from 2:1 in control plants to 1:1 in plants exposed to root herbivores. At the same time, there
28 was a slight increase in the *N*-oxide concentration in the roots, mostly due to an increase in the concentration of
29 senecionine *N*-oxide, while the total tertiary amine concentration in the roots remained constant. The
30 concentration of *N*-oxides of major PAs such as jacobine *N*-oxide, jacoline *N*-oxide and erucifoline *N*-oxide did

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

9

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

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

3

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

26

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

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

4
5 The diet of a herbivorous host may also affect parasitoids that develop in this host by exposing them to
6 unmetabolized defensive chemicals (Ode 2006). In our study, root herbivory did not affect the performance of
7 the parasitoid *M. mediator*. Interestingly, although we did not detect a relationship between PA concentrations
8 and *M. brassicae* performance, in our study parasitoids developed faster when the concentration of jacobine-type
9 PAs, such as jacobine *N*-oxide and jacoline *N*-oxide in the plant was higher. This suggests that *N*-oxides indeed
10 could have less adverse effects on the performance of insects than tertiary amines. Future studies should examine
11 whether there is a true causal positive relationship between jacobine-type *N*-oxides and parasitoid performance,
12 or whether this is merely a coincidental correlation, and what the mechanisms are that underlie these
13 interactions.

14
15 In summary, this study shows that root herbivory by wireworms has a strong negative effect on the concentration
16 of PAs in the leaves of *J. vulgaris* possibly via the mechanism of restricted transport of PA *N*-oxides from roots
17 to leaves. However, this does not result in a positive effect on the performance of the generalist insect herbivore
18 *M. brassicae* or its parasitoid *M. mediator*. In contrast, *M. brassicae* tends to grow slower on plants exposed to
19 root herbivory. This decline in herbivore performance can be explained by changes in foliar PA composition in
20 plants exposed to root herbivory whereby the relative concentration of less toxic PAs decreases. Moreover, in
21 our study the performance of parasitoids was also positively correlated with the concentration of less toxic PAs.
22 Further research should aim at elucidating the putative role of individual PAs in aboveground-belowground
23 multitrophic interactions.

24
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REFERENCES

- 1
2
3 AWMACK, C. S. and LEATHER, S. R. 2002. Host plant quality and fecundity in herbivorous insects. *Annu.*
4 *Rev. Entomol.* 47:817-844.
- 5 BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., and WÄCKERS, F. L. 2003. Interactions between
6 above- and belowground insect herbivores as mediated by the plant defense system. *Oikos* 101:555-562.
- 7 BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., VAN DER PUTTEN, W. H., and WÄCKERS, F. L.
8 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following
9 root and leaf injury. *J. Chem. Ecol.* 30:53-67.
- 10 BEZEMER, T. M., DE DEYN, G. B., BOSSINGA, T. M., VAN DAM, N. M., HARVEY, J. A., and VAN DER
11 PUTTEN, W. H. 2005. Soil community composition drives aboveground plant-herbivore-parasitoid
12 interactions. *Ecol. Lett.* 8:652-661.
- 13 BEZEMER, T. M. and VAN DAM, N. M. 2005. Linking aboveground and belowground interactions via
14 induced plant defenses. *Trends Ecol. Evol.* 20:617-624.
- 15 BLOSSEY, B. and HUNT-JOSHI, T. R. 2003. Belowground herbivory by insects: Influence on plants and
16 aboveground herbivores. *Annu. Rev. Entomol.* 48:521-547.
- 17 DE BOER, N. J. 1999. Pyrrolizidine alkaloid distribution in *Senecio jacobaea* rosettes minimizes losses to
18 generalist feeding. *Entomol. Exp. Appl.* 91:169-173.
- 19 BROWN, V. K. and GANGE, A. C. 1990. Insect herbivory below ground. *Adv. Ecol. Res.* 20:1-58.
- 20 CHENG, D. D., KIRK, H., VRIELING, K., MULDER, P. P. J., and KLINKHAMER, P. G. L. 2011a. The
21 relationship between structurally different pyrrolizidine alkaloids and western flower trips resistance in F₂
22 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. *J. Chem. Ecol.* 37:1071-1080.
- 23 CHENG, D. D., KIRK, H., MULDER, P. P. J., VRIELING, K., and KLINKHAMER, P. G. L. 2011b.
24 Pyrrolizidine alkaloid variation in shoots and roots of segregating hybrids between *Jacobaea vulgaris* and
25 *Jacobaea aquatica*. *New Phytol.* 192:1010-1023.
- 26 DREYER, D. L., JONES, K. C., and MOLYNEUX, R. J. 1985. Feeding deterrence of some pyrrolizidine,
27 indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrtosipon pisum*) and evidence for phloem
28 transport of indolizidine alkaloid swainsonine. *J. Chem. Ecol.* 11:1045-1051.
- 29 ERB, M., ROBERT, C. A. M., HIBBARD, B. E., and TURLINGS, T. C. J. 2011. Sequence of arrival determines
30 plant-mediated interactions between herbivores. *J. Ecol.* 99:7-15.

- 1 ERB, M., LENK, C., DEGENHARDT, J., and TURLINGS, T. C. J. 2009. The underestimated role of roots in
2 defense against leaf attackers. *Trends Plant Sci.* 14:653-659.
- 3 ERB, M., TON, J., DEGENHARDT, J., and TURLINGS, T. C. J. 2008. Interactions between arthropod-induced
4 aboveground and belowground defenses in plants. *Plant Physiol.* 146:867-874.
- 5 FRISCHKNECHT, P. M., SCHUHMACHER, K., MÜLLER-SCHÄRER, H., and BAUMANN, T. W. 2001.
6 Phenotypic plasticity of *Senecio vulgaris* from contrasting habitat types: growth and pyrrolizidine alkaloid
7 formation. *J. Chem. Ecol.* 27:343-358.
- 8 GODFRAY, H. C. J. 1994. Parasitoids: Behavioral and Evolutionary Ecology. Princeton University Press,
9 Princeton.
- 10 HANOUNIK, S. B. and OSBORNE, W. W. 1977. The relationships between population density of *Meloidogyne*
11 *incognita* and nicotine content of tobacco. *Nematologica* 23:147-152.
- 12 HARTMANN, T. and DIERICH, B. 1998. Chemical diversity and variation of pyrrolizidine alkaloids of the
13 senecionine type: Biological need or coincidence? *Planta* 206:443-451.
- 14 HARTMANN, T. 1999. Chemical ecology of pyrrolizidine alkaloids. *Planta* 207: 483-495.
- 15 HARVEY, J. A. and GOLS, R. 2011. Population-related variation in plant defense more strongly affects survival
16 of an herbivore than its solitary parasitoid wasp. *J. Chem. Ecol.* 37:1081-1090.
- 17 HOL, W. H. G., MACEL, M., VAN VEEN, J. A., and VAN DER MEIJDEN, E. 2004. Root damage and
18 aboveground herbivory change concentration and composition of pyrrolizidine alkaloids of *Senecio*
19 *jacobaea*. *Basic Appl. Ecol.* 5:253-260.
- 20 JOHNSON, S.N., BEZEMER, T.M., and JONES T.H. 2008. Linking aboveground and belowground herbivory,
21 pp. 153-170, in S. N. Johnson and P. J. Murray (eds.). Root Feeders. An Ecosystem Perspective. CABI,
22 UK.
- 23 JOOSTEN, L., MULDER, P. P. J., KLINKHAMER, P. G. L., and VAN VEEN, J. A. 2009. Soil-borne
24 microorganisms and soil-type affect pyrrolizidine alkaloids in *Jacobaea vulgaris*. *Plant Soil* 325:133-143.
- 25 JOOSTEN, L., CHENG, D. D., MULDER, P. P. J., VRIELING, K., VAN VEEN, J. A., and KLINKHAMER, P.
26 G. L. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea*
27 *vulgaris*. *Phytochemistry* 72:214-222.
- 28 KAPLAN, I., HALITSCHKE, R., KESSLER, A., REHILL, B. J., SARDANELLI, S., and DENNO, R. F. 2008a.
29 Physiological integration of roots and shoots in plant defense strategies links above- and belowground
30 herbivory. *Ecol. Lett.* 11:841-851.

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

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

6 LEISS, K. A., CHOI, Y. H., ABDEL-FARID, I. B., VERPOORTE, R., and KLINKHAMER, P. G. L. 2009.
7 NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. *J. Chem. Ecol.*
8 35:219-229.

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

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

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

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

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

20 MORAN, M. D. 2003. Arguments for rejecting the sequential bonferroni in ecological studies. *Oikos* 100:403-
21 405.

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

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

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

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

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

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

7 REIDINGER, S., ESCHEN, R., GANGE, A. C., FINCH, P., and BEZEMER, T. M. 2012. Arbuscular
8 mycorrhizal colonization, plant chemistry, and aboveground herbivory on *Senecio jacobaea*. *Acta Oecol.*
9 38:8-16.

10 SOLER, R., VAN DER PUTTEN, W. H., HARVEY, J. A., VET, L. E. M., DICKE M., and BEZEMER, T. M.
11 2012. Root herbivore effects on aboveground multitrophic interactions: patterns, processes and
12 mechanisms. *J. Chem. Ecol.* 38: 755-767.

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

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

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

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

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

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

- 1 VAN DAM, N. M., RAAIJMAKERS, C. E., and VAN DER PUTTEN, W. H. 2005. Root herbivory reduces
2 growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol. Exp. Appl.*
3 115:161-170.
- 4 VAN DAM, N. M. 2009. Belowground herbivory and plant defenses. *Annu. Rev. Ecol. Evol. Syst.* 40:373-391.
- 5 VAN DER MEIJDEN, E., DE BOER, N. J., and VAN DER VEEN-VAN WIJK, C. A. M. 2000. Pattern of
6 storage and regrowth in ragwort. *Evol. Ecol.* 14:439-455.
- 7 WELCH, B. L. 1947. The generalization of "student's" problem when several different population variances are
8 involved. *Biometrika* 34: 28-35.
- 9 WHITE, T.C.R. 1984. The abundance of invertebrate herbivores in relation to the availability of nitrogen in
10 stressed food plants. *Oecologica* 63: 90-105.
- 11 WHITTAKER, J. B. 2003. Root-animal interactions, pp. 363-385, in H. de Kroon and E. J. W Visser (eds.).
12 Root Ecology. Springer, New York.
- 13 WURST, S., VAN DAM, N. M., MONROY, F., BIERE, A., and VAN DER PUTTEN, W. H. 2008.
14 Intraspecific variation in plant defense alters effects of root herbivores on leaf chemistry and aboveground
15 herbivore damage. *J. Chem. Ecol.* 34:1360-1367.
- 16

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

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

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

1 TABLE 2 Mean concentration (\pm SE, mg g⁻¹ dw) of individual PAs detected in shoots and roots of control (-RH) plants and plants exposed to belowground herbivory (+RH).
2 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
3 exposed to root herbivore compare to control plants. AcEr – Acetylerucifoline, AcSp – Acetylseneciphylline, DADn – Desacetyldoronine, DHJn – Dehydrojaconine, Er –
4 Erucifoline, Ir – Integerrimine, Jb – Jacobine, Jl – Jacoline, Jn – Jaconine, Jz – Jacozine, On – onetine, Ot – otosenine, Rd – Riddelliine, Rt – Retrorsine, Sk – Senkirikine, Sn
5 – Senecionine, Sp – Seneciphylline, St – Spartioidine, Sv – senecivernine, Us – Usaramine, -ox – *N*-oxide form of the corresponding PA

PA	Shoot					Root				
	-RH	+RH	% difference	<i>t</i>	<i>P</i> ^a	-RH	+RH	% difference	<i>t</i>	<i>P</i> ^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

1 ^aAsterisks indicate significant differences analyzed by *t-test* *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns – not significant

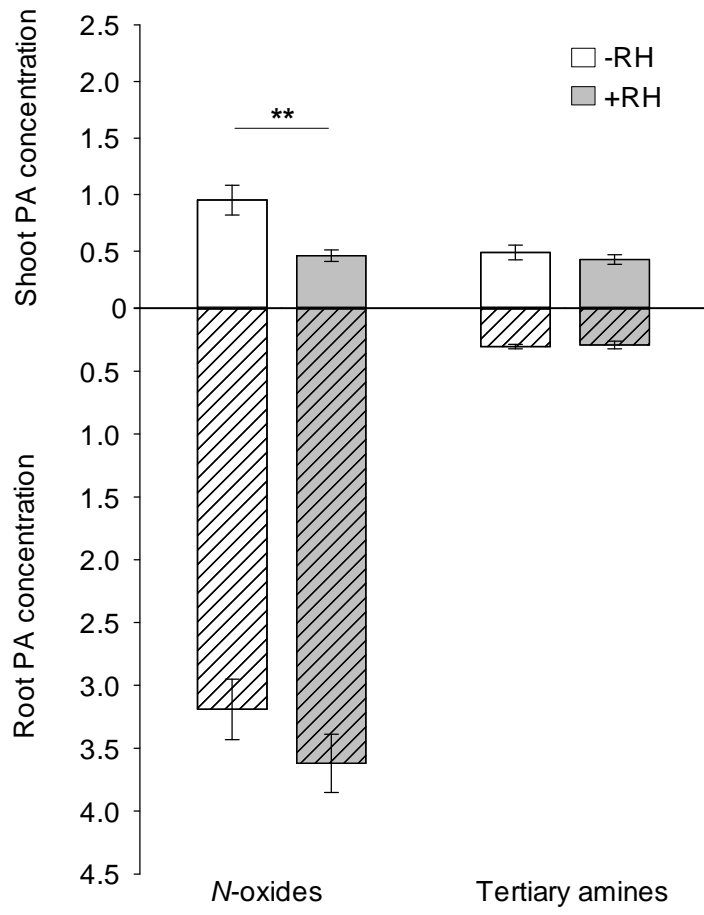
1 **Fig. 1** Mean *N*-oxide and tertiary PAs concentration (N =40; ± SE, mg g⁻¹ dw) of *J. vulgaris* shoots (bars without
2 pattern) and roots (hatched bars) in plants kept without root herbivory (-RH, white bars) and plants exposed to
3 root herbivory by *A. lineatus* (+RH, grey bars). Asterisks indicate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

5
6 **Fig. 2** Ratio of *N*-oxides to tertiary amines (± SE) of individual pyrrolizidine alkaloids of *J. vulgaris* shoots and
7 roots in plants kept without root herbivory (-RH, white bars) and plants exposed to root herbivory by *A. lineatus*
8 (+RH, grey bars). The ratio was calculated as $\ln \left(\frac{[\text{total } N\text{-oxide concentration}]}{[\text{total tertiary amine concentration}]} \right)$. Values larger than 0 indicate that the concentration of the *N*-oxide form of a PA is higher than
9 that of the tertiary amine form, and values less than 0 indicate that the concentration of the *N*-oxide form is lower
10 than that of the tertiary amine form. Dehydrojaconine and otosenine-type PAs occurred only as tertiary amine
11 and therefore were not included in the figure. For the legend of PA names see Table 2

12
13
14 **Fig. 3** Biplot showing the first and second axis of a principal component analysis (PCA) of the shoot
15 pyrrolizidine alkaloid profiles. The mean (± SE) sample scores of undamaged control plants (-RH, open circles)
16 and plants exposed to root herbivory by *A. lineatus* (+RH, filled circles) are shown, and all PAs with more than
17 30% fit. The numbers between brackets show the amount of variation explained by each axis. For the legend of
18 PA names see Table 2

19

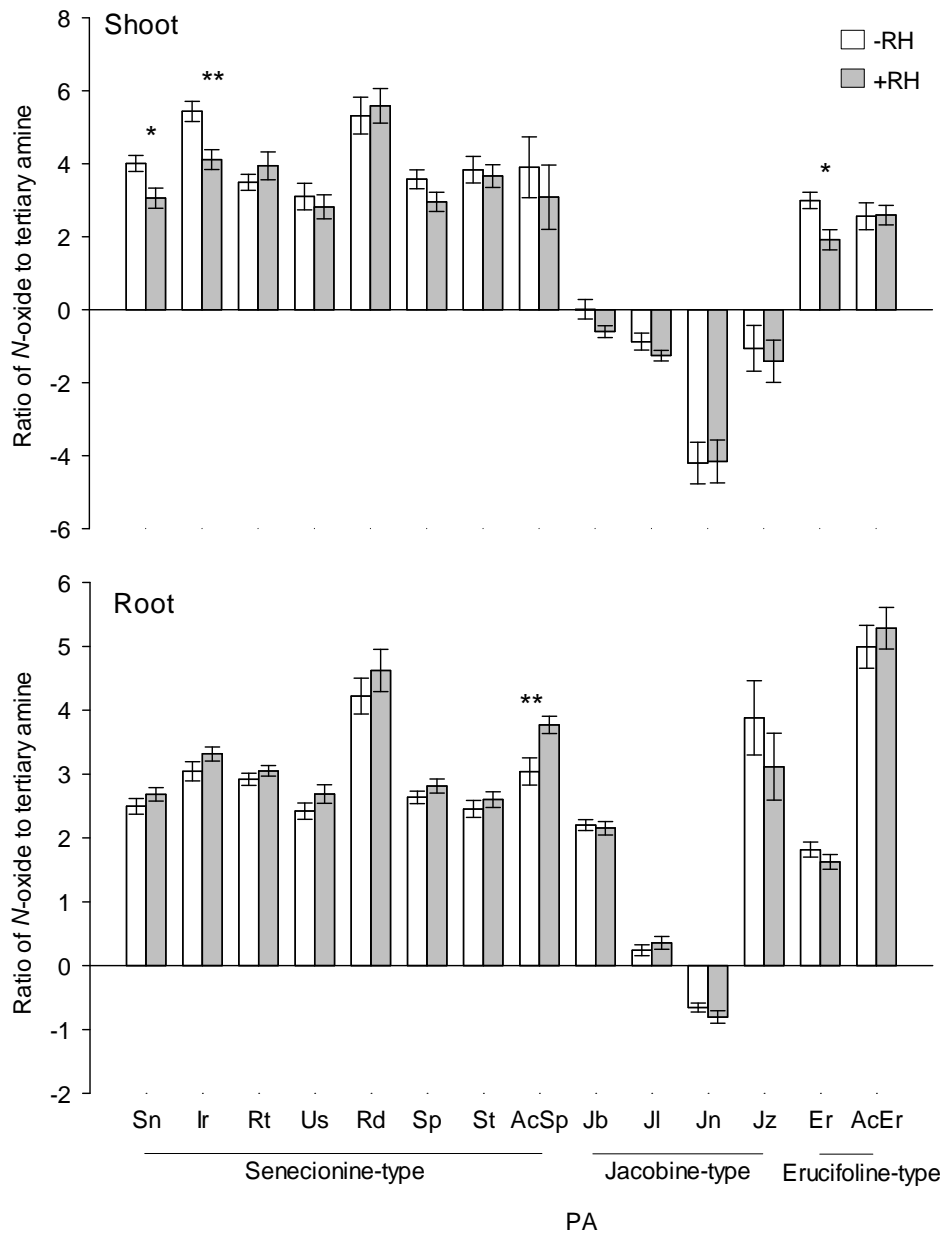
1 FIG. 1



2

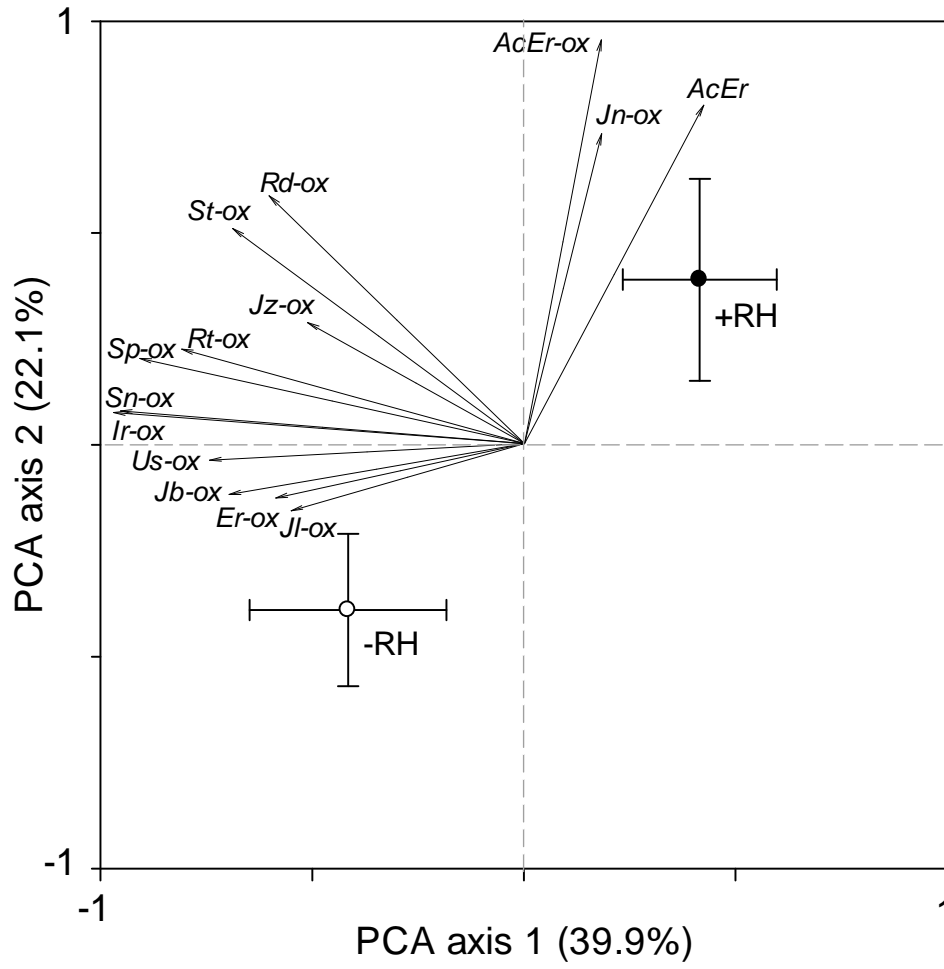
3

1 FIG. 2



2
3

1 FIG. 3



2