Trade-offs between chemical defence and regrowth capacity in *Plantago lanceolata*

J. H. Reudler [1, 2], S. C. Honders[1], H. Turin[1] and A. Biere[1]

[1]*Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB Wageningen, The Netherlands, current address:* [2]*Centre of Excellence in Biological Interactions Research, Department of Biological and Environmental Science, University of Jyväskylä, Finland, email: jhtalsma@hotmail.com*

Running title: Trade-offs between defence and regrowth

Keywords - fitness costs, iridoid glycosides, nutrient stress, *Plantago lanceolata*, regrowth capacity, selection lines

Total word count: 5758
Abstract: 298
Introduction: 1153
Material and Methods: 1725
Results: 1049
Discussion: 1458
Acknowledgements: 75

Cited references: 77
Figures: 4
Supplementary Tables: 2

Abstract - Resistance and tolerance are different strategies of plants to deal with herbivore attack. Since resources are limited and resistance and tolerance serve similar functions for plants, trade-offs between these two strategies have often been postulated. In this study we investigated trade-offs between resistance and one aspect of tolerance, the ability to regrow after defoliation. In order to minimize confounding effects of genetic background and selection history, we used offspring derived from artificial selection lines of ribwort plantain (*Plantago lanceolata*) that differed in their levels of leaf iridoid glycosides (IGs), allelochemicals that confer resistance to generalist herbivores, to study genetic associations with regrowth ability. We tested whether high-IG plants 1) suffer allocation costs of resistance in terms of reduced shoot and root growth, 2) have reduced regrowth ability (tolerance) after defoliation compared to low-IG plants, and 3) whether such costs are more pronounced under nutrient stress. High-IG plants produced fewer inflorescences and side rosettes than low-IG plants and showed a different biomass allocation pattern, but since neither the vegetative, nor the reproductive biomass differed between the lines, there was no evidence for a cost of IG production in terms of total biomass production under either nutrient condition. High-IG plants also did not suffer a reduced capacity to regrow shoot mass after defoliation. However, after regrowth, root mass of high-IG plants grown under nutrient-poor conditions was significantly lower than that of low-IG plants. This suggests that under these conditions shoot regrowth of high-IG plants comes at a larger expense of root growth than in low-IG plants. We speculate therefore that if there is repeated defoliation, high-IG plants may eventually fail to maintain shoot regrowth capacity and that trade-offs between resistance and tolerance in this system will show up after repeated defoliation events under conditions of low resource availability.

Introduction

Two of the general defence strategies of plants against herbivores are resistance, reducing the amount of herbivore damage, and tolerance, reducing the detrimental effects of herbivore damage on plant fitness (Crawley 1983; Rosenthal and Kotanen 1994; Strauss and Agrawal 1999; Stowe et al 2000). Resistance mechanisms include the production of secondary metabolites and morphological structures that reduce herbivore preference or performance. Tolerance mechanisms include increased photosynthetic activity, compensatory growth, utilization of stored reserves, activation of dormant meristems and phenological and architectural traits that contribute to maintaining high fitness in the face of damage (Tiffin 2000a). Tolerance may be an effective strategy against specialist herbivores that are unaffected by chemical defences in the food plants to which they have adapted (Crawley 1983; Giamoustaris and Mithen 1995; van der Meijden 1996).

Since both resistance and tolerance traits can incur fitness costs (e.g. Fineblum and Rausher 1995; Stinchcombe 2002; Strauss et al 2002; Fornoni et al 2004a) and both defence strategies are likely to draw upon the same pool of resources, trade-offs between these mechanisms have often been postulated (van der Meijden et al 1988; Fineblum and Rausher 1995; Mauricio et al 1997; Leimu and Koricheva 2006), depending on relative costs and benefits of these strategies (Tiffin 2000b; Fornoni et al 2004b; Restif and Koella 2004). Most plants seem to exhibit a mixed pattern of defence, allocating resources to both types of defence strategies (Núñez-Farfán et al 2007; Fornoni 2011) and meta-analyses of published studies on associations between resistance and tolerance have not yielded strong support for general trade-offs between these two defence strategies (Leimu and Koricheva 2006, see also Muola et al 2010; Tucker and Avila-Sakar 2010, but see Oduor et al 2011), illustrating that conditions for negative associations

between resistance and tolerance may be more restricted than previously thought (de Jong and van der Meijden 2000).

One factor that may have strong impact on whether trade-offs between resistance and tolerance occur is the strength of resource limitation (Agrawal 2011). In this paper we study the impact of nutrient availability on the costs of chemical defence in terms of growth and regrowth ability, one of the mechanisms underlying tolerance, within a species. Costs of defence are expected to increase under stress conditions such as low light or nutrient stress and competition for two reasons (Bergelson and Purrington 1996; Strauss et al 2002). First, trade-offs between different functions such as growth and defence are more pronounced when resources are more severely limiting (Herms and Mattson 1992). Second, environmental stress can cause increased production of defence chemicals (Gershenzon 1984; Hirata et al 1993; Dixon and Paiva 1995), incurring larger costs. Thus far, reviews of studies addressing costs of defence in relation to stress have not revealed a general pattern of more pronounced costs in stressful environments (Bergelson and Purrington 1996; van Dam and Baldwin 2001). Two factors may be responsible for this. First, increased production of secondary metabolites under severe resource limitation may not always have a substantial extra cost. For instance, nutrient shortage may lead to a relative excess of fixed carbon in the plant that can be transferred into carbon-based secondary metabolites at virtually no extra cost (Herms and Mattson 1992). Secondly, competitive stress may not result in enhanced costs of defence, if the production of these defences also provides a benefit in competitive interactions (Siemens et al 2002).

In this study we investigate whether constitutive levels of two defence compounds in *Plantago lanceolata*, the iridoid glycosides (IG) aucubin and catalpol, affect the plant's ability to grow and regrow and whether such costs depend on nutrient conditions. Aucubin and catalpol are carbon-based secondary metabolites in which *P. lanceolata* invests up to 9% of the dry weight

100 (Bowers 1991). Variation in the constitutive foliar concentrations of IGs within and among
101 populations of *P. lanceolata* has a strong genetic component (Bowers and Stamp 1992, 1993;
102 Adler et al 1995; Marak et al 2000). The biosynthetic costs of these IGs are high (Gershenzon
103 1984), but previous studies of fitness costs of IGs in *P. lanceolata* in the absence of herbivores
104 and pathogens have produced mixed results. No costs of IGs could be detected in terms of
105 negative among-genotype correlations between the level of IGs and aboveground biomass of
106 plant growth (Adler et al 1995), but in another study costs were found in terms of lower
107 reproductive dry weight and a smaller number of inflorescences produced by plants selected for
108 high levels of leaf IGs (Marak et al 2003). None of these studies addressed effects of IG on the
109 ability to regrow after defoliation. Regrowth is one of the mechanisms underlying tolerance that
110 involves e.g. the use of resources stored in plant parts that are relatively free from herbivore
111 attack (van der Meijden et al 1988; Iwasa and Kubo 1997).

112 So far, studies addressing trade-offs between resistance and tolerance have been mainly
113 performed either as cross-species comparisons (e.g. Agrawal and Fishbein 2008), or by
114 comparing genotypes within a species (see e.g. Manzaneda et al 2010). As argued before (Strauss
115 et al 1999; Siemens et al 2002; Stowe and Marquis 2011), costs and trade-offs in such
116 comparisons are difficult to study, as they are confounded by differences in selection history and
117 variation in genetic background between individuals. Hence such studies are not able to address
118 whether trade-offs result from a process in which particular allele combinations involved have
119 been brought together by selection (but can easily be broken down again by recombination), or
120 whether such associations are due to pleiotropic effects that really pose a constraint. Artificial
121 selection studies offer the advantage that they can assess pleiotropic costs of resistance in terms
122 of tolerance, or vice versa, in the absence of confounding effects of linkage disequilibrium. To
123 our knowledge, this approach has only been used once before to study associations between

chemical resistance and tolerance traits. Stowe (1998) showed that annual, rapidly cycling, *Brassica rapa* selected for high foliar glucosinolate levels suffered reduced fruit and seed production following partial defoliation compared to control and low-glucosinolate lines. It is unknown how this trade-off is affected by resource availability or how selection for high levels of chemical defence affects the regrowth ability, another aspect of tolerance, in longer lived species. For this study we therefore used *P. lanceolata* plants that had been artificially selected for high and low leaf IG concentrations (Marak et al 2000). We investigated whether plants selected for high levels of IG (resistance trait) (1) suffer allocation costs in terms of reduced shoot and root growth, (2) have reduced regrowth ability (tolerance), and (3) whether such costs are more pronounced under nutrient stress. In addition we assessed whether defoliated plants were able to attain similarly high levels of IGs in their regrown leaves as in non-defoliated plants (no cost of regrowth in terms of resistance), and whether the ability to do so was as strong in plants from high IG lines as in plants from low IG lines.

Materials and methods

Plants

Plantago lanceolata L., ribwort plantain, is a rosette-forming, self-incompatible, wind-pollinated, perennial herb that overwinters as a basal rosette and produces numerous leaves and spiked inflorescences at the end of fibrous stalks in the spring and summer (Cavers et al 1980; Primack and Antonovics 1982). It has a worldwide distribution and large ecological amplitude. Among the secondary plant compounds produced by *P. lanceolata* are the two iridoid glycosides (IGs) aucubin and catalpol (Duff et al 1965; Bowers and Stamp 1992; Adler et al 1995). Apart from two caffeoyl phenylethanoid glucosides (Fons et al 1999), these two IGs are the main known defence compounds of *P. lanceolata* (Suomi et al 2001). They are known to have a deterrent effect on pathogens (Marak et al 2002), and generalist insect herbivores of *P. lanceolata* (Bowers and Puttick 1988; Bowers 1991; Biere et al 2004). Previous feeding experiments have shown that both IGs prolong the development time of the generalist lepidopteran species *Spodoptera exigua* and *Chrysodeixis chalcites* and reduce their pupal and adult weight (Reudler et al 2011) and that catalpol is the more toxic of the two IGs to generalist herbivores (Bowers and Puttick 1988). However, both IGs also function as oviposition cues and feeding stimulants for specialist herbivorous insects. For instance, aucubin is used as oviposition cue by *Melitaea cinxia* (Nieminen et al 2003; Reudler Talsma et al 2008) whereas both IGs are used as oviposition cue by *Junonia coenia* (Pereyra and Bowers 1988) and as feeding stimulants by a number of specialist checkerspot butterflies (Bowers 1983). In some parts of its distribution *P. lanceolata* is heavily defoliated by herbivores such as caterpillars of the specialist nymphalid butterfly *Melitaea cinxia*, whose larvae feed gregariously in communal webs on the plant.

The *P. lanceolata* plants used for this experiment were the offspring of 16 full-sib crosses. Eight of these crosses were made between parents with low levels of leaf IGs ('low-IG crosses') and eight between parents with high levels of leaf IGs ('high-IG crosses'). The parents originated from two selection lines previously created after four generations of artificial selection for low and high leaf IG concentrations ('low-IG line' and 'high-IG line') from a common base population (Marak et al 2000). Previous studies have shown that average leaf IG levels differ ca. four-fold between these lines, but that considerable variation is present among different plant families within the lines as well (Marak et al 2000, 2003). On average, offspring from low-IG crosses are thus expected to have lower leaf IG levels than offspring from high-IG crosses, but considerable variation is expected within these sets of crosses as well, providing a range of IG levels among crosses used in this study.

Set up

Sixty seeds from each of the 16 crosses were germinated on water agar in a growth cabinet (L:D 14:10h; 25ºC/15ºC). After 14 days, seedlings were transplanted into plastic pots (diameter 13.0 cm, height 11.2 cm and volume 970 ml) with sand. All pots were placed on saucers so that nutrient solution spilled from the bottom of the pot could be reabsorbed by the soil and plant.

The experiment consisted of three treatments: one clipping-and-regrowth treatment (R) and two unmanipulated growth treatments, harvested at two different time points (U8 and U13). In treatment R, shoots were clipped just above the caudex and harvested 8 weeks after transplantation (T=8). This harvest is referred to as "R8". Roots were then allowed to regrow new shoots for another 5 weeks until roots and regrown shoots were harvested 13 weeks after transplantation (T=13), when plants started to senesce under low nutrient conditions ("R13"). One set of unmanipulated plants was harvested at the time of clipping ("U8"), the other

simultaneously with the final harvest thirteen weeks after transplantation ("U13"). Each treatment was performed under two nutrient conditions. Nutrient treatments ('rich' and 'poor') were performed by adding different strengths of nutrient solution to the pots. The first two weeks all the plants received the same amount of nutrients (50ml of a 1/32 strength Hoagland solution) to facilitate good establishment. Nutrient treatments were started two weeks after transplantation. Half of the pots of each growth treatment (R, U8, U13) received a low nutrient level: 50 ml of a 1/32 strength Hoagland's solution from T=2 to T=4 and a 1/16 strength Hoagland's solution from T=4 to T=13 (poor). The other half of the pots received a high nutrient level: 50 ml of a 1/8 strength Hoagland's solution from T=2 to T=13 (rich). Full strength Hoagland's solution contained: 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 174 μM $C_{10}H_{12}FeN_2O_8Na$, 93 μM H_3BO_3, 18 μM $MnCl_2$, 1.5 μM $ZnSO_4$, 0.6 μM $CuSO_4$ and 1.0 μM Na_2MoO_4.

All plants were watered individually 3 times a week. During the last five weeks (after the first harvest of treatment R), pots of treatment U13 (not clipped) received 100ml solution to meet their increased water demands without lowering soil nutrient concentration. In total the experiment consisted of 16 genotypes x 3 treatments x 2 nutrient levels x 6 replicates, 576 plants, that were grown in a randomized block design with three blocks, each containing two of the six replicates per treatment combination, located on different benches within the same greenhouse compartment. Plants within blocks were rotated every week and rearranged after harvest of the U8 plants.

Measurements

At T=8, 8 weeks after transplantation, the shoots of all the plants in treatment R8 and the complete plants of treatment U8 were harvested. At T=13, 13 weeks after transplantation,

complete plants of treatment R13 and U13 were harvested. For all plants we measured the day that they produced their first inflorescence, and at harvest we measured the number of rosettes, number of leaves and the length and width of their longest leaf. After harvest, roots were rinsed clean and plants were separated into roots, vegetative shoots and reproductive parts. All fractions per plant were put at -80˚C before freeze-drying. After freeze-drying, the plant parts were weighed to determine their dry mass.

At T=8, we had two sets of plants from which aboveground tissues were harvested, R8 plants and U8 plants. Plants from U8 were used for analyses of biomass and size-related traits, since from these plants also roots were harvested. Plants from R8 were used for leaf chemical analyses, since this allowed comparison of leaf IG concentrations from the same plants before and after defoliation. Sample sizes for leaf chemical analyses differed among treatments. Leaf samples from all plants (six replicates per treatment combination) of R8 (clipped leaves) and U13 plants were used for leaf chemical analyses. For plants of treatment R13 (regrown leaves) part of the samples were lost and only three replicates per treatment combination were used for leaf chemical analyses.

Since plants from the R treatment were left to regrow new leaves after defoliation, we lack measurements of individual root weights of these plants at T=8. As we wanted to relate the regrowth ability of plants from different crosses in treatment R to both their IG levels and their root dry weight at the time of defoliation, we estimated dry weights of roots in treatment R at T=8 as follows. First, we fitted general linear models to estimate how well ln-root dry weight of plants in treatment U8 (harvested at the same time as defoliation of R plants took place) could be predicted from their ln-shoot dry weight and the cross from which they originated. Both ln-shoot dry weight and cross significantly affected ln-root dry weight under nutrient-poor conditions ($F_{1, 62} = 358.8$, $P<0.001$ and $F_{15, 62} = 4.76$, $P<0.001$, respectively, interaction n.s.) and under nutrient-

rich conditions ($F_{1,59}$ = 89.23, P<0.001 and $F_{15, 59}$ = 3.91, P<0.001, respectively, interaction n.s.), explaining 84% and 68% of variation in ln-root dry weight under nutrient-poor and rich conditions, respectively. Then, the parameter estimates from these analyses were used to estimate ln-root dry weight of plants in treatment R from their ln-shoot dry weight in treatment R and the cross from which they originated.

Chemical analyses

Samples of the freeze-dried leaves were ground to a fine powder with a ball mill (type MM 301, Retsch GmbH & Co., Haan, Germany). Ground leaf material (25mg) was extracted in 10 ml of 70% MeOH and was shaken overnight (15°C, 100RPM). The crude extract was filtered using Whatman filter paper #4 and the filtrate was diluted ten times with Milli-Q water. The concentrations of the IG aucubin and catalpol were analysed by HPLC using a Bio-Lc (Dionex Corp., Sunnyvale, USA) equipped with a GP40 gradient pump, a Carbopac PA 1 guard (4 x 50 mm) and analytical column (4 x 250 mm), and an ED40 electrochemical detector for pulsed amperimetric detection (PAD). NaOH (1M) and Milli-Q water were used as eluents (10:90, 1ml/min). Retention times were 3.25 min and 4.40 min for aucubin and catalpol, respectively. Peaks were analyzed using Chromeleon version 6.60 (Dionex Corp., Sunneyvale, USA). Leaf concentrations of aucubin and catalpol were expressed as a percentage of total leaf dry weight. In addition to aucubin and catalpol, we also analysed the concentration of total IGs (i.e., the sum of aucubin and catalpol) and the ratio of catalpol to the total IGs. Since catalpol is the more toxic of the two components towards generalist insects (Bowers and Puttick 1988; Bowers 1991, 1992), this ratio is likely to reflect an important aspect of direct defense in addition to the total concentration of IGs.

Statistical analyses

The effects of Block, Line (selection line from which parents of the crosses originated), Cross (nested within Line), Nutrient level and their interaction effects on the different plant traits were analyzed using GLM (Generalized Linear Models) (SAS v. 9.2, procedure GENMOD, SAS Institute, Cary, NC) with a normal error distribution and an identity link function. Dependent variables were transformed prior to analysis if necessary to improve normality. The number of leaves and all weight measurements were ln-transformed and the IG levels were square root-transformed. In addition, we used (Pearson) correlations to analyse associations between (cross means for) leaf IG levels and (cross means for) growth, reproduction, or regrowth capacity. Allocation costs of chemical defence in terms of growth, reproduction, or regrowth would show up either as Line effects for these traits in GLM (lower performance by plants of the high-IG crosses), or as negative correlations between cross-means for these traits and cross-means for leaf IG levels.

Results

Differences in leaf IG between crosses

Leaf IG concentrations at T=8 were approximately two-fold higher in crosses from the high-IG line than in crosses from the low-IG line, under both nutrient conditions (Table S1; Fig. 1a). Both aucubin (Fig. 1b) and catalpol (Fig. 1c) contributed to this difference (Table S1). Leaf IG concentrations at T=8 also varied significantly among crosses within lines, resulting in a three- to four-fold range of variation in mean leaf IG concentrations among offspring groups from the 16 different crosses. Interestingly, the ratio of catalpol-to-total IG, which did not differ significantly between lines (Table S1, Fig. 1d) did show large variation among the crosses within these lines (Table S1). Leaf IG concentrations were on average 1.7 fold higher under nutrient-poor conditions than under nutrient-rich conditions (Table S1, Fig. 1a). This difference could be mainly attributed to higher leaf concentrations of aucubin under these conditions. The relative difference in IG concentration between crosses from the high and low line was not significantly affected by nutrient supply (no interaction line x nutrient, Table S1).

At T=13, unmanipulated plants from the high-IG line still had significantly higher leaf IG concentrations than plants from the low-IG line (Table S1, Fig. 1a). Leaf IG concentrations showed a clear ontogenetic pattern. Under nutrient-poor conditions, plants from both lines had higher leaf concentrations of aucubin at T=13 than at T=8 (Fig. 1b), whereas concentrations of catalpol were similar between time points (Fig. 1c), resulting in a decline in the ratio of catalpol-to-total IG (Fig. 1d) over time. Under nutrient-rich conditions, only plants from the high line appeared to have higher leaf aucubin concentrations at T=13 than at T=8, whereas plants from the low line had lower leaf catalpol concentrations than at T=8, also resulting in a decline in the ratio of catalpol-to-total IG over time (Fig. 1d).

Growth costs of iridoid glycosides

Total dry weight of plants varied significantly among crosses, both at T=8 and T=13 (Table S2), but plants from crosses from the high-IG line did not have a lower total dry weight than plants from crosses from the low-IG line (Table S2; Fig. 2a), indicating that there was no general growth cost of producing high levels of leaf IG under the conditions of the experiment. This is confirmed by the absence of negative correlations between cross means for leaf IG and total dry weight at T=8 (nutrient-poor: r=-0.31, n=16, P=0.24; nutrient-rich: r=-0.13, n=16, P>0.5) or T=13 (nutrient-poor: r=-0.17, n=16, P>0.5; nutrient-rich r=0.34, n=16, P=0.20). However, biomass allocation patterns and plant architecture differed between plants from the high-IG and low-IG line. First, plants from the high-IG line invested a smaller fraction of their biomass into roots: at T=8 their root mass fraction (root/total biomass) was significantly lower than that of plants from the low-IG line (Table S2; Fig. 2b), although the difference in absolute biomass of roots between the lines was not significant (Table S2; Fig. 2c). Second, at T=13, when plants under nutrient-rich conditions had invested on average more than 30% of their aboveground dry weight in reproduction, plants from the high line had produced fewer inflorescences than plants from the low line (Table S2; Fig. 2d). This could indicate a potential reproductive cost of high leaf IG production, however the reduced number of inflorescences was not accompanied by a significantly lower reproductive biomass (Table S2; Fig. 2e). Plants under nutrient rich conditions developed their first inflorescence significantly earlier than plants under nutrient poor conditions, for both selection lines (nutrient effect, $F[1,163] = 176.8$, $P < 0.001$). Third, shoot architecture differed between plants from the high-IG and low-IG line. Plants from the high line produced significantly fewer side rosettes and leaves per plant (Table S2; Fig. 2f,g) but the leaf area (length x width) of their longest leaf was significantly larger than that of plants from the low line (Table S2; Fig. 2h).

Regrowth costs of iridoid glycosides

Plants defoliated at T=8 were not able to achieve full growth compensation by T=13, i.e. they had lower biomass at T=13 than plants that had been allowed to grow undisturbed until T=13 (Fig. 2a). On average, the biomass of regrown shoots on plants defoliated at T=8 was 27% and 51% lower than the shoot biomass of unclipped control plants at T=13 under nutrient-poor and nutrient-rich conditions, respectively (Fig. 2i). Shoot biomass at T=13 produced by plants that had been defoliated at T=8 (regrowth) was positively correlated with their estimated root biomass at the time of defoliation (Fig. 3), but did not differ between plants from crosses originating from the high-IG and the low-IG line (Table S2; Fig. 2i), indicating that there is no trade-off between production of direct defence chemicals and the regrowth of shoot biomass. However, plants from the high-IG line did produce significantly fewer inflorescences after defoliation than plants from the low-IG line (Table S2, Fig. 2d), as was observed for untreated plants at T=13. Moreover, at low nutrient supply, the root biomass of high-IG plants, that already tended to be lower than that of low-IG plants at the time of their defoliation (low nutrient supply, line effect: $F[1,14] = 3.48$, $P = 0.08$; Fig. 2c), was now significantly lower than that of low-IG plants ($F[1,14] = 8.19$, $P = 0.013$; Fig. 2c). Accordingly, there was a significantly negative correlation between the mean IG concentration of the different crosses at T=8 and their mean root biomass five weeks after defoliation at T=13, under nutrient-poor conditions (Fig. 4; $r = -0.55$, $n=16$, $P = 0.03$). At high nutrient supply, the difference in root weight between plants from low- and high-IG lines was not significant.

IG of regrown leaves following defoliation

IG concentrations of regrown, new, leaves of plants at T=13 whose leaves had been defoliated at T=8 (Fig. 1, R) differed from those of unmanipulated plants at T=13. Under nutrient-poor conditions, regrown leaves contained similar concentrations of aucubin (Fig. 1b), but significantly lower levels of catalpol (Fig. 1c), resulting in lower catalpol-to-total ratios compared to unmanipulated plants at T=13 (Fig. 1d). This could indicate that there is a trade-off between regrowth and the production of catalpol under nutrient-poor conditions. By contrast, under nutrient-rich conditions, regrown leaves of plants from the low line contained higher IG (both aucubin and catalpol) concentrations than leaves from unmanipulated plants, without affecting the catalpol-to-total ratio.

Discussion

Regrowth costs of IGs

Several studies have reported costs of chemical defence in terms of tolerance to herbivory. For instance, high glucosinolate levels were associated with reduced tolerance of damage by caterpillars of *Pieris rapae* in wild radish (Strauss et al 2003) and with reduced tolerance to partial defoliation in *Brassica rapa* (Stowe 1998), costs of nicotine were observed in terms of tolerance of damage by nematodes (Preisser et al 2007), and proteinase inhibitors incurred a cost in terms of regrowth in *Nicotiana attenuata* (Zavala and Baldwin 2006). Regrowth capacity depends in part on energy and nutrients that are saved and stored in organs that are relatively free from attack. These reserves can be reallocated after herbivory. Plants from high-IG crosses had lower root mass fractions than plants from the low-IG crosses, but their absolute root biomass was not significantly lower than that of low-IG crosses and consequently they did not have a lower regrowth capacity after defoliation. This indicates that there was no cost of high IG production in terms of reduced shoot regrowth after defoliation under the competition-free conditions of our experiment. However, especially under nutrient-poor conditions, plants that had high levels of leaf IG at the time of defoliation had significantly lower root biomass at the final harvest (T=13). This likely indicates that under nutrient-poor conditions, plants from high-IG crosses are well able to regrow new shoots, but do so at a slightly larger expense of new root growth than plants from low-IG crosses. After a single defoliation event we would therefore not see a negative effect of high IG production on shoot regrowth. But as shoot regrowth is strongly and positively correlated with root mass at the time of defoliation (Fig. 3), we expect that after repeated defoliation the progressively stronger reduction of root mass of high-IG plants will eventually result in a reduced capacity of shoot regrowth compared to low-IG plants, i.e. will

eventually result in a cost of resistance in terms of regrowth capacity under nutrient-poor conditions. This conclusion should be viewed with caution since our study is based on experimental defoliation using scissors. Contact with oral secretions of herbivorous insects can profoundly alter a plant's response to defoliation. For instance, down-regulation of systemin after contact with oral secretion of *Manduca sexta* caterpillars results in increased root allocation in *Solanum nigrum* (Schmidt and Baldwin 2009), which may actually prevent depletion of stored reserves by shoot regrowth following defoliation.

Chemical defence of regrown leaves

Numerous studies have shown that the defence chemistry of leaves that have regrown on plants following partial or complete defoliation can considerably differ from the defence chemistry of corresponding leaves on undamaged plants (e.g. Katjiua and Ward 2006; Rooke and Bergstrom 2007; Verges et al 2008; Roitto et al 2009). Together with concomitant changes in primary metabolites, such differences in leaf chemistry may have large effects on the palatability of the newly formed leaves. Barton (2008) observed that 4-week old *P. lanceolata* plants that suffered 50% defoliation by caterpillars of the specialist lepidopteran *Junonia coenia* showed a transient, 80%, decrease in IG concentrations one week after damage, but that IG concentrations had increased to levels not different from control plants five weeks later. Our results largely confirm the absence of differences in total IG concentrations between regrown and control leaves 5 weeks after damage, under nutrient-rich conditions. Under these conditions, IG concentrations in leaves that had regrown on plants from low-IG crosses were even slightly higher than those of control plants at similar plant age. However, under nutrient-poor conditions, the relative amounts of aucubin and catalpol were strongly affected by defoliation. Regrown leaves on plants that had been defoliated at T=8 had both a significantly lower amount of catalpol and a significantly lower

ratio of catalpol to total IG at T=13 than control plants at T=13. The latter result is consistent with a previous study showing a reduced catalpol to total IG ratio in *P. lanceolata* five weeks after defoliation (Stamp and Bowers 1994). Aucubin and catalpol differ in their biological activity (Bowers 1991; Marak et al 2002). Catalpol is considered the more toxic of the two for generalist herbivores (Bowers and Puttick 1988; Bowers 1991, 1992). Consequently, the relative contribution of aucubin and catalpol to the total IG pool is biologically relevant. After defoliation, regrown shoots may therefore be more susceptible to generalist herbivores than shoots of plants that were not defoliated.

Growth and reproduction costs of IGs

In addition to the, at least initial, absence of regrowth costs of chemical defense, we also did not observe strong growth (biomass production) costs of chemical defense under the conditions of the experiment. A fundamental assumption of most plant defence theories is that limited resources lead to allocation constraints, often depicted as trade-offs between the production of secondary plant compounds and other fitness-enhancing traits. In the absence of natural enemies the production of these defence chemicals will then have fitness costs. In our study we did not find evidence for a trade-off between chemical defence and biomass production at either nutrient level. Plants from the high-IG line had fewer but larger leaves than plants from the low-IG line, resulting in similar leaf and shoot biomass. Plants from the high-IG line invested a smaller fraction of their total biomass in roots, which might suggest a potential cost of chemical defence in environments where low investment in roots is disadvantageous. However, for undamaged plants, differences in the absolute amount of root mass between plants from high- and low-IG lines were not significant, partly because of the large variation in root biomass between crosses within each of the lines. The large variation in root mass among crosses within the high- and low-

IG lines may also explain why previous studies that used just one cross from each line found either no difference (de Deyn et al 2009) or even higher (Wurst et al 2008) root mass in plants from a high-IG cross compared to plants from a low-IG cross.

A consistent potential fitness cost of IG production that we observed was the smaller number of inflorescences and side rosettes produced by plants from high-IG crosses compared to plants from low-IG crosses under nutrient-rich conditions. This finding is consistent with other studies reporting reproductive costs of chemical defence (e.g. Berenbaum et al 1986; Zangerl and Berenbaum 1997, Stowe and Marquis 2011) and confirms results from a previous study in *P. lanceolata* based on the same selection lines (Marak et al 2003; Wurst et al 2008). However, since the reduction in inflorescence number was not accompanied by a lower reproductive biomass, further studies are necessary to investigate whether the expected reduction in the number of seeds produced is partly compensated by a higher individual seed mass and the fitness consequences of these differences under different environmental conditions.

Increased costs of chemical defence under conditions with environmental stress are expected, based on the arguments that a given investment in defence more strongly constrains other fitness-enhancing traits at low than at high resource levels. However, the precise effects will depend on the type of stress and the type of chemicals involved (Agrawal 2011). IGs are carbon-based secondary metabolites. According to the carbon-nutrient balance hypothesis (Bryant et al 1983; Bryant et al 1988; Tuomi et al 1988), plants in resource-limited environments could divert the carbon reserves that they accumulate beyond growth requirements to secondary metabolism without a trade-off in growth (Bryant et al 1985). Indeed, we observed higher levels of IGs under nutrient poor conditions, as previously shown in *P. lanceolata* (Darrow and Bowers 1999; Marak et al 2003), without observing stronger reductions in growth or reproduction, than under nutrient-rich conditions. This confirms results from Marak et al (2003) who did not find

evidence for enhanced costs of chemical defence in this system under low nutrient conditions. Some studies have reported that costs of carbon-based chemical defence can even be higher under nutrient-rich conditions (Stevens et al 2007) where the resistance chemicals directly compete with growth for limited carbon resources. It is therefore likely that stress-enhanced costs of carbon based defences such as IGs are more readily observed under light than under nutrient stress.

In summary, we detected allocation costs of chemical defence in terms of reduced numbers of side rosettes and inflorescences by high-IG plants. After a single defoliation event, we did not observe a trade-off between resistance and tolerance, i.e., plants with high levels of a trait that confers resistance to generalist insects (IG level) did not have lower tolerance (shoot regrowth capacity) than plants with lower IG levels. However, since high IG plants did suffer reduced root biomass after a single defoliation and since root mass strongly determines shoot regrowth capacity, we expect that trade-offs between resistance and tolerance will become apparent after repeated defoliation.

Acknowledgements

We thank E. van der Meijden for his constructive comments on an earlier version of the manuscript. L. Reudler Talsma and R. M. Niermeyer are acknowledged for their help with weighing and grinding of the samples and Francesca Rinaldi for her help with the HPLC-analyses. This study was supported by a grant from the Earth and Life Science Foundation (ALW) of the Netherlands Organization for Scientific Research (NWO). Publication 5342 Netherlands Institute of Ecology (NIOO-KNAW).

References

Adler LS, Schmitt J, Bowers MD (1995) Genetic-variation in defensive chemistry in *Plantago lanceolata* (Plantaginaceae) and its effect on the specialist herbivore *Junonia coenia* (Nymphalidae). Oecologia 101:75-85

Agrawal AA (2011) Current trends in the evolutionary ecology of plant defence. Funct Ecol 25:420-432

Agrawal AA, Fishbein M (2008) Phylogenetic escalation and decline of plant defense strategies. Proc Natl Acad Sci USA 105:10057-10060

Barton KE (2008) Phenotypic plasticity in seedling defense strategies: compensatory growth and chemical induction. Oikos 117:917-925

Berenbaum MR, Zangerl AR, Nitao JK (1986) Constraints on chemical coevolution: wild parsnip and the wild parsnip webworm. Evolution 40:1215-1228

Bergelson J, Purrington CB (1996) Surveying patterns in the cost of resistance in plants. Am Nat 148:536-558

Biere A, Marak HB, van Damme JMM (2004) Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? Oecologia 140:430-441

Bowers MD (1983) The role of iridoid glycosides in host-plant specificity of checkerspot butterflies. J Chem Ecol 9:475-793

Bowers MD (1991) Iridoid glycosides. In: Rosenthal GA, Berenbaum MR (eds) Herbivores: their interactions with secondary plant metabolites. Academic Press, San Diego, pp 297-325

Bowers MD (1992) Evolution of unpalatability and the cost of chemical defense in insects. In: Roitberg BD, Isman MB (eds) Insect chemical ecology: an evolutionary approach. Chapman and Hall, New York, pp 216-244

492 Bowers MD, Puttick GM (1988) Response of generalist and specialist insects to qualitative
493 allelochemical variation. J Chem Ecol 14:319-334
494 Bowers MD, Stamp NE (1992) Chemical variation within and between individuals of *Plantago*
495 *lanceolata* (Plantaginaceae). J Chem Ecol 18:985-995
496 Bowers MD, Stamp NE (1993) Effects of plant-age, genotype, and herbivory on *Plantago*
497 performance and chemistry. Ecology 74:1778-1791
498 Bryant JP, Chapin FS, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to
499 vertebrate herbivory. Oikos 40:357-368
500 Bryant JP, Chapin FS, Reichardt P, et al (1985) Adaptation to resource availability as a
501 determinant of chemical defense strategies in woody plants. Recent Adv Phytochem
502 19:219-237
503 Bryant JP, Tuomi J, Niemelä P (1988) Environmental constraint of constitutive and long-term
504 inducible defenses in woody plants. In: Spencer KC (ed) Chemical medation of
505 coevolution. Academic Press, San Diego, pp 367-389
506 Cavers PB, Bassett IJ, Crompton CW (1980) The biology of Canadian weeds. 47. *Plantago*
507 *lanceolata* L. Can J Plant Sci 60:1269-1282
508 Crawley MJ (1983) Herbivory. Blackwell, Oxford.
509 Darrow K, Bowers MD (1999) Effects of herbivore damage and nutrient level on induction of
510 iridoid glycosides in *Plantago lanceolata*. J Chem Ecol 25:1427-1440
511 de Deyn GB, Biere A, van der Putten WH, et al (2009) Chemical defense, mycorrhizal
512 colonization and growth responses in *Plantago lanceolata* L. Oecologia 160:433-442
513 de Jong G, van der Meijden E (2000) On the correlation between allocation to defence and
514 regrowth in plants. Oikos 88:503-508

515 Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085-
516 1097
517 Duff RB, Bacon JSD, Mundie CM, et al (1965) Catalpol and methylcatalpol: naturally ocurring
518 glycosides in *Plantago* and *Buddleia* species. Biochem J 96:1-5
519 Fineblum WL, Rausher MD (1995) Tradeoff between resistance and tolerance to herbivore
520 damage in a morning glory. Nature 377:517-520
521 Fons F, Tousch D, Rapior S, et al (1999) Phenolic profiles of untransformed and hairy root
522 cultures of *Plantago lanceolata*. Plant Physiol Biochem 37:291-296
523 Fornoni J (2011) Ecological and evolutionary implications of plant tolerance to herbivory. Funct
524 Ecol 25:399-407
525 Fornoni J, Valverde PL, Núñez-Farfán J (2004a) Population variation in the cost and benefit of
526 tolerance and resistance against herbivory in *Datura stramonium*. Evolution 58:1696-
527 1704
528 Fornoni J, Núñez-Farfán J, Valverde PL, et al (2004b) Evolution of mixed strategies of plant
529 defense allocation against natural enemies. Evolution 58:1685-1616-1695
530 Gershenzon J (1984) Changes in the levels of plant secondary metabolites under water and
531 nutrient stress. Recent Adv Phytochem 18:273-320
532 Giamoustaris A, Mithen R (1995) The effect of modifying the glucosinolate content of leaves of
533 oilseed rape (*Brassica napus* ssp. *oleifera*) on its interaction with specialist and generalist
534 pests. Ann Appl Biol 126:347-363
535 Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. Q Rev Biol 67:283-
536 335
537 Hirata K, Asada M, Yatani E, et al (1993) Effects of near-ultraviolet light on alkaloid production
538 in *Catharanthus roseus* plants. Planta Med 59:46-50

Iwasa Y, Kubo T (1997) Optimal size of storage for recovery after unpredictable disturbances. Evol Ecol 11:41-65

Katjiua MLJ, Ward D (2006) Resistance and tolerance of *Terminalia sericea* trees to simulated herbivore damage under different soil nutrient and moisture conditions. J Chem Ecol 32:1431-1443

Leimu R, Koricheva J (2006) A meta-analysis of tradeoffs between plant tolerance and resistance to herbivores: combining the evidence from ecological and agricultural studies. Oikos 112:1-9

Manzaneda AJ, Prasad KVSK, Mitchell-Olds T (2010) Variation and fitness costs for tolerance to different types of herbivore damage in *Boechera stricta* genotypes with contrasting glucosinolate structures. New Phytol 188:464-477

Marak HB, Biere A, van Damme JMM (2000) Direct and correlated responses to selection on iridoid glycosides in *Plantago lanceolata* L. J Evolution Biol 13:985-996

Marak HB, Biere A, van Damme JMM (2002) Two herbivore-deterrent iridoid glycosides reduce the in-vitro growth of a specialist but not of a generalist pathogenic fungus of *Plantago lanceolata* L. Chemoecology 12:185-192

Marak HB, Biere A, van Damme JMM (2003) Fitness costs of chemical defense in *Plantago lanceolata* L.: effects of nutrient and competition stress. Evolution 57:2519-2530

Mauricio R, Rausher MD, Burdick DS (1997) Variation in the defense strategies of plants: Are resistance and tolerance mutually exclusive? Ecology 78:1301-1311

Muola A, Mutikainen P, Laukkanen L, et al (2010) Genetic variation in herbivore resistance and tolerance: the role of plant life-history stage and type of damage. J Evol Biol 23:2185-2196

562 Nieminen M, Suomi J, van Nouhuys S, et al (2003) Effect of iridoid glycoside content on
563 oviposition host plant choice and parasitism in a specialist herbivore. J Chem Ecol
564 29:823-844
565 Núñez-Farfán J, Fornoni J, Valverde PL (2007) The evolution of resistance and tolerance to
566 herbivores. Annu Rev Ecol Evol Syst 38:541-566
567 Oduor AMO, Lankau RA, Strauss SY, et al (2011) Introduced *Brassica nigra* populations exhibit
568 greater growth and herbivore resistance but less tolerance than native populations in the
569 native range. New Phytol 191:536-544
570 Pereyra PC, Bowers MD (1988) Iridoid glycosides as oviposition stimulants for the buckeye
571 butterfly, *Junonia coenia* (Nymphalidae). J Chem Ecol 14:917-928
572 Preisser EL, Gibson SE, Adler LS, et al (2007) Underground herbivory and the costs of
573 constitutive defense in tobacco. Acta Oecol 31:210-215
574 Primack RB, Antonovics J (1982) Experimental ecological genetics in *Plantago.* VII.
575 Reproductive effort in populations of *P. lanceolata* L. Evolution 36:742-752
576 Restif O, Koella JC (2004) Concurrent evolution of resistance and tolerance to pathogens. Am
577 Nat 164:E90-E102
578 Reudler Talsma JH, Biere A, Harvey JA, et al (2008) Oviposition cues for a specialist butterfly:
579 plant chemistry and size. J Chem Ecol 34:1202-1212
580 Reudler JH, Biere A, Harvey JA, et al (2011) Differential performance of a specialist and two
581 generalist herbivores and their parasitoids on *Plantago lanceolata*. J. Chem. Ecol 37:765-
582 778
583 Roitto M, Rautio P, Markkola A, et al (2009) Induced accumulation of phenolics and sawfly
584 performance in Scots pine in response to previous defoliation. Tree Physiol 29:207-216

585 Rooke T, Bergstrom R (2007) Growth, chemical responses and herbivory after simulated leaf
586 browsing in *Combretum apiculatum*. Plant Ecology 189:201-212
587 Rosenthal JP, Kotanen PM (1994) Terrestrial plant tolerance to herbivory. Trends Ecol Evol
588 9:145-148
589 Siemens DH, Garner SH, Michell-Olds T, et al (2002) Costs of defense in the context of plant
590 competition: *Brassica rapa* may grow and defend. Ecology 83:505-517
591 Schmidt S, Baldwin IT (2009). Down-regulation of systemin after herbivory is associated with
592 increased root allocation and competitive ability in *Solanum nigrum*. Oecologia, 159:473-
593 482
594 Stamp NE, Bowers MD (1994) Effects of cages, plant age and mechanical clipping on plantain
595 chemistry. Oecologia 99:66-71
596 Stevens MT, Waller DM, Lindroth RL (2007) Resistance and tolerance in *Populus tremuloides*:
597 genetic variation, costs, and environmental dependency. Evol Ecol 21:829-847
598 Stinchcombe JR (2002) Can tolerance traits impose selection on herbivores? Evol Ecol 16:595-
599 602
600 Stowe KA (1998) Experimental evolution of resistance in *Brassica rapa*: correlated response of
601 tolerance in lines selected for glucosinolate content. Evolution 52:703-712
602 Stowe KA, Marquis RJ (2011) Costs of defense: correlated reponses to divergent selection for
603 foliar glucosinolate content in *Brassica rapa*. Evol Ecol 25:763-775
604 Stowe KA, Marquis RJ, Hochwender CG, et al (2000) The evolutionary ecology of tolerance to
605 consumer damage. Annu Rev Ecol Syst 31:565-595
606 Strauss SY, Agrawal AA (1999) The ecology and evolution of plant tolerance to herbivory.
607 Trends Ecol Evol 14:179-185

608 Strauss SY, Siemens DH, Decher MB, et al (1999) Ecological costs of plant resistance to
609 herbivores in the currency of pollination. Evolution 53:1105-1113
610 Strauss SY, Rudgers JA, Lau JA, et al (2002) Direct and ecological costs of resistance to
611 herbivory. Trends Ecol Evol 17:278-285
612 Strauss SY, Watson W, Allen MT (2003) Predictors of male and female tolerance to insect
613 herbivory in *Raphanus raphanistrum*. Ecology 84:2074-2082
614 Suomi J, Wiedmer SK, Jussila M et al (2001) Determination of iridoid glycosides by micellar
615 electrokinetic capillary chromatography-mass spectrometry with use of the partial filing
616 technique. Electrophoresis 22:2580-2587
617 Tiffin P (2000a) Mechanisms of tolerance to herbivore damage: what do we know? Evol Ecol
618 14:523-536
619 Tiffin P (2000b) Are tolerance, avoidance, and antibiosis evolutionarily and ecologically
620 equivalent responses of plants to herbivores? Am Nat 155:128-138
621 Tucker C, Avila-Sakar G (2010) Ontogenetic changes in tolerance to herbivory in *Arabidopsis*.
622 Oecologia 164:1005-1015
623 Tuomi J, Niemela P, Chapin FS, et al (1988) Defensive responses of trees in relation to their
624 carbon/nutrient balance. In: Mattson WJ, Levieux J,Bernard-Dagan C (eds) Mechanisms
625 of woody plant defenses against insects: search for pattern. Springer-Verslag, New York,
626 pp 57-72
627 van Dam NM, Baldwin IT (2001) Competition mediates costs of jasmonate-induced defences,
628 nitrogen acquisition and transgenerational plasticity in *Nicotiana attenuata*. Funct Ecol
629 15:406-415
630 van der Meijden E (1996) Plant defence, an evolutionary dilemma: contrasting effects of
631 (specialist and generalist) herbivores and natural enemies. Entomol Exp Appl 80:307-310

van der Meijden E, Wijn M, Verkaar HJ (1988) Defense and regrowth, alternative plant strategies in the struggle against herbivores. Oikos 51:355-363

Verges A, Perez M, Alcoverro T, et al (2008) Compensation and resistance to herbivory in seagrasses: induced responses to simulated consumption by fish. Oecologia 155:751-760

Wurst S, van Dam NM, Monroy F, et al (2008) Intraspecific variation in plant defense alters effects of root herbivores on leaf chemistry and aboveground herbivore damage. J Chem Ecol 34:1360-1367

Zangerl AR, Berenbaum MR (1997) Cost of chemically defending seeds: furanocoumarins and *Pastinaca sativa*. Am Nat 150:491-504

Zavala JA, Baldwin IT (2006) Jasmonic acid signalling and herbivore resistance traits constrain regrowth after herbivore attack in *Nicotiana attenuata*. Plant Cell Environ 29:1751-1760

644 **Figure legends**

645

646 **Figure 1**. Leaf concentrations (% of dry weight) of total iridoid glycosides (IG) (a), aucubin (b),
647 catalpol (c) and catalpol-to-total-IG ratio (d) of *Plantago lanceolata* lines selected for low (white
648 bars) and high (black bars) leaf iridoid glycoside concentrations. Plants were grown under
649 nutrient poor or rich conditions and leaves were harvested at an age of eight weeks (T=8;
650 treatment R8), thirteen weeks (T=13, treatment U13), or after regrowth from T=8 until T=13
651 following defoliation (R; treatment R13). Non-shared letters indicate significant differences
652 among bars (lines or time points) within nutrient treatments (LSD post-hoc tests, $P<0.05$). Values
653 are back-transformed means ± 1 s.e.m.

654

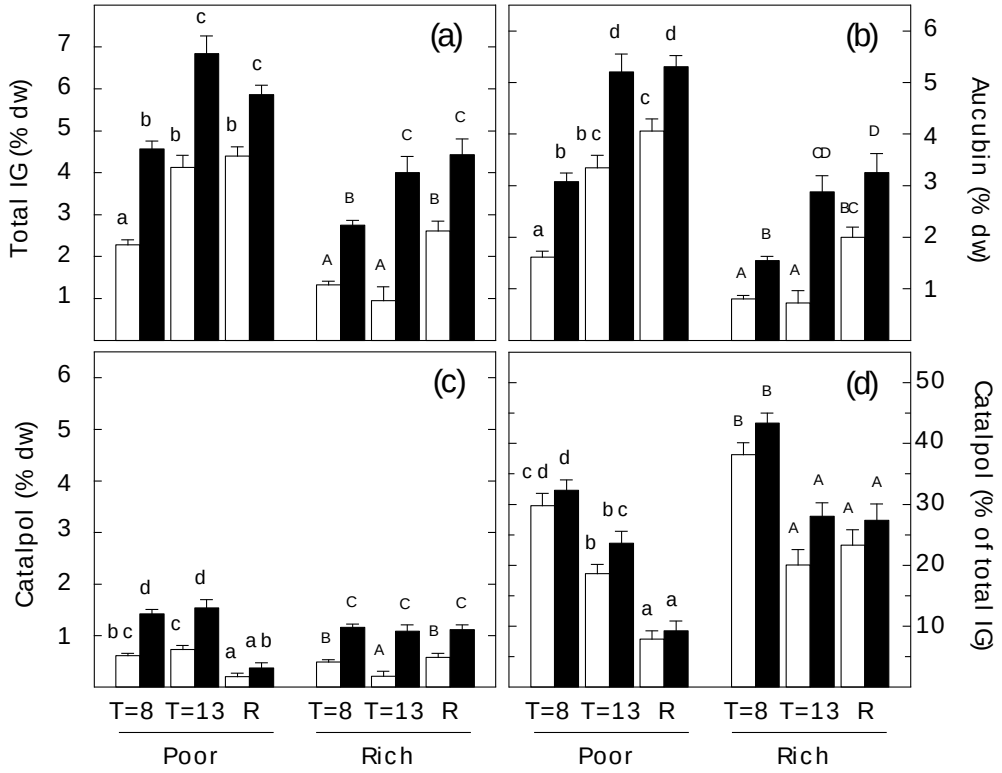
655 **Figure 2**. Total dry weight (a), ratio of root-to-total-biomass (b), total root dry weight (c),
656 number of rosettes (d), number of leaves (e), leaf area of the longest leaf (f), number of
657 inflorescences (g), dry weight of inflorescences (h), and total vegetative shoot dry weight (i) of
658 *Plantago lanceolata* lines selected for low (white bars) and high (black bars) leaf iridoid
659 glycoside concentrations. Plants were grown under nutrient poor or rich conditions and harvested
660 at an age of eight weeks (T=8; treatment U8), thirteen weeks (T=13; treatment U13) or after
661 regrowth from T=8 until T=13 following defoliation (R; treatment R13). Asterisks indicate
662 significant differences between lines for each treatment combination (line effect tested over cross
663 within line). + $P<0.10$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Values are back-transformed means ±
664 1 s.e.m.

665

666 **Figure 3**. Mean weight of *P. lanceolata* shoots at T=13 that had regrown following defoliation at
667 T=8, as a function of their mean estimated root dry weight at the time of defoliation (T=8).

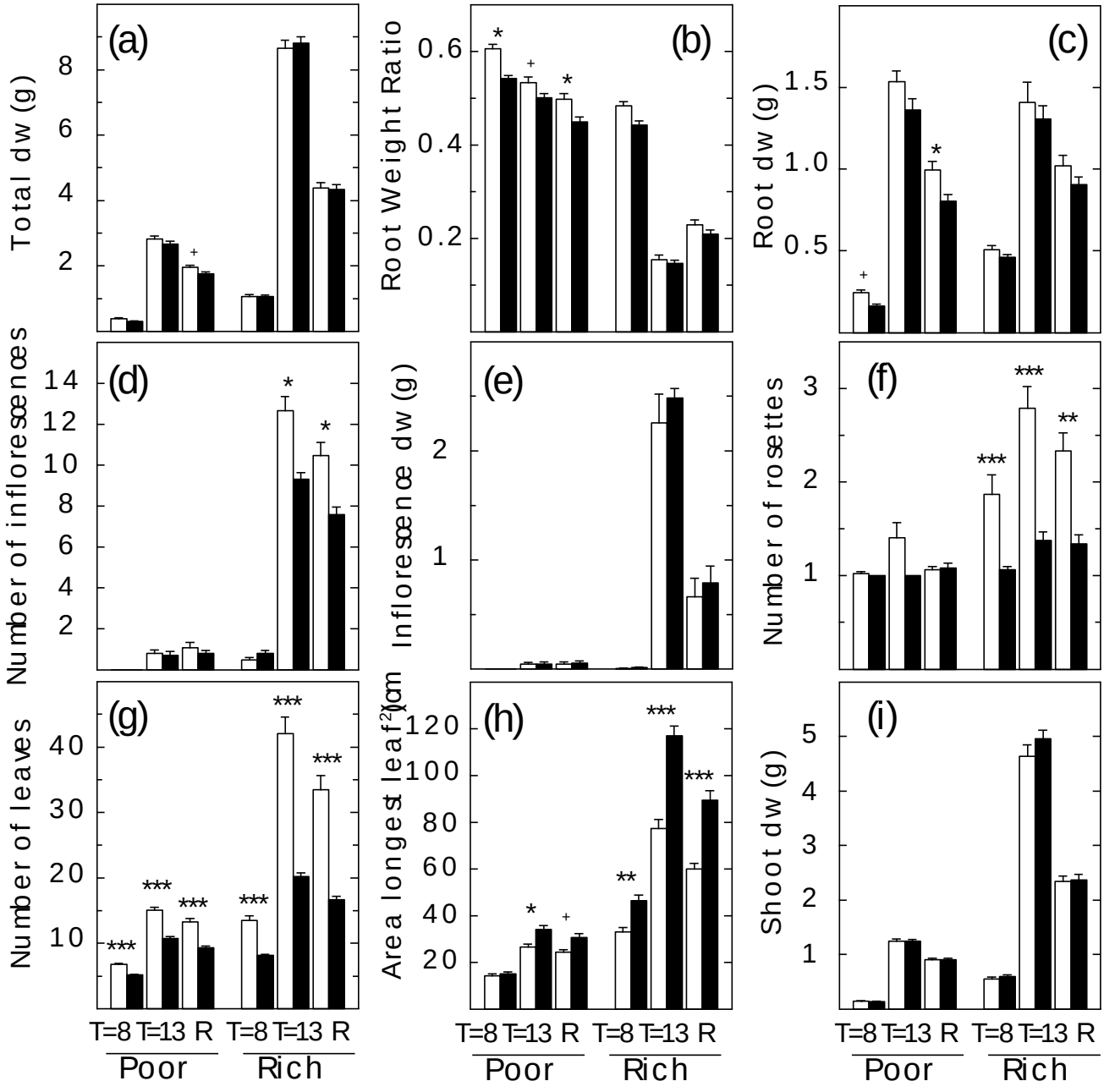
668 Values are mean values for progeny of eight crosses derived from a low-IG line (open symbols)
669 and eight crosses from a high-IG line (closed symbols) grown under nutrient-poor (circles) or
670 rich (squares) conditions. Note the log-scale of both axes.
671
672 **Figure 4**. Mean dry weight of roots at T=13, five weeks after defoliation, as a function of the
673 mean iridoid glycoside (IG) concentration in the leaves of the same *P. lanceolata* plants at T=8,
674 the time of defoliation. Values are mean values for progeny of eight crosses derived from a low-
675 IG line (open symbols) and eight crosses from a high-IG line (closed symbols) grown under
676 nutrient-poor conditions. Note the log-scale of both axes.
677

678 Figure 1

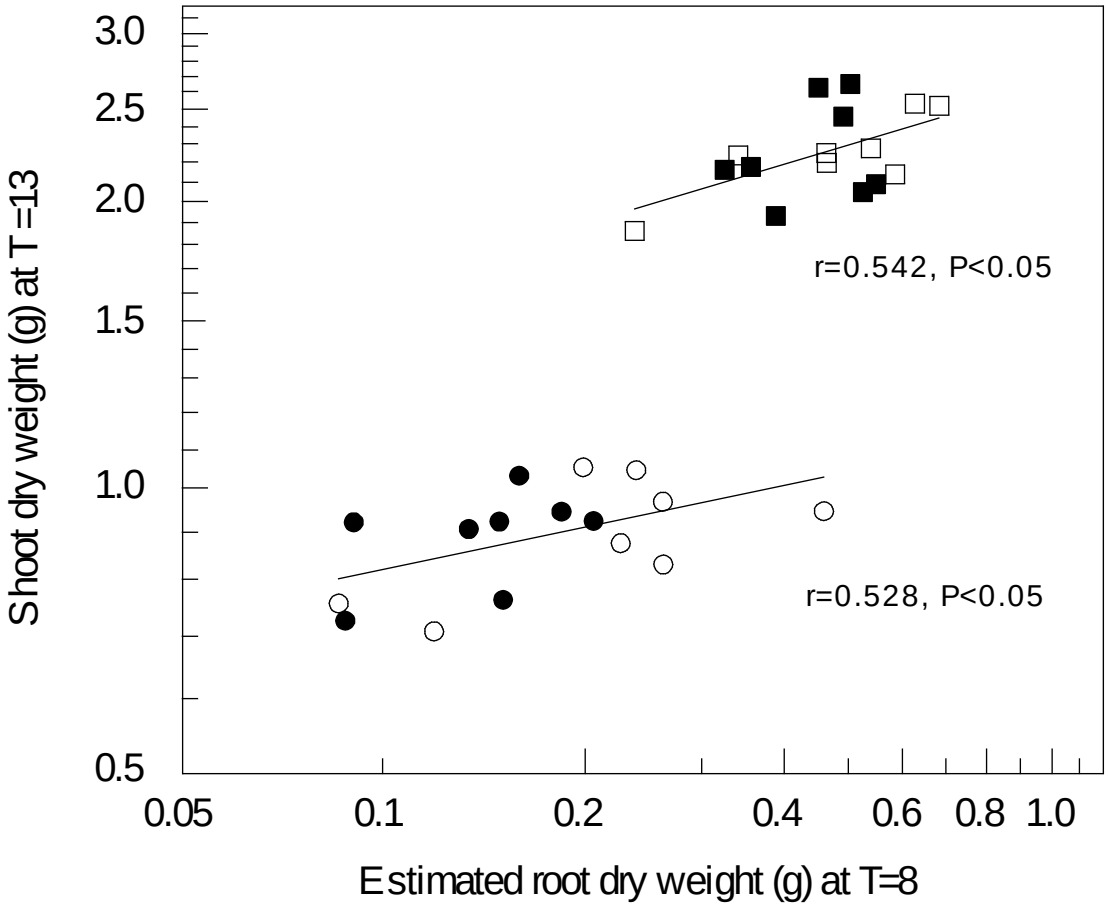


679
680

681 Figure 2
682

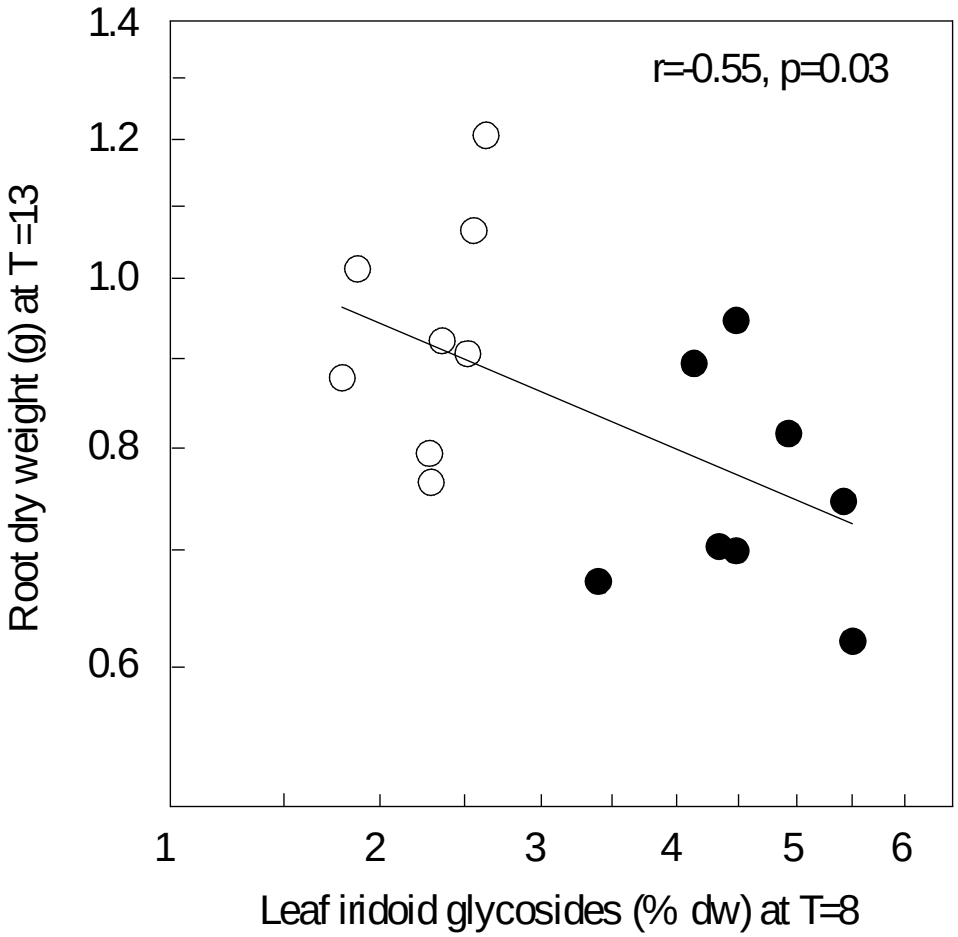


683 Figure 3



684

685 Figure 4



686