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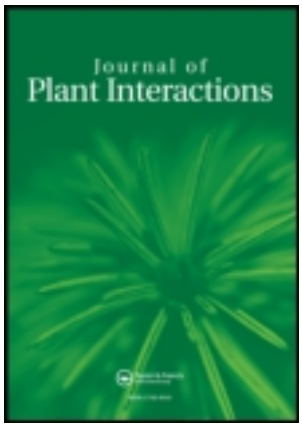

Journal of Plant Interactions
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tjpi20

Changes in Senecio grisebachii pyrrolizidine alkaloids abundances and profiles as response to soil quality

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Accepted author version posted online: 21 Jun 2011.Published online: 27 Jun 2011.

To cite this article: Margarita A. Yaber Grass & Silvia R. Leicach (2012) Changes in Senecio grisebachii pyrrolizidine alkaloids abundances and profiles as response to soil quality, Journal of Plant Interactions, 7:2, 175-182, DOI: 10.1080/17429145.2011.591504

To link to this article: http://dx.doi.org/10.1080/17429145.2011.591504

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Journal of Plant Interactions
Vol. 7, No. 2, June 2012, 175–182

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ORIGINAL ARTICLE

Changes in *Senecio grisebachii* pyrrolizidine alkaloids abundances and profiles as response to soil quality

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(Received 30 April 2011; final version received 23 May 2011)

Senecio grisebachii Baker is an invasive weed considered to be toxic due to the presence of pyrrolizidine alkaloids (PA) in its tissues. The PA production by *S. grisebachii* aerial parts was evaluated in samples grown in two Argentinean Rolling Pampa fields with the same kind of soil but differing in the length of their exploitation period by conventional tillage practices and, consequently, in their deterioration level.

We found significant differences in the relative concentrations of seven alkaloids between samples taken from the two fields. Seneciphylline was the most abundant alkaloid in inflorescences from less deteriorated soil (LD) while senecionine was the major one in those from highly deteriorated soil (D) being followed by seneciphylline, integerrimine, and minor amounts of spartiodine, jacobine, jacozine and retrorsine. A significant increase in total alkaloid content (TAC) was observed in inflorescences from samples growing in D soil (3.52 ± 0.20 mg/g DW) when comparing with those from samples grown in LD one (3.23 ± 0.26).

Keywords: *Senecio grisebachii*; pyrrolizidine alkaloids; soil deterioration

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Introduction

Plant-environment interactions can induce changes on the relative allocation of resources to primary and secondary metabolites pathways, and agricultural practices have been demonstrated to affect the abiotic and biotic environmental characteristics determining them. Intensive exploitation of Argentinean Rolling Pampa cropping fields by conventional tillage practices during the last decades have resulted in deleterious effects on soil physicochemical and biological properties (de la Fuente et al. 2003). It has seriously affected soil quality increasing compaction, lowering porosity, and leading to lower values in soil aggregates diameter, organic matter content and nutrient availability. The levels of soil deterioration have been correlated to the length of the agricultural history (Maddonni et al. 1999, 2000). It has been demonstrated that organic carbon, total nitrogen, extractable phosphorus, pH, aggregates mean weighted diameter (directly associated to soil aggregate stability), and infiltration rate are good indicators for soil quality. However, aggregates mean weighted diameter and extractable phosphorus seem to be the most critical abiotic parameters to be considered when evaluating soil deterioration (Uricarriet and Lavado 1999).

In this scenario, different species within weed communities, *Senecio grisebachii* among them, have developed a considerable adaptability towards agroecosystems changes leading to the colonization of Rolling Pampa fields, particularly those areas undergoing continuous agricultural exploitation (Suárez et al. 2001). Their easy adjustment to environmental changes includes the development of resistance towards different control management practices (Leguizamón 2007).

Pyrrolizidine alkaloids (PA), a group of nitrogenated secondary metabolites chemically classified as esters of necic acid and 1-hydroxymethyl dehydropyrrolizidine, are found in several families including Asteraceae (*Senecio*), Fabaceae (*Crotalaria*) and Boraginaceae (*Heliotroprium*, *Trichodesma* and *Symphytum*). More than 360 PA present as free bases or *N*-oxides have been identified in over 6000 species (Stegelmeier et al. 1999). These compounds represent a significant carcinogenic hazard to mammals, mankind included, that directly consume plant material or related foodstuffs, such as honey produced by bees that collect nectar from the inflorescences of producing species or flours obtained from grains grown in invaded crop fields (Mattocks 1986; Winter and Segall 1989; Cheeke 1989; Röder 1995).

Cyclic diesters such as retrorsine are the most toxic with an oral LD_{50} value of 34 mg/kg (Huxtable 1989). The first recorded example of human disease caused by their hepatotoxic activity was reported in South Africa, where multiple cases of cirrhosis occurred following consumption of bread made from flour contaminated with *S. grisebachii* Baker seeds (Willmot and Robertson 1920). Humans are exposed to PA not only through consumption of contaminated grains, honey, milk, offal and eggs but

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ISSN 1742-9145 print/ISSN 1742-9153 online
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http://dx.doi.org/10.1080/17429145.2011.591504
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also through plant species used in herbal medicine and dietary supplements (Gaul et al. 1994).

The presence of PA contaminating honey represents a serious problem to beehives in Argentine, seriously affecting its commercialization. The fact that *S. grisebachii* is now a common weed in crop lands near beehives, suggests that it may contribute to the contamination of honey- and grain-derived foodstuffs, particularly if its chemical defenses production can be modulated by the soil deterioration that characterizes these fields. Increments in defensive chemicals have also proved to occur in other common weeds (Gil et al. 2002; de la Fuente et al. 2003; Leicach et al. 2003; Chludil et al. 2008).

The primary precursor in the PA pathway in *Senecio* species is the water soluble senecionine *N*-oxide, which represents their backbone structure (Hartmann and Ober 2000). It is translocated via the phloem into the shoots (Hartmann et al. 1989), where it is transformed into the derivatives that characterize each species PA profile, and stored in vacuoles of inflorescences cells and peripheral stem tissues. Since PA do not undergo turnover or degradation (Ehmke et al. 1988; Hartmann and Dierich 1998), their localization contributes to demonstrate their potential role as chemical defenses (Hartmann 1999). The PA content depends on the species, some of them (*S. boissier* and *S. abrotanifolius*) exhibit low concentrations (0.1 and 0.9 mg/g DW, respectively) in their inflorescences, others (*S. jacobaea* and *S. leucophyllus*) accumulate large amounts (2.2 and 4.3 mg/g DW, respectively; Macel et al. 2004; Pelser et al. 2005).

Insects from various non-related taxa sequester PA and maintain them as *N*-oxides (less toxic than the free bases) as a way to defend themselves from predators. The PA toxicity is bioactivated by microsomal liver cytochrome P-450 enzymes into unstable pyrrolic intermediates that are known as reactive alkylating agents (Winter and Segall 1989). In fact, *N*-oxidation is a detoxification mechanism to avoid its occurrence (Cheeke 1994).

The aim of this work was to evaluate changes in PA concentrations and profiles in *S. grisebachii* aerial parts from samples grown in two Rolling Pampa fields with two different soils deterioration levels, differentiating inflorescences from leaves and stems.

Material and methods

Plant samples

The *S. grisebachii* Baker cuttings were obtained from randomly selected plants within a weed population growing in San Pedro Experimental Station fields, National Institute of Agricultural Technology (INTA) in Argentina. Cuttings were placed in containers filled with sterile earth and supplied with ample water in order to promote root development. After a week, 10 cm tall plantlets were transplanted (one per pot) to plastic containers (15 cm depth, 20 cm diameter) filled with the corresponding soil sample. A voucher specimen was deposited with the No. 17431 in the Herbarium 'Gaspar Xuarez', Facultad de Agronomía, UBA, Buenos Aires, Argentina (BAA).

Soil samples

Twenty soil samples were collected to a depth of 12 cm, from two plots in a field located in Buenos Aires, Argentina (31°44′S, 60°32′W), characterized by silty clay loam soil (Typic Argiudoll) corresponding to Rojas soil series (INTA 1974). Two locations were chosen based on the different length of their cropping history, one continuously submitted to conventional tillage practices for 15 years, D (deteriorated soil), and the other for 10 years, less deteriorated soil (LD). Five years prior to soil sample collections both plots were submitted to a soybean/corn rotation using direct seeding with the addition of fertilizers and herbicides to control weeds. Soil analysis of samples (performed by Laboratory of Soil Analysis, School of Agronomy, University of Buenos Aires) from both plots indicated significant differences in their degradation level.

Treatments

Bioassay started at the beginning of winter in a greenhouse. Forty pots containing the soil samples were kept in a non-controlled environment chamber, maintaining their maximum field-capacity, and rotating their positions to make sure that plants received similar light distribution. Plants were randomly allocated to grow in the two soil qualities (LD or D). Weeds were hand-controlled; no pesticides were used to avoid interferences with the study. By the beginning of spring plants grew up to flowering stage and were harvested 45 days later. Inflorescences were separated from leaves and stems, and plant materials were oven-dried at 40°C under ventilation to constant weight.

Phytochemical analysis

Analytical solvents were purchased from Sintorgan (Chemical Center SRL, Argentina). Kieselgel 60 F_{254} TLC aluminum sheets for thin layer chromatography were purchased from Merck (Research AG, Buenos Aires, Argentina). A commercial sample of Senecionine was purchased from Sigma-Aldrich, Argentina. Two g of dry aerial material (inflorescences or leaves and stems), were milled and submitted to continuous extraction with methanol (4 h) using a Soxhlet apparatus. Methanol crude extract, which contained lipidic material besides PA, was evaporated to dryness under reduced pressure at 40°C, and kept under vacuum conditions until constant weight. The dry residue was suspended in 5% HCl up to pH 1 and washed with $CHCl_3$ (3×30 mL) to remove lipids and

pigments. Since PA can be present as free bases or *N*-oxides, the defatted aqueous solution was reduced with an excess of zinc dust overnight to transform *N*-oxides into free bases. Twenty-five percent NH_4OH was added to the solution obtained by filtration, raising pH value up to 11, and then extracted with $CHCl_3$ (3×30 mL). Chloroformic extracts were dried over Na_2SO_4, evaporated to dryness, and kept at $-18°C$.

Alkaloids were characterized by thin layer chromatography on Kieselgel 60 F_{254} TLC aluminum sheets using CH_2Cl_2: MeOH: 25% NH_4OH (85:14:1, v/v/v) as mobile phase. Chromatograms were visualized by UV-light at 254 nm and by chromogenic reaction with Draggendorff reagent.

Gas chromatography analysis

A weighted fraction of each PA dry extract was dissolved in $CHCl_3$ and analyzed by gas chromatography (GC) using a Hewlett-Packard gas chromatograph 5890 A (Avondale, PA), equipped with a HP-Ultra 2 capillary column (50 m, i.d.: 0.32 mm, film thickness: 0.25 μm) (Agilent Technologies Inc.) and a flame ionization detector (FID). Samples were injected (1 μL) with split mode (split ratio, 1:20). The injector temperature was 250°C and the detector temperature 280°C. Helium was used as carrier gas with a flow rate of 1.0 mL/min. Temperature program: 150°C isothermal for 2 min, 150–260°C at a rate of 4°C/min, then 260°C isothermal for 15 min. Hexadecane was used as internal standard. The response factor of an authentic sample of senecionine was near unity. Total alkaloid content (TAC) and individual relative abundances were calculated based on alkaloid peak areas in relation to that of the internal standard. Each value was then referred to the corresponding weighted dry matter.

Gas chromatography – mass spectrometry analysis

The GC-MS analysis was performed on a Shimadzu GC-17A chromatograph (Shimadzu Corporation, Kyoto, Japan) coupled with a CMS-QP 5050A spectrometer with the same column and conditions used to perform GC analysis, except the He flow rate that was 1.6 mL/min. Ionization voltage: 70 eV, mass range: m/z 40–400. The PA retention indices were determined relative to a homologous series of *n*-alkanes C_7–C_{25}. Identification of individual alkaloids was based on their retention indices by comparison of their molecular ions and fragmentations patterns with those from the spectrometer software (NIST Mass Spectral Database MS Search v. 1.6d. 98.L L) and confirmed by comparison with literature data.

Data and statistical analysis

Statistical methods were performed by means of InfoStat/Professional software, 1.1 version (Universidad Nacional de Córdoba, Argentina, 2002). Tests of normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene test) were applied in each analysis. Data were evaluated by analysis of variance (ANOVA) and differences between means were compared using Tukey's test with a 0.05 confidence interval in a completely randomized design with 10 repetitions and two treatments: LD and highly deteriorated soil (D). Numerical data are accompanied by standard deviation. Significant differences between samples from the two treatments were further visualized performing principal component analysis (PCA).

Results

Table 1 shows data corresponding to soil analysis of the two samples, summarizing the parameters related to their degradation levels. As was expected, samples from the plot characterized by highly deteriorated soil (D) presented lower values in mean weighted diameter of soil aggregates, extractable phosphorus, organic carbon, total nitrogen, and pH, than those corresponding to the less deteriorated one (LD).

Separation and identification of alkaloids

Alkaloids were characterized by TLC, by means of the chromogenic reaction with Draggendorff reagent. The R_F values of senecionine and retrorsine (0.60 and 0.28, respectively) agreed with those reported in literature (Luthy et al. 1981). Seven PA were detected and identified by GC-MS in *S. grisebachii* samples (Table 2). Mass fragmentation pattern of the eight PA (Table 2), produced by *Senecio* species, share peaks corresponding to ions at m/z 220, 136, 120, and 94 (Bredenkamp 1991).

Senecionine, seneciphylline, integerrimine, spartioidine, jacobine, jacozine, and retrorsine were found in inflorescences and leaves and stems. Transformation

Table 1. Soil analysis.

Analysis	Method	LD	D
Organic carbon (%)	Walkley Black	2,05	1,66
Total nitrogen (%)	micro-Kjeldhal	0,22	0,15
Extractable phosphorus (ppm)	Kurtz and Bray 1	18,56	6,99
pH	1:2.5 soil/water ratio	6,25	5,72
Aggregates mean weighted diameter (mm)	De Leehneer and De Boodt	2,55	1,54

LD = less deteriorated soil; **D** = deteriorated soil.

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Table 2. Mass spectra of identified pyrrolizidine alkaloids.

Alkaloid	RI	M^+ m/z	Characteristic ions (abundance percentage)	References
Senecivernine (**8**)	2283	335 (6)	43 (52), 67 (21), 80 (28), 93 (75), 94 (65), 95 (44), 119 (90), 120 (100), 136 (99), 220 (30), 246 (12)	Liu and Zhao 1999
Senecionine (**1**)	2294	335 (9)	43 (21), 67 (10), 80 (30), 93 (63), 94 (61), 106 (13), 119 (62), 120 (90), 136 (100), 220 (39), 246 (16)	Asres et al. 2008 Pelser et al. 2005
Seneciphylline (**2**)	2303	333 (3)	43 (30), 67 (9), 80 (28), 93 (56), 94 (60), 119 (75), 120 (100), 136 (72), 220 (1), 246 (9)	Asres et al. 2008 Pelser et al. 2005
Integerrimine (**3**)	2350	335 (3)	43 (28), 67 (13), 80 (45), 93 (83), 95 (58), 119 (95), 120 (100), 136 (99), 220 (20), 246 (5)	Asres et al. 2008 Pelser et al. 2005
Spartioidine (**4**)	2347	333 (2)	43 (25), 67 (10), 93 (95), 94 (57),120 (100), 136 (70), 220 (1), 246 (10)	Macel et al. 2004
Jacobine (**5**)	2432	351 (3)	43 (30), 67 (8), 80 (38), 93 (62), 94 (47), 95 (65), 120 (100), 136 (35), 220 (3), 246 (1), 269 (10)	Macel et al. 2004 Gardner et al. 2006
Jacozine (**6**)	2460	349 (1)	43 (20), 67 (11), 80 (26), 93 (50), 94 (45), 95 (40), 119 (98), 120 (100), 136 (30), 220 (3), 290 (18)	Macel et al. 2004 Gardner et al. 2006
Retrorsine (**7**)	2515	351 (13)	41 (16), 67 (8), 80 (30), 93 (65), 94 (55), 95 (45), 119 (85), 120 (98), 136 (100), 220 (30), 246 (20)	Gardner et al. 2006 Bredenkamp 1991

RI = retention index; M^+ = molecular ion.

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of senecionine into the other PA can be explained through four simple single-step reactions: Z/E-isomerization at C_{20}, 13, 19-dehydrogenation, site-specific hydroxylation, and site-specific epoxidation (Figure 1) (Hartmann 1999).

Another pyrrolizidine alkaloid, senecivernine, was only detected in leaves and stems in trace amounts. However, senecivernine is not considered a senecionine derivative because of the different orientation one of the two isoleucine-derived C_5-units in their necic acid moiety that results in two structural differences: the lack of methyl group at C_{20} and the presence of an extra methyl group at C_{14} (Figure 1) (Hartmann 1999).

Biomass of leaves and stems, and inflorescences

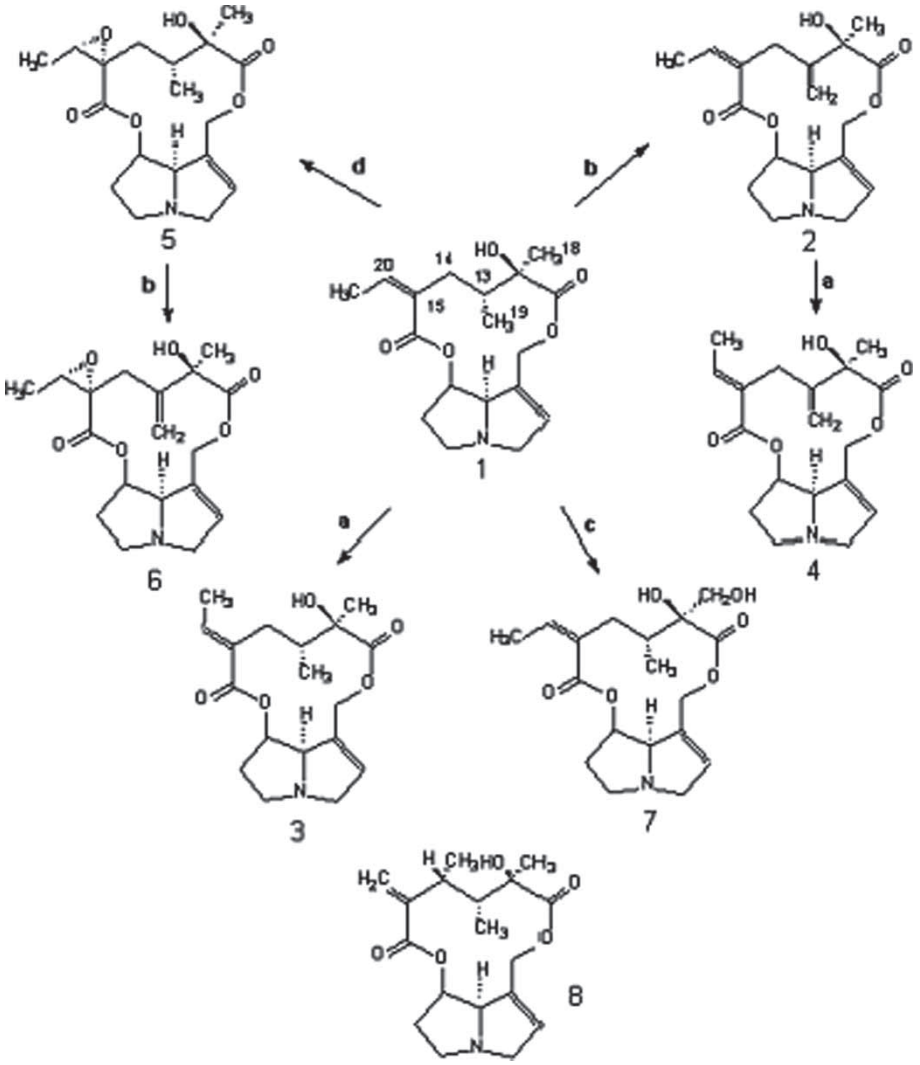
Dry weight values did not show significant differences between plant samples growing in soils with different level of deterioration. Dry weight mean values (DW) for leaves and stems and inflorescences in samples grown in LD were 1.99 ± 0.43 g and 1.49 ± 0.24 g, respectively, while those corresponding to highly deteriorated soil (D) were 1.89 ± 0.49 g and 1.39 ± 0.33 g, respectively.

Total alkaloid content

The GC analysis was used to determine TAC and alkaloids relative abundances. A significant increase in TAC was observed only in inflorescences from samples that grew in D soil (3.52 ± 0.20 mg/g DW) compared with those grown in LD soil (3.23 ± 0.26). No significant differences were found in leaves and stems, as the same value (0.05 mg/g DW) was obtained for samples grown in either soil (Table 3).

Alkaloid relative abundances

Seneciphylline was the major alkaloid in inflorescences from samples grown in LD soil ($47.90 \pm 8.04\%$) followed by senecionine ($38.94 \pm 6.60\%$). On the contrary, the latter was the major one in samples from D soil ($48.78 \pm 10.28\%$) followed by seneciphylline ($38.41 \pm 9.04\%$). Senecionine was also the major alkaloid in leaves and stems in samples from both soils, always followed by seneciphylline integerrimine



1. senecionine **2.** seneciphylline **3.** integerrimine **4.** spartioidine
5. jacobine **6.** jacozine **7.** retrorsine **8.** senecivernine.
a: *Z/E*-isomerization at C_{20} **b**: dehydrogenation between C_{13} and C_{19}
c: hydroxylation at C_{18} **d**: epoxidation between C_{15} and C_{20}.

Figure 1. Alkaloids found in inflorescences and leaves and stems of *S. grisebachii*.

and minor amounts of spartiodine, jacobine, jacozine and retrorsine, without significant differences between treatments. Low concentrations of senecivernine were only detected in leaves and stems (Table 3).

PA principal components analysis

Multivariate analysis was performed on data related to alkaloids relative abundances in inflorescences and in leaves and stems; however, it did not allow differentiations related to soil quality in the latter. Figure 2 shows a biplot representation of the PA present in the inflorescences grouped by their abundance. Note the clear differences between samples from LD and D soils and the association of seneciphylline to the former and senecionine to the latter.

The first principal component, which accounted for 89% of total variance, clearly separated LD and D treatments. Seneciphylline was the most important variable associated to the first component, exhibiting the highest and only positive eigenvector in LD soil. Senecionine was strongly associated with D treatment, showing the higher negative eigenvector, followed by lower negative values for integerrimine, spartiodine, jacobine, jacozine, and retrorsine. These results are in agreement with data showed in Table 2 where senecionine concentration was significantly higher than seneciphylline in D soil.

The second principal component accounted for the rest of total variance. The most important variable associated to the second component was integerrimine, which exhibited the higher positive value associating it to LD treatment.

Discussion

We have previously reported data on the enhancement of chemical defenses in several species as a

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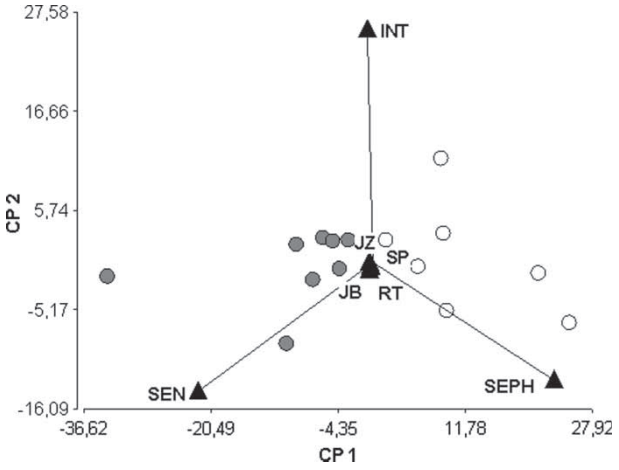

Figure 2. Inflorescences PA principal component analysis ($P < 0.05$).

response to different kinds of environmental stress (Pomilio et al. 2000; Vilariño et al. 2005; Chludil et al. 2009). Some studies have described the impact of soil degradation caused by agricultural management on the relative production of allelochemicals (Gil et al. 2002; Leicach et al. 2003; Chludil et al. 2008); however, there is no available data about its impact on alkaloid production.

Nutrient availability, an important feature in soil quality, can affect plant development and secondary metabolites biosynthesis in different ways, nitrogen deficit being particularly relevant for alkaloid production. Alkaloid concentration in plant tissues depends on environmental factors such as soil and climate conditions and on plant developmental stage and physiological condition. Diurnal fluctuations have also been reported for *Lupinus albus* quinolizidine alkaloids (Wink and Witte 1984).

Drought, extreme temperatures, low soil quality, presence of pesticides, pathogens and herbivores are now common features in intensively cultivated agroecosystems. Several examples in the literature report higher alkaloid levels induced by herbivorous attack or artificial defoliation mimicking it (Baldwin 1988; Wink 1992, 1993; Zhang et al. 1997). The response is genetically determined for each species and particular cultivar. We found that defoliation produced by *Anticarsia gemmatali* induced larger accumulation of alkaloids in a *L. albus* sweet cultivar than in a bitter one; on the other hand none of the *Lupinus angustifolius* varieties exhibited changes in alkaloid content (Vilariño et al. 2005).

In a previous work we reported that *S. grisebachii* samples submitted to N deficiency under hydroponic conditions increased their PA content compared to control samples, being almost three fold higher in inflorescences and 70% in stems and leaves (Yaber Grass et al. 2009). Kirk and coworkers (2010) reported similar results for *S. jacobaea* and *S. aquaticus* samples grown in dune sand soil under excessive amounts of water or nutrient deficiency.

Besides nutrients availability, degradation indicators such as organic carbon and soil aggregates stability have proved to affect plant development and defenses, their values exhibiting striking differences when comparing continuously cultivated soils to pristine ones (Gil et al. 2002; Leicach et al. 2003; Chludil et al. 2008). In the present work we show that soil degradation indicators can exhibit significant differences depending on the length of continuous exploitation period (Table 1). Significant losses in organic carbon (19.0%), total nitrogen (31.8%), extractable phosphorus (62.3%), and aggregates mean weighted diameter (39.6%) were determined in soil samples with longer cropping history (D) compared to those with a shorter one (LD).

The *S. grisebachii* seems to allocate more resources to chemical defenses as soil degradation increases. Significantly higher values for inflorescences TAC (8%) were obtained for samples grown in D soil compared to those grown in LD ones, without significant changes in leaves and stems. Alkaloid profiles also showed noticeable differences. Despite soil deterioration level, samples used in the present research exhibited for inflorescences TAC almost threefold values (3.52 and 3.23 mg/g DW for D and LD soils, respectively) compared with those

Table 3. Alkaloids total content and relative abundances in *S. grisebachii*.

	Inflorescences		Leaves and stems	
Alkaloids	LD	D	LD	D
TAC (mg/gr DW)	3.23 ± 0.26 a	3.52 ± 0.20 b	0.05 ± 0.03 a	0.05 ± 0.02 a
Senecionine (%)	38.94 ± 6.60 a	48.78 ± 10.28 b	50.87 ± 6.39 a	56.64 ± 10.16 a
Seneciphylline (%)	47.90 ± 8.04 b	38.41 ± 9.04 a	33.06 ± 11.18 a	25.45 ± 12.09 a
Integerrimine (%)	12.27 ± 4.20 a	11.51 ± 3.17 a	12.85 ± 5.40 a	13.31 ± 4.34 a
sp + jb + jz + rt (%)	0.66 ± 0.02	1.69 ± 0.90	3.22 ± 0.90	4.60 ± 0.90
Senecivernine (%)	–	–	Traces	traces

LD = less deteriorated soil; **D** = highly deteriorated soil; sp = spartiodine; jb = jacobine; jz = jacozine; rt = retrorsine.
Traces: < 0.1%.
Data given as mean ± standard deviation, $n = 10$.
Different letters indicate significant differences ($p \leq 0.05$).

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previously reported in samples submitted to N and P deficiencies under hydroponic conditions (1.33 and 1.34 mg/g DW, respectively; Yaber Grass et al. 2009).

We did not find significant differences in leaves and stems and inflorescences biomass between samples grown in LD (1.99 ± 0.43 g and 1.49 ± 0.24 g, respectively) and D soils (1.89 ± 0.49 g and 1.39 ± 0.33 g). However leaves and stems and inflorescences DW mean values were considerably higher in samples submitted to N deficiency (7.99 ± 2.21 g and 2.45 ± 0.22 g, respectively) or P deficiency (6.95 ± 1.13 g and 2.31 ± 0.35 g, respectively) under hydroponic conditions.

The increment in inflorescences TAC and the significant reduction in aerial biomass observed in samples grown in soils with cropping history compared to those submitted to N deficiency under hydroponic condition seem to suggest a strong influence of other soil deterioration indicators on the development of this weed.

Senecionine, metabolic precursor of seneciphylline, was the major alkaloid in inflorescences from *S. grisebachii* samples grown in D soils, and in leaves and stems grown in both fields, while seneciphylline was the most abundant in inflorescences from samples grown in LD soil. These findings suggest that higher abundances of senecionine under stress conditions might be related to a lower metabolic energy investment in alkaloid synthesis, since the dehydrogenation step between C_{13} and C_{19} that transforms senecionine in seneciphylline is obviated (Hartmann 1999). The biplot representation of individual PA abundance in the inflorescences clearly associated seneciphylline to the LD soil and senecionine to D soil. The latter result is consistent with our previous findings where, under hydroponic conditions, senecionine was the major alkaloid under nitrogen deficiency (Yaber Grass et al. 2009).

Quantitative and qualitative changes in *S. grisebachii* PA might represent an important hazard considering the invasive potential of this weed for crops, pastures, and fields near beehives. Such changes may not only affect herbivory by insects, but increase the potential harm associated with its ingestion as a contaminant of foodstuff related to cereals, offal, eggs, milk, and honey.

Acknowledgements

We thank Universidad de Buenos Aires for financial support by grants UBACYT G055 and G067.

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