COMMUNAUTÉ FRANÇAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Plant traits variability within and among populations in the context of calcareous grassland restoration

Mélanie HARZE

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs : Grégory MAHY et Arnaud MONTY

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ABSTRACT

In Western Europe, abandonment of traditional forms of agriculture has caused the fragmentation of semi-natural grasslands, affecting ecosystems functioning as well as population survival. Habitat restoration has become a crucial aspect of grasslands conservation, and one of the main issues is evaluating restoration success and setting appropriate criteria to do so. Indicators used to judge whether a restoration has been successful may concern a wide range of organisms. They may be defined at different geographical scales and may concern various levels of biodiversity organization. Among them, population parameters are less represented despite their usefulness. To consider restoration as a success, restored populations of targeted species should demonstrably possess characteristics allowing their dispersal, reproduction, growth and adaptation to the environment.

In this thesis, the use of plant population parameters for evaluating grassland restoration was assessed based on a literature review. Then, the success of calcareous grassland restoration was determined regarding colonization of restored grasslands by five calcareous grasslands species; *Helianthemum nummularium*, *Hippocrepis comosa, Potentilla tabernaemontani, Sanguisorba minor* and *Scabiosa columbaria*. Population establishment and intra-specific functional trait variability in response to the environment were observed and compared among restored and reference populations of those species, through field inventories and in-situ traits measurements in calcareous grasslands of south Belgium. Moreover, a glasshouse experiment was set-up with the aim to evaluate the intra-specific variability in response to drought stress in reference populations of *P. tabernaemontani*.

The results indicated that population parameters were less well studied for evaluating grassland restoration success compared to indicators related to ecosystems and communities. The population parameters used to assess the success of calcareous grassland restoration showed that the study species colonized restored sites and established new populations. The fitness of the restored populations was very high. High intra-specific trait variability was highlighted at a very local scale in the reference habitat. Individuals exhibited high leaf dry matter content (LDMC), low specific leaf area (SLA) and low vegetative height in relation to low soil depth and high potential incident radiation (PDIR). These environmental variables are possibly linked to the availability of soil moisture. Further, the relationship between traits and environmental variables was not always consistent in restored sites. However, the intra-specific variability of plant traits was similar in the reference and restored grasslands. Finally, the findings from the glasshouse experiment suggested that individuals originating from drier parts of calcareous grasslands better survive drought stress.

The overall conclusion of this thesis is rather optimistic concerning restored populations of the five specialist plant species studied, specifically regarding their colonization, persistence and response to the environment or to potential climate change. The conclusions must, however, be modified depending on the reference ecosystem used as a model. This approach has to be integrated into a multi-scales and a multi-species approach to fully evaluate restoration outputs. Yet, this thesis has contributed to the understanding of population responsiveness to habitat restoration, as well as to the evaluation of restoration success of calcareous grasslands in Belgium.

RÉSUMÉ

L'abandon des pratiques d'agriculture traditionnelle a mené au déclin des pelouses semi-naturelles en Europe de l'Ouest. S'en est suivie une fragmentation intense de ces habitats, qui affecte le fonctionnement des écosystèmes et des populations qui les composent. La restauration de ces pelouses est dès lors nécessaire et fait partie intégrante des stratégies de conservation de ces écosystèmes. Evaluer le succès de ces restaurations est un grand challenge et divers indicateurs peuvent être utilisés à cette fin. Ceux-ci concernent une grande variété d'organismes ainsi que diverses échelles géographiques et différents niveaux d'organisation de la biodiversité (individus, populations, communautés, écosystèmes). Parmi ces indicateurs, ceux qui concernent les populations semblent avoir été moins utilisés, malgré leur pertinence. Dans ce contexte, on peut considérer une restauration comme réussie lorsque les populations d'espèces ciblées sont capables de se disperser dans les zones restaurées, se reproduire, croitre et s'adapter aux changements de leur environnement.

Dans le cadre de cette thèse, l'utilisation de paramètres populationnels pour jauger la réussite de restaurations de pelouses a été évaluée grâce à une revue de la littérature. Ensuite, le succès de restauration de pelouses calcicoles a été étudié dans le sud de la Belgique. Des populations de cinq espèces - *Helianthemum nummularium*, *Hippocrepis comosa, Potentilla tabernaemontani, Sanguisorba minor* et *Scabiosa columbaria* - ont été étudiées, en termes de colonisation des sites restaurés, d'établissement au sein de ces sites et de variabilité intra-spécifique de traits fonctionnels en réponse à l'environnement. Cela a été réalisé à l'aide d'inventaires de terrain dans des zones restaurées et des zones références de pelouses calcicoles. De plus, une expérience a été réalisée sous serre dans le but d'évaluer la variabilité intra-spécifique de la réponse au stress hydrique dans des populations de référence de *P. tabernaemontani*.

Les résultats de cette thèse ont montré que pour évaluer des restaurations de pelouses, les paramètres populationnels ont été moins utilisés que les indicateurs relatifs aux communautés ou aux écosystèmes. L'utilisation d'une approche populationnelle pour évaluer le succès de restaurations de pelouses calcicoles en Belgique a démontré que les espèces étudiées étaient capables de coloniser les sites restaurés et d'y établir de nouvelles populations caractérisées par un très bon succès reproducteur. Sur les pelouses de référence, les populations des espèces étudiées étaient caractérisées par une très grande variabilité fonctionnelle intra-spécifique à l'échelle locale. Cette variabilité des traits était fortement liée aux paramètres de l'environnement. En effet, les individus sur sols peu profonds et soumis à un rayonnement solaire important étaient caractérisés par de faibles hauteurs végétatives, une « specific leaf area » (SLA) faible et un contenu de matière sèche dans les feuilles (LDMC) élevé.

Ce lien entre les traits mesurés et leur environnement n'a pas été confirmé dans les pelouses restaurées. Par contre, la variabilité fonctionnelle intra-spécifique élevée qui caractérisait les populations de référence était similaire dans les populations restaurées. Enfin, les résultats de l'expérience réalisée en serre ont montré que les individus issus des zones les plus sèches des pelouses étaient davantage capables de survivre à un stress hydrique important.

Les résultats de cette thèse sont optimistes concernant la restauration des populations des cinq espèces étudiées. Ces résultats doivent néanmoins être nuancés par rapport au choix des populations de référence. Cette approche mérite d'être intégrée dans une approche globale d'évaluation des restaurations de pelouses calcicoles, considérant différentes échelles géographiques et écologiques. Quoi qu'il en soit, ce travail contribue à l'évaluation du succès de restauration des pelouses calcicoles en Belgique et à la compréhension de la réponse des populations à la restauration de leur habitat.

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Avant de vous lancer dans la lecture des quelques 130 pages qui composent ma thèse de doctorat (et je ne compte ni les annexes ni les références), j'espère que vous sourirez en vous reconnaissant dans les quelques lignes qui suivent. Si ce n'est pas le cas, sachez que je vous remercie d'ores et déjà de l'intérêt porté à ce document, même si cela s'arrête au titre, au résumé ou à cette section de remerciements.

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CHAPTER 1:

Introduction, objectives and scientific strategy

Introduction

Land use changes worldwide

Recent decades have been dominated by unprecedented rates of habitat perturbations by human activities, reducing the capacity of ecosystems to provide valuable services, such as the maintenance of soil fertility, climate regulation, water supply and pollination (Chapin III et al. 2000; Cardinale et al. 2012). It has been estimated that more than one-third of the world's ecosystems have been converted for human use and at least another third have been heavily degraded through fragmentation, unsustainable harvesting, pollution or exotic species invasion (Millennium Ecosystem Assessment 2005). Those phenomena have increased the rates of species extinction and have threatened biodiversity worldwide (Saunders et al. 1991; Dobson et al. 1997; Balmford et al. 2005). The relative importance of these threats has differed among biogeographical regions. In Western Europe, abandonment of traditional forms of agriculture is a major past and current pressure on habitats (Fig. 1), notably on semi-natural grasslands that have declined sharply over the last decades (Watt et al. 2007).

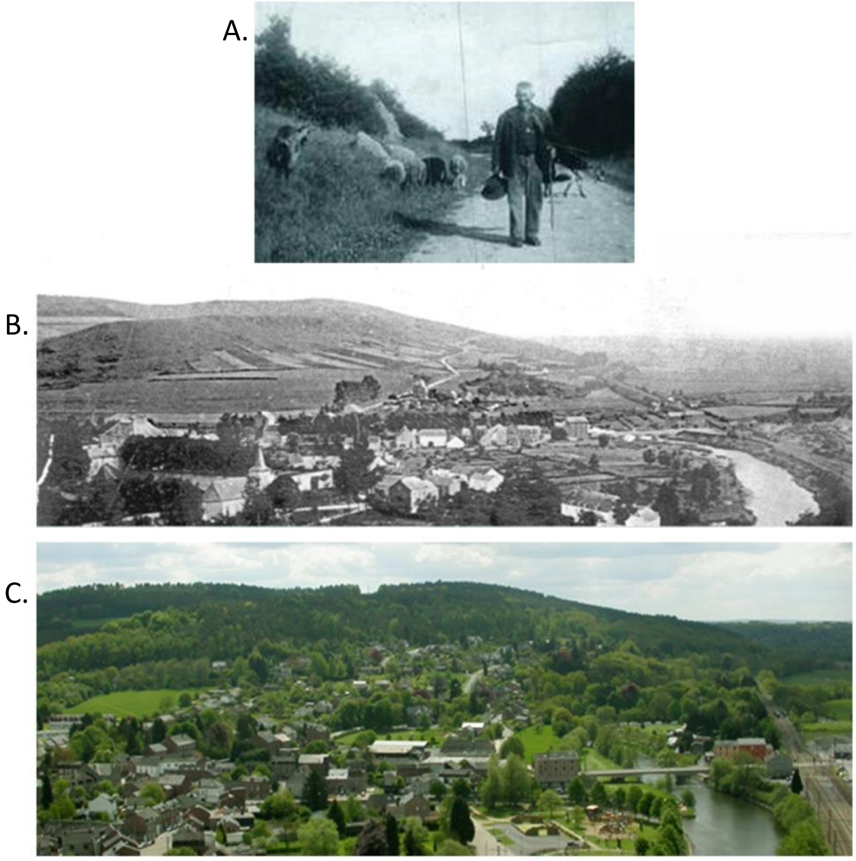

Figure 1 – Abandonment of traditional forms of pastoralism (A) have transformed the landscape. Semi-natural open areas have declined sharply over the last decades. Those pictures illustrate landscape changes in Bomal (Belgium) between the beginning of the twentieth century (B.) and the beginning of the twenty-first century (C.).

Habitat fragmentation

Habitat fragmentation (Fig. 2) is a process during which a continuous habitat is reduced to several small, spatially isolated remnants (Young et al. 1996). This implies four effects on habitat pattern: a reduction in habitat amount, an increase in the number of habitat patches, a decrease in the size of habitat patches and a rise in isolation of habitat patches within the landscape (Fahrig 2003).

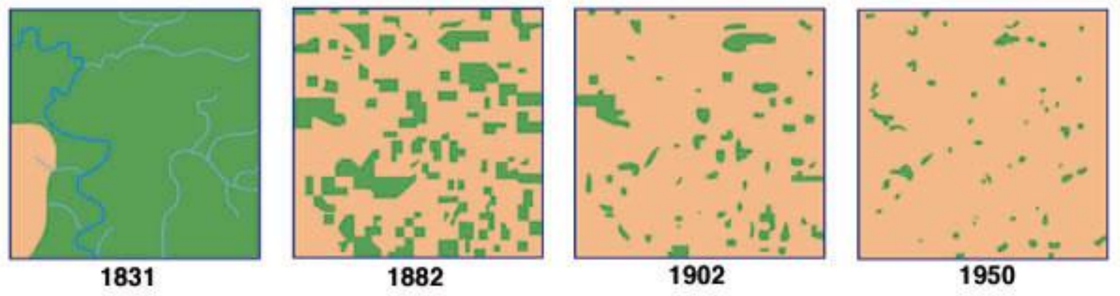

Figure 2 – Fragmentation of woodland habitat at Cadiz Township, Wisconsin. The forest has been progressively reduced from a nearly continuous cover in 1831 to isolated woodlots in 1950 covering less than 1% of the original area (Conservation biology – Enhancement chapter: Johnson's The Living World, Third Edition[1]).

Habitat fragmentation strongly affects ecosystems, species and populations. It may alter the environmental conditions of habitat patches, compromise species interactions, increase edge effects, raise the incidence of invasive species, diminish population sizes and disrupt genetic exchanges (Young et al. 1996; Hobbs and Yates 2003; Lienert 2004). As sessile organisms, plants are particularly threatened by fragmentation (Young et al. 1996). Small and isolated populations are more exposed to environmental and demographic stochasticity, genetic drift and inbreeding that can negatively impact their genetic structure, fitness and demography, leading to greater risks of extinction (Lienert 2004; Leimu et al. 2006). Ultimate consequences of fragmentation are local species extinction, decreased species richness and modified community composition (Butaye et al. 2005a).

The need for restoration

Conservation of remnant habitats is necessary to stop the fragmentation process and ensure habitat persistence across the landscape (Kahmen et al. 2002). However, conservation alone is often not sufficient to guarantee the long-term survival of many plant species, when populations are already small and isolated (Rodrigues et al. 2004). Thus, habitat restoration increasingly becomes a crucial aspect of biodiversity conservation (Dobson et al. 1997; Hobbs and Harris 2001). Restoration aims to improve ecological networks of habitats and to diminish fragmentation along with its negative effects (Jongman and Pungetti 2004).

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[1] Available from http://www.mhhe.com/biosci/genbio/tlw3/enhancement_chapters/conservation.html.

Ecological restoration

Ecological restoration is the process of assisting the recovery of damaged, degraded or destroyed ecosystems (Society for Ecological Restoration (SER) 2004). It ranges from local to regional actions and encompasses a wide variety of interventions, such as restoring disturbance regimes, fighting against invasive species or reintroducing native ones after restoration of abiotic conditions (Hobbs and Cramer 2008; Laughlin 2014).

One main challenge in restoration ecology is to evaluate restoration success and define appropriate criteria to do so (Ruiz-Jaen and Aide 2005a; Zedler 2007). Restoration objectives and results can vary widely with respect to economic, aesthetic, recreational, educational or ecological aspects of restoration (Suding 2011). Two crucial steps that must be considered in the evaluation of restoration are: 1) the definition of a reference for the restoration; and 2) the selection of indicators for the evaluation (SER 2004; Cristofoli and Mahy 2010).

The need for a reference

The restoration success is primarily assessed by comparing restored ecosystems to a reference considered a “model”. This reference can be a description of restored site prior to damage (if historical records exist), a list of target species (characteristic, rare or endangered) or remnant sites still present within the landscape and representing the self-sustaining current ecosystem (SER 2004).

Using historical information to describe a reference has been challenging - it is difficult to justify the choice of a specific time period when landscapes have experienced centuries of modifications (Thorpe and Stanley 2011). Moreover, climate change and other significant global change processes influencing current ecosystems are making those historically less relevant (Harris et al. 2006; Choi et al. 2008; Hobbs et al. 2009). The objectives of ecological restoration should consider restoring resilient assemblages for the future and improving ecosystem functioning in environments that may have no historical analogue (Harris et al. 2006; Choi et al. 2008; Suding 2011).

However, ecosystems that still exist within the landscape have been widely employed as references for restoration. They may reflect species assemblages that evolved in response to natural climate as well as past and current disturbances and have been considered a natural sustainable state (Jackson et al. 1995; Choi et al. 2008; Hiers et al. 2012).

Indicators of success: different approaches in restoration ecology

The ecological criteria utilized to judge whether a restoration is successful are numerous. Of note, they can be classified according to the level of biodiversity organization they refer to, i.e. individual, population, community or ecosystem. Moreover, they may concern a broad spectrum of organisms, such as plants, fungi, arthropods, birds or mammals (Hobbs and Norton 1996; Zedler 2007; Cristofoli and Mahy 2010).

The Society for Ecological Restoration (SER 2004) provided a list of nine ecosystem attributes that can be used to determine when restoration has been accomplished; (1) similar species assemblage and community structure in comparison with the reference ecosystem; (2) indigenous species; (3) functional groups necessary for long-term stability of the ecosystem; (4) physical environment capable of sustaining reproducing species populations; (5) normal functioning; (6) integration into a larger ecological matrix or landscape with which it interacts; (7) potential threats from the surrounding eliminated or reduced; (8) resilience of the ecosystem to normal disturbances; and (9) self-sustaining to the same degree as the reference ecosystem. Measuring these attributes may provide a complete assessment of restoration success regarding ecosystems in their entirety. In practice, however, few studies have enough financial and time resources to monitor such a large body of attributes. Most studies have utilized measures of restoration success that can be classified into three major classes: (1) species diversity - mainly assessed through plant species richness and abundance; (2) vegetation structure - primarily gauged by vegetation cover, density or biomass; and (3) ecological processes - mostly biological interactions and nutrient cycling (Ruiz-Jaen and Aide 2005a; Wortley et al. 2013). Among the nine ecosystem attributes proposed by the SER, three are rarely measured in restoration projects, especially number 4 concerning sustaining reproducing populations (Ruiz-Jaen and Aide 2005a).

Population parameters seem less represented among indicators employed to evaluate restoration success. A population approach usually refers to one or more target species, often rare or threatened (Carignan and Villard 2002; Cristofoli and Mahy 2010). The population parameters accounted for are, for example, the number of individuals, the genetic diversity or the population growth rate (Cristofoli and Mahy 2010). This approach has often been criticized because the information recorded is considered too restricted compared to the diversity of ecosystem components (Franklin 1993). However, population biology can offer valuable indicators of restoration success. Montalvo et al. (1997) emphasized the application of population biology evaluating ecological restoration and advocated that restoration should be deemed successful when species are re-established to a level that permits their long-term persistence within the landscape. Restored populations must therefore possess characteristics that allow their dispersal, reproduction, growth and adaptation to new environments.

A population approach to restoration ecology

Evaluating restoration success at the population scale is relevant, specifically because restoration impacts plant population processes in many ways. After habitat restoration, plant species must first colonize restored patches of habitats and establish viable populations over the long-term through offspring production (Montalvo et al. 1997). Species colonization depends on its presence in the regional species pool, the presence of dispersal agents and the spatial position of other habitats across the landscape (Bakker and Berendse 1999; Helsen et al. 2013a). In the case of limited dispersal in space and time, founding populations can be small and poorly diversified genetically (Montalvo et al. 1997; Hufford and Mazer 2003). This may lead to increased risks of inbreeding depression and decreased adaptive potential for restored populations (Barrett and Kohn 1991; Ellstrand and Elam 1993). Moreover, loss of genetic variation in founding populations can result in lower fitness (Shaffer 1981; Lande 1988; Williams 2001; Reed and Frankham 2003; Matthies et al. 2004). Additionally, small populations are more exposed to random environmental fluctuations (Menges 1991b; Widen 1993; Heschel and Paige 1995), may be less attractive to pollinators (Sih and Baltus 1987; Hendrix and Kyhl 2000) and could consequently have lower reproductive success.

Species performance might also be affected by the environmental conditions of restored habitats. Reproductive success of restored populations has been acknowledges to be impacted by a loss of habitat quality (Vergeer et al. 2003; Quintana-Ascencio et al. 2007; Adriaens et al. 2009). In new environments, plant populations able to exhibit adaptive responses through local adaptation or phenotypic plasticity will probably be more prone to survive in the long-term (Bolnick et al. 2003; Bellard et al. 2012). Plants’ capacity to adapt to their environment will also be of primary importance in the context of climate change.

Plant populations usually consist of phenotypically diverse individuals. Individuals can vary with respect to traits related to abiotic tolerance, resource use or competitive ability, and that may influence demographic parameters at the population level (Bolnick et al. 2003; Lankau and Strauss 2007). Intra-specific trait variation, hence, merits assessment when studying plant population functioning along with environmental conditions. As high trait variability may allow species to grow and reproduce under variable degrees of environmental conditions (Weiner 2004; Fort et al. 2014), the extent of intra-specific trait variability may indicate the ability of a species to better cope with global change (Almeida et al. 2013; Mitchell and Bakker 2014b). Intra-specific trait variability can be addressed at different scales; between populations (e.g. Jung et al. 2010; Baruch 2011; Andersen et al. 2012; Almeida et al. 2013; Pakeman 2013) as well as within populations (Bolnick et al. 2011; Mitchell and Bakker 2014b), the latter being poorly addressed thus far based on the literature.

Among plant traits, a number of those that are functional are recognized as reliable predictors of species resistance to disturbance (Gamfeldt and Kallstrom 2007) and response to environmental conditions (Pakeman et al. 2009). Functional traits can be defined as morpho-physiophenological traits that impact fitness indirectly via their effects on growth, reproduction and survival (Violle et al. 2007). They express plant responses to environmental changes (Lavorel and Garnier 2002) and may have an effect on ecosystem functioning (Díaz and Cabido 2001; Lavorel and Garnier 2002). A core list of functional traits is widely recognized; they are relevant to understand plant response to a broad range of environmental conditions and to address fundamental population processes, such as dispersal, establishment and persistence (Weiher et al. 1999). This common core list includes: seed mass, seed shape, dispersal mode, clonality, specific leaf area (SLA), leaf water content, height, aboveground biomass, life history, onset of flowering, stem density and resprouting ability (Weiher et al. 1999).

Study model: calcareous grasslands

Calcareous grasslands comprise dry grasslands present on limestone, chalk and calcareous loess (Butaye et al. 2005b). In Central and Western Europe (Fig. 3), they are distributed from Ireland and South Sweden to Northern Spain (Wolkinger and Plank 1981; Royer 1991). They are predominantly semi-natural habitats dependent on extensive grazing (usually with sheep) or on annual mowing. Calcareous grasslands originated from different time periods since the Neolithic age, established from various types of land uses. The maximum spread of these habitats is related to the period of large sheep flock migrations and transhumance between the fifteenth and twentieth centuries. During this period, calcareous grasslands were widespread in the hilly calcareous regions of Europe. In the twentieth century, the extent of calcareous grasslands decreased largely because of the abandonment of traditional shepherding and hay-making, urbanization, afforestation and fertilization. This has brought about a profound change not only in the number, but also in the size of remnant calcareous grasslands that are now embedded within a landscape matrix of intensively managed farmlands, forests or roads (Poschlod and WallisDeVries 2002).

Figure 3 – Distribution of calcareous grasslands in Central and Western Europe at the end of the twentieth century (Wolkinger and Plank 1981).

Today, calcareous grasslands are a central issue for habitat conservation in Europe. They are listed in the EU Habitat directive and considered vulnerable according to the European Red List of habitats (Janssen et al. 2016). They are among the most species-rich habitats in Western Europe, at both the local and regional scales, regarding plants but also insect species (Bobbink et al. 1987; Willems 2001; Poschlod and WallisDeVries 2002). As well, they deliver crucial ecosystem services, like pollination via native insects that are valuable for crop production (Kremen et al. 2004; Meyer et al. 2009). Calcareous grasslands continue to be highly threatened, predominantly by the fragmentation of the remaining patches of habitats across the landscape (Fischer and Stöcklin 1997). Between 1993 and 2015, 37 European LIFE projects targeted calcareous grassland restoration (Fig. 4) through many locations in Europe[2], based on an investment of tens of millions Euros, with the goal of enhancing ecological networks of those habitats at the European scale.

Figure 4 – Results of calcareous grassland restoration as part of the European LIFE project “Hélianthème” in Belgium (Coteau de Spy, Ferrières, 50°23’ N, 5°32’ E, alt. 140–200m).

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[2] http://ec.europa.eu/environment/life/project/Projects/

Calcareous grasslands are a stressful environment for plant species. They are typified by nutrient poor soils and are periodically water-limited (especially in the summer and sometimes in the spring) because of their underlying soils characterized by rapid drainage and poor water storage (Buckland et al. 1997; Kalicka et al. 2008). The amount of available water for plants varies notably according to habitat topography and exposure, with poor water content on sunny steep slopes as a result of high evaporation (Bennie et al. 2006; Bennie et al. 2008). Shallow soils also diminish water storage (Alard et al. 2005). Some typical plant species are naturally present throughout this microclimatic gradient (Fig. 5), from south-facing slopes with xerophilous grasslands to plateaus and north-facing slopes covered by mesophilous grasslands (Butaye et al. 2005b; Piqueray et al. 2007).

Figure 5 – Diversity of calcareous grasslands in terms of topography and exposure, some south-facing slopes with xerophilous grasslands and some plateaus covered by mesophilous grasslands.

In Belgium, calcareous grasslands are mainly localized in five regions (Fig. 6): (1) Viroin valley; (2) Meuse valley and tributaries (Molignée, Fonds de Leffe); (3) Lesse and Lomme valleys; (4) Ourthe and Aisne valleys and (5) Montagne-Saint-Pierre (Visé).

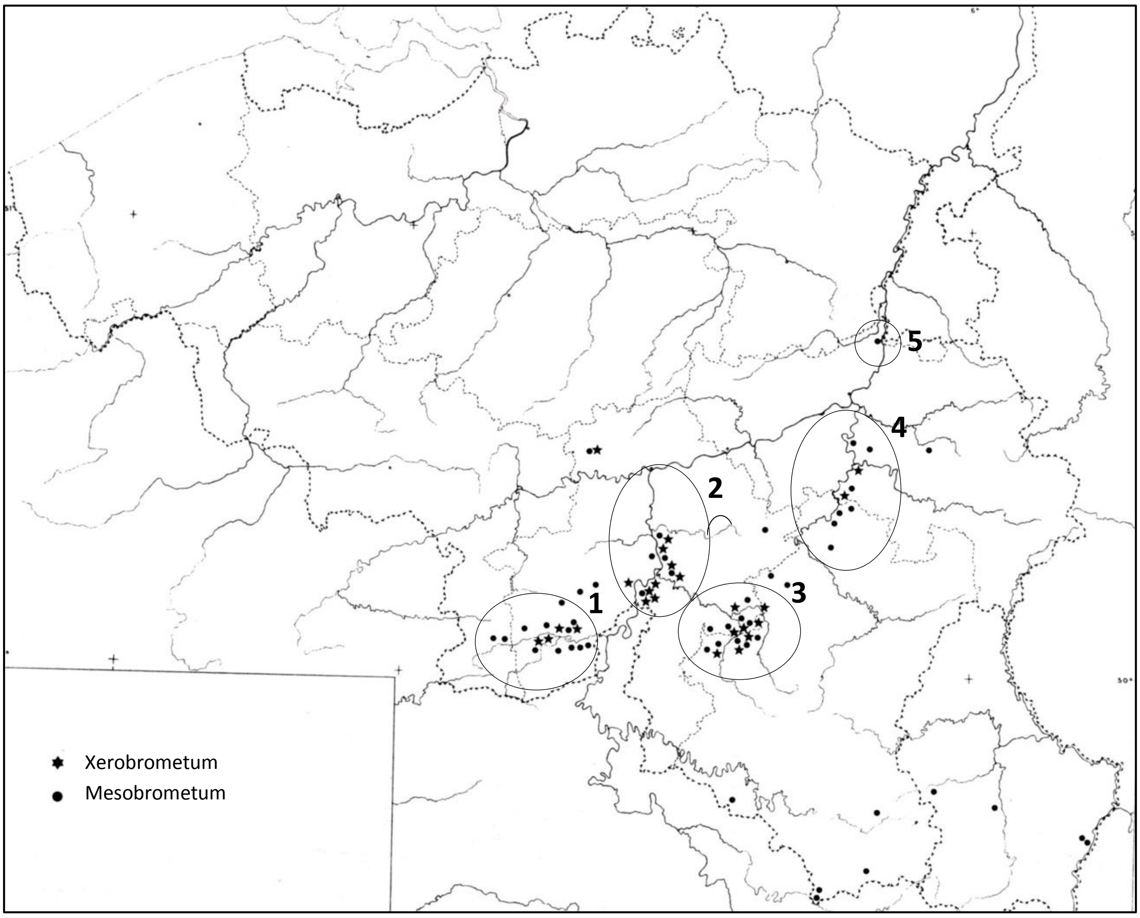

Figure 6 – Location of main Belgian calcareous grasslands (from Gehu 1984): (1) Viroin valley, (2) Meuse valley and tributaries, (3) Lesse and Lomme valleys, (4) Ourthe and Aisne valleys and (5) Montagne-Saint-Pierre (Visé).

Belgian calcareous grasslands emanated from traditional agro-pastoral practices, primarily sheep herding (WallisDeVries et al. 2002). They are semi-natural communities requiring management by grazing or mowing to prevent successional dynamic toward forest (WallisDeVries et al. 2002). Since the eighteenth century, a large proportion of calcareous grasslands has been afforested and other areas have been progressively replaced by oak woodlands through natural succession following abandonment of traditional agro-pastoral practices (WallisDeVries et al. 2002). In Belgium, more than 90% of the areas have been lost since the nineteenth century (WallisDeVries et al. 2002; Piqueray et al. 2011c). In the Viroin valley, the total area of calcareous grasslands decreased from more than 4000 ha in 1775 to less than 75 ha in 2003 (Fig. 7; Adriaens et al. 2006).

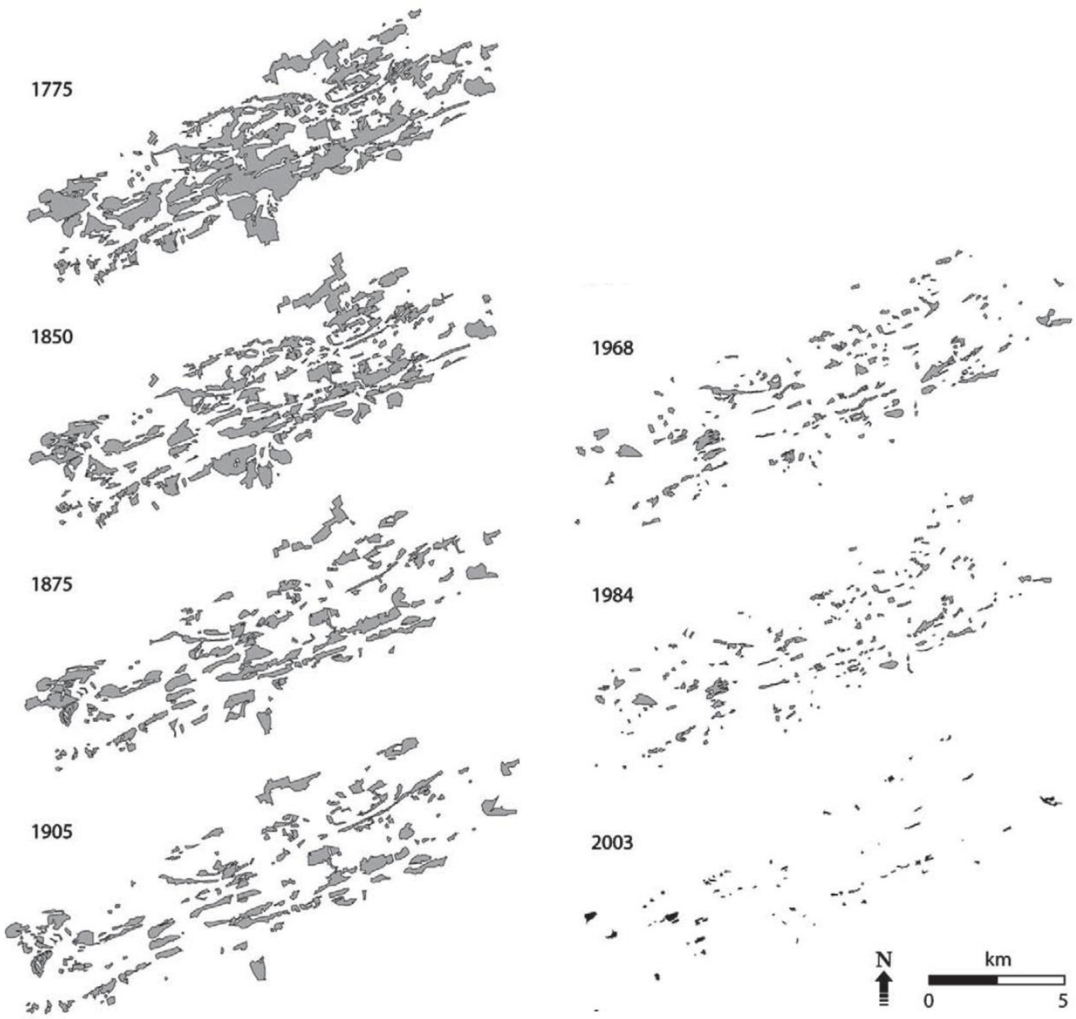

Figure 7 – Calcareous grasslands fragmentation in the Viroin valley between 1775 and 2003 (Adriaens et al. 2006).

Thanks to restoration projects that took place in Belgium between 2000 and 2014, especially via three European LIFE projects targeting calcareous grassland restoration, calcareous grassland area has increased gradually. In the Viroin valley, calcareous grasslands reached more than 150 ha in 2010. All remaining and restored grasslands are managed through grazing and mowing.

Belgian calcareous grasslands belong to the *Festuco-Brometea*. They typically consist of a mixture of dominant grasses and dicotyledons. Dominant grasses are *Brachypodium pinnatum, Bromus erectus* and *Festuca ovina*. *Avenula pubescens*, *Briza media* and *Koeleria macrantha* are also frequent, but usually less abundant. Sedges that occur more or less frequently are *Carex caryophyllea* and *Carex flacca*. Among the dicotyledons, hemicryptophytes dominate, e.g. *Galium pumilum*, *Hieracium pilosella*, *Lotus corniculatus*, *Plantago lanceolata*, *Potentilla tabernaemontani*, *Sanguisorba minor* and *Scabiosa columbaria*. Chamaephytes generally number little, but *Helianthemum nummularium, Teucrium chamaedrys* and *Thymus praecox/pulegioides* are frequent within the community and can be locally dominant (Butaye et al. 2005b)[3].

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[3] Species name follows the nomenclature of LAMBINON et al. (2012)

Objectives and scientific strategy

Objectives

Grasslands have been targeted by restoration projects in many places around the world (Gibson-Roy et al. 2007) with the objective of improving ecological networks of habitats and reducing fragmentation along with its negative effects (Jongman and Pungetti 2004).

The first objective of this thesis was to assess the use of plant population parameters for evaluating grassland restoration.

We reviewed the scientific literature - based on more than 3000 papers published between 1986 and 2015 - to determine to what extent a population approach has been applied to evaluate the success of grassland restoration compared to higher levels of biodiversity organization, such as communities and ecosystems. We then detailed papers using population parameters in order to establish which parameters have primarily been examined and which were disregarded.

The second objective of this thesis was to assess calcareous grassland restoration success by comparing plant population density, reproductive performance and intra-specific functional variability in reference and restored populations, taking into account environmental variability. Our models were calcareous grasslands specialist plant species and calcareous grassland sites in Belgium.

Populations of calcareous grasslands plant species were chosen for this study due to three main reasons. First, calcareous grasslands have undergone habitat fragmentation followed by large-scale habitat restoration. Both processes are known to highly influence population functioning (Montalvo et al. 1997; Lienert 2004). Then, those ecosystems are characterized by a certain environmental variability at the local scale, particularly in terms of water availability and drought stress (Bennie et al. 2008; Dujardin et al. 2012). Overall, this represents an opportunity to study variability of plant traits at the local scale in response to environmental heterogeneity. Finally, these dry ecosystems are already subjected to drought stress and could be even more threatened by future climate change. It is probable that the frequency of summer heat waves will increase in large parts of Europe (Pachauri et al. 2014). Studying the functioning of plant populations of calcareous grassland is therefore of prime importance for species conservation in a context of fragmentation, restoration and climate change.

Study area and sites

The calcareous grassland sites selected for this work were located in two Belgian regions (Fig. 8). The first was the Calestienne region, a five-km wide and 100-km long belt of Devonian geological formations with limestone or calcareous-siliceous rocks. This region is characterized by a hilly landscape with isolated calcareous grasslands within a matrix of forests and enriched meadows or arable land. Calcareous grasslands are present on limestone hills originated from former coral reefs. The second region, the Meuse valley, is a carboniferous formation characterized by limestone-enriched schist with higher soil acidity. Despite the loss of calcareous grassland since the nineteenth century, these regions are considered the core areas for calcareous grassland conservation in Belgium.

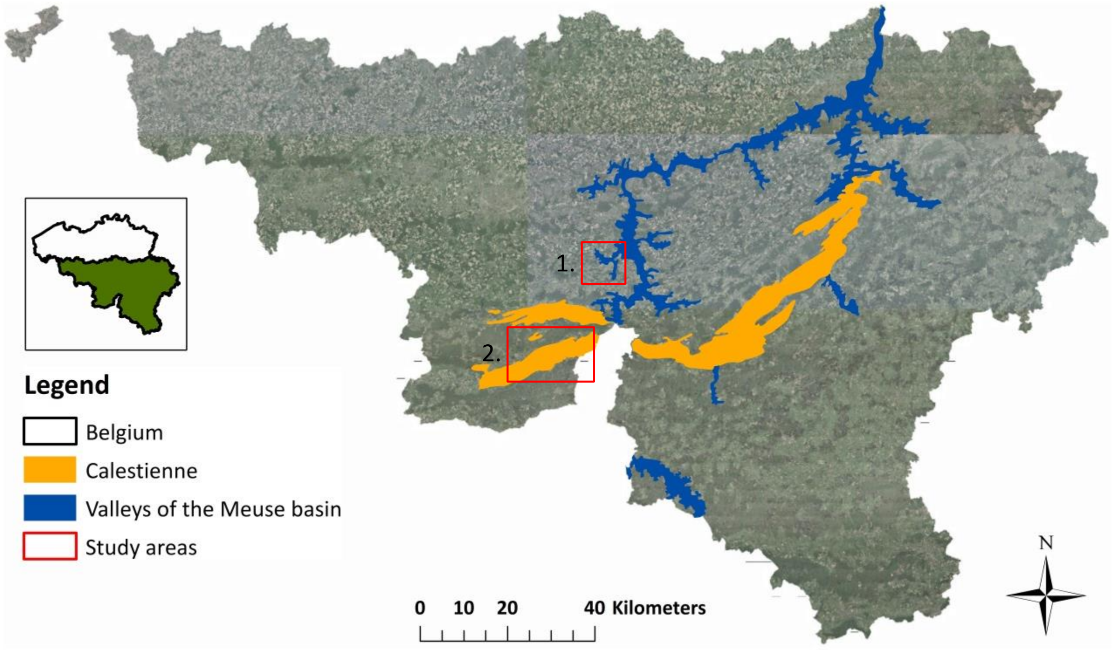


Figure 8 – Study regions and study areas in Belgium: 1) the Molignée valley in the Meuse basin; and 2) the Viroin valley in the Calestienne region.

The study sites were localized in two areas of those regions - the Molignée valley (Meuse basin) and the Viroin valley (Calestienne region). Six calcareous grasslands were selected in the two study areas (Fig. 9–10): the Sosoye hill (50°17′ N, 4°46′ E, alt. 170–200m) in the Molignée valley and the Tienne Breumont (50°04′ N, 4°32′ E, alt. 200–215m), the Abannets (50°04′ N, 4°33′ E, alt. 220–225m), the Montagne-aux-buis (50°05′ N, 4°34′ E, alt. 215–240m), the Tienne Delvaux (50°05′ N, 4°36′ E, alt. 220–230m) and the Rivelottes (50°05′ N, 4°39′ E, alt. 170–180m) in the Viroin valley. Climate of the two areas is characterized by annual mean rainfall comprises between 900 mm and 1000 mm and annual mean temperature comprises between 9°C and 10°C.

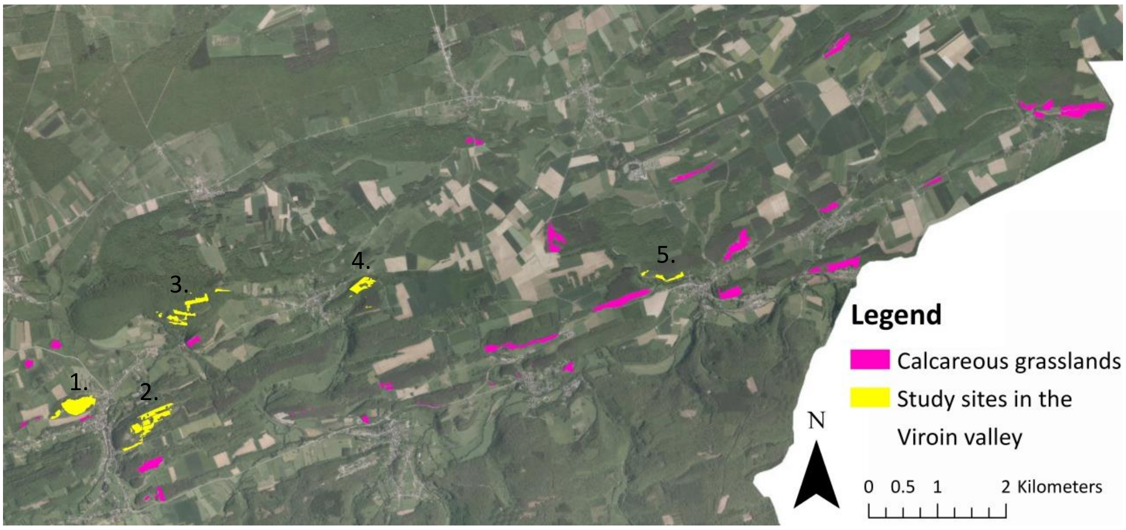


Figure 9 – Calcareous grasslands (pink) and selected sites (yellow) in the Viroin valley: 1) Tienne Breumont, 2) Abannets, 3) Montagne-aux-buis, 4) Tienne Delvaux and 5) Rivelottes.

At each site, reference grasslands coexist with restored grasslands (Fig. 10). Our reference are calcareous grasslands known to have existed for more than two centuries and considered the reference ecosystem for restoration (SER 2004). These communities were previously described by Butaye et al. (2005b) in the Viroin valley. Restored grasslands have been derived from 40–100 year-old forests of oak coppices or pine stands established on former calcareous grasslands. Pine stands were *Pinus nigra* or *Pinus sylvestris* plantations. Dense shrub oak coppices were mainly formed with *Prunus spinosa*, *Crataegus monogyna* and *Corylus avellana*, with intermingled scarce trees of *Quercus robur* (Piqueray et al. 2011b). Restored grasslands communities were described in the Viroin valley by Piqueray et al. (2011b). Main restorations have been realized between 2002 and 2006 as part of a European LIFE project (LIFE “Haute-Meuse” focusing on dry grassland restoration). Restoration protocols included trees and shrubs clearing followed by sheep and goat grazing (Fig. 11). Trees and shrubs were exported from the site, but tree stumps remained and a management of stump sprouts was applied the first years following restoration (Delescaille 2005). Restoration relied on seed rain from the surrounding grasslands, soil seed bank and migrating sheep flocks. No seeds were sown manually.

Reference and restored grasslands are currently managed by extensive grazing of migrating sheep or goat flocks (duration: two to three weeks per year, resulting in a grazing intensity of one to two sheep ha^{-1} $year^{-1}$). Restored grasslands are grazed each year, reference grasslands every two or three years depending on site.

Figure 10 – Study sites with restored grasslands (open areas) around reference grasslands (in red) within a matrix of forests and improved meadows or arable lands. Selected sites are: a) the Sosoye hill, in the Molignée valley, and b) the Montagne-aux-buis, c) the Tienne Delvaux, d) the Rivelottes, e) the Abannets, and f) the Tienne Breumont, in the Viroin valley.

Figure 11 – Illustrations of calcareous grassland restoration protocols including (from top left to bottom right) trees and shrubs clearing followed by sheep grazing and management of stump sprouts.

Piqueray et al. (2011b) studied soil characteristics of twelve calcareous grassland sites in the Viroin valley and in the Lesse and Lomme valleys. The Montagne-aux-buis, the Rivelottes and the Abannets sites were among selected sites. Soil characteristics of reference grasslands were described (Table 1) and compared with restored grasslands (3 ages: 2–4 years, 5–8 years and 10–15 years) and with pre-restoration forests. They uncovered no significant differences in soil nitrogen, phosphorous, potassium, carbon or iron content between restored and reference grasslands, but observed a decrease in the mineralization rate indicators in recent grassland restorations, which was resorbed in older restorations. Soil pH and soil depth were similar among reference and restored grasslands (Table 2).

Table 1 – Mean values and standard deviation (SD) of soil parameters in reference grasslands (modified from Piqueray et al. 2011b).

	N (g/100g)	P (mg/100g)	K (ppm)	C (g/100g)	Fe (ppm)	C:N ratio	pH	Soil depth (cm)
Mean	0.575	0.963	15.97	7.26	15.15	12.7	5.7	8.19
SD	0.119	0.263	5.22	1.4	2.96	1.4	1.2	2.74

Table 2 – Mean differences for soil parameters between reference grasslands, restored grasslands of different age classes and pre-restoration forests. (−) indicates a deficiency, (+) indicates a surplus. P-values are the result of ANOVA (soil parameter ~ type: restored grasslands of different ages, reference grasslands or pre-restoration forests). Significant p-values are in bold. Different letters indicate significant differences (modified from Piqueray et al. 2011b).

	Forest	Restored grasslands			Reference	p
		2–4 years	5–8 years	10–15 years		
N (g/100g)	-0.050	−0.026	+0.016	+0.002	0	0.660
P (mg/100g)	-0.012	+0.032	+0.032	+0.111	0	0.941
K (ppm)	+0.376	−0.7	+0.765	−0.63	0	0.811
C (g/100g)	-0.054	+0.463	+0.909	−0.04	0	0.882
Fe (ppm)	+3.95	+5.76	+2.84	+2.77	0	0.107
C:N ratio	+1.32^{a}	+1.35^{a}	+1.40^{a}	−0.093^{b}	0^{b}	**0.016**
pH	-0.5	−0.6	+0.3	+0.5	0	0.053
Soil depth (cm)	+0.66	+1.78	-0.91	−1.46	0	0.153

The same authors (Piqueray et al. 2011b) described vegetation parameters of the same sites and compared those parameters between reference and restored grasslands (Table 3). Species richness and the cover of grassland species was higher in reference grasslands than in recent restoration and tended to increase with increasing age of restored grasslands. Shrub cover was higher in recent restoration and decreased with increasing age of restoration. Bare ground cover was reduced in oldest restorations and was higher in recent restoration.

Table 3 – Mean values of vegetation parameters and comparison between reference grasslands, restored grasslands of different age classes and pre-restoration forests. P-values are the result of ANOVA (vegetation parameter ~ type: restored grasslands of different ages, reference grasslands or pre-restoration forests). Significant p-values are in bold. Different letters indicate significant differences (modified from Piqueray et al. 2011b).

	Forests	Restored grasslands			Reference	p
		2–4 years	5–8 years	10–15 years		
Species richness	6.3^{a}	15.0^{b}	16.8^{bc}	18.9^{bc}	20.3^{c}	**<0.001**
Grassland species cover (%)	24.6^{a}	37.8^{ab}	62.0^{bc}	72.9^{c}	73.6^{c}	**<0.001**
Forest species cover (%)	0.4^{b}	0.8^{b}	0.1^{a}	0.1^{a}	0.0^{a}	**0.001**
Shrubs cover (%)	22.0^{c}	12.4^{b}	8.7^{bc}	5.2^{ab}	2.3^{a}	**0.005**
Bare ground cover (%)	51.8^{c}	15.9^{b}	2.8^{a}	2.4^{a}	7.0^{ab}	**<0.001**

Study species: specialist forbs

Calcareous grasslands are one of the most species-rich ecosystems in temperate regions, especially concerning plant species (Prendergast et al. 1993; WallisDeVries et al. 2002). This plant diversity is mainly owing to forb species that account for the larger component of species richness (Butaye et al. 2005b; Piqueray et al. 2007). Grasses generally have higher cover, particularly in restored or successional sites with reduced grazing pressure (Piqueray et al. 2011b). Further, grasses typically establish well in restored grasslands, while the key difficulty in restoration is principally enhancing the performance of forb species (Pywell et al. 2003).

Based on a phytosociological classification of calcareous grasslands in the Viroin valley (Table 4, Butaye et al. 2005b), we selected five species specialist of the *Festuco-Brometea* (Fig. 12).

- *Helianthemum nummularium* (Cistaceae), a hermaphrodite herbaceous chamephyte with yellow flowers characterized by a protogynous maturation and pollinated by insects. Autogamy is possible for this species;
- *Hippocrepis comosa* (Fabaceae), a hermaphrodite herbaceous chamephyte with yellow flowers pollinated by insects;
- *Potentilla tabernaemontani* (synonyms *P. neumanniana*, *P. verna*) (Rosaceae), a hermaphrodite hemicryptophyte with yellow flowers pollinated by insects. Apogamy is possible for this species;
- *Sanguisorba minor* (Rosaceae), a polygamous hemicryptophyte with green or red flowers pollinated by wind and insects; and
- *Scabiosa columbaria* (Dipsacaceae), a gynodioecious hemicryptophyte with blue flowers characterized by a protandrous maturation and pollinated by insects.

Table 4 – Frequency (%) table of species of the *Festuco-Brometea* in relevés perfomed in the Viroin valley (modified from Butaye et al. 2005b). Species selected in this thesis are in bold.

Vegetation type	I	II	III	IV	V	VI	VII
Number of relevés	7	28	51	167	94	44	10
Festuco-Brometea							
Helianthemum nummularium	**29**	**89**	**65**	**72**	**46**	**25**	**-**
Hippocrepis comosa	**43**	**43**	**20**	**11**	**41**	**11**	**-**
Polygala vulgaris	29	32	-	5	30	9	10
Thymus pulegioides	29	4	18	17	46	-	-
Inula conyzae	43	39	10	5	2	16	-
Carex caryophyllea	14	-	22	59	35	11	10
Carex flacca	43	-	14	55	96	86	10
Festuca ovina	-	32	45	88	90	18	40
Sanguisorba minor	**-**	**7**	**39**	**92**	**93**	**36**	**10**
Potentilla tabernaemontani	**-**	**75**	**75**	**89**	**65**	**20**	**10**
Scabiosa columbaria	**-**	**11**	**8**	**41**	**45**	**2**	**-**
Thymus praecox	-	11	20	60	19	7	-
Teucrium chamaedrys	-	86	88	85	17	25	-
Koeleria macrantha	-	7	18	43	35	7	-
Brachypodium pinnatum	-	-	51	96	93	98	50
Allium oleraceum	-	-	10	3	-	5	10
Polygala comosa	-	-	2	9	6	-	-
Eryngium campestre	-	-	-	1	-	-	-
Phleum bertolonii	-	-	-	1	2	-	-
Euphorbia cyparissias	-	-	2	1	-	2	-
Platanthera bifolia	-	-	-	-	1	-	-

I = *Fragaria vesca-Atropa bella-donna* community; II = Carex humilis-Geranium sanguineum community; III = *Sesleria caerulea* community; IV = *Teucrium chamaedrys-Thymus praecox community*; V = *Brachypodium pinnatum* dominated community; VI = *Leontodon hispidus-Cirsium acaule* community; VII = *Agrostis capillaris-Cytisus scoparius* community.

Figure 12 – Specialists forbs selected: *Helianthemum nummularium* (a–d)*, Hippocrepis comosa* (e–h), *Potentilla tabernaemontani* (i–l), *Sanguisorba minor* (m–p) and *Scabiosa columbaria* (q–t). For each species, from left to right: buds, flowers, fruits, individuals.

These are forb species, i.e. herbaceous flowering plants that are not graminoids (grasses, sedges or rushes). Selected species are specialist plants of the *Festuco-Brometea*, which means that they mainly occur on dry grasslands of the *Festuco-Brometea* in the study regions, but may occasionally inhabit other habitat types (Krauss et al. 2004), such as low fertilized dry meadows. Among selected species, some are more specialized than others. Indeed, *S. minor* and *P. tabernaemontani* are more widely distributed in Belgium than are calcareous grasslands. Those two species are therefore less specialized as compared to *H. nummularium*, *S. columbaria* and *H. comosa*, which have a restricted range in Belgium, closely linked to calcareous grasslands distribution (Fig. 13). Selected species are also considered calcareous grassland specialists in other European regions, especially in Southern and Central Germany, in Switzerland and in the Netherlands (Willems 1982; Krauss et al. 2004). Specialist species have been selected because they are expected to be more affected by habitat degradation, fragmentation and environmental change and are experiencing higher extinction risk than generalist species (Devictor et al. 2008; Clavel et al. 2011).

All selected species are frequent in Belgian calcareous grasslands (more than 75% occurrence in plots in the Belgian calcareous grasslands; Adriaens et al. 2006) and have been chosen because they occurred on reference as well as on restored grasslands of the selected sites, which permitted multi-sites comparison.

These species are mainly pollinated by insects. They may therefore be particularly threatened by fragmentation and its effects, notably pollination failure (Goodwillie 1999; Wilcock and Neiland 2002). The species investigated are not dispersed effectively by wind (Poschlod et al. 1998) and seeds are mainly dispersed through epizoochory thanks to sheep and goats flocks migration among reference and restored calcareous grasslands (Müller-Schneider 1954; Fischer et al. 1996; Poschlod et al. 1998). Seed persistence in the soil has proven to be very long (> 25 years) for *P. tabernaemontani* and *S. minor*, moderate (6-20 years) for *H. comosa* and rather short (2-5 years) for *H. nummularium* and *S. columbaria* (Poschlod et al. 1998).

None of the selected species is protected by national laws. They are study models that permitted field sampling and experimental manipulations, which would not be possible with rare or protected species.

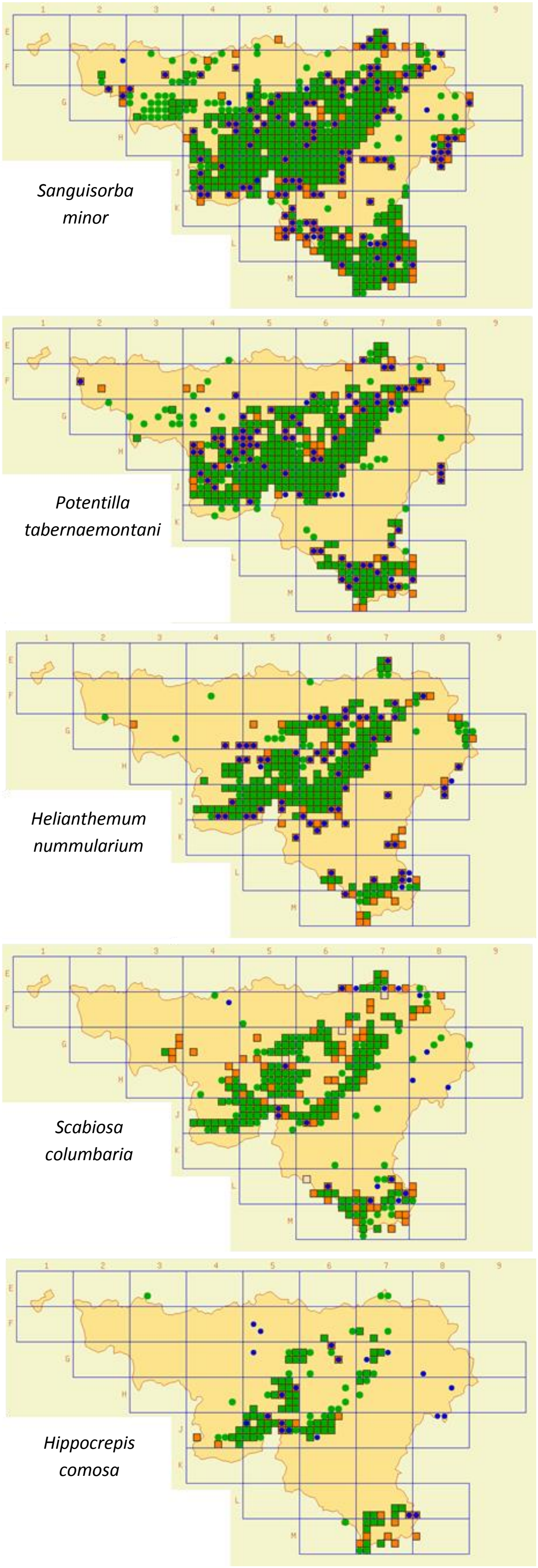


Figure 13 – Distribution range of selected species in south Belgium (Wallonia). Data from the "Atlas de la flore" (available from http://biodiversite.wallonie.be). Species occurrence (records: <1930=pink square, >1930=green square and blue circle, >1980=green circle).

Selected traits

The main traits that have been measured on selected plants were: 1) the specific leaf area (SLA), i.e. the one-sided area of a water-saturated leaf divided by its oven-dried mass (in $mm^2\ mg^{-1}$); 2) the leaf dry matter content (LDMC), i.e. the oven-dried mass of a leaf divided by its water-saturated fresh mass (in $mg\ g^{-1}$); 3) the vegetative height, i.e. the shortest distance between the upper boundary of the photosynthetic tissues (excluding inflorescences and inflorescence stalks, including leaves on inflorescence stalks) of the plant and the soil surface (in cm); and 4) the number of seeds produced by a plant (Weiher et al. 1999; Cornelissen et al. 2003; Pérez-Harguindeguy et al. 2013).

They are fundamental traits for comparative ecology across regions and scales (Weiher et al. 1999). SLA and plant height are functional traits linked to a plant's response to its environment and are linked to stress tolerance and resource use efficiency (Wilson et al. 1999; Garnier et al. 2001a; Westoby et al. 2002; Ackerly 2004). They represent essential trade-offs regulating plant strategies, such as opportunities for rapid growth and coping with disturbances, for example grazing and water deficit (Westoby 1998; Weiher et al. 1999). Conservative species that exhibit low SLA and low plant height (corresponding to dense leaf tissues and low growth rates) exhibit high resource conservation, notably that of water (Buckland et al. 1997; Volaire 2008; Poorter et al. 2009; Albert et al. 2010a). Inversely, exploitative species that exhibit high SLA and high growth rate are dominant in fertile areas and have a high resource acquisition capacity (Reich et al. 1999; Albert et al. 2010a). SLA and plant height are major traits related to plant establishment. This includes tolerating changes in resource availability, competing for or tolerating the competitive effects of plant neighbours and avoiding or coping with disturbances (Weiher et al. 1999). The number of seeds produced reflects plant fitness, i.e. a population's ability to produce offspring (Begon et al. 1990), one principal measure of plant performance and survival.

Over the past decades, functional traits have been increasingly used as reliable predictors of community responses to environmental gradients (McGill et al. 2006; Ackerly and Cornwell 2007; Pakeman et al. 2009). SLA and vegetative height have been widely studied in functional approach targeting plant communities, but they can also vary substantially within species (Albert et al. 2010a; Hulshof et al. 2013), and notably between (e.g. Jung et al. 2010; Baruch 2011; Andersen et al. 2012; Almeida et al. 2013; Pakeman 2013) and within populations (Bolnick et al. 2011; Mitchell and Bakker 2014b), the latter being poorly addressed thus far based on the literature.

Selected environmental variables

Like others dry ecosystems, calcareous grasslands are a stressful environment for plant species in term of water and nutrient availability in soils (Buckland et al. 1997; Kalicka et al. 2008). The amount of available water for plants varies notably according to habitat topography, exposure and soil depth (Alard et al. 2005; Bennie et al. 2006; Bennie et al. 2008).

The environmental conditions of the selected study sites were mostly distinguished by two variables linked to soil resource availability – soil depth (in cm) and the potential direct incident radiation (PDIR) index. PDIR is based on the ‘folded’ aspect (slope orientation), slope and latitude. It is dimensionless and ranges in value from 0.03 to 1.11, with higher values signifying high potential direct incident radiation (on south-facing steep slopes). Details of this equation are provided in McCune and Keon (2002). Soil depth and PDIR gradients are considered reliable proxies of soil water availability for plants (Alard et al. 2005; Bennie et al. 2008; Dujardin et al. 2012), but they may also influence soil nutrient availability and vegetation competition (Alard et al. 2005).

Thesis statements

The thesis is divided in two parts. The first part is a review of the scientific literature (**Chapter 2**). It aims to determine to what extent a population approach has been applied to evaluate the success of grassland restoration. In this review, only grasslands were considered. These ecosystems are endangered by land-use changes worldwide (Veldman et al. 2015a; Veldman et al. 2015b; Koch et al. 2016) and have been targeted by restoration projects in many places across the globe (Gibson-Roy et al. 2007). Grasslands are the second ecosystem most represented in restoration studies, just after forests (Wortley et al. 2013).

The second part of this thesis assesses calcareous grassland restoration success using a population approach. Our models were calcareous grasslands specialist plant species and calcareous grassland sites in Belgium. Traits that were studied are related to population demography (**Chapter 3**), reproductive performances (**Chapter 3**) and intra-specific functional response to environmental heterogeneity (**Chapters 4–6**).

Extent of intra-specific functional variability was observed and structured within and among populations. However, the source of observed variations was not addressed in this thesis. Observed intra-specific traits variability may stem from phenotypic plasticity, or it can be the result of variability between genotypes originating from sexual reproduction and evolutionary processes such as local adaptation (Fisher 1930; Joshi et al. 2001; Hughes et al. 2008). Carefully designed experiments are needed to highlight the source of the observed intra-specific variation. Those aspects were not in the scope of in this thesis but will be discussed in **Chapter 7** (general discussion).

The functional variability was within and among populations while environmental heterogeneity was considered within (local scale) and among (landscape scale) study sites.

Chapter structure

The strategy of the thesis is represented through a conceptual framework (Fig. 14), illustrated by chapter structure.

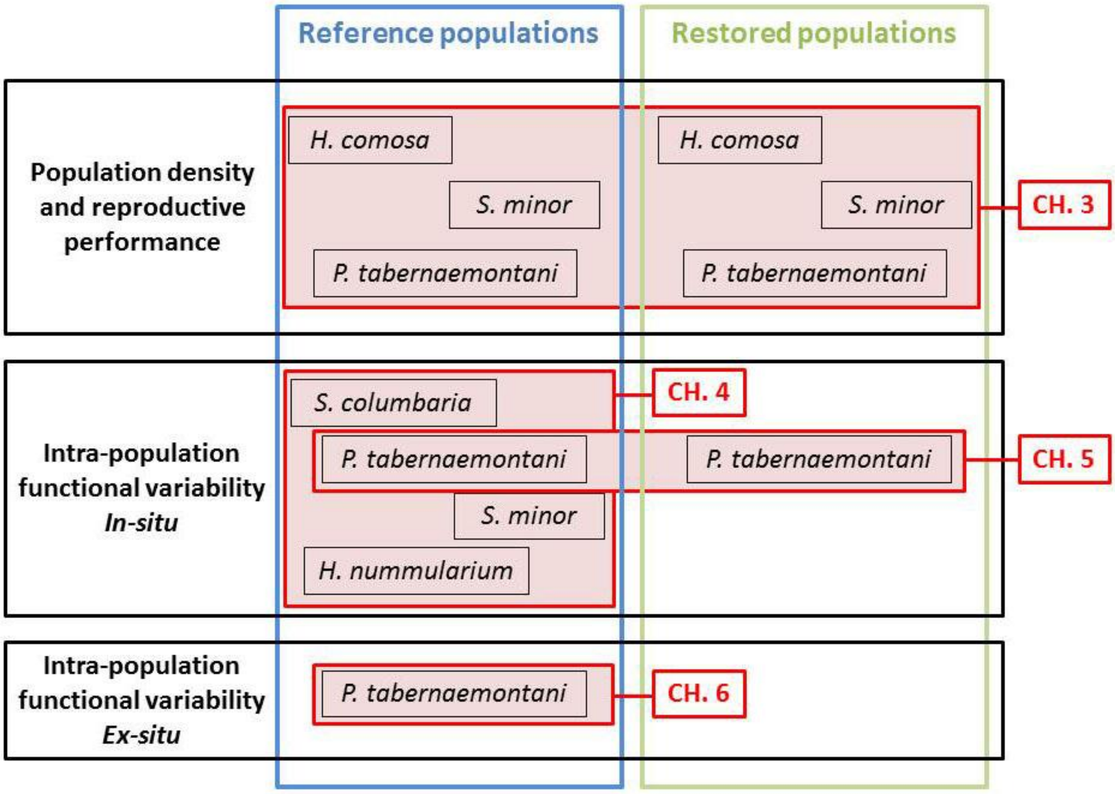


Figure 14 – Conceptual framework of the PhD thesis and chapter structure.

Population density and reproductive performance were compared in three sites, among reference and restored calcareous grasslands, for *H. comosa*, *S. minor* and *P. tabernaemontani* (**Chapter 3**).

Intra-population functional variability was characterized in situ, in three reference sites, for *S. columbaria*, *P. tabernaemontani, S. minor* and *H. nummularium* (**Chapter 4**).

Intra-population functional variability was characterized in situ, and compared among restored and reference grasslands located in five sites, for *P. tabernaemontani* (**Chapter 5**). The local and the landscape scales have been taken into account in the analysis.

Finally, the ex-situ functional variability of *P. tabernaemontani* was observed in a glasshouse experiment exposing individuals to high levels of drought stress (**Chapter 6**).

List of published, accepted or submitted papers

During the thesis, two papers have been published, one paper has been accepted with minor revisions (May 2017) and one paper has been submitted (March 2017):

- "Specialist plant species harbour higher reproductive performances in recently restored calcareous grasslands than in reference habitats"; Mélanie Harzé, Grégory Mahy, Jean-Philippe Bizoux, Julien Piqueray and Arnaud Monty; **published** in the journal **Plant Ecology and Evolution** in **2015** (volume 148, pages 181–190)

- "Functional traits are more variable at the intra- than inter-population level: a study of four calcareous dry-grassland plant species"; Mélanie Harzé, Grégory Mahy and Arnaud Monty; **published** in the journal **Tuexenia** in **2016** (volume 36, pages 321–336)

- "Towards a population approach for evaluating grassland restoration – a systematic review"; Mélanie Harzé, Arnaud Monty, Sylvain Boisson, Carline Pitz, Julia-Maria Hermann, Johannes Kollmann and Grégory Mahy; **accepted with minor revision** in the journal **Restoration Ecology** in May **2017**

- "Individuals from drier zones of calcareous grasslands survive drought stress better"; Mélanie Harzé, Grégory Mahy and Arnaud Monty; **submitted** in the journal **Plant Ecology and Evolution** in March **2017**.

CHAPTER 2:

Towards a population approach for evaluating grassland restoration – a review

The content of this chapter has been accepted (with minor revisions) as a review article in “Restoration Ecology”

(May 2017)

Abstract

Persistence of restored populations depends on growth, reproduction, dispersal, local adaptation, and a suitable landscape pattern to foster meta-population dynamics. While the negative effects of habitat fragmentation on plant population dynamics are well understood, particularly in grasslands, the population parameters that control grassland restoration are less known. We reviewed the use of population parameters for evaluating grassland restoration success based on 141 publications (1986–2015). The results demonstrated that population demography was relatively well-assessed but detailed studies providing information on key stages of the life cycle were lacking despite their importance in determining population viability. Vegetative and generative performances have been thoroughly investigated, notably the components of plant fitness, such as reproductive output, while genetic and spatial population structures were largely ignored. More work on population response to ecological restoration would be welcome, particularly with a focus on population genetics. Evaluation of restoration success should be conducted at different levels of biodiversity organization, but so far, communities and ecosystems are over-represented. More focus should be directed toward a population approach as population parameters are essential indicators of restoration success.

Introduction

Over the past decades, there has been an increased destruction and fragmentation of natural and semi-natural habitats in many parts of the world (Balmford et al. 2005; Kuussaari et al. 2009; Deák et al. 2016). Fragmentation has negative effects on population size and connectivity, thus affecting plant fitness and leading to elevated risks of extinction (Lienert 2004; Leimu et al. 2006). Small and isolated populations are more exposed to environmental and demographic stochasticity, genetic drift and inbreeding, that can negatively impact their genetic structure, fitness and demography (Lienert 2004). As sessile organisms, plants are particularly threatened by habitat fragmentation (Young et al. 1996), and the consequences for plant populations have been intensely studied, e.g. in grasslands (Lienert 2004; Bowman et al. 2008; Adriaens et al. 2009; Vanden Broeck et al. 2015). Many grasslands are endangered by land-use change, such as arable utilization, afforestation and urban sprawl (Veldman et al. 2015a; Veldman et al. 2015b; Koch et al. 2016). Those changes are most prominent in temperate grasslands that are also influenced by eutrophication (Hoekstra et al. 2004). In addition to conservation measures, ecological restoration has become necessary to ensure long-term viability of threatened grassland plants. Grasslands have been targeted by restoration projects in many places of the world (Gibson-Roy et al. 2007) with the aim of improving ecological networks and reducing fragmentation along with its negative effects (Jongman and Pungetti 2004).

Restoration includes of a wide range of actions, from increasing the number of individuals of endangered species to recreating the reference ecosystem after land-use changes. According to the Society for Ecological Restoration (SER 2004), the ultimate goal of restoration projects is to develop an ecosystem that is self-supporting and resilient to perturbations. Numerous criteria can be employed to evaluate the success of restoration interventions (Hobbs and Norton 1996; Piqueray et al. 2011b; Harzé et al. 2015), including different disciplines (Wortley et al. 2013) as well as various levels of biodiversity organization, i.e. individuals, populations, communities, ecosystems and landscapes (Ruiz-Jaen and Aide 2005a; Cristofoli and Mahy 2010). According to Ruiz-Jaen and Aide (2005a), most studies use three community or ecosystem attributes to assess restoration success, i.e. vegetation structure, species diversity and ecosystem processes, while population parameters are less represented. Among the nine attributes listed by the Society for Ecological Restoration (SER 2004) to determine when restoration has been accomplished, none directly relate to population attributes. Indirectly, however, attributes number five ("restored ecosystem apparently functions normally for its ecological stage of development, and signs of dysfunction are absent") and eight ("restored ecosystem is sufficiently resilient to endure the normal periodic stress events in the local environment that serve to maintain the integrity of the ecosystem") may include population parameters. However, population biology can provide useful indicators of restoration success, and already, Montalvo et al. (1997) highlighted the application of population biology to ecological restoration. They advocated that a restoration should be considered successful when species are re-established to a level that allows their long-term persistence in the landscape. Thus, restored populations must possess characteristics allowing their reproduction, growth, dispersal and adaptation to the new environment.

Montalvo et al. (1997) also identified gaps in population biology that could be addressed in the context of ecological restoration. They proposed five research areas of particular importance linked to questions posed by restoration practitioners. One of these areas is related to population dynamics in fragmented landscapes, i.e. "the influence of the spatial arrangement of landscape elements on meta-population dynamics and population processes such as migration". They advocated that there was a lack of knowledge concerning the effects of isolation on processes, like adaptation and gene flow, and their impacts on the survival and dynamic of restored populations or meta-populations. They underscored the use of demographic data and, notably, transition matrix models to explore population viability (Menges 1990) and argued that studies on genetic diversity and structure are necessary to better comprehend meta-population dynamics and long-term population viability (Hastings and Harrisson 1994).

Twenty years after Montalvo et al. (1997), we assessed how these recommendations have been taken into account with respect to grassland restoration. Specifically, we have addressed the following questions: i) To what extent has a population approach been used to evaluate the success of grassland restoration?; ii) Which population parameters have mainly been examined? and iii) How often have meta-population dynamics been considered in ecological restoration? To this end, we reviewed the scientific literature and concentrated on grassland restoration and plant species population indicators.

Material and methods

A review of the literature was conducted using the electronic database “Scopus” with the following search terms (1986–2015): “grassland* AND (restoration OR reclamation OR rehabilitation)” in the title, keywords or abstract; only publications with an English abstract were selected.

To what extent has a population approach been used to evaluate the success of grassland restoration?

The resulting papers were individually screened and classified into four categories based on title, abstract and content if needed with a dichotomous key (Fig. 1). (1) Papers that were not evaluating the results of grassland restoration were discarded as “not grassland restoration”. An ecosystem was considered a “grassland” when the authors employed the term grassland either in the title, keywords or abstract. Certain actions must have been realized in the context of a degraded ecosystem with the aim to restore, create or rehabilitate grassland, irrespective of the state of the ecosystem before. (2) Papers evaluating the results of grassland restoration but not dealing with plant species were discarded as “not plant species”. (3) Papers evaluating the results of grassland restoration, addressing plant species but not using a population approach were removed as “not population approach”. (4) Finally, papers assessing the results of grassland restoration with plant species and that took a population approach were selected (“selected paper”).

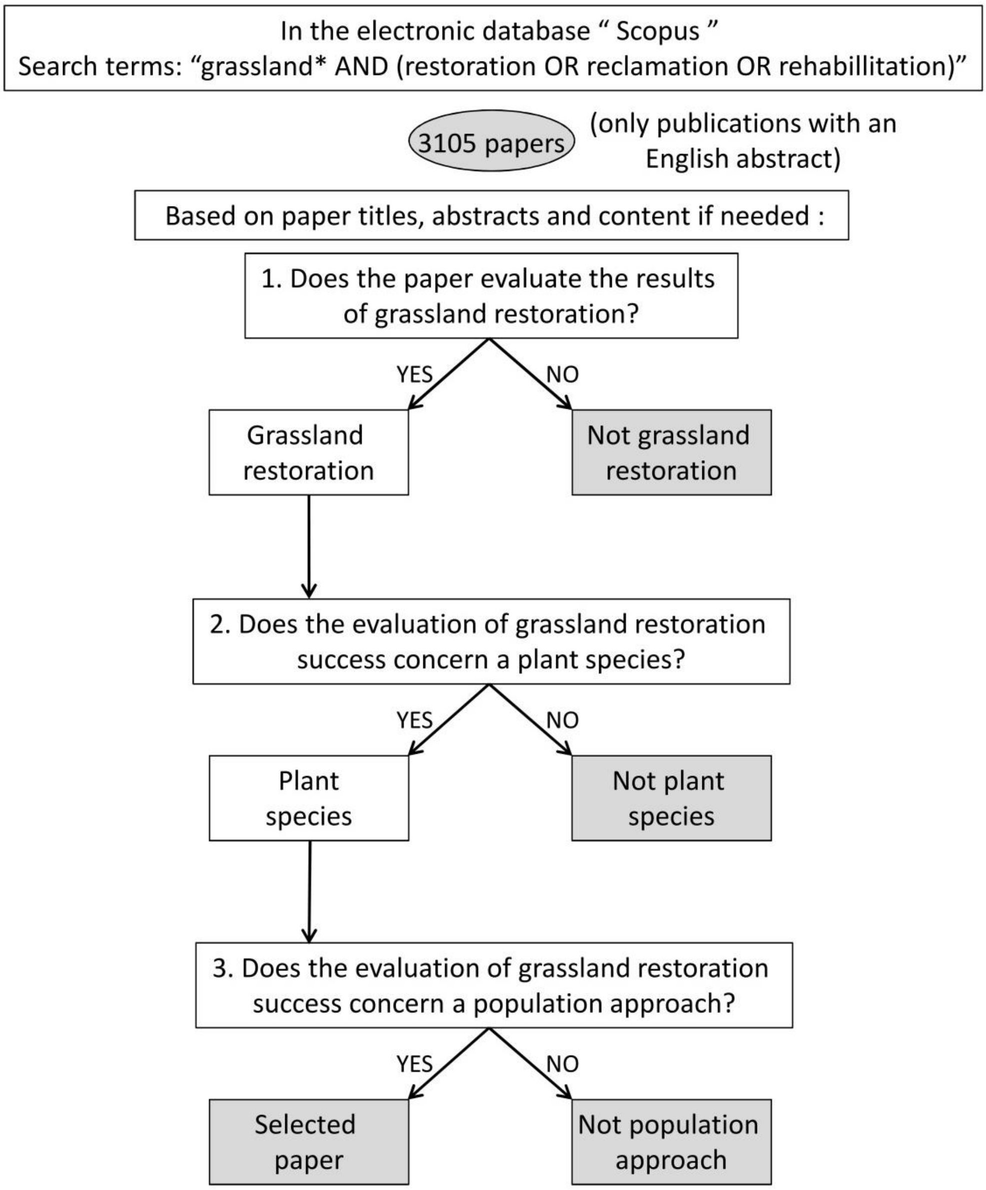


Figure 1 – A dichotomous key was utilized to classify papers into four categories ("not grassland restoration", "not plant species", "not population approach" and "selected paper") based on titles, abstracts and content if needed.

All papers were sorted by the first author. To determine reproducibility, a subset of 315 papers (ca. 10%) was independently classified by three other scientists (Sylvain Boisson and Carline Pitz from the University of Liège, Gembloux Agro-Bio Tech and Julia-Maria Hermann from the Technic University of Munich) following the same protocol. The quality of reviewing these papers was established by the percentage of agreement between the reviewers using Cohen's Kappa statistic (κ), which adjusts the proportion of records for which there is agreement by the amount of agreement expected by chance alone (Cohen 1960). Agreement among the reviewers was substantial ($\kappa > 0.6$) between one pair, and moderate ($\kappa > 0.4$) between five pairs of reviewers; agreement can be considered "fair" when $\kappa > 0.2$ (Landis and Koch 1977).

Which population parameters have mainly been examined and how often have meta-population dynamics been considered in ecological restoration?

The selected papers were carefully examined and the following information was traced in the text: (1) study location; (2) study species, including number, functional type (grass, forb, shrub, fern) and plant descriptors (native, invasive, weed, rare, common, dominant, typical); (3) population parameters for evaluating restoration success; (4) time since restoration and years of post-restoration monitoring; and (5) research area of the paper. The population parameters recorded were grouped into six classes according to the literature (Silvertown and Charlesworth 2001; Ricklefs and Miller 2005) and expert recommendations; individual papers may have utilized more than one parameter class (Table 1). The first three classes of population parameters concerned population structure and were divided into: 1.) genetic structure (allele frequency and genotypes); 2.) spatial structure (distance between individuals, localization, density, etc.); and 3.) demographic structure (population size, age and stage structure, etc., at one time only: static information). Population performance was then divided into: 4.) vegetative performance and 5.) reproductive performance. The last class of parameters concerned changes in the demographic structure through time based on population dynamics, also called 6.) population demography (Silvertown and Charlesworth 2001; Ricklefs and Miller 2005). All selected papers are listed in annex (Annex 1: Table S1) with the classes of parameters used for the population approach of each paper.

Table 1 – Population parameters were grouped into six classes according to the literature and expert recommendations.

Classes of population parameters	
1.	Genetic structure
2.	Spatial structure
3.	Demographic structure
4.	Vegetative performance
5.	Reproductive performance
6.	Demography

Results and discussion

A total of 3105 papers with English abstract have been screened and classified using a dichotomous key (Fig. 2); 66% were not dealing with grassland restoration.

Few papers employed a population approach to evaluate grassland restoration

Among the 1056 papers evaluating grassland restoration success surveyed, 66.3% used plant taxa as indicators (Fig. 2). As interventions to restore grasslands are essentially focused on vegetation management or plant species addition, and as targeted habitats are mostly described by vegetation composition, it is consistent that restoration evaluation focused on plants. It was already noted by Ruiz-Jaen and Aide (2005a) that plant richness was the most common measure of diversity recovery.

Among the 700 papers utilizing plant taxa to assess grassland restoration, 79.9% did not include population parameters, and instead mainly focused on community or ecosystem attributes (Fig. 2). Hence, population biology is still not fully included in the evaluation of grassland restoration success. This may be explained by the fact that the standardized methods proposed by the Society for Ecological Restoration (SER 2004) to determine accomplishing restoration are related to higher levels of biodiversity organization than populations. The European Commission also concentrated on restoration in terms of ecosystem and their services (Decleer 2012). Moreover, population studies, primarily addressing several target species, have often been criticized because the information recorded is considered too restricted compared to the diversity of ecosystem components (Franklin 1993). A population approach was considered particularly relevant when targeting keystone, umbrella, indicator, rare or threatened species (Carignan and Villard 2002; Cristofoli and Mahy 2010). Roughly 60% of the selected papers considered one or two species, while only 12% of the selected papers considered rare or threatened species. Other targeted species were typical, common or dominant native species or invasive species.

Plant invasions are a major threat to ecosystems (Usher 1988; Hobbs 2000) and can drastically diminish the chances of successful restoration (Johnston 2011). Therefore, weeds and invasive species were particularly well-studied - one-third of the selected papers evaluated the success of restoration of invaded habitats - predominantly testing protocols for their reduction. This was related to 31 different taxa (12 forbs, 11 grasses, 7 woody species and 1 fern). California grasslands experienced one of the most drastic biological invasions, with almost complete conversion from native to exotic annuals (Hamilton 1997). Restoration of invaded grasslands and the way local grassland populations dealt with invasive was therefore quite extensively investigated, particularly in North America (e.g. Kimball and Schiffman 2003; Gillespie and Allen 2004; Moyes et al. 2005; Cox and Allen 2011).

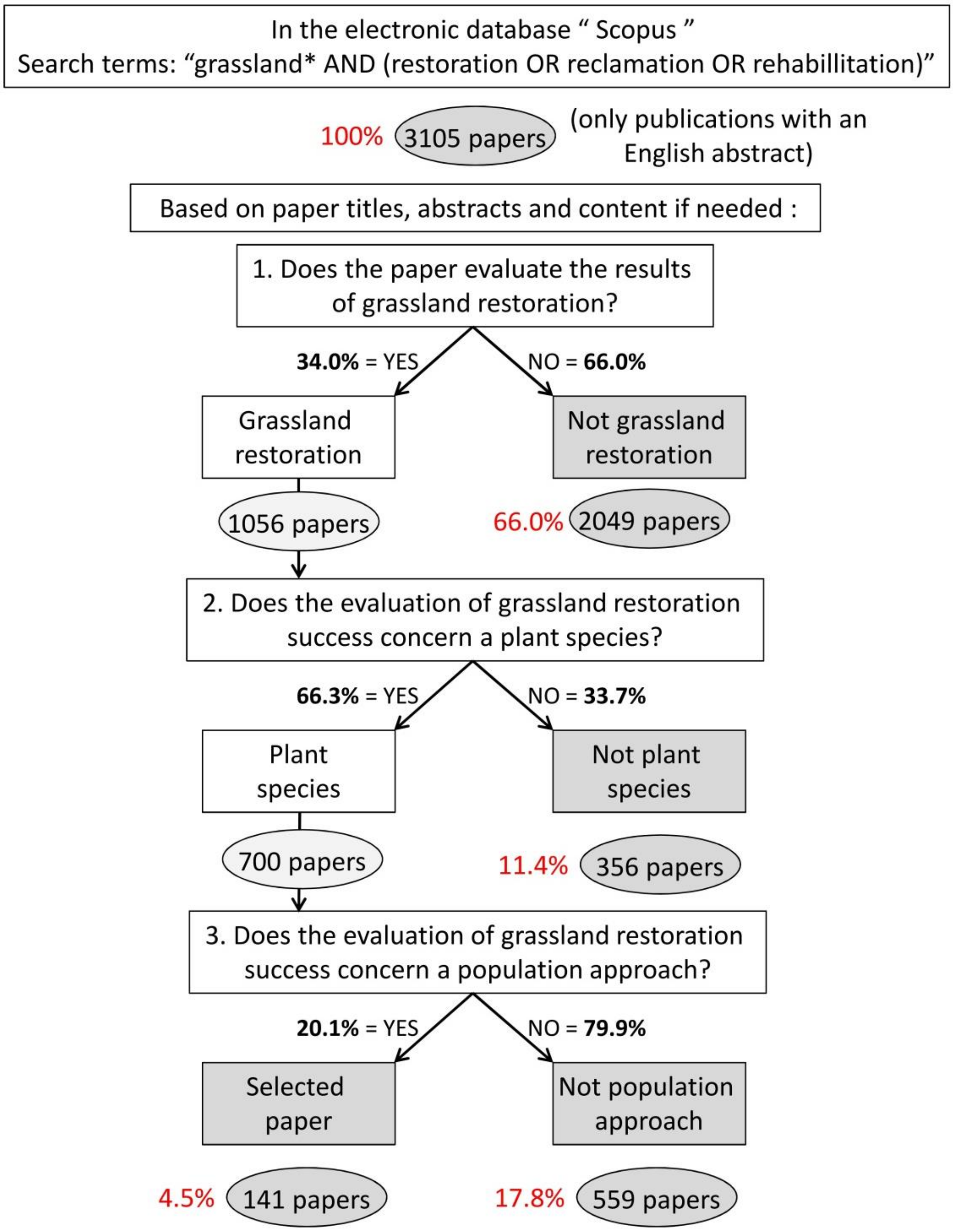


Figure 2 – Results of papers classification into: "not grassland restoration", "not plant species", "not population approach" and "selected paper", using the dichotomous key.

Study sites of selected papers were principally localized in North America and Europe, with three main regions where study took place: California (USA), South England (UK) and Bavaria (South Germany; Fig. 3)

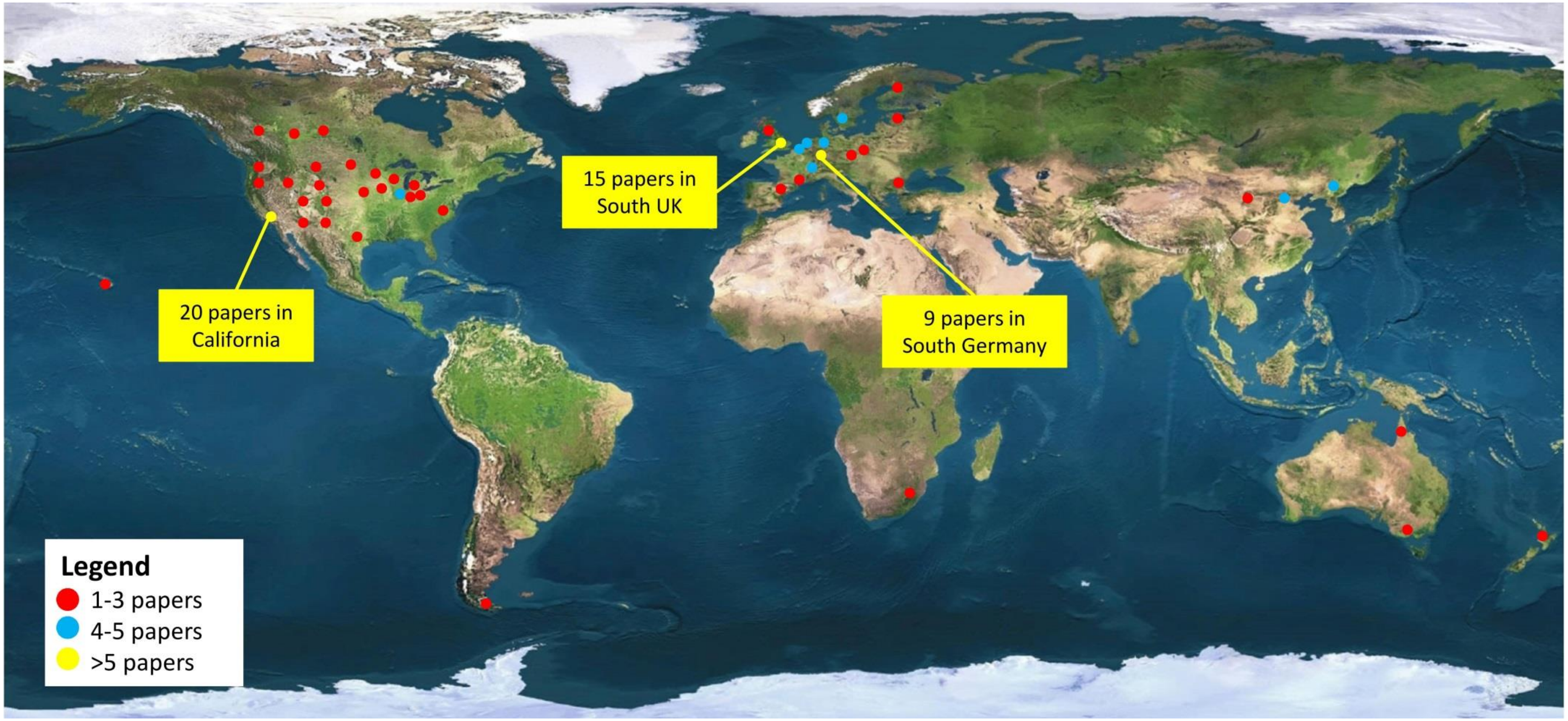


Figure 3 – Approximate location of study sites (grasslands) in selected papers (red points represent one, two or three papers, blue points represent four or five papers and yellow points represent more than five papers).

Genetic and spatial population structures are largely ignored

Demography describes changes in population size or changes in the demographic structure through time. It was the class of population parameters most often employed to evaluate grassland restoration (77%, Fig. 4). Specifically, this concerned parameters including number of emerged seedlings or individual survival through time, using one to 64 different species, mostly forbs. Detailed demographic processes were poorly studied - just 8% of the selected papers followed individual fate and, among them, only 3% detailed demographic models of population growth and identified critical life history stages for population survival. Demographic studies providing information on the most crucial stages of the life cycle are useful for evaluating population viability (Lande 1988; Oostermeijer et al. 2003), and allow predictions of future growth of populations (Schemske et al. 1994; Oostermeijer et al. 2003). Moreover, demographic studies are usually considered of more immediate importance than population genetics for determining short-term population viability (Lande 1988). Indeed, decreases in the success of a critical step during the life cycle may directly affect population recruitment many generations before negative genetic effects appear (Lande 1988; Ouborg et al. 1991; Morgan 1998). On the other hand, such pluriannual demographic studies are time-consuming and much effort is expended by researchers compared to less detailed demographic studies or those targeting simply one stage of population dynamic.

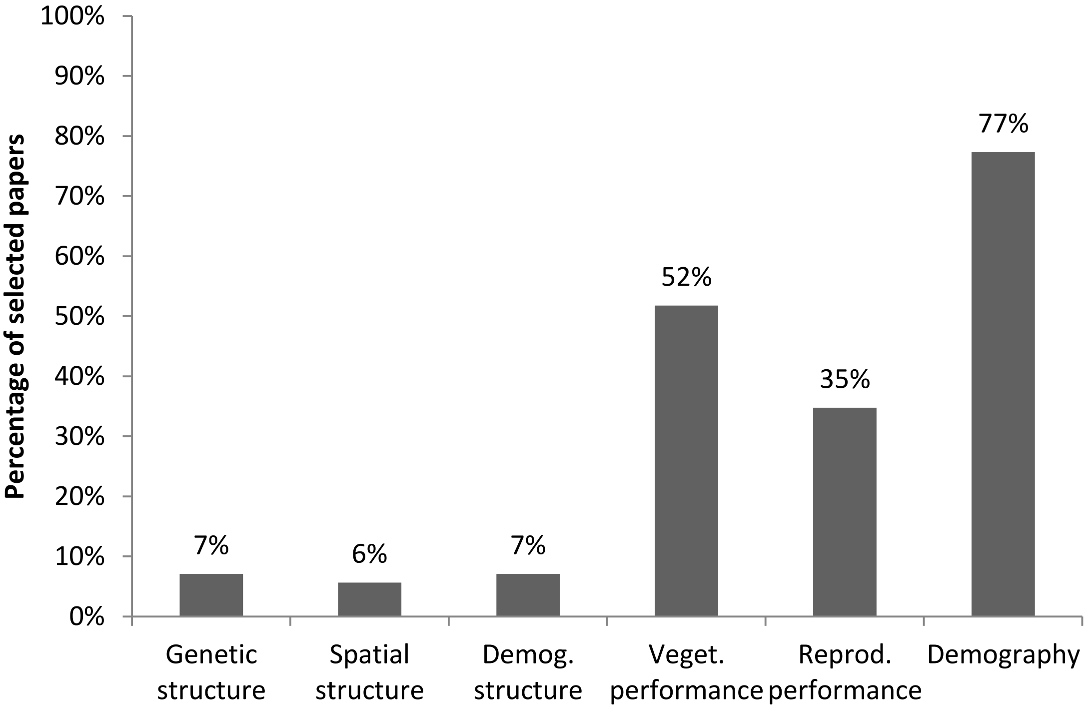


Figure 4 – Percentage of selected papers (n_{tot} = 141) using population parameters to evaluate restoration success. Parameters were grouped in six classes according to the literature and experts recommendations. One paper may use more than one class of parameters.

Population performances were the second most assessed classes of parameters, primarily encompassing vegetative performance (52%, Fig. 4), mainly described through above-ground biomass and vegetative height (Table 2). Reproductive performance (35%, Fig. 4) was principally described through flower, seed and fruit production (Table 2). Vegetative and reproductive performances are often linked, and the final measure of population performance is individual fitness, i.e. population ability to produce offspring (Begon et al. 1990). Morphological traits influence reproductive traits and, in turn, final fitness. According to Violle et al. (2007), the three major components of plant fitness are vegetative biomass, reproductive output (number of seeds produced) and plant survival, and all have been relatively well-assessed with regards to their applicability in evaluating success of grassland restoration to date.

Table 2 – Traits used to describe vegetative or reproductive plant performances (measured at the individual level). A paper may have employed more than one trait to describe population performances, including traits describing both vegetative and reproductive performances.

Traits used to describe vegetative performance	**Papers (%, n=73)**	**Traits used to describe reproductive performance**	**Papers (%, n=49)**
Aboveground biomass	55	Flower production	63
Vegetative height	48	Seed production	31
Number of stems	22	Fruit production	18
Size (basal diameter or area)	16	Flower or seed or fruit size	16
Belowground biomass	10	Seed germinability	14
Leaves number or size	8	Reproductive height	12
Observed vegetative vigour	5	Reproductive biomass	8
		Recruitment	6

The genetic structure of restored populations was addressed in 10 papers (7%, Fig. 4) via two different approaches.

First, five papers assessed the impact of restoration by seeding and/or the impact of seed origin (local vs non-local) on the population genetic structure of one to three species, including seven forbs and six grasses. Baer et al. (2014) observed that cultivars of *Sorghastrum nutans* were genetically different from populations of the regional ecotype, while the genetic diversity of the two seed sources was similar. In contrast, Selbo and Snow (2005) as well as Gustafson et al. (2004) found no differences in genetic diversity between local remnant and seeded populations or cultivars of *Andropogon gerardii* and *Sorghastrum nutans*. Smulders et al. (2000) detected a significant founder effect caused by the reintroduction of a limited number of seeds for *Cirsium dissectum* and *Succisa pratensis*. Finally, Delaney and Baack (2012) assessed the risk of hybridisation for 38 species (11 grasses and 27 forbs) between remnant and introduced genotypes that would potentially lead to outbreeding depression. They found that restorations were likely to create mixed ploidy populations, leading to lower reproductive success. Restoration genetics is a field that arose from the increasing need for species translocation with the intent of restoration (Young et al. 2005). It has provoked new questions concerning the consequences of seed sampling protocols or local adaptation (Hufford and Mazer 2003). More genetic research combined with long-term monitoring is necessary to establish the success of plant species' population restoration and to evaluate their evolutionary potential in the face of future environmental change.

Secondly, five papers addressed the genetic consequences for populations restored in fragmented landscapes. We will discuss this point later (p 48).

The class of population parameters least employed to evaluate grassland restoration was the spatial structure of populations. It was addressed in eight papers (6%, Fig. 4) that primarily focused on forbs. Overall, those papers assessed the recolonization ability of targeted species and tested whether species dispersal was the limiting factor for a successful restoration. This was the case for *Silaum silaus* and *Serratula tinctoria* in restored floodplain grasslands (Bischoff 2000; Bischoff 2002) and for 16 species of semi-natural grasslands (Öster et al. 2009a). The role of mobile links, such as sheep (Freund et al. 2014), was also studied with regards to grassland restoration. In the appraisal of grassland restoration, recruitment ability as a limiting factor to restore populations was more often evaluated than species dispersal. This may be explained by the fact that out of the 141 papers selected, 21% examined grassland restoration without any species addition while 79% did so after introducing seeds, hay or transplant, thus short-cutting the dispersal filter in restoration. Out of the eight papers addressing spatial structure of populations, only one investigated the role of spatial isolation of populations on restoration success (Moore et al. 2011).

The more frequent use of simple parameters related to population demography (seedling or juvenile stages) or vegetative performance may be explained by the fact that detailed genetic studies are more costly, and that long-term demographic studies are time-consuming and laborious. Moreover, when the number of study species increases, a choice must be made among population processes that could be taken into account.

Research on restoration impacts in the context of population fragmentation is scarce

Five of the 10 papers assessing the genetic structure of restored populations, analysed the genetic consequences for populations restored in fragmented landscapes. Gustafson et al. (2002) showed that restored grassland populations of *Dalea purpurea* had greater genetic diversity relative to remnant populations in a highly fragmented landscape. They advocated that meta-populations are maintained throughout the landscape by frequent local gene flow and because restorations have been realized with seeds from multiple source populations. In the same way, Helsen et al. (2013b) did not observe a decrease in genetic diversity in recently restored populations of *Origanum vulgare*, indicating that spontaneous colonization after habitat restoration can lead to viable populations within a short time, especially when several source populations are located nearby. However, Aavik et al. (2013) found there was low gene flow between sown and natural populations of *Lychnis flos-cuculi*. Jacquemyn et al. (2010) emphasized the absence of gene flow between remnant populations of *Cirsium acaule* in a severely fragmented landscape that affected genetic diversity of plant populations and decreased the success of restoration - none of the recently restored areas was occupied by the study species. Finally, Rico et al. (2014) tested the effect of rotational shepherding on the demographic and genetic connectivity of a calcareous grassland species, demonstrating that populations of ungrazed sites (not linked by sheep grazing) had lower genetic diversity than grazed populations. Those five studies targeted the key research area proposed by Montalvo et al. (1997) regarding the influence of the spatial arrangement of landscape elements on meta-populations, centred around one or two insect-pollinated forb species. The genetic structure of wind-pollinated species is less likely to be affected by fragmentation, and grasses generally perform better than forbs in restored habitats (Pywell et al. 2003). This of course does not answer the question of why other restoration studies that included forbs did not take meta-populations into account.

The negative consequences of habitat fragmentation on plant populations are well-known (Lienert 2004), particularly those on genetic structure (Hufford and Mazer 2003). Along these lines, restoration is needed to enhance ecological networks of habitats, to reduce the genetic threats of fragmentation on plant species and to ensure long-term viability of threatened plant populations. However, restoration may also represent a threat for populations. Indeed, when restoration relies on the spontaneous colonization of restored habitats, founder populations can be small and represent just a minor proportion of the genetic diversity of the source population (Montalvo et al. 1997; Hufford and Mazer 2003). This can be based on a lack of seed source, low dispersal capabilities in space and time, the absence of dispersal agents or germination problems (Bakker and Berendse 1999; Coulson et al. 2001; Öster et al. 2009b; Helsen et al. 2013a).

Small and genetically less diverse populations have diminished survival over the long-term because of the effects of demographic, genetic and environmental stochasticity (Menges 1991a; Ellstrand and Elam 1993). In this context, the evaluation of restoration success considering the genetic structure of restored populations is necessary but still largely deficient. This research area represents a considerable gap based on the literature on the evaluation of grassland restoration success. Knowing the potentially deleterious outcomes, and especially the genetic effects, of habitat fragmentation on plant species populations, it is now necessary to determine the efficacy of grassland restoration protocols to counteract this threat.

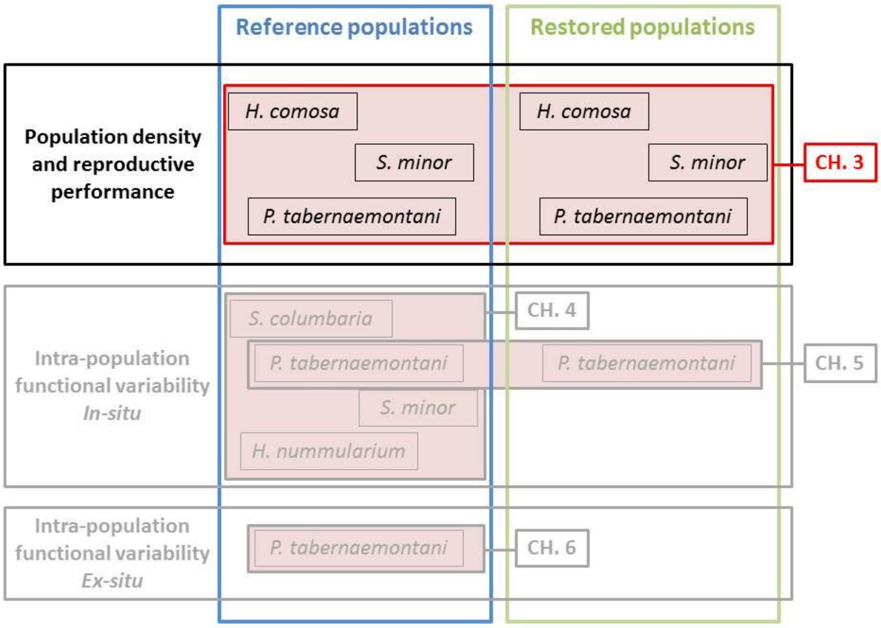
Conclusions

Twenty years after Montalvo et al. (1997) identified gaps in population biology research that could be addressed in the context of ecological restoration, population parameters are still unfrequently used for the evaluation of grassland restoration success. As well, despite knowing the consequences of habitat fragmentation on plant populations, the success of restoration in enhancing meta-population dynamics through the creation of a connected network of habitats has only been rarely taken into account in grassland ecological restoration efforts. Hopefully, our review stimulates future research into grassland restoration and the assessment of restoration success over the next twenty years.

CHAPTER 3:

Specialist plant species harbor higher reproductive performances in recently restored calcareous grasslands than in reference habitats

The content of this chapter has been published as a research article in “Plant Ecology and Evolution” (2015), Annex 2



CHAPTER 3: Specialist plant species harbor higher reproductive performances in recently restored calcareous grasslands than in reference habitats

Abstract

Calcareous grasslands are local biodiversity hotspots in temperate regions that have suffered intensive fragmentation. Ecological restoration projects on calcareous grasslands took place all over Europe. Their success has traditionally been assessed using a plant community approach. However, population ecology can also be useful to assess restoration success and to understand underlying mechanisms. We took advantage of three calcareous grassland sites in Southern Belgium, where reference grasslands coexist with grasslands restored in the late twentieth century and with more recently restored grasslands. We evaluated the colonization stage of three specialist species (*Sanguisorba minor*, *Potentilla tabernaemontani* and *Hippocrepis comosa*) using occurrence data. We also measured the reproductive traits of 120 individuals per species and compared components of fitness between recent restorations, old restorations and reference habitats. We found that the occurrence of *H. comosa* was similar in the different restoration classes, whereas both *P. tabernaemontani* and *S. minor* occurrences decreased from reference grasslands to recent restorations. In contrast, these two latter species exhibited a much higher reproductive output in recent restorations, thanks to an increased production of flowers and seeds. Our results suggest that, during colonization of recently restored calcareous grasslands, favourable environmental conditions, low competition and sufficient genetic mixing may lead to increased fitness components of individuals and a faster population growth than in the reference habitat. These results demonstrate how population processes can increase ecological resilience and highlight the interest of a population-based approach to assess the success of ecological restoration.

Introduction

Recent decades have been dominated by unprecedented rates of habitat perturbations by human activities. Critical changes in land use have led to the destruction and fragmentation of (semi)natural habitats, threatening biodiversity worldwide (Saunders et al. 1991; Balmford et al. 2005). As the conservation of remnant habitats is not sufficient to guarantee the long-term survival of several plant species (Rodrigues et al. 2004), active habitat restoration has become a necessity to preserve biodiversity worldwide. However, habitat restoration can be cost prohibitive, and efforts to reach restoration goals must demonstrate their success (Fagan et al. 2008).

Calcareous grasslands are local biodiversity hotspots in temperate regions (Prendergast et al. 1993; WallisDeVries et al. 2002; Jacquemyn et al. 2003). These habitats have suffered intensive fragmentation due to the abandonment of traditional agropastoral systems and the resulting encroachment, reforestation, urbanization or transformation into arable lands (Poschlod and WallisDeVries 2002; Piqueray et al. 2011a). In order to preserve and enhance the ecological value of those habitats, ecological restoration projects have taken place all over Europe.

Criteria used in judging whether a restoration is successful are numerous (Hobbs and Norton 1996; Ruiz-Jaen and Aide 2005a; Zedler 2007; Cristofoli and Mahy 2010; Piqueray et al. 2015). Most studies evaluating calcareous grasslands' restoration success focused on the recovery of plant species diversity and composition (Lindborg and Eriksson 2004; Kiehl and Pfadenhauer 2007; Fagan et al. 2008; Maccherini et al. 2009; Piqueray et al. 2011b; Maccherini and Santi 2012; Redhead et al. 2014). Few studies have evaluated calcareous grasslands' restoration success by a population approach (Endels et al. 2005). The discipline of population biology provides one perspective on what might be considered a successful restoration, namely that "populations must be restored to a level that allows them to persist over the long-term" (Montalvo et al. 1997). Restored populations must therefore possess attributes necessary for dispersal, growth, reproduction and adaptive evolutionary changes (Montalvo et al. 1997).

For a successful habitat restoration, species must first colonize newly created patches and establish new populations. The probability for a species to colonize a new habitat notably depends on its presence in the local species pool, the presence of dispersal agents, the ability of seeds to germinate, the longevity of the soil seed bank and the spatial position of patches in the landscape (Bakker and Berendse 1999; Helsen et al. 2013a). Most calcareous grasslands species are badly represented in the persistent seed bank (Kalamees and Zobel 1998; Bisteau and Mahy 2005). However, some species can persist for several decades in the soil seed bank and emerge after restoration by clear cutting (Poschlod et al. 1998). Grazing by sheep and goats plays a major role in species dispersal in the landscape (Poschlod et al. 1998). Accordingly, grasslands management practices imitating traditional shepherding may enhance species colonization on restored grasslands. In the absence of itinerant grazing, long-distance dispersal is thought to be limited for most calcareous grasslands species even if some species are potentially wind-dispersed (Tackenberg et al. 2003).

After colonization, restored populations must be able to persist over the long-term through offspring production (Montalvo et al. 1997). In the case of limited dispersal in space and time, founding populations can be small and represent only a minor portion of the genetic diversity of the source population (Montalvo et al. 1997; Hufford and Mazer 2003). This leads to increased risks of inbreeding depression and decreased adaptive potential of the restored population (Barrett and Kohn 1991; Ellstrand and Elam 1993). Small populations are more exposed to random environmental fluctuations (Menges 1991b; Widen 1993; Heschel and Paige 1995), may be less attractive to pollinators (Sih and Baltus 1987; Hendrix and Kyhl 2000) and may consequently have lower reproductive success. Loss of genetic variation in founding populations can lead to a lower fitness (Shaffer 1981; Lande 1988; Williams 2001; Reed and Frankham 2003; Matthies et al. 2004). Nevertheless, rapid population growth and expansion can also decrease risks of reducing fitness in newly created populations (Nei et al. 1975; Leimu and Mutikainen 2005; Bizoux et al. 2011).

Finally, species performances in restored habitats may be affected by environmental conditions that gently differ from the reference habitat of the species and subsequently affect fitness (Vergeer et al. 2003; Quintana-Ascencio et al. 2007; Adriaens et al. 2009). Colonization of a new habitat by maladapted genotypes can lead to a population sink (Pulliam 1988). Conversely, colonization by genotypes able to survive and reproduce in the new created habitat could increase species persistence in the landscape (Blais and Lechowicz 1989).

In this context, we took advantage of three calcareous grasslands sites in Southern Belgium, where reference grasslands coexist with grasslands restored in the last twenty years and with more recently restored grasslands. Using occurrence data, we evaluated the colonization stage of three plant species in the different sites. We then compared components of fitness between recent restorations, old restorations and reference habitats.

Material and methods

Study area and sites

The study was conducted in the Viroin valley. Three sites were selected in the region: “La Montagne-aux-buis” in Dourbes, “Les Abannets” in Nismes and “Les Rivelottes” in Treignes. At each site, three calcareous grasslands sub-sites were selected (Fig. 1): (1) Reference grassland, i.e. calcareous grassland known to have existed for more than two centuries and considered the reference ecosystem for the restoration (SER 2004); (2) old restoration, i.e. grassland restored between 1990 and 2000; and (3) recent restoration, i.e. grassland restored between 2004 and 2006. Restored grasslands derived from 40–100 year-old forests of oak coppices (Montagne-aux-buis, Abannets) or pine stands (Rivelottes, Abannets) established on former calcareous grasslands. Pine stands were *Pinus nigra* or *Pinus sylvestris* plantations. Dense shrub oak coppices were mainly formed with *Prunus spinosa*, *Crataegus monogyna* and *Corylus avellana*, with intermingled scarce trees of *Quercus robur*. Restoration protocols included trees and shrubs clearing, mainly followed by sheep and goat grazing.

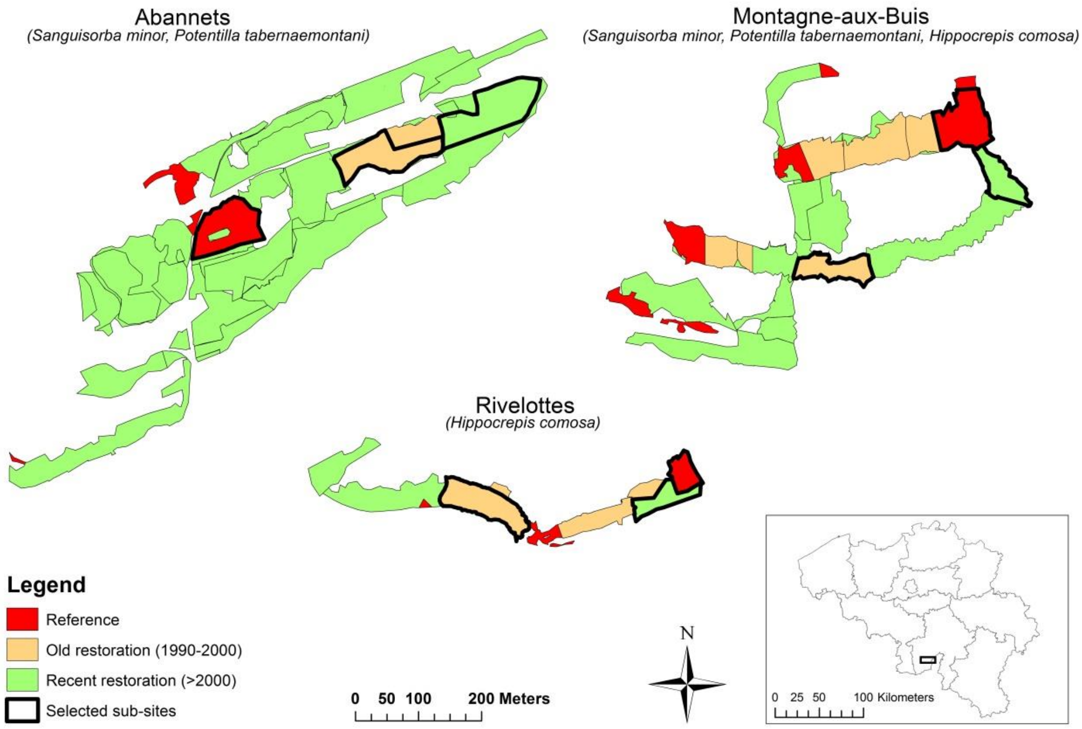


Figure 1 – Study region (Viroin Valley, Southern Belgium) and selected sub-sites of the three study sites (Montagne-aux-buis: 50°05’N–4°34’E, Abannets: 50°04’N–4°34’E, Rivelottes: 50°05’N–4°40’E). Occurrence of study species was observed in each sub-site. Fitness components were measured in only two sites for each study species (as indicated under sites names).

Study species

Three plant species were selected: *Hippocrepis comosa* L. (Fabaceae), a perennial legume producing yellow entomophilous flowers; *Potentilla tabernaemontani* Rchb. (Rosaceae), a creeping perennial forbs producing yellow entomophilous flowers; and *Sanguisorba minor* Scop. (Rosaceae), a polycarpic perennial forbs producing flower heads consisting of 15–30 wind-pollinated flowers. All three species are frequent in the study area (Adriaens et al. 2006).

Data collection

In order to evaluate the success of calcareous grasslands restoration, indicators of species colonization and fitness components were compared between the reference and restored grasslands (old and recent restorations).

The occurrence of species at the sub-site scale was evaluated by observing species presence or absence in 1-m^2 plots systematically placed in sub-sites according to a grid (one plot every four meters) representing a sampling rate of ca. 6% of each sub-site area (between 123 and 505 observed plots per sub-site, depending on the sub-site for a total of 2303 plots). For each study species, 20 plots (1-m^2) were randomly selected in each sub-site (among plots were the species was present) of two sites (Fig. 1), and one individual was randomly selected (in each plot) for measuring fitness components (Fig. 2).

The method used to measure fitness components depended on the species (see details of the recording method in Annex 3: Table S2). For *H. comosa* and *P. tabernaemontani* the number of flowers per individual and the number of seeds per fruit were recorded. For *S. minor*, the number of inflorescences per individual and the number of seeds per inflorescence were recorded. The total seeds production over one season was either directly measured (*S. minor*, Fig. 2) or estimated from other fitness components (*H. comosa* and *P. tabernaemontani* ; see details of the recording method in Table S2, Annex 3).

As competition and environmental variability can directly affect species performances we evaluated the bare ground cover in 1-m^2 plots around each selected individual. We estimate the bare ground cover using van der Maarel (1979) scale (0 = 0%; 1 = < 0.1%; 2 = 0.1–1%; 3 = 1–5%; 5 = 5–25%; 7 = 25–50%; 8 = 50–75%; 9 = 75–100%).

Figure 2 – Illustration of data collection for *S. minor* individuals. From top left to bottom right: one marked individual of S. minor, one inflorescence and measurement of the total seed production per inflorescence.

Data analysis

In order to evaluate species colonization in restored grasslands, a generalized linear model was fitted to the occurrence data (binomial family), followed by an analysis of deviance with restoration classe [(1) reference grassland, (2) old restoration, (3) recent restoration] and site as crossed factors. The analysis was followed by a pairwise comparison of proportions (number of plot where the species was present/total number of plot, using the “pairwise.prop.test” function in R) among restoration classes.

In order to compare fitness components of each study species in restored and reference grasslands, two-way ANOVAs were performed (with restoration classe and site as crossed factors) on each variable (fitness components, Table S2, Annex 3). In the case of a significant interaction with site effect, one-way ANOVAs were performed for each site separately. If a significant difference was observed between restoration classes, a Tukey’s comparison test was performed.

To compare bare ground cover between study sites and restoration classes, one-way ANOVAs were performed for each site with restoration classe as fixed factor [(1) reference grassland, (2) old restoration, (3) recent restoration]. If a significant difference was observed between restoration classes, a Tukey’s comparison test was performed. Response variables were arcsine-square root- or log- transformed when needed to meet the assumptions of statistical analyses. All analyses were performed with R 2.14.0 (R Development Core Team 2014).

Results

Colonization of restored grasslands

The occurrence of *P. tabernaemontani* (Fig. 3B) was higher in reference grasslands and old restorations than recent restorations (df = 2,2300; deviance = 46.81; $p < 0.001$), except in the Montagne-aux-buis site, where the occurrence of *P. tabernaemontani* was significantly higher in the recent restoration than in other grasslands (df = 2,717; deviance = 74.73; $p < 0.001$). For *S. minor*, the occurrence was significantly higher in reference grasslands than in restored grasslands (Fig. 3C). It was also higher in old restorations than in recent restorations (df = 2,2300; deviance = 1177; $p < 0.001$). *H. comosa* was less abundant than the two other species, and no significant differences were observed between reference and restored grasslands, except in the Montagne-aux-buis site, where the occurrence of *H. comosa* was significantly higher in the recent restoration (df = 2,717; deviance = 163.02; $p < 0.001$) than in other grasslands (Fig. 3A).

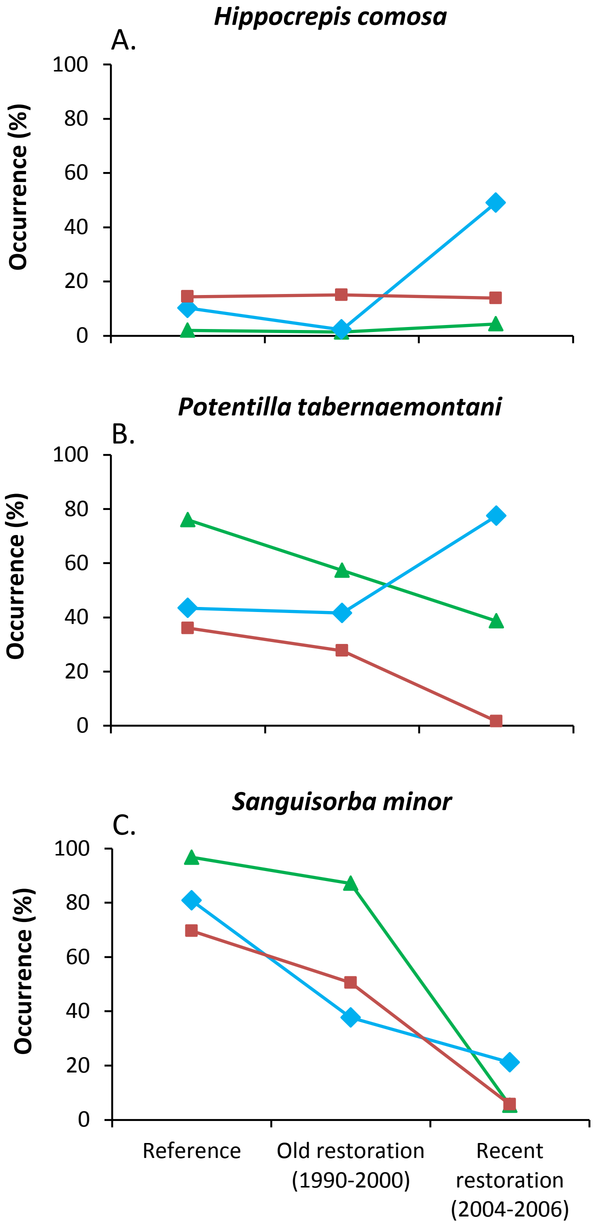


Figure 3 – Species occurrence (% of presence in 1-m^2 plots placed systematically in sub-sites representing a sampling rate of ~6% of each sub-site area) in selected sub-sites of the three study sites (Abannets, Montagne-aux-buis and Rivelottes sites are represented by green triangles, blue diamonds and red squares respectively).

Reproductive success

Individuals of *H. comosa* produced significantly more flowers (df = 2,114; F = 8.40; p < 0.001; Fig. 4A) in recent restorations (826.0±128.0 flowers) than in old restorations (244.8±26.5 flowers). For this species, differences of seeds production per fruit were site dependent. Data was missing for one sub-site of the Montagne-aux-buis site because the sub-site was grazed before measurements. No differences were observed between reference and old restoration in this site. At the Rivelottes site, individuals of the old restoration produced significantly more seeds per fruit (df = 2,56; F = 4.32; p = 0.018; Fig. 4D) than individuals of the recent restoration (respectively 2.3±0.2 and 1.6±0.2 seeds/fruit). Individuals of *P. tabernaemontani* and *S. minor* produced significantly more inflorescences, or flowers, in recent restorations than others grasslands (respectively df = 2,114; F = 37.40; p < 0.001; and df = 2,114; F = 18.49; p < 0.001; Fig. 4B–C). *P. tabernaemontani* individuals produced 33.9±6.9 flowers in recent restorations, compared to 2.5±0.4 flowers in reference grasslands and 2.8±1.0 flowers in old restorations (Fig. 4B). *S. minor* individuals produced 8.3±1.7 inflorescences in recent restorations, compared to 1.9±0.5 inflorescences in reference grasslands and 0.9±0.3 inflorescences in old restorations (Fig. 4C). For *P. tabernaemontani*, no differences were found for the number of seeds per fruit between the reference and the restored grasslands (Fig. 4E). For *S. minor*, the production of seeds per inflorescence was site dependent. For this species, the seeds production per inflorescence was significantly higher for the reference grassland than for the recent restoration of the Montagne-aux-buis site (df = 2,25; F = 4.75; p = 0.018; Fig. 4F).

Total seeds production was site dependent for individuals of *H. comosa* (Fig. 4G). Individuals of the recent restoration of the Rivelottes site produced significantly more seeds (df = 2,56; F = 4.39; p = 0.017; 913.0±135.0 seeds) than individuals of the old restoration (379.0±81.6 seeds). Data was missing for one recently restored sub-site of the Montagne-aux-buis site. At this site, no difference of seeds production was observed between the reference and the old restoration. *P. tabernaemontani* and *S. minor* individuals had a significantly higher seeds production (respectively df = 2,114; F = 23.1; p < 0.001; and df = 2,114; F = 13.9; p < 0.001) in recent restorations than other grasslands (Fig. 4H–I). *P. tabernaemontani* individuals produced 458.0±106.0 seeds in recent restorations, compared to 38.4±5.9 seeds in reference grasslands and 60.1±16.4 seeds in old restorations (Fig. 4H). *S. minor* individuals produced 147.5±35.6 seeds in recent restorations, compared to 29.5±7.1 seeds in reference grasslands and 18.4±5.8 seeds in old restorations (Fig. 4I).

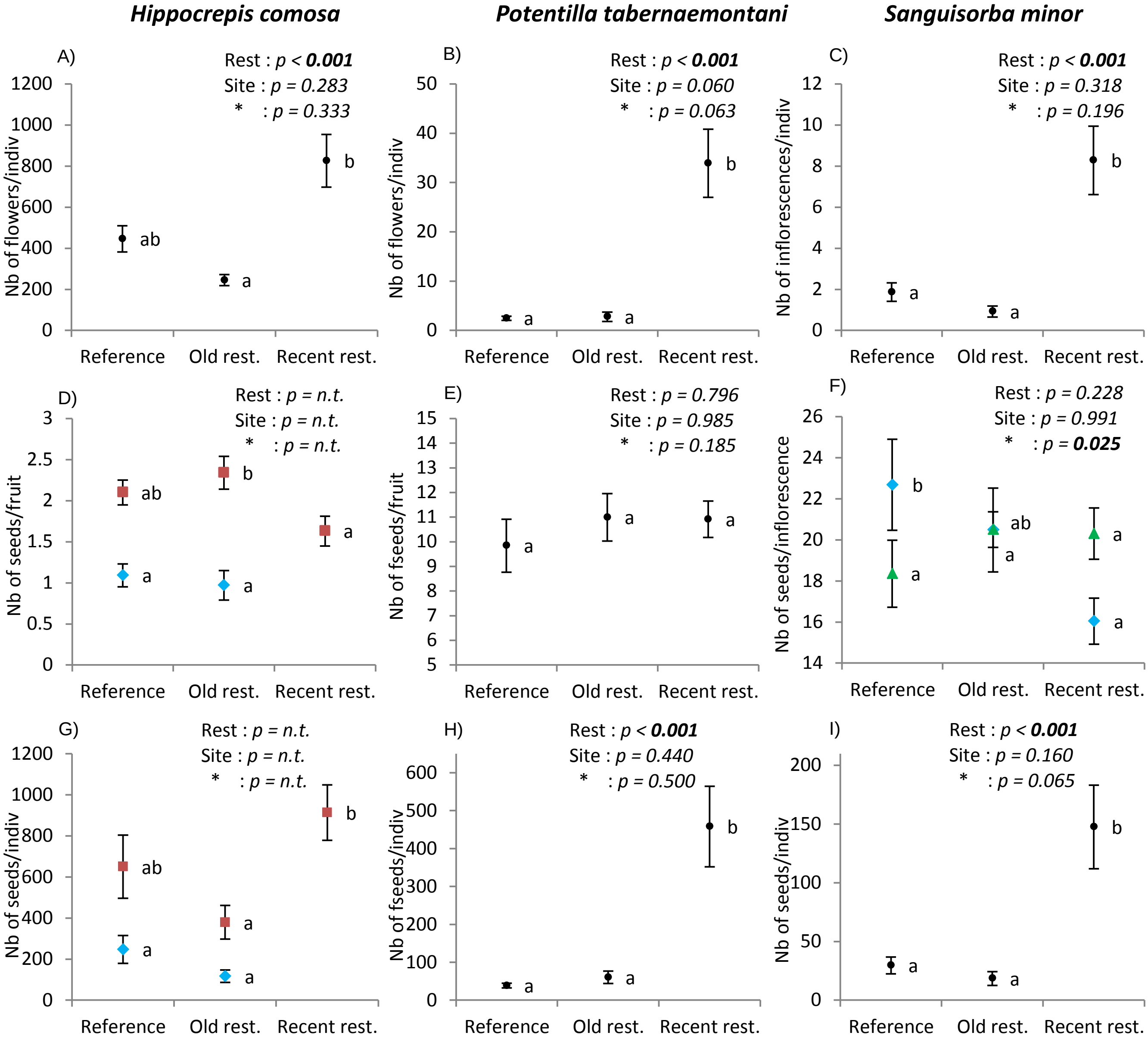


Figure 4 – Means and standard error of fitness components of study species for reference grasslands, old and recent restorations. For a same symbol, significantly different means are followed by different letters. P-values of the two ways ANOVAs for the different factors (restoration class, site and their interaction) are given up-right on each graph, and significant p-values are in bold (n.t. = not tested). When a significant interaction between the two factors was pointed out, results were drawn for each site separately. Results of AV2 for two sites are represented by black points, results of AV1 for the Abannets, Montagne-aux-buis and Rivelottes sites are represented by green triangles, blue diamonds and red squares respectively. Data were missing for *Hippocrepis comosa* in one sub-site because it was grazed before fruits and seeds were collected.

Bare ground cover

In the Montagne-aux-buis site, the bare ground cover was significantly higher on the recent restoration compared to other grasslands, whatever the species (df = 2,57; F = 7.27; p = 0.002 for *H. comosa*; df = 2,57; F = 5.55; p = 0.006 for *P. tabernaemontani*; df = 2,57; F = 7.06; p = 0.002 for *S. minor*; Table 1). In the Abannets site, the bare ground cover was not significantly different around *P. tabernaemontani* individuals (df = 2,57; F = 0.12; p = 0.883; Table 1) but significantly higher in the recent restoration and the reference around *S. minor* individuals (df = 2,57; F = 11.61; p < 0.001; Table 1). In the Rivelottes site, there was significantly more bare ground cover in the reference than in the old restoration (df = 2,57; F = 3.67; p = 0.032; Table 1).

Table 1 – Differences of bare ground cover between sub-sites. Percentage of bare ground cover was estimated in 1-m^2 plots placed around each individual. The van der Maarel (1979) scale was used to estimate de bare ground percentage in the plot (0 = 0%; 1 = < 0.1%; 2 = 0.1–1%; 3 = 1–5%; 5 = 5–25%; 7 = 25–50%; 8 = 50–75%; 9 = 75–100%). Means and standard errors of bare ground cover are given for reference grasslands, old and recent restorations. Significantly different means are followed by different letters. Significant p-values are in bold.

Species	**Sites**	**Reference**	**Old restorations**	**Recent restorations**	**p**
H. comosa	Mont.-aux-buis	0.30±0.21 ^{a}	0.00±0.00 ^{a}	1.35±0.41 ^{b}	**0.002**
	Rivelottes	2.50±0.44 ^{b}	1.00±0.34 ^{a}	1.70±0.39 ^{ab}	**0.032**
P. tabernaemontani	Mont.-aux-buis	0.15±0.15 ^{a}	0.25±0.25 ^{a}	1.40±0.42 ^{b}	**0.006**
	Abannets	0.60±0.28	0.45±0.25	0.45±0.21	0.883
S. minor	Mont.-aux-buis	0.15±0.15 ^{a}	0.15±0.15 ^{a}	1.50±0.46 ^{b}	**0.002**
	Abannets	0.85±0.25 ^{b}	0.00±0.00 ^{a}	1.50±0.30 ^{b}	**<0.001**

Discussion

Colonization of restored grasslands

The first step to evaluate restoration success using a population approach is to determine if species have been able to colonize restored grasslands. Like other ecological processes, colonization and species expansion can require long periods of time. Following restoration, grasslands may exhibit a colonization credit due to a time lag for species dispersal (Cristofoli et al. 2009); restored patches exhibit a lower species richness than expected by the area and connectivity of patches (Jackson and Sax 2009). Some species can therefore not yet be observed in the vegetation but can be expected to colonize grasslands in ensuing years. In the calcareous grassland of our study region, however, Piqueray et al. (2011c) showed that there was no colonization credit for our study species. Those species have been able to colonize restored habitats. This is in agreement with the observations of Delescaille (2006; 2007). However they were over less present on restored than on reference grasslands.

There are probably multifactorial causes explaining patterns of occurrence of each target species on study sites, including emergence from soil seed bank, multiple dispersal agent (like wind, sheep or human management), spatial distribution of grasslands patches and environmental variability. Although restored sites were afforested 40–100 years ago, it is conceivable that *P. tabernaemontani* and *S. minor* emerged from the soil seed bank, as showed by a study conducted at the Abannets site (Delescaille et al. 2006). Indeed, those two species have a long-term persistent seed bank (over several decades, > 25 years) (Poschlod et al. 1998). Moreover, *P. tabernaemontani* and *S. minor* could have colonized restored sites through dispersal by sheep or goats that grazed different calcareous grasslands. Those species are not well dispersed by wind (Poschlod et al. 1998), and sheep play a major role in dispersal across the landscape (Fischer et al. 1996; Poschlod et al. 1998). The seed bank of *H. comosa* persists only 6–20 years in the soil (Poschlod et al. 1998), and seeds of this species are not efficiently dispersed by wind (Poschlod et al. 1998). The presence of this species on restored grasslands could be explained by the management, as dispersal by goats has been observed for this species (Müller-Schneider 1954). *H. comosa* occurrences on restored and reference grasslands were similar. However, it has to be noted that reference grasslands are remnants that have been isolated for more than one century. These grasslands surely represent a core area for the calcareous grasslands ecological network, but they may also exhibit an extinction debt (Piqueray et al. 2011c). Indeed, some species may respond rapidly to fragmentation, but a time lag in the response may also occur, creating an extinction debt: a condition in which populations are still present in a habitat, but is expected to go extinct (Tilman et al. 1994; Hanski and Ovaskainen 2002).

Interestingly, in one site (Montagne-aux-buis), we found the occurrence of *P. tabernaemontani* and *H. comosa* to be higher in the recent restoration than the reference grasslands and the old restoration (Fig. 3). Species recolonization can be influenced by the distance between the restored grassland and the nearest reference grassland (Helsen et al. 2013a). In this site, the recent restoration was close to the reference and the old restoration was 120 meters away from the nearest reference habitat. However, study sub-sites were always close to another calcareous grassland sub-site. No sub-sites were isolated in a forest or agricultural landscape. Environmental variability between sites or sub-sites could also explain differences in patters of species occurrence. In a previous work, Piqueray et al. (2011b) pointed no significant differences in soil conditions between sites of the same study area. However, our results showed that the bare ground cover was higher in this recent restoration of the Montagne-aux-buis site. This could increase availability of microsites for germination (Piqueray et al. 2013) and seedlings emergence (Kotorová and Lepš 1999; Zobel et al. 2000) and modify competition regimes as compare to reference grasslands.

Reproductive success of restored populations

A second step in evaluating the restoration success using a population approach comprises assessing performances of recently established individuals as compared to reference ones. In this study, all three studied species produced more flowering units and more seeds per individual in recent restorations than in the reference grasslands (with a less obvious pattern for *H. comosa*, Fig. 4). These results are hopeful concerning population persistence in restored grasslands, as individuals with higher fitness components are prone to increase population dynamics and decrease extinction risks. We must however be careful as we did not test seeds quality and viability. Rosaceae are known to produce a significant proportion of non-viable seeds (ENSCONET 2009). Moreover, seeds could be predated before germination occurs. The observation of higher fitness components in recently founded populations can be explained by a high genetic diversity of created populations (Leimu and Mutikainen 2005). Recent populations can indeed have been created from multiple colonization events from several sources populations through grazing, since sheep herds typically graze alternatively in many calcareous sites. In addition, the seed bank may be a source of genetic diversity (Templeton and Levin 1979) that could be restored when habitat conditions are suitable again. A high genetic diversity associated with a rapid demographic extension may have promoted reproductive success in recent populations (Leimu and Mutikainen 2005).

The number of seeds per inflorescence or per fruit was not significantly different between reference and restored grasslands. This shows that the key trait influencing fitness is the number of flowers, or inflorescences and not the number of seeds produced by floral unit. This suggests that pollination is not a limiting factor for seed production.

Finally, higher reproductive performances in recent restoration may be explained by environmental conditions. In our study sites, Piqueray et al. (2011b) showed that soil conditions have been restored and do not differ according to previous land use (oak or pine forests). They pointed out low differences between reference and restored grasslands in terms of soil fertility, but the mineralisation rate was higher on restored sites and may explain individual success. In addition, recently restored grasslands generally exhibit higher bare ground cover that offers microsites for germination and decreases competition (Piqueray et al. 2013). However, the higher bare ground cover was not the only variable that can explain seeds production variability between grasslands. Indeed, in the Abannets site, the fitness components of *P. tabernaemontani* were significantly different between sub-sites despite no differences of bare ground cover. And for *H. comosa*, the seeds production was higher on the recent restoration of the Rivelottes site despite a reduction of bare ground cover compared to reference grasslands (Fig. 4 and Table 1).

Implications for the future

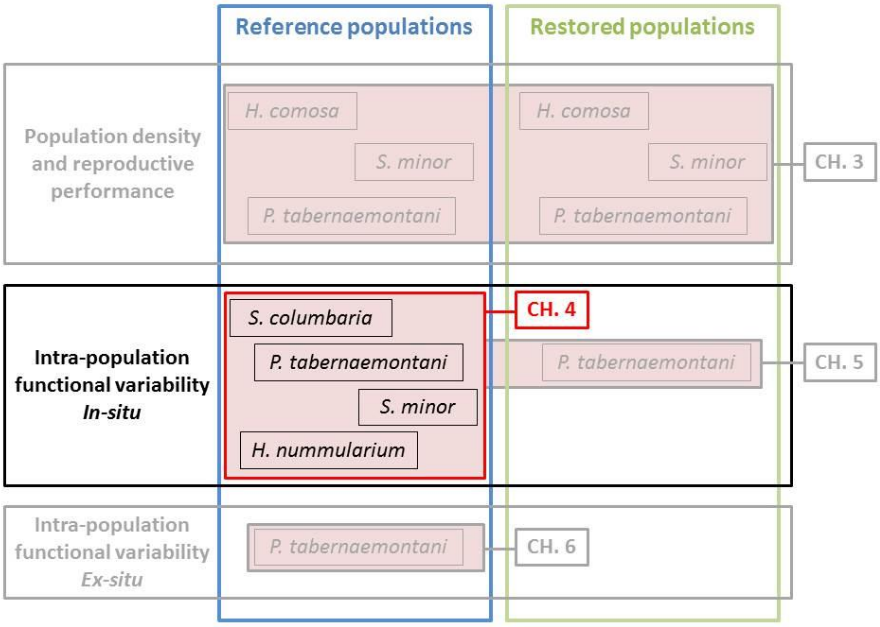
Our results suggest that, during the colonization of recently restored calcareous grasslands, individual fitness is hardly affected by any process that could reduce their reproductive capacity like unfavourable environmental conditions or insufficient genetic mixing. In contrast, the high reproductive output of individuals in restored grasslands is expected to enhance population growth, which may finally compensate for the lower initial occurrence. These results therefore demonstrate how population processes can increase ecological resilience (sensus Suding et al. 2004). When the massive colonization is over, both abiotic and biotic conditions in the restored habitat should approach those of the reference habitats (Piqueray et al. 2011b), and the fitness of individuals should be reduced to levels similar to the reference grasslands. This final situation seems to almost be reached for *H. comosa* that exhibit similar occurrences in all restorations classes. That is the species for which final seeds production was similar between recent restorations and reference habitats (Fig. 4). *S. minor* and *P. tabernaemontani*, in contrast, exhibit slower colonization still in progress, with occurrences of respectively 82±8% and 52±12% in reference habitats, 58±15% and 42±9% in old restorations and only 11±5% and 39±22% in recent restorations. For these two species, final seeds production was respectively five times and eleven times higher in recent restorations than in reference habitats.

From a management point of view, these processes are encouraging. Seed dispersal seems sufficient to establish satisfactorily diverse populations. One must keep in mind, however, that the patterns observed in this study might not be true for rarer species and/or those whose seed dispersal relies on agents not related to management schemes and/or those relying on declining populations of specific pollinators for successful reproduction. Using a population approach for these species will be necessary to fully assess the success of restoration programs of calcareous grasslands in Belgium.

CHAPTER 4:

Functional traits are more variable at the intra- than inter-population level: a study of four calcareous dry-grassland plant species

The content of this chapter has been published as a research article in "Tuexenia" (2016), Annex 4



CHAPTER 4: Functional traits are more variable at the intra- than inter-population level: a study of four calcareous dry-grassland plant species

Abstract

The majority of studies investigating plant functional traits have used species average trait values, and assumed that average values were sufficiently representative of each species considered. Although this approach has proven valuable in community ecology studies, plant functional traits can significantly vary at different scales, i.e. between but also within populations. The study of species functional trait variability can facilitate increasingly accurate studies in community ecology. Nevertheless, the current extent of within-site plant trait variability has been poorly addressed based on the literature. Calcareous grasslands are ecosystems well-suited to study plant trait variation at small spatial scales. Many species are present on heterogeneous calcareous sites, with significant differences in hydric status due to variations in soil depth, soil moisture, aspect, and slope. This study assesses the extent of intra-population functional trait variability and tests the hypothesis that this variability can be explained by within-site environmental heterogeneity. Three functional traits (SLA-specific leaf area, LDMC-leaf dry matter content, and plant vegetative height) were assessed in three populations of four calcareous grassland species totaling 950 individuals. The heterogeneity in soil depth and potential direct incident radiation (PDIR) was also quantified and related to plant functional trait variability. The intra-population functional trait variability was compared to the inter-population variability of collected data and overall inter-population variability data obtained from the worldwide TRY functional traits database. The results showed that SLA, LDMC, and plant height are characterized by considerable intra-population variation (SLA: 72–95%, LDMC: 78–100% and vegetative height: 70–94% of trait variability). The results also indicate higher plant height and larger SLA for individuals located in plots with deeper soils or lower potential direct incident radiation, on gentle slopes or north-facing slopes. Our findings additionally support the concept that higher plant height, higher SLA, and lower LDMC are related to higher availability of soil water. Individuals on shallow soils or in more exposed areas are better equipped to cope with environmental stress. Our results indicate plasticity or local adaptation in individuals to environmental heterogeneity. This study suggests that detailed analyses involving plant functional traits require measurements in situ from a large number of individuals, as the degree of individual response strongly depends on an individual’s location and its micro-environmental conditions. Neglecting intra-population trait variability may be critical, as intra-specific variation can be very high at the population scale, and is likely to be driven by local environmental heterogeneity.

Introduction

Over the past decades, functional traits have been increasingly used as reliable predictors of species and communities responses to environmental gradients (McGill et al. 2006; Ackerly and Cornwell 2007; Pakeman et al. 2009). The majority of studies investigating functional traits have focused on differences between species, using species average trait values (e.g. Ackerly and Cornwell 2007; Duru et al. 2010; Sandel et al. 2010; Sonnier et al. 2010; Gong et al. 2011; de Bello et al. 2013; Amatangelo et al. 2014), assuming that average values were sufficiently representative of each considered species. Despite the widespread use of this approach in community ecology, plant functional traits can vary substantially within species (Albert et al. 2010b; Hulshof et al. 2013). Taking intra-specific trait variation into account may therefore improve the accuracy and resolution of studies of community ecology (Siefert et al. 2015). This intra-specific trait variation can have crucial implications for species coexistence (Jung et al. 2010; Long et al. 2011) or ecosystem functions (Pontes et al. 2007; Lecerf and Chauvet 2008), and is an essential element of functional diversity approaches (Cianciaruso et al. 2009; Albert et al. 2012). Although usually defined as the functional trait variation within a species, intra-specific functional trait variation can be studied at different scales, notably between (e.g. Jung et al. 2010; Baruch 2011; Andersen et al. 2012; Almeida et al. 2013; Pakeman 2013) and within populations (Bolnick et al. 2011; Mitchell and Bakker 2014b), the latter being poorly addressed thus far based on the literature.

Calcareous grasslands are well-suited ecosystems to study plant trait variation at small spatial scales. Calcareous grassland species are present on heterogeneous sites, with noticeable differences in soil depth, soil moisture, aspect, and slope, mainly resulting in differences in hydrological status (Butaye et al. 2005b; Piqueray et al. 2007). The total amount of available water for plants was proven to be lower for grasslands characterized by thin soils (<10 cm) and low plant cover compared to grasslands with higher vegetation cover and slightly deeper soils (Alard et al. 2005; Bennie et al. 2008; Dujardin et al. 2012). Topography and exposure represent additional decisive factors influencing water supply in calcareous grasslands (Bennie et al. 2006). Slope inclination and exposure strongly affect the amount of solar radiation intercepted by the surface, and subsequently influence soil water content (Ackerly et al. 2002).

Leaf traits are key functional traits that are linked to plant responses to environmental variability (Wilson et al. 1999; Garnier et al. 2001a; Ackerly 2004) and to stress tolerance (Westoby et al. 2002). The regulation of water loss through leaves can be expressed by several key leaf functional traits (Wright et al. 2001; Ackerly 2004) and, for example, can be reflected in higher leaf dry matter content (LDMC) and/or lower specific leaf area (SLA) (Buckland et al. 1997; Volaire 2008; Poorter et al. 2009). At large scales, SLA generally declines along gradients of decreasing soil nutrients or water availability (Cunningham et al. 1999; Reich et al. 1999; Fonseca et al. 2000; Pérez-Harguindeguy et al. 2013; Sánchez-Gómez et al. 2013; Monty et al. 2013), while LDMC generally decreases with greater water availability (Cornelissen et al. 2003). Moreover, trade-offs exist between plant height and environmental stress tolerance (Cornelissen et al. 2003). SLA, LDMC and plant height are strong indicators of plant resource use strategies (Weiher et al. 1999; Cornelissen et al. 2003; Westoby and Wright 2006).

This study assesses the extent of intra-population functional trait variability at local scale, and tests the hypothesis that this variability can be explained by within-site environmental heterogeneity.

Material and methods

Study sites

We selected three calcareous grassland sites located in Southern Belgium for the study: the “Montagne-aux-buis” in Dourbes (50°05'N, 4°34'E), the “Tienne Breumont” in Nismes (50°04'N, 4°32'E), and “Sosoye hill” in Sosoye (50°17'N, 4°46'E). The Montagne-aux-buis and the Tienne Breumont are located in the Calestienne region and the Sosoye hill is situated in the Meuse basin.

Study species

From a list of calcareous grassland plant species (Piqueray et al. 2007), we selected four perennial species that are generally abundant in European calcareous grasslands (Adriaens et al. 2006): *Helianthemum nummularium* (Linnaeus) Miller 1768 (Cistaceae), *Potentilla tabernaemontani* Ascherson 1891 (Rosaceae), *Sanguisorba minor* Scopoli 1771 (Rosaceae), and *Scabiosa columbaria* Linnaeus 1753 (Dipsacaceae), following the nomenclature of Lambinon et al. (2012). Those species are not protected by local or national law.

Data collection

Measurements of functional traits and within-site environmental heterogeneity were made along transects placed perpendicularly to the contour of the hills (i.e. in the direction of the slope) in order to meet the whole range of within-site environmental heterogeneity. At each of the three sites, between 3 and 5 transects were used (depending on transect length) in order to find around 90 individuals for each species. Along transects, between 87 and 91 individuals of each study species were selected at each site, except for *S. columbaria*, which was less abundant in the Montagne-aux-buis and the Tienne Breumont sites (48 and 22 individuals selected, respectively). Individuals of one species located on one site are considered as one population. Following the methodology of Cornelissen et al. (2003), only healthy, robust, fully grown adult plants that have their foliage exposed to full sunlight were selected.

For each selected individual, three functional traits were assessed: (i) the vegetative height; (ii) the specific leaf area (SLA, in mm² mg^{-1}) and (iii) the leaf dry matter content (LDMC, in mg g^{-1}). Leaf traits were measured on two leaves for each selected individual. Our sample size was much larger than the one required by standard protocols (Cornelissen et al. (2003) recommended 10 individuals for SLA and LDMC, and 25 individuals for plant height) in order to encompass the environmental heterogeneity of sampling sites. We followed the leaf trait measurement protocol of Garnier et al. (2001b): leaves were weighed to the nearest 10^{-3} g (Mettler Toledo®, Viroflay, France) following a minimum of 6 hours of rehydration, and subsequently scanned (Fig. 1). Leaf area was measured using ImageJ imaging software (Abràmoff et al. 2004). Leaves were then oven-dried at 60°C for 72 hours prior to the second weighing.

As plant traits may be affected by grazing (Noy-Meir et al. 1989), individuals located in calcareous grassland areas that were exposed to the same grazing pressure and frequency were selected. All selected individuals were located on grasslands managed by grazing once every three years with approximately the same number of sheep (by hectare) and not grazed during the year in which measurements were taken.

Along transects, the within-site heterogeneity of two variables linked to the potential drought stress was characterized: soil depth (in cm) and the potential direct incident radiation index (PDIR). The aspect (in degrees) and the slope (inclination in degrees) were measured at the exact position of each individual sampled for trait measurements. Soil depth was measured at all four corners of a 1-m^2 plot placed around each sampled individual in order to calculate a mean soil depth value for each plot.

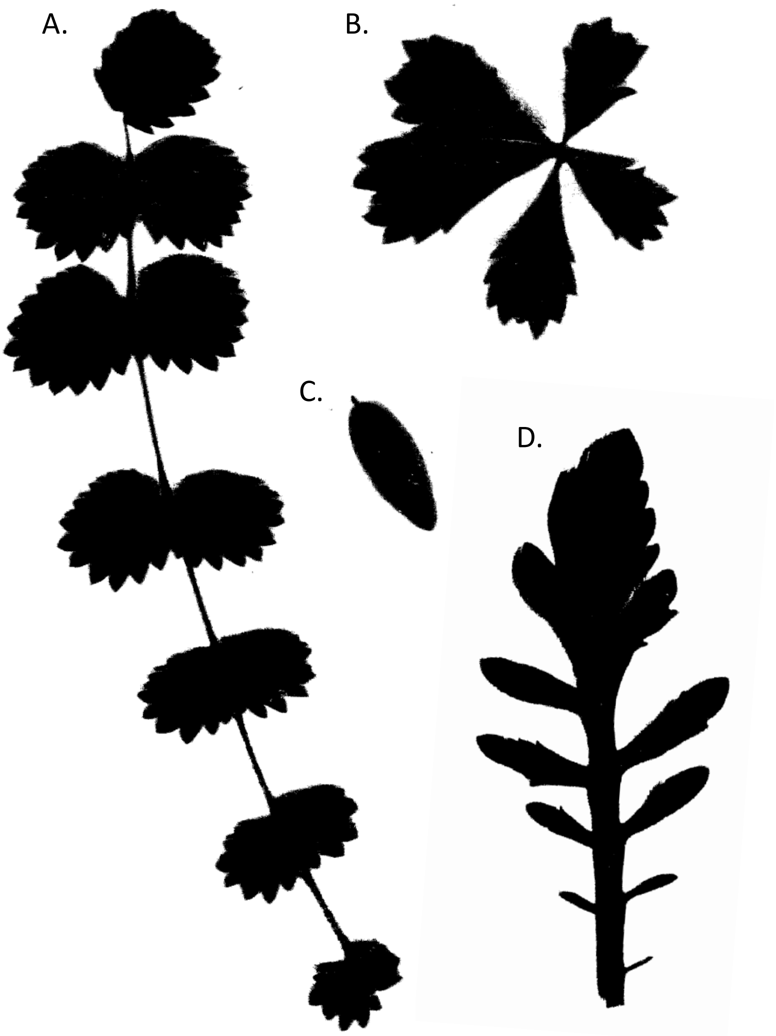


Figure 1 – Leaf scans of *S. minor* (A), *P. tabernaemontani* (B), *H. nummularium* (C) and *S. columbaria* (D).

Data analysis

The extent of intra-population functional variability was displayed using a boxplot and a kernel density plot (a non-parametric method of estimating the probability density function of a random variable) for each trait and population (each species present at a site).

Intra-population functional variability was compared to:

a) the inter-population functional variability of collected data. Linear mixed models (Bolker et al. 2009) were used with “site” designated as a random effect to quantify the contribution of each hierarchical level (intra-population vs. inter-population) to the total variation in each trait for each species. The “site” effect provides the inter-population functional variability of data, and intra-population functional variability is provided by the estimated standard error of the model.

b) the inter-population functional variability that can be expected for the species and traits selected in this study. For this, data from the worldwide TRY functional traits database was used (Kattge et al. 2011). Data entries for SLA, LDMC, and vegetative height of our four study species were selected, and only data that encompassed an average of at least 10 individuals were used. Each data entry from the database therefore represents one trait value for one population of the species in the world (i.e. an average trait value taken from at least 10 individuals of one population).

The coefficients of variation (CV = standard deviation/mean) were calculated for each trait of each species at a site (intra-population variability) and in the database (inter-population variability) in order to assess and compare the degree of trait variation. The CV has the advantage of being dimensionless and comparable between species and traits (Albert et al. 2011). It has been frequently used in the context of trait variation and interpretation of intra-specific variation (Jung et al. 2010; Fajardo and Piper 2011; Lemke et al. 2012; Fu et al. 2013; Wellstein et al. 2013; Mitchell and Bakker 2014b; García-Cervigón et al. 2015).

Linear regressions were conducted for all species traits and environmental variables at each site separately in order to highlight each population’s functional response to within-site environmental heterogeneity.

Mixed models were performed using lmer function in the lme4 library. All analyses were performed using R.2.14.0 (R Development Core Team 2014).

Results

Extent of intra-population functional variability

We found large intra-population functional variability for SLA in all species (Fig. 2A–D) and LDMC (Fig. 2E–H), but particularly in *H. nummularium*. For this species, intra-population SLA values ranged from 10.2 to 42.5 mm^2 mg^{-1} (Fig. 2A), and LDMC values ranged from 118 to 545 mg g^{-1} (Fig. 2E) at the Sosoye hill site. Coefficients of variation (CVs) ranged from 0.13 to 0.30 for SLA (Fig. 2A–D) and from 0.08 to 0.20 for LDMC (Fig. 2E–H), depending upon the species and site. The intra-population variability of vegetative height was high for each species (Fig. 2I–L), with coefficients of variation ranging from 0.30 to 0.67, depending upon the species and site.

A comparison of our measured intra-population functional variability to inter-population variability from the TRY database (DB inter-population variability) exhibited contrasting results, depending upon the species and traits considered. For SLA, DB inter-population variability was lower than our measured intra-population variability for two study species: *H. nummularium* (inter-population database CV = 0.20, compared to 0.20–0.30 for our measured intra-population variability, Fig. 2A) and *S. columbaria* (inter-population database CV = 0.16, compared to 0.17–0.20 for our measured intra-population variability, Fig. 2D). For *S. minor* and *P. tabernaemontani*, DB inter-population variability was higher than our measured intra-population variability (inter-population database CV = 0.25, compared to 0.18–0.19 for our measured intra-population variability for *P. tabernaemontani* and inter-population database CV = 0.27, compared to 0.13–0.14 for our measured intra-population variability for *S. minor*, Fig. 2B–C). For LDMC, DB inter-population variability was lower than our measured intra-population variability for all species except *S. minor* (inter-population CV = 0.18, compared to 0.08–0.09 for our measured intra-population variability, Fig. 2G). For vegetative height, DB inter-population variability and intra-population variability were similar (sometimes higher, sometimes lower depending on the site), except in *H. nummularium*. For this species, DB inter-population variability was higher than our measured intra-population variability (CV = 74, compared to 0.30–0.51 for our measured intra-population variability, Fig. 2I). For this trait, database values were higher than the study population values. Species vegetative height ranged from 5 to 80 cm in the database, while it ranged from 1 to 26 cm in our study populations.

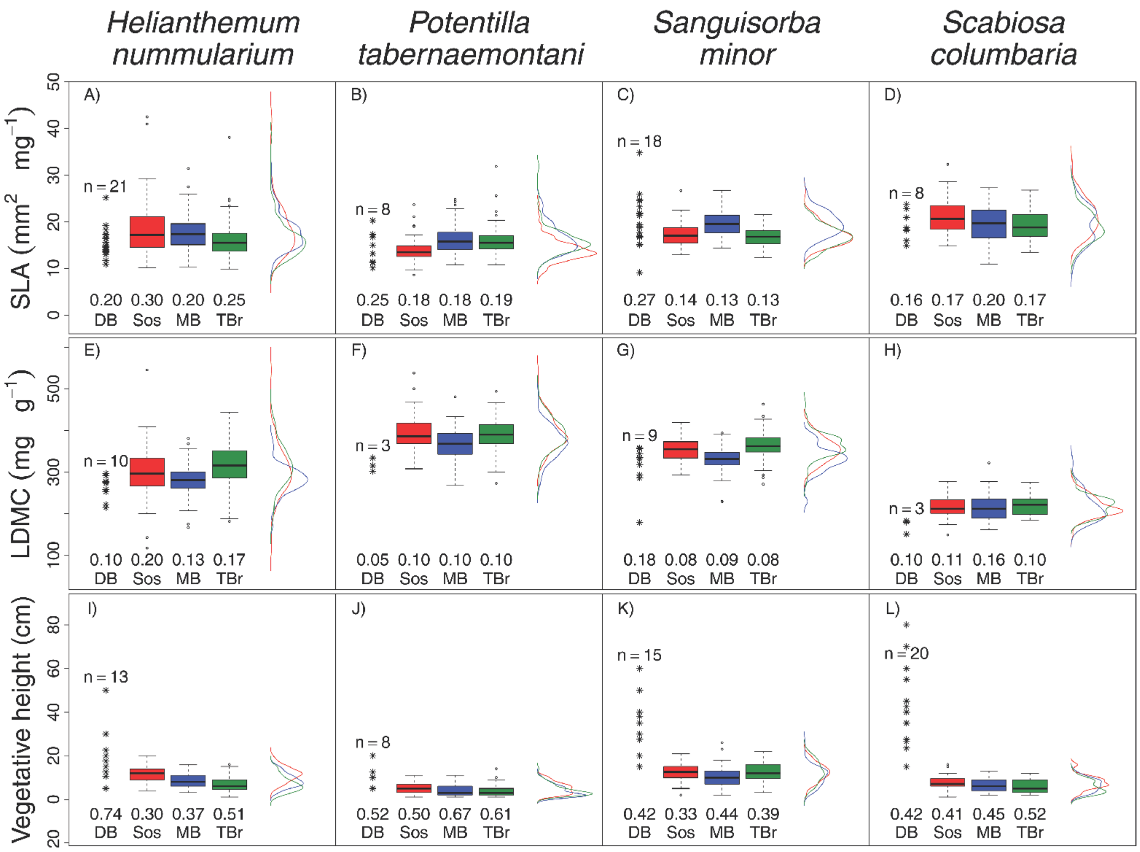


Figure 2 – Extent of intra-population functional variability. Boxplots and kernel density plots for each species and each trait in a site (Sos = Sosoye hill in red, MB = Montagne-aux-buis in blue and TBr = Tienne Breumont in green). Kernel density plots represent the density of data estimated by kernel method (Sosoye hill in red, Montagne-aux-buis in blue and Tienne Breumont in green) and boxplots show the median value (black line) and the inter-quartile ranges: between Q2–Q3 (box) and between Q1–Q2 and Q3–Q4 (dotted segments). Database values are represented for each species and each trait by asterisks and the number of data (n) are given above asterisks. Coefficients of variation (CV) for each trait of each species in a site and in the database are given under boxplots or asterisks.

Partition of our measured total trait variability between intra- and inter-population variability indicates that the intra-population variability accounts for 70 to 100% of the total variance, depending on the trait and species (Table 1), while 0 to 30% of the variance was due to differences between populations.

Table 1 – Variance partitioning of traits studied between (inter) and within (intra) populations. Percentage of the total trait variability is partitioned between intra- and inter-population trait variability for each study species. SLA, specific leaf area, LDMC, leaf dry matter content.

		Helianthemum nummularium	***Potentilla tabernaemontani***	***Sanguisorba minor***	***Scabiosa columbaria***
SLA	Intra-pop.	95	83	72	93
	Inter-pop.	5	17	28	7
LDMC	Intra-pop.	89	88	78	100
	Inter-pop.	11	12	22	0
Vegetative height	Intra-pop.	70	94	94	92
	Inter-pop.	30	6	6	8

Functional response to environmental heterogeneity

At the within-population level (within-site), a total of 36 regressions were conducted for the soil depth data, and a total of 36 were also conducted for PDIR data (four species, three populations per species and three traits measured). Of the 36 regressions, 20 were significant ($p < 0.05$) for soil depth (Table 2 and Fig. 3), while 23 were significant ($p < 0.05$) for PDIR (Table 3 and Fig. 4). For all species, significant regressions always indicated the same trend: SLA and vegetative height increased with soil depth (Fig. 3A–D and 3I–L), and decreased when PDIR increased (Fig. 4A–D and 4I–L). LDMC decreased when soil depth increased (Fig. 3E–H) and increased with PDIR (Fig. 4E–H).

Across populations, regressions were conducted between study traits and environmental variables for the four study species (Table 4). A total of 12 regressions were conducted for the soil depth data, and a total of 12 were also conducted for PDIR data (four species and three traits measured). Of the 12 regressions, 10 were significant ($p<0.05$) for soil depth and 7 were significant ($p<0.05$) for PDIR. Regressions always indicated the same trend whatever the species considered, similar to the trends observed at the within-site level.

Table 2 – P-values and R-squared of the regressions between soil depth and study traits (SLA, LDMC and vegetative height) in each of the three study sites (Sos = the Sosoye hill, MB = the Montagne-aux-buis and TBr = the Tienne Breumont). Significant p-value (p<0.05) are in bold.

		SLA (mm² mg^{-1})		LDMC (mg g^{-1})		Vegetative height (cm)	
Species	Site	p	R^2	p	R^2	p	R^2
H. nummularium	Sos	0.236	0.005	0.103	0.020	0.211	0.007
	MB	**<0.001**	0.157	0.576	0.008	**0.004**	0.079
	TBr	0.329	0.0004	0.645	0.009	**<0.001**	0.302
P. tabernaemontani	Sos	**<0.001**	0.360	**<0.001**	0.286	**<0.001**	0.116
	MB	**0.025**	0.044	0.102	0.019	**<0.001**	0.135
	TBr	**<0.001**	0.133	0.052	0.033	**<0.001**	0.194
S. minor	Sos	**<0.001**	0.136	**<0.001**	0.269	0.151	0.012
	MB	0.659	0.009	0.299	0.001	0.270	0.003
	TBr	0.374	0.002	0.329	0.0004	**<0.001**	0.147
S. columbaria	Sos	**0.015**	0.056	**<0.001**	0.123	0.641	0.009
	MB	**<0.001**	0.354	**<0.001**	0.250	**0.001**	0.195
	TBr	**0.024**	0.192	0.074	0.108	**0.041**	0.152

Table 3 – P-values and R-squared of the regressions between PDIR and study traits (SLA, LDMC and vegetative height) in each of the three study sites (Sos = the Sosoye hill, MB = the Montagne-aux-buis and TBr = the Tienne Breumont). Significant p-value (p<0.05) are in bold.

		SLA (mm² mg^{-1})		LDMC (mg g^{-1})		Vegetative height (cm)	
Species	Site	p	R^2	p	R^2	p	R^2
H. nummularium	Sos	**0.019**	0.052	**0.008**	0.069	0.244	0.004
	MB	**<0.001**	0.118	0.647	0.009	**0.037**	0.037
	TBr	0.806	0.011	0.425	0.004	**<0.001**	0.579
P. tabernaemontani	Sos	**<0.001**	0.269	**<0.001**	0.210	**0.012**	0.063
	MB	**<0.001**	0.160	**0.015**	0.054	**<0.001**	0.184
	TBr	**<0.001**	0.146	0.425	0.004	**0.001**	0.445
S. minor	Sos	**<0.001**	0.209	**<0.001**	0.335	0.413	0.004
	MB	0.901	0.011	0.335	0.0007	0.894	0.011
	TBr	0.934	0.012	0.119	0.017	**<0.001**	0.368
S. columbaria	Sos	**<0.001**	0.128	**<0.001**	0.210	0.722	0.010
	MB	**<0.001**	0.320	**<0.001**	0.319	0.092	0.040
	TBr	**0.012**	0.241	**0.005**	0.299	**<0.001**	0.484

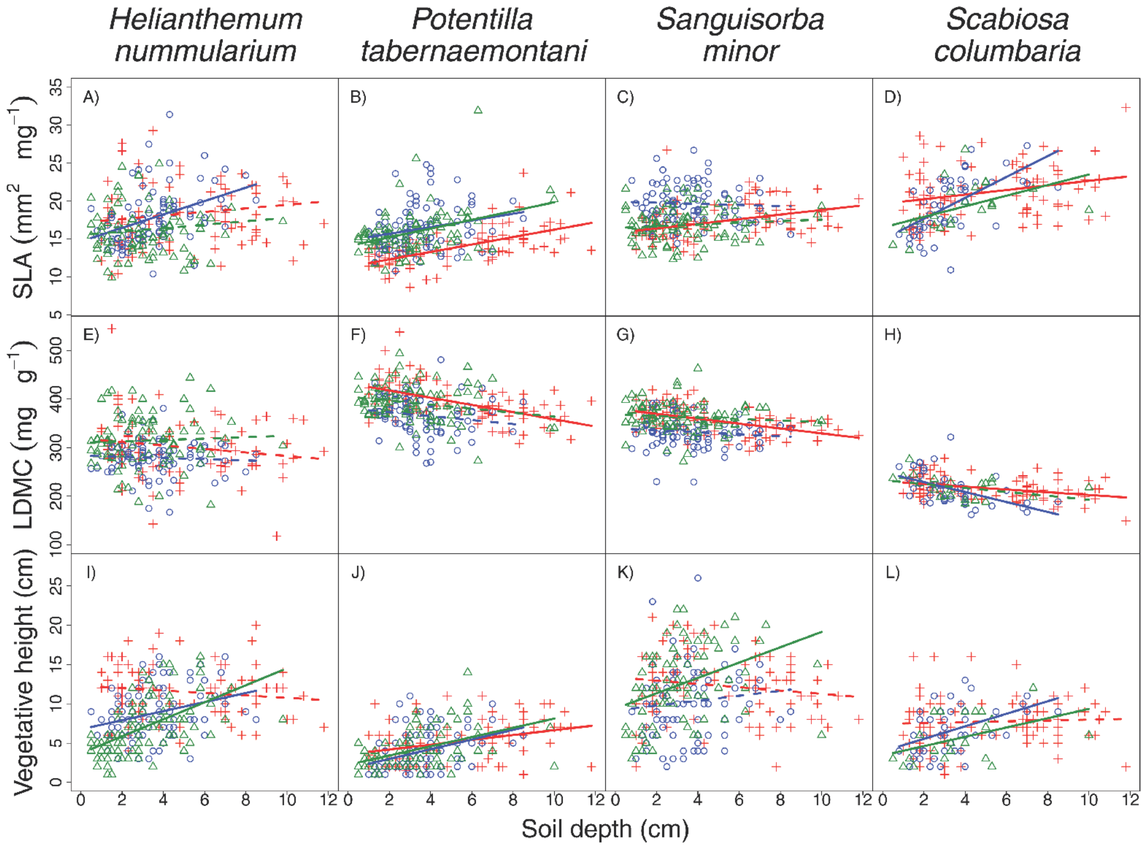


Figure 3 – Population responses to the within-site soil depth heterogeneity. Individual trait values (SLA, LDMC and vegetative height) and the related soil depth (measured at the four corners of a 1-m^2 plot placed around each sampled individual in order to calculate a mean soil depth value for each plot) were drawn for each species individual in a site (the Sosoye hill in red crosses, the Montagne-aux-buis in blue circles and the Tienne Breumont in green triangles). Linear regressions between each species traits and the soil depth were drawn for each species in a site. Regression lines are solid if significant ($p < 0.05$) and dashed if not.

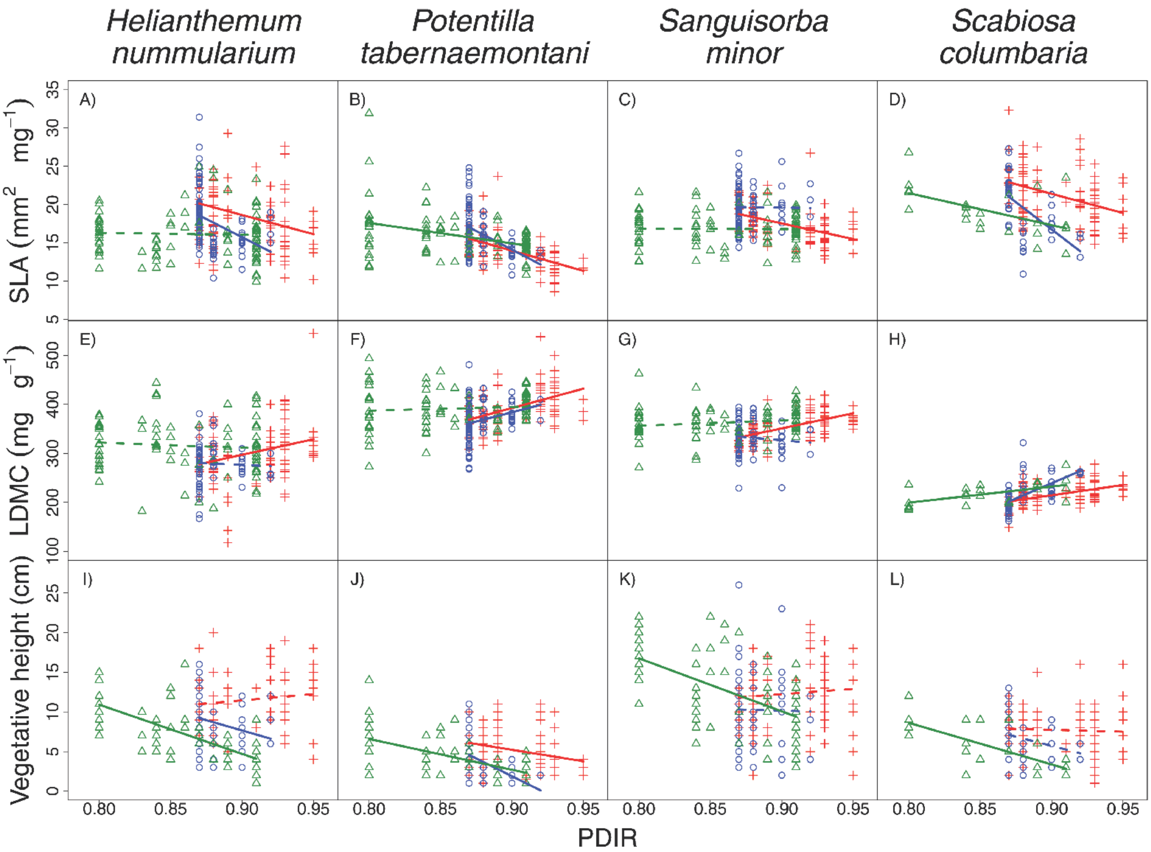


Figure 4 – Population responses to the within-site potential direct incident radiation (PDIR) heterogeneity. Individual trait values (SLA, LDMC and vegetative height) and the related PDIR (index based on the aspect and the slope measured at the exact position of each individual sampled) were drawn for each species individual in a site (the Sosoye hill in red crosses, the Montagne-aux-buis in blue circles and the Tienne Breumont in green triangles). Linear regressions between each species traits and the PDIR were drawn for each species in a site. Regression lines are solid if significant ($p < 0.05$) and dashed if not.

Table 4 – Coefficients of regression, P-values and R-squared of the regressions between environmental variables (A. - soil depth, B. - PDIR) and study traits (SLA, LDMC and vegetative height) across populations. Significant p-value ($p<0.05$) are in bold.

A.	Soil depth								
	SLA (mm^2 mg^{-1})			LDMC (mg g^{-1})			Vegetative height (cm)		
Species	Coef.	p	R^2	Coef.	p	R^2	Coef.	p	R^2
H. nummularium	0.421	**<0.001**	0.046	-1.960	0.155	0.004	0.486	**<0.001**	0.084
P. tabernaemontani	0.279	**<0.001**	0.046	-4.376	**<0.001**	0.064	0.452	**<0.001**	0.170
S. minor	0.136	**0.049**	0.011	-3.136	**<0.001**	0.051	0.192	0.101	0.006
S. columbaria	0.532	**<0.001**	0.145	-3.542	**<0.001**	0.121	0.255	**0.006**	0.041

B.	PDIR								
	SLA (mm^2 mg^{-1})			LDMC (mg g^{-1})			Vegetative height (cm)		
Species	Coef.	p	R^2	Coef.	p	R^2	Coef.	p	R^2
H. nummularium	-3.619	0.641	0.003	11.590	0.899	0.004	-4.490	0.503	0.002
P. tabernaemontani	-41.60	**<0.001**	0.210	252.58	**0.001**	0.039	-24.61	**<0.001**	0.094
S. minor	-8.715	0.060	0.010	148.79	**0.007**	0.023	-30.49	**<0.001**	0.052
S. columbaria	-24.23	**0.010**	0.036	325.96	**<0.001**	0.139	-2.960	0.715	0.006

Discussion

To date, the majority of trait-based research has utilized mean trait values to describe a given species. However, this may hide functional variation at different scales, both among and within populations (Bolnick et al. 2011). This study highlights the extent of intra-population variability of functional traits at local scale in response to within-site environmental variability within the same habitat type (calcareous grasslands).

The results indicate that SLA, LDMC, and vegetative height are not only characterized by significant variation between species, as previously suggested (Westoby 1998), but that significant variations can also be highlighted at the intra-population level. Congruent with the results of this study, Wellstein et al. (2013) studied the intra-specific variability of the same traits for four perennial species representative of mountain grasslands, discovering strong variation of those traits within populations, particularly in SLA and plant height. At local scale, our study highlighted CV values ranging from 0.13 to 0.30 for SLA, 0.08 to 0.20 for LDMC, and 0.30 to 0.67 for vegetative height. This can be compared to a larger scale study by Albert et al. (2010b) who characterized the intra-specific variability of LDMC and vegetative height for sixteen terrestrial species, discovering CV values ranging from 0.08 to 0.25 for LDMC, and from 0.19 to 0.49 for vegetative height, with a large portion of intra-specific variability representing differences between populations. By comparing the extent of intra-population functional variability with large database values, this study suggests that within-population variation represents a significant component of the overall variance in these traits, except maybe for the vegetative height that was more variable in the database compared to our measured values. This could be due to a methodologic bias when measuring vegetative height. Indeed, values can differ if inflorescence stalks are or not taking into account in vegetative height measurements. In this study, we considered leaves in the inflorescence stalk but we did not take the stalk length into account, what may be different for some values coming from the TRY database.

In this study, over 70% of trait variability was explained at the intra-population level, which is higher than values reported by previous studies. For example, Mitchell and Bakker (2014b) discovered that for SLA, differences between populations explained 58% of the observed variation for *Hypochaeris radicata*, while only 42% of variation was due to differences at the intra-population level. Additionally, Albert et al. (2010a) discovered that approximately 50% of the trait variability (SLA, LDMC, LNC-leaf nitrogen concentration, and LCC-leaf carbon concentration) in alpine species was due to differences between populations, and 50% was due to differences within populations.

Among traits measured in this study, SLA exhibited higher CV values than LDMC. This is consistent with other studies of trait variation at the intra-specific level, indicating that SLA (or its inverse leaf mass per area) is one of the most variable leaf functional traits both at broad and at narrow spatial scales (Garnier et al. 2001a; Albert et al. 2010a; Fajardo and Piper 2011; Auger and Shipley 2013; Boucher et al. 2013; Wellstein et al. 2013; García-Cervigón et al. 2015; Siefert et al. 2015). While some traits are fixed and do not vary within species, others can vary substantially, as is notably the case for leaf traits (Hulshof and Swenson 2010; Albert et al. 2010b).

The findings of this study demonstrated high functional variation within calcareous grassland species populations at fine spatial scales. As differences in abiotic tolerances or resource use between individuals can generate variance in demographic parameters of populations (Bolnick et al. 2003), large intra-population functional variability can have a large impact on population functioning within the site. Moreover, this study showed that the within-population functional variability was a major component of the overall variance in study traits and therefore challenged the assumption that average trait values are sufficiently representative of a given species. This intra-specific functional trait variability at fine spatial scale can be related to microhabitat environmental conditions such as light, soil temperature or availability of nutrients (Wellstein et al. 2013). In our case, the intra-population functional trait variability was related to within-site soil depth and PDIR variation.

We found higher plant height and higher SLA for individuals located in plots with deeper soils or low PDIR (less potential direct incident radiation, on gentle slopes or north-facing slopes), and the inverse response for LDMC, with higher values in shallow soil or high potential direct incident radiation. This supports the idea that higher plant height, higher SLA, and lower LDMC may be related to significantly higher availability of soil moisture, as has been previously shown for herbaceous species (Meziane and Shipley 1999; Wellstein et al. 2013). Individuals on shallow soils are characterized by lower plant height and higher leaf tissue density, which ensure more successful performance under stressful conditions. Indeed, high LDMC and low SLA are related to high investment in structural tissues, which allow plants to maintain leaf turgor under drought stress (Niinemets 2001; Siefert 2012). Conservative species that exhibit low SLA (high LDMC), corresponding to dense leaf tissues and low growth rates, exhibit high resource conservation (Albert et al. 2010a).

Trait differentiation with respect to fine scale variation in soil depth has already been suggested by Ravenscroft et al. (2014) who indicated a significant soil depth effect whereby LDMC was lower in deeper soils. Ackerly et al. (2002) showed that SLA declined with increasing insolation, indicating that species with thicker and/or denser leaves preferentially occupied more exposed, south-facing slopes. These intra-specific patterns are the same as those shown in inter-specific trends (Cornwell and Ackerly 2009; Pakeman et al. 2009).

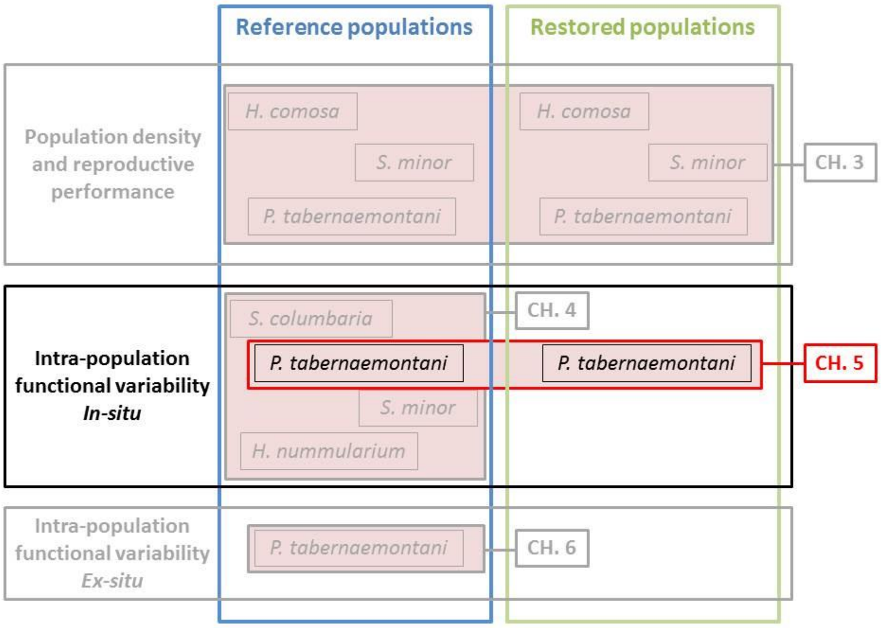
For P. tabernaemontani, the functional response to environmental variables was the most significant with 15 significant regressions (out of 18) at the within site scale (Tables 2–3) while all regressions were significant across populations for this species (Table 4). Therefore, this species has been selected as a model for the next chapters of this thesis (**Chapter 5–6**).

Carefully designed experiments are needed to test whether the observed intra-specific variation is due to plasticity (the observed trait variation is due to the direct response of individuals to environmental conditions) or local adaptation (the observed trait variation is due to the presence of genetic variation between individuals resulting from natural selection). In our study, gene flow between individuals is certainly high as spatial distance between them is quite short and because species are pollinated by flying insects. The possibility of genetic differentiation between individuals is therefore reduced and plasticity is the likely cause of observed intra-specific variability.

Our results indicated that intra-population functional variability is not only highly variable at local scale but that this variability is likely to be driven by local environmental heterogeneity. Our study therefore challenges the use of published mean values of functional trait to describe species behaviour. Measuring traits in situ on a large number of individuals is of primary importance, as the degree of species response strongly depends on individuals’ location and direct environmental influences. Species mean trait values should be replaced by a distribution of traits along environmental gradients.

CHAPTER 5:

Intra-specific functional variability for one calcareous grasslands plant species after habitat restoration



Abstract

The success of habitat restoration depends on the reformation and maintenance of suitable environmental conditions for target species as well as species capacity to colonize restored area and to cope with new environmental conditions. Calcareous grasslands are characterized by a high environmental variability at local scale which has been proven to be related to high intra-specific functional variability for some specialist plant species. Observed intra-specific variability in functional traits expresses the response of a species to its environment through local adaptation or phenotypic plasticity. A large intra-specific functional variability is often considered as crucial for future species adaptation to environmental changes. Objectives of this study were to quantify the intra-specific functional variability in restored populations of *P. tabernaemontani*, a calcareous grassland specialist. Moreover, we aimed to compare the intra-specific functional variability among restored and reference populations of this species, both at the local and the landscape scale. At the local scale, the variability of functional traits (range) was similar among reference and restored populations. At the landscape scale, vegetative height was as variable in restored as in reference populations, though specific leaf area (SLA) was more variable in restored populations. Restored grasslands covered the entire range of soil depth and PDIR variability characterizing reference grasslands. Plant height was higher for individuals located in deeper soils or at microsites with low PDIR, while SLA was not related to measured environmental gradients. Restoration protocols that have been applied to restore calcareous grasslands in the study region could be considered successful when regarding the landscape scale as intra-specific variability of study functional traits characterizing reference populations was fully represented in restored populations of *P. tabernaemontani*. We argue that conserving and restoring calcareous grasslands with high environmental heterogeneity can be decisive to maintain large intra-specific functional variability in populations and thereby a variety of phenotypes that can potentially buffer against future environmental changes. Future research should address whether or not the observed intra-specific functional variability induces fitness differences among populations, thus influencing their long-term persistence in restored habitats.

Introduction

Calcareous grasslands are local biodiversity hotspots in temperate regions that have suffered from intensive habitat fragmentation and destruction during the last century (Prendergast et al. 1993; Poschlod and WallisDeVries 2002; WallisDeVries et al. 2002; Adriaens et al. 2006). In order to preserve and enhance the ecological value of those habitats, restoration projects have taken place all over Europe.

Restoration success depends primarily on the reformation and maintenance of suitable environmental conditions for target species, as well as species capacity to colonize restored areas and to cope with new environmental conditions (Piqueray and Mahy 2010; Andrade et al. 2014). After grassland restoration, species are transferred or they colonized sites that may still differ in terms of abiotic conditions compared to the source habitat (Ouro et al. 2001; Vergeer et al. 2003; Walker et al. 2004; Piqueray et al. 2011b; Andrade et al. 2014). In order to survive and reproduce in the recreated habitat, occurring plant species should show some trait plasticity relevant to the environmental conditions (Andrade et al. 2014) or should be locally well adapted to those conditions (Pulliam 1988; Blais and Lechowicz 1989).

Observed intra-specific variability in functional traits express the response of a species to its environment through local adaptation or phenotypic plasticity (Joshi et al. 2001; Byars et al. 2007; Albert et al. 2011). Intra-specific functional variability may be measured at different scales including among populations (e.g., Jung et al. 2010; Baruch 2011; Andersen et al. 2012; Almeida et al. 2013; Pakeman 2013; Monty et al. 2013) or among individuals within populations (e.g., Bolnick et al. 2011; Mitchell and Bakker 2014b).

Studies dealing with intra-specific variability of functional traits mainly considered that species ability to display a large intra-specific functional variability in response to environmental heterogeneity can potentially represent an important feature for species to be able to adapt to a changing environment (Albert et al. 2010a; Albert et al. 2012; Almeida et al. 2013; Wellstein et al. 2013).

In that context, the overall objective of this study was to quantify intra-specific functional trait variation in restored populations of a plant species, *Potentilla tabernaemontani*, as a primarily way to assess its ability to cope with varying levels of environmental conditions. Therefore, (1) we quantified the intra-specific functional variability among individuals in restored grasslands and in reference grasslands and (2) we tested the hypothesis that this functional variability was a response to the environmental heterogeneity.

Calcareous grasslands in the study region, the Viroin valley in the Calestienne region of Southern Belgium, have mainly been restored by expanding areas of still-existing remnants sites. Restoration objectives, however, were also considered at the landscape scale, with the aim to restore a connected network of habitats. Therefore, functional variability and environmental heterogeneity were assessed at two different spatial scales: at local scale (within sites), and at landscape scale (across sites).

Two functional traits were assessed; specific leaf area (SLA) and vegetative height, both in reference and restored populations of *P. tabernaemontani*. Two environmental variables related to resource use efficiency were investigated: the soil depth and the potential direct incident radiation (PDIR).

P. tabernaemontani has been selected among four species studied in the previous chapter (*H. nummularium*, *P. tabernaemontani*, *S. minor* and *S. columbaria*) because the functional response to environmental variables was the most significant for this species (**Chapter 4**).

Material and methods

Study area

Five calcareous grassland sites were selected in the Viroin valley: the Rivelottes, the Tienne Delvaux, the Tienne Breumont, the Montagne-aux-buis and the Abannets. Historical and current maps in addition to a field survey were used to select two sub-sites in each site (Fig. 1): (1) one reference grassland, a historical calcareous grassland known to have existed for more than two centuries and considered the reference ecosystem for restoration (SER 2004) and (2) one restored grassland, which derived from 40–100 year-old forests of oak coppices or pine stands established on former calcareous grassland. Restoration protocols took place around still-existing reference grasslands in order to enlarge existing areas and included trees and shrubs clearing, mainly followed by sheep and goat grazing for management (André and Vandendorpel 2004; Graux 2004; Delescaille 2007). Selected restored grasslands were restored between 2000 and 2006. Reference and restored grasslands are managed by grazing, using migrating sheep or goat flocks (duration: two to three weeks per year, resulting in a grazing intensity of one to two sheep ha^{-1} year^{-1}). Restored grasslands are grazed each year, reference grasslands every two or three years depending on site. Selected sub-sites were not grazed before data collection the year of the study. On the Montagne-aux-buis site, two restored and two reference sub-sites were selected.

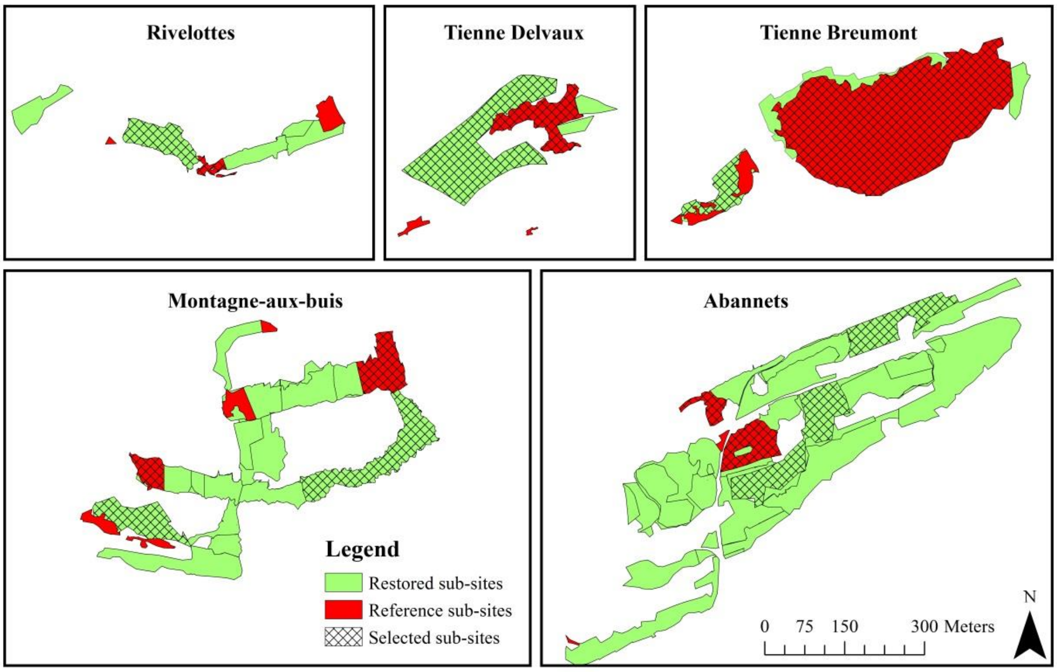


Figure 1 – Selected sub-sites of the five study sites. One reference grassland (historical calcareous grassland considered the reference ecosystem for the restoration) and one restored grassland (restoration between 2000 and 2006) were selected on each study site, except at one site, Montagne-aux-buis, where two reference and two restored sub-sites were selected.

Traits measurements

On each selected sub-site, 30 individuals of *P. tabernaemontani* were randomly selected. Following the methodology of Pérez-Harguindeguy et al. (2013), only healthy, robust, fully grown adult plants that had their foliage exposed to full sunlight were selected. All measurements were made at less than 10 days interval. For each selected individual, two functional traits were assessed: (i) the vegetative height and (ii) the specific leaf area (SLA, in $mm^2\ mg^{-1}$), measured on two leaves for each selected individual (Weiher et al. 1999; Cornelissen et al. 2003; Pérez-Harguindeguy et al. 2013). Leaves were scanned following a minimum of six hours of rehydration (Garnier et al. 2001b). Leaf area was measured using ImageJ imaging software (Abràmoff et al. 2004). Leaves were then oven-dried at 60°C for 72 hours and weighed to the nearest 10^{-1} mg (Mettler Toledo®, Viroflay, France).

Environmental conditions

Environmental conditions experienced by selected individuals were characterized by two variables linked to resource use efficiency: soil depth (in cm) and the potential direct incident radiation index (PDIR). The aspect (in degrees), the slope (inclination in degrees) and the soil depth were measured at the exact position of each individual sampled for trait measurements.

Data analysis

Variability of functional traits and environmental heterogeneity were quantified and compared among reference and restored grasslands, at the local and the landscape scales.

At the local scale (for each site separately), equality of means (ANOVA) and homogeneity of variance (Levene’s test) were tested for each functional traits (SLA and vegetative height) and each environmental variables (soil depth and PDIR), among reference and restored grasslands. P-values of ANOVAs and Levene’s tests were adjusted for multiple comparisons (Bonferroni correction for 6 comparisons – 6 sub-sites; $p<0.0083$).

At the landscape scale (across sites), the extent of functional variability and environmental heterogeneity was displayed using a kernel density plot (a non-parametric method of estimating the probability density function of a random variable) for reference and restored grasslands. Homogeneity of variance (Levene’s test) was tested and coefficients of variation (CV = standard deviation/mean) were calculated for each functional trait and each environmental variable among reference and restored grasslands.

Linear regressions between functional traits and environmental variables were performed at the local and at the landscape scales, for reference and restored grasslands separately, in order to highlight intra-specific functional response to the environmental heterogeneity.

Regressions and Levene’s tests were performed with R.2.14.0 (R Development Core Team 2014). ANOVAs were performed with Minitab® 17.1.0.

Results

Functional traits variability

At the local scale (within populations), functional traits were quite different among reference and restored populations (Table 1). SLA was significantly different among reference and restored grasslands in two sites while it was the case in three sites for vegetative height. However, the variability of functional traits (range) was similar among reference and restored grasslands on nearly all sites. Homogeneity of variance among reference and restored grasslands was met in five sites for SLA and in four sites for vegetative height (Table 1).

Table 1 – Equality of means (ANOVA) and homogeneity of variance (Levene's tests) tested for functional traits (SLA and vegetative height), among reference and restored grasslands, at local scale (for each site separately). Significant p-values are in bold (p<0.0083, Bonferroni correction for 6 comparisons).

	Abannets	Rivelottes	Tienne Breumont	Tienne Delvaux	Montagne-aux-buis 1	Montagne-aux-buis 2
Equality of means (ANOVA): p-values						
SLA	0.148	0.070	**<0.001**	0.034	0.683	**<0.001**
Vegetative height	0.015	**<0.001**	**<0.001**	0.016	0.758	**<0.001**
Homogeneity of variance (Levene's tests): p-values						
SLA	0.412	**0.003**	0.614	0.162	0.221	0.075
Vegetative height	0.414	0.129	**0.006**	0.925	0.774	**<0.001**

At the landscape scale, SLA was more variable in restored grasslands (from 8.26 to 26.12 mm^2 mg^{-1}) than in reference grasslands (from 8.05 to 20.51 mm^2 mg^{-1}; Fig. 2A). Coefficients of variation were barely different among reference and restored grasslands for SLA ($CV_{reference}$ = 0.17 and $CV_{restored}$ = 0.21; Fig. 2A), but variance was significantly different among reference and restored grasslands (Levene's test p = 0.001; Fig. 2A). The intra-specific variability of vegetative height was similar among reference and restored grasslands (Fig. 2B). Coefficients of variation were barely different among grasslands for this trait ($CV_{reference}$ = 0.56 and $CV_{restored}$ = 0.57; Fig. 2B) and equality of variance among reference and restored grasslands was met (Levene's test p = 0.407).

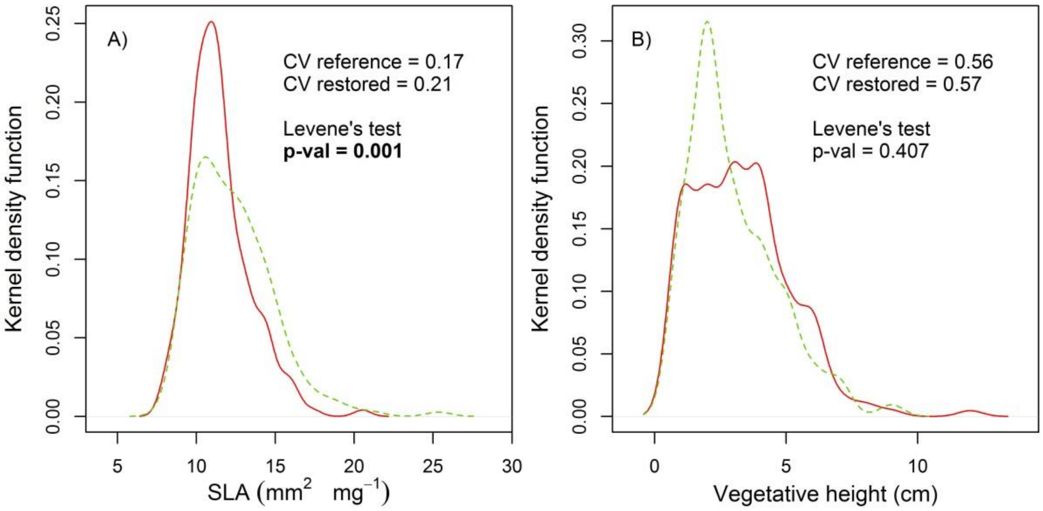


Figure 2 – Kernel density plot of functional traits (A: SLA and B: vegetative height) at the landscape scale (reference grasslands in solid red line and restored grasslands in dashed green line). Coefficients of variation (CV) are given in each graph for the reference and restored grasslands. Homogeneity of variance (Levene's test) was tested for each functional trait, among reference and restored grasslands. P-values are given on graphs and significant p-values are in bold.

Heterogeneity of environmental conditions

At the local scale, environmental conditions of reference and restored grasslands were different as shown by comparison of means (ANOVA, Table 2). Soil depth was significantly different among reference and restored grasslands in four sites, while PDIR was significantly different among grasslands in three sites. However, the range of soil depth values was similar among reference and restored grasslands. Indeed, soil depth variances were homogenous for all sites (p-values of Levene's tests ranged between 0.092 and 0.932; Table 2). Conversely, PDIR variance was significantly different among reference and restored grasslands in four sites. In the two other sites, PDIR variance was null within reference and restored grasslands (slope, orientation and latitude did not vary within a sub-site at the Rivelottes and at the Montagne-aux-buis 2), such that p-values of Levene's test could not be computed.

Table 2 – Equality of means (ANOVA) and homogeneity of variance (Levene's tests) tested for environmental variables (soil depth and PDIR), among reference and restored grasslands, at local scale (for each site separately). Significant p-values are in bold (p<0.0083, Bonferroni correction for 6 comparisons). PDIR variance was null within reference and restored grasslands of two sites (slope, orientation and latitude did not vary within a sub-site at the Rivelottes and at the Montagne-aux-buis 2), such that p-values of Levene's test could not be computed (NA).

	Abannets	Rivelottes	Tienne Breumont	Tienne Delvaux	Montagne-aux-buis 1	Montagne-aux-buis 2
Equality of means (ANOVA): p-values						
Soil depth	0.585	**<0.001**	0.011	**0.003**	**0.005**	**<0.001**
PDIR	**<0.001**	NA	**<0.001**	**<0.001**	0.094	NA
Homogeneity of variance (Levene's tests): p-values						
Soil depth	0.932	0.110	0.558	0.207	0.092	0.170
PDIR	**<0.001**	NA	**<0.001**	**0.001**	**<0.001**	NA

At the landscape scale (across sites), the range of environmental conditions was similar among reference and restored grasslands (Fig. 3B–B). Ranges of soil depth and PDIR values found in reference grasslands (from 1 to 15 cm for soil depth and from 0.86 to 0.95 for PDIR) were similar to those found in restored grasslands (from 1 to 17 cm for soil depth and from 0.84 to 0.95 for PDIR). Coefficients of variation were equal among reference and restored grasslands for PDIR ($CV_{reference} = CV_{restored} = 0.04$; Fig. 3B) and barely different for soil depth ($CV_{reference} = 0.70$ and $CV_{restored} = 0.64$; Fig. 3A). Variance was homogenous among reference and restored grasslands for soil depth and PDIR (Levene's test p = 0.145 and 0.433, respectively; Fig. 3A–B).

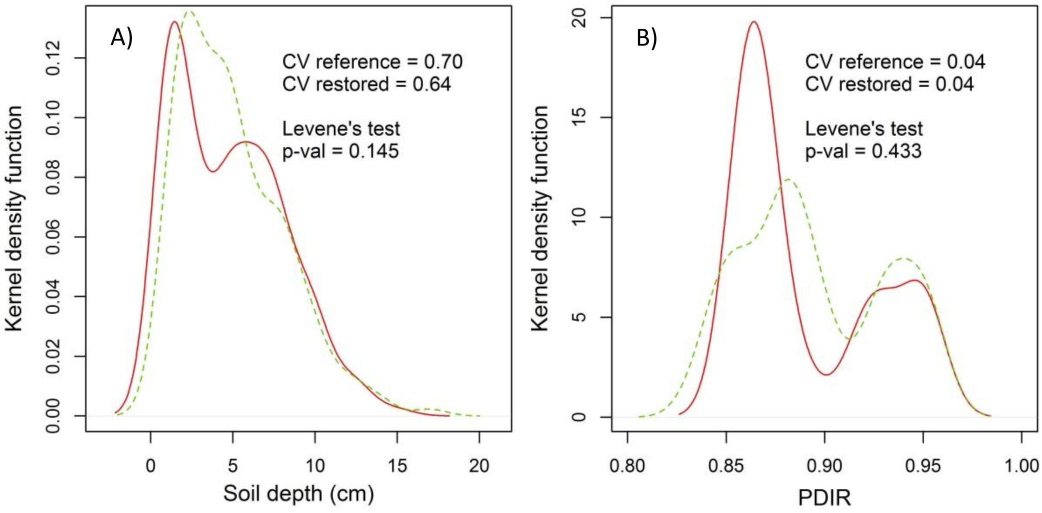


Figure 3 – Kernel density plot of environmental conditions (C: soil depth and D: PDIR) at the landscape scale (reference grasslands in solid red line and restored grasslands in dashed green line). Coefficients of variation (CV) are given in each graph for the reference and restored grasslands. Homogeneity of variance (Levene's test) was tested for each each environmental variable, among reference and restored grasslands. P-values are given on graphs and significant p-values are in bold.

Functional response to environmental variability

At the local scale, regressions between functional traits and environmental variables were not significant (Tables 3–4), except for three sub-sites out of 12. In the restored sub-site on the Tienne Delvaux and on the reference sub-site on the Montagne-aux-buis 1, vegetative height increased with soil depth (Table 3). In both sub-sites on the Montagne-aux-buis 1, vegetative height decreased when PDIR increased while SLA increased with PDIR in the restored sub-site and decreased when PDIR increased in the reference sub-site (Table 4).

Table 3 – Coefficients of regression, P-values and R-squared of the regressions between soil depth and functional traits (SLA and vegetative height) at the local scale (within sub-site). Significant p-value (p<0.05) are in bold.

		SLA ($mm^2 mg^{-1}$)			Vegetative height (cm)		
Sites	Sub-sites	Coef.	p	R^2	Coef.	p	R^2
Abannets	Reference	-0.065	0.692	0.030	-0.052	0.589	0.025
	Restored	0.015	0.939	0.035	0.030	0.708	0.030
Rivelottes	Reference	0.049	0.446	0.014	0.042	0.599	0.025
	Restored	-0.299	0.160	0.036	0.040	0.525	0.021
Tienne Breumont	Reference	0.067	0.624	0.624	0.074	0.229	0.017
	Restored	-0.126	0.225	0.018	-0.053	0.597	0.025
Tienne Delvaux	Reference	-0.095	0.663	0.029	0.167	0.181	0.030
	Restored	-0.048	0.595	0.025	0.242	**0.003**	0.253
Montagne- aux-buis 1	Reference	0.164	0.318	0.001	0.779	**<0.001**	0.453
	Restored	-0.167	0.175	0.031	0.199	0.088	0.068
Montagne- aux-buis 2	Reference	0.030	0.723	0.031	-0.145	0.184	0.029
	Restored	-0.147	0.360	0.005	0.084	0.171	0.033

Table 4 – Coefficients of regression, P-values and R-squared of the regressions between PDIR and functional traits (SLA and vegetative height) at the local scale (within sub-site). Significant p-value (p<0.05) are in bold.

		SLA ($mm^2 mg^{-1}$)			Vegetative height (cm)		
Sites	Sub-sites	Coef.	p	R^2	Coef.	p	R^2
Abannets	Reference	NA	NA	NA	NA	NA	NA
	Restored	-39.310	0.248	0.013	2.778	0.843	0.034
Rivelottes	Reference	NA	NA	NA	NA	NA	NA
	Restored	NA	NA	NA	NA	NA	NA
Tienne Breumont	Reference	11.481	0.378	0.378	-8.140	0.168	0.033
	Restored	NA	NA	NA	NA	NA	NA
Tienne Delvaux	Reference	NA	NA	NA	NA	NA	NA
	Restored	-90.850	0.197	0.025	130.160	0.052	0.097
Montagne- aux-buis 1	Reference	-26.380	**0.030**	0.128	-50.000	**0.001**	0.309
	Restored	169.100	**<0.001**	0.470	-170.000	**<0.001**	0.521
Montagne- aux-buis 2	Reference	NA	NA	NA	NA	NA	NA
	Restored	NA	NA	NA	NA	NA	NA

Across sites, regressions between environmental variables (soil depth and PDIR) and SLA were not significant (Fig. 4A–B) while regressions between environmental variables and vegetative height were significant. Vegetative height increased with soil depth (Fig. 4C) and decreased when PDIR increased (Fig. 4D).

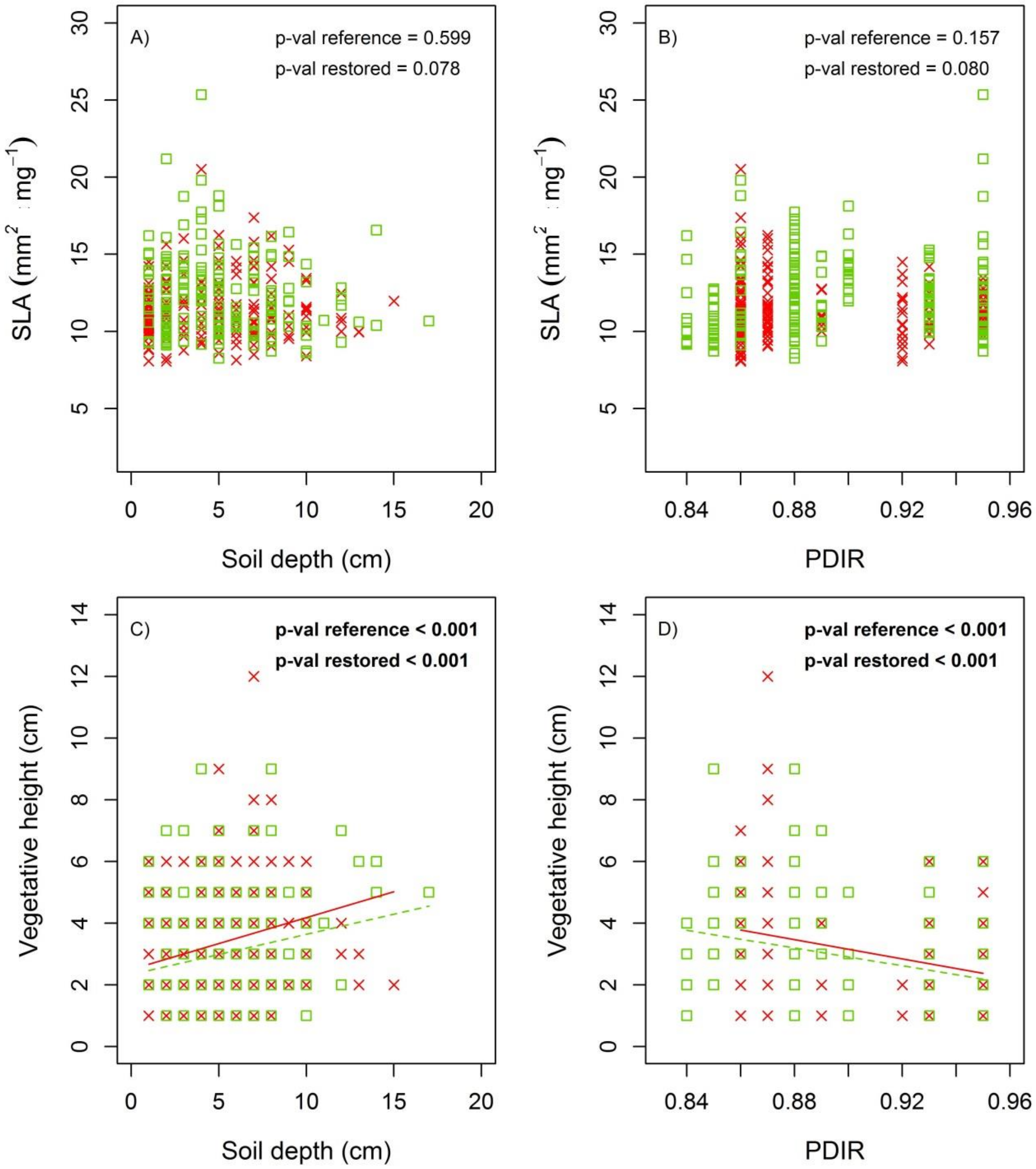


Figure 4 – Linear regressions between functional traits (SLA and vegetative height) and environmental variables (soil depth and PDIR, measured at the exact position of sampled individuals) were performed for reference and restored grasslands. Reference grasslands are represented by red crosses and restored grasslands by green square. Only significant regression lines have been drawn, regressions are red solid lines for reference grasslands and dashed green lines for restored grasslands. P-values of each regression are given on the graphs. Significant p-values ($p<0.05$) are in bold.

Discussion

The first objective of this study was to quantify the intra-specific functional variability among restored and reference populations of *P. tabernaemontani*, at the local and at the landscape scales. This was a primarily way to assess species ability to cope with varying levels of environmental conditions. The second aim was to assess if intra-specific functional variability of populations growing in restored areas responded to the environmental heterogeneity of their habitat as it has been shown for reference populations in the previous chapter of this thesis (**chapter 4**).

Functional traits variability

Vegetative height was similar in terms of mean trait values and range values among reference and restored grasslands. Concerning SLA, the intra-specific variability was higher in restored than in reference populations. High trait variability allows species to grow and reproduce under variable degrees of environmental stress (Weiner 2004; Fort et al. 2014). As differences in stress tolerance or resource use among individuals can, in certain case, generate variance in population demographic parameters (Bolnick et al. 2003), large intra-specific functional variability may be essential for the functioning of species populations within restored sites. The extent of intra-specific trait variability may indicate the ability of a species to cope with changes in environmental conditions (Almeida et al. 2013; Mitchell and Bakker 2014b). High functional variability is therefore crucial for species evolving in stressful environments, as can be the case in newly restored sites where environmental conditions may differ locally from the historic habitat of the species (Vergeer et al. 2003; Quintana-Ascencio et al. 2007). In this study, intra-specific variability of study functional traits characterizing reference populations was fully represented in restored populations of *P. tabernaemontani* what can be considered favorable for population functioning in restored grasslands.

Heterogeneity of environmental conditions

Ecological restoration of calcareous grassland should take into account the whole range of environmental variability that characterized historical habitats. In this study, at the local scale, a high variability of environmental conditions was shown within sub-sites. Environmental conditions characterizing reference grasslands in a site was mainly not represented in restored grasslands at the same site. As restoration protocols took place around remnants of historical reference in order to enlarge still-existing areas of calcareous grasslands, soil depth and PDIR are driven by the topography of areas selected for restoration purpose. We showed that opportunistic restoration around reference did not restore environmental conditions characterizing those reference habitats.

However, as restoration aims to improve ecological networks of suitable habitats for target species at the landscape level (Jongman and Pungetti 2004), an analysis at higher geographical scale is needed. At the landscape scale, restored grasslands covered the entire range of soil depth and PDIR variability characterizing reference grasslands. This showed that opportunistic restoration protocols that have been applied to restore calcareous grasslands in the study region could be considered successful at the overall scale. The high environmental variability shown between sub-sites in a site is counterbalanced when considering the landscape scale.

Functional response to environmental variability

It is not surprising that plant vegetative height was highly related to soil depth and PDIR, with smaller individuals on shallow soils with high PDIR, expressing stressful conditions as is has already been observed and discussed in reference calcareous grassland sites in the previous chapter (**Chapter 4**).

In our study, however, the SLA was not related to environmental gradients of soil depth or PDIR. However many studies showed that SLA may be related to soil moisture or incident radiation (Meziane and Shipley 1999; Ackerly et al. 2002; Wellstein et al. 2013; Harzé et al. 2016). Under stressful conditions, conservative species, which are characterized by low SLA, dense leaf tissues and low growth rates, exhibit high resource conservation (Albert et al. 2010a). However, Pescador et al. (2015), who studied the variation of intra-specific plant functional traits along an altitudinal gradient for 11 grassland species, did not detect any significant changes in SLA related to soil moisture gradient, despite a severe drought stress gradient that decreased with altitude. In our case, the season and location in which data were measured, the summer of 2014 in Belgium, was characterized by abnormally higher precipitation (2014 was the second-most rainy summer since 1981, with 348.2 mm of precipitation and 49 days of rain) than normal (224.6 mm of precipitation and 43.9 days of precipitation). This could explain why SLA did not respond to our proxy of soil moisture. Indeed, soil depth and potential direct incident radiation are potentially not a good proxy of soil moisture if summer precipitations are excessive. In the previous chapter, the study realized in the same study region, in the summer of 2012 (considered as normal for the season regarding precipitation) showed a clear plant leaf trait response along PDIR and soil depth gradients (Harzé et al. 2016). In the present study, a higher plant height was found for individuals located in plots with deeper soils or low PDIR (less potential direct incident radiation, on gentler or north-facing slopes). This could support the idea that higher plant height may be related to higher availability of soil moisture, as has already been shown for herbaceous species (Wellstein et al. 2013). In our case, as soil moisture may not be a limiting factor, the plant height response to soil depth could also be related to soil fertility (Alard et al. 2005).

Conclusion

Reference populations of *P. tabernaemontani* have been characterized in the previous chapter of this thesis (**Chapter 4**) and a high intra-population functional variability was shown at a very local scale, regarding SLA and vegetative height. The results of the present chapter showed similar intra-specific functional variability among reference and restored populations. Therefore, restoration of calcareous grasslands in the study area can be considered successful regarding the intra-specific functional variability of restored populations of the specialist plant species, *P. tabernaemontani*. Future research should address whether or not the observed intra-specific functional variability induces fitness differences among populations inhabiting these sites, thus influencing their long-term persistence in restored habitats.

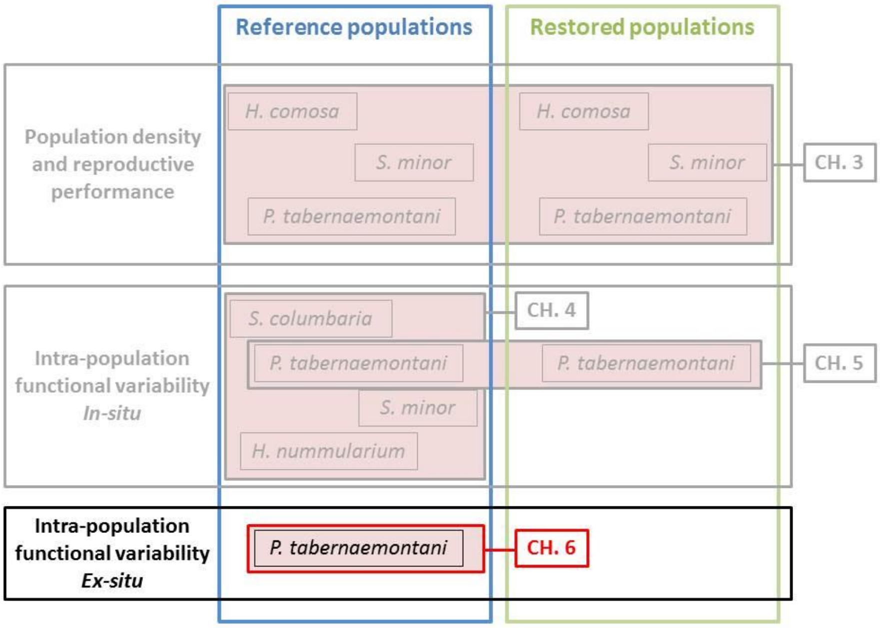
This work deserves to be completed for other species, as results could be different for rarer species or species evolving in narrower environmental conditions. We argue that conserving and restoring habitats with high environmental heterogeneity can be decisive to maintain large intra-specific variability in the landscape and thereby a variety of phenotypes that can potentially buffer plant species from future environmental changes (Wellstein et al. 2013; Mitchell and Bakker 2014b).

CHAPTER 6:

Intra-specific variability in the response to drought for *P. tabernaemontani*

The content of this chapter has been submitted as a short communication to "Plant Ecology and Evolution"

(March 2017)



Abstract

Calcareous grasslands are among the most species-rich habitats in Western Europe. Populations of plant species characterizing those ecosystems are naturally submitted to high variability in environmental conditions at the very local scale, resulting in pronounced variation in functional traits. Individuals located on xeric parts of calcareous grasslands are characterized by plant traits that potentially ensure more successful performance under stressful conditions. In the context of increased frequency of summer heat waves based on climate change, our aim was to characterize a possible intra-specific variability in drought response among individuals of one specialist plant species. A greenhouse experiment was set up to follow the survival of *Potentilla tabernaemontani* individuals according to their habitat of origin (xeric or mesic parts of Belgian calcareous grasslands) and the treatment applied (low or high drought stress). The results demonstrated that individuals originating from xeric parts of grasslands survived drought stress better than individuals from mesic parts. Specific leaf area (SLA) of all individuals was very low in the experiment, allowing them to decrease water loss during drought stress. Leaf production was lower for individuals exposed to high drought stress but flowers production was higher, potentially expressing a trade-off between tolerance to water stress, individual growth and reproduction. Local scale environmental heterogeneity deserves to be considered in conservation and restoration plans as it induces intra-specific functional variability between individuals and impacts individuals' ability to survive drought stress.

Introduction

Calcareous grasslands are recognized as local biodiversity hotspots in temperate regions, hosting rare and specialist plant species (Krauss et al. 2004; Adriaens et al. 2006). Those ecosystems suffered from intensive habitat destruction and fragmentation during the last century (Prendergast et al. 1993; Poschlod and WallisDeVries 2002; WallisDeVries et al. 2002; Adriaens et al. 2006) and are today a central issue in habitat conservation in Europe (Janssen et al. 2016). Calcareous grasslands are also characterized by high environmental variability at the very local scale, notably in terms of soil depth, soil moisture, slope and exposure, identified as factors that may influence soil hydrological status (Butaye et al. 2005b; Bennie et al. 2006; Piqueray et al. 2007). The total amount of available water for plants is demonstrably lower for xeric parts of grasslands, featuring thin soils (<10 cm), mostly present on steep and rocky slopes with a southerly aspect and excessive drainage. Those conditions lead to high soil desiccation, high evaporation and higher drought stress for herbaceous species (Alard et al. 2005; Butaye et al. 2005b; Bennie et al. 2008; Dujardin et al. 2012). Xeric parts of grasslands are characterized by lower herbaceous cover and fewer litter accumulation than mesic parts (Butaye et al. 2005b).

Climate change will likely become one of the major threats to biodiversity over the next few decades (Thuiller 2007; Bellard et al. 2012). Climate change predictions forecast that the frequency of summer heat waves will probably increase in large parts of Europe (Pachauri et al. 2014). These changes could strongly affect the structure and functions of grassland communities. Specialist plant species that will be able to reach more suitable habitats or produce phenotypic adaptive responses to climate change through local adaptation or phenotypic plasticity will probably be more prone to survive on the long-term (Bolnick et al. 2003; Bellard et al. 2012).

In calcareous grasslands, high intra-specific phenotypic variability has been exhibited at the very local scale (Harzé et al. 2016). Plant functional traits related to resource-use efficiency may vary greatly among individuals for a number of plant species, including *Potentilla tabernaemontani*, a perennial forbs that is considered calcareous grassland specialist in Belgium (Piqueray et al. 2007; Harzé et al. 2016). Specifically, individuals located on xeric parts of calcareous grasslands have been characterized by lower plant height and higher leaf tissue density (Harzé et al. 2016). Those traits potentially permit plants to maintain leaf turgor and foster more successful performance under drought stress (Niinemets 2001; Siefert 2012).

The current study aimed to evaluate if the high intra-specific phenotypic variability observed *in situ*, at the very local scale, for a specialist calcareous grassland species, may induce intra-specific differences in response to drought stress. More specifically, the study aimed to evaluate if individuals located on xeric parts of calcareous grasslands survive drought stress better. Individuals of *P. tabernaemontani* were collected from xeric and mesic parts of grasslands in order to test their survival under two levels of induced drought stress in a greenhouse experiment.

Material and method

Experimental design

Three sites were selected in southern Belgium; the Montagne-aux-buis (50°05' N, 4°34' E), the Tienne Breumont (50°04' N, 4°32' E) and the Sosoye hill (50°17' N, 4°46' E). They are calcareous grasslands developed through traditional agropastoral practices, where xeric and mesic grasslands coexist (Butaye et al. 2005b; Piqueray et al. 2007). At each site, 30 genets of *P. tabernaemontani* were randomly selected in mesic parts of grasslands and 30 genets were randomly selected in xeric parts (Fig. 1). Those genets were randomly selected among individuals of the **Chapter 4**. Genets were collected at a minimal distance of five meters from each other to assure collecting different genets.

For each selected genet, two ramets were collected between the 19th and the 26th of November 2013. Collected ramets were linked by stolon on fields and separated to be transplanted in pots (8 cm length x 8 cm width x 8 cm depth). Pots were previously filled with a substrate composed by ¼ of white sand and ¾ of a commercial lime compost (turf, fertilizer and lime) and water saturated. Ramets were then placed in a greenhouse in Gembloux (Belgium; 50°3'57''N; 4°42'11''E; annual mean temperature ca. 9°C). The number of leaves of each ramet was counted at the beginning of the experiment (L_1). The greenhouse was heated during winter to avoid frost (Fig. 2).

One week after transplantation, treatments were applied. For each selected genet, the two ramets were submitted to two distinct drought stress treatments (Fig. 1) during six months. The first ramet was submitted to a low stress treatment and the second ramet was submitted to a high stress treatment. Stress treatments were applied by allowing volumetric water content to decrease to a level of 30% (for the low stress) and of 15% (for the high stress) before watering the pots. Soil water content was measured three times a week using a volumetric moisture probe (TRIME®-PICO32, IMKO, Fig. 2) in 36 randomly selected pots of each treatment. All pots of the treatment were watered if the mean volumetric water content of the 36 pots was below 30% for the low stress treatment and below 15% for the high stress treatment.

A study realized in similar habitats indicated that soil moisture reached a maximum of approximately 50% in late autumn and winter and decreased to a minimum of roughly 20% during summer drought stress periods (Niklaus et al. 1998). Position of pots in the greenhouse was randomly changed every week.

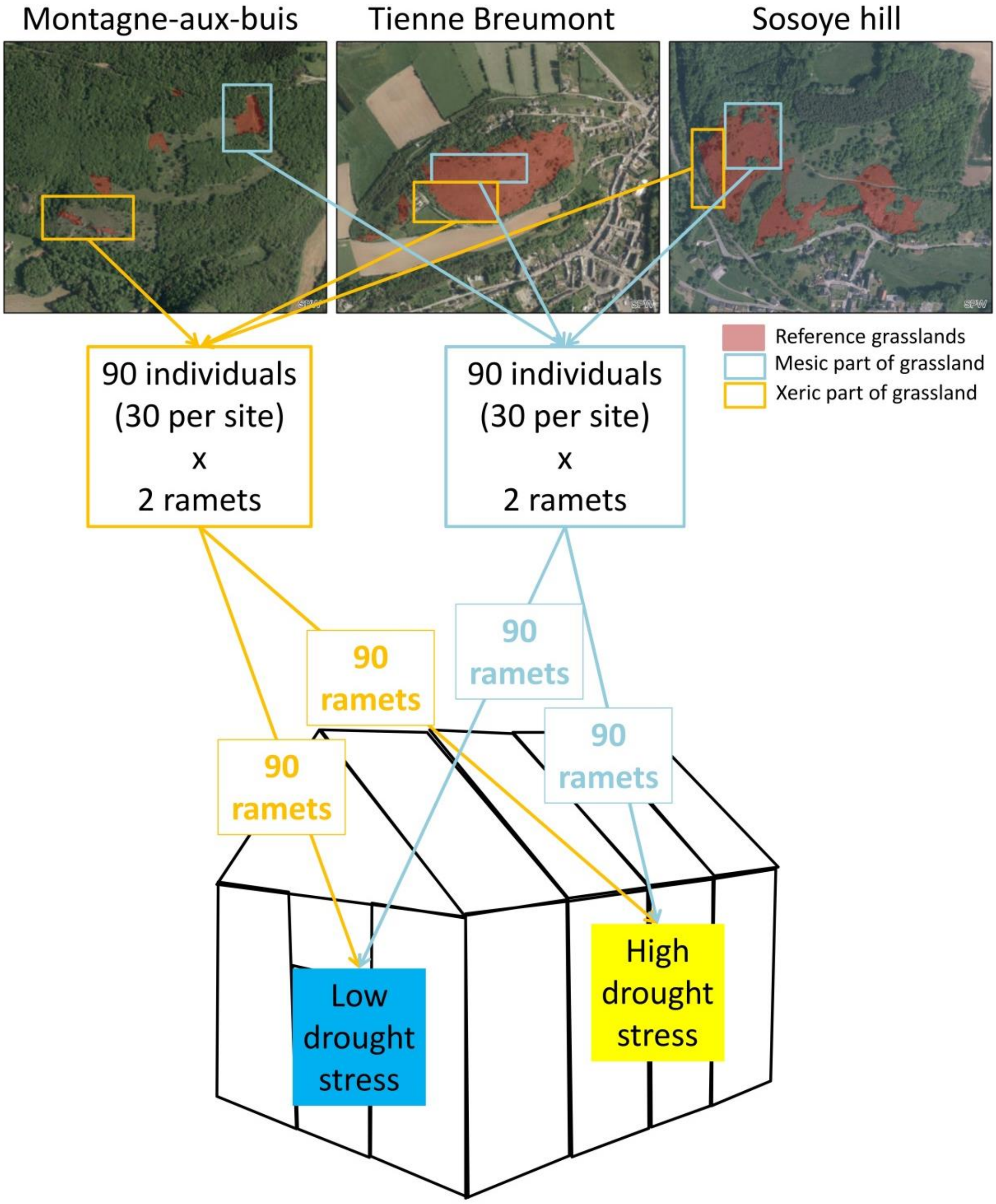


Figure 1 – Schema of the field survey and experimental design.

Figure 2 – Illustrations of the greenhouse experiment. From top left to bottom right: the greenhouse; the experimental design; pots at the beginning of the experiment; flowering individuals, flower and volumetric moisture probe (TRIME®-PICO32, IMKO).

Data collection and analysis

Ramet survival and flower production were monitored three times a week over six months. Survival was defined as complete leaf senescence and no ramet regrowth during two weeks after plant rehydration. The number of days between the beginning of the experiment and the production of the first flower of each ramet was calculated (first flowering) and the total number of flowers produced by each ramet was counted (no of flowers produced). The number of leaves of each ramet was counted at the beginning and at the end of the experiment for surviving ramets in order to calculate a foliar increase ratio: ($\ln L_1 - \ln L_0$), where L_0 and L_1 were the number of leaves at the beginning and at the end of the experiment, respectively (Hunt 2017).

The regulation of water loss through leaves can be expressed by several key leaf functional traits (Wright et al. 2001; Ackerly 2004) and, for example, be reflected through lower specific leaf area (SLA; Volaire 2008; Poorter et al. 2009). After six months, two leaves produced during the experiment by surviving ramets were collected for SLA measurement (in $mm^2\ mg^{-1}$). Fresh leaves were scanned and leaf area was measured using ImageJ imaging software (Abràmoff et al. 2004). Leaves were then oven-dried at 60°C for 72 hours and weighed to the nearest 10^{-1} mg (Mettler Toledo®, Viroflay, France).

In order to compare ramet performance (no of flowers produced, first flowering, foliar increase ratio and SLA) according to their habitat of origin (xeric or mesic parts of grasslands) and the drought stress treatment applied (low or high drought stress), three-way ANOVAs were performed based on site (random, three modalities), habitat of origin (fixed, two modalities) and stress treatment (fixed, two modalities) as crossed factors. As survival was a binary variable, a generalized linear model was fitted using the binomial family followed by an analysis of deviance with site, habitat of origin and stress treatment as crossed factors. The site effect was never significant (all p-values > 0.05), so we conducted the same analysis with habitat of origin and stress treatment as crossed factors. Deviance analysis and ANOVAs were followed by a pairwise comparison of means (Tukey's test). Foliar increase ratio and SLA were log transformed to meet the assumptions of the statistical analyses. ANOVAs were performed with Minitab® 17.1.0 and analysis of deviance were performed with R 3.1.0 (R Development Core Team 2014).

Results

The interaction between habitat of origin (mesic or xeric parts of grasslands) and stress treatment (low or high) was not significant for any measurements (Table 1). Survival probabilities were significantly higher for individuals collected from xeric parts of grasslands compared to individuals coming from mesic parts (p = 0.017, Table 1) and were significantly reduced for individuals exposed to high drought stress compared to low drought stress (p = 0.016, Table 1). Individuals from xeric parts of grasslands growing under low drought stress survived better (Fig. 3A).

Table 1 – Deviance analysis (survival) and two-way ANOVAs (no of flowers produced, first flowering, foliar increase ratio and SLA) performed with habitat of origin (fixed, two modalities) and stress treatment (fixed, two modalities) as crossed factors. Means are given according to habitat of origin or stress treatment. Significant p-values (p<0.05) are in bold.

	Interaction (habitat of origin * stress treatment)	Habitat of origin			Stress treatment		
	p	Xeric parts	Mesic parts	p	Low stress	High stress	p
Survival	0.627	0.85	0.75	**0.017**	0.85	0.75	**0.016**
no of flowers prod.	0.819	6.0	6.7	0.237	5.8	6.9	**0.028**
First flowering	0.496	88.9	88.0	0.789	85.9	91.1	0.108
Foliar increase ratio	0.116	1.08	1.20	0.064	1.23	1.05	**0.008**
SLA	0.297	7.8	8.1	0.157	7.7	8.2	0.067

Habitat of origin had no effect on the number of flowers produced, first flowering (number of days between the beginning of the experiment and the production of the first flower), foliar increase ratio and SLA, while stress treatment had no effect on first flowering and SLA (Table 1). The foliar increase ratio was lower for individuals exposed to high drought stress (p = 0.008, Table 1), particularly for individuals from xeric parts of grasslands (Fig. 3D). The number of flowers produced was higher for individuals exposed to high drought stress (p = 0.028, Table 1) but no significant differences between means were highlighted by the pairwise comparison of means (Tukey's test, Fig. 3B). First flowering and SLA were similar among habitat of origin and treatment (Fig. 3C and 3E). SLA values were particularly low for both treatments with values comprised between 3.6 and a maximum of 18.4 $mm^2 mg^{-1}$ with a mean of approximately 8.0 $mm^2 mg^{-1}$ for both treatments.

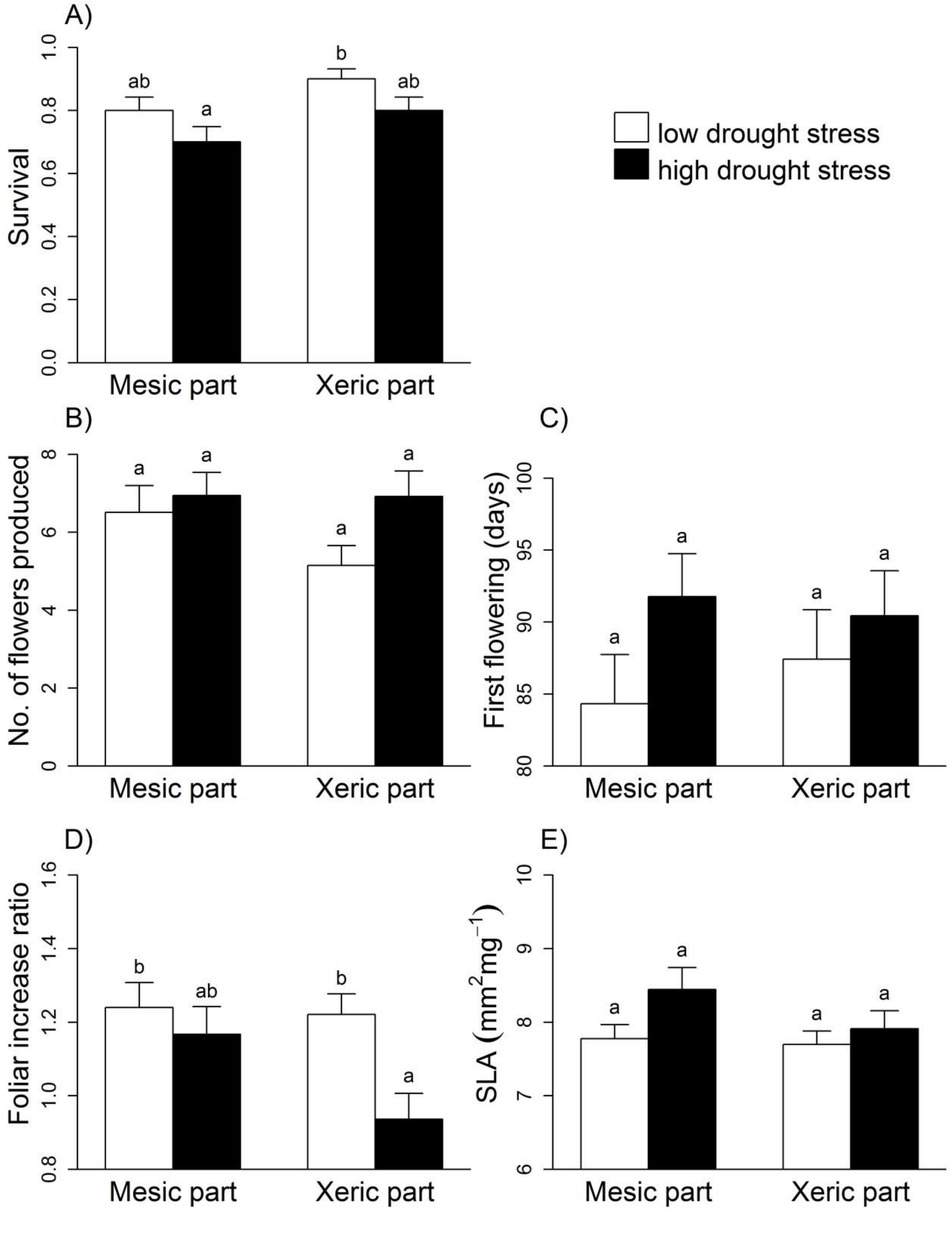


Figure 3 – Effects of habitat of origin (mesic or xeric parts of grasslands) and stress treatment (low stress in white and high stress in black) on survival (A), number of flowers produced (B), first flowering (C), foliar increase ratio (D) and SLA (E) of *P. tabernaemontani* individuals. On each graph, means and standard errors are drawn and significantly different means (crossed Tukey's test) are followed by different letters

Discussion

Populations of *P. tabernaemontani* are naturally exposed to high small-scale variability in environmental conditions in calcareous grasslands (Bennie et al. 2008; Harzé et al. 2016), leading to high *in situ* variability of functional traits at the very local scale (Harzé et al. 2016). Individuals grown in potentially more xeric parts of grasslands (low soil depth and high potential direct incident radiation) are smaller and characterized by lower specific leaf area (SLA) than individuals in potentially more mesic parts of grasslands, which should ensure more successful performance under stressful conditions. Indeed, low SLA is related to high investment in structural tissues, which allow plants to maintain leaf turgor under drought stress (Niinemets 2001; Siefert 2012). Conservative species that exhibit low SLA, corresponding to dense leaf tissues and low growth rates, exhibit high resource conservation (Albert et al. 2010a).

Intra-specific variability in drought stress responses was evaluated in a greenhouse experiment for a specialist plant species, *P. tabernaemontani*. The findings confirmed that drought response was variable according to the habitat of origin of the species. Individuals originating from xeric parts of grasslands survived better both low and high drought stress conditions. This could be because of low SLA values, allowing individuals to decrease water loss under drought stress. SLA were particularly low for all individuals at the end of the experiment (maximum value: 18.36 mm^2 mg^{-1}) compared to *in situ* observation (32 mm^2 mg^{-1}) (Harzé et al. 2016). However, in this experiment, SLA did not differ across habitats of origin or stress treatments and therefore did not explain the observed differences in individual survival. Resistance to drought stress could be understood by variability in other (not measured) physiological traits linked to water use efficiency, such as root traits or stomatal density (Cornelissen et al. 2003; Pérez-Harguindeguy et al. 2013). Indeed, previous slowly increasing stresses may induce physiological adjustments in plant that may protect them from a future stress abruptly imposed (Kozlowski and Pallardy 2002).

Although phenological changes are more heavily investigated in response to mean change in climate than to extreme events, plant species may respond to drought by modifying their phenology, such as earlier onset of leaf development or flowering (Parmesan and Yohe 2003; Root et al. 2003; Reyer et al. 2013). This was not the case for *P. tabernaemontani* individuals as the period of time before the first flowering was similar for individuals from xeric or mesic parts of grasslands and among treatments.

The foliar increase ratio also presented high variability among individuals, illustrating the ability of species to produce distinct phenotypes in response to the environment. In arid environments, a negative relationship between plant capacity to tolerate drought and growth potential can be predicted. This could be because off a trade-off between tolerance to water stress and individual growth (Chapin 1980; Reznick 1985; Loehle 1987). Plants with a higher growth potential may be characterized by physiological traits that, in principle, should result in poor performance under intense drought conditions and vice versa (Fernández and Reynolds 2000). This could justify the differences in leaf production between treatments with individuals reducing their production of leaves under high drought stress. Reducing leaves production under stressful environment could also be the results of an allocation strategy between growth and reproduction. Indeed, plants may allocate greater proportion of their resource to reproduction than to vegetative growth under environmental stress (Aronson et al. 1993; Ravenscroft et al. 2014). That could explain why *P. tabernaemontani* produced more flowers under high drought stress.

The observed variability in plant traits can stem from multiple factors. It can be the result of variability between genotypes originating from evolutionary processes and local adaptation (Fisher 1930; Hughes et al. 2008), or it can be based on acclimation or phenotypic plasticity; i.e. the potential of each individual genotype to produce multiple phenotypes under various environmental conditions (DeWitt et al. 1998). Those mechanisms may act simultaneously and the observed variability in plant traits can be the consequence of different combinations of genetic and environmental variabilities (Bolnick et al. 2003). Carefully designed experiments are necessary to assess whether the observed intra-specific variation is brought about by plasticity or local adaptation. However, whatever the causes of the observed traits variability in calcareous grassland specialist plant species, the greenhouse experiment demonstrated that individuals from very stressful environments survive drought stress better. It represents a challenge for species conservation in the face of future climate change.

CHAPTER 7:

General discussion

Major findings and statements of the discussion

Growing concerns surrounding the ongoing loss of biodiversity worldwide has resulted in increased efforts to conserve endangered ecosystems (Balmford et al. 2005). Although crucial, conservation measures alone are not sufficient enough to halt the decline of many species (Ozinga et al. 2009) and ecological restoration is widely recognized as one optimistic solution to face the challenge of biodiversity loss (Dobson et al. 1997; Suding et al. 2015; Perring et al. 2015).

Best restoration practices can be determined by the evaluation of restoration success, and the criteria used to determine this success are numerous. Indicators may focus on different ecological groups (e.g. plant, insects, birds) along with various levels of biodiversity organization (e.g. individuals, populations, species, communities, ecosystems). Moreover, they can be considered at various spatial and temporal scales (SER 2004; Ruiz-Jaen and Aide 2005b; Cristofoli and Mahy 2010).

Major findings of the first part of this thesis (**Chapter 2**) showed that population parameters, though pertinent to evaluate restoration success, have been less examined compared to indicators related to higher levels of biodiversity components. Population demography and performances are widely employed to evaluate grassland restoration success while considering the genetic structure of restored populations in a context of fragmentation is largely deficient.

Grassland restoration essentially concentrates on the recovery and maintenance of suitable environmental conditions and the recolonization of target species (Perrow and Davy 2002; Piqueray and Mahy 2010). Community composition as well as abiotic components of the restored ecosystem are therefore key parameters to assess restoration success (SER 2004; Ruiz-Jaen and Aide 2005a). The community approach evaluates species presence and/or abundance subsequent to habitat restoration. Such an approach notably permits identifying several species that were not able to colonize restored areas, mainly typical species of the reference habitat, sometimes rare and protected by local laws (Kiefer and Poschlod 1996; Piqueray et al. 2011b). When restoration goals focus on the recovery of those species, population parameters are fully relevant to gauge restoration success (Carignan and Villard 2002; Cristofoli and Mahy 2010). Restoration practices impact plant species populations in many ways; from colonization to establishment, growth and reproduction.

In the second part of this thesis (**Chapters 3–6**), calcareous grassland restoration was evaluated through a population approach for five specialist plant species. From a population biology perspective, restored populations must possess characteristics that foster dispersal, growth, reproduction and adaptation to the environment in order to enhance species persistence over the long-term in restored areas (Montalvo et al. 1997).

Major findings of the second part of this thesis showed that:

1. Populations of targeted species were able to colonize the restored areas;

This statement will be discussed regarding the targeted species and the protocols applied to restore studied grasslands (p 119–120).

2. Populations of targeted species were able to establish and to reproduce in the restored areas;

This statement will be discussed in term of population dynamics over the long term in restored areas (p 121). Moreover, the quality of the selected reference will be nuanced (p 122–123).

3. Reference and restored populations of targeted species were characterized by high intra-population functional variability in response to the variability of their environment.

The relevance of considering high intra-specific trait variability a success in restoration will be discussed, notably in a context of climate change (p 124–125). Functional trait syndrome will be described along the environmental gradient (p 126) and discussed in the light of the specialization of study species (p 127). Moreover, the consistency of trait response to environmental gradients will be nuanced (p 128–130).

Species response to environmental stress and especially to drought stress will be discussed regarding underlying mechanisms and trade-off between observed traits (p 131).

Finally, findings of the thesis will be discussed in a larger context, including the landscape scale (p 132), regarding previous studies evaluating calcareous grassland restoration in Belgium and elsewhere in Europe (p 133–134) and proposing some practical implications and perspectives (p 135–136).

Targeted species colonized the restored sites

The first step to evaluate restoration success with a population approach is to determine if species have been able to reach restored sites in order to form a new population. For the species studied here, seed dispersal may be interpreted as successful as the study species colonized the restored calcareous grasslands. This first statement is quite obvious as, for methodological reasons, we selected plant species that occurred on reference and restored grasslands of the selected sites.

The colonization patterns observed for our study species might, however, not be the same for all calcareous grassland species. In a study comparing reference and restored calcareous grassland communities in Belgium, Piqueray et al. (2011b) found that 76% of species inhabiting the reference grasslands also occurred in restored grasslands, while 24% of species failed to colonize the restored sites. Those results were similar to other studies concerning calcareous grassland restoration in Estonia, Germany and Poland notably (Pärtel et al. 1998; Von Blanckenhagen and Poschlod 2005; Dzwonko and Loster 2007). Those poor colonizers are, for example, *Anthericum liliago*, *Pulsatilla vulgaris*, *Trifolium montanum* and *Veronica prostrata* (Piqueray et al. 2011b). There are multifactorial causes that can explain patterns of species occurrence on restored sites, including emergence from the soil seed bank, dispersal agents and spatial distribution of the species within the landscape. Those species described as poor colonizers are much rarer in Belgium (Fig. 1), which reduce the amount of local seed source for the colonization of restored sites, compared to our selected species. Moreover, persistence of their seeds in the soil is relatively short (Poschlod et al. 1998).

Many calcareous grassland species are capable to colonize spontaneously restored sites. It was the case of our study species. Deeper research on the cause of colonization or germination failure is needed for those non-recolonizing species and the conclusions of this thesis cannot be generalized to rarer and more specialized species.

To avoid restoration failure for poor colonizers, an input of seeds or other propagules might be necessary to establish populations in restored areas. This technique has been applied numerous times before in grassland restoration. Indeed, out of the 141 papers selected in **Chapter 2**, only 21% examined grassland restoration without any species addition, while 79% did so after introducing seeds, hay or transplants, thus short-cutting the dispersal filter to restoration. In our study, calcareous grasslands were restored without any species addition, relying solely on spontaneous colonization of restored sites and enhanced by management with migrating sheep flocks. In calcareous grasslands, sheep often play a major role in species dispersal across the landscape (Fischer et al. 1996; Poschlod et al. 1998).

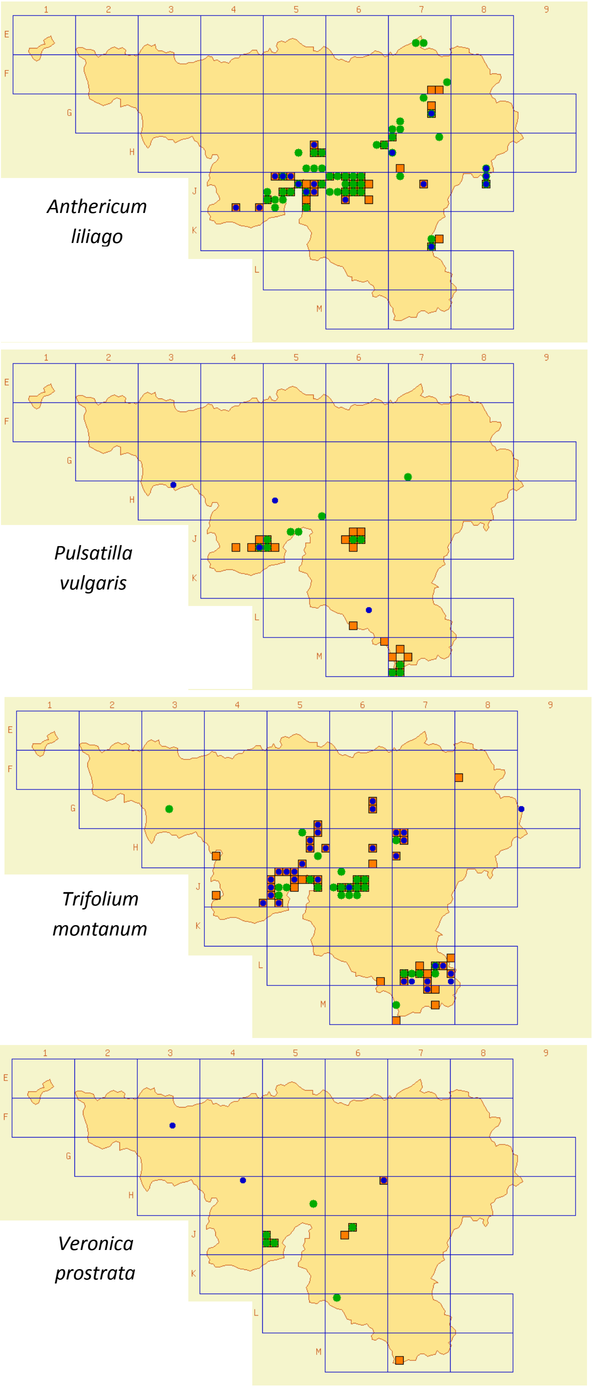


Figure 1 – Distribution range of *Anthericum liliago*, *Pulsatilla vulgaris*, *Trifolium montanum* and *Veronica prostrata* in south Belgium (Wallonia). Data from the "Atlas de la flore" (available from http://biodiversite.wallonie.be). Species occurrence (records: <1930=pink square, >1930=green square and blue circle, >1980=green circle).

Population establishment in restored sites

In a previous study on restored Belgian calcareous grasslands (12 sites located in two areas: the Viroin valley and the Lesse and Lomme valleys), Piqueray et al. (2011b) demonstrated that the five species studied in this thesis were present at restored sites but that they that they were less frequent at restored sites than at references sites and locally less abundant. This was also the case in our study sites, as our study species were able to colonized restored grasslands but were overall less locally abundant on restored grasslands than on reference grasslands (**Chapter 3**). Study species were therefore successful in reaching restored areas, though population processes that permit population growth and establishment were not completely successful.

Seed production was not the deficient process as restored populations produced a large amount of flowering units and seeds (**Chapter 3**). However, in plants, essential trade-off exists between traits linked to growth, survival and reproduction. If resources are limited, the organisms allocating energy to one process must decrease energy allocated to the other(s) (Obeso 2002). Higher seed production may therefore indicate reduced plant survival in the population.

A complete overview of population demography can be revealed by the use of population matrix analysis. Matrix projection models are potentially powerful tools to assess population viability, to identify the less efficient life stages (Lande 1988; Menges 1990; Oostermeijer et al. 2003), to predict future growth of populations (Schemske et al. 1994; Oostermeijer et al. 2003) or to assess the effects of past or future changes in the management or in the environment (Crone et al. 2011). Following the fate of individuals within populations over the course of several years could facilitate determining if restored populations are still in a colonizing state, with high seed production and high juvenile density, compared to reference populations potentially denoting a stable dynamic with higher adult survival and less reproductive outputs. Burmeier et al. (2011) showed that seed production was greater at restored compared to remnant sites for one grasslands plant species but that juvenile mortality was higher and adult density similar. They put forth that the lower vegetation cover of restored sites impacted seedling survival and that the soil seed bank contributed more to reproduction for populations of remnant sites. Despite their interest, demographical studied using matrix projection models need long term data acquisition, which represent a constraint for ecologists (Crone et al. 2011).

In restored calcareous grasslands, when the initial colonizing processes will be over, both abiotic and biotic conditions should reach those of the reference habitats (Piqueray et al. 2011b). The fitness of individuals should then be similar to those of reference grasslands and population dynamics should approximate those of remnants.

Another possibility is that remnant populations are actually experiencing a declining dynamic. Indeed, one of the first indications of population decline might be lower reproductive success (Endels et al. 2005). Remnant populations may be in a senescent state, signified by the presence of several adults and few juveniles, with therefore limited potential viability on the long-term (Endels et al. 2004).

It should be noted that this could be because of a lack of management of reference grasslands (Fig. 2). Many species of open habitats need small gaps for regeneration by seeds. A denser vegetation structure resulting from less frequent management often leads to lower population densities (Oostermeijer et al. 1994) and declining populations (Oostermeijer et al. 1994; Brys et al. 2004; Schleuning et al. 2009; Schleuning and Matthies 2009; Walker and Pinches 2011). For *Helianthemum nummularium*, Poschlod et al. (2011) demonstrated that the highest density of individuals was found in grazed populations and the lowest in grasslands without management.

Figure 2 – A lack of management on reference grasslands may decrease availability of regeneration gaps and lead to grassland encroachment (example in a reference grassland on the Tienne Breumont, Viroin valley, Belgium).

Local reference sites are recognized as truly conducive to evaluate restoration projects (White and Walker 1997; Ruiz-Jaen and Aide 2005a). Calcareous grassland remnants have been widely employed as references for restoration (e.g. Hutchings and Booth 1996; Fagan et al. 2008; Piqueray et al. 2011b; Olsson and Ödman 2013; Helsen et al. 2013b). Remnant populations have persisted across the landscape with the past and current disturbances as well as climate change, and could therefore be deemed sustainable and resilient (Jackson et al. 1995). However, one must bear in mind that those reference are remnants that have been isolated within the landscape for more than a century, which could have negative consequences on plant species populations and their functioning (Young et al. 1996; Hobbs and Yates 2003; Lienert 2004). Remnant calcareous grasslands that have been affected by habitat fragmentation in the past may exhibit an extinction debt (Piqueray et al. 2011a). Indeed, some species may almost immediately respond to fragmentation, but a time lag in the response may also occur, creating an extinction debt: a condition in which populations are still present in a habitat, even if the population is expected to go deterministically extinct (Tilman et al. 1994; Hanski and Ovaskainen 2002; Kuussaari et al. 2009). In the Viroin valley, however, Adriaens et al. (2006) supplied no evidence of an extinction debt as opposed to studies on other regions (Lindborg and Eriksson 2004; Piqueray et al. 2011a; Piqueray et al. 2011c). Finally, species performances may be affected by environmental conditions of remnant patches that may affect their fitness (Vergeer et al. 2003; Quintana-Ascencio et al. 2007; Adriaens et al. 2009).

Hopefully, the restored populations of the study species were in a better population dynamic state compared to declining remnant populations. More in-depth demographic studies encompassing several years and following population growth rates are required to get a more complete picture of the population dynamics.

High intra-specific variability in response to the environment

Calcareous grasslands are typified by local-scale variability in soil water availability (Bennie et al. 2008; Dujardin et al. 2012). Local variability of environmental conditions may induce variability in plant traits at the local-scale, thereby allowing species to grow and reproduce under variable degrees of environmental stress (Weiner 2004; Fort et al. 2014).

As restored environments may differ from reference habitats (Vergeer et al. 2003; Piqueray et al. 2011b), phenotypically diverse individuals may compose reference and restored plant species populations. Further, individuals may differ in their response to abiotic tolerance or resource use, which may in turn impact population demographic parameters (Bolnick et al. 2003; Lankau and Strauss 2007). In new environments, plant populations able to elicit a phenotypic adaptive response will probably be more prone to survive in the long-term (Bolnick et al. 2003; Bellard et al. 2012).

Studies dealing with intra-specific variability of functional traits often regard high trait variability being necessary to enhance species persistence in the context of a changing environment (Albert et al. 2010a; Albert et al. 2012; Almeida et al. 2013; Wellstein et al. 2013). However, this assumption is not necessarily true and depends on the mechanism underlying intra-specific functional variability. Indeed, observed intra-specific trait variability may stem from phenotypic plasticity, i.e. the potential of each individual genotype to produce multiple phenotypes under various environmental conditions (DeWitt et al. 1998), or it can be the result of variability between genotypes originating from sexual reproduction and evolutionary processes, such as local adaptation (Fisher 1930; Joshi et al. 2001; Hughes et al. 2008).

If the observed trait variability is based on genetic variation, then, a high intra-specific variability may be a clue for highly adaptive potential to a changing environment (Ravenscroft et al. 2014). In that case, it is meaningful to preserve high trait variation with the objective of conserving high population genetic diversity (Joshi et al. 2001; Bolnick et al. 2003; Ravenscroft et al. 2014; Mitchell and Bakker 2014a).

In contrast, if the observed trait variability is not related to genetic variation, but to a direct plastic response of individuals to variable conditions, this variability may not necessarily enhance species persistence in a changing environment. For instance, high trait variability can arise from the presence in the population of stressed and non-stressed individuals. At the individual level, high phenotypic plasticity may aid in coping with an environmental change (Richards et al. 2006). Yet, high trait variability at the population level does not mean that all individuals have high plasticity.

Carefully designed experiments are required to demonstrate that plasticity is adaptive. It is necessary to demonstrate that plasticity is heritable, that there is variation for it and that this variation is related to fitness. Practically speaking, if plasticity in functional traits enhances survival and reproduction, this plasticity could be considered adaptive (Sultan 1987; Sultan 1995; Griffith and Sultan 2005).

In this thesis, the observed intra-specific functional variability was probably largely attributable to plasticity. Indeed, gene flow between studied individuals was certainly high as spatial distance between them was quite short and because the study species are pollinated by flying insects. The possibility of genetic differentiation between individuals is therefore reduced. Moreover, in the greenhouse experiment with *P. tabernaemontani* (**Chapter 6**), no drought stress was strong enough to cause exceedingly high mortality, confirming that the individuals were able to tolerate a wide range of abiotic conditions.

As we did not test whether observed phenotypic plasticity was linked to plant performance (fitness), we must keep in mind that high intra-specific trait variability is not necessary to enhance population persistence within the context of environmental change.

Very high trait variability in response to the environment has been detected in populations of specialist species growing in reference calcareous grasslands (**Chapter 4**). The extent of intra-specific variability in plant traits from our study sites, at a very local scale, exceeded ranges of the same traits in studies covering several kilometers and large environmental gradient such as climatic gradients in alpine valleys (Hulshof and Swenson 2010; Albert et al. 2010b). Across study sites, the extent of intra-specific variability of the studied plant traits was similar between reference and restored populations (vegetative height) or more variable (SLA) in restored than in reference grasslands. To conclude to restoration success, we should assess whether or not this observed intra-specific variability of plant traits in reference and restored populations impacted their performance. Indeed, the potential link between intra-specific functional variability and reproductive performance deserves to be studied in order to fully grasp the importance of intra-specific functional variability for population dynamics and restoration success, especially in a context of climate change.

Climate change predictions forecast that the frequency of summer heat waves will probably increase in large parts of Europe (Pachauri et al. 2014). Specialist plant species that will be able to reach more suitable habitats or produce phenotypic adaptive responses to climate change through local adaptation or phenotypic plasticity will probably be more prone to survive on the long-term (Bolnick et al. 2003; Bellard et al. 2012).

Functional trait syndrome along environmental gradients

Observed intra-specific variability of plants traits was related to environmental conditions in reference populations (**Chapter 4**). Individuals exhibited high LDMC, low SLA and low vegetative height in relation to low soil depth and high PDIR, potentially related to low availability of soil moisture (**Chapter 4**). This trait syndrome expressed resource conservation under stressful conditions, potentially ensuring better population performance (Niinemets 2001; Bolnick et al. 2003; Albert et al. 2010b; Siefert 2012).

In this work, three functional traits were measured to reflect population response to potential soil water availability: SLA, LDMC and vegetative height. These traits are recognized as reliable predictors of species resistance to disturbance (Gamfeldt and Kallstrom 2007) and response to environmental conditions (Pakeman et al. 2009). They are also widely acknowledged as relevant for understanding plant response to a broad assortment of environmental conditions (Weiher et al. 1999; Lavorel and Garnier 2002). Other plant traits are most directly related to soil water availability, though they are also more difficult to measure. They often involve quantification over long periods of time or complex experimental manipulations, therefore called “hard” traits (Weiher et al. 1999). For example, water use efficiency (WUE) is the ratio of the net photosynthetic and transpiration rates and is determined through infrared gas analysis. In practice, “soft” traits are often measured in the field, namely traits that are easier to assess and considered analogous to hard traits, which is the case for our selected traits (Weiher et al. 1999; Hodgson et al. 1999).

SLA and plant height were chosen because they are fundamental traits connected to plant response to their environment and are linked to stress tolerance and resource use efficiency (Wilson et al. 1999; Garnier et al. 2001a; Westoby et al. 2002; Ackerly 2004). The relationship between SLA or height and soil water availability has been previously verified in practice (Miao et al. 1992; Craufurd et al. 1999; Anyia and Herzog 2004; Liu and Stützel 2004; Gross et al. 2008; Jung et al. 2010). It has also been confirmed ex-situ as individuals of *P. tabernaemontani* exhibit very low SLA in response to drought stress (**Chapter 6**). SLA and height may also vary based on temperature, light and soil nutrients (Craufurd et al. 1999; Meziane and Shipley 1999; Mendes et al. 2001; Ackerly et al. 2002; Navas and Garnier 2002; Gross et al. 2007; Pérez-Harguindeguy et al. 2013; Wellstein et al. 2013).

Herein, soil depth and PDIR gradients were considered reliable proxies of soil water availability for plants. However, soil depth may also impact other abiotic and biotic factors, notably soil nutrient availability and competition by the vegetation (Alard et al. 2005). Our gradient may therefore be considered as a stress gradient, encompassing soil resource availability, water as well as nutrients.

Study species occurred all along environmental gradients encompassed in this thesis and they showed a similar functional response to these gradients. Those species are specialists of calcareous grassland of the *Festuco-Brometea* (Butaye et al. 2005b). However, the degree of specialization of a species can be defined at very different spatial scales.

In Belgium, study species are certainly specialists of the *Festuco-Brometea*, including mesic and xeric part of grasslands. Although among selected species some are more specialized than others (Fig. 13, **Chapter 1**), they shared a similar response to the gradient studied in this thesis.

Compared to other species occurring in calcareous grasslands, the five selected species are certainly more generalists than, for example, *Melica ciliata* and *Arabis hirsuta* that rarely occur on mesic parts of grasslands and are more specialists of the *Xerobromion*, or *Pimpinella saxifraga* and *Centaurea scabiosa* that rarely occur on xeric parts of grasslands and are specialist of the *Mesobromion* (Butaye et al. 2005b; Piqueray et al. 2007).

Selecting species occurring on xeric and mesic parts of grasslands permitted us to observe their response to the variability of their environment. As highly specialist species and species with restricted ranges are expected to be more sensitive to changes of the environment and less favored in disturbed environments (Johnson 1998; Thuiller et al. 2005), it would be interesting to assess the functional response of more specialized species and the consequences of environmental heterogeneity on their demography, especially in a context of climate change and habitat restoration.

Consistency of trait response to environmental variables

Initially, a very clear functional response according to soil depth and PDIR gradients was observed for our study species (**Chapter 4**), supporting the idea that greater plant height, higher SLA, and lower LDMC was related to greater availability of soil resources, water or nutrients (Meziane and Shipley 1999; Wellstein et al. 2013). What was much more difficult to explain was the complete disappearance of the response of SLA based on the same gradients two years later (**Chapter 5**).

In order to better understand the relationship between the study traits and examined environmental variables, supplementary analyses were realized. Functional traits (SLA and vegetative height) and environmental variables (soil depth and PDIR) measured at exactly the same study sites (Montagne-aux-buis and Tienne Breumont, only reference grasslands) were compared between 2012 (**Chapter 4**) and 2014 (**Chapter 5**) for *P. tabernaemontani* individuals. I performed linear regressions between functional traits and environmental variables for both years separately.

Vegetative height increased significantly with soil depth (Fig. 3B and 3F) and decreased when PDIR increased (Fig. 3D and 3H), and these results were consistent between 2012 and 2014 and between sites (except at the Montagne-aux-buis in 2014; Fig. 3F). In 2012, SLA rose significantly in relation to soil depth (Fig. 3A) and was reduced when PDIR increased (Fig. 3C), while the same regressions were not significant in 2014 (Fig. 3E and 3G). This could not be interpreted via a narrower range of environmental conditions in 2014 versus 2012 at the study sites. Indeed, the ranges of soil depth and PDIR in 2014 were even wider compared to the 2012 measurements. Ranges of SLA, however, were narrower in 2014 than in 2012, with maximum values being much smaller in 2014 (Fig. 3).

A potential climatic effect could be one factor, as soil depth and PDIR were potentially not as reliable proxies of soil moisture in 2014 as precipitation was excessive compared to 2012 (**Chapter 5**). No response of SLA to soil moisture has been observed for certain alpine grassland species despite a huge environmental gradient (Albert et al. 2010b; Kichenin et al. 2013; Pescador et al. 2015). Justification proposed in those publications was that competition and facilitation between plant species may both vary along environmental gradients and affect intra-specific variation more than abiotic factors (Kichenin et al. 2013).

Another reason could be the absence of a relationship between SLA and environmental gradients because of a problem in gradient selection. Indeed, when studying the lower end of an environmental gradient, one may find a positive relationship between trait value and gradient, while at the upper end, a negative relationship may be expected (Albert et al. 2010b). These explanations are not really convincing in our case as a clear relationship between traits and environmental variables was found in 2012. Maybe other not investigated environmental variables impacted SLA in 2014 more so than those selected did.

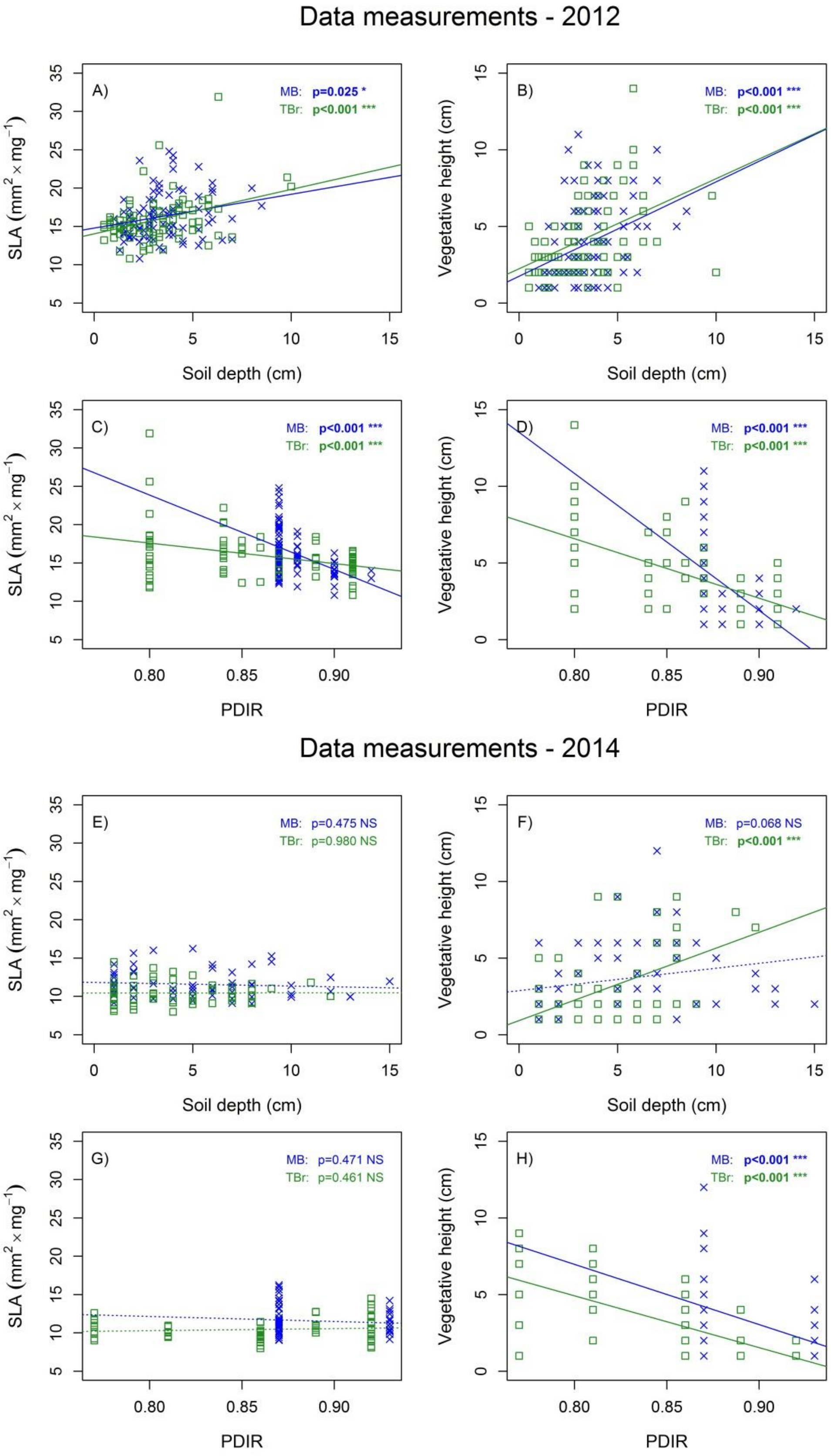


Figure 3 – Linear regressions between functional traits (SLA and vegetative height) and environmental variables (soil depth and PDIR) in 2012 and 2014 on the Montagne-aux-buis (blue) and Tienne Breumont (green) sites, concerning *P. tabernaemontani* individuals. Significant regression lines are solid and non-significant regression lines are dashed. P-values of each regression are given on the graphs and significant p-values ($p<0.05$) are in bold.

Species response to drought stress and allocation strategies

The findings from this thesis showed that reference populations were not only characterized by a very high intra-specific variability of specific plant traits (**Chapter 4**), but also by intra-specific variability in response to drought stress. In the experiment exposing individuals of *P. tabernaemontani* to drought stress, a higher stress induced variability in plant survival, flowers production and vegetative growth while the specific leaf area was particularly low for all plants in the experiment (**Chapter 6**).

In this thesis, we observed morphological plant response to drought stress. Morphological mechanisms of drought stress response notably involve changes that reduce water loss from plants, due to stomatal control of transpiration, and improve water uptake through an extensive and prolific root system (Turner et al. 2001; Kavar et al. 2008). It may be observed through a wide variety of traits, and notably leaf traits such as SLA, leaf area or leaf pubescence. Root traits are other deterministic traits involved in drought stress response, for example root biomass or density (Farooq et al. 2009).

However, plant response to drought is a complex phenomenon that involves a number of morphological, physiological and biochemical processes at genes, cells, tissues, organs and whole-plant levels (Farooq et al. 2009). Moreover, as previously discussed, essential trade-off exists between traits linked to growth, survival and reproduction (Obeso 2002). The energy organisms allocate to one process varies over time, across environments and among species. Under drought stress, plants may allocate greater proportion of their resource to water uptake by increasing root biomass, or they may allocate more resource to reproduction (Aronson et al. 1993; Ravenscroft et al. 2014).

We should also notice that effects of environmental stress may be beneficial for plants. Slowly increasing stresses may induce physiological adjustments in plant that may protect them from a future stress abruptly imposed. Moreover, water deficit may promote allocation to root growth, resulting in plants with a higher capacity to absorb water but also nutrients (Kozlowski and Pallardy 2002).

More in depth measurements are therefore needed to fully understand plant response to soil water availability and to drought stress, but also to understand how morphological traits such as SLA respond to water limitation and how these changes may subsequently affect plant growth, survival or reproduction (Poorter et al. 2012).

Considering multiple populations within the landscape

Local-scale variability of environmental conditions as well as local-scale variability in plant traits can be very high in calcareous grasslands (**Chapters 4 and 5**). However, in the context of calcareous grassland restoration, a multi-site approach revealed that environmental conditions exemplifying reference grasslands at a site was, for the most part, not represented in restored grasslands at the same site (**Chapter 5**). This highlighted the importance of considering a landscape approach that encompasses several sites in order to counterbalance local-scale differences between reference and restored grasslands.

Landscape connectivity is increasingly seen as a key conservation and restoration goal, particularly because of a changing climate. Deciding what and where to restore is clearly a major challenge for future restoration efforts (Perring et al. 2015). Choosing to restore sites with maximum environmental variability at the local scale may potentially result in high local-scale functional variability in plant species populations while decreasing restoration investment. However, the selection and prioritization of sites for restoration is an arduous matter and always depends on restoration goals and the scale considered for success evaluation (Marignani et al. 2008).

Evaluation of calcareous grassland restoration in Belgium and in Europe

The results of this thesis are quite optimistic concerning restored populations of specialist plant species and their colonization, persistence, dynamics and response to their environment. Studies concentrating on fewer number of species have been criticized because the information recorded is considered too limited compared to the diversity of ecosystem components (Franklin 1993). However, this approach has to be interpreted as integrated with other studies evaluating restoration outputs. Restoration success must be accounted for in any multi-disciplinary and multi-scale approach. Indicators of success should reflect the whole ecosystem; plants, birds, arthropods, microbes, soils and abiotic conditions as well as individual, population, community and landscape scales.

In Belgium, several investigations examined calcareous grassland restoration success at different spatial and ecological scales.

At the landscape scale, Piqueray et al. (2011c) discerned the presence of a colonization credit in restored patches of calcareous grasslands; meaning that restored patches had not reached their final species richness and exhibited a lower species richness than expected by their area and connectivity (Jackson and Sax 2009). It was confirmed by Gijbels et al. (2012) who found that several orchid species were missing from restored grasslands because of an existing colonization credit for them.

Regarding abiotic conditions, Piqueray et al. (2011b) uncovered no significant differences in soil nitrogen, phosphorous, and potassium content between restored and reference grasslands, but did find a decrease in mineralization rate indicators in restored sites.

In terms of species richness, Bisteau and Mahy (2005) established that restored communities display a species richness similar to their reference calcareous grassland. This was also demonstrated by Piqueray et al. (2011b) for older restored grasslands. In terms of community composition, Piqueray et al. (2011b) further revealed that older restorations were the most similar to reference grasslands. However, several differences persisted. A few rare species did not colonize restored grasslands and the cover of native dominant grasses was higher in restored grasslands versus references. Additionally, Bisteau and Mahy (2005) showed differences between restored and reference communities based on a larger abundance of pioneer and ruderal species in restored grasslands. In terms of functional traits, Piqueray et al. (2015) oberved that many differences remained in restored compared to reference grasslands. In particular, geophyte, mycorrhizal and evergreen species abundance did not reach reference grassland values.

At the population scale, Piqueray et al. (2013) studied seedling emergence patterns in reference and restored areas and concluded that seeds and microsite availability can be limiting factors for site colonization by *Pulsatilla vulgaris, Trifolium montanum* and *Veronica prostrata*. They stated that lower seedling emergence in restored versus reference grasslands was probably a consequence of lower habitat quality at restored sites. Furthermore, in a survey of 63 calcareous grassland sites in south-western Belgium, Jacquemyn et al. (2010) demonstrated that no recently restored area was occupied by the specialist, *Cirsium acaule*.

Helsen et al. (2013b) studied restored populations of *Origanum vulgare* and observed that the genetic diversity was not reduced in restored populations. A higher inbreeding coefficient, however, was determined in recent populations but this was not associated with reduced fitness. They contended that spontaneous colonization after habitat restoration can lead to viable populations over a relatively short time when several source populations were nearby. Moreover, Endels et al. (2005) showed that removing canopy resulted in an immediate flowering response and increased both growth and seedling recruitment the year after restoration for *Primula veris*.

Results of those studies are rather positive in term of restoration success with many features that could be regarded as encouraging.

Elsewhere in Europe, studies evaluating calcareous grassland restoration outputs are numerous regarding plant communities (e.g. Zobel et al. 1996; Pärtel et al. 1998; Willems and Bik 1998; Bisteau and Mahy 2005; Bossuyt and Honnay 2008; Fagan et al. 2008; Piqueray et al. 2011b; Maccherini et al. 2014), but also arthropods communities (e.g. Maccherini et al. 2009; Fagan et al. 2010; Woodcock et al. 2010; Rakosy et al. 2011), abiotic conditions (e.g. Fagan et al. 2008; Piqueray et al. 2011b) or landscape patch connectivity (e.g. Knop et al. 2011; Piqueray et al. 2011c; Helsen et al. 2013a).

In addition, several studies have assessed a number of population parameters in response to calcareous grassland restoration, particularly intra-specific trait variability (Andrade et al. 2014), species colonization and establishment (Hutchings and Booth 1996; Smith et al. 2005; Olsson and Ödman 2013; Piqueray et al. 2013; Freund et al. 2014), plant species demography (Endels et al. 2005; Koch et al. 2011; Walker et al. 2015; Wagner et al. 2016) or genetic aspects (Jacquemyn et al. 2010; Helsen et al. 2013b; Rico et al. 2014). The latter merits additional inquiry – the genetic structure of restored populations from a fragmented landscape has been highlight as largely understudied to date (**Chapter 2**).

The way forwards

As a contribution to grassland restoration, this thesis has some practical implications:

- Measurements of population parameters provide a more complete understanding of grassland restoration success and may complete evaluations realized at the community and ecosystem levels;
- Population demography and population performances are widely used to evaluate grassland restoration success while considering the genetic structure of restored populations requires to be deeply studied in a context of population fragmentation;
- Successful species dispersal on restored calcareous grassland sites can be reached without seeding or individual transfer (probably because off nearby donor populations, adequate management with migrating sheep or goats flock and/or reliable soil seed bank), especially for species that are frequent in the surroundings. Restoration is therefore recommended close to ancient grasslands to facilitate the spontaneous establishment of target species;
- Concerning rarer species, conservation and restoration activities in calcareous grasslands should consider the low probability of dispersal and an input of seeds or other propagules may be considered as a restoration tool;
- High proportion of bare ground cover in calcareous grasslands seems important to enhance population performances (it may increase availability of microsites for germination and seedlings emergence and modify competition regimes);
- One should take care when using local calcareous grassland remnants as reference sites to serve as a model in restoration. Populations in reference sites may experience a declining dynamic (due to a lack of management or as a consequence of their fragmentation within the landscape);
- To evaluate restoration success, it is important to consider a landscape approach that encompasses several sites in order to counterbalance local-scale differences between sites;
- Choosing to restore sites with high environmental variability at the local scale may potentially result in high local-scale functional variability in plant species populations while decreasing restoration investment;
- The within-population variability of some functional traits may be a major component of the overall variance in those traits. Moreover, species response strongly depends on individuals' location and direct environmental influences. This challenged the use of published mean values of functional trait to describe species behavior;
- On calcareous grasslands, individuals from very stressful environments survive drought stress better. It represents a challenge for species conservation in the face of future climate change.

Ultimately, this thesis serves as a novel contribution to the evaluation of calcareous grassland restoration success considering five specialist plant species. Our study species shared similar patterns of reproductive performance and responses to the environment, as expanded upon throughout each chapter. Using a population approach targeting rarer species, those with seed dispersal that relies on agents not related to grassland management and/or those depending on specific pollinators will be needed to fully determine the success of restoration programs of calcareous grasslands. Moreover, evaluation of restoration success based on other species, populations, sites, regions and traits will support a more full understanding of plant species response to restoration.

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ANNEXES

Annex 1: Table S1 (Chapter 2)

List of selected papers (141 papers) and classes of population parameters that were used to evaluate restoration success (“1”=class has been used in paper, “0”=class has not been used in paper). If research area of the paper corresponds to the research area proposed by Montalvo et al. (1997) concerning “the influence of the spatial arrangement of landscape elements on meta-population dynamics and population processes”; “1” is indicated in the last column.

	Classes of population’s parameters used to evaluate restoration success						Montalvo et al. 1997
Reference	**Genetic structure**	**Spatial structure**	**Demographic structure**	**Vegetative performance**	**Reproductive performance**	**Demography**	**Landscape and meta-populations**
1	1	0	1	0	0	0	1
2	0	0	0	1	1	0	0
3	0	0	0	0	0	1	0
4	0	0	0	1	0	0	0
5	0	0	0	1	0	1	0
6	0	0	0	1	0	1	0
7	1	0	0	0	0	0	0
8	0	0	0	0	0	1	0
9	0	0	0	0	0	1	0
10	0	1	0	0	1	1	0
11	0	1	0	0	1	1	0
12	0	0	0	0	0	1	0
13	0	0	0	0	0	1	0
14	0	0	0	1	0	1	0
15	0	0	0	1	0	1	0
16	0	0	0	1	0	1	0
17	0	0	0	1	1	1	0
18	0	0	0	1	1	0	0

Classes of population's parameters used to evaluate restoration success							Montalvo et al. 1997
Reference	Genetic structure	Spatial structure	Demographic structure	Vegetative performance	Reproductive performance	Demography	Landscape and meta-populations
19	0	0	0	1	1	1	0
20	0	0	0	0	0	1	0
21	0	0	0	0	0	1	0
22	0	0	0	0	1	1	0
23	0	0	0	1	0	1	0
24	0	0	0	0	0	1	0
25	0	0	0	0	0	1	0
26	0	0	0	1	0	1	0
27	0	0	0	0	0	1	0
28	0	0	0	1	1	1	0
29	1	0	0	0	0	0	0
30	0	0	0	0	0	1	0
31	0	0	0	0	0	1	0
32	0	0	0	1	1	0	0
33	0	0	0	1	0	1	0
34	0	0	0	1	0	1	0
35	0	0	0	1	1	1	0
36	0	0	0	1	0	0	0
37	0	0	0	1	1	1	0
38	0	0	0	1	1	1	0
39	0	0	0	0	1	1	0
40	0	0	0	1	0	1	0
41	0	0	0	1	0	1	0
42	0	0	0	1	1	1	0
43	0	0	0	1	1	1	0

Classes of population's parameters used to evaluate restoration success							**Montalvo et al. 1997**
Reference	**Genetic structure**	**Spatial structure**	**Demographic structure**	**Vegetative performance**	**Reproductive performance**	**Demography**	**Landscape and meta-populations**
44	0	1	0	0	0	1	0
45	0	0	0	0	1	1	0
46	0	0	0	1	1	1	0
47	0	0	0	1	0	1	0
48	0	1	0	0	0	1	0
49	0	0	0	0	0	1	0
50	1	0	0	1	1	0	1
51	1	0	0	0	0	0	0
52	0	0	0	1	0	1	0
53	0	0	1	0	1	0	0
54	0	0	1	0	0	0	0
55	0	0	0	0	0	1	0
56	0	0	1	1	0	0	0
57	1	0	1	0	1	0	1
58	0	0	0	1	0	0	0
59	0	0	0	0	0	1	0
60	0	0	0	0	0	1	0
61	0	0	0	0	1	1	0
62	0	0	0	0	0	1	0
63	0	0	0	0	0	1	0
64	0	0	0	0	0	1	0
65	1	0	1	0	0	0	1
66	0	0	0	0	0	1	0
67	0	0	0	1	1	0	0
68	0	0	0	0	0	1	0

Classes of population's parameters used to evaluate restoration success							Montalvo et al. 1997
Reference	Genetic structure	Spatial structure	Demographic structure	Vegetative performance	Reproductive performance	Demography	Landscape and meta-populations
69	0	0	0	0	1	1	0
70	0	0	0	0	1	1	0
71	0	0	0	1	1	0	0
72	0	0	0	1	0	1	0
73	0	0	0	0	0	1	0
74	0	1	0	1	1	1	0
75	0	0	0	1	0	1	0
76	0	0	0	0	0	1	0
77	0	0	0	1	1	1	0
78	0	0	0	0	0	1	0
79	0	0	0	1	0	1	0
80	0	0	0	1	1	1	0
81	0	0	0	1	0	1	0
82	0	0	0	1	0	1	0
83	0	0	0	1	1	1	0
84	0	0	0	1	1	1	0
85	0	0	0	1	1	1	0
86	0	0	0	1	0	0	0
87	0	0	0	1	0	0	0
88	0	0	1	0	0	0	0
89	0	0	1	1	1	0	0
90	0	0	0	1	0	1	0
91	0	0	0	1	0	0	0
92	0	0	0	1	0	1	0
93	0	0	0	1	0	1	0

Classes of population's parameters used to evaluate restoration success							Montalvo et al. 1997
Reference	Genetic structure	Spatial structure	Demographic structure	Vegetative performance	Reproductive performance	Demography	Landscape and meta-populations
94	0	0	0	1	1	1	0
95	0	0	0	1	1	1	0
96	0	0	0	0	0	1	0
97	0	1	0	0	0	1	1
98	0	0	0	1	1	1	0
99	0	0	0	0	1	0	0
100	0	0	0	1	1	1	0
101	0	0	0	0	1	1	0
102	0	0	0	0	0	1	0
103	0	0	0	0	0	1	0
104	0	0	0	0	0	1	0
105	0	0	0	0	1	1	0
106	0	0	0	0	0	1	0
107	0	1	0	0	0	1	0
108	0	1	0	0	0	1	0
109	0	0	0	1	1	1	0
110	0	0	0	1	1	1	0
111	0	0	0	0	0	1	0
112	0	0	0	0	0	1	0
113	0	0	0	0	0	1	0
114	0	0	0	1	0	1	0
115	0	0	0	0	0	1	0
116	0	0	0	0	0	1	0
117	0	0	0	1	0	0	0
118	0	0	1	1	1	0	0

	Classes of population's parameters used to evaluate restoration success						**Montalvo et al. 1997**
Reference	**Genetic structure**	**Spatial structure**	**Demographic structure**	**Vegetative performance**	**Reproductive performance**	**Demography**	**Landscape and meta-populations**
119	0	0	0	1	0	0	0
120	0	0	0	1	1	1	0
121	1	0	1	0	0	0	1
122	0	0	0	1	0	0	0
123	0	0	0	1	0	1	0
124	0	0	0	1	0	1	0
125	0	0	0	0	0	1	0
126	1	0	0	0	1	0	0
127	0	0	0	1	0	1	0
128	0	0	0	1	1	1	0
129	0	0	0	0	0	1	0
130	1	0	0	0	0	1	0
131	0	0	0	1	0	0	0
132	0	0	0	1	0	1	0
133	0	0	0	0	0	1	0
134	0	0	0	1	0	0	0
135	0	0	0	0	1	1	0
136	0	0	0	0	1	1	0
137	0	0	0	1	1	1	0
138	0	0	0	1	1	1	0
139	0	0	0	0	0	1	0
140	0	0	0	1	0	1	0
141	0	0	0	1	0	1	0

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REGULAR PAPER

Specialist plant species harbour higher reproductive performances in recently restored calcareous grasslands than in reference habitats

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Background and aims – Calcareous grasslands are local biodiversity hotspots in temperate regions that suffered intensive fragmentation. Ecological restoration projects took place all over Europe. Their success has traditionally been assessed using a plant community approach. However, population ecology can also be useful to assess restoration success and to understand underlying mechanisms.
Methods – We took advantage of three calcareous grassland sites in Southern Belgium, where reference parcels coexist with parcels restored in the late twentieth century and with more recently restored parcels. We evaluated the colonization stage of three specialist species (*Sanguisorba minor*, *Potentilla neumanniana* and *Hippocrepis comosa*) using occurrence data. We also measured the reproductive traits of 120 individuals per species and compared components of fitness between recent restorations, old restorations and reference habitats.
Key results – We found that the occurrence of *H. comosa* was similar in the different restoration classes, whereas both *P. neumanniana* and *S. minor* occurrences decreased from reference grasslands to recent restorations. In contrast, these two latter species exhibited a much higher reproductive output in recent restorations, thanks to an increased production of flowers and seeds.
Conclusions – Our results suggest that, during colonization of recently restored calcareous grasslands, favourable environmental conditions, low competition and sufficient genetic mixing may lead to an increased fitness of individuals and a faster population growth than in the reference habitat. These results demonstrate how population processes can increase ecological resilience and highlight the interest of a population-based approach to assess the success of ecological restoration.

Key words – Plant population, specialist, fitness, reproductive success, ecological restoration, ecological resilience, calcareous grassland.

INTRODUCTION

Recent decades have been dominated by unprecedented rates of habitat perturbations by human activities. Important changes in land use have led to the destruction and fragmentation of (semi)natural habitats, threatening biodiversity worldwide (Saunders et al. 1991, Balmford et al. 2005). As the conservation of remnant habitats is not sufficient to guarantee the long-term survival of many plant species (Rodrigues et al. 2004), active habitat restoration has become a necessity to preserve biodiversity worldwide. However, habitat restoration can be cost prohibitive, and efforts to reach restoration goals must demonstrate their success (Fagan et al. 2008).

Calcareous grasslands are local biodiversity hotspots in temperate regions (Prendergast et al. 1993, WallisDeVries et al. 2002, Jacquemyn et al. 2003). These habitats have suffered intensive fragmentation due to the abandonment of traditional agropastoral systems and the resulting encroachment, reforestation, urbanization or transformation into arable lands (Poschlod & WallisDeVries 2002, Piqueray et al. 2011a). In order to preserve and enhance the ecological value of those habitats, ecological restoration projects have taken place all over Europe.

Criteria used in judging whether a restoration is successful are numerous (Hobbs & Norton 1996, Ruiz-Jaen & Aide 2005, Zedler 2007, Cristofoli & Mahy 2010, Piqueray et al. 2015). Most studies evaluating calcareous grasslands' restoration success focused on the recovery of plant species diversity and composition (e.g. Lindborg & Eriksson 2004, Kiehl & Pfadenhauer 2007, Fagan et al. 2008, Maccherini et al. 2009, Piqueray et al. 2011b, Maccherini & Santi 2012, Redhead et al. 2014). Few studies have evaluated calcareous grasslands' restoration success by a population approach (but see Endels et al. 2005). The discipline of population biology provides one perspective on what might be considered a successful restoration, namely that "populations must be restored to a level that allows them to persist over the long term" (Montalvo et al. 1997). Restored populations must therefore possess attributes necessary for dispersal, growth, reproduction and adaptive evolutionary changes (Montalvo et al. 1997).

For a successful habitat restoration, species must first colonize newly created patches and establish new populations. The probability for a species to colonize a new habitat notably depends on its presence in the local species pool, the presence of dispersal agents, the ability of seeds to germinate, the longevity of the soil seed bank and the spatial position of patches in the landscape (Bakker & Berendse 1999, Helsen et al. 2013). Most calcareous grasslands species are badly represented in the persistent seed bank (Kalamees & Zobel 1998, Bisteau & Mahy 2005). However, some species can persist for several decades in the soil seed bank and emerge after restoration by clear cutting (Poschlod et al. 1998). Grazing by sheep and goats plays a major role in species dispersal in the landscape (Poschlod et al. 1998). Accordingly, grasslands management practices imitating traditional shepherding may enhance species colonization on restored grasslands. In the absence of itinerant grazing, long distance dispersal is thought to be limited for most calcareous grasslands species even if some species are potentially wind dispersed (Tackenberg et al. 2003).

After colonization, restored populations must be able to persist over the long term through offspring production (Montalvo et al. 1997). In the case of limited dispersal in space and time, founding populations can be small and represent only a minor portion of the genetic diversity of the source population (Montalvo et al. 1997, Hufford & Mazer 2003). This leads to increased risks of inbreeding depression and decreased adaptive potential of the restored population (Barrett & Kohn 1991, Ellstrand & Elam 1993). Small populations are more exposed to random environmental fluctuations (Menges 1991, Widén 1993, Heschel & Paige 1995), may be less attractive to pollinators (Sih & Baltus 1987, Hendrix & Kyhl 2000) and may consequently have lower reproductive success. Loss of genetic variation in founding populations can lead to a lower fitness (Shaffer 1981, Lande 1988, Williams 2001, Reed & Frankham 2003, Matthies et al. 2004). Nevertheless, rapid population growth and expansion can also decrease risks of reducing fitness in newly created populations (Nei et al. 1975, Leimu & Mutikainen 2005, Bizoux et al. 2011).

Finally, species performances in restored habitats may be affected by environmental conditions that gently differ from the reference habitat of the species and subsequently affect fitness (Vergeer et al. 2003, Quintana-Ascencio et al. 2007, Adriaens et al. 2009). Colonization of a new habitat by maladapted genotypes can lead to a population sink (Pulliam 1988). Conversely, colonization by genotypes able to survive and reproduce in the new created habitat could increase species persistence in the landscape (Blais & Lechowicz 1989).

In this context, we took advantage of three calcareous grasslands sites in Southern Belgium, where reference parcels coexist with parcels restored in the last twenty years and with more recently restored parcels. Using occurrence data, we evaluated the colonization stage of three specialist species in the different parcels. We then compared components of fitness between recent restorations, old restorations and reference habitats.

MATERIAL AND METHODS

Study area, sites and parcels

The study was conducted in the Viroin valley, located in the Calestienne region (southern Belgium), a 5 km-wide and 100 km-long belt of Devonian limestone hills and plateaus, with a SW-NE orientation (fig. 1). Calcareous grasslands of this area were developed under traditional agropastoral practices. Due to the abandonment of traditional agriculture, urbanization and afforestation in the nineteenth century, calcareous grasslands have declined dramatically in the region (Adriaens et al. 2006, Piqueray et al. 2011a). Thanks to restoration projects, the calcareous grasslands area increased gradually from less than 40 ha in the 1980s to more than 150 ha today. All remaining and restored grasslands are now managed with grazing and mowing in order to keep the habitat open. Despite the huge loss of habitat since the nineteenth century, this region is considered one of the core areas for calcareous grasslands conservation in Belgium.

Three sites were selected in the region: "La Montagne-aux-buis" in Dourbes, "Les Abannets" in Nismes and "Les Rivelottes" in Treignes. At each site, three calcareous grasslands parcels were selected (fig. 1): (1) **Reference grassland**, i.e. calcareous grassland known to have existed for more than two centuries and considered the reference ecosystem for the restoration (SER (Society for Ecological Restoration International Science & Policy Working Group) 2004), (2) **Old restoration**, i.e. grassland restored between 1990 and 2000, (3) **Recent restoration**, i.e. grassland restored between 2004 and 2006. Restored parcels derived from forty to one hundred-year-old forests of oak coppices (Montagne-aux-buis, Abannets) or pine stands (Rivelottes, Abannets) established on former calcareous grasslands. Pine stands were *Pinus nigra* or *Pinus sylvestris* plantations. Dense shrub oak coppices were mainly formed with *Prunus spinosa*, *Crataegus monogyna* and *Corylus avellana*, with intermingled scarce trees of *Quercus robur*. Restoration protocols included trees and shrubs clearing mainly followed by sheep and goat grazing.

Study species

Three species considered calcareous grassland specialists (Piqueray et al. 2007) were selected: *Sanguisorba minor* Scop. (Rosaceae), a polycarpic perennial forbs producing flower heads consisting of fifteen to thirty wind-pollinated flowers; *Potentilla neumanniana* Rchb. (Rosaceae), a creeping perennial forbs producing yellow entomophilous flowers; and *Hippocrepis comosa* L. (Fabaceae), a perennial legume producing yellow entomophilous flowers. All three species are abundant in the study area (Adriaens et al. 2006).

Data collection

In order to evaluate the success of calcareous grasslands restoration, indicators of species colonization and individual fitness were compared between the reference and restored grasslands (old and recent restorations).

The occurrence of species at the parcel scale was evaluated by observing species presence or absence in 1 m^2 plots systematically placed in parcels, representing a sampling rate of ca. 6% of each parcel area (between 123 and 505 observed plots, depending on the parcel for a total of 2303 plots).

For each study species, twenty plots (1 m^2) were randomly selected in each parcel (among plots were the species was present) of two sites (fig. 1), and one individual was randomly selected (in each plot) for fitness components measurements.

The method used to measure fitness components depend on the species (see details of the recording method in table 1). For *S. minor*, the number of inflorescences per individual (Sm_inflos) and the number of seeds per inflorescence (Sm_seeds/inflo) were recorded. For *P. neumanniana* and *H. comosa* the number of flowers per individual (respectively Pn_flowers and Hc_flowers) and the number of seeds per fruit (respectively Pn_seeds/fruit and Hc_seeds/fruit) were recorded. The final fitness was measured as the total seeds produced per individual (see details of the recording method in table 1).

As competition and environmental variability can directly affect species performances we evaluated the bare ground cover in 1 m^2 plots around each selected individual. We estimate the bare ground cover using van der Maarel (1979) scale (0 = 0%; 1 = < 0.1%; 2 = 0.1–1%; 3 = 1–5%; 5 = 5–25%; 7 = 25–50%; 8 = 50–75%; 9 = 75–100%).

Data analysis

In order to evaluate species colonization in restored parcels, a generalized linear model was fitted to the occurrence data (binomial family), followed by an analysis of deviance with restoration classes [(1) reference grassland, (2) old restoration, (3) recent restoration] and site as crossed factors. The analysis was followed by a pairwise comparison of proportions (number of plot where the species was present/total number of plots, using the "pairwise.prop.test" function in R) among restoration classes.

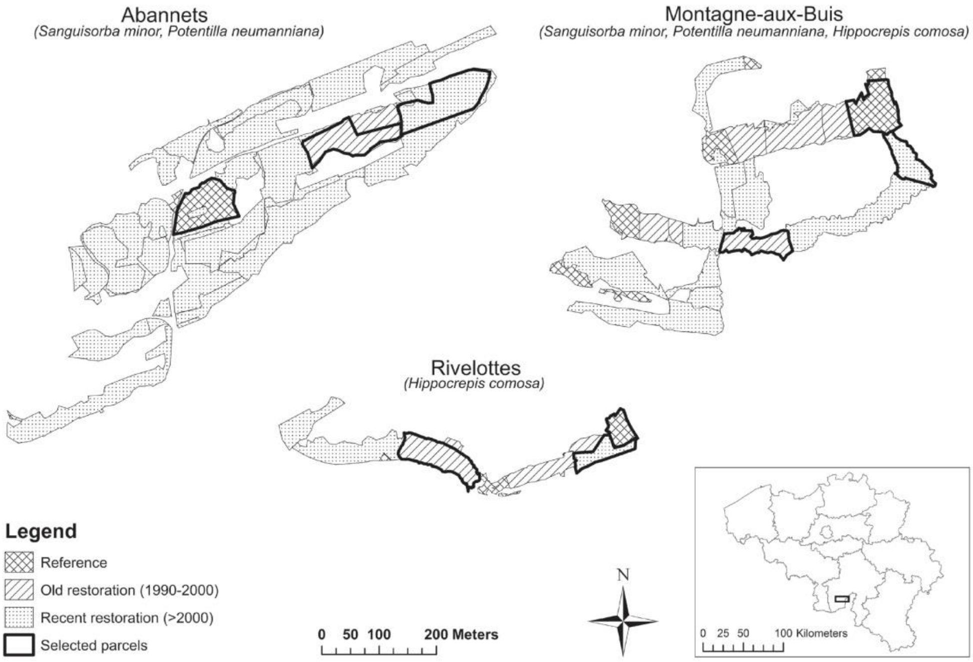

Figure 1 – Study region (Viroin Valley, Southern Belgium) and selected parcels of the three study sites (Montagne-aux-buis: 50°05′N–4°34′E, Abannets: 50°04′N–4°34′E, Rivelottes: 50°05′N–4°40′E). Occurrence of study species was observed in each site. Fitness components were measured in only two sites for each study species (as indicated under sites names).

Table 1 – Fitness traits evaluated on study species.
Final fitness, i.e. seeds production over one season, was either directly measured (*S. minor*) or estimated from fitness components. Fitness components (in bold) allow for a better understanding of reproductive performance variation.

	Sanguisorba minor	*Potentilla neumanniana*	*Hipocrepis comosa*
Fitness components	**Sm_inflos**: Number of inflorescences per individual	**Pn_flowers**: Number of flowers per individual (Pn_flowers/stem × Pn_stems)	**Hc_flowers**: Number of flowers per individual (Hc_flowers/inflo × Hc_inflos)
		Pn_flowers/stem: Mean number of flowers per stem (measured on 5 randomly selected stems per individual)	Hc_flowers/inflo: Mean number of flowers per inflorescence (measured on 20 randomly selected inflorescences per individual)
		Pn_stems: Number of stems per individual	Hc_inflos: Number of inflorescences per individual
	Sm_seeds/inflo: Mean number of seeds per inflorescence (measured on all the inflorescences of each individual)	**Pn_seeds/fruit**: Mean number of seeds per fruit (measured on 20 randomly selected fruits per individual)	**Hc_seeds/fruit**: Mean number of seeds per fruit (measured on 20 randomly selected fruits per individual)
			Hc_fruits/inflo: Mean number of fruits per inflorescence (measured on 20 randomly selected inflorescences per individual)
Final fitness	**Sm_seeds**: Number of seeds per individual	**Pn_seeds**: Number of seeds per individual (Pn_seeds/fruit × Pn_flowers)	**Hc_seeds**: Number of seeds per individual (Hc_seeds/fruit × Hc_fruits/inflos × Hc_inflos)

In order to compare fitness components and the final fitness of each study species in restored and reference parcels, two-way analyses of variance were performed (with restoration classes and site as crossed factors) on each variable (fitness components and final fitness, table 1). In the case of a significant interaction with site effect, one-way ANOVAs were performed for each site separately. If a significant difference was observed between restoration classes, a Tukey's comparison test was performed.

To compare bare ground cover between study sites and parcels, one-way ANOVAs were performed for each site with restoration classes as fixed factor [(1) reference grassland, (2) old restoration, (3) recent restoration]. If a significant difference was observed between restoration classes, a Tukey's comparison test was performed.

Response variables were arcsine-square root- (Pn_seeds, Hc_seeds) or log- (Sm_inflos, Sm_seeds, Pn_flowers, Hc_seeds/fruit, Hc_flowers) transformed when needed to meet the assumptions of statistical analyses. All analyses were performed with R 2.14.0 (The R Foundation for Statistical Computing 2011).

RESULTS

Colonization of restored parcels

The occurrence of *S. minor* was significantly more important in reference grasslands than in restored grasslands. It was also more important in old restorations than in recent restorations (*df* = 2,2300; *deviance* = 1177.11; $P < 0.001$) (fig. 2). For *P. neumanniana*, the occurrence was more important in the reference grasslands and the old restoration than the recent restoration (*df* = 2,2300; *deviance* = 46.81; $P < 0.001$), except in the Montagne-aux-buis site, where the occurrence of *P. neumanniana* was significantly higher in recent restoration than in other parcels (*df* = 2,717; *deviance* = 74.73; $P < 0.001$). *H. comosa* was globally less abundant than the two other species, and no significant differences were observed between reference and restored grasslands except in the Montagne-aux-buis site, where the occurrence of *H. comosa* was significantly higher in recent restoration (*dF* = 2,717; *deviance* = 163.02; $P < 0.001$) than in other parcels (fig. 2).

Reproductive success

Fitness components – Individuals of *S. minor* and *P. neumanniana* produced significantly more inflorescences, or flowers, in recent restorations than others parcels (respectively *df* = 2,114; *F* = 18.49; $P < 0.001$; and *df* = 2,114; *F* = 37.40; $P < 0.001$; fig. 3A & B). *S. minor* individuals produced 8.3±1.7 inflorescences in recent restorations, compared to 1.9±0.5 inflorescences in reference grasslands and 0.9±0.3 inflorescences in old restorations (fig. 3A). *P. neumanniana* individuals produced 33.9±6.9 flowers in recent restorations, compared to 2.5±0.4 flowers in reference grasslands and 2.8±1.0 flowers in old restorations (fig. 3B). For *S. minor*, the production of seeds per inflorescence was site dependent. For this species, the seeds production per inflorescence was significantly higher for the reference grassland than for the recent restoration of the Montagne-aux-buis site (*df* = 2,25; *F* = 4.75; *P* = 0.018; fig. 3D). For *P. neumanniana*, no differences were found for the number of seeds per fruit between the reference and the restored grasslands (fig. 3E). Concerning *H. comosa*, the number of flowers was significantly higher (df = 2,114; F = 8.40; $P < 0.001$; fig. 3C) for recent restorations (826.0±128.0 flowers) than for old restorations (244.8±26.5 flowers). For this species, differences of seeds production per fruit were site dependent. Data was missing for one parcel of the Montagne-aux-buis site because the parcel was grazed before measurements. No differences were observed between reference and old restoration parcels in this site. At the Rivelottes site, individuals of the old restoration produced significantly more seeds per fruit (df = 2,56; F = 4.32; P = 0.018; fig. 3F) than individuals of the recent restoration (respectively 2.3±0.2 and 1.6±0.2 seeds/fruit).

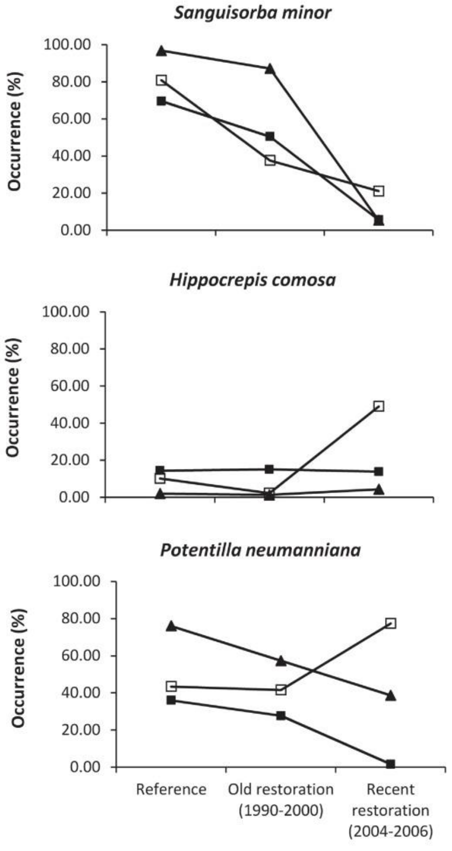

Figure 2 – Species occurrence (% of presence in 1m^2 plots placed systematically in parcels representing a sampling rate of ~6% of each parcel's area) in selected parcels of the three study sites (Abannets, Montagne-aux-buis and Rivelottes sites are represented by triangles, white squares and black squares respectively).

Final fitness – Individuals of *S. minor* and *P. neumanniana* had a significantly higher final fitness (respectively df = 2,114; F = 13.9; $P < 0.001$; and df = 2,114; F = 23.1; $P < 0.001$) in recent restorations than other parcels. *S. minor* individuals produced 147.5±35.6 seeds in recent restorations, compared to 29.5±7.1 seeds in reference grasslands and 18.4±5.8 seeds in old restorations. *P. neumanniana* individuals produced 458.0±106.0 seeds in recent restorations, compared to 38.4±5.9 seeds in reference grasslands and 60.1±16.4 seeds in old restorations (fig. 3H). For *H. comosa*, differences in seeds production per individual were site dependent. Individuals of the recent restoration of the Rivelottes site produced significantly more seeds (df = 2,56; F = 4.39; P = 0.017; 913.0±135.0 seeds) than individuals of the old restoration (379.0±81.6 seeds). Data was missing for one recently restored parcel of the Montagne-aux-buis site. At this site, no difference of final fitness was observed between the reference and the old restoration (fig. 3I).

Bare ground cover

In the Montagne-aux-buis site, the bare ground cover was significantly higher on the recent restoration compared to other parcels, whatever the species (df = 2,57; F = 7.06; P = 0.002 for *S. minor*; df = 2,57; F = 5.55; P = 0.006 for *P. neumanniana*; df = 2,57; F = 7.27; P = 0.002 for *H. comosa*; table 2). In the Abannets site, the bare ground cover was not significantly different around *P. neumanniana* individuals (df = 2,57; F = 0.12; P = 0.883; table 2) but significantly higher in the recent restoration and the reference around *S. minor* individuals (df = 2,57; F = 11.61; $P < 0.001$; table 2). In the Rivelottes site, there was significantly more bare ground cover in the reference parcel than in the old restoration (df = 2,57; F = 3.67; P = 0.032; table 2).

DISCUSSION

Colonization of restored parcels

The first step to evaluate restoration success using a population approach is to determine if species have been able to form new populations on restored grasslands. Like other ecological processes, colonization and species expansion can require long periods of time. Following biotope restoration, grasslands may exhibit a colonization credit due to a time lag for species dispersal (Cristofoli et al. 2010). Some species can therefore not yet be observed in the vegetation but can be expected to colonize restored grasslands in ensuing years. In the calcareous grassland of our study region, however, Piqueray et al. (2011c) showed that there was no colonization credit for our three study species. The three species considered in the present study have been able to colonize restored habitats. This is in agreement with the observations of Delescaille (2006, 2007) in the Abannets site. However they were globally less present on restored grasslands than on reference grasslands. There are probably multifactorial causes explaining patterns of occurrence of each target species on study sites, including emergence from soil seed bank, multiple dispersal agent (like wind, sheep, goats or human management), spatial distribution of grasslands patches in the landscape and environmental variability. Although restored sites were

Table 2 – **Differences of bare ground cover between study parcels.**
Percentage of bare ground cover was estimated in 1m^{2} plots placed around each individual. The van der Maarel (1979) scale was used to estimate de bare ground percentage in the plot (0 = 0%; 1 = < 0.1%; 2 = 0.1–1%; 3 = 1–5%; 5 = 5–25%; 7 = 25–50%; 8 = 50–75%; 9 = 75–100%). Means and standard errors of bare ground cover are given for reference grasslands, old and recent restorations of study sites. Significantly different means are followed by different letters. Significant p-values are in bold.

Species	Sites	Reference	Old restorations	Recent restorations	*P*-value
Sanguisorba minor	Mont.-aux-buis	0.15±0.15 ^{a}	0.15±0.15 ^{a}	1.50±0.46 ^{b}	**0.002**
	Abannets	0.85±0.25 ^{b}	0.00±0.00 ^{a}	1.50±0.30 ^{b}	**<0.001**
Potentilla neumanniana	Mont.-aux-buis	0.15±0.15 ^{a}	0.25±0.25 ^{a}	1.40±0.42 ^{b}	**0.006**
	Abannets	0.60±0.28	0.45±0.25	0.45±0.21	0.883
Hipocrepis comosa	Mont.-aux-buis	0.30±0.21 ^{a}	0.00±0.00 ^{a}	1.35±0.41 ^{b}	**0.002**
	Rivelottes	2.50±0.44 ^{b}	1.00±0.34 ^{a}	1.70±0.39 ^{ab}	**0.032**

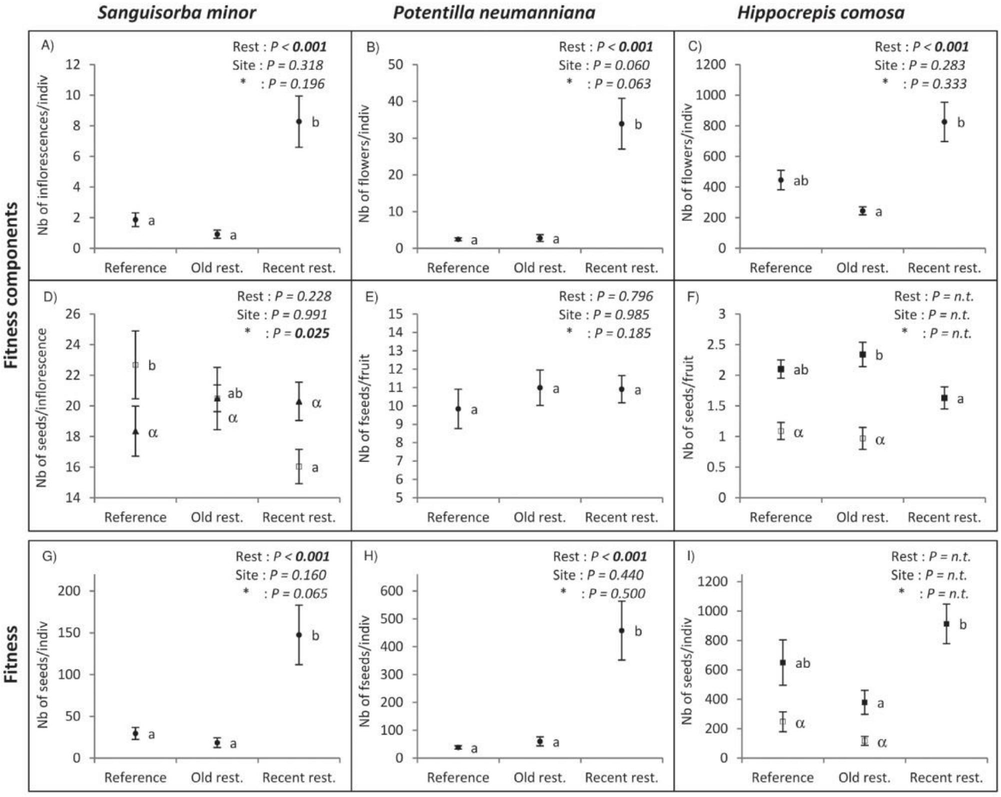

Figure 3 – Means and standard error of fitness components (Graphs A to F) and fitness traits (Graphs G to I) of study species for reference grasslands, old and recent restorations. For a same symbol, significantly different means are followed by different letters. P-values of the two ways variance analyses for the different factors (restoration class, site and their interaction) are given up-right on each graph, and significant p-values are in bold (n.t. = not tested). When a significant interaction between the two factors was pointed out, results were drawn for each site separately. Results of AV2 for two sites are represented by black points, results of AV1 for the Abannets, Montagne-aux-buis and Rivelottes sites are represented by triangles, empty squares and full squares respectively. Data were missing for *Hippocrepis comosa* in one parcel because the parcel was grazed before fruits and seeds were collected.

afforested forty to one hundred years ago, it is conceivable that *P. neumanniana* and *S. minor* emerged from the soil seed bank, as showed by a study conducted at the Abannets site (Delescaille et al. 2006). Indeed, those two species have a long-term persistent seed bank (over several decades, > 25 years) (Poschlod et al. 1998). Moreover *P. neumanniana* and *S. minor* could have colonized restored parcels through dispersal by sheep or goats that grazed parcels of different calcareous grasslands sites during the grazing season. Those species are not well dispersed by wind (Poschlod et al. 1998), and sheep play a major role in dispersal across the landscape (Fischer et al. 1996, Poschlod et al. 1998). *H. comosa* seed bank persists only between six and twenty years in the soil (Poschlod et al. 1998), and seeds of this species are not efficiently dispersed by wind (Poschlod et al. 1998). The presence of this species on restored grasslands could be explained by parcels management, as dispersal by goats has been observed for this species (Müller-Schneider 1954). *H. comosa* occurrences on restored and reference grasslands were comparable. However, it has to be noted that reference grasslands are remnant grasslands that have been isolated in the landscape for more than one century. These grasslands surely represent a core area for the calcareous grasslands ecological network, but they may also exhibit an extinction debt that can affect specialist plant species (Piqueray et al. 2011c).

Interestingly, in one site (the Montagne-aux-buis), we found the occurrence of *P. neumanniana* and *H. comosa* to be higher in the recent restoration than the reference grasslands and the old restoration parcels (fig. 2). Species recolonization can be influenced by the distance between the restored parcels and the nearest reference grassland (Helsen et al. 2013). In this site, the recent restoration is close to the reference parcel and the old restoration is 120 meters away from the nearest reference habitat. However, study parcels are always close to another calcareous grassland parcel. No parcel is isolated in a forest or agricultural landscape. Environmental variability between sites or parcels could also explain differences in patters of species occurrence. In a previous work, Piqueray et al. (2011b) pointed no significant differences in soil conditions between parcels or sites of the same study area. However, our results showed that the bare ground cover was higher in this recent restoration of the Montagne-aux-buis site. This could increase availability of microsites for germination (Piqueray et al. 2013) and seedlings emergence (Kotorová & Lepš 1999, Zobel et al. 2000) and modify competition regimes as compare to reference grasslands.

Reproductive success of restored populations

A second step in evaluating the restoration success using a population approach comprises assessing performances, e.g. the fitness of recently established individuals as compared to reference ones. In this study, all three studied species produced more flowering units and more seeds per individual in recent restorations than in the reference grasslands (with a less obvious pattern for *H. comosa*, see fig. 3). These results are hopeful concerning population persistence in restored grasslands, as individuals with a higher fitness are prone to increase population dynamics and decrease extinction risks. We must however be careful as we did not test seeds quality and viability. Rosaceae are known to produce a significant proportion of non-viable seeds (ENSCONET 2009). Moreover, seeds could be predated before germination occurs. The observation of a high fitness in recently founded populations can be explained by a high genetic diversity of created populations (Leimu & Mutikainen 2005). Recent populations can indeed have been created from multiple colonization events from several sources populations through grazing, since sheep herds typically graze alternatively in many calcareous sites. In addition, the seed bank may be a source of genetic diversity (Templeton & Levin 1979) that could be restored when habitat conditions are suitable again. A high genetic diversity associated with a rapid demographic extension may have promoted reproductive success in recent populations (Leimu & Mutikainen 2005).

The number of seeds per inflorescence or per fruit was not significantly different between reference and restored grasslands. This shows that the key parameter influencing final fitness is the number of flowers, or inflorescences and not the number of seeds produced by floral unit. This suggests that pollination is not a limiting factor for seed production.

Finally, higher reproductive performances in recent restoration may be explained by environmental conditions. In our study sites, Piqueray et al. (2011b) showed that soil conditions have been restored and do not differ according to previous land use (oak or pine forest). They pointed out low differences between reference and restored grasslands in terms of soil fertility, but the mineralisation rate was higher on restored sites and may explain individual success. In addition, recently restored parcels generally exhibit higher bare ground cover that offers microsites for germination and decreases competition (Piqueray et al. 2013). However, the higher bare ground cover was not the only parameter that can explain fitness variability between parcels. Indeed, in the Abannets site, the fitness of *P. neumanniana* was significantly different between parcels despite no differences of bare ground cover. And for *H. comosa*, the fitness was higher on the recent restoration of the Rivelottes site despite a reduction of bare ground cover compared to reference grasslands (fig. 3 & table 2).

Implications for the future

Our results suggest that, during the colonization of recently restored calcareous grasslands, the fitness of individual is hardly affected by any process that could reduce their reproductive capacity like unfavourable environmental conditions or insufficient genetic mixing. In contrast, the high reproductive output of individuals in restored parcels is expected to enhance population growth, which may finally compensate for the lower initial occurrence. These results therefore demonstrate how population processes can increase ecological resilience (sensus Suding et al. 2004). When the massive colonization is over, both abiotic and biotic conditions in the restored habitat should approach those of the reference habitats (Piqueray et al. 2011b), and the fitness of individuals should be reduced to levels comparable to the reference grasslands. This final situation seems to almost be reached for *H. comosa* that exhibit similar occurrences in all restorations classes. That is the species for which final fitness was comparable between recent restorations and reference habitats (fig. 3). *S. minor* and *P. neumanniana*, in contrast, exhibit slower colonization still in progress, with occurrences of respectively 82±8% and 52±12% in reference habitats, 58±15% and 42±9% in old restorations and only 11±5% and 39±22% in recent restorations. For these two species, final fitness was respectively five times and eleven times higher in recent restorations than in reference habitats.

From a management point of view, these processes are encouraging. Seed dispersal seems sufficient to establish satisfactorily diverse populations. One must keep in mind, however, that the patterns observed in this study might not be true for rarer species and/or those whose seed dispersal relies on agents not related to management schemes and/or those relying on declining populations of specific pollinators for successful reproduction. Using a population approach for these species will be necessary to fully assess the success of restoration programmes of calcareous grasslands in Belgium.

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Annex 3: Table S2 (Chapter 3)

Fitness components evaluated on study species. The method used to measure fitness components depend on the species. For *H. comosa* and *P. tabernaemontani* the number of flowers per individual (respectively **Hc_flowers** and **Pn_flowers**) and the number of seeds per fruit (respectively **Hc_seeds/fruit** and **Pn_seeds/fruit**) were recorded. For *S. minor*, the number of inflorescences per individual (**Sm_inflos**) and the number of seeds per inflorescence (**Sm_seeds/inflo**) were recorded. The total seeds production over one season was either directly measured (*S. minor*) or estimated from other fitness components.

Hipocrepis comosa	***Potentilla tabernaemontani***	***Sanguisorba minor***
Hc_flowers: Number of flowers per individual (Hc_flowers/inflo x Hc_inflos) Hc_flowers/inflo: Mean number of flowers per inflorescences (measured on 20 randomly selected inflorescences per individual) Hc_inflos: Number of inflorescences per individual	**Pn_flowers**: Number of flowers per individual (Pn_flowers/stem x Pn_stems) Pn_flowers/stem: Mean number of flowers per stem (measured on 5 randomly selected stems per individual) Pn_stems: Number of stems per individual	**Sm_inflos**: Number of inflorescences per individual
Hc_seeds/fruit: Mean number of seeds per fruit (measured on 20 randomly selected fruits per individual)	**Pn_seeds/fruit**: Mean number of seeds per fruit (measured on 20 randomly selected fruits per individual)	**Sm_seeds/inflo**: Mean number of seeds per inflorescence (measured on all the inflorescences of each individual)
Hc_fruits/inflo: Mean number of fruits per inflorescence (measured on 20 randomly selected inflorescences per individual)		
Hc_seeds: Number of seeds per individual (Hc_seeds/fruit x Hc_fruits/inflos x Hc_inflos)	**Pn_seeds**: Number of seeds per individual (Pn_seeds/fruit x Pn_flowers)	**Sm_seeds**: Number of seeds per individual

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Functional traits are more variable at the intra- than inter-population level: a study of four calcareous dry-grassland plant species

Funktionelle Merkmale variieren stärker innerhalb von Populationen als zwischen Populationen: Eine Studie an vier Pflanzenarten der Kalkmagerrasen

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Abstract

The majority of studies investigating plant functional traits have used species average trait values, and assumed that average values were sufficiently representative of each species considered. Although this approach has proven valuable in community ecology studies, plant functional traits can significantly vary at different scales, i.e. between but also within populations. The study of species functional trait variability can facilitate increasingly accurate studies in community ecology. Nevertheless, the current extent of within-site plant trait variability has been poorly addressed in the literature. Calcareous grasslands are ecosystems well-suited to study plant trait variation at small spatial scales. Many species are present on heterogeneous calcareous sites, with significant differences in hydric status due to variations in soil depth, soil moisture, aspect, and slope. This study assesses the extent of intra-population functional trait variability and tests the hypothesis that this variability can be explained by within-site environmental heterogeneity. Three functional traits (SLA-specific leaf area, LDMC-leaf dry matter content, and plant vegetative height) were assessed in three populations of four calcareous grassland species totalling 950 individuals. The heterogeneity in soil depth and potential direct incident radiation was also quantified and related to plant functional trait variability. The intra-population functional trait variability was compared to the inter-population variability of collected data and global inter-population variability data obtained from the worldwide TRY functional traits database. The results showed that SLA, LDMC, and plant height are characterized by considerable intra-population variation (SLA: 72–95%, LDMC: 78–100% and vegetative height: 70–94% of trait variability). The results also indicate higher plant height and larger SLA for individuals located in plots with deeper soils or lower potential direct incident radiation, on gentle slopes or north-facing slopes. Our findings additionally support the concept that higher plant height, higher SLA, and lower LDMC are related to higher availability of soil water. Individuals on shallow soils or in more exposed areas are better equipped to cope with environmental stress. Our results indicate plasticity or local adaptation in individuals to environmental heterogeneity. This study suggests that detailed analyses involving plant functional traits require measurements in situ from a large number of individuals, as the degree of individual response strongly depends on an individual's location and its micro-environmental conditions. Neglecting intra-population trait variability may be critical, as intraspecific variation can be very high at the population scale, and is likely to be driven by local environmental heterogeneity.

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Keywords: Environmental heterogeneity, intraspecific variability, leaf trait, local scale, soil depth, water availability, within-species variation

Erweiterte deutsche Zusammenfassung am Ende des Artikels

1. Introduction

Over the past decades, functional traits have been increasingly used as reliable predictors of species and community responses to environmental gradients (MCGILL et al. 2006, ACKERLY & CORNWELL 2007, PAKEMAN et al. 2009). The majority of studies investigating functional traits have focused on differences between species, using species average trait values (e.g. ACKERLY & CORNWELL 2007, DURU et al. 2010, SANDEL et al. 2010, SONNIER et al. 2010, GONG et al. 2011, DE BELLO et al. 2013, AMATANGELO et al. 2014), assuming that average values were sufficiently representative of each considered species. Despite the widespread use of this approach in community ecology, plant functional traits can vary substantially within species (ALBERT et al. 2010a, HULSHOF et al. 2013). Taking intraspecific trait variation into account may therefore improve the accuracy and resolution of studies of community ecology (SIEFERT et al. 2015). This intraspecific trait variation can have important implications for species coexistence (JUNG et al. 2010, LONG et al. 2011) or ecosystem functions (PONTES et al. 2007, LECERF & CHAUVET 2008), and is an important element of functional diversity approaches (CIANCIARUSO et al. 2009, ALBERT et al. 2012). Although usually defined as the functional trait variation within a species, intraspecific functional trait variation can be studied at different scales, notably between (e.g. JUNG et al. 2010, BARUCH 2011, ANDERSEN et al. 2012, ALMEIDA et al. 2013, PAKEMAN 2013) and within populations (BOLNICK et al. 2011, MITCHELL & BAKKER 2014), the latter being poorly addressed thus far in the literature.

Calcareous grasslands are well-suited ecosystems to study plant trait variation at small spatial scales. Calcareous grassland species are present on heterogeneous sites, with noticeable differences in soil depth, soil moisture, aspect, and slope, mainly resulting in differences in hydrological status (BUTAYE et al. 2005, PIQUERAY et al. 2007). The total amount of available water for plants was proven to be lower for grasslands characterized by thin soils (<10 cm) and low plant cover compared to grasslands with higher vegetation cover and slightly deeper soils (ALARD et al. 2005, BENNIE et al. 2008, DUJARDIN et al. 2012). Topography and exposure represent additional decisive factors influencing water supply in calcareous grasslands (BENNIE et al. 2006). Slope inclination and exposure strongly affect the amount of solar radiation intercepted by the surface, and subsequently influence soil moisture content (ACKERLY et al. 2002).

Leaf traits are key functional traits that are linked to plant responses to environmental variability (WILSON et al. 1999, GARNIER et al. 2001a, ACKERLY 2004) and stress tolerance (WESTOBY et al. 2002). The regulation of water loss through leaves can be expressed by several key leaf functional traits (WRIGHT et al. 2001, ACKERLY 2004) and, for example, can also be reflected in higher leaf dry matter content (LDMC) and/or lower specific leaf area (SLA) (BUCKLAND et al. 1997, VOLAIRE 2008, POORTER et al. 2009). At large scales, specific leaf area (SLA) generally declines along gradients of decreasing nutrients or moisture availability (CUNNINGHAM et al. 1999, REICH et al. 1999, FONSECA et al. 2000, PÉREZ-HARGUINDEGUY et al. 2013, SÁNCHEZ-GÓMEZ et al. 2013, MONTY et al. 2013), while LDMC generally decreases with greater water availability (CORNELISSEN et al. 2003). Moreover, important trade-offs exist between plant height and environmental stress tolerance

(Cornelissen et al. 2003). SLA, LDMC and plant height are strong indicators of plant resource use strategies (Weiher et al. 1999, Cornelissen et al. 2003, Westoby & Wright 2006).

This study assesses the extent of intra-population functional trait variability at local scales, and tests the hypothesis that this variability can be explained by within-site environmental heterogeneity.

2. Material and methods

2.1 Study sites

We selected three calcareous grassland sites located in southern Belgium for the study (Fig. 1): the "Montagne-aux-buis" in Dourbes (50°05'N, 4°34'E), the "Tienne Breumont" in Nismes (50°04'N, 4°32'E), and "Sosoye hill" in Sosoye (50°17'N, 4°46'E). The Montagne-aux-buis and the Tienne Breumont are located in the Calestienne region, a 5 km wide and 100 km long belt of Devonian limestone hills and plateaus, with a SW-NE orientation. Sosoye hill is situated in the Meuse valley, a carboniferous formation characterized by limestone-enriched schist. The Calestienne region and the Meuse valley are the two core areas for calcareous grasslands conservation in Belgium. The calcareous grasslands of those regions historically developed under traditional agro-pastoral practices.

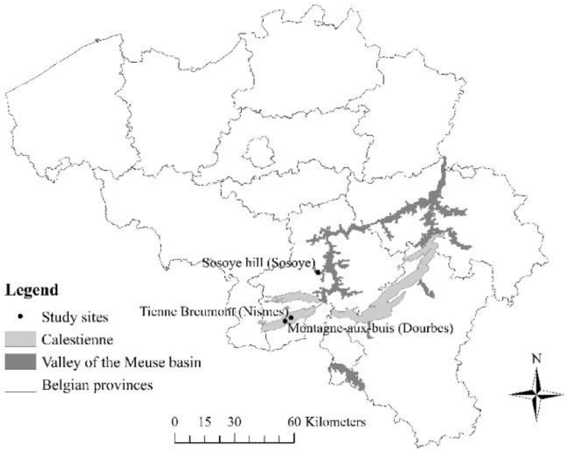

Fig. 1. Study sites locations in Belgium. Three calcareous grassland sites were selected in southern Belgium. The Montagne-aux-buis in Dourbes (50°05' N, 4°34' E) and the Tienne Breumont in Nismes (50°04' N, 4°32' E) are located in the Calestienne region and the Sosoye hill in Sosoye (50°17' N, 4°46' E) is located in the Meuse valley.

Abb. 1. Lage der untersuchten Kalkmagerrasen in Belgien. Die beiden Gebiete Montagne-aux-buis in Dourbes (50°05' N, 4°34' O) und Tienne Breumont in Nismes (50°04' N, 4°32' E) liegen in der Calestienne-Region und der Sosoye hill in Sosoye (50°17' N, 4°46' E) im Meusetal.

2.2 Study species

From a list of plant species identified as being calcareous grassland specialists (Piqueray et al. 2007), we selected four perennial species that are generally abundant in European calcareous grasslands for this study (Adriaens et al. 2006): *Helianthemum nummularium* (Linnaeus) Miller 1768 (*Cistaceae*), *Potentilla tabernaemontani* Ascherson 1891 (*Rosaceae*), *Sanguisorba minor* Scopoli 1771 (*Rosaceae*), and *Scabiosa columbaria* Linnaeus 1753 (*Dipsacaceae*), following the nomenclature of Lambinon et al. (2012). The selected species are not protected by local or national law.

2.3 Data collection

Measurements of functional traits and within-site environmental heterogeneity were made along transects placed perpendicularly to the contour of the hills (i.e. in the direction of the slope) in order to meet the whole range of within-site environmental heterogeneity. At each of the three sites, between 3 and 5 transects were used (depending on transect length) in order to find around 90 individuals for each species. Along transects, between 87 and 91 individuals of each study species were selected at each site, except for *Scabiosa columbaria*, which was less abundant in the Montagne-aux-buis and the Tienne Breumont sites (48 and 22 individuals selected, respectively). Individuals of one species located on one site are considered as one population. Following the methodology of Cornelissen et al. (2003), only healthy, robust, fully grown adult plants that have their foliage exposed to full sunlight were selected.

For each selected individual, three functional traits were assessed: (i) the vegetative height, i.e. the shortest distance between the upper boundary of the photosynthetic tissues (excluding inflorescences) of the plant and the soil surface (in cm); (ii) the specific leaf area (SLA), i.e. the one-sided area of a fresh leaf, divided by its oven-dried mass (in $mm^2 \times mg^{-1}$); (iii) the leaf dry matter content (LDMC), i.e. the oven-dried mass of a leaf divided by its water-saturated fresh mass (in $mg \times g^{-1}$) (Weiher et al. 1999, Cornelissen et al. 2003, Pérez-Harguindeguy et al. 2013). Leaf traits were measured on two leaves for each selected individual. Our sample size was much larger than the one required by standard protocols (Cornelissen et al. (2003) recommended 10 individuals for SLA and LDMC, and 25 individuals for plant height) in order to encompass the environmental heterogeneity of sampling sites. We followed the leaf trait measurement protocol of Garnier et al. (2001b): leaves were weighed to the nearest 10^{-3} g (Mettler Toledo®, Viroflay, France) following a minimum of 6 hours of rehydration, and subsequently scanned. Leaf area was measured using ImageJ imaging software (Abràmoff et al. 2004). Leaves were then oven-dried at 60 °C for 72 hours prior to the second weighing. As plant traits may be affected by grazing (Noy-Meir et al. 1989), individuals located in calcareous grassland areas that were exposed to the same grazing pressure and frequency were selected. All selected individuals were located on parcels managed by grazing once every three years with approximately the same number of sheep (by hectare) and not grazed during the year in which measurements were taken.

Along transects, the within-site heterogeneity of two variables linked to the potential drought stress was characterized: soil depth (in cm) and the potential direct incident radiation index (PDIR). PDIR is based on the folded aspect, the slope, and the latitude. It is dimensionless and ranges in value from 0.03 to 1.11, with higher values representing high potential direct incident radiation (on south-facing steep slopes). Details of this equation are given in McCune & Keon (2002). The aspect (in degrees) and the slope (inclination in degrees) were measured at the exact position of each individual sampled for trait measurements. Soil depth was measured at all four corners of a 1 m^2 plot placed around each sampled individual in order to calculate a mean soil depth value for each plot.

2.4 Data analysis

The extent of intra-population functional variability was displayed using a boxplot and a kernel density plot (a non-parametric method of estimating the probability density function of a random variable) for each trait and population (each species present at a site).

Intra-population functional variability was compared to:

a) the inter-population functional variability of collected data. Linear mixed models (BOLKER et al. 2009) were used with "site" designated as a random effect to quantify the contribution of each hierarchical level (intra-population vs. inter-population) to the total variation in each trait for each species. The "site" effect provides the inter-population functional variability of data, and intra-population functional variability is provided by the estimated standard error of the model.

b) the global inter-population functional variability that can be expected for the species and traits selected in this study. For this we used data from the worldwide TRY functional traits database was used (KATTGE et al. 2011). Data entries for SLA, LDMC, and vegetative height of our four study species were selected, and only data that encompassed an average of at least 10 individuals were used. Each data entry from the database therefore represents one trait value for one population of the species in the world (i.e. an average trait value taken from at least 10 individuals of one population).

The coefficients of variation (CV = standard deviation/mean) were calculated for each trait of each species at a site (intra-population variability) and in the database (inter-population variability) in order to assess and compare the degree of trait variation. The CV has the advantage of being dimensionless and comparable between species and traits (ALBERT et al. 2011). It has been frequently used in the context of trait variation and interpretation of intraspecific variation (JUNG et al. 2010, FAJARDO & PIPER 2011, LEMKE et al. 2012, FU et al. 2013, WELLSTEIN et al. 2013, MITCHELL & BAKKER 2014, GARCÍA-CERVIGÓN et al. 2015).

Linear regressions were conducted for all species traits and environmental variables at each site separately in order to highlight each population's functional response to within-site environmental heterogeneity.

Mixed models were performed using lmer function in the lme4 library. All analyses were performed using R.2.14.0 (R DEVELOPMENT CORE TEAM 2008).

3. Results

3.1 Extent of intra-population functional variability

We found large intra-population functional variability for SLA in all species (Fig. 2A–D) and LDMC (Fig. 2E–H), but particularly in *Helianthemum nummularium*. For this species, intra-population SLA values ranged from 10.2 to 42.5 $mm^2 \times mg^{-1}$ (Fig. 2A), and LDMC values ranged from 118 to 545 $mg \times g^{-1}$ (Fig. 2E) at the Sosoye hill site. Coefficients of variation (CVs) ranged from 0.13 to 0.30 for SLA (Fig. 2A–D) and from 0.08 to 0.20 for LDMC (Fig. 2E–H), depending upon the species and site. The intra-population variability of vegetative height was high for each species (Fig. 2I–L), with coefficients of variation ranging from 0.30 to 0.67, depending upon the species and site.

A comparison of our measured intra-population functional variability to inter-population variability from the TRY database (DB inter-population variability) exhibited contrasting results, depending upon the species and traits considered. For SLA, DB inter-population variability was lower than our measured intra-population variability for two study species: *H. nummularium* (inter-population database CV = 0.20, compared to 0.20–0.30 for our measured intra-population variability, Fig. 2A) and *Scabiosa columbaria* (inter-population database CV = 0.16, compared to 0.17–0.20 for our measured intra-population variability, Fig. 2D). For *Sanguisorba minor* and *Potentilla tabernaemontani*, DB inter-population variability was higher than our measured intra-population variability (inter-population database CV = 0.25, compared to 0.18–0.19 for our measured intra-population variability for *P. tabernaemontani* and inter-population database CV = 0.27, compared to 0.13–0.14 for our

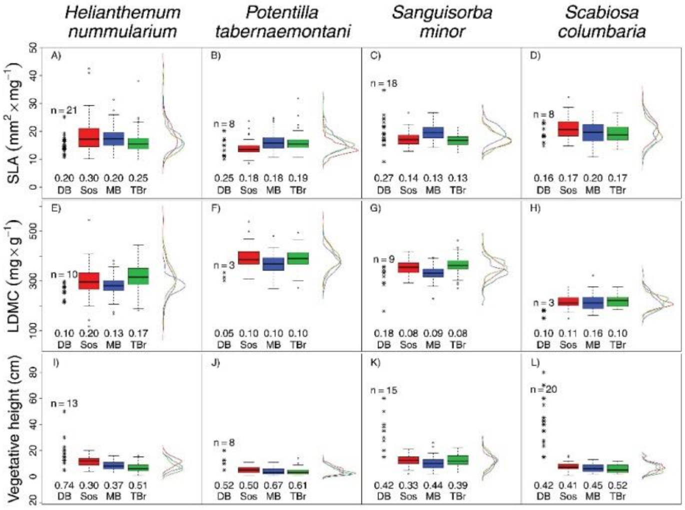

Fig. 2. Extent of intra-population functional variability. Boxplots and kernel density plots for each species and each trait in a site (Sos = Sosoye hill in red, MB = Montagne-aux-buis in blue and TBr = Tienne Breumont in green). Kernel density plots represent the density of data estimated by kernel method (Sosoye hill in red, Montagne-aux-buis in blue and Tienne Breumont in green) and boxplots show the median value (black line) and the inter-quartile ranges: between Q2–Q3 (black box) and between Q1–Q2 and Q3–Q4 (dotted segments). Database values are represented for each species and each trait by asterisks and the number of data (*n*) are given above asterisks. Coefficients of variation (CV) for each trait of each species in a site and in the database are given under boxplots or asterisks.

Abb. 2. Variabilität von drei funktionellen Merkmalen (Spezifische Blattfläche: SLA–*specific leaf area*, Trockenmasseanteil der Blätter: LDMC–*leaf dry matter content* und Höhe der vegetativen Pflanzen: *vegetative height*) bei vier Arten der Kalkmagerrasen in drei Gebieten: Sos = Sosoye hill (rot), MB = Montagne-aux-buis (blau) und TBr = Tienne Breumont (grün). Boxplots und Kerndichteschätzungen (*kernal densities*: Kurven) sind dargestellt. Die Kerndichteschätzungen zeigen die mit der *Kernel*-Methode vorhergesagte Datendichte und die Boxplots den Median (fette Linie), die Quartile (Q): Q2–Q3 als Box und Q1–Q2 und Q3–Q4 als gestrichelte Linien sowie Ausreißerwerte als schwarze Kreise an. Sterne zeigen aus der Datenbank TRY gewonnene Werte mit der entsprechenden Stichprobenzahl *n* an. Unter den Boxplots oder Sternen sind für jedes Merkmal und jede Art die Varianzkoeffizienten (CVs) dargestellt.

measured intra-population variability for *S. minor*, Fig. 2B–C). For LDMC, DB inter-population variability was lower than our measured intra-population variability for all species except *S. minor* (inter-population CV = 0.18, compared to 0.08–0.09 for our measured intra-population variability, Fig. 2G). For vegetative height, DB inter-population variability and intra-population variability were comparable (sometimes higher, sometimes lower depending on the site), except in *H. nummularium*. For this species, DB inter-population variability was higher than our measured intra-population variability (CV = 74, compared to

Table 1. Variance partitioning of traits studied between (inter) and within (intra) populations. Percentage of the total trait variability is partitioned between intra- and inter-population trait variability for each study species. SLA, specific leaf area, LDMC, leaf dry matter content.

Tabelle 1. Aufteilung der Varianz von drei funktionellen Merkmalen bei vier Pflanzenarten auf innerhalb (intra) und zwischen (inter) Populationen. SLA, Spezifische Blattfläche, LDMC, Trockenmasseanteil der Blätter, *Vegetative height*, Höhe der vegetativen Pflanzen.

		Helianthemum nummularium	*Potentilla tabernaemontani*	*Sanguisorba minor*	*Scabiosa columbaria*
SLA	intra-population	95	83	72	93
	inter-population	5	17	28	7
LDMC	intra-population	89	88	78	100
	inter-population	11	12	22	0
Vegetative height	intra- population	70	94	94	92
	inter- population	30	6	6	8

0.30–0.51 for our measured intra-population variability, Fig. 2I). For this trait, database values were higher than the study population values. Species vegetative height ranged from 5 to 80 cm in the database, while it ranged from 1 to 26 cm in our study populations.

Partition of our measured total trait variability between intra- and inter-population variability indicates that the intra-population variability accounts for 70 to 100% of the total variance, depending on the trait and species (Table 1), while 0 to 30% of the variance was due to differences between populations.

3.2 Functional response to within-site environmental heterogeneity

A total of 36 regressions were conducted for the soil depth data, and a total of 36 were also conducted for PDIR data (four species, three populations per species and three traits measured). Of the 36 regressions, 20 were significant ($p < 0.05$) for soil depth (Fig. 3), while 23 were significant ($p < 0.05$) for PDIR (Fig. 4). For all species, significant regressions always indicated the same trend: SLA and vegetative height increased with soil depth (Fig. 3A–D and 3I–L), and decreased when PDIR increased (Fig. 4A–D and 4I–L). LDMC decreased when soil depth increased (Fig. 3E–H) and increased with PDIR (Fig. 4E–H).

4. Discussion

To date, the majority of trait-based research has utilized mean trait values to describe a given species. However, this may hide functional variation at different scales, both between and within populations (Bolnick et al. 2011). This study highlights the extent of intra-population variability of functional traits at local scales in response to within-site environmental variability within the same habitat type (calcareous grasslands).

The results indicate that SLA, LDMC, and vegetative height are not only characterized by important variation between species, as previously suggested (Westoby 1998), but that significant variations can also be highlighted at the intra-population level. Congruent with the results of this study, Wellstein et al. (2013) studied the intraspecific variability of the same traits for four perennial species representative of mountain grasslands, discovering-strong variation of those traits within populations, particularly in SLA and plant height. At

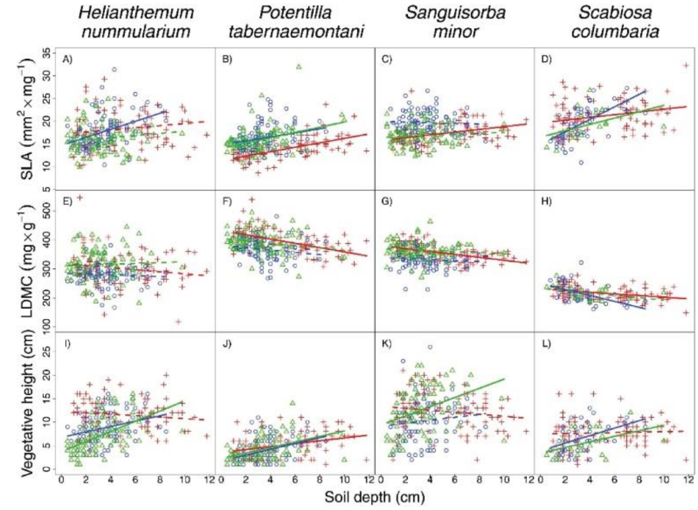

Fig. 3. Population responses to the within-site soil depth heterogeneity. Individual trait values (SLA, LDMC and vegetative height) and the related soil depth (measured at the four corners of a 1m^2 plot placed around each sampled individual in order to calculate a mean soil depth value for each plot) were drawn for each species individual in a site (the Sosoye hill in red crosses, the Montagne-aux-buis in blue circles and the Tienne Breumont in green triangles). Linear regressions between each species traits and the soil depth were drawn for each species in a site. Regression lines are solid if significant (*p*-values < 0.05) and dashed if not.

Abb. 3. Lineare Zusammenhänge zwischen drei funktionellen Merkmalen (Spezifische Blattfläche: SLA–*specific leaf area*, Trockenmasseanteil der Blätter: LDMC–*leaf dry matter content* und Höhe der vegetativen Pflanzen: *vegetative height*) und der Bodengründigkeit bei vier Arten der Kalkmagerrasen in drei Gebieten: Sosoye hill (rote Kreuze), Montagne-aux-buis (blaue Kreise), Tienne Breumont (grüne Dreiecke). Durchgezogene Regressionsgeraden zeigen signifikante ($p < 0,05$) Zusammenhänge und gepunktete Regressionsgeraden keinen Zusammenhang ($p > 0,05$) an.

local scales, our study highlighted CV values ranging from 0.13 to 0.30 for SLA, 0.08 to 0.20 for LDMC, and 0.30 to 0.67 for vegetative height. This can be compared to a larger scale study by ALBERT et al. (2010a) who characterized the intraspecific variability of LDMC and vegetative height for sixteen terrestrial species, discovering CV values ranging from 0.08 to 0.25 for LDMC, and from 0.19 to 0.49 for vegetative height, with a large portion of intraspecific variability representing differences between populations. By comparing the extent of intra-population functional variability with large database values, this study suggests that within-population variation represents a significant component of the overall variance in these traits. In this study, over 70% of trait variability was explained at the intra-population level, which is higher than values reported by previous studies. For example, MITCHELL & BAKKER (2014) discovered that for SLA, differences between populations explained 58% of the observed variation for *Hypochaeris radicata*, while only 42% of variation was due to differences at the intra-population level. Additionally, ALBERT et al. (2010b)

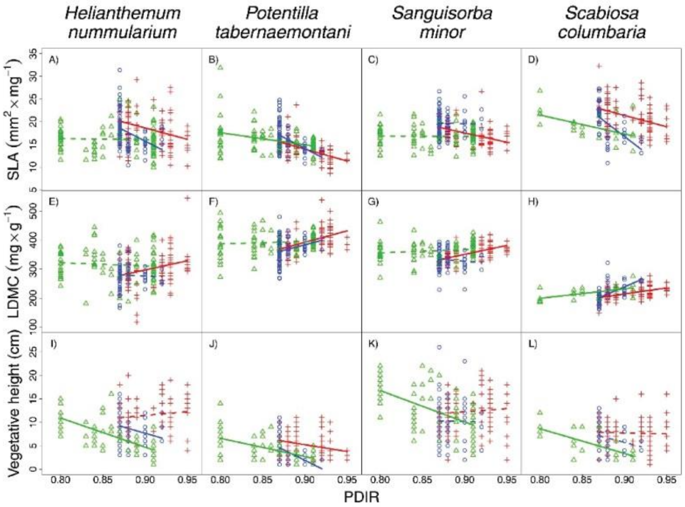

Fig. 4. Population responses to the within-site potential direct incident radiation (PDIR) heterogeneity. Individual trait values (SLA, LDMC and vegetative height) and the related PDIR (index based on the aspect and the slope measured at the exact position of each individual sampled) were drawn for each species individual in a site (the Sosoye hill in red crosses, the Montagne-aux-buis in blue circles and the Tienne Breumont in green triangles). Linear regressions between each species traits and the PDIR were drawn for each species in a site. Regression lines are solid if significant (p-values < 0.05) and dashed if not.

Abb. 4. Lineare Zusammenhänge zwischen drei funktionellen Merkmalen (Spezifische Blattfläche: SLA–*specific leaf area*, Trockenmasseanteil der Blätter: LDMC–*leaf dry matter content* und Höhe der vegetativen Pflanzen: *vegetative height*) und der potenziellen Einstrahlung (PDIR–*potential direct incident radiation*) bei vier Arten der Kalkmagerrasen in drei Gebieten: Sosoye hill (rote Kreuze), Montagne-aux-buis (blaue Kreise) und Tienne Breumont (grüne Dreiecke). Durchgezogene Regressionsgeraden zeigen signifikante Zusammenhänge ($p < 0{,}05$) und gepunktete Regressionsgeraden keinen Zusammenhang ($p > 0{,}05$) an.

discovered that approximately 50% of the trait variability (SLA, LDMC, LNC-leaf nitrogen concentration, and LCC-leaf carbon concentration) in alpine species was due to differences between populations, and 50% was due to differences within populations.

Among traits measured in this study, SLA exhibited higher CV values than LDMC. This is consistent with other studies of trait variation at the intraspecific level, indicating that SLA (or its inverse leaf mass per area) is one of the most variable leaf functional traits both at broad and at narrow spatial scales (Garnier et al. 2001a, Albert et al. 2010b, Fajardo & Piper 2011, Auger & Shipley 2013, Boucher et al. 2013, Wellstein et al. 2013, García-Cervigón et al. 2015, Siefert et al. 2015). While some traits are fixed and do not vary within species (such as photosynthetic pathway, for example), others can vary substantially, as is notably the case for leaf traits (Albert et al. 2010a, Hulshof & Swenson 2010).

The findings of this study demonstrate high functional variation within calcareous grassland species populations at fine spatial scales. As differences in abiotic tolerances or resource use between individuals can generate variance in a population's demographic parameters (BOLNICK et al. 2003), large intra-population functional variability can have a large impact on a population's functioning within the site. Moreover, this study showed that the within-population functional variability was a major component of the overall variance in study traits and therefore challenged the assumption that average trait values are sufficiently representative of a given species. This intraspecific functional trait variability at fine spatial scale can be related to microhabitat environmental factors such as light, soil temperature or availability of nutrients (WELLSTEIN et al. 2013). In our case, the intra-population functional trait variability was related to within-site soil depth and PDIR variation.

We found higher plant height and higher SLA for individuals located in plots with deeper soils or low PDIR (less potential direct incident radiation, on gentle slopes or north-facing slopes), and the inverse response for LDMC, with higher values in shallow soil or high potential direct incident radiation. This supports the idea that higher plant height, higher SLA, and lower LDMC may be related to significantly higher availability of soil moisture, as has been previously shown for herbaceous species (MEZIANE & SHIPLEY 1999, WELLSTEIN et al. 2013). Individuals on shallow soils are characterized by lower plant height and higher leaf tissue density, which ensure more successful performance under stressful conditions. Indeed, high LDMC and low SLA are related to high investment in structural tissues, which allow plants to maintain leaf turgor under drought stress (NIINEMETS 2001, SIEFERT 2012). Conservative species that exhibit low SLA (high LDMC), corresponding to dense leaf tissues and low growth rates, exhibit high resource conservation (ALBERT et al. 2010b). Trait differentiation with respect to fine scale variation in soil depth has already been suggested by a study by RAVENSCROFT et al. (2014) who indicated a significant soil depth effect whereby LDMC was lower in deeper soils. ACKERLY et al. (2002) showed that SLA declined with increasing insolation, indicating that species with thicker and/or denser leaves preferentially occupied more exposed, south-facing slopes. These intraspecific patterns are the same as those shown in interspecific trends (CORNWELL & ACKERLY 2009, PAKEMAN et al. 2009).

Carefully designed experiments are needed to test whether the observed intraspecific variation is due to plasticity (the observed trait variation is due to the direct response of individuals to environmental conditions) or local adaptation (the observed trait variation is due to the presence of genetic variation between individuals resulting from natural selection). In our study, gene flow between individuals is certainly high as spatial distance between individuals is quite short and because species are pollinated by flying insects. The possibility of genetic differentiation between individuals is therefore reduced and plasticity is the likely cause of observed intraspecific variability.

Our results indicate that intra-population functional variability is not only highly variable at local scale but that this variability is likely to be driven by local environmental heterogeneity. Our study therefore challenges the use of published mean values of functional trait to describe species behaviour. Measuring traits in situ on a large number of individuals is of primary importance, as the degree of species response strongly depends on individuals' locations and direct environmental influences. Species mean trait values should be replaced by a distribution of traits along environmental gradients.

Erweiterte deutsche Zusammenfassung

Einleitung – Die meisten Studien zu funktionellen Merkmalen von Pflanzenarten (sog. *plant functional traits*) bedienen sich Art-Mittelwerten und setzen damit voraus, dass Art-Mittelwerte die tatsächliche Ausbildung der funktionellen Merkmale ausreichend gut repräsentieren. Obwohl sich solche Art-Mittelwerte in ökologischen Studien als grundsätzlich geeignet erwiesen haben, können funktionelle Merkmale innerhalb der Arten stark variieren (ALBERT et al. 2010a). Die Berücksichtigung dieser innerartlichen Variabilität in ökologischen Studien könnte sinnvoll oder auch notwendig sein (SIEFERT et al. 2015). Innerartliche Variabilität kann auf verschiedenen Ebenen untersucht werden, zum Beispiel zwischen Populationen (JUNG et al. 2010) oder innerhalb von Populationen (MITCHELL & BAKKER 2014a); die letztere Möglichkeit wurde bisher kaum wahrgenommen. Auf kleinräumiger Skala sind Kalkmagerrasen zur Untersuchung funktioneller Merkmale von Pflanzenarten besonders geeignet. Auf den heterogenen Kalkstandorten sind einerseits viele Arten vertreten und andererseits unterscheiden sich diese Arten oftmals in ihren Umweltansprüchen, z. B. in ihren Ansprüchen an die Bodengründigkeit, Bodenwasserversorgung, Hangexposition oder Hangneigung. Die vorliegende Studie untersucht die kleinräumige Variabilität funktioneller Merkmale in Populationen ausgewählter Pflanzenarten der Kalkmagerrasen und testet die Hypothese, dass die Variabilität dieser Merkmale hauptsächlich durch Umweltheterogenität erklärt werden kann.

Material und Methoden – In Süd-Belgien wurden drei Kalkmagerrasengebiete (Dourbes, Nismes und Sosoye) sowie vier Pflanzenarten ausgewählt: *Helianthemum nummularium* (L.) Mill. (*Cistaceae*), *Potentilla tabernaemontani* Asch. *(Rosaceae)*, *Sanguisorba minor* Scop. (*Rosaceae*) und *Scabiosa columbaria* L. (*Dipsacaceae*). In drei Populationen jeder Art wurden die funktionellen Merkmale (1) Spezifische Blattfläche (SLA–*specific leaf area*), (2) Trockenmasseanteil der Blätter (LDMC–*leaf dry matter content*) und (3) Höhe der vegetativen Pflanzen an insgesamt 950 Individuen gemessen. Die Variabilität der Bodengründigkeit und der potenziellen Einstrahlung an den Wuchsorten wurden bestimmt und in Beziehung zur Variabilität der funktionellen Merkmale der Pflanzen gesetzt. Die funktionellen Merkmale und die Umweltvariabilität innerhalb der Gebiete wurden entlang von Transekten aufgenommen, die senkrecht die Hügel hinunter verliefen. Die gemessene Variabilität der funktionellen Merkmale innerhalb der Populationen und zwischen den Populationen wurde anhand eines linearen gemischten Modells pro Art bestimmt. Zusätzlich wurden die gemessene und die „globale" Variabilität der funktionellen Merkmale miteinander verglichen wobei die „globale" Variabilität auf Grundlage der Werte aus der Datenbank TRY bestimmt wurde. Dazu wurden für jedes funktionelle Merkmal pro Art und Gebiet Varianzkoeffizienten (CV) berechnet. Um die Reaktion der Populationen auf unterschiedliche Umweltbedingungen einzuschätzen, wurden die Zusammenhänge zwischen funktionellen Merkmalen und Umweltbedingungen mit linearen Regressionen für jedes Gebiet separat untersucht.

Ergebnisse – Die Ergebnisse zeigen eine starke Variabilität der funktionellen Merkmale innerhalb der Populationen wobei die Höhe der vegetativen Pflanzen mit Varianzkoeffizienten zwischen 0,30 und 0,67 (je nach Art und Gebiet) besonders stark variierte (Abb. 2). Je nach Art und Merkmal konnten 70–100 % der Varianz auf Unterschiede zwischen den Pflanzen innerhalb der Populationen und lediglich 0–30 % der Varianz auf Unterschiede zwischen den Populationen zurückgeführt werden (Tab. 1). Die spezifische Blattfläche (SLA) und Höhe der vegetativen Pflanzen nahm bei allen Arten mit der Bodengründigkeit zu und mit der potenziellen Einstrahlung an den Standorten ab. Im Vergleich dazu nahm der Trockenmasseanteil der Blätter (LDMC) mit der Bodengründigkeit ab und mit der potenziellen Einstrahlung zu (Abb. 3–4).

Diskussion – In unserer Studie an vier Kalkmagerrasenarten variierten die spezifische Blattfläche (SLA), der Trockenmasseanteil der Blätter (LDMC) und die Höhe der vegetativen Pflanzen nicht nur zwischen Arten und Pflanzengesellschaften (wie es bereits WESTOBY 1998 zeigen konnte), sondern auch innerhalb von Populationen erheblich. Diese Variabilität der funktionellen Merkmale ist die Folge direkter Reaktion der Pflanzen auf eine heterogene Umwelt. Die Variabilität eines funktionellen Merkmales einer Pflanzenart innerhalb ihrer Populationen hat dabei einen erheblichen Anteil an der Gesamtvariabilität des Merkmales, wie der Vergleich der in den Populationen gemessenen Variabilität mit der „globalen Variabilität“ auf Grundlage einer weltweiten *Trait*-Datenbank zeigen konnte. Konkret zeigen unsere Ergebnisse, dass eine bessere Wasserversorgung in Kalkmagerrasen zu einem höheren Wuchs, einer größeren spezifischen Blattfläche und einem geringeren Trockenmasseanteil der Blätter der Pflanzen führen. Im Umkehrschluss bedeuten unsere Ergebnisse, dass diejenigen Pflanzen, die in Kalkmagerrasen auf besonders flachgründigen Standorten oder Standorten mit besonders hoher Einstrahlung wachsen, den hier höheren Umweltstress durch gezielte Ausbildung ihrer funktionellen Merkmale besser ertragen. Vermutlich handelt es sich dabei um plastische Reaktionen der Pflanzen auf Umweltstress; es können aber auch lokale Anpassungen vorliegen. Weiterhin zeigt unsere Studie, dass bei der Untersuchungen von funktionellen Merkmalen die Untersuchung einer großen Anzahl an Individuen notwendig ist, da die Individuen auf die unterschiedlichen Standortbedingungen ihrer Wuchsorte mit ihren funktionellen Merkmalen stark reagieren können. Die Nichtbeachtung der durch solche Umweltheterogenität bedingten hohen Variabilität der funktionellen Merkmale kann ökologische Zusammenhänge verschleiern oder auch zu Fehleinschätzungen führen.

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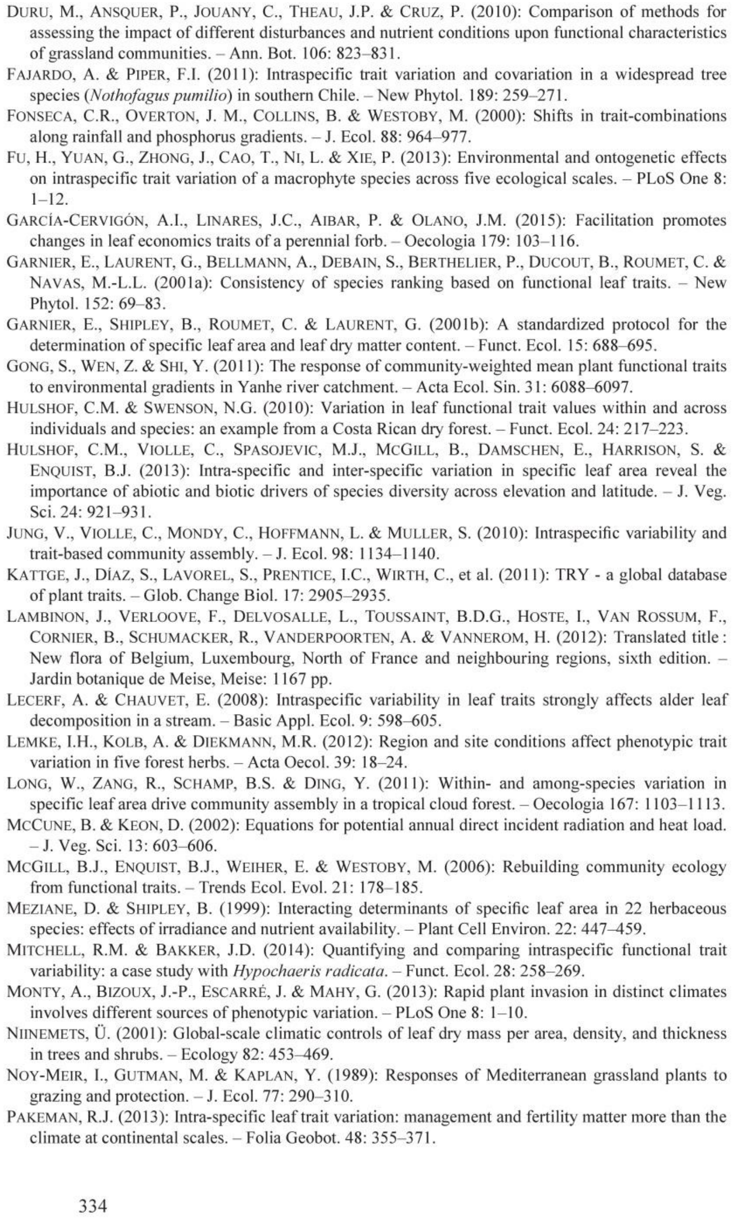

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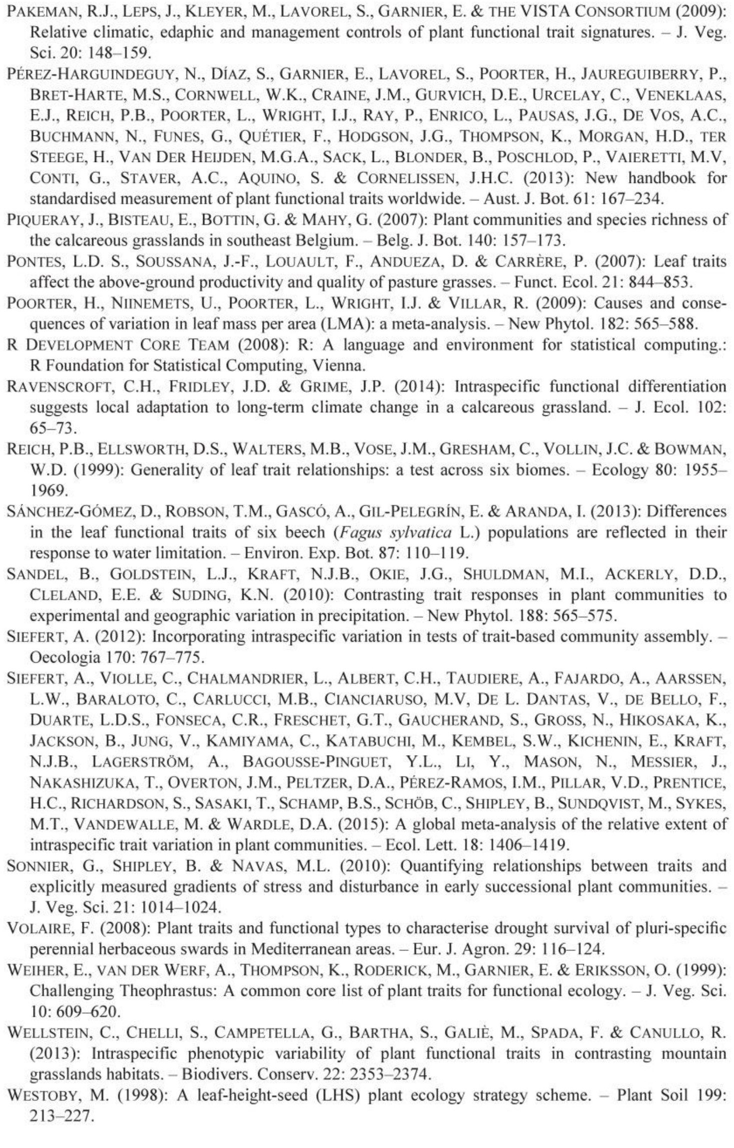

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