Natural hybridization between Senecio jacobaea and Senecio aquaticus : ecological outcomes and evolutionary consequences

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Plant hybridization and secondary metabolite expression: a case study of pyrrolizidine alkaloids in the genus *Senecio*

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Among other consequences in plants, hybridization may influence the expression and evolution of resistance to natural enemies. Plant resistance to parasites, including both microbial pathogens and herbivores, is often mediated by the composition of secondary metabolites expressed by the plant. Here, we focus on pyrrolizidine alkaloids (PAs), a class of secondary metabolites that exhibits high structural diversity among species within the plant genus *Senecio*. We examine PA composition in *Senecio jacobaea*, *S. aquaticus*, artificially generated F_1 hybrids, and also later generation natural hybrids between these two species. We test the hypothesis that hybridization may contribute to PA diversity within plants, by comparing PA expression in hybrids to that in parents across a range of water and nutrient treatments. We report that hybrids produce a putatively novel PA, and that this PA is conserved in natural hybrids, which are highly backcrossed to *S. jacobaea*. Also, the range of PA concentration and diversity is more extreme in the roots and shoots of artificial hybrids over various environmental conditions, and high ranges are preserved in the shoots of natural hybrids. These results suggest that hybridization may partially explain the diversity of PAs found within the *Senecio* genus. Also, hybridization may increase the variation upon which selection can act in hybrid populations, such that some hybrids have the potential to be more resistant to natural enemies than parental individuals.

Key words: hybridisation, pyrrolizidine alkaloids, *Senecio jacobaea*, *Senecio aquaticus*, secondary metabolite diversity, plant resistance

INTRODUCTION

Over the past decade it has become evident that hybridization may play a much larger role in organic evolution than earlier anticipated. Especially in plants, research into the role of hybridization in the evolution of novel traits and new species is gaining momentum (Arnold, 1997; Buerkle, 2000; Rieseberg, 2003; Vellend *et al.*, 2007; Abbott *et al.*, 2008). It is now clear that hybridization can have various roles in plant evolution, from the homogenization of divergent species (McCarthy *et al.*, 1995), to the generation of novel, sometimes adaptive traits (Rieseberg *et al.*, 1999), to speciation (Rieseberg *et al.*, 2003; Seehausen, 2004; Abbot *et al.*, 2008). Yet, it is not yet clear how often hybridization plays an evolutionary role, or under what circumstances hybridization is most likely to be adaptive.

Among other consequences, hybridization may be involved in the evolution of resistance to natural enemies in plants. Many studies, especially in *Eucalyptus* (e.g., Dungey *et al.*, 2000) and willows (Hochwender & Fritz, 2004), have been directed at the effects of plant hybridization on phytophagous insect communities (Whitham *et al.*, 1999). Such studies have reported high variation in expression of secondary metabolites within hybrid zones and classes. Also, resistance among hybrids to herbivores is often highly variable, and hybrid zones may thus support highly diverse herbivore communities (Hochwender & Fritz, 2004; Drew *et al.*, 2005; Bangert *et al.*, 2006).

From a plant's perspective, attack by natural enemies can constitute an enormous selection pressure (Kover & Caicedo, 2001), and hybridization may provide a mechanism for rapid evolution of resistance to parasites, including herbivores and pathogens. Evolution of resistance through hybridization could function via a number of mechanisms. Fritz *et al.* (1994, 1999), showed that F_1 hybrids can demonstrate extreme levels of resistance in relation to parental species. Yet, it was not clear whether extreme resistance could be found in later generation hybrids, and in natural hybrid populations. In 1999, Rieseberg *et al.* showed that transgressive segregation, or the expression of extreme traits in later generation hybrids, occurs in an overwhelming majority of hybrid populations. More recently, Fritz & Hochwender (2004) have shown that F_1 and F_2 hybrids between two willow species have different patterns of resistance against 14 different herbivores, such that resistance to some herbivores is greater in hybrids than parental species.

Increased resistance in hybrids may be attributable to a number of different genetic and molecular processes (Fritz, 1999). Firstly, epistatic interactions between genes combined from both parents may create unique resistance traits, such as unique secondary metabolites. If hybridization can generate entirely new resistance traits, or new combinations of resistance traits to which natural enemies have never been exposed, resistance may be increased, and hybrid lines or hybrid genes may be selectively favored. For example, Ehrlich & Raven (1964) proposed that the diversity of secondary metabolites in plants can be explained if production of novel secondary metabolites increases plant resistance to specialist herbivores, and thus allows plants to escape attack. This hypothesis has received strong support by the wild parsnip and parsnip webworm system (Berenbaum *et al.*, 1989; Berenbaum &

Zangerl, 1998), in which different furanocoumarins have different specificity, and therefore different deterrent effects on the specialist herbivore. Similar effects have been found in a number of systems (Linhart & Thompson, 1999; Shonle & Bergelson, 2000; Adler & Kittelson, 2004; Macel *et al.*, 2005; Albrechtsen *et al.*, 2007). If hybridization leads to the production of novel secondary metabolites, which in turn leads to increased resistance to natural enemies, hybridization may be adaptive.

Also, if resistance traits exhibit higher genetic variation among hybrid individuals than among parental species, hybrids may be more responsive to high selection pressure than pure parental species. This hypothesis has been supported empirically (though not in relation to parasite resistance) in *Drosophila*, for which several studies have shown that variation in abiotic stress resistance can be higher in hybrid lines than in parental lines (Hercus & Hoffman, 1999; Wisseman, 2007), such that hybrids may evolve more quickly in response to stress than parental species.

In general, secondary metabolites provide good examples of anti-herbivore/pathogen systems that can be strongly affected by hybridization. Many classes of secondary metabolites, including alkaloids, flavonoids, terpenoids, etc. are known to mediate resistance to natural enemies. Studies from various plant genera have shown that in both artificial, and later generation natural hybrids, secondary metabolites can be expressed in extreme concentrations in relation to parental species (O'Reilly-Wapstra *et al.*, 2005; Kirk *et al.*, 2005). Furthermore, Orians (2000) showed that hybridization indeed often facilitates the production of secondary metabolites that are novel (not present in either parental species).

The *Senecio* genus is a useful system for studies of hybridization, herbivore resistance, and secondary metabolite diversity. *Senecio* contains more than 1500 species, and hybridization may play an important role in the species diversity within the genus (e.g., Harris & Ingram, 1992; Abbott *et al.*, 2000; Kirk *et al.*, 2004). Moreover, the genus is notorious for the production of pyrrolizidine alkaloids (PAs), secondary metabolites that are highly toxic to generalist mammalian (Cheeke, 1988) and insect (Frei *et al.*, 1992; Macel *et al.*, 2005) herbivores, and which may play a role in pathogen resistance (Hol & van Veen, 2002).

PAs are an interesting and diverse example of secondary metabolites, which occur in a number of families including Asteraceae, Boraginaceae, and Orchidaceae (Hartmann & Witte, 1995). These nitrogen containing compounds have been particularly well studied in the genus *Senecio* (Asteracaea), in which PAs are synthesized as senecionine N-oxide in plant roots (Hartmann & Toppel, 1987; Toppel *et al.*, 1987). Senecionine N-oxide is transported through the phloem to the shoots (Hartmann & Dierich, 1998) where diversification into a number of related PA structures occurs. PA diversification is species specific, such that the suite of PAs within a plant species is generally unique. Furthermore, significant variation in PA composition and concentration within *Senecio* species is observed (Macel *et al.*, 2004). Also, PA composition, including concentration and diversity, can alter in response to plant developmental cues (Schaffner *et al.*, 2003), abiotic environment (Macel *et al.*, 2009), and interactions with natural enemies (Hol *et al.*, 2004).

Senecio jacobaea (Asteraceae) has been particularly well studied, since this species is an invasive pest in North America, Australia, and New Zealand. *Senecio jacobaea* individuals can contain more than 10 PAs (Witte *et al.*, 1992), which play a role in interactions with specialist (Macel & Vrieling, 2003) and generalist herbivores (Macel, 2003), and with root-associated fungi (Hol & van Veen, 2002). Selection pressure by specialist herbivores on *S. jacobaea* may be quite high, since a number of specialist herbivores, including *Tyria jacobaeae* and *Longitarsus jacobaeae*, cause extreme damage to above ground and below ground plant parts.

Senecio jacobaea hybridizes in nature with *Senecio aquaticus* (Kirk *et al.*, 2004), a species that is much less susceptible to specialist herbivore attack in nature (personal observation). *Senecio aquaticus* is usually found in seasonally water-logged, humic, chalk-poor, and nutrient-rich soils (Weeda & Deursen, 1994), while *S. jacobaea* prefers sunny environments with sparse vegetation (Weeda & Deursen, 1994). Natural hybrids, first observed at the Zwanenwater in 1979 (R van der Meijden, personal communication) can be found in a narrow zone spanning a bank at the edge the lake, which appears to be intermediate to parental sites with regards to soil organic content and humidity. Very little overlap seems to occur between the local distributions of these species (personal observation). Genotype × environment interactions may be relevant in this study because *S. jacobaea* and *S. aquaticus* inhabit substantially different abiotic and biotic environments, and environmental factors can have a significant impact on PA expression in at least *S. jacobaea* (Macel, 2003; Hol *et al.*, 2004; Macel & Klinkhamer, 2009).

Here, we investigate the quantitative and qualitative expression of PAs in both natural and artificial hybrids of *S. jacobaea* and *S. aquaticus*. We ask (1) is there evidence that novel PAs are produced in hybrids? (2) Do hybrids express extreme concentrations of PAs compared to parental species? (3) Do hybrids produce a higher diversity of PAs than parents? (4) Do genotype × environment factors affect PA expression of hybrids in relation to parental species.

MATERIALS AND METHODS

Study system

Viable hybrids between *S. jacobaea* L. and *S. aquaticus* Hill have been reported from a number of locations including the United Kingdom (Stace, 1975), Germany (Christian Düring, personal communication), and The Netherlands (Kirk *et al.*, 2004). In this investigation, we studied natural *S. jacobaea* × *S. aquaticus* hybrids from the Zwanenwater reserve (The Netherlands). Composed mostly of sand dunes, the Zwanenwater reserve contains a small lake around which a hybrid population exists (see Kirk *et al.* 2005 for diagram). *Senecio jacobaea* are abundant in the dunes surrounding the lake, while *S. aquaticus* occurs infrequently at the lake fringe. *Senecio jacobaea* is susceptible to severe herbivory by the specialist lepidopteran *T. jacobaeae*, which is stimulated to oviposit by PAs (Macel & Vrieling, 2003). *Senecio aquaticus* is not subject to attack by *T. jacobaeae*, and other specialists that pupate in the soil around the plant, because pupae do not survive in the moist environments

where *S. aquaticus* is found (personal observation). Thrips appear to be common in the *S. aquaticus* population (personal observation). Preliminary tests show that in climate chamber experiments, *S. aquaticus* and *S. jacobaea* are equally susceptible to *T. jacobaeae* (Macel *et al.*, 2002), and the generalist lepidopteran herbivore *Spodoptera exigua* (personal observation), but *S. aquaticus* is much more resistant to the generalist thrips species *Thrips tabaci* than is *S. jacobaea* (Kirk *et al.*, 2005).

Seeds of *S. jacobaea*, *S. aquaticus*, and natural hybrids were collected from plants in the field during 2001 and 2002. Putative hybrids were identified in the field based on leaf lobe and flower morphology, and were later confirmed to be hybrids based on diagnostic amplified fragment length polymorphism (AFLP) markers (Kirk *et al.*, 2004).

F_1 hybrids were produced by collecting second year rosettes of parental plants, exhibiting the development of flowering stems, from the field. To minimize chances that introgressive genes were present in experimental parents, *S. aquaticus* individuals were collected from a marshy agricultural grassland approximately 500 meters from the hybrid zone, and *S. jacobaea* individuals were collected from dunes located approximately 300 meters from the hybrid zone. Plants from both species were placed in a greenhouse, allowed to flower, and were crossed in pairs of *S. jacobaea* × *S. aquaticus* by rubbing flower heads together. Seeds were harvested from both parental plants.

Plant growth

We selected five *S. aquaticus* genotypes, five *S. jacobaea* genotypes, and five natural hybrid genotypes for experimental use. We also included 10 genotypes from F_1 producing crosses, from both parental plants in the reciprocal cross, such that genotypes originated from five crosses, and ten parental plants (five *S. aquaticus* mothers, and five *S. jacobaea* mothers). F_1 genotypes were unrelated to parental genotypes used in this experiment.

One equal sized clonal plantlet from most genotypes was transplanted into each of six experimental columns (1 m length, 15 cm diameter), yielding a total of 136 experimental plants. We aimed at 150 plants but due to variance in sizes and difficulties with cloning 136 plants were used. One plant from each genotype was thus subjected to each of six experimental treatments.

The experiment was established to test a combination of two nutrient and three water treatments. We used sieved dune sand to fill all columns. In half the columns, the dune sand was mixed with 'Osmocote' slow release fertilizer (N:P:K = 15:11:13 + 2 MgO) at a concentration of 1.3 g/L sand to provide a nutrient rich medium. After establishment of seedlings, columns were partially submerged in water of three different depths: 5, 50, and 100 cm.

All columns were given sufficient water at the beginning of the experiment to allow for seedling establishment. Therefore at the beginning of the experiment, soil throughout the total length of the column was moist. Experimental conditions were established two weeks after seedlings were transplanted to columns, and the experiment was subsequently continued for ten weeks. Roots and shoots were harvested separately, and were dried in an oven for three days at 50 °C.

PA extraction

All dried leaves and roots from each plant were separately milled to a fine powder and homogenized. Milled samples were stored in a freezer at –80 °C until use. For extraction, approximately 15 mg of plant material was extracted according to a modified version (de Boer, 1999) of the acid-base extraction method (Hartmann & Zimmer, 1986). Extractions were dissolved in methanol containing heliotrine (Latoxan, France) as an internal standard and analysed using gas chromatography (GC). Conditions (injector 250 °C, temperature programme 0-22-5-250, split mode 1-30, carrier gas N_2 0.9 ml min^{-1}, pressure 56 kPa; detector NPD) were controlled by a Hewlett Packard gas chromatographer (model 6890). GC traces were compared with known references to identify sample composition.

Statistical analysis

We tested PA concentrations and diversity separately for roots and shoots, since PAs may interact both with root and with shoot pathogens and herbivores. We quantified PA diversity by counting the number of PAs present in each sample. Differences in both diversity and total PA concentration were analyzed using ANOVAs, for which we identified water, nutrient and plant group's treatments as fixed factors. Plant group denotes the both parental species, the F_1 and the natural hybrids. Shoot to root ratio was used as a covariate because it is assumed that root biomass is proportional to PA production and the size of the shoots is not related to PA production. Relatively larger shoots therefore may lead to a dilution of PAs. We first tested for maternal effect on PA expression in F_1 hybrids, by including only F_1 hybrids in the analysis. Since we found that maternal parent was never a significant factor in the analyses, we treated all F_1 hybrids as one group for subsequent ANOVAs using the entire data set.

We also applied principal component analysis (PCA) to quantitative PA data. PCA reduces a large number of variables into a smaller number of uncorrelated principal components (PCs), while preserving most of the variance in the data set. We applied ANOVAs to each PC, to test whether nutrient and water treatments and plant group had significant effects on PA composition. We then identified individual PAs that were highly correlated with each PC.

RESULTS

Eleven PAs were identified during our analysis (Table 1). We found eight PAs in *S. jacobaea* (eight in the shoots, and eight in the roots). Additionally, PA1 was present in low amounts in two plants of *S. jacobaea* and florosenine in one plant. To our knowledge, neither of these PAs have previously been reported from *S. jacobaea* individuals, and may represent PAs introgressed from the Zwanenwater hybrid zone. Ten PAs were present in *S. aquaticus*, of which otosenine appeared to be specific to *S. aquaticus* compared to *S. jacobaea* although it is also rare in *S. aquaticus*. In contrast to findings for *S. jacobaea*, PA1 is almost always present in *S. aquaticus* shoots. The reverse is true for jacobine. All eleven PAs were present among both natural

Table 1 Presence of 11 pyrrolizidine alkaloids in the roots (R), and shoots (Sh) of *S. jacobaea*, *S. aquaticus*, natural hybrid, and F_1 hybrid genotypes

Retention time	Alkaloid	*Senecio jacobaea*	*Senecio aquaticus*	Natural hybrid	F_1 hybrid
3.97	PA1	Sh/R	Sh/R	Sh/R	Sh/R
7.07	Senecivernine	Sh/R	Sh/R	Sh/R	Sh/R
8.015	Senecionine	Sh/R	Sh/R	ShR	Sh/R
8.257	Seneciphylline	Sh/R	Sh/R	Sh/R	Sh/R
8.874	Integerrimine	Sh/R	Sh/R	Sh/R	Sh/R
10.386	Jacobine	Sh/R	Sh	Sh/R	Sh/R
10.852	Jacoline	Sh/R	Sh/R	Sh/R	Sh/R
11.30	PA2	R	Sh/R	Sh	R
12.01	Erucifoline	Sh/R	Sh/R	Sh/R	Sh/R
12.50	Otosenine		R	R	R
14.31	Florosenine	R		Sh/R	Sh

hybrid and artificial hybrid genotypes. Florosenine (Fig. 1) appeared to be specific to hybrids, though trace amounts were expressed by one *S. jacobaea* plant.

Some hybrid individuals had extreme PA concentrations compared to parents, though on average, PA concentrations were not extreme in hybrids compared to parental species (Table 2). There was no significant effect of, or interaction involving plant group on either shoot or root PA concentrations (Table 3, Fig. 2). PA concentrations in *S. jacobaea* and in the natural hybrids were very similar in both roots and shoots. In wet sandy conditions low PA concentrations in the shoot were found as indicated by the significant main effect of water and the interaction between nutrients and water. The roots in sandy soils have higher PA concentrations. Generally, PA concentrations in the roots are lower in dryer soils (Table 3, Fig. 2).

PA diversity in the shoots differed significantly among groups. Averaged over all treaments the number of PAs per plant (± SE) were respectively, 3.96 ± 0.35, 3.59 ± 0.33, 4.13 ± 0.33, 4.80 ± 0.24 for *S. jacobaea*, *S aquaticus*, natural hybrids and F_1 hybrids. In shoots, PA diversity was affected by an interaction between nutrient and water treatments and interactions between nutrient treatment and plant group (Table 2 and 4). The latter interaction is mainly caused by extreme low diversity of *S. aquaticus* in medium and dry nutrient rich conditions. In the roots, water was the only significant factor; PA diversity was higher in wetter conditions (Fig. 3).

PCA yielded two components (Table 5) for PAs in plant shoots, which cumulatively explained 85.6% of the variation in PA expression. The first component mostly explained variation in the least metabolically derived alkaloids identified in this experiment (i.e., significantly correlated with) senecionine (r = 0.68), seneciphylline

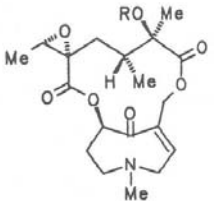

Figure 1 Chemical structures of otosenine (R = H) and florosenine (R = Ac).

Table 2 Effect of nutrient and water treatment, and plant group (fixed factors) on pyrrolizidine alkaloid concentration in the roots and shoots of *S. jacobaea*, *S. aquaticus*, natural hybrids, and F_1 hybrids. Shoot to root ratio (SRratio) of the plant was used as a covariate

Source	Type III SS	df	MS	*F*	*P*
Shoots					
Covariate SRratio	0.640	1	0.640	1.528	0.219
group	0.726	3	0.242	0.578	0.631
nutrient	1.465	1	1.465	3.497	0.063
water	6.632	2	3.316	7.917	0.001
group*nutrient	0.408	3	0.136	0.325	0.807
group*water	4.152	6	0.692	1.652	0.140
nutrient*water	10.123	2	5.062	12.085	0.000
group*nutrient*water	2.846	6	0.474	1.132	0.348
Error	46.490	111	0.419		
Roots					
Covariate SRratio	0.794	1	0.794	1.292	0.258
group	4.059	3	1.353	2.202	0.092
nutrient	6.884	1	6.884	11.204	0.001
water	6.549	2	3.275	5.329	0.006
group*nutrient	1.900	3	0.633	1.031	0.382
group*water	1.507	6	0.251	0.409	0.872
nutrient*water	0.639	2	0.320	0.520	0.596
group*nutrient*water	7.858	6	1.310	2.132	0.055
Error	68.203	111	0.614		

Table 3 Effect of nutrient and water treatment, and plant group (fixed factors) on pyrrolizidine alkaloid diversity in the roots and shoots of *S. jacobaea*, *S. aquaticus*, natural hybrids, and F_1 hybrids. Shoot to root ratio (SRratio) of the plant was used as a covariate

Source	Type III SS	df	MS	*F*	*P*
Shoots					
Covariate SRratio	1.758	1	1.758	0.577	0.449
group	30.530	3	10.177	3.339	0.022
nutrient	0.439	1	0.439	0.144	0.705
water	5.421	2	2.711	0.889	0.414
group*nutrient	31.988	3	10.663	3.499	0.018
group*water	24.382	6	4.064	1.333	0.248
nutrient*water	67.319	2	33.660	11.044	0.000
group*nutrient*water	8.652	6	1.442	0.473	0.827
Error	338.292	111	3.048		
Root					
Covariate SRratio	2.364	1	2.364	0.806	0.371
group	28.433	3	9.478	3.233	0.025
nutrient	1.727	1	1.727	0.589	0.444
water	42.868	2	21.434	7.312	0.001
group*nutrient	22.180	3	7.393	2.522	0.061
group*water	21.237	6	3.539	1.207	0.308
nutrient*water	1.975	2	0.988	0.337	0.715
group*nutrient*water	7.419	6	1.236	0.422	0.863
Error	325.386	111	2.931		

(*r* = 0.99) and integerimine (*r* = 0.74) and to a lesser extend variation in jacobine (*r* = 0.21), jacoline (*r* = 0.47) and erucifoline (*r* = 0.38) (Table 5). The second component explained variation in jacobine (*r* = 0.98), florosenine (*r* = 0.44), PA1 (*r* = -0.29) and senecionine (*r* = -0.31) (Table 5). Component 1 was significantly affected by all abiotic conditions and their interaction. Scores for component 1 were higher under sandy conditions and were low in wet and high in intermediate wet conditions. The second component (PC2) was significantly affected by group with the parents showing the most extreme scores (*S. jacobaea* 0.34 ± 0.17a), *S. aquaticus* (-0.61 ± 0.17b) and hybrids intermediate (natural hybrids -0.04 ± 0.17b, F_1 hybrids 0.16 ± 0.12a)

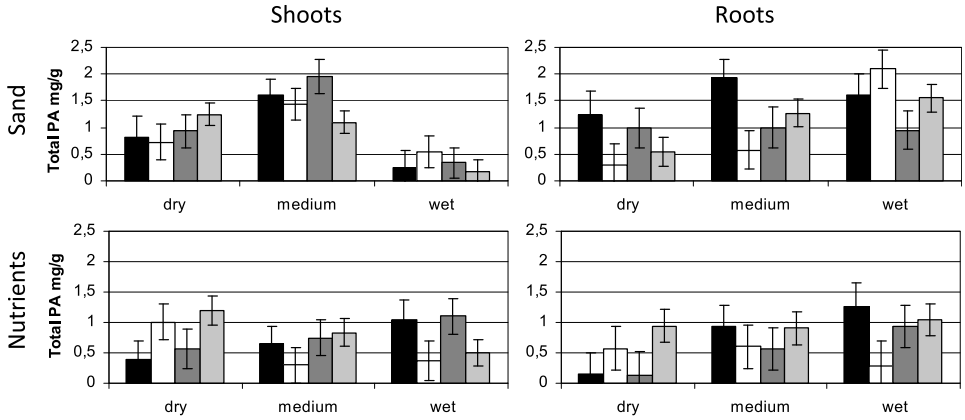

Figure 2 PA concentration in the shoots and roots of *S. jacobaea* (black), *S. aquaticus* (white), natural hybrids (dark grey), and F_1 hybrids (light grey) in a factorial design with two nutrient levels and three water levels. Vertical bars represent standard error.

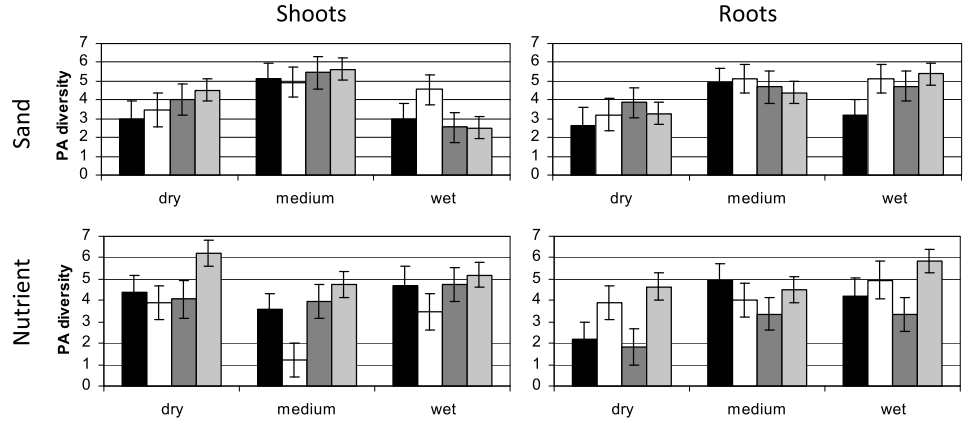

Figure 3 PA diversity, defined as the number of PAs detected within each sample, in the shoots and roots of *S. jacobaea* (black), *S. aquaticus* (white), natural hybrids (dark grey), and F_1 hybrids (light grey) in a factorial design with two nutrient levels and three water levels. Vertical bars represent standard error.

Table 4 Range of PA diversity and concentration among individuals within plant groups. Bold numbers in natural and F_1 hybrids indicate values that are extreme in relation to both parental ranges

Treatment	*S. jacobaea*	*S. aquaticus*	Natural hybrid	F_1 hybrid
Shoot diversity				
Dry sand	2-4	1-8	**0**-5	2-8
Medium sand	4-6	4-6	**3**-**8**	4-**8**
Wet sand	2-4	1-5	2-**6**	2-**8**
Dry nutrients	4-6	3-4	3-**7**	**2**-**8**
Medium nutrients	1-6	0-2	3-5	2-**8**
Wet nutrients	3-6	3-5	3-5	**1**-**10**
Shoot concentration				
Dry sand	0.03-0.51	0.09-1.79	**0**-1.45	**0**-1.02
Medium sand	0.79-1.85	0.49-2.39	**0.23**-**3.45**	**0.21**-**3.26**
Wet sand	0.60-1.26	0.12-1.54	0.16-**2.35**	0.27-**2.29**
Dry nutrients	0.25-0.91	0.23-0.57	0.31-**2.64**	**0.16**-0.91
Medium nutrients	0.25-1.46	0.10-0.52	0.45-1.16	0.23-**2.44**
Wet nutrients	0.03-0.82	0.53-1.62	0.27-0.96	**0.02**-**3.02**
Root diversity				
Dry sand	0-5	4-7	4-7	2-**8**
Medium sand	4-6	3-7	3-7	3-7
Wet sand	2-3	2-4	2-**5**	2-**7**
Dry nutrients	3-5	4-6	**0**-5	4-**8**
Medium nutrients	3-7	2-7	2-5	**0**-**9**
Wet nutrients	0-7	3-4	0-3	2-**8**
Root concentration				
Dry sand	0-3.03	0.60-4.69	0.35-2.23	0.35-3.16
Medium sand	1.10-3.36	0.10-0.85	0.43-1.59	0.57-2.91
Wet sand	0.09-3.01	0.29-0.48	0.24-2.41	**0.04**-1.86
Dry nutrients	0.36-1.98	0.21-0.40	**0**-1.80	0.23-**2.18**
Medium nutrients	0.05-1.82	0.01-2.06	0.05-1.63	**0**-**2.31**
Wet nutrients	0-0.49	0.02-1.19	0-0.28	0.11-**2.18**

(different letters indicate significant differences). Another significant main effect was found for nutrients with scores for nutrient rich conditions (0.31 ± 0.11) higher than for sandy soils (-0.38 ± 0.11).

Overall PA composition in the shoots was often extreme in both natural and artificial hybrids, as revealed by extreme PC scores from the PCA (Fig. 4).

Two PCs accounted for 88.9% of the variation in root PA expression. Like in the shoots, the first component mostly explained variation in the least metabolically derived alkaloids (i.e., senecionine (r = 0.95), seneciphylline (r = 0.66) and integerimine (r = 0.77)) and to a lesser extend variation in jacoline (r = 0.47) and erucifoline (r = 0.25) (Table 5). The second component explained variation in senecionine (r = -0.31), seneciphylline (r = 0.75), integerimine (r = 0.26), jacoline (r = 0.6), PA2 (r = 0.5) and erucifoline (r = 0.48). All three main effects (group, water, nutrients) were significant for component 1. Again parents showed the most extreme scores (*S.*

Table 5 Effects of nutrient treatment, water treatment, and plant group on principal components (PC) that are correlated with PA expression in roots and shoots

	PC	% variation[1]	Correlated PAs[2]	Significant factors ($P < 0.05$)[3]
Shoots	1	53.0	(+)Senecionine	Nutrient
			(+)Seneciphyllin	Water
			(+)Integerrimine	Water*nutrient
			(+)Jacobine	
			(+)Jacoline	
			(+)Erucifoline	
	2	32.6	(-)PA1	Group
			(-)Senecionine	Nutrient
			(+)Jacobine	
			(+)Florosenine	
Roots	1	64.7	(+)Senecionine	Group
			(+)Seneciphyllin	Water
			(+)Integerrimine	Nutrient
			(+)Jacoline	
			(+)Erucifoline	
	2	24.2	(-)Senecionine	Group
			(+)Seneciphyllin	Nutrient
			(+)Integerrimine	Water*nutrient
			(+)Jacoline	
			(+)PA2	
			(+)Erucifoline	

[1]Indicates percent of total PA variation explained by each PC.
[2]Indicates PAs that are highly correlated with each PC ($P < 0.05$).
[3]Significant factors represent single factors and/or interactions that had significant effects on PC values in ANOVA analyses.

jacobaea 0.53 ± 0.18a, *S. aquaticus* -0.31 ± 0.17b) and hybrids intermediate (natural hybrids -0.21 ± 0.17b, F_1 hybrids 0.01 ± 0.12ab) (different letters indicate significant differences). Scores for component 1 decreased with dryer conditions and were lower in nutrient rich soils. Component 2 showed significant mains effects of group and nutrients and a significant interaction of water and nutrients. For 'group' the parents showed the most extreme scores (*S. jacobaea* -0.84 ± 0.17a, *S. aquaticus* 0.42 ± 0.16b) and hybrids intermediate (natural hybrids -0.01 ± 0.16b, F_1 hybrids 0.15 ± 0.11b) (different letters indicate significant differences).

Analysis of PC1 and PC2 for roots and shoots yield that group factor is significant in both shoots and roots indicating that PA diversity is influenced by the group factor. In contrast total PA concentration is not influenced by plant group (Table 3).

DISCUSSION

It has recently been stated that the possibility that 'synergistic host resistance result[ing] from formation of F_2 hybrids... is unlikely because recombination is not predicted to enhance plant defences against herbivores' (Hochwender & Fritz, 2004). This statement may be inaccurate if epistatic interactions lead to the forma-

tion of novel structural variations of secondary metabolites, to which herbivores are unadapted. We cannot say with 100% certainty that novel secondary metabolites arose from hybridization here. However, our findings strongly indicate that hybridization may indeed lead to unique patterns of secondary metabolite expression, which may very well lead to increased resistance to herbivores after recombination.

Both F_1 and natural hybrids produce the PA florosenine, which may be a novel product of hybridization in the Zwanenwater population. Florosenine has been previously reported from the South American *Senecio* species *S. glaber* (Reina *et al.*, 1993) and *S. leptolobus* (Mendez *et al.*, 1990), as well as from a Swiss population of *S. aquaticus* (Pelser *et al.*, 2005). Florosenine was never found in *S. aquaticus* from the Zwanenwater nature reserve during this current study, or during previous (Kirk *et al.*, 2004) studies. Trace amounts of florosenine were found in one *S. jacobaea* individual here, but this occurrence may represent introgression, since this PA has never been reported from other *S. jacobaea* populations, and was not found in either Zwanenwater *S. jacobaea* genotypes, or control populations from a variety of European locations analysed in a previous study (Kirk *et al.*, 2004).

Florosenine is the O-acetyl derivative of otosenine. Mechanistically, it is possible that hybridization combines the ability of *S. jacobaea* to acetylate PAs, and the ability of *S. aquaticus* to synthesize otosenine. If such inter-specific epistatic interactions between enzymes and substrates can result in the production of unique PA structures, then hybridization within the genus *Senecio* may be a mechanism for structural PA diversification

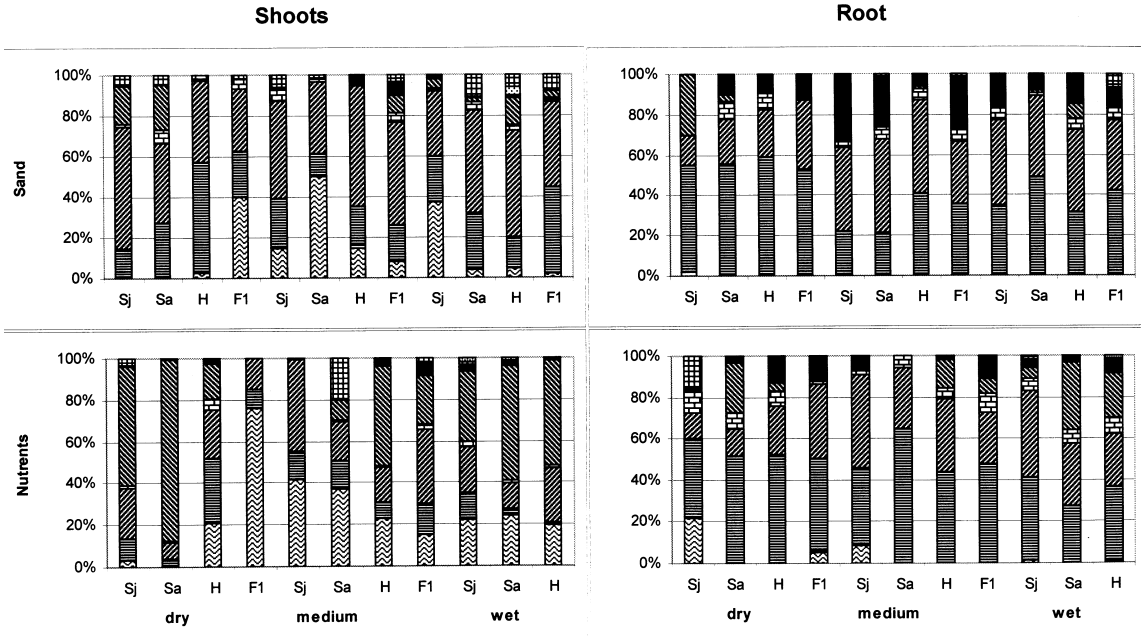

Figure 4 Relative PA composition in the shoots and roots of *S. jacobaea*, *S. aquaticus*, natural hybrids, and F_1 hybrids in a factorial design with two nutrient levels and three water levels.

Regardless of whether florosenine is a novel PA resulting from hybridization, hybridization clearly alters patterns of PA expression in hybrids compared to parental individuals. On average, hybridization did not lead to higher PA concentration and diversity within individual hybrids compared to parental individuals. Yet, there was greater variation in PA concentration and diversity in artificial hybrids, and this variation seemed to be preserved in the shoots, though not in the roots of natural hybrid individuals. This high variation suggests that there is more adaptive potential among both natural and artificial hybrids than in parental species.

Interestingly the ANOVA's of the factors of the PCA analyses (Table 5) show that the natural hybrids which are backcrossed several times to *S. jacobaea* (Kirk *et al.*, 2004) are with regard to PA diversity significantly different from *S. jacobaea* but not from *S. aquaticus*. The F_1s are in all cases more similar to *S. jacobaea*. This suggests that PA diversity is under the influence of natural selection.

Although it is not yet clear whether increased PA diversity is adaptive, hybridization may provide a partial explanation for the high diversity of PAs within *Senecio*, even if such increased diversity is selectively neutral. In order to test whether unique PA patterns generated by hybridization provide selective advantages to plants, it will be interested to test whether hybrid individuals that express unique PA compositions are more resistant to natural enemies than parental individuals.

High variation in PA expression within hybrid classes supports the conclusion that it most useful to study hybrid ecology and evolution by focusing on genotypic differences between individuals in hybrid populations, rather than fitness differ-

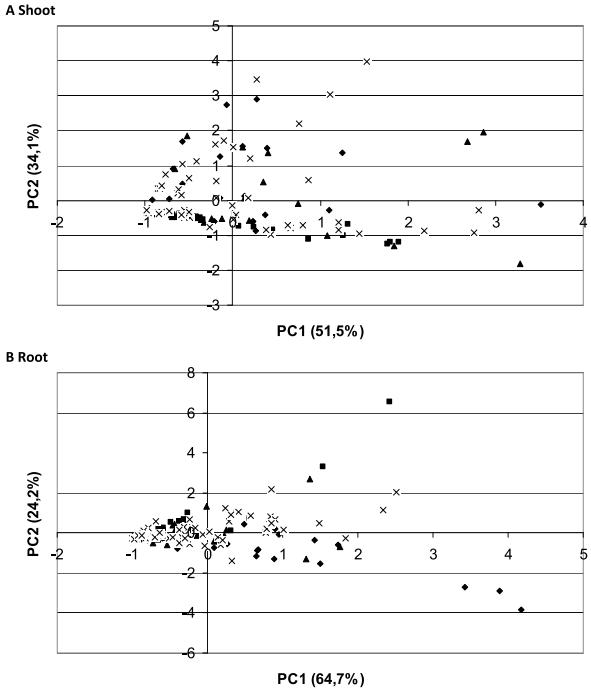

Figure 5 Plots of the two principal components accounting for most of the variation in PA composition in the shoots (A) and roots (B) of in a factorial design with two nutrient levels and three water levels. *Senecio jacobaea* (diamonds), *S. aquaticus* (squares), natural hybrids (triangles) and F_1 hybrids (crosses) are shown.

ences between hybrid classes (i.e., F_1, F_2, BC, etc.). Variation between hybrid individuals can be extremely high, and the fate of hybrid individuals and/or hybrid gene combinations may differ widely. In future studies, evaluating natural selection on different hybrid genotypes possessing different combinations of PAs may be extremely useful for elucidating the role of PA diversity in plants (i.e., Lexer *et al.*, 2003).

Also, hybridization between *S. jacobaea* and *S. aquaticus* may have different effects on herbivore/pathogen resistance in different environments, since PA concentrations are plastic depending species-specific reaction to soil nutrient and water content. For example, natural hybrids seemed to have lower PA concentrations than parental species in nutrient rich soils, which may reflect negative selection on PA concentration in nutrient rich habitats. It is well known that abiotic environmental factors such as light, water, and nutrient availability can alter the defensive ability of plants with regard to insect and pathogen resistance (Kytö *et al.*, 1996; Glynn *et al.*, 2003). Also, different pathogens and herbivores are present in different environments, and may thus constitute significantly different selection pressures in differing habitats. Researchers studying the effects of hybridization on secondary metabolite expression should incorporate abiotic factors occurring in parental and hybrid habitats, especially when abiotic factors differ greatly across habitats.

Overall, our findings indicate that hybridization can putatively lead to the production of novel PAs, and to the expression of extreme and/or unique PA compositions within hybrid individuals. Environmental conditions may play an important role in the resistance of hybrids to natural enemies, since species interact with environmental condition to determine expression of secondary metabolites. Though PAs are clearly involved with plant-parasite interactions, the consequences of increased PA diversity, concentration, and novelty through hybridization are not yet understood. Research into the ecological and adaptive consequences of unique PA patterns in hybrids may lead to an increased understanding of the role of hybridization in the evolution of plant resistance.

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