Jacobaea through the eyes of spectroscopy

Identifying plant interactions with the (a)biotic environment by chemical variation effects on spectral reflectance patterns

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Thesis

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Contents

"Estes descobrimentos não se fizeram indo a acertar."

Pedro Nunes (1537)

(*"These discoveries were not done using guesswork."*)

Abstract

Plants interact with a wide array of aboveground and belowground herbivores, pathogens, mutualists, and their natural enemies. These interactions are important drivers of spatio-temporal changes in vegetation, however, they may be difficult to be revealed without extensive sampling. In this thesis I investigated the potential of visible and near-infrared spectral measurements to detect plant chemical changes that may reflect interactions between plants and biotic or abiotic soil factors. First, I examined the relative contribution of pyrrolizidine alkaloids (PAs; these are defence compounds of Senecio-type plants against generalist herbivores) to the spectral reflectance features in the visible and short-wave infrared region. My hypothesis was that PAs can be predicted from specific spectral features of aboveground plant tissues. Since PA profiles and their relation to spectral features could be species specific I compared three different species, *Jacobaea vulgaris*, *J. erucifolia* and *S. inaequidens* subjected to nutrient and water treatments to stimulate plant chemical variation. Pyrrolizidine alkaloids were predicted best by spectral reflectance features in the case of *Jacobaea vulgaris*. I related the better results obtained with *J. vulgaris* to the existence of the correlation between PAs and nitrogen and the presence of the epoxide chemical structure in *J. vulgaris*.

I also examined if different soil microbial communities influenced plant shoot spectral reflectance. I grew the same three plant species as before in sterilized soil and living soil collected from fields with *J. vulgaris*. I expected that soil biota would change shoot defence content and hyperspectral reflectance in plant species-specific ways. Indeed, the exposure to different soils caused plant chemical profiles to change and both chemical and spectral patterns discriminated plants according to the soil biotic conditions.

I studied how primary and secondary plant metabolites varied during the growing season and vegetation successional stages. I used a well-studied chronosequence of abandoned arable fields and analysed the chemistry of both leaves and flowers of *Jacobaea vulgaris* throughout the seasons in fields of different successional status. My general hypothesis was that seasonal allocation of nutrients and defence metabolites to reproductive organs fitted the optimal defence theory, but that pattern was dependent on the successional stage of the vegetation. I found

an interaction between season and succession stage, as plants from longer abandoned fields generally had flowers and leaves with higher N-oxides, especially in late Summer. Independent of the succession stage there was a seasonal allocation of nutrients and defence metabolites to flowers. Analyses of spectral reflectance of the field plants showed that defence compounds could be estimated more reliably in flowers, while in leaves primary compounds could be predicted best. Succession classes were successfully discriminated by the spectral patterns of flowers. Both chemical and spectral findings suggested that flowers are more sensitive to field ageing processes than leaves.

Conclusions

- The estimation of pyrrolizidine alkaloids by spectral reflectance features was better in *Jacobaea vulgaris* than in *Senecio inaequidens* or *Jacobaea erucifolia* (chapter 2).
- Differences in soil communities affect plant leaves' chemistry and spectral reflectance patterns (chapter 3).
- *Jacobaea vulgaris* plants from recent and longer-abandoned fields showed the largest differences in chemical concentration, composition of defence compounds, and spectral reflectance patterns. Flowers were more discriminatory than leaves (chapters 4 and 5).
- There is a potential to detect plant-biotic interactions by analyzing spectral reflectance patterns (this thesis).

Introduction

1

Plants are continuously influenced by a wide array of aboveground and belowground herbivores, pathogens, mutualists, and their natural enemies. However, the influence of soil organisms on plant population dynamics is often overlooked. Although they are cryptic, soil organisms do influence plants, with a range of symbiotic to pathogenic interactions that have important roles in driving spatio-temporal changes in vegetation (Clements, 1963; Price, 1984; Blomqvist *et al.*, 2000; Bardgett & Wardle, 2010). The influence of soil organisms on vegetation dynamic becomes particularly interesting in outbreak plant species. We often don not know the impact of soil organisms on a species' success to occupy a new niche or its decline thereafter. When introduced in another range, some plant species may become invasive as a result of their release from the native soil biota that control plant population abundance (van der Putten *et al.*, 2007). With respect to enemy release of introduced exotic plant species, some researchers have suggested that when time proceeds, soil biota can gradually exert natural biological control (Diez *et al.*, 2010). The challenge for invasion ecologists is to understand and predict when and where in the new range introduced exotic plant species may become controlled by soil-borne biota. The aim of my thesis research was to test whether remote sensing could help detect where plant species with invasive characteristics are controlled by soil-borne biota. I have used *Jacobaea vulgaris*, a native outbreak plant species, as a model.

Since plant primary and secondary metabolites respond to plant interactions with mutualists, pathogens, and herbivores, plant chemistry could be an important aspect to understand when cryptic biota influence plants. However, it is difficult to determine where soil-borne biota will become active without disturbing field sites or performing destructive and time consuming analyses. Detection of biotic resistance of soil biota to plants might benefit from rapid non-destructive approaches such as hyperspectral reflectance, provided that plant reflectance is a reliable indicator of such interactions. Yet, little is known about how interactions between plants and soil biota may translate into hyperspectral reflectance patterns.

The potential of spectroscopy in the visible and infrared wavelengths for

studying changes in plant chemistry is high. In this thesis I focus on its applicability to ecological questions related to plant interactions with either soil biotic or abiotic factors. According to Graetz (1990) the problems ecologists face are global, yet they require solutions that are local and highly site specific. Certainly, cryptic factors such as the impact of soil biota on plants fit Graetz' view. This spectral reflectance approach has the potential towards, for example, monitoring plant stress, plant invasion processes (enemy release hypothesis, see box 1), competition and soil microbial influence. The technological developments in remote sensing of the last decades provide the possibility to study temporal and spatial scales of local systems that can be integrated in global systems due to high dataset handling capacity. Understanding how spectral patterns may indicate specific biotic interactions between plants and their soil-borne enemies could be important in future studies, especially when the challenge of spatial and temporal scale is considered, (Schmidt & Skidmore, 2003; Mutanga *et al.*, 2004; Beck *et al.*, 2008; Knox *et al.*, 2010; Ramoelo *et al.*, 2011a) as often these plant-soil biota interactions might depend on location and season (Jaeger *et al.*, 1999; Van Der Putten, 2003). In this thesis I investigated the potential of spectroscopy of the visible and infrared wavelengths to detect plant chemical changes resulting from plant interactions with either biotic or abiotic factors.

Box1

Enemy release hypothesis - ERH - (Keane & Crawley 2002) suggests that plants, when introduced in a new habitat, will be released from their native specialist predators and the plants will experience reduced herbivore pressure in the newly invaded area. This released pressure provides an advantage to invade the new area. The ERH also relates to the evolution of increased competitive ability – EICA – hypothesis, proposed by Blossey and Notzold (1995). It states that under reduced enemy pressure, invasive plants species will be selected to shift the allocation of resources from defence to growth giving the invasive plants a competitive advantage when compared to native plant species.

The role of chemical components in plant interactions

There are two main aspects of plant chemistry that are important for plant interactions with the environment: i) primary compounds that relate to plant

nutrition, source of energy and growth, and ii) secondary compounds related to plant defence and communication. As such, during their life span plants have to continually balance between allocation of resources to growth and reproduction or to defence (Siemens *et al.*, 2002; Dicke & Baldwin, 2010). The trade-off between growth and defence culminates in an array of evolutionary strategies: Some species spend little energy in defences, but more in faster growth; in the other extreme are species that spend much energy in defence compounds with the trade-off that such plants are slow growing (Herms & Mattson, 1992). So, although we perceive plants as static they are far from "sitting ducks".

Chemicals such as nitrogen, carbon and phosphorous are building blocks for any necessary metabolite required for growth and maintenance. Included in primary compound category are the photosynthetic pigments, such as chlorophyll or carotenoids that influence the metabolite productivity by the plants. To grow, plants must allocate nutrients to photosynthesis. Access to nutrients can be a limiting factor affecting processes such as the photosynthetic rate, plant growth or defence efficiency. Plants that are adapted to low-nutrient environments are generally slow growers, with, among others, low photosynthetic rates and low leaf turnover.

Secondary compounds enclose an array of chemicals with numerous functions, such as defence against herbivores and pathogens or mediation of interactions with competitors and mutualists (Iason *et al.*, 2012). In this thesis I focus on secondary metabolites with defence characteristics. Phenolics, alkaloids, glucosinolates and tannins are examples of plant secondary compounds with defence functions. Their defence function against numerous intermediaries both below and aboveground communities. There is a great diversity in secondary compounds including the individual compounds and its mixtures. Besides their function in organ defence, secondary compounds are also know to influence other processes like litter decomposition (Grime *et al.*, 1996; Bardgett & Wardle, 2010), which ultimately influences nutrient cycling and soil microbial composition.

Soil microbial communities can affect and are affected by nutrient availability, and plant nutrient uptake and decomposition are affected by the soil community composition and functioning. Plants are often infected by a large range of soil microorganisms. Some are symbionts, for example *Rhizobium* bacteria in legume nodules that help nutrient uptake. Others can be necrotrophic fungi and bacteria that destroy plant cells and release nutrients for pathogens to take up (Walters, 2011). When attacked by soil microorganisms, plants can change their defence metabolism

(Macel *et al.*, 2005; Joosten *et al.*, 2009) affecting both belowground and aboveground organisms (Wardle *et al.*, 2004b). Several studies have shown that differences in soil community composition can affect nutrient cycles, which influences plant performance, chemical profiles and vegetation composition (Bever *et al.*, 1997; Hedlund *et al.*, 2003; de Vries & Bardgett, 2012; Wardle *et al.*, 2012).

Optimal defence theory (McKey, 1974; Rhoades, 1979) states that aboveground plant parts that are more valuable, such as young leaves and flowers or seeds, are best protected, in order to avoid major damage by herbivores. Several defence compounds are known to increase during the flowering stage and to decline by the time of senescence. The multiple functions of defence compounds in biotic and abiotic interactions create a very dynamic system throughout the plants' lifespan. During their life history plants are exposed to numerous ecological interactions, such as herbivory, pollination, competition, etc. Especially if addressing outbreak species that suffer different pressure from pathogen and herbivore attacks than native populations. Different pressures across the life span of a plant increase the need to include the temporal scale to understand plant-interactions. The legacy of past interactions can also influence plant resistance and resilience towards biotic attacks or nutrient depletion (Joosten *et al.*, 2009; Kostenko *et al.*, 2012b).

Temporal dynamics of plant-interactions

Plant metabolite composition and levels are space and time dependent (Van der Putten *et al.*, 2001; Koricheva & Barton, 2012) as populations and communities change, diseases appear or herbivores attack. Consequently there are temporal changes in chemical defence allocation to plant tissues during plant life history. Life strategies could vary at short-term and long-term scales. As short temporal scales we can consider hours, days or even seasons and long-term we perceive years, decades or centuries. An extreme example of quick temporal change is found in *Nicotiana attenuata* plants adapted to decrease the levels of flower chemical defence during the night to the benefit of pollination (Euler & Baldwin, 1996). Flowers are important organs for reproduction and several examples show that nutrients and defences are allocated to flowers to enhance their defence against generalist herbivores (Fagerstrom *et al.*, 1987). This generally pressures herbivores to feed in seasons when plant organs are least toxic (Boyd & Goodyear, 1971; de Boer, 1999; Van der Meijden & Klinkhamer, 2000; Mysterud *et al.*, 2011).

While seasonal variation of plant chemistry may be partially determined by optimized defences, variation during vegetation succession cycles (longer time scales) may be related to genetic differentiation as well. Different antagonists can lead to genetic variation in defences within a population (Van der Meijden, 1996). With proceeding succession, the legacy of biotic interactions may change vegetation composition (Korthals *et al.*, 2001; Bezemer *et al.*, 2003). If so, at every sampling time we may find chemical variation due to the impact of previous plant interactions (Zhang *et al.*, 2009). This chemical shift due to legacy effects is an interesting issue to study in outbreak species. Another question is how these plants are affected by the legacy of native plant-soil interactions? Further, when are plant species with invasive characteristics controlled by cryptic systems such as soil-borne biota?

Plant species' defence expression can affect several ecological processes and may be affected by them with consequences for the concentration and/or composition of secondary metabolites. Such intricate aspects are essential to understand but are also difficult to monitor. Systems affected by exotic species are of especial interest to understand as new interactions can result of these species outbreak. This need for continuous evaluation involves laborious work, which can often be expensive. Currently, new techniques that range from detailed metabolomics to extensive remote sensing are under construction. In this thesis I have examined how spectroscopy could be used to detect differences in plant defence chemistry. I also studied what could be achievable at different temporal scales.

Spectroscopy

The main goal of most remote sensing studies is to attain high resolution images with accurate estimation of the variables under study. A less known aspect of remote sensing is spectroscopy, the study of physics and electromagnetic radiation of the elements that constitute the object of study. Generally spectroscopy studies are the prelude to airborne or satellite imagery studies or handheld machinery for quick monitoring purposes. Spectroscopy provides the spectral feature of a material at each wavelength of the electromagnetic field, which can vary depending on whether the material is in solid, liquid or gas form. In essence atoms react to particular wavelengths with distortion of their electron orbit fields. As such the target material may generate different reflectance frequencies that can be measured by

spectrometers. Reflectance measurements are a common choice as they are sensitive to specific chemical bonds and shift with variation in the chemical composition of the object measured. The response of the materials at every wavelength is defined as its spectral properties (Colwell *et al.*, 1983; Kumar *et al.*, 2001).

Plant spectral properties

Photosynthesis can be well recognized in its general spectral pattern (Fig. 1). The incoming radiation of the sun when reaching the earth's surface is highest in the visible region of the electromagnetic spectrum (450 – 750 nm). The ultraviolet radiation is mostly absorbed by the atmosphere (< 450nm) and the infrared region (>800nm) is highly affected by the atmospheric water molecules. The water in the plants is also used at cellular level to produce thermal energy (Leopold & Kriedemann, 1975; Kumar *et al.*, 2001). The plants' photosynthetic processes make use of the most energetic radiation between the ultraviolet and the infrared regions.

In plants the spectral reflectance pattern can be subdivided into 4 important regions (Kumar *et al.*, 2001): the visible (450nm-700nm) the red-edge (700-800nm), the near-infrared (700-1300nm) and the mid-infrared (1300-2500nm) region. All provide information on different physiological characteristics of plants.

The visible region

This region is mostly influenced by the photosynthetic pigments. Chlorophylls, carotenoids and xantophylls use electron transitions as aits mechanism to absorb radiation that can be used for photosynthesis. The wavelengths where most of the radiation is absorbed are around the 400-450nm and, in the case of chlorophyll, also at 650nm (Table 1). These electrons transitions occur in what is called the spectral absorption maxima. Consequently, the lowest reflectance in the visible area is found in the 400-500nm and the 600-700nm regions (Ollinger, 2011). Although high chlorophyll concentration may result in high radiation absorption, it does not necessarily relate to high photosynthetic rates and translation to higher biomass. This discrepancy is generally attributed to intraspecific differences in photosynthetic efficiency (Leopold & Kriedemann, 1975; Kumar *et al.*, 2001). However, according to ecological studies, this photosynthesis- spectral absorption discrepancy could also be a result of the plant need to allocate photosynthetic products to production of defence compounds, which is a common trade-off for growth. This trade-off of

growth against defence is predicted by optimal defence theory (Fagerstrom *et al.*, 1987; VanDam *et al.*, 1996).

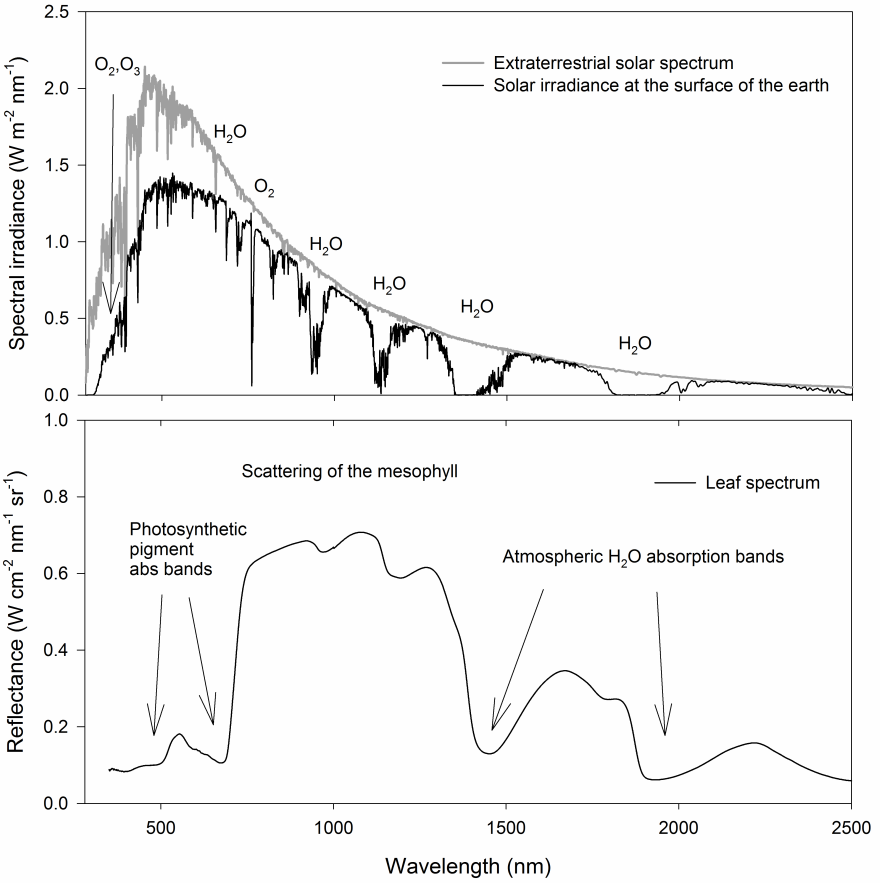

Figure 1: Top panel represents the incoming solar radiation outside earth atmosphere and at the surface. Major absorption features are identified with its responsible atmosphere molecule. Bottom panel represents a characteristic green leaf reflectance pattern. Major features are identified with its responsible leaf chemical or cell structure

The red-edge

The red-edge is a characteristic feature in plants, which occurs in the spectral reflectance shift between pigment absorption areas and the infra-red area affected by water and cell structure (or plant canopy structure in the case of integral plant measurements)(Horler *et al.*, 1983). The inflection point in the red-edge has often been correlated with, among others, chlorophyll content in the leaf and plant stress resultant of water and nitrogen limitations, pesticides and ozone damage (Boochs *et*

al., 1990; Carter *et al.*, 1996; Knox *et al.*, 2010). Such correlations could also be of interest in plant-biotic interactions resulting in plant stress, but such tests are rare, if existing at all.

Table 1: Leaf chemical components with related absorption features in the visible and infrared spectral region, the wavelength of the feature absorption maximum and its mechanism. The information has been compiled from literature by Kumar et al. (2001).

Chemical	Wavelength (nm)	Absorption mechanism
Cellulose	1736	O-H stretch
Cellulose	1820	O-H and C-O stretch
Cellulose	1924	O-H stretch and deformation
Cellulose	2340	C-H stretch, O-H deformation
Cellulose, nitrogen, protein	2350	H-C-H rotation, C-H deformation
cellulose, sugar	1490	O-H stretch, 1st Overtone
Cellulose, sugar, starch	1780	C-H stretch, O-H stretch 1st overtone, H-O-H deformation
Cellulose, sugar, starch	2270	C-H and O-H stretch, C-H and H-C-H rotation
Chlorophyll a	430	Electron transition
Chlorophyll a	660	Electron transition
Chlorophyll b	460	Electron transition
Chlorophyll b	640	Electron transition
Lignin	1120	C-H stretch, 2nd Overtone
Lignin	1420	C-H stretch and deformation
Lignin, starch, protein	1690	C-H stretch, 1st overtone
Oil	930	C-H stretch, 3rd Overtone
Oil	1040	C-H stretch and deformation
Oil	2310	C-H bend, 2nd Overtone
Protein	910	C-H stretch, 3rd Overtone
Protein	1020	N-H stretch
Protein	1510	N-H stretch, 1st Overtone
Protein	1730	C-H stretch
Protein	1980	N-H asymmetry
Protein	2130	N-H stretch
Protein	2240	C-H stretch
Protein, nitrogen	2060	N-H stretch, N=H rotation
Protein, nitrogen	2180	N-H rotation, C-H, C-O and C=O stretch
Protein, nitrogen	2300	C-H rotation, C=O and N-H stretch
Starch	990	O-H stretch, 2nd Overtone
Starch	1530	O-H stretch, 1st Overtone
Starch	1900	O-H and C-O stretch
Starch	2000	O-H and C-O deformation
Starch	2250	O-H stretch and deformation
Starch	2320	C-H stretch, H-C-H deformation
Starch, cellulose	1540	O-H stretch, 1st Overtone
Starch, cellulose	2100	O-H rotation and deformation, C-O-C stretch

Table 1 (continued):

Chemical	Wavelength (nm)	Absorption mechanism
Starch, cellulose	2280	C-H stretch, H-C-H deformation
Starch, sugar	1580	O-H stretch, 1st Overtone
Starch, sugar	1960	O-H stretch and bend
Starch, sugar	2080	O-H stretch and deformation
Starch, sugar, water lignin	1450	O-H stretch, C-H stretch and deformation
Water	1400	O-H bend, 1st Overtone
Water, cellulose, starch	1200	O-H bend, 1st Overtone
Water, protein, lignin, cellulose, starch, nitrogen	1940	O-H stretch and deformation
Water, Starch	970	O-H bend, 1st Overtone
Xanthophyll	450	Electron transition
Xanthophyll	475	Electron transition
α-Carotene	440	Electron transition
α-Carotene	470	Electron transition
β-Carotene	450	Electron transition
β-Carotene	480	Electron transition

Near -infrared region

When focused on leaf measurements, the mid-infrared region is associated with cell structure, its water content and intercellular airspaces (Kumar *et al.*, 2001; Ollinger, 2011) . The higher the amount of airspaces the more radiation scatters and reflects, and the larger the amount of water the lower the scattering and reflection. This dual effect results in complex intraspecific variation that depends on the internal structure of species leaves (Ollinger, 2011) A clear example arises from comparing leaves of conifers and deciduous trees. Due to the influence of water influence in this region of the spectrum, many absorption features of organic compounds decline in strength, causing difficulties in interpretation.

Mid-infrared region

The mid-infrared region has two well-demarked absorption dips characteristic of the water content in plants and the surrounding atmosphere (around 1400 and 1900nm). Since in the near-infrared region water is a main influence of the spectral pattern, most other spectral features found in this region are weakened (Wessman *et al.*, 1988a). While in the visible range the spectral features relate to electron transitions in atoms, in the infrared the features relate to the stretching and bending of the bonds in the molecules. Molecular vibration occurs when quantum energy is absorbed by

the molecule, which causes a change of state. In spectroscopy these changes of state are called overtones and harmonics of the molecule (Wessman *et al.*, 1988a; Curran, 1989). The overtones and harmonics of bonds like C-H or O-H cause minor absorption features that are generally depicted in the infrared regions of the plant spectral reflectance patterns (Table 1). Although spectral features from bonds such as C-H and O-H are weak in the infrared region, it is still an important spectral region to study. For example, some spectral features relate to bonds that belong to proteins, sugars, and cellulose, which are chemicals that are relevant to ecological processes (Kumar *et al.*, 2001). In ecological studies, variation in such type of molecules has been related to processes of plant defence, growth and decomposition.

Spectral reflectance over plant life time

As plants architecture and chemical composition changes, the optical properties of plants change as well during their life-time (Wessman, 1992). The most common studies on temporal spectral changes involve seasonal fluctuations of nutrients in agronomic landscapes (for animal forage or food production) (Bartholomeus *et al.*, 2011; Ramoelo *et al.*, 2011a). Studies in savannas have shown that considering seasonal changes benefits nutrient levels estimation and improves monitoring of migratory routes of large herbivores (Ramoelo *et al.*, 2011a; Knox *et al.*, 2012). The predictive models of nutrients in savannas have improved by taking plant age into account (Knox *et al.*, 2010). This shows the importance of combining ecological and spectral knowledge to create realistic empirical models. To be able to map plant chemical processes continuously we need to understand the physical and spectral changes of plants through time, which could, in future, improve monitoring of outbreak species.

How vegetation succession (i.e. a longer time scale) impacts plant spectral patterns is less known, probably due to the increased level of complexity and the effect of subjective group classification. For instance, the categories old and young are often a human classification of an object within a category that is generally continuous, time. It is not well understood how succession causes shifts in spectral reflectance of a specific plant. For example, it has been suggested that carbon assimilation by forest stands depends on their succession stage (Law *et al.*, 2001), although the mechanism that might explain this pattern is not clear. An accurate

discrimination of the succession stages of plant populations by spectral patterns would be an added element to studies on chemical mediation of invasive species.

Over longer time periods, in spite of time being a continuous variable, researchers often resort to simplified fixed classes, due to limitations of temporal monitoring. This subjective factor might increase the difficulty to accurately discriminate succession in vegetation. More studies including both plant chemical processes and its spectral information should increase our capacity to study changes in vegetation resulting from plant-(a)biotic interactions (Graetz, 1990). Cryptic processes, such as in plant-soil biotic interactions that change over time, could benefit from such spectral discrimination quality. Understanding the temporal build-up of soil biota that exerts biological control of plant outbreaks would benefit from understanding the spectral reflectance features affected by temporal factors. There is also a lack of knowledge on effects of other trophic levels on seasonal or successional changes in spectral reflectance patterns, beyond large aboveground herbivores. Soil microorganisms and insects can be drivers of ecosystem changes (de Beurs & Townsend, 2008) and hyperspectral reflectance could be an interesting avenue to study these concealed biotic effects on plant fitness.

Spectroscopy and its potential in ecology studies

Herbivory, pollination, and biotic communities in general - both above and belowground - can play a role in plant chemical dynamics through time. Spectroscopy has the potential to measure chemical bonds by non-destructive methods. These chemical bonds often belong to the plant metabolites that influence and are influenced by biotic interactions. With such potential information in spectral measurements, there is a need to link chemical bond measurements and shifts in spectral patterns to plant interactions.

At the start of this thesis study, several questions were raised about the possibility to use spectral measurements to determine the exposure of outbreak or exotic plants to soil-microorganims and the sensitivity of spectral patterns to intra-specific variation. If chlorophyll evolved to absorb specific wavelengths, how significant is the influence of other relevant chemicals on the spectral signature of plant reflectance? If we can estimate chemicals of interest, or even understand spectral patterns, can such spectral patterns be used in studying interactions between plants and their enemies? How sensitive are spectral patterns to changes over time

and could we find shifts in biological control of plant species? In the last two decades models, such as the PROSPECT radiative transfer model, have been introduced to study the leaf optical properties (Jacquemoud *et al.*, 2009). Nonetheless, only in the last 5 years the plant evolutionary link to spectral patterns was proposed with models such as spectranomics (Asner & Martin, 2009). In spectranomics it is suggested that plant taxonomic separation should be achieved with a fusion of chemical and spectral knowledge in order to accomplish highly accurate images of species specific distribution. Several researchers also start to suggest new plant functional groups that include spectral pattern information, in order to improve imagery accuracy (Graetz, 1990; Ustin & Gamon, 2010; Ollinger, 2011). If spectral information is relevant for plant functional groups one could expect it to be also relevant for intra-specific variation. *Jacobaea vulgaris* and a long-term monitored field area were chosen to test a multidisciplinary approach to study chemical variation in the context of plant-interactions and spectral shifts.

Jacobaea vulgaris and closely related species

Jacobaea vulgaris Gaertn. (syn. *Senecio jacobaea L.,* Asteraceae) is a monocarpic biennial to short-lived perennial. In the first year this species forms a rosette, whereas flowering occurs in the second year if conditions are favourable (Harper & Wood, 1957; Wesselingh & Klinkhamer, 1996). If not, then flowering can be postponed for one or more years (Van der Meijden & van der Waals-Kooi, 1979). In Spring this species has a circular rosette structure (Fig. 2) with basal stalked leaves that are obovate to pinnately lobed and generally 2 to 6 cm wide. During early and late Summer the rosette leaves senesce and a single central stem develops with pinnate lobed leaves. The flower heads are the characteristic capitulum of Asteraceae with bright yellow flowers and small green bracts and are present throughout the Summer.

Although native to the Netherlands, *Jacobaea vulgaris* is considered a noxious outbreak weed as it is highly dominant in abandoned arable fields and toxic towards humans and livestock (Mattocks, 1986; Bezemer *et al.*, 2006a). *Jacobaea vulgaris* is also invasive in other parts of the world (Kempf *et al.*, 2010b; Wiedenfeld & Edgar, 2011). Its toxicity is mainly due to its pyrrolizidine alkaloids (PA) that are present in all organ types, from leaves to flower heads and seeds. *Jacobaea vulgaris* has been a model

species for many ecological studies due to this hepatotoxic characteristic towards humans, cattle and invertebrates (Mattocks, 1968; Kempf *et al.*, 2010a; Wiedenfeld & Edgar, 2011). Up to 30 different PAs can be found in this species in all plant organ types, from roots, stems and leaves to flower heads and seeds (Mattocks, 1986). *J. vulgaris* populations also can differ in their PA profiles of flowers (Hartmann & Witte, 1995) and leaves (Macel *et al.*, 2004). It is known that defence chemicals of *J. vulgaris* plants can be affected by soil nutrient and microbial composition (Macel *et al.*, 2004; Joosten *et al.*, 2009; Kostenko *et al.*, 2012b). The plant species has also antifungal activity due to its PAs (Hol & Van Veen, 2002) that can affect, and be affected by, soil biota and insects (Macel *et al.*, 2005; Joosten *et al.*, 2009).

Figure 2: *Jacobaea erucifolia* (left panel), *J. vulgaris* (middle panel) and *Senecio inaequidens* (right panel).

Jacobaea erucifolia Gaertn. Mey and Scherb. (syn. *Senecio erucifolius L.,* Asteraceae) is a much rarer perennial species that is closely related to *J. vulgaris*. Besides its morphologic similarity (Fig. 2) to *J. vulgaris* little is known about this species. It does not form a rosette, but stems are woody at its base with leaves of similar shape of *J. vulgaris*. It is also native in the Netherlands, but interactions with soil organisms have not yet been studied. Little is known about the toxicity of this plant species however, the diversity of PAs is much lower than of its congener *J. vulgaris*.

Senecio inaequidens DC. is a perennial species originally from South Africa and invasive in several European countries (Ernst, 1998; Lachmuth *et al.*, 2010). Its stems are woody at its base and their leaves are linear between 1 to 7 mm wide. PAs are a major defence metabolite of this species and its hepatotoxic character is a threat to many native herbivores. Like *J. vulgaris*, PAs in *S. inaequidens* can be poisoning and position itself as a likely contaminant of human products, such as honey or milk, in Europe.

Pyrrolizidine Alkaloids

With a dual role, that attracts specialist insect herbivores but deters most vertebrate and invertabrates, pyrrolizidine alkaloids (Fig. 3) are a very diverse group of plant defence compounds (Harper & Wood, 1957; Mattocks, 1986; Lindigkeit *et al.*, 1997; Macel, 2011). In Senecionaea species the pillar structure of PAs is the Senecionine N -oxide type from which all other PAs derive (Hartmann & Dierich, 1998). In plants PAs are present in two main chemical forms: Tertiary-amines and N-oxides (Rizk, 1990). As such, all PAs in *J. vulgaris* and congeners share similarities in there chemical structure that could be relevant for accurate estimation using spectroscopy.

Several bonds of these PA structures are expected to affect spectral reflectance features: the double bond between C-1 and C-2, an esterified allylic hydroxyl group at C-9 and the esterified secondary hydroxyl group at C-7 in the necine base (Hartmann & Witte, 1995). The pyrol and the amine rings are also expected to influence some spectral feature in the infrared region due to their vibrational states (Weyer, 1985). The spectral regions that are affected by the PAs are currently unknown and will be analysed in this thesis.

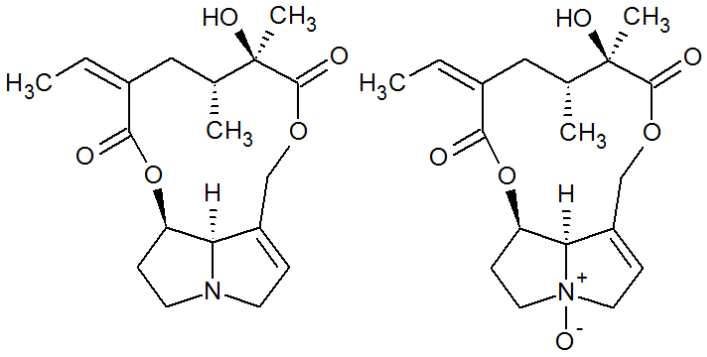

Figure 3: Two pyrrolizidine alkaloid molecular structures commonly found in the Senecionaea tribe. Senecionine (left) and senecionine N-oxides (right).

Study area and its relation to *J. vulgaris*

The relation between PA concentrations and reflected electromagnetic radiation is, at this moment, unknown. Therefore a controlled laboratory experiment was set-up to explore the specific spectral regions that interact with PAs. However for other questions of this thesis we selected a well-studied field area that has been monitored during the past decade (Table 2 and Fig. 4). The selected fields belong to a

chronosequence of abandoned arable-fields (Kardol *et al.*, 2006) located on south Veluwe, Gelderland Province, the Netherlands. Agricultural production stopped between 5 and 30 years ago and the fields are currently part of a large nature reserve aiming at re-creating biodiverse open grasslands. This chronosequence provides an interesting system to study differences in plant chemistry in different temporal scales such as season or succession. We can use this series of abandoned fields to substitute space for time (Bezemer *et al.*, 2006b).

Jacobaea vulgaris (ragwort) is a natural outbreak plant species that shows increased plant cover in the first 5 to 7 years whereas a steep decrease occurs thereafter (Fig. 5), leading to a hump-shaped population development when time since land abandonment increases (van de Voorde *et al.*, 2012). This pattern was attributed, to some extent, to the relationship between *J. vulgaris* density and the level of control by soil biota and negative feedback effects from other plant species that gradually colonize the old fields (Kostenko *et al.*, 2012b; van de Voorde *et al.*, 2012). We used information on site characteristics and responses of *J. vulgaris* to soil biota (van de Voorde *et al.*, 2011) to selected 8 fields for this thesis study (Table 2). Plant-soil feedback in this chronosequence depends of the soil origin with negative feedback in early years, neutral thereafter and positive in later successional soils (Kardol *et al.*, 2006).

Table 2: Selected fields for the studies in chapter 4 and 5, its code, age, years of abandonment at the time of the study, and succession class.

Field	Code	Age (abandonment time)	Year of abandonment	Succession class	Latitude (N)	Longitude (E)
Oud reemst	OR	5	2005	Young	52.02	5.48
Reyerskamp	R	5	2005	Young	52.01	5.47
Telefoonweg	T	7	2002	Young	52.00	5.45
Assel	A	7	2002	Young	52.12	5.49
Mossel	M	15	1995	Medium	52.03	5.45
Nieuw Reemst	NR	20	1990	Medium	52.04	5.47
Wolfheze	W	22	1988	Old	51.60	5.47
Dennenkamp	D	27	1982	Old	52.02	5.48

Aims and thesis outline

Since little was known about the contribution of Senecionaea species defence metabolites to the spectral reflectance pattern of these plants, in Chapter 2 I examined the relative contribution of its defence chemicals (total PAs, as well as the

tertiary amines and N-oxides) to the spectral reflectance features in the visible and short-wave infrared. The hypothesis was, that PAs influenced specific spectral features and that PA prediction using the visible and short-wave infrared regions of the electromagnetic field was possible.

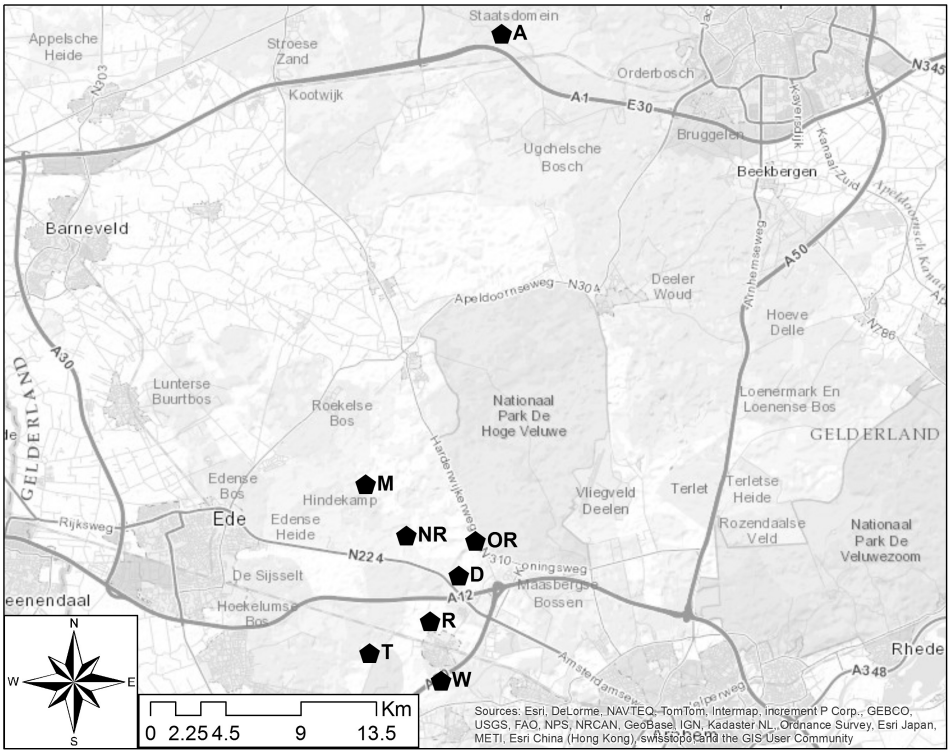

Figure 4: Map of the field study area

In Chapter 3 I examined the contribution of soil microbial communities to changes in shoot spectral reflectance in both native and exotic plant species. I studied plant-soil biota interactions and analysed their influences on shoot chemistry and hyperspectral reflectance patterns. I expected that soil biota would change species shoot defence content and that hyperspectral reflectance would enable detection of the effects of soil biota on plants.

Besides the biotic interactions, I was also interested in determining if the temporal factor was relevant to the chemical variation in *Jacobaea vulgaris*. I focused on how primary and secondary plant metabolites variation may depend on seasonal and vegetation successional stages. In Chapter 4 I analysed chemistry of both leaves and flowers of *Jacobaea vulgaris* in order to investigate how this depended on the

position of the plants in secondary successional environments. The general hypothesis was that seasonal allocation of nutrients and defence metabolites to reproductive organs fitted the optimal defence theory, but that patterns were dependent on the successional stage of the vegetation and the build-up of biological control.

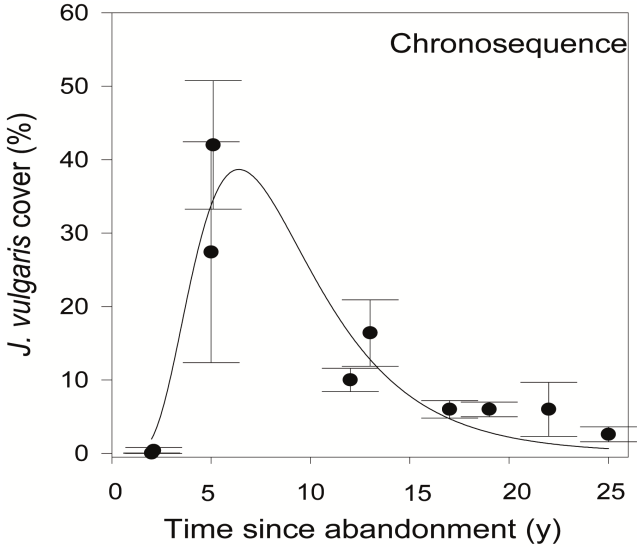

Figure 5: *Jacobaea vulgaris* cover percentage in the selected study area established by Van de Voorde et al (2012) with its estimated relationship based on a log-normal fit (represented by the line). *Image provided by the author*

After examining the chemical shifts of *J. vulgaris* in this secondary successional gradient, I analysed in Chapter 5 the spectral changes in *J. vulgaris* across time. The potential of spectral reflectance to discriminate temporal variation of *J. vulgaris* in different stages of secondary succession was investigated in two plant organs: leaves and flowers. I expected that seasonality and succession stage of *J. vulgaris* were expressed in the spectral reflectance of both leaf and flower organs and were related to the chemical variation (according to chapter 4) in both leaves and flowers.

Changes in plant defence chemistry (pyrrolizidine alkaloids) revealed through high-resolution spectroscopy

2

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Abstract

Plant toxic biochemicals play an important role in defence against natural enemies and often are toxic to humans and livestock. Hyperspectral reflectance is an established method for primary chemical detection and could be further used to determine plant toxicity in the field. In order to make a first step for pyrrolizidine alkaloids detection (toxic defence compound against mammals and many insects) we studied how such spectral data can estimate plant defence chemistry under controlled conditions. In a greenhouse, we grew three related plant species that defend against generalist herbivores through pyrrolizidine alkaloids: *Jacobaea vulgaris*, *Jacobaea erucifolia* and *Senecio inaequidens*, and analysed the relation between spectral measurements and chemical concentrations using multivariate statistics. Nutrient addition enhanced tertiary-amine pyrrolizidine alkaloids contents of *J. vulgaris* and *J. erucifolia* and decreased N-oxide contents in *S. inaequidens* and *J. vulgaris*. Pyrrolizidine alkaloids could be predicted with a moderate accuracy. Pyrrolizidine alkaloid forms tertiary-amines and epoxides were predicted with 63% and 56% of the variation explained, respectively. The most relevant spectral regions selected for prediction were associated with electron transitions and C-H, O-H, and N-H bonds in the 1530 and 2100 nm regions. Given the relatively low concentration in pyrrolizidine alkaloids concentration (in the order of $mg.g^{-1}$) and resultant predictions, it is promising that pyrrolizidine alkaloids interact with incident light. Further studies should be considered to determine if such a non-destructive method may predict changes in PA concentration in relation to plant natural enemies. Spectroscopy may be used to study plant defences in intact plant tissues, and may provide managers of toxic plants, food industry and multitrophic-interaction researchers with faster and larger monitoring possibilities.

Introduction

Plant defence compounds provide valuable insights into the interactions between plants and their (a)biotic environment. Determining toxic plant defence chemistry in a non-destructive way could significantly contribute for managers of toxic plants, food industry and plant ecology researchers with faster and larger monitoring capabilities. However, available methods, such as field spectroscopy, rarely have been used to analyse plant defence chemistry at a specific level (Ferwerda *et al.*, 2006; Izaguirre *et al.*, 2006). Pyrrolizidine alkaloids (PAs) are a group of chemical defence compounds that are toxic to humans and most cattle, and are found in *Senecio* species (Mattocks, 1986; Høy *et al.*, 1998). In this paper we examine the potential of spectral reflectance to estimate pyrrolizidine alkaloids (PAs) of three plant species from the Senecionaea tribe that have contrasting ecology and life history strategy and are generally considered noxious weeds in arable fields.

Field spectroscopy is a widely accepted method to measure the spectral reflectance of plants under natural conditions (Kumar *et al.*, 2001), with high capacity for imagery upscale. In addition, it facilitates the study biophysical and biochemical properties of (plant) materials (Curran, 1989; Ollinger, 2011) and many efforts are being made to improve the interpretation of spectral features (Ollinger, 2011). To date, field spectroscopy has been applied in order to quantify chlorophyll, nitrogen, phosphorus among other chemicals from laboratory to imagery settings (Wessman *et al.*, 1988b; Curran, 1989; Curran *et al.*, 1992; Fourty *et al.*, 1996; Asner, 1998; Kokaly *et al.*, 2009; Ustin *et al.*, 2009; Schlerf *et al.*, 2010; Skidmore *et al.*, 2010). However, studies regarding specific secondary compounds related to plants chemical toxicity and defence mechanisms are rare (Martens & Martens, 2000; Ferwerda *et al.*, 2006; Izaguirre *et al.*, 2006).

The *Senecio* species used for the present study are an example of a well-known group of plants that express high levels of toxicity, chemical plasticity and spatial dynamics (Lindigkeit *et al.*, 1997; Hol *et al.*, 2003; Macel *et al.*, 2004; van de Voorde *et al.*, 2010). Pyrrolizidine alkaloids (PAs) are the main reason why *Senecio* species are so well known, since PAs are their defence against natural enemies (Mattocks, 1968; Wiedenfeld & Edgar, 2011). Therefore, food safety has been a major concern associated with these species because of their PAs, as several *Senecio* species can affect human and livestock health upon ingestion (Mattocks, 1968). Some *Senecio* species are invasive outside their native range and pose particular problems for

agronomists (Mattocks, 1986; McEvoy *et al.*, 1991; Wardle *et al.*, 1995; Kempf *et al.*, 2010b). In order to determine if PAs can be detected by field spectroscopy, the spectral reflectance of the three species *Senecio inaequidens, Jacobaea erucifolia* (syn. *Senecio erucifolius*) and *Jacobaea vulgaris* (syn. *Senecio jacobaea*) was analysed under controlled conditions as measurement of PAs using spectroscopy have never been tested before.

The three species chosen to study the relation between PA and spectroscopy are well known (Hartmann & Witte, 1995; Joosten *et al.*, 2009). Up to 30 different PAs appear in these species, which are present in the plant in two main chemical forms: Tertiary-amines and N-oxides. The PA diversity is species-specific (Hartmann & Witte, 1995) and suggested to be an adaption to the numerous biotic interactions of plants with fungal pathogens, herbivorous insects, and mammals (Lindigkeit *et al.*, 1997; Hol *et al.*, 2004; Macel & Klinkhamer, 2010). As PA variation can also be affected by nutrient availability (Hol *et al.*, 2003), in this experiment plants have been grown under low and high nutrient supply to the soil in order to enhance chemical variation for during spectral measurements.

In the present study, we examined the relative contribution of total PAs, as well as the tertiary amines and N-oxides concentrations, to the reflectance spectra of the three *Senecio* species. Both these necine-base groups are frequently considered in plant-animal interaction studies. We hypothesize that PAs will influence specific spectral features and allow for PA prediction in the visible to short-wave infrared regions. Based on an earlier study (Weyer, 1985) we expect higher influence of PAs in the infrared region, at 1550 nm and 2050 nm. To test this hypothesis, we determined the best overall predictive regression models and identified its most relevant spectral regions in the visible and short-wave infrared for PAs.

Materials and Methods

Species description

Jacobaea vulgaris is a monocarpic biennial or short-lived perennial species that can dominate early successional grasslands, while *J. erucifolia* is a much rarer perennial species. Both these species are native to the Netherlands, but *J. vulgaris* is considered a noxious weed (Mattocks, 1968; van de Voorde *et al.*, 2010). *Senecio inaequidens* is also a perennial plant species, but is native in South Africa and invasive in the Netherlands and other parts of Europe (Ernst, 1998; Lachmuth *et al.*, 2010).

Experimental design

The relation between PA concentrations and reflected signal is, at the moment, unknown, so a field experiment represents an intermediate environment with high resource investment. Therefore a controlled laboratory experiment was arranged to explore the specific spectral regions that interact with PAs.

In order to generate a large chemical variation seeds from *J. vulgaris*, *S. inaequidens* and *Jacobaea erucifolia* were grown for 10 weeks under four different conditions of varying water and nutrient availability: i) Low water - Low nutrients (25 ml demineralized water); ii) Low water - High nutrients (25 ml 0.5 % Hoagland nutrient solution); iii) High water - Low nutrient (50 ml demineralized water); **iv**) High water - High nutrient (50 ml 0.5 % Hoagland nutrient solution) resulting in a factorial experiment. There were 20 replicates of each treatment, resulting in 240 plants (3 species x 20 replicate plants per species x 4 nutrient/water treatments).

Spectral measurements

Spectral reflectance varies depending on whether fresh or dry leaves, or the entire canopy are measured (e.g. Asner, 1998 and ; Skidmore *et al.*, 2010), because, frequently, weaker absorption features may be better detected at the canopy level than at individual leaves (Roberts *et al.*, 2004). Furthermore canopy structures have been found to influence nitrogen and chlorophyll optical patterns (Ollinger *et al.*, 2008). We evaluated whether PA regression models and optical patterns vary between these three types of measurements. The dry-leaf measurements were used to assess the effect of water presence/absence to PA regression models, as the short-wave infra-red region (1000 - 2500 nm) of the spectra is highly dominated by water features (Kumar *et al.*, 2001). No pure chemicals were measured as PA signals in leaves will be incorporated with other leaf components, reacting to chemical cues and even overshadowed by cell structure. Individual chemical NIRS measurements do not guarantee the same reflected signal relation in such intact leaves, but in general such correlations frequently exist (Kumar *et al.*, 2001).

Canopy level setup

Canopy reflectance of all plants was measured in a dark room with a field spectrometer (Fieldspec 3, ASD Inc., Boulder, USA) fitted with a 8° optic probe secured by a tripod at nadir. The spectral range of the spectrometer was 350 - 2500

nm with spectral resolution of 3 nm in the visible and 10 nm in the infrared. The illumination was carried out by a halogen light bulb with a 15° angle positioned at the same level of the optic. The halogen bulb emits a near-solar light spectrum (ASD Pro Lamps, ASD Inc., Boulder, Colorado, USA). Between every plant canopy measurement, all the reflectance measurements were standardized using a Spectralon white reference-panel (Labsphere Inc., North Sutton, USA). Since the fertilizer treatments created differences in plant growth, the spectrometer set-up was kept constant and each plant was individually adjusted to guarantee a field-of-view (FOV) of 12 cm diameter by the optic probe, which is a constant target-sensor distance. All pot surfaces were covered by the same black mat paper at soil surface to ensure a constant background reflectance and avoid effects from the possible variation in soil radiance. Of every plant, 10 reflectance readings were made four times with a rotation of 90º each time to reduce directional effects on reflectance (Mutanga *et al.*, 2003; Knox *et al.*, 2010). Each spectral reading was offset corrected and the 40 readings of each plant were averaged in a single reflectance measurement.

Fresh and dry leaf level setup

Immediately after the canopy reflectance measurements the shoots were clipped at the soil surface and the leaf reflectance measurements were performed with a RTS-3ZC Integrating sphere (ASD Inc., Boulder, USA) connected to the same FS-3 spectrometer used for the canopy measurements. Four leaves were measured individually with care to avoid primary veins. For each leaf, 10 spectral measurements were made and the 40 measurements thus obtained were averaged in order to produce a leaf sample measurement for every individual plant. For calibration, the Spectralon reference panel was measured between each leaf measurement. Immediately after the fresh spectral readings the leaves were freeze-dried for 96 hours and the reflectance of the dried leaves was measured in the same way as was done for the fresh leaves.

Spectral data processing

The 350 - 359 nm and 2250 - 2500 nm spectral ranges were removed after visual inspection because they were highly noisy. The spectral range used for further analysis was the same for all leaf and canopy samples in order to ensure that the predictive models and band selection could be compared within and among leaf and canopy level reflectance measurements. All samples' reflectance measurements were mean center-corrected and the Savitzky-Golay smoothing filter (Savitzky & Golay,

1964) was applied to remove random noise. This filter was applied with a third degree polynomial and a symmetrical seven-band window.

Pyrrolizidine alkaloids extraction

The leaves of each individual plant used for fresh-leaf spectral measurements were freeze-dried and ground. PA extraction was done following the protocol by Joosten *et al.*(2011). Quantification was performed against a blank plant extract containing a set of available PA reference standards as described by Cheng *et al.* (2011a).

Data analysis

Differences in the spectral reflectance signals between treatment or species groups were analysed with ANOVA and pair-wise *t*-test statistics in R 2.13.1 for Windows (http://www.r-project.org./). Relations between spectral reflectance patterns and PAs were analysed by partial least square regression (PLSR). A Hotelling T2 test was performed within the principal component analysis (PCA) to detect any atypical sample. This procedure was performed in Unscrambler X 10.1 for Windows (Camo Software AS, Oslo, Norway).

The obtained total PA, tertiary-amines and N-oxides concentrations were analysed by ANOVA with species, nutrient and water treatments as fixed factors. Type IV sums of squares and Gabriel Post hoc test were selected to better handle any missing values. Pearsons' correlation was used to analyse the possible correlation between defence compounds and primary, such as chlorophyll and nitrogen. The whole ANOVA analysis and Pearson correlation was performed in SPSS 17.0 for Windows.

Partial least squares regression

Jacobaea erucifolia was excluded from the regression analysis since the PA concentrations of the majority of samples of this species were residual to non-detectable, particularly for the low nutrient treatments. At the three levels of spectral measurements (canopy, fresh and dry leaf) the results for the other two species, *S. inaequidens* and *J. vulgaris*, were analysed both individually and together. Within each species the total PA concentration and that of its two main chemical forms (tertiary-amines and N-oxides) were analysed independently.

PLSR was used because it maximizes the covariance between dependent (plant chemistry) and independent (spectral reflectance) variables, taking care to

gather the projection of the minimum error with the fewest principal components possible. PLSR has not only the capability to handle a high number of independent variables, but also minimizes overfitting and data multi-collinearity problems (Wold *et al.*, 2001). The best models were assessed in terms of minimum root mean square error (RMSE) and the highest coefficient of determination using cross validation (r^2 cv). Cross-validation consisted of a three-group random segmentation of the full data set where iteratively two segments were selected for the calibration of the regression model while the other was reserved for testing the calibrated model. Thereafter the total prediction error for all individuals was calculated within all previous models and the regression model with the lowest root mean square error of prediction was selected (Wold *et al.*, 2001; Westerhuis *et al.*, 2008). This procedure was carried out in Unscrambler X 10.1 for Windows.

Interpretation of selected wavebands

The waveband variables selected in the regression models were examined by their regression coefficients and normalized loading weights, which express how much each coefficient contributes to the modeling of PA variation with a 95% confidence. Additionally, the variables important for PA prediction in the model are attained through a modified *t*-test to determine whether the regression coefficients of the calibration model are significantly different from zero. The procedure identifies regression coefficients that are sufficiently stable across cross-validation segments to guarantee precise estimates (Horler *et al.*, 1983).

To interpret the relevance of the selected bands for the prediction of PAs by PLSR we considered known chemical absorption features and electron transitions or bond vibrations that occur in the visible and short-wave infrared (Weyer, 1985; Curran, 1989; Billaud *et al.*, 2002; Huang *et al.*, 2004). The general molecular composition of PAs (Rizk, 1990) was also acknowledged in order to discern if the selected spectral regions were consistent with the general PA chemical configuration and known absorption features.

Results

Species-specific spectral differences

Reflectances of *J. vulgaris* and *S. inaequidens* remained different, irrespective of the source of reflectance, i.e. fresh or dry leaves or canopy (ANOVA $P = 0.05$; Fig. 1,

Fig. S1). *Jacobaea vulgaris* had overall higher reflectance values than *S. inaequidens* (Fig. 1). The spectral reflectance of *J. erucifolia*, however, differed in a non-systematic way from the other two species (see supporting information S1). The fresh and dry-leaf reflectances of *J. erucifolia* always were greater than of *J. vulgaris* and *S. inaequidens* through the entire short-wave infrared (ANOVA P = 0.05; Fig. 1a, c, Fig. S1). The canopy reflectance of *J. erucifolia,* however, only showed significant differences from *J. vulgaris* and *S. inaequidens* in the 700-1300 nm and 1300-2200 nm region, respectively (P=0.05; Fig. S1).

Nutrient addition also caused significant differences in spectral reflectance within each species (P = 0.05; Figs.1, 2). Without nutrients added, *J. vulgaris* and *S. inaequidens* had higher reflectance values in the visible region, but lower reflectance in the short wave infrared region (Fig. 2a and c). Low nutrient availability affected *J. erucifolia* reflectance in the visible region, but not in the short-wave infrared region (Fig. 2b).

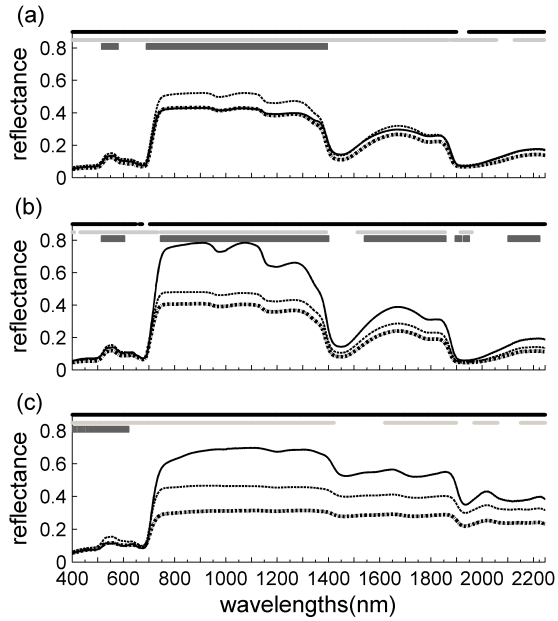

Figure 1: Mean spectral reflectance of each species at a) canopy, b) fresh-leaf and c) dry-leaf. *J. vulgaris* is represented by dashed bold line, *S. inaequidens* by simple dashed line and *J. erucifolia* by bold line. The significant differences between the three species (3-way ANOVA P=0.05) are shown on the top of each panel. Black line for species differences, light grey line for nutrient treatment effects and a dark grey line for water treatment effects.

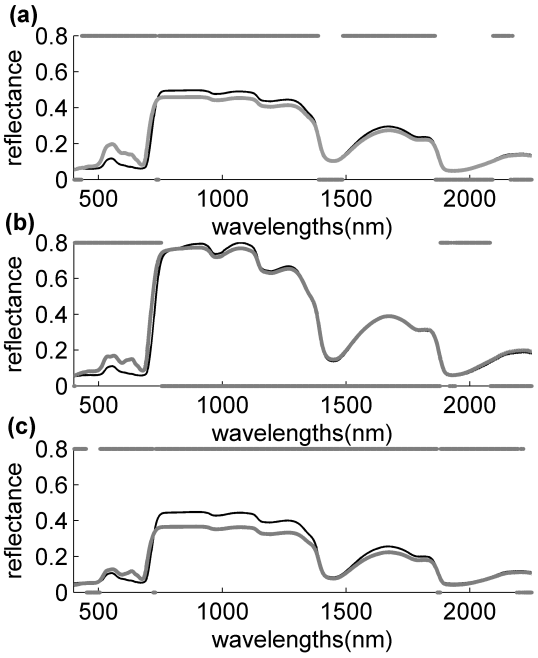

Figure 2: The effects of low and high nutrient treatments on mean spectral reflectance in fresh leaves. Low nutrient treatment plants are the grey line and high nutrient the black line. a) *Jacobaea vulgaris* b) *J. erucifolia* and c) *S. inaequidens*. Significant differences (t-test P=0.05) are represented in dark grey lines on the top of the each panel.

Pyrrolizidine alkaloids regression models

The strong variation in PA concentrations between plants with and without added nutrients and a good spectral separation of the three species allowed us to study the relation between the spectral bands and PA concentrations in the visible and short-wave infrared regions.

For *J. vulgaris* there was a positive correlation between the spectral reflectance and total PA concentration with a r^2cv of 0.46 for dry leaf, 0.52 for fresh leaf, and 0.55 for canopy reflectance (Fig. 3a, Fig. S2) and with RMSE of 3.23, 3.01 and 2.91 $mg.g^{-1}$ dry weight, respectively. However, *S. inaequidens* showed only a weak correlation with total PAs, with the best r^2cv acquired for fresh leaves, $r^2cv = 0.21$ and RMSE of 3.19 $mg.g^{-1}$ dry weight (Fig. 3d and S2). When only the tertiary amines of *J. vulgaris* were considered, a stronger correlation with reflectance was obtained ($r^2cv = 0.63$, RMSE 2.81 $mg.g^{-1}$ dry wt) than for total PA ($r^2cv = 0.52$, RMSE 3.01 $mg.g^{-1}$ dry wt). For *S. inaequidens* a slightly improved correlation was found when only the N-oxides were considered (Fig. 3d and f). However, the correlation ($r^2cv = 0.26$, RMSE 3.0 $mg.g^{-1}$ dry wt) was still quite weak. The analyses of tertiary-amines in *S. inaequidens* and N-oxides in *J. vulgaris* separately did not improve predictions (Fig. 3c

and e). Overall, *J. vulgaris* showed the highest correlation with both total PAs and tertiary-amines. Tertiary-amines specifically were best predicted by PLSR.

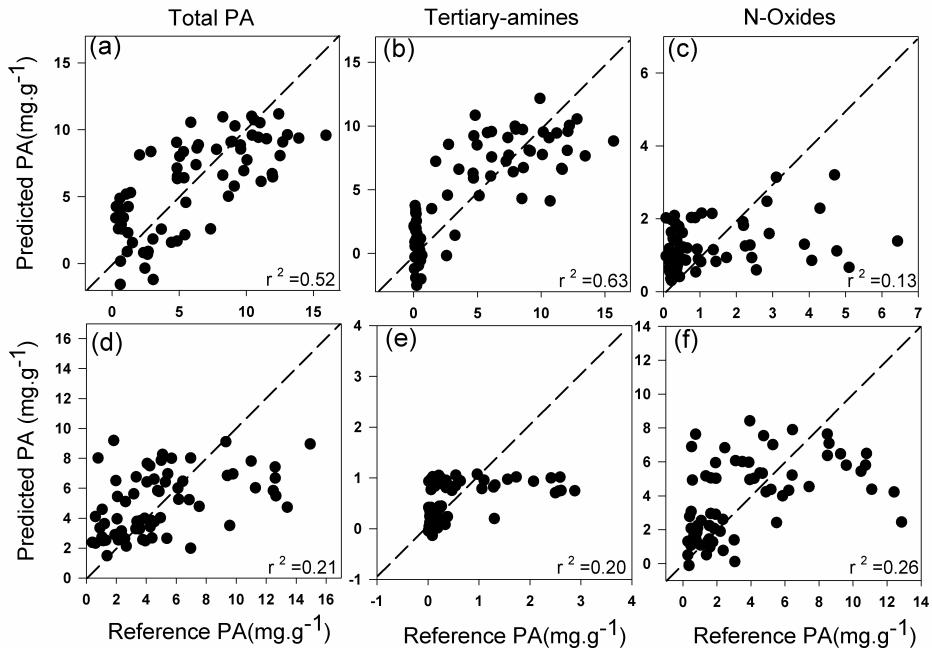

Figure 3: Predicted versus reference PA concentrations in fresh leaves spectral measurements. a) *J. vulgaris* total PAs b) tertiary-amines, c) N-oxides, d) *S. inaequidens* total PAs, e) tertiary-amines, f) N-oxides. Dashed lines represent the one to one regressions.

Although *J. vulgaris* and *S. inaequidens* both have tertiary amines and N-oxides the prediction results were not comparable. A considerably higher correlation between reflectance and PAs in *J. vulgaris* suggested that *J. vulgaris* could have a specific PA functional group affecting the spectral reflectance better than the other groups. The functional group typical for *J. vulgaris* is the epoxide ring in the necine acid region (Fig. 4), whereas *S. inaequidens* has high concentrations of non-epoxides. PLSR of the epoxides in *J. vulgaris* resulted in a r^2cv of 0.56, whereas no correlation was found for *S. inaequidens* samples (Fig. 5 a and b). In general the r^2cv for the epoxide group was slightly lower than the tertiary-amines, but its RMSE was better (in the 1.70 $mg.g^{-1}$ dry wt region). The non-epoxide PLSR analysis resulted in the expected weak correlation (r^2cv between 0.31 and 0.13; Fig. 5 c and d).

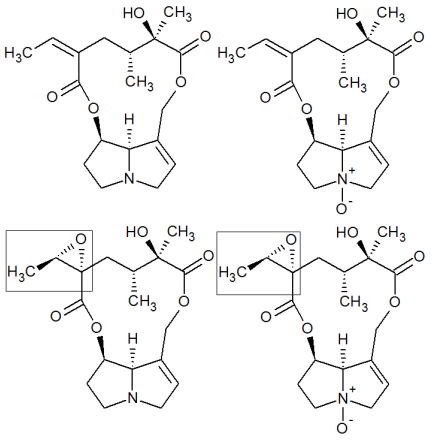

Figure 4: Chemical structure of senecionine (top left) and senecionine N-oxides (top right), two PAs commonly found in Senecionaea tribe. The most common *J. vulgaris* PAs, Jacobine (bottom left) and jacobine N-oxide (bottom right) with the epoxide structure highlighted in the box.

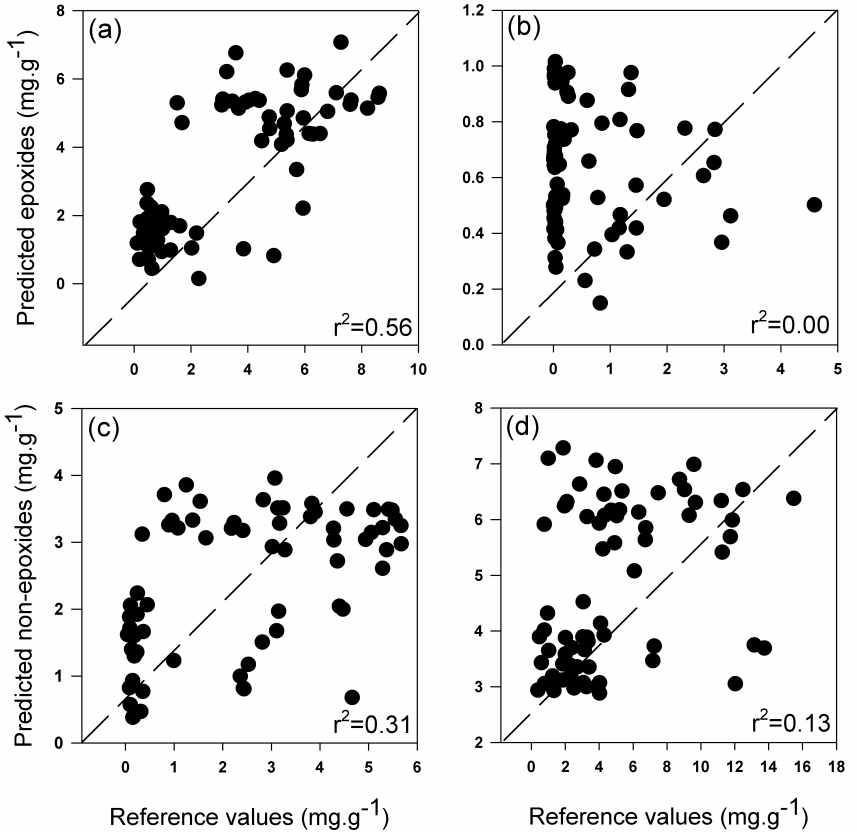

Figure 5: Predicted versus reference epoxide and non-epoxide PAs for fresh leaf partial least square regression results. Top row panels represent epoxide results, bottom row panels represent non-epoxide results. a) and c)- *J. vulgaris,* b) and d) *S. inaequidens*. Dashed lines represent the one to one regressions.

Interpretation of PLS selected wavebands

The regression coefficient illustrates the contribution of each individual wavelength to the PLSR: the more positive or negative a specific regression coefficient is, the stronger contribution it has to the PLSR development. The smaller regression coefficients were found in the infrared region (Fig.6). The N-oxide wavelength selection is quite complex for interpretation, as many bands from the visible and infrared spectra, were selected (Fig.6). Nonetheless the largest (in absolute value) regression coefficients were also in the visible and red-edge regions.

Five specific regions were commonly selected independently of the chemical group or species considered (Fig. 6 and S3); One selected region was in the visible (around 600 nm), one in the red-edge region (700 - 750 nm) and two other regions in the infrared (about 1550 nm and 2100 nm). The last common region selected by

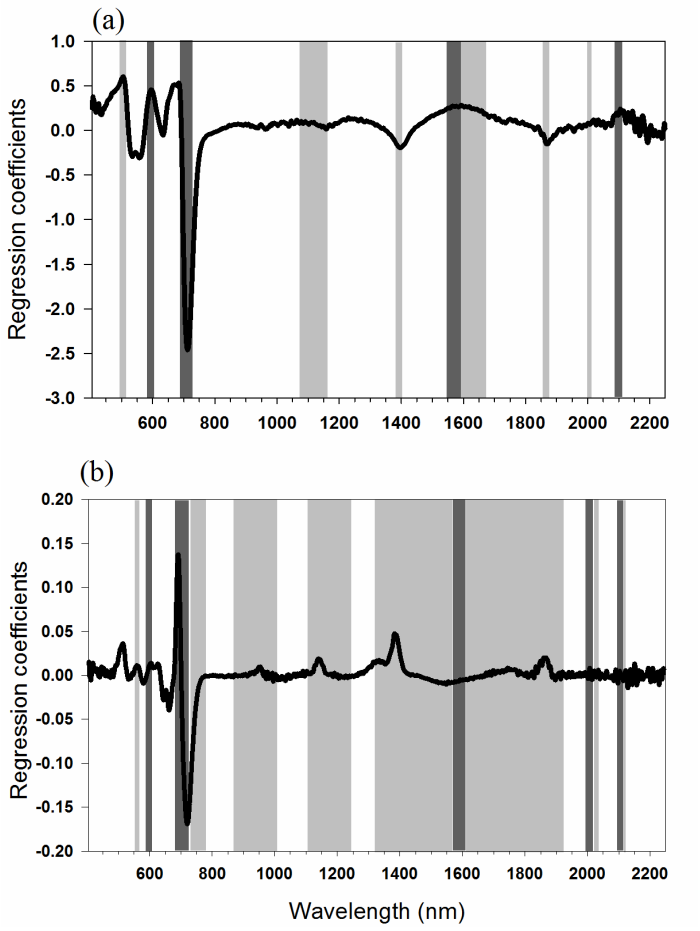

Figure 6: Partial least square regression coefficients (bold line) for each wavelength in the chemical estimation models. Regression coefficients of a) tertiary-amines in *J. vulgaris* and b) N-oxides in *S. inaequidens*. Grey boxes highlight the selected bands by the partial least square regression; dark grey highlights the selected regions that co-occur throughout the several PLSR analyses.

PLSR was in the 2000 nm region. The comparison between the tertiary-amines and N-oxides coefficients revealed that not only the six mentioned regions were shared by the two regression models, but also that the 1100 - 1150 nm, the1400 nm and 1800 nm regions were shared (Fig 6a and b). Several other regions were distinctively selected by the different regressions, such as the 950-1000 nm for the *J. vulgaris* total PA prediction (see supporting information S2) or 550-560 nm areas in *S. inaequidens* N-oxides prediction (Fig. 6 and S3b).

The analysis of the epoxide structure revealed similar outcomes to the *J. vulgaris* regression coefficients for the total PA model (Fig. S3).

Pyrrolizidine alkaloids (PA) treatment effects

The total PA concentrations of *J. vulgaris* and *S. inaequidens* varied between non-detectable and 16 $mg.g^{-1}$ dry weight, while for *J. erucifolia* PA concentrations varied between non-detectable and 10 $mg.g^{-1}$ dry weight (Fig. 7). The mean total PA concentration of *J. erucifolia* (1.47 $mg.g^{-1}$ dry wt) was significantly lower than for the other species (6.24 $mg.g^{-1}$ dry wt for *J. vulgaris* and 5.48 mg. g^{-1} dry wt for *S. inaequidens*). As expected, nutrient addition affected the total PA concentration within each species (ANOVA, Table 1) but there was no significant effect of water treatment. The total PA concentration of *J. vulgaris* and *J. erucifolia* was lower when the plants were grown without nutrient additions, whereas in *S. inaequidens* the total PA concentration was significantly higher when grown without added nutrients. In *J. vulgaris* and *J. erucifolia* the addition of nutrients strongly stimulated the production of tertiary-amines while reducing the levels of N-oxides. In *S. inaequidens* N-oxide levels were strongly reduced by addition of nutrients as well, but the tertiary amines levels were not increased (Fig. 7). Therefore, nutrient addition enabled us to achieve an increased range of composition and concentration of PAs, which was the aim for testing PA-predictive spectral reflectance models.

The correlations demonstrated both *J. vulgaris* and *S. inaequidens* had a positive correlation between total chlorophyll and nitrogen (Table 2). However, *J. vulgaris* and *S. inaequidens* showed opposite correlations between defence compounds and nitrogen. While *J. vulgaris* had a positive correlation *S. inaequidens* showed a negative correlation between these two properties.

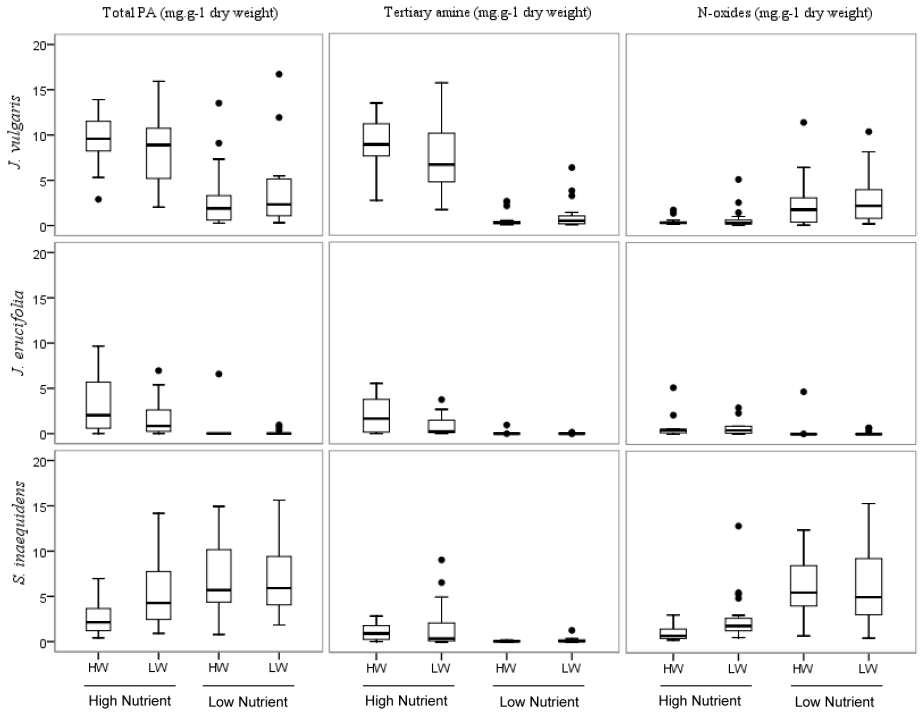

Figure 7: Total pyrrolizidine alkaloids, tertiary-amines and N-oxides concentration within *Jacobaea vulgaris*, *J. erucifolia* and *Senecio inaequidens* treatments. Rows from top to bottom represent *J.vulgaris*, *J. erucifolia and S. inaequidens* respectively*;* from left to right columns- total PAs, tertiary-amines and N-oxides concentrations, respectively. The experimental treatments are represented in the x-axis; HW- High water, LW-low water. Full circles (•) indicate possible outliers in the treatment.

Table 1: The effects of species, nutrient and water treatment on total pyrrolizidine alkaloids, tertiary-amines and N-oxides concentration by ANOVA.

Fixed factors	df	F total PA	P Total	F tertiary-amines	P tertiary-amines	F N-oxides	P N-oxides
Corrected Model	11	13.75	<0.001	50.06	<0.001	14.89	<0.001
Intercept	1	334.91	<0.001	239.93	<0.001	153.32	<0.001
species	2	33.10	<0.001	98.55	<0.001	37.88	<0.001
water treatment	1	0.14	0.71	0.84	0.36	1.10	0.30
nutrient treatment	1	11.73	0.01	162.56	<0.001	32.59	<0.001
species X water treatment	2	1.84	0.16	1.26	0.29	0.68	0.51
species X nutrient treatment	2	28.07	<0.001	62.88	<0.001	15.64	<0.001
species X watertreat X nutrient treatment	3	2.54	0.58	2.43	0.09	1.20	0.30
Mean square error	188	3.39		3.39		5.11	
Total	200						
Corrected Total	199						

Table 2: Pearson correlation matrix between several plant chemical variables. Chl – total Chlorophyll; N - Nitrogen; C – Carbon; t. amines – tertiary amines. In bold are the significant correlations between variables ($P < 0.05$).

		water	chl	chl	N	C	epoxide	non-epoxide	total PA	t. amines	N-oxides
J. vulgaris	water (g/g FW)	1									
	chl (mg.(cm²)⁻¹)	0.00	1								
	chl (mg.g FW⁻¹)	-0.07	**0.85**	1							
	N (%)	0.07	**0.76**	**0.70**	1						
	C (%)	0.01	0.05	0.06	**0.51**	1					
	epoxide	-0.01	**0.59**	**0.54**	**0.72**	0.09	1				
	non-epoxide	-0.01	**0.45**	**0.38**	**0.50**	0.02	**0.71**	1			
	total PA (mg.g DW⁻¹)	0.00	**0.59**	**0.54**	**0.68**	0.09	**0.83**	**0.70**	1		
	t. amines (mg.g DW⁻¹)	-0.01	**0.62**	**0.58**	**0.71**	0.09	**0.82**	**0.66**	**0.97**	1	
	N-oxides (mg.g DW⁻¹)	0.05	-0.24	-0.26	-0.27	-0.02	-0.12	0.04	-0.07	**-0.32**	1
S. inaequidens	water (g/g FW)	1									
	chl (mg.(cm²)⁻¹)	0.08	1								
	chl (mg.g FW⁻¹)	0.01	**0.91**	1							
	N (%)	0.07	**0.80**	**0.74**	1						
	C (%)	-0.20	-0.18	-0.11	0.14	1					
	epoxide	0.02	0.17	0.21	**0.25**	0.08	1				
	non-epoxide	0.19	**-0.37**	**-0.36**	-0.20	**0.24**	0.03	1			
	total PA (mg.g DW⁻¹)	0.14	**-0.34**	**-0.31**	-0.13	**0.28**	0.14	**0.93**	1		
	t. amines (mg.g DW⁻¹)	0.12	**0.30**	**0.25**	**0.36**	-0.06	0.17	**0.28**	**0.37**	1	
	N-oxides (mg.g DW⁻¹)	0.12	**-0.53**	**-0.49**	**-0.37**	**0.29**	-0.17	**0.89**	**0.85**	-0.11	1

Discussion

The examination by spectral reflectance of macro- and micro-nutrients or leaf area and mass (Curran, 1989; Ollinger, 2011) is a well-established procedure (Ustin *et al.*, 2009). Here, we analyse whether defence compounds concentration and composition correlates with visible and short-wave infrared reflectance spectra. Our hypothesis was that specific pyrrolizidine alkaloid (PA) chemical bonds would affect plant spectral reflectance and allow non-destructive prediction of PAs. Our use of PLSR provided new insights into the chemical properties that affect several regions of the visible and infrared region and revealed positive correlations between tertiary-amines and spectra with the strongest prediction outcome, however, only in *J. vulgaris*. All PA regressions selected 5 spectral regions. The electron transition features in the 600 nm region, which is attributed to nitrogen and chlorophyll content (Curran, 1989;

Knox *et al.*, 2010). The red-edge slope is also a feature related to nitrogen (Cho & Skidmore, 2006; Knox *et al.*, 2010) while the 1500 nm – 1580 nm region is affected by stretch overtones of O-H and combination bands of N-H with C=O, and C-N stretches associated with amine structures (Weyer, 1985; Curran, 1989; Billaud *et al.*, 2002). Another region shared was the 2100 nm, which is influenced by a C-H combination band of terminal chemical bonds (Weyer, 1985), and the most pertinent region selected in the PLS models was the 2020 nm (Fig. 6). According to Weyer (1985) and Billaud *et al* (2002) that region is affected by cyclic amines and pyrroles first overtones, which are building blocks of all PAs. Regions affected by O-H overtones (1450, 1900, 1960 and 2070 nm) and C-H second overtones (1140 nm), common bonds in almost all PAs, were also selected in some of the regressions.

The outcomes with lowest explained variance for a specific species always concerned the chemical forms that had residual to low concentrations. This suggests that the spectral features in these species have a detection limit of approximately 2 $mg.g^{-1}$ dry weight. The prediction of PAs using canopy measurements was lower than leaf measurements. We attribute that to the loss of minor, but important, spectral features such as the 1900-2100nm spectral region affected by pyrrole bonds. Two factors will reduce detection limits at canopy scales. First, this is a region of minimal multiple scattering due to strong liquid water absorption in plants. As a result, subtle absorption features will not be enhanced by multiple scattering, but rather obscured by other factors such as shadows and plant architecture (Roberts *et al.*, 2004). In addition, 1900-2100 nm is region of strong atmospheric absorption, further complicating detection at canopy scales.

Many studies addressing spectral features relation to chemical compounds have achieved similar outcomes (r^2 of 0.5-0.7) although much higher concentrations were considered when compared with the ones now addressed. For example, the r^2cv's obtained for tertiary-amines and N-oxides are comparable to results found for other plant chemical components such as starch (Curran *et al.*, 2001) and phenols (Cartelat *et al.*, 2005). Our results, therefore suggest that even at such low concentrations, i.e. milligram per gram, sensitive regression models could be achieved.

A subsequent analysis of PAs based of the presence of the epoxide and non-epoxide structures revealed that the spectral signal influence is probably caused by the presence of the epoxide in the PA structure, hence the better models for *J. vulgaris* as *S. inaequidens* lacks epoxide structures. Epoxide - amine structures assigned

to hydroxyl groups are mostly known to influence the 1428, 1610 and 1970-2209 nm regions (Billaud *et al.*, 2002) which were generally selected in the PLSR predictive models. Since *J. vulgaris* is typical for its epoxide containing PAs, especially in its invasive regions (Joshi & Vrieling, 2005), the capacity to predict epoxide structured PAs may help to study the defence chemistry of this species both in its native and invasive range. Epoxide groups are much less common in nature than e.g. tertiary amines. Therefore this spectral methodology should be relevant for monitoring how the defence strategy of *J. vulgaris* may develop since the time of introduction and under enhanced pressure of natural enemies. Still improvements are necessary to develop more accurate models as several of the selected spectral regions overlap with spectral regions already affect by other chemicals (Curran *et al.*, 1992).

The strong correlation between primary and secondary compounds in our study confirms the overlap of the selected regions. However, in spite of both species showing correlations between PA, nitrogen and chlorophyll only *J. vulgaris* provided good PA estimations. The strong correlation between PAs and nitrogen in *J. vulgaris* cannot be disregarded and suggests the additive effect of several collinear compounds in the features selected by the PLS models in the visible region. Nonetheless the significant effect of amine compounds in the infrared regions suggests that PAs may affect the spectra, even if mixed with other compounds. Currently, our study is limited by the inability to link "pure" PA measurements to spectral features. Both *J. vulgaris* and *S. inaequidens* contain PAs, but each plant species contains a different mixture of these compounds, which are also present in several chemical forms, and the levels may depend on environmental conditions. In addition, we cannot disregard the potential impact of soil background on such spectral measurements. The black background used in this experimental design might be a requirement for successful replication. Since we are aware of the regions of influence by the PAs, future measurements could consider the impact of natural background soil on such estimations. Another question to address in subsequent studies is the correlation of the chemical compounds: does this occur in natural environments and how strong may such correlations influence the spectral features in such systems. In case of a positive correlation an indirect estimation could still become feasible, especially if we consider the increasing capacity in remote sensing technology (Asner & Martin, 2011; Asner *et al.*, 2012).

Chemical and spectral properties

The plants grown without nutrient addition revealed a different reflectance pattern than plants to which nutrients had been added. Higher reflectance was analogous to previous studies with vegetation that had lower concentration of photosynthetic pigments and cellular structure (Ollinger, 2011). The low nutrient plant patterns, therefore, suggest that they have a lower concentration of photosynthetic pigments. A shift of the red-edge slope (located at roughly 700 nm) to shorter wavelengths is also consistent with patterns of environmental stress (Kokaly *et al.*, 2009; Ustin *et al.*, 2009).

Nutrient addition enhanced tertiary-amine concentrations but not N-oxides. Thus, tertiary-amines might be an indication of enhanced nutrient supply, while a pronounced presence of N-oxides could indicate nutrient limitation. Nutrient addition led to a large variation in total as well as in tertiary amines and N-oxide PA contents, which was essential to produce extensive prediction models. In contrast to earlier findings, our results show an increase of PA concentrations with increasing nutrient levels for *J. vulgaris* and *J. erucifolia* (Hol *et al.*, 2003; Hol, 2011), whereas *S. inaequidens* showed a decrease in PA concentration. We had more extreme treatments compared to previous studies (Hol *et al.*, 2003) and thus reached a wider range of PA concentrations (non-detectable to 16 $mg.g^{-1}$ dry weight) than in previous studies. For example, the highest total PA shoot concentrations found by Hol *et al.* (2003) was approximately 6 $mg.g^{-1}$ dry weight.

The fact that nutrient stress causes PA chemical expression to shift in a species-specific way was essential information to understand the relationship between the plant defence compounds and spectral reflectance patterns. With these findings we were able to show that another group of chemicals beyond the ones already acknowledged (e.g chlorophyll, nitrogen, phosphorus) can affect known spectral features directly or indirectly. Curran and co-workers (1989; 1992) suggest that predictions through such methods will only achieve high accuracy when all chemicals that interact within the same regions of the incident light are known, pyrrolizidine alkaloids are an addition to the list of chemical that can affect spectra. In conclusion, significant correlations of PAs with known spectra were obtained, which supports our hypothesis. This was especially valid if we consider the relatively low PA concentrations and the specific PA chemical forms, tertiary-amine, and N-oxides, separately. The presence of epoxide PA structures in *J. vulgaris* explained why

the prediction models worked better in this species than in *S. inaequidens*. The correlation between PAs and nitrogen or chlorophyll cannot be disregarded, however we are able to show that PAs could interact in the same regions, possibly contributing to the additive effect suggested by Curran and co-workers (1989; 1992).

Conclusion and perspectives

Remote sensing and spectroscopy have long studied primary constituents of plants to monitor food quality, photosynthetic processes etc.(Asner, 1998; Ebbers *et al.*, 2002; Mutanga & Skidmore, 2004; Skidmore *et al.*, 2010). Predictions similar to the ones obtained in this study, were found in earlier laboratory studies of spectral data (e.g. Curran *et al.*, 1992). Currently, fast nitrogen and chlorophyll mapping (among other chemicals) has been achieved with some success and chlorophyll and photosynthetic rates can be monitored *in situ* with portable spectral machines (Ebbers *et al.*, 2002; Ferwerda *et al.*, 2005; Darvishzadeh *et al.*, 2008b; Schlerf *et al.*, 2010). Defence chemistry, however, has seldom been addressed, nevertheless the technology and computing capacity continues to increase and spectral techniques could be applied into plant defence and toxicity studies (Ebbers *et al.*, 2002). Therefore, we conclude that, in the long term, field spectroscopy could offer some new possibilities to study Senecionaea defence mechanisms, its toxicity and its species-nutrient interactions in the field. Currently the sensitivity of remotely sensed measurements is not as accurate as highly sensitive analytical methods (e.g. LC - MS), though it could complement it by detecting this toxic chemical presence in the field, widening the temporal - spatial monitoring scale. The effects of smoothing filters, sampling set-up and nutrient effect differences between lab and field settings are some of several limitations to the extrapolation from lab to field and imagery. Therefore additional field tests for validation of our findings are necessary and currently under consideration. Such field- and imagery-based approaches are important to be tested and studied as it could provide managers of toxic plants with relevant and preventive information, such as is currently provided for primary chemicals (e.g. nitrogen, chlorophyll). Food management could quickly monitor toxic contaminations into their crops. Therefore, with the pyrrolizidine alkaloids results found and its upscale potential to the field, we suggest that further studies in the laboratory and field are necessary to validate and develop a thorough and practical field monitoring method.

Supplementary Information

Methods (seed germination process), Table S1, Figures S1—S3

Methods - *Seed germination process*:

Seeds from *Jacobaea vulgaris* were collected from one mother plant at Mossel, Veluwe, The Netherlands while *Senecio inaequidens* seeds were collected from one single plant at Oosterhuis, The Netherlands. *Jacobaea erucifolia* seeds were obtained from a specialized seed supplier (Blaufelden-Raboldshausen, Germany) who collects seeds from wild plant populations. Seeds of the three species were sterilized in 2% bleach solution and germinated on glass beads in a climate chamber with 16 h 25 ºC day conditions and 8 h 15 ºC at night. Once germinated, 80 seedlings of each species were transferred to individual 1.5 L pots with sterilized sandy soil. The selected soil was poor in nutrients to allow for the nutrient manipulation. The sterilization was done by Gamma radiation ($\geq$ 25 KGray, Isotron, Ede, the Netherlands) to keep all the soil biotic effects to a minimum. The soil was sieved through a 5 mm mesh size and homogenized. All plants were grown for 10 weeks in the greenhouse with a 14 h 21 ºC day and 10 h 16 ºC night regime with all atmospheric conditions constant at 70 % relative humidity.

Table S1: Cross validation r^2 and root mean square error (RMSE) in $mg.g^{-1}$ dry weight related to each tested chemical group in *J. vulgaris* (JV), *S. inaequidens* (SI) and *J. vulgaris* and *S. inaequidens* combined (JV+SI).

PLSR	JV r2cv	JV RMSE	SI r2cv	SI RMSE	JV + SI r2cv	JV + SI RMSE
Total PA	0.52	3.01	0.21	3.19	0.15	3.76
Tertiary amines	0.63	2.81	0.2	0.7	0.59	2.54
N-oxides	0.13	1.36	0.26	3.03	0.4	2.43
Epoxides	0.56	1.74	0	0	0.48	1.72
Non-epoxides	0.31	1.66	0.13	3.49	0.21	2.88

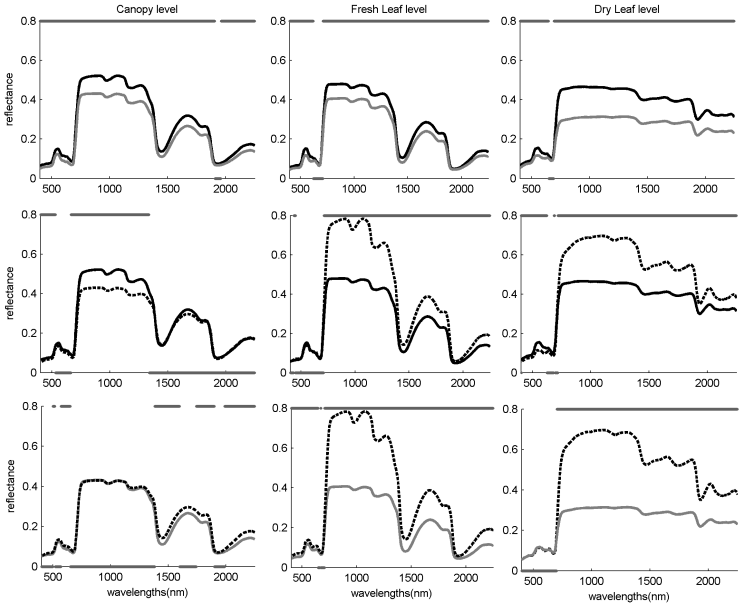

Figure S1: Species-pair mean spectral reflectance for the canopy, fresh and dry leaves (left to right columns respectively). *J. vulgaris* in black, *S. inaequidens* in dark grey and *J. erucifolia* in dashed line. Significant differences (t-test P=0.05) in dark grey lines on the top of the each panel.

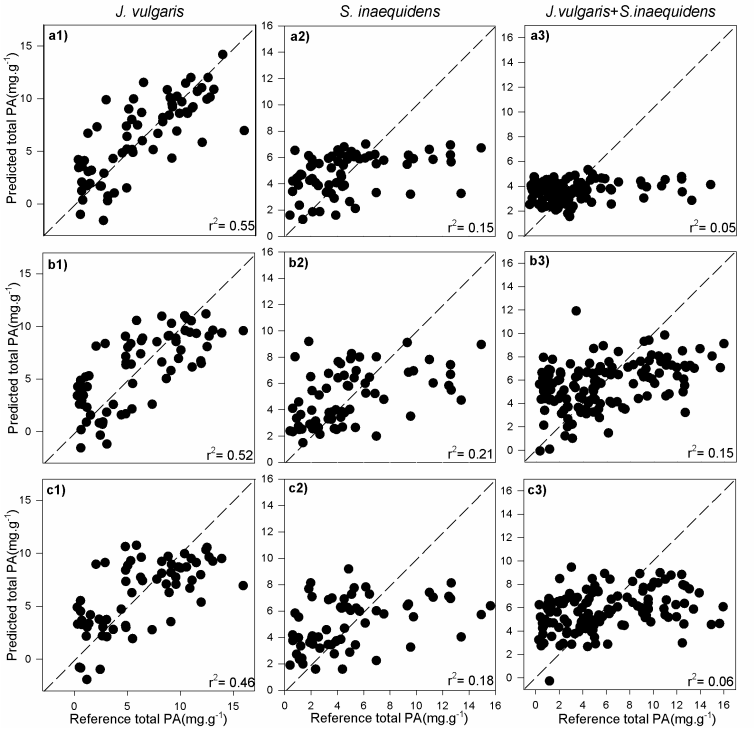

Figure S2: Partial least square regression predicted versus reference total PA concentrations for a) canopy, b) fresh leaves and c) dry leaves. a1, b1, c1) *J. vulgaris*, a2, b2, c2) *S. inaequidens,* a3, b3, c3) *J.vulgaris* and *S. inaequidens* combined. Dashed line represents the one to one relationship.

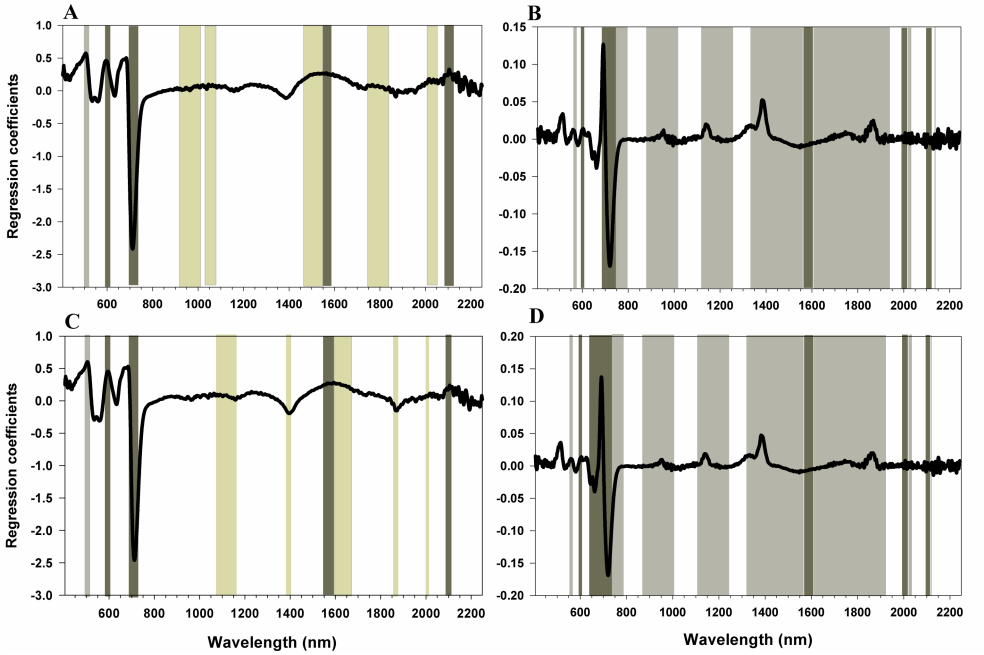

Figure S3: Partial least square regression coefficients (bold line) for each wavelength in the chemical estimation models. Regression coefficients of a) total PA in *J. vulgaris,* b) total PA in *S. inaequidens,* c) tertiary-amines in *J. vulgaris,* d) N-oxides in *S. inaequidens*. Grey boxes highlight the selected bands for the development of the Partial least square regression by its modified t-test (section 2.5.3); dark grey highlights the selected regions that co-occur throughout the several PLSR analyses.

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

3

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Abstract

Recent studies revealed that plant-soil biotic interactions may cause changes in aboveground plant chemistry. It would be a new step in belowground-aboveground interaction research if such aboveground 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 greater than 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 metabolites (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 non-destructive 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 intra-specific plant chemical variation due to 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.*, 1988b; Ferwerda *et al.*, 2005; Knox *et al.*, 2010; Skidmore *et al.*, 2010; Asner & Martin, 2011) and recently even canopy phylogeny (Asner & Martin, 2009; Asner & Martin, 2011). Additionally, remote sensing studies of aboveground 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 be used as well 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 plant responses aboveground through inducing primary

and secondary metabolite accumulation in the shoots (Masters *et al.*, 1993; van Dam, 2009; Kostenko *et al.*, 2012b). It has also been argued that belowground interactions between plants and soil biota may influence aboveground multitrophic interactions between 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 aboveground factors alone (Joosten *et al.*, 2009; Bartelheimer *et al.*, 2010). Therefore, our hypothesis was that soil biota can induce changes in aboveground plant chemistry and that these changes can be detected by spectral reflectance patterns.

In our study we used three closely related Senecionaea 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 chlorophyll and nitrogen affecting photosynthesis (e.g.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.*, 1988b; 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 soil-borne

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 levels (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.

Material 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.). *Jacobaea vulgaris* is a monocarpic biennial to 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 to 6 cm wide. *Jacobaea 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 of *J. vulgaris*. It is also native in the Netherlands, but interactions with soil organisms have not yet been studied. *Senecio 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 to 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 (≥ 25KGray, Isotron, Ede, the Netherlands) and used it as substrate where the soil samples from both other fields were inoculated into.

All soils were sieved with a 5mm mesh size 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 non-sterilized, D non-sterilized 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 : 15ºC, day : night regime. After 3 weeks, 24 seedlings of each species were transferred individually to 1-L pots prepared with one of the soils. The experiment set-up consisted of three species x four soil treatments (D sterilized, D non-sterilized, W sterilized and W non-sterilized). Of 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 weeks all plants were grown in a greenhouse under a 14 : 10h, 21 : 16º C, day : night regime at 70% relative humidity. All plants were watered every two days 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 nm to 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 by 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 degrees (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 (4 rotations x 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 the 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 on 10 nm intervals. All reflectance measurements were brightness-normalized (Feilhauer *et al.*, 2010) and mean centre corrected. Brightness normalization is a sample-wise pre -processing transformation that attempts to scales 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 square regression (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 hours while all roots were carefully rinsed with water to remove soil particles. The roots were oven-dried at 70°C for 96 hours. 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), N (%), C (%) and the PA defence compounds (mg/g).

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 dymethyl sulfoxide (DMSO) and stored in a dark room for three days at constant room temperature. Absorbance (Abs) measurements

at 649 nm and 665 nm were carried out in a spectrophotometer (Genesys 20 spectrophotometer 4001/4, Thermo Fisher Scientific Inc., Waltham, MA, USA) and chlorophyll concentrations (initially in ug/ml) were calculated using the equation:

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

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

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.

PAs 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). 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.*(2011a).

Statistical analysis

The effects of soil biota on, plant chemistry, shoot and root dry weight 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, non-sterilized) as fixed factors. Shoot dry weight 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 x 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 Analyses (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 more than 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 and 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 non-sterilized 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 non-sterilized 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 non-detectable to 20.5 mg/g DW in the live soil and from non-detectable to 13.0 mg/g DW in the sterilized soils. PA concentrations in non-sterilized inoculum were thus 100 to 400% higher, depending on species, than in sterilized inoculum (Fig. 2, Supplementary information Table S4). In *J. vulgaris,* on average these defence compound concentrations did not differ between sterilized and non-sterilized 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 non-sterilized 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 to 15% lower in non-sterilized soils for all species. The largest decrease occurred in *J. vulgaris* (Fig.2, Table S4). Soil origin did not affect N content. Chlorophyll contents ranged from 0.03 $mg.g^{-1}$ fresh weight to 1.62 $mg.g^{-1}$ fresh weight and were highest in *J. vulgaris* and *J. erucifolia* species. Chlorophyll was not affected by soil origin or sterilization treatment.

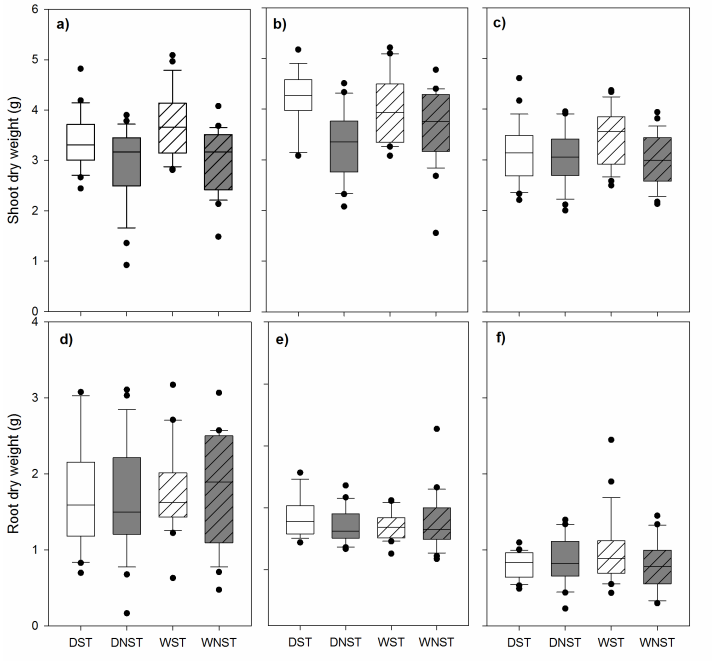

Figure 1: Species shoot (top panels) and root (bottom panels) dry weight per soil treatment (g). Soil origins are Dennekamp (D) and Wolfheze (W). Dark grey-black boxes, non-sterilized (NST) soil; white boxes, sterilized (ST) soil treatments. On average there were 23 samples per species per treatment. There were no significant differences in roots (ANOVA, all $P > 0.05$). An effect of sterilized versus non-sterilized 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) *J. erucifolia;* (c,f) *Senecio inaequidens*.

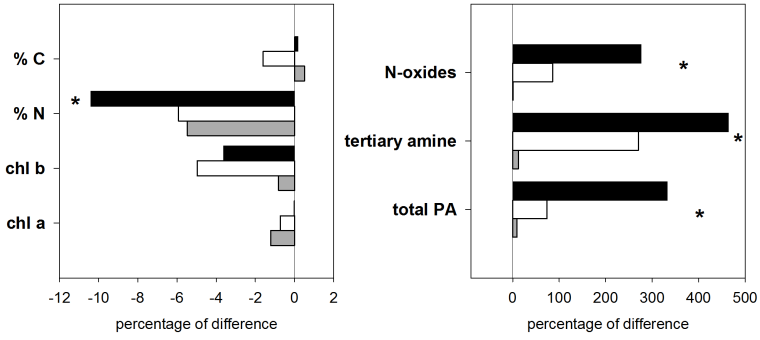

Figure 2: Percentage of difference in plant shoot mean chemical content between non -sterilized (NST) and sterilized (ST) treatment. Left panel, primary chemicals; right panel, pyrrolizidine alkaloids (PA). Grey bar, *Jacobaea vulgaris*; black bar, *J. erucifolia*; white bar, *Senecio inaequidens*. The 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)

Table 1: ANOVA analysis of the effect of species (*Jacobaea vulgaris*, J. *erucifolia* and *Senecio inaequidens*), soil origin (Denenkamp or Wolfheze) and soil treatment (sterile or non-sterile) on the shoot chemical concentrations. The shoot dry weight was included as a covariate in the analysis. The significant *P*-values are highlighted in bold. Chl, chlorophyll; N, Nitrogen; C, Carbon; PA, pyrrolozidine alkaloids; tertiary-amines and N-oxides are two different types of PAs.

Fixed factors	df	F Chl*a*	P value Chl*a*	F Chl*b*	P value Chl*b*	F Chl total	P value Chl total	F N%	P value N	F C%	P value C	F total PA	P value total PA	F tertiary amines	P value tertiary amines	F N-oxides	P value N-oxides
Corrected	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
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
Shoot DW	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
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**
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	0.94	0.33	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**
Species x Soil	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	0.59	0.26	0.77
Species x Soil	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**
Soil origin x	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	0.59
Species x origin	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
Mean Square		0.02		0.04		0.07		0.29		16.15		9.82		2.11		6.44	

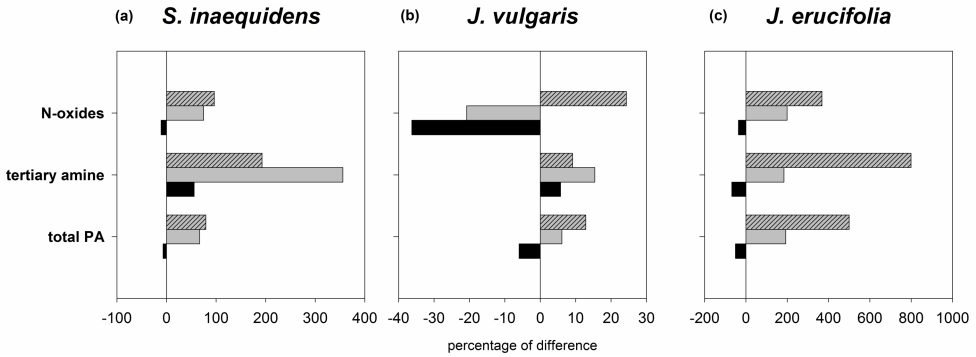

Figure 3: Percentage of differences in pyrrolizidine alkaloids between plant shoots grown in different soil origin treatments, Wolfheze (W) and Dennenkamp (D): grey bars, differences between W non-sterilized (WNST) and W sterilized (WST); dashed grey bars, differences between D non-sterilized (DNST) and D sterilized treatments (DST). Black bar, differences between DNST and WNST treatments. Ratios for *Jacobaea vulgaris* (lest panel), *J. erucifolia* (middle panel) and *Senecio inaequidens* (right panel). 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.

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).

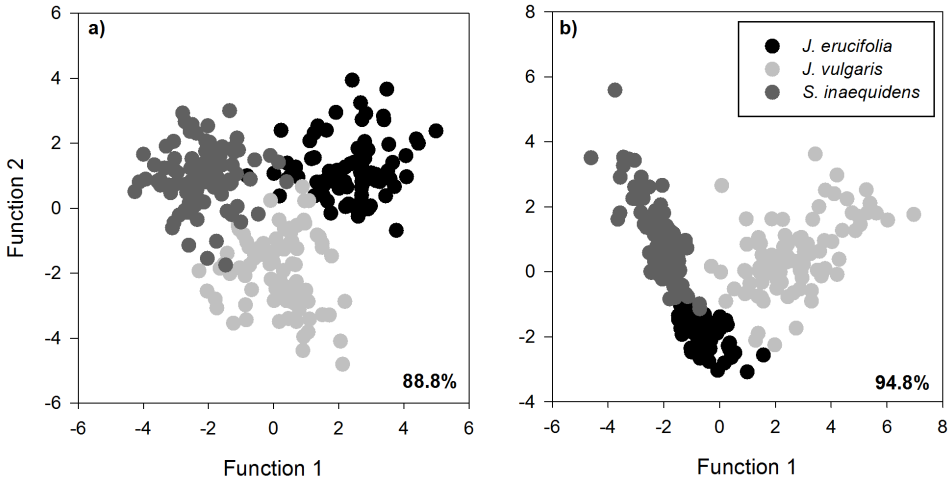

Figure 4: Scatterplot of the species linear discriminant scores generated by spectral reflectance (left) and chemical content analysis (right). Discriminant function 1 and 2 discriminate among *Senecio inaequidens* (dark grey dots*), Jacobaea vulgaris* (light grey dots) and *J. erucifolia* (black dots). 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 is notified the percentage of correct classification of 'unknown' samples to the LDA is noted. For more details see Table S1.

Soil treatment discrimination

The separation of the effects on soil treatments per species by LDA, either on chemical or spectral properties, was in general relatively low (Figure 5, Tables S1 and 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, Table S1 and S2).

Spectral data LDA within *J. vulgaris* achieved an overall percentage of correct classification of 'unknown' samples of 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. 5 c, 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 was c. 41%. The first function appeared to differentiate between the sterilized group and the other two non-sterilized groups in *J. erucifolia* and *S. inaequidens* (Fig. 5c and 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' samples 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 and 5f, Table S2). Both species had an overall cross-validation correct classification of the soil treatment > 60%. All soil treatment discriminant functions in the chemical data were again non-significant ($P>0.1$) with exception of the first discriminant function of *S. inaequidens* ($P<0.001$). This function differentiated between the plants in sterilized and non-sterilized soils (Fig. 5f), clearly highlighted by the groups centroid (which is the center of the samples distribution ellipsoid).

A reanalysis of both spectral and chemical datasets considering just non-sterilized versus 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 and 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 to 570 nm, 650 to 750 nm, 1350 to 1450 nm and 1800 to 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 versus non-sterilized) 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 nm and 2000 nm-2400 nm regions respectively (Fig. 6 b and c), while *J. vulgaris* sterilized treatment variation decreased in the visible region between 600 and 700 nm (Fig. 6 a). 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.

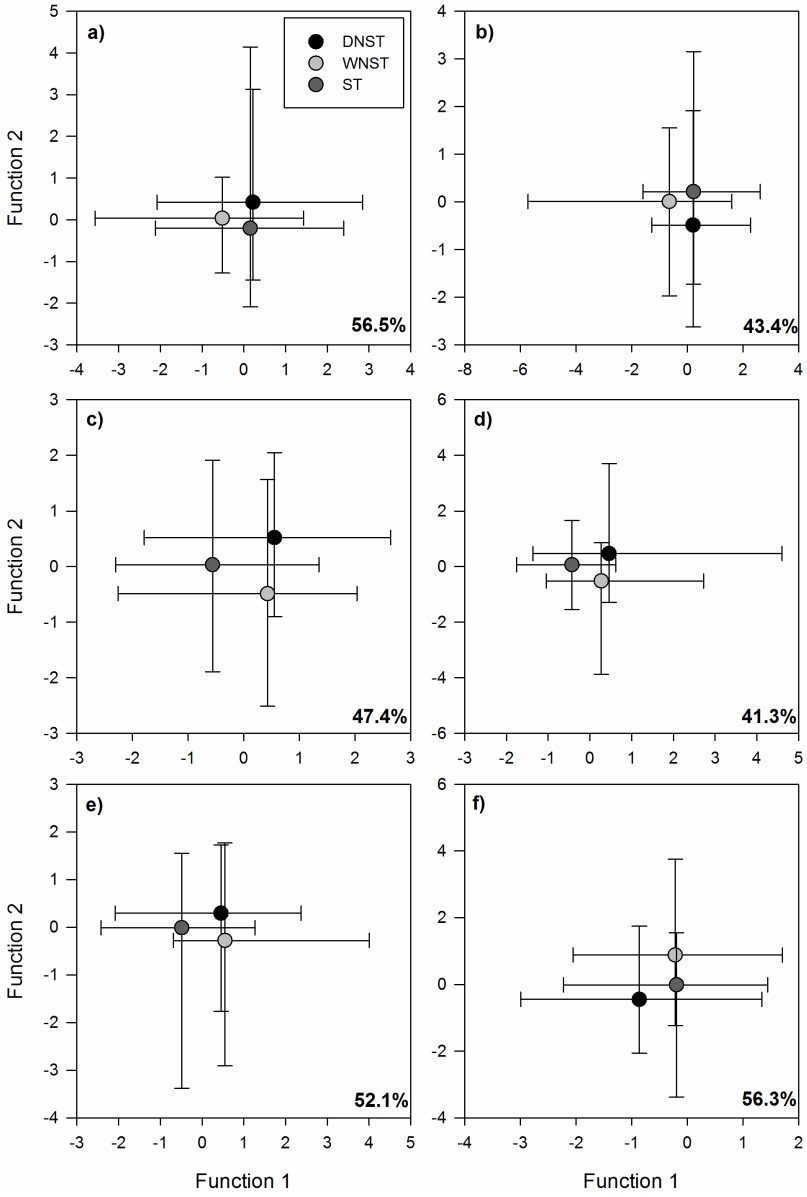

Figure 5: Linear discriminant mean scores (± minimum or maximum) generated by spectral reflectance (left panels) or chemical content analysis (right panels) for the species intraspecific soil treatment grouping: Sterilized (dark grey), Dennekamp non-sterilized (black) and Wolfheze non-sterilized (light grey). (a, b) *Jacobaea vulgaris* linear discriminanr analysis (LDA); (c, d) *J. erucifolia*; (e, f) *S. inaequidens*. The percentages of correct sample cross-validation classification 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 classification of 'unknown' samples to the LDA is given. For more details see table S1, S2.

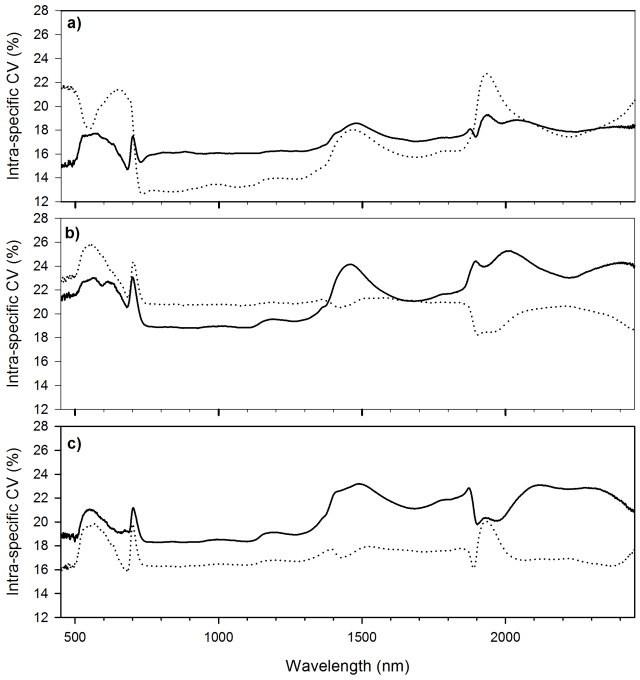

Figure 6: The mean intra-specific coefficient of variation (CV) as a percentage of the canopy spectral reflectance for each species soil inoculum treatment. (a) *Jacobaea vulgaris*; (b) *J. erucifolia*; and (c) *Senecio inaequidens*. The bold line represents non-sterilized soil and the dashed line represents sterilized soil. CV is calculates as (mean/standard deviation) x 100.

Plant chemical prediction

The estimation of species' chemical content by PLSR ranged between non-detectable and $r^2 = 0.54$ values (Table 2). The best estimations were for nitrogen in *J. vulgaris* ($r^2 = 0.53$) and *J. erucifolia* ($r^2 = 0.54$) while *S. inaequidens* achieved the best estimations for Chlb ($r^2 = 0.40$). 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 non-sterilized 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 non-adapted to the soils used in the experiment, so the enhanced expression of PAs may be a stress response. An

Table 2: Estimation of species chemical concentration by partial least square regression (PLSR) using their spectral reflectance signatures. 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 exception of nitrogen which is percentage.

	Jacobaea vulgaris				*Jacobaea erucifolia*				*Senecio inaequidens*			
Chemical	mean	r^2	RMSE	%RMSE	mean	r^2	RMSE	%RMSE	mean	r^2	RMSE	%RMSE
Chl*a*	0.99	0.29	0.13	13.13	0.92	0.29	0.07	7.61	0.72	0.38	0.11	15.28
Chl*b*	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

interesting finding was the shift in *J. vulgaris* N-oxide in to the different non-sterilized 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 and 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.*, 2011b). Our results showed that soil biotic conditions have the potential to influence plant reflectance patterns. Separation of soil biotic treatments (sterilized versus inoculated with non-sterilized field soil) within each species by either spectral reflectance or chemical content resulted in 50 – 60% of unknown (new) samples being 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 aboveground 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 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 (NIR) of green leaves as a result of species evolutionary strategies (Asner, 1998). The wavelength variation between sterile and non-sterile soils suggests that the biotic aspect of the soils can contribute to the spectral variation in the NIR. 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.*, 2004a). 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.* (2008a) 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 concentration was also lower compared to our pilot study (Carvalho S. 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.*, 2011b). 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 non-existent, 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 "unkown" 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.

Supplementary Information

Tables S1 - S4 and Figure S1

Table S1: Percentage of correct classification of samples by the spectral reflectance linear discriminant cross-validation and test dataset analysis (i.e. unknow samples to the LDA model).

		3 soil treatments				2 soil treatments		
training data set	species (%)	DNST (%)	WNST (%)	Sterile (%)	overall 3treat (%)	non-sterile (%)	sterile (%)	*overall NSTvsST (%)*
J. vulgaris	98.3	82.6	61.9	57.5	67.3	57.1	87.8	73.5
J. erucifolia	98.3	68.4	60.9	58.3	62.5	66.7	71.4	69.1
S.inaequidens	96.9	47.8	50	60.9	52.9	63	75.6	69.2
overall correct	97.8				60.92			70.60
% correct classification of unkown samples	**88.8**							

% correct classification of unkown samples	3 soil treatments (%)	*NST vs ST (%)*
J. vulgaris (23)	56.5	47.8
J. erucifola (19)	47.4	57.9
S.inaequidens (23)	52.1	56.5

Table S2: Percentage of correct classification of samples by the chemical concentration linear discriminant cross-validation and test dataset analysis (i.e. unknow samples to the LDA model).

		3 soil treatments				2 soil treatments		
training data set	species (%)	DNST (%)	WNST (%)	Sterile (%)	overall 3treat (%)	non-sterile (%)	sterile (%)	*overall NSTvsST (%)*
J. vulgaris	94.7	70.6	57.9	42.5	52.6	58.3	65.0	61.8
J. erucifolia	98.4	37.5	17.6	70	61.9	60.6	83.3	71.4
S.inaequidens	93.0	44.4	53.3	78.9	64.8	75.8	81.6	78.9
overall correct	95.2							
% correct classification of unkown samples	**94.8**							

% correct classification of unkown samples	3 soil treatments (%)	*NST vs ST (%)*
J. vulgaris (23)	43.4	47.4
J. erucifola (19)	41.3	57.1
S.inaequidens (23)	56.3	69.0

Table S3: Complete Descriptive statistics of each species chemical concentration in sterile and non-sterile inoculum.

	Sterile inoculum					Non-sterile inoculum				
Jacobaea vulgaris										
	N	Minimum	Maximum	Mean	Std. Deviation	Variance	N	Minimum	Maximum	Mean	Std. Deviation	Variance
shoot dry weight (g)	47	2.444	5.090	3.520	0.608	0.370	46	0.925	4.079	2.990	0.683	0.467
root dry weight (g)	47	0.630	9.202	1.881	1.246	1.552	46	0.167	3.108	1.721	0.717	0.513
totalDryweight	47	3.642	12.915	5.401	1.582	2.502	46	1.092	6.648	4.711	1.299	1.687
chla (mg/mm2)	47	0.021	0.026	0.022	0.001	0.000	46	0.021	0.023	0.022	0.000	0.000
chlb(mg/mm2)	47	0.001	0.033	0.020	0.006	0.000	46	0.012	0.029	0.020	0.004	0.000
chla(mg/g fresh)	47	0.731	1.406	0.997	0.152	0.023	46	0.738	1.407	0.985	0.154	0.024
chlb(mg/g fresh)	47	0.031	1.485	0.888	0.244	0.060	46	0.542	1.303	0.881	0.185	0.034
% N	47	2.617	7.339	3.993	0.725	0.526	46	2.669	5.132	3.839	0.652	0.425
% C	40	40.049	47.919	43.586	1.515	2.296	37	41.075	49.215	43.809	1.930	3.723
total PA	47	1.447	13.591	5.646	2.874	8.260	44	1.083	14.324	6.172	2.964	8.784
tertiary amine	47	0.786	12.142	4.200	2.510	6.301	44	0.132	10.953	4.721	2.394	5.733
N-oxides	47	0.172	7.151	1.434	1.411	1.991	44	0.145	5.143	1.444	1.173	1.377
Jacobaea erucifolius	N	Minimum	Maximum	Mean	Std. Deviation	Variance	N	Minimum	Maximum	Mean	Std. Deviation	Variance
shoot dry weight (g)	37	3.080	5.215	4.060	0.629	0.395	44	1.557	4.776	3.508	0.720	0.518
root dry weight (g)	37	0.261	1.568	0.756	0.286	0.082	44	0.175	2.273	0.743	0.381	0.145
totalDryweight	37	3.523	6.506	4.816	0.849	0.720	44	1.732	6.451	4.251	1.002	1.004
chla (mg/mm2)	37	0.020	0.025	0.022	0.001	0.000	44	0.020	0.023	0.022	0.001	0.000
chlb(mg/mm2)	37	0.019	0.036	0.027	0.005	0.000	44	0.012	0.036	0.026	0.005	0.000
chla(mg/g fresh)	37	0.620	1.405	0.920	0.157	0.025	44	0.667	1.391	0.920	0.154	0.024
chlb(mg/g fresh)	37	0.713	1.605	1.114	0.188	0.035	44	0.699	1.626	1.074	0.231	0.053
% N	37	2.668	5.022	3.738	0.454	0.206	43	1.884	4.813	3.491	0.475	0.225
% C	31	10.398	47.557	42.040	6.091	37.100	33	41.960	48.885	43.390	1.295	1.678
total PA	37	0.003	0.524	0.064	0.097	0.009	44	0.001	2.534	0.276	0.549	0.302
tertiary amine	37	0.000	0.216	0.019	0.042	0.002	44	0.000	1.569	0.110	0.291	0.085
N-oxides	37	0.001	0.307	0.044	0.062	0.004	44	0.001	1.238	0.166	0.286	0.082

Table S3 (cont.) : Complete Descriptive statistics of each species chemical concentration in sterile and non-sterile inoculum.

	Sterile inoculum					Non-sterile inoculum				
Senecio inaequidens	N	Minimum	Maximum	Mean	Std. Deviation	Variance	N	Minimum	Maximum	Mean	Std. Deviation	Variance
shoot dry weight (g)	48	2.209	4.610	3.299	0.592	0.351	49	2.001	3.953	3.016	0.523	0.273
root dry weight (g)	48	0.435	2.448	0.890	0.360	0.129	49	0.229	1.450	0.830	0.316	0.100
totalDryweight	48	2.741	5.821	4.189	0.815	0.664	49	2.451	5.390	3.846	0.791	0.625
chla (mg/mm2)	48	0.020	0.022	0.022	0.001	0.000	49	0.020	0.023	0.022	0.001	0.000
chlb(mg/mm2)	48	0.021	0.038	0.027	0.004	0.000	49	0.017	0.037	0.026	0.005	0.000
chla(mg/g fresh)	48	0.561	0.872	0.718	0.085	0.007	49	0.131	0.981	0.713	0.122	0.015
chlb(mg/g fresh)	48	0.544	1.314	0.892	0.144	0.021	49	0.201	1.395	0.848	0.190	0.036
% N	48	2.882	5.848	4.326	0.686	0.471	49	2.426	5.567	4.137	0.738	0.545
% C	40	37.615	45.300	41.123	1.749	3.060	35	37.487	43.858	40.464	1.258	1.583
total PA	47	0.134	13.005	3.708	3.170	10.048	47	0.574	20.487	6.561	5.184	26.877
tertiary amine	47	0.011	0.440	0.068	0.082	0.007	47	0.026	3.496	0.257	0.577	0.333
N-oxides	47	0.123	11.453	3.084	2.919	8.519	47	0.516	18.100	5.855	4.830	23.324

Table S4: Mean values of each treatment per species and the percentage of difference in plant shoots within each soil origin treatments. W- Wolfheze, D- Dennenkamp, nst- non-sterilized and st- sterilized. For species JV- *Jacobaea vulgaris*, JE- *J. erucifolia* and SI- *S. inaequidens*. 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. With such we study how much larger/smaller is the chemical content in the NST treatment plants when compared with its ST treatment plants within each soil origin.

MEAN	**JVW**	**JVD**	**JVst**	**JVnst**	**SIW**	**SID**	**SIst**	**SInst**	**SEW**	**SED**	**SEst**	**SEnst**
chla(mg/g fresh)	0.99	0.98	1.00	0.99	0.73	0.70	0.72	0.71	0.91	0.93	0.92	0.92
chlb(mg/g fresh)	0.87	0.89	0.89	0.88	0.88	0.82	0.89	0.85	1.08	1.07	1.11	1.07
% N	3.81	3.78	4.02	3.80	4.18	3.97	4.32	4.07	3.37	3.43	3.79	3.40
% C	44.07	43.53	43.59	43.81	40.17	40.71	41.12	40.46	41.19	43.15	42.04	42.11
total PA	5.99	6.37	5.65	6.17	6.18	6.65	3.71	6.42	0.19	0.38	0.06	0.28
tertiary amine	4.85	4.58	4.20	4.72	0.31	0.20	0.07	0.25	0.06	0.17	0.02	0.11
N-oxides	1.14	1.78	1.43	1.44	5.38	6.05	3.08	5.73	0.13	0.21	0.04	0.17

%DIFFERENCE	**JVW:JVD**	**JVW:JVst**	**JVD:JVst**	**JVnst:JVst**	**SIW:SID**	**SIW:SIst**	**SID:SIst**	**SInst:SIst**	**SEW:SED**	**SEW:SEst**	**SED:SEst**	**SEnst:SEst**
chla(mg/g fresh)	0.41	-1.02	-1.42	-1.22	5.09	1.80	-3.13	-0.72	-2.98	-1.40	1.63	-0.02
chlb(mg/g fresh)	-1.52	-1.57	-0.06	-0.81	6.92	-1.73	-8.09	-4.97	1.28	-3.07	-4.29	-3.62
% N	0.64	-5.18	-5.78	-5.47	5.15	-3.36	-8.09	-5.92	-1.76	-11.15	-9.56	-10.40
% C	1.25	1.12	-0.13	0.51	-1.31	-2.31	-1.01	-1.60	-4.52	-2.01	2.63	0.18
total PA	-5.98	6.10	12.84	9.32	-7.06	66.66	79.31	73.25	-51.13	193.03	499.57	332.37
tertiary amine	5.75	15.40	9.12	12.40	55.64	355.55	192.70	270.73	-68.42	184.03	799.32	463.71
N-oxides	-36.27	-20.79	24.30	0.73	-11.07	74.57	96.30	85.89	-36.16	198.93	368.23	275.88

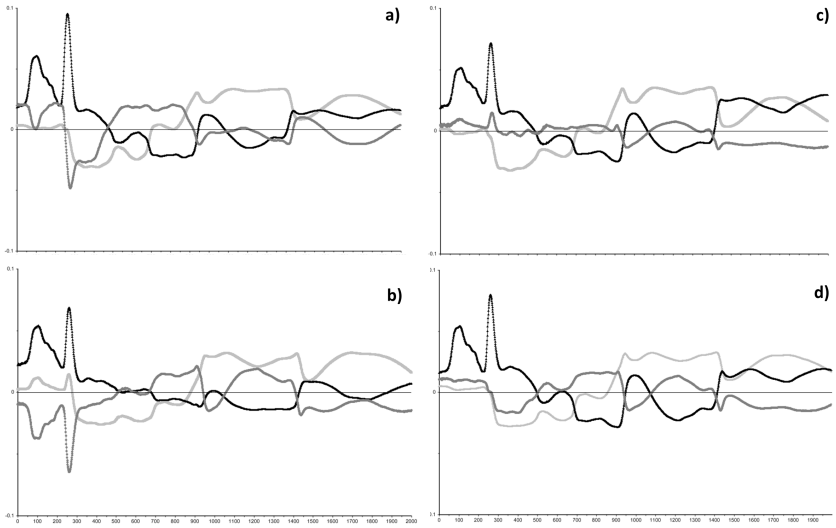

Figure S1: PCA-LDA Loadings of the spectral reflectance linear discriminant analysis. The loadings of the PCA will indicate how the LDA functions relate to the original variables, so that we understand the relation between the spectra and the group membership. The black line is the spectral loadings given by LDA to PCA 1 one, dark grey line PCA 2 and light grey line PCA3. The lower or higher the loadings are in each wavelength the stronger the wavelength influence in the PCA-LDA. a) PCA-LDA for *J. vulgaris* soil treatments; b) PCA-LDA for *J. erucifolia* soil treatments; c) PCA-LDA for *S. inaequidens* soil treatments and d) PCA-LDA for species groups discrimination.

Chemical variation in *Jacobaea vulgaris* as influenced by succession stage of vegetation and seasonal growth

4

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In revision

Abstract

Knowledge on plant chemistry variation is important to assess spatio-temporal dynamics of plant nutrient and defence allocation under natural conditions. Plant chemistry is known to vary in relation to both plant seasonal development and vegetation successional stage, however, in few studies these two sources of variation have been examined in combination. Here we show how primary and secondary chemistry of *Jacobaea vulgaris* (Asteraceae) varies with season during secondary succession. We investigated both leaves and flowers, as these may differ in chemical defence during seasonal (phenological) development. We analysed chemistry during secondary succession by collecting plants from a well-established chronosequence of abandoned arable fields in The Netherlands. The chemical concentration of *J. vulgaris* varied throughout the season and was affected by vegetation succession stage. Concentrations of pyrrolizidine alkaloid (PA) tertiary-amines were highest in flowers during early Summer and in fields that had been abandoned ten to twenty years ago. PA N-oxide concentrations of both leaves and flowers increased with the progression of both seasonal growth and secondary succession. In Spring and early Summer chlorophyll concentrations were highest, especially in the oldest fields of the chronosequence. During phenological development, nitrogen concentration increased in flowers and decreased in leaves revealing allocation of nutrients from vegetative to reproductive plant parts throughout the growing season. Patterns of plant chemistry in *J. vulgaris* during growing season are in line with predictions on optimal defence strategy: flowers are better defended than leaves. Our results suggest that plant defence can vary with successional stage as well. The highest concentrations of N-oxides and chlorophylls in older fields suggest that competition pressure increases when vegetation complexity increases. Thus, our results suggest that defence and photosynthesis of *J. vulgaris* may be optimized depending on the stage of vegetation succession.

Introduction

Numerous studies have examined variation in plant secondary chemistry within plants (e.g. Hartmann & Zimmer, 1986; Zangerl & Bazzaz, 1992; Pichersky & Gang, 2000), among plant development stages (e.g. Walters, 2011; Iason *et al.*, 2012), or among environments (e.g. Pyšek *et al.*, 2005; Gols *et al.*, 2008). Plant population structure can change substantially across successional gradients, however, very few studies have related variation in plant chemistry during plant development to successional stage. Here, we examine how chemical concentration and composition of the early secondary succession plant species *Jacobaea vulgaris* Gaertn. (syn. *Secenio jacobaea* L.*, Asteraceae*) varies with phenological development during the growing season and successional development in an abandonment chronosequence of ex-arable fields.

Optimal defence theory assumes that there are costs involved in allocating resources to growth and defences (McKey, 1974; Rhoades, 1979). Allocation of defences within a plant is therefore expected to change during a plants' life time. The timing of seasonal activity (i.e. phenology) may play an important role in plant species composition and even influence invasion success (Wainwright et al., 2012). The interaction of plants with (a)biotic factors further enhances seasonal variation in defences dependent on the life history strategy of the plant (Zhang *et al.*, 2009). As the growing season of a plant species can have a different time span than surrounding species, plants are exposed continuously differing conditions of light, temperature etc., which further promotes variations in primary and secondary chemistry (Gols et al., 2007; Walters, 2011).

Plants are 'smart' investors (Van Dam et al., 1996) and strong seasonal herbivore pressure can induce plant responses (Shiojiri & Karban, 2008). Often, plant defence levels increase throughout the season (Brooks & Feeny, 2004; Iason *et al.*, 2012). Even in greenhouse-controlled conditions, secondary plant chemistry was shown to change in plants grown at different times in the year (Gols et al., 2007). During reproduction plants frequently show a relocation of defences from leaves to flowers (Hartmann & Zimmer, 1986; Çıtrak *et al.*, 2007). Besides defence compounds also nutrients and photosynthetic compounds vary throughout plant phenological development. However, such seasonal variation will depend on plant life history strategy. For example, Amsellem and Mckey (2006) showed two contrasting tree strategies: *Leonardoxa Africana* delayed greening the leaves until full leaf expansion

while *Barteria nigritana* photosynthesized during leaf expansion. Relocation and/or shift of chemical content, therefore, is advantageous for plant fitness and will vary during seasonal development.

Herbivory pressure can alter plant size and shape and, consequently, change the expression of defence chemistry by plant species (Korthals *et al.*, 2001; de Bie *et al.*, 2011; Walters, 2011). Therefore, defence compounds may change throughout succession (Walters, 2011), when plant competition and interactions with aboveground and belowground biota may intensify. The nitrogen and phosphorus content in the soil is known to shift along most chronosequences (Richardson et al., 2005). For example, in an Australian chronosequence nitrogen tends to limit plant growth in relatively young fields while ancient fields tend to be phosphorous limited (Lambers et al., 2008). Plants are plastic in an array of traits, which helps to overcome nutrient limitations. These plastic responses include effective mechanisms of resorption, internal recycling, allocation and use of nitrogen and phosphorous in growth and defence (Richardson *et al.*, 2005; Lambers *et al.*, 2008; Walters, 2011). Mason and co-workers (2012) showed that in a chronosequence contrasting growth forms (angiosperms, conifers, tree ferns) all declined in leaves nitrogen and phosphorous concentrations. The declines mounted between 67% and 88% along the soil chronosequence. In addition to soil nutrient limitation plants face aboveground light competition as plant community composition shifts during vegetation succession and seasonal growth. Models have suggested that low nutrient and little disturbed habitats tend to have vegetation succession from light specialists to nutrient specialists, while vegetation in high nutrient or highly disturbed habitats evolve to light specialists (Tilman, 1982; Tilman, 1987; Rees & Bergelson, 1997). As such we want to study how important the stage of vegetation succession is for the variation of primary and secondary metabolites of *J. vulgaris*.

For our study, we used a chronosequence of ex-arable fields near Veluwe National Park in the Netherlands, as this enabled to study differences in plant chemistry between successional stages of vegetation and *J. vulgaris* population dynamics during the growing season. Management aiming at re-creating open grasslands with a high biodiversity, by abandoning previously cultivated land, has resulted in a series of abandoned fields that can be used to reconstruct the performance of *J. vulgaris* during time since abandonment at one moment in time (van de Voorde et al., 2012). These ex-arable fields are colonized by *J. Vulgaris*,which

is an outbreak plant species that has a hump-shaped population development booming during the first five-seven years and then busting during the next ten-twenty years following land abandonment (van de Voorde *et al.*, 2012). This pattern was attributed, to some extent, to level of control by soil biota and negative feedback effects from other plant species that gradually colonize the old fields (Kostenko et al., 2012b; van de Voorde et al., 2012).

An earlier garden experiment with *J. vulgaris* (Aplin & Rothschild, 1972) showed that pyrrolizidine alkaloids (PAs) in leaves reached highest concentrations in June with a steep decrease later in the season. De Boer (1999) found that nitrogen percentage and PAs tend to decrease with leaf age in the plant. Within the reproductive season studies on *Senecio vulgaris* and *S. vernalis* also showed the highest PA concentrations to be found in the flowers probably due to the optimal defence strategy of *J. vulgaris*. As such it is important to consider both leaves and flower organs to study the seasonal chemistry variation. Additionally, attacks by herbivores can result in reduced rates of photosynthesis and change of carbon and nutrients balance (Bryant *et al.*, 1983; Tuomi *et al.*, 1984). Since in natural conditions temporal variation of nitrogen or chlorophyll in *J. vulgaris* is, as far as we are aware of, unknown, we studied both defence compounds and nutrient and chlorophyll contents. As this chronosequence is not nitrogen limited we should expect that vegetation develops towards light competitive specialist (Rees & Bergelson, 1997; van der Wal *et al.*, 2006; van de Voorde *et al.*, 2012), thus *J. vulgaris* should shift towards higher chlorophyll contents.

Our goal was to investigate if seasonal shifts of *J. vulgaris* primary and secondary metabolites may depend on vegetation successional stage. We analysed chemistry of both leaves and flowers, in order to investigate if defence may be optimized depending on the position of plants in secondary successionalfields. We addressed the following questions: i) how did field age (time since abandonment) affect plant chemistry, ii) was the seasonal variation consistent between succession stages, and iii) did *J. vulgaris* flowers in the field had higher concentrations of pyrrolizidine alkaloids than leaves, and were these concentrations affected by succession stage? Our hypotheses were that: a) there was a seasonal allocation of nutrients and defence metabolites to reproductive organs that fitted the optimal defence theory; b) this seasonal/organ variation in chemistry would be dependent on the successional stage of the vegetation.

Material and Methods

Species description

Jacobaea vulgaris Gaertn (syn. *Senecio jacobaea L.,* Asteraceae) is a monocarpic biennial to short-lived perennial. In the first year this species forms a rosette, whereas flowering occurs in the second year if conditions are favourable (Harper & Wood, 1957; Wesselingh & Klinkhamer, 1996). If not, then flowering can be postponed for one or more years (Van der Meijden & van der Waals-Kooi, 1979). Although native to the Netherlands, *Jacobaea vulgaris* is considered a noxious weed, as it is a pioneer species that can become highly dominant in arable fields, unless it is controlled by mechanical or chemical means (van de Voorde *et al.*, 2011). This species contains pyrrolizidine alkaloids (PAs) that have hepatotoxic as well as carcinogenic properties towards many herbivores, including livestock (Mattocks, 1986; Macel, 2011). The plant species has also antifungal activity (Hol & Van Veen, 2002) that can affect, and is affected by, soil biota and insects (Macel *et al.*, 2005; Joosten *et al.*, 2009). Toxicity of *J. vulgaris* is largely due to its PA content. More than 30 different PAs can be found in this species in all plant organ types, from roots, stems and leaves to flower heads and seeds (Mattocks, 1986, Table S1). *Jacobaea vulgaris* populations can differ in their PA concentration in flowers (Hartmann & Witte, 1995) and leaves (Macel et al., 2004).

Field selection

We selected 8 fields (Table 1) located at south Veluwe, the Netherlands, where agricultural production had ceased between 5 and 30 years ago. Currently, these fields are being subjected to nature restoration (Bezemer et al., 2006). *Jacobaea vulgaris* density in these fields increases in the first five to seven years of land abandonment and then declines according to a hump-shaped pattern (van de Voorde *et al.*, 2012). This typical population dynamics is attributed, at least to some extent, to plant-soil interactions (Bezemer et al., 2006; van de Voorde et al., 2012). Based on previous findings of negative, neutral, and positive plant-soil feedback in early-, mid- and late-successional fields (Kardol *et al.*, 2006) we grouped fields in successional age classes: Young (0-10 years of abandonment), Medium (10-20) and Late (20-30).

Table 1: Selected ex-arable fields, their code, age, years of abandonment at the time of the study, and succession class. For plant community characteristics of the different fields see van de Voorde *et al.* (2011) and soil characteristics see Kardol *et al.* (2006)

Field	Code	Age (abandonment time)	Year of abandonment	Succession class	Latitude (N)	Longitude (E)
Oud reemst	OR	5	2005	Young	52.02	5.48
Reyerskamp	R	5	2005	Young	52.01	5.47
Telefoonweg	T	7	2002	Young	52.00	5.45
Assel	A	7	2002	Young	52.12	5.49
Mossel	M	15	1995	Medium	52.03	5.45
Nieuw Reemst	NR	20	1990	Medium	52.04	5.47
Wolfheze	W	22	1988	Old	51.60	5.47
Dennenkamp	D	27	1982	Old	52.02	5.48

Sample collection

In each field we followed a sampling scheme similar to van de Voorde et al (2011) by establishing an imaginary W-shaped transect that covered the whole field. Every 5 m a plant sample was collected resulting in a total of 20 plant samples per field. As the fields were not similar in size, the samples were collected from the centre of the fields in an area of 30 x 100 m. Sampling was carried out three times during the growing season: the rosette stage (Spring), the flowering stage (early Summer) and the senescing/seed stage (late Summer). In the sampling year (2010) the Spring rosette was sampled in May, flowering took place in June, and senescence occurred in late August. In early and late Summer the flowers were collected from the same plant as the leaves, but each season different plants were randomly sampled. In total 8 fields x 3 seasons were planned to be sampled. However, two fields (Telefoonweg and Assel; see Table 1) were mown before flowering stage, so that it was impossible to collect plant samples from these two fields during early and late Summer.

Chemical analysis

From each individual plant we collected five leaves from basis to top of the shoot in order to include leaves of various ages. We also collected four flower heads when plants were flowering. Leaves and flowers were analysed separately for chlorophyll *a* and *b* (mg/g), nitrogen (% of dry weight - dw), carbon (% dw) and pyrrolizidine alkaloids (mg/g dw). We did not measure chlorophyll content of flowers. Per individual plant the 5 leaves or the 4 flowers were pooled together to extract an

average organ chemical content.For the chlorophyll extraction one 10-mm diameter disc was collected from each leaf. The discs were cut out, immersed immediately in 3ml of dimethyl sulfoxide (DMSO) and stored in a dark room for three days at constant room temperature. In a spectrophotometer (Genesys 20 spectrophotometer 4001/4, Thermo Fisher Scientific, Waltham, MA, USA) absorbance (Abs) at 649 nm and 665 nm was measured and chlorophyll concentrations (initially in μg/ml) were calculated using the equation:

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

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

The leaves were freeze-dried for 96 hours after chlorophyll analysis. Thereafter the leaf samples were homogenized and fast-ground to a fine powder for PA analysis and Nitrogen (N) and Carbon (C) analyses. For the C:N analysis 6 mm diameter metal cups were selected and 3-5 mg of dried powder used. Combustion-reduction was done in a C:N analyser (Thermo flash EA 1112, Thermo Fisher Scientific, Waltham, MA, USA) for C:N percentage estimation.

Pyrrolizidine alkaloids were extracted according to Joosten *et al.* (2011). We extracted 10 mg of powdered material with 1 ml of 2% formic acid solution containing heliotrine as internal standard (1 μg/ml). The extract was then filtered and 25 μL were diluted 40 times with 10 mM ammonium hydroxide. The PA content was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Waters Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). Separation was achieved on a Waters C18 BEH column (150 x 2.1 mm, 1.7 μm particles) using 5 mM ammonium hydroxide as mobile phase and acetonitrile as organic modifier (0-50%) in a 12-min linear gradient. The mass spectrometer was operated in positive electrospray mode and the samples were screened for a total of 45 PAs. Details on the mass spectrometric conditions can be found in Table S1. PAs were quantified against a set of PA standards added to *Tanacetum vulgare* plant extract (which itself is free of PAs) to minimize matrix effects that otherwise could play a role when using standards in solvent only. The calibrant solution was injected every 25 samples to check for variations in detector response. Samples were injected in a randomized order. Data were processed using Masslynx 4.1 software (Waters, Milford, MA, USA). For a number of PAs no reference standard was available. For these compounds quantification was performed by comparison with a structurally related compound, indicated in Table S1. Data on individual compounds were summed to

obtain total PA, tertiary-amine and N-oxide content and the concentration of the main PA types present in *J. vulgaris*.

Statistical analysis

The effects of vegetation succession class (Table 1) and season on chemical content of leaves and flowers were examined by analysis of variance (ANOVA). Succession class and season were added in the model as fixed factors. To accurately test the effect of succession the fields were nested in the factor successional class as a random term. Differences between groups were analysed with Post-hoc Tukey HSD tests. To meet the assumptions of normality and homoscedasticity total pyrrolizidine alkaloids, tertiary-amines and N-oxides were log-transformed. The ANOVA analyses were performed in SPSS 17.0 for Windows.

Since PAs in *Jacobaea vulgaris* are highly diverse, leaf and flower PA composition were analysed with multivariate statistics to study changes in composition. The most appropriate multivariate analyses were chosen by detrended correspondence analysis (DCA). With DCA we tested for linear (principal component analyses – PCA, and redundancy analyses - RDA) or Unimodal (correspondence analyses – CA co-correspondence analyses - CCA). As all gradients were smaller than 3, linear analysis was chosen (Lepš & Šmilauer, 2003). The explanatory power of season, field origin and succession class for the variation in the PA composition was evaluated by RDA. Monte Carlo permutations (999 permutations) were used to test the significance of all axes. All multivariate analyses were performed in CANOCO 4.5 for Windows.

The differences between plant organs (leaf vs. flower) were analysed per season. In each season we examined the chemical concentration differences between flowers and leaves by analysis of variance (ANOVA). Field and plant organ were fixed factors. PCA and RDA were applied to examine the chemical composition differences of the organs within each season.

Results

Plant chemical concentrations

The factors season and succession stage had significant effects on leaf and flower chemical content, ($P<0.05$; Table 2 and 3) and the interaction of these factors was

Table 2: Effect of season, vegetation succession stage and field location on the concentrations of primary and secondary compounds of *Jaubuea vulgaris* leaves. PA- pyrrolizidine alkaloids; tertiary-amines and N-oxides are two different forms of PAs. Table entries are F values of ANOVA. PA data were log transformed. *** P < 0.001, **P <0.01 and *P<0.05.

Factors	df	Chl*a*	Chl*b*	Chl total	Nitrogen	Carbon	Total PA	PA T-amines	PA N-oxides
Season	2	27.385***	35.940***	41.699***	40.323***	9.002***	6.165**	17.456***	12.209***
Succession class	2	6.016**	7.357**	7.211**	5.023**	1.2	3.608	7.229**	1.816
Field (nested in succession)	5	1.706	2.459*	2.052	2.616*	13.618***	0.676	8.236***	1.699
Season x succession	10	3.325***	3.257**	3.674***	3.268***	5.551***	2.192*	2.316*	1.848*
Error	326	0.002	0.002	0.006	0.244	2.3	14.923	0.755	14.126

highly significant in the leaves chemical content (P<0.002, Table 2 and 3). Mid succession flowers had higher tertiary-amines than flowers in Young or Old fields, especially in early Summer. Flowers of older succession stages showed the highest N-oxides in end Summer. When comparing flowers and leaves we found that in early Summer flower heads had higher levels of PA tertiary-amines and N-oxides than leaves. In late Summer, however, the tertiary-amines and N-oxides contents were higher in leaves than in flower heads (Fig. 1, Fig. S1).

Leaves in general had higher concentrations of chlorophyll a, nitrogen and PA tertiary-amines in Spring, while in early Summer leaves had higher content of chlorophyll b. There was an increase of PA N-oxides concentration throughout the seasons. Concentrations were highest in late Summer. PA tertiary-amines showed an opposite trend with levels being highest in Spring (Fig 2 and 3, Fig S1). Interaction between the factors season and succession showed that in late Summer *J. vulgaris* plants from Medium succession fields (10-20 years abandoned) were richer in PA tertiary -amines, while plants collected in early Summer from Old succession fields (20-30 years abandoned) were richer in PA N-oxides (Fig. 2). Leaves from Old fields had higher chlorophyll a content than leaves from the Medium or Young succession fields during Spring and early Summer while leaves from Young fields had the lowest chlorophyll b content in all seasons (Fig. 2).

In flowers concentrations were also significantly affected by season and succession class (P<0.001, Table 3), although the interaction between the factors was only significant for pyrrolizidine alkaloids. Flowers of Medium aged fields (10-20

years) had the highest PA tertiary-amine content, particularly in early Summer season while Old succession fields (20-30) were significantly higher in PA N-oxides if late Summer season was considered (Fig. 1). The nitrogen in flowers was highest in late Summer independent of succession. The chemical variation in flower nitrogen concentrations within fields was relatively large, whereas no significant differences were found in flower N concentrations between fields.

Table 3: The effect of season, vegetation succession stage and field on the chemical concentration of primary and secondary compounds of *Jacobaea vulgaris* flowers. PA - pyrrolizidine alkaloids; tertiary-amines and N-oxides are two different types of PAs, all Log transformed. Table entries are F values of ANOVA *** P < 0.001, **P <0.01 and *P<0.05.

Factors	df	Nitrogen	Carbon	Total PA	PA T-amines	PA N-oxides
Season	1	49.69***	2.67	253.01***	226.3***	176.37***
Succession	2	0.03	2.65	4.13*	6.46**	5.58**
Field (nested in succession)	3	3.53*	1.17	4.91**	7.59***	7.91***
Season x Succession	5	1.15	0.96	5.34***	8.19***	4.11***
Error	219	0.1	11.44	0.03	0.01	0.03

Pyrrolizidine alkaloid composition

Although small, the redundancy analyses (RDA) revealed an effect of season and succession class in both leaves and flowers. In leaves 6.7 % of the variation was explained by season (P = 0.002), whereas succession class explained 4.2% of the variation (P = 0.042). The PCA for leaves revealed a complex interaction between season and succession (Fig. 3). In early and late Summer leaves from mid-succession fields tended to be distinguished by the lack of hydroxyjacobine (HOJb) and hydroxyjacoline (HOJl), and a stronger presence of jacobine-type PAs. Acetylerucifoline (AcEr), erucifoline (Er) distinguished young fields and early season leaves.

In flowers 15.6% of the variation was explained by season (P = 0.001) and 4.8% by time since abandonment, (P = 0.007) (Fig. 4). In early Summer, flowers had relatively high levels of AcEr, Er, their N-oxides and senecionine (Sn) compared to late Summer flowers. In late Summer, Young fields also correlated positively with less common PAs, such as dehydroeruciflorine (DHEf) and its N-oxide (DHEf-ox), whereas there was a negative correlation with the main PAs, such as jacobine. Medium succession fields correlated with jacobine (Jb), jacoline (Jl) and jaconine (Jn) and their corresponding N-oxides and hydroxy-PA metabolites especially in the case of late Summer.

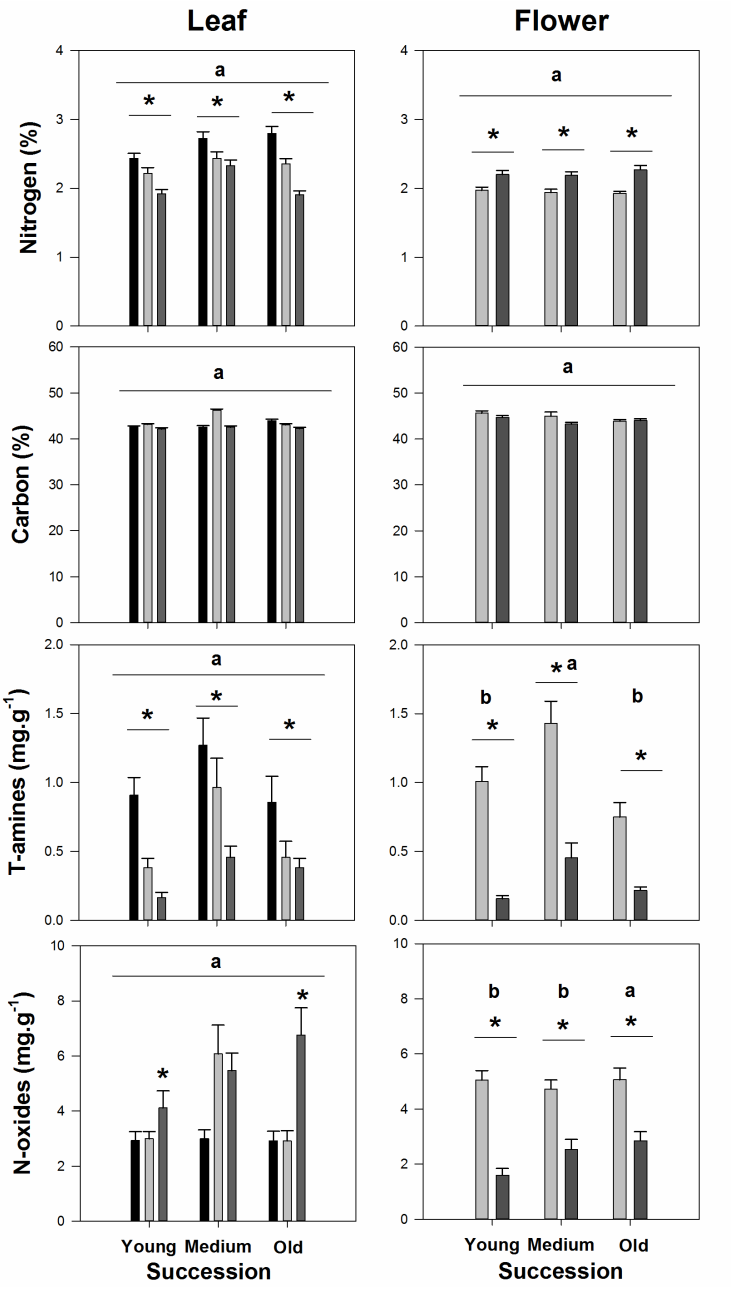

Figure 1: Chemical content in leaves (left column) and flowers (right column) of *Jacobaea vulgaris* by field succession stage. Bars represent seasons, black – Spring; light grey – early Summer, dark grey – late Summer. Star symbol (*) represent significant differences between seasons (ANOVA< leaf $P<0.005$, flower $P<0.001$), letters represent significant differences between succession classes (ANOVA, leaf $P<0.05$, flower $P<0.005$). Error bars are standard errors.

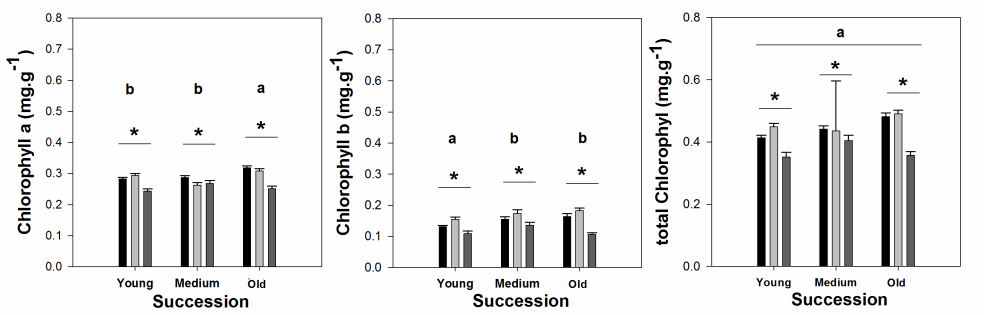

Figure 2: Mean chlorophyll content in leaves of *Jacobaea vulgaris* by field succession stage. Bars represent seasons, black - Spring; light grey - early Summer, dark grey -late Summer. * represents significant differences between seasons (ANOVA, P<0.001), letters represent significant differences between field succession stages (ANOVA, P<0.005). Error bars are standard errors.

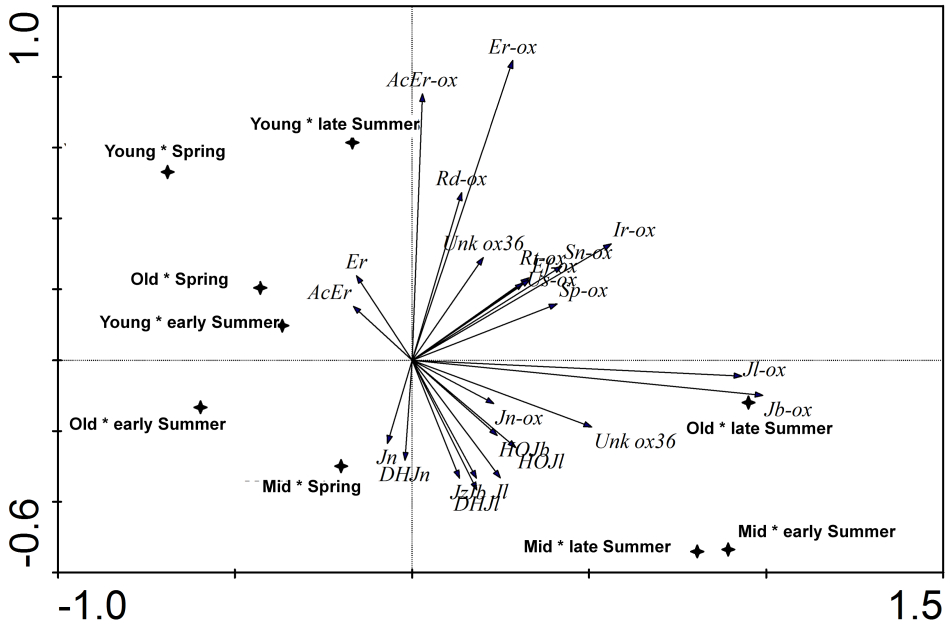

Figure 3: Principal component analysis (PCA) of the pyrrolizidine alkaloid (PA) composition in leaves of *Jacobaea vulgaris*. PCA was done on log-transformed concentrations with succession class and season interaction. Axis 1 explains 48.8% of the variation, axis 2 explains 19.9%. Redundancy analysis showed 6.7% of the variation was significantly explained by the effect of season (P=0.002) and 4.8% by succession class (P=0.007). Star symbols represent (age category x seasons) group position. Arrows represent the different PAs (inclusion rule 20%; see Table S1 for list of PA names).

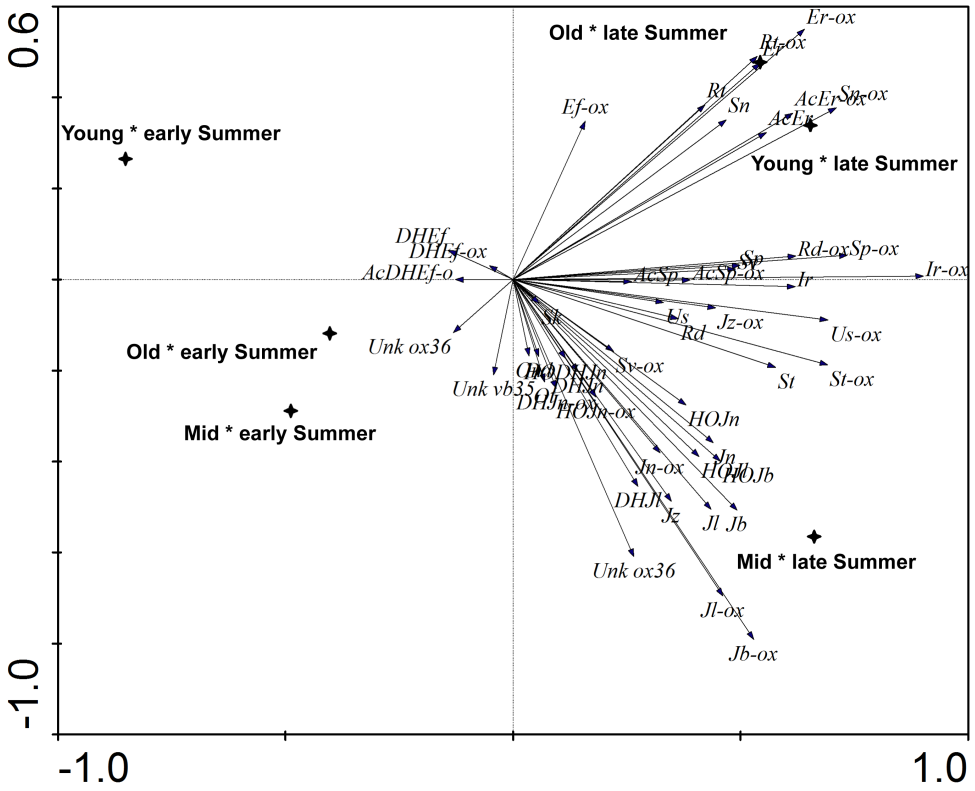

Figure 4: Principal component analysis (PCA) of the pyrrolizidine alkaloid (PA) composition of flowers of *Jacobaea vulgaris*. PCA was done on log-transformed concentrations with succession stage and season interaction. Axis 1 explains 37.6% of the variation, axis 2 explains 29.7%. Redundancy analysis showed that 15.3% of the variation was significantly explained by the effect of season (P = 0.001), the succession class explained 4.2%, but significance value was near the boundary (P = 0.042). Star symbols represent (succession x seasons) group position. Arrows represent the different PAs (inclusion rule 20%; see Table S1 for list of PA names).

PA composition in leaves and flowers

In both seasons, it was possible to differentiate between leaf and flower samples based on PA composition (Fig.5, Fig. S2). RDA analysis showed that more than 25% of the variation was explained by the effect of organ (P = 0.001). Acetylerucifoline (AcEr) and its N-oxide (AcEr-ox), senecionine (Sn), senkirkine (Sk), integerrimine (Ir) and its N-oxide (Ir-ox), jacoline (Jl), among others, contributed to the separation between flowers and leaves. Leaves correlated with higher concentrations of jacobine (Jb) and its N-oxide (Jb-ox), jacoline N-oxide (Jl-ox), and lower concentrations of erucifoline N-oxide (Er-ox) concentration. The PA composition in the flowers and in the leaves remained similar throughout the seasons.

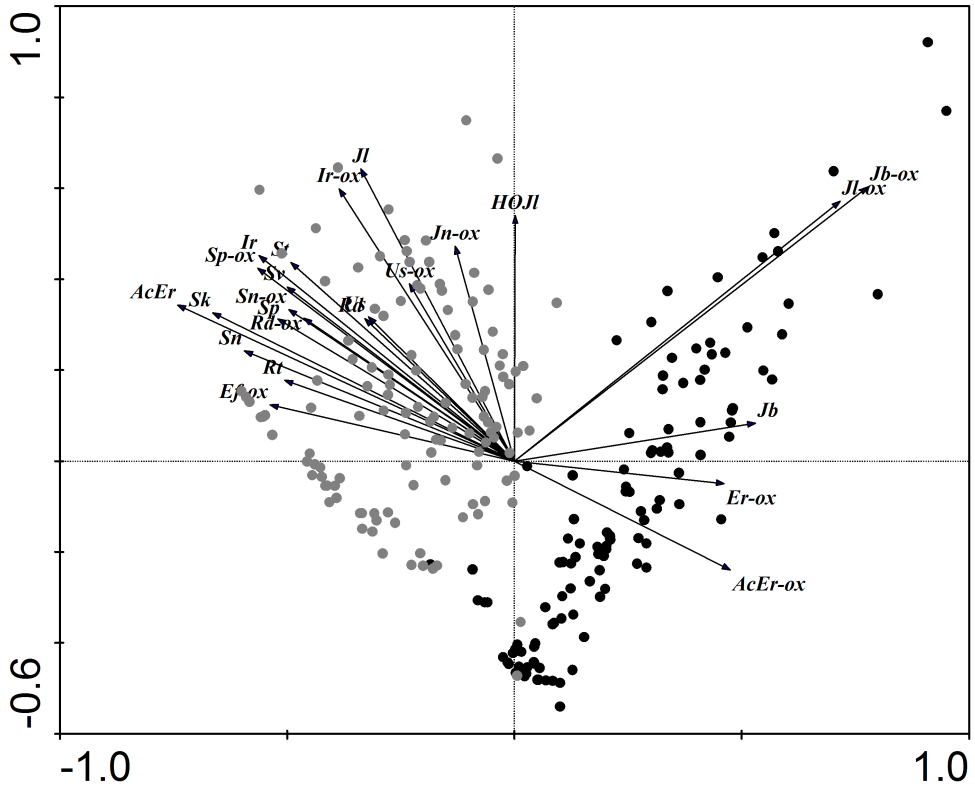

Figure 5: Principal component analysis (PCA) of the pyrrolizidine alkaloid (PA) composition of leaves versus flowers of *Jacobaea vulgaris* in early Summer (for late Summer see Fig. S3). PCA was done on log-transformed concentrations. Axis 1 explains 36.7% of the variation, axis 2 explains 21.3%. Redundancy analysis showed that 25.6% of the variation was significantly explained by the effect of organ (P=0.001). Colours represent organ: black circles- leaves; dark grey circles- flowers. Arrows represent the PAs with highest fit to the model (inclusion rule 20%), the list of PA names is in table S2

Discussion

In this study we analysed the temporal chemical variation in *Jacobaea vulgaris*. We observed seasonal changes in chemical concentrations, with a different trend between leaves and flowers. Interestingly, the composition of defensive chemicals in *J. vulgaris* plants depended on the succession stage of the abandoned arable fields in secondary succession. Our data also suggest allocation of defensive and nutritional compounds from leaves to flowers during early Summer. Although the interaction effect between season and successional position resulted in a complex pattern of plant defence variation, our results suggest that the allocation of defence compounds from leaves to flowers depends, to some extent, on successional position.

Seasonal variation of nitrogen found in *J. vulgaris* was consistent with other studies on the related species *Senecio vulgaris* (Qasem & Hill, 1995), but also with grasses (Jaeger et al., 1999). Interestingly Jaeger and co-workers (1999) related the seasonal decrease of nitrogen in grasses to inability of the soil microbial community to sequester soil nitrogen in Spring, while later in the seasons soil microbes could limit the plants access to nitrogen. A similar soil process could be occurring in *J. vulgaris*, as in the studied chronosequence nutrient mineralization by the soil food web has been shown to vary during the growing season (Holtkamp et al., 2011). Most likely such decrease of nitrogen content in the leaves is due to its allocation to flower heads, which show a seasonal increase. Chlorophyll concentration was lowest in late Summer, which is to be expected as *J. vulgaris* is a monocarpic biennial species that dies after seed release. Other studies have shown that chlorophyll variation in plants depends on field site and chlorophyll amounts being generally lower in late growing season (Zhao *et al.*, 2005; Joiner *et al.*, 2011). Chlorophylls also showed an interaction between season and successional position. Despite chlorophyll a and b concentrations were higher in Spring and early Summer chlorophyll a was highest in old fields and chlorophyll b increased with succession. It is known that vegetation structure and composition creates differences in plant competition for light (Tilman, 1988). With increasing time since abandonment the vegetation structure becomes more complex and denser vegetation (Kardol et al., 2005). The ground cover of *J. vulgaris* also decreases with field age (van de Voorde et al., 2012). The interaction between successional position and season suggests that the increase chlorophylls a and b early in the growing season is relevant for the phenology and growth of *J. vulgaris*, but the higher contents in older fields suggest increased light competition with surrounding vegetation when time since abandonment increases. Still further tests are necessary to verify how and why the photosynthetic system changes as a consequence of combination of season and time since abandonment.

Although seasonal effects were stronger, interaction with succession stage also had an effect on the concentration of plant defence compounds. In leaves, the N-oxide concentration increased with time since abandonment and tertiary-amines concentrations were highest in the Mid succession stage suggesting that different (a) biotic pressures might occur in those fields. Earlier studies have shown that PA content can be affected by both aboveground and belowground biota (Joosten *et al.*, 2009; Macel & Klinkhamer, 2010). These aboveground and belowground biota are known to change throughout secondary succession (van de Voorde *et al.*, 2011),

which can contribute variation in plant chemistry among successional stages. Since defence compounds concentration in flower heads also shifted with succession, such chemistry changes in the plant organs may well have multiple defence purposes. PAs are considered a defence compound especially effective towards generalist herbivores, whereas N-oxides might be more effective in defence than tertiary-amines (Leiss *et al.*, 2009; Cheng *et al.*, 2011b). The significant interaction of season and successional position on flowers defence compounds concentration suggests that as the fields age and *J. vulgaris* cover decreases plants are under higher pressure from generalist herbivores.

In flowers PA concentrations were higher in early than in late Summer. This might coincide with the peak of herbivory (Van der Meijden *et al.*, 1989; de Boer, 1999). Hartmann and Zimmer (1986) related such a trend to the loss of seeds from the flower heads, but we collected flower heads still intact, so loss of seeds did not play a role in the trends observed in our study. Another probable explanation is that most herbivores prefer leaves above flowers setting seed. A third factor could be that during the ripening of the flowers in late Summer nutrient flux is reduced and the influx of new PAs perhaps as well. These two possibilities need further testing.

When analysing PA composition it was evident that jacobine had strong influence on *J. vulgaris* plant defence composition. High levels of jacobine are associated with plant toxicity towards generalist herbivores, but also with a lower fungal diversity in the rhizosphere (Kowalchuk et al., 2006; Leiss et al., 2009). Yet, jacobine is also known for having a positive effect on the feeding patterns of the specialist herbivore *T. jacobaea* (Macel & Klinkhamer, 2010). As flowering plants from especially mid and older fields showed a higher affinity for jacobine-type PAs, flowering plants from older fields appear to be under relatively large pressure of generalist herbivores. Additionally, acetylerucifoline has been associated with soil legacy effects from past plant herbivory history (Kostenko et al., 2012b). The plants in our study showed a higher concentration of acetylerucifoline in Spring than in Summer, which supports Kostenko *et al.*(2012b) greenhouse experiment suggestion of a soil legacy effect. However acetylerucifoline shows a continuous decrease in concentration when time since abandonment increases. Apparently, soil legacy effects may act at the scale of months to years, rather than at the scale of decades.

A number of PAs were also represented more in *J. vulgaris* flower heads than in leaves, such as senecionine, integerrimine, seneciphyline and their N-oxides. Although the concentrations of senecionine, integerrimine and seneciphyline are

lower when compared to erucifoline or jacobine, their presence in the flower heads suggests that these PAs still have a role towards flower protection (Hartley *et al.*, 2012). Senecionine and seneciphylline are deterrents of generalist insect herbivores (Dreyer et al., 1985; Macel et al., 2005) and integerrimine has shown to be an effective fungal inhibitor (Hol & Van Veen, 2002). Interestingly, the flower PA composition could be attractive to the specialist herbivore *T. jacobaea* thus enhancing oviposition (Macel & Vrieling, 2003). It is known that *T. jacobaea* also shows preference for highest concentrations of Senecionine-type PAs (Lindigkeit *et al.*, 1997; Macel & Klinkhamer, 2010), whereas retrosine does not stimulate oviposition. Interestingly, the preferred *T. jacobaea* PA combination is also deterrent to snails (Speiser et al., 1992). The strongest senecionine, integerrimine, seneciphyline concentrations were found in both leaves and flowers of late Summer especially in young and old fields, suggesting that *T. jacobaea* and snail herbivory pressure may be highest in later Summer flowers of older fields. In one study, *T. jacobaea* was not found in the rosette stage (Spring season)(Kostenko et al., 2012a).The differences in PA composition of leaves and flowers may suggest different roles of PAs towards damage by herbivores and pathogens. Defence of flowers has a trade-off, as mutualists and pollinators of the plant should not be deterred whilst preventing herbivory. Further experiments should be done with both organs considered, as most studies so far have focussed almost exclusively on herbivory impact on leaves.

Pathogens and herbivores, both belowground and aboveground may cause selective pressure on PA composition and/or concentration. The variability of PAs in organs and seasons supports Macel and Klinkhamers' (2010) suggestion of an ecological cost involving PA production, possibly leading to a specific production of defensive chemistry depending of function and attack pressures. While data from early Summer clearly supports the optimal defence theory with flowers richer in PAs than leaves, data from samples collected in late Summer do not support that theory, especially if we consider the Old succession stage. Perhaps the ripening flowers are not attractive to most generalist herbivores, and in that case flowers need less protection. That would support optimal defence but not the assumption that flowers should be the most important organ of the plant at this stage.

Our study does not distinguish whether the effects in plant chemistry are solely due to seasonal and succession factors or if the genetic variation in among plants may play a role as well. Succession and season interactions explained only a limited percentage of the variation. PA profiles and concentrations are at least partly

genetically determined in *J. vulgaris* (Vrieling *et al.*, 1993; Macel *et al.*, 2004). Nevertheless, the effects of genetic variation might, at least in part, be ruled out by the replicate sites per successional stage. Since PA profiles alter with soil biota (Joosten *et al.*, 2009; Carvalho *et al.*, 2012) and both specialist and generalist herbivores (Speiser *et al.*, 1992; Macel & Vrieling, 2003; Macel *et al.*, 2005) these chemical changes could be partly attributed to phenotypic plasticity. To distinguish between genetic differentiation (selection on particular genotypes) and phenotypic plasticity further studies are needed.

In conclusion, both concentration and composition of chemicals in *J. vulgaris* varied throughout the growing season, whereas several chemicals were affected by successional stage as well. Chemicals in leaves and flowers did not vary in the same way, which could be due to optimization of defence. There was a relocation of PAs and nitrogen from leaves to flowers. Whether the influence of successional stage on plant chemistry is indicative of more complex optimal defence strategies, or of unidentified environmental factors interfering with plant defensive chemistry is unknown. The effect of successional position on chemical compounds of *J. vulgaris* was especially evident in flowers with higher PA tertiary-amine concentrations in fields that had been abandoned 10 to 20 years ago. When fields had been abandoned longer ago, flowers and leaves contained higher concentrations of N-oxides, especially in late Summer. Chlorophylls were affected by interaction between season and successional position. Chorophyll concentrations were highest in early Spring and Summer in the oldest fields, possibly due to higher competition from surrounding vegetation for available light.

Supplementary Information

Tables S1, Figures S1 - S2

Table S1: All *Jacobaea vulgaris* pyrrolizidine alkaloids (PA) analysed and the codes used for identification in the multivariate analyses

PA name	PA code	Parent mass	Cone	Fragment 1	Collision	Fragment 2	Collision
Senecionine-type							
senecionine	Sn	336,2	40	94	40	120	30
senecionine-N-oxide	Sn-ox	352,2	40	94	40	120	30
integerrimine	Ir	336,2	40	94	40	120	30
integerrimine-N-	Ir-ox	352,2	40	94	40	120	30
senecivernine	Sv	336,2	40	94	40	120	30
senecivernine-N-oxide	Sv-ox	352,2	40	94	40	120	30
retrorsine	Rt	350,2	40	94	40	120	30
retrorsine-N-oxide	Rt-ox	268,2	40	94	40	120	30
usaramine	Us	352,2	40	94	40	120	30
usaramine-N-oxide	Us-ox	368,2	40	94	40	120	30
eruciflorine-N-oxide	Ef-ox	368,2	40	94	40	120	30
riddelliine	Rd	350,2	40	94	40	138	30
riddelliine-N-oxide	Rd-ox	366,2	40	94	40	118	30
dehydroeruciflorine	DHEf	350,2	40	94	40	138	30
dehydroeruciflorine-N-oxide	DHEf-ox	366,2	40	94	40	118	30
seneciphylline	Sp	334,2	40	120	30	138	30
seneciphylline-N-oxide	Sp-ox	350,2	40	94	40	138	30
spartioidine	St	334,2	40	120	30	138	30
spartioidine-N-oxide	St-ox	350,2	40	94	40	120	30
acetylseneciphylline	AcSp	376,2	40	120	30	138	30
acetylseneciphylline-N-oxide	AcSp-ox	392,2	40	94	40	118	30
Jacobine-type							
jacobine	Jb	352,2	40	120	30	155	30
jacobine-N-oxide	Jb-ox	368,2	40	120	30	296	25
jacoline	Jl	370,2	40	94	40	138	30
jacoline-N-oxide	Jl-ox	386,2	40	94	40	120	30
jaconine	Jn	388,2	40	94	40	120	30
jaconine-N-oxide	Jn-ox	404,2	40	94	40	120	35
jacozine	Jz	350,2	40	94	40	120	30
jacozine-N-oxide	Jz-ox	366,2	40	94	40	118	30
dehydrojacoline	DHJl	368,2	40	94	40	120	30

Table S1 (continued)

Jacobine-type							
dehydrojaconine	DHJn	386,2	40	94	40	120	30
hydroxyjacobine	HOJb	368,2	40	120	30	296	25
hydroxyjacoline	HOJl	386,2	40	94	40	120	30
hydroxyjaconine	HOJn	404,2	40	94	40	120	35
Erucifoline-type							
erucifoline	Er	350,2	40	94	40	138	30
erucifoline-N-oxide	Er-ox	366,2	40	94	40	118	30
acetylerucifoline	AcEr	392,2	40	94	40	118	30
acetylerucifoline-N-oxide	AcEr-ox	408,2	40	94	40	120	30
Otosenine-type							
senkirkine	Sk	366,2	30	122	30	168	25
otosenine	Ot	382,2	30	268	25	122	30
onetine	On	400,2	30	122	30	168	30
desacetyldoronine	Dd	418,2	30	122	30	168	30
florosenine	Fs	424,2	30	122	35	168	30
floridanine	Fd	442,2	30	122	30	168	30
doronine	Do	460,2	30	122	35	168	30
Unknown-type							
unknown tertiary amine	Unk fb 350	350,2	40	94	40	138	30
unknown N-oxide	Unk ox 366	366,2	40	94	40	118	30
Heliotrine (IS)	Hel	314,2	30	138	25		

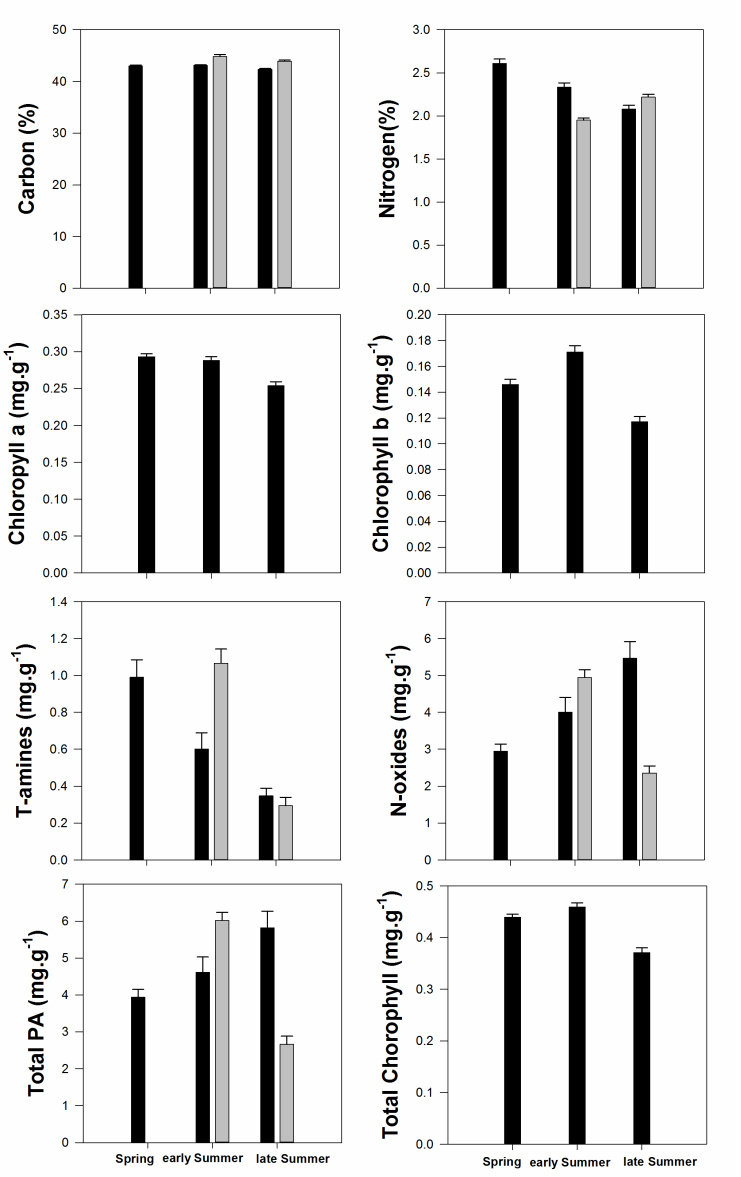

Figure S1: Chemical concentrations in leaves (black) and flowers (grey) per season. All seasons are significantly different. Figure in Carvalho S *et al* ., 2013.

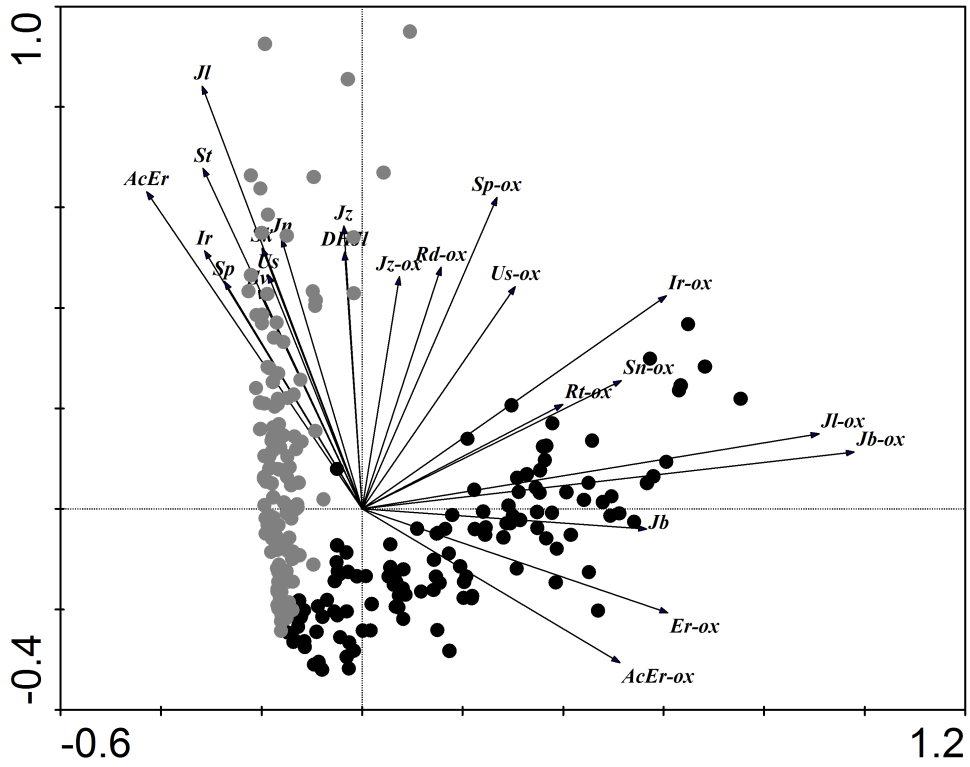

Figure S2: Principal component analysis (PCA) of the pyrrolizidine alkaloid (PA) composition of plant organs (leafs versus flowers) of *Jacobaea vulgaris* in season 3 (late Summer). PCA was done in log-transformed concentrations. Axis 1 explains 55.3% of the variation, axis 2 explains 15.2%. Redundancy analysis showed 29.0% of the variation was significantly explained by the effect of organ (P = 0,001). Colours represent organ: black circles- leafs; dark grey circles- flowers. Arrows represent the different PAs.

Hyperspectral reflectance of leaves and flowers of an outbreak species discriminates season and successional stage of vegetation.

5

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Abstract

Spectral reflectance can be used to assess large-scale performances of plants in the field based on plant nutrient balance as well as composition of defence compounds. However, plant chemical composition is known to vary with season – due to its phenology- and it may even depend on the succession stage of its habitat. Here we investigate i) how spectral reflectance could be used to discriminate successional and phenological stages of *Jacobaea vulgaris* in both leaf and flower organs and ii) if chemical content estimation by reflectance is flower or leaf dependent. We used *J. vulgaris*, which is a natural outbreak plant species on abandoned arable fields in north-western Europe and studied this species in a chronosequence representing successional development during time since abandonment. The chemical content and reflectance between 400 and 2500 nm wavelengths of flowers and leaves were measured throughout the season in fields of different successional ages. The data were analysed with multivariate statistics for temporal discrimination and estimation of chemical contents in both leaf and flower organs. Two main effects were revealed by spectral reflectance measurements: i) both flower and leaf spectra show successional and seasonal changes, but the pattern is complex and organ specific ii) flower head pyrrolizidine alkaloids, which are involved in plant defence against herbivores, can be detected through hyperspectral reflectance. We conclude that spectral reflectance of both leaves and flowers can provide information on plant performance during season and successional stages. As a result, remote sensing studies of plant performance in complex field situations will benefit from considering hyperspectral reflectance of different plant organs. This approach may enable more detailed studies on the link between spectral information and plant defence dynamics both aboveground and belowground.

Introduction

Spatial and temporal variation in plant chemical properties results from exposure to biotic and abiotic factors in the environment. To study how plant interactions with the environment result in vegetation patterns, ecological research develops hypotheses based on observations in temporal or spatial transects such as occurring in field chronosequences. These hypotheses are tested by experimental manipulations of plants under controlled conditions in fields, greenhouses or in the laboratory (Clements, 1963; van Dam *et al.*, 1993; Kardol *et al.*, 2006). However, studies on changed species interactions through time are often laborious and difficult to carry out on a large spatial scale. Visible and near infra-red (VNIR) spectroscopy offers the opportunity to study the biochemical and phenological state of plants and investigate how spectral input can aid the understanding of plant temporal processes that vary with ecosystem succession (Liu *et al.*, 2008; Rautiainen *et al.*, 2009; Knox *et al.*, 2010; Zeng *et al.*, 2010).

Hyperspectral sensors provide comprehensive spectral information of plants that allow for e.g. identifying species (Martin *et al.*, 1998; Schmidt & Skidmore, 2001; Mutanga *et al.*, 2003; Buddenbaum *et al.*, 2005; Asner & Martin, 2011), analyses of plant chemical content (Card *et al.*, 1988; Wessman *et al.*, 1988b; Curran *et al.*, 1992; Fourty & Baret, 1998; Knox *et al.*, 2010; Ramoelo *et al.*, 2011a) and soil nutrient impact on a plant's (Asner & Martin, 2011; Pretorius *et al.*, 2011). So far, few studies have addressed temporal spectral variation as a result of species responses to stages of vegetation succession. Although many temporal studies have discerned seasonal impacts on issues such as vegetation quality for food for mammals (Lechowicz & Koike, 1995; Hall-Beyer, 2003; Mutanga *et al.*, 2004; Cartelat *et al.*, 2005; Gilmore *et al.*, 2008; Zeng *et al.*, 2008; Knox *et al.*, 2010; Skidmore *et al.*, 2010; Zeng *et al.*, 2010), the lack of such succession-seasonal studies in other trophic levels, such as insects and soil herbivores, is certainly constrained by elegant detection methods that reveal the nature and strength of these biotic stressors in the field. Nevertheless soil microorganisms and insects are drivers of ecosystem changes (de Beurs & Townsend, 2008) and using hyperspectral reflectance could be an interesting avenue to study this more concealed biotic effects on plant performance (Carvalho *et al.*, 2012). One such site currently available is a chronosequence of abandoned, ex-arable fields in the Netherlands, that has been studied and monitored in order to study the role of soil biota in secondary vegetation succession (van de Voorde *et al.*, 2012).

Numerous studies have been undertaken to understand the ecological processes driving vegetation succession (Walker & del Moral, 2003). More recently, the role of soil biota (such as fungi, bacteria and invertebrate root herbivores) and their impact on plant quality and community composition have been taken into account (Bezemer *et al.*, 2006; Kardol *et al.*, 2006; Van de Voorde *et al.*, in press). *Jacobaea vulgaris* has been a model species for many of these ecological studies, amongst others because of its hepatotoxic characteristics towards humans, cattle and invertebrates. It is known that *J. vulgaris* biomass changes throughout seasons and succession and can be affected by soil nutrient and microbial composition (Macel *et al.*, 2004; Joosten *et al.*, 2009; Kostenko *et al.*, 2012b). According to Kardol *et al.* (2006) species succession in the chosen chronosequence may depend substantially on the accumulation of harmful and beneficial organisms in the rhizosphere and not only on abiotic soil properties. Such rhizosphere communities may cause negative soil feedback in early succession, neutral in mid succession and positive feedback to late succession plants (Kardol *et al.*, 2006).

Plant-soil biota interactions may affect the leaf chemical properties and canopy structural properties of a plant, which then possibly translates into a change in spectral reflectance. In a recent greenhouse study we found that such soil biotic effects can, to some extent, affect leaf chemical composition and spectral reflectance (Carvalho *et al.*, 2012). Examining hyperspectral reflectance might provide researchers with further avenues to study plant exposure to ecological processes such as competition, plant diseases, invasiveness and soil biological control of plant abundance, through the, so far, limited temporal scale.

It is essential to understand if the spectral changes that may take place in plants through time could relate to the ecological changes that have already been demonstrated in these plants. As such, we investigated the potential of spectral reflectance to discriminate temporal variation of *J. vulgaris* during the secondary succession stages of abandoned fields. We studied hyperspectral reflectance of both leaves and flowers in order to determine if these two plant organs separately can provide additive information on plant fitness. We tested the hypotheses that: i) Seasonality and succession stage of *J. vulgaris* are expressed in spectral reflectance of both leaf and flower organs and iii) the chemical variation resulting from successional and seasonal plant development can be detected in both leaves and flowers.

Material and Methods

Species description

Although native to the Netherlands, *Jacobaea vulgaris* is considered a noxious outbreak weed as it is toxic towards humans and livestock and highly dominant in recently abandoned arable fields (Mattocks, 1986; Bezemer *et al.*, 2006). When time of abandonment increases, *J. vulgaris* dominance declines and it largely disappears from the vegetation (van de Voorde *et al.*, 2012). Its toxicity is mainly due to its pyrrolizidine alkaloids (PA) that are present in all organ types, from leaves to flower heads and seeds. In Spring this species has a rosette structure, it flowers via a single central stem throughout the Summer, if conditions are favourable (Fig. 1). The circular rosette has basal stalked leaves obovate to pinnately lobed, generally 2 to 6 cm wide. During early and late Summer the rosette leaves senesce and stems develop with pinnate lobed leaves. Flower heads are the characteristic Asteraceae capitulum with bright yellow flowers and green bracts.

Figure 1: Representative leaf and flower heads of *Jacobaea vulgaris*. Top row characterises early Summer bottom row late Summer.

Field sampling

The selected fields belong to a chronosequence of abandoned arable-fields (Kardol *et al.*, 2006). We used information on site characteristics and responses of *J. vulgaris* to soil biota (Bezemer *et al.*, 2006; Kardol *et al.*, 2006; van der Wal *et al.*, 2006; van de Voorde *et al.*, 2011) to selected 6 fields (Table 1). These fields were all located on south Veluwe, Gelderland Province, the Netherlands. Agricultural production had stopped between 5 and 30 years ago and the fields are currently part of a large nature reserve (Bezemer *et al.*, 2006).

Table 1: Names of fields selected, code names, time since abandonment (in years) its classified succession class and geo-location. Soil and plant community characteristics are available in Kardol at al. (2005), van de Voorde et al. (2012).

Field	Field Code	Time since abandonment	Year of abandonment	Succession class	Latitude (°N)	Longitude (°E)
Oud Reemst	OR	5	2005	Young	52.02	5.48
Reyerskamp	R	5	2005	Young	52.01	5.47
Mossel	M	15	1995	Medium	52.03	5.45
Nieuw Reemst	NR	20	1990	Medium	52.04	5.47
Wolfheze	W	22	1988	Old	51.6	5.47
Dennenkamp	D	27	1982	Old	52.02	5.48

Since the fields were of different sizes, in each field we set a W-shaped transect that covered the field central area of 30 m by 100 m. We sampled one plant every 5 m to a total of 20 plants per field. This process was repeated two times throughout the Summer season. As such we covered 2 phenological stages: the flowering and the senescing stage. At each sampling date in all fields, of each individual plant five leaves were measured positioned from base to top of the stem. Five flower heads (the full capitulum, Fig. 1) in the centre of the inflorescence were measured in a lateral perspective to incorporate both the flower petals and the bracts of the capitulum. Both leaves and flowers of each plant were measured still intact and attached to the plant. We used a plant probe and leaf-clip attached to the ASD Fieldspec 3 fieldspectrometer (ASD Inc., Boulder CO, USA) to collect the spectral reflectance data. The measured leaves and flowers were immediately collected and stored in ice for chemical extractions in the laboratory thereafter.

Leaf spectral measurements and processing

Spectral data were collected with an ASD Fieldspec 3 spectrometer with an ASD plant-probe and leaf-clip device (ASD Inc., Boulder CO, USA). The instrument has a spectral range between 350 and 2500 nm with 3nm spectral resolution in the 350 nm – 1000 nm and 10 nm between 1000 nm and 2500 nm wavelengths. The plant-probe was designed for non-destructive data collection from live plants with heat sensitive halogen light bulb (colour and temperature 2901 ± 10º% K) and spectral measurement spot size of 10 mm radius. The leaf-clip has a gentle gripping system designed for the plant-probe to hold the sample in place without inflicting damage or removing the sample. Since we were interested in spectral reflectance measurements the black panel face of the leaf-clip was used in each measurement. In each leaf or flower 4 single reflectance measurements were undertaken resulting in a 20-fold composite leaf or flower spectral sample per plant (Ramsey, 1997). All spectral measurements were calibrated with the white reference face of the leaf-clip. The reflectance measurements were offset corrected and its composite average calculated with software ViewSpec Pro 5.6.10 (ASD Inc. Boulder, USA). In pre-processing we realized that a technical error occurred with the first season measurements, requiring the rosette leaf measurements to be removed from further analysis.

Chemical extraction

Chlorophyll *a* and *b* (mg.g^{-1}), nitrogen (%), carbon (%) and the defence compounds pyrrolizidine alkaloids (mg.g^{-1}) were extracted from the five leaves (base to top) while in the flower-heads chlorophyll content was not considered.

The chlorophyll extraction was done using four leaf discs of 10 mm diameter each. The leaf discs were immersed in 3 ml of dymethyl sulfoxide (DMSO) and stored in a dark room for three days at constant room temperature. In a spectrophotometer (Genesys 20 spectrophotometer 4001/4, Thermo Fisher Scientific Inc., Waltham, USA) the 649 nm and 665 nm absorbance (Abs) was measured and chlorophyll concentrations were calculated.

A fine homogenized powder from freeze-dried samples was used for pyrrolizidine alkaloids (PA), nitrogen (N) and carbon (C) estimations. Metal cups of 6 mm diameter were used with 3-5 mg sample powder for combustion-reduction in a C:N analyser (Thermo flash EA 1112, Thermo Fisher Scientific Inc., Waltham ,USA) to estimate C:N percentage.

Pyrrolizidine alkaloids (PAs) were extracted according to Joosten *et al.* (2009) and quantified as described by Cheng *et al.*(2011a). The PA content was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Waters UPLC system (Waters, Milford, USA) coupled to a Waters Premier XE tandem mass spectrometer (Waters, Milford, USA).

Statistical analysis

- Analysis of variance (ANOVA test) and Tukey post-hoc test

One-way ANOVA was performed in the spectral measurements, first with season and succession classes and secondly with season and field as factors. After a significant ANOVA test, a post-hoc test Tukey honestly significance difference (Tukey HSD) was performed to test each wavelength in a pairwise manner. This test is conservative for unequal sample sizes and accounts for type I errors by reducing the significance level (α) of each test so that the group-wise type I error rate stays at the chosen level, in this case, $\alpha = 0.05$ (Quinn & Keough, 2002). This multiple comparison permits to find those wavelengths that are significantly different between succession groups (young vs medium, young vs late, medium vs late) and seasons with reduced error. The ANOVA analyses were performed for both leaves and flower, using R 2.13.2 for Windows.

- Discriminant analysis

While ANOVA tests for differences between groups, discriminant analysis can be applied to generate a combination of features that maximizes the probability of correctly assigned objects to their defined groups (Naes *et al.*, 2002; Quinn & Keough, 2002). Additionally, the discriminant analyses can be used to classify observations into the groups of interest. In this study we applied quadratic discriminant analysis (QDA) since it does not assume equal within-group covariance. The spectra can be highly collinear and QDA is sensitive to such effects (Naes *et al.*, 2002). To correct for multicollinearity the spectral reflectance was mean-centred and principal component analyses (PCA) was computed (Naes *et al.*, 2002). The resulting PCA scores were then used for performing the QDA. We used 20 principal components as it explained 99.9% of the variance, thus including all the information existent in the original data. Discriminant analysis can have a problem with unequal number of samples per group, overestimating a correct classification, thus prior

probabilities were calculated based on the observed group sizes to reduce the random correct classification. The prior probability of the groups describes what is known *a priori* about the groups to be estimated in the analysis, is based on the Bayes' theorem and is integrated in the discriminant analysis to infer the posterior probabilities (Naes *et al.*, 2002). Success of the classification of the QDA equation was assessed by the quality of the cross-validation confusion matrix and by its success to classify new observations into the groups. The first discriminant function is the combination of variables that maximize the ration between-group to within-group variation in MANOVA, so that the analysis was considered to test statistical significant differences between the groups (Quinn & Keough, 2002).

By chance classification of samples (also called the error of commission or specificity) is often raised as a problem in spectral data analysis. Different measures of accuracy consider different assumptions and one standardized method to overcome all problems is still challenging (Foody, 2002). By considering the MANOVA statistical test alongside the discriminant analysis we allocate a statistical power to the groups discriminant functions. Highly significant functions should assure that groups compared have lower by-chance classifications. Additionally the Tau index was computed as it provides a standardized measure of the proportional improvement over a model's classification error rate established by chance (Klecka, 1980). The formula applied was:

$$\tau = \frac{Nc - \sum_{i=1}^{G} PiNi}{Nc - \sum_{i=1}^{G} PiNi}$$

Nc is the number of samples correctly classified, N_i is the number of samples in the i^{th} group, N is the total number of samples, G is the number of groups and P_i is the by chance probability of allocating the sample to that group. The groups of interest in this study were the succession classes (Table 1). The QDA was analysed in Unscrambler X 10.1 and the MANOVA was processed in SPSS 17.0 for Windows.

- Partial least square regression (PLSR)

While discriminant analysis tests the possibility for classification of samples into the groups of interest (in this case succession class), with partial least square regression (PLSR) we tested if specific spectral band data can be used as predictors of chemical concentrations in individual samples (Naes *et al.*, 2002). Therefore we examine the linkage between the sample chemical content and its spectral signature. The cross-

validation procedure selected was 'leave-one-out' sampling that iteratively generates regression models with 1 random sample reserved for validation of the model. This was done in a training-set with approximately 70% of the samples to determine the optimal number of factors and lowest root mean square error (RMSEcv) of cross-validation. The accuracy of the model for prediction of chemical concentrations was assessed in terms of minimum root mean square error of prediction (RMSEp) and the highest coefficient of regression (r^2) of the test-set (i. e the reserved 30% of the samples). The entire procedure was done in Unscrambler X 10.1 for Windows.

Results

Spectral reflectance differences in leaf and flowers

It was possible to significantly differentiate between leaves and flowers by spectral reflectance patterns (Figs. 2 and 3). The analysis of *Jacobaea vulgaris* leaves and flowers revealed statistical significant differences, which were more prominent in the flower spectral reflectance than in leaves. Both leaf and flower spectral reflectance showed variations in relation to succession stage and season.

Hyperspectral reflectance of leaves from plants in old succession stages was significantly different from young succession stages both in the visible range (500-650 nm) and in the inflection between the visible and near-infrared (700 nm region) (Fig. 3). Flowers of plants from younger fields reflected significantly less in the 500 - 650 nm range than flowers from medium or old succession stages (Fig. 3). No significant differences were found between flower reflectance of medium and old succession classes ($P > 0.05$).

There were differences between early and late Summer in the red edge area (690 - 710 nm region) of leaves ($P < 0.05$; Fig. 3). Flower spectra in early and late Summer were significantly different through the entire visible and infra-red region ($P < 0.05$; Fig. 3).

Succession class discrimination.

It was possible to discriminate successional classes with high accuracy (of cross-validation) for both flower and leaf spectral reflectance patterns. The successional classes underlying dimensions of the cross-validated model were highly significant ($P < 0.001$) in explaining differences. In addition, Tau results indicate that the

classification analysis obtained between 90% and 100% fewer errors than what would be expected by chance.

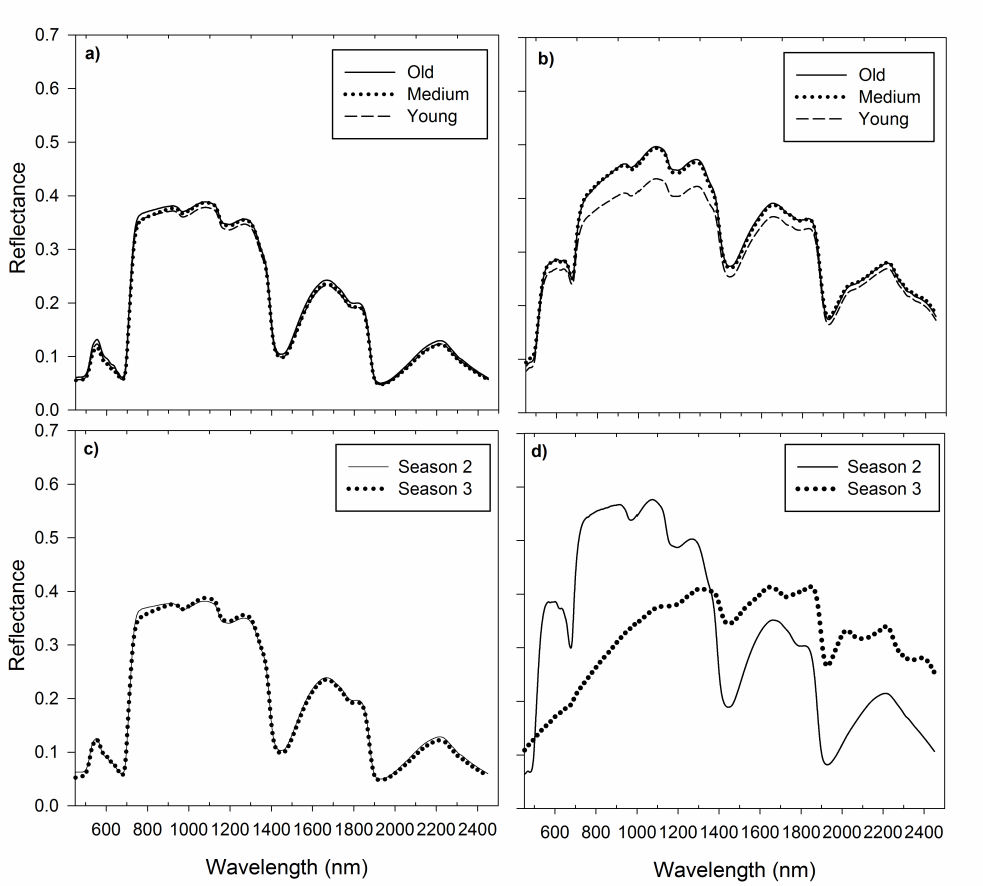

Figure 2: Mean spectral reflectance of a) succession class in leaves, b) succession class in flower heads c) seasons in leaves and d) seasons in flowers heads.

The Succession classes discrimination analyses with both seasons together resulted overall classification accuracy, by the quadratic discriminant analysis, of 92.3% for the leaf cross-validation model and 94.4% for the flowers (Table 2). Even so, succession had only moderate prediction accuracy in the test-set with 56.1% for leaf and 65% for flower correct prediction of unknown samples.

The discrimination analyses with early and later Summer separately resulted in 100% overall correct classification in the cross-validation model, for both leaves and flowers (Table 3). However early Summer provided a low prediction accuracy for the

validation samples for flowers (32.5%) and leaves (50%) while late Summer was low to moderate with 52.6% for leaves and 60% for flowers correct prediction for the validation samples.

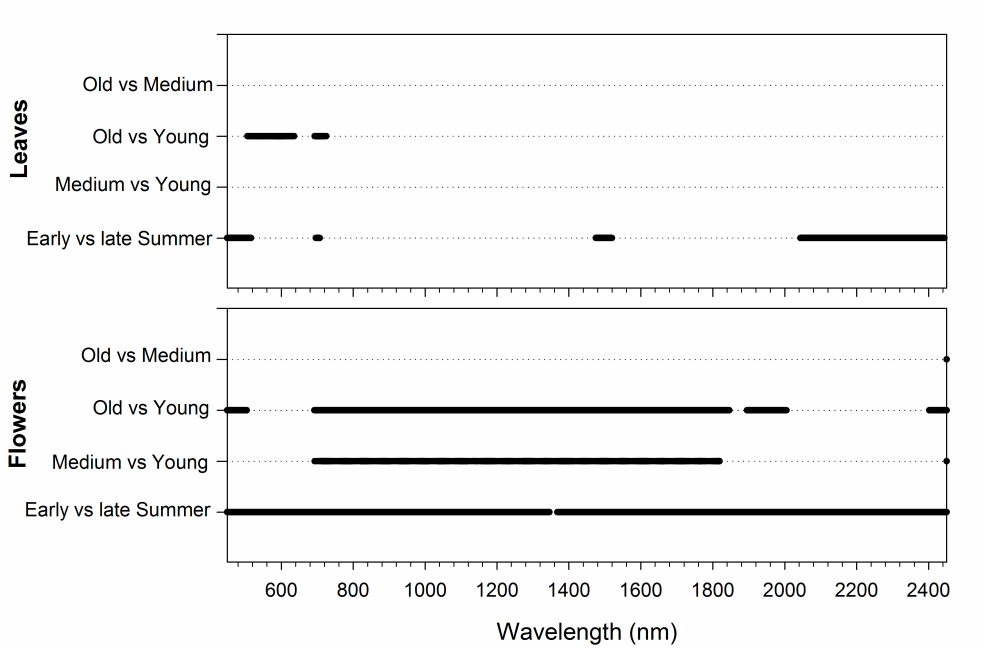

Figure 3: One way ANOVA and Tukey HSD test results for leaf and flower reflectance measurements. Dark circles indicate wavebands that were significantly different ($P<0.05$) in each pair comparison. Early Summer vs late Summer - early Summer and late Summer comparison; Old vs Young- Old succession vs Young succession fields. Old vs Medium – Old and Medium succession comparison; Medium vs Young- Medium and Young succession comparison.

Chemical content estimation in leaves and flowers

There were significant differences in chemical content between the seasonal and successional classes (Figs. 4 and 5). The PA concentrations were affected by both season and succession stage, whereas nitrogen was significantly affected only by season. This pattern was found in both leaf and flower organs, and partial least square regression (PLSR) was successful in the estimation of several chemical concentrations.

The most successful foliar estimates occurred in the primary compounds, nitrogen and chlorophyll (Table 4). The prediction of unknown samples by such models resulted in moderate correct estimations of foliar content with root mean square errors of prediction (RMSEp) in the 12 to 15% range of the mean. The PLSR failed prediction of foliar PA content (Table 4).

Table 2: Confusion matrix of the quadratic discriminant analysis with both seasons considered. Succession stage was used as the discrimination group. The first 20 principal components of the spectral reflectance were used in the QDA. The bold number along the diagonal represents the number of correct classified samples. The results display the cross validation (leave-one-out) and separate test set for classification of unknown samples for both leaf (tau= 0.88) and flower heads (tau=0.92). All discriminant functions were highly significant (P<0.001). In brackets is the number of samples used in each dataset.

both seasons

		Crossvalid (168)			Predict (59)			
		Old	Medium	Young		Old	Medium	Young
Leaf	Old	**54**	2	2	Old	**14**	6	5
	Medium	1	**47**	0	Medium	3	**9**	4
	Young	3	5	**54**	Young	5	4	**9**

		Crossvalid (178)			Predict (60)			
		Old	Medium	Young		Old	Medium	Young
Flower	Old	**57**	2	3	Old	**12**	2	4
	Medium	2	**57**	2	Medium	4	**14**	2
	Young	0	1	**54**	Young	4	4	**13**

Overall accuracy in cross-validation was 92.3% in leafs, 94.4% in flowers with predicted accuracy of 56.1% and 65% respectively.

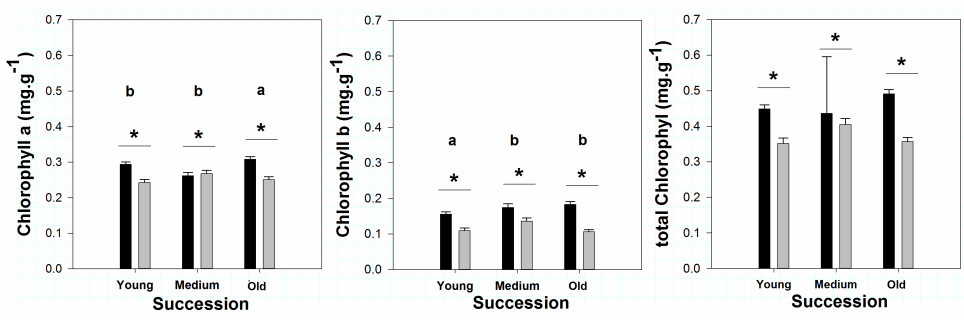

Figure 4: Chlorophyll content per succession stage (young, intermediate, old) in leaves, for both early (black bars) and late Summer (grey bars). Asterisk represents significante differences between seasons. Letters represent significant differences between succession categories (P<0.05). Error bars are standard errors.

While the most accurate estimations of leaf chemicals concerned the primary compounds, in flowers the highest accuracy was obtained for estimates of the defence compounds (Table 4). Using PLSR we were able to estimate unknown samples between 38 and 61% accuracy. The best estimation accuracies were 23% and 29% of the mean for total PA and N-oxides, respectively. Tertiary amines presented the highest error with 61.6% of the mean. Contrary to the foliar PLSR, predictive

models for flower contents failed to estimate primary compounds (Table 4). The most accurate PLSR models, both for leaves and flowers, consistently selected the spectral regions known from literature (data not shown), such as reported by Curran (1989), Kumar *et al.* (2001) and Carvalho *et al.* (2013).

Table 3: Confusion matrix of the quadratic discriminant analysis per season. Succession stage was used as the discrimination group. The first 20 principal components of the spectral reflectance were used in the QDA. The bold number along the diagonal represents the number of correct classified samples. The results display the cross validation (leave-one -out) and separate test set for classification of unknown samples for both leaf (tau= 1) and flower heads (tau=1). All discriminant functions were highly significant (P<0.001). In brackets is the number of samples used in each dataset.

			Crossvalid (74)				Predict (38)		
			Old	Medium	Young		Old	Medium	Young
Early Summer	Leaf	Old	**25**	0	0	Old	**6**	2	4
		Medium	0	**25**	0	Medium	1	**8**	3
		Young	0	0	**24**	Young	6	2	**5**
			Crossvalid (80)				Predict (40)		
			Old	Medium	Young		Old	Medium	Young
	Flower	Old	**27**	0	0	Old	**3**	3	1
		Medium	0	**27**	0	Medium	6	**5**	2
		Young	0	0	**26**	Young	5	4	**5**

Overall accuracy in cross-validation was 100% in leafs and flowers with predicted accuracy of 50% and 32.5% respectively.

			Crossvalid (74)				Predict (38)		
			Old	Medium	Young		Old	Medium	Young
Old Summer	Leaf	Old	**25**	0	0	Old	**6**	2	4
		Medium	0	**25**	0	Medium	1	**8**	3
		Young	0	0	**24**	Young	6	2	**5**
			Crossvalid (78)				Predict (40)		
			Old	Medium	Young		Old	Medium	Young
	Flower	Old	**25**	0	0	Old	**10**	2	2
		Medium	0	**27**	0	Medium	5	**6**	2
		Young	0	0	**26**	Young	3	2	**8**

Overall accuracy in cross-validation was 100% in leafs and flowers with predicted accuracy of 50% and 52.6% respectively.

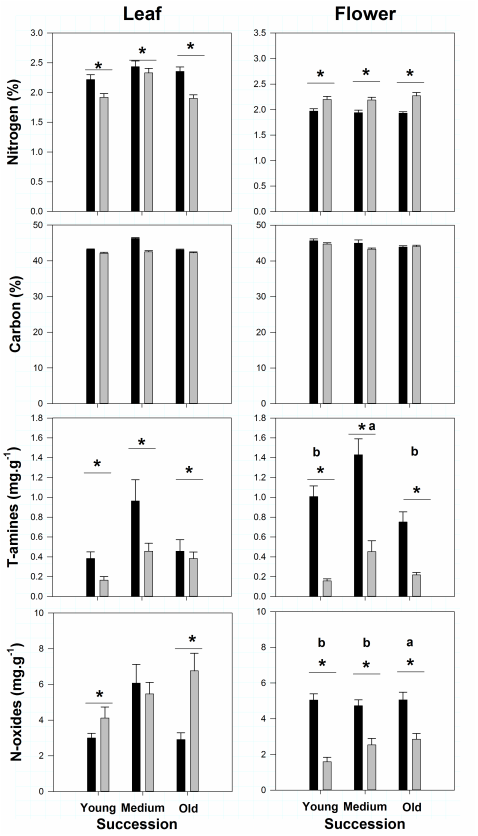

Figure 5: Chemical content (primary and secondary) per succession stage (young, intermediate, and old) in leaves and flowers, for both early (black bars) and late Summer (grey bars). The asterisk represents significante differences between seasons. Letters represent significant differences between succession categories ($P<0.05$). Error bars are standard errors.

Discussion

In this study of temporal variation in the hyperspectral reflectance of *Jacobaea vulgaris,* two main effects were revealed: i) there are successional and seasonal variations in spectral reflectance of leaves and flowers ii) the defence chemical content of flowers can be detected through hyperspectral reflectance.

We had expected seasonal differences in hyperspectral reflectance between flowers throughout the whole visible and infrared range, as in early Summer the flower capitula were still yellow, whereas in late Summer flowers were replaced by dry white papus. Such clear differentiation in leaves was far less obvious. This lack of full differentiation of spectral information between leaves from different seasons might be influenced by spectral measurements done only in live leaves. Brown and dead leaves have not been considered in this study. Another limitation to this seasonal study is the lack of the Spring season data, when plants were in the rosette

stage. This lack of data is due to an initial technical problem, so that we were unable to include this early seasonal stage in our analyses. Nevertheless, we believe that Spring season data could very well improve the discrimination ability of our approach, as it is a fully different ontogenetic stage of the life cycle of *J. vugaris*. However, further tests are necessary.

Table 4: Species chemical concentration estimations by partial least square regression using leaf or flower spectral reflectance signatures. r^2 calib represents r^2 of model calibration, r^2 cv represents r^2 of cross-validation (leave-one-out) and r^2predict represents how good is the fit of the cross validated model to predict new samples from a separate test-set. RMSEp is root mean square error of prediction and % RMSEp is the error as a percentage of the mean value of the chemical concentration. Num.Factors indicates the number of factors selected by the model. Dash represents PLSR models that failed prediction and consequently the RMSE presented is of cross-validation. PA - pyrrolizidine alkaloids, which act as defensive components in *J. vulgaris*.

Leaf	mean	St. Dev.	r2 calib	r2 cv	r2 predict	RMSEp	% RMSEp	num. Factors
Nitrogen (%)	2.30	0.58	0.73	0.66	0.62	0.29	12.77	9
Carbon (%)	42.77	1.76	0.22	0.13	0.06	1.89	4.43	5
Chlorophyll a (mg.g-1)	0.28	0.05	0.59	0.50	0.26	0.04	14.80	7
chlorophyll b (mg.g-1)	0.15	0.06	0.65	0.60	0.60	0.03	21.38	5
chlorophyll total (mg.g-1)	0.42	0.09	0.68	0.62	0.37	0.06	15.13	5
Total PA (mg.g-1)	0.69	0.27	0.02	0.00	-	0.30	42.96	1
Tertiary-amines (mg.g-1)	0.17	0.18	0.02	0.00	-	0.15	85.96	1
N-oxides (mg.g-1)	0.62	0.29	0.01	0.00	-	0.30	48.95	1

Flower	mean	St. Dev.	r2 calib	r2 cv	r2 predict	RMSEp	%RMSEp	num. Factors
Nitrogen (%)	2.09	0.35	0.26	0.25	-	-	-	1
Carbon (%)	44.50	3.37	0.06	0.02	-	3.45	-	1
Total PA (mg.g-1)	0.65	0.26	0.73	0.67	0.61	0.15	23.55	3
Tertiary-amines (mg.g-1)	0.19	0.16	0.41	0.40	0.38	0.11	61.62	2
N-oxides (mg.g-1)	0.60	0.25	0.63	0.56	0.47	0.18	29.77	3

The successional class of old field succession defined by Kardol *et al.*(2006) resulted in a successful differentiation using the hyperspectral information of flowers. This suggests that flower composition is more sensitive to field ageing processes than that of leaves. The hyperspectral reflectance of leaves showed significant differences between old and young succession fields in the visible region, suggesting that differences in the photosynthetic process may occur at larger temporal scales than usually thought. The spectral changes in the visible

wavelengths are supported by the significant differences found in concentration of chlorophyll a and b between these succession groups. The resulting spectral changes between plants found in the present study can, at least to some extent, be related to the chemical changes in the plants. This suggests that changes in plant chemical properties during succession possibly translate into the changes in spectral reflectance and that plant successional position in the field might be identified through analysis of hyperspectral reflectance data. The results support, to some extent, our hypothesis as the reflectance by flowers was sensitive to succession stages, and both leaf and flower spectral patterns reflected, to some degree, the plant chemistry.

Our study suggests that succession category affect, albeit slightly, the plant spectral reflectance. The high cross-validation results in our study and significant explained variation by the discrimination functions suggest that even within species the statistical significant discrimination of groups (succession) is possible. However the natural continuum range of variation has some level of erroneous prediction of new samples due to groups that overlap. Since the accuracy of our cross-validation discriminant analysis is similar to results from some other studies (Asner & Martin, 2011; Ramoelo *et al.*, 2011b), we suggest that there should be caution in the interpretation of the discrimination power based solely on cross-validation models. A test set for prediction of new samples should always be considered due to the continuum variation naturally occurring in field conditions. The discrimination accuracy by the spectra of the plant organs was also season dependent, whether we group the season or use them individually, but in general, succession class discrimination achieve similar levels of accuracy.

We demonstrated that chemical concentration estimation using spectral data is possible for both leaves and flowers. The cross-validation results obtained for foliar estimation of nitrogen and chlorophyll were consistent with earlier studies, including the spectral features selected for the models (Curran *et al.*, 2001; Darvishzadeh *et al.*, 2008b; Knox *et al.*, 2010; Asner & Martin, 2011; Ramoelo *et al.*, 2011b). Its accuracy provided a moderate predictive ability of chemical concentration in new leaf samples, showing that even in controlled field measurements such chemical concentration estimations by hyperspectral reflectance are challenging. The flower estimation results were the reverse of results on leaves. Nitrogen contents could not be predicted in flowers, whereas estimations of PA compounds were moderately accurate. Concentration wise both flowers and leaves were relatively similar in

nitrogen concentration variation. The differences between flowers and leaves were therefore unexpected. Both Asner and Martin (2008) and Kokaly and Clark (1999) highlighted that vegetation structure can affect spectral features and influence chemical detection. It could be that the flower structure (i. e. the capitulum shape) affects the spectral features associated with nitrogen estimations resulting in such estimation inconsistencies between organs. The flower structure combined with lower water content in flower could have aided the features associated with PA estimations. However, using the same technology, Carvalho *et al.* (2013) were able to estimate foliar PAs with moderate accuracy in laboratory conditions. Other studies might be necessary to analyse what could cause such leaf and flower predictive differences. Nevertheless, the two plant organs can affect the chemical estimation by spectral features differently. This is important to study further as upscale and temporal studies are foreseen in remote sensing.

Differences in *J. vulgaris* successional position might be detected by spectral reflectance and this old-field chronosequence has been proposed to be driven, at least in part, by soil biota (Kardol *et al.*, 2006; van de Voorde *et al.*, 2012). Our results show that spectral reflectance could add information to temporal studies in the field on complex ecological processes driven by for example cryptobiota that influence plant performance, which go beyond the impact of large vertebrate herbivores. Moreover, we show that different plant organs may vary in an organ-specific way during secondary succession. More insight into plant-soil interactions and their impacts on spectral reflectance patterns might be considered as having potential for studies on biological control of invasive species or soil-borne diseases. An important issue to solve is the multiple information aspect of spectral signals in imagery. In imagery both leaf and flower information will be detected during flowering season. What the spectral signal of a plant as a whole may tell about interactions, successional situation, its adaptation, etc., needs to be established in subsequent studies to improve the development of accurate extrapolations to imagery.

We conclude that the results are organ dependent and spectral overlap may be the main reason for some of the lowest outcomes. Yet the spectral variation in both flowers and leaves is supported by the variation in chemical concentration of *J. vulgaris*. Defence compounds could be estimated more reliably in flowers, whereas of leaves primary compounds could be predicted best. As such we suggest that remote

sensing studies should consider the effect not only of phenology but also of different organs.

Supplementary Information

Figures S1 - S6

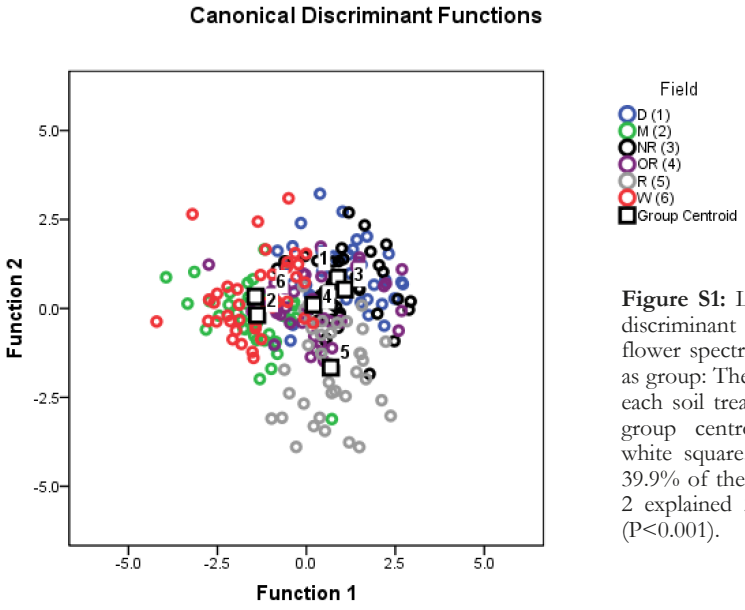

Figure S1: Leave-one-out Quadratic discriminant scores generated by flower spectral reflectance with field as group: The mean group scores for each soil treatment are given by the group centroid represented by a white square. Function 1 explained 39.9% of the variation and Function 2 explained 24.1% of the variation (P<0.001).

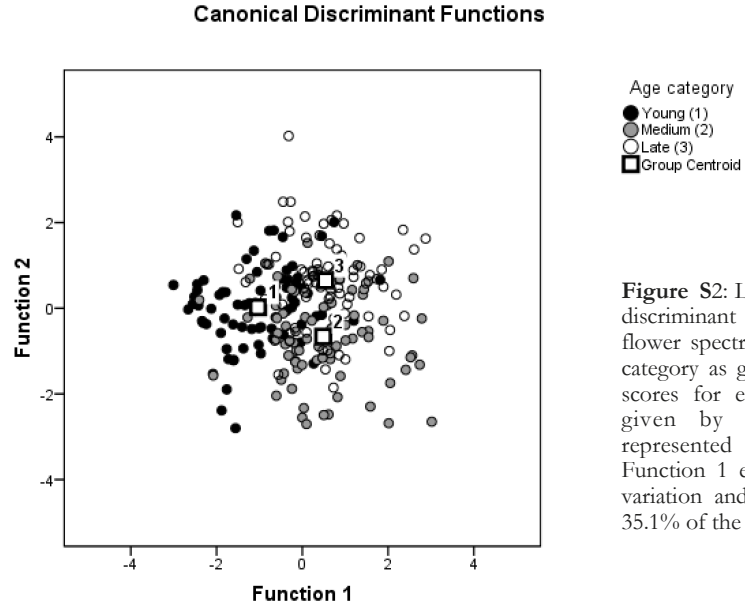

Figure S2: Leave-one-out Quadratic discriminant scores generated by flower spectral reflectance with Age category as group: The mean group scores for each soil treatment are given by the group centroid represented by a white square. Function 1 explained 64.9% of the variation and Function 2 explained 35.1% of the variation (P<0.001).

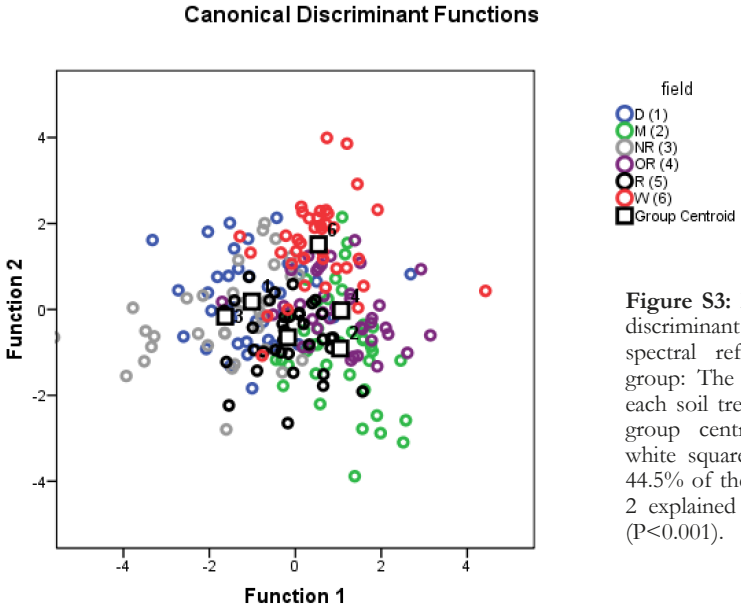

Figure S3: Leave-one-out Quadratic discriminant scores generated by leaf spectral reflectance with field as group: The mean group scores for each soil treatment are given by the group centroid represented by a white square. Function 1 explained 44.5% of the variation and Function 2 explained 27.1% of the variation (P<0.001).

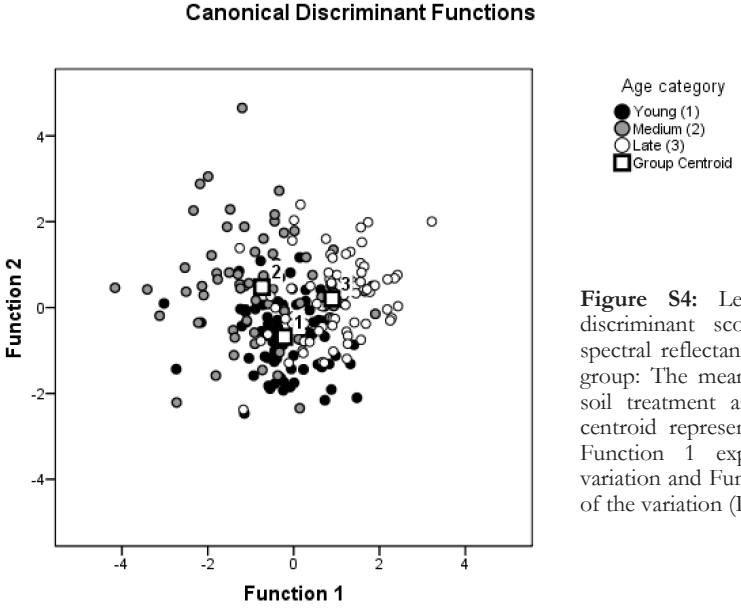

Figure S4: Leave-one-out Quadratic discriminant scores generated by leaf spectral reflectance with Age category as group: The mean group scores for each soil treatment are given by the group centroid represented by a white square. Function 1 explained 66.1% of the variation and Function 2 explained 33.9% of the variation (P<0.001).

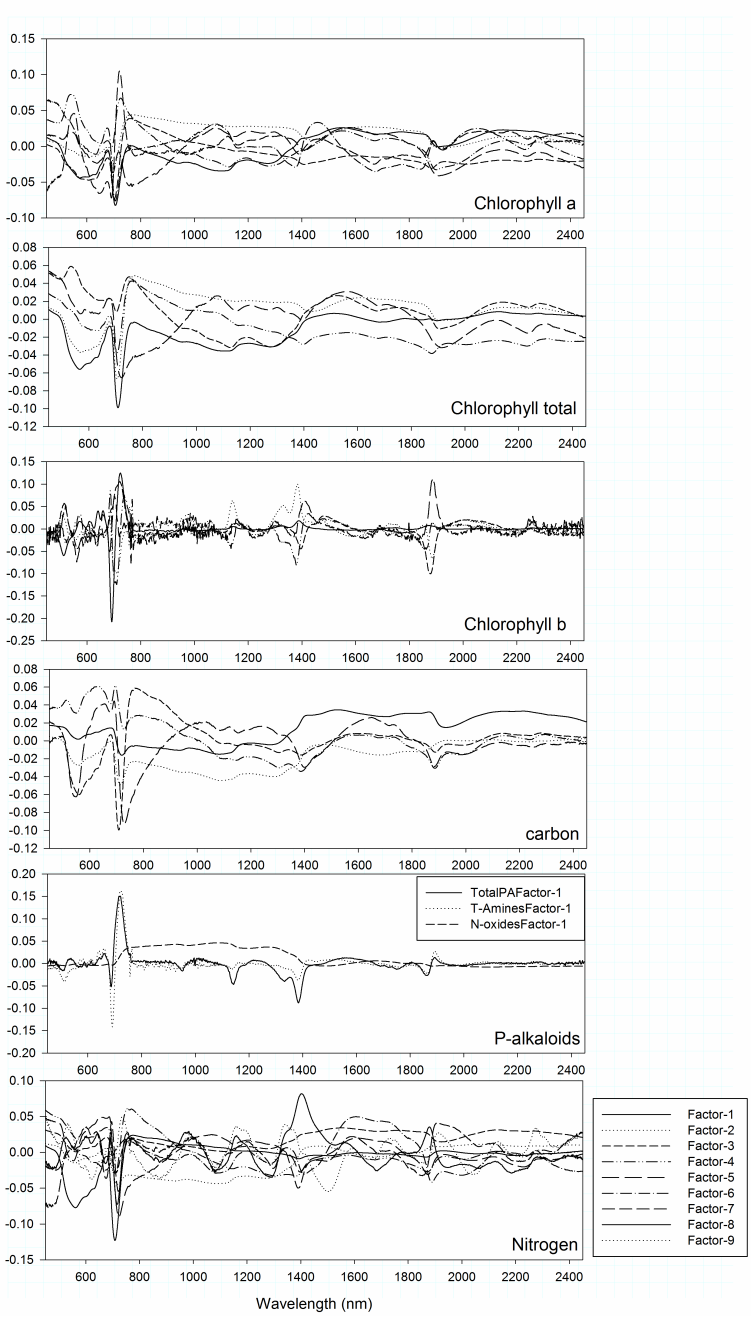

Figure S5: Partial least square regression loadings showing the contribution of each wavelength into the model for the estimation of the chemical content of leaves. The more positive or negative the more it contributes towards the development of the estimation model.

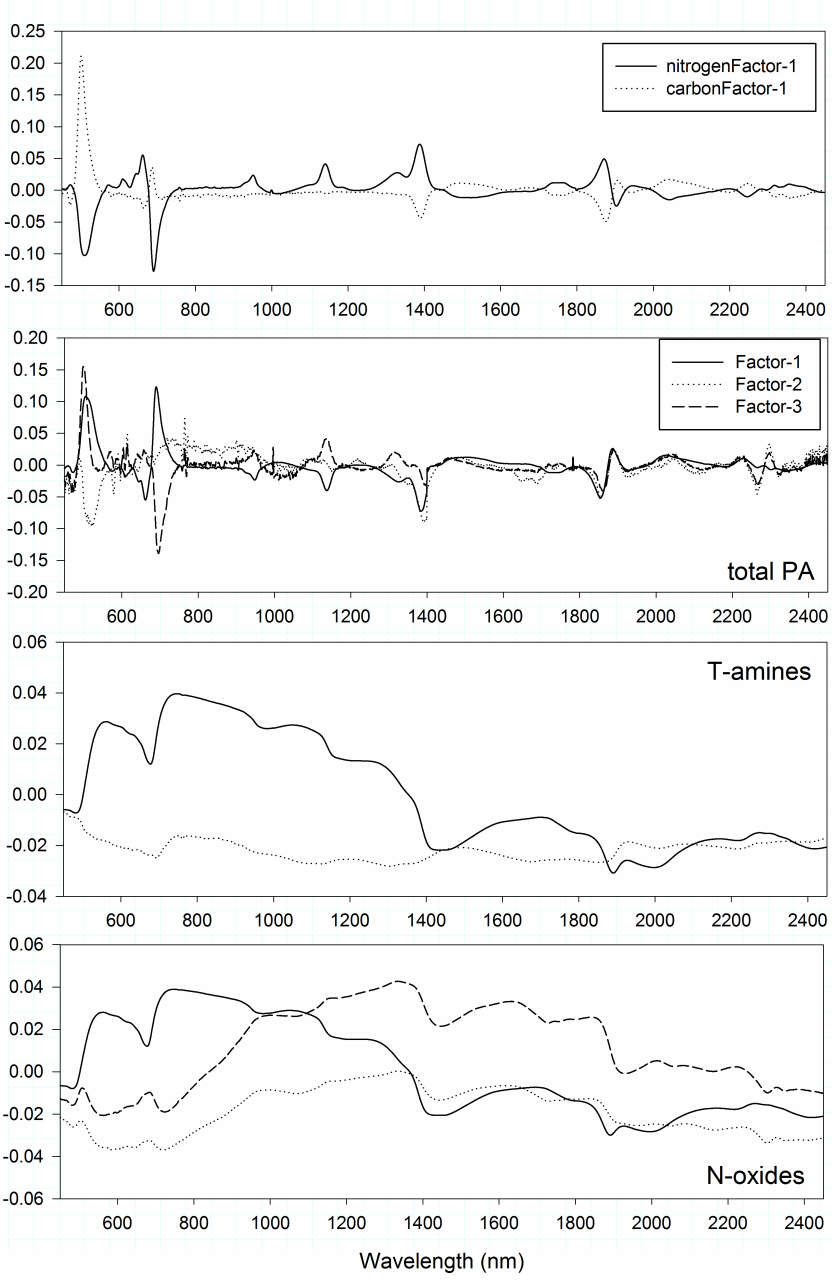

Figure S6: Partial least square regression loadings showing the contribution of each wavelength into the model for the estimation of the chemical content of flowers. The more positive or negative the more it contributes towards the development of the estimation model.

Discussion and Synthesis

6

In this thesis I investigated the potential of visible and near-infrared spectral measurements to detect plant chemical changes as a result of plant-soil interactions with either biotic or abiotic factors. Soil organisms are known to influence plants, and a range of symbiotic to pathogenic interactions can be important drivers of spatio-temporal changes in vegetation (Clements, 1963; Price, 1984; Blomqvist *et al.*, 2000). We often do not know the influence of soil organisms on the success of plant species to occupy a new niche or decline from those niches. With an increasing number of exotic plants species invading new habitats the question is if and how these plants may become controlled over time (Van der Putten, 2012). Based on one recent study, the impact of soil biota controlling plant performance is expected to increase over time

Ideally spectral reflectance patterns would allow larger surveys and faster assessment of plant-soil interactions and evaluate shifts in biological control. However, the control of introduced exotic plant species by soil biota might take one or two orders of time more than of native plant species. Therefore, in this study I used Senecionaea species as a model system, of which one species, *Jacobaea vulgaris* Gaertn. (Syn. *Senecio jacobaea* L.), is a native breakout species that has a rapid boom-bust pattern of 5-10 years in early secondary succession systems in the Netherlands (van de Voorde *et al.*, 2012). For that species it is known that soil microbial communities often caused a decrease in biomass as a consequence of negative plant-soil feedback (Kostenko *et al.*, 2012b; van de Voorde *et al.*, 2012).

Studies in a chronosequence of ex-arable fields have also shown that the population density and ground cover of *J. vulgaris* first increases and then decreases as part of the process of vegetation succession (van de Voorde *et al.*, 2011). Since this pattern was attributed, to some extent, to the soil microbial communities (van de Voorde *et al.*, 2012) this chronosequence seemed appropriate to study the spectral patterns during rise and fall of plant abundance as a result of plant-soil interaction under natural conditions. With this model system I expected to be able to test if plant spectral reflectance patterns can reveal soil feedback effects, not only in

greenhouse experiments (chapters 2 and 3), but also in field conditions during a growing season and during time since abandonment (chapters 4 and 5).

Variation and estimation of Senecionaea defence compounds by spectral reflectance

Defence compounds play an important role during plant-biota interactions. Often, the level of defences in plants increases during herbivore or pathogen attacks, both aboveground and belowground (Wurst & van der Putten, 2007; van Dam, 2009; Iason *et al.*, 2012). Since little was known about the contribution of pyrrolizidine alkaloids (PAs) to the spectral pattern in Chapter 2 I tested the hypothesis that PAs influenced specific spectral features of the study species. The aim was to determine if estimation of PA foliar content by spectral features was possible and whether differences in PA foliar content and, subsequently, in spectral reflectance would be indicative of negative feedback effects from the soil community to the model plants. My study showed that tertiary-amines, a specific type of PAs, may have a moderate effect on foliar spectral features. Interestingly, this effect was strongest for *J. vulgaris*. This species-specific effect was most likely related to a particular chemical structure found in *J. vulgaris* PAs, the epoxide ring. However, the positive correlation of the PAs of *J. vulgaris* with nitrogen content could not be disregarded as a likely indirect influence on the spectral regions selected for PA estimation. Both PAs and Nitrogen interact with several spectral regions, possibly contributing to the additive effect suggested by Curran and co-workers (1989; 1992).

The differences in soil biotic communities had higher impact on the variation of the foliar chemistry of the exotic *S. inaequidens* and native *Jacobaea erucifolia* than of *J. vulgaris* (Chapter 3). Since *J. vulgaris* already occurred in these soils I associated this trend to its optimal defence strategy and *S. inaequidens* and *J. erucifolia* reaction to its growth in unfamiliar soil communities. Such an increase of PAs in the exotic species does not support the enemy release hypothesis (ERH), which proclaims a decrease of defence compounds when a plant is released from its enemies. However, PAs are mostly associated with defences against generalist herbivores and pathogens (Van der Meijden, 1996; Macel, 2011) and the release of specialist herbivores should not affect the PAs content. A similar trend was seen in range-expanding species exposed to novel herbivores. The range expanders produced more phenolics than natives (Engelkes *et al.*, 2008). Our results supported the 'shift in defence' hypothesis as *S.*

inaequidens and *J. erucifolia* increased the production of "low-cost" but still toxic compounds while growing in unfamiliar soils (Joshi & Vrieling, 2005; Doorduin *et al.*, 2010).

Variation in chemical content was also found in *J. vulgaris* in fields from the chronosequence (Chapter 4 & 5). Despite the observed chemical variation the explained variance of PA content by spectral reflectance features was low in both greenhouse and field studies. This result supports the assumption in chapter 2 that a correlation between PAs and nitrogen needed to occur in order to achieve accurate PA estimation from spectral reflectance patterns. Nevertheless, I was able to show that chemical content estimations can be achieved in more plant organs than just leaves (chapter 4). Chemical content estimation by spectral features using *Jacobaea vulgaris* flowers achieved comparable accuracy to other studies performed in plant leaf and canopy (Curran *et al.*, 2001; Cartelat *et al.*, 2005; Ramoelo *et al.*, 2011b).

Throughout this thesis the estimation of plant chemistry by spectral features proved to be inconsistent and organ-dependent (chapters 2, 3 and 5). The fact that some estimations are successful while others are not suggests that we still have insufficient information on the processes that link the chemicals to the spectral features of a plant or plant organ. In which situations is a spectral feature affected by the chemical bonds and if this link is dependent on environmental conditions still needs further investigation. This is imperative to improve the remote sensing models derived to estimate chemical content of plants.

Species discrimination

The first experiment (chapter 2) also allowed a simple trial to evaluate if the introduced exotic *S. inaequidens* reflects differently from natives *J. vulgaris* and *J. erucifolia*, or if species with invasive character (*J. vulgaris* and *S. inaequidens*) had similar reflectance patterns. It was expected that similarity in leaf area and canopy structure in the two natives would play a major role in the differentiation between the natives and the exotic *S. inaequidens*. At canopy level this was indeed the case, however, the removal of the canopy factors (i.e. fresh and dry leaf measurements) suggested more resemblance between the spectral patterns of the two species with invasive character. The causality for such spectral trend is yet to be tested. In chapter 3, however, I show that this similarity between *J. vulgaris* and *S. inaequidens* will be less strong if *J. vulgaris* grows in soil with familiar (native) soil biota.

The potential of spectral reflectance to discern between invasive and non-invasive species is not new. Underwood *et al.* (2003) were able to discern *Carpobrotus edulis* with high accuracy using hyperspectral images. Asner and co-workers (2008) were able to discriminate between both native and non-native tree species of Hawaii, as well as their nitrogen-fixing and non-fixing life strategies. However, the differences between canopy and leaf level spectral patterns found in the present study suggest that we should consider more often the leaf versus canopy spectral level.

Soil biota effects on plant reflectance patterns

The exposure of plants to soil biota can result in plant responses aboveground by both shoot primary and secondary metabolites (Masters *et al.*, 1993; van Dam, 2009; Kostenko *et al.*, 2012b). Consequently the intermittently distributed soil biota will enhance plant spatial heterogeneity (Masters & Brown, 1997; Van der Putten *et al.*, 2001). In chapter 3 I tested the impact of different soil biotic composition on shoot chemical content and spectral reflectance. I expected that soil biota would increase species shoot defence content (Bezemer & van Dam, 2005; Joosten *et al.*, 2009) and that hyperspectral reflectance would enable detection of the effects of soil biota on plant properties. With spectral reflectance data it was possible to accurately predict species identity and discriminate, to some degree, plants growing in the different soil biotic conditions (especially with vs without soil-borne pathogen activities). The capacity of spectral reflectance to discriminate plant-soil interactions was equivalent to the traditional discrimination analyses using plant chemical profiles. This ability of spectra to discern between species-specific plant-soil interactions opens new opportunities to study plant interactions with cryptic belowground communities. A major challenge will be to discriminate between reflectance patterns from biotic versus abiotic soil influences, and to tease apart effects of various different soil biota.

Temporal dynamics in plant chemistry and spectral reflectance

I was also interested in the temporal dynamics of plants and how primary and secondary compounds, as well as spectral patterns may be affected by season and successional stages of the vegetation. In the case of *J. vulgaris* the shift in plant

population structure during succession was attributed, to some extent, to the build-up of biological control factors among the soil biota (Macel & Klinkhamer, 2010; van de Voorde *et al.*, 2011; Kostenko *et al.*, 2012b). I tested how plant-soil interactions affect chemistry and spectral patterns in relation to season and time since land abandonment.

The implications of different plant organs in seasonal and succession studies

In Chapter 4 I focused on the chemical variation of both leaves and flowers of *J. vulgaris* throughout the chronosequence. This allowed me to investigate how dependent the temporal pattern was of the organ we studied. The general hypothesis was that seasonal allocation of nutrients and defence metabolites to reproductive organs fitted the optimal defence theory, but that such patterns were dependent on the successional stage of the vegetation.

Both chemical concentration and composition varied throughout the season and PAs and chlorophyll content were also affected by successional stage. The effect of succession stage on chemical contents of *J. vulgaris* was especially evident in flowers with higher PA tertiary-amine concentrations in fields that had been abandoned 10 to 20 years ago. Plants from longer abandoned fields generally had flowers and leaves with higher N-oxides, especially in the late Summer season. The results also suggested the allocation of defensive and nutritional compounds to flowers during early Summer. Although the interaction between the factors season and succession resulted in a complex chemical variation pattern, the allocation of defence compounds from leaves to flowers depended, to some extent, on successional position. This trend supported the hypothesis of optimal defence and our expectations, flowers and plants growing in larger population cover were better protected.

High levels of jacobine (a specific PA) were found in *J. vulgaris* growing in the chronosequence. This compound is associated with defence against herbivores and a lower fungal diversity in the rhizosphere (Kowalchuk *et al.*, 2006; Leiss *et al.*, 2009). Yet jacobine is also known to be attractive to the specialist herbivore *T. jacobaea* (Macel & Klinkhamer, 2010). In both leaves and flowers acetylerucifoline was also an important PA. Its higher concentration in Spring supported greenhouse findings associating it with soil legacy effect from previous plant herbivory (Kostenko *et al.*, 2012b). Still, to support the proposed 'legacy effect' (Kardol *et al.*, 2007; Kostenko *et*

al., 2012b) one would need to test if herbivory in *J. vulgaris* is higher in early years than in later succession years.

The spectral reflectance ability to discern seasonal and succession patterns within plant-specific organs

After examining the chemical shifts of *J. vulgaris* in the secondary successional gradient, in Chapter 5 I analysed if the spectral variation found in the plants could be related to the ecological changes already reported in Chapter 4. I expected that seasonality and vegetation succession stage would be expressed in the spectral reflectance of both leaf and flower organs. Surprisingly, leaf reflectance was little affected by seasonality, which could be due to spectral measurements done in living leaves only. Yet, if we consider the significant leaf variation in chemical content the lack of seasonal differentiation by the spectra of leaves was not expected. On the other hand the successional stages defined by Kardol *et al.*(2006) were successfully discriminated by the spectral patterns of flowers. This result was consistent with chapter 4 findings suggesting that flower composition is more sensitive to field ageing than leaves. The hyperspectral reflectance of leaves showed significant differences between old and young successional fields in the visible region, suggesting that differences in the photosynthetic process may occur even at large temporal scales explored in my study. Once more this finding was supported by the chemical analysis presented in chapter 4.

The cross-validation models showed potential for detecting seasonal and successional differences in plants using hyperspectral reflectance patterns. However, the results were organ dependent and the continuum range of variation suggested spectral overlap to be a reason for some of the lowest outcomes.

The link between spectral reflectance and plant-soil interactions

It is accepted that soil organisms influence plants, with a range of symbiotic to pathogenic interactions that have considerable impact in the spatio-temporal dynamics of plant populations (Van der Putten *et al.*, 2001). Many studies use remotely sensed chemical products to explore its capacity to manage agroecosystems and study large herbivores-plant interactions (Graetz, 1990; Blackburn & Steele,

1999; Ebbers *et al.*, 2002; Mutanga *et al.*, 2004; Cho *et al.*, 2006; Gray *et al.*, 2010; Ramoelo *et al.*, 2011b). It has also been established that soil nutrients affect plant quality and its reflectance (Ebbers *et al.*, 2002; Pretorius *et al.*, 2011; Ramoelo *et al.*, 2011a; Knox *et al.*, 2012). However, at the start of my thesis study little was known about the impact of soil biota on spectral reflectance patterns of plants. Deriving remote sensing products that could be used to study plant-soil interactions could benefit many fields of research. Some examples are biological control of invasive species, soil-borne diseases, nutrient cycling and litter decomposition, all processes affected by soil biota (Grime *et al.*, 1996; Van der Putten & Peters, 1997; Van der Putten *et al.*, 2001; De Deyn *et al.*, 2003; Bezemer *et al.*, 2006; Hol *et al.*, 2010; Inderjit & van der Putten, 2010).

The discrimination of plants due to soil biotic factors either by chemical profiles (chapters 3 and 4) or spectral reflectance pattern (chapters 3 and 5) were comparable. These studies demonstrated that plant reflectance patterns could provide information about the soil biota interactions with the plant. The fact that chemical estimations were inconsistent did not reflect on the capacity of spectral reflectance to differentiate plant-biotic interactions. Despite the less successful PA estimation in chapters 3 and 5, discrimination of soil communities and its temporal factors by the same spectral features was, to some extent, successful.

The empirical approach for spectral analysis (Skidmore, 2001) shows several constraints to study plant PA chemicals by remote sensing. First, it assumes that the spectral reflectance of leaves represents the sum of each chemical weighted by its concentration (Kumar *et al.*, 2001). Second, it needs the multivariate analyses to have a near-linear relation between spectra and chemical concentration without any confounding factor (Curran, 1989). Several confounding factors may affect accurate chemical estimation such as the sensor-specific measurements and its dependence on sampling conditions. In principle, models that take physical processes into account are not sensitive to such factors because they are established on physical laws. However, the physical approach needs model inversion of the parameters often causing various combinations of canopy parameters to result in similar spectra outcomes (Baret & Guyot, 1991; Darvishzadeh *et al.*, 2011). Although physical models such as SAIL and PROSPECT are easier to transfer often they provide non-specific results, this demands extra information that empirical approaches do not necessarily need to study spectral patterns variation, ideal for plant precision analysis (Colombo *et al.*, 2003). Both approaches have advantages and disadvantages, but due

to the specificity of plant-soil interactions the versatility of the empirical approach was regarded as ideal for the questions in this thesis.

At the start of my thesis study, little was known about the impacts of soil biota on spectral reflectance patterns of plants. I demonstrate that plant reflectance patterns can discriminate plant-soil biota interactions, even when different plant organs (leaf or flower) are considered. Despite the inconsistent estimation of pyrrolizidine alkaloids by spectral reflectance features, the successful discrimination of plant- (a)biotic interactions illustrate that spectral patterns might contain more information than the sum of its chemicals. This spectral independence could provide exciting avenues for remote sensing to study plant-soil interactions in broader scales and benefit several fields of research with its additional knowledge.

Conclusions

- The estimation of pyrrolizidine alkaloids by spectral reflectance features had higher accuracy in *Jacobaea vulgaris*, possibly as a result of the presence of the epoxide chemical structure and its correlation with nitrogen in abiotic conditions (chapter 2).
- Differences in soil communities affect plant leaves chemistry and spectral reflectance patterns (chapter 3).
- *Jacobaea vulgaris* plants from recent and longer-abandoned fields showed the largest differences in chemical concentration, composition of defence compounds, and spectral reflectance patterns. Flowers were more discriminatory than leaves (chapters 4 and 5).
- There is a potential to detect plant-biotic interactions through analyzing plant spectral reflectance patterns (this thesis)

Future research avenues

During the research period of this thesis several other aspects were raised as interesting avenues to further develop the perspective of plant-soil interactions studies via spectral reflectance patterns. Here I summarise some possibilities.

The implication of plant physical properties

It might be necessary to study how to better integrate several methods to improve the analyses of the influence of plant-soil interactions on the spectral patterns. Radiative transfer models, such as SAIL and PROSPECT (Verhoef, 1984; Jacquemoud & Baret, 1990; Jacquemoud *et al.*, 2009), take physical processes into account when studying the influence of chemicals on spectra. Models that integrate foliage structure and inclination could improve our understanding of the reflectance patterns variation as a function of, not only chemical content, but also non-chemical processes such as plant-biotic interactions. Such studies may help improve in the transfer of fitted models between sites and/or species without extensive ground calibration.

Manipulation of soil communities (native versus exotic plants)

Pathogenic soil biota that impact on plants have the potential to affect plant spectral reflectance. I propose that further studies should manipulate soil infections more specifically in order to study if specific plant-soil interactions may be identified by analysing hyperspectral reflectance patterns. In the specific case of exotic invasive species reflectance analyses could further help detect hot-spots of biotic resistance against exotic invaders. Since spectral measurements are non-destructive, studies over time should also be considered in order to assess the feasibility for early detection. Plants also have a complex network of interactions including both belowground and aboveground. It is important to test the spectral differences between below-, above-ground and combined interactions.

The implication of existing spectral databases for plant-soil interactions

There is an increasing number of databases with high-fidelity spectral measurements. In this thesis the significant classification of "unknown" spectral samples into the correct species group suggest an extended potential for spectral libraries to become the *GenBank* (digital library for nucleotide and protein sequences association) of remote sensing measurements. Expansions into temporal scales and plant-soil interactions should be further analysed. The implications of spectral measurements standardization for such spectral databases should be considered in sensitive boundaries as soil biotic effects on reflectance patterns of plants.

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A

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Summary

Soil organisms can influence plants by symbiotic or pathogenic interactions, and by decomposition of organic matter. Belowground interactions play important roles in driving spatio-temporal changes in vegetation. However, the influence of soil organisms on plant population dynamics is often difficult to assess due to the cryptic nature of the interactions. It is increasingly acknowledged that release from belowground interactions enhances plant invasiveness and that over time introduced exotic plant species become increasingly controlled by the soil biota from the introduced range. A challenge for invasion ecologists is to understand and predict when and where plant species may become controlled by soil biota. Soil biota are known to change plant primary and secondary chemistry in aboveground tissues. Changes in plant chemistry may be detected through analysing hyperspectral reflectance patterns of aboveground plant tissues. In this thesis I investigated the potential of visible and near-infrared spectral measurements to detect plant chemical changes that result from plant-soil interactions with either biotic or abiotic factors and that may relate to boom-bust patterns in plant population dynamics.

In this thesis, Senecionaea species were used as a model system, especially *Jacobaea vulgaris* Gaertn. (Syn. *Senecio jacobaea* L.), which is a native breakout species in early secondary successional ecosystems in the Netherlands. Studies in a chronosequence of ex-arable fields have shown that *J. vulgaris* density and cover increases during early years of land abandonment, and then decreases during further vegetation development. According to previous work, this boom-bust pattern was attributed, at least to some extent, to soil biota. Therefore, I used this chronosequence to study if the spectral patterns were sensitive to the rise and fall of plant abundance as affected by plant-soil interaction. If successful, the current study may become a good prelude to the study of increased biotic control of invasive exotic plant species by the soil community when time since introduction passes on.

Spectroscopy provides the spectral feature of an object at each wavelength of the electromagnetic field. Depending on the condition or chemical composition of the object different reflectance frequencies may be generated that can be measured by spectrometers. Reflectance measurements are common as they are sensitive to

specific chemical bonds and shift with variation in the chemical composition of the object measured. PAs are the defence compounds of Senecionaea species toward generalist herbivores and play an important role during biotic interactions between plants and (soil-borne) enemies. As such, it becomes important to understand the contribution of defence compounds to the spectral features of the plants during their interactions with soil biota and influences of soil abiotic conditions.

First, I examined the relative contribution of pyrrolizidine alkaloids (PAs) to the spectral reflectance features in the visible and short-wave infrared region. In my first experiment I showed that tertiary-amines, a specific type of PAs, may affect foliar spectral features. However, the study of three different species - *Senecio inaequidens*, *Jacobaea vulgaris* and *J. erucifolia* – showed that the results were species-specific. I postulate that this species-specific effect is most likely related to the epoxide ring present in the PA chemical structures of *J. vulgaris* and its positive correlation with nitrogen content. In subsequent experiments (chapters 3 and 5) the explained variation of PA content by spectral reflectance was low. Since in chapters 3 and 5 the correlation between PAs and nitrogen was very low, this supports my suggestion in chapter 2 that there should be a correlation between PAs and nitrogen in order to achieve a successful PA estimation when using spectral reflectance patterns. Additionally, I was able to show that chemical content estimations using spectral reflectance can be achieved in more plant organs than just leaves (chapter 5); *J. vulgaris* flower head PAs were also detectable through reflectance.

In chapter 3 I tested the impact of different soil microbial communities on chemical content and spectral reflectance of shoots. I grew the 3 species in sterilized soil and living soil collected from fields with *J. vulgaris*. I analysed the soils' influences on shoot chemistry and hyperspectral reflectance patterns. I expected that soil biota would increase shoot defence content and that hyperspectral reflectance would enable discrimination between different used soils. The living soils had higher impact on the variation of the foliar chemistry of the exotic *S. inaequidens* and native *Jacobaea erucifolia* than on the native *J. vulgaris*. Since *J. vulgaris* already occurred in these soils I related this trend to a species-specific response to the soil community that has been developed in fields with *Jacobaea vulgaris*. The capacity of spectral reflectance to discriminate plant-soil interactions was equivalent to the traditional discrimination analyses using plant chemical profiles. This ability of spectra to discern plant-soil biotic interactions supports the potential of spectral reflectance patterns to study plant interactions with cryptic soil communities.

In chapter 4 and 5 I studied the temporal dynamics of primary and secondary plant compounds, as well as the plant spectral patterns during seasonal and successional stages of vegetation development. The effect of succession stage on chemical contents of *J. vulgaris* was especially evident in flowers, which had the highest PA tertiary-amine concentrations in fields that had been abandoned 10 to 20 years ago. Plants from longer abandoned fields generally had flowers and leaves with higher N-oxides, especially in the late Summer. The results also showed that chemical variation during the growing season is in line with optimal defence strategy: flowers have higher defence content than leaves. Surprisingly the lack of seasonal differentiation in the spectral patterns of leaves was not expected. On the other hand the various stages of succession were successfully discriminated by the spectral patterns of flowers. This result was consistent with chapter 4 findings suggesting that flower composition is more sensitive to field ageing processes than leaves. The spectral patterns showed potential for detecting seasonal and successional differences in plants but, most importantly, I was able to show that more organs than the frequently considered ones (leaves versus leaves and flowers) can provide temporal information about plant-interactions.

At the start of my thesis study, little was known about the impact of soil biota on spectral reflectance patterns of plants. This thesis demonstrates that plant reflectance patterns may provide information about the soil biotic interactions with the plant, even if we consider different plant organs. I conclude that despite the variation in strength to estimate pyrrolizidine alkaloids by spectral reflectance features, soil biotic communities can affect shoot spectral reflectance. The consistent discrimination of plant-soil biota interactions by spectra shows that it might not be necessary to fully understand specific chemical changes in order to study plant-biota interactions using spectral reflectance. Successful differentiation of plant-interactions with soil biota and abiotic soil conditions illustrate that spectral patterns might contain more information than the sum of the plant chemicals. This spectral independence could provide interesting avenues for remote sensing to study plant-soil interactions at broader scales. If operational, the spin-off might benefit many fields (e.g biological control, monitoring soil quality for agriculture) of research with its additional knowledge.

Samenvatting

Bodemorganismen beïnvloeden planten op verschillende manieren: zowel door symbiotische en ziekteverwekkende interacties, als door vrijmaken van nutriënten door vertering van dood organisch materiaal. Deze ondergrondse interacties spelen tevens een belangrijke sturende rol in het veranderen van de vegetatiesamenstelling, zowel in tijd (successie) als in ruimte. Een belangrijke oorzaak van veranderingen in vegetatiesamenstelling is dat de populatiedynamica van planten niet constant is in ruimte en tijd. Echter, het blijkt vaak lastig om de exacte invloed van bodemorganismen op de populatiedynamica van planten in te schatten, omdat deze interacties zich meestal aan het oog onttrekken. Voor invasieve exoten geldt dat deze zich vaak losgemaakt hebben van de negatieve interacties met ondergrondse pathogenen uit hun oorspronkelijke verspreidingsgebied, maar gedurende de tijd in hun nieuwe verspreidingsgebied toch in toenemende mate last krijgen van een toename van ondergrondse vijanden.

Het is momenteel een uitdaging voor ecologen om beter te begrijpen en voorspellen onder welke condities invasieve exotische plantensoorten te maken krijgen met een opbouw van ondergrondse vijanden. Een van de manieren waarop dat gedaan zou kunnen worden is met behulp van hyperspectrale reflectiepatronen van planten. Omdat bodemorganismen direct invloed kunnen hebben op de primaire en secundaire plantenstoffen in bovengrondse weefsels van planten en omdat hyperspectrale metingen gebruikt zouden kunnen worden om deze stoffen te traceren, zou remote sensing een kunnen worden toegepast om veranderingen in bodemgemeenschappen in kaart te brengen door middel van de reactie daarop van de plant. In dit proefschrift heb ik de mogelijkheden van hyperspectrale reflectiepatronen uitvoerig getest met behulp van metingen aan reflectiespectra in het zichtbare licht en het nabije infrarood, die ik vervolgens vergeleken heb met verschillen in de chemische samenstelling van planten. Daarbij heb ik me voornamelijk gericht op interacties van de plant met zowel biotische als abiotische bodemfactoren, aangezien deze factoren bepalend kunnen zijn voor de plotselinge toe- en afname (zgn. boom-bust dynamiek) van plantenpopulaties gedurende successie. Deze ontwikkelingen staan volgens mijn visie model voor wat zich op langere termijn afspeelt met invasieve exotische planten. Ook zij kunnen een toe- en afname in populatiedichtheid doorlopen, maar aangezien dat proces bij exoten

ongeveer tien keer zo lang duurt, is het minder geschikt voor experimenteel onderzoek.

In dit proefschrift werden verschillende soorten Senecionaea gebruikt om de relatie tussen bodemgesteldheid, primaire en secundaire chemie en hyperspectraallijnen te onderzoeken: de inheemse soorten *Jacobaea vulgaris* Gaertn. (syn. *Senecio jacobaea*) en *J. erucifolia* Gaertn., Mey and Scherb. (syn. *Senecio erucifolius*) en de invasieve exoot *Senecio inaequidens* DC. De nadruk ligt op *J. vulgaris*, een inheemse, maar gedurende de afgelopen decennia rap toegenomen plantensoort in Nederland, die vooral voorkomt in jonge secundaire successiestadia. Eerdere studies aan een reeks uit gebruik genomen landbouwgronden van verschillende leeftijd (een zgn. chronosequentie) lieten zien dat de dichtheid en bedekking van *J. vulgaris* sterk toeneemt nadat de soort zich explosief had ontwikkeld tijdens vroege successie. Afgaande op eerder werk aan de ecologie van deze soort kan dit 'boom-bust'-patroon ten dele worden toegeschreven aan de opbouw van negatieve interacties tussen planten en bodemorganismen. Deze chronosequentie biedt daarom goede mogelijkheden voor het bestuderen van de relatie tussen veranderingen in gemeenschappen van bodemorganismen en veranderingen in spectrale reflectiepatronen van planten. In het geval de beoogde methode toepasbaar is, zouden de resultaten tevens gebruikt kunnen worden als een voorzet voor het bestuderen van toenemende ondergrondse controle van invasieve exotische soorten.

Spectroscopie resulteert voor ieder voorwerp in een reflectiepatroon, en geeft voor iedere golflengte een andere waarde. Afhankelijk van de chemische samenstelling van een object kunnen met behulp van een spectrometer verschillen in spectrale reflectie gemeten worden, omdat verschillende chemische bestanddelen in een andere golflengte reflecteren. Senecionaea bevatten chemicaliën, zogenaamde pyrrolizidine alkaloïden (PA's), die als verdediging tegen generalistische herbivoren worden aangemaakt. Deze stoffen spelen een belangrijke rol bij interacties tussen de plant en boven- en ondergrondse vijanden. Het meten van deze stoffen met behulp van een spectrometer kan daarom iets zeggen over de mate waarin biotische of abiotische (bodem)factoren deze plant beïnvloeden.

Om de relatie tussen de plant en haar biotische en abiotische factoren te bestuderen, heb ik in hoofdstuk 2 eerst de bijdrage van PA's aan het reflectiespectrum van *J. vulgaris* bepaald, zowel in het zichtbare als nabije infrarode deel van het lichtspectrum. In het eerste experiment laat ik zien dat tertiaire amines, een specifiek soort PA's, verschillende delen van het bladspectrum kunnen

beïnvloeden. Echter, uit een vergelijkende studie tussen verschillende nauw verwante soorten (*J. vulgaris, J. erucifolia, S. inaequidens*) blijkt dat deze effecten soortsafhankelijk zijn. Ik postuleer dat deze soortsafhankelijke effecten worden veroorzaakt door de epoxide-ring in de PA's van *J. vulgaris* en de positieve correlatie tussen de hoeveelheden PA's en stikstof in de plant.

In opvolgende experimenten (hoofstukken 3 en 5) vind ik de variatie in PA-gehaltes nauwelijks terug in verschillen in spectrale reflecties. Ook blijkt de correlatie tussen de hoeveelheid PA's en stikstof in de planten in deze experimenten veel minder sterk. Dit ondersteunt de bovenstaande suggestie. Variatie in PA's kan blijkbaar alleen goed gemeten worden als er tegelijkertijd ook duidelijke verschillen in stikstofgehaltes tussen de planten zijn. Verder laat ik in deze hoofdstukken zien dat met behulp van spectrale technieken verschillen in PA's ook traceerbaar zijn in de bloemhoofdjes van *J. vulgaris*.

In hoofdstuk 3 heb ik onderzocht hoe verschillende microbiële bodemgemeenschappen de chemische samenstelling en de spectrale reflectie van planten beïnvloeden. De drie eerdergenoemde soorten werden gezaaid in gesteriliseerde en niet-gesteriliseerde bodems uit verschillende successiestadia. De nadruk van dit experiment lag andermaal op de invloed van de bodem op de chemische samenstelling en de spectrale reflectiepatronen van de verschillende planten. Hierbij verwachtte ik dat aanwezigheid van bodembiota de verdedigingsmechanismen van de planten zou activeren, wat zou resulteren in een hogere hoeveelheid PA's. Inderdaad bleken de levende bodems grotere invloed op de hoeveelheid gemeten verdedigingsstoffen te hebben dan gesteriliseerde bodems, maar voornamelijk in *S. inaequidens* en *J. erucifolia* en veel minder in *J. vulgaris*. Omdat *J. vulgaris* reeds voorkwam op de door mij gebruikte bodems kan dit resultaat tevens worden toegeschreven aan een soortsafhankelijke respons op de bodemgemeenschap. Deze analyses lieten tevens zien dat de spectrale reflectiepatronen en de chemische analyses zeer vergelijkbare resultaten geven. Dit onderstreept de potentie van deze remote sensingmethode voor het traceren van interacties tussen planten en complexe bodemgemeenschappen

In de hoofdstukken 4 en 5 richt ik me op de temporele dynamiek van primaire en secundaire plantenstoffen. Hiervoor heb ik de spectrale reflecties van *J. vulgaris* in verschillende successiestadia en verschillende seizoenen vergeleken. Het effect van verschillende successiestadia op de chemische samenstelling was vooral waarneembaar in de bloemhoofdjes. Daar waren de PA concentraties het hoogst in

successiestadia op landbouwgronden die 10 tot 20 jaar geleden uit productie werden genomen. In planten afkomstig uit velden die latere successiestadia representeerden, werden hogere concentraties stikstofoxiden gemeten, zowel in bloemen als bladeren. Dit effect was het sterkst aan het eind van de zomer, hetgeen suggereert dat de variatie in verdedigingsstoffen gedurende het groeiseizoen overeenkomt met de optimale verdedigingsstrategie: bloemen hadden hogere concentraties verdedigingsstoffen dan bladeren. Het ontbreken van seizoensveranderingen in concentraties chemicaliën in de bladeren was een onverwacht resultaat. Met behulp van spectrale technieken konden we dus wel meten dat concentraties verdedigingsstoffen in de bloemen duidelijk veranderden, maar niet in de bladeren. Dit resultaat is in overeenstemming met de conclusies van hoofdstuk 4, waarin ik laat zien dat gedurende secundaire successie, de chemische samenstelling van bloemen meer verandert dan de chemische samenstelling van bladeren. De gevonden patronen tonen het potentieel van de door mij gebruikte methode voor het opsporen van temporele variatie in de chemie van veldpopulaties van kruiskruidachtigen en mogelijk ook van andere wilde plantensoorten. Echter, en wellicht belangrijker, dit resultaat laat zien dat andere plantenorganen dan alleen bladeren informatie kunnen verschaffen over de interacties tussen planten en de bodem.

Aan het begin van mijn promotie-onderzoek was weinig bekend over de rol van bodembiota op de spectrale reflectie van planten. Dit proefschrift laat zien dat reflectiepatronen van planten veel informatie over de interacties met de bodem en het bodemleven kunnen verschaffen, zeker als verschillende plantenorganen worden gebruikt. Daarom concludeer ik dat, ondanks de variatie in het succes waarmee PA's door spectrale reflectie gemeten kunnen worden, bodemorganismen spectrale reflectie van planten kunnen beïnvloeden.

Mijn onderzoek laat zien dat het misschien niet uitdrukkelijk nodig is om de specifieke chemische veranderingen in planten te begrijpen om plant-bodem-interacties te bestuderen. Met behulp van spectrale technieken kan ik namelijk consistent hetzelfde onderscheid maken. Mijn onderzoek opent daarom nieuwe invalshoeken voor vervolgonderzoek aan plant-bodem-interacties. Als blijkt dat de door mij gevonden resultaten geëxtrapoleerd kunnen worden naar andere plantensoorten kunnen spin-off's van dit project ook in andere systemen gebruikt worden. Bijvoorbeeld in de biologische bestrijding van ziekten en plagen, of voor onderzoek aan bodemkwaliteit in de landbouw. Zo kunnen de resultaten van dit onderzoek in de toekomst op vele plekken van pas komen.

Sumário

Os organismos do solo podem influenciar as plantas por meio de interacções simbióticas ou patogénicas e através da decomposição da matéria orgânica. Estas interacções a nível do solo desempenham um papel importante a induzir alterações quer espaciais, quer temporais (sucessão) na vegetação. No entanto, a influência dos organismos do solo sobre a dinâmica de populações das plantas é muitas vezes difícil de avaliar devido à natureza enigmática destas interacções. É cada vez mais reconhecido que a redução das interacções entre plantas e microorganismos pode aumentar a capacidade de invasão de espécies vegetais. Por outro lado, também se sabe que, ao longo do tempo, espécies exóticas introduzidas podem ser controladas pelos organismos do solo onde foram introduzidas. Um desafio recorrente para os ecologistas no estudo de invasões é conseguir compreender e prever quando e onde estas espécies de plantas podem ser controladas por estes organismos do solo. Os organismos presentes no solo têm capacidade de influenciar a produção de químicos primários e secundários nos tecidos superiores das plantas, uma vez que estes organismos podem providenciar ou limitar o acesso das plantas a nutrientes. Estas mudanças químicas nas plantas podem ser detectadas através da análise de padrões de reflectância hiperespectral de órgãos superiores das plantas (ex. folhas e flores). Nesta tese, eu investiguei o potencial da utilização de padrões hiperespectrais, na zona do visível e do infravermelho, para detectar alterações químicas nas plantas que tenham resultado de interacções plantas-solo; interacções estas relacionadas com factores bióticos ou abióticos, e que possam ter influência nos padrões de abundância e dispersão de plantas durante os ciclos de sucessão.

Para o trabalho desenvolvido nesta tese foram seleccionadas várias espécies da tribo Senecionaea como modelo de estudo, com especial ênfase na *Jacobaea vulgaris* Gaertn. (Syn. *Senecio jacobaea* L.) que é uma espécie nativa e pioneira no início de estados secundários de sucessão em vários ecossistemas dos Países Baixos. Estudos de uma cronossequência de campos agrícolas abandonados têm demonstrado que a densidade de *J. vulgaris* aumenta durante os primeiros anos após o abandono das terras e, em seguida, diminui com o desenvolvimento e sucessão da vegetação. De acordo com trabalhos anteriores, este padrão "boom-bust" foi atribuído, pelo menos em parte, aos organismos do solo. Assim, seleccionou-se esta cronossequência de

campos abandonados para estudar se os padrões espectrais são sensíveis ao aumento e diminuição da abundância destas plantas em função das suas interacções com o solo. Este estudo pode tornar-se um bom prelúdio para estudos que monitorizam o controlo biológico de espécies vegetais exóticas invasoras por parte de organismos do solo, desde o momento da sua introdução.

A técnica da espectroscopia permite obter o padrão espectral de um objecto em múltiplos comprimentos de onda do campo electromagnético. Dependendo da condição ou composição química do objecto, podem ser geradas frequências de reflectância diferentes e estas podem ser medidas por espectrómetros. É comum recorrer-se à medição da reflectância (i.e. a proporção da radiação electromagnética incidente que é reflectida após interagir com uma superfície), através de espectroscopia, uma vez que é sensível às vibrações de várias ligações químicas específicas do objecto medido e à composição química deste. Os alcalóides pirrolizidínicos (PAs, sigla inglesa na tese) são importantes compostos de defesa presentes em espécies da tribo Senecionaea, usados contra herbívoros generalistas, e desempenham um papel importante nas interacções bióticas entre as plantas e os seus inimigos (também a nível do solo). Como tal, torna-se importante compreender de que modo a concentração destes compostos de defesa, presentes nos tecidos superiores das plantas, contribui para as características espectrais das plantas durante as interacções bióticas e abióticas das plantas com o solo.

Em primeiro lugar, eu investiguei a contribuição relativa dos alcalóides pirrolizidínicos (PAs) para o padrão de reflectância hiperespectral na região do visível e do infravermelho (capítulo 2). Na minha primeira experiência eu demonstrei que as aminas terciárias, um tipo específico de PAs, podem afectar o padrão espectral das folhas. No entanto, este estudo feito em três espécies diferentes - *Senecio inaequidens*, *Jacobaea vulgaris* e *J. erucifolia* - mostrou que os resultados são específicos para cada espécie. Eu inferi que este efeito específico está provavelmente relacionado com uma estrutura cíclica, o anel de epóxido, presente nos PAs da espécie *J. vulgaris* e da sua correlação positiva com o teor de azoto. Em experiências posteriores (capítulos 3 e 5) a estimativa da concentração de PAs através da reflectância foi baixa. Uma vez que nos capítulos 3 e 5 a correlação encontrada entre PAs e azoto foi muito reduzida, estes resultados apoiam a conclusão obtida no capítulo 2: é necessário haver uma correlação entre PAs e azoto a fim de conseguir uma estimativa correcta do conteúdo em PAs usando os padrões de reflectância espectral. Além disso, eu mostrei que é possível estimar a concentração de químicos de defesa utilizando a

reflectância de outros órgãos da planta para além das folhas (Capítulo 5): nos capítulos das plantas de *J. vulgaris* foi também possível detectar PAs através de reflectância.

No capítulo 3, eu testei a influência de diferentes comunidades microbianas do solo sobre o conteúdo químico e os padrões de reflectância das plantas. Eu cultivei as três espécies em solo esterilizado e em solo vivo recolhido em campos onde *J. vulgaris* estava presente. A minha hipótese era que a presença de comunidades microbianas no solo provocaria o aumento das defesas na parte superior das plantas e que a reflectância hiperespectral iria permitir a discriminação dos diferentes solos utilizados. De facto, os solos vivos provocaram um maior impacto sobre a variação química foliar na exótica *S. inaequidens* e na nativa *J. erucifolia* do que na nativa *J. vulgaris*. Uma vez que *J. vulgaris* já ocorria nos campos de origem destes solos eu inferi que esta resposta diferencial era específica da espécie, já que a comunidade do solo desenvolveu-se em campos onde *J. vulgaris* estava presente. A capacidade da análise de reflectância para discriminar as interacções plantas-solo foi equivalente à das análises de discriminação tradicionais que utilizam perfis químicos. Esta possibilidade de utilizar padrões espectrais para discernir interacções bióticas entre as plantas e o solo demonstra o potencial destas técnicas para estudar as interacções das plantas com as comunidades crípticas existentes no solo.

Nos capítulos 4 e 5 eu estudei a dinâmica temporal dos compostos químicos primários e secundários bem como os padrões espectrais de *J. vulgaris* durante o desenvolvimento sucessivo da vegetação. O efeito do estado da sucessão nos conteúdos químicos de *J. vulgaris* foi especialmente evidente nas flores, tendo as maiores concentrações de aminas terciárias sido detectadas em campos que haviam sido abandonados entre 10 a 20 anos atrás. Plantas de áreas abandonadas há mais tempo apresentavam geralmente flores e folhas com mais N-óxidos, especialmente no final do Verão. Os resultados também mostraram que a variação química sazonal está em linha com a estratégia de optimização de defesa: as flores contêm mais defesas do que as folhas quando medidas na mesma época. Surpreendentemente, não era esperada a ausência de diferenciação sazonal nos padrões espectrais das folhas, no entanto as várias fases da sucessão foram discriminadas com sucesso pelos padrões espectrais das flores. As análises espectrais corroboram as análises químicas, ambas sugerindo que a composição química das flores é mais sensível ao processo de sucessão da vegetação do que folhas. Os padrões espectrais mostraram potencial para serem utilizados na detecção de diferenças sazonais e de sucessão da vegetação.

Mais importante ainda, estes resultados evidenciaram que outros órgãos além das folhas podem fornecer informação temporal sobre as interacções entre plantas e solo.

No início da investigação presente nesta tese pouco se sabia sobre o impacto dos organismos do solo nos padrões de reflectância espectral das plantas. Esta tese demonstrou que os padrões de reflectância das plantas podem fornecer muitas informações sobre as suas interacções com o solo, mesmo usando diferentes órgãos da planta. Daí eu concluir que, apesar da fraca consistência ao utilizar padrões de reflectância espectral para estimar a concentração de alcalóides pirrolizidínicos, as comunidades bióticas do solo podem afectar a reflectância das plantas. Ao conseguir discriminar, de modo consistente, as interacções entre as plantas e os organismos do solo, demonstrei que se pode usar os padrões espectrais para estudar as interacções entre plantas e biota do solo mesmo sem a necessidade de compreender as mudanças químicas específicas que ocorrem. Os resultados desta investigação sobre as interacções das plantas com os microorganismos e as condições abióticas do solo demonstraram que os padrões espectrais podem fornecer mais informações do que a simples soma dos produtos químicos de uma planta. Portanto, os resultados desta tese abrem caminho para a utilização de detecção remota das interacções plantas-solo em escalas maiores, fazendo uso de, por exemplo, fotografias aéreas ou imagens de satélite. Os desenvolvimentos futuros nesta investigação poderão beneficiar diversas outras áreas, como, por exemplo, o controle biológico ou a monitorização da qualidade do solo para a agricultura.

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help was to have access to tools such as Skype, Viber, Facebook, Email and very fancy phones to keep me closer to everyone I cherished at home. I would like to thank all the researchers, nerds, geeks and alikes, the brilliant minds that created such tools, such worlds as the internet. Your quest for an answer, your thrill and creativity makes our world better and closer, and mine for sure was (and is) much happier for that.

Despite being far away from my family I got a new one here as well and I would like thank Madeleine for that, as she happily shared her home and family with me, I will take Sinterklaas traditions with me and become a weird Portuguese family on the 5th of December.

À minha família: Obrigada por me apoiarem mesmo estando longe. Aos meus pais por tudo o que me ensinaram mesmo antes de eu cortar os cordelitos e seguir a vida. Obrigada pela muita força que me deram nesta vida de emigrante. No dia que vi a minha mãe chorar quando eu disse que ia para a Holanda fazer estágio e vi o meu pai a aceitar a ideia de olho franzido pensei só que era mania de pais galinhas (era tão inocente na altura, querem o quê?). Vocês viram-me seguir o vosso percurso, ir sozinha (vá com o namorado que virou maridito do coração :p) para um país distante que não reconhece a nossa cultura e costumes. E depois disso ainda mais sozinha quando o Nuno voltou para Portugal. Como vocês os dois aguentaram essa separação anos a fio à 25 anos atrás ainda estou para descobrir! Se me é dificil com esta tecnologia toda em volta, vocês sem nada disso são a minha inspiração, de que mesmo com estes percalços um casamento pode ser sólido, feliz e sobreviver :). Obrigada por toda a força que me dão. Maninho do coração e famelga, muito obrigado por estarem sempre aí! À minha família por anexo, que me fizeram rir imenso e me integraram num lar ribatejano-espanholado com todo o amor e carinho do mundo: Muito obrigada por tudo e desculpem lá ainda não ter feito adições à família, vamos ver como corre nos próximos anos. À avó Méme (RIP) em especial, porque foi uma inspiração incrível de felicidade e sorriso, mesmo nos momentos mais complicados.

Club farinheira!!! My beloved friends Joana, Ricardo, Paola, Alberto and Annika! I miss you so much, without you the Netherlands is not the same, but even far you were always there for me, for us! I cannot express how much I love you all! Paola and Alberto, the strongest couple I have met in the last few years, I wish you all the best and that you get closer to home after such a troublesome time in the other side of the world. Preferably closer to Portugal so we can enjoy our awesome

dinners once again, and with, at least, one new member, the pequenito Martin! Joana e Ricardo o casal maravilha que nos suporta as crises mesmo longe! Obrigada por tudo, obrigada Joana, por tudo mesmo, és uma amiga espectacular! Ao pessoal da Liga da Protecção da Natureza que me recebeu de braços abertos e me tratou como parte da família desde o primeiro dia! Obrigada por terem tomado tão bem conta do meu rapaz. Um grande obrigada ao grupo do CEF por me receber sempre tão bem nas minhas rápidas visitas, adoro a vossa companhia e as mais variadas conversas que partilhamos, espero muito em breve fazer isso com mais frequência. Um especial agradecimento às irmãs Marchante, Beta e Hélia, vocês mostraram-me o mundo das invasoras e, mesmo sem querer, mudaram o rumo da minha vida, os vossos conselhos levaram-me ao doutoramento que aqui fiz! E claro que agradeço aos meus amigos portugueses todos porque fizeram o seu melhor para me manter nas suas vidas, muito obrigada! Em especial à Ana, Samuel, Carla, Marta e Bruno, Ana, Catarina e Dário, Margarida, Moisés, Miguel e Mifá, Sílvia, Anita, Cláudia, Joana e Andreia.

E claro que a pessoa mais importante nisto tudo fica para o fim, o meu melhor amigo, o meu amor, o meu suporte, alma gémea, o meu marido. :,) que te digo a ti que tanto fizeste por mim? Tu é que és o dotado da escrita e não eu. Mas a coisa boa de partilhar 11 anos da minha vida contigo é que sei que basta olhar para ti e tu vais sempre perceber o quanto tudo isto significa para mim. Fazes-me muito feliz, estás aqui para mim, respeitas-me e incentivas os meus sonhos mais doidos, e eu adoro-te por isso. Fizeste-me prometer que acabava isto, está feito, demorou mas já está. E não só está feito, como está feito por ti com um layout de livro que muito pacientemente e com agrado fizeste para mim. O que este doutoramento fez foi amar-te ainda mais. Agora prometo que farei tudo para voltar a estar ao teu lado (e a reclamar por tropecar nos teus sapatos a toda a hora). Obrigada por fazeres parte da minha vida, és o meu "estado natural" e é muito dificil imaginar algo diferente. Sem ti nada disto fazia sentido e a vida tinha pouca graça. Imaginar-nos velhotes resmungões com 60 anos de memórias partilhadas é algo que me aquece o coração. 49 to go! Amo-te!

Curriculum Vitae

Sabrina was born in Lille, France. But she did not really like it there, so left after only a year. She was raised in the high mountains of Northern Portugal, in the deep countryside. She played with ducklings and bunnies, and ran in the cornfields – much to her grandmother's fury – and made mud cakes with her cousins. Outside, she watched her family take care of their small farm, and learned the true flavor of meat and vegetables. Inside, she listened to her parents French vinyl collection, and sneakily read her sister's books. Her determination and courage she got from her Father. Her firmness and temperament came from her Mother. All set on a background of a rural, conservative society of a small village, which made her irreverence and independence from "what should be" stand out even more. And she studied hard! Well, when she was not busy skipping class with her friends to go swim in the river, she did…

At the age of 18 she wanted to get as far as possible from her village, but all she managed was to get to Aveiro. That was close enough to go home every weekend… In Aveiro, she spent four years studying Biology, in one of the most beautiful university campus of the country. Oh, and there she met her handsome and lovely (ahem!) husband-to-be.

After graduation, her Dutch adventure started! First with an internship at the Institute of Biodiversity and Ecosystem Dynamics, at the University of Amsterdam, where she studied the population viability of *Gentianella amarella* in the Dutch dune areas. Afterwards, with an MSc in Spatial Ecology, during which she researched and wrote a thesis on the genetic variation in the *Gentianella amarella* group using AFLP markers and its implications for taxonomy and conservation. Her superior baking skills also developed during this time, resulting in the granting of an Honorific Title by her supervisor, Dr. Gerard Oostermeijer. During this first year she also learned what Frikandel, Dropjes and Haring were, and how to best avoid them.

Not having yet experienced enough bad weather, bad food or bad music, she decided to remain in the Netherlands for her PhD. This time going deeper into the

countryside, sharing her time between Heteren (and later Wageningen), in the Department of Multi-trophic Interactions (now Terrestrial Ecology) of the Netherlands Institute of Ecology (NIOO-KNAW), and Enschede, in the Faculty of Geo-Information Science and Earth Observation (ITC) of the University of Twente.

Her PhD research was on something so outstanding and multidisciplinary, in the realms of plant biochemistry and spectroscopic remote sensing, that even I, also a Biologist, had difficulty in explaining it to our friends. Or even just understanding it… Sometimes… After a lot of hard work, sweat and tears, her research resulted in this thesis, in which she used hyperspectral reflectance patterns to identify and discriminate chemical variations in plants relating it to plant interactions with the biotic and abiotic soil environment.

Sabrina served as the President of the ITC PhD students Committee for a year, which resulted in the activation of certain "political skills", which had, until now, been lying dormant. The political beast had been unleashed!

She now has a position as a Post-Doc in the Department of Terrestrial Ecology of the NIOO-KNAW, in another project so revolutionary and boundary-crossing that I will have to keep asking her what it's about all the time. Which I did again, and she told me she is working on assessing the impacts of bio-based economy on the legacy of plant-soil interactions and its relation to spectral pattern changes. So, some more time in the Netherlands to enjoy the bicycle-riding, the vintage shops in Amsterdam, Sinterklaas, homemade stroopwafels, and, with some luck, the Elfstedentocht!

After that… well, get ready Portugal, and World beware, 'cause what's coming is going to hit you in the face, turn you upside down, and still leave you with a smile on your face! I should know.

Nuno Curado
(Yes, the husband)

PE&RC PhD Training Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)
- Studying of biochemical and biophysical properties of *Senecio* and *Jacobaea* species: is there a link to invasiveness?

Writing of project proposal (4.5 ECTS)
- Studying of biochemical and biophysical properties of *Senecio* and *Jacobaea* species: is there a link to invasiveness?
- The Establishment of Invasive Plant Species in the European Mediterranean Basin: Life-history Traits Analysis and Future Trends in a Climate Change Scenario.

Post-graduate courses (6.8 ECTS)
- Advanced statistics
- Art of Modelling
- Multivariate data analysis of spectroscopic data
- Introduction to R
- Soil biodiversity and Life

Laboratory training and working visits (4.5 ECTS)
- Pyrrolizidine alkaloid extraction
- Plant invasions

Deficiency, refresh, brush-up courses (2 ECTS)
- Basic Statistics
- ASD remote sensing training

Competence strengthening / skills courses (1.4 ECTS)
- Scientific writing course

PE&RC annual meetings, seminars and the PE&RC weekend (3 ECTS)
- PE&RC weekend
- Netherlands Ecology meeting
- ITC PhD day
- NIOO seminars
- Remote sensing of the environment
- Spectro-directional sensing of vegetation
- Plant insect interactions workshop

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)
- PhD Tutorial at ITC-Natural Resources department
- PhD discussion group at NIOO
- Ecological Theory and Application Discussion Group - WUR
- TEDx Wageningen

International symposia, workshops and conferences (7.9 ECTS)
- Neobiota European conference on Biological invasions
- 10th International conference Ecology and Management of Alien Plant invasions, Stellenbosch, South Africa.
- World Conference on biological invasions and ecosystem functioning, Porto, Portugal.
- Art, Science and Applications of reflectance spectroscopy, USA.
- Plant population biology – crossing borders, Nijmegen, the Netherlands.

Supervision of a MSc student
- Fatemeh Eghbali Moghaddam. "Estimation of pyrrolizidine alkaloids in native and invasive weed species of the Netherlands using reflectance spectroscopy".

The research presented in this thesis was conducted at the department of Terrestrial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW) in Wageningen and at the department of Natural Resources of the ITC Faculty of the University of Twente.

This is NIOO thesis 111.

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