

Soil biotic impact on plant species shoot chemistry and hyperspectral reflectance patterns

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Summary

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- Recent studies revealed that plant–soil biotic interactions may cause changes in above-ground plant chemistry. It would be a new step in below-ground–above-ground interaction research if such above-ground chemistry changes could be efficiently detected. Here we test how hyperspectral reflectance may be used to study such plant–soil biotic interactions in a nondestructive and rapid way.
- The native plant species *Jacobaea vulgaris* and *Jacobaea erucifolius*, and the exotic invader *Senecio inaequidens* were grown in different soil biotic conditions. Biomass, chemical content and shoot reflectance between 400 and 2500 nm wavelengths were determined. The data were analysed with multivariate statistics.
- Exposing the plants to soil biota enhanced the content of defence compounds. The highest increase (400%) was observed for the exotic invader *S. inaequidens*. Chemical and spectral data enabled plant species to be classified with an accuracy > 85%. Plants grown in different soil conditions were classified with 50–60% correctness.
- Our data suggest that soil microorganisms can affect plant chemistry and spectral reflectance. Further studies should test the potential to study plant–soil biotic interactions in the field. Such techniques could help to monitor, among other things, where invasive exotic plant species develop biotic resistance or the development of hotspots of crop soil diseases.

Introduction

Plant chemistry plays a central role in ecological interactions between plants and their abiotic and biotic environment. Chemical variation within and between plant species is based on genetic differences (Pichersky & Gang, 2000), phenological stage of the plants (Hartmann & Zimmer, 1986), climate, herbivory or soil biota (Meijden *et al.*, 1988; Macel *et al.*, 2004; Soler *et al.*, 2005; Shiojiri & Karban, 2008; Joosten *et al.*, 2009). This chemical variation can be found over all classes of primary (e.g. nitrogen (N), Chlorophyll (Chl)) and defence compounds, such as alkaloids (Baldwin, 1999) and glucosinolates (Kliebenstein *et al.*, 2001). The detection of these primary and secondary metabolites requires destructive sampling and analysis with specialized laboratory equipment, whereas ecological studies in the field would benefit from rapid nondestructive approaches. This is especially true when plants are exposed to cryptic environmental factors, such as soil biota, for instance fungi, bacteria and invertebrate root herbivores. Here, we examine how spectral information, provided by the light reflected from the plant canopy, may reveal interspecific chemical differences among plant species, as well as intraspecific

plant chemical variation as a result of plant exposure to pathogenic effects from soil communities.

Remotely sensed spectra of reflected light have been frequently linked to plant chemical traits and related to soil fertility (Wessman *et al.*, 1988; Ferwerda *et al.*, 2005; Knox *et al.*, 2010; Skidmore *et al.*, 2010; Asner & Martin, 2011) and recently even canopy phylogeny (Asner & Martin, 2009, 2011). Additionally, remote sensing studies of above-ground insect infestation in forests have proven successful (de Beurs & Townsend, 2008; James *et al.*, 2011). This is possible through hyperspectral reflectance measurements in hundreds of narrow spectral bands that reveal subtle features associated with certain plant chemical compounds (Card *et al.*, 1988; Curran, 1989; Curran *et al.*, 1992; Fourty *et al.*, 1996). The aim of our study was to test if shoot chemical and spectral properties may also be used to distinguish different plant species and their responses to contrasting soil biotic conditions. Using spectral patterns to reveal biotic soil conditions has, as far as we know, not yet been demonstrated. If operational, this approach may open up new avenues for studying plant exposure to soil biota in the field.

An increasing number of studies have shown that soil organisms (e.g. root herbivores) can influence above-ground plant responses through inducing primary and secondary

metabolite accumulation in the shoots (Masters *et al.*, 1993; van Dam, 2009; Kostenko *et al.*, 2012). It has also been argued that below-ground interactions between plants and soil biota may influence above-ground multitrophic interactions among plants, herbivores and their enemies (Van der Putten *et al.*, 2001; Bezemer *et al.*, 2005; Kaplan *et al.*, 2008; Erb *et al.*, 2009). In the field, soil biota are often patchily distributed (Masters & Brown, 1997; Van der Putten *et al.*, 2001). As a result, spatial heterogeneity in soil biotic communities may enhance spatial variation in the chemical constitution within the same plant species that cannot be explained by genetic or above-ground factors alone (Joosten *et al.*, 2009; Bartelheimer *et al.*, 2010). Therefore, our hypothesis was that soil biota can induce changes in above-ground plant chemistry and that these changes can be detected by spectral reflectance patterns.

In our study we used three closely related Senecioneae species (Asteraceae): *Jacobaea vulgaris* (syn. *Senecio jacobaea*) and *Jacobaea erucifolia* (syn. *Senecio erucifolius*), both native to the Netherlands, and the invasive *Senecio inaequidens*. We chose these species, because their primary and secondary chemistry is quite well known (Meijden *et al.*, 1989; Hartmann & Witte, 1995) and two of them have been shown to respond to soil biotic communities in specific ways (Engelkes *et al.*, 2008; Joosten *et al.*, 2009). Soil–plant interaction studies of species such as *J. vulgaris* have shown that soil type and biotic community can change the composition and concentration of pyrrolizidine alkaloids (PAs) in shoots (Joosten *et al.*, 2009; Macel & Klinkhamer, 2010). Invasive exotic species may be more efficient in photosynthesis (Garcia-Serrano *et al.*, 2009; Feng *et al.*, 2011). The effect of root damage could cause changes in Chl and N, affecting photosynthesis (Garcia-Serrano *et al.*, 2009), but this response may differ between native and exotic species, as they are differently susceptible to root damage. Therefore, secondary metabolite concentrations of an exotic species could respond differently to soil biota than related natives (Engelkes *et al.*, 2008).

Soil fertility studies have shown that reflectance signal is sensitive to abiotic changes (Wessman *et al.*, 1988; Asner & Martin, 2011). However, the contribution of soil organisms to changes in spectral reflectance of exotic or native plant species is an open question. Therefore, we exposed plants to contrasting soil biotic conditions and studied the influence on shoot chemistry and hyperspectral reflectance patterns. This approach has the potential to assess exposure of exotic and native plants to biotic resistance in natural conditions, or where crops become exposed to soilborne diseases. We tested the hypotheses that the exotic species changes in chemistry will be more affected by soil biota than will native species; that hyperspectral reflectance will enable detection of the effects of soil biota on plants; and that spectral reflectance patterns will correlate with shoot chemistry. To test our hypotheses, the three species were grown in living and sterilized soils from fields with known pathogenic concentrations (van de Voorde *et al.*, 2011). We measured canopy spectra between the wavelengths of 400 and 2500 nm and related those to Chl, N and C content in the shoots as well as the secondary defence compounds (Pas), as these may be influenced by soil biota.

Materials and Methods

Species description

We selected three Asteraceae species to test the soil effects on biomass production, chemistry and spectral signatures: *Senecio inaequidens* DC., *Jacobaea vulgaris* Gaertn. ssp. *vulgaris* (syn. *Senecio jacobaea* L.) and *Jacobaea erucifolia* Gaertn. Mey and Scherb. (syn. *Senecio erucifolius* L.). *J. vulgaris* is a monocarpic biennial to a short-lived perennial noxious weed in early-succession grasslands that develops on open soil, for example on recently abandoned arable land. In the first year, *J. vulgaris* develops a circular rosette with basal stalked leaves, obovate to pinnately lobed, in general 2–6 cm wide. *J. erucifolia* is a perennial species that is closely related to *J. vulgaris*. It does not form a rosette but stems are woody at its base with leaves of similar shape to those of *J. vulgaris*. It is also native in the Netherlands, but interactions with soil organisms have not yet been studied. *S. inaequidens* is a perennial species originally from South Africa and invasive in several European countries (Lachmuth *et al.*, 2010). Its stems are woody at its base with slim, linear leaves between 1 and 7 mm wide. Seeds of *J. vulgaris* were collected from one mother plant at Mossel, Veluwe, the Netherlands, while *S. inaequidens* seeds were collected from one mother in Oosterhuis, the Netherlands. Seeds of *J. erucifolia* were obtained from a specialized seed supplier (Blaufelden-Raboldshausen, Germany) that collects seeds from wild populations.

Soil collection and experimental design

Soil was collected from two abandoned arable fields in the Netherlands: Dennekamp (D) and Wolfheze (W). From both locations, 20 kg of soil were collected in bulks, as intact as possible, and stored in sterile plastic bags. Soil from these fields was specifically chosen, as they are known to have different negative impacts on the biomass of *J. vulgaris* (van de Voorde *et al.*, 2011). In addition, we collected 400 kg of soil from a nearby field, in Mossel, the Netherlands, sterilized it with gamma radiation (≥ 25 kGray; Isotron, Ede, the Netherlands) and used it as substrate into which the soil samples from both other fields were inoculated.

All soils were sieved with a 5 mm mesh to homogenize any spatial variation without obliteration of the soils' biotic characteristics (van de Voorde, 2011). The two live soil treatments consisted of 80% sterilized soil from Mossel and 20% field soil inoculum from either Dennekamp (D) or Wolfheze (W). As a control treatment, we used 80% sterilized background soil and 20% sterilized soil from either field. In total, four treatments were prepared: W nonsterilized, D nonsterilized and their sterilized controls, W sterilized and D sterilized.

All seeds were sterilized in 2% bleach solutions and seedlings were germinated in transparent boxes with sterile glass beads in a climate chamber under a 16 : 8 h, 25 : 15°C day : night regime. After 3 wk, 24 seedlings of each species were transferred individually to 1 l pots prepared with one of the soils. The experiment setup consisted of three species \times four soil treatments (D sterilized, D nonsterilized, W sterilized and W nonsterilized). In each

treatment, there were 24 seedlings per species. This resulted in a total of 288 plants, of which 11 died during the experiment. Over 10 wk all plants were grown in a glasshouse under a 14 : 10 h, 21 : 16°C day : night regime at 70% relative humidity. All plants were watered every 2 d with a 0.5% Hoagland nutrient solution to reduce any possible biomass effects resulting from nutrient limitations in the soil (Van der Putten & Peters, 1997).

Canopy spectral measurements

In a darkroom, we measured the canopy reflectance (350–2500 nm) of the plants with a field spectrometer (ASD Fieldspec 3; ASD, Inc., Boulder, CO, USA) fitted with an 8° optic probe secured by a tripod at nadir. At the level of the optic probe, one halogen light bulb was positioned with a 15° angle for the illumination of the plant. The emitted light has a near-solar light spectrum (ASD Pro Lamps; ASD, Inc.). All the reflectance measurements were calibrated with a Spectralon white reference-panel (Labsphere Inc., North Sutton, NH, USA) used between each plant canopy measurement. A constant target-sensor distance was kept in order to achieve a field-of-view (FOV) of 12 cm diameter by the optic probe. A matt-black paper shade was used to cover all pots at soil level to secure a constant background reflectance and prevent possible effects by soil radiance. To reduce reflectance-directional effects in canopy measurements, the samples were measured four times with a rotation of 90° (Mutanga *et al.*, 2003; Knox *et al.*, 2010). In each rotation, 10 single-reflectance measurements were done and all measurements were then averaged per sample, resulting in a 40-fold composite sample (mean (four rotations × 10 measurements) = one mean reflectance measurement per plant (Ramsey, 1997)). All reflectance measurements were offset corrected and the 40 measurements of each individual plant were averaged in a single reflectance measurement by the instruments software ViewSpec Pro 5.6.10 (ASD Inc.).

Only reflectance bands between 450 and 2450 nm were used. The remaining bands were considered highly noisy following visual inspection and were removed; this resulted in 2000 wavebands in the visible and infrared regions each at 10 nm intervals. All reflectance measurements were brightness-normalized (Feilhauer *et al.*, 2010) and mean centre-corrected. Brightness normalization is a sample-wise preprocessing transformation that attempts to scale spectral samples in order to clear biomass and canopy structure effects from the canopy spectra. This allows a better estimation of chemical contents in canopy spectra with partial least-squares regression (PLSR; Feilhauer *et al.*, 2010).

Plant biomass and chemical analysis

After the spectral readings, the shoots were cut at the soil surface and freeze-dried for 96 h while all roots were carefully rinsed with water to remove soil particles. The roots were oven-dried at 70°C for 96 h. All dried roots and shoots were weighed and the net effects of the soil on plant biomass were determined. Shoot samples were analysed to determine Chl a and Chl b (mg g⁻¹), N (%), carbon (C, %) and the PA defence compounds (mg g⁻¹).

Chlorophyll extraction was done using fresh shoot samples immediately after performing the spectral measurements. In each individual plant, four discs of 10 mm diameter were cut randomly from four different leaves and weighed. Thereafter, the discs were immersed in 3 ml of dimethyl sulphoxide (DMSO) and stored in a dark room for 3 d at constant room temperature. Absorbance (Abs) measurements at 649 and 665 nm were carried out in a spectrophotometer (Genesys 20 spectrophotometer 4001/4; Thermo Fisher Scientific Inc., Waltham, MA, USA) and Chl concentrations (initially in µg ml⁻¹) were calculated using the equation:

$$\text{Chl}a = 12.19 \times \text{Abs}(665 \text{ nm}) - 3.45 \times \text{Abs}(649 \text{ nm})$$

$$\text{Chl}b = 21.99 \times \text{Abs}(649 \text{ nm}) - 5.32 \times \text{Abs}(665 \text{ nm})$$

Five random leaves of the same age were selected from each sample, homogenized and fast-ground to a fine powder for PAs and C : N estimations. Percentages of N and C were estimated with a C : N analyser (Thermo flash EA 1112; Thermo Fisher Scientific Inc.) by combustion-reduction. In this analysis, 3–5 mg of dried powdered were used in well isolated 6-mm-diameter metal cups.

Pyrrolizidine alkaloids were extracted following the protocol by Joosten *et al.* (2011), using 10 mg of material dissolved in 1 ml of 2% formic acid solution with heliotrine internal standards (1 µg ml⁻¹). We diluted 25 µl of the solution 40 times with 10 mM ammonium hydroxide and determined PAs by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Waters UPLC system (Waters, Milford, MA, USA) coupled to a Waters Premier XE tandem mass spectrometer (Waters). Quantification was performed against a control plant extract containing a set of available PA reference standards as described by Cheng *et al.* (2011).

Statistical analysis

The effects of soil biota on plant chemistry, shoot and root DW were analysed in a full-factorial three-way ANOVA with plant species (*J. vulgaris*, *J. erucifolia*, *S. inaequidens*), soil origin (D, W) and soil treatment (sterilized, nonsterilized) as fixed factors. Shoot DW was used as covariate in the ANOVA for the chemical content. The ANOVA analyses were performed in SPSS 17.0 for Windows, SPSS inc., Chicago, USA. *Post-hoc* Tukey tests were performed to analyse which groups (species, species × soil treatments) were different from each other in significant main effects or interactions.

Linear discriminant analysis (LDA) was applied in order to find functions that best separate our samples to species (*J. vulgaris*, *S. inaequidens* or *J. erucifolia*) or to soil biotic treatment. LDA was done separately for chemical variables and spectra, in order to compare discrimination results, as we were interested in whether spectra can discriminate soil effects, as well as main chemical profiles. LDA uses the chemistry or spectra and its category (group) to find discriminant functions that best separate between the sample categories. These functions were built in two-thirds of the dataset with cross-validation of a randomly selected 10% of the samples, while one-third was reserved as the testing set. The testing set consisted of samples unknown to the LDA that were used to measure the success of correct classification of new samples within

the LDA model. The strength of discriminant analysis is its capacity to create functions that best explain the relationship between the variables we have and the groups we are interested in, in order to classify 'unknown' samples. The better the functions are, the better the classification of unknown samples into the groups defined. Since the spectra can be highly collinear and LDA is sensitive to such effects, a principal component analysis (PCA) was computed with the spectral reflectance data. The resulting PCA scores were then used to perform the LDA. PCA is an ordination method that attempts to express the response variables' association without taking any categorical classification into account. This enabled us to reduce the number of variables without decreasing the response variables' association. The LDA was performed in the first 10 PC scores that explained 99% of the response variables' variation. Mahalanobis distance was used as the distance measure for group discrimination. LDA is sensitive to the number of samples used. To guarantee that our LDA results had the lowest impact possible from the sample size, the smallest groups analysed had > 20 samples. Thus, this exceeded the number of predictors used in the cross-validation LDA. Additionally, to reduce the impact of different group sizes, the prior probabilities were considered. A full description of the method is provided by Naes *et al.* (2002) and Quinn & Keough (2002). This procedure was done in Unscrambler X 10.1 (CAMO software AS, Oslo, Norway) and SPSS 17.0 for Windows. The discrimination of soil treatments was performed within each species to disentangle it from the stronger species effect.

Whereas in LDA we tested the possibility for classification of samples into important ecological groups (in this case, species and soil conditions), with PLSR we tested whether spectral data can be used as predictors of chemical concentrations in individual samples (Naes *et al.*, 2002). Therefore, we applied PLSR analysis into the brightness-normalized spectra to examine the linkage between the samples' chemical content and their spectral signature independent of their sample category. The PLSR was performed separately on the data for each plant species. PLSR maximizes the covariance between chemical and spectral reflectance, taking care of overfitting by means of a projection of the minimum error with the fewest number of factors. This is done by minimizing the residual error of the sum of squares. Once more, we selected a cross-validation procedure that iteratively generated regression models where 10% of the samples were randomly reserved for validation of the model. The most accurate predictions of chemical concentrations were assessed in terms of minimum root mean square error (RMSE) and the highest coefficient of regression (r^2) of cross-validation statistics. The entire procedure was done in Unscrambler X 10.1 for Windows.

Results

Soil treatment effects on plant chemical content

The biomass of roots and shoots of each plant species was little affected by soil treatments (Fig. 1). Although plant biomass in nonsterilized soils tended to be lower, this effect was not statistically significant for any of the plant species. All three species were significantly different from each other for all chemicals studied (Table 1). In general, plants grown in nonsterilized soil had

increased concentrations of defence compounds and slightly decreased percentage N. The total PA and N-oxide concentration in *S. inaequidens* and *J. erucifolia* ranged from nondetectable to 20.5 mg g⁻¹ DW in the live soil and from nondetectable to 13.0 mg g⁻¹ DW in the sterilized soils. PA concentrations in nonsterilized inoculum were thus 100–400% higher, depending on species, than in sterilized inoculum (Fig. 2, Supporting Information, Table S4). In *J. vulgaris*, on average these defence compound concentrations did not differ between sterilized and nonsterilized soil but did differ significantly between the live soil origins. There was no effect of soil origin (D or W soils) on the PA content of *S. inaequidens* and *J. erucifolia*. PA concentrations in *J. vulgaris* depended on soil origin. Compared with the sterilized soils, N-oxide content in plants with nonsterilized soil inoculum from Dennekamp was increased, whereas it was decreased in soil with Wolfheze inoculum (Fig. 3). N content ranged from 2.43 to 5.85% and was generally 10–15% lower in nonsterilized soils for all species. The largest decrease occurred in *J. vulgaris* (Fig. 2, Table S4). Soil origin did not affect N content. Chlorophyll content ranged from 0.03 to 1.62 mg g⁻¹ FW and was highest in *J. vulgaris* and *J. erucifolia* species. Chlorophyll was not affected by soil origin or sterilization treatment.

Species discrimination

In the LDA analyses, the correct discrimination of 'unknown' samples into the correct species, with either spectra or chemical data, was above 85%. The discriminant analysis of species by the canopy spectral data revealed two discriminant functions, which explained 59.6 and 40.4% of the species variance and significantly differentiated the species ($P < 0.001$). The first discriminant function clearly separated *J. erucifolia* from the other two species, whereas the second separated *J. vulgaris* from the other species (Fig. 4a). The overall correct classification of the cross-validation LDA was 97.8%, which decreased to 88.8% when applied to the test dataset of 'unknown' samples to the LDA model (Table S1).

With the species LDA analysis using the chemical content, two discriminant functions were once more selected with 73.4 and 26.6% of the variance significantly explained ($P < 0.001$). The first discriminant function separated *J. vulgaris* from the other species, whereas the second function discriminated *J. erucifolia* from the other two (Fig. 4b). The overall correct classification in the cross-validation was 95.2%, which slightly decreased to 94.8% when applied to 'unknown' samples to the LDA model (Table S2).

Soil treatment discrimination

The separation of the effects on soil treatments per species by LDA, on either chemical or spectral properties, was, in general, relatively low (Fig. 5, Tables S1, S2). The separation was only significant for *S. inaequidens*. The LDA of soil treatments within each species achieved a classification of 'unknown' samples into the soil groups between 47 and 56% (Fig. 5, Tables S1, S2).

Spectral data LDA within *J. vulgaris* achieved an overall percentage of correct classification of 'unknown' samples of

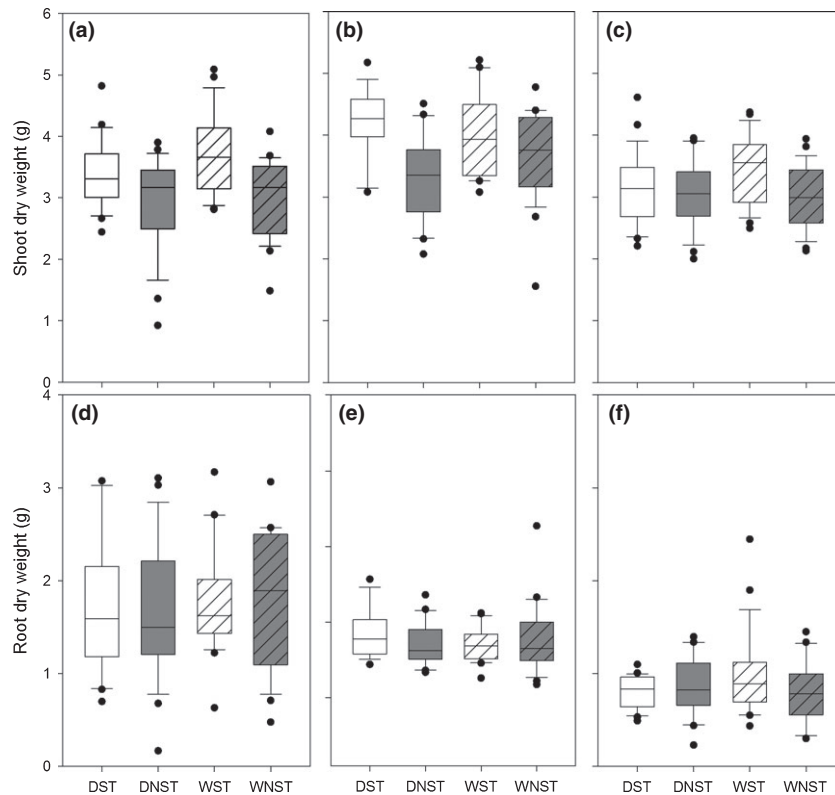


Fig. 1 Species shoot (a–c) and root (d–f) dry weight per soil treatment (g). Soil origins are Dennekamp (D) and Wolfheze (W). Dark grey-black boxes, nonsterilized (NST) soil; white boxes, sterilized (ST) soil treatments. On average, there were 23 samples per species per treatment. There were no significant differences in roots (ANOVA, all $P > 0.05$). An effect of sterilized vs nonsterilized soil treatment was found in shoots (ANOVA, $P < 0.001$); sterilized treatments (both DST and WST) were not significantly different from each other (ANOVA, $P > 0.05$). (a, d) *Jacobaea vulgaris*; (b, e) *Jacobaea erucifolia*; (c, f) *Senecio inaequidens*.

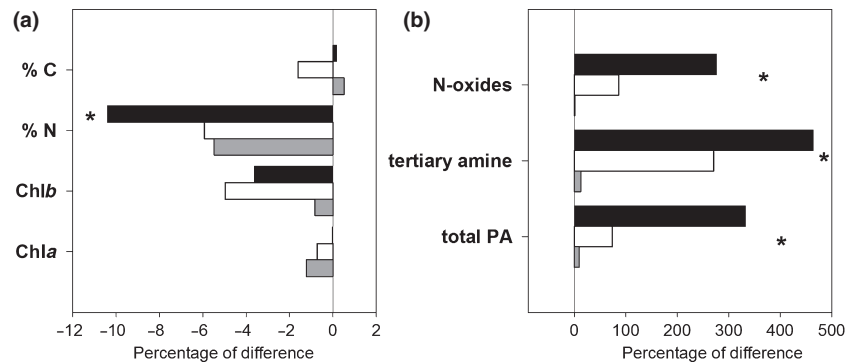


Fig. 2 Percentage of difference in plant shoot mean chemical content between nonsterilized (NST) and sterilized (ST) treatments. (a) Primary chemicals; (b) pyrrolizidine alkaloids (PAs). Grey bar, *Zacobaea vulgaris*; black bar, *J. erucifolia*; white bar, *Senecio inaequidens*. The percentage of difference in chemical content between soil treatments was calculated as: (mean NST – mean ST)/mean NST (see Table S4). *, $P < 0.05$ for differences between the species (ANOVA, Table 1)

56.5% (Fig. 5a, Table S1). The discriminant analysis of *J. erucifolia* by spectra provided an overall correct classification of ‘unknown’ samples of 47.4% (Fig. 5c, Table S1), while in *S. inaequidens* this was 52%. The largest contribution to this outcome was given by correct classification of the sterilized samples (Fig. 5e, Table S1). In each discriminant analysis the first function explained *c.* 59% of the variance while the second explained *c.* 41%. The first function appeared to differentiate between the sterilized group and the other two nonsterilized groups in *J. erucifolia* and *S. inaequidens*

(Fig. 5c,e). However, none of these functions was significant ($P > 0.1$).

The same soil treatment discriminant analysis applied to the chemical dataset achieved within *J. vulgaris* an ‘unknown’ sample correct classification of 43.4% (Fig. 5b, Table S2). Within *J. erucifolia*, only 41.3% of the ‘unknown’ samples were correctly classified to their soil treatments, whereas *S. inaequidens* achieved 56.3% correct classification (Fig. 5d,f, Table S2). Both species had an overall cross-validation correct classification of the soil treatment

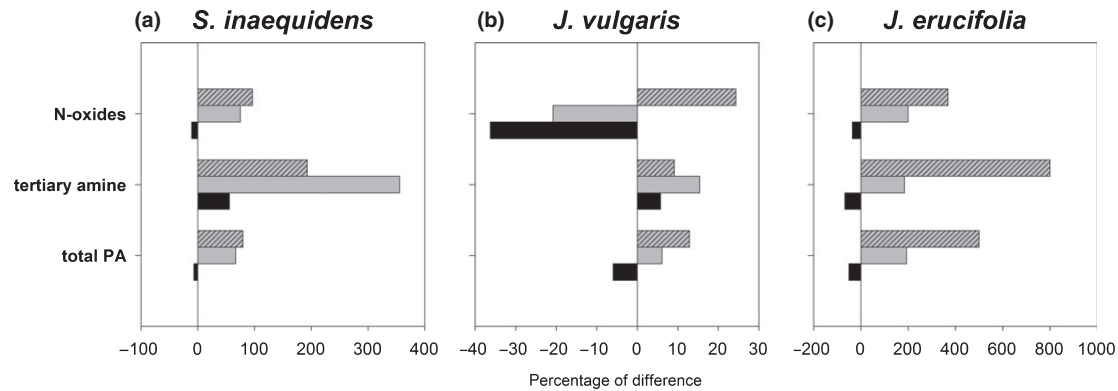


Fig. 3 Percentage of differences in pyrrolizidine alkaloids between plant shoots grown in different soil origin treatments, Wolfheze (W) and Dennenkamp (D): grey bars, differences between W nonsterilized (WNST) and W sterilized (WST); dashed grey bars, differences between D nonsterilized (DNST) and D sterilized (DST) treatments. Black bar, differences between DNST and WNST treatments. Ratios for *Jacobaea vulgaris* (a), *J. erucifolia* (b) and *Senecio inaequidens* (c). The percentage of difference in chemical content between soil treatments was calculated as: (mean of group NST – mean of group ST)/mean of group NST. For statistics, see Table 1. PA, pyrrolizidine alkaloids.

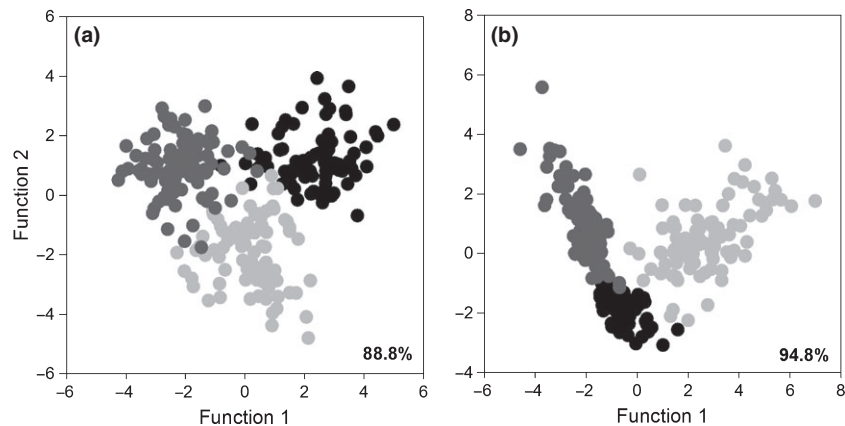


Fig. 4 Scatterplot of the species linear discriminant scores generated by spectral reflectance (a) and chemical content analysis (b). Discriminant functions 1 and 2 discriminate among *Senecio inaequidens* (dark grey dots), *Jacobaea vulgaris* (light grey dots) and *Jacobaea erucifolia* (black dots). The percentages of correct sample cross-validation classification in the linear discriminant analysis (LDA) were 97.8% (a) and 95.2% (b). In each panel, the percentage of correct classifications of ‘unknown’ samples to the linear discriminant analysis (LDA) is noted. For more details, see Table S1.

> 60%. All soil treatment discriminant functions in the chemical data were again nonsignificant ($P > 0.1$) with the exception of the first discriminant function of *S. inaequidens* ($P < 0.001$). This function differentiated between the plants in sterilized and nonsterilized soils (Fig. 5f), clearly highlighted by the group’s centroid (which is the centre of the sample’s distribution ellipsoid).

A reanalysis of both spectral and chemical datasets considering just nonsterilized vs sterilized treatments, disregarding soil origin, achieved similar results within each species, when either spectral or chemical data were considered. The overall correct classification of ‘unknown’ samples was between 47 and 57%, except for *S. inaequidens* (with 69%) when using chemical data (Tables S1, S2). Once again, only in *S. inaequidens* was the soil treatment discriminant function significant ($P < 0.05$), for both chemical and spectral data.

The greater the loading scores of the PCA, the more important the wavelengths were for the discrimination functions. The highest loading scores in the PCA-LDA (in the discriminant analysis by the spectral data) were in the 500–570, 650–750, 1350–1450 and

1800–1900 nm wavelengths (Fig. S1). The selected areas for the loadings were consistent with the areas of shifts in intraspecific variation in the spectral reflectance (Fig. 6). The soil treatment (sterilized vs nonsterilized) caused no clear differential effect throughout the spectra variation pattern. However, for both *J. erucifolia* and *S. inaequidens*, the sterilized treatments showed 50% greater spectral variation in 1900–2000 and 2000–2400 nm regions, respectively (Fig. 6b,c), while *J. vulgaris* sterilized treatment variation decreased in the visible region between 600 and 700 nm (Fig. 6a). These small fluctuations in the coefficient of variation and the ‘unknown’ sample correct classification results are an indication that the soil treatments may affect the spectral reflectance of plants, albeit weakly.

Plant chemical prediction

The estimation of species’ chemical content by PLSR ranged between nondetectable and $r^2 = 0.54$ (Table 2). The best estimations were for N in *J. vulgaris* ($r^2 = 0.53$) and *J. erucifolia*

Table 1 ANOVA analysis of the effect of species (*Jacobaea vulgaris*, *Jacobaea erucifolia* and *Senecio inaequidens*), soil origin (Denenkamp or Wofftheze) and soil treatment (sterile or nonsterile) on the shoot chemical concentrations

Fixed factors	df	F Chla		P value Chla		F Chlb		P value Chlb		F Chl total		P value Chl total		F total PAS		P value total PAS		F tertiary amines		P value tertiary amines		F N-oxides		P value N-oxides	
		F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Corrected model	12	18.08	< 0.001	5.94	< 0.001	10.52	< 0.001	17.26	< 0.001	2.17	0.01	15.42	< 0.001	45.73	< 0.001	13.66	< 0.001	45.73	< 0.001	13.66	< 0.001	13.66	< 0.001	13.66	< 0.001
Intercept	1	436.30	< 0.001	168.12	< 0.001	417.79	< 0.001	966.12	< 0.001	777.61	< 0.001	8.67	0.00	1.23	0.27	8.28	0.00	1.23	0.27	8.28	0.00	8.28	0.00	8.28	0.00
Shoot DW (covariate)	1	6.57	0.01	0.49	0.48	0.63	0.43	143.29	< 0.001	0.08	0.78	0.20	0.65	3.96	0.05	0.42	0.52	3.96	0.05	0.42	0.52	0.42	0.52	0.42	0.52
Species	2	105.03	< 0.001	25.34	< 0.001	55.40	< 0.001	3.11	0.05	9.99	< 0.001	70.61	< 0.001	265.89	< 0.001	56.25	< 0.001	265.89	< 0.001	56.25	< 0.001	56.25	< 0.001	56.25	< 0.001
Soil origin	1	0.50	0.48	1.07	0.30	1.29	0.26	1.90	0.17	0.18	0.67	1.07	0.30	0.89	0.33	0.76	0.39	0.89	0.33	0.76	0.39	0.76	0.39	0.76	0.39
Soil treatment	1	1.27	0.26	0.85	0.36	1.61	0.21	57.14	< 0.001	0.03	0.87	8.81	< 0.001	4.64	0.03	6.38	0.01	4.64	0.03	6.38	0.01	6.38	0.01	6.38	0.01
Species × soil origin	2	0.91	0.40	0.81	0.45	1.29	0.28	0.31	0.73	0.03	0.97	0.16	0.85	0.53	0.26	0.77	0.85	0.53	0.26	0.77	0.26	0.77	0.26	0.77	
Species × soil treatment	2	0.08	0.92	0.25	0.78	0.05	0.95	1.36	0.26	0.52	0.59	3.94	0.02	0.70	0.50	7.95	0.001	0.70	0.50	7.95	0.001	7.95	0.001	7.95	0.001
Soil origin × treatment	1	0.04	0.84	0.12	0.73	0.13	0.72	0.06	0.80	2.59	0.11	0.01	0.93	1.50	0.22	0.28	0.93	1.50	0.22	0.28	0.28	0.28	0.28	0.28	0.28
Species × origin × treatment	2	0.69	0.50	0.05	0.95	0.08	0.93	0.21	0.81	1.87	0.16	0.09	0.91	1.68	0.19	0.16	0.85	1.68	0.19	0.16	0.85	0.16	0.85	0.16	0.85
Mean square error		0.02		0.04		0.07		0.29		16.15		9.82		2.11		6.44		2.11		6.44		6.44		6.44	

The shoot DW was included as a covariate in the analysis. The significant *P*-values are highlighted in bold. N, nitrogen; C, carbon; PAs, pyrrolizidine alkaloids; tertiary amines and N-oxides are two different types of PAs.

($r^2 = 0.54$), while *S. inaequidens* achieved the best estimations for Chl *b* ($r^2 = 0.40$). Although estimations were low, the RMSEs were consistently between 6 and 20% of the mean (Table 2). There was no correlation between PA and spectral reflectance patterns in this study.

Discussion

Both nonsterilized soil treatments caused *J. erucifolia* and *S. inaequidens* to increase their PA content. *J. vulgaris*, which occurs naturally in the selected field sites, only increased PAs when grown in soil from Dennekamp, the most pathogenic of the selected soils (van de Voorde *et al.*, 2011). The increase in PA content of *J. vulgaris* plants as a response to soil sterilization was minor when compared with that of *S. inaequidens* and *J. erucifolia*. The latter two species are nonadapted to the soils used in the experiment, so the enhanced expression of PAs may be a stress response. An interesting finding was the shift in *J. vulgaris* N oxide into the different nonsterilized soils. It suggests that the two PA forms (tertiary amines and N oxides) may be different responses to particular soil biota.

The shoot chemical analyses confirmed that soil biota affected the PA concentrations. In our study, biomass was not affected, because we supplied relatively high concentrations of nutrients, which is known to reduce soil biota effects on plant biomass (Van der Putten & Peters, 1997). Moreover, we did not condition the soils before the experiment, which may have limited the soil biota influences on plant biomass (van de Voorde *et al.*, 2011). We used this approach to minimize plant size or ontogeny differences that would complicate the comparison of spectral patterns affected by soil biota. Nevertheless, at the end of the growth period, plants will have been fully exposed to soil biota that have developed on the plant roots during the experiment.

Both spectral reflectance and chemical contents could be used to significantly discriminate between the plant species with high accuracy. The dimensionality reduction in spectral data as suggested by Adam & Mutanga (2009), which in our case was PCA followed by its loading weights in the LDA, might indeed be important for studies considering closely related species. As a result, spectral data can be a good source for accurate classification of new samples into species without destructive procedures even within closely related species. This can be an elegant method for species identification in the field, especially when specific (e.g. flower) characteristics are absent during the field surveys. The high percentage of correct classifications of unknown samples also supports the potential of the spectral libraries available worldwide for species identification.

Several studies have shown that plant spectra can be influenced by soil abiotic effects, such as the soil fertility (Mutanga *et al.*, 2004; Asner & Martin, 2011; Pretorius *et al.*, 2011; Ramoelo *et al.*, 2011). Our results showed that soil biotic conditions have the potential to influence plant reflectance patterns. Separation of soil biotic treatments (sterilized vs inoculated with nonsterilized field soil) within each species by either spectral reflectance or chemical content resulted in 50–60% of unknown (new) samples being

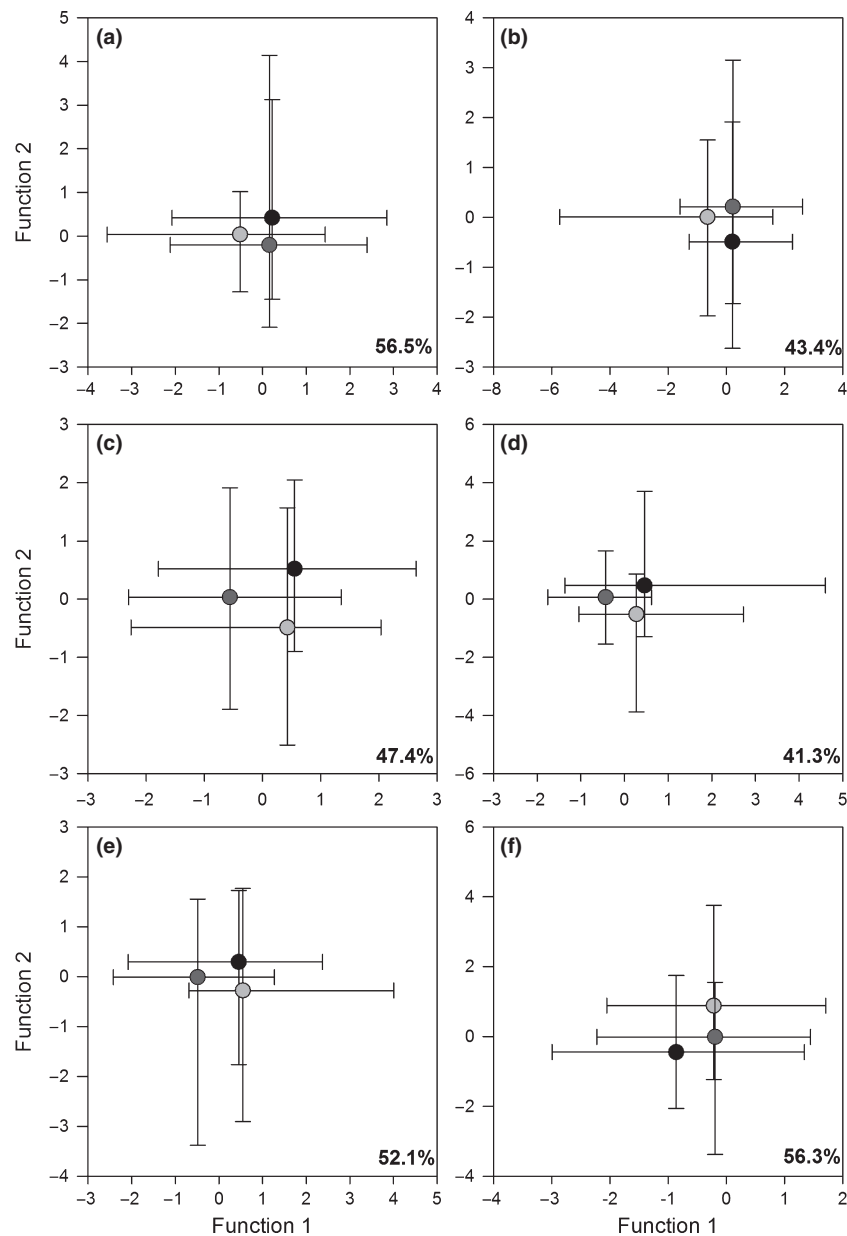


Fig. 5 Linear discriminant mean scores (\pm minimum or maximum) generated by spectral reflectance (a, c, e) or chemical content analysis (b, d, f) for the species intraspecific soil treatment grouping: sterilized (dark grey), Dennekamp nonsterilized (black) and Wolfheze nonsterilized (light grey). (a, b) *Jacobaea vulgaris* linear discriminant analysis (LDA); (c, d) *Jacobaea erucifolia*; (e, f) *Senecio inaequidens*. The percentages of correct sample cross-validation classifications in the LDA were: (a) 67.3%, (b) 52.6%, (c) 62.5%, (d) 61.9%, (e) 52.9%, and (f) 64.8%. In each panel, the percentage of correct classifications of 'unknown' samples to the LDA is given. For more details, see Tables S1, S2.

correctly classified. This was a moderate result, yet, considering the low sensitivity of the plants to soil biotic conditions in this experiment, it is a highly conservative estimate. Therefore, this first attempt to study the effects of soil community on reflectance patterns has shown some promising prospects. When conditions are such that plants are more sensitive to soil biotic effects, or when they grow in more extreme conditions, it may be expected that the responses to soil biota are stronger and that spectral reflectance estimates may also have higher accuracy. Even though a better understanding of the effects of soil biota in plant spectral patterns is still needed, the results of this study outline further avenues of study

in relation to plant ecological processes, such as competition, plant diseases, invasiveness and biological control using spectral reflectance patterns in the field. From earlier work by van de Voorde *et al.* (2011), we were aware of the negative soil feedback in *J. vulgaris*. To disentangle single microorganisms, their function and their impact on plant above-ground chemistry, and subsequently on reflectance, is highly complex at present (Cortois & De Deyn 2012). Having said that, soil community effects on spectral patterns were our first interest, which are the natural condition in the field. Hence, we did not attempt to elucidate which soil (micro)organisms may have caused such effects. Further tests

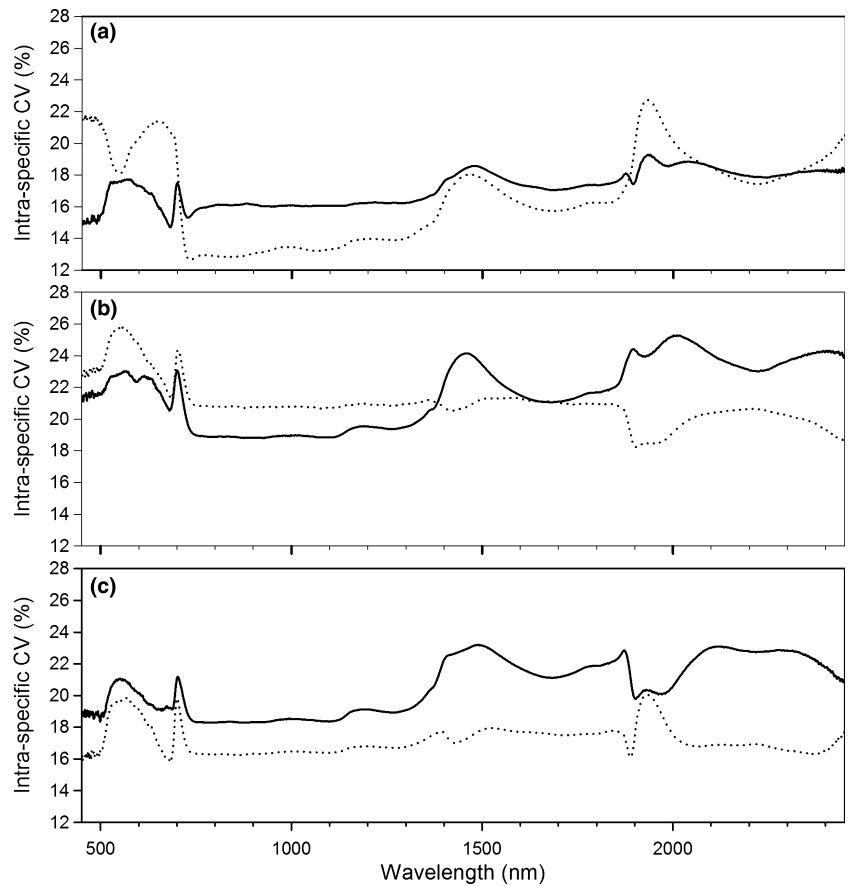


Fig. 6 The mean intraspecific coefficient of variation (CV) as a percentage of the canopy spectral reflectance for each species soil inoculum treatment. (a) *Jacobaea vulgaris*; (b) *Jacobaea erucifolia*; and (c) *Senecio inaequidens*. The bold line represents nonsterilized soil and the dashed line represents sterilized soil. CV is calculated as (mean/standard deviation) \times 100.

Table 2 Estimation of species chemical concentration by partial least-squares regression (PLSR) using their spectral reflectance signatures

Chemical	<i>Jacobaea vulgaris</i>				<i>Jacobaea erucifolia</i>				<i>Senecio inaequidens</i>			
	Mean	r^2	RMSE	%RMSE	Mean	r^2	RMSE	%RMSE	Mean	r^2	RMSE	%RMSE
Chla	0.99	0.29	0.13	13.13	0.92	0.29	0.07	7.61	0.72	0.38	0.11	15.28
Chlb	0.89	0.06	0.19	21.35	1.09	0.30	0.12	11.01	0.87	0.40	0.16	18.39
Chl total	1.87	0.10	0.27	14.44	2.02	0.36	0.14	6.93	1.59	0.30	0.21	13.21
Nitrogen	3.90	0.53	0.41	10.51	3.59	0.54	0.48	13.37	4.23	0.28	0.41	9.69
Total PAs	5.90	0.00	0.00	0.00	0.18	0.00	0.00	0.00	5.05	0.00	0.00	0.00
Tertiary amines	4.45	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.16	0.00	0.00	0.00
N-oxides	1.44	0.00	0.00	0.00	0.11	0.00	0.00	0.00	4.38	0.00	0.00	0.00

RMSE, the cross-validation root mean square error of PLSR with the units of the original chemical concentration; % RMSE, the error as a percentage of the mean value of the chemical concentration; r^2 , represents quality of the fit of PLSR; PAs, pyrrolizidine alkaloids. All chemicals are in units of mg g^{-1} with the exception of nitrogen which is a percentage.

should be considered now that we have shown that soil biota can alter spectral reflectance.

In the course of the past two decades, research on hyperspectral reflectance has shown an increased potential for studies on variability in vegetation. As early as 1998 it was possible to find a wavelength-dependent variation in the near-infrared region of green leaves as a result of species evolutionary strategies (Asner, 1998). The wavelength variation between sterile and nonsterile soils suggests that the biotic aspect of the soils can contribute to the spectral variation in the near-infrared. This is feasible if we consider, for example, the role of soil organisms in nutrient cycling

and succession (Gange *et al.*, 1990; Ekschmitt & Griffiths, 1998; De Deyn *et al.*, 2004; Wardle *et al.*, 2004). The response of *S. inaequidens* to soil biota and how this translates into hyperspectral reflectance patterns opens up opportunities at the field scale where biotic resistance may develop against invasive exotic species. For example, it may be possible to detect where hotspots of biotic resistance will develop to counteract invasion. Such hotspots may develop, for example, when soil pathogens of the invaded range become more virulent (Reinhart *et al.*, 2010). Since the exotic species increased their PA content in tested conditions, we postulate that when species no longer respond by increasing metabolite

content or shifting defence properties, this could indicate a location where biotic resistance to invasive exotic plant species is developing. Such shifts in soil biota–exotic plant interactions across time–since-introduction gradients have been suggested in recent studies (Lankau *et al.*, 2009; Diez *et al.*, 2010).

While we achieved highly accurate species discrimination with both spectral and chemical data, PLSR within species resulted in weak estimations of their chemical content. Nevertheless, we worked within single species, whereas most published surveys occurred between species. The chemical concentration range in our study was much narrower than is usual in the field. While studies by Doughty *et al.* (2011) and Darvishzadeh *et al.* (2008) presented Chl concentrations between 1 and 14 mg g⁻¹, we only achieved a range between 1 and 3 mg g⁻¹. The PLSR models failed to predict plant PA concentration, but the current study had many samples below the suggested threshold for correct predictions (2 mg g⁻¹) and the range of PA concentrations was also lower than in our pilot study (S. Carvalho *et al.*, unpublished). The most accurate predictions achieved occurred with foliar N, which had concentration ranges similar to previous studies (Knox *et al.*, 2010; Ramoelo *et al.*, 2011). These results suggest that, within a species, there may be some limitations in using spectral measurements for chemical predictions at relatively low concentrations with a narrow concentration range.

In conclusion, we showed that the foliar chemistry of the exotic invader *S. inaequidens* was more affected by soil biotic conditions than was the native *J. vulgaris* (the species that was already occurring in these field sites). Using spectral reflectance data, we were able to accurately predict species identity and, to some extent, distinguish between plants growing in different soil biotic conditions. Correlation between canopy hyperspectral signal and the measured plant chemical composition was moderate to nonexistent, depending on the types of chemicals. Although the effect was weak, we established that pathogenic soil biota effects have the potential to affect the spectral reflectance of plants. We propose that further studies are needed to develop this method for use at the field scale as part of a strategy to detect hotspots of biotic resistance against exotic invaders, or to detect early development of soilborne diseases in fields of crops. The increasing number of databases with high-fidelity spectral measurements and significant classification of ‘unknown’ spectral samples into the correct species group suggests an extended potential for spectral libraries to become the GenBank (digital library for nucleotide and protein sequence association) of remote sensing measurements for temporal developments in plant–soil interactions.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 PCA-LDA loadings of the spectral reflectance LDA for *J. vulgaris*, *J. erucifolia* and *S. inaequidens*.

Table S1 Percentage of correct classification of samples by the spectral reflectance LDA

Table S2 Percentage of correct classification of samples by the LDA model with plant chemical concentration

Table S3 Descriptive statistics of each species' chemical concentration in sterile and nonsterile inoculum

Table S4 Mean values of each treatment per species and the percentage of difference in plant shoots within each soil origin treatment

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